COLD SPRING HARBOR LABORATORY
THE CENTENNIAL YEAR

ANNUAL REPORT 1990

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Cold Spring Harbor Laboratory
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Front cover: Jones Laboratory, during the Centennial fireworks display. This building, completed in 1893, was the first structure built expressly for science at Cold Spring Harbor. (Photograph by Ross Meurer and Margot Bennett.)

Back Cover: (*Top*) Centennial reenactment of the first biology class at Cold Spring Harbor on board the launch "Rotifer." (*Inset*) The original class of 1890. Reenactments for the Centennial were directed by Rob Gensel. (Photograph by Randy Wilfong.) (*Bottom*) Centennial reenactment of the Laboratory's first biology class on the Jones Laboratory porch. (*Inset*) The original 1890 class on the porch of the old Fish Hatchery building before the completion of Jones Laboratory. (Photograph by Margot Bennett.)

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(*Front row*) Mrs. G.G. Montgomery, J.D. Watson, Mrs. J.H. Hazen, L.J. Landeau (*Middle row*) W.S. Robertson, D.L. Luke, W. Everdel, T. Knight, D. Sabatini, T. Maniatis, B.D. Clarkson, J.R. Warner, T. Silhavy, G.W. Cutting, F.M. Richards (*Back row*) W.H. Page, O.R. Grace, T.M. Jessell, W.R. Miller, J. Darnell, S. Strickland, O.T. Smith, J.R. Reese, D. Botstein, D.B. Pall *Not shown*: C.F. Dolan, M. Lindsay, T. Whipple, M Cowan, S. Tilghman

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Representation on the Board of Trustees itself is divided between community representatives and scientists from major research institutions. Ten such institutions are presently represented on the Board of Trustees: Albert Einstein College of Medicine, Columbia University, Harvard University, Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York University Medical Center, Princeton University, The Rockefeller University, The State University of New York at Stony Brook, and Yale University.

Also represented as a participating institution is the Long Island Biological Association (LIBA). LIBA's origins began in 1890 when members of the local community became involved as friends and with membership on the Board of Managers of the Biological Station in Cold Spring Harbor, under the aegis of the Brooklyn Academy of Arts and Sciences. When the Brooklyn Academy withdrew its support of the Biological Station, community leaders, in 1924, organized their own association that actually administered the Laboratory until the Laboratory's reorganization as an independent unit in 1962. Today, LIBA remains a nonprofit organization organized under the Department of Social Welfare of the State of New York and represents a growing constituency of "friends of the Laboratory." Its 900 members support the Laboratory through annual contributions and participation in fund drives to raise money for major construction projects.

The Laboratory is chartered as an educational and research institution by the Board of Regents of the Education Department of the State of New York. It is designated as a "public charity" by the Internal Revenue Service.

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Edward Pulling
(1898–1991)

Edward Pulling, who died on April 17, 1991 at the age of 92, was a truly remarkable man whose career embodied the virtues of education, family life, and devotion to service for the public good. In his dual roles, as a trustee of this Lab and as Chairman of the Long Island Biological Association, he played an invaluable role in the Laboratory's achievements over the past two decades. My wife and I will always remember the gracious way Ed and his wife Lucy brought us into their world when we first arrived in this community in the summer of 1968. By then, he had retired as the founding headmaster of the Millbrook School and they had moved to Oyster Bay to live in "Redcote," their home on Yellowcote Road which Lucy had inherited upon the death of her father.

To this charming extended farmhouse, sited on its accompanying estate, we were first invited by Ed on a late June evening to meet Lucy and several of their neighbors. There, below the terrace off the drawing rooms, I first appreciated the *ha ha*, the sunken fence of stone that the English have long used to keep their horses at proper distance from their living quarters. Later, Liz and I came often to Redcote and its book-lined rooms to hear Ed and Lucy speak of their lives and of the events that formed their aspirations, and soon we felt at ease as denizens of their fabled world of Long Island's Oyster Bay. In particular, I still remember our flying down from Cambridge during an interterm break at Harvard and soon becoming so delightfully snowbound that we missed the opening days of the spring academic term.

Ed's erect 6'3" body with its strong-featured warm face and voice gave instantly the impression of a leader toward goodness and responsibility, first tested in battle conditions when he served as a destroyer officer in the British Navy toward the end of the First World War. He was born on June 10, 1898, in the London suburb of Ealing. His father, of Devonshire yeoman stock, was then the British agent for the New York Life Insurance Company. As a youth, he had emigrated to the United States and there had married Elizabeth Douglass, whose Ohio-based family had come several generations before from Scotland. After their marriage, Ed's parents moved to England, where his father established a thriving business that lasted over a decade. Then a change in American insurance company regulations forced New York Life to close down its English office, and at the age of ten, Ed and his parents returned to the states, first to Pittsburgh and then to Baltimore. In the latter city, Ed prepared at the Gilman School for Princeton, where his college education was interrupted by his service in the British Royal Navy.

Upon leaving the Royal Navy he was offered a position at the Bethlehem Steel Company but happily, when stopping at Princeton on his way home from England, he was persuaded by Professor Charles Kennedy to give a try to teaching. After an interview at Groton, then headed by the renowned Endicott Peabody, Ed became spellbound by the Rector's wonderful personality and quickly signed on to be a Master of History. For six subsequent happy years, he learned the art of teaching under Mr. Peabody's aegis. Interrupting this very formative period were two years spent in Trinity College at Cambridge, where he read English and displayed his mastery of golf through his membership on the Cambridge University team. It was while at Groton that he first became befriended by Franklin Roosevelt, whose son James was one of his students and whom he tutored at Hyde Park over the Christmas 1920 vacation period. It was Franklin Roosevelt who first suggested that Ed start his own school. Ed remembered being counseled "You ought to start a school someday. Promise to let me know when you do, because I want to help."

On a trip to Europe during the summer of 1926, he first met Lucy Leffingwell, the only daughter of the banker Russell Leffingwell, a partner of J.P. Morgan, who subsequently was to become Chairman of the Board of J.P. Morgan and Company. Ed and Lucy both were passengers on the Cunard Liner *Aquitania* and saw each other first as partners in a game of deck tennis. Their marriage occurred two years later in St. John's Church here in Cold Spring Harbor, with their departing from their reception at the Beach Club aboard the yacht *Corsair* which J.P. Morgan had put at their disposal.

In 1929, while searching a site for their proposed school, Franklin Roosevelt, by then governor of New York State, offered them property on Cream Street adjacent to his Hyde Park estate. This most generous proposal, however, was declined both because they feared Hyde Park would soon be less rural than they wanted and also because they feared that the parents of many prospective students might hesitate to favor a school founded under the aegis of such a famous Democrat. Years later, after President Roosevelt's death, Eleanor Roosevelt made her home on the Cream Street site. To this site, Ed and Lucy made many visits during and after the Roosevelt's grandson Haven was a Millbrook student.

The site Ed and Lucy finally chose was near the town of Millbrook in the rolling countryside of Duchess County. Even today it is totally unspoilt. Their first class of 21 boys came together in the fall of 1931, and over subsequent years they presided over an ever-expanding school of great beauty which purposefully offered a less traditional curriculum than Groton. Rather than putting faith in grades or admission tests, Ed hired teachers and accepted students on a hunch. Always paramount was a close relationship between the faculty and the students. Equally important was the participation of all its students, through community service, in the school's daily functioning. The 34-year partnership of Ed and Lucy was extraordinarily productive, being from its start to their leaving a joint venture. Much later Ed was to write that while he believed his students respected him, he knew they loved Lucy. During their Milbrook years, they raised and educated their four children—Joan, Patricia, Lucy, and Tom, with whom they would go each summer to their house at West Chop on Martha's Vineyard.

Ed was an early beneficiary of the surgical procedures for hip replacements developed in the 1950s in England. In 1963, a severe attack of osteoarthritis threatened to confine him to a wheelchair for the rest of his life. To his rescue came Dr. Otto Aufranc of the Massachusetts General Hospital (MGH), who through two operations performed nine days apart gave Ed two steel hips. He was the first person operated upon at MGH to have both hips replaced during one hospitalization. Happily, the surgery was amazingly successful, as within 10 months Ed was again riding and playing golf.

It was Walter Page who sensed the great opportunity offered to the Cold Spring Harbor Laboratory by Ed and Lucy's movement into our community following his retirement in 1965. Sensing Ed's vast experience in public relations and fund raising gained while he was a headmaster, Walter, then the President of LIBA, persuaded Ed in 1968 to become a LIBA director. In so joining the LIBA board, Ed followed in the tradition of his late father-in-law Russell Leffingwell, who in 1924 became a founding director of LIBA. A year later, in 1969, Ed assumed the chairmanship, a position he was to hold for 17 highly effective years. Soon the LIBA membership was doubled to nearly 500 families, and conditions were created for the subsequent holding of major fund drives. The first, conducted during 1972–1973, raised $250,000 toward (I) construction of

the west addition to James Lab for cancer virus research, (2) winterization of Blackford Hall, and (3) purchase and renovation of the Takami residence as a dormitory, now called Olney House. In 1977, Ed mounted his second fund drive, which raised $220,000 to allow Williams House to be completely rebuilt as a year-round residence with five apartments.

Just as this project was finished, Ed received the devastating news that Lucy was striken with a potentially fatal disease. Fortunately, the initial therapy sent her disease into complete remission, and Lucy could actively participate in the celebrations held at the Piping Rock Club to mark their 50th wedding anniversary. Unfortunately, Lucy's disease reoccurred the following year, culminating in her death on February 15, 1979. With her passing, Ed grieved for his gentle companion who so well personified loyalty and open-minded intelligence.

At no time, however, did Ed's ability to work for this institution falter. Over the following years, he led his third fund drive, which raised $200,000 to purchase 20 acres of land from the Carnegie Institution of Washington. Its purchase made possible the 1982 completion of the Harris Building, as well as the subsequent siting of the Oliver and Lorraine Grace Auditorium. Funds for the construction of this later building were the objective of Ed's last major fund-raising effort. Through his efforts, $600,000 was raised from individuals who previously had expressed interest in the Lab. In addition, it was he who attracted the Graces to make their seven figure gift which allowed us to proceed with the project. Ed, as chairman of the Building Committee of our Board of Trustees, also played a very active role in the design process, drawing upon his many years of experience in the building projects of the Millbrook School.

A mere chronicling of the LIBA fund drives does not adequately reflect the many important gifts to the Lab itself that came in large measure through his actions. I particularly remember Ed tracking me down in California in late June of 1982 to arrange a date when Charles S. Robertson would make his first visit to the Lab and have lunch at Osterhout Cottage where Liz and I were then living. This was the most important lunch ever held at the Lab, initiating the subsequent measures which led to the creation of the Robertson Research Fund and the Banbury Center. Ed's enormous enthusiasm for the Lab was also the major factor leading the Doubleday Trust to give us the Doubleday shares that made possible the creation of the Doubleday professorship for advanced cancer research.

To honor Ed's enormous contributions to this institution as well as those of Walter Page, Liz and I hosted a party on the Airslie lawn in June 1983 to mark their combined 40 years of service (25 by Walter and 15 by Ed). To it came some 200 guests who took much pleasure in our good fortune of being served by two such illustrious neighbors. By then, we feared that Ed was considering passing on his mantle, wishing to retire while he still had complete command of his LIBA role. This moment formally came in January 1986 when Ed transferred his responsibilities to George Cutting, Jr. In this his final major act of help, Ed's instincts were again right on mark, with George knowing from the start how to carry on the responsibilities so ably handled before. Later, Ed took delight in continuing to manage the dinner parties hosted by LIBA members for Symposium participants. Much pleasure also came to him from the books he put together that recorded the earlier years of his life. Happily, he was to remain in good health almost to the very end of his life. Up until he was stricken with a bad cold that affected his heart, he maintained an active social life and greatly enriched the many occasions when he went to neighboring homes for drinks or dinner.

His final days were peaceful and serene. He knew that his time had come and his heart ceased to beat after he was read to by his daughter Lucy. Two days later, T. Carleton Lee conducted the funeral services at St. John's, the very church in which Ed was married 63 years before and to which he regularly came to hear Carleton give the Sunday service. Afterwards, the assembled set of bereaved friends went back to Redcote to convey their condolences to the family over which he presided with such love and dignity.

In his passing, we have lost an extraordinary force for enlightenment and decency. We will continue to cherish his accomplishments for the remainder of our lives.

April 29, 1991 **James D. Watson**

DIRECTOR'S REPORT

Cold Spring Harbor Laboratory has now just passed its 100th birthday. Our centenary celebrations are over; so instead of looking backward to take pride in our past achievements, we must again be oriented toward the future. In looking ahead, we must ask not only what science we should be doing, but also, equally important, what sort of institution do we want to be—what are our roles and what do we really cherish? We must ask these questions because our reputation owes as much to the way we do science as it does to the sterling research accomplished here over the past century. Now there are many other universities and research institutions with stellar track records, and they also are justifiably proud of what they have become. Each has had its own recipe for success, and if asked what lies behind these triumphs, each would have its own unique story to tell. Here, I will try to evaluate the ingredients that have made us what we are. In so doing, I can call on a direct involvement that goes back more than 40 years.

When I first came here for the summer of 1948, I heard much local gossip about what Cold Spring Harbor was like from my two most important mentors, Salvador Luria and Max Delbrück. They were first invited here for the summer of 1941, only a few months after the Yugoslav-born, Cornell-trained geneticist Milislav Demerec (1895–1966) became the Director. Until then, Cold Spring Harbor Laboratory was very much a Yankee institution, with both the strength of an ethic for hard work and the limitation of prejudice against newly arrived immigrants. Suddenly, it was a truly international laboratory playing host to many superb scientists who came to the United States as refugees and then remained here after the war ended. I still remember my first day at Cold Spring Harbor eating in the Blackford Hall where French was spoken by many, and I felt acutely the cultural limitations of my Midwestern background.

Cold Spring Harbor's Main Intellectual Goal Has Been the Gene

What most made Cold Spring Harbor Laboratory then an extraordinary institution was its commitment to understanding the gene. Then, as now, the most important ingredient for greatness was the setting up of an important goal that would make one leap into the unknown as opposed to following others along a well-worn path toward predictable success. Milislav Demerec's goal for Cold Spring Harbor in 1941 was to find out what the gene was and how it functioned. Although for several years more he kept *Drosophila* as his major research tool, by the end of the war he had become committed to the genetically then much more exploitable bacteria and their viruses—the bacteriophages. Demerec thus

chose not only the right goal for his era, but also the right system to move toward it.

Equally important was his commitment to the first class in selecting his scientific colleagues. It is impossible to imagine two better appointments than his of Barbara McClintock in 1941 and Alfred Hershey in 1950. Also crucial was his choice of Max Delbrück to refocus (with his bacteriophage course) the summer teaching program away from the prewar emphasis on physiology and ecology toward genetics. And by having the likes of Theodosius Dobzhansky, Ernst Mayr, Leonor Michaelis, Salvador Luria, Rollin Hotchkiss, and Ephraim Racker as summer guests in the unheated whaling-era tenements along Bungtown Road, Demerec gave to Cold Spring Harbor summers an air of intellectual brilliance and integrity unmatched anywhere else in the biological world.

We Have Been Led by Scientific Entrepreneurs

To direct a scientific institution inspiringly, one must not only have a feel for where science can go and who should do it, but also be in a real sense an entrepreneur, scientific-style. Monies must be found both for new equipment and buildings. Successful leaders of science must accept the fact that they are managers as well as doers of science. Demerec, for example, knew that postwar Cold Spring Harbor had to be more than a plant and animal- breeding institution and had to have research facilities for studying genetics at the molecular level. As soon as the war ended, he began planning for the 1953 building now known as Demerec Laboratory. At the same time, he knew that the Symposium could only continue to thrive if a lecture hall became available, and he successfully sought out the Carnegie Foundation funds needed for the construction of what we now call the Vannevar Bush Lecture Hall. And to make the one-storied 1930 James Biophysics Laboratory suitable for molecular genetics, he planned for a second-story addition.

Even more the scientific entrepreneur was Charles B. Davenport (1866–1944), who in 1898 became our third Director. At that time, we were still a small, marine-oriented biology station at the head of the inner harbor of Cold Spring Harbor, founded in 1890, two years after the Marine Biological Laboratory at Woods Hole was started. Immediately after his appointment, Davenport dreamed of expanding Cold Spring Harbor into a year-round research and teaching center. In this way, he would have a much greater impact on biology than if he were to remain a member of the zoology faculty of the University of Chicago.

He labored mightily to persuade the newly established Carnegie Institution of Washington to establish a year-round research "Station for the Study of Experimental Evolution" (the name was changed in 1918 to the Department of Genetics) adjacent to the summer-oriented Biological Laboratory. Davenport had the great foresight to concentrate his Carnegie monies on extending the 1900s rediscovery in Europe of Mendel's laws independently by Hugo de Vries, Carl Correns, and T. Tschermak. In this way, he quickly made Cold Spring Harbor one of the world's leading sites for genetics, with its earliest great triumph being the 1908 discovery by George Shull of the increased yield of hybrid corn over that produced by highly inbred corn strains. Davenport's crusading personality also led him to persuade Mrs. E.M. Harriman, the widow of the powerful railroad founder and the mother of one of his earlier students, to support (1910) and later richly endow (1918) his much more poorly thought out eugenics pro-

gram aimed at first identifying and then, through sterilization, eliminating deleterious genes from the human population.

Davenport brought in the best architects of this area to design the new buildings for his year-round research program. To proclaim the standards he hoped to reach, he had his friend Julian Peabody copy features of the Zoological Station at Naples for the design of the 1914 Animal House (now McClintock Laboratory). Davenport's first building for Carnegie, a new (1905) laboratory (now Carnegie Library), was also in the Italianate style, and the 1906 Blackford Hall dining hall and dormitory had classical Grecian features overlaying its advanced reinforced concrete construction.

Davenport's entrepreneurial zeal was passed on in the mid-1920s to his son-in-law, Reginald Harris (1898–1936), who took on the task of reinvigorating the Biological Laboratory. At that time, its summer courses and research compared very poorly to those of the Marine Biological Laboratory at Woods Hole. Assistance was thus sought from the major estate owners of the North Shore, such as Marshall Field and William Kissan Vanderbilt, to create a year-round research program at the Biological Laboratory aimed at understanding cells at the chemical level. Harris was as equally successful as Davenport had been in finding the right long-term goals for his era, focusing research at the Biological Laboratory on hormones and on the electrical properties of cell membranes. For the new buildings to house these research programs and the more research-oriented summer courses, Harris utilized the highly talented architect Henry Saylor. He designed Nichols and Davenport Laboratories in the colonial revival style, and for the more hidden James Biophysics Laboratory, he adapted the idiom of early modernism.

Harris, however, was soon faced with the impact of the Great Depression, which greatly lessened financial support from the estate owners for year-round research. To combat his growing frustration over his inability to maintain a thriving experimental effort, Harris initiated in 1933 the Cold Spring Harbor Symposia—comprehensive meetings that initially extended over a month-long interval. These meetings proclaimed our difference from Woods Hole in our partiality to quantitative, as opposed to descriptive, approaches to science. Central to Harris's vision was the need for physicists and chemists to join forces with biologists in attacking until then unsolvable problems such as the structure and function of proteins and the propagation of signals along nerve fibers. Support for the Symposium and the summer research that went along with it came from the Rockefeller Foundation, which continued its assistance even after Harris's untimely and premature death from pneumonia early in 1936. Only through this Rockefeller help did the Biological Laboratory's program on cells manage to survive through the Laboratory's 50th anniversary in 1940. The year following, Demerec, who had joined the Carnegie staff in 1924, was appointed the Director of the Biological Laboratory as well as Director of the Department of Genetics of the Carnegie Institution. In effect, the two institutions were merged, thereby creating an institution where both the summer and year-round research programs focused on genetics.

Max Delbrück Created Our Tradition of Intellectual Fearlessness and Honesty

While Demerec was the manager who skillfully moved Cold Spring Harbor into the modern world, the spirit that dominated the transition was that of Max Del-

brück (1906–1981), German-born and a product of the Protestant intellectual elite of Berlin. Delbrück was an extraordinary mixture of arrogance and decency. He despised all forms of pretense and had equally little use for tortoise-like minds that remained anchored to the past and could not share his love affair with the little phages of *Escherichia coli*. But he could also relax from being deadly serious and took pleasure in tennis, canoeing, camping, and the fun that came from practical jokes on those who took themselves too seriously. Giving a seminar before Delbrück was for most scientists a frightening experience—he actually wanted to learn if you had a "take home message." Unclear talks could provoke his scorn, as did unreadable scientific papers. Any form of secrecy or hint of intellectual dishonesty also upset him, and those who seemed to be in science for personal gain as opposed to intellectual excitement never became accepted into the inner circle of the "Phage Group" that he was to lead with Luria and Hershey.

Delbrück's approach to science pervaded the early postwar Cold Spring Harbor Symposia, particularly those that had genetics themes and at which the speakers had to face front rows occupied by sharp minds that would not hesitate to interrupt when the message was unclear or patently erroneous. Occasionally, an unschooled mind would present an outrageous talk, but for the most part, everyone respected each other, and no one was accorded special privileges to speak because of either age or past accomplishment. Postwar Cold Spring Harbor thus became *the* site to which the best and brightest of the gene-dominated scientific world came to meet for the exchange of ideas or to which their brightest proteges were sent for one of its expanding list of summer courses. In coming here, there was the expectation that everyone would benefit, with the giving of ideas to be as important as the receiving of them. Helping to maintain these ideals were the relatively small number of scientists then occupying the DNA world. Those who behaved badly and who were selfish with their ideas soon found themselves not wanted and chose to spend their summers elsewhere.

This Delbrück-dominated style continued through the five years that John Cairns was the Director (1963–1968). His was a very tough assignment indeed, since substantial Carnegie Institution of Washington funds were no longer coming to Cold Spring Harbor. The year previous to his appointment, the Carnegie Institution decided to close down their Department of Genetics, providing monies only to allow Barbara McClintock and Alfred Hershey to remain working in Demerec Laboratory. Suddenly, there was no endowment to provide funds to attract younger scientists or to provide them with permanency if their experiments succeeded. Cairns worried that unless the newly created Cold Spring Harbor Laboratory (the name given to the new body formed to take over the facilities of the Biological Laboratory and the Department of Genetics) acquired a substantial endowment of its own, it could not survive in a world where it was facing increasing competition from many other institutions that began to follow its lead in making the pursuit of the gene their major research objective.

Opting for Scientific Achievements as Opposed to Tenured Stability

I thus decided when I became the Director in 1968 that our survival depended on a radical shift in our main goal so that we would once again be perceived as a leader in science. My choice was to push research on the small DNA tumor viruses like SV40 and the adenoviruses. In so moving, I took the gamble that

their cancer-inducing properties would reflect the presence along their chromosomes of one or more cancer-causing genes (oncogenes). If my hunch was correct, then the pursuit of these viruses could provide the best way to get at the essence of cancer at the molecular level. In turning to tumor virus research, I also solved, at least temporarily, the Laboratory's financial crisis. Then, there was much more money voted by Congress for cancer research than could be used effectively. Joe Sambrook took virtually no financial risk in deciding to move here in the summer of 1969 from Renato Dulbecco's laboratory at The Salk Institute. The grant application we submitted to the National Institutes of Health was quickly funded, as was a much larger request for $1,000,000 per year that we submitted in 1971.

Moreover, no difficulty existed in recruiting many other first-class younger scientists to collaborate with Joe, despite the fact that the total endowment of the Laboratory was then only $20,000. Fortunately for us, it soon became clear that accomplished postdocs were more concerned with moving to positions that would allow them to do important science, rather than to less-exciting opportunities that carried potential long-term job security. We thus made the decision to use Robertson Research funds created by Mr. Charles S. Robertson's 1973 gift of $8,000,000 to support the careers of young scientists, rather than to create tenured research professorships to guarantee the salaries of more senior scientists.

Our belief was that those who already had been here for several years would have by then built up highly productive research groups that would allow them to compete well for outside research grants. In the 1970s, before it became possible to clone oncogenes, there was still more money for cancer research than good proposals to use it. So worrying about money over the short term was not then a serious concern. Nonetheless, we wanted to provide some umbrella of help for those more-senior scientists whose grant support might not come through immediately. To provide such support, we began in 1978 to give them Rolling 5 appointments. When they were promoted to this position, generally around the age of 35 after they had been here some five years, their salaries were guaranteed for a period of five years and this guarantee would continually roll forward if they continued as successful scientists. In retrospect, we put ourselves at little risk with this sort of salary guarantee. During the 13 years that it has been in existence, only twice have we had to provide such salary support, and both times only for very limited periods. In each case, the science involved was connected not to the DNA tumor viruses, but to the intellectually very important movable genetic elements. Unfortunately, support through the National Institute of General Medical Sciences has proved increasingly inadequate to fund all of the new, high-powered, pure genetics done in the United States.

Although the Rolling 5 position has allowed us to maintain an energetically charged group of productive scientists in their thirties, for the most part it has not allowed us to keep them through their forties. By the time this age approaches, most scientists have families, and the prospects of educating their children makes them more security conscious than they were in their early thirties. The result has been a steady flow of these still highly productive scientists away from Cold Spring Harbor to tenured university positions. Although such departures have caused me great anguish, both because of friendships formed and because of the fear that we might not be able to recruit

an equivalent younger scientist whose career will blossom equally well here, we have taken pride in the high quality of the universities and medical schools to which they have moved. The net result, however, is that as we have grown in size, we have spent more and more of our time in recruiting young scientists. So we have found ourselves ever more frequently asking whether we should move gradually to establish a number of truly tenured positions.

A cadre of high-powered tenure appointments would immeasurably help me to appoint outstanding young scientists fresh from their postdoctoral positions. On the other hand, it very likely would create more long-term problems than it would solve. Conceivably, our greatest advantage now is the relatively young age of even our more senior scientists. In recent national ratings of major universities and research institutions that measure the number of citations to published research papers, we ranked at the top or very close to it. So why change from a system that has served us so well to one that is bound to make us more aged and conservative? The unknown will always be scary, and those individuals we may need the most may very well be those who worry about how to do their science as opposed to whether they will go on being successful.

Continuing to Emphasize the Young and the Underdogs

We must thus remain an institution where young scientists join our staff with the expectation that they will be treated fairly. Toward this objective, our own resources should continue to be directed predominantly toward them, providing the funds to attract the graduate students and postdocs on which their careers will depend and to secure the special pieces of equipment needed to allow them to be competitive with their peers elsewhere. Furthermore, we should continue to have flexible terms of appointment and never permit talented minds to seek jobs elsewhere below their abilities. We must also avoid the danger of excessive preoccupation with the judging of whether a junior staff member is worthy of promotion. While a member of the Harvard faculty, I participated in all too many such actions and seldom saw anyone who was the better for it. In reality, most scientists, like members of virtually all other occupations, seldom live up to their inherent talents. Our highest objective must be to maintain an environment, moral as well as physical, where our scientific abilities can be maximally expressed.

Our concerns should always embrace the well-being of all scientists, not only those here, but also those who strive to achieve scientific enlightenment elsewhere. We are rightfully admired for the selflessness shown in the ways we organize and finance our advanced teaching courses, as well as for the inherently democratic way we host our now very wide variety of meetings both large and small. Through our meetings, with their packed schedules from early morning to late evening, we try to extend recognition to all who do good science as opposed to providing still another forum where old-boy networks dominate the lecture platforms. Nothing could cause more harm to our reputation than to be regarded as an instrument of a senior establishment concerned with the maintenance of its power over the fate of others. If we are ever to be accused of favoritism, it should be that directed toward the talented underdogs as opposed to those well entrenched in science.

If we can continue to so act and dream, we shall move into our next century with our heads high and our eyes level to those who need our help.

HIGHLIGHTS OF THE YEAR

The Laboratory's Hundredth Birthday Party

The Centennial celebration held on July 14th was a splendid day of festivities for the Laboratory and all of its guests. From the gathering of sailing ships to the endless variety of music, food, and fireworks, all went remarkably well. I, for one, had a grand time. It was on July 7, 1890 that eight students and seven teachers gathered for three weeks to participate in the Laboratory's first biology class. On the Centennial day, members of that original class were portrayed by Rob Gensel, from our Purchasing Department, and a group of Laboratory friends, who set forth on the small launch *Rotifer* to reach our shore while storyteller Heather Forest narrated the occasion. Four majestic sailing vessels also took part in the gala. On Blackford lawn, more than 1500 people enjoyed a delicious feast, which included a giant birthday cake, as the Old Bethpage Village Restoration Brass Band paraded through the crowd playing music from a century ago. Throughout the day, we received a number of proclamations in honor of the Centennial, including a letter from President George Bush commending the Laboratory and its staff. "Your devotion to the pursuit of knowledge has led to some of the most important scientific discoveries of our time," the President's letter stated.

Barbara McClintock and Susan Cooper being interviewed by Cablevision at the Centennial

In the evening, Liz and I hosted a gala dinner party for our corporate friends who helped support the celebration. Our guests were greeted with sea chanteys by Stephen Sanfilipo, and they danced to the big band music of *High Society*. Fireworks by the famed Grucci family lit up the misty skies, and the Clan Gordon Highlander's Pipe Band of Locust Valley marched across the fog-enshrouded campus. No finer day of revelry could have been planned.

In September, as part of our Centennial celebration, the Laboratory offered a very special concert. Opera virtuoso Frederica von Stade performed a selection of music ranging from Mozart to Gershwin that filled Grace Auditorium. Her breathtaking performance raised more than $70,000 for the Laboratory, most of which will go toward endowing a Frederica von Stade Undergraduate Research Internship. With her voice, "Flicka" brought joy to all who heard her.

Skillfully orchestrating all of the centennial year events was Susan Cooper, who energized her Public Affairs and Library staffs to heights of imagination and hard work that virtually exceeded the capabilities of the human mind and body. In pursuing her multifold objectives, Susan was most ably assisted by her administrative aide Emily Eryou, her valuable Library co-worker Laura Hyman, her very imaginative designer Margot Bennett, and her writer Dan Schechter.

All the funds needed to host the centennial events, as well as for two more specialized scientific meetings (on cancer and evolution), were raised by the centennial committee headed by Robert McMillan and Tom Doherty. Ably assisting them in their solicitations was Judith Carlson. Through their *Partners for the Future* campaign, specifically aimed at the Long Island business community, they raised almost $200,000. To immortalize these events, a highly attractive *Partners for the Future* booklet, over 200 pages in length, was prepared by Susan Cooper and her staff. In it is a very informative "time line," prepared by Elizabeth Watson, which recalls key events in each of our 100 years of existence.

Frederica von Stade

Neuroscience Center, nearing completion

The Neuroscience Center

With the Neuroscience Center on the upper campus nearing completion, the stage is now set for a major new effort at Cold Spring Harbor Laboratory in molecular neurobiology. Our hope is to open new pathways in the fight against neurological disease and to unlock the mysteries of learning and memory. Over the next few years during the "decade of the brain" (so named by President Bush), up to 75 additional researchers will join us, most of them setting up shop in the Center's Arnold and Mabel Beckman Laboratory. The new facility encompasses the W.M. Keck Structural Biology Laboratory; the Hughes Teaching Laboratories; Dolan Hall, a 60-room guest lodge for visiting scientists; and a 150-car garage. In Hazen Tower, a unique symbol of biology's quest, a helical staircase winds its way upward to a bell almost within one's reach. Charming in its blend of styles and materials, the Neuroscience Center fits beautifully into the Laboratory's eclectic 19th and 20th century architecture.

Symposium—The Brain

The Brain was the subject of our 55th Symposium on Quantitative Biology, held in late May and early June. Eric Kandel, Terrence Sejnowski, Charles Stevens, and I endeavored to bring together the top researchers in all aspects of neuroscience.

F. Crick and J.D Watson on the porch of Airslie during the 1990 Symposium on The Brain

Among them was my early cohort Francis Crick, who presented his latest findings on memory and the way the brain processes vision. Francis spoke to an overflow crowd at the LIBA-sponsored Dorcas Cummings Memorial Lecture. During his visit, Francis and I were persuaded to pose beside a model of the double helix in the DNA Learning Center, in a reenactment of a photograph made more than 35 years ago at the Cavendish Laboratory. While we were at the Learning Center, we encountered a high school biology class from Dobbs Ferry, New York, and its avid teacher, Carl Shiavo. Both Francis and I enjoyed meeting some of the younger DNA science enthusiasts.

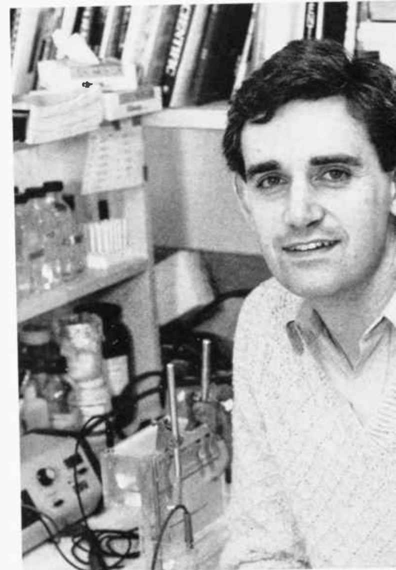
Centennial Meetings

In addition to our Symposium on *The Brain*, two other meetings commemorated the Laboratory's first hundred years. In early September, some of the world's best minds in cancer research were brought together by Joan Brugge, Tom Curran, Ed Harlow, and Frank McCormick. In the fifteen years since our first *Origins of Human Cancer* meeting, enormous progress has been made here and elsewhere in identifying the genetic mechanisms of that dreaded disease. Fighting cancer remains as much our priority today as it was in 1975. Our second hundred years will surely see a successful solution to this struggle. Saturday evening during the *Cancer* meeting, our guests were treated to a special centennial performance by world-class saxophonist Christopher Hollyday. Christopher entertained scientists and staff on Grace Terrace with his 1950s-style jazz. The crisp fall air, cold beer, and bebop proved most enjoyable.

In late September, a meeting entitled *Evolution: Molecules to Culture* embraced a variety of fields, ranging from molecular biology to anthropology and linguistics. Attendees came away from that gathering with new insights into how the evolution of living things compares with those of culture and language. Richard Dawkins, who organized the meeting with Jared Diamond, presented his own intriguing views on Darwinian theory during a LIBA-sponsored lecture for the general public.

Assistant Director Bruce Stillman

With my commitment to the human genome initiative taking up much of my time, finding a capable second at the Laboratory became essential. On September 1, the appointment of Bruce Stillman as Assistant Director of Cold Spring Harbor Laboratory was announced. A gifted researcher and organizer with endless energy, his help will be invaluable in the recruitment of new scientists during this critical period. Bruce first came to us in 1979 as a postdoc from the Australian National University and has held the position of Senior Staff Scientist since 1985. His work over the last decade in identifying the key cellular proteins involved in DNA replication has elevated him to the highest ranks of his field. I am certain that he will continue to make such strides while ensuring that our research staff maintains its vitality and diversity.



B. Stillman

David Micklos Honored

For his pioneering achievements as founder and Director of the Laboratory's DNA Learning Center (the first such facility in the world), David Micklos received the Charles A. Dana Foundation award, given each year to outstanding individuals in health-related education. Amazingly, in the few years since the Learning Center's inception, its instructors have taught over 10,000 high school students and have trained nearly 2000 teachers to conduct experiments using some of the latest techniques in biotechnology. Some of the experiments developed by Dave and his colleagues for the Learning Center have been incorporated into the Advanced Placement Biology curriculum, reaching more than 50,000 students across the country every year. In May 1990, Dave and DNA Learning Center Assistant Director Mark Bloom journeyed to Moscow to set up a similar center there.

D. Micklos and C. A. Dana

Mark made a second trip to the Soviet Union in January 1991 to inaugurate the new center and conduct its first training sessions in DNA technology.

A Good Year for CSHL Press

Our publications continued making strides in 1990. The second edition of *Molecular Cloning: A Laboratory Manual* by Joe Sambrook, Ed Fritsch, and Tom Maniatis surpassed the 50,000 mark in sales. Another manual destined to become a standard, *Antibodies*, by Ed Harlow and David Lane, has already sold more than 20,000 copies. The first printing of the high school/college text *DNA Science: A First Course in Recombinant DNA Technology*, written by David Micklos and Greg Freyer, sold out in short order. Among our journals, *Cancer Cells* successfully completed its first full year in a highly competitive field. A study of citation frequency found *Genes & Development*, now in its fourth year of publication, to have the greatest impact of any journal in either genetics or developmental biology. *"G&D"* also won a Gold Award in the 1990 Neographic competition for excellence in cover and graphic design.

D. Marshak

Daniel Marshak Receives Prize

For his work in tracking down the protein S100β, a major factor in Down's syndrome, Senior Staff Investigator Daniel Marshak was presented with a Science Scholar Award from the National Down's Syndrome Society. Dan is among those at the Laboratory pursuing a greater understanding of the brain. His research has come far in elucidating the protein chemistry and molecular biology of neurites (the physical circuits between nerve cells). Soon his group will occupy a space in the Neuroscience Center.

Banbury Meetings Cover a Wide Range

Last year, a remarkable variety of topics were discussed at the Laboratory's Banbury Center, located on the beautiful estate donated by the late Charles S. Robertson. Each year, Banbury Director Jan Witkowski outdoes himself in focusing on topics in biology that are of concern to scientists and to the public at large. This year was no exception, with meetings ranging from basic research, such as *Computational Aspects of Protein Folding*, to topics of broader interest,

including *The Impact of Human Molecular Genetics on Society*. A workshop for science journalists on *Women and Cancer* surveyed the latest research on cancers unique to women and explained diagnostic tests and treatments in development. At a meeting on *Addiction*, congressional staffers learned about the physiological and psychological components of a disorder that has become epidemic in this country. In January 1991, another congressional workshop dealt with the numerous scientific aspects of aging.

Baring Brothers Executive Conference

Last October, the investment firm Baring Brothers & Co., Inc., and Cold Spring Harbor Laboratory cohosted the fifth annual weekend meeting at Banbury Center for executives interested in biotechnology. The subject this time was *Genetic Pathways to Cancer*. Attendees included Laboratory Trustee William Miller, Vice Chairman of the Board of Bristol-Myers Squibb, and Henry Wendt, Chairman of SmithKline Beecham. Top scientists in all areas of cancer research presented the current status of work on oncogenes, cell-cycle control, genetic prognosis, the prospects for gene-specific therapies, and related topics. The executives, who spent much of one day at the Laboratory's main campus, learned the way the work of our scientists fits with other frontline research around the world.

Major Gifts/Second Century Campaign

Through the efforts of the seven committees of the Second Century Campaign and the help of numerous staff members and volunteers, the Laboratory is nearing its goal of $44 million set in 1986. Over $40 million has been raised to date, helping to ensure the Laboratory's financial health, providing support for our scientists, and propelling our expansion. The completion of the Neuroscience Center, the renovation of Blackford Hall, much-needed improvements in our infrastructure, and the addition of new cabins for visiting scientists have all become a reality because of the generosity of our many individual, corporate, and foundation donors. In 1990, major corporate support totaling more than $2 million came from Boehringer Mannheim Corporation, Burroughs Wellcome Co., E.I. du Pont de Nemours & Company, Hoffman-La Roche Inc., Merck & Company, Pall Corporation, Rorer Group, Schering Plough Corporation, and SmithKline Beecham Pharmaceuticals.

The largest foundation gift announced in 1990 was from the Lucille P. Markey Charitable Trust, which donated $4 million toward the Neuroscience Center. The Markey Charitable Trust, established by the will of Mrs. Markey in 1983, supports and encourages basic medical research. The Trust's generous donation will benefit our new scientists in their fight against disorders and disabilities of the brain.

Other foundation grants received in 1990 include a $500,000 challenge grant from an anonymous donor, to be awarded if we can raise an additional $1 million in unrestricted funds; a $300,000 grant from the Ira W. DeCamp Foundation; and $75,000 from the Nichols Foundation. George Garfield, a gentleman who as a teenager was taught and inspired by Laboratory Director Charles Davenport in 1914, graciously contributed $70,000 from the Garfield Foundation. In 1990 and early 1991, the William and Maude Pritchard Charitable Trust gave the Laboratory a total of $75,000.

A Vibrant Board of Trustees

Our Board continues to exemplify the knowledge, expertise, and generosity that are needed to run an institution such as ours. In November, President George Bush awarded Laboratory Trustee Dr. David Pall the National Medal of Technology, the highest honor for technology in the United States, if not the world. David was recognized for his vast contributions as founder and Chairman of Pall Corporation, through which he has developed filters for use in industry, medicine, air travel, and numerous aspects of life. One of his latest inventions is a biomedical device that removes leukocytes from blood and platelet concentrate during transfusions, bringing the benefits of such to patients in several thousand hospitals.

Last year, Trustee Helen Ann Dolan left the Board after serving six years, having been a great help to the Building, Education, and Robertson House Committees. At the same time that Helen departs, her husband Charles F. Dolan joins us. Charles is the Chairman and CEO of Cablevision Systems Corporation and the founder of a number of premium cable services, such as Home Box Office, with tens of millions of subscribers. His years of experience and acumen in business will be an invaluable asset to the Finance and Investment Committee.

Wendy Hatch, a good friend and tireless champion of the Laboratory, has worked on the Development, Executive, Finance and Investment, Building, LIBA, and Banbury Committees. Having served two consecutive terms on the board, Wendy is required to take a two-year leave, but she will continue to act as Chair of the Major Gifts Committee of the Second Century Campaign and will work with many of her other committees as well. Her energy and talents have proved vital to the Laboratory and will continue to be a treasured resource.

Departing Trustee Thomas J. Silhavy, a gifted professor of molecular biology at Princeton University, has for many years taught a course in advanced bacterial genetics at Cold Spring Harbor Laboratory. Also from Princeton, new trustee Shirley M.C. Tilghman is a leader in mouse genetics research and an investigator of the Howard Hughes Medical Institute. As a member of the Tenure and Appointments Committee, Dr. Tilghman will help in the tenure and approval process for staff promotions.

Owen T. Smith, an accomplished environmental lawyer and expert on taxation and real estate, joins the Board as a caring neighbor, having lived in nearby Oyster Bay for 40 years and, more recently, in Laurel Hollow. As a member of the DNA Learning Center Committee, Owen will be a high-profile advocate for that vital conduit of biology education.

Mary D. Lindsay returns to us after a two-year absence during which she accompanied her husband to England while he was there on business. Mary has served three previous terms as a Laboratory Trustee and has been a Director and Vice Chairman of LIBA. We are fortunate to have her back, working on the Nominating and Building Committees.

Sadly, 1990 marked the passing of a dear friend and wonderful advocate of the Laboratory, Elizabeth B. Schneider. Betty served on the board for six years and on the LIBA board for 25 years. Among the many activities to which her enthusiasm extended were sailing, flying, and especially her work here. She will be sorely missed.

Elizabeth B. Schneider

Robertson Research Fund

Our largest endowment, the Robertson Research Fund, has maintained its integrity amidst the volatile markets of the last year, allowing the continued support

of younger scientists at the Laboratory who have yet to secure their own grants. In some instances, the Fund also bolsters the research of more senior scientists and provides other miscellaneous support. In 1990, the Robertson Fund contributed housing and supplemental stipends for postdoctoral fellows, aided our plant genetics program, and provided relocation expenses for 24 scientists and salary support for visiting scientists.

Blackford Renovation

The renovation of Blackford Hall, the Laboratory's dining facility, proceeds apace. The basement is now completed, including the Blackford lounge and new restrooms. The original foundation, part of the first poured concrete structure built on Long Island in 1907, was reinforced in preparation for the addition, which will bring Blackford's one-sitting dining capacity to 400 from the present 150. After this year's meeting season has ended, workers will begin the second phase of this project. We anticipate completion by spring 1992.

New Cabins Begun

Five new cabins, slightly larger than the six completed in 1989, are now under construction. Like the others, each new cabin will comfortably house eight, but in addition, each will have a full basement containing heating equipment and storage space. Thus, the living areas will have more room, without sacrificing any of the home-like charm of the original design. The two connecting cabin complexes, located above the Neuroscience Center, provide a relaxing retreat for scientists attending our meetings and courses. Toward the cost of these cabins,

Renovation of Blackford Hall

One of five new cabins under construction

as toward those built earlier, we have specifically sought the help of scientific alumni, in particular those who have helped create the increasingly successful biotechnology industry. Most pleasing this last year was a major gift received from our Trustee Tom Maniatis, who with Mark Ptashne founded Genetics In-

stitute. Earlier on, Mark made a gift toward the first set of cabins. This support from my former colleagues in the Harvard Department of Biochemistry and Molecular Biology pleases me deeply.

LIBA and Associates

J. Eisenman

Since its founding in 1924, the Long Island Biological Association has been an invaluable source of support for the Laboratory. In 1990, LIBA provided funds for six postdoctoral fellows and granted a New Investigator Start-up Fund to Senior Staff Investigator Nicholas Tonks. Nick comes to us with an impressive body of work on tyrosine phosphatases and their relation to cell growth.

Last year, Jim Eisenman retired as Treasurer of LIBA, after serving for 27 years. Arthur Crocker, Penny Chenery, and Lon Chaiken also retired. Succeeding them were Jack Evans, Missy Geddes, Alan Kisner, and Alec Thayer.

The LIBA Annual Meeting in January of 1990 included an interesting panel discussion on science in the Soviet Union. Four of our visiting scientists and their wives spoke of differences in our cultures, disparities in education, the funding of science, and the slow exchange of information from abroad. Joint efforts like the establishment of the Moscow DNA Learning Center provide some hope of greater communication in the future.

There were 139 Cold Spring Harbor Associates (contributors of $1000 or more) in 1990, thanks to the efforts of the LIBA Directors, the Development Office and our volunteers. An Associates lecture, held on November 30, dealt with new technologies and opportunities in the life sciences industry. Laboratory Trustee John Reese moderated an informative discussion on the problems and prospects for fledgling biotechnology firms. Panelists included David Bonagura, partner at Ernst and Young; John R. Drexel IV, president of Concord Management International, and Douglas E. Rogers, a Director at Baring Brothers & Co., Inc.

Undergraduate Research Program

Since 1959, up to 20 undergraduates from the United States and overseas have come each summer to the Laboratory to work with our scientists. In 1990, 18 students participated in the Undergraduate Research Program, coming from as far away as Tartu University in Estonia and the University of Glasgow in the United Kingdom. In addition, two students came from Cambridge University. Each student was able to complete a ten-week project on topics such as telomere length (with Bruce Futcher and Carol Greider) and the use of computer software to assist in restriction mapping (with Kim Arndt). Although National Science Foundation support was unavailable for the program in 1990, funds were provided by a number of corporations, primarily Burroughs Wellcome and Baring Brothers.

Partners for the Future

We inaugurated a program in 1990 that enables a select group of high school students to conduct research at the Laboratory, naming it *Partners for the Future* after the centennial year's cooperative program involving Long Island businesses. From the more than 150 high schools on Long Island, five excep-

tional students were chosen to assist some of our researchers. Along with a stipend, the interns receive an unforgettable initiation into the world of science. As funding permits, more high school students will join us each year, opening the path toward constructive and rewarding careers. The following is a list of the 1990 interns, their high schools, and their Laboratory mentors: David Adelman, Commack High School (Dr. Jeffrey Kuret); Cathy Andorfer, East Islip High School (Dr. Venkatesan Sundaresan); Eugene Choi, Garden City High School (Dr. Richard Roberts); Susie Hong, Syosset High School (Dr. Robert Franza and Dr. Yuchi Li); and Jennifer Munneyyirci, Plainview High School (Dr. Nouria Hernandez).

Changes in Scientific Staff

With keen regret we say goodbye to Senior Scientist Ed Harlow, who is establishing a new base at the Massachusetts General Hospital Cancer Center in Charlestown, Massachusetts. Ed's invaluable work in the study of cancer-suppressing genes and the retinoblastoma anti-oncogene–E1A protein interaction signified a great breakthrough in the quest to demystify cancer's origins in cells. Several major honors attest to the significance of his findings, namely, two awards of $50,000 each, one from the Milken Foundation and the other from Bristol-Myers Squibb, both for distinguished achievement in cancer research.

E. Harlow

Senior Staff Scientist Yakov Gluzman, who first arrived here in 1977 to work with Joe Sambrook, has also ended his direct affiliation with the Laboratory. For the past several years, Yasha has served as the Director of the molecular biology research section of the American Cyanamid Company, Medical Research Division, Lederle Laboratories, in Pearl River, New York. He continues to probe the molecular biology of viruses and oncogenes and the mechanism of antibiotic activity and resistance.

Y. Gluzman

Senior Staff Investigators Guilio Draetta, Loren Field, and Andrew Rice accepted new positions elsewhere. Guilio, who was appointed Robertson Fellow at Cold Spring Harbor in 1986, collaborated with David Beach on cell-cycle control in yeast and vertebrates. He is now at the European Molecular Biology Laboratory in Heidelberg, Germany. Loren has taken up new duties as associate professor in the Department of Medicine, Division of Cardiology, Krannert Institute of Cardiology in Indianapolis, Indiana. His field of study is the molecular biology of the cardiovascular system. Also assuming new responsibilities as associate professor is Andrew Rice, who continues his research at the Division of Molecular Virology, Baylor College of Medicine in Houston, Texas. Andy came to us from the Imperial Cancer Research Fund and focused his work on the regulation of human immunodeficiency virus (HIV) gene expression.

In the fall of 1990, Staff Investigator Eileen White left us to join the staff of Rutgers' Center for Advanced Biotechnology and Medicine in Piscataway, New Jersey. After receiving her PhD from the State University of New York, Stony Brook, in 1983, she joined Bruce Stillman's laboratory as a postdoc studying the function of adenovirus E1B tumor antigens in transformation and lytic infections. She was appointed Staff Investigator in 1986.

Staff Associates John Collicelli, Nicholas Dyson, Ken Ferguson, and Toshiki Tsurimoto also departed in 1990. John became assistant professor at the School of Medicine in the Department of Biological Chemistry at the University of California, Los Angeles. Nick is an assistant professor at the Cancer Center of the Massachusetts General Hospital in Charlestown, Massachusetts, and Ken has

become director of the program in signal transduction at ICOS Corporation in Seattle, Washington. Toshiki has returned to Japan, where he is assistant professor at the Institute for Molecular and Cellular Biology at Osaka University.

New Staff Members

We welcome to our ranks Nicholas Tonks, who joins us as Senior Staff Investigator in James Laboratory, and Hong Ma, who joins us as Staff Investigator in Delbrück Laboratory. Nick examines signal transduction mechanisms. Before coming to us, he was at the University of Washington's Department of Biochemistry in Seattle. One of David Botstein's doctoral students, Hong Ma left the biology division of the California Institute of Technology in Pasadena to pursue further research here. He focuses on plant signal transduction, plant flower development, and transcription factors.

New visiting scientists in 1990 include Igor Garkavtsev, Catherine Jessus, Janos Posfai, and Natalia Yenikolopov. Igor and Natalia add to the numbers of our Russian scientific contingent. Working out of Demerec and James laboratories, respectively, Igor comes to us from the Medical Genetics Center in Moscow and Natalia comes from the Institute of Molecular Biology at the Academy of Science, also located in Moscow. Catherine Jessus, from the Centre Nationale de la Recherche Scientifique in Paris, is in David Beach's laboratory researching cell-cycle regulation, particularly the M-phase or mitosis stage. Janos Posfai from Hungary has made two previous visits to Rich Roberts' laboratory, first in 1987 and again in 1989. He is here for a third time, continuing his exploration of biological sequence analysis using mathematical methods. Another addition to David Beach's laboratory is Japanese scientist Toru Mizukami, who comes to us from the Tokyo Research Laboratories, where he was researching amino acid fermentation and enzyme production.

Several visiting scientists completed their studies at Cold Spring Harbor, including Robin Allshire who returned to Scotland to take up responsibilities as staff associate at the MRC Human Genetics Unit in Edinburgh's Western General Hospital. Clement Echetebu accepted a position as research associate in the division of molecular biology, AIDS wing, Baylor College of Medicine in Houston, Texas, and Valentin Shick returned to the Institute of Molecular Biology at Moscow's Academy of Science.

Staff Promotions

Former Staff Investigator Jeff Kuret has moved up to the position of Senior Staff Investigator. In 1984, he received his PhD in pharmacology from Stanford University in California and then joined the Department of Biochemistry at the Medical Sciences Institute in Dundee, Scotland. His postdoctoral interest involved the structure and mechanism of enzymes in signal transduction. Jeff, whose current work focuses on protein kinases, first came to Cold Spring Harbor in 1987 as a postdoc in Mark Zoller's laboratory.

Rob Martienssen has also been promoted to Senior Staff Investigator. A native of England, he completed his doctorate in plant molecular genetics at Cambridge University. As a postdoc at Berkeley in California, he worked with Michael Freeling and William Taylor. Rob works out of Delbrück Laboratory in plant developmental genetics, specifically investigating the high mutation rate in the maize transposable elements system.

Former visiting scientist Yan Wang, from Beijing, China, has been made Staff Associate. After completing his MD and before coming to us, he completed postdoctoral studies at Stony Brook, Department of Biotechnology. Postdocs Graeme Bolger and Tamar Michaeli, who both work in Mike Wigler's laboratory in Demerec, have also been advanced to Staff Associates. Graeme arrived here in 1985 and has worked in phosphodiesterases and human neurological diseases, and Tamar has pursued her research in the functions of RAS in *Saccharomyces cerevisiae* and the characterization of phosphodiesterase enzymes in the human brain.

Postdoctoral Fellows

The following scientists have departed after having completed their postdoctoral studies at Cold Spring Harbor Laboratory: Young-Seuk Bae to Kyung-Pook National University in Daegu, Korea; Jennifer Brown to Miami Beach, Florida; Karen Cochrane to the Krannert Institute of Cardiology, Indianapolis, Indiana; Greg Conway to Harvard University Biological Laboratories, Cambridge, Massachusetts; John Diffley to the Imperial Cancer Research Fund, Herts, England; Bernard Ducommon to the European Molecular Biology Laboratory in Heidelberg, Germany; and Christine Hermann to the Department of Molecular Pathology, Anderson Cancer Center at the University of Texas in Houston.

Also departed are Emma Lees and Jackie Lees, both to continue their work at the Massachusetts General Hospital Cancer Center in Charlestown, Massachusetts, and William Ryan, to join the firm of Smith, Barney in New York City as an associate in corporate finance. Mark Steinhelper departed to the Krannert Institute of Cardiology in Indianapolis; Li Lhui Tsai to Massachusetts General Hospital Cancer Center in Charlestown, Massachusetts; Ilse Weiland to the Institute for Cell Biology, University of Essen, Germany; Zhang Ming to Emery University Medical School in Atlanta, Georgia; and Zuo Zhao to Pioneer Hi-Bred International, Plant Breeding Division, Johnston, Iowa.

Several graduate students completed their doctorates and moved on, namely: Karen Buchkovich-Sass to the Department of Biochemistry, New York University Medical Center, New York City; Duncan McVey who returned to Falcon Heights, Minnesota, to pursue an entrepreneurial career, and Susan Smith to the Cell Biology Laboratory at Rockefeller University in New York City.

Employees Recognized for Long-term Service

In 1990, three employees reached the milestone of 20 years' service to the Laboratory: Jack Richards, Director, Buildings and Grounds; Barbara Ward, Course Registrar; and Madeline Wisnewski, Scientific Secretary. Two others, Vincent Carey, Grounds Foreman, and Robert McGuirk, Senior Lab Technician, have completed 15 years' service.

I particularly wish to mention here the extraordinary role Jack Richards has played in the physical rebuilding of the Laboratory. Jack first came to my attention when he was an independent contractor. In April 1970, he was one of three contractors asked to make bids for the construction of the annex to James Laboratory in which my office is located. After the bids were in and Jack the obvious choice to do the job, he asked us whether instead of acting as the outside contractor, he could become the head of our Buildings and Grounds Depart-

J. Richards, V. Carey, M. Wisnewski, B. Ward, J.D. Watson

ment. In that capacity, he would find subcontractors to do the job. We immediately accepted, and ever since then Jack, in effect, has been our contractor in residence. His extraordinary devotion to the Laboratory, coupled with his innate good taste, has already left a mark on the Laboratory equal to that of our most distinguished scientists.

My NIH Position Continues to be Both Time- and Emotion-consuming

Effectively, I have spent almost half my time during the past year in my capacity as the Director of the National Center for Human Genome Research in Bethesda, Maryland. But because of the FAX, much of the time occurred while I was in my office here at the Laboratory. Initially, I thought I would regularly spend the first part of the week in Bethesda, but my visits there have evolved into a process where I try to overlap my time there with essential meetings and conferences. That I have been able to direct the Genome Office owes much to the high quality of my staff at the National Institutes of Health, in particular to Dr. Elke Jordan, the Deputy Director of the Center, and to Dr. Mark Guyer, the Assistant Director for Program Affairs. In addition, we now have our own study section to handle our major grants, and I was very pleased when we were able to persuade Eric Lander of the Whitehead Institute of the Massachusetts Institute of Technology to chair this most important body. I was also greatly assisted by the help that Norton Zinder of Rockefeller University gave to me as Chairman of our Program Advisory Board during its first two years of operation. In fiscal 1990, we had a budget of $58 million, a sum that has increased to $88 million for fiscal 1991. Already I take great pleasure in the establishment of our first genome centers at six major academic institutions (Washington University School of Medicine; University of Michigan Medical School; University of Utah Medical School; University of California, San Francisco, Medical School; Massachusetts Institute of Technology; and Baylor University Medical School) as well as the awarding of the four large grants to commence megabase sequencing of selected regions of the genomes of the model organisms *Escherichia coli*, *Mycoplasma capricolum*, *Saccharomyces cerevisiae*, and *Caenorhabditis elegans*.

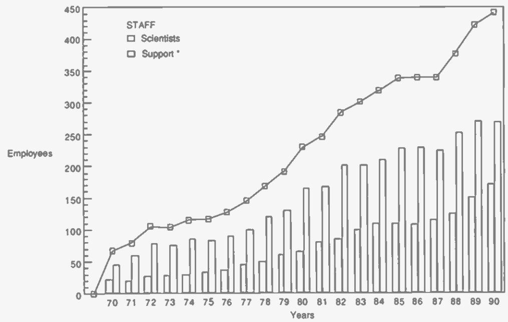
Our Nation Needs Its Scientists More Than Ever

The recent congressional hearings on abuses in the way federal research funds have been spent has created in many scientists' minds the fear that we are witnessing the start of an anti-intellectual phase in Congress that could badly damage our nation's capacity for high-class intellectual pursuits. In my dealings with Congress, however, I sense no such mood, only a bipartisan sense of sorrow and indignation over individual acts of misbehavior, sometimes by scientists and at other times by university administrators, that badly discredit the image of science as an endeavor devoted to the pursuit of truth. Science, however, is no longer a small elite enterprise; rather, it is a major aspect of our nation's fabric populated by far more individuals than can occupy the largest of athletic arenas. So it is not surprising that misdeeds, some very black, have been done in the past, and we must expect more in the future. After all, scientists like everyone else are human, and we have never expected any other occupation to be a paragon of virtue.

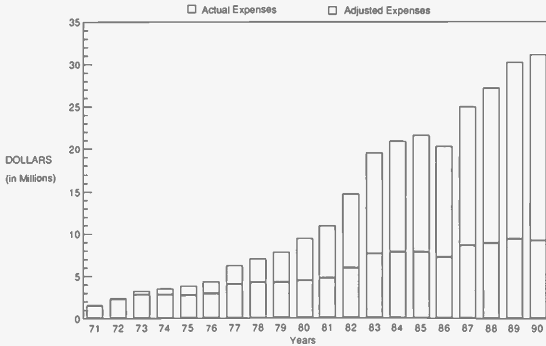
At the same time, however, we must never forget that in the past, our nation has treated us very well. It has valued us for our objective analyses of the natural world, not for bending the facts to suit our own personal needs and prejudices. If our charlatans are not treated for what they are, our nation's scientists risk being perceived as just another lobby group more interested in their own private gain than in the perpetuation of our nation's greatness. Drawing the wagons around us to protect our sinners from punishment is not the way to ensure that the voices of scientists are heard more, not less, in the Washington corridors of power.

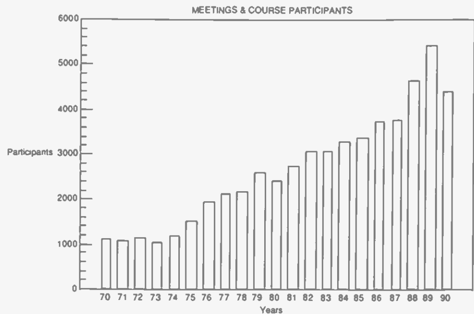
In our past as in the present, we as scientists have much to be proud about. We thus must not falter in seeing that we continue to be known for our integrity as well as our brains.

April 19, 1991 **James D. Watson**



* Consists of Full time and Part time Technical Support, Core Services, Publications, Meetings, Library, Public Affairs, Buildings and Grounds, Administrative Personnel, Banbury Center and DNA Learning Center





DEPARTMENTAL REPORTS

ADMINISTRATION

Cold Spring Harbor Laboratory completed 100 years at the forefront of biological science in 1990. Across a broad spectrum, the Laboratory's research and education programs continue to be outstandingly productive. Not surprisingly, this has resulted in satisfactory operating results and an overall strong financial condition.

The year ended with a small surplus of revenues over expenditures after fully providing for $1.5 million of depreciation and raising the reserve for future start-up expenses of the Neuroscience Center to $650,000. The positive operating numbers at the Laboratory reflect the continued success of our remarkable young scientific staff in commanding a good share of the now very competitive Federal funding available for science. Foundations, corporations, and private individuals continue to support the Laboratory's programs at a high level.

Cold Spring Harbor Laboratory Press had another excellent year, with sustained demand for its best-selling books and good sales of new 1990 titles. The Journal Department contributed to the good results, and it is particularly noteworthy that *Genes & Development* achieved its first surplus after four years of existence. The Meetings Department held two fewer major scientific meetings during 1990 than is ordinary, and this resulted in lower revenues. Several meetings planned especially for the Centennial, particularly the Symposium on "The Brain" and the fall meetings on "The Origins of Human Cancer" and "Evolution: Molecules to Culture," were outstanding scientific events.

Banbury Center and the DNA Learning Center had memorable years as well. Banbury, strongly supported by the thriving Corporate Sponsor Program, held 17 meetings at the Robertson Estate in Lloyd Harbor, with a fall meeting on "*Impact of Human Molecular Genetics on Society*" being particularly noteworthy. The DNA Learning Center's year was highlighted by Dave Micklos' receipt of the Charles A. Dana Foundation Award for Pioneering Achievement in Education and by the opening of a DNA Learning Center in Moscow.

The Laboratory's endowment, consisting of the Robertson and Cold Spring Harbor Funds, reached a new high level of just over $44 million by the end of the year. This was the result of the reinvestment of a substantial portion of the income from the Funds to provide for inflation and future growth of the Laboratory, some new gifts, and very good investment results accomplished by our investment advisers, Miller Anderson and Sherrerd. They succeeded in achieving a positive investment return in a year when many popular market averages declined by as much as 15–20%.

The Second Century Fund capital campaign completed the second year of its planned three-year fund-raising effort with more than $40 million of its $44 million goal already in hand. One cannot overstate the contribution to the Laboratory's future made by David Luke III, Chairman of the Fund, the Campaign Committee Chairs, Oliver Grace, Wendy Hatch, George Cutting, Taggart Whipple, Bill Miller, and Townsend Knight, and the many campaign volunteers. The effectiveness of their efforts is clearly demonstrated by the impressive dollar totals already accomplished. They have been ably supported by Konrad Matthaei and the Cold Spring Harbor Laboratory Development Office. The marvelous facilities and growing endowment made possible by the Second Century Fund will be treasured by the next generations of scientists here as the Laboratory's second century of science unfolds.

1990 might well be termed the year of the Laboratory's Buildings and Grounds Department, so very ably led by Jack Richards. They have managed, refurbished, or

largely constructed no less than five major projects, including the Neuroscience Center, Blackford Hall (Phase I), five new guest cabins, and the Frame House and Northview House. In addition to attending to innumerable Laboratory alterations, renovations, and general repairs, all of the above-mentioned work was accomplished with skill, on budget, and within reasonable time frames. The cost savings have been substantial, and the high quality of the work is a great tribute to the professional abilities of Russ Allen, Lou Jagoda, John Meyer, Lane Smith, and others too numerous to mention. The Laboratory's 30 separate buildings have been well maintained under the leadership of Peter Stahl, Danny Jusino, and Dorothy Youngs. As in the past, but particularly this Centennial year, the beauty of the entire Laboratory grounds has reflected the care and pride of such men as Buck Trede, Vinny Carey, Chris McEvoy, Danny Miller, and Tim Mulligan.

Now as we look ahead to the great promise of the next 100 years, we must first deal with the realities of 1991. Cold Spring Harbor Laboratory Press book sales in the early months of 1991 have been adversely affected by the Gulf War, the national recession, and severe constraints on grants at academic institutions. Federal grants for the Laboratory's research programs are being arbitrarily reduced on renewal by as much as 20% instead of being adjusted upwards as expected to compensate for inflation. This seriously affects the direct research funding available to the scientific staff and also reduces indirect cost recovery, which is the major source of funds available to pay for overhead. The cost of major overhead components such as maintenance, depreciation, and hazardous waste disposal continues to increase. Postponing or ignoring them is only a form of mortgaging the future. Other overhead components such as utilities and administrative expenses can be controlled and every effort is being made to do so.

Recent instances of improper practices at some institutions regarding indirect cost recovery promise to bring about across-the-board limitations on overhead reimbursement that could seriously threaten the infrastructure of much of the nation's basic research establishment. We shall have to live with whatever Federal decisions result from the current reevaluation of the whole subject of indirect costs, but in all likelihood, new non-Federal sources of funding will have to be found. Such costs are a very real part of doing research and cannot be legislated away.

Despite the immediate challenges created by the recent explosive growth of the Laboratory and the unusual number of uncertainties for the year immediately ahead, we view the future with much optimism and anticipation. I feel most fortunate in being able to rely on an administrative staff of extraordinary motivation and skill to guide the Laboratory soundly into its second century of science.

G. Morgan Browne

BUILDINGS AND GROUNDS

The Annual Report for the Buildings and Grounds Department is again one neverending story of construction, renovation, and repair. Every nook and cranny of the Laboratory has been touched by us, and in fact, we are probably the only department that knows every square inch of the campus, both indoors and outdoors.

Centennial Celebration

Most, if not all, of the Buildings and Grounds staff were involved in the preparation of the Laboratory for the celebration of its 100th birthday. The carpenters built a stairway and bridge between Demerec Laboratory and Grace Auditorium, as well as a bandstand on the back lawn of Davenport House. Where needed, the painters painted and the groundskeepers landscaped, manicured, and in general cleaned up, all contributing to making the Laboratory look its best. The electricians supplied all of the extra lighting needed for the celebration, which went on into the late evening. All of the special events were manned by the custodial staff, with other segments of the department lending a hand in setting up chairs and tents on the lawns of Blackford and Airslie, parking cars, acting as guides, helping to prepare the fireworks display, and cleaning up after the celebration was over.

Neuroscience Center

Our highest priority for the year was the construction of the new Neuroscience Center, which is located on the site of the now-defunct Page Motel. One of our responsibilities when we entered the construction in progress during the year was to help the contractor keep on schedule. Our in-house architect, Leslie Allen, spent much time solving problems inherent in coordinating our crew and the construction site crew. Job report meetings were held every week to review the work and to keep to the schedule, and by the end of the year, more than 90% of the construction was completed. Two new housekeepers have been hired so far, and we will need at least three more staff for maintenance.

Log Cabins

By the end of the year, four of the planned five new cabins were erected and enclosed. We discovered after the first six cabins were built that more room was needed for the utilities and the hot water heaters. Full basements were thus included in the design of this second complex of cabins, providing additional space in the living areas plus increased storage areas and better insulation for the heating systems.

Blackford Dining Hall

Phase I of the Blackford expansion began after the summer meetings season. The 3-foot-deep concrete piers originally supporting the building were reinforced and extended down another 10 feet to prepare for the new addition. In the basement below the dining area, we demolished the bar, restrooms, and of-

fices to make room for the Blackford lounge and new restrooms. Phase II will begin after the 1991 meetings season.

Landscaping and Paving

It is becoming abundantly clear that parking is inadequate to meet the needs of our ever-increasing staff. Additional parking spots furnished by the large parking area for the Neuroscience Center may alleviate some of the problem, but we are always on the lookout for other areas. This year, the parking lot above the pond was made larger to accommodate about 25 more cars.

We also added a waterfall to the pond this year. About a ten-foot-wide area on the north end of the pond was excavated and lined with concrete, and large flat rocks were set in tiers to form the waterfall. A pump was added to recirculate the water from the pond to the pool above the waterfall. A little further up from the pond, the sculpture "Transform" had begun to sink because it had been placed in the pond meadow which retains a lot of water. To prevent it from sinking further, we provided better drainage, fashioned a concrete base, and bolted the sculpture to this base. In the early summer, two of the older cabins near the Neuroscience Center were demolished, and this area was also graded and landscaped.

Wastewater Treatment Facilities

The Laboratory provides many utilities, and perhaps the least popular is wastewater treatment plant. To accommodate the increased flow from the new buildings, the wastewater treatment plant was renovated to improve its capacity and reliability. Our own plumbers and mechanics re-piped the underground systems, which now include an additional equalization tank, a new distribution box, and a relocated sludge tank. After the pumps were relocated, the sludge removal area was landscaped. All of this work was done before the Centennial celebration, and our staff did an excellent job of completing this project on schedule.

Alterations and Renovations

Cairns Laboratory: The north microscope room at Cairns was renovated to make room for a laser microscope and to provide additional space for nitrogen storage for Dr. Spector.

Demerec Laboratory: We rebuilt an entire office complex for Dr. Wigler by gutting the original area and building new offices for the postdocs, a secretary, and Dr. Wigler. A new entrance was also created to allow more light to enter the narrower foyer area caused by the enlargement of this office complex.

Harris Research Facility: We completed the remodeling of the kitchen and repainted the entire building. We also installed door guards and chair rails to prevent further damage by lab carts.

Hershey Building: The offices for Dr. Beach's postdocs were renovated to accommodate 12 postdocs and a secretary. We also installed new built-in furniture, which included new desks, and new telephone lines.

McClintock Laboratory: On the south end of the second floor of McClintock we completed Dr. Beach's laboratory. The library seminar room was gutted and rebuilt into a laboratory, which included new utilities, cabinets, and exhaust hoods.

Bonn House: After two years of debate, the Historical Society gave us permission to install a new asphalt roof on Bonn House. This historical Huntington house on Route 108 is owned by New York State and is maintained for the State by the Laboratory.

Davenport House: Like all exterior porches on older houses, Davenport's porch showed signs of structural deterioration. We replaced all of the old porch columns and repaired the hand rail.

Frame House: All interior walls, piping, and electrical wiring were removed from this house, which is situated in Cold Spring Harbor on Route 25A. The gutted interior was reworked into five bedrooms, four bathrooms, a living room, dining room, kitchen, and library. The project was completed by the summer.

Northview House: We renovated this entire house purchased from the U.S. Government to provide accommodations for Laboratory personnel. All of the window glass had to be replaced, and since the heater had not been drained properly, there was much damage to the pipes over the winter and the whole system had to be replaced, including the radiators.

Nichols Building: The installation of another built-in desk allowed one more person to work in the Grants Department office. After making the computer room smaller, we were able to build another office for the Personnel Department.

Banbury Center: To conform with the requirements of the Board of Health, we renovated the kitchen in Robertson House to create a more efficient facility. This included the installation of a heavy-duty commercial dishwasher and a large refrigerator-freezer.

Uplands Farm: We refurbished the Basset house and the Superintendent's cottage with a coat of bright white paint.

I would like to take this opportunity to thank all of the people in the department for another year of a job well done!

Jack Richards

DEVELOPMENT

Second Century Campaign

1990 was a year of challenge and growth for the Development Department. Reductions in government funding and new urgent social and environmental issues meant increased competition for the philanthropic dollar. Despite these issues, the Laboratory was able to add over $4 million to its Second Century Campaign. Initiated in 1986, this $44 million capital drive, which was the Laboratory's first broad, public-funding campaign, has now raised $40.4 million or 92% of its goal. National and local foundations, those new to the Laboratory and those familiar with it, have accounted for slightly more than 50% of this amount. Laboratory Trustees and other individuals have accounted for approximately 38%, and an ever-widening group of corporations have contributed nearly 10%. Campaign Chairman David L. Luke III and Steering Committee members Oliver R. Grace, George W. Cutting, Jr., Mrs. Sinclair Hatch, Townsend J. Knight, and Taggart Whipple continue to provide dynamic leadership for the Campaign. Other diligent and persistent volunteers ably man the six different Campaign Committees and are listed later in this report. Through their efforts, three foundation challenge grants have been matched or are well on their way to being matched: the Fannie E. Rippel Foundation, the Kresge Foundation, and an anonymous foundation.

The Second Century Campaign was designed to raise money for new and renovated facilities and for staff endowment. It is this latter area that we must emphasize in the remainder of the Campaign. The Neuroscience Center, which will be inhabited in March and dedicated in May, is powerful proof of the Campaign's success in realizing the first of these goals.

Several areas in the Neuroscience Center have been named as a result of significant campaign gifts: Dolan Hall (The Dolan Family Foundation), Hazen Tower (Lita Annenberg Hazen), Keck Structural Biology Laboratory (The W.M. Keck Foundation), The Plimpton Seminar Room (Mrs. Pauline A. Plimpton and Mr. Amyas Ames), The Gardner Neuroscience Library (Mr. and Mrs. Robert B. Gardner, Jr.), The Lucy and Edward Pulling Seminar Room (Mr. and Mrs. George W. Cutting, Jr.), and The F. Thomas Powers Room (Elaine E. and Frank T. Powers, Jr., Foundation).

Another remarkable Campaign gift was a benefit concert performed in the Laboratory's Oliver and Lorraine Grace Auditorium in September by world renowned Metropolitan Opera mezzo-soprano Frederica von Stade. In a program entitled *From Mozart to Broadway*, Miss von Stade enthralled the sold-out auditorium with selections that ranged from Mozart's *Don Giovanni* to Gershwin's *Porgy and Bess*. The benefit raised more than $70,000, which will help match the Kresge Foundation challenge and will endow the Frederica von Stade Undergraduate Research Internship. Patrons who played such an important part in the success of this event are listed later in this Annual Report.

Annual Giving

Unrestricted annual giving provides strong underpinning for the Laboratory's fiscal needs. It is money that can be used at the Trustee's discretion for specific un-

expected, and sometimes pressing, needs. Almost 80% of the Laboratory's annual unrestricted income comes from Cold Spring Harbor Laboratory Associates, and the remainder comes from the Long Island Biological Association. Early in the year, at George Cutting's behest, John Reese agreed to Chair a new committee to expand and strengthen the Associates membership. Through the efforts of Associate Committee members Jack Evans, Charles Gay, Missy Geddes, Harry Lee, and Jordan Saunders, the Laboratory has the highest number of Associates it has ever had and the number is growing steadily. These programs are enthusiastically and effectively guided by George W. Cutting, Jr., and 28 directors. (A complete report of their 1990 activities may be found in Financial Support of the Laboratory, which appears later in this Annual Report).

Planned Giving

In addition to making outright gifts to the Laboratory that take effect immediately (cash, securities, insurance policies, and real estate), gifts may also be made to take effect in the future. These gifts can be made in trust or by will. The mechanism for one of these "planned gifts" has just been established by the Laboratory with the U.S. Trust Company. **The Cold Spring Harbor Laboratory Pooled Income Fund** allows an individual to give cash or stock to the Laboratory which will be pooled with other such gifts. These funds are then invested, and the donor or the person the donor designates will receive the net income from his or her share of the Fund for life. Afterward, the principal amount of the gift is paid to the Laboratory. There are several significant benefits of making such a gift: It provides long-term stability for Cold Spring Harbor Laboratory, it increases income for the donor or the donor's relative, capital gains tax is avoided, and it provides an immediate charitable deduction for income tax purposes. **With a low-cost-based stock, for instance, it is now possible to make a gift to the Laboratory (and the Campaign) and increase your income**!

Staff

The Development Department staff (Claire Fairman, Joan Pesek, and Debra Mullen) has become a cohesive, effective fund- and friend-raising team. I wish to acknowledge their skill, dedication, and diligence and the fact that they always approach their tasks with creativity and a sense of humor. Trustee George Cutting wears many hats at the Laboratory and has put in long and productive volunteer hours. Without him, the Development Department would not be nearly as effective as it is.

We continue to be grateful to all of those who have helped us make such progress toward our development goals. Second Century Campaign contributors are listed under Financial Support of the Laboratory. We invite those who have not yet participated in the Campaign to do so before the end of 1991. **What better way to help secure the future of Cold Spring Harbor Laboratory!**

Konrad Matthaei

Active Archives in Centennial Year

The Laboratory archives was an extraordinarily busy place in 1990, our Centennial year. The news media needed to know a great deal about our early years and the accomplishments and growth from 1890 to the present. The archives, a true working archives in constant use under the dedicated supervision of Lynn Kasso, contains a well-preserved wealth of information and photographs. In December, a New York State archives examiner came for a routine half-hour visit; he was so impressed with the organization and content of the archives that he stayed the entire day and was totally engrossed.

In addition to the media and book publishers who constantly access our picture collection, the archives was used extensively for the following: the centennial commemorative book; research on a new CSHL Press book in progress by Elizabeth Watson; a 1990 book from our press, *The Emergence of Bacterial Genetics* by Thomas Brock; Jan Witkowski's articles in *Current Contents* and *Cancer Cells; DNA Science* by David Micklos and Greg Freyer; and by Judy Cuddihy for *Genes & Development*.

The archives also played an important part in the Centennial exhibit of paintings and prints by Jane Davenport Harris DeTomasi, which opened in January in Bush Lecture Hall. This was the Laboratory's first indoor art show and was most successful. The exhibit was critically acclaimed, and several paintings and prints were sold. Since then, an abbreviated version of the exhibit has traveled to the Union Savings Bank of Huntington and the Cold Spring Harbor Public Library. Matted works and wood blocks were also sold as part of the Laboratory's third annual arts and crafts fair. What began as a great idea by two scientific secretaries, the arts and crafts fair has grown in size, popularity, and profit and is managed by Laura Hyman, whose undaunted energy and talent contribute to so much at the Laboratory beyond her job description. Without her support, both in the Library and in the Public Affairs Department, my work on the Centennial would not have been possible.

Automated Reference Center

Our automated reference center has exceeded expectations in meeting the needs of individual Laboratory scientists. This system is capable of downloading machine-readable searches from library equipment to their personal databases lab-wide. The Medline database on compact disk provides key word searches by end users for citations and abstracts published from 1966 to the present. *Current Contents* on diskette allows "current awareness" scanning of journals. In 1990, 540 different queries were done by scientists and staff on Medline CD and 68 on *Current Contents*. Genemary Falvey, Head of Library Services, gives excellent, personalized instruction in the use of the new technologies, and she reports that most Laboratory scientists like this direct involvement in literature searching. The library subscribes to these services just as they do to journals; therefore, no additional telecommunication costs are passed on to the user. Comprehensive online searching of other databases is still available from the librarian.

Our computerized interlibrary loan service continues to provide fast access to articles requested by staff scientists. Photocopies of 70 tables of contents from journals received are sent to 44 scientists.

New Storage Facility in Plainview

Our storage facility was moved from West Side School to a warehouse in Plainview this summer. Approximately 5800 volumes and 1500 linear feet of shelving were relocated. Although the distance is greater, a two-day retrieval time is still possible.

A Special Staff

I want to thank the entire library staff: Genemary Falvey, Head of Library Services; Laura Hyman, Administrative/Business Manager; Helena Johnson, Library Assistant; Lynn Kasso, Archives Assistant; Wanda Stolen, Senior Library Assistant; and our newest member, Clare Bunce, Library Clerk, for all of their assistance and support during 1990. I would not have been able to concentrate so much of my time and energy on the Centennial had I not known that the library was in extremely capable hands.

Future

The future of library and information services at the Laboratory depends in large measure on three factors: the availability of funds for an ever-inflating subscription budget, the unknown advances in information technology, and sufficient space for quality library service.

Susan Cooper

The Centennial Celebration

By the end of 1991, more than three years filled with a variety of events will stand as a reminder of the Laboratory's first 100 years. Add to that two years of planning preceding the dedication of the DNA Learning Center in 1988 and a total of five years will have been spent focusing on this scientifically and historically important event. It was a unique privilege to work with Emily Eryou, Dan Schechter, Margot Bennett, Herb Parsons, and Ed Campodonico. Their grit and dedication created the events of the Centennial.

July 14, 1990 marked the 100th birthday of Cold Spring Harbor Laboratory. On July 7, 1890, 100 years and 7 days earlier, the first biology class was held on the shore of Cold Spring Harbor. That first class was immortalized by Rob Gensel and his "Rotifer" cast members (see back cover illustration). This tiny launch was joined by four magnificent, sailing vessels, *Black Pearl*, *Zarefah*, *Ernestina*, and *Little Jennie*. At 2:00 p.m. that afternoon, staff members and guests were gently propelled back in time for a grand celebration. Storyteller Heather Forest narrated the arrival of the historic launch and later told related stories to children and adults alike. A hearty meal, adeptly prepared by Jim Hope and his food service staff, complete with ale on tap, was served on Blackford lawn. There, the Old Bethpage Village Restoration Brass Band played "Happy Birthday" and marched through the crowd to traditional turn-of-the-century music. "Sparkles the Clown" kept the little ones enchanted with her balloon art and magic.

The evening's festivities were divided between the gala dinner party held at Airslie for our corporate friends who supported the Centennial and a concert on Davenport lawn. The latter featured the highly acclaimed sea chanteymen, The Forebitter, from Mystic Seaport, and starred Kim Strongin, a superb folk-rock singer-songwriter. Although mother nature threatened to rain on our festivities, all agreed that the misty fireworks by Grucci provided the evening's crescendo. Then out of the mist came the noble sound of bagpipes; the Clan Gordon Highlanders Pipe Band of Locust Valley marched majestically from Airslie to Grace Auditorium. Our guests, who waited patiently for the buses at day's end, were joyously entertained by Synergy, a Barbershop Quartet.

A general sense of well-being dominated the day—people were relaxed and joyous and basked in the sense of history surrounding this great institution.

Two Very Special Concerts

On September 8, jazz musician Christopher Hollyday, the 20-year-old alto saxophone sensation, performed on the Grace Auditorium terrace for the attendees of the "Origins of Human Cancer" meeting. A month later, Hollyday was pictured in *Time* magazine and extolled as "a veteran who began performing at age 13." Then on September 16, mezzo-soprano Frederica von Stade graciously performed a benefit, "From Mozart to Broadway," in Grace Auditorium. It was one of the most enchanting evenings that was ever experienced at the Laboratory.

Centennial Materials Expand Library of Lab Information

"Cold Spring Harbor Laboratory & Long Island: Partners for the Future," our commemorative book, was compiled to document the Laboratory's past, present, and future. A specially designed section called "Partners for the Future," contained congratulatory messages from Long Island business. Special thanks go to Judith Carlson who was commissioned to coordinate this important aspect of the book. Together with her cochairmen, Robert McMillan of Rivkin, Radler, Bayh, Hart, & Kremer, and Thomas Doherty of Norstar Bank, she encouraged a group of prominent business figures to contribute nearly $215,000 in support of the Laboratory and its Centennial. Born out of this program were the new internships for qualified Long Island high school seniors. Appropriately named for the initial contributors, the "Partners for the Future" program allowed five gifted seniors to work with five laboratory scientists after regular school hours from October to March.

In addition to the Centennial literature, which included an art catalog, special commemorative meetings posters, and a variety of programs, flyers, tickets, and invitations, 1990 saw the complete redesign and revision of *FYI*, the little flyer that packs a lot of information about Cold Spring Harbor Laboratory into a number 10 envelope. Three *Harbor Transcripts* were completed, and the second and third issues were combined to make way for the Centennial. Photographs of new staff members hired in 1990 were completed for inclusion in the 1990 *F.A.C.E.S.*, which is being published in February 1991. *F.A.C.E.S.*, the staff directory, contains 435 photos; just 5% of the staff were camera shy. In 1989, 10% were not represented. We look forward to a time when all will be pictured.

A Banner Year for Press Coverage

As expected, the Centennial provided the perfect focus for press attention. The Laboratory was featured in a dozen television programs and appeared in more than 200 magazine and newspaper articles. It has been gratifying to see that Cold Spring Harbor Laboratory is no longer the best kept secret on Long Island. Especially noteworthy was Cablevision's special documentary, *A Century of Discovery*, which aired to all of the company's affiliates. Of the numerous and wonderful articles that appeared in print, the following were of particular interest: *The Scientist*, "Cold Spring Harbor Tops Among Independent Labs;" *Newsday*, "The Land of DNA," a major article on the Lab and its history; and *Innovations in Oncology*, "At the Cold Spring Harbor Laboratory," which is a comprehensive lay report featuring lab scientists involved in cancer research.

Staff Changes

After two and one-half years, we said goodbye to the multitalented Dan Schechter, who in September joined Darby & Darby, a law firm in Manhattan, as a patent agent. As the Laboratory's first science writer, Dan established a standard for multitasking in Public Affairs. To this day, it is hard to figure out just how he did what he did with a computer. His cancer brochure, *The Good Fight*, became the mainstay of our effort to reach the Laboratory's lay audience.

Ed Campodonico joined the staff in April 1990 to assist both Herb Parsons, Director of Audiovisual, and Margot Bennett, Artist/Photographer. Ed's background includes a BFA from Long Island University, C.W. Post campus, and extensive work in audiovisual and photography both in school and in the private sector.

Our new science writer, David Siegel, joined the staff in October. Dave has an eclectic background. He received his BS in physics and astronomy from the State University of New York at Stony Brook in 1984; an MS in astronomy from Yale; and an MA in journalism, science, and environmental reporting from New York University in 1990. David worked as a news intern at CBS, as a research intern at WNET Innovations, and was a feature writer for *Yale Science Magazine*.

Rounding out the staff, Clare Bunce joined us in early 1990 as a part-time assistant. Clare does a great job with all the office tasks that filter down from an overworked and crowded staff. She was that extra edge we needed during the final stages of Centennial planning.

Extra Effort Appreciated

I have received appreciation in abundance for my part in planning the Centennial—there are always so many who need to be thanked, and a list would not begin to express my gratitude. Those people know just how hard they worked, and I want them to know that the Laboratory will always be thankful for their extra effort on its behalf. This was a special time at a very special place.

Susan Cooper

RESEARCH
ROTIFER

TUMOR VIRUSES

At the close of 1990, the Tumor Virus Section was preparing to apply for renewal of the large program project grant that has supported research in this area since 1972. As can be seen from the reports of the nine constituent groups, the ground covered by this section has expanded considerably over the years. It now includes two new viruses, bovine papillomavirus and human immunodeficiency virus, in addition to the time-honored adenovirus and simian virus 40, and it encompasses the cellular processes with which these viruses come into contact. These processes include DNA replication, transcription, mRNA splicing, translation, and posttranslational modification, all of which are potential sites of disturbance in malignancy as well as fundamental aspects of gene regulation in the cells of humans and other animals. Also at the end of 1990, we prepared to bid farewell to Ed Harlow, a member of this section for more than 8 years. His work on protein-protein interactions, and particularly his discovery of the complex between adenovirus E1A proteins and the product of the cellular retinoblastoma susceptibility gene, epitomized the power of the tumor viruses as probes of cellular function and have made an indelible mark in fields as diverse as the cell cycle, transformation, and transcription.

DNA SYNTHESIS

B. Stillman	E. White	J.F.X. Diffley	F. Bunz	T. Macdougall
	T. Tsurimoto	S.-U. Din	K. Fien	N. Kessler
	G. Bauer	A. Dutta	Y. Marahrens	P. Sabbatini
	S.P. Bell	T. Melendy	S. Smith	L. Mellon
	S. Brill	J.M. Ruppert		

In dividing cells, the duplication of the genetic material is a key event that is normally precise and tightly controlled. There is a growing appreciation of the notion that a breakdown in the control of cell division increases the chance of damage to the genome, including accumulation of mutations that contribute to cancer progression. For this reason, studies on DNA and chromosome replication may provide valuable insight into the mechanisms of carcinogenesis. We continue to investigate both the mechanism and regulation of DNA replication using both human cells and the yeast, *Saccharomyces cerevisiae*. As in previous years, these two systems are wonderfully complementary, resulting in substantial progress.

Mechanism of DNA Replication

G. Bauer, S. Brill, F. Bunz, K. Fien, N. Kessler, T. Melendy, J.M. Ruppert, T. Tsurimoto, B. Stillman

The best system currently available to study the mechanism of DNA replication in eukaryotic cells is simian virus 40 (SV40). SV40 contains a small circular genome that harbors a single unique origin of DNA replication. The viral genome encodes a protein, the SV40 large tumor antigen (T antigen), that plays a number of essential roles in the replication of SV40 DNA. T antigen recognizes, binds to, and locally unwinds the origin of DNA replication and also functions as a DNA helicase (DNA unwind-

ing enzyme) during DNA synthesis. The remaining replication machinery is provided by the host cell, and it is the identity and functions of these cellular proteins that have been the focus of our attention during the past 5 years. In the past year, we have reconstituted the essential, core DNA replication machinery with highly purified proteins and have determined the general mechanism of initiation and elongation. In addition, we continue to identify functional homologs of the human cellular DNA replication proteins from yeast cells to allow a genetic analysis of the replication apparatus.

REPLICATION FACTORS

In last year's Annual Report, we described in detail the cellular proteins required for SV40 DNA replication in vitro. These proteins are: replication factor A (RF-A), a multisubunit single-stranded DNA-binding protein and polymerase α and δ auxiliary protein; replication factor C (RF-C), a DNA-dependent ATPase that is also a primer/template-binding protein and DNA polymerase α and δ auxiliary protein; proliferating cell nuclear antigen (PCNA), a polymerase δ auxiliary protein that stimulates RF-C ATPase activity; DNA polymerase α and its associated primase subunits; DNA polymerase δ; and topoisomerases I and II. Although polymerase δ had been implicated in DNA replication by the requirement for RF-C and PCNA, its participation in DNA replication had not been directly shown. In the past year, we have purified an essential DNA replication factor (replication factor D, RF-D) and subsequently identified it as DNA polymerase δ. Together with the previously described replication factors and immunopurified polymerase α/primase complex, we have used this highly purified polymerase δ to reconstitute DNA replication in vitro.

MECHANISM FOR BIDIRECTIONAL DNA REPLICATION

With the seven purified cellular replication factors and SV40 T antigen, a series of detailed biochemical analyses were conducted to determine their role in DNA replication. The principle used was to omit individual factors or combinations of factors from the reaction and characterize the resulting "phenotype" by studying replication products. It was found that in the absence of either T antigen, RF-A, or polymerase α/primase complex, no DNA replication was observed, suggesting that polymerase α/primase was responsible for the synthesis of the first strand at the replication origin. It had been demonstrated that T antigen and RF-A cooperate to unwind the replication origin, and recent studies have demonstrated that addition of the polymerase α/primase complex is sufficient for synthesis of the first nascent DNA at the origin. In contrast, omission of either RF-C, PCNA, or polymerase δ, or combinations of these, yielded some DNA replication, but the products were abnormal. Short nascent DNA strands that correspond to products from only one DNA strand (lagging strand) were observed. DNA replication of the opposite strand (leading strand) did not occur. These results concur with observations made in previous years when PCNA and RF-C were omitted from replication reactions with crude extracts. Moreover, they clearly demonstrate that the polymerase α/primase complex is responsible, at least in part, for replication of the lagging strand and that polymerase δ synthesizes the leading strand at a replication fork.

Last year, we noted a striking similarity between the replication factors RF-C and PCNA and the bacteriophage T4 proteins encoded by genes 44/62 and 45, respectively. These phage-encoded proteins function with the T4 DNA polymerase (gene 43) to synthesize the leading strand at a replication fork. We have now demonstrated that the phage T4 proteins can substitute for RF-C, PCNA, and polymerase δ in a hybrid replication system. The phage proteins function with T antigen, human RF-A, and polymerase α/primase to replicate DNA. These observations suggest an extraordinary evolutionary conservation of the replication functions. More importantly, because mutations in the genes encoding the phage proteins debilitate phage DNA replication in vivo, we suggest that our biochemical studies using the SV40 system have identified homologous proteins that function in the replication of human cell DNA.

A MODEL FOR DNA REPLICATION

The studies described above suggested a novel mechanism for the initiation of SV40 DNA replication that might be generalized for initiation of bidirectional DNA replication in eukaryotic chromosomes. The novel feature of this model, shown in Figure 1, involves the switching of DNA polymerases at the replication origin during initiation of leading-strand DNA replication. We propose that polymerase α/primase synthesizes the first nascent strand at the

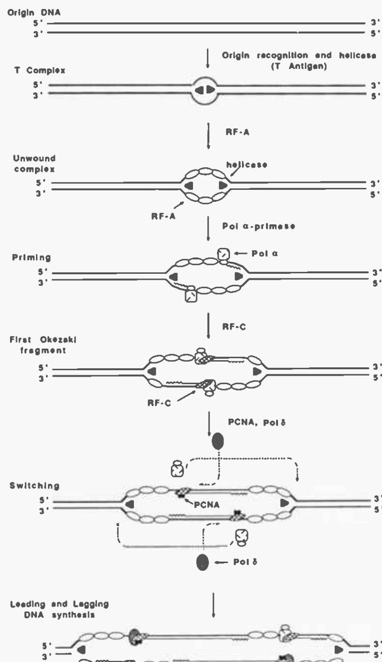


FIGURE 1 Initiation of bidirectional DNA replication by two DNA polymerases.

replication origin. Because RF-C is a primer/template recognition protein, it recognizes the 3′end of this nascent strand and then binds PCNA. The RF-C/PCNA complex displaces polymerase α and recruits polymerase δ. The RF-C/PCNA/polymerase δ complex functions as a processive replication complex and continuously synthesizes the leading strand (see Fig. 1).

Detailed biochemical analysis of the function of RF-C and PCNA, as well as their effect on the function of polymerase α and δ, provides strong support for this mechanism of initiation of DNA replication. These studies include nuclease protection footprinting and native gel electrophoresis of RF-C and RF-C/PCNA complexes with DNA. RF-C is an unusual DNA-binding protein that recognizes a specific structure (a primer/template junction) in a sequence-independent manner. Once RF-C is bound to the DNA, PCNA recognizes the RF-C/DNA complex and binds adjacent to RF-C. This is an interesting complex because PCNA does not, by itself, recognize DNA. We have shown that formation of the RF-C/PCNA complex at a primer/template junction, in cooperation with RF-A, functions in the polymerase switching step, thereby preventing polymerase α from copying the leading-strand template.

Our studies to date have reconstituted the rudimentary core of the eukaryotic DNA replication apparatus in vitro with purified proteins. We still need to identify factors that are required for formation of complete, covalently closed daughter DNA.

REPLICATION FACTORS FROM YEAST

To investigate the role of these replication factors during cell chromosomal DNA replication, we have searched for functional homologs of the human factors in the yeast, *S. cerevisiae*. This organism offers the ability to combine biochemical and genetic methods to the problem of DNA replication. We have previously described the purification of yeast RF-A (yRF-A) as a complex of three proteins with molecular weights of 69K, 36K, and 13K. In the past year, the genes encoding the three subunits of yRF-A have been cloned and sequenced. All three genes are essential; mutants lacking any one of them fail to grow. The production of conditionally defective mutations in these genes will enable us to determine if they have a defect consistent with a block in chromosomal DNA replication.

The gene encoding the 69K subunit is identical to a gene, RPA1, that was isolated by R. Kolodner's group at Harvard because it encodes a protein implicated in genetic recombination. The gene encoding the 36K subunit has previously not been identified, and curiously, it is one of the few genes in *S. cerevisiae*, other than ribosomal protein genes, that is spliced. The predicted sequence of the yeast 36K subunit shows 29% identity and 40% similarity with the human 34K RF-A subunit, the sequence of which was determined in T. Kelly's laboratory (Johns Hopkins). The gene encoding the RF-A 13K subunit is also a novel gene, and the protein is unrelated to others present in sequence databases.

In addition to the yRF-A replication factor, we have identified and purified the *S. cerevisiae* RF-C

replication factor, yRF-C. This factor cooperates with yRF-A and yPCNA to stimulate the activity of yeast DNA polymerase δ in much the same way as RF-C, RF-A, and PCNA cooperatively stimulate human cellular DNA polymerase δ. Isolation of the genes encoding the yRF-C subunits will allow a genetic analysis of the function of this replication factor in cellular DNA replication.

Regulation of DNA Replication

S. Brill, S.-U. Din, A. Dutta, B. Stillman

In the past year, we reported that RF-A from human and yeast cells was phosphorylated on serine residues and that this posttranslational modification was cell-cycle-regulated. The 34K subunit was not phosphorylated in the G_1 phase, but it was phosphorylated later in the cell cycle. We have more precisely timed the onset of the phosphorylation event to just prior to the onset of DNA replication in S phase. This places the onset of RF-A phosphorylation at an interesting and critical regulatory stage in the cell cycle.

We have pursued a two-pronged approach to determine whether the phosphorylation of RF-A plays a role in the control of DNA replication. The first, which is currently under way, is to make mutants in both the human and yeast genes encoding the 34K subunit that affect phosphorylation and study the phenotype. A second approach is to identify the RF-A kinases and determine if they perform a regulatory function and influence RF-A activity. Considerable progress has been made toward this goal.

An RF-A kinase was found in an extract from human cells that phosphorylated RF-A on a subset of the serines phosphorylated in vivo. Importantly, this kinase activity was absent in extract prepared from cells in the G_1 phase of the cell cycle but was present at later stages. This kinase was purified to homogeneity. The kinase activity cofractionated with a multisubunit protein complex consisting of a 62K subunit and a doublet at 34K, with a 45K protein that may associate with the other proteins. This subunit structure suggested that the protein kinase was identical to the $p34^{cdc2}$ kinase associated with a cyclin subunit (62K), and subsequent studies with antibodies confirmed this identification of the enzyme as the cyclin B/$p34^{cdc2}$ kinase complex. The *cdc2* protein kinase has been shown both genetically and biochemically to be a major regulator of cell cycle progression by its association with a number of cyclins, proteins that are synthesized and degraded in a temporally controlled manner through the cell cycle (see D. Beach's report in the Genetics Section). We have demonstrated, in collaboration with D. Beach and G. Draetta, that both the cyclin A and cyclin B forms of *cdc2* kinase will phosphorylate RF-A. The challenge now is to determine what form of cyclin/*cdc2* kinase phosphorylates RF-A and at what time in the cell cycle, in addition to identifying the function of the phosphorylation.

Along these lines, we have shown a modest increase in RF-A single-stranded DNA binding following phosphorylation in vitro by purified *cdc2* kinase. Furthermore, a stimulation of origin unwinding by RF-A and T antigen was also attributable to RF-A phosphorylation in vitro by the $p34^{cdc2}$ kinase, probably reflecting increased DNA binding. Current studies are directed toward determining whether these effects occur in SV40-infected cells during SV40 DNA replication and in uninfected cells at S phase.

Initiation of Chromosomal DNA replication

S.P. Bell, J.F.X. Diffley, Y. Marahrens, B. Stillman

The SV40 system for studing DNA replication has been invaluable for examining the mechanism of DNA synthesis. These studies have relied on the SV40 T antigen and are therefore of limited value for understanding initiation of DNA replication in eukaryotic chromosomes. To complement our studies on yeast replication proteins, we have studied the structure and function of a chromosomal origin of replication in *S. cerevisiae* called ARS1. ARS1 was selected because it confers on plasmid DNAs the ability to replicate as a circular, minichromosome in synchrony with the 16 cellular chromosomes and has been shown by other investigators to function as a chromosomal origin of DNA replication.

Comparison of the sequences of a number of ARS elements revealed a highly conserved 11-bp "ARS consensus sequence" that is essential, but not sufficient, for ARS function. Several laboratories performed deletion analyses on various ARS elements

including ARS1 to identify additional functional domains. These studies, however, managed to define only vaguely broad regions next to the ARS consensus sequence as important.

To define this origin further, we substituted 8-bp ARS sequences with the sequence 5′-CCTCGAGG-3′ that can be cut with the restriction enzyme *Xho*I. Thirty-four such mutations were constructed to cover 179 bp of a 185-bp ARS1 clone. Two substitutions, both disrupting the ARS consensus sequence, abolish origin function entirely. We tested origin efficiency of the remaining 32 mutants by measuring plasmid levels in yeast cultures after 14 generations of cell growth. Clusters of mutants with reduced plasmid levels defined three additional short sequences that contribute to origin function. We named these additional domains B1, B2, and B3 in descending order of importance. Disrupting combinations of domains either greatly reduces or abolishes ARS1 function.

DNA was synthesized that contained the four important domains, all correctly spaced, but with all other DNAs scrambled. This "synthetic ARS" functioned nearly as well as the natural origin, proving that the four domains are sufficient for efficient ARS function. Moreover, none of the domains (B1, B2, or B3) successfully substituted for each other, suggesting that they are functionally distinct.

Domain B3 overlapped with the footprint for ARS-binding factor 1 (ABF1), a protein purified in our laboratory and discussed in previous Annual Reports. Point mutations that abolished ABF1 binding also abolished B3 activity, demonstrating that ABF1 binding at ARS1 was responsible for this activity. Because ABF1 also has a known role as a transcription factor, we replaced the ABF1-binding sequence with a sequence that binds a homologous transcription factor called RAP1. Interestingly, the binding of RAP1 restored domain B3 function. These results suggest that transcription factors that are capable of binding to DNA may influence initiation of DNA replication at chromosomal origins in a manner similar to activation of a number viral origins.

We have continued to study the function of the ABF1 protein, particularly its role in DNA replication and transcriptional regulation. A clone of the ABF1 gene has been used for defining a functional domain structure for the ABF1 protein. An initial surprising result was that ABF1 contained two distinct regions of the protein for site-specific DNA binding. One region, when expressed alone, bound specifically to the ABF1-binding site, but with greatly reduced affinity. Another region, when expressed alone, bound nonspecifically to DNA in a zinc-dependent manner. The two regions, when coexpressed on the same polypeptide, recognized the ABF1-binding site with the same specificity and affinity as the intact ABF1 protein. These results suggest a novel bipartite DNA recognition structure containing two different, cooperating, DNA-binding domains. Further characterization of this structure is under way.

Chromatin Assembly

G. Bauer, S. Smith, B. Stillman

In previous years, we had described a cell-free system for assembly of correctly spaced chromatin during DNA replication. In addition to the essential DNA replication factors, novel chromatin assembly factors are required to assemble the histones onto the DNA. Last year, we described the purification and characterization of one such factor, CAF-1. This multisubunit protein is required for the assembly of histones onto the replicating DNA.

Biochemical fractionation of the remaining cell extract has allowed separation of the chromatin assembly reaction into two steps. During the first step, CAF-1 targets the deposition of newly synthesized histones H3 and H4 to the replicating DNA. This reaction is dependent on and coupled with DNA replication and utilizes the newly synthesized forms of histones H3 and H4, which, unlike bulk histone found in chromatin, do not bind to DNA by themselves. The H3/H4-replicated DNA complex is a stable intermediate that exhibits a micrococcal-nuclease-resistant structure and can be isolated by sucrose gradient sedimentation. In the second step, this replicated precursor is converted to mature chromatin by the addition of histones H2A and H2B in a reaction that can occur after DNA replication. On the basis of these results, we have suggested a model for the DNA-replication-dependent assembly of chromatin and a role for CAF-1 in this process (Fig. 2). The requirement for CAF-1 in at least the first step of the reaction suggests a level of cellular control for this fundamental process. It is of particular interest that CAF-1 is modified by phosphorylation, and we are currently determining whether this modification plays a regulatory role during chromatin assembly.

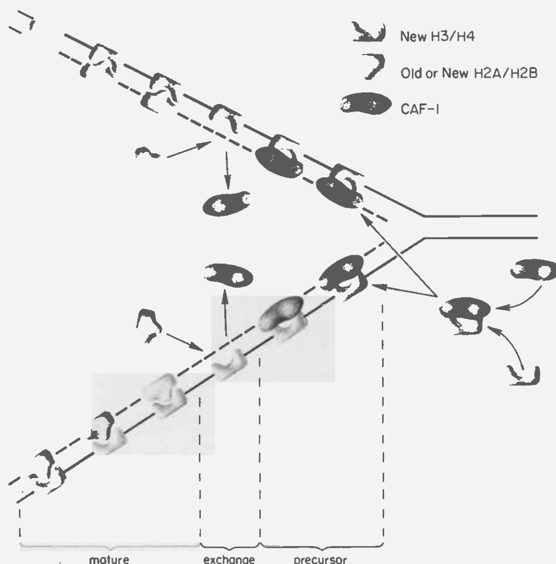


FIGURE 2 A model for a two-step mechanism for chromatin assembly by CAF-1.

Functions of Adenovirus E1B Protein

E. White, P. Sabbatini

The adenovirus E1B gene is required for regulation of viral gene expression in productively infected cells and for oncogenic transformation. Last year, we made some interesting discoveries that have greatly enhanced our understanding of E1B protein fuction. The E1B gene of adenovirus encodes two major tumor antigens, the 19K and 55K proteins. The coding regions for these proteins have been placed under the control of heterologous promoters to ascertain the role of individual E1B proteins in transformation and their primary biological function. These E1B plasmid expression vectors have led to two significant findings: (1) Both E1B proteins possess transforming activity when cotransfected with a plasmid encoding E1A, but it is the 19K protein that confers anchorage-independent growth, and (2) the E1B 19K protein has the unique and unusual ability to associate with and disrupt the organization of two distinct classes of intermediate filaments, cytoplasmic vimentin filaments and nuclear lamin filaments. We propose and are preparing to test the hypothesis that the biological function of the 19K protein in transformation and productive infection is a direct consequence of the perturbation of intermediate filaments.

This year, we have expanded upon these results in three areas. First, we have been identifying cellular proteins, most likely intermediate filament proteins, that directly interact with the 19K protein. Second, we have continued our investigation of the role of E1B proteins in transformation, establishing whether or not E1 gene products are capable of cooperating with oncogenes other than E1A. Third, a mutational analysis of the 19K coding region was performed to identify important funtional domains responsible for the activity of the protein in transformation and productive infection.

We have begun to test the ability of E1B proteins to cooperate with the products of other viral oncogenes. In a collaborative effort with Peter Howley at the National Cancer Institute, we have discovered that the E1B 19K protein can enhance the transforming activity of the human papillomavirus type-16 E6 and E7 transforming genes. This finding establishes that the transforming activity of the E1B 19K protein is not restricted to cooperation with the adenovirus E1A gene and may represent a more universal function related to the development of human cancer.

Finally, mutational analysis has so far identified an important region of the 19K protein required for both transforming activity and intermediate filament disruption. With one exception, mutations within a conserved central region of the 19K protein eliminate transforming activity of the protein. Therefore, a single domain in the 19K protein may encode the transforming function. Furthermore, a single substitution of an acidic glutamic acid residue for the basic lysine at position 44 of the 19K coding region results in simultaneous loss of transforming activity and the ability to associate with and disrupt intermediate filaments. This is readily apparent in double-label indirect immunofluorescence of transfected cells transiently expressing either the wild-type 19K protein or the mutant protein. Whereas the wild-type protein localizes to the nuclear envelope and cytoplasm and disrupts intermediate filaments, the mutant protein is defective for nuclear localization and the organization of intermediate filaments is completely unaffected. Whether this sequence of the 19K protein is a bone fide nuclear localization signal is being explored.

In summary, the E1B 19K protein represents a transforming protein with a novel function that will be useful both for understanding a unique mechanism of oncogenic transformation and for determining the function of those cellular structures with which it interacts, intermediate filaments.

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MOLECULAR BIOLOGY OF BOVINE PAPILLOMAVIRUS

A. Stenlund M. Ustav J. Alexander
P. Szymanski E. Ustav

The papillomaviruses are a family of viruses that have been isolated from a large variety of mammals. Initially believed to be related to the polyomaviruses, it is now clear that they form their own distantly related group. The group as a whole has some distinctive characteristics. All of the papillomaviruses have what appears to be a very high degree of tissue specificity; a given virus type very frequently infects only one tissue type. None of the papillomaviruses give rise to a full productive (or lytic) cycle in tissue culture cells. In some virus types, however, a part of the viral life cycle can be studied. Bovine papillomavirus type 1 (BPV-1) has become the prototype virus for the papillomavirus group, since the BPV genome can transform mouse C127 cells in culture. In these transformed cells, the viral DNA is maintained and replicates, as an episome, providing model systems for gene expression, replication, and transformation.

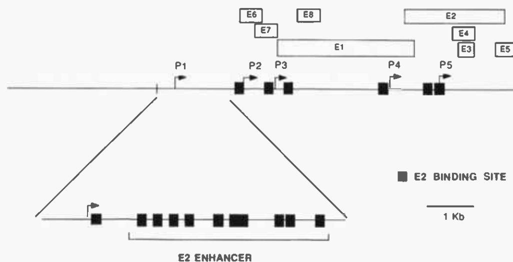
Apart from its importance as a prototype virus for the pathogenic human viruses that cannot be readily studied at the molecular level, BPV has attracted considerable attention for its replication properties in tissue culture cells. BPV shares with one other virus, Epstein-Barr virus, the property that the viral DNA can replicate as an episome with a stable copy number in transformed cells. The study of BPV DNA replication offers several unique and interesting aspects fundamentally different from replication in the lytic viral systems which have been studied in detail. These include questions about the control of copy number and segregation and the involvement of cellular mechanisms in regulation of viral replication.

Development of a Transient Assay for BPV Replication In Vivo and Determination of Viral Gene Requirement for Replication

M. Ustav, A. Stenlund

One of the essential tools required for studies of BPV DNA replication is an efficient short-term replication assay. The main problem in detecting short-term replication of BPV in C127 cells is the low copy number compared to that of lytic viruses. Therefore, to detect replicating DNA, a large number of cells must take up and replicate the DNA. Conventional procedures for transfection of C127 cells yielded very low transfection efficiencies. We therefore developed an electroporation procedure that could be used to deliver DNA into C127 cells with very high efficiency. In fact, 10–20% of the cells expressed transfected markers. Using this procedure, we developed a short-term replication assay for BPV that consisted of transfecting BPV DNA by electroporation, followed by harvest of low-molecular-weight DNA at time points ranging from 2 to 6 days after transfection and analysis of the DNA by gel electrophoresis and Southern blotting. Replicated DNA was scored after digestion with *Dpn*I, which will cleave nonreplicated DNA. Using this assay, we have reexamined some of the properties of BPV replication. Interestingly, the accumulation of replicated DNA is biphasic. Initially, replication during the first 48–72 hours proceeds at a faster rate than at later times, when accumulation of replicated DNA appears to increase at the same rate as the cellular DNA. This result is consistent with the results expected from a two-stage model in which initial amplification of the viral DNA is followed by stable maintenance.

We determined which viral gene products were required for replication by assaying a series of mutants located throughout the early region of BPV in the short-term replication assay. Frameshift mutations in the E6, E7, and E5 open reading frames had no effect on replication, whereas mutations in the E1 and E2 open reading frames abolished replication completely (Fig. 1, top). Using complementation experiments, we showed that the mutations affected *trans*-acting factors and mapped to two different complementation groups. The same mutants were tested in stable replication assays with similar results. The E2 open reading frame encodes three related polypeptides that all share the carboxy-terminal part of the open reading frame. To determine which of these polypeptides were important for replication, we



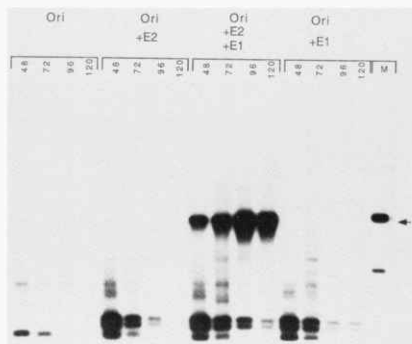


FIGURE 1 (*Top*) Schematic figure showing the organization of the early region of the BPV genome. (*Open bars*) Early open reading frames E1–E8. Arrows labeled P1 through P5 represent the five known promoters that transcribe the early region. (*Closed boxes*) Location of mapped binding sites for the gene products from the E2 open reading frame. (*Bottom*) Short-term replication assay using E1 and E2 expression vectors as the only source of viral *trans*-acting factors. A 3.2-kb *ori* fragment from BPV, lacking the coding sequence, was transfected either alone or together with either E1 or E2 expression vectors or together with both E1 and E2 expression vectors. The arrow marks the position of the replicating *ori* fragment.

generated expression vectors encoding these three polypeptides. To determine that the expression vectors produced the predicted polypeptides, we analyzed the protein products produced in transient expression assays by in vivo labeling and immunoprecipitation with polyclonal antiserum raised against E2. When tested for complementation of an E2 mutant in a short-term replication assay, only the expression vector expressing the full-size *trans*-activator form of E2 could complement the defect, indicating that this form, a 48-kD polypeptide, was required for replication.

The results of assaying mutations throughout E1 indicated that the replication function was encoded by the entire open reading frame. We therefore constructed several expression vectors and assayed them for their ability to complement mutations in the E1 open reading frame. Several of the vectors could complement the E1 defect, and we could identify by immunoprecipitation the complementing polypeptide as a 72-kD protein that was encoded by the entire E1 open reading frame. A polypeptide of similar molecular mass, 69–72 kD, has recently been identified in BPV-transformed cells. To determine whether

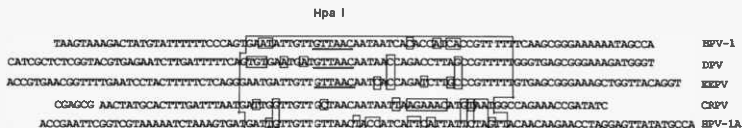


FIGURE 2 Comparison of the sequences at the BPV *ori* with the corresponding region from four other papillomaviruses. The boxed sequences represent the region with the highest degree of homology.

any other viral gene products were required for replication, we deleted all of the early coding sequences from the viral genome and assayed the resulting fragment for its ability to replicate in the presence of the E1 and E2 expression vectors (Fig. 1, bottom). This 3.5-kb fragment replicated to wild-type levels. Replication was completely dependent on both E1 and E2, showing that the two proteins were necessary and sufficient for replication of BPV in C127 cells.

cis-Acting Sequences Required for BPV Replication

M. Ustav, E. Ustav, A. Stenlund

In conjunction with the determination of which viral factors are required in *trans* for replication, we have also conducted a search for the *cis*-acting viral elements that are involved in replication. Our approach has been very simple. We have cotransfected C127 cells with E1 and E2 expression vectors to provide *trans*-acting factors (see above), together with subgenomic restriction fragment mixtures of the BPV genome. Replication was then assayed in the short-term replication assay, and fragments that were capable of replication under these conditions could easily be identified. We found that for any given digest, only one fragment was replicated, indicating that a single region was required for replication. In addition, all fragments capable of replication had some sequences in common. For technical reasons, detection of replication of very small fragments is difficult, i.e., small fragments are obscured by the *Dpn*I cleavage products. With this approach, therefore, the smallest replicating fragment that we could detect was 1.5 kb.

Deletions in the 1.5-kb region in the context of a larger fragment showed that the sequences required for replication were confined to a small region around a unique *Hpa*I site. That this region was both necessary and sufficient for replication was shown by the fact that a cloned 105-nucleotide-long *Alu*I fragment, which included the unique *Hpa*I site, replicated in an E1- and E2-dependent manner in C127 cells. Replication of this construct was destroyed by insertion of an *Xba*I linker into the *Hpa*I restriction site, confirming the importance of the sequence at the conserved *Hpa*I site (Fig. 2). To confirm the importance of the putative *ori* region in the context of the whole viral genome, we inserted an *Xba*I linker into the *Hpa*I site of the viral DNA and assayed the mutated construct for replication in C127 cells. The construct failed to replicate even when cotransfected with the wild-type BPV genome as a source of *trans*-acting factors. The mutant also failed to replicate in stable transformation assays when cotransfected with the wild-type BPV genome, indicating that this region plays an important role at all stages of the viral life cycle.

The sequences surrounding the minimally required *Alu*I fragment in BPV have quantitative effects on replication even though these sequences are not essential for replication. This is especially true for the sequences located on the upstream side of the *Hpa*I site, in the so-called upstream regulatory region (URR) that serves as an E2-dependent enhancer for transcription. The relatively small effects on replication of deletions in this region are enhanced considerably when the wild-type genome is present as a competitor for replication. Our preliminary results thus indicate that the sequences within the URR can augment replication and that in addition to its role in transcription, the URR region can serve as an auxiliary sequence for replication.

Host Range for BPV Replication

M. Ustav, A. Stenlund

A characteristic of the papillomaviruses is the very limited host and cell-type range that they exhibit.

Very little is known about what determines this restriction, since only a few types of papillomaviruses will reproduce any part of their life cycle in tissue culture cells. BPV has the widest host range and has been shown to replicate in, and transform, mouse C127 and NIH-3T3 cells, Rat 2 cells, hamster embryo fibroblasts (HEFs), and primary bovine conjunctival cells. For SV40 and polyomavirus, the restriction on host range is at least partly due to restrictions directly on viral DNA replication. It has been shown that the interaction between T antigen and DNA polymerase/primase appears to be species-specific. To determine whether this was the also case for BPV, we tested several established primate cell lines for their ability to support BPV replication in a short-term replication assay. None of these cell lines (CV-1, COS-7, 293, HeLa) replicated the viral DNA to detectable levels. To test if this restriction was due to limitations in gene expression, we utilized the E1 and E2 expression vectors that are driven by the cytomegalovirus (CMV) immediate-early promoter (which is active in most cell types) and cotransfected these with a BPV restriction fragment containing the *ori* elements. In these cases, we could detect replication in all tested primate cells, including HeLa, 293, CV-1, and COS-7 cells. The efficiency of replication was similar to the efficiency seen in C127 cells, assuming that the transfection efficiency for these different cell lines was not widely different. This result indicated that some aspect of expression of the viral factors E1 and E2 are limiting for replication. When these two viral factors are provided, the cellular machinery for replication is capable of replicating BPV DNA. Further analysis using mutants in the BPV genome has shown that the expression of both E1 and E2 from the viral genome appears to be restricted in primate cells, since both of these gene products must be supplied from heterologous expression vectors to allow for replication.

Role of the E2 Enhancer in Viral Gene Expression

P. Szymanski, A. Stenlund

Analysis of viral gene expression has been performed mainly in BPV-transformed cells or with isolated subgenomic fragments linked to the chloramphenicol acetyltransferase (CAT) gene. This means that even though viral gene expression in transformed cells has been extensively studied, very little is known about immediate-early gene expression from the intact viral genome. With the aid of the highly efficient electroporation procedure, we have for the first time been able to analyze transient gene expression from the in-

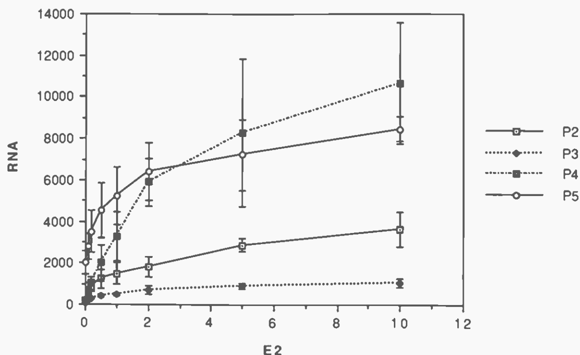


FIGURE 3 Response of the P2–P5 promoters to the *trans*-activator E2. The BPV genome was cotransfected with varying amounts of E2 expression vector, and transcription was measured from each promoter using an RNase protection assay. The RNA levels in arbitrary units are plotted against the quantity (micrograms) of cotransfected E2 expression vector.

tact viral genome in mouse C127 cells. We have developed specific RNA probes to detect transcripts initiated at four of the early promoters, which allows us to analyze expression from these promoters simultaneously. Using this procedure, we have shown that the transient expression pattern from the viral genome is similar to the expression pattern that is seen in stably transformed cells. Furthermore, we have shown that the E2 inducible enhancer present in the upstream regulatory region of BPV is responsible for activating the four early promoters in an E2-dependent fashion (Fig. 3). In addition, by analyzing a large number of deletion mutants in the URR for their effect on the four promoters, we have determined that the enhancer is functionally redundant. Our studies have also shown that none of the other known gene products from the viral genome have any effect on transcription from the viral promoters. This establishes that the E2 system functions as the master regulator for viral gene expression.

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ADENOVIRUS TRANSFORMING FUNCTIONS

T. Grodzicker S. Abraham P. Yaciuk
E. Moran Y. Rikitake M. Carter
H.-G.H. Wang

For the past several years, our primary goal has been to understand the genetic and biological mechanisms that are the basis of adenovirus E1A transforming functions. In previous years, we have demonstrated that the E1A protein products encode two autonomous transforming functions localized in separate regions of the E1A proteins (see Fig. 1). The transforming function associated with domain 2 is linked with the ability of domain-2 residues to bind the product of the cellular retinoblastoma (RB) tumor suppressor gene. The transforming function localizing to the E1A amino terminus is linked with the ability of the E1A products to bind an unidentified cellular product of 300 kD. The extraordinary degree of independence that exists between the two functional domains is emphasized by the ability of domain-2 and amino-terminal deletion mutants to cooperate in *trans*, in a translation-dependent manner, to induce cellular transformation.

T-antigen Complementation of the E1A Amino-terminal Function

P. Yaciuk, M. Carter, E. Moran

In previous years, we have shown that E1A domain 2 constitutes a discrete genetic element that is common to the transforming proteins of other classes of DNA tumor viruses. We demonstrated this relationship specifically in SV40 T antigen, and it was subsequently shown by other investigators to exist in the human papillomavirus E7 gene products as well. As the autonomous nature of the E1A amino-terminal transforming function became clearer, it became of interest to determine whether other transforming products such as SV40 T antigen encode a functional analog of the E1A amino-terminal function. In last year's Annual Report, we described preliminary evidence in-

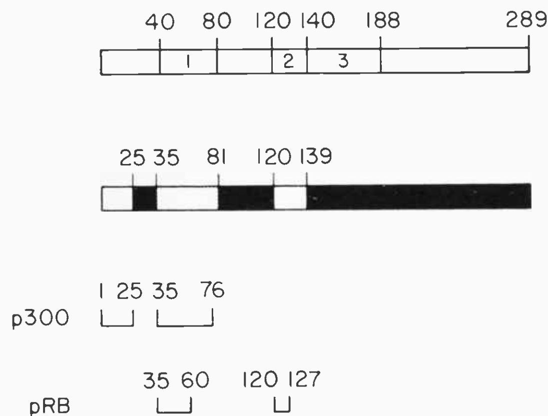

FIGURE 1 Summary of E1A protein structure. The E1A proteins contain three domains of highly conserved amino acid sequence (indicated in the upper bar) alternating with less-conserved regions. Conserved domain 3 is unique to the largest (13S) E1A splice product. Large segments of the E1A proteins can be deleted without impairing transformation function severely. Three such deletions are indicated by the black areas in the lower bar. The numbers above the bars indicate the positions of amino acid residues. The boundaries of the E1A sequences required for p300 and pRB binding are indicated below the bars. Various other E1A-associated proteins require similar sequences as pRB, but no associated proteins other than p300 appear to require the extreme amino-terminal sequences.

dicating that SV40 T antigen does, indeed, encode a function complementary to the biological activity encoded by the amino-terminal region of the E1A products. During the past year, we have confirmed and extended these results.

We have found that an E1A amino-terminal mutant can be complemented in *trans* for transforming activity by a T antigen mutant that lacks the entire domain-2 homology, shows no pRB-binding activity, and, by itself, cannot transform primary cells. Neither the pRB nor the p53-binding activity of T antigen is sufficient to substitute for the E1A amino-terminal function. The complementing T-antigen function can, however, be inactivated by a deletion near the T-antigen amino terminus (construct obtained from Dr. J.M. Pipas at the University of Pittsburgh) . The *trans*-cooperating activity shown by the heterologous combination of E1A and T-antigen mutants is as efficient as the *trans*-cooperating activity shown by the combination of individual E1A domain mutants. The demonstration that SV40 T antigen encodes a biological activity that is functionally equivalent to the transforming activity lost by deletion of the E1A p300-binding region significantly extends the known relationships between the DNA tumor-virus-transforming proteins and supports the prediction that p300 plays an important role in the regulation of cell growth.

E1A-dependent Cell Cycle Effects Correlated with Induction of cdc2 Expression

H.-G.H. Wang, E. Moran

The recognition that E1A encodes two separate functions with specific cell cycle effects provides important genetic tools that will now allow us to separate and analyze the biochemical steps by which the E1A products control the expression of cellular products in such a way as to activate resting cells.

A major goal for us now is to identify and characterize the specific cellular targets with which E1A interacts to mediate cell cycle activity. Several years ago, our laboratory and others showed that the cell-growth-regulating activities of the E1A gene are independent of the major E1A transcriptional *trans*-activation activity that localizes to domain 3. Thus, it is likely that study of cellular products whose expression is induced independently of domain-3 function will lead to a better understanding of the E1A-mediated activities underlying the transition from quiescence to active proliferation. In recent years, we have identified two cellular products, intimately associated with cell growth, whose expression is induced by E1A independently of the well-characterized E1A *trans*-activation function. One of these is the DNA replication factor known as proliferating cell nuclear antigen (PCNA). The other is the mammalian homolog of the yeast cell-cycle-regulating gene, *cdc2*, which is believed to play a central role in mammalian cell cycle regulation.

Because the $p34^{cdc2}$ serine/threonine kinase is believed to play such a central role in cell cycle control, the regulation of expression and activity of this product in response to E1A transforming domains is now a particular focus of this project. Of special interest is the recent evidence from several laboratories suggesting that $p34^{cdc2}$ is the pRB kinase: The RB protein sequence includes several consensus $p34^{cdc2}$ substrate recognition sites, pRB can be phosphorylated by $p34^{cdc2}$ in vitro, and cell extracts can be depleted of pRB kinase activity by addition of α-

p34^{cdc2} antibodies. Current models of pRB function propose that phosphorylation inactivates the cell-growth-suppressing effect of pRB. The link between p34^{cdc2} and pRB phosphorylation suggests the possibility that, in addition to any direct effects the E1A products may exert through actual physical association with the RB products, the E1A products may be able to release pRB-mediated growth suppression indirectly by inducing *cdc2* activity. It has long seemed likely that the E1A products have the capacity to mediate cell cycle activity in the absence of the pRB-binding function; even before the principal E1A domain-2-associated cellular protein was identified as the pRB product, we had shown that E1A domain 2, although absolutely required for transforming activity and pRB binding, is largely dispensable for E1A-mediated induction of DNA synthesis in quiescent cells. E1A-mediated induction of PCNA expression is independent of both domain-2 and domain-3 function. If E1A-mediated induction of *cdc2* is similarly independent of domain-2 and domain-3 function, this might represent an alternative mechanism by which E1A could mediate entry into the cell cycle.

To explore this possibility, and to elucidate the

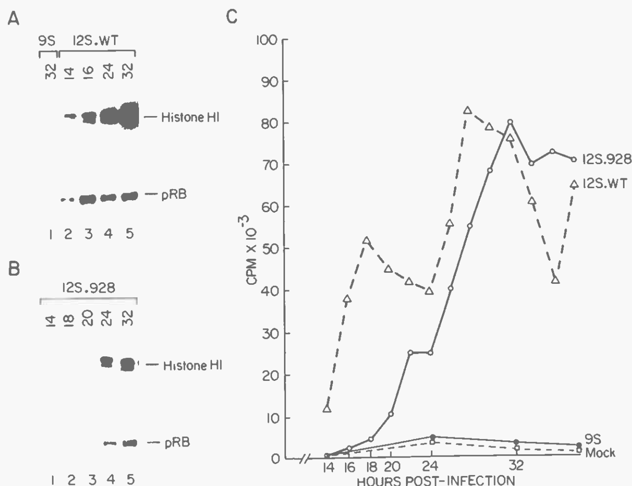

FIGURE 2 Induction of *cdc2* activity and pRB phosphorylation mediated by a domain-2 negative mutant. At various times after infection of primary BRK cells with 12S.WT or 12S.928 (a domain-2 mutant, non-RB-binding virus), cdc2 kinase activity was assayed by immunoprecipitation of the cdc2 protein from specific cell extracts, followed by reaction with histone H1 substrate in the presence of [γ-^{32}P]ATP. The phosphorylation state of pRB was monitored in a duplicate set of infections by in vivo ^{32}P-pulse-labeling, followed by specific immunoprecipitation. Induction of cdc2 kinase activity correlates closely with pRB phosphorylation in both sets of infections. Clearly, though, induction of cdc2 activity and pRB phosphorylation occur several hours later in the 12S.928 infection compared with the 12S.WT infection. A third set of infected cells was labeled for 2-hr pulses with [^{3}H]thymidine to monitor the induction of DNA synthesis. The slower induction of cdc2 kinase activity and pRB phosphorylation in the 12S.928-infection correlate closely with a slower onset of DNA synthesis in 12S.928-infected cells. The level of DNA synthesis in 12S.928-infected cells eventually reaches the same level as that in 12S.WT-infected cells, and at this point, the degree of pRB phosphorylation is similar in either infection. The cdc2 histone kinase activity in 12S.928-infected cells does not increase to the same levels reached in 12S.WT infection, presumably because beyond 24 hr postinfection, the 12S.WT levels of cdc2 kinase activity reflect a contribution from mitotic cdc2 activity, which represents a dramatic increase.

mechanisms underlying the biological events directed by E1A, we have monitored the expression of *cdc2* and the RB products in primary cells stimulated by wild-type E1A and by selected E1A mutants that induce abortive cell cycle progression. (Some of these experiments were done in collaboration with Dr. G. Draetta at the EMBL Institute in Heidelberg.) We have found that, in addition to binding the RB product, the 12S E1A products can actually induce phosphorylation of pRB in normal quiescent cells. The induction of pRB phosphorylation correlates with E1A-mediated induction of $p34^{cdc2}$ expression and kinase activity, consistent with the possibility that $p34^{cdc2}$ is an RB kinase. Expression of SV40 T antigen induces similar effects. Induction of pRB phosphorylation is independent of the pRB-binding activity of the E1A products: E1A domain-2 mutants, which do not bind detectable levels of pRB, remain competent to induce pRB phosphorylation and to activate *cdc2* protein kinase expression and activity (see Fig. 2). Domain-2 mutants induce wild-type levels of pRB phosphorylation and host-cell DNA synthesis, yet fail to induce cell division. These results imply that direct physical interaction with pRB is not required in the early stages (pre-S phase) of E1A-mediated cell division. Significantly, these results also imply that pRB phosphorylation occurring during S phase is not, of itself, sufficient to allow quiescent cells to divide. We infer from these results that the E1A products do not need to bind pRB in order to stimulate resting cells to enter the cell cycle. Indeed, a more important role of the pRB-binding activity of the E1A products may be to prevent dividing cells from returning to G_0.

The demonstration that the E1A products can induce expression of G_1 products independently of domain 2 (and domain 3) suggests that this activity can be expressed by the amino-terminal active site, which is linked with p300 binding. It is possible that induction of G_1 genes is a property of the amino-terminal active site, whereas pRB binding and the ability to induce progression from S phase through mitosis are properties of domain 2. However, we have found that deletion of either the amino-terminal region or domain 2 leaves the ability to induce *cdc2* expression and activity and to induce pRB phosphorylation intact, albeit with slower kinetics. Only a double mutant, combining mutations in both the amino terminus and domain 2, is devoid of these activities. These results imply that the ability to induce G_1 gene expression and pRB phosphorylation is a redundant function, a property of either the amino-terminal or the domain-2 active sites, whereas the mitogenic activity of E1A requires that both sites be functionally intact. These results suggest that p300 binding, like pRB binding, is linked both with induction of G_1 gene expression and with some function required for progression from S phase through mitosis. Our plans now are to identify and delineate the role of the p300 product. Preliminary progress toward that goal is described further below.

Use of E1A to Analyze Steps in the TGFβ1-induced Signaling Pathway

S. Abraham, M. Carter, E. Moran

Separation of the amino-terminal and domain-2 transforming functions has also provided compelling genetic evidence for a role of the retinoblastoma tumor suppressor gene in the pathway of growth inhibition induced by transforming growth factor β1 (TGFβ1). During the past year, we have found, in collaboration with Drs. Peter Howley and Hal Moses, that expression of the wild-type E1A products completely blocks the growth-suppressing effects of TGFβ1 in epithelial cells, including the ability of TGFβ1 to repress expression from the *myc* promoter. The ability to block TGFβ1-mediated repression of *myc* promoter activity is lost if the domain-2 pRB-binding function is inactivated in E1A. The ability to block the *myc*-suppressing effects of TGFβ1 appears to be a specific effect of domain 2, because this ability is not lost in an E1A amino-terminal deletion, even though this mutant has lost all ability to induce cell growth or transformation. These results imply that TGFβ1 may function by activating pRB, presumably by preventing pRB phosphorylation (possibly by decreasing *cdc2* kinase activity), and that E1A binding to pRB negates the effects of dephosphorylation. Suppression of pRB phosphorylation in the presence of TGFβ1 was demonstrated subsequently in Dr. J. Massague's laboratory, and supression of *cdc2* kinase activity in the presence of TGFβ1 has been reported recently from Dr. E. Leof's laboratory.

We are continuing to study the relationship between the TGFβ1-blocking activity of E1A and the ability of the E1A products to induce DNA synthesis. Our results suggest that E1A-mediated induction of $p34^{cdc2}$ kinase activity may play a role in the ability of E1A to counteract the effects of TGFβ1. In partial

contrast to the *myc*-CAT (chloramphenicol acetyltransferase) transient expression results, we have found that infection with domain-2 mutant viruses is sufficient to protect DNA synthesis activity substantially in TGFβ1-treated cells, implying indirectly that physical binding of pRB by E1A is not absolutely required for E1A to block pRB function. On the basis of the studies described immediately above, we postulated that E1A induction of *cdc2* kinase activity may be a means by which E1A can abrogate the effects of TGFβ1. We have found that E1A does indeed induce *cdc2* kinase activity in TGFβ1-sensitive cells. In the presence of E1A, high levels of kinase activity are maintained even in the presence of TGFβ1, whereas in the absence of E1A, *cdc2* kinase activity is sharply reduced by treatment with TGFβ1. To explore the mechanism of TGFβ1-mediated suppression of *cdc2* activity, we assayed *cdc2* expression levels. These results show that TGFβ1 suppresses *cdc2* expression as well as *cdc2* kinase activity; *cdc2* expression levels are maintained, however, if E1A is present during TGFβ1 treatment.

Expression of both wild-type and domain-2 mutant (non-RB-binding) E1A resulted in the maintenance of $p34^{cdc2}$ histone H1 kinase activity, as well as the maintenance of phosphorylation and synthesis of $p34^{cdc2}$, in the presence of TGFβ1. In addition, the domain-2 mutant virus was also capable of maintaining pRB in a phosphorylated state even in the presence of TGFβ1. These results suggest that adenovirus E1A may function both directly by sequestering pRB, preventing pRB activity, and by intervening in the natural cellular pathway by which TGFβ1 activates pRB. Either mechanism of intervention may be sufficient to overcome TGFβ1-mediated growth inhibition.

p300: Purification and Antibodies

P. Yaciuk, E. Moran

The results of our cell cycle studies imply strongly that the unidentified p300 cellular product plays a major role in the biological mechanisms by which E1A induces cell-cycle-specific gene expression and cell growth. The indication that other DNA tumor virus proteins, such as SV40 T antigen, encode a function biologically analogous to p300 binding supports the evidence that p300 is involved in a central control pathway. The identification and characterization of the E1A-associated p300 product are now a major goal of our laboratory.

Our goal of identifying and characterizing p300 has focused largely on obtaining specific antibodies that recognize this product. We have recently succeeded in raising high-affinity p300-specific rabbit polyclonal antiserum. The antiserum, raised against human p300, recognizes p300 in both human and rodent cells. Peptide digest analysis confirms that the 300-kD protein species recognized by the antiserum is authentic E1A-associated p300 (see Fig. 3). Comparison of antibody-bound p300 with E1A-associated p300 indicates that the majority of detectable p300 is associated with E1A in both newly infected cells and stably transformed cells. p300 is synthesized in both quiescent and proliferating cells and appears to be a fairly stable cellular protein with a half-life greater than 10 hours in either the presence or absence of E1A.

p300 is a phosphoprotein, and we find that it is actively phosphorylated in both quiescent and growing cells. However, in partially purified mitotic cell populations, we detect a form of p300 with decreased electrophoretic mobility, suggesting that there is an additional modification specific to mitotic cells. We have found a similar slower-migrating species coprecipitating from ^{32}P-labeled infected mitotic cells isolated either by nocodazole treatment, by mitotic shake off, or by elutriation, so this effect does not appear to be an artifact of drug treatment. We have also seen this form in all cell lines examined and in both the presence and absence of E1A expression. Analysis by silver-staining indicates that at least half of E1A-associated p300 is in the higher-molecular-weight form in mitotic cells. The mitotic-specific form of p300 is greatly reduced by treatment with potato acid phosphatase, implying that a phosphorylation event is involved in the generation of the mitotic-specific form. The mitotic-specific form of p300 shows a near-identical partial proteolytic digest pattern to p300 from unsynchronized cells. Significantly, however, we observe two additional peptide fragments that appear to be specific for the mitotic form of p300. The specific appearance of these peptide fragments in digests of the mitotic p300 form is reproducible, and we are continuing to investigate the nature of these peptides. We also plan to determine whether any biological or biochemical properties of p300 vary with its phosphorylation state.

The availability of specific antiserum represents an important tool that we can now use to probe ex-

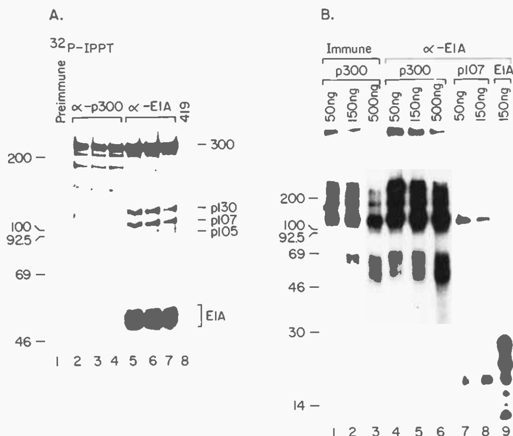

FIGURE 3 Analysis of p300-specific rabbit immune serum. (*A*) An immune bleed was withdrawn from a rabbit 2 weeks after a second injection of p300. ^{32}P-labeled 293 cell extracts were immunoprecipitated with preimmune serum (lane *1*), serum from the test bleed (lanes *2–4*), or E1A-specific monoclonal antibody supernatant (lanes *5–7*). An immunoprecipitate with control monoclonal antibody supernatant (pAb419) is shown in lane *8*. The positions of the E1A products, and the predominant E1A-associated products including p300, are shown on the right. Reactions were done in triplicate to provide material for V8 peptide digests. The immune serum specifically immunoprecipitates a phosphoprotein species migrating indistinguishably from the E1A-associated p300 phosphoprotein. (*B*) The 300-kD bands (lanes *2–7*) were cut out of the gel shown in *A* and subjected to V8 protease digestion. As a control, p107 and an E1A band were also excised and subjected to V8 digestion. (Lanes *1–3*) Digests of the immune-serum-precipitated bands; (lanes *4–6*) E1A-affinity-precipitated p300 bands. The amounts of V8 protease used in the reactions are indicated. The peptide pattern generated from the immune-serum-precipitated p300 species exactly matches that generated from E1A-affinity-precipitated p300. The control digests generate very different patterns.

pression libraries in order to obtain a cDNA clone corresponding to p300. Obtaining this clone will enable us to identify and sequence p300 and to probe its molecular function with a variety of approaches.

p300: DNA-binding Activity

Y. Rikitake, E. Moran

With the availability of purified p300, we have already begun to characterize the in vitro biochemical properties of this product. A variety of approaches to this question are possible, but the genetic evidence suggesting that p300 plays a basic role in the regulation of gene expression prompted us to concentrate on the possibility that p300 is a DNA-binding protein. We have now obtained a great deal of preliminary biochemical evidence suggesting that p300 does, indeed, bind to DNA with at least some degree of specificity. We have found that p300 binds double-stranded DNA in a nitrocellulose-binding assay. Gel-shift assays have confirmed the ability of p300 preparations to bind DNA, even in the presence of high levels of competing nucleic acid.

Analysis of total cellular protein extracts on DNA-cellulose columns indicates that a significant portion of p300 is retained specifically on the columns until the high-salt elution fractions, confirming that p300 has DNA-binding activity. Purified p300 shows similar salt-stable binding affinity on DNA-cellulose. p300 binds both single- and double-stranded DNA with similar efficiency. We are engaged in additional studies to determine whether there is a specific nucleotide recognition sequence involved in the interaction of p300 with DNA. To examine the sequence specificity of p300 binding to DNA, we selected possible binding sites for p300 using a polymerase chain reaction (PCR)-amplified oligonucleotide selection method. We synthesized a 50-bp oligonucleotide encoding terminal restriction enzyme recognition sites bracketing random sequences. This double-stranded oligonucleotide was chromatographed through a cyanogen bromide (CNBr)-linked p300-Sepharose column. p300 affinity-selected oligonucleotides were cloned and sequenced. Of 17 sequenced clones, 16 contained a sequence closely related to a 5-bp consensus sequence. p300 shows stronger DNA-binding activity with the affinity-selected oligonucleotides than with random sequence oligonucleotides in a nitrocellulose-filter-binding assay.

Although these results indicate that p300 DNA-binding activity is relatively nonspecific under less-stringent conditions, preferential binding to specific sequences can be detected under more-stringent binding conditions. We are continuing to explore the biological significance of the DNA-binding activity we observe in p300.

Fine-structure Analysis of the Amino-terminal Active Site and Conserved Region 1

M. Carter, E. Moran

One goal of our laboratory is to define the functional boundaries of the E1A amino-terminal active site by constructing and analyzing deletion mutations. Our deletion analysis, combined with others, has now restricted the boundaries of this active site to two regions, residues 1–25, and a noncontiguous segment from residue 36 to 76 that coincides with conserved region 1 (see Fig. 1). The surprising finding that the extreme amino terminus of E1A (outside the highly conserved sequences) is essential for transforming function has interesting implications. The localization of the amino-terminal active site allowed us to demonstrate, in agreement with other studies, that the amino-terminal function is independent of the RB-product-binding function and correlates with binding of the unidentified p300 product. The required region from residue 1 to 25 is linked with binding only p300, whereas the region from residue 36 to 76 appears to be involved in the binding of almost all E1A-associated proteins, including p300 and pRB.

The observation that the E1A region extending from residue 36 to 76 is involved in binding both the amino-terminal-binding protein, p300, and the domain-2-binding proteins that include pRB raises several interesting questions. One important question is whether p300 and pRB bind to the same, or different, residues within the region from 36 to 76. The deletion mutants that have been examined to date are not sufficiently specific to address this question. We have approached this question by constructing and analyzing smaller deletions and specific single missense mutations within the area of interest. This analysis is still in progress; however, substantial preliminary results indicate that p300 and pRB associate with distinctly different subregions of conserved region 1. Although we have not formally ruled out the possibility of some overlap in required binding sequences, it appears that the required pRB-binding region is limited to residues between 36 and 55, whereas the required p300-binding region is limited to residues between 55 and 73. Deletions or missense mutations that are confined to either of these subregions abrogate the binding of only one or the other of these two E1A-associated proteins. This conclusion is supported by analysis of the ability of specific monoclonal antibodies that bind to E1A within conserved region 1 to block the binding of p300 or pRB. A monoclonal antibody whose binding site lies within E1A residues 15–50 blocks E1A association with pRB but not with p300. Conversely, a monoclonal antibody whose binding site lies within E1A residues 68–85 blocks the binding of p300 but not pRB.

A second important question that arises from the observation that p300- and pRB-binding sites within conserved region 1 can be separated is whether conserved region 1 is required in E1A transforming functions for its p300-related binding activity or its pRB-related binding activity or both. Experiments designed to answer this question are now in progress.

Influence of E1A on E2F Cellular Transcription Factor Complexes

M. Carter, E. Moran [in collaboration with
P. Raychaudhuri, S. Bagchi, and J.R. Nevins,
Duke University Medical Center]

The generation of new and more-specific E1A mutations has also proved useful in a collaborative study done with Dr. Joseph Nevins aimed at understanding the significance of the ability of the E1A products to dissociate, or inhibit formation of, transcription complexes involving the E2F cellular transcription factor. The ability of E1A to activate E2F is independent of domain 3, raising the possibility that activation of this transcription factor may be related to E1A-mediated gene activation involved in cell cycle activation. We have examined the ability of a panel of E1A constructs expressing mutations covering the entire region upstream of domain 3 to influence the formation of E2F transcription complexes. We have found that E1A mutants with defects in conserved region 1 or conserved region 2 are inactive for E2F complex dissociation, whereas deletions within the amino-terminal region have no effect on the activity, nor do mutations localized between the two conserved domains.

Isolation and partial purification in the Nevins laboratory of a cellular factor designated E2F-BP, which associates with E2F to generate the E2F complex, have permitted a more-detailed analysis of the mechanism of E1A action. Mutations within both conserved region 1 and domain 2 impair the ability of E1A to dissociate the reconstituted E2F/E2F-BP complex. In contrast, if the E1A protein is added at the same time that E2F and E2F-BP are combined, only mutations within conserved region 1 block formation of the complex. In addition to E2F-BP, the Nevins laboratory has also found an activity designated E2F-I that inhibits E2F binding to DNA, again apparently through the formation of a complex with E2F. This inhibitory activity is also blocked by E1A, dependent on the same elements of the E1A protein that disrupt the interaction with E2F-BP. These results thus suggest a complexity of E2F interactions, at least one of which inhibits E2F function. Moreover, since the E1A sequences that are important for releasing E2F from these complexes are also sequences necessary for mitogenic activity, this activity may be a critical component of the cell-cycle-regulating activity of E1A.

SUMMARY

This year, we have made significant progress in our goal of analyzing the mechanisms of regulation of cellular products controlled in response to E1A expression. Elucidation of the pathways activated by E1A is giving us valuable insight into the mechanisms of cell growth control. A particular advantage of the E1A system is that the good genetics permit us to correlate the gene control pathways with specific functional domains of E1A. These functional domains, in turn, have already been linked by direct physical association with specific cellular products. At least in the case of pRB, these E1A-associated products have been implicated as important cell-growth control proteins. The availability of E1A mutants that induce S phase without progressing through cell division provides a special advantage in studying the cellular events that control progression through these later phases of the cell cycle. For example, these mutants have shown that pRB phosphorylation can be dissociated from progression through mitosis, and that a G_1 level of *cdc2* kinase activity can be detected in cells that do not enter mitosis or activate mitotic levels of *cdc2* kinase activity. The E1A mutants will facilitate more detailed study of the role of $p34^{cdc2}$ kinase, pRB, and other cell-growth-regulating proteins involved in the $S/G_2/M$ transitions. This information is basic to our long-term goal of understanding the mechanism of carcinogenesis.

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PROTEIN IMMUNOCHEMISTRY

E. Harlow

S. Allan, C. Bautista, K. Buchkovich, N. Dyson, B. Faha, M. Falkowski, A. Giordano, C. Herrmann, P. Guida, Q. Hu, E. Lees, J. Lees, C. McCall, L.-K. Su, L.-H. Tsai

The Protein Immunochemistry Laboratory is divided into two different sections. One section is a basic research group that has been studying the interaction of the transforming proteins of small DNA tumor viruses with the products of tumor suppressor genes, such as the retinoblastoma protein. The other section of the Protein Immunochemistry Laboratory is the Monoclonal Antibody Facility.

MONOCLONAL ANTIBODY FACILITY

The Monoclonal Antibody Facility is a service group that prepares and characterizes monoclonal antibodies for Cold Spring Harbor Laboratory scientists. The facility provides all of the reagents as well as the expertise for the production and characterization of hybridomas. When needed, the facility personnel can handle all of the steps in this process. Typically, antigens are prepared by scientists and then the facility staff does immunizations, test bleeds, fusions, screening, and single-cell cloning of the resultant hybridomas. Where appropriate, the Monoclonal Antibody Facility can be used as a central site for the production of hybridomas, e.g., when Cold Spring Harbor scientists who are skilled in the hybridoma fusions lack a suitable place for this work in their own laboratory. The facility is managed by Carmelita Bautista and staffed by Margaret Falkowski and Susan Allan. In the last year, they have prepared more than 100 different hybridoma lines, secreting antibodies specific for over 15 different antigens. The use of the various antibodies is discussed within the individual research reports in this volume.

PROTEIN IMMUNOCHEMISTRY LABORATORY

For the past several years, we have been studying transformation by the small DNA tumor viruses. Small viruses face a number of unique problems following infection of cells. One of the most challenging is how to use a limited amount of genetic information to reprogram the host-cell metabolism into an environment more suited for virus replication. To help initiate these changes, small DNA tumor viruses such as the adenoviruses, polyomaviruses, and papillomaviruses have evolved potent cell-cycle-modifying proteins. An analysis of the viral proteins that promote cell cycle changes indicates that they all share properties of potent oncogenes. During the last several years, some of the mechanisms used by these viruses to immortalize cells in culture have begun to be understood. It appears that one key mechanism used by these viral proteins to manipulate host cells is to bind to cellular proteins and alter their function. Perhaps not surprisingly, several of these cellular proteins are key players in the control of the eukaryotic cell cycle. The fact that these complexes exist and are relatively stable provides an experimental avenue to their study. Antibodies that are specific for members of these complexes can be used to isolate the associated proteins and to investigate their function.

Perhaps the best characterized of these viral/host protein complexes involves the adenovirus E1A proteins. The E1A polypeptides bind to a number of cellular proteins, known originally by their molecular weights of 300K, 130K, 107K, 105K, 90K, 80K, 60K, 50K, 40K, 33K, and 28K. Some of these proteins are known to interact directly with E1A, whereas others may bind indirectly. In many cases, identical or similar interactions have been detected with the large T antigens of the polyomaviruses as well as with the E7 proteins of the papillomaviruses.

Genetically, the same regions of E1A that are needed to bind to the cellular proteins are also those that are required for E1A to act as an oncogene. This suggests that these cellular proteins, either individually or collectively, are the cellular targets for the role of E1A in transformation. Although the functional significance of all of these interactions is not known, several lines of evidence suggest that these proteins are closely connected with the cell-cycle-controlling pathways of a cell.

Two of the cellular proteins are now known by name. p105 is the product of the retinoblastoma tumor suppressor gene, and p60 is the human cyclin A. The retinoblastoma gene is the best characterized of the tumor suppressor genes or anti-oncogenes. The protein products of these genes are thought to act as negative regulators of cell proliferation. Their loss during tumorigenesis removes a block to cell division and thereby indirectly contributes to the loss of growth control. Because E1A functions as a potent oncogene when introduced into primary cells and appears to act by association with proteins like pRB, it has been suggested that E1A inactivates the function of pRB by binding. The interaction and inhibition of pRB function would then mimic the loss of pRB in human tumors.

Another view of the role of E1A in transformation comes from consideration of the function of cyclin A. Cyclins are proteins whose levels oscillate dramatically through the cell cycle. They appear to play an important regulatory role in the cell cycle, mediated at least in part by physical association with cdc2. This interaction appears to modulate the activity of the cdc2 kinase in a cell-cycle-dependent manner. Cyclin A is one of the originally identified proteins of this class. It interacts with cdc2 to produce a kinase that is active in interphase cells. Why cyclin A appears in E1A-immune complexes is unknown at present.

It is convenient to divide our work into the following two major divisions: (1) the analysis of the retinoblastoma and related proteins and (2) the study of the control of the mammalian cell cycle. Our entry into both of these areas was the association of key proteins in these fields with the E1A proteins of adenovirus. An underlying goal of all our work is to learn how interactions with E1A affect the function of these cellular proteins.

The Retinoblastoma Protein Is Targeted by the Large T Antigens of Many Polyoma-Type Viruses

N. Dyson, P. Guida, E. Harlow [in collaboration with R. Bernards and S. Friend, Massachusetts General Hospital Cancer Center; L. Gooding, Emery University; J. Hassel, McMaster University; E. Major, National Institutes of Health; J. Pipas and T. VanDyke, University of Pittsburgh; A. Larose, M. Sullivan, and M. Bastin, Sherbourg]

The E1A proteins of adenovirus type 5, the large T antigen of SV40, and the E7 protein of human papillomavirus type 16 (HPV-16) all form stable complexes with the protein product of the retinoblastoma susceptibility gene. In each case, genetic studies have shown that regions of the viral protein that are necessary for association with the retinoblastoma protein (pRB) also contribute to transformation by the viral oncoproteins. The correlation between the loss of pRB binding and the loss of transforming ability suggests that the association with pRB is an important component in virus-mediated transformation.

Similar sequences can also be found in the large T antigens of other polyomaviruses. The large T antigens of all polyomaviruses are thought to perform similar roles during virus infections, and each has potent transforming or immortalizing properties when introduced into appropriate host cells. However, the oncogenic properties of these proteins vary considerably, both in tissue culture assays and in their association with naturally occurring malignancies. We were interested in determining the pRB-binding abilities of these viral proteins. Stable protein complexes between the large T antigens of mouse, monkey, baboon, or human polyomaviruses and the retinoblastoma protein were detected using an in vitro coprecipitation assay. All of the large T antigens tested were able to bind to both human and mouse retinoblastoma polypeptides, showing that these interactions have been conserved during evolution.

In collaboration with M. Bastin and his colleagues, we have investigated further the function of

the pRB-binding region of polyomavirus large T antigen. A series of mutants with small deletion or single amino acid substitutions were prepared in the two regions of the protein that show sequence similarity to conserved regions 1 and 2 of adenovirus E1A. The pRB-binding properties of the large T mutants were assessed with an in vitro coprecipitation assay. pRB binding was readily detected with wild-type large T antigen, but coprecipitation was completely abolished by a number of single amino acid substitutions (Asp-141 to Glu or Glu-146 to Asp) in region 2 of the polyomavirus large T antigen. Mutants defective in pRB binding were unable to immortalize primary rat embryo fibroblasts, suggesting that association with pRB is an important component of immortalization mediated by polyomavirus large T. The mutations in region 1 affected pRB binding only marginally, yet some of them severely impaired immortalization, indicating that for polyomavirus large T antigen, like other viral oncoproteins, pRB binding may be essential but not sufficient for deregulated growth.

A Short Region of Adenovirus E1A Provides Interaction with a Family of Cellular Proteins

N. Dyson, P. Guida

Adenovirus E1A will readily bind in vitro to cellular proteins isolated in detergent lysates. We have exploited this observation to investigate the physical parameters of the association of E1A with several host proteins and to compare the binding properties of homologous sequences from several viral oncoproteins. Synthetic peptides (prepared in Dan Marshak's laboratory at Cold Spring Harbor Laboratory) were designed to contain sequences that are predicted by genetic studies to be important for binding to pRB. Using E1A peptides, we have shown E1A contains at least two regions that make direct and distinct contact with pRB.

Peptides comprising the pRB-binding regions of E1A were assayed for their ability to compete with E1A for binding to cellular proteins. An E1A peptide containing amino acids 37–49 and 117–132 efficiently blocks binding between E1A and pRB. This same peptide inhibits the binding of several other cellular polypeptides in addition to pRB. This group of proteins includes p130, p107, and cyclin A. In a complementary assay, peptides representing regions of E1A were covalently bound to Sepharose beads. These peptide beads also were shown to interact with the same set of cellular proteins. Together, these data show that small regions of E1A are sufficient for protein association with the pRB, p107, p130, and p300 proteins.

We have performed similar experiments using peptides comprising SV40 large T antigen or HPV-16 E7 protein sequences that show homologies with the pRB-binding regions of E1A. The peptides from large T antigen or E7 generally behaved in manner similar to that of the E1A peptides, suggesting that the physical parameters that allow these viral oncoproteins to bind to cellular proteins, such as pRB, are conserved between these viruses. Nevertheless, these peptides did show several intriguing differences in their binding properties that are being investigated further: T-antigen peptides were able to bind to the E1A-associated protein p300, both E7 and T-antigen peptides showed a reduced affinity for cyclin A, and the T peptide, like the full-length protein, binds preferentially to the unphosphorylated forms of pRB.

The Adenovirus E1A-associated p300 Protein Binds to the Transformation-controlling Region of SV40 Large T Antigen

N. Dyson, E. Harlow [in collaboration with Jeff Morgan, John Ludlow, Nazanine Modjtahedi, and David Livingston, Dana-Farber Cancer Center]

Recent experiments carried out in collaboration with David Livingston's group suggest that the E1A-associated p300 proteins also interact with SV40 large T antigen. This interaction has been shown for protein complexes formed both in vivo and in vitro.

Both biochemical and genetic means have been used to identify the regions of large T antigen involved in complex formation. In the first, a synthetic peptide, containing amino acids 6–19 and 98–113 joined together through a glutamine (T;6-19-Q-98-113), was coupled covalently to beads and incubated in a lysate prepared from cells labeled with radioisotopes. The beads were washed, and the proteins that remained bound were analyzed on SDS-polyacrylamide gels; 300-kD proteins were observed that comigrated with the heterogeneous E1A-associated p300 bands. These proteins were not seen when beads carrying control peptides were used. A

comparison was made of the partial digestion patterns generated by these bands after proteolytic digestion with *Staphylococcus aureus* V8 protease. These patterns matched, indicating that this small portion of T antigen is sufficient for stable binding to p300. In complementary experiments, T-antigen peptides were used to compete the interaction of ^{32}P-labeled p300 proteins from human cell lysates with E1A provided from a cold cell lysate. Large T antigen peptides containing as little as 102–115 amino acids of wild-type sequence were able to block the E1A-p300 binding completely. Amino acids 121–134 of E1A are highly homologous to amino acids 102-115 of T antigen, but we have been unable to show that these E1A sequences interact with p300, even though both regions efficiently bind to pRB and p107.

Genetic data support the idea that the region of SV40 large T antigen that interacts with pRB is also essential for the association of large T antigen with p300. Stably expressed mutants of T antigen that delete this region of T fail to bind to p300. These data suggest that in addition to its association with adenovirus E1A, p300 is also able to bind to SV40 large T antigen. In both cases, p300 binds to regions of the viral proteins that are essential for transformation activity. Thus, binding to p300 may be a further common mechanism used by these viral oncogenes to transform cells. An unexplained feature of these results is that E1A and SV40 large T antigen seem to be using different structures to bind to p300.

Monoclonal Antibodies Specific for the Retinoblastoma Protein Identify a Family of Related Proteins

Q. Hu, C. Bautista [in collaboration with Gwynneth Edwards, Deborah Defeo-Jones, and Raymond Jones; Merck, Sharp, and Dohme Research Laboratories]

To help in the analysis of the retinoblastoma protein (pRB), we have prepared a new set of anti-human pRB monoclonal antibodies. Previously, we mapped the regions of pRB that allow binding to adenovirus E1A or SV40 large T antigen. The sequences needed for binding consist of two distinct regions of pRB, the first from amino acid 393 to 573 and the second from 646 to 772. These regions appear to form an independent structural domain, as even small deletions across any of the boundaries destroy the ability of E1A or large T antigen to bind. More importantly, this region also appears to be important in pRB function as 23 of the 25 naturally occurring pRB mutations identified to date alter sequences in these regions. This is true even for missense or short in-frame deletions, all of which map to these regions.

A carboxy-terminal fragment of pRB that contains the binding regions was cloned and overexpressed in bacteria. This pRB fragment was purified and used to immunize BALB/c mice. Approximately 30 hybridomas that secrete antibodies specific for this region of pRB were isolated and characterized. In addition to being useful reagents for the study of human pRB, these antibodies display several unexpected properties. All of the antibodies were able to recognize the native forms of pRB, although some had preference for the status of pRB phosphorylation. Antibodies, such as XZ77 and XZ91, precipitated all the forms of pRB, whereas others, such as XZ19 and XZ55, preferred the underphosphorylated forms of pRB. In contrast, antibodies, such as XZ121, were only able to recognize unphosphorylated pRB. These preferences were also seen in immunoblot analysis.

Some of the antibodies were able to recognize pRB homologs in other species, including mouse, rat, chicken, and *Xenopus*. The identity of pRB in these other species was confirmed by several methods. In several cases, cDNAs for pRB are available. When a cloned gene for the homolog was not available, we identified pRB candidates by mixing with E1A or large T antigen. Such a high degree of structural conservation between pRB from these species suggests that pRB is likely to play a similar role in many vertebrate species. Since pRB function appears to be critical for the control of division in many human cells, similar key roles can be expected in all vertebrates.

Genetic studies have indicated that E1A uses similar regions to target several cellular proteins, including p300, p130, p107, and pRB. Therefore, it is possible that these cellular proteins share some structural similarities, at least in the E1A-targeted regions. Using the XZ antibodies, we have shown that at least one antibody, XZ37, can directly recognize both pRB and p107. More surprisingly, a second antibody, XZ77, can directly recognize both pRB and p300. Although the properties of p107 and pRB had suggested that they might be structurally related, there was little indication that p300 and pRB also shared homology. These results suggest that pRB may be one member of a family of cellular proteins that show at least limited homology, particularly in the E1A/large T antigen interaction domains.

The Retinoblastoma Protein Binds to Human cdc2

Q. Hu, J. Lees, K. Buchkovich

Using a new panel of antibodies specific for the retinoblastoma protein (pRB), we have investigated the biochemical properties of pRB and its counterparts in human cells. Immune complexes prepared with some of these antibodies contained a potent kinase activity. The kinase was able to phosphorylate pRB itself as well as histone H1 when it was added as an exogenous substrate. Since this kinase was detected by immunoprecipitation with anti-pRB antibodies, there were three possible ways to account for pRB phosphorylation. First, pRB itself might be the kinase. This seemed unlikely as the pRB sequence did not show any of the characteristic hallmarks of a kinase. Second, the anti-pRB antibodies might cross-react to another cellular protein that either was a kinase itself or was bound to a kinase. Third, the kinase might be bound to pRB and precipitated by virtue of this association. To distinguish between the second and third possibilities, we tested whether the kinase activity copurified with pRB. Using glycerol gradients, we found that the pRB kinase activity could not be separated from pRB itself, supporting the notion that pRB was bound to the active kinase. This was further tested by analyzing cell lines that carry a homozygous deletion of the retinoblastoma gene. In these cells, no histone H1 kinase activity could be immunoprecipitated with the pRB antibodies. Again, these experiments suggest that the kinase activity is closely linked with the presence of pRB. Finally, we tested whether the associated phosphorylating activity had any characteristics of known kinases. Immunoblot analysis showed that pRB immunoprecipitations contained the human cdc2 kinase or a closely related kinase. In addition, in vitro kinase assays performed with anti-cdc2 antibodies contained a phosphorylated band of pRB. Together, these results indicate that the pRB forms a complex with a subset of the cdc2 kinase from human cells.

When the in-vitro-phosphorylated pRB sites were compared by two-dimensional tryptic peptide mapping, it was found that the sites that are phosphorylated in vitro were a subset of the sites that were phosphorylated in vivo. This suggests that the associated kinase activity represents an authentic pRB in vivo event and a potential regulatory step. In preliminary experiments, we have tested the cell cycle regulation of the associated activity and have shown that the associated kinase is found in all stages of the cell cycle in which pRB is naturally phosphorylated. These data in conjunction with the data of Lees et al. (see the following report) strongly implicate the cell-cycle-controlling kinase, cdc2, as a major regulator of pRB function.

The Retinoblastoma Protein Is Phosphorylated by cdc2

J. Lees, K. Buchkovich [in collaboration with
Carl Anderson, Brookhaven National Laboratory, and
G. Binns and D. Marshak, Cold Spring Harbor Laboratory]

Although the retinoblastoma tumor suppressor gene product is postulated to inhibit cellular proliferation, it continues to be expressed in actively dividing cells. When the levels of pRB are analyzed throughout the cell cycle, little change is seen at the various stages. However, a number of studies have shown that pRB becomes phosphorylated on transition from the G_1 to S phase of the cell cycle, suggesting that the tumor suppressor function of the retinoblastoma protein is regulated by phosphorylation and that the unphosphorylated form is the active repressor. To understand the significance of these phosphorylation events better, we have begun to study the kinases that are responsible for these phosphorylations and to determine the exact amino acids that are phosphorylated. One of the first kinases that was tested for its ability to phosphorylate pRB in vitro was the human cdc2 kinase. cdc2 kinase was purified from human cells arrested at mitosis with nocodozole and was kindly provided by Jim Bischoff, Leonardo Brizuela, and David Beach (Cold Spring Harbor Laboratory). This preparation of purified cdc2 was able to phosphorylate pRB in vitro, and two-dimensional tryptic maps showed that the pattern of phosphorylation is very similar to that found in vivo. To determine which residues were phosphorylated in vivo, a strategy that compares potential sites in vivo with sites phosphorylated in vitro was employed. Synthetic peptides representing all serine or threonine residues followed by a proline (representing the weakest cdc2 consensus phosphorylation sequence) were prepared and phosphorylated by the purified cdc2 kinase. These peptides were then digested with trypsin and compared to the authentic in vivo proteolytic fragments. Comigration of the spots in

two dimensions was taken as strong evidence that the site phosphorylated in vitro corresponded to the same site in vivo. To identify the correct residue in the digested peptides, the phosphorylated trypsin digestion products were purified, their mass was determined by spectroscopy, and the position of the labeled phosphate group was located by sequencing. From this type of analysis, five sites on pRB that are phosphorylated in vivo have been identified. All are surrounded by sequences that fit well with the cdc2 consensus phosphorylation sequence. These results, together with those of Hu et al. (see above) showing that pRB is physically associated with cdc2, suggest that either cdc2 or a cdc2-like kinase acts to regulate pRB in vivo.

Adenovirus E1A Associates with a Serine/Threonine Kinase

C. Herrmann, L.-K. Su, E. Harlow

Many proteins are regulated during physiological changes by phosphorylation. The addition or removal of a phosphate group is a common mechanism used in all cells to regulate key proteins. Many of the proteins that bind to E1A, as well as E1A itself, are phosphorylated when isolated from cells metabolically labeled with ^{32}P orthophosphate. Several years ago, we noticed that immunoprecipitations prepared with antibodies to E1A contained an active protein kinase activity. In these experiments, anti-E1A antibodies are used to isolate E1A and its associated proteins from lysates of cells. The immune complexes are then incubated with appropriate buffers containing γ-labeled ATP. Associated kinases are scored by the transfer of the labeled phosphate group to any of the E1A-bound polypeptides or to an exogenously added substrate. The target of these phosphorylations are the E1A-associated 107K and 130K proteins.

Our initial experiments have been directed at determining which of the proteins in the immune complexes might be the active kinase. Although we do not yet know which kinase is responsible for the phosphorylation, its association with E1A has provided us with an easy assay to characterize the kinase activity. The optimal conditions for the kinase reaction have been established, and we have begun to determine whether the kinase activity can be attributed to any of the well-characterized E1A-associated proteins. This has been done by studying E1A mutants that can interact with only a subset of the cellular proteins. By choosing mutants that fail to bind to a particular cellular protein and assaying for associated kinase activity, we can eliminate various E1A-bound proteins as candidates for the kinase. In addition, we have been able to prepare specific antibodies for some of the cellular proteins that bind to E1A. These antibodies can be used to preclear lysates of all immunoreactive proteins prior to the kinase assay. This also has been used to eliminate certain proteins as candidates for the kinase. Together, these experimental approaches have shown that the kinase cannot be the retinoblastoma protein or the E1A-associated p300 polypeptide. These assays also indicate that the p130 or p107 proteins are unlikely to be responsible for the kinase activity.

Using E1A as a molecular handle to purify and study this kinase, we have also examined the cell cycle control of the associated kinase. The levels of the substrate 107K and 130K proteins do not change throughout the cell cycle, whereas the kinase activity shows dramatic changes. The associated kinase activity appears to be maximally activated during the transition between the G_1 and S phase. With some protocols, we have seen a low level of kinase activity in G_1, but with other protocols, the activity is specifically activated when cells cross the G_1/S boundary. Further work will be needed to characterize this kinase fully, but recently, A. Giordano et al. (Molecular Genetics of Eukaryotic Cells Section) have shown that E1A associates with a member of the cdc2 kinase family. This protein then would become an excellent candidate for the E1A-associated kinase activity characterized here. Giordano et al. have also seen similar properties of this kinase, further indicating that both groups have been studying the same kinase activity. Although it is still not certain, this kinase is probably one of the kinases described by B. Faha (see below).

Structure/Function Studies of Human Cyclin A

E. Lees

One of the cellular proteins that is found in immunoprecipitations with anti-E1A antibodies is human cyclin A. Originally, this protein was known

only by its molecular weight of 60,000. Two sets of results have helped this protein gain an identity. First, A. Giordano and others at Cold Spring Harbor Laboratory were able to show that the E1A-associated p60 protein was associated with a cdc2 or cdc2-like kinase in cells that do not have adenovirus E1A. Then, Pines and Hunter (*Nature 346:* 760 [1990]) cloned the human cyclin A and went on to demonstrate that it was identical to the E1A-associated p60 protein. We have begun to study the structure/function relationships of human cyclin A in some detail. Using an in vitro mixing technique, we have begun to map the binding site on cyclin A for cdc2. These experiments have been done in two ways. In the first approach, wild-type cyclin A or mutated cyclin A is prepared in vitro by translation in rabbit reticulocyte lysates and then mixed with a cold cell lysate as a source of cdc2. Any complexes that form are recovered with anti-cdc2 antibodies or with a cdc2 adsorbent, p13 beads. Using these reagents, we have shown that it is possible to remove the amino-terminal 173 amino acids without affecting the ability of cyclin A to bind to cdc2. Analysis of carboxy-terminal deletions showed that only 12 amino acids could be removed and still retain binding. However, internal deletions that removed amino acids 241–275 allowed good binding. Somewhat surprisingly, we have shown that in the presence of this internal deletion, it is possible to remove further amino- and carboxy-terminal sequences without abolishing binding. This study suggests that two small regions, 173–241 and 275–320, are sufficient to bind to cdc2. We are not yet certain why this internal deletion allows further deletions at the amino and carboxyl termini to bind more efficiently. In the second experimental approach, we have tested the same cyclin A mutants for their ability to interact with a bacterially expressed fusion protein between glutathione-*S*-transferase (GST) and the human cdc2-coding region. This fusion protein binds tightly to cyclin A mutations. We are currently analyzing which mutants can bind the GST-cdc2 fusion construct.

To compare the structural characteristics of these interactions with their biological functions, we have shown that human cyclin A can cause maturation of the *Xenopus* oocytes when RNA for cyclin A is microinjected. In preliminary experiments, any mutant that can bind tightly to cdc2 retains the ability to stimulate maturation of the oocytes. As the binding characteristics of the cyclin A mutants become more clear, we will continue to examine this correlation.

Cyclin A Forms Complexes with Several Cellular Proteins

B. Faha

We have investigated the cellular proteins that coprecipitate with cyclin A in the absence of adenovirus E1A using a new panel of monoclonal antibodies specific for cyclin A. One of the proteins that is bound to cyclin A is the E1A-associated p107 protein. The complex between cyclin A and p107 occurs in cells whether or not E1A or the retinoblastoma protein is present. The association between E1A and p107 has been shown to be important for oncogenic transformation by adenoviruses. In addition, p107 has been shown to associate with the large T antigen of SV40 and JC viruses. The significance of the association of cyclin A with p107 has yet to be determined. However, the identification of p107 in anti-cyclin A immune complexes suggests that it may play a role in the regulation of the cell cycle.

We have also shown that cyclin A associates with the human cdc2 protein and p33, a protein originally described by Pines and Hunter (*Nature 346:* 760 [1990]) and Giordano et al. (Molecular Genetics of Eukaryotic Cells Section). Comparison of these bands with authentic human cdc2 (either precipitated by anti-cdc2 antibodies or translated in vitro from the human cdc2 cDNA) by V8 partial proteolysis has shown that the two slower-migrating bands are authentic cdc2. The retarded migration of these bands has been shown to be due to phosphorylation. The fastest-migrating band, p33, has a V8 partial proteolytic pattern distinct from that of cdc2. The p33 proteins associated with cyclin A are phosphorylated on threonine and tyrosine. The p33 band is also found in anti-E1A immune complexes, as first described by Giordano et al. However, we have not been able to detect any cdc2 in the E1A immune complexes. It is unknown whether these proteins, p107, cdc2, and p33, are part of one complex or whether they constitute separate interactions with cyclin A.

Cloning of Multiple cDNAs That Encode Human cdc2-like Kinases

L.-H. Tsai [in collaboration with Matthew Meyerson, Massachusetts General Hospital Cancer Center]

Recently, several *cdc2*-related genes have been cloned from *Schizosaccharomyces pombe*, *Droso-*

phila, Xenopus, and mammalian cells. Although these genes encode proteins that share close sequence homology with *cdc2*, most fail to rescue null mutants of *cdc2* in *S. pombe* or *CDC28* in *Saccharomyces cerevisiae* and thus are postulated to perform a distinct function during the cell cycle. One cdc2-related kinase in which we have become particularly interested is known as p33 (see report by B. Faha above). p33 is bound to cyclin A and is structurally related to cdc2, but represents a distinct family member. In an attempt to isolate a cDNA for p33, we performed polymerase chain reaction amplification of human cDNA, using degenerate oligonucleotides derived from conserved regions of *cdc2* genes. Using this strategy, we have cloned a number of unique *cdc2*-like DNA sequences from the human cell lines HeLa, NALM-6, T84, and RAC. All of these clones encode protein sequences that are characteristic of protein kinases. A computer search of GenBank reveals that these genes are more similar to the *cdc2/CDC28* class of genes than to other kinases. Some of them contain *cdc2*-specific motifs such as the PSTAIR region. We are currently characterizing these clones and are particularly interested in determining whether any of them are capable of encoding the cyclin-A-associated p33 kinase.

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PROTEIN CHEMISTRY

D.R. Marshak W.B. Benjamin G.L. Russo
G.E. Binns M.T. Vandenberg
H. Mehmet I.J. Yu
M.R. Meneilly

The Protein Chemistry Laboratory is engaged in research pertaining to the structure and function of proteins involved in fundamental mechanisms of normal and abnormal growth of cells. We use advanced instrumentation such as protein sequencers, mass spectrometers, and peptide synthesizers to take a biochemical approach to the analysis of proteins and, particularly, in the analysis of modifications on proteins. One class of modifications, the phosphorylation of serine, threonine, and tyrosine residues, and the enzymes that catalyze them, protein kinases, appears to modulate many of the regulatory mechanisms in cellular growth.

Mapping Phosphorylation Sites on Nuclear Oncoproteins

D.R. Marshak, M.T. Vandenberg, G.E. Binns

During the past year, our work on mapping phosphorylation sites has concentrated on the human recessive oncogene products p53 and RB (retinoblastoma). As part of this work, we have assembled a spectrum of protein kinases to use for in vitro phosphorylation of the overexpressed proteins. These kinases include casein kinases I and II (CK-I and CK-II); protein kinase C (PK-C); cAMP-dependent protein kinase (PK-A); myosin-light-chain kinase (MLCK); calcium-calmodulin kinase II (CaM K-II); *raf* kinase (raf); p34^{cdc2}; glycogen synthase kinase 3 (GSK-3); phosphorylase *b* kinase; and pp60$^{c\text{-}src}$ and pp60$^{c\text{-}src(+)}$. These protein kinases are enzymes that catalyze the transfer of phosphate from adenosine triphosphate to the hydroxyl moiety of serine, threonine, or tyrosine residues. The recessive oncogenes refer to human cellular genes that give rise to tumors in which both alleles of the gene are mutated, usually by partial deletion. Normally, such genes are thought to play a role in suppressing and regulating nuclear processes that would lead to abnormal cell growth and tumorigenesis. The recessive oncogene products p53 and RB are phosphoproteins with apparent molecular weights of 53K and 105K, respectively. It has been only in the last few years that the concept of recessive oncogenes has changed the paradigm under which research in the biology of cancer is performed. Understanding the mechanisms by which these recessive oncogene products control cell proliferation and are themselves regulated by phosphorylation is the central goal of this research.

The focus of the phosphorylation work has been to uncover the sites of modification by p34^{cdc2}, the cell-division-cycle-regulated protein kinase that is critical to the G_1/S transition and the onset of mitosis in eukaryotic cells. The phosphorylation site on p53 was determined by isolating the peptide from the phosphorylated protein and subjecting it to automated Edman degradation on an Applied Biosystems 473A protein sequencer. By collecting the reaction products of each cycle, the amino acid residue containing the radioactive phosphate was determined. Figure 1 shows a plot of the radioactivity recovered from each cycle of the analysis. The site of phosphorylation turned out to be the serine residue 315 in the sequence Lys-Arg-Ala-Leu-Pro-Asn-Asn-Thr-Ser-Ser-Ser-Pro-Gln-Pro-Lys-Lys. To confirm this assignment further, a synthetic peptide containing the native sequence of the p53 site was synthesized by solid-phase methods, phosphorylated with p34^{cdc2}, and subjected to two-dimensional peptide maps. The phosphorylated synthetic peptide migrated to exactly the same position as that of the protein phosphorylated in vivo. These studies demonstrated that this cell-cycle-regulated protein kinase, p34^{cdc2}, phosphorylates the recessive oncogene product, p53, on serine both in vivo and in vitro. However, it is not yet clear what the physiological consequences of this modification are. One intriguing possibility is based on the observation that the p34^{cdc2} site in p53 is adjacent to the nuclear localization sequence in the protein, exactly as in the case of the SV40 large T antigen protein. Last year, similar biochemical analyses demonstrated the phosphorylation of T antigen by p34^{cdc2} and the effects of this phosphorylation to stimulate DNA replication in vitro. We suspect that

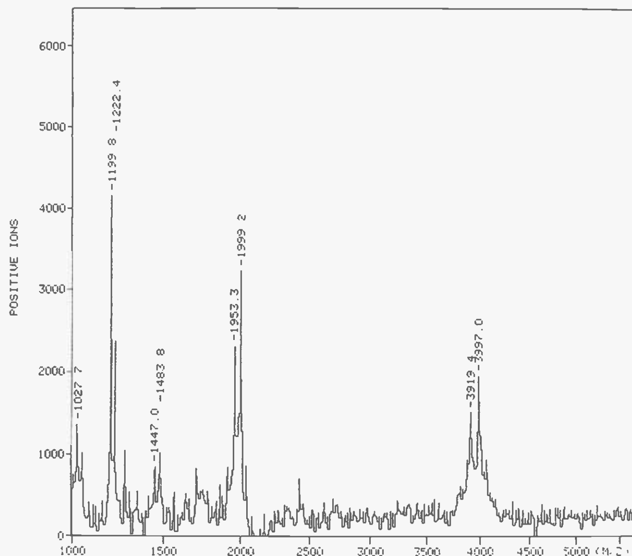

FIGURE 1 Radioactive phosphate recovered from automated Edman degradation of a synthetic peptide substrate for p34^{cdc2} based on the recessive oncogene p53 sequence. Data taken from Bischoff et al. (1990).

the role of p53 in DNA replication might be affected by the analogous phosphorylation by p34^{cdc2}. Further effort in this area is in progress in collaboration with C. Prives (Columbia University) and D. Beach (Cold Spring Harbor Laboratory).

Phosphorylation of the retinoblastoma gene product, RB, by the cell-cycle-regulated protein kinase, p34^{cdc2}, has been studied in collaboration with Ed Harlow, Jackie Lees, and Karen Buchkovich (Cold Spring Harbor Laboratory). The approach to this project has been somewhat different. RB is a very large protein that has a molecular mass of more than 100,000 daltons and dozens of potential phosphorylation sites. We chemically synthesized peptides corresponding to sites in the carboxy-terminal domain of RB that contain several potential targets for the kinase. The consensus phosphorylation sites contain either serine or threonine, followed by a proline, two neutral residues, and one or more basic residues. Although it is not a strict requirement, this consensus Ser/Thr-Pro-Xxx-Xxx-Lys/Arg is very often followed in nuclear oncogene products. We have used advanced instrumentation, including mass spectrometry, to analyze the synthetic peptides and to compare their phosphorylations with those of the protein in vivo. Mass spectrometry measures the precise molecular weight of the peptide fragment containing the phosphate modification, calculated as a mass-to-charge ratio (m/z). Compared to the unmodified peptide, the phosphorylated peptide is 80 atomic mass units (amu) larger for each phosphate group attached. Because the resolution of the spectrometer is about 2 amu, the addition of phosphate is very easy to identify. With one charge ($z = 1$), the difference in mass should be 80, whereas charges of 2 or 3 should produce differences of 40 and 27, respectively. Figure 2 shows one of these RB synthetic peptides following phosphorylation with p34^{cdc2}. The peaks at m/z = 3997, 1999, and 1222 correlate well with the expected peaks for the singly,

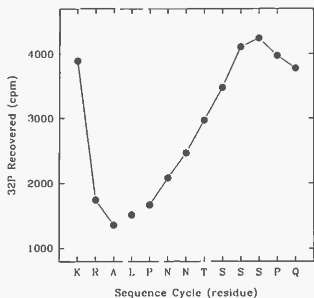

FIGURE 2 Partial mass spectrum of a peptide based on the RB sequence after phosphorylation with p34^{cdc2} in vitro.

doubly, and triply charged (z = 1, 2, or 3) species of the phosphorylated peptide. In each case, the peak below represents the unphosphorylated peptide. This project exemplifies our application of state-of-the-art biochemical approaches to questions of paramount biological interest.

Phosphorylation of Transcription Factors

D.R. Marshak, G.E. Binns, G.L. Russo, M. Vandenberg, I.J. Yu

Transcription factors are multiple families of proteins that either bind to regulatory elements of genes or interact with protein complexes, which, in turn, promote gene transcription. Many of these transcription factors appear to be phosphorylated, mainly by attachment of phosphate to serine and threonine residues, catalyzed by several candidate nuclear protein kinases. Of these, we have studied casein kinase II, p34^{cdc2}, glycogen synthase kinase 3, protein kinase C, and cAMP-dependent protein kinase. Using this panel of enzymes, our strategy has been to phosphorylate transcription factors in vitro and to test how individual phosphorylation events might alter the ability to bind to the appropriate DNA element or to direct transcription. To this end, we require purified proteins produced in relatively large amounts, unlike the vanishingly small quantities of natural products. Expression of the cDNAs for these proteins in bacteria or in insect cells is necessary to obtain sufficient quantities of protein for our biochemical analyses.

We have continued to study regulatory proteins involved in the control of transcription of the c-*fos* gene. Two regulatory elements have been defined by M. Gilman and his colleagues (Cold Spring Harbor Laboratory) as the serum response element and the cAMP responsive element, and the corresponding proteins that bind to these elements have been termed the serum response factor (SRF) and the cAMP element binding protein (CREB). The phosphorylation of SRF by CK-II appears to increase the binding affinity of the factor to its cognate DNA sequence. This result has been slightly confusing, since the time course of CK-II induction on serum stimulation follows that of induction of the c-*fos* gene transcription. Thus, the physiological role for CK-II phosphorylation ought to be in the turn-off of c-*fos* transcription. However, increased binding affinity for DNA has been generally assumed to be associated with a turn-on of gene transcription. Therefore, further studies of the exact mechanism of SRF action at the c-*fos* serum response element are necessary to sort out this quandry.

In another project related to gene transcription, we have collaborated with J. Kadonaga at the University of California, San Diego, on the structural analysis of a transcriptional repressor. This repressor activity was identified in the supernatant of an ammonium sulfate precipitation of the basal transcription mixture. In this fractionated in vitro transcription mixture, basal rates of transcription are high, but sequence-specific factors do not activate the rate of transcription. The repressor lowers the basal rate of transcription and restores the abilities of several sequence-specific DNA-binding proteins to activate the system. Because the repressor activity possessed the ability to rejuvenate previously inactive mixtures of transcriptional activators, the factor was given the operational nickname Elvis, to commemorate the deceased singer who is reported to reappear in odd places by the popular press. The goal of this project has been to determine the structure of Elvis. Following purification, the protein was digested with proteases, and the fragments were isolated by chromatography. Each fragment was analyzed by sequencing, amino acid analysis, and mass spectrometry. Upon collating all the data, Elvis was found to have a primary structure identical to that of histone H1, as shown in Figure 3.

This was an exciting finding because it suggested that the mechanism of transcriptional activation, in

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1
S D S A V A T S A S P V A A P P A T V E K K V V Q K K A S G
31
S A G T K A K K A S A T P S H P P T Q Q M V D A S I K H L K
61
E A G G S S L L A I K K Y I T A T Y K C D A Q K L A P F I K
91
K Y L K S A V V N G K L I Q T K G K G A S G S F K L S A S A
121
K K E K D P K A K S K V L S A E K K V Q S K K V A S K K I G
151
V S S K K T A V G A A D K K P K A K K A V A T K K T A E N K
181
K T E K A K A K D A K K T G I I K S K P A A T K A K V T A A
211
K P K A V V A K A S K A K P A V S A K P K K T V K K A S V S
241
A T A K K P K A K T T A A K K
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FIGURE 3 Sequence analysis of the basal transcriptional repressor, histone H1. The heavy lines represent portions sequenced by chemical means, and the light lines indicate sequences confirmed by mass spectrometry. Data taken from Croston et al. (1991).

many cases, is not by true activation of a basal activity, but by antirepression. By analogy, one can think of it in the same way as we turn on lights, either from an on/off switch or from a dimmer switch. True activation in the absence of Elvis would be analogous to turning up the lights in a room with a dimmer switch, progressing from low levels to high levels of light. On the other hand, antirepression, mediated by Elvis, is analogous to turning on the lights in a room that is initially dark because the switch is off. In other words, Elvis keeps the system off, just as the switch keeps the lights completely off, and a sequence-specific transcription factor turns the system on by antagonizing Elvis, just as the new occupant of a room would "antirepress" (i.e., turn on) the light switch. It is clear from this analogy that the antirepression mechanism in theory allows the mechanism of transcriptional activation to be an on/off proposition, rather than having continuously intermediate levels of action.

Role of Casein Kinase II in Cell Growth

I.J. Yu, H. Mehmet, D.R. Marshak

We are continuing our studies of CK-II in cell growth and viral transformation of cells. In two different cell systems, a human lung fibroblast cell line (WI38) and a human epithelial carcinoma cell line (HeLa), increases in CK-II activity appear to be correlated with cell growth phenomena. Initial studies on cell cycle control of CK-II in HeLa cells indicate that CK-II activity is high in the G_1 phase of the cell cycle and decreases during S phase to very low levels. In mitosis, CK-II is reactivated to approximately 30% of the G_1 levels. These cyclic patterns of CK-II activity are consistent with our previous observations of CK-II stimulation early in serum activation of fibroblasts. H. Mehmet worked as a visiting scientist in our laboratory for 3 months, introducing the Swiss 3T3 fibroblast cell line to these studies. The signal transduction pathways in these cells have been dissected by Drs. Mehmet and Rozengurt in London, and the role of CK-II in those systems was investigated. No clear results have been obtained, but the response appears to be different from earlier work in epithelial cells, suggesting that protein kinase C activation was positively linked to CK-II, and the cAMP pathway was negatively linked. These studies point to the possibility that fibroblasts and epithelial cells may utilize CK-II in different parts of their signaling mechanisms.

To investigate the cell cycle regulation of CK-II further, Il Je Yu has raised polyclonal antibodies that recognize specific epitopes on various subunits of the enzyme. The structure of CK-II is highly conserved among eukaryotes, and we had trouble developing high-titer useful antibodies to the intact purified holoenzyme. Polyclonal antibodies to holo-CK-II reacted poorly on Western blots, and all attempts to produce mouse monoclonal antibodies to the bovine protein failed. Therefore, we synthesized peptides representing epitopes on each of the α, α', and β subunits. These were coupled to either keyhole limpet hemocyanin or ovalbumin with maleimidobenzoyl-*N*-hydroxysuccinimide. Antisera were raised in rabbits that react with the individual subunits of CK-II. This has been demonstrated on immunoblots of whole-cell extracts of purified CK-II. In summary, we have antibodies—specific for α (the catalytic subunit) and specific for α' (an alternate form of the catalytic subunit)—that react with both α and α' at different epitopes and that react exclusively with β (the regulatory subunit). The antibodies were used to localize CK-II subunits in asynchronous HeLa cells. The α and β subunits stain cytoplasm, with little or no staining of the nucleus. However, antibodies that react with α' as well as α stain the nucleus and cytoplasm. In addition, the antibodies that recognize α' fail to stain 18% of the asynchronous cells' nuclei. After treatment of cells with hydroxyurea to

arrest at the G_1/S transition, 5% of the cells are negative for α' in their nuclei. Upon release of the hydroxyurea block, there is an increase of α'-negative nuclei during the first 4 hours, followed by diffuse staining for all of the subunits when the nuclear envelope breaks down in prophase of mitosis. Following mitosis, the original G_1 distribution of CK-II subunits is reestablished. To localize CK-II in HeLa cells during mitosis, we have used immunoelectron microscopy because the immunofluorescence patterns were not interpretable due to diffuse staining patterns. At the electron microscopic level, it is clear that the α subunit decorates the spindle fibers of the mitotic apparatus as well as other nuclear structures in metaphase and anaphase. Figure 4 shows a representative photograph of HeLa cells in metaphase and anaphase, indicating immunoreactivity of CK-II α on spindle fibers. The chromosomes are clearly negative, and there is diffuse staining in other parts of the cells.

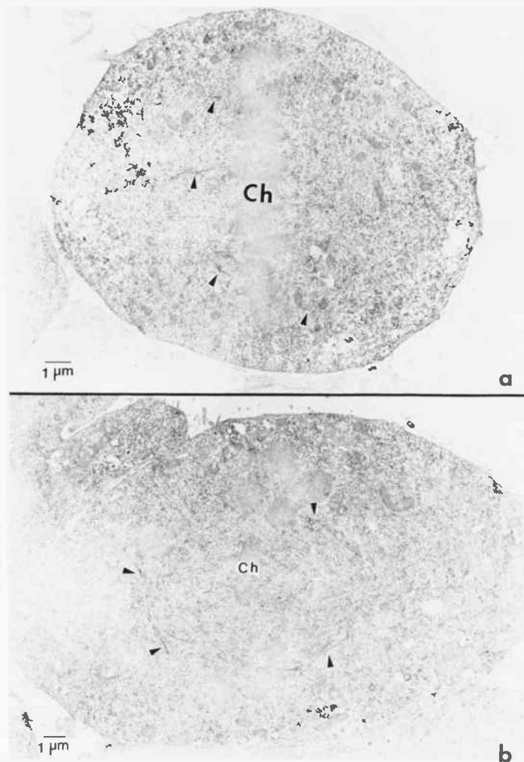

FIGURE 4 Electron micrographs of HeLa cells in mitosis immunostained using an antibody to casein kinase II α subunit. (Panel *a*) Cell in metaphase; (panel *b*) cell in anaphase. Arrowheads point to spindle fibers. (Photos courtesy of I.J. Yu and D. Spector.)

Our working hypothesis is that the α′ subunit is the major nuclear form of the enzyme during G_1 and that it must exit the nucleus at the onset of S phase to allow DNA replication. The change in localization of α′ during S phase is concomitant with the decrease in activity found in extracts of elutriated cells from S phase. We are continuing to investigate the role of CK-II in DNA replication, possibly as an antagonistic activity. The activity of CK-II is to a large extent reciprocal in level to $p34^{cdc2}$; i.e., CK-II is high in G_1 and undergoes a large decrease at the G_1/S transition, whereas $p34^{cdc2}$ kinase activity is low in G_1 and undergoes a large increase during the progression of the cell through S and into G_2 and M phase. During the past year, in collaboration with J. Roberts (Fred Hutchinson Cancer Center, Seattle), we found that $p34^{cdc2}$/cyclin complexes can induce replication in extracts of G_1 cells. Previously, we showed (with D. Beach, Cold Spring Harbor Laboratory) that $p34^{cdc2}$ phosphorylates SV40 T antigen and stimulates viral DNA replication. The control of DNA replication by $p34^{cdc2}$ and potentially by CK-II is a continuing interest of the members of our laboratory.

Synthetic Peptide Substrates for Protein Kinases

D.R. Marshak, M.T. Vandenberg, G.E. Binns, M.R. Meneilly

We have been actively pursuing the design and synthesis of peptides that are phosphate acceptors on serine or threonine residues for CK-II and $p34^{cdc2}$ kinases. Peptides are synthesized by chemical methods on the basis of the original Merrifield approach using *N*-α–Boc-protected amino acids on polystyrene supports. Synthesis is done using automated instruments, and they are extensively characterized by mass spectrometry, sequence analysis, and amino acid analysis to ensure purity. Peptides are analyzed as kinase substrates by carrying out a phosphorylation reaction with radioactively labeled ATP and then separating the radioactivity incorporated into the peptide by immobilization on phosphocellulose paper.

Synthetic peptide substrates for the cell-division-cycle-regulated protein kinase, $p34^{cdc2}$, have been developed and characterized. These peptides are based on the sequences of two known substrates of the enzyme, SV40 large T antigen and the human cellular recessive oncogene product, p53. The peptide sequences are H-A-D-A-Q-H-A-T-P-P-K-K-K-R-K-V-E-D-P-K-D-F-OH (T antigen) and H-K-R-A-L-P-N-N-T-S-S-S-P-Q-P-K-K-K-P-L-D-G-E-Y-NH_2 (p53), and they have been employed in a rapid assay of phosphorylation in vitro. Both peptides show linear kinetics and an apparent K_m of 74 and 120 μM, respectively, for the purified human enzyme. The T-antigen peptide is specifically phosphorylated by $p34^{cdc2}$ and not by seven other protein serine/threonine kinases, chosen because they represent major classes of such enzymes. The peptides have been used in whole-cell lysates to detect protein kinase activity, and the cell cycle variation of this activity is comparable to that measured with specific immune and affinity complexes of $p34^{cdc2}$. In addition, the peptide phosphorylation detected in mitotic cells is depleted by affinity adsorption of $p34^{cdc2}$ using antibodies to either $p34^{cdc2}$ or immobilized p13, a $p34^{cdc2}$-binding protein. Purification of peptide kinase activity from mitotic HeLa cells yields an enzyme indistinguishable from $p34^{cdc2}$. These peptides should be useful in the investigation of $p34^{cdc2}$ protein kinases and their regulation throughout the cell division cycle. Table 1 shows the utility of these peptides in their abilities to detect $p34^{cdc2}$ activity in whole-cell extracts, and it compares the activities of the peptides and histone H1 with enzyme prepared by three preciptation methods.

Protein Chemistry Core Facilities

G.E. Binns, M.R. Meneilly, D.R. Marshak

During the last 5 years, the growth of the protein chemistry core services has increased dramatically. The major areas of chemistry required are the structural analysis of proteins and the synthesis of peptides. We are now seeing the number of these procedures performed per year begin to plateau as we reach the physical capacity of the instruments and the limited personnel. At present, scientists must wait weeks to months for their analyses because of these limitations in resources. Figure 5 demonstrates graphically the plateau effect in sequencing and synthesis, due to limited resources. In the coming year, we will move to the Keck Foundation Laboratory of Structural Biology in the new Neuroscience Center. This will allow more space, personnel, and

TABLE 1 Peptide Kinase Activity in Elutriated HeLa Cell Extracts

Sample	Cell cycle stage	Phosphate incorporated (pmole/10 min/10^7 cells)					
		CSH103		CSH133		histone H1	
Cell lysate	G_1	138		204		n.d [b]	
	S	536		586		n.d.	
	G_2/M	890	[6.5][a]	822	[4.0]	n.d.	
Immunoprecipitate $p34^{cdc2}$ antibody	G_1	29.0		13.2		36.9	
	S	117		87.4		169	
	G_2/M	155	[5.4]	155	[11 7]	258	[7.0]
Immunoprecipitate p60 antibody	G_1	0.19		0.15		0.09	
	S	0.65		0.55		0.50	
	G_2/M	0.96	[5.1]	0.72	[4.8]	0.92	[10.2]
Precipitate with p13-Sepharose	G_1	120		59.3		55.3	
	S	493		407		370	
	G_2/M	646	[5.4]	552	[9.3]	578	[10.5]

From Marshak et al. (*J Cell. Biochem.* [1991] in press).

[a] The value shown in brackets is the relative increase in phosphate incorporation, calculated as the ratio between the values for the G_2/M and G_1 cell extracts.

[b] n.d. indicates not determined.

the possibility of new equipment for the constantly increasing demands for protein chemistry. In addition, we have instituted several new methods, such as mass spectrometry, high-performance electrophoresis, and quantitative chemical analyses to maintain the facility on the leading edge of new technology. The growth of the use of protein chemistry is illustrated in Table 2.

As part of the core protein chemistry support facility, peptide synthesis is performed to assist researchers throughout the programs at Cold Spring Harbor Laboratory in their work. In general, there are three uses of peptides: (1) as antigens for antibody production, (2) as enzyme substrates, and (3) for biological studies. Approximately half the peptides synthesized are used as antigens by coupling to carrier proteins or by direct injection into animals. The antibodies produced in this way allow an investigator to prepare a reagent that reacts very specifically with a site on the protein of interest. Such "site-directed" antibodies are of tremendous value in structure-function studies of proteins. To facilitate the rapid synthesis of these peptides, we have employed small-scale (0.1 mmole) rapid-cycle chemistry using an automated synthesis instrument. By optimizing our procedures, we have been able to synthesize, purify, characterize, and deliver 79 peptides during the 1990 calendar year. This rate is constantly increasing, and our annual production with the single instrument now available is 100 peptides per year (two per working

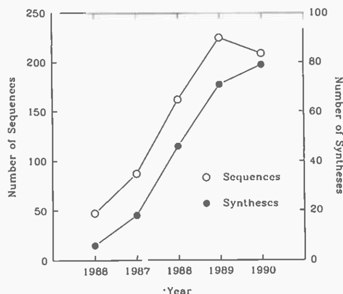

FIGURE 5 Growth of protein sequencing and peptide synthesis procedures at Cold Spring Harbor Laboratory, 1986–1990. Data based on the calendar years.

TABLE 2 Growth of Protein Chemistry

Procedure	1986	1987	1988	1989	1990
Protein sequences	47	87	162	225	205
Peptide synthesis	6	18	46	71	79
Amino acid analysis		357	389	400	339
Chromatography		61	68	75	67
Mass spectrometry			256	666	1064

week). This is in addition to the other procedures carried out in the facility.

One of the recent developments has been the sequence analysis of peptides using mass spectrometry. This has resulted in fewer peptides requiring chemical sequencing, which makes the sequencer available for other projects. Sequence analysis of peptides by plasma desorption mass spectrometry (PDMS) has been accomplished for an assortment of peptides. Peptides ranging in molecular weight from 1100 to 3600 amu displayed useful sequence ions under PDMS. Acidic, basic, and neutral peptides all showed sequence fragment ions, and carboxy-terminal sequence information was readily obtained on all peptides (A, B, or C′′ ions). The A ion series was most abundant for the basic peptides, whereas the C′′ ion series was most abundant for the acidic peptides. Sequence-specific fragment ion patterns appear to occur. For example, peptide CSH084 showed dehydration (−18 amu) of all the Y′′ ions in the internal basic region (residues 10–16). Subsequent examples of peptides containing aromatic side chains indicate that loss of the indole moiety from the side chain is common under PDMS. Similar generation of these w′ ions from histidine residues through loss of imidazole has also been detected. We suggest that PDMS is generally useful for sequence analysis of peptides of various compositions. In particular, PDMS is useful as a complementary tool to classical methods of protein chemistry, and PDMS should be considered an important part of a multidisciplinary approach to the structural analysis of peptides and proteins. It is clear from Table 2 that there has been a huge increase in the use of mass spectrometry, reflecting the growing capability of the instrument to analyze samples, both natural and synthetic.

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PROTEIN SYNTHESIS

M.B. Mathews O. Echetebu, R. Galasso, S. Green, S. Gunnery, C. Kannabiran, M. Kessler, C. Labrie, M. Laspia, Y. Ma, K.H. Mellits, L. Manche, G.F. Morris, R. Packer, T. Pe'ery, A.P. Rice, M. Sullivan, P Wendel

Studies have continued on the regulation of gene expression in three systems: translational control by small viral RNAs, the regulation of a cellular growth-related replication factor, and transcription of the human immunodeficiency virus (HIV) genome. With the departure in the fall of Andrew Rice and Odi Echetebu for Baylor College of Medicine, we have discontinued work on the structure of the Tat protein, but studies of its function are still being actively pursued.

Adenovirus VA RNA and Translational Control

T Pe'ery, Y. Ma, L. Manche, K.H. Mellits,
M.B. Mathews

The interferons are products of a multigene family that can affect a wide range of cellular functions including cell growth, the immune response, and viral infectivity. Exposure of cells to interferon can induce an antiviral state in which the cells become refractory to infection by a variety of viruses. This induction involves the transcriptional activation of a number of genes whose protein products interfere with viral replication at stages ranging from uncoating to assembly. One of the most studied interferon-induced proteins is the protein kinase DAI, an acronym for the *d*ouble-stranded (ds) RNA-*a*ctivated *i*nhibitor of protein synthesis. This enzyme is present in most cells at a basal level and in an inactive (latent) form; it is activated in a process that is accompanied by autophosphorylation and requires dsRNA. Once activated, the enzyme becomes able to phosphorylate eukaryotic initiation factor eIF-2 on its α-subunit. Phosphorylation of eIF-2 hampers its function and brings protein synthesis to a halt.

It appears that DAI activation is mediated by dsRNA generated as a consequence of viral metabolism. No requirement for specific dsRNA sequences is apparent, but the length and concentration of the dsRNA are critical. Duplexes of more than 50 bp activate the enzyme, whereas shorter duplexes block activation. Activation requires very low concentrations of dsRNA, and high concentrations prevent DAI activation, giving rise to a bell-shaped activation curve. Two hypotheses have been advanced to explain the paradoxical inhibitory action of high concentrations of dsRNA (Fig. 1). These hypotheses make different predictions about the number of sites for the dsRNA ligand (one or two), the molecularity of the autophosphorylation reaction (uni- or bimolecular), and whether phosphorylation is inter- or intramolecular. Our binding assays detected only a single site for dsRNA, and kinetic measurements indicate that the activation reaction is second order with respect to DAI. These data are consistent with the one-site/bimolecular model (model 1), although they do not prove it, and some data in the literature are more compatible with the two-site/unimolecular model (model 2). Further support for model 1 comes from the finding that adenovirus VA RNA_I competes with dsRNA for binding to DAI and *vice versa*. This result implies that the sites for these two RNA ligands are probably one and the same; alternatively, if they are different, they must communicate with one another in such a way as to ensure mutually exclusive occupancy. Additionally, mixing experiments indicated that the activated form of the enzyme, which phosphorylates eIF-2, is not capable of activating latent DAI. Thus, activation results in a change in substrate preference, probably brought about by an alteration in enzyme conformation. Presumably, this switch guards against runaway autocatalytic activation of the enzyme that could block protein synthesis inopportunely.

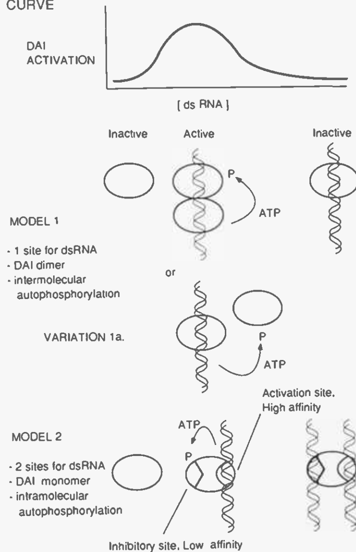

FIGURE 1 Models for the binding of dsRNA to DAI and its consequences for enzyme activation (from Mathews et al. 1990). See text for explanation.

Uninfected cells possess detoxification mechanisms to dispose of dsRNA that accumulates as a result of normal metabolism. Presumably, these safety mechanisms are saturated or otherwise incapacitated during viral infection, allowing dsRNA to accumulate and trigger the activation of DAI and

other dsRNA-dependent enzymes. For their part, several viruses have acquired the means to evade this antiviral defense. In the case of adenovirus, an abundant small RNA, VA RNA_I, blocks activation of the kinase and the resultant inhibition of translation. As described in last year's Annual Report, this RNA is highly structured, comprising a central domain flanked by two stems. One of these stems, the apical stem, is required for efficient binding of VA RNA to DAI, and because of its extensive predicted secondary structure, it was thought that VA RNA might function as a short RNA duplex to prevent DAI activation. Surprisingly, the molecule's function is preserved in mutants that contain alterations in the apical stem, but mutations that perturb the structure of the central domain abrogate this function whether or not the site of mutation is in the central domain. Foreign sequences inserted into the apical stem are tolerated provided the central domain and the base of the apical stem remain intact, although the sequence in the stem can be varied. Consideration of other mutational data argues that the terminal stem is also nonessential for function, emphasizing that the central domain plays the determinative role in blocking DAI activation.

We have begun to explore the functional consequences of changes in the central domain using an in vitro kinase assay. Our data are still preliminary, but some conclusions can already be drawn. First, mutations in the central domain are permitted, even some that delete or substitute relatively large stretches of sequence. Second, some of the mutants that retain functionality cannot adopt the conformation depicted in last year's report, so it is possible that alternative structures for the central domain are (or can be) active. Third, there are signs of tertiary interactions within the central domain, and these may also be important for function. We expect that more light will be shed on the structure-function relationships within this important region when our current series of mutagenic and phylogenetic analyses are completed.

Regulation of PCNA

G.F. Morris, C. Kannabiran, C. Labrie, R. Packer,
M.B. Mathews

Activation of the cellular DNA replication machinery is central to the process of neoplastic transformation. The proliferating cell nuclear antigen (PCNA) functions in conjunction with DNA polymerase δ as an essential DNA replication factor in leading-strand DNA synthesis (see DNA Synthesis section). In accord with this function, the cellular PCNA level increases in response to mitogenic agents such as growth factors and viral oncogenes, including the adenovirus E1A oncogene. From this pattern of synthesis and its critical role in DNA replication, it seems likely that activation of PCNA gene expression is a necessary component of the transition from the quiescent state to the proliferating state. The goal of this project is to explore the induction of PCNA gene expression by the E1A oncogene product and thereby to elucidate the relationship between PCNA induction and oncogenic transformation. Initially, we focused on identification of the *cis*-acting E1A-responsive elements of the PCNA promoter by use of cotransfection assays in HeLa cells. We have initiated experiments to determine if the findings with HeLa cells also apply to other cell types.

Alternative splicing generates five different mRNAs from the primary E1A transcript, with the two largest mRNAs, 13S and 12S, as the major products. Comparison of the predicted amino acid sequences for the E1A proteins of several adenovirus serotypes reveals three regions of relatively high sequence conservation. Two of the conserved regions, CR1 and CR2, occur in both the 12S and 13S products, whereas CR3 is unique to the 13S transcript. Under most circumstances, the 13S product is a much more efficient *trans*-activator of gene expression than the 12S product, and 13S is responsible for the activation of other early viral genes during infection. Nevertheless, the E1A 12S product can activate expression of a number of cellular growth-regulated genes, including the gene encoding PCNA. Just as CR3 is dispensable for induction of the PCNA gene, functions of the first and second conserved domains are largely dispensable for the viral gene *trans*-activation function. This observation implies that the PCNA gene represents a different class of targets, activated in response to an E1A function different from that of the viral early genes.

We determined that cotransfection of a plasmid that expresses the E1 region of adenovirus *trans*-activates a chimeric gene consisting of the PCNA promoter fused to the bacterial chloramphenicol acetyltransferase (CAT) reporter gene. Because the products of both E1 genes can influence gene expression (including their own), it was necessary to express the products of the E1 region independently and

efficiently in order to address their role in the stimulation of the PCNA promoter. We obtained from E. White (DNA Synthesis section) vectors in which the highly active cytomegalovirus (CMV) immediate-early promoter drives expression of the products of the E1B region individually or together (CMV19K, CMV55K, and CMVE1B) and prepared similar clones to express E1A gene products (CMV13S, CMV12S, and CMVE1A). We found that the CMV55K plasmid has no effect on the PCNA promoter upon cotransfection with PCNA-CAT into HeLa cells. The CMV19K plasmid stimulates the expression of PCNA-CAT and of two control plasmids, E3CAT and SV2CAT. Furthermore, cotransfection with CMV19K stimulates CAT expression from each of the PCNA promoter deletion mutants. These observations are consistent with our previous observations that 19K stabilizes transfected plasmid DNAs. The 13S and 12S expression clones both stimulate expression of the PCNA promoter at all concentrations tested, but they have different effects on the E3CAT and SV2CAT constructs. Low amounts of the CMV13S plasmid stimulate expression from E3CAT or SV2CAT, whereas higher amounts stimulate little or give a negative response, presumably because of the E1A repression function. The CMV12S plasmid represses CAT expression from the E3 or SV40 promoters at all concentrations tested.

These results demonstrate that the E1A 12S product can activate transcription from the PCNA promoter in the absence of other adenovirus genes. Examination of the promoter sequence revealed a number of potential E1A-responsive elements. To distinguish elements in the PCNA promoter that respond to 13S, 12S, or both, deletion constructs of the PCNA promoter were cotransfected with the 13S and 12S expression plasmids. As a control, we employed a construct containing a frameshift mutation just downstream from the translation initiation site of the E1A protein (CMV12S.FS). The analysis suggests that an element(s) responsive to both E1A 12S and 13S resides within the region located 87–46 nucleotides upstream of the transcription initiation site. This region includes a sequence (TGACGTCG) that has homology with an ATF site (TGACGTCA) centered about 50 nucleotides upstream of the start of transcription. Additional mutations that eliminate the ATF site from the full-length promoter reduce the basal level of PCNA-CAT mRNA about fivefold and abolish *trans*-activation by 12S, suggesting that the ATF site plays a role in the basal level of transcription and in the response to E1A.

To confirm this result, we mutated the ATF sequence in the background of a shortened PCNA promoter, the –87 to +60 PCNA-CAT construct that contains only 147 nucleotides of PCNA gene sequence. This construct is *trans*-activated to a greater degree by cotransfection of pCMV12S, but its basal activity is low. We therefore included the CMV19K plasmid in the transfections to boost basal levels of transcription from the –87 to +60 constructs. The results allow us to conclude (1) that the principal target for the 13S product in the PCNA promoter is the ATF site at –50, but another element in the region from –46 to +60 or possibly within the vector sequences can also respond to the *trans*-activating effect of the E1A 13S protein, and (2) that the sole responsive element in the –87 to +60 region that responds to *trans*-activation by the E1A 12S product is the ATF site at –50. ATF elements are widespread in promoters, but only the PCNA ATF site and possibly an AP-1 site in the c-*jun* promoter have been shown to be 12S-responsive. The selective response of the PCNA promoter to 12S may be conferred by some contextual feature of the PCNA promoter, by a complex involving specific members of the ATF family, or by a combination of these two possibilities. Our present work is aimed at addressing these issues.

The preceding experiments were conducted in HeLa cells and may not reveal all the mechanisms by which the PCNA gene can be stimulated by E1A. It is possible that there are other pathways for E1A to activate PCNA gene expression that predominate in other cell types. We have begun experiments to evaluate the generality of the ATF-mediated stimulation, to explore the roles of other possible E1A-responsive elements in the PCNA promoter that might not be addressed in the HeLa cell assay, and to extend our findings to cell types with more normal growth properties. In preliminary studies of the response of the human PCNA promoter in primary cells, we cotransfected baby rat kidney (BRK) cells with the PCNA-CAT and E1A constructs employed previously. We found that the activity of the human PCNA promoter is reduced by cotransfection of any CMV E1A expression plasmid (12S, 13S, or E1A). This repression seems to require PCNA promoter sequences located between 249 and 172 nucleotides upstream of the cap site. It is unclear at present how to reconcile these observations with prior transformation assays and our HeLa cell results. Perhaps species differences or differences in the state of cell growth or other cell-type-specific factors can account for the disparate results in the two cell types. It is also pos-

sible that other adenovirus genes participate, since adenovirus early gene products other than E1A can elicit transcriptional effects. In the case of viral infection of BRK cells, the requirement for E1A sequences could be due to the *trans*-activating effects of the E1A protein on other viral genes or possibly to a requirement for E1A sequences in *cis* for the expression of other viral early gene products. In the context of a viral infection, it is difficult to determine whether the effect on the cellular PCNA gene is directly attributable to the 12S product or is mediated through effects of 12S on other viral genes.

The demonstration that the 12S E1A product can activate transcription of a growth-related gene directly could provide important clues to its transforming functions. The adenovirus E1-responsive human DNA polymerase-β promoter possesses an ATF site centered 45 nucleotides upstream of the transcription initiation point, and this site displays homology (12/13 nucleotides) with the ATF site in the PCNA promoter. It is possible that recognition of this extended ATF site by an E1A-responsive factor assumes a key role in the induction by E1A of the components of the DNA replication machinery. Indeed, the PCNA gene could serve as a paradigm for the induction of a large number of coordinately controlled cellular proteins (see Quest Protein Database in the Molecular Genetics of Eukaryotic Cells Section).

Mechanism of *trans*-Activation by the HIV-1 Tat Protein

M.F. Laspia, M. Kessler, P. Wendel, M.B. Mathews

The human immunodeficiency viruses (HIVs) appear to be the etiologic agents of acquired immunodeficiency syndrome (AIDS). HIV infects and kills $CD4^+$ helper T cells, which are an essential component of the immune system required for fighting microbial and viral infections. This produces an immunocompromised state in infected individuals, rendering them susceptible to infection by opportunistic pathogens. HIV also infects nervous system cells, and, as a result, patients often manifest neurological disorders. An important pathogenic component of HIV infection is the establishment of a state of viral latency, lasting several years, between infection and the onset of clinical symptoms.

An area of intense interest in HIV research and one that continues to be a primary focus of our research is elucidation of the molecular basis of regulation of HIV gene expression by the virus-encoded *trans*-activator protein Tat. In addition to the structural proteins Gag, Pol, and Env, the genome of HIV encodes several structural and regulatory proteins not found in other retroviruses. One of these, Tat, is an essential protein that greatly increases the expression of genes linked to the long terminal repeat (LTR) of the virus. Tat is a novel activator that binds to a structured RNA, known as TAR, present in the 5′-untranslated region of all HIV mRNAs. As discussed in previous Annual Reports, we have utilized a recombinant adenovirus model system to explore the nature of *trans*-activation by Tat. This system employs a recombinant adenovirus vector (HIV-1CATad) containing the HIV-1 LTR, including TAR sequences required for *trans*-activation by Tat, fused to a reporter gene (CAT) to infect HeLa cells that stably express Tat. For comparative purposes, we have also analyzed regulation of HIV-1 gene expression by the adenovirus E1A *trans*-activator protein.

Earlier work led us to propose that Tat stimulates HIV-1 transcription bimodally, by increasing transcriptional initiation and stabilizing transcriptional elongation. We discovered that in the absence of Tat, the basal rate of initiation of HIV-1 transcription by RNA polymerase II is low and elongation is unstable such that the number of transcriptional complexes declines with increasing distance from the transcriptional start site (polarity). Tat interacts with TAR and stimulates transcription by increasing the frequency of transcriptional initiation and also by suppressing elongational polarity, resulting in a greater fraction of initiating RNA polymerases reaching the 3′ end of the transcription unit. E1A, on the other hand, stimulates initiation with little effect on elongation and does not require TAR. On the basis of these findings, our working model, shown in Figure 2, is that TAR RNA functions much like a DNA enhancer element, providing a binding site for Tat at the HIV-1 promoter. Tat causes the efficient formation of an initiation complex at the HIV-1 promoter that is capable of stable elongation.

The polarity of HIV-directed transcription in the absence of Tat suggests that elongation away from the promoter is impaired. We have mapped the distribution of transcription complexes by sizing the run-on transcripts to determine if the impairment of transcription is due to a specific elongation block within the promoter proximal sequences or to poor processivity on the part of complexes formed at the HIV

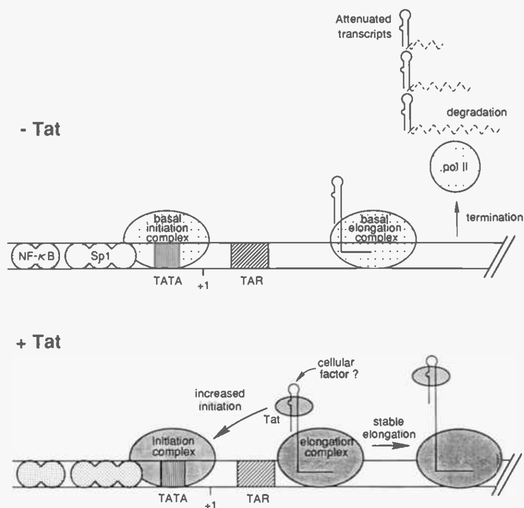

FIGURE 2 Model for *trans*-activation of HIV-1 gene expression by Tat. The basal level of transcription (−Tat) is influenced by cellular and viral transcriptional activators (such as NF-κB, Sp1, and E1A) as well as the position of an origin of DNA replication. In HeLa cells, for example, the basal level of transcriptional initiation is low, but it can be increased by adenovirus E1A or PMA. In the absence of Tat, elongating transcriptional complexes are unstable, and the density of transcribing RNA polymerases declines with increasing distance from the promoter. The RNA products of prematurely terminated RNA polymerases may be trimmed back to the base of the TAR stem. Tat elevates the frequency of transcriptional initiation and also stabilizes elongation, leading to a large relative increase in transcription in the 3′ end of the gene. We propose that Tat bound to the HIV-1 leader via TAR causes the efficient formation of a unique RNA polymerase complex at the HIV-1 promoter that is capable of efficient elongation.

promoter. This was accomplished by isolating pulse-labeled HIV-1-specific run-on transcripts from transfected cells by hybrid selection, followed by analysis in sequencing gels. Pulse labeling of the nascent RNA in the absence of Tat led to the detection of several RNA transcripts of 40–80 nucleotides. In the presence of Tat, the same short transcripts accumulated as well as longer RNAs. When pulse labeling was followed by 30 minutes of chase transcription in the presence of unlabeled precursors, the 40–80-nucleotide transcripts elongated, implying that their accumulation during the pulse is due to the transcriptional complexes pausing, but not terminating, transcription. The accumulation of transcripts of several different sizes suggests that polarity is due not to an elongation block at a specific site but to a propensity of the complexes formed at the HIV promoter to stop within the promoter proximal sequences. Interestingly, RNase protection analysis of cytoplasmic RNA in transient assays has revealed that in the absence of Tat, the predominant HIV-directed products are short transcripts of 55–60 nucleotides. We are now studying the processes involved in the accumulation of the short cytoplasmic transcripts and the relationship of these transcripts to those produced in the run-on reactions.

To investigate the discrepancy between our findings—that Tat both stimulates transcriptional initiation and suppresses polarity, and the observation from other laboratories that Tat acts solely on elongation—we turned to transient expression experiments. Plasmids containing an HIV-1 LTR-directed reporter gene and an SV40 replication origin region were transfected into COS cells, monkey cells that express SV40 T antigen and permit plasmid amplification as a result of DNA replication. Surprisingly, we found that the mode of action of Tat depends on the structure of the plasmid containing the HIV LTR-CAT cassette. The position and orientation of the origin region, the source of the LTR (LTRs from two different viral isolates were tested), the presence of additional R and U5 sequences downstream from TAR, and the nature of the plasmid vector were all considered. We found that the determinative feature is the position of the SV40 DNA replication origin relative to the LTR, but not its orientation. Nuclear run-on analysis indicated that, in the absence of Tat, placement of the origin region downstream from the HIV LTR-CAT cassette results in tenfold more promoter proximal transcription than when the origin is placed upstream of the LTR. When the origin is in the upstream location, Tat increases both the number of initiation events and the efficiency of elongation; on the other hand, when the origin is downstream, and the initiation rate is already high, Tat functions primarily to increase the efficiency of transcriptional elongation. These observations are compatible with results obtained by analysis of accumulated cytoplasmic RNA using an RNase protection assay. Thus, the effect of Tat on initiation is diminished when the basal transcription level is high.

Analysis of plasmid DNA levels indicated that these transcriptional differences were not due to differences in copy number related to the position of the origin. Moreover, although the origin region contains portions of both the early and late SV40 promoters, RNase protection analysis indicated that transcription from the origin region does not occur to any significant extent and cannot explain its effect on expression from the HIV LTR. Nevertheless, it is possible that sequences in the 200-bp origin region exert a transcriptional effect on the HIV LTR from a distance. Alternatively, the origin could influence transcription from the LTR or the effect of Tat by altering template structure through replication. Data from experiments in CV-1 cells (monkey cells lacking SV40 T antigen) favor the idea that replication is involved. In the presence of T antigen, supplied by cotransfection, the position effect is observed as in COS cells; in the absence of T antigen, no such effect is seen. These findings suggest that T antigen is required, most likely to allow replication, although other interpretations are not yet excluded. Replication has been reported to activate transcription in several systems; if confirmed in the case of HIV-1, this mechanism of transcriptional activation could contribute in an important way to the transition from latency to active infection when resting T cells are induced to proliferate.

Viral latency may result in part from transcriptional quiescence of HIV provirus in infected resting T cells. We and other investigators have found that Tat cooperates with general viral *trans*-activators and T-cell activators to elevate HIV gene expression synergistically. Since the interaction between Tat and other transcriptional activators may be involved in the transition from low levels of transcription early in infection or during viral latency to high levels of expression during active viral growth, we examined the basis of synergy. As described in last year's Annual Report, Tat and adenovirus E1A or phorbol esters elevate LTR-directed RNA levels to a greater extent than expected for an additive action. Analysis of transcription rates indicated, unexpectedly, that synergy is not due to elevated levels of transcriptional initiation which appeared to saturate. To determine whether transcriptional synergy results from suppression of elongational polarity, we constructed DNA probes that enabled us to measure transcription rates across the entire LTR-directed transcription unit by nuclear run-on assay. This analysis indicated that synergy can be accounted for by increased levels of transcription in the 3′ portions of the HIV-1-directed transcription unit, suggesting that synergy is due principally to stabilization of transcriptional elongation.

An important focus of our efforts is to establish a cell-free system for *trans*-activation by Tat that mimics the effects we have observed in vivo. Recently, Phil Sharp and his colleagues at the Massachusetts Institute of Technology reported that Tat protein is capable of stimulating HIV-1 transcription in vitro. Such a system provides a powerful means to identify and characterize cellular factors involved in *trans*-activation by Tat. To this end, we purified Tat to approximately 80% homogeneity from *Escherichia coli* using a bacterial expression system. To test the biological activity of Tat preparations, we utilized the technique of scrape loading to introduce Tat into cells infected with the recombinant adenovirus. Uptake of

Tat protein resulted in a large stimulation of HIV-1-directed gene expression: Analysis of reporter gene activity and cytoplasmic RNA levels demonstrated that the bacterially expressed Tat stimulated LTR-directed transcription levels in a fashion similar to that produced by Tat stably expressed in an HIV-1CATad-infected HeLa/*tat* cell line. Thus, our purified preparations of bacterially expressed Tat protein display the same biological properties as Tat expressed in mammalian cells.

In agreement with results obtained in Phil Sharp's laboratory, we have also found that partially purified Tat is capable of stimulating transcription from the HIV-1 promoter in vitro in a nuclear extract prepared from HeLa cells. We have optimized conditions for transcription by Tat in vitro and have found that Tat stimulates transcription up to 25-fold from an HIV-1 DNA template digested with a restriction enzyme so as to produce a correctly initiated 747-nucleotide runoff transcript. Tat stimulated transcription in a dose-responsive manner from a wild-type HIV-1 LTR template but not from a TAR mutant template or an adenovirus major late promoter (MLP) template. Tat produced a smaller, fourfold stimulation of transcription when the HIV-1 template was digested with a restriction enzyme so as to produce a shorter 333-nucleotide runoff transcript. The finding that the magnitude of stimulation of transcription by Tat increases for longer runoff transcripts is consistent with Tat acting to stimulate transcriptional elongation. We are currently attempting to determine if Tat stimulates transcriptional initiation in the cell-free system: Future efforts will be directed toward fractionating nuclear extracts and attempting to identify cellular factors involved in stimulation of transcription by Tat.

Translational Effects of HIV-1 TAR RNA

S. Gunnery, S Green, M B. Mathews

The 5′ LTR of human immunodeficiency virus type 1 (HIV-1) contains a *cis*-acting element called TAR that confers responsivity to the *trans*-acting protein Tat. A transcript of the TAR sequence (TAR RNA) is present in the 5′-untranslated region of all HIV-1 mRNAs and adopts a stable stem-loop structure. It is also present as a free cytoplasmic form of about 60 nucleotides. It has been reported that TAR RNA can exert an inhibitory effect on protein synthesis both in *trans* and in *cis*: As a result, mRNA with a TAR leader is translated poorly in vitro compared with that without the TAR structure. The stem of the TAR RNA is also reported to inhibit initiation of translation in vitro by activating the dsRNA-activated inhibitor, DAI, which phosphorylates initiation factor eIF-2.

As mentioned above, the protein kinase DAI requires 30–50 bp of perfect dsRNA for activation: Shorter duplexes or imperfect duplexes do not activate the kinase. Secondary structure analysis of TAR RNA indicates that the stem region has only 11 bp of continuous duplex, so it would not be expected to activate DAI. On the contrary, it is more likely to inhibit the activation of DAI as other short duplexes do. On the basis of our experience with VA RNA, we thought that the reported activation of DAI by TAR RNA might be due to dsRNA that often contaminates RNA transcribed in vitro using bacteriophage RNA polymerase. To test this idea, we synthesized TAR RNA (+1 to +82 of HIV-1 LTR) in vitro using T7 RNA polymerase. In agreement with earlier results, we observed that partially purified TAR RNA, fractionated through a denaturing polyacrylamide gel, does activate DAI. Although TAR RNA is sensitive to single-strand-specific RNase T1 and insensitive to the double-strand-specific RNase III, the DAI activation capability of partially pure TAR RNA exhibits the opposite sensitivities. This suggests that the activation of DAI is not due to TAR RNA per se, but is due instead to a contaminant whose nuclease sensitivity is like that of dsRNA. Moreover, the DAI activation capacity is eliminated by further purification through a nondenaturing gel and chromatography on cellulose CF-11.

At high concentrations, purified TAR RNA inhibits the activation of DAI by dsRNA in a manner similar to that of adenovirus VA RNA. This result was observed in an in vitro kinase assay, where activation was monitored by autophosphorylation of the enzyme. The nuclease sensitivity of the inhibition matches that of TAR RNA, and the antisense complement of TAR RNA does not affect DAI activation except at extremely high concentrations, suggesting that the effect is specific. We have also tested the inhibitory property of TAR RNA in a translation assay using a rabbit reticulocyte lysate. This cell-free protein-synthesizing system affords a reasonable approximation to the in vivo situation as DAI is ribosome-associated and is activatable by dsRNA, leading to inhibition of protein synthesis. In this sys-

tem, the dsRNA-mediated inhibition of translation is blocked by both TAR RNA and VA RNA. We are now testing a variety of mutants of TAR RNA in order to correlate the structure of the molecule with its functions.

Structural Analysis of HIV-1 and HIV-2 Tat Proteins

A.P. Rice, C.O. Echetebu, M. Sullivan, R. Packer

Last year, we reported the use of the wheat-germ cell-free system to analyze structural features of wild-type and mutant HIV-1 Tat proteins. The wild-type protein consisting of 86 residues was found to form a highly fold, protease-resistant structure consisting of the first 72 residues contained in the first coding exon. This structure is likely to be relevant to Tat function, as all mutant proteins unable to form this structure are defective for *trans*-activation activity in vivo. In the past year, we have continued to exploit the wheat-germ system to investigate the structures of Tat proteins. We have shown that an HIV-1 Tat protein consisting of only the first 57 residues is able to form a protease-resistant structure. However, a Tat protein consisting of the first 48 residues is unable to form such a protease-resistant structure. In addition, we have used the techniques of gel filtration, velocity sedimentation, and glutaraldehyde cross-linking to demonstrate that wheat-germ Tat exists predominantly, if not exclusively, as a monomer. This finding contrasts with initial publications which presented evidence that the HIV-1 Tat protein expressed and purified from *E. coli* existed as a dimer. Recent modified purification procedures, however, have demonstrated that *E. coli*-expressed Tat can exist as a monomer.

We have also continued our analysis of the Tat protein from HIV-2, a human immunodeficiency virus thus far found largely only in western Africa. The HIV-2 Tat protein that we have analyzed contains 99 residues and is structurally related to the HIV-1 Tat protein; the proteins are 64% identical and 90% homologous in the centers of the molecules, which include cysteine-rich and basic regions. To determine how much of the carboxyl terminus can be deleted before *trans*-activation activity is lost, we used site-directed mutagenesis to construct a series of truncation mutants. We found that an HIV-2 Tat protein truncated immediately after the basic region (residue 90) possesses approximately 30% of the *trans*-activation activity of full-length HIV-2 Tat. Further deletion of five basic residues results in almost complete loss of *trans*-activation activity. Finally, we have initiated structural studies of the HIV-2 Tat protein expressed in the wheat-germ system. Under conditions of limiting trypsin digestion, the HIV-2 Tat protein appears to be significantly more sensitive to proteolysis than the HIV-1 Tat protein. We also found that the HIV-2 Tat protein elutes in gel-filtration columns with a Stokes' radius significantly greater than that of HIV-1 Tat; because these proteins differ by only 13 residues, this large difference in Stokes' radii cannot be explained simply by the additional residues contained in the Tat HIV-2 protein. These preliminary results therefore indicate that the closely related HIV-1 and HIV-2 Tat proteins possess significantly different structures.

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NUCLEIC ACID CHEMISTRY

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The long-term aim of our research is to obtain a detailed understanding of the mechanism of pre-mRNA splicing in mammalian cells. In particular, we are interested in determining how the RNA cleavage-ligation reactions are catalyzed, and how the specificity of splice site selection is achieved. As part of this effort, we are purifying several of the nucleoprotein and protein factors that are necessary for cleavage of the pre-mRNA at the 5′ splice site and for lariat formation. Our general strategy is to develop complementation assays for individual activities, such that one or both cleavage-ligation reactions are strictly dependent on the presence of the active component in question. The factors responsible for these activities are purified and then characterized to determine their mode of action. More recently, we have begun to characterize activities that modulate the selection of alternative splice sites in vitro. The identification and detailed characterization of splicing factors should provide crucial insights into the mechanism of pre-mRNA splicing, the specificity of splice site selection, and the origin and evolution of the splicing machinery and of pre-mRNA introns.

SF2 PURIFICATION

Previously, we purified a protein factor called SF2 from HeLa cells and showed that it was an essential splicing factor. SF2 was purified on the basis of a biochemical complementation assay, taking advantage of our observation that cytoplasmic fractions prepared in hypotonic buffers are inactive in splicing because they lack only this activity. SF2 can be extracted from nuclei at higher salt concentrations, probably due to its association with large heterogeneous nuclear ribonucleoprotein (hnRNP) complexes. The most purified fractions of SF2 contain a doublet of 33-kD polypeptides that have very similar amino acid compositions. In addition, a 32-kD polypeptide is also present, but it is not sufficient for activity because it peaks in an adjacent chromatographic fraction that is both inactive and devoid of the 33-kD polypeptides. This smaller polypeptide may nevertheless represent a subunit of SF2 that may or may not be limiting in the S100 extract.

ROLE OF SF2 IN SPLICEOSOME ASSEMBLY

The complementation assay employed for the purification of SF2 indicates that this activity is required for cleavage of the pre-mRNA at the 5′ splice site and for lariat formation. This reaction is normally preceded by the assembly of specific pre-spliceosome and spliceosome complexes, in which pre-mRNA interacts in a stable manner with multiple components of the splicing apparatus, such as the U small nuclear ribonucleoproteins (snRNPs). To determine the requirement for SF2 in this assembly pathway, RNP complexes were analyzed by a gel-retardation assay.

These experiments showed that SF2 is required for the assembly or the stabilization of the earliest specific complex in the spliceosome assembly pathway, which is thought to be the A complex.

SF2 HAS AN RNA ANNEALING ACTIVITY

To determine whether SF2 has unwinding or annealing activities, we incubated the purified protein with complementary RNAs and digested the RNA with ribonuclease T1, which is specific for single-stranded RNA. We found that SF2 promoted annealing of complementary RNAs in an ATP-independent manner. No unwinding or ATP-dependent helicase activities were detected under these conditions. Thus, SF2 lowers the activation energy for intermolecular RNA duplex formation and thus behaves as an RNA annealing activity. To assess the significance of this RNA annealing activity, we tested other proteins known to promote intermolecular RNA annealing, such as the 35-kD hnRNP A1 protein (S. Munroe, pers. comm.). We found that concentrations of A1 that were active in RNA annealing did not substitute for SF2 in the splicing complementation and splice site selection assays. These observations suggest that if RNA annealing activity is integral to SF2 and necessary for splicing, it is not sufficient, implying that specific protein-protein or protein-RNA interactions are involved in mediating the role of SF2 in splicing and splice site selection.

Further experiments are necessary to elucidate the precise substrate specificity, if any, of the SF2 RNA annealing activity. However, we can envisage three types of complementary RNA targets upon which the SF2 RNA annealing activity could act to mediate its effects on splicing and splice site selection. First, at least two snRNA–pre-mRNA helices are known to be formed during splicing and to contribute to splice site selection; these involve base-pairing interactions between the 5′ terminus of U1 snRNA and pre-mRNA 5′ splice sites and between an internal region of U2 snRNA and pre-mRNA branchpoint sequences. Second, U4 and U6 snRNAs coexist in a single snRNP particle held together by intermolecular base pairs, and there is some evidence that the U4 subunit may be released from the spliceosome during the course of splicing, possibly to be recycled. Third, pre-mRNA may be recognized as a proper splicing substrate when it adopts a defined secondary and tertiary structure. Some of the intramolecular RNA helices may preferentially expose the splice sites and branchpoint.

RNA-BINDING PROPERTIES OF SF2

To determine whether purified SF2 binds RNA, several types of analyses were carried out. UV cross-linking demonstrated binding to pre-mRNA and to mRNA (Fig. 1). In this type of analysis, when the cross-linked material is extensively digested with ribonuclease A, the protein-oligoribonucleotide adducts usually comigrate or migrate slightly behind the untreated polypeptides on SDS-PAGE. In the case of SF2, the labeled adducts comigrate with the 33-kD doublet, as expected if both polypeptides bind RNA. No RNA binding was detected by Northwestern blotting, but this could be due to poor renaturation after SDS-PAGE. We have not been able to separate the two polypeptides by nondenaturing techniques, and thus we cannot presently determine whether only one or both are responsible for the observed activities. However, amino acid analysis of the individual electrophoretically separated polypeptides suggests that they are highly related.

The RNA-binding properties of SF2 were studied further by RNase H protection experiments (Fig. 2). Pre-mRNA was incubated in the presence of SF2 and then briefly digested in the presence of a large amount of RNase H and one of several complementary deoxyoligonucleotides. RNase H specifically cleaves the RNA strand in RNA-DNA hybrids. Bound protein may protect the pre-mRNA from digestion by interfering with oligonucleotide and/or

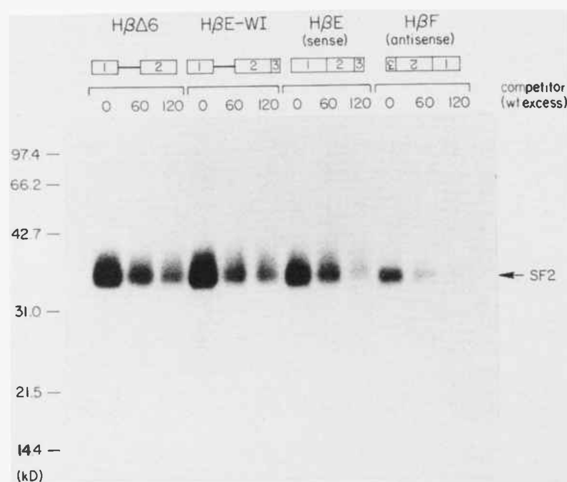

FIGURE 1 UV cross-linking assay. Purified SF2 was incubated with the indicated radioactively labeled RNAs under splicing reaction conditions, irradiated at 254 nm, digested with RNase A, and analyzed by SDS-PAGE and autoradiography. The indicated amount of rRNA was present as competitor.

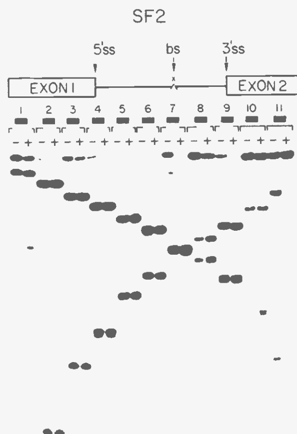

FIGURE 2 RNase H protection assay. Pre-mRNA was mixed with purified HeLa SF2 in splicing buffer and kept on ice (-) or incubated at 30°C (+) for 30 min The indicated oligonucleotide (*1-11*) was added in excess together with RNase H, and the reactions were incubated for 5 min at 30°C. Under these hybridization and digestion conditions, complete cleavage is observed in the absence of SF2.

RNase H binding. In the absence of SF2, all of the oligonucleotides resulted in complete pre-mRNA cleavage (not shown). In the presence of SF2 but without preincubation, several regions were substantially protected from digestion. When the pre-mRNA was preincubated with SF2, the cleavage pattern was different, and several protections and enhancements were observed. For example, the branch site is more susceptible to cleavage after incubation with SF2 (oligo 7). In contrast, a region in exon 2 becomes more resistant to cleavage (oligo 11). These experiments suggest that binding may occur at multiple sites in the pre-mRNA and that there may be sites of preferred binding. The observed results may also reflect conformational rearrangements of the pre-mRNA induced by SF2.

SF2 INFLUENCES 5′ SPLICE SITE SELECTION

U1 and U2 snRNAs play important roles in splice site selection by virtue of their ability to interact by intermolecular RNA-RNA base pairing with 5′splice sites and branchpoint sequences, respectively. However, these interactions are not sufficient to account for the specificity of splice site selection. Splice sites are often surrounded by cryptic splice sites that are only activated upon mutation of the natural splice sites. It is not known whether the same set of factors that recognize natural splice sites are also capable of recognizing the cryptic sites when the natural sites are mutated. Furthermore, although 5′and 3′splice sites from different introns are usually compatible, the splicing machinery normally avoids exon skipping by an unknown mechanism. Many genes are capable of expressing multiple protein isoforms by using alternative 5′and/or 3′splice sites, often in a regulated manner. Again, it is not known how this type of mechanism is controlled. Finally, it appears that in addition to the conserved 5′, 3′, and branchpoint sequence elements, additional, poorly defined, sequences in exons and introns can also influence splice site selection and splicing efficiency. It is not known how these context elements exert their effects.

We previously showed that not only is SF2 an essential general splicing factor, but it is also capable of influencing splice site selection in vitro in a concentration-dependent manner. Thus, when multiple 5′splice sites are present in *cis*-competition, high concentrations of SF2 promote the use of the downstream, or proximal, 5′splice site. We have extended our initial observations to a large number of substrates and have consistently observed a polarity toward proximal 5′splice sites. So far, the splice site selection effect appears to be restricted to 5′splice sites. Interestingly, even high levels of SF2 result in proper discrimination between authentic and cryptic 5′splice sites. These properties are consistent with a role of SF2 in ensuring the specificity of splice site selection during constitutive splicing in vivo, by helping to avoid aberrant exon skipping. Furthermore, SF2 may play a role in the tissue-specific or developmental regulation of certain alternatively spliced genes. In collaboration with D. Helfman (Molecular Genetics of Eukaryotic Cells Section), SF2 was shown to be able to modulate the pattern of splicing of a natural alternatively spliced pre-mRNA in vitro. High levels of SF2 led to the preferential utilization of the proximal skeletal-muscle-specific tropomyosin 5′splice site at the expense of the fibroblast-specific 5′splice site. Thus, high levels of SF2 can overcome tissue-specific differences between 5′splice sites at least in some cases. In addition, these studies showed that SF2 can prevent aber-

rant exon skipping in vitro. In collaboration with J. Manley (Columbia University), we showed that SF2 is identical with ASF, a factor isolated independently in Dr. Manley's laboratory, which can modulate alternative splicing of the SV40 tumor antigens.

Current experiments are aimed at determining whether the in vivo levels or activity of SF2 vary among different cell lines and whether posttranslational modification can play a role in regulating SF2 activity.

MOLECULAR CLONING OF SF2

To study the structure, function, and in vivo expression of SF2, we have cloned the corresponding cDNAs, expressed them in functional form, and obtained antibodies against this factor. The most abundant polypeptides present in purified SF2 preparations from HeLa cells have been analyzed in detail. The overall amino acid composition of a highly purified fraction was determined, as well as the individual composition of each of the two major 33-kD bands following SDS-PAGE and electroblotting to a polyvinylidene difluoride (PVDF) membrane. Both bands have a similar amino acid composition, characterized by an extremely high content of arginine (~20%). The same fraction was subjected to automated sequencing and yielded a single sequence. Our preparations of SF2 contain variable amounts of a 32-kD polypeptide, which peaks in an adjacent fraction that is free of the 33-kD polypeptides, and is by itself inactive in the complementation assay for splicing. This smaller polypeptide, which is not arginine-rich, yielded the same amino-terminal sequence as the active fraction, and since larger quantities were available, we were able to obtain a reliable sequence for the first 31 amino acids. These observations indicate that the two 33-kD polypeptides either share the same amino terminus with the 32-kD polypeptide or are both blocked at the amino terminus. Although we could not exclude the possibility that the 32-kD polypeptide is unrelated to SF2, we set out to obtain cDNAs and antibodies for this protein to allow probing of its function.

32-kD Putative Subunit. Degenerate oligonucleotides were designed on the basis of the most reliable and least degenerate portions near the ends of the 31-amino-acid sequence. The carboxy-terminal antisense oligonucleotides were used to prime cDNA synthesis on HeLa poly(A)$^+$ mRNA, and polymerase chain reaction (PCR) products of the expected length were obtained after 30 rounds of amplification. The products were subcloned and sequenced. The unique sequence bridging the degenerate primers matched the experimentally determined amino acid sequence, and an oligonucleotide corresponding to this unique sequence was used to screen a λ1149 HeLa cDNA library. A single clone was obtained from a screen of 3×10^5 recombinants, and the cDNA was sequenced. The sequence has no significant homologies with current entries in the protein and DNA databases. It appears to be full-length and encodes a highly acidic protein of 21.4 kD that initiates with a leucine CTG codon, which corresponds to the amino terminus of the purified HeLa protein, an unusual but not unprecedented finding. Following mutagenesis of this codon to ATG, the protein has now been overproduced in soluble form in *Escherichia coli*, using the T7 expression system (Fig. 3). This recombinant product has the correct electrophoretic mobility, i.e., 32 kD. We have also obtained antisera to a fusion protein containing the PCR amplified portion and, more recently, to the complete protein. These antibodies will be used in immunofluorescence studies and in inhibition and immunodepletion studies to investigate whether this polypeptide may represent an essential subunit of SF2 or an associated protein.

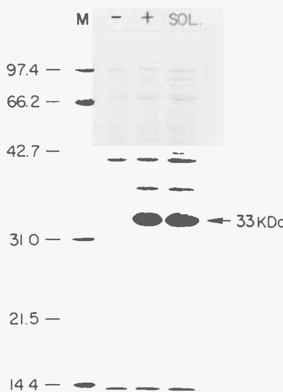

FIGURE 3 Bacterial expression of the 32-kD putative subunit The full-length cDNA was expressed as an authentic protein in *E. coli* with a T7 gene-10 promoter/ribosome-binding site vector. Crude proteins are analyzed by SDS-PAGE and Coomassie blue staining before induction (-) and after addition of IPTG to induce the T7 RNA polymerase (+). The soluble fraction (SOL) is also shown.

33-kD Subunit(s). More recently, we obtained a partial amino acid sequence of several peptides generated by V8 protease digestion of highly purified 33-kD polypeptides. Appropriate degenerate oligonucleotides were designed and then tested in each of the two possible relative positions for each pair of peptides. One particular combination generated a PCR product from HeLa poly(A)$^+$ mRNA. Its sequence matched the experimentally determined amino acid sequence immediately adjacent to the primers and provided an additional unique sequence between the two peptides.

An oligonucleotide corresponding to the unique nucleic acid sequence was synthesized and used to probe a λ1149 HeLa cDNA library. Two independent clones were obtained from a screen of 3 x 10^5 recombinants. They contain the same apparently complete coding sequence but different lengths of 5′-and 3′-untranslated sequences. They encode a basic 27.7-kD protein with the expected high content of arginine residues and no sequence relationship to the acidic 32-kD polypeptide (Fig. 4). Several clusters of alternating arginine and serine residues constitute regions of high homology with the *Drosophila* proteins *tra*, *tra*2, and suppressor of white apricot, all of which are involved in the regulation of specific alternative splicing pathways. A similar region is present in the U1 snRNP-specific 70-kD protein from several species and in a phylogenetically conserved region of the *trans*-activating domain of the papillomavirus E2 protein. This cDNA clone also contains a region that has strong homology with the 80-amino-acid RNA recognition motif, including the two internal RNP1 and RNP2 consensus elements, which is common to a large family of RNA-binding proteins. These include several hnRNP and snRNP proteins, the *Drosophila* alternative splicing regulators *sxl* and *tra2*, and many other prokaryotic and eukaryotic proteins. This putative RNA-binding domain near the amino terminus is followed by a stretch of multiple glycines, which separates it from the carboxy-terminal R/S-rich region. The U1 snRNP 70-kD protein exhibits similar organization, despite its larger size and lack of strong global homology. There is, in addition, a stretch of eight identical amino acids present in SF2 and in the 70-kD protein, located in different positions in the two proteins relative to the RNA recognition motif, which in the case of the 70-kD protein corresponds to a true RNA-binding domain.

The fact that this extremely basic protein copurified through ion exchange columns with the acidic 32-kD protein suggests an interaction between these polypeptides, although its physiological relevance is uncertain. We are presently trying to express the R/S-rich polypeptide in *E. coli* and to generate the corresponding polyclonal and monoclonal antibodies. One or both of the 33-kD bands are the most likely to be SF2 because UV cross-linking experiments show that they can bind RNA (see Fig. 1). Independent studies on ASF have yielded a very similar polypeptide profile, including the arginine-rich polypeptides, using a different purification protocol. As mentioned above, the two 33-kD polypeptides have similar or identical amino acid compositions, and thus they probably differ by posttranslational modification, or they may represent two different isoforms generated by gene duplication or alternative splicing. Consistent with the possibility that the heterogeneity is due to posttranslational modification, the above cDNAs generate doublets of the expected electrophoretic mobility upon in vitro transcription, followed by translation in reticulocyte or wheat-germ extracts (Fig. 5).

The conserved sequence motifs identified in the

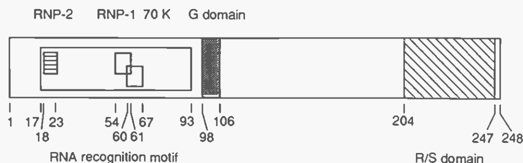

FIGURE 4 Structure of SF2 subunit. The 248-amino-acid SF2 polypeptide (33 kD apparent molecular mass) is drawn to scale. The RNA recognition motif, glycine-rich domain, and arginine plus serine-rich domain are indicated. Within the RNA recognition motif, the smaller boxes denote the RNP1 and RNP2 consensus elements and an eight-amino-acid identity to a region in the U1 snRNP-specific 70K polypeptide.

FIGURE 5 In vitro translation of recombinant SF2. SF2 mRNA was generated by in vitro transcription with T7 RNA polymerase from the cloned full-length SF2 cDNA (33-kD polypeptide). The mRNA was translated in wheat-germ or rabbit reticulocyte extracts in the presence of [^{35}S]-methionine. The proteins were detected by SDS-PAGE and fluorography.

SF2 cDNAs can account in theory for some of the observed biochemical properties. For example, the RNA-binding properties of SF2 are fully consistent with the presence of an RNA recognition motif. Furthermore, this element is probably responsible for the ATP-independent RNA annealing activity of SF2, since a homologous element is found in several proteins with comparable activities, e.g., hnRNP A1, *Drosophila* helix destabilizing protein, and T4 gene-32 protein. The R/S domain is especially intriguing because it is found in several known regulators of alternative splicing, although its precise function remains unknown. We plan to examine the roles of these domains in splicing, splice site selection, RNA binding, and RNA annealing by mutagenesis and biochemical analysis of the recombinant protein.

PURIFICATION OF HELA SF5 AND OTHER BIOCHEMICAL STUDIES

We have recently identified an activity in HeLa cell nuclear extracts, termed SF5, that specifically counteracts the effects of SF2 on splice site selection. SF5 is not a general inhibitor of SF2, because the latter activity is essential for splicing and a large excess of SF5 still results in efficient splicing. However, the ratios of these two activities precisely determine which splice site is used in vitro (Fig. 6). Thus, high levels of SF5 result in utilization of distal 5′ splice sites, whereas high levels of SF2 favor use of proximal 5′ splice sites.

An assay was designed for the purification of this activity. An alternatively spliced pre-mRNA is used as the substrate, and S100 and partially purified SF2 are added in precisely titrated amounts to result in equal utilization of each of the two 5′ splice sites. Chromatographic fractions are added, and SF5 activity is located by the resulting switch to the distal 5′ splice site. On the basis of this activity assay, SF5 has been partially purified from sonicated HeLa nucleoplasm through three chromatographic steps,

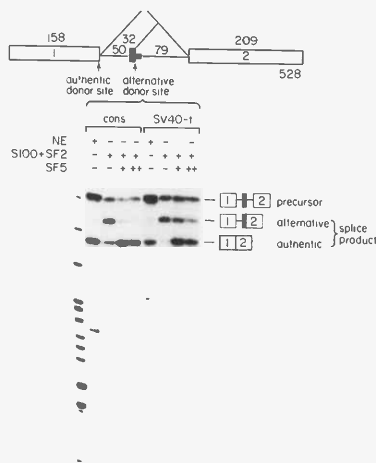

FIGURE 6 SF5 activates distal 5′ splice sites. Synthetic pre-mRNAs containing alternative 5′ splice sites (a distal authentic β-globin 5′ splice site, and either a proximal consensus 5′ splice site or a proximal SV40 small T antigen 5′ splice site) were spliced in vitro in the presence of nuclear extract (NE), S100 extract, partially purified SF2, and/or partially purified SF5, as indicated. The electrophoretic mobilities of the pre-mRNA and of the alternative mRNAs are indicated.

with good recovery of activity. It has been separated from SF2 activity and from snRNAs, and it does not require RNA for its activity. Partially purified SF5 is active in assays employing several different alternatively spliced pre-mRNAs. Thus, this activity does not operate in a substrate-specific manner, but rather it has a general effect on the polarity of 5′ splice site selection. We believe that the intracellular ratios of SF2 and SF5 may control the specificity of splice site selection. Furthermore, in vivo regulation of one or both of these activities may play an important role in the tissue-specific or developmental regulation of alternative splicing.

Another ongoing project concerns the analysis of spliceosome assembly in nuclear extracts from which large endogenous RNP complexes have been selectively removed. Although a number of essential factors are associated with these endogenous complexes, they can be released in an ATP-dependent manner. The resulting extracts are competent for splicing, but they display very different properties in the assembly of spliceosomes. In contrast to crude extracts, the RNP-depleted extracts do not form nonspecific complexes that are largely due to hnRNP proteins, and they form a single spliceosome complex that contains the pre-mRNA and the intermediates of splicing. These experiments suggest that hnRNP proteins are dispensable for splicing and that the kinetics of spliceosome assembly in standard extracts may reflect the binding of nonessential factors that delay the formation of an active complex.

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TRANSCRIPTIONAL REGULATION

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Transcriptional regulation in eukaryotes is complex. A large variety of *cis*-acting promoter elements are recognized by an even larger number of transcriptional activators. Commonly, families of transcriptional activators recognize the same *cis*-acting element. This phenomenon is no better exemplified than in the regulation of development by homeodomain proteins. One of the current challenges in the study of transcriptional regulation in development is to understand how transcription factors such as homeodomain proteins that recognize the same DNA sequence can differentially activate transcription.

Homeodomain proteins were first discovered as regulators of development in *Drosophila*. Subsequently, the 60-amino-acid homeodomain was shown to be a DNA binding structure similar to the helix-turn-helix motif found in prokaryotic transcriptional regulators. In *Drosophila*, homeodomain proteins that elicit very different developmental pathways can share very similar DNA binding properties. Thus, it

is likely that in some instances, it is the different interactions between proteins that are responsible for the differences in transcriptional regulation observed in vivo. Some of these types of interactions have been the focus of our studies of the human homeodomain proteins Oct-1 and Oct-2, which recognize the same octamer motif ATGCAAAT found in a variety of cellular and viral promoters.

VIRAL PROMOTERS AS PROBES FOR TRANSCRIPTIONAL REGULATION IN MAMMALIAN CELLS

As probes to study transcriptional regulation in mammalian cells, we use three primate viruses, the simian DNA tumor virus SV40 and the human pathogens herpes simplex virus (HSV) and human immunodeficiency virus (HIV). Our past studies of the enhancer within the SV40 early promoter, which can activate transcription over large distances, showed that the restricted or unrestricted cell-specific activity of enhancers results from interactions between multiple enhancer modules; seldom is a single element responsible for the full activity of the enhancer. The SV40 enhancer is typical of broadly active promoter elements. It contains many individual elements that can be categorized into two different types of organizational units called enhansons and proto-enhancers. Enhansons are the fundamental structural unit of enhancers and correlate with protein-binding sites. As structural units, enhansons are not always active on their own, but instead, they need to be intimately associated with a second enhanson to be active. Active enhancer elements, composed of either one or more enhansons, are called proto-enhancers. Proto-enhancers, however, can still only create an effective enhancer (i.e., activate transcription at a distance) when present in multiple copies, but, unlike enhansons, here there is no strict spacing requirement between proto-enhancers.

Our past genetic dissections of the SV40 enhancer revealed that it contains three proto-enhancers called A, B, and C, all of which are active in the permissive simian cell line CV-1. Currently, we are using strategies similar to those that allowed us to dissect the SV40 enhancer to analyze the structure of the HIV-1 promoter, including the characterization of HIV-1 promoter sequence rearrangements in HIV-1 variants. To study general aspects of transcriptional regulation in mammalian cells, we have focused on the transcriptional activation properties of Oct-1 and Oct-2, which recognize an octamer motif within the B proto-enhancer. The SV40 B proto-enhancer consists of two overlapping sets of motifs, a tandemly repeated 9-bp "sph" motif and an octamer motif formed by the junction of the two sph motifs. The SV40 octamer motif does not normally activate transcription in cells permissive for SV40 infection (indeed, it may repress transcription), but instead, it displays activity in B cells, which express the Oct-2 activator. We are studying how Oct-1, which is ubiquitously expressed, and Oct-2, which is expressed primarily in B cells, differentially activate transcription. It turns out that a major player in this story is an activator of HSV immediate-early gene expression called VP16 (also referred to as Vmw65) that modifies the activity of Oct-1, but not Oct-2, by discriminating between the homeodomains of these two activators. VP16 associates with Oct-1 to form a multiprotein-DNA complex that alters the activation potential of Oct-1 and that can alter the regulatory specificity of Oct-1 by recruiting it to new DNA binding sites.

Oct-1 Recognizes a Diverse Set of Regulatory Sites through Interdependent Interactions by Multiple Regions of the POU Domain

R. Aurora

The octamer motif-binding proteins Oct-1 and Oct-2 are POU proteins. The POU proteins are a unique subfamily of homeodomain proteins that are distinguished by a 150–160-amino-acid bipartite DNA binding domain, called the POU domain. The 60-amino-acid homeodomain lies in the carboxy-terminal portion of the POU domain; the amino-terminal region contains a 75-amino-acid motif particular to the POU proteins called the POU-specific region. Linking these two conserved motifs together is a 15–27-amino-acid hypervariable segment. Although the POU homeodomain can bind DNA weakly on its own, the entire POU domain is required for high-affinity and sequence-specific DNA binding.

Oct-1, along with many eukaryotic transcriptional regulators, possesses the ability to recognize a diverse set of DNA sequences. The ability of site-specific transcriptional regulators to recognize

diverse sequences has important consequences on the flexibility with which promoters can be assembled with overlapping binding sites and potentially on how transcription factor activity can be modified by the sequence of the binding site. Oct-1 recognizes and activates transcription through two regulatory elements of very different sequence, the octamer motif ATGCAAAT, through which both Oct-1 and Oct-2 can activate transcription of cellular promoters, and the TAATGARAT motif, through which Oct-1 activates HSV immediate-early transcription in association with VP16. We showed previously that Oct-1 is able to bind to these two very different sequences because it can recognize DNA through flexible interactions with DNA. Few if any Oct-1/DNA contacts are essential for Oct-1 binding. Instead, it is the sum of many individual Oct-1/DNA contacts that result in effective and sequence-specific DNA recognition.

To understand the mechanism of divergent DNA sequence recognition by Oct-1. we have assayed the relative contribution of the different regions of the Oct-1 POU domain for binding to a series of different octamer- and TAATGARAT-related DNA binding sites. We took advantage of the fact that the related pituitary-specific POU protein Pit-1 also recognizes the octamer and TAATGARAT motifs; but whereas Oct-1 binds preferentially to the octamer motif, Pit-1 displays the opposite binding site preference. To assay the relative contribution of different segments of the POU domain—the POU-specific region (which can be subdivided into A and B segments), the hypervariable linker, and the homeodomain—to binding to these two different types of sites, we prepared a large series of Oct-1/Pit-1 POU-domain chimeras (in the context of the Oct-1 protein) by reciprocally exchanging Oct-1 and Pit-1 POU segments. All of the chimeras can bind DNA well and in fact frequently bind better than either parent on particular sites. This analysis has furthermore shown that the contribution of a particular subsegment to sequence recognition, including even the nonconserved linker between the POU-specific and POU-homeodomain regions, is dependent on the origin of the other segments in the POU domain. These results suggest that the POU domain is a dynamic structure in which DNA binding specificity is generated by multiple interdependent POU domain—DNA contacts as well as intraprotein—protein contacts within the POU domain. Such flexible interactions may influence the structure of Oct-1 on different binding sites, affecting its ability to interact with other proteins involved in transcriptional regulation.

Differential Transcriptional Regulation by Oct-1 and Oct-2

M. Tanaka. W. Clouston, G. Das, J.-S. Lai, W. Thomann, K. Visvanathan, K. Zito

Although Oct-1 and Oct-2 bind DNA with very similar, if not identical, sequence specificity, they are implicated in the regulation of different promoters. Consistent with expectation, the DNA binding domains of these two proteins, the POU domain, are very similar in sequence. Outside of the POU domains, Oct-1 and Oct-2 also display sequence similarity within the amino-terminal region, which are both rich in glutamine residues, a feature shared with the ubiquitous activator Sp1, where such regions constitute transcriptional activation domains. The carboxyl termini of Oct-1 and Oct-2 display minimal sequence similarity, but B cells express a variant form of Oct-2, called Oct-2B, that through alternative splicing of the primary Oct-2 mRNA transcript acquires a carboxy-terminal extension of 135 amino acids that is closely related to the carboxyl terminus of Oct-1.

The DNA target of transcriptional regulation by Oct-1 and Oct-2, the octamer motif, is unusual in that it is found in a large variety of promoters, either ubiquitously or cell-specifically expressed cellular promoters, as well as viral regulatory regions. The octamer motif is implicated in cell cycle regulation of the ubiquitously expressed histone H2B gene, where it is located close to the transcriptional start site, and it also functions as an enhancer of small nuclear RNA (snRNA) genes. snRNAs are short, nonpolyadenylated structural RNAs involved in RNA processing and can be synthesized either by RNA polymerase II, as in the case of the U2 snRNA, or by RNA polymerase III, as in the case of the U6 snRNA (see N. Hernandez in the Genetics Section). Curiously, in these promoters, a TATA box, which is usually associated with RNA polymerase II transcription, is responsible for directing RNA polymerase III transcription. Among cell-specific cellular promoters, the octamer motif functions as an enhancer and promoter-proximal element in immunoglobulin promoters, and it functions as a B-cell regulatory element in the SV40 enhancer. It is also associated with some of the TAATGARAT motifs that serve as the target of VP16 activation in HSV immediate-early promoters.

A series of experiments we performed some years ago in collaboration with N. Hernandez (Cold Spring

Harbor Laboratory) showed that the octamer motif displays either cell-specific or ubiquitous enhancer activities, depending on the structure of the elements proximal to the transcriptional start site. Thus, in the context of mRNA transcription units such as the β-globin promoter, the octamer motif displays B-cell-specific activity, suggesting that the B-cell Oct-2 protein is responsible for this activity. In the context of an snRNA promoter, however, the octamer motif is able to activate transcription in all cell types tested, suggesting that the ubiquitous Oct-1 protein is responsible for this activity. During the past year, we have established in transfection studies that indeed Oct-1 and Oct-2 possess different intrinsic abilities to activate snRNA and mRNA promoters: Oct-1 can activate the U2 snRNA promoter, whereas Oct-2 can readily activate the β-globin mRNA promoter.

We have shown previously that Oct-2, after introduction into the non-B-cell HeLa cell line, can readily activate transcription from an mRNA promoter. In contrast, Oct-1 does not readily activate such promoters, but instead its activation potential is dependent on promoter context. For example, in preliminary experiments, we have found that whereas in other promoter contexts Oct-1 fails to activate mRNA transcription, in the context of the human histone H2B promoter, where the octamer motif is located near the TATA box, overexpression of either Oct-1 or Oct-2 leads to equivalent further activation of this promoter. We plan to dissect this promoter in detail to understand how Oct-1 is able to activate transcription in this promoter context.

A major success in the past year has been our further understanding of how Oct-1 is able to activate the U2 snRNA promoter. It proved difficult to assay transcriptional activation by Oct-1 because of the ubiquitous endogenous Oct-1 activity already present in mammalian cells. To circumvent the endogenous activity, we reprogrammed the DNA binding specificity of Oct-1 (and Oct-2) without changing the gross structure of the protein by replacing the Oct POU domains with the Pit-1 POU domain, thus creating the chimeras Oct-1.P.1 and Oct-2.P.2 with different DNA binding specificities to Oct-1 and Oct-2. Using a multimerized Pit-1-binding site from the prolactin gene promoter in place of the octamer motif in the β-globin and U2 snRNA promoters, we assayed the ability of Oct-1.P.1 and Oct-2.P.2 to stimulate transcription from these promoters. Consistent with the differential ability of Oct-1 and Oct-2 to activate the β-globin mRNA promoter, these Oct/Pit-1 chimeras also showed that Oct-2 possesses an intrinsic ability, lacking in Oct-1, to activate an mRNA promoter. In striking contrast, however, in the context of the U2 snRNA promoter, the activities of Oct-1.P.1 and Oct-2.P.2 are reversed. Now, the Oct-1.P.1 protein can activate transcription more than tenfold, whereas the Oct-2.P.2 protein does not detectably activate the U2 promoter. Although Oct-2 does not activate the U2 snRNA promoter, the Oct-2 variant, Oct-2B, which carries an Oct-1-related carboxyl terminus, is able to activate the U2 snRNA promoter. Thus, here it is promoter-selective activation domains, separate from the DNA binding domain, that confer the transcriptional specificity of the activator, and an alternative splice can alter the promoter selectivity of a transcriptional activator without affecting the DNA binding domain.

The major determinants for Oct-1 activation of the U2 promoter lie in the carboxy-terminal region, although the Oct-1 amino-terminal region can also independently activate U2 transcription. This result differs from the analysis of Oct-2 activation of mRNA transcription, where two interdependent activation domains, the glutamine-rich amino terminus and a serine/threonine/proline-rich carboxy-terminal segment, cooperate to stimulate transcription. Oct-1 fails to activate transcription of the β-globin promoter readily because it lacks a domain equivalent to the carboxy-terminal Oct-2 activation domain. It does, however, possess a glutamine-rich activation domain that can cooperate with the carboxyl terminus of Oct-2 in an Oct-1/Oct-2 chimera to activate β-globin transcription. We hypothesize that Oct-1 can activate mRNA transcription in particular mRNA promoter contexts, where the requirement for the carboxyl terminus of Oct-2 is relieved. This activity has probably been conserved for activation of mRNA transcription, because although the Oct-1 amino terminus can functionally replace the Oct-2 amino terminus for activation of an mRNA promoter, unexpectedly, the Oct-2 amino terminus cannot substitute for the Oct-1 amino terminus to activate the U2 promoter. This result suggests that the amino terminus of Oct-1 has conserved two different types of activation surfaces: one for mRNA promoters and the other for snRNA promoters.

Analysis of the activation of the U6 RNA polymerase III promoter by the Oct/Pit-1 chimeras has revealed unexpectedly that both Oct-1 and Oct-2 are able to activate this promoter. Thus, these two activators, which display different abilities to activate two different types of RNA polymerase II promoters, can both activate the same RNA polymerase III

promoter. Possibly, this is due to the fact that the U6 promoter contains elements in common with both snRNA and mRNA RNA polymerase II promoters. The U6 promoter contains both an snRNA-specific proximal element in common with the U2 snRNA promoter and a TATA box in common with mRNA promoters.

Transcriptional Modification of Oct-1 through Association with VP16

S. Stern, M. Cleary, W. Herr, M. Tanaka

We continue to dissect in molecular detail the interactions between Oct-1 and VP16. On its own, VP16 does not bind to DNA well, but instead, in association with Oct-1 and a second less well-characterized host-cell factor we refer to as HCF, VP16 can direct formation of a multiprotein-DNA complex. Because VP16 contains a very effective acidic mRNA activation domain, the association of Oct-1 and VP16 results in activation of mRNA promoters that would otherwise not be activated by Oct-1 alone. Thus, VP16 effectively serves as an adaptor to convert Oct-1 into an activator of mRNA promoters. Last year, we showed that the Oct-1 homeodomain is responsible for directing formation of the VP16-induced multiprotein-DNA complex. Oct-2 fails to complex with VP16 because of seven amino acid differences between the Oct-1 and Oct-2 homeodomains. We are now dissecting the different associations between Oct-1, VP16, and HCF in the VP16-induced complex. Although Oct-1 and VP16-like activities are not present in invertebrate cells, the HCF activity is highly conserved because it is also present in *Drosophila* cell extracts. VP16 can form a stable heteromeric complex with either human or *Drosophila* HCF in the absence of Oct-1. We have analyzed the ability of VP16 mutants that fail to form the VP16-induced complex to bind to HCF. Surprisingly, three of the four mutants analyzed still retain the ability to bind to HCF. This result suggests that the other three mutants are defective in other aspects of the formation of the VP16-induced complex.

To study the influence of the VP16 mutations on associations with DNA and Oct-1, we have assayed VP16-induced complex formation in the absence of HCF by using higher concentrations of VP16 in gel-retardation assays. At the highest VP16 concentrations, VP16 can bind to DNA in the absence of either HCF or Oct-1. At intermediate concentrations, VP16 only forms a gel-retardation complex in the presence of the Oct-1 POU domain. Under these conditions, Oct-2 still fails to associate with VP16, thus showing that VP16 directly discriminates between the Oct-1 and Oct-2 homeodomains. At lower VP16 concentrations, VP16 only forms the VP16-induced complex in the presence of HCF. Two of the VP16 mutants that can bind HCF but fail to form the VP16-induced complex are defective for binding DNA at the high concentrations of VP16. The remaining mutant, however, binds DNA as well as the wild-type VP16 protein; instead, it has only lost the ability to interact with the Oct-1 homeodomain. Thus, we apparently have uncovered the region of VP16 responsible for interacting with the Oct-1 homeodomain. Consistent with this conclusion, a synthetic peptide representing the region of VP16 affected by this particular mutant can associate itself with Oct-1 bound to DNA.

The DNA binding specificity of the VP16-induced complex differs from the binding specificity of Oct-1 itself. VP16 can readily form complexes on certain DNA sequences, called TAATGARAT, that themselves are poor binding sites for Oct-1. Although we do not detect differences in the bases contacted by Oct-1 alone and the VP16-induced complex on such sites, VP16 stabilizes Oct-1 on these otherwise weak Oct-1-binding sites. Thus, VP16 effectively recruits Oct-1 to new targets; Oct-2 is not recruited because it does not interact with VP16. In vivo activity assays also reveal that Oct-1 is not active on the TAATGARAT motif unless VP16 is present, presumably because of the low Oct-1 affinity for these sites. These results demonstrate that VP16 alters not only the transcriptional activation properties of Oct-1, but also its DNA binding properties. The association of VP16 with Oct-1 serves as a model system to study the reprogramming of the transcriptional activity of a homeodomain protein by a cell-specific adaptor protein.

Genetic Dissection of the HIV-1 Promoter

W. Phares

Our earlier studies focused on the similarities between the transcriptional control elements in HIV-1 and SV40, in particular, on the relationship between

the κB motif sequence GGGACTTTCC found twice in the HIV-1 promoter and once in the SV40 C proto-enhancer. Both transcriptional activation studies and DNA binding studies showed that the HIV-1 and SV40 κB motifs are functionally indistinguishable. During the past year, we studied the proteins that bind to the HIV-1 and SV40 κB motifs by using gel-retardation analysis. The protein-binding patterns in all cell lines we have tested (i.e., HeLa cells and the human T-cell lines Jurkat and H9) were complicated and included many complexes in addition to the one formed by the previously described κB-binding protein NF-κB. The patterns of complex formation also varied considerably between Jurkat and H9 T cells. Of particular interest was that in H9 cells, there was no evident NF-κB DNA binding activity even though the κB motif is very active in these cells, suggesting that other κB-binding activities can activate expression by this motif.

In our more recent studies, we have focused on the analysis of sequence variation in the HIV-1 long terminal repeat (LTR) in infected individuals. Characterization of retroviral pathogenicity in other retroviruses has shown that rearrangements within regulatory sequences can alter the progression of retrovirus-induced disease. To understand how such alterations may affect the progression of AIDS in persons infected with HIV-1, we are characterizing the LTR sequences present in infected individuals. When viruses isolated in vivo are passaged in cell culture, there is a strong selection for those viruses that can grow in tissue culture. To avoid this bias, we are collaborating with Dr. J. Gold (Bronx-Lebanon Hospital) to analyze LTR sequences isolated directly from blood samples by first amplifying the LTR sequences with the polymerase chain reaction (PCR). In addition to being direct, this strategy has the advantage that we can also study the regulatory sequences from defective viruses that would not grow in culture but may be important in the progression of AIDS.

To date, we have analyzed samples from five HIV-1 seropositive individuals of which two had AIDS. Sequence analysis of the amplified LTR sequences isolated from one of these patients has revealed a 16-bp sequence duplication just upstream of the κB elements. This sequence duplication is reminiscent of duplications that arise in SV40 to improve both enhancer potential and viral growth. In SV40, such sequence duplications permitted us to identify functional SV40 enhancer elements. Consistent with the significance of the HIV-1 duplication we have identified, three other HIV-1 isolates grown in culture have been shown to contain duplications in the same region of the HIV LTR, and analysis of one of these showed it resulted in improved growth in culture. We plan to continue the analysis of HIV-1 LTR sequences from infected individuals and to dissect the regulatory function of the HIV-LTR duplication.

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MOLECULAR GENETICS OF EUKARYOTIC CELLS

Research in this section is generally directed at questions of signal transduction or cell regulation, the mechanisms by which cells respond to external change. This encompasses the subject of cell growth and differentiation, processes that are aberrant in cancer, diseases of the immune system, and in neurological diseases.

The laboratory of Nicholas Tonks (Structure, Function, and Regulation of Protein Tyrosine Phosphatases) studies the newly discovered superfamily of protein tyrosine phosphatases (PTPases). This family is diverse in structure and function and contains proteins with all of the hallmarks of extracellular receptors.

The laboratory of Dafna Bar-Sagi (RAS Oncogenes and Signal Transduction) studies the role of RAS proteins and phospholipase A_2 in signal transduction. The involvement of RAS in lymphocyte activation is being studied, and the physiological role of phospholipases in membrane signaling is being probed with newly developed monoclonal antibodies.

The laboratory of Michael Wigler (Genetics of Cell Proliferation) studies the mechanisms by which oncoproteins transmit growth proliferation signals and uses yeast systems to discover and characterize components of mammalian signal transduction pathways.

The laboratory of Michael Gilman (Nuclear Signal Transduction) is directed at the problem of how multiple, independent pathways converge on the control of a single genetic element, the "serum response element." This is representative of the more general problem of how signals are transduced from the cytoplasm to nucleus.

The laboratory of David Spector (Cell Biology of The Nucleus) uses advanced microscopic techniques to observe the structure of the cell nucleus during gene activation. Recently, the pathways of nascent RNA molecules have been followed from their sites of transcription to the cytoplasm using confocal laser scanning microscopy. The functional interaction between the nucleus and the cytoskeleton has long been a focus at Cold Spring Harbor Laboratory.

The laboratory of David Helfman (Molecular Biology of The Cytoskeleton) studies the multiple forms of cytoskeletal components and how isoforms become specifically expressed. Recently, a protein has been identified that may determine the pattern of alternative splicing of a tropomyosin isoform. One general approach to the study of cell regulation depends on the tools of two-dimensional gel electrophoresis, a method that has the power to discern changes in the population of protein species expressed in cells. The development of these tools and the building of a comprehensive protein data base are the objects of Quest Protein Database Center, directed by Jim Garrels. Robert Franza (Cellular *trans*-Activators of Gene Expression) uses this approach to study the early changes in gene expression. Many complex cellular interactions can best be studied in the intact animal.

Methods for the germ-line manipulation of Transgenic Mice are applied by Jacek Skowronski and co-workers to study problems in the pathogenicity of the human immunodeficiency virus and the function of the central nervous system.

TRANSGENIC MICE

J. Skowronski R. Mariani L. Usher
N. Peunova G. Yenikolopov

The recently developed technology of gene transfer into the mouse germ line has provided a powerful way to address diverse biological problems. Developmental and regulatory processes that involve complex interactions between different cell types can now be probed in transgenic animals that carry new, defined genetic traits. Transgenic mice also provide one of the most comprehensive assays to assess the pathogenic potential of viral genes and to generate animal models where complex disease processes are reproduced and accessible to study. Our studies have focused on two separate problems. First, we are assessing the disease-inducing potential of the human immunodeficiency virus (HIV) regulatory genes and characterizing their effects on development and function of the immune system. Second, Grisha Yenikolopov is now developing strategies to perturb signaling pathways in the central nervous system (CNS) of transgenic animals and consequently deregulating those functions of the CNS that are involved in learning and memory processes.

HIV-1 Genes in Transgenic Mice

R. Mariani, L. Usher, J. Skowronski

HIV-1 infection of humans is associated with a complex disease most frequently manifested by multiple immunologic defects and an increased risk of developing neoplastic lesions. T cells and antigen-presenting cells are the major targets of HIV-1, and it is conceivable that infection of these cells results in disease development. In addition to the *gag*, *pol*, and *env* genes found in all retroviruses, HIV-1 contains at least seven accessory genes (*tat*, *rev*, *tev*, *vif*, *nef*, *vpr*, and *vpu*). *tat*, *rev*, *tev*, and perhaps *nef* are important for regulation of expression and replication of the viral genome, and *vif* plays a role in viral assembly. The function of the other HIV-1 gene products is not clear, as are the consequences of HIV-1 *nef* and other regulatory gene expression on the infected cell and the biological effects of these genes on host-cell function.

Recent evidence has implicated three of the HIV-1 genes, *env*, *tat* and *nef*, as potentially important for HIV-1 pathogenicity. The *nef* gene is found in all members of the lentivirus family and is well conserved between different viral isolates (64% invariable amino acid residues; *tat* and *rev* show 48% and 54%, respectively). Accumulating evidence indicates that the HIV-1 *nef* gene can perturb cellular signaling when expressed in cultured cells. Results of in vitro experiments suggest that at least some variant *nef* genes (e.g., SF2, NL43, and HxB3) may down-regulate transcriptional activity of the viral long terminal repeat (LTR) and viral replication in established cells. In T-cell lines, these effects are relatively subtle; however, they may reflect defects that have a profound effect on T-cell function in vivo. To test these possibilities, several lines of transgenic mice that express the HIV-1 *nef* gene in lymphoid and nonlymphoid cell types have been recently generated. Our data suggest that the HIV-1 *nef* gene can perturb T-cell development/function when expressed in transgenic animals.

TARGETED EXPRESSION OF THE HIV-1 *NEF* GENE TO LYMPHOCYTES

When the experiments described here were initiated, T-cell-specific expression vectors characterized in transgenic mice were not available. Therefore, in initial experiments, transcription control elements derived from the murine immunoglobulin μ heavy-chain gene (IgHμ) were used to direct expression of the HIV-1 *nef* gene to lymphoid cells. IgHμ control elements were chosen because previous studies had demonstrated their transcriptional activity not only in the B cell, but also in the T-cell lineage including adult and fetal thymocytes. Although the IgH promoter is not absolutely lymphoid-specific and may direct low-level transgene expression into nonlymphoid cells types, this promoter was the best choice at that time. A hybrid transcriptional control region composed of the IgH enhancer placed upstream of the SV40 early promoter is much more active in T cells than the IgH enhancer/promoter con-

structs (R. Grosschedl, pers. comm.). Two hybrid genes composed of the HIV-1 *nef* gene (isolate HxB3) placed under the control of the IgH enhancer/SV40 early promoter (μSV Nef) or the IgH enhancer/promoter (μμ Nef) were used to construct transgenic mice.

Fifty-three mice were born as a result of microinjection experiments with μμ Nef and μSV Nef insert DNA, but no transgenic mice were identified by analysis of DNA from tail biopsies (yield less than 2%). This was a highly unusual phenomenon because in our hands, on average, 10–40% of the pups that develop from microinjected embryos are transgenic when other constructs are used. To exclude the possibility of a technical error, three independent preparations of insert DNA were used in these experiments. At the same time, we also also generated H-2K Nef transgenic mice without any difficulty (see below). The unusually low efficiency of generating transgenic mice with the μμ Nef and μSV Nef constructs suggested that expression of the HIV-1 *nef* gene directed by the IgH enhancer may have a deleterious developmental effect on transgenic mice that prevents their normal development to term. This is a valid possibility since it is known that the IgHμ gene is expressed in the fetal hematopoietic tissue from day 11 of embryonic development.

LETHALITY AND THYMIC ALTERATIONS IN pμμ NEF MICE

The lethal effects of the μμ Nef may have resulted from a high level of transgene expression. We therefore attempted to attenuate expression of the μμ Nef vector. It was anticipated that this would result in attenuation of the lethal phenotype and would allow us to construct viable transgenic mice and observe their phenotypes. It has been observed that the presence of plasmid sequences in microinjected hybrid gene constructs frequently results in significantly lower levels of transgene expression (10–100-fold lower) than when plasmid sequences are removed from the hybrid expression constructs prior to microinjection. Therefore, the complete μμ Nef construct, including the plasmid sequences, was used to generate transgenic mice. Embryos microinjected with the pμμ Nef construct gave rise to a total of 82 animals. Analysis of DNA isolated from tail biopsies identified four of them as transgenic. It appears that the attenuation strategy was partially successful; however, the yield of transgenic animals was still remarkably low (5% yield). We believe that this low frequency of transgenic animals reflects transgene integration at particular chromosomal loci where transgene expression and its consequences are further attenuated.

The conditions of two of the four pμμ Nef transgenic mice deteriorated rapidly within the first 3 days of life (mice 3 and 4), and these animals were collected for analysis when moribund. Perinatal death was observed only at a low frequency among nontransgenic littermates (3 of 78) when compared to transgenic animals generated in this experiment (50%; 2 of 4). Histologic analysis performed on tissues of transgenic animals 3 and 4 and two nontransgenic controls (which died in the same period) identified characteristic alterations in the thymuses of both transgenic pups and in the bone marrow and spleen of animal 3, but not in the nontransgenic controls. Hematoxylin/eosin-stained sections showed disruption of normal thymic architecture. The T-cell content in thymuses of both transgenic animals was low (pμμ Nef animal 3, ~10–20% of that in the thymus collected from the control animal). The bone marrow cell count of animal 3 was also low. No abnormalities were observed in other tissues of these transgenic mice.

Two lines of transgenic mice were established with the remaining two founder mice (pμμ Nef animals 1 and 2). These animals carry approximately 5 and 20 copies of the pμμ Nef transgene in an apparently nonrearranged conformation. Transgene expression analysis was performed with the RNase protection assay and total RNA isolated from major organs of 6-week-old animals. Correctly initiated transgenic transcripts were detected in RNA isolated from the heart and brain of pμμ Nef animal 1. In pμμ Nef animal 2, a high level of Nef transcripts was observed in the spleen and lymph node. Nef transcripts were also detected in the thymus and other tissues, but at a lower level. Transgene expression was also high in Peyer's patches, which are specialized intestinal lymphoid organs that contain large numbers of B cells. This pattern is consistent with predominantly B-cell-specific expression of the pμμ Nef animal 2 transgene. RNase protection experiments and polymerase chain reaction (PCR) amplification of cDNA prepared to RNA isolated from tissues of animals 1 and 2 indicated that pμμ Nef transcripts were processed correctly. We are currently characterizing the B-cell, T-cell, and immune functions of the pμμ Nef transgenic mice.

Low yield of the μSV/μμ/pμμ Nef animals is

likely to reflect perturbations elicited by these transgenes during prenatal development. This conclusion is supported by a limited success of experiments with an attenuated pμμ Nef transgene. The known specificity of the IgHμ enhancer suggests that thymic T-cell depletion of the pμμ Nef 3 and 4 mice resulted from transgene expression in T cells, or perhaps their precursors. It is possible that the *nef* gene expression affected normal progression of the thymic T-cell maturation process, generation of bone marrow precursors, and/or their migration to the thymic tissue. These possibilities have been addressed in additional transgenic experiments with T-cell-specific expression vectors and attenuated μμ Nef constructs.

CD2 NEF AND H-2K NEF TRANSGENIC MICE TO DIRECT *NEF* GENE EXPRESSION TO T CELLS AND TO A WIDE VARIETY OF CELL TYPES

To exclude technical reasons for our inability to generate transgenic mice with the μμ Nef vector, and to define consequences of *nef* gene expression in selected cell types of transgenic mice, additional transgenic animals were generated. First, the H-2K histocompatibility class I gene promoter was used to direct expression of the HIV-1 *nef* gene into a broad variety of cell types in transgenic mice. Second, the CD2 expression vector was used to direct high-level *nef* gene expression to T cells, a cell type that apparently was affected in the pμμ Nef 3 and 4 transgenic animals. These experiments were performed in parallel with the pμμ Nef experiments.

The H-2K gene is one of the class I genes of the major histocompatibility complex of the mouse and encodes the heavy chain of the cell-surface H-2K antigen. It is constitutively expressed on thymic medullary T cells and stromal structures but not on cortical lymphocytes or epithelial cells. In addition, the H-2K promoter directs low-level constitutive expression in keratinocytes and several cell types in spleen and lymph nodes. The H-2K enhancer/promoter fragment sufficient to direct expression in transgenic mice is contained within the 2-kb 5′-flanking region of the H-2K gene. A fusion gene composed of the HIV-1 Nef protein-coding region placed under control of the H-2K gene enhancer/promoter and followed by the SV40 small T antigen intron and polyadenylation signal (H-2K Nef) was microinjected into one cell embryos. Four lines of transgenic mice (H-2K Nef 1 to 4) were established without any unusual difficulties. Analysis of transgene expression by an RNase protection assay detected correctly initiated transgenic transcripts in total RNA isolated from thymuses, spleens, and lymph nodes of all four lines of transgenic mice.

The murine CD2 antigen (T11, or erythrocyte receptor) is one of the earliest developmental markers expressed on all T cells following migration of the bone marrow T-cell precursors to the thymus. The CD2 expression vector comprises 22 kb of the native CD2 gene, including approximately 5 kb of 5′-flanking sequences and 5 kb of the 3′-flanking sequence. The precise location of transcription regulatory elements is not known. Therefore, in order to preserve the native structure of the CD2 gene, the CD2 initiator ATG located in the second exon was disrupted by site-specific mutagenesis and replaced with a small polylinker containing a unique *Cla*I cloning site (vector provided by D. Littman, University of California, San Francisco), into which the HxB3 HIV-1 *nef* gene was cloned. Four transgenic founder animals were generated with the CD2 Nef construct. Recently, the CD2 Nef 1 and 2 mice became available for analysis. Relatively high levels of correctly initiated and processed fusion CD2 Nef transcripts were detected in RNA isolated from thymuses isolated from mice of both transgenic lines. Currently, we are characterizing phenotypes of transgenic animals and of transgenic T cells in particular.

In conclusion, it appears that the HIV-1 HxB3 *nef* gene, when placed under control of the IgHμ enhancer, elicits several specific effects in transgenic mice. First, transgenic animals are generated at a very low frequency. Second, T cells are depleted in thymuses of transgenic mice that die in early postnatal life. It appears that these consequences of the HIV-1 *nef* gene are specifically associated with the IgHμ Nef transgene, but not with the H-2K Nef or CD2 Nef transgenes. It is conceivable that this promoter specificity of perturbations elicited by the *nef* gene reflects its expression in specific cell types, e.g., lymphocytes or perhaps their bone marrow precursors. Specific alterations in the T-cell compartments may be relevant to the mechanisms of the T-cell depletion that invariably ensues in HIV-1-infected humans. Our observations suggest that the HIV-1 *nef* gene may be an important determinant of the HIV-1-induced disease and that specific defects conferred by the *nef* gene and their biological consequences can be analyzed in transgenic mice. Experiments to address these issues are currently under way.

Recombinant Pseudosubstrate Inhibitors of the Protein Kinases

G. Yenikolopov, N. Peunova

The aim of our study is to obtain dominant interfering mutations, by reverse genetics techniques, that would affect specific structures involved in memory and learning processes in mice. Currently, genetic analysis of memory and learning in higher animals is prevented by the lack of appropriate single-gene mutations. Our general approach is to use transgenic technology to introduce into the mouse germ line recombinant genes that would impose a dominant phenotype in the neuronal cells of the hippocampus and the cortex of the brain by perturbing the relevant signal transduction pathways. Transgenic animals will be tested for neurophysiological changes in these areas of the brain, in particular, the long-term potentiation in the hippocampus, and for the behavioral correlates of the introduced molecular changes. The rationale for this strategy is based on the neurophysiological studies on *Aplysia*, genetic data from *Drosophila*, and studies of long-term potentiation in mammals, which together suggest that secondary messenger systems are crucially involved in synaptic plasticity and thus in the acquisition and storage of new information. It appears that the memory deficit that occurs in these systems is induced by defects in the adequate response to the incoming signal, rather than a change in the overall amount of second messenger per se, and that various types of perturbations introduced into the signaling machinery of a neuronal network may lead to similar effects on learning and memory processes.

Of the numerous potential points of interference with the signaling pathways in neurons, we have chosen the protein kinase level of signal transduction because protein kinases are located downstream from many signaling events, and in some cases, they have been shown to interact directly with the last targets in the pathway. It is known that several protein kinases, including cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and Ca^{++}/calmodulin-dependent protein kinase II (CaMKII), are regulated by an autoinhibitory mechanism. This suggests a way of altering the activity of these enzymes by introducing into the cells either the gene(s) coding for the inhibitory ("pseudosubstrate") sequences or the gene(s) coding for the truncated catalytic domain (subunit) of the kinase, devoid of the autoinhibitory sequences. These protein kinases are especially challenging options for manipulation because of their known direct involvement in the modulation of the synaptic plasticity in the brain. To date, our efforts have concentrated on the construction of recombinant pseudosubstrate inhibitors of protein kinases and on the analysis of the disruption of specific signaling pathways in cultured cells by these inhibitors.

We have made a series of recombinant genes containing peptide sequences that are known to act as protein kinase inhibitors. The recombinant PKA inhibitors involve two types of agents: one containing the active part of a natural PKA inhibitor from skeletal muscle (PKI) and the second containing a mutated murine PKA regulatory subunit (RIα) that is unable to release the catalytical subunit in the presence of cAMP. We have matched the structures of the recombinant PKC and CaMKII inhibitors to the sequences of the short conserved peptides located at positions 19–36 in various forms of PKC and positions 273–309 of CaMKII, respectively. Such peptides act as powerful pseudosubstrate inhibitors of these enzymes in vitro. We have based the selection of the most potent recombinant inhibitors on their ability to antagonize activation of the c-*fos* gene promoter in cultured cells by various stimuli (cAMP, TPA, Ca^{++}, depolarization); activation by these agents is mediated by the corresponding protein kinases. Mutant forms of the c-*fos* promoter that no longer respond to particular signals were used as experimental controls. A series of reporter constructs with the chloramphenicol acetyltransferase (CAT) gene under the control of the c-*fos* promoter (from M. Gilman, Cold Spring Harbor Laboratory) were cotransfected along with the recombinant protein kinase inhibitors, and CAT activity was monitored. Comparison of CAT activities in control cells and cells stimulated by forskolin, TPA, or K^+-induced depolarization has shown that the recombinant inhibitors of PKA, PKC, and CaMKII, respectively, were very efficient in blocking the induction of transcription. Quantitative comparison permitted us to select particulary strong inhibitors of PKA and PKC from a series of prepared fusion genes and point mutants. For instance, we have found particular substitutions of the Arg-27 residue in the PKC inhibitor that greatly augmented the inhibitory activity of the construct. Likewise, we have shown that some recombinant PKA inhibitors (e.g., RIα/PKI and influenza virus hemagglutinin epitope/PKI chimeras) were 10–100 times more potent in blocking PKA activity than the fragments of

the natural PKI gene. At present, we do not know whether this observed enhancement is due to the lower K_i or to the increased stability of the peptides. These results have demonstrated the feasibility of our approach in cultured cells and have proven directly the potential of the pseudosubstrate-coding genes to block the action of PKC and CaMKII in vivo.

Recombinant inhibitors show a remarkable degree of specificity. Our experiments demonstrate that the PKA inhibitors do not block the activation of the c-*fos* promoter by TPA and, reciprocally, that the PKC inhibitors do not block the induction of c-*fos* by forskolin. Furthermore, in vitro tests by other investigators with the synthetic peptide inhibitors and purified enzymes show a very high degree of specificity of each inhibitor for its cognate kinase. Taken together, these data suggest that the recombinant inhibitors provide a powerful approach to dissect points of overlap and divergence among the signal transduction pathways.

Recombinant pseudosubstrate inhibitors represent a new generation of in vivo protein kinase inhibitors that, in contrast to the low-molecular-weight synthetic compounds and peptides, can be introduced into the genome of the cell and can act in a transgenic organism. We have constructed and tested the recombinants designed to target the expression of the inhibitors for PKA, PKC, and CaMKII to the neuronal cells of transgenic mice, using neuron-specific transcriptional control elements of neurofilament H and L, β-amyloid, and SCG10 genes. We now plan to generate transgenic lines of mice using the most potent and specific recombinant reagents.

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GENETICS OF CELL PROLIFERATION

M. Wigler

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J. Colicelli	R. Ballester	I. Wieland	C. Nicolette	L. Rodgers
K. Ferguson	E. Chang	A. Vojtek	N. Chester	J. Brodsky
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The focus of our group remains largely on the signal transduction pathways involved in growth control, with a special emphasis on pathways involving RAS proteins. The RAS proteins were first discovered as the products of retroviral oncogenes. They are low-molecular-weight GTP- and GDP-binding proteins. We and other investigators have found mutant RAS proteins in a large number of human tumor cells. Closely related proteins are found in yeast, and we have attempted to explore the function of these proteins both in the budding yeast *Saccharomyces cerevisiae* and in the fission yeast *Schizosaccharomyces pombe*. RAS function is best understood in *S. cerevisiae*, where we identified its major effector as adenylyl cyclase. However, the mechanism by which RAS activates adenylyl cyclase remains largely unknown. A new component of the RAS/adenylyl cyclase system has been discovered, called CAP, which may integrate this signaling system with remodeling of the cellular cytoskeleton. In *S. pombe*, where little is understood about RAS function, we have identified several genes that appear to encode

components of the RAS signaling system. We use both yeasts to explore the function of mammalian RAS and putative RAS-related proteins, such as the product of the neurofibromatosis locus, NF1, and we have developed genetic screens to clone novel mammalian cDNAs which encode proteins that function in signal transduction pathways. Among these proteins are candidates for mammalian RAS effectors. This work has also led to the cloning of genes encoding cAMP phosphodiesterases, enzymes of considerable physiological importance. Finally, we continue work aimed at developing methods for genomic difference cloning that will enable us to discover recessive oncogenes and novel pathogens.

S. cerevisiae Adenylyl Cyclase

J. Field, J. Colicelli, T. Michaeli, K. Ferguson,
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The *CYR1* gene encodes adenylyl cyclase, the one known target for RAS protein action (Kataoka et al., *Cell 43:* 493 [1985]; Toda et al., *Cell 40:* 27 [1985]). This large (220-kD) protein has two distinct domains: a 40-kD carboxy-terminal catalytic domain and a 60-kD leucine-rich domain with a repeating motif, located in the center of the molecule. In the past year, we completed two related studies of adenylyl cyclase/RAS interactions. In the first study, a series of in-frame deletion and insertion mutations were made in *CYR1*, and the encoded product was tested for RAS responsiveness (Colicelli et al., *Mol. Cell. Biol. 10:* 2539 [1990]). Most of the amino-terminal region, to within 100 amino acids of the leucine-rich repeat domain, is not required for retention of RAS responsiveness. Large deletions or small insertions within the repeat domain destroy RAS responsiveness. Large deletions in the region between the repeat and the catalytic domain also destroy RAS responsiveness, but small insertions are tolerated throughout this region. We conclude from these data that the structure of the leucine-rich repeat domain is very sensitive to perturbation and is critical for RAS responsiveness. The latter conclusion is confirmed by the second study. We discovered that overexpression of catalytically defective adenylyl cyclase molecules inhibits RAS function in yeast (Field et al., *Science 247:* 464 [1990]). Truncated genes encoding fragments of adenylyl cyclase were tested for this effect. The smallest fragment capable of inhibiting RAS function was precisely the leucine-rich repeat domain. These studies further emphasize the importance of this domain in RAS responsiveness. Future studies will focus on determining what other proteins bind to the leucine-rich repeat.

S. cerevisiae CAP

J. Field, A. Vojtek, J. Gerst, K. Ferguson,
M. Kawamunkai, M. Riggs, L. Rodgers, M. Wigler

To explore the mechanism by which RAS stimulates adenylyl cyclase, we designed an epitope fusion-immunoaffinity protocol to purify the adenylyl cyclase complex from yeast (Field et al., *Mol. Cell. Biol. 8:* 2159 [1988]; *Cell 51:* 219 [1990]). The complex is RAS-responsive and contains at least one other protein, with an apparent mobility of 70 kD, which we have called CAP. Antibodies raised to CAP were used to screen expression libraries, resulting in the cloning of *CAP*. Sequence and restriction endonuclease analysis indicates that *CAP* is identical to the gene we prevously called *SUPC*. We had previously identified an allele of *SUPC* as a suppressor of the activated $RAS2^{val19}$ allele. Gene disruption indicates that CAP function is required by cells to be fully responsive to RAS. However, cap^{-} cells have other phenotypic defects. They are round, often enlarged and multinucleated, sensitive to nitrogen deprivation, and fail to grow in rich medium or in the presence of an excess of certain amino acids, such as valine (Gerst et al., *Mol. Cell. Biol. 11:* 1248 [1991]). CAP is in fact a bifunctional protein; the amino terminus is required for full RAS responsiveness and the carboxyl terminus is required for the diverse growth and morphological effects.

To gain insight into the nature of the carboxyl terminus, we have sought genes on multicopy plasmids that suppress the phenotype that results from the loss of CAP. One strong suppressor has been found so far: *PFY*, the gene encoding profilin (A. Vojtek et al., in prep.). Profilins are low-molecular-weight proteins that can block actin polymerization by binding the actin monomer (Lassing and Lindberg, *Nature 314:* 472 [1985]). This binding is released by interaction with lipid micelles containing phosphatidyl inositol phosphates.

The surprising connection between profilin and CAP led us to examine the phenotypes of pfy^{-} and

cap⁻ cells more closely. These studies were performed in collaboration with Susan Brown and Brian Haare at the University of Michigan. *pfy*⁻ cells look like *cap*⁻ cells. They are round and enlarged. They also grow less well in rich medium. *cap*⁻ cells, like *pfy*⁻ cells, have no discernible actin cables and bud randomly from their surface. Overexpression of *PFY* in *cap*⁻ cells restores most of *cap*⁻ phenotypic defects, save one: Such cells grow in rich medium, survive a nitrogen starvation, are smaller, bud asymmetrically from one pole, and have actin cables, but they are not RAS-responsive. These results suggest that the carboxyl terminus of CAP is required for proper profilin function and that the phenotype which results from its loss is mainly due to loss of profilin function.

Some biochemical insight into CAP emerges from these experiments. In our collaboration with the group at the University of Michigan, we have found mutant *PFY* genes that can suppress a *pfy*⁻ cell but not a *cap*⁻ cell. These mutant genes encode proteins that apparently bind actin with affinities equal to those of wild-type profilins, so some function beside actin binding is required for suppression of *cap*⁻ cells, perhaps phospholipid binding. To test this, we expressed form I and form II *Acanthamoeba* profilins in *pfy*⁻ and *cap*⁻ yeasts. Form II profilin binds phosphatidyl inositol phosphates with high affinity and suppresses both *cap*⁻ and *pfy*⁻ cells. However, form I profilin, which binds phospholipids with lower affinity, suppresses *pfy*⁻ cells but not *cap*⁻ cells. These results suggest that CAP effects profilin function, and hence the cellular cytoskeleton, by effecting the metabolism of phosphatidyl inositol phosphates.

We do not yet know if CAP is a regulatory molecule, and if so, what regulates it. However, it physically connects two major pathways: (1) the cAMP pathway, presumably involved in growth signaling, and (2) a pathway in phospholipid metabolism effecting the remodeling of the cell. CAP is therefore a prime candidate for integrating these pathways. Its proximity to adenylyl cyclase means it is proximal to RAS, and although we have no evidence yet that CAP is controlled by RAS, we note that the types of changes in mammalian cells induced by oncogenic RAS, namely, morphological transformation with its dissolution of actin cables and alterations in phospholipid metabolism, resemble the effects of altered CAP function in yeast.

Future studies will be directed to finding CAP homologs in other organisms, exploring the precise biochemical function of CAP, and looking for further genetic clues into its interactions with other proteins.

Mammalian cDNAs Selected for Interfering with RAS Function in *S. cerevisiae*

J. Colicelli, C. Nicolette, E. Chang, L. Rodgers, M. Riggs, M. Wigler

The observation that dysfunctional forms of adenylyl cyclase interfere with RAS function inspired the design of a genetic screen for mammalian cDNAs that encode products which can interact with yeast RAS proteins. Cells expressing the *RAS2*^{val19} gene are heat-shock-sensitive, one of the phenotypic hallmarks of a constitutively activated RAS pathway. The same phenotype results from overexpressing adenylyl cyclase or the catalytic subunits of the cAMP-dependent protein kinase, encoded by the *TPK* genes, or from the disruption of the cAMP phosphodiesterases, encoded by the *PDE1* and *PDE2* genes. We designed a high-copy yeast shuttle vector that can express mammalian cDNA inserts from a strong yeast promoter. cDNA libraries constructed in this vector were screened for clones that could suppress the heat-shock-sensitive phenotype of *RAS2*^{val19} strains. Several clones were so isolated, and these fell into two sets (Colicelli et al., *Proc. Natl. Acad. Sci. 86:* 3599 [1989]; *Proc. Natl. Acad. Sci. 88:* 2913 [1991]). The first set of clones could also suppress the heat-shock-sensitive phenotype of *pde*⁻ strains. These cDNAs encode cAMP phosphodiesterases (see below). The second set failed to suppress *pde*⁻ phenotypes and hence contained candidates encoding proteins that interact with RAS proteins.

Three genes were represented among this second set: *JC99*, *JC265*, and *JC310*. Sequence analysis revealed no relationship between these genes and genes in the known data banks; however, *JC99* and *JC265* encode proteins that are clearly related to each other and presumably are members of a family. Further genetic analysis in *S. cerevisiae* indicates that all three genes can interfere with the activity of *RAS1* and *RAS2* and the mammalian Ha-*ras* genes expressed in *S. cerevisiae*. The mechanism of action is not known.

Future studies will aim at expressing products of the genes in mammalian cells, *Xenopus* oocytes, and the yeast *S. pombe* in an attempt to understand

whether they interact with RAS proteins directly and to determine if they are effectors of RAS action.

Function of the Von Recklinghausen Neurofibromatosis Gene Product, NF1

R. Ballester, M. Wigler

The gene for the Von Recklinghausen neurofibromatosis locus *NF1* was recently identified (Cawthon et al., *Cell 62:* 193 [1990]; Viskochil et al., *Cell 62:* 187 [1990]; Wallace et al., *Science 249:* 181 [1990]). People afflicted with this disease have widespread disorders of the proliferation and differentiation of tissue of neuroectodermal origin. The partial sequence of the *NF1* cDNA was determined and found to encode a protein with sequence similarities to the mammalian GAP and yeast IRA proteins (Buchberg et al., *Nature 347:* 291 [1990]; Xu et al., *Cell 62:* 599 [1990]). GAP (*G*TPase *a*ctivation *p*rotein) is a factor that accelerates GTP hydrolysis by RAS and is capable of down-regulating wild-type RAS proteins (Trahey and McCormick, *Science 238:* 542 [1987]; Ballester et al., *Cell 29:* 681 [1989]; Zhang et al., *Nature 246:* 754 [1990]). Oncogenic RAS proteins are generally resistant to GAP. The IRA proteins are structurally related proteins with similar functions (Tanaka et al., *Cell 60:* 803 [1990]). They are encoded by the *IRA1* and *IRA2* loci, and disruption of either loci leads to a heat-shock-sensitive phenotype, the result of activation of the RAS pathway. The NF1 protein bears about the same relatedness to GAP as do the IRA proteins, but NF1 shows a greater global similarity to the IRA proteins. In collaboration with F. Collins and D. Marchuk at the University of Michigan, we have begun to examine the function of the human NF1 protein when expressed in yeast (Ballester et al., *Cell 63:* 851 [1990]). Expression of NF1 can restore heat-shock resistance to yeast defective in IRA function, as we have previously demonstrated for GAP, and can down-regulate mammalian Ha-ras when they are co-expressed in yeast. Unlike GAP, NF1 appears to be capable of interfering with the function of the activated Ha-ras^{val12} mutant protein in yeast. These results indicate that NF1 can interact with a wide range of RAS proteins. We directly demonstrated that NF1 possesses GAP-like activity: Extracts of yeast cells expressing NF1 accelerate the GTP hydrolysis of purified Ha-ras, but not of Ha-ras^{val12}, proteins. In the future, we intend to examine whether NF1 shares other functions with the IRA proteins.

S. pombe Adenylyl Cyclase

D. Young, M. Kawamukai, K. Ferguson, M. Wigler

We have begun studying *S. pombe* as an experimental system to explore RAS function. *S. pombe* contains a single *ras1* gene encoding a RAS homolog (Fukui and Kaziro, *EMBO J. 4:* 687 [1985]). *ras1* does not regulate *S. pombe* adenylyl cyclase (Fukui et al., *Cell 44:* 239 [1986]). In search of clues to RAS function, we have begun to explore the difference between the regulation of adenylyl cyclase in the two organisms. First, we cloned and sequenced the *S. pombe cyr1* gene (Young et al., *Proc. Natl. Acad. Sci. 86:* 7989 [1989]). It encodes a large protein with about 30% sequence identity to *S. cerevisiae* CYR1 overall. The homology between the catalytic domains is stronger. Like *S. cerevisiae CYR1*, the *S. pombe* gene encodes a leucine-rich repeat domain, and the catalytic domain is carboxy-terminal. Thus, the overall structures of the two yeast cyclases are very similar and differ radically from the structures of the mammalian adenylyl cyclases that have been discovered recently (Krupinski et al., *Science 244:* 1558 [1989]).

Disruption of *S. pombe cyr1* produces a phenotype we call hypersexed (Kawamukai et al., *Cell Regul. 2:* 155 [1991]). *cyr1*$^-$ cells are perfectly viable, but mate prematurely in rich medium, unlike wild-type cells that mate upon starvation. Overexpression of *cyr1* leads to a partial sterile phenotype. Overexpression of the repeat domain encoded by *cyr1* also leads to a hypersexed phenotype, suggesting that expression of this fragment of adenylyl cyclase interferes with the function of the wild-type protein, much as we have found in our studies of the *S. cerevisiae* cyclase. This study suggests that both molecules share a conserved regulatory mechanism. Other evidence points to the conclusion that *S. pombe* cyr1 is regulated.

Full-length cyr1 molecules can be purified from *S. pombe*, using the epitope addition immunoaffinity method, and these preparations have high levels of enzymatic activity when assayed in the presence of Mn^{++} ion. The ratio of Mn^{++} to Mg^{++} activities is much higher for *S. pombe* cyr1 than it is for *S. cerevisiae* CYR1. We see no stimulation of activity in the presence of guanine nucleotides. We have clear evidence that a protein antigenically related to *S.*

cerevisiae CAP is associated with *S. pombe* cyr1, and we are in the midst of characterizing this molecule.

An *S. pombe* Kinase in the ras1 Pathway

Y. Wang, H.-P. Xu, M. Riggs, L. Rodgers, M. Wigler

We have sought genes in *S. pombe* that encode components of the ras1 signaling pathway. *ras1⁻* cells are viable but fail to sporulate or conjugate and are round in shape rather than cylindrically elongated (Fukui et al., *Cell 44:* 329 [1986]). We selected for *S. pombe* genes present on high-copy shuttle vectors that could restore conjugation to cells expressing a dominant interfering *S. cerevisiae* $RAS2^{ala15}$ gene. In this way, we isolated *byr2* (*by*pass of *r*as, no. 2, also known as *sir1*) (Wang et al., *Cell Biol.* [1991] in press). *byr2* can also suppress the sporulation defects of *ras1⁻/ras1⁻* cells but not the cell-shape abnormalities or the conjugation defects of a *ras1⁻* cell. *byr2* has the potential to encode a serine/threonine kinase, with a carboxy-terminal catalytic domain. In size and structure, byr2 resembles the protein kinases C, the raf kinases, and the cGMP-dependent protein kinases, but it has no particularly close relationship to any individual kinases. In its genetic properties, *byr2* most closely resembles *byr1*, an *S. pombe* gene encoding another protein kinase that was identified previously (Nadin-Davis and Nasim, *EMBO J. 7:* 985 [1988]). Expression of *byr1* can likewise suppress the sporulation defects of *ras1⁻/ras1⁻* diploids. Like *byr1⁻* cells, *byr2⁻* cells are perfectly viable and of normal shape but are absolutely defective in conjugation and sporulation. *byr1* and *byr2* are thus both good candidates for genes encoding downstream components of the ras1 signaling pathways. Since overexpression of *byr1* can complement the sporulation defects of *byr2⁻* cells, but not vice versa, we can place the site of action of byr1 downstream from byr2.

A Putative Nucleic-acid-binding Protein in the ras1 Pathway

H.-P. Xu, Y. Wang, M. Riggs, L. Rodgers, M. Wigler

We directly selected for *S. pombe* genes that on high-copy shuttle vectors are capable of suppressing the sporulation defects of *ras1⁻/ras1⁻* diploids. One of these genes, called *byr3* (also known as *prs* and *HP18*), was found to encode a protein comprised almost in its entirety of a zinc finger repeat motif (H.-P. Xu, Ph.D. Thesis [1990]). This metal-binding motif is commonly found in proteins that bind double- or single-stranded DNA, or RNA. The motif structure of byr3 resembles most closely the metal-binding motif of CNBP, a putative sterol regulatory element that binds single-stranded DNA (Rajavashisth et al., *Science 245:* 640 [1989]). On the basis of its structure, we imagine that *byr3* encodes an inhibitor of gene transcription.

The genetic characterization of *byr3* is as follows. High-copy expression of *byr3* suppresses the sporulation defects of *ras1⁻/ras1⁻* diploids, but not the shape abnormalities or conjugation defects of *ras1⁻* cells, and none of the phenotypic defects of *byr2⁻* or *byr1⁻* cells. *byr3⁻* cells are perfectly viable and normally shaped, but they are partially sterile. This defect is restored by high-copy wild-type *ras1* or *byr2*, but not by *byr1*. This result indicates again that the byr2 kinase lies closer to the root of the ras1 signaling pathway than does the byr1 kinase. This result further suggests that byr2 and byr1 have common but perhaps parallel functions and that byr1 acts at least partly through byr3. Further studies will focus on resolving this model and delineating the role, if any, that byr3 plays in transcriptional regulation.

GAP-like Molecules in *S. pombe*

Y. Wang, H.-P. Xu, M. Riggs, L. Rodgers, M. Wigler

We have sought genes that on high-copy shuttle vectors are capable of inhibiting the phenotype of *S. pombe* cells expressing $ras1^{val17}$, the activated allele of *ras1*. Such cells are partially sterile, highly agglutinable, and develop long exaggerated conjugation tubes. One gene was isolated that we called *sar1* (*s*uppressor of *a*ctivated *r*as) (Wang et al., *Mol. Cell. Biol.* [1991] in press). Upon DNA sequencing, it eventually became clear that *sar1* encoded another member of the family of proteins that included the GAP, IRA, and NF1 proteins. The homology with other members of this family resides strictly in the "catalytic" domain, and sar1 is closest in sequence to NF1.

Genetic evidence indicates that *sar1* down-regulates *ras1* in *S. pombe*. *sar1⁻* cells have the same

phenotype as $ras1^{val17}$ cells, and $ras1^-$ mutations are epistatic to $sar1^-$; i.e., $ras1^-$ $sar1^-$ double mutants have the same phenotype as $ras1^-$ cells. Expression of sar1, like expression of GAP and NF1, can complement yeast defective in the *IRA* genes.

Many investigators have speculated that GAP-like molecules encode RAS effectors. There is some evidence for this in mammalian cells (Tatani et al., *Cell 61:* 769 [1990]), but no evidence of this for the *S. cerevisiae* IRA proteins. There is also no support for this hypothesis from studies of sar1 function in *S. pombe.*

An RNase in the Regulation of *S. pombe* Sexual Function

H.-P. Xu, Y. Wang, M. Riggs, L. Rodgers, M. Wigler

Disruption of ras1 function leads to failure to conjugate and sporulate. We therefore selected for genes on high-copy plasmids that caused failure to sporulate. One such gene was isolated and called *hcs* (*h*igh *c*opy *s*terile) (Xu et al., *Nucleic Acids Res. 18:* 5304 [1990]). Disruption of *hcs* causes lethality. Overexpression causes no diminution in growth rate or change in shape, but it renders cells unable to sporulate or conjugate. Sequence analysis reveals the *hcs* can encode 363 amino acids with 24% sequence identity to *Escherichia coli* RNase III. RNase III is a double-strand-specific RNase involved in processing of ribosomal RNA and mRNAs in *E. coli* (Nashimoto and Uchida, *Mol. Gen. Genet. 201:* 25 [1985]; Portier et al., *EMBO J. 6:* 2165 [1987]; Takiff et al., *J. Bacteriol. 171:* 2581 [1989]). This work suggests that some control of the pathways regulating sexual differentiation may be exerted at the level of RNA stability or availability and implicates regulation of RNases in the control of gene expression in eukaryotes.

Mammalian cDNAs Selected for RAS Function in *S. pombe*

H.-P. Xu, M. Riggs, L. Rodgers, M. Wigler

It is altogether possible that the function of *ras1* in *S. pombe* resembles its function in mammalian cells. To explore this possibility, we screened mammalian cDNA libraries cloned into *S. pombe* expression vectors for genes capable of inducing sporulation in $ras1^-/ras1^-$ diploids. Many classes of candidates were obtained. The first class comprised mammalian *RAS* genes, which was expected (Xu et al., *Cell Regul. 1:* 763 [1990]). The second class comprised mammalian *RAP* genes, which was unexpected, including *RAP1A*, *RAP1B*, and *RAP2* (Xu et al., *Cell Regul. 1:* 763 [1990]). The *RAP* genes encode members of the RAS superfamily (Pizon et al., *Oncogene 3:* 201 [1990]). RAP proteins show approximately 50% identity with RAS proteins. RAP1A is also known as Ki-*rev*-1, which was discovered as a weak inhibitor of the morphologic transformation induced by oncogenic *RAS* (Kitayama et al., *Cell 56:* 77 [1989]). These mammalian *RAP* genes can induce sporulation in $ras1^-/ras1^-$ diploid *S. pombe* and can restore normal cell shape to $ras1^-$ haploid cells, but they fail to restore conjugal competence to $ras1^-$ cells. We next explored the function of mammalian *RAP1A* in *S. cerevisiae.* In that yeast, expression of *RAP1A* was not able to provide RAS-like functions. Indeed, expression of *RAP1A* weakly antagonized the phenotype of the activated $RAS2^{val19}$ allele. These studies indicate that mammalian RAP can interact with RAS targets, sometimes productively and sometimes nonproductively.

The third class of cDNAs comprise two genes known as *ATG16* and *ATG29* (H.-P. Xu, Ph.D. Thesis [1990]). In virtually all its genetic aspects, *ATG29* resembles a weaker form of *ATG16*. In high copy, both genes can induce sporulation in $ras1^-/ras1^-$ diploid yeast. They fail to correct shape and conjugation defects of $ras1^-$ cells, but both can correct the conjugation defects of $byr3^-$ yeast. Neither can correct the phenotypic defects of $byr1^-$ or $byr2^-$ yeast. These results suggest that ATG16 and ATG19 can each carry out a portion of ras1 function lying upstream of the byr1 and byr2 kinases and also suggest that they operate on the same pathway. Strikingly, both *ATG16* and *ATG29* can induce haploid sporulation in cells containing the activated $ras1^{val17}$ allele. Haploid sporulation is a very rare event in wild-type yeast. This result again strongly suggests the relatedness of the function of these gene products, which can evidently act synergistically with $ras1^{val17}$. Finally, coexpression of *ATG16* and *ATG29* can induce haploid sporulation in wild-type *S. pombe.* Thus, their gene products can cooperate. Future studies will focus on the characterization of the *ATG16* and *ATG29* gene products and their function in mammalian cells.

Mammalian cAMP Phosphodiesterase Genes

J. Colicelli, T. Michaeli, G. Bolger, M. Riggs, L. Rodgers, M. Wigler

In the course of selecting for mammalian cDNAs that could suppress the phenotype of *RAS2*^{val19} cells, a rat cAMP phosphodiesterase (PDE) gene was cloned (Colicelli et al., *Proc. Natl Acad. Sci. 86:* 3599 [1989]) and named rat *DPD* because it was homologous to the D*rosophila melanogaster d*unce *p*hosphodiesterase. A great variety of cAMP phosphodiesterases are expressed in mammalian tissues and are of considerable importance, since cAMP regulates many physiological processes. A better understanding of this complex family of enzymes will emerge once the genes encoding them are cloned and characterized. Approaching this problem through biochemical purification and sequencing has been a slow process, and yeast selection for cDNAs encoding PDEs provides a way to accelerate this discovery.

By screening a cDNA library made from a human cell line, we have discovered three different human PDE genes. Two of these are closely related and encode rolipram-inhibitable, high affinity, cAMP-specific PDEs. These two genes are homologous to the rat *DPD*. We discovered other members of this family by using the rat *DPD* as a probe. Using degenerate oligonucleotide probes to conserved regions as primers in polymerase chain reactions, we have concluded that there are probably only four genes that comprise this family. The third human PDE cDNA cloned by complementation in yeast has biochemical properties that suggest it represents a previously undiscovered family of PDEs. We plan to characterize the human PDE families further.

Genomic Difference Cloning

I. Wieland, G. Asouline, K. O'Neill, M. Wigler

We have been in the process of developing a method of "genomic difference cloning." This method allows the identification by cloning a DNA sequence present in one genome that is absent in an otherwise similar or identical genome (Wieland et al., *Proc. Natl. Acad. Sci. 87:* 2720 [1990]). Such differences arise in important situations: when an individual or a tissue is infected with a DNA-based pathogen or when a neoplastic cell has lost sequences from both alleles of a recessive oncogene. The outline of one method for difference cloning was described in last year's Annual Report and was published this past year. This method is not yet powerful enough to be used routinely for our purposes. We have concentrated this year on the incremental improvement of our methodologies.

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RAS ONCOGENES AND SIGNAL TRANSDUCTION

D. Bar-Sagi N. Gale, L. Graziadei, K. Degenhardt, S. Kaplan, S. Palmer, J. Yates, Y. Yeh

Our major interest is in transmembrane signals that control cell proliferation. Our research focuses on two components of the signal transduction machinery in mammalian cells: (1) *ras* proteins and (2) phospholipase A_2 (PLA_2). *ras* proteins are small guanine-nucleotide-binding proteins that reside on the inner surface of the plasma membrane. They have been highly conserved in evolution and play a critical role in mediating signals that control cell growth. PLA_2 is a calcium-dependent esterase that catalyzes the hydrolysis of fatty acid ester bond specifically at the *sn-2* position of glycerophospholipids (hence, the designation A_2). Our interest in the action of PLA_2 derives from its implication in mediating signal transduction through the release of arachidonic acid from phospholipids in the plasma membrane. Arachidonate is the precursor for a number of intracellular messengers involved in growth control, including leukotrienes, thromboxanes, and prostaglandins. In addition, earlier studies from our laboratory indicate a possible functional link between PLA_2 activation and *ras*-induced cell transformation. During the past year, we have continued to analyze the molecular properties of PLA_2. A particularly useful development in this area has been the generation of monoclonal antibodies against PLA_2. These antibodies are being extensively used to characterize the involvement of PLA_2 in signal transduction. We have also continued to study the mechanisms of signal transduction by *ras* proteins. Through the use of signaling events in the immune system, we have obtained some new insights into the possible function of these proteins.

Participation of *ras* Proteins in T-cell Activation

N. Gale, D. Bar-Sagi

To understand the regulatory function of *ras* proteins, it is essential to identify both upstream and downstream components in the *ras* signaling pathway. To this end, we have been investigating the role of *ras* proteins in T-cell activation. Activation of T cells by interaction of antigen with the T-cell receptor (TCR) proceeds via a well-documented sequence of transduction events that leads to cellular differentiation and proliferation. The interleukin-2 (IL-2) gene is expressed in T cells in a strictly regulated manner. In resting T cells, IL-2 transcripts are not detectable; however, triggering of the TCR by lectins or antibodies in combination with protein kinase C stimulation leads to the activation of the IL-2 gene. We reasoned that if *ras* proteins are involved in these signaling events, then their overexpression may have an effect on IL-2 gene activation. To test this possibility, Jurkat cells were transfected with a plasmid expressing the proto-oncogenic form of *ras* (Fig. 1, pNV-WT) and control plasmid (Fig. 1, pNV-VECTOR). To monitor *ras* effects on the level of IL-2 mRNA, a quantitative polymerase chain reaction (PCR)-based assay was employed. To visualize and quantitate the amplified products, ^{32}P-end-labeled primers were used. In these experiments, mRNA levels of three genes were evaluated using specific primer sets for each. IL-2 mRNA was evaluated for inducibility under the various stimulatory conditions.

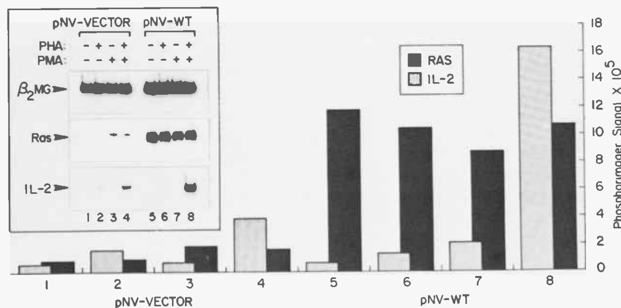

FIGURE 1 Effects of *ras* overexpression on IL-2 gene inducibility. Jurkat cells were transfected with vector alone (pNV-VECTOR) or with a wild-type c-Ha-*ras* (pNV-WT). Twenty hours after transfection, cells were stimulated with PHA, PMA, PHA+PMA, or none for 8 hr. Cells were then harvested, and RNA was prepared and processed for PCR. To visualize and quantitate the PCR products, ^{32}P-end-labeled primers were used in the reaction. Equal aliquots of the reactions were electrophoresed on TBE-PAGE gels and exposed to XAR film (*inset*) or phosphorimaging screen. To correct for any differences in input cDNA among the various conditions, results were normalized according to β_2MG expression. Numbers under bar graphs correspond to lane numbers on the autoradiogram. Note that overexpression of *ras* induces a fourfold stimulation of the induction of IL-2 by PMA+PHA (compare IL-2, lane *4*, and IL-2, lane *8*).

ras mRNA levels were assayed to verify the expression of the exogenous construct. β_2 microglobulin (β_2MG) mRNA levels were used as internal controls for the quantity and quality of RNA used in each assay. As shown in Figure 1, in cells transfected with control plasmid, a very low level of *ras* expression was detected (*Ras*, lanes 1–4). In contrast, a high level of *ras* expression was detected in cells transfected with the *ras* expression plasmid (*Ras*, lanes 5–8). As expected, IL-2 transcription was induced when cells were stimulated with both PHA (a mitogenic lectin) and PMA (a protein kinase C activator) (IL-2, lane 4). We found that *ras* overexpression had no effect on IL-2 transcription in unstimulated cells or in cells stimulated with PHA or PMA alone (IL-2, lanes 5–7). However, in Jurkat cells treated with both PHA and PMA, the induction of IL-2 expression was potentiated by *ras* overexpression (compare IL-2, lanes 4 and 8). Quantitative analysis of the results (Fig. 1, bar graphs) indicates that *ras* overexpression results in a fourfold stimulation of IL-2 induction. These observations provide evidence for the involvement of *ras* in signaling events relevant to IL-2 induction. The signaling pathways that contribute to the induction of the IL-2 gene have been extensively characterized. Therefore, we anticipate that the effects of *ras* on IL-2 expression will constitute a useful experimental system to elucidate the *ras* pathway.

Posttranslational Processing of *ras* Proteins: Role of Carboxyl Methylation

K. Degenhardt, D. Bar-Sagi

Protein carboxyl methylation is a posttranslational modification found in both prokaryotic and eukaryotic cells. In mammalian cells, this methylation reaction is part of a series of posttranslational modifications of proteins originally synthesized with a –Cys-AAX carboxy-terminal sequence, which involve the proteolytic removal of the last three amino acids, the lipidation of the cysteine sulfhydryl group with an isoprenyl moiety, and the methyl esterification of the newly exposed α-carboxyl group. The protein substrates for these reactions include molecules involved

in sensory transduction (the α-subunit of retinal cGMP phosphodiesterase), in nuclear membrane structure (lamin B), and in transmembrane signaling (small [21–24 kD] G proteins).

One of these G proteins has been clearly identified as *ras*. We have developed an in vitro assay system for the methyl esterification of the α-carboxyl group of the carboxy-terminal cysteine residue of *ras* proteins. In this assay system, membrane preparations are incubated with the methyl donor [^{3}H]*S*-adenosyl-methionine for various intervals. *ras* polypeptides are isolated by immunoprecipitation using anti-*ras* monoclonal antibody (Y13-259). The immunoprecipitates are resolved on polyacrylamide gels. The *ras* band is then cut from the gel, and the radioactively labeled methyl group is hydrolytically released by the addition of 1 N NaOH and counted by scintillation counting. Using this assay system, we have found that more than 90% of the incorporation of ^{3}H-methyl group onto *ras* was inhibited by *S*-adenosylhomocysteine, a specific inhibitor of methyltransferase enzyme. This result demonstrates that the methylation of *ras* in vitro is specifically catalyzed by protein carboxyl methyltransferase. In addition, we have found by pulse-chase experiments that the carboxyl methyl group on *ras* turns over with a half-life of approximately 60 minutes. These results document, for the first time, the turnover of the carboxyl methyl group on *ras* and are consistent with a dynamic methylation-demethylation cycle catalyzed by methyl transferase and methyl esterase, respectively. We plan to use this in vitro assay system to investigate the functional significance of the carboxyl methylation of *ras* proteins. Our current hypothesis is that methyl esterification of *ras* proteins leads to a tighter association of these proteins with the plasma membrane.

Establishment of a Method for the Introduction of *ras* or Other Proteins into a Large Number of Cells and the Isolation of Protein-loaded Cells

L. Graziadei, D. Bar-Sagi

We have recently shown that *ras* proteins co-cap with surface immunoglobulins (sIg) in mitogenically stimulated B lymphocytes. The functional significance of this redistribution event is being studied in our laboratory. To assess the possible role of *ras* in the clustering of sIg, we plan to introduce mutationally activated *ras* proteins into B cells and then monitor sIg distribution. These experiments require that cells which contain introduced protein can be distinguished from those which do not. B cells grow in suspension and are very small, with large nuclei and relatively little cytoplasm. This makes them unsuitable for microinjection. In addition, the redistribution phenomenon observed in stimulated B cells persists for only minutes, thus making the DNA transfection approach not applicable. Therefore, we have established a method for introducing proteins into B cells. This method involves the use of electroporation to load purified proteins into a large number of cells with high efficiency. A unique feature of this method is that dextran-FITC is included in the electroporation medium and is thus cointroduced with the protein of interest. This enables the purification of cells containing dextran-FITC using fluorescence-activated cell sorting (FACS) and yields a population that is composed almost entirely of cells containing the protein of interest. The features of the method can be summarized as follows: (1) Subjecting cells to electroporation in the presence of fluorescently labeled dextran leads to the efficient uptake of dextran into the cytoplasm. (2) Electrical parameters of the electroporation can be adjusted to optimize for uptake and cell viability. (3) Dextrans with molecular masses of 19–150 kD display equivalent capacity to enter cells by this technique, as measured by the fluorescence intensity of loaded cells. (4) Using FACS, it is possible to separate the live loaded cells from the nonloaded cells. (5) After electroporation, cells remain capable of capping sIg upon proper stimulation.

To establish that dextran-FITC can be used as a reliable indicator of protein entry into electroporated cells, we co-loaded dextran-FITC with a fluorescent protein and directly monitored by FACS the coincidence of the two molecules on a per cell basis. The protein we used for this analysis is B-phycoerythrin (PE), a 240-kD member of the family of plant Phycobiliproteins which has an intrinsic fluorescence. As indicated by the two-color flow cytometric analyses shown in Figure 2, there is a linear correlation between the amount of dextran-FITC and the amount of PE loaded per cell. Thus, dextran-FITC uptake can be used as a quantitative indicator of protein loading. This method has been used on various cell types in our laboratory and should prove ap-

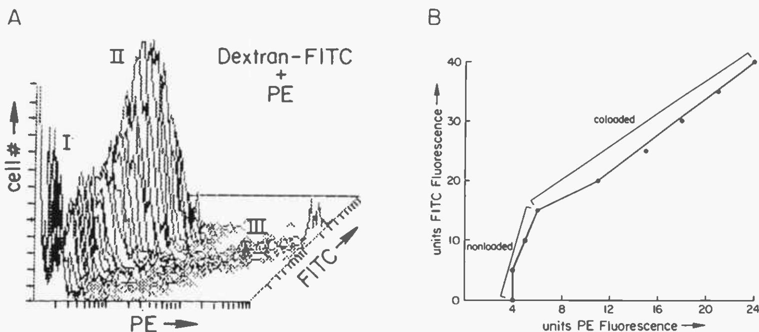

FIGURE 2 Use of dextran-FITC as a marker for the loading of proteins by electroporation. (*A*) Three-dimensional biparameter histogram of cells that have been co-loaded with dextran-FITC and PE. The peak height along the *z*-axis represents cell number. Population I is composed of nonloaded cells. Population II represents the double-loaded cells and constitutes 70% of total cells. Population III is composed mostly of dead cells and debris to which PE tends to stick nonspecifically. (*B*). Fluorescence intensities of PE versus FITC measured at positions of maximum peak height on the histogram shown in *A*. Numerical values on the axes are derived from the channel number on the log fluorescence scale and represent equivalent units of fluorescence intensity on each axis. Nonloaded and coloaded populations are indicated. The plot yields a straight line indicating a linear correlation between the amount of dextran-FITC and that of PE loaded per cell.

plicable for the introduction of proteins into many cell types. It is especially useful in situations where a large number or pure population of protein-loaded cells are required.

Tissue-specific Expression of Phospholipase A_2 Enzymes

N. Gale, S. Palmer, J. Yates, D. Bar-Sagi

Phospholipases A_2 (PLA_2) are a growing family of enzymes implicated in the biosynthesis of the prostaglandins, leukotrienes, and related eicosanoids. PLA_2 hydrolyze membrane phospholipids to liberate arachidonic acid, which is the rate-limiting factor in the synthesis of these important regulators of cell growth and proliferation. All of the structural information available to date about PLA_2 is derived from secreted forms of the enzyme. The two most abundant forms of secreted PLA_2 identified in mammalian cells have been termed pancreatic (type I) and splenic (type II) PLA_2. Catalytically, these two forms appear indistinguishable despite significant sequence differences primarily in the noncatalytic domains of the enzyme. In the past year, we have searched for novel forms of cellular PLA_2 using PCR amplification. To this end, we have synthesized several degenerate primer pairs, the sequence of which corresponded to two conserved domains found in both the pancreatic and splenic enzymes. cDNAs made from total RNA from various rat tissue sources were then used as targets in low-stringency PCRs to hunt for novel PLA_2 clones. Sequence analysis of a large number of amplified products revealed that all of them were identical to either the pancreatic enzymes or the splenic enzymes. However, we found that we were able to amplify these two types of PLA_2 clones with different apparent frequencies, depending on the tissue source examined, and in several instances were able to observe transcription of PLA_2 in tissues where they had been heretofore unseen. In light of these observations, we have undertaken the quantitative analysis of the expression of the pancreatic and the splenic enzymes in various rat tissues. For these experiments, pairs of primers that match exactly one or the other type of PLA_2 were synthesized. These primer pairs were used in high-stringency PCRs, using the cDNAs from 14 rat tissues as templates. Because there were significant differences in the qualities of RNA that we were able to prepare from different tissues, we incorporated an internal control for quality and quantity of input RNA into the assay.

For this control, we employed exact match primer pairs to β_2MG, a gene whose transcription is thought to be ubiquitous and nearly equal in all cell types. As shown in Figure 3, the levels of PLA_2 expression vary significantly among various tissues. As expected, pancreatic PLA_2 (type I) is expressed highly in the pancreas, greater than two orders of magnitude higher than in any other tissue examined. In other tissues, pancreatic PLA_2 expression is relatively high or low, with no true instances of intermediate levels of expression. There are notably high levels of pancreatic PLA_2 expression in ovary, lung, testis, and spleen that had not been previously observed; in fact, this PLA_2 was thought to be restricted in expression to only the pancreas and the lung. As has been previously reported, the levels of splenic PLA_2 (type II) expression are high in spleen and even more abundant in the intestine. Splenic PLA_2 is also expressed at intermediate levels in many of the tissues surveyed, including liver, heart, lung, and pancreas. Note that in contrast to the expression of pancreatic PLA_2, splenic PLA_2 was expressed at low levels in nearly all tissues examined, with the exception of muscle tissue and adrenal glands where its expression is absent or extremely low. Previously, splenic PLA_2 was thought to be restricted in expression to spleen, intestine, and platelets. We have found that splenic PLA_2 expression occurs at comparable levels in pancreas and liver as well. Overall, the pattern of expression of the PLA_2 enzymes indicates that in a given tissue, one form of the enzyme is preferentially expressed. In addition, the abundance of PLA_2 appears to be correlated with the proliferative capacity of the tissue. Thus, brain and muscle tissues exhibit low levels of expression, whereas spleen, pancreas, and intestine display high levels of expression. It is our hope that these new findings will be of value in our ongoing research concerning the role of PLA_2 in transducing intracellular signals, as well as in research in other laboratories concerning the structure and function of this enzyme.

Identification of a Novel Form of Cellular Phospholipase A_2

S. Kaplan, Y. Yeh, D. Bar-Sagi

In the past year, we have succeeded in generating a monoclonal anti-phospholipase A_2 (PLA_2) antibody using the pancreatic secreted enzyme as an antigen.

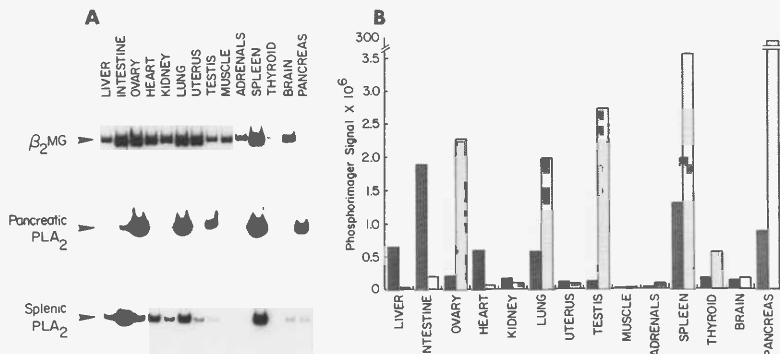

FIGURE 3 Expression of pancreatic and splenic PLA_2 enzymes in various rat tissues. The relative abundance of PLA_2 transcripts was determined in a variety of tissues using PCR. Three separate reactions were performed on cDNA from each tissue using different primer pairs in each reaction. Primer pairs were to β_2MG, pancreatic PLA_2, and splenic PLA_2. Results of these reactions are shown in panel *A*, and the quantitative phosphorimager analysis of these results, corrected for differences in β_2MG expression, is shown in panel *B*. (*Gray bars*) Pancreatic PLA_2; (*black bars*) splenic PLA_2.

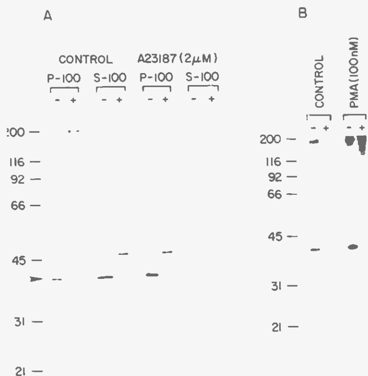

FIGURE 4 Immunoprecipitation of a novel polypeptide by a monoclonal anti-PLA_2 antibody. (*A*). [^{35}S]Methionine-labeled HeLa cells were fractionated to S-100 and P-100. Detergent lysates were prepared from each fraction and immunoprecipitated with anti-PLA_2 antibody (–) or with anti-PLA_2 antibody blocked with an excess of purified antigen (+). Under normal conditions (panel *A*, control), the antibody specifically immunoprecipitates a 39-kD protein (arrowhead) present in both the soluble and particulate fractions. In cells treated with the calcium ionophore A23187 prior to fractionation, the proportion of the 39-kD protein associated with the P-100 fraction is significantly increased. (*B*) Immunoprecipitation from ^{32}P-labeled HeLa cells. The 39-kD protein is a phosphoprotein (control), and the stimulation of PKC by PMA treatment results in the significant enhancement of its phosphorylation.

This antibody specifically immunoprecipitates a 39-kD protein from metabolically labeled cells. This 39-kD protein has been detected in a variety of cell lines. Cell fractionation experiments show that this protein is found in both the soluble (S-100) and the particulate (P-100) fractions (Fig. 4A). The association with the particulate fraction is enhanced by an increase in the intracellular calcium concentration (Fig. 4A) and is abolished by calcium removal. These results indicate that the 39-kD protein is most likely a PLA_2, since it has been long recognized that the association of PLA_2 with the cell membrane is calcium-dependent. Indirect immunofluorescence staining with this antibody shows that the protein is located in both the membrane and the cytoplasm. Specific enrichment of staining is found in membrane ruffles and in perinuclear regions. This staining pattern is consistent with the biochemical fractionation data published by other investigators. The 39-kD protein has a pI of approximately 5.0 and appears to consist of multiple forms. In addition, the 39-kD protein is a phosphoprotein, and its phosphorylation is significantly enhanced by treatment of cells with the phorbol ester PMA. These results indicate that the 39-kD protein is a protein kinase C substrate. Immunoaffinity purification experiments are under way to test whether the protein exhibits a PLA_2 activity. Purification of the protein will be the focus of our effort in the coming year.

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NUCLEAR SIGNAL TRANSDUCTION

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We are interested in how extracellular signals are communicated to the nucleus, resulting in stable and specific changes in the pattern of cellular gene expression. The major focus of our work is the proto-oncogene c-*fos*, which plays a critical role in cellular signaling. The c-*fos* gene is rapidly activated in cells within minutes of receiving a signal. Activation does not require new protein synthesis and therefore proceeds by posttranslational modification of preexisting proteins. A major goal of our work is to define the proteins and other molecular messengers through which these signals travel from the cell surface to the c-*fos* gene. In particular, we are interested in how the specificity of signaling is achieved. Since a cell will respond in a distinct fashion to a given growth factor, how is the message delivered by one factor different from another? Thus, we wish to know how signals from discrete individual signal transduction pathways reach the c-*fos* gene and how the function of the Fos protein complex itself might vary depending on the activating signal.

We are taking several approaches to this problem, as described in the sections below. We are performing mutagenesis on the c-*fos* promoter and other regulatory sequences to identify the elements that respond to individual signal transduction pathways. We are using these sequences to identify cellular DNA-binding proteins that mediate the activities of these elements. We are also developing genetic approaches that we hope will accelerate our efforts to isolate intracellular mediators along these signaling pathways. Finally, we are using high-resolution two-dimensional gel electrophoresis to study the composition of the Fos protein complex induced in cells by different stimuli, and we are actively isolating novel components of the complex.

Functional Analysis of the SRE

R. Graham, M. Gilman

The serum response element (SRE) is a major target through which growth factors activate c-*fos* transcription. We and other investigators have shown that the SRE is the target for at least two distinct signaling pathways, one dependent on protein kinase C (PKC) and one independent of PKC. In addition, the SRE appears to be a major target for the active repression of c-*fos* transcription that begins approximately 30 minutes after induction. Not surprisingly, the SRE is a binding site for several different cellular proteins. We have undertaken a detailed mutagenesis of the SRE in an effort to correlate the binding of these proteins with individual regulatory activities of the SRE.

We isolated one important class of SRE mutants. These mutants bind one of the SRE-binding proteins, SRF, with wild-type affinity, but they fail to form a ternary protein-DNA complex, consisting of SRE, SRF, and a second protein termed TCF (ternary complex factor). We found that these mutants were specifically defective in response to one of the signals that act on c-*fos* through the SRE, the PKC pathway. Response to PKC-independent signals was unaffected in these mutants (Fig. 1). Thus, we conclude that the ternary complex, SRF and TCF bound to the SRE, comprises the target for the PKC signal, whereas PKC-independent signals are able to target SRF alone or in conjunction with proteins we have not yet identified.

Our observations suggest a model to account for the specificity of action of growth factors on genes regulated by SRE sequences. It is clear that an SRF-binding site alone is not sufficient for response to the PKC pathway. Only when the site is situated within a sequence context that permits the recruitment of TCF is it responsive to this signal. We generalize this observation to suggest that all signals require accessory proteins that interact with SRF. An important ramification of this model is that virtually any spectrum of signal response can be generated in an SRE-containing promoter simply by varying the sequence context near, or perhaps distant from, the SRE. A critical prediction of this model is that we will find additional sequences in the c-*fos* promoter required for response to PKC-independent signals. We are now seeking such sequences.

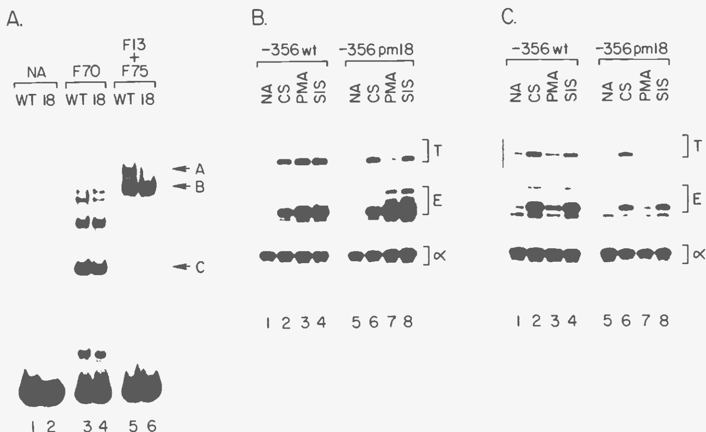

FIGURE 1 Binding properties in vitro and inducibility in vivo of a c-*fos* promoter carrying a single nucleotide substitution, pm18, that abolishes ternary complex formation. (*A*) Mobility-shift assay. DNA probes carried c-*fos* sequences from -356 to -275, either wild-type (WT) or mutant (pm18). Assays were performed with no added protein (lanes *1* and *2*), partially purified p62DBF (lanes *3* and *4*), and partially purified TCF and SRF (lanes *5* and *6*). (*B*) Transient expression assay of c-*fos* promoters. BALB/c 3T3 cells were transfected with plasmids carrying a wild-type (lanes *1–4*) or pm18 (lanes *5–8*) promoter and starved for serum for 48 hr. The cells were treated for 45 min with nothing (lanes *1* and *5*), 10% calf serum (lanes *2* and *6*), 50 ng/ml phorbol myristate acetate (lanes *3* and *7*), or 40 ng/ml c-*sis* protein (lanes *4* and *8*). T, E, and α indicate the positions of probe fragments protected by RNA from the transfected gene, the endogenous c-*fos* gene, and a human α-globin gene internal control, respectively. (*C*) Identical to *B*, except that cells were treated for 48 hr with 200 ng/ml phorbol dibutyrate to remove active PKC.

Regulation of c-*fos* Transcription by cAMP

L. Berkowitz

Among the many second-messenger systems that activate c-*fos* is the well-studied cAMP pathway. Previously, we identified three elements in the c-*fos* promoter required for induction by cAMP. These three cAMP response elements (CRE) all contain the CRE consensus core sequence, TGACG, yet they differ in transcriptional strength. The hierarchy of strength correlates with the elements' affinity for cellular binding protein(s), thus reflecting their intrinsic activities and not their relative positions within the promoter.

A likely candidate for the transcription factor acting through these CREs is the CRE-binding protein (CREB). This protein is phosphorylated in vitro by the cAMP-dependent protein kinase, the biological effector of cAMP, and can activate transcription of a CRE-containing gene in vitro. Using sequences from a published CREB clone, we isolated two classes of CREB cDNA clones by the polymerase chain reaction (PCR). The two types of CREB proteins differed by the insertion of 14 amino acids, presumably the result of alternative splicing. The two CREB proteins, produced by in vitro transcription/translation, showed no difference in their binding affinities to the strongest c-*fos* CRE. Both forms are expressed in all tissue-culture cell lines examined and in several mouse tissues. Studying the in vivo function of the cloned CREB proteins is complicated by the presence of endogenous CREB and related proteins. Therefore, we reprogrammed the DNA-binding specificity of CREB by attaching the DNA-binding domain of the yeast transcription factor *GAL4*. Correspondingly, the CRE in the reporter plasmid was changed to a *GAL4*-binding site. Cotransfection of the *GAL4*-CREB fusion expression plasmid and the *GAL4* reporter showed that both forms of CREB are able to confer cAMP responsiveness on the reporter. This re-

sponsiveness required CREB proteins in *trans* and the *GAL4*-binding site in *cis*. Thus, both forms of CREB are capable of transcriptionally activating a gene in response to cAMP.

Although CREB can confer cAMP responsiveness on a heterologous promoter and can bind to the c-*fos* promoter, it is not known if it plays a direct role in c-*fos* induction by cAMP in vivo. To determine this, we are attempting to block CREB activity by microinjecting specific CREB antibodies into cells and measuring their effect on c-*fos* expression. Several monoclonal antibodies against CREB have been generated. Alternatively, mutations made in the basic region create a dominant interfering CREB, which should interact with the endogenous CREB but create a nonfunctional complex. This construct will be transfected and microinjected into cells, and c-*fos* response will be assessed. We will also explore the basis of the growth inhibitory effect of CREB overexpression in cells.

The Synergistic Action of Protein Kinase C and Calcium on c-*fos* Transcription in T Lymphocytes

G. Lee

A fourth pathway that regulates c-*fos* transcription (after PKC, PKC-independent, and cAMP) is calcium. Calcium is a particularly important second messenger in the response of T lymphocytes to antigen. In fact, treatment with phorbol esters (which activate PKC) and calcium ionophores (which elevate intracellular calcium concentrations) is sufficient to mimic the effect of antigen. A key feature of this response is the synergistic interaction between these two signals. Together, they elicit a substantially stronger response than either does separately. We are interested in investigating the mechanism of this synergistic interaction.

Transfected c-*fos* genes are strongly activated by the combination of PMA and ionomycin at concentrations at which each has little effect. Thus, we observe this synergistic interaction on genes transiently expressed in T cells. We are currently considering two general molecular models that account for the synergistic response. First, the two signals may converge prior to reaching the c-*fos* gene, perhaps by synergistically activating a protein kinase, which in turn acts on a single element in the promoter. In this case, we would expect the induction to be abolished by mutation of one element, probably the SRE, which we already know to be a target for the PKC pathway. Alternatively, the two signals may act independently on the c-*fos* gene but be integrated in a synergistic fashion at the level of transcription. In this scenario, we anticipate that mutation at any of two unrelated sites would block induction. Our preliminary data suggest that induction is indeed blocked by mutations in two different sites—the SRE and an as yet uncharacterized element in the first intron of the gene. We are planning to map this intron element and determine if it is the target for the calcium signal.

Biochemical Characterization of Multiprotein Complexes at the Serum Response Element

H. Sadowski

Transcription of the c-*fos* gene is stimulated by the activation of a large number of cellular signal transduction pathways (i.e., protein tyrosine kinases, protein kinase C, protein kinase A, and calcium). Dissection of regulatory sequences within the c-*fos* promoter has led to the identification of sequence elements that confer responsiveness to specific stimuli through specific signal transduction pathways. The SRE is required for the large stimulation of c-*fos* transcription by serum in quiescent cultured cells. Several proteins are capable of binding specifically to the SRE sequence in vitro. Serum response factor (SRF) is a 67-kD phosphoprotein that binds to the SRE as a dimer. Mutational analysis indicates that the binding of SRF is required for c-*fos* induction by serum. In vitro, a 62-kD protein (TCF) can also bind to this site in the presence of bound SRF, forming a ternary complex. In vivo DNA footprinting assays suggest that these proteins are bound to the SRE in the presence or absence of stimulation, and no new complexes can be detected in gel-shift assays using extracts from stimulated cells versus unstimulated cells. Although SRF is phosphorylated by casein kinase II in vitro (and possibly in vivo as well), SRF is constitutively phosphorylated in cells (its phosphorylation state does not change after serum stimulation).

Serum is a complex mixture of growth factors,

hormones, and bioactive components that activate several signal transduction pathways in cells. Serum activation of *c-fos* transcription occurs through both PKC-dependent and -independent pathways, and maximal activation requires an intact SRE. Recent experiments from this laboratory have demonstrated that TCF is the target for PKC-dependent activation of *c-fos* transcription, as mutants that bind SRF but not TCF lose response to phorbol esters while retaining a PKC-independent response.

In the simplest model, serum stimulates TCF phosphorylation/dephosphorylation through PKC activation, leading to altered activity of the ternary complex in the absence of new binding activities. Using both wild-type and mutant SREs, DNA affinity precipitation (DNAP) assays of [^{32}P]orthophosphate-labeled nuclear extracts from phorbol-ester-stimulated and unstimulated cells are being performed to determine whether TCF phosphorylation is altered. If this is the case, biochemical fractionation of nuclear and cytoplasmic proteins from stimulated cells will be performed to purify the kinase/phosphatase responsible. Furthermore, these types of DNAP experiments, as well as gel-shift assays, will be perfomed with nuclear extracts from cells overexpressing epitope-tagged SRF to faciliate the detection of potential "quaternary" complex formation. In addition, these complexes can be subjected to chemical cross-linking in vitro (in the DNAP assays), and subsequent immunoprecipitation with anti-epitope monoclonal antibodies should allow the identification of nearest neighbors of SRF bound to the DNA template.

Cloning of a Novel SRE-binding Protein

R. Attar

As described above, the SRE is a target for multiple signals that activate and repress *c-fos* transcription. The SRE is a binding site for at least three cellular DNA-binding proteins. Until now, only one of these proteins (SRF) has been cloned. To achieve a better understanding of the complex mechanism by which the SRE regulates *c-fos* transcription, we have attempted to clone other SRE-binding proteins. We screened a HeLa cell cDNA expression library for phage expressing proteins that specifically bound an SRE oligonucleotide. For this study, a variant SRE sequence was used that binds a protein previously characterized in this laboratory, p62 DBF, with high affinity and SRF with low affinity. We isolated a phage that encoded a *lacZ* fusion protein that specifically bound this SRE oligonucleotide but not a mutant site that does not bind the human protein in vitro. The fusion protein binds specifically to the SRE in both a Southwestern blot and a mobility-shift assay.

Analysis of the partial DNA sequence of this clone revealed that it was a previously unidentified gene that belongs to the family of zinc-finger-containing proteins related to the *Drosophila Kruppel* gene. The clone contains seven tandem repeats in the carboxyl terminus that match the zinc finger consensus for this gene family. In the zinc finger region, the clone shares 65% sequence identity with several human and mouse *Kruppel*-related genes. This gene is expressed in a variety of human cell lines at a very low level. Expression is induced by serum in HeLa cells with a maximum level at 6 hours of stimulation. Antibodies raised against this cloned protein do not recognize p62DBF in Western blot or mobility-shift assays. These observations, together with the methylation interference pattern obtained with the cloned protein, suggest that this clone does not encode p62DBF, but instead identifies a novel SRE-binding protein. Immunofluorescence assays on transfected COS cells show that this protein is localized to the nucleus (Fig. 2). Preliminary results suggest that the cellular counterpart has an apparent molecular mass of 70 kD. The presence of zinc fingers in this clone strongly suggests that it is a DNA-binding protein and probably a transcription factor. After preliminary characterization of the protein, we will address the role of this protein in the regulation of the *c-fos* transcription.

Isolation of Human Genes Encoding SRE Complex Proteins by Functional Complementation in *Saccharomyces cerevisiae*

D. Grueneberg

We are studying the intracellular pathways by which signals travel from cellular receptors to the SRE of the *c-fos* proto-oncogene. There are multiple proteins

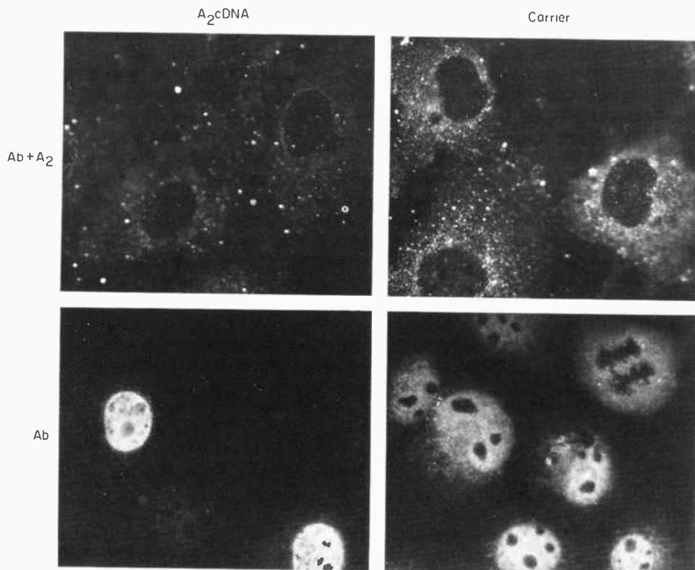

FIGURE 2 Indirect immunofluorescence demonstrating the nuclear localization of the zinc finger SRE-binding protein. All panels show COS cells fixed and stained with a rabbit polyclonal antiserum raised against *E. coli*-expressed protein. In the upper panels, the antiserum was blocked with antigen prior to use, demonstrating the specificity of staining. The panel on the lower left shows COS cells transfected with a vector expressing the cDNA. Note the strong staining in the nuclei of two transfected cells in the field. The panel on the lower right shows untransfected cells photographed with a longer exposure time, revealing nuclear fluorescence due to endogenous immunoreactive protein.

that bind to the SRE in order to both positively and negatively regulate the expression of c-*fos*. One DNA-binding protein, SRF, has been cloned and characterized. Recently, an SRF homolog, termed *MCM1*, has been cloned in the yeast *Saccharomyces cerevisiae*. Like SRF, *MCM1* acts in conjunction with accessory proteins encoded by *MATα1* and *MATα2*. The complex formed by the MCM1 and MATα1 proteins is highly similar to the SRF-TCF ternary complex. And like the SRF-TCF complex, the MCM1-MATα1 complex constitutes the target for activation by an extracellular signal, the a-factor mating pheromone. Therefore, we are attempting to clone a cDNA encoding TCF by complementation of a *MATα1* mutation.

We have constructed a yeast strain in which the expression of a selectable marker (*HIS3*) is under the control of a yeast upstream activating sequence (UAS) that binds the MCM1-MATα1 complex. These cells are His^-. We have deleted the active *MATα1* gene in this strain, which extinguishes expression of the *HIS3* marker gene, making the cells His^-. Finally, we have transformed this strain with a human cDNA library contained within a yeast expression vector and selected colonies that restored *HIS3* function. We obtained 60 His^+ colonies, of which approximately 70% were plasmid-dependent (Fig. 3). Sequence analysis revealed that almost all of these clones contained cDNAs derived from the same human gene. The gene, called *phox1*, encodes a

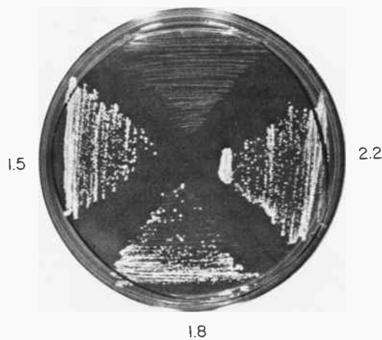

FIGURE 3 Activation of a *MATα1*-dependent UAS by a human cDNA. Three independent yeast transformants (labeled 1.5, 1.8, and 2.2—all derived from the same human gene) were streaked on minimal plates lacking histidine. The parental strain, labeled matα1::TRP1, does not grow on these plates, whereas the three transformants all grow.

homeodomain protein. The *phox1* homeodomain shares 70% homology with the homeodomain of the *Drosophila paired* gene. However, *phox1* does not share homology with *MATα1*. It appears to be more similar to *MATα2*, which is a homeodomain protein.

DNA-binding assays were performed using extracts prepared from yeast cells. Control cells contain the yeast transcriptional activator, *MCM1*, which binds the SRE, forming an MCM1-SRE complex. When extracts are prepared from yeast cells expressing *phox1*, we observed a new complex of lower mobility. We are testing whether the phox1 protein interacts with MCM1 and SRF. We hope that complementation in yeast will prove to be a fruitful approach for isolating human signal transduction genes.

Changes in the Fos/AP-1 Complex during Human Fibroblast Aging

K. Riabowol, J. Schiff

Human diploid fibroblasts (HDFs) undergo a finite number of population doublings in vitro that are inversely proportional to the in vivo age of the donor. In addition, fibroblasts explanted from individuals with premature aging syndromes such as Werners and Progeria show decreased abilities to divide in culture. These and other observations have led to the fibroblast being used as a model for cellular aging. After isolation from a donor, HDFs undergo a period of exponential growth followed by a gradual decline in growth rate that culminates in cells entering a state of cellular senescence. Senescent HDFs are unable to proliferate in response to a variety of normally mitogenic extracellular stimuli, even though receptors for growth factors and initial ligand-receptor events such as receptor autophosphorylation are not markedly altered. In addition, the majority of senescent HDFs contain a 2 N complement of DNA, and serum-stimulated senescent HDFs show a DNA-staining pattern similar to that shown by proliferation-competent cells in late G_1. Thus, it appears that the major block to cell cycle progression occurs during mid to late G_1 of the cell cycle, which is also the period of the cell cycle during which activity of Fos is required for entry into DNA synthesis (Riabowol et al., *Mol. Cell Biol. 8:* 1670 [1988]). The results of several different experiments suggest that this loss of proliferative potential with increased age is a result of a genetic program that may involve expression of "anti-proliferative" genes and/or decreased expression of genes whose functions are required for proliferation. Several genes, including those encoding c-*fos*, c-Ha-*ras*, c-*myc*, and c-*myb*, have been identified whose expression or active protein products are necessary for cells to progress through the G_1 phase of the cell cycle and enter into DNA synthesis. Transcription of these and a variety of other genes is not appreciably reduced in senescent cells with the exception of c-*fos*.

Since Fos binds DNA and affects transcription of genes containing AP-1 sites only upon formation of heterodimers with members of the Jun protein family, we investigated the ability of young (Y), old (O), and senescent (S) fibroblasts to express Fos in response to several stimuli, and asked if the Fos expressed in young and old fibroblasts formed heterodimers with Jun. Fibroblasts were deprived of serum for 48 hours and refed with medium containing [^{35}S]methionine and agents known to induce Fos expression through different signal transduction pathways. Cells were harvested 1 hour later, and lysates containing identical numbers of input counts were immunoprecipitated under nondenaturing conditions in antibody excess. Figure 4 shows that in the absence of stimula-

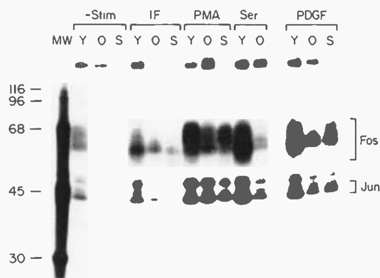

FIGURE 4 Reduced induction of Fos and Jun proteins in old and senescent human fibroblasts. Cultures of young (40 mean population doublings [MPD]), old (76 MPD), and senescent (80 MPD) were serum-deprived for 48 hr and then stimulated with isobutyl methylxanthine and forskolin (IF), phorbol myristate acetate (PMA), fetal calf serum (Ser), or c-*sis* protein (PDGF) in the presence of [^{35}S]methionine. Lysates were prepared, and immunoprecipitations were performed using affinity-purified polyclonal antibodies against Fos. Immunoprecipitates were analyzed by SDS-PAGE and visualized by fluorography.

tion (–Stim), young fibroblasts expressed a basal level of Fos greater than the amounts expressed by old and senescent cells. Stimulation with cAMP (IF), the phorbol ester PMA, platelet-derived growth factor (PDGF), and 10% serum (Ser) all induced expression of Fos to much higher levels in young fibroblasts than in old or senescent fibroblasts. Since Jun (recovered due to heterodimer formation with Fos), actin (a nonspecific contaminant), and FRA1 (recognized by virtue of shared epitopes with Fos) show similar electrophoretic mobilities, the proportion of Fos forming heterodimers with Jun was tested by immunoprecipitation of nondenatured lysates from young and old cells using Jun antibodies. We observed that a considerable proportion of the Fos protein expressed in response to 1 hour of serum stimulation in young fibroblasts is recovered in complex with Jun. In contrast, little, if any, Fos is recovered from old fibroblasts at this time point. This difference is not due to different kinetics of Fos induction or of heterodimer formation, since no Fos is recovered in complex with Jun at time points up to 8 hours following serum stimulation. We are now in the process of examining the AP-1 binding and transcriptional activity in young and old fibroblasts directly to better understand the consequences of decreased Fos expression during cellular aging.

Isolation, Cloning, and Characterization of Fos-related and Fos-associated Proteins

K. Riabowol, D. Girgenti

Fos is one member of a family of proteins consisting of c-*fos*, FosB, and the Fos-related antigens FRA1 and FRA2 that form heterodimers with members of the Jun protein family (c-Jun, JunB, and JunD). By itself, Fos is unable to bind DNA efficiently, but upon heterodimer formation, Fos-Jun complexes avidly bind AP-1 and related sites, affecting transcription of genes containing these sequence motifs.

Fos expression is rapidly and transiently elevated by a wide variety of mitogenic and nonmitogenic stimuli to varying degrees in different cell types. One possible explanation for the different cellular effects seen upon stimulation of cells with agents that all increase Fos expression is that the activity of the AP-1 complex is regulated by varying the ratios of the seven known members of the complex and by additional cell-type-specific and stimulus-specific proteins. To address this possibility, we have taken an immunological approach to purify proteins on the basis of their being directly recognized by Fos antibodies or by virtue of being complexed with Fos or Fos-related proteins through leucine zippers or other regions of protein-protein association. One such protein with an apparent molecular mass of 45 kD was present in relatively high levels in human T and B lymphocytes, but was not detected in 15 other cell types examined. This protein was not recovered in immunoprecipitations from denatured cell extracts, suggesting that p45 associates with Fos or another protein directly recognized by the affinity-purified Fos antibody. When run under nondenaturing conditions, p45 sediments in glycerol gradients with a relative molecular mass of approximately 250 kD.

We have now isolated sufficient amounts of the p45 protein from the human H9 T lymphoblast line to obtain partial protein sequences from several peptides generated by V8 proteolysis. Degenerate oligonucleotide primers were synthesized and used to prime the polymerase chain reaction using double-stranded

cDNA from H9 cells as substrate. Several clones representing approximately 40% of a 45-kD protein were isolated and sequenced. Initial sequence comparison of this portion of p45 to available databases shows limited homology with an enzyme of nucleotide metabolism and with histone H2A. Experiments are under way to isolate a full-length clone of p45 as well as to isolate other proteins recognized directly or indirectly by Fos antibodies.

PUBLICATIONS

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STRUCTURE, FUNCTION, AND REGULATION OF PROTEIN TYROSINE PHOSPHATASES

N.K. Tonks Q. Yang
P. Guida

It is now well established that the phosphorylation of proteins on tyrosyl residues is an essential feature of many cellular processes, including the control of both normal and neoplastic cell growth. In the 10 years since protein tyrosine phosphorylation was first observed, considerable progress has been made in the characterization of the structure, function, and mode of regulation of the protein tyrosine kinases (PTKs). The PTKs exist as low relative molecular weight (low-M_r) enzymes, as typified by the *src* gene product, and as transmembrane molecules, such as the receptors for a variety of hormones and growth factors. Nevertheless, one should not lose sight of the fact that protein tyrosine phosphorylation is a reversible, dynamic process. The net level of phosphate in a target substrate depends on the balance between the activity of the kinase that phosphorylates it and the activity of the phosphatase that catalyzes the dephosphorylation reaction. The main theme of the research in this laboratory is the elucidation of the structure, properties, and mode of regulation of members of the protein tyrosine phosphatase (PTPase) family of enzymes. A complete understanding of the physiological role of tyrosine phosphorylation and its potential as a mechanism for the reversible modulation of enzyme activity must necessarily encompass the characterization of the PTPases.

It is becoming apparent that the family of PTPases will rival that of PTKs in terms of the structural diversity of its members. The PTPases represent a rapidly expanding family of enzymes found in a diverse array of tissues and cell lines, which includes both nontransmembrane low-M_r forms and integral membrane proteins with the hallmarks of receptor molecules. The occurrence of receptor-like PTPases raises the exciting possibility of a novel mechanism of signal transduction, in which the early events involve the ligand-modulated dephosphorylation of tyrosyl residues in proteins. However, such ligands remain to be identified and their effect on activity ascertained.

I joined Cold Spring Harbor Laboratory in mid-October 1990. Consequently, rather than presenting a description of work carried out in 1990, I will use this Annual Report to discuss briefly some of the directions my laboratory will follow in the coming years.

REGULATION OF PTPASE ACTIVITY

When assayed in an in vitro reaction, the PTPases display a very high V_{max} (one to three orders of magnitude higher than the PTKs) and a high affinity for substrate (K_m values in the submicromolar range). Consequently, one would expect them to control effectively the level of phosphotyrosine in the cell; furthermore, they must be tightly regulated so as to permit the normal function of the PTKs.

The structure of the low-M_r PTPases, PTP1B and TCPTP (see Fig. 1), can be described in terms of two segments. In these ~50-kD molecules, the amino-terminal approximately 300 residues contain the catalytic domain, whereas the carboxy-terminal ~11-

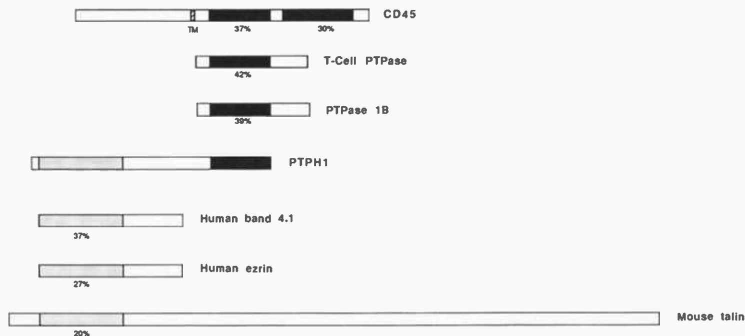

FIGURE 1 Schematic diagram illustrating the structure of PTPH1 and its relationship to other proteins. PTPH1 comprises three segments: an amino-terminal segment (*shaded bars*) that has homology with the membrane localization domains in band 4.1, ezrin and talin, a putative regulatory segment in the middle of the protein (*open bars*), and a carboxy-terminal PTPase related segment (*closed bars*). TM denotes the transmembrane domain in CD45. The length of each protein is shown in proportion to the number of amino acid residues. The homology among domains is indicated by percentage identity. (Reproduced from Yang and Tonks, *Proc. Natl. Acad. Sci.* [1991] in press.)

kD segment appears to serve a regulatory function. This latter segment is involved in controlling the intracellular localization of the enzymes and modulating their activity. It appears to direct the association of the ~50-kD PTPases into a very high-molecular-weight complex that elutes from FPLC gel-filtration columns with an apparent relative molecular weight in excess of 700K. We are attempting to identify the binding proteins with which the PTPases associate in these high-M_r complexes and to characterize the effects of such putative regulatory proteins on activity.

In addition, the PTPases are themselves phosphorylated. Both the low-M_r enzymes and CD45, the first receptor PTPase to be identified, are phosphorylated on tyrosyl residues in vitro. In addition, phosphotyrosine has been detected in CD45 in vivo (D.R. Stover, H. Charbonneau, N.K. Tonks, and K.A. Walsh, unpubl.). Furthermore, many of the PTPases display canonical sequence determinants for phosphorylation by a variety of protein serine/ threonine kinases. Thus, CD45 is known to be phosphorylated by protein kinase C in vitro and in vivo and is also a substrate for casein kinase II in vitro. We have shown that the low-M_r PTPases are phosphorylated on serine/threonine residues in vivo and are currently investigating the possibility that the kinase involved is the cell cycle control element $p34^{cdc2}$.

An appreciation of the mechanisms for control of PTPase activity will be essential for understanding how the level of cellular phosphotyrosine is regulated. The characterization of regulators of the PTPase will illustrate a new tier of control, in addition to the direct effects of kinases and phosphatases, through which the amount of phosphotyrosine in the cell can be modulated.

PTPASES AND THE CYTOSKELETON

We have isolated cDNA for a novel PTPase, which we term PTPH1, from a HeLa cell library. Its structure is summarized in Figure 1. It is a member of the family of proteins that includes band 4.1, talin and ezrin, proteins that participate in the interaction between the plasma membrane and the cytoskeleton. They possess a homologous amino-terminal domain of approximately 340 residues that appears to associate with protein components in the plasma membrane (Rees et al., *Nature 347:* 685 [1990]). In band 4.1, which promotes the association of actin and spectrin in erythrocytes, this domain interacts with the transmembrane protein, glycophorin. A similar model has been proposed for the interaction of talin with the integrin family of extracellular matrix receptors. In the case of ezrin, which displays a submembranous

localization in brush border cells, the details of its interaction with other proteins remain to be established.

The structure of PTPH1 can be described in terms of three segments (Fig. 1): (1) the amino-terminal segment displays homology with the membrane localization domains of band 4.1, talin and ezrin; (2) there is a central segment bearing putative phosphorylation sites for protein serine/threonine kinases, including $p34^{cdc2}$ and casein kinase II; and (3) a segment that is homologous to members of the PTPase family is located at the carboxyl terminus. The protein has been expressed, and intrinsic phosphatase activity has been demonstrated. In view of the structural homology with the band-4.1 family, we propose that PTPH1 will also display a submembranous localization at interfaces between the cytoskeleton and the plasma membrane, such as focal adhesion plaques.

Focal adhesion plaques are specialized regions of the plasma membrane through which cells in culture adhere to their external substrate (Burridge et al., *Ann. Rev. Cell Biol. 4:* 487 [1988]). On their internal face, these structures anchor actin stress fibers, which are important in determining cell shape. Within focal adhesions, the integrins provide the transmembrane link between components of the extracellular matrix, such as fibronectin, and the cytoskeleton. Their intracellular segments interact indirectly with actin cables through a multiprotein complex that comprises talin, vinculin, and α-actinin; however, this picture is undoubtedly incomplete, and additional focal adhesion proteins remain to be identified and their function established. Similar but less well characterized structures have also been implicated in attachment between neighboring cells and adherence to the extracellular matrix in vivo.

Oncogenic transformation is frequently associated with a less-adherent, rounded morphology, which results from a disruption of cytoskeletal integrity and a reduction in the number of focal adhesions (Kellie, *Bio-Essays 8:* 25 [1988]). Furthermore, residual adhesion plaques are invariably associated with the transforming PTK, such as *src*. In cells transformed by Rous sarcoma virus, for example, it has been postulated that a contributing factor to the generation of the transformed phenotype is the aberrant phosphorylation by $pp60^{v\text{-}src}$ of tyrosyl residues in key focal adhesion proteins, including vinculin, talin, ezrin, paxillin, and the β-subunit of the integrins. The exact role of the tyrosine phosphorylation in this process is unclear as yet; however, recent studies have implicated the phosphorylation of the integrins as a crucial step (Horvath et al., *Oncogene 5:* 1349 [1990]). The adherens junctions also represent a major target for $pp60^{v\text{-}src}$, and the protein connexin43 has been shown recently to be an important substrate for tyrosine phosphorylation in these structures (Swenson et al., *Cell. Reg. 1:* 989 [1990]; Volberg et al., *Cell. Reg. 2:* 105 [1991]). Immunocytochemical staining with antibodies to phosphotyrosine has indicated that tyrosine phosphorylation of focal adhesion and apical junction proteins also occurs in nontransformed cells, suggesting that PTKs act at these sites during normal cell function. However, the kinases involved have yet to be identified. In view of the potentially disastrous effects of aberrant tyrosine phosphorylation, it is expected that the activity of these tyrosine kinases will be tightly controlled. The localization of a PTPase to these structures is one means by which such regulation may be achieved. We are currently investigating the possibility that PTPH1 may play such a role.

Antibodies to PTPH1 are being generated to look at intracellular localization and also to identify proteins with which this phosphatase interacts in vivo. We will also be pursuing the question of whether, in view of the fact that its structure suggests that PTPH1 will localize to expected sites of action *src*, overexpression of this PTPase will reverse the transformed phenotype induced by src. If so, PTPH1 should prove to be a powerful probe with which to ascertain the precise role of tyrosine phosphorylation in the transformation process.

Clearly, the elevated levels of intracellular phosphotyrosine associated with transformation can be generated through either the activation of a PTK or the inactivation of a PTPase. Thus, the question arises as to whether the genes for PTPases function as growth suppressor genes or anti-oncogenes; i.e., will deletion of the PTPH1 gene lead to transformation? We will be addressing this issue and also trying to ascertain the chromosomal localization of the PTPH1 gene in an attempt to correlate its position with chromosomal abnormalities that characterize some of the known malignancies.

PTPASES AND NONINSULIN-DEPENDENT DIABETES MELLITIS

This work will be performed as a collaboration with Dr. J. Sommercorn, National Institutes of Health, Phoenix, Arizona. Noninsulin-dependent diabetes mellitis (NIDDM) is a life-threatening disease that af-

fects about 10% of the general population over the age of 40, and it is particularly prevalent in the Pima Indians of Arizona, in which 50% of adults over the age of 35 are diabetic. Because NIDDM is associated with aging and the population as a whole is getting older, the prevalence of NIDDM is likely to increase. Longitudinal studies of the development of NIDDM have demonstrated that subjects with normal glucose tolerance, who subsequently become diabetic, first develop impaired glucose tolerance, a condition that is characterized by reduced capacity of skeletal muscle to respond to insulin. This condition of insulin resistance may be an early manifestation of a genetic lesion that causes NIDDM in Pima Indians and in other racial groups. Understanding the biochemical basis of insulin resistance should therefore help to elucidate the cause and to suggest potential treatments of NIDDM.

Although mutations in the insulin receptor gene are associated with insulin resistance and NIDDM in rare disease syndromes, the predicted primary structure of the receptor is normal in subjects with the more common form of NIDDM. Furthermore, studies conducted by the Phoenix group show that properties of insulin binding and regulation of the PTK activity of the receptor from muscle of insulin-resistant humans do not account for insulin resistance. Nevertheless, insulin resistance limits the ability of the hormone to influence activities of various muscle enzymes, including S6 kinase, S6 peptide kinase, kemptide kinase, myelin basic protein kinase, and type-1 protein phosphatase, which are thought to mediate insulin action intracellularly. Because numerous responses are affected, the cause of insulin resistance likely involves an early postreceptor step in the mechanism of insulin action.

Recently, we have shown that insulin infusion in vivo produced a rapid 25% inhibition of soluble PTPase activity in skeletal muscle of insulin-sensitive subjects. This response was severely impaired in subjects who were insulin-resistant. Insulin did not affect the particulate PTPase activity; however, the basal level of activity was 33% higher in resistant subjects than in sensitive subjects. Given that signal transduction in response to insulin absolutely requires the phosphorylation of proteins on tyrosyl residues by the insulin receptor kinase, we anticipate that either or both of these effects on skeletal muscle PTPase, higher basal particulate activity, and the lack of insulin-induced suppression of soluble activity may antagonize the action of insulin in resistant subjects.

Although the observed effects of insulin on PTPase activity are small, it is possible that we are looking at a modification of one isoform in a background of several PTPases. We will expand on these observations by using both molecular biology and protein chemistry approaches to characterizing the PTPases involved. It is possible that the defect that causes insulin resistance will not be in the PTPase itself, but rather in its mode of regulation by insulin, for instance, either through interaction with regulatory proteins or via covalent modifications such as phosphorylation. Again, this highlights the importance of understanding the mechanisms through which the activity of these enzymes is controlled in vivo.

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CELL BIOLOGY OF THE NUCLEUS

D.L. Spector S. Huang, S. Henderson, R. O'Keefe, A. Ryan, G. Lark, R. Derby

Our research program has continued to focus on the structural and functional organization of the mammalian cell nucleus. We have continued to evaluate the localization and nuclear associations of factors involved in pre-mRNA processing, and we have begun to study the intranuclear organization of specific pre-mRNA molecules. The use of the electron microscopy core facility has continued to expand, and a large number of collaborations are under way with the excellent technical expertise of Robert Derby.

Differential Distribution and Nuclear Associations of Spliceosomal Factors during Interphase and Mitosis

D.L. Spector, G. Lark [in collaboration with X.-D. Fu and T. Maniatis, Harvard University]

We have examined the intranuclear distribution and nuclear associations of SC-35, a non-snRNP (small nuclear ribonucleoprotein particle) factor that is associated with spliceosomes (Fu and Maniatis, *Nature 343:* 437 [1990]). Our studies have shown that although these factors colocalize within a specific nuclear domain (nuclear network) during interphase, they appear to be associated with nuclear components by different molecular interactions. Using confocal microscopy and immunoelectron microscopy (Fig. 1), we found that, similar to snRNPs, the SC-35 protein appears to be organized in a nuclear network that

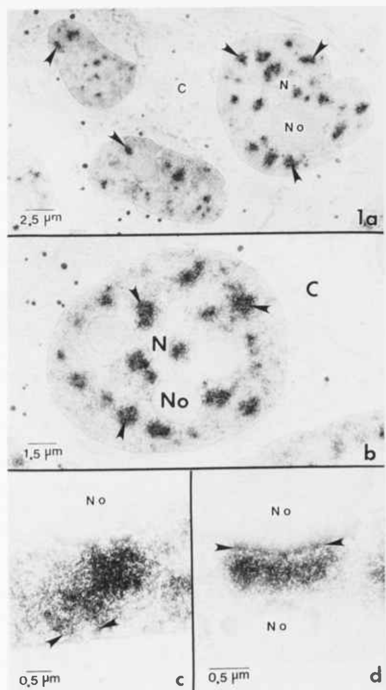

FIGURE 1 Immunoelectron microscopic distribution of SC-35. (*a*) Low-magnification view showing several immunostained cells. The protein distribution is observed by immunoperoxidase staining (arrowheads). SC-35 appears in clusters within the nucleoplasm (N); it is not present in the nucleoli (No) or in the cytoplasm (C) (*b*). Regions of SC-35 immunostaining extend out to the nuclear lamina envelope (*c*, arrowheads) and come into direct contact with the nucleolus (No) (*d*, arrowheads).

contacts the nucleolar surface and the nuclear envelope at several locations (Fig. 1). However, the additional diffuse nuclear staining observed with anti-snRNP antibodies is not observed with antibodies directed against SC-35. The associations of the SC-35 protein with nuclear components were investigated further by in situ nuclease digestions. The distribution of the SC-35 antigen in a speckled pattern is not changed by prior treatment of cells with DNase I or RNase A, in contrast to the distribution of snRNPs that become diffusely distributed after RNase A digestion. Furthermore, although snRNPs become diffusely distributed after cells are heat-shocked, this treatment does not disrupt the speckled distribution of SC-35 but causes minor changes in its localization pattern. Studies on cells undergoing mitosis (Fig. 2) show that the speckled staining pattern breaks up in prophase, and speckles containing SC-35 re-form in the cytoplasm of anaphase cells. However, snRNPs do not organize into a speckled staining pattern until late in telophase. Thus, although both types of antigens, snRNPs and SC-35, are integral components of the spliceosome, and they colocalize to the same nuclear domains, their molecular interactions with nuclear components appear to be fundamentally different. We interpret the interphase localization of pre-mRNA processing factors to specific nuclear domains to mean that spliceosome assembly and/or RNA processing events take place within these nuclear regions.

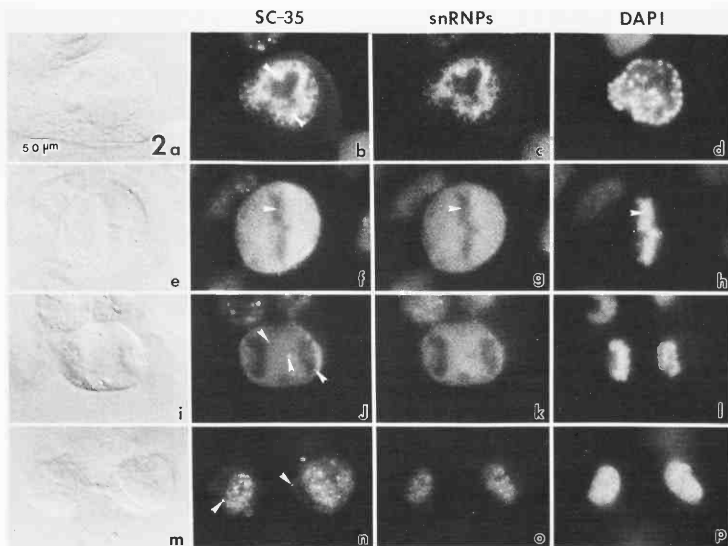

FIGURE 2 Redistribution of SC-35, snRNPs, and DNA during mitosis. As cells enter prophase (*a–d*), most of the speckles break up; SC-35 (*b*) and snRNPs (*c*) are more uniformly distributed between the condensing chromosomes (seen by immunofluorescence in *d*). In addition, some diffuse immunoreactivity appears to be present in the cytoplasm of prophase cells. During metaphase (*e–h*), both SC-35 (*f*) and snRNPs (*g*) are uniformly distributed throughout the cytoplasm. However, these antigens are not associated with the interior of the chromosomes in the metaphase plate (arrowheads). During anaphase (*i–l*), SC-35 begins to reassociate into speckles (arrowheads *j*), whereas snRNPs are still uniformly distributed throughout the cytoplasm (*k*). During telophase (*m–p*), the nuclear envelope is re-formed, and although all of the snRNP immunoreactivity is contained within the nucleus (*o*), in many cells, several SC-35 speckles (arrowheads in *n*) appear in the cytoplasm in addition to the typical nuclear immunostaining.

Localization of c-*fos* mRNA in the Cell Nucleus

S. Huang, D.L. Spector

Although much biochemical information is currently available with regard to the events associated with pre-mRNA processing, little to no information is available as to where these events occur within the cell nucleus. We have been interested in the localization of specific pre-mRNAs and their association with nuclear domains and factors. We have chosen to study a cellular mRNA, the mouse c-*fos* message, since this message can be induced to high levels upon serum stimulation of quiescent NIH-3T3 cells. This system allows us to trace the message from its site of transcription through the nucleus.

c-*fos* mRNA has been localized in the interphase nucleus by in situ hybridization using a biotinylated genomic probe and streptavidin fluorescein. Conventional fluorescence microscopy indicates that the message localizes to two dots in the interphase nucleus of cells that have been stimulated with 20% fetal calf serum (Fig. 3a). However, upon further analysis by confocal laser scanning microscopy, we have determined that the dots extend in the *z*-axis of the nuclei forming "tracks" (Fig. 4). This signal is not observed in cells that have been serum starved for 24 hours (Fig. 3b). Upon serum stimulation, the c-*fos* message can be detected within 5 minutes. To confirm that the signal we have observed represents mRNA, we have evaluated the effect of nuclease digestion upon the localization of the signal. We have found that the signal is sensitive to RNase A but is resistant to DNase I digestion. These data support the idea that we are indeed observing the localization of RNA molecules. Ongoing studies are evaluating the association of the c-*fos* message with other cellular components, such as factors involved in pre-mRNA splicing and the nuclear envelope. In addition, we are evaluating the localization of other mRNAs, some of which lack introns, in order to determine how mRNA is organized in the nucleus.

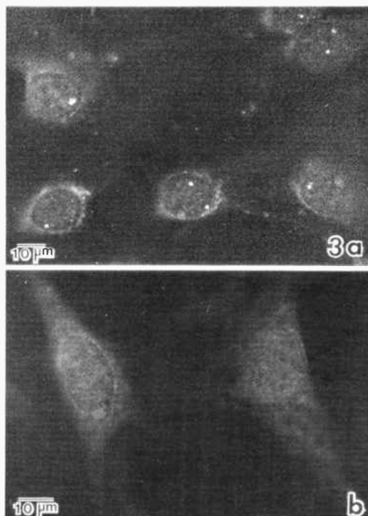

FIGURE 3 In situ hybridization using a 3.4-kb biotinylated c-*fos* probe. c-*fos* mRNA localizes to two dots in the nuclei of NIH-3T3 cells that have been stimulated with 20% fetal calf serum for 15 min (*a*). Cells that have not been serum stimulated do not exhibit any hybridization signal (*b*).

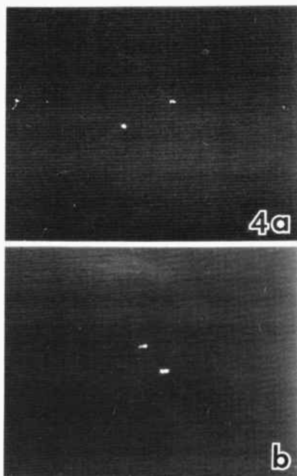

FIGURE 4 Transverse view of a three-dimensional reconstruction of the localization of the c-*fos* message (*a*) provides data identical to that obtained by standard fluorescence microscopy. However, if this model is rotated 90°, it is apparent that the dot forms a "path" that extends in the *z*-axis of the cell nucleus (*b*).

Organization of DNA Replication Sites in the Interphase Nucleus

R. O'Keefe, D.L. Spector

The sites of DNA replication were identified in nonsynchronous and synchronous cultures of HeLa cells by incorporation, for 10 minutes, of 10 μM 5-bromodeoxyuridine (BrdU). BrdU is a thymidine analog that is incorporated into DNA at active replication sites by the cell's replication machinery. The incorporated BrdU was then detected by monoclonal antibodies that are either directly or indirectly conjugated to FITC. The sites of DNA replication were visualized by standard fluorescence microscopy and confocal laser scanning microscopy. Five distinct patterns of DNA replication have been identified during S phase (Fig. 5). The five patterns correspond to different sites of DNA replicating at different times. During early S phase, replicating chromatin appears to be diffusely distributed throughout the nucleoplasm, excluding the nucleolus (Fig. 5a). As replication continues, the more peripheral regions of heterochromatin appear to replicate (Fig. 5b), followed by perinucleolar chromatin (Fig. 5c) and more internal patches of heterochromatin (Fig. 5d,e). Similar or identical patterns have been identified in a variety of mammalian cell types. These results are consistent with the idea that euchromatin replicates early in S phase and that heterochromatin replicates late in S phase. Laser scanning confocal microscopy has allowed us to elucidate the three-dimensional spatial organization of the sites of DNA replication throughout S phase. Quantitation of the different patterns of DNA replication during S phase in synchronous cells and the ultrastructural localization of these sites by electron microscopy are now in progress. These studies will provide information on the timing of each pattern in S phase and the exact nuclear localization of these patterns. Future studies will attempt to identify specific single-copy genes replicating within the context of the BrdU-staining patterns.

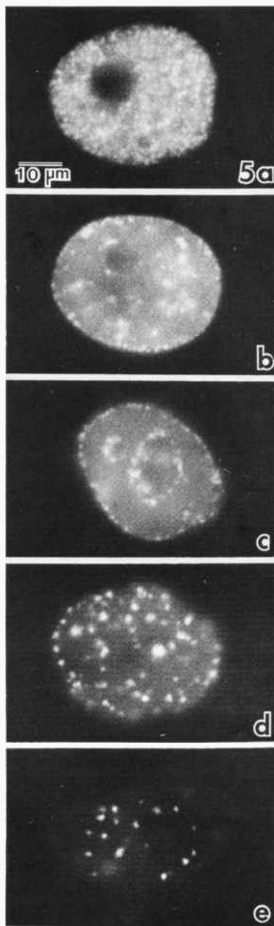

FIGURE 5 Localization of DNA replication sites in HeLa cells. Observations of nonsynchronous HeLa cells indicate that five patterns of replication are present as the cells progress through S phase.

Immunolocalization of Nuclear Antigens in Cryofixed Cells Prepared by Freeze-Substitution

R. Derby, D.L. Spector

Studies in our laboratory are involved in evaluating the structural and functional organization of the mammalian cell nucleus. Since several major classes (U1, U2, U4/U6, and U5) of snRNPs and another non-snRNP factor, SC-35 (Fu and Maniatis, *Nature*

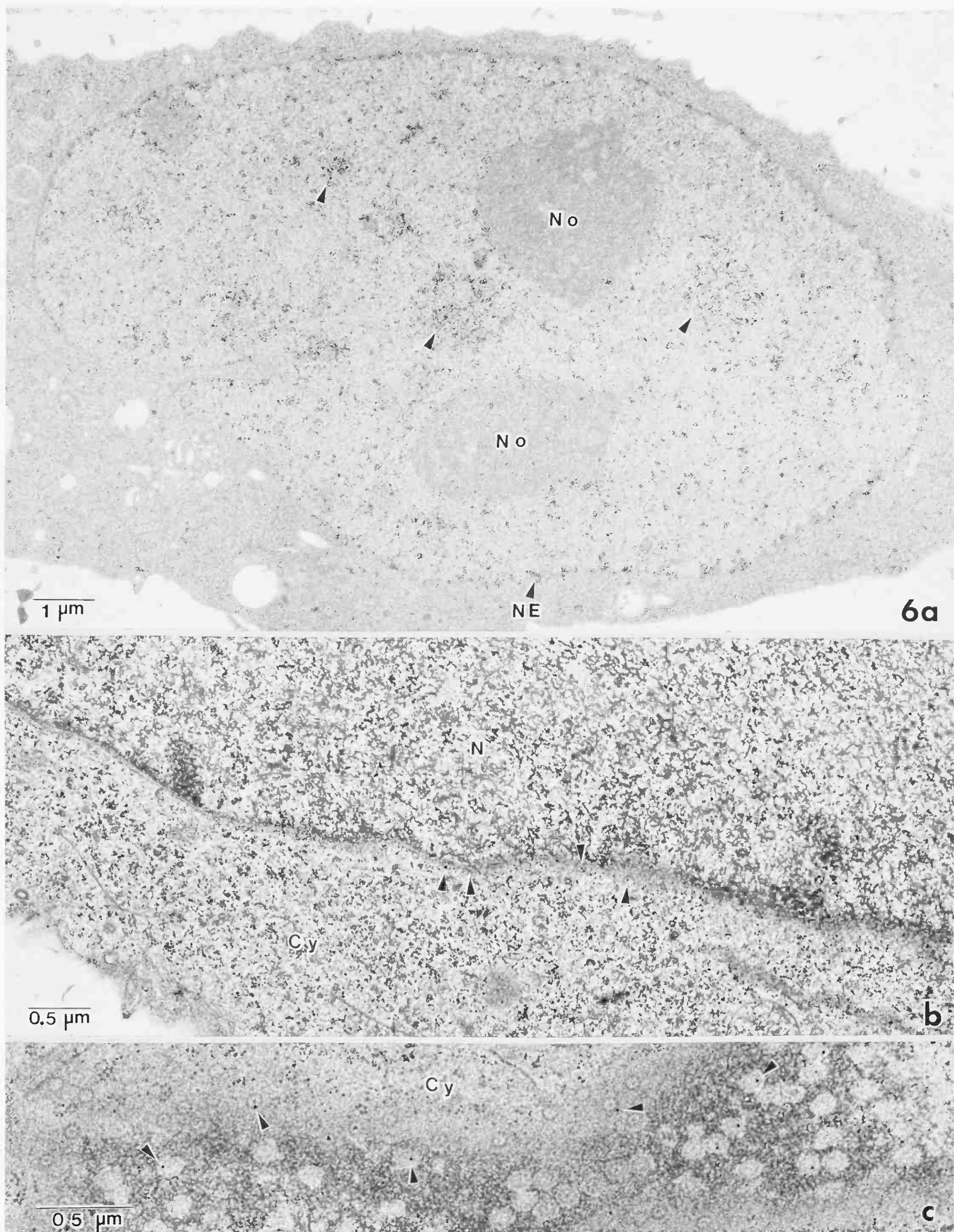

FIGURE 6 Cryofixed and freeze-substituted CHOC 400 cell section immunolabeled with anti-SC-35 monoclonal antibody followed by 15 nm colloidal-gold-conjugated goat anti-mouse IgG (*a*). SC-35-conjugated particles are present in clusters within the nucleoplasm (*a*, arrowheads). In addition, smaller concentrations of this protein are found within the nucleoplasm and come into direct contact with the nucleolus (No) or the nuclear lamina envelope (NE). Cryofixed and substituted (0.5% OsO_4 in acetone) CHOC 400 cell sections were immunolabeled with a monoclonal antibody against a 62-kD nuclear pore complex protein followed by 15 nm colloidal-gold-conjugated goat anti-mouse IgG (*b–c*). Nuclear pores in which an antigenic epitope was accessible in the plane of section are each labeled with a colloidal gold particle (*b–c*, arrowheads).

343: 437 [1990]), play a crucial role in the processing of pre-mRNA molecules, we have been interested in the localization of these factors within the cell nucleus. Using preembedding immunoperoxidase labeling combined with three-dimensional reconstruction, we have recently shown that nuclear regions enriched in snRNPs form a reticular network within the nucleoplasm that extends between the nucleolar surface and the nuclear envelope (Spector, *Proc. Natl. Acad. Sci. 87:* 147 [1990]). In the present study, we were interested in extending these nuclear localizations using cell preparation techniques that avoid slow penetration of fixatives, chemical cross-linking of potential antigens, and solvent extraction. CHOC 400 cells were cryofixed using a CF 100 ultrarapid cooling device. After cryofixation, cells were substituted in 0.5% OsO_4 in acetone for 72 hours at −80°C and embedded in Epon-Araldite. Thin sections (70 nm) were etched with 5% H_2O_2 for 3 minutes and immunolabeled with an anti-SC-35 monoclonal antibody that recognizes a 35-kD non-snRNP protein associated with spliceosomes (Fu and Maniatis, *Nature 343:* 437 [1990]). Antibody-antigen complexes were identified by labeling with 15 nm colloidal-gold-conjugated secondary antibody. Clusters of SC-35-conjugated colloidal gold are found within the nucleoplasm, and some clusters are observed to attach to the nucleolar surface or the nuclear lamina envelope (Fig. 6a). This immunolocalization appears to be identical to that which we reported using preembedding immunoperoxidase staining. However, with the present procedure, preservation of cellular structure is significantly increased due to rapid cryofixation and elimination of routine dehydration protocols. In addition, detergents were not needed to permeabilize cells, and resins such as Lowicryl K_4M or LR White, which extract membrane lipids, were not needed in order to achieve antibody labeling.

A 62-kD protein associated with nuclear pores (Davis and Blobel, *Cell 45:* 699 [1990]) was localized on sections of cryofixed cells which were substituted in 0.5% OsO_4 in acetone for 72 hours at −80°C. Cell sections were immunolabeled with anti-p62 followed by 15 nm colloidal-gold-conjugated secondary antibody. A grazing section of the nuclear envelope showed numerous pores in cross-section (Fig. 6b–c). Pores in which an antigenic epitope was accessible in the plane of section were each labeled by a single colloidal gold particle (Fig. 6b–c). Little to no background labeling was observed. In summary, cryofixation followed by freeze-substitution provides conditions for immunolabeling of several nuclear antigens such that cellular preservation does not have to be sacrificed in order to maintain antigenicity. We hope to use this technique in high-resolution studies to elucidate the functional organization of the cell nucleus.

In Situ Localization of the Adenovirus Genome in Infected HeLa Cells

A. Ryan, D.L. Spector

The adenovirus genome is a linear molecule of double-stranded DNA (35 kb) that codes for 20–30 polypeptides. The adenovirus genome is particularly interesting since the E1A region is capable of immortalizing cells and, with the E1B region, complete oncogenic transformation can occur. We have been interested in evaluating the subcellular localization of the adenovirus-5 (Ad5) genome in infected HeLa cells using a biotinylated probe prepared from the entire Ad5 genome. The probe is visualized in the fluorescence microscope after conjugation with streptavidin-FITC. The goals of this study are to elucidate the pathway by which the virus enters the nucleus, the localization of the viral DNA within the nucleus, and the pathways by which specific viral RNAs move from their sites of transcription through the nucleus and into the cytoplasm.

Uninfected HeLa cells that were hybridized with the Ad5 probe show no hybridization signal (Fig. 7b). Cells infected with wild-type Ad5 at a multiplicity of infection of 60 pfu/cell were examined by in situ hybridization at various times postinfection. At 7 hours postinfection, hybridization signal was observed as several fine dots within the cytoplasm (Fig. 7d); 8–10 hours postinfection, viral DNA is localized as dots throughout the nucleoplasm (Fig. 7f,h). The viral signal moves to the nuclear periphery and forms doughnut-shaped inclusions at 14 hours postinfection (Fig. 7j). Since the hybridization signal is significantly increased at this time point, we assume that viral DNA replication is occurring. At 26 hours postinfection, the nucleus appears to be filled with viral DNA (Fig. 7l). Electron and confocal microscopic studies are currently under way to identify the precise pathway of the Ad5 genome into the cell nucleus and the cellular components with which it interacts.

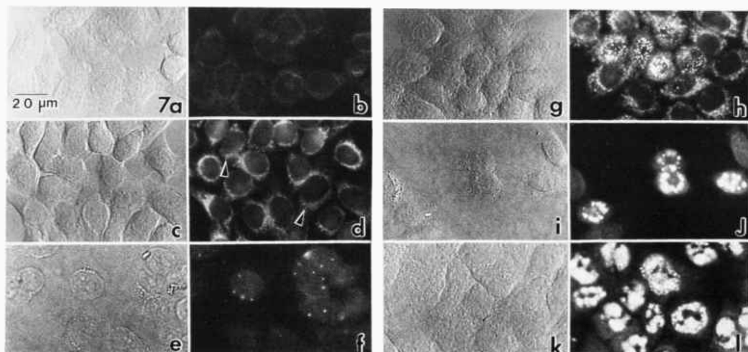

FIGURE 7 In situ hybridization localizing the adenovirus-5 genome in infected HeLa cells. See text for details.

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CELLULAR *TRANS*-ACTIVATORS OF GENE EXPRESSION

B.R. Franza A. Giordano G. Mak
J.-H. Lee J. Ross
Y. Li J.A. Scheppler

We study cellular proteins involved in the growth control of mammalian cells. The mammalian cell we study most comprehensively is the human T lymphoblast. When an interesting event is observed in the T lymphoblast, we then compare other mammalian cells, including lymphoblasts and fibroblasts from different species, to determine the generality or specificity of the proteins involved in the change. The QUEST protein database system is the tool that permits such comparative qualitative and quantitative observations.

During the past several years, we have focused on three systems. One is the effect that expression of oncogenes has on the entire complement of proteins present in a particular cell type. The second is the induction of protein alterations when quiescent cells are stimulated to proliferate. These studies are directed at determining the molecules involved in conveying signals to the genome and the earliest responses of the genome to these signals. The third is the control of gene expression at the level of regulation of transcription of mRNA encoding genes.

The goal, implicit in all of our studies, is to construct a molecular map of the networks involved in regulating transcription as well as other genome responses to a change in the cell's growth status. This project is in its infancy. The results of the initial studies appear to justify a comprehensive application of the strategy we have used during our early investigations. The strategy employs several tools, including recombinant or synthetic fragments of DNA containing genetically defined transcription control elements; antibodies to proteins identified either to associate with the control elements or to respond to stimuli that activate quiescent cells; enrichment of cells at different stages of the cell division cycle; high-resolution two-dimensional protein gels, a computer-accessible protein database; and in vitro assays of transcriptional activation/repression and kinase activity in order to determine the biological function of identified proteins. Because of the formidable complexity of a living cell, the strategy is designed to enable multiple investigators to make observations that ultimately can be linked.

Several projects, utilizing the strategy, were initiated or further developed during 1990. These projects are directed at the elucidation of the molecular mechanisms of the following processes: (1) the immediate-early responses of cells to different extracellular stimuli, (2) the regulation of transcription of human immunodeficiency virus type 1 (HIV-1), and (3) the activities of adenovirus early region 1A (E1A) protein that result from association with cellular proteins implicated in the regulation of the cell division cycle. We work as a group and *everyone* listed above contributes to each of the projects. Those individuals contributing substantially to one project more than another are recognized by being first author of published papers (see Publication list).

IMMEDIATE-EARLY CELLULAR RESPONSES

Our past investigation (in collaboration principally with T. Curran's group at the Roche Institute, Nutley, New Jersey) revealed that the products of two immediate-early genes, Fos and Jun, formed protein complexes with each other and cooperatively bound two different DNA transcription control elements, the activator protein-1 (AP-1) and cAMP response element (CRE) sites. The term immediate-early gene designates mammalian genes whose transcription (and the induction thereof) is not apparently dependent on de novo cellular protein synthesis. This provides the cell with a mechanism for rapid response to signals generated when the cell encounters other cells or soluble factors that interact with various receptors on the cell surface or alter the membrane potential of the cell.

The initial two-dimensional gel characterization of Fos was accomplished using an anti-Fos peptide rabbit antisera to isolate Fos from different rat cell lines. The Fos immune complex was indeed *complex*. Numerous cellular peptides, apparently not the product of the *fos* gene, were reproducibly found in the Fos immune complex. Subsequently, one set of isoforms (different migratory behavior in the two-dimensional gel because of chemical alteration of the primary gene product) was shown to be the product of *jun*. Another set was found to share a region of primary structure with Fos but to be the product of a distinct gene, *fra1*. FRA stands for Fos-related antigen and the peptide region of Fos to which the rabbit antisera reacted fortuitously was the region of FRA1 common to Fos. FRA1 is therefore one of the many non-Fos spots in the two-dimensional gel pattern of the Fos immune complex.

At the time we were studying the Fos immune complex, we were interested in pursuing mechanisms involved in the transcriptional regulation of inducible genes like the c-*fos* gene. We wanted to apply the rigorous analytic power of QUEST analysis of proteins separated on two-dimensional gels. We speculated that the protein interaction with control elements just beginning, at that time, to be defined in promoter regions for genes like c-*fos* might be complex enough to justify such analysis. We therefore developed a sensitive and specific DNA affinity precipitation (DNAP) assay using biotin-labeled DNA and streptavidin-agarose to capture proteins that interact with specific nucleic acid control elements.The assay is designed for a small scale, rapid, and flexible identification and analysis of cellular proteins bound to the nucleic acid sequence being studied. Use of this assay has led to the identification of several proteins that interact with κB sites in several promoters, including the AIDS virus, HIV-1. In addition, using this assay, the transcription factor AP-1 was shown to interact with AP-1 sites in numerous cellular and viral promoters, including the HIV-1 long terminal repeat (LTR), and to be composed of both Fos and Jun and related oncoproteins. We have not been disappointed by the lack of complexity of DNA-protein interactions of transcription control elements.

We are extending our analysis of the Fos immune complex and the members of the complex that are found to associate with AP-1 and CRE sites in dif-

ferent promoters. Although much has been learned regarding the interaction of Fos, Jun, and DNA through the work of numerous investigators, we continue to find evidence for relatively interesting and unexplored aspects of the Fos immune complex and the AP-1 and CRE DNAP complexes. In all of the two-dimensional gel analyses of the Fos complex that we have published, several proteins other than Fos, FRA, and Jun are detected. Because of the rapid transcriptional activation of the *fos* gene and the equally rapid and transient synthesis of Fos itself, most experiments were confined to a single time point for labeling and measuring the Fos protein, thereby yielding the equivalent of a snapshot of a moment in a very dynamic protein. That snapshot of Fos and the proteins associated with it or immunologically related to it is obviously complicated and has been very informative. However, we knew that, ultimately, a detailed time-course analysis of cells being stimulated by agents that induce Fos would have to be

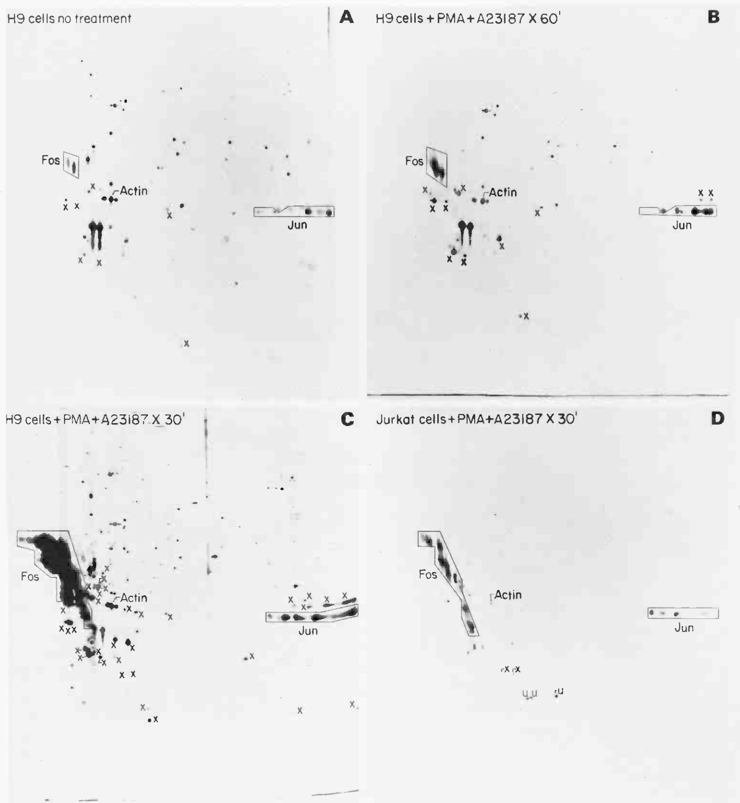

FIGURE 1 Two-dimensional gel analysis of inducible proteins in two different human T-lymphocyte cell lines that are members of the Fos immune complex. Cells were labeled with [^{35}S]methionine for the times indicated in each panel. A23187 is a calcium ionophore. See text for further details.

done not only to complete the characterization of Fos and the proteins associated with it, but also to observe what else the cell was doing. Another fact that we knew we would have to experimentally pursue is whether the response of cells in one stage of the cell division cycle was the same or different at other stages. Typically, examination of the Fos complex has been performed in serum-deprived, growth-arrested cells or asynchronously dividing cells. So much remains to be studied.

We are now pursuing a more comprehensive analysis of the regulation of Fos and the other members of the Fos immune complex and comparing those results with proteins interacting with AP-1 and CRE sites isolated from the same cellular extracts. We have chosen to compare two different human T-lymphoblast lines and a fibroblast line. The fibroblast line was chosen because it exhibits serum dependence and minimal, if any, spontaneous transformation from contact inhibition growth in culture. For each cell line, we are establishing conditions for centrifugal elutriation. This procedure permits the considerable enrichment of cells at the G_1 and G_2/M stages of the cell division cycle. A simple comparison of the Fos complex isolated from one of the $CD4^+$ lymphoblast lines labeled at two time points after induction of *fos* reveals significant differences in the extent not only of Fos synthesis, but also of the synthesis of several of the other proteins in the Fos immune complex. Comparison of the two $CD4^+$ lymphoblast lines at one time point after induction of *fos* has already revealed proteins unique to the Fos complex from one of the cell lines. Figure 1 presents two-dimensional gel images of these comparisons. The labeling of spots has been generalized to convey three points: (1) The difference in extents of Fos and Jun synthesis (encircled areas); (2) the complexity of the associated or antigenically related proteins (marked simply by an X); and (3) the induced spots unique to the Jurkat cell Fos immune complex compared to the H9 (HuT78) complex (spots marked by a U). What should be conveyed by this *simple* comparison is that the investigation of something as restricted as a single immune complex, isolated from cells at different time points, contains multiple gene products each of which is being processed at some unique rate. In all of our studies, we are pursuing mechanisms that coordinate transcription of cellular genes as cells traverse the nonproliferative state to the point of initiation of replication of DNA (G_0-G_1 to S phase). The combinatorial possibilities of different gene products apparent in the comparison of the Fos immune complex isolated at different moments during this period may reveal some molecular aspects of this timing mechanism. In any case, the extent of change and the number of molecules changing evident in this one type of immune complex suggest that definitive statements as to the regulatory function(s) for any of these proteins must account for how the fluctuating combinations of multiple proteins modulate the function of each of them. Perhaps it is better to discern what is actually happening before claiming either *the* function or mechanism of regulation of macromolecules like Fos.

REGULATION OF TRANSCRIPTION OF HIV-1

*HIVEN86A, a Member of the Set of κB-binding Proteins, Is a Product of the Human c-*rel *Proto-oncogene.* Several years ago, using the DNAP assay system we developed to study protein interaction with transcriptional control DNA elements, we identified HIVEN86A as an inducible member of a set of cellular proteins that specifically bind to the κB enhancer. This enhancer motif has been detected in numerous cellular and viral transcriptional control domains. Recently, cDNAs have been cloned that encode the 50-kD DNA-binding subunit of murine NF-κB and the closely related human κ-binding factor (KBF-1). A 350-amino-acid domain at the amino terminus of these proteins was found to be homologous to the v-*rel* oncogene from the avian reticuloendotheliosis virus, strain T (REV-T), as well as a maternal effect gene, *dorsal*. *dorsal* is known to activate transcription of certain *Drosophila* genes. The v-Rel oncoprotein has been identified as a transcriptional activator in certain assay systems and has been shown to be induced by the tumor promoter, phorbol-12-myristate-13-acetate (PMA) in avian cells. HIVEN86A is also inducible by PMA. It was therefore logical to determine if HIVEN86A was encoded by the human c-*rel* proto-oncogene. To do so, in collaboration with W. Greene's group at Duke University, we transcribed mRNA in vitro from a full-length human *rel* cDNA and translated the Rel protein product in vitro. We demonstrated its ability to bind the HIV-1 κB sites by DNAP assay. We resolved the in-vitro-translated Rel on two-dimensional gels and showed that it comigrated with cellular HIVEN86A. We excised the Rel and HIVEN86A proteins from the gel (see Fig. 2) and, by chemical cleavage with *N*-chlorosuccinimide (NCS), proved that the two proteins were indistinguishable structurally at this level of resolution. We will dis-

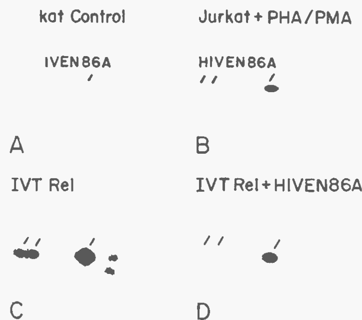

FIGURE 2 Microscale DNA-affinity precipitation assays of Jurkat cells and in-vitro-translated human c-*rel* as resolved on two-dimensional gels. (*A*) Unstimulated Jurkat cell extract plus HIVEN86A probe that contains a duplication of the κB sites in the HIV-1 LTR. (*B*) Extract from PHA+PMA-stimulated Jurkat cells plus HIVEN86A probe. (*C*) In-vitro-translated hc-Rel plus HIVEN86A probe. (*D*) Mixture of one-half of the volume of samples *B* and of *C* that were loaded on the two-dimensional gels represented in panels *B* and *C*. Jurkat cells were extracted after treatment with PHA and PMA and metabolic labeling with [^{35}]Smethionine. Prior to metabolic labeling for 30 min, 4 x 10^8 cells were treated with PMA (100 ng/ml) and PHA (1 μg/ml) for 3.5 hr.

cuss, a bit further on in this report, that an anti-carboxy-terminal Rel peptide antibody reacts with HIVEN86A. In collaboration with P. Enreitto's group at Stony Brook, we showed that v-Rel binds the HIV-1 κB site using the DNAP procedure.

At least two different cellular genes have now been identified that encode κB-specific binding proteins: NF-κB/KBF-1 and human Rel. NF-κB/KBF-1 encodes a 105-kD product that in its full-length form appears to lack DNA-binding activity. However, a truncated form of this protein, with a molecular size of approximately 50-kD, does indeed interact specifically with the κB enhancer. In addition, this 50-kD protein appears to interact with at least two other proteins, including a p65 polypeptide and a cytoplasmic inhibitory factor termed I-κB. It will be interesting to examine whether immunologic reagents specific for NF-κB/KBF-1 will also react with QJ11 or one of the other 40–50-kD proteins we have identified by DNAP analysis to interact with the κB enhancer. Whether all of the members of the set of NF-κB-binding proteins identified by DNAP analysis are to some extent homologous to c-*rel* remains to be determined. However, it is interesting that genes involved in transcription (HIVEN86A/hc-*rel*, NF-κB/KBF-1), oncogenesis (v-Rel), and patterned development (*dorsal*) are structurally related and that their binding specificity for DNA has been conserved.

Development of Immunologic Reagents That React with κB-binding Proteins. One disadvantage of the DNAP assay is that it only permits analysis of proteins that bind DNA or that complex with DNA-binding proteins. We need to be able to study κB-binding proteins that may at some stage not bind DNA because of posttranslational modification or the lack thereof. Given the Baeuerle and Baltimore I-κB model of inhibition of NF-κB (i.e., a cytoplasmic anchor of a transcriptional regulator that upon the cell receiving certain signals releases the regulator), we also need immunologic reagents to study proteins associated with κB-binding proteins when they are not in a DNA-binding state. Furthermore, we need immunologic reagents to perform subcellular localization studies, to screen cDNA expression libraries, and to use for affinity purification of the κB-binding proteins. We have begun preparing both polyclonal and monoclonal antibodies to proteins identified to interact with the κB site.

While we were determining whether HIVEN86A was the product of the *rel* gene, we had a peptide synthesized (CSHL protein chemistry) based on the sequence of the carboxyl terminus of human Rel. N. Rice and colleagues had already demonstrated that antisera to this region of Rel immunoprecipitated a cellular protein with with an apparent molecular size of 82 kD. Our antipeptide serum has been partially characterized thus far and has already revealed that it can (1) immunoprecipitate both HIVEN86A and in-vitro-translated c-Rel, (2) immunoblot both HIVEN 86A/Rel isolated by DNAP assay and several cellular proteins that are not present in the DNAP assays, and (3) immunoprecipitate proteins other than Rel. We are currently investigating if the coimmunoprecipitated proteins are Rel-related (antigenically) or Rel-associated proteins. This peptide antiserum has already revealed that total levels of Rel in unstimulated cell extracts are significantly larger than the amount of Rel that binds to the κB site in the DNAP assays when either whole-cell or nuclear extracts are used. Upon stimulation, the total levels of Rel increase somewhat in 4 hours, but the amount of Rel that associates with the κB site increases substantially. This suggests that the ability of Rel to associate with the κB site is controlled by some posttransla-

tional process perhaps by mechanisms similar to the association of NF-κB with I-κB.

Having identified multiple cellular proteins that associate with κB sites in in vitro DNAP assays, we decided to use the DNAP procedure to prepare a complex immunogen analogous to the approach our colleague Ed Harlow used to prepare antibodies to E1A-associated protein. Upon completion of a κB DNAP assay, we rinsed the beads and then injected the avidin agarose-biotin κB-protein suspension into the peritoneum of mice. After five injections, at intervals of 14 days, the antiserum immunoprecipitated HIVEN86A, and cells stimulated with PMA showed increased nuclear immunofluorescence. A fusion to murine NS1 cells was accomplished, and 1 hybridoma from 187 screened was positive by immunoprecipitation and immunoblot analysis for reaction to HIVEN86A. It was subsequently single-cell-cloned, and ascites was generated by injection of the cloned cells into mice. This antibody (139.6) reveals a distribution of immunofluorescence in the cytoplasm and occasional nuclei in the human osteosarcoma (Hos) cell line ATCC CRL1543. Upon stimulation of these cells with PMA for 2 hours or more, there is a clear increase in the number of positive nuclei and in the intensity of signal from both the nuclei and cytoplasm. Under similar conditions, HOS cells produce Rel as determined by immunoprecipitation with 139.6 antibody and κB DNAP assays.

Immunoblot comparisons of cellular extracts and DNAP-enriched κB complexes reveal a startling set of results with monoclonal antibody 139.6. In addition to the detection of Rel, two cellular proteins are readily detected by the antibody in crude extracts of Jurkat cells. These proteins exhibit no obvious fluctuation in extracts from PMA-stimulated Jurkat cells and they also bind DNA. However, the association of these proteins with DNA is relatively nonspecific; e.g., it is easily competed by increasing the amount of competitor DNA and therefore they behave opposite to HIVEN86A/Rel under the same conditions. These proteins migrate with apparent molecular sizes of 70 kD and 40 kD, respectively. Another protein recognized by the monoclonal antibody is significantly induced by phytohemagglutinin (PHA) and PMA in Jurkat extracts and binds DNA with considerable affinity; however, it binds the mutant κB site CTCACTTTCC significantly better than the wild-type site GGGACTTTCC and migrates with an apparent molecular size of 60 kD. We have previously reported the fact that certain proteins bind the mutant site better than the wild type. This may indicate that the κB motif is really a composite of different binding sites and that the mutation actually enhances the interaction with one member of the set of potential κB-binding proteins. It therefore appears that the antibody recognizes an epitope that is conserved among several cellular proteins that exhibit different site-specific interactions within the 10-bp κB site as well as relatively nonspecific interactions with the site.

An In Vitro Transcription Analysis of Early Responses of the HIV-1 LTR to Different Transcriptional Activators. We have developed a simple, fast, and reliable method to prepare whole-cell or nuclear extracts from small numbers of cells to study in vitro transcriptional activation of promoters such as the HIV-1 LTR. Our results reveal that the time course of activation of extracts derived from cells stimulated with the mitogenic lectin PHA or the tumor promoter PMA is different. PMA induces a rapid (20–30 min) onset of increased in vitro transcription from the HIV-1 LTR, whereas PHA causes a slow (4–6 hr) and sustained response. The biochemical relevance of protein synthesis inhibition by cycloheximide treatment of cells has been investigated using this approach. Cycloheximide has been used by several investigators to dissect cellular responses that apparently occur in the absence of de novo protein synthesis; e.g., if transcription of a gene can be induced in the presence of cycloheximide, the gene falls in the class of the immediate-early gene as discussed above. In these studies, PMA induction of a change in in vitro transcriptional activity was not dependent on de novo protein synthesis. Cycloheximide alone was insufficient to induce activation. Oligonucleotide-mediated site-directed mutagenesis demonstrated that mutation of the TATA box in the HIV-1 LTR ablated initiation of both basal level transcription and activation by PMA. Surprisingly, mutation of both κB sites in the LTR was shown to reduce but not extinguish the in vitro response to extracts from either PHA- or PMA-stimulated cells. The reduction was greater in extracts derived from cells treated with PMA. However, in both cases, other regions of the LTR clearly must be contributing to the in vitro transcriptional activity induced by each agent. Deletion analysis of the HIV-1 LTR reveals at least one region (–464 to –252) capable of suppressing in vitro transcription in extracts from Jurkat cells stimulated by PMA. This result is consistent with early studies of the HIV-1 LTR in transient transfection assays of mammalian cells with HIV-1 LTR reporter

gene constructs. We therefore have been able to observe distinct regulatory events at early time points after cells are exposed to agents known to induce transcription of both the HIV-1 LTR reporter gene constructs and the HIV-1 provirus itself.

ADENOVIRUS E1A PROTEIN INTERACTION WITH CELLULAR PROTEINS

We are interested in understanding the molecular mechanisms by which an oncogene alters the growth status of human cells. The adenovirus early region 1A (E1A) gene products have been very useful in the search for the cellular processes and the actual proteins involved in growth regulation. The identification of the product of the retinoblastoma-linked gene and a cyclin A gene as members of the set of proteins complexed with E1A indicates the power of E1A-affinity precipitation to isolate cellular growth regulatory proteins. The fact that numerous mutants of E1A exist that are deficient in one or more of the biological activities ascribed to E1A contributes to the ability to probe experimentally the function of each associated cellular protein. We are now focusing on a detailed definition of the cell-division-cycle-modulated association of E1A with cellular proteins and of the biochemical activities of the complexes isolated from cells throughout the cell cycle. As part of these studies, we will extend the analysis of the E1A-associated protein, p130. We look to an understanding of the interaction of E1A with these cellular proteins to reveal mechanisms not only of E1A-induced oncogenesis, but also of the normal growth regulatory properties of these proteins. For some of the studies reported below, we have continued to enjoy the productive collaboration with David Beach, Ed Harlow, and members of their respective research groups.

Human Cyclin A and the Retinoblastoma Protein Interact with Similar but Distinguishable Sequences in the Adenovirus E1A Gene Product. The adenovirus E1A proteins associate with several cellular proteins in adenovirus-infected or -transformed cells. Recently, two of the cellular proteins that bind to E1A have been identified. p105 has been shown to be the product of retinoblastoma tumor suppressing gene. p60 has been shown to be a human cyclin A. Previously, studies have shown that E1A protein sequences encoded by conserved domains 1 and 2 are required for interactions with the retinoblastoma protein (pRB). We have now demonstrated that amino acids 30–60 and 121–127 within E1A are required for interaction with p60/cyclin A. These are the same sites within conserved domains 1 and 2 that are required for E1A to associate with pRB. However, the association of p60/cyclin A does not appear to require pRB. We also demonstrated that another cellular protein, p130, interacts with E1A at essentially the same sites. It is interesting that mutations in these regions destroy the ability of E1A to function as an oncogene, thereby raising the possibility that interaction with several different cellular proteins may be needed for transformation by E1A.

The strategy we employed to determine the physical requirements for complex formation with p60/cyclin A and 130K was to use adenovirus mutants to identify the regions of E1A required to bind these proteins. This strategy has been used successfully to map the sites of protein-protein interactions such as E1A with pRB. Such knowledge permits correlation with existing data on those regions of E1A that exert specific activities, such as morphogenic transformation and altered growth control of mammalian cells. HeLa cells were infected with recombinant mutants of adenovirus type 5, and analysis of E1A–cellular protein complexes was accomplished by immunoprecipitation and subsequent separation of the proteins by gel electrophoresis (for summary of results, see Fig. 3). The anti-E1A monoclonal antibodies, M73 and M58, were used in each study. Two-dimensional gel electrophoresis was used to investigate the p60/cyclin A interaction with E1A. The results obtained in this study indicate that there is a close relationship between the specific biological activities of E1A and the interaction with p60/cyclin A and p130. Previous mutagenesis studies had indicated that three regions of E1A, amino acids 1–25, 36–76, and 121–129, are required for efficient transformation. The region from 1 to 25 is correlated with binding of p300 but does not appear to be required for binding p130, p107, pRB, or p60/cyclin A. Binding of this set plus p300 is also dependent on sequences in conserved domain 1 within residues 30 to 60. They also interact with sequences in conserved domain 2. The fact that p60/cyclin A and p130 require a subset of the E1A-transforming regions (i.e., amino acids 30–60 and 121–127) for binding suggests that interaction with these cellular proteins may contribute to the role of E1A as an oncogene. One issue is whether p60/cyclin A or p130 require expression of pRB for association with E1A. We answered

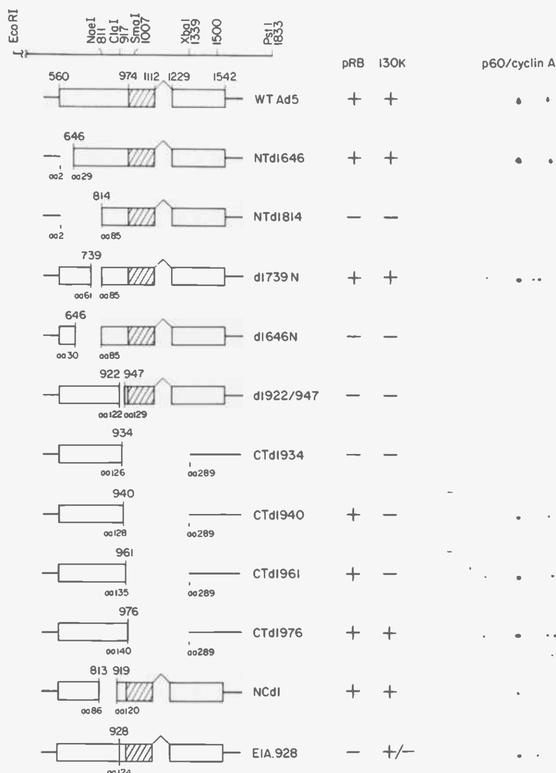

FIGURE 3 Two-dimensional gel electrophoretic analysis of p60 interactions with different recombinant Ad5 E1A mutants. This figure presents a schematic diagram of the genetic structure of the wild-type Ad5 E1A region and the structure of different recombinant Ad5 E1A mutants and a summary of data regarding the interaction of pRB (derived by P Whyte and E. Harlow) and p130 with each mutant. The region from individual two-dimensional gels in which the p60 isoforms migrate is shown for each M73 or M58 immunoprecipitation of the respective Ad5-infected HeLa cell lysate.

this by determining E1A association with p60/cyclin A and p130 in adenovirus-infected cells in which pRB is not expressed. Both alleles of the retinoblastoma gene have been deleted from the genomes of these cells.

p60 has been found to associate with the p34 protein product of the human homolog of the yeast *cdc2* gene. Expression of *cdc2* is required in yeast for cell cycle progression from G_1 to S phase (DNA synthesis) and for transition from G_2 to mitosis (M phase). It has now been demonstrated that mammalian *cdc2* expression is required not only for the

G_2 to M transition, but also for the G_1 to S transition. The interaction of p60 with *cdc2* may play a role in regulating the activity of this protein. This speculation is strengthened by the identification of p60 as a human cyclin A. Cyclins are a family of proteins, rapidly degraded at mitosis. The association of p60/cyclin A with *cdc2*, coupled with the demonstration that the region of E1A to which p60/cyclin A binds is required for its transformation function, suggests a direct link between regulation of the cell division cycle and acquisition of malignant growth properties when mammalian cells express E1A. Biochemical elucidation of the actual function of E1A may thereby reside, at least partially, in understanding its interaction(s) with p60/cyclin A and $p34^{cdc2}$ (or related gene products).

Cell Cycle Regulation of an Adenovirus E1A-associated Histone H1 Kinase Activity. As stated above, several cellular proteins have been shown to form stable protein-protein complexes with E1A in extracts derived from adenovirus-infected or -transformed cells. We have extended an analysis of the interaction of E1A with cellular proteins by demonstrating that two members of the E1A immune complex are very related to, if not actual products of, the gene encoding $p34^{cdc2}$. We have determined that this E1A complex displays histone H1-specific kinase activity, that this activity is modulated throughout the cell division cycle, and that there is apparently no requirement for pRB association with E1A for this activity.

To attempt to prove that the E1A-associated p34 isoforms were products of the human *cdc2* gene, we performed the following analyses. Adenovirus-infected HeLa cells were labeled with [^{32}P]orthophosphate, and extracts were prepared. The extracts were immunoprecipitated with an anti-$p34^{cdc2}$ pep-

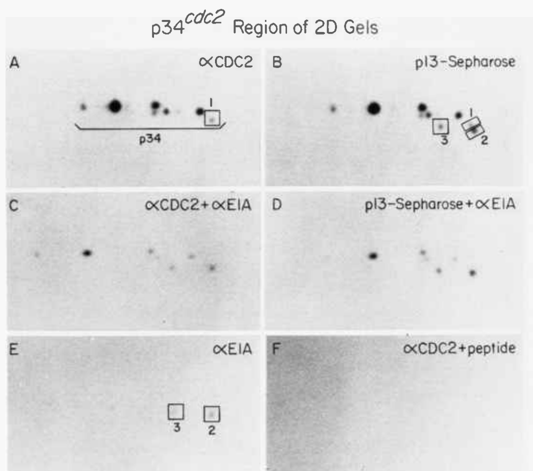

FIGURE 4 Two-dimensional gel analysis of the different $p34^{cdc2}$ isoforms. (*A*) Precipitates of $p34^{cdc2}$ from HeLa cells infected with wild-type Ad5 using an anti-*cdc2* serum (G6); (*B*) precipitates of $p34^{cdc2}$ from HeLa cells infected with wild-type Ad5 using p13-Sepharose beads; (*C*) mixture of proteins from precipitates shown in *A* and *E*; (*D*) mixture of proteins from precipitates shown in *B* and *E*; (*E*) HeLa cells infected with wild-type Ad5 and immunoprecipitated with the M73 monoclonal antibody; (*F*) anti-*cdc2* immunoprecipitation of HeLa cells infected with wild-type Ad5, performed in the presence of 100 nmoles of the antigenic carboxy-terminal *cdc2* peptide.

tide antibody (G6, carboxy-terminal peptide) or were exposed to p13-Sepharose beads. Figure 4A, represents the region of the two-dimensional gel in which the immunoprecipitated p34^{cdc2} isoforms are resolved. Comparison with the p13-Sepharose-precipitated p34^{cdc2} isoforms (see Fig. 4B) reveals a strikingly similar pattern with two exceptions (spots in boxes 2 and 3 in panel B). These same two spots are seen in p13-Sepharose precipitates of uninfected HeLa cells. Clearly, G6 does not immunoprecipitate these forms of p34. The spot in box 1 corresponds exactly to the spot in the box in Figure 4A. Mixture of the M73 immunoprecipitate from adenovirus-infected cells with either the G6 immunoprecipitate (Fig. 4C) or the p13-Sepharose precipitate (Fig. 4D) reveals that the two p13-Sepharose unique spots precisely comigrate with the p34 spots in the E1A immune complex. Figure 4E represents the two spots in the M73 immunoprecipitate of infected HeLa cells and Figure 4F represents the complete loss of all of the G6 p34^{cdc2} isoforms when the immunizing peptide was included in the lysate prior to immunoprecipitation.

The structures of the unique p13-Sepharose and E1A-immune-complexed p34 isoforms were compared to the p34^{cdc2} structure. The results of peptide analysis using *N*-chlorosuccinamide (NCS) are presented in Figure 5. NCS chemically cleaves proteins at tryptophan residues. Spots from the G6 immunoprecipitation (Fig. 4B, spot in box), the M73 immunoprecipitation (Fig. 4E, spot in box), and the p13-Sepharose precipitation (see Fig. 4B, spots in boxes 1 and 2) were excised from the two-dimensional gel and digested with NCS. The resultant pattern for each spot is identical to the p34^{cdc2} pattern. This is the pattern for NCS cleavage of mammalian and *Schizosaccharomyces pombe* p34^{cdc2} previously reported. In all cases, the spots represent phosphoproteins. The reason for the difference in two-dimensional gel migration of the p13-Sepharose/E1A unique isoforms of p34^{cdc2} remains to be determined, but the NCS results demonstrate that the primary structure of the E1A immune-complex-putative p34^{cdc2} isoforms are virtually indistinguishable from the forms immunoprecipitated by the G6 antibody. However, as noted above, these isoforms are not recognized by a p34^{cdc2} carboxy-terminal peptide polyclonal antisera, G6, but they do bind p13-Sepharose. It may be that they represent products of a gene highly related to the human *cdc2* gene, such as the Eg1 cDNA from *Xenopus laevis*. Perhaps the unique isoforms contain different phosphorylated amino acids or some other modification that affects their migration in the two-dimensional gels and blocks their recognition by the G6 antibody. The apparent size difference between these two forms is no greater than that between each of the other "layers" of p34^{cdc2} isoforms, so it is possible that they all are structurally identical but modified differently, and it is the modification that prevents access of G6 to the forms that associate with E1A. Another explanation for the inability of G6 to recognize these forms is that one or two amino acid carboxy-terminal truncations of these isoforms have occurred. The size of the NCS products is such that one or two amino acid deletions might not be detected. A protein sequence comparison between the two isoforms and other isoforms of p34^{cdc2} is required to establish the primary structural difference if one exists.

Histone H1 Kinase Activity of the E1A Immune Complex Is Modulated through the Cell Division Cycle. The histone H1 kinase activity precipitated with the

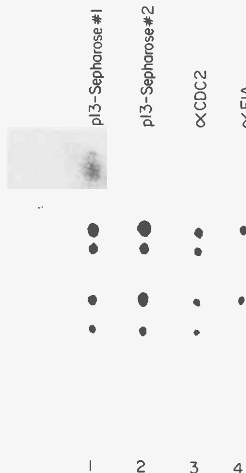

FIGURE 5 NCS digest of p34 spots from two-dimensional gels. (*1*) p13-Sepharose spot 1, see Fig. 2A, panel B, box 1; (*2*) p13-Sepharose spot 2, see Fig. 2A, panel B, box 2; (*3*) anti-p34^{cdc2} spot, see Fig. 2A, panel A, spot in box; (*4*) p34 spot in anti-E1A precipitate, Fig. 2A, panel E, spot in box.

anti-*cdc2* (G6) and the anti-p60/cyclin (C160) antibodies displays a distinctive pattern of cell cycle regulation in mammalian cells. The *cdc2*-p60/cyclin complex has properties distinct from the *cdc2*-p62/cyclin B complex in HeLa cells. We therefore compared the E1A immune complex histone H1 kinase activity with the activities of the C160 and the G6 immune complexes from populations of 293 enriched at different stages of the cell division cycle by centrifugal elutriation. Cell lysates were prepared from equivalent numbers of elutriated cells. Lysate protein concentrations were determined, and equivalent amounts of extract were immunoprecipitated with either Pab416, M73, G6, or C160 antibodies. The results of the elutriation and kinase assays are graphically represented in Figure 6. As shown in Figure 6 (top), a substantial enrichment of cells at G_1 and G_2/M phases was achieved. At the transition from G_1 to S between fractions 3 and 4, we see an increase in histone H1 kinase activity in the E1A immune complex that peaks between fractions 6 and 7. The histone H1 kinase activity patterns for the C160, anti-p60/cyclin A, and G6 anti-p34^{cdc2} immune complexes are consistent with what we previously observed in cell division cycle phase-enriched HeLa cell extracts.

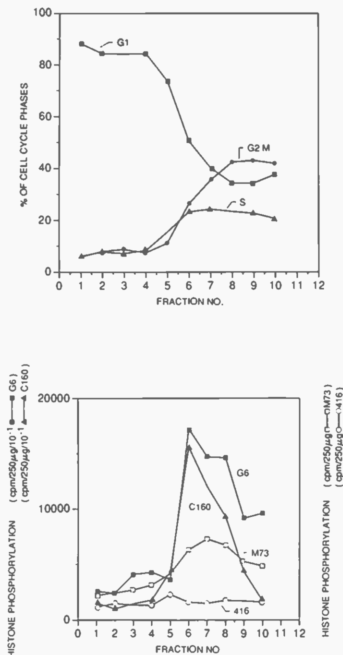

FIGURE 6 Centrifugal elutriation and E1A-immune complex histone H1 kinase activity of 293 cells. Cell fractions, collected by centrifugal elutriation, were fixed and stained with propidium iodide and analyzed by flow cyto-metry (*top*). The remaining cells in each fraction were extracted and immunoprecipitated with either M73, anti-*cdc2* (G6), anti-p60/cyclin A (C160), or Pab416 antibodies, and histone H1 kinase was performed for each immune complex (*bottom*).

The pattern of cell division cycle modulation of the E1A immune complex histone H1 kinase activity suggests that one mechanism for E1A-induced transformation may reside in its ability to associate with cell cycle regulatory proteins at specific moments during the transition of cells from G_1 to S phase. Interestingly, the *dl*922/947 deletion mutant (deletion in domain 2, see Fig. 3) is defective for oncogenic activity and binds significantly less p34^{cdc2} and p60/cyclin A. Consistent with decreased protein-protein interaction is a significant 80% reduction in the *dl*922/947 E1A immune complex H1 kinase activity. It remains to be determined if p34^{cdc2} and p60/cyclin A association with E1A can occur independently. All E1A mutants examined thus far that affect the binding of p34^{cdc2} and p60/cyclin A do so equivalently.

At least three other E1A-associated proteins, the 130-kD, 107-kD, and pRB proteins, require sequences within domain 1 and/or domain 2 of E1A for complex formation, and each of these proteins is phosphorylated. It has been demonstrated that pRB is phosphorylated at serine and threonine residues. It is possible that p34^{cdc2} is the kinase responsible for pRB phosphorylation in cells. Interestingly, the *dl*928 mutant that does not bind pRB maintains full histone H1 kinase activity and binds the isoforms of p34^{cdc2} and p60/cyclin A. This raises the possibility that the association of the *dl*928 product with p34^{cdc2} contributes to the induction of viral and cellular DNA synthesis that has been observed when this mutant E1A protein is expressed. We would hypothesize that mechanistically, the *dl*928 E1A-p34^{cdc2} complex phosphorylates pRB sometime during the G_1 to S phase transition and thereby is able to contribute to induction of DNA synthesis because the phos-

phorylation of pRB results in release of suppression of DNA synthesis. Of course, other activities of the E1A-p34^{cdc2}-p60/cyclin A complex may contribute to the mitogenic activity of E1A. The physical association of E1A with pRB would represent an additional component of the transformation phenotype resultant from expression of E1A. Such a model would be consistent with E1A having pRB-binding-independent and pRB-binding-dependent effects on cellular growth control.

Much remains to be learned regarding the regulation of the modifications of the cellular proteins associated with E1A and of the E1A gene products, as well. For instance, the extensive, rapid posttranslational modification of E1A may be due at least partly to the presence of p34^{cdc2} in the E1A complex. The resolution of the same two specific forms of p34^{cdc2} in both the E1A and p60/cyclin A immune complex focuses our effort on determining what is special about these two proteins and if one of their activities is to modify E1A itself. Because it is now known that p34^{cdc2} is required for G_1 to S transition as well as G_2 to M transition in mammalian cells, it is important to determine if these two isoforms contribute to the effects of E1A on the regulation of DNA synthesis. We hypothesize that these two isoforms are involved in the regulation of the mammalian equivalent of "START" in yeast. An extension of this hypothesis is to determine not only if these two isoforms participate in the regulation of initiation of DNA synthesis, but also if they contribute to the posttranslational modification of RNA polymerase II, resulting in modulation of transcription initiation. We will therefore be studying the activity of extracts from cells either expressing E1A or not at different stages of the cell cycle to determine if in vitro transcription of promoters known to be E1A-sensitive is growth-regulated. We will simultaneously identify the proteins associated with the promoters during the time course analysis of transcriptional activity. Further study of the activity of different E1A–cellular protein complexes may therefore uncover mechanisms for the coordination of activation of transcription and initiation of DNA synthesis when resting mammalian cells are propelled into the cell division cycle.

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MOLECULAR BIOLOGY OF THE CYTOSKELETON

D.M. Helfman W. Guo, G. Henry, J. Kazzaz, A. Kistler, M. Leonard, J. Lees-Miller, G. Mulligan, M. Pittenger, R. Roscigno, T. Tsukahara, S. Wormsley

The molecular and biochemical bases for related cellular processes such as cell motility and contractility, organelle movement, chromosome movement, cytokinesis, and the generation of cell shape are important problems in biology. These processes are all dependent on a complex macromolecular set of protein fibers found in the cytoplasm, termed the cytoskeleton. The cytoskeleton of vertebrate cells is composed of three major filamentous systems: actin filaments, intermediate filaments, and microtubules. Each of these filamentous systems contains a number of different protein components, although different cell types and tissues express specific protein isoforms that comprise these structures. The research in our laboratory is focused on two related problems in molecular and cell biology: (1) the mechanisms responsible for tissue-specific and developmentally regulated patterns of gene expression and (2) the functional significance of cell-type-specific protein isoform expression. Specifically, our laboratory has been interested in understanding the regulation and function of tropomyosin gene expression in muscle and nonmuscle cells. Tropomyosins are a diverse group of actin-binding proteins with distinct isoforms present in striated muscle (skeletal and cardiac), smooth muscle, and nonmuscle cells. We now know that at least 12 different tropomyosin isoforms are expressed from three separate genes in rat. The α-tropomyosin (α-TM) gene encodes nine isoforms (Fig. 1), the β-tropomyosin (β-TM) gene encodes two isoforms (Fig. 2), and the tropomyosin-4 (TM-4) gene encodes a single isoform. We have been study-

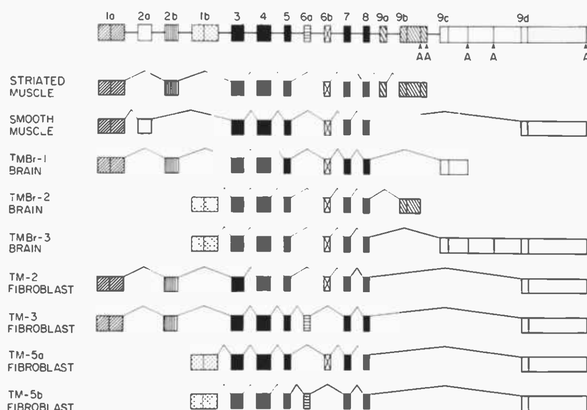

FIGURE 1 Schematic diagram of the rat α-tropomyosin gene and nine different isoforms expressed from this gene. Boxes represent exons and horizontal lines represent introns; they are not drawn to scale. The gene contains two alternative promoters which result in expression of two different amino-terminal coding regions (exons 1a and 1b), two internal mutually exclusive exon cassettes (exons 2a and 2b and 6a and 6b), and four alternatively spliced 3′ exons that encode four different carboxy-terminal coding regions (exons 9a, 9b, 9c, and 9d). The different polyadenylation signals are also indicated (A).

ing the expression of these genes with particular attention to understanding the mechanisms of their regulation at the posttranscriptional level, i.e., tissue-specific alternative RNA splicing. In addition, the expression of a diverse group of tropomyosin isoforms in a highly tissue-specific manner via alternative RNA processing strongly suggests that each isoform is required to carry out specific functions in conjunction with the actin-based filaments of various muscle and nonmuscle cells. The function of these different isoforms is not known and is under study. We have also recently cloned a gene from the fission yeast *Schizosaccharomyces pombe* that encodes a distantly related member of the actin family and have begun to study the function of this actin-like protein. Below is a summary of our present studies.

Identification of *cis*-Elements and Cellular Factors Involved in Regulated Alternative Splice Site Selection

W. Guo, G. Mulligan, S. Wormsley, R. Roscigno, D. Helfman

The generation of protein isoform diversity by alternative RNA processing is a fundamental process which contributes to tissue-specific and developmentally regulated patterns of gene expression. In addition, alternative splicing pathways have also been demonstrated for a number of viral genes. At present, the molecular basis for tissue-specific alternative splicing is poorly understood. We are using the rat β-TM gene as a model system to investigate the molecular basis for developmental and tissue-specific RNA splicing (Fig. 2). The β-TM gene expresses both skeletal muscle β-TM and fibroblast TM-1 by an alternative RNA splicing mechanism. The gene contains 11 exons. Exons 1 through 5 and exons 8 and 9 are common to all mRNAs expressed from this gene. Exons 6 and 11 are used in fibroblasts, as well as smooth muscle cells, whereas exons 7 and 10 are used exclusively in skeletal muscle. Furthermore, the alternative RNA splicing patterns of the β-TM gene are regulated during myogenesis. Myoblasts express the fibroblast-type tropomyosin isoform (TM-1). During differentiation, the pattern of pre-mRNA splicing changes such that there is repression of the fibroblast-type splice products and induction of the muscle-specific splice products, i.e., skeletal muscle β-TM.

During the past year, we have focused most of our efforts on understanding why exon 7 is not normally used in nonmuscle cells. There are at least two models to explain why exon 7 (skeletal-muscle-type splice) is not used in HeLa cells. One is that there is an RNA secondary structure, which is predicted by computer modeling, formed between intron sequences upstream and downstream from exon 7. This structure would sequester this exon from the splicing machinery in HeLa cells and thereby prevent its use. A second model is that cellular factors interact with specific sequences in the pre-mRNA and block the use of exon 7 in nonmuscle cells. On the basis of our experiments described below, we are in favor of the second model.

Using both in vitro and in vivo systems, we have identified a number of *cis* elements involved in alternative splice site selection. We recently identified two distinct elements in the intron upstream of exon 7 involved in splice site selection (Helfman et al., *Genes Dev. 4:* 98 [1990]). The first element is a polypyrimidine tract located 89–143 nucleotides upstream of the 3′ splice site, which specifies the location of the lariat branchpoints located an unusually long distance (144–153 nucleotides) upstream of exon 7. The second element is composed of intron sequences located between the polypyrimidine tract and

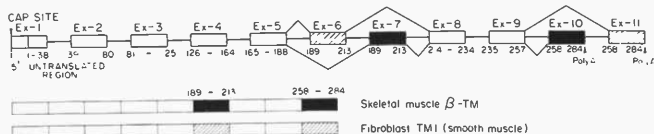

FIGURE 2 Schematic diagram of the rat β-tropomyosin gene and the two different isoforms expressed from this gene. The amino acids encoded by each exon are indicated. The cap site and polyadenylation sites are also indicated.

3′ splice site of exon 7. This element contains an important determinant in alternative splice site selection, because deletion of these sequences resulted in the use of the skeletal-muscle-specific exon in nonmuscle cells.

To analyze further the role of specific nucleotides within this region that are required for inhibiting the use of exon 7 in nonmuscle cells, we have introduced a series of clustered point mutations in the 3′ end of intron 6 and within exon 7. For example, it was important to determine if the deletion mutants in our previous studies resulted in activation of the skeletal-muscle-type splice in nonmuscle cells due to simply altering the distance between the 3′ splice site and upstream branchpoint or removal of an inhibitory sequence. These experiments have identified a number of specific sequences in the intron and exon that, when mutated, lead to the use of exon 7 in nonmuscle cells. These mutations appear to act by disrupting the interaction of cellular factors with the pre-mRNA that normally prevent the use of exon 7 (see below). We have also found that transfection of tropomyosin minigenes containing mutations in the 5′ or 3′ splice sites of exon 6 (fibroblast-type splice) does not result in the increased use of exon 7 (skeletal-muscle-type splice) in nonmuscle cell systems. These results suggest that splice site selection in vivo is not regulated by a simple *cis*-competition mechanism, but rather by a mechanism that inhibits the use of the skeletal muscle exon in nonmuscle cells. As mentioned above, we believe that nonmuscle cells express factors that block the use of exon 7. We then reasoned that it should be possible to overcome the effects of these putative blocking factors by expressing large amounts of the pre-mRNA. Accordingly, transfecting increasing amounts of plasmid containing a wild-type minigene into HeLa cells resulted in use of exon 7.

As described above, we have identified a number of critical regions in the intron upstream of exon 7 (skeletal-muscle-specific exon) and exon that, when mutated, led to the use of exon 7 in nonmuscle cells. These results strongly suggested that these sequences in the pre-mRNA interact with cellular factors in nonmuscle cells and thereby inhibit the use of the skeletal muscle exon. Using UV photochemical cross-linking, native gel electrophoresis, and binding competition experiments, we have identified RNA-binding proteins that interact with the pre-mRNA, which likely influence the use of exon 7 in nonmuscle cells. HeLa nuclear extracts were fractionated using a variety of chromatographic techniques, and the fractions were analyzed by an RNA-protein gel mobility shift assay to determine if there are any proteins that bind to the intron sequences upstream of the 3′ splice site of exon 7. We have identified an activity that binds specifically to the 3′ splice site of exon 7, but not to similar regions of exon 6 or exon 8. Furthermore, competition studies demonstrate that this activity does not bind strongly to the mutants that exhibit activation of exon 7 in vivo. We propose that the interaction of this factor with the pre-mRNA prevents the use of exon 7 and is therefore involved in suppressing the use of the skeletal muscle exon in nonmuscle cells.

Purification and Characterization of a Protein That Binds to Regulatory Sequences in the β-TM Pre-mRNA

G. Mulligan, W. Guo, S. Wormsley, D. Helfman

As described above, we have identified a number of *cis* elements critical to regulated splicing of the β-TM pre-mRNA. Specifically, there is a polypyrimidine stretch upstream of the muscle-specific exon 7 that is required for use of the adjacent branchpoints by the 5′ splice site of exon 5 (the muscle-specific-splicing pattern). We and other investigators have used UV cross-linking to detect a 62-kD polypeptide that binds specifically to long stretches of pyrimidines. This protein is expressed in muscle and nonmuscle cell types, and its role in splicing remains unclear. Adjacent to this region of the transcript is another *cis* element that appears to represent a binding site for cellular factors in HeLa cells (see above). Deletion of, or substitutions within, this element allows the cellular machinery of a nonmuscle cell (HeLa) to splice the muscle-specific exon 7. We propose that the binding of a protein to these sequences between the polypyrimidine stretch and the muscle-specific exon inhibits the use of this exon in nonmuscle cells. The use of UV cross-linking and gel mobility shift techniques has allowed identification of a cellular factor that binds to this inhibitory element with high specificity. Separation techniques have allowed the isolation of this protein to at least 90% purity. Work is currently under way to develop in vitro biochemical assays to study the function of this protein. We are also developing antibodies and cDNA clones that will be valuable tools in studying the role of this factor in alternative splicing systems.

Development of In Vitro Splicing Systems from Myogenic cells

T. Tsukahara, D. Helfman

To identify cellular factors and mechanisms involved in muscle-specific alternative RNA splicing, it will be necessary to develop an in vitro system from myogenic cells. We have begun studies to establish such a system using the mouse myogenic cell line, BC3H1. The undifferentiated cells express the non-muscle (fibroblast-type) tropomyosin isoforms from the α-TM and β-TM genes, e.g., TM-1 and TM-2. Upon differentiation, the pattern of pre-mRNA splicing changes such that there is repression of the fibroblast-type splice products and induction of the muscle-specific splice products, i.e., skeletal muscle α-TM and β-TM. We have prepared nuclear extracts from undifferentiated BC3H1 cells that are able to splice β-TM pre-mRNAs in the appropriate cell-type pattern. Experiments are in progress to prepare nuclear extracts from differentiated cells.

Alterations in Transcription and Alternative RNA Splicing Are Involved in the Changes in Tropomyosin Expression in Transformed cells

J. Lees-Miller, M. Leonard, D Helfman

One of our aims is to study the effects of different nuclear and cytoplasmic oncogenes on tropomyosin expression. We have found that rat fibroblasts transformed with Kirsten virus and Rous sarcoma virus have undetectable levels of TM-2 and TM-3 mRNAs but elevated levels of TM-5a and TM-5b mRNAs (Goodwin et al., *J. Bio. Chem.* [1991] in press). Analysis of the genetic basis for the expression of the four isoforms (TM-2, TM-3, TM-5a, and TM-5b) revealed that all are expressed from the α-TM gene via the use of alternative promoters and alternative RNA splicing (Fig. 1). Interestingly, mRNAs for TM-2 and TM-3 are transcribed from a 5′ proximal promoter, whereas mRNAs for TM-5a and TM-5b are transcribed from an internal promoter. The lack of mRNAs for TM-2 and TM-3 in cells transformed by the Kirsten and Rous sarcoma viruses correlates with the absence of proteins for these isoforms. Thus, the absence of mRNAs for these two isoforms is likely due to the inhibition of transcription from the upstream promoter of this gene. In addition, we have determined that cells transformed by adenovirus and SV40 have increased levels of mRNA encoding TM-3 and TM-5b, relative to TM-2 and TM-5a, which correspond to changes in the relative use of alternative exons 6a and 6b (Goodwin et al., *J. Bio. Chem.* [1991] in press). At present, it is not known if these changes in exon usage are due to alterations in the concentration or activity of splicing factors or due to differences in the stability of the respective mRNAs. Experiments are under way to determine the precise mechanism(s) responsible for these alterations in tropomyosin expression. Understanding these mechanism(s) will provide new insights into the pleiotropic action of the oncogenes associated with these various RNA and DNA tumor viruses.

Transcriptional Control of Tropomyosin Gene Expression

J. Kazzaz, D. Helfman

The rat β-TM gene expresses two distinct isoforms via an alternative splicing mechanism (Fig. 2). Although the gene is expressed in muscle (skeletal, cardiac, and smooth) and nonmuscle cells, a single transcription initiation site is used in the various cell types that express the gene. We now wish to determine if the same *cis*-acting elements are used in muscle (skeletal, cardiac, and smooth) and nonmuscle cells (e.g., fibroblasts) and if the same or different *trans*-acting factors are involved in transcriptional control of the gene in different cell types. Sequence analysis of the 5′ end of the β-TM gene has already revealed a number of potentially important elements involved in transcriptional control in skeletal muscle cells. The gene contains three copies of a skeletal-muscle-specific enhancer element (MRE) located approximately 300, 350, and 400 bp upstream of the transcriptional start site. The consensus sequence of this 14-nucleotide-long element (C/GNG/AG/ACAC/GC/GTGC/TC/TNC/G) has been found in a number of genes expressed in skeletal muscle including the muscle creatine kinase, δ-subunit of the acetylcholine receptor, myosin light chain 1/3, desmin, and vimentin (Buskin and Hauschka, *Mol. Cell. Biol. 9:* 2627 [1989]). This element is believed to be the site of action of the myogenic regulatory factors such as MyoD and myogenin. Since these myogenic

regulatory factors are expressed in skeletal muscle, but not in cardiac and smooth muscle or nonmuscle cells, it is likely that expression of the β-TM gene in cells other than skeletal muscle will require a different set of transcriptional regulatory proteins.

For the initial characterization of the promoter, we have cloned 300–1800 bp of the upstream promoter region into a chloramphenicol acetyltransferase (CAT) expression vector. We used this vector to transfect a variety of cell lines and tested for CAT activity in transient assays. Results from transfections into a rat fibroblast cell line (REF52) and a mouse fibroblast cell line (NIH-3T3) indicate that 1800 bp of the upstream promoter region are not sufficient to drive transcription of CAT in fibroblasts. To determine if the muscle-specific regulatory elements (MRE) identified by DNA sequence analysis are functional, REF52 and NIH-3T3 cells were cotransfected with a plasmid that contains a copy of the MyoD cDNA clone (Davis et al., *Cell 51:* 987 [1987]; courtesy of A. Lassar) and the CAT-promoter constructs described above. These experiments demonstrated that the MyoD binding sites between positions −300 and −400 are functionally active. Fur-

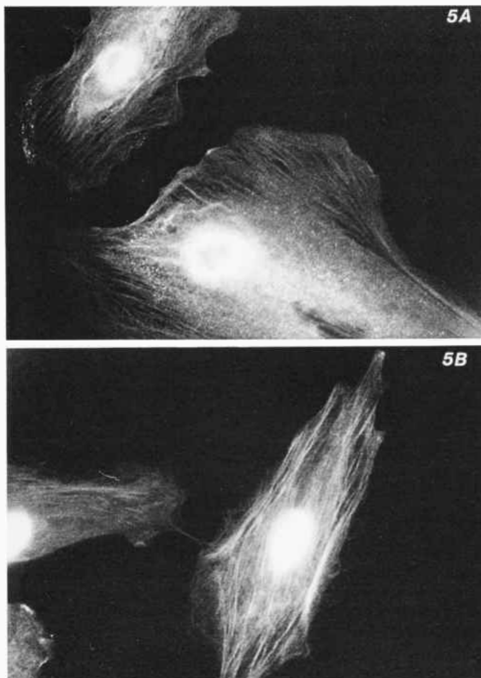

FIGURE 3 Localization of tropomyosin isoforms in fibroblasts. Bacterially produced fibroblast isoforms TM-5A and TM-5B were fluorescently tagged with rhodamine and microinjected into rat fibroblasts (REF52). Three hours after injection, the cells were fixed and visualized by fluorescence microscopy. Both isoforms localized to actin filaments.

ther deletion analysis confirmed that this region is sufficient to confer responsiveness to MyoD. To determine if these sequences can activate transcription in a muscle cell line, C_2C_{12} myotubes were transfected with the various CAT-promoter constructs. This cell line upon induction with low serum differentiates into myocytes and fuses to form myotubes. Transfection into differentiated myotubes indicates that the β-TM MRE is sufficient for activation of CAT in differentiated muscle cell lines. Experiments are in progress to determine if all three elements are required for muscle-specific gene expression and if the four different myogenic regulatory factors, i.e., myoD and myogenin, mrf4, and myf5, interact with these sequences in distinct ways.

In addition to the myogenic regulatory region, sequence analysis has also revealed a 124-bp direct repeat within the first intron of the β-TM gene. This repeat has a 45-nucleotide purine/pyrimidine (dA-dC) stretch. A poly(dA-dC) repeat has been shown to have enhancer properties in SV40 (Berg et al., *Mol. Cell. Biol. 9:* 5248 [1989]). To determine if this element has enhancer-like activity, we have cloned both copies of this element upstream of the 5′ promoter proximal elements in the tropomyosin-CAT constructs indicated above. Preliminary evidence suggests that this element does function as an enhancer in both fibroblast and muscle cell lines. Cotransfection with MyoD indicates that the effect of the internal enhancer is additive to that of the muscle regulatory region. Further investigation will determine whether the enhancer activity resides in the poly(dA-dC) repeat, in the flanking regions, or a combination of both.

Tropomyosin Isoform Diversity and Cellular Function: Microinjection of Fluorescently Labeled Tropomyosins

M. Pittenger, A. Kistler, D. Helfman

Rat fibroblasts express at least six distinct forms of TMs. As a step toward understanding the function of this TM isoform diversity, we have utilized the cDNA clones generated in our laboratory to produce homogeneous preparations of the individual isoforms in a bacterial expression system. The purified proteins were labeled with fluorescent reagents and then microinjected into cultured rat fibroblasts. Five of the isoforms we injected could be readily detected to bind actin filaments; e.g., TM-5A and TM-5B both localized to actin filaments (Fig. 3). In the coming year, we plan to study the individual isoforms during such events as mitosis, cell spreading, and migration into a monolayer wound. We will attempt to inject transformed rat cells to reintroduce those isoforms that are missing to see if there are any effects on cell morphology and microfilament structure.

Are There Specific Tropomyosin-binding Proteins in Tissues?

M. Pittenger, D. Helfman

At present, it is not known whether TMs in nonmuscle cells bind to proteins other than actin. Using ^{125}I-labeled Bolton-Hunter reagent, we labeled purified TM-5a and TM-5b, which differ in only a 24-amino-acid internal sequence (see Fig. 1). The ^{125}I-labeled proteins were used to probe a nitrocellulose blot of proteins from various rat tissues separated by SDS-PAGE. We have detected distinct bands in most tissues that bind to the labeled TM probes. Interestingly, some bands exhibit preferential binding to each TM probe. We are in the process of identifying these proteins, some of which are probably known cellular proteins, whereas others may prove as yet unidentified. This approach will also be used to identify and map the regions in the TM isoforms that are involved in binding to specific cellular proteins in this type of assay. In addition, experiments are in progress to determine whether these putative TM-binding proteins are effected in transformed cells.

Why Do Transformed Cells Alter Tropomyosin Expression?

M. Pittenger, D. Helfman

Dramatic shifts in TM isoform expression occur in cells transformed by such diverse agents as DNA and RNA tumor viruses and chemical carcinogens. Moreover, there are two recent reports of spontaneous revertants from a transformed phenotype to a nontransformed cell type which then express the normal complement of TMs. The underlying reasons for these alterations in TM expression are not understood, but these findings suggest a role for TM in maintaining normal cell phenotype. Morphological alterations are the most obvious and immediate char-

acteristics of transformed cells in culture. It is not known whether these gross abnormalities are directly related to transformation (e.g., by interfering with cell-cell communication and/or signal transduction pathways) or whether they occur only as an indirect consequence. We are interested in determining if morphological alterations are directly related to changes in TM isoform expression. This also requires an understanding of the normal regulation of TM expression.

We have used a pulse-chase experiment, high-resolution gel electrophoresis, and quantitative analysis to look at the half-life of TM proteins in normal rat embryo fibroblasts (REF52). All TM isoforms were found to have long half-lives, but the curves appeared to be biphasic, with a initial high slope and a more gradual slope after approximately 8 hours. We are testing the possibility that the initial rate reflects unbound TMs, whereas the slower degradation rate reflects that of TMs that have bound (reversibly) to actin filaments and are therefore not degraded. Transformed rat cell lines will then be analyzed in a similar way to see if alterations in half-life of particular isoforms can account for the alteration in their abundance.

Transformed cells generally have a more rapid transit through the cell cycle than normal cells. To analyze TM expression through the cell cycle, we have synchronized populations of cells by thymidine block and mitotic shake-off. The newly synthesized proteins in each population were pulse-labeled and resolved by two-dimensional gel electrophoresis. Only a modest change was detectable in the newly synthesized TMs. This alone would not account for the changes seen in transformed cells. However, it will now be interesting to see if there is any change in the degradation rate of isoforms through the cell cycle.

To address the question of whether morphological alterations are directly related to changes in TM expression, we have plated normal rat fibroblasts on a substrate that has been treated with polyHEMA to prevent cell attachment. This treatment can be varied to alter the extent of cell spreading. Newly synthesized TM isoforms were then analyzed after two-dimensional gel electrophoresis. We found that cells that cannot attach and spread properly show synthetic patterns more similar to those of transformed cells than to the patterns of well-spread cells. We will now test transformed cells on the polyHEMA substrate to test whether this produces any further changes in TM protein synthesis.

Identification and Characterization of an Actin-like Protein from *Schizosaccharomyces pombe*

J. Lees-Miller, G. Henry, D. Helfman

Actins are a family of highly conserved proteins that are ubiquitously distributed in all eukaryotic cells. They form the structural core of thin filaments in muscle and microfilaments in nonmuscle cells. Through the interaction with a host of cellular proteins, they participate in several essential cellular functions such as muscle contractility, cell motility, cytokinesis, cell structure, and organelle movement. We have recently cloned a gene from the fission yeast *S. pombe* that encodes a distantly related member of the actin family. Unlike all other known actins from a broad range of phyla, including vertebrates (human), yeast, plants, and protozoa, that have between 60% and 90% amino acid sequence identity and are 374–376 amino acids in length, this actin-like protein is only 30–35% identical to other actins, including that of *S. pombe*, and is 427 amino acids in length. Because it is not more similar to *S. pombe* actin than to actins from a variety of other organisms, including mammals, there is a strong possibility that this protein diverged from actin early in eukaryotic evolution and will be found in other species.

Our present goal is to determine the function of this actin-like protein and in so doing to better understand the complex set of interactions that underlie the cellular roles of conventional actins. Experiments are in progress to determine the phenotypes resulting from both knock-out and overexpression of the actin-like protein gene in *S. pombe*. On the basis of the results of this analysis, we will attempt to generate a selectable phenotype that can be used to identify potential homologs encoded within a mammalian cDNA expression library and to identify interacting proteins within fission yeast. In addition, we plan to purify the actin-like protein. It will be functionally characterized in regard to homotypic and heterotypic protein-protein interactions, and antibodies will be made for the purpose of cellular localization and as an additional tool in the search for a mammalian homolog.

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The QUEST Protein Database Center began an expansion in 1990 with renewed support from the Biomedical Research Technology Program of the National Institutes of Health. Our computer facility has been expanded with the hiring of Gerald Latter and Pat Monardo. Their immediate mission is to develop an enhanced version of the QUEST software for quantitative two-dimensional gel analysis that can be distributed to other scientists. Further expansion began in 1990 with the hiring of Scott Patterson to head a new laboratory for the identification and characterization of proteins from two-dimensional gels.

A major upgrade to the QUEST computer facility began in 1990. Since the present QUEST facility was established in 1985, computer hardware has improved tremendously, and so have the system software and database tools now available to all users of modern workstations. Jerry Latter, who comes to us from the Linus Pauling Institute where he had a great deal of experience in two-dimensional gel analysis, now leads our effort to produce an updated software package that can be used by investigators anywhere who have Sun or compatible workstations. Pat Monardo, who joined Jerry's team early in the year, brings a tremendous knowledge of modern software tools. He and Jerry have designed and partially implemented a new structure for the QUEST system, as described below.

Jerry Latter has also taken charge of the daily operations of the QUEST Center. Some of the functions of the QUEST Center are to perform routine two-dimensional gel analysis for investigators at Cold Spring Harbor Laboratory and elsewhere and to train and assist users in the analysis of their data. Cecile Chang continues to interact heavily with the users, helping to analyze, manage, and interpret their data. Phyllis Myers tirelessly carries out the routine aspects of pattern matching and editing for virtually all data passing through the system. Jim Kos has maintained the user software and has begun a relational database

to manage the records on every gel run in the QUEST system. The sections below describe much of the work that has passed through the QUEST center in 1990.

A new scientist, Scott Patterson, was hired in late 1990. In January, 1991, he will join the QUEST team as the protein chemist in charge of protein identification and characterization from two-dimensional gels. Scott is a young researcher from Australia with experience in many forms of electrophoresis and in protein characterization. New laboratory space being prepared for Scott will include both his protein chemistry facility and an upgraded two-dimensional gel electrophoresis laboratory. To train him in the latest methods of protein sequencing from two-dimensional gels, plans were made for Scott to work in the laboratory of Dr. Ruedi Aebersold in Vancouver during the first 2 months of 1991.

The two-dimensional gel laboratory in the McClintock building continues under the supervision of Heidi Sacco, with assistance from Shuling Fang and Kris Duhamel. Kris replaces Christy Blanchford, who departed for medical school.

Our scientific aims continue to be the construction and analysis of protein databases for mammalian cells and for yeast. The yeast database, carried out largely by Dr. Calvin McLaughlin of the University of California at Irvine, was described fully in last year's Annual Report and is being readied for publication and dissemination in 1991. In the following sections, we report in detail on recent developments in the rat REF52 database and the mouse embryo database. Dr. Robert Franza has continued to add new information on regulatory proteins to the databases, as described briefly below. His work is covered more fully in a separate report entitled "Cellular *trans*-Activators of Gene Expression."

QUEST Software for Two-dimensional Gel Analysis

J. Garrels, G. Latter, P. Monardo, J. Kos

The QUEST software package is a very complex entity that includes such diverse functions as film scanning, conversion of film density to units of radioactivity, combining image data from separate film exposures, spot detection and quantitation, pattern matching, interactive editing, selection of quantitative or qualitative changes, statistical analysis of data, graphic display of data, annotation of data, and comparison of data from experiments performed at different times and by different investigators. In the 1980s, we developed strategies for each of these tasks, we developed and tested algorithms for each phase of analysis, and we wrote a large software package for use on Unix-based (Masscomp) workstations. This software package was used to build our REF52 database, which was published in 1989.

The QUEST software package for the 1990s must be different in several major respects. It must incorporate software standards for graphics and for the user interface that did not exist a few years ago. It must take advantage of fast modern workstations that often are interconnected on networks. Finally, it must be a robust, easy-to-use, documented package that can be distributed widely to other scientific users.

The software upgrade has proceeded in two stages. First, the old body of code was converted to run on Sun workstations using the X windows graphics system. This conversion has involved (1) recoding complex algorithms for spot fitting and for image displays that formerly were designed to run in an array processor, (2) simplification of the older code that had become overly complex due to a proliferation of features and tricks that were then necessary to increase performance, and (3) adding new algorithms that were impractical in the slower environment of the older workstations. At the same time, the main algorithms for pattern matching and spot fitting have been significantly improved. Finally, an entirely new body of code, the Cluster Analysis functions, has been developed for database analysis (see below). Jim Garrels, who developed most of the original software package, has been primarily focused on this stage of the upgrade.

The second stage of software upgrade has proceeded simultaneously. Jerry Latter and Pat Monardo have taken the converted software and reimplemented it as small independent modules that communicate in a distributed (networked) environment. This structure ensures that the software can be easily maintained because each module is independent of the others, and this design is efficient because all users can take advantage of data and processing power anywhere on their network. Jerry, Pat, and Jim Kos further enhance the software by adding data management tools, utility functions, and, most of all, a standardized user interface. Finally, they develop the documentation and training materials that all users will require. The design and much of the coding of the final system were completed in 1990. The new

system is targeted for release to off-site users by August, 1991.

The REF52 Database Analyzed by Cluster Analysis

J.I. Garrels, R. Franza, C. Chang, G. Latter

The REF52 database contains a wealth of information about the patterns of protein synthesis and modification in the REF52 rat cell line as it responds to growth regulators and transforming agents. This large body of data consists of 12 major experiments, each containing 6–10 different gels, each of which has been quantitatively analyzed at the level of 1600 protein spots. Finding the biologically important patterns in this data set has been both rewarding and challenging. During 1990, we implemented a new tool, Cluster Analysis, in the QUEST system in order to find the patterns of coordinate regulation more easily.

Figure 1 presents one of the REF52 gel images, and the inset shows an enlarged region containing two proteins of interest. The expression profiles of these two proteins, caldesmon and a nuclear protein of 85 kD, are elaborated in Figure 2. For this experiment, cells were labeled at various times after plating, and some cultures were refed with fresh medium and serum before labeling. The expression profile for 85K is typical of proteins that are repressed at confluence (day 6) and at quiescence (day 12) and that

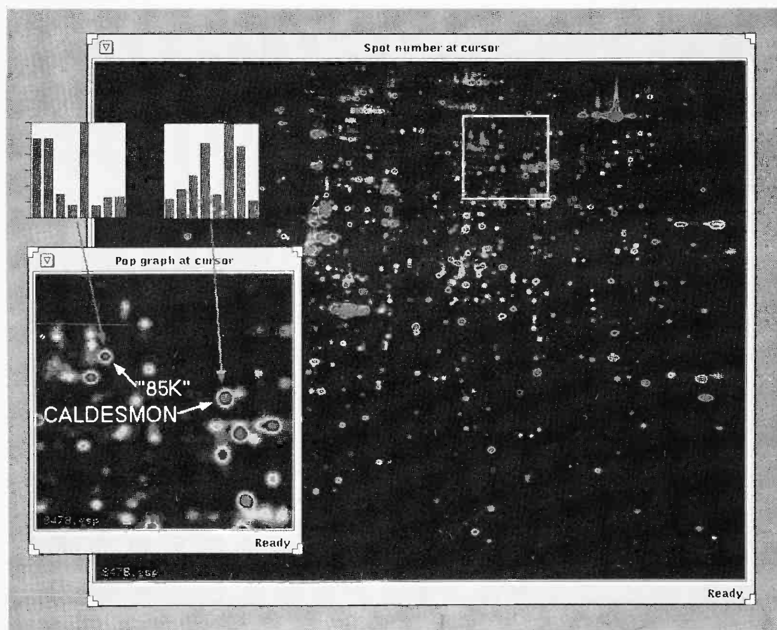

FIGURE 1 A two-dimensional gel, displayed on the Sun workstation, is shown as a whole-gel image in the large window, and the region in the box is enlarged in the smaller window. The expression profiles for two spots of interest, caldesmon and "85K," are shown. Each bar of the graphs shows the relative protein intensity from one sample, and the samples are identified in Fig. 2.

are induced to higher rates of synthesis as confluent cells re-enter S phase. The expression profile for caldesmon is typical of proteins that are induced as cells reach confluence and that have repressed rates of synthesis after refeeding.

Cluster analysis is a method used to find proteins with similar expression profiles in a given experiment. The algorithm compares the expression profile of every protein to that of every other protein, generating a matrix of similarity scores. Clustering is then the process of grouping the spots with high similarity scores. In the QUEST system, cluster analysis can be used either to find those proteins with expression profiles most similar to a given protein or to find all of the inherent expression profiles (clusters) ranked by the number of proteins they contain. The latter method was used to analyze the experiment depicted in Figure 2, and the most common expression profiles are shown in Figure 3.

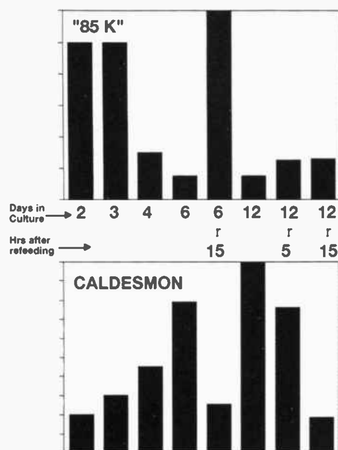

FIGURE 2 These expression profiles are taken from an experiment in the REF52 database. This experiment is designed to reveal changes in the relative rates of protein synthesis as REF52 cells proliferate, reach quiescence, and respond to refeeding. Each bar represents quantitation of one protein from one gel, and the vertical axis is normalized to the highest bar. The labels in the center apply to both graphs. These graphs show some of the highly characteristic protein expression profiles that exist in the database.

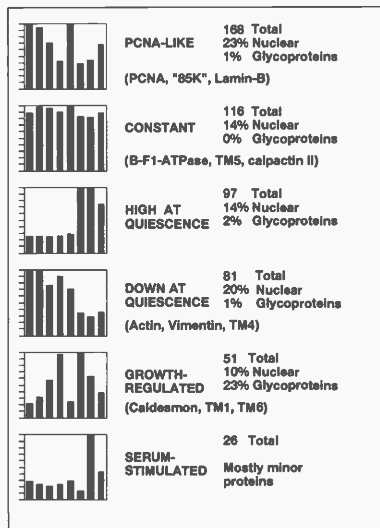

FIGURE 3 Cluster analysis of the protein expression profiles. The eight-sample experiment described in Fig. 2 was fully analyzed by cluster analysis, as described in the text. Each cluster is a group of proteins with similar expression profiles, and the graphs shown are the average expression profiles for the proteins in each cluster. Some known members and some biological properties of each cluster are given. Each cluster is discussed further in the text.

The largest cluster in Figure 3 is called "PCNA-like" because it has an expression profile similar to that of PCNA. The 85K protein is a member of this cluster, as was PCNA and lamin-B. The rate of synthesis of proteins in this cluster correlates well with the number of cells in S phase, and interestingly, this cluster has a high content of nuclear proteins. The next largest cluster (116 proteins) is the set with constant expression. The synthesis of these proteins, which comprise about 13% of all proteins analyzed, was not significantly regulated as REF52 cells entered quiescence and after cells were stimulated by refeeding.

The next two clusters shown in Figure 3 are characterized by high or low expression in confluent (day

12) cells, but with little change noted during growth to confluence. These patterns of expression, which were not recognized before the application of cluster analysis, show that many proteins are specifically induced or repressed as REF52 cells enter the G_0 state, and that these changes are not substantially reversed at 5 or 15 hours after refeeding. The "growth-regulated" cluster has the caldesmon-like pattern, and it includes several of the tropomyosin isoforms. These proteins are synthesized at elevated rates in both confluent (day 6) and quiescent (day 12) cells, and their synthesis is rapidly reduced after refeeding. Glycoproteins make up 23% of the proteins in this cluster, in contrast to a glycoprotein content of only 1–2% in other clusters.

The final cluster in Figure 3 contains 26 proteins that are induced early (5 hr) but not late (15 hr) after refeeding. Many of these proteins are undetectable in nonstimulated cells, and therefore could not be easily scored as nuclear or cytoplasmic. They are not significantly growth-regulated (during days 2–6), but appear to belong specifically to the early response to growth factors.

The application of cluster analysis to this relatively simple experiment has led to the identification of six fundamental patterns of regulation for the proteins of REF52 cells. The other key experiments in the REF52 database have likewise been subjected to cluster analysis, and the results are published elsewhere (Garrels et al., *Electrophoresis 11:* 1114 [1990]). Cluster analysis is now applied as a standard method in the QUEST system to recognize the major patterns of coordinate regulation in any quantitative experiment.

Mouse Embryo Database

J. I. Garrels, C. Chang, P. Myers [in collaboration with K. Latham and D. Solter, Wistar Institute]

The mouse embryo database is a quantitative study of synchronized embryos labeled every 3 hours from fertilization through the blastocyst stage. Additional samples from 6.5- and 7.5-day embryos separated into germ layers are now being processed. This baseline study of normal embryos serves as the control against which other studies are being compared. The database of normal development through the four-cell stage has been completed and analyzed using cluster analysis. The number of changes that occur during either the one-cell stage or the two-cell stage is much greater than the number of differences (quantitative and qualitative) that were found between proliferating and quiescent REF52 cells.

The clusters for the two-cell stage are most revealing. A large cluster of proteins was discovered for which the expression profile shows a steady rise in rate of synthesis between 6 and 18 hours of the two-cell stage. Many of these proteins were not detected at earlier times. On average, these proteins are induced by fivefold, although for proteins not detected at early times, this number reflects only the induction above the level of sensitivity. Members of this cluster correspond to the proteins encoded by embryonic mRNA, which is known to be first synthesized during the two-cell stage.

The next two clusters, in order of size, have expression profiles that decrease rapidly between 3 and 6 hours into the two-cell stage. One cluster (20% of all proteins analyzed) continues to decrease throughout the two-cell stage. The other cluster (16% of proteins analyzed) decreases sharply (fivefold) between 3 and 6 hours, but then returns to somewhat higher rates of synthesis by the end of the two-cell stage. The members of these two clusters are thought to be proteins encoded by maternal mRNAs that are inactivated or degraded in the two-cell stage. Some of these proteins are apparently not encoded by embryonic mRNA, whereas the synthesis of others is probably restored by the activation of embryonic transcription.

Two other clusters of proteins reveal transient changes peaking at 6–9 hours into the two-cell stage. Six percent of the proteins analyzed have sharply elevated levels of synthesis (tenfold on average) at 9 hours relative to 0 hour, but by the end of the two-cell stage, these proteins have fallen fivefold on average from their peak rate of synthesis. Conversely, 4% of the proteins have sharply lower rates of synthesis (sixfold on average) at 6 hours relative to 0 hour. The meaning of these two clusters is not clear, but it is interesting that many transient events occur coincident with embryonic gene activation.

To summarize the findings from the two-cell stage, 37% of the proteins belong to the cluster of induced proteins, 36% of the proteins belong to the two clusters of repressed proteins, and 10% of proteins show coordinate but transient changes. No cluster of proteins with constant synthesis during the two-cell stage was found. At the four-cell stage, in contrast, the largest cluster (63% of proteins analyzed) has a constant expression profile, and the other clusters

show inductions or repressions of twofold or less.

Further experiments are in progress to analyze the protein patterns of androgenones and gynogenones, which are embryos possessing either two male or two female pronuclei, respectively. The two-dimensional gel patterns reveal a block in the progression of changes in protein synthesis of androgenones that accompanies an observed morphological block. Other experiments in progress include the analysis of embryos exposed to protein kinase (PK-A and PK-C) inhibitors that are known to interfere with development.

Regulatory Protein Database

B.R. Franza

Proteins involved in regulation of cell growth and cell division are being studied in human cells. A variety of techniques have been used to identify, clone, and characterize these genes and their protein products (see Cellular *trans*-Activators of Gene Expression). The maps of these regulatory proteins, their expression profiles, and their patterns of modification are important contributions to the mammalian, and especially human, databases that now exist and to those that are planned for the future.

In recent work, the E1A-associated protein p60/cyclin A was shown to be associated with $p34^{cdc2}$. The p60/cyclin A complex also appears in immunoprecipitates complexed with significant amounts of the major protein kinase C substrate, MARCKS. In another line of research, it was shown that the DNA-binding protein, HIVEN86A, originally discovered using the DNA precipitation assay (DNAP) followed by two-dimensional gel analysis, is actually the product of the c-*rel* proto-oncogene. These and other regulatory proteins have been characterized on two-dimensional gels with respect to their patterns of induction, modification, and association with other regulatory proteins in several different human cell lines.

Service Projects

C. Chang, P. Myers, G. Latter, J Garrels

During 1990, the QUEST center has engaged in 11 collaborative and service projects as part of its mission as a resource facility. A few of the these projects are summarized here. Studies of the human HOS cell line and several transformed derivatives were carried out with Dr. C. Kumar from Schering Research. HOS, K-HOS (Ki-*ras*-transformed), MNNG-HOS (containing the *met* oncogene), and HOS 312 (K-HOS revertant) were analyzed at days 2, 4, 7, and 11 after plating. We had known that transformation shuts off the synthesis of a myosin light-chain isoform, but it was important to understand the regulation of this protein in normal and transformed HOS cells at all stages of proliferation and at confluence. Contrary to our expectation, we found that in normal cells, this cytoskeletal protein is synthesized most rapidly in proliferating cells and is repressed at confluence. Knowing the stage of maximum expression helped us to evaluate meaningfully the altered patterns of expression in transformed cells (Kumar et al., *EMBO J.* [1991] submitted).

Another study of human cells was carried out by Dr. J. Quigley and S. Nielson of SUNY, Stony Brook. They have examined HEp3 epidermoid carcinoma cells that can be reversibly cycled between metastatic and nonmetastatic states depending on growth conditions. Protein differences between metastatic and nonmetastatic cells have been identified with the QUEST system. More detailed studies are in progress to determine the timing of these changes and the cellular fractions in which they occur, prior to biochemical analysis of the most interesting proteins.

A mouse project, which interfaces with the mouse embryo database base (see above), is a study of normal, androgenetic, and gynogenetic ES cell lines. This study is carried out with Dr. Colin Stewart of the Roche Institute and Dr. K. Latham of the Wistar Institute. Although the developmental potential of these cell lines is markedly different, the protein synthesis differences are subtle. No qualitative differences were found, but about 20 proteins were found to exhibit quantitative differences of 1.5–2-fold. These studies may help to locate differences in regulatory pathways in stem cells that ultimately affect the course of development.

PUBLICATIONS

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profiles in responses to growth regulation, serum stimulation, and viral transformation. *Electrophoresis* 11: 1114–1130.

In Press, Submitted, and In Preparation

Giordano, A., C. McCall, P. Whyte, and B.R. Franza, Jr. 1991. Human cyclin A and the retinoblastoma protein interact with similar but distinguishable sequences in the adenovirus E1A gene product. (Submitted.)

Kumar, C.C., C. Chang, and J.I. Garrels. 1991. Human smooth muscle myosin light chain-2 gene expression is repressed in *ras*-transformed cells. *EMBO J.* (Submitted.)

Latham, K.E., J.I. Garrels, C. Chang, and D. Solter. 1991. Quantitative analysis of protein synthesis in mouse embryos. I. Extensive reprogramming at the one- and two-cell stages. *Development* (in press).

GENETICS

"The most interesting proteins are not found by biochemistry: Genetics is a much more powerful approach." "Genetics without biochemistry is sterile." This discussion occurs in many guises. The protagonists are those on one side who approach a biological problem by grinding up a cell or tissue and taking the resulting extract to the cold room, and on the other are those who begin by exposing a living cell to a mutagen. Curiously, each side now chases its own tail. Having identified a mutant phenotype of interest, the geneticist hopes to isolate the relevant gene, obtain its nucleotide sequence, and express its protein product in a suitable host for biochemical investigation. The biochemist goes the other way. Having purified a factor or enzyme, a small amount of protein sequence is obtained and used to design an oligonucleotide to isolate the respective gene. The biochemist becomes a geneticist and vice versa. This will not stop the argument that is as much about temperament as it is about scientific method. This section describes the work of those researches with a primarily genetic outlook.

EUKARYOTIC CELL CYCLE CONTROL

D. Beach S. Allan, J. Bischoff, M. Caligiuri, D. Casso, T. Connolly, G. Cottarel, S. Davey, U. Deuschle, H. Feilotter, K. Galaktionov, I. Garkavtsev, C. Gawel, C. Jessus, D. Lombardi, K. Lundgren, T. Matsumoto, T. Mizukami, L. Molz, B. Nefsky, A. Tesoro, N. Walworth, Y. Xiong

1988 and 1989 were extraordinary years for the cell cycle field and for our laboratory in particular. A group consisting primarily of Robert Booher, Leonardo Brizeula, Giulio Draetta, and David Beach (with outside collaborators, especially John Newport and Joan Ruderman) uncovered the connection between the *cdc2* protein kinase and a class of proteins called cyclins. Each cyclin was found to act as a subunit of the protein kinase resulting in a family of enzymes, each of which has a particular role in regulating progression through the cell division cycle. This discovery has triggered an explosive growth in the field, as more cyclins are discovered and efforts are made to piece each new cell cycle kinase into the overall picture.

In 1990, this work continued apace in our laboratory (two new classes of cyclin are described below). We were most fortunate in having our efforts recognized by the Howard Hughes Medical Institute of which David Beach is now an Investigator. This has allowed our work to branch out in new directions, some of which are described below. During the course of the year, Suzie Dembski left to take a position with Ed Harlow in Boston, and Joanne Lamonica entered medical school. We were joined by Catherine Jessus (visiting scientist), Adrienne Tesoro (lab manager), Craig Gawel (technician), Susan Allan (technician), David Casso (graduate student), Dianne Lombardi (computer programmer), and several postdoctoral fellows, Maureen Caligiuri, Harriet Feilotter, Igor Garavtsev, Toru Mizukami, Brad Nefsky, Nancy Walworth, and Yue Xiong.

Human D-type Cyclin

Y. Xiong, T. Connolly, D. Beach
[in collaboration with B. Futcher, Cold Spring Harbor Laboratory]

Two classes of cyclins, A type and B type, have been previously identified from various eukaryotic species. The B-type cyclin has been shown to act in mitosis

by serving as an integral subunit of the *cdc2* protein kinase. The A-type cyclin also independently associates with the *cdc2* kinase, forming an enzyme that appears to act earlier in the division cycle than mitosis. A third class of cyclin, named CLN type, was identified in budding yeast and was shown to be essential for progression through the G_1/S phase transition of the cell cycle. In *Saccharomyces cerevisiae*, there are three Cln proteins. Disruption of any one *CLN* gene function alone has little effect on growth; however, if all three genes are disrupted, the cells arrest in G_1.

To pursue our continuing study of the mammalian cell cycle, a general strategy was developed to isolate previously unidentified human cyclin genes. A yeast strain was constructed that contained insertional mutations in the *CLN1* and *CLN2* genes to render them inactive. The remaining *CLN3* gene was further altered to allow for conditional expression from a glucose repressible promoter *GAL1*. A cDNA library prepared from a human glioblastoma cell line was then introduced into the budding yeast strain. A previously unidentified human cyclin gene was rescued that we have termed cyclin D1. Compared to the other three classes, *CYCD1* encodes the shortest cyclin (34 kD). A comprehensive amino acid sequence comparison of 17 cyclin genes was carried out to construct an evolutionary tree of the cyclin family. This analysis established that human *CYCD1* represents a new class of cyclin. The expression of both the cyclin D1 gene transcript and its 34-kD product appears to be present at very low levels in several cell lines examined, but they are both abundant in a glioblastoma cell line. The role of cyclin D in the cell cycle of human cells is presently being investigated.

A New Fission Yeast Cyclin

L. Molz, D. Beach

In fission yeast, the *cdc2* protein kinase is required in G_1, prior to DNA synthesis, and in G_2, prior to mitosis. Recessive mutations that abolish *cdc2* gene function result in cell-cycle-arrest cells at either the G_1 or the G_2 control points. There exist dominant alleles of *cdc2* (the so-called *wee* alleles) that shorten G_2 and result in a small cell size. The combination of a particular *wee* allele of *cdc2* (*cdc2-3w*) and a loss of function allele of the *wee1* gene (a mitotic inhibitor) results in cells that divide at a very small cell size and undergo aberrant cellular and nuclear divisions, leading to cell death. This phenotype has been termed mitotic catastrophe.

To identify new genes interacting with the *cdc2* protein kinase, we have isolated extragenic mutations that suppress mitotic catastrophe (*mcs* mutations: *m*itotic *c*atastrophe *s*uppressors). Subsequent genetic analysis revealed that two of the *mcs* mutations (*mcs2-75* and *mcs6-13*) interacted in an allele-specific manner with the various *wee* alleles of *cdc2*. This suggests that the *mcs2* or *mcs6* genes may be substrates or subunits of the *cdc2* protein kinase. Two genes were cloned by complementation of the *mcs2-75* defect. One was demonstrated by linkage analysis to be the authentic *mcs2* gene, whereas the second was a high-copy suppressor of the *mcs2-75* defect. The *mcs2* gene was shown to encode a 37.5-kD protein that displays limited homology with cyclins (known subunits of the *cdc2* protein kinase). The high-copy suppressor of the *mcs2-75* mutation was shown to encode a putative 34.5-kD serine/threonine protein kinase. Null alleles of these genes created by gene disruption display a mid-mitotic arrest. In particular, the *mcs2* gene disruption was demonstrated to arrest with compact nuclei and condensed chromosomes. We are currently raising antibodies against these proteins for a molecular analysis of their gene products.

cdc2 Tyrosine Kinase

K. Lundgren, S. Dembski, N. Walworth, D. Beach

cdc2 activity is required for the entry of cells into mitosis. Dephosphorylation of the Tyr-15 residue of the protein kinase is at least one step that is required for activation of the enzyme. Genetic analysis has shown that the *wee1* protein kinase acts antagonistically to a mitotic inducer, *cdc25*, in the regulation of *cdc2* activity. *cdc25* is required for entry into mitosis and has been shown to be involved in the tyrosine dephosphorylation of *cdc2*. *wee1* acts as a mitotic inhibitor, yet its substrate has previously been obscure.

In a genetic screen designed to isolate inhibitors of mitosis, we identified *mik1*, a new protein kinase that is closely related to *wee1* itself. The loss of *mik1* function does not confer a phenotype, but a mutant in which *mik1* and *wee1* functions are both lost is lethal.

Microscopic examination of the cells reveals a great range of mitotic abnormalities (Fig. 1). The cells are literally dividing themselves to death.

We have found that not only do *mik1wee1* cells enter mitosis prematurely, but, in doing so, they can bypass the normal requirements for "START," S-phase, and the *cdc25* function. In addition, we have found that *cdc2* is tyrosine-dephosphorylated in these cells (Fig. 2)

We have shown that *mik1* acts redundantly with *wee1* in the negative regulation of *cdc2*. The simplest hypothesis is that *wee1* and *mik1* comprise the inhibitory *cdc2* tyrosine kinase. This model is now being tested.

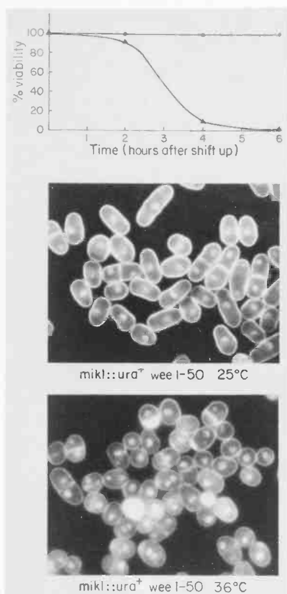

FIGURE 1 Lethal mitosis in the *mik1 wee1* double mutant. (*Top*) Viability plot of wild-type (*circles*) and *mik1:ura*+ wee1-50 (*triangles*) strains after intervals of incubation at 36°C. Viability was assayed by the ability to form colonies at 25°C. (*Middle*) DAPI-stained *wee-1-50 mik1::ura4*+ strain at 25°C. (*Bottom*) DAPI-stained cells 3 hr after transfer to 36°C. Bar, 5 μm.

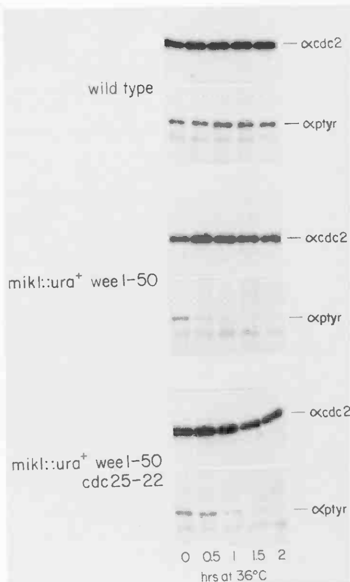

FIGURE 2 Phosphotyrosine content of *cdc2*. Lysates from wild-type (*top*), *mik1::ura*+ *wee1-50* (*middle*), and *cdc25-22 mik1::ura*+ *wee1-50* (*bottom*) cells, transferred from 25°C to 36°C for the indicated times, were immunoprecipitated with anti-*cdc2* (G8) antibody, separated on SDS-PAGE gels, transferred to nitrocellulose, and probed with either anti-*cdc2* or antiphosphotyrosine antibodies.

Involvement of a Novel GTPase in Cell Cycle Dependency

T. Matsumoto, D. Beach

In fission yeast, abnormalities of nuclear morphology are readily detectable under a fluorescence microscope. To find new mutants defective in cell cycle regulators, we constructed a temperature-sensitive mutant library that consists of more than 700 individual mutants and then screened each mutant microscopically. We have concentrated on one particular mutant *pim1*. *pim1* mutants display hyper-condensed chromosomes under restrictive conditions. Even when cells prearrested at the G_1/S boundary by hydroxyurea, they show hyper-condensed chromo-

somes. The phenotype suggests that *pim1* mutants can bypass DNA synthesis and enter mitosis directly.

We cloned the *pim1*$^{+}$ gene and a multicopy suppressor gene (*spi1*$^{+}$) by complementation. Sequence analysis of *pim1*$^{+}$ revealed that the gene product has significant homology with human Rcc1. Mammalian cells deficient in Rcc1 have a phenotype very similar to that of the fission yeast *pim1* mutants. Interestingly, the *spi1*$^{+}$ gene product has 80% amino acid identity with the human TC4 gene. TC4 and *spi1* comprise the only presently known members of a new class of GTPase signaling protein. These results suggest that a GTPase is involved in maintaining a coordinated cell cycle.

Control of Meiosis in Fission Yeast

S. Davey, D. Beach

Fission yeast respond to starvation by leaving the mitotic cell cycle and undergoing meiosis and sporulation. We are currently investigating the molecular controls that constitute the switch from the mitotic cell cycle to the meiotic cell cycle.

A number of genes involved in the control pathway are known, and of primary importance to our current work are the meiotic inhibitor *ran1* and the meiotic activators *mei2* and mei3. The *ran1* gene product ($p52^{ran1}$) is a protein kinase that is required for maintenance of the mitotic cell cycle; loss of *ran1* function causes cells to enter meiosis immediately. Starvation initiates a molecular cascade that leads to the production of the *mei3* gene product, which binds and inactivates $p52^{ran1}$. The role of *mei2* is not understood, but it is required for the switch from the mitotic to the meiotic cell cycle.

Research is proceeding on two fronts. We are currently undertaking genetic screens to identify novel suppressors of *ran1* and *mei2*. In addition, we are screening mammalian expression libraries for genes that can functionally replace the meiotic control genes in *Schizosaccharomyces pombe*. This work will further our understanding of meiotic control in fission

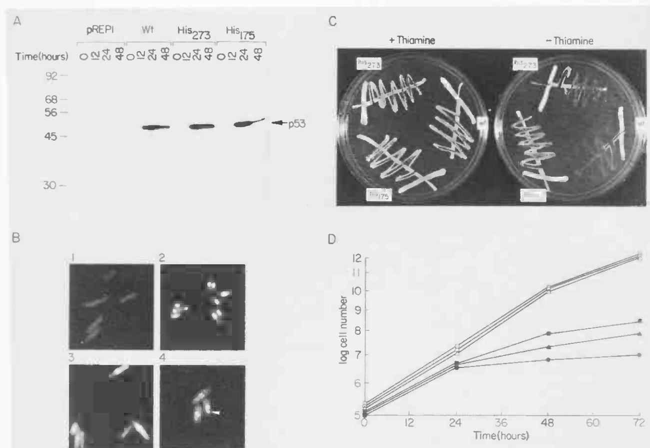

FIGURE 3 Expression of human p53 in *S. pombe* inhibits growth. (*A*) Western blot of wild-type (wt) p53, p53 with an arginine to histidine mutation at amino acid residue 273 (His-273), and p53 with an arginine to histidine mutation at amino acid residue 175 (His-175). (*B*) Immunofluorescence of *S. pombe*. (*1*) Control strain; (*2*) strain expressing wild-type p53; (*3*) strain expressing His-273; (*4*) strain expressing His-175 (arrow denotes extracellular staining). (*C*) Strains expressing wild-type p53, His-273, and His-175 struck on plates containing thiamine (no p53 expression) and plates with no thiamine (p53 expression). Growth curve of wt p53 (*circles*), His-273 (*triangles*), and His-175 (*squares*) in the presence (*open symbols*) or absence of (*closed symbols*) thiamine .

yeast and determine whether a similar cascade operates in mammalian cells.

A Yeast System to Study Human p53

J. Bischoff, D. Casso, D. Beach

p53 is a tumor suppressor gene that is involved in many types of human cancer. It is thought that p53 may normally act as a negative regulator of cell division. To investigate the possible role of p53 in the cell cycle, we have introduced human p53 into *S. pombe*. The overexpression of human wild-type p53 inhibits growth in *S. pombe* as it does in mammalian cells. Two mutant alleles of p53 also inhibit growth, but less severely (Fig. 3). Using a strain in which wild-type human p53 is integrated into the genome of *S. pombe*, we have identified a mutant allele of p53. It is a point mutation at amino acid residue 175, a mutation frequently found in tumor biopsies. This is direct evidence for a dominant mutant allele of a tumor suppressor gene. This implies that certain point mutations in a single allele of p53 could inactivate the wild-type allele, which could be important in the process of cellular transformation.

We are now using two approaches to identify genes that might interact with p53. One approach is to transform the p53-expressing *S. pombe* strain with cDNA libraries and look for cDNAs that relieve the negative effect of p53 on growth. Such cDNAs have been found and are being investigated. The other approach is to mutagenize chemically the p53-expressing strain to isolate extragenic suppressors of p53. We hope that by identifying genes that interact with p53, we will be able to understand the mechanism of action of this protein.

Fission Yeast Genome Mapping

I. Garkavtsev, T. Mizukami, D. Lombardi, D. Beach
[in collaboration with T. Marr, Cold Spring Harbor Laboratory].

A stage has been reached in the genetic analysis of model organisms such as the fission yeast that it is now appropriate to undertake a comprehensive approach toward the genome. Ideally, one would determine the nucleotide sequence of the entire genome, and efforts could then refocus on trying to understand what genes are doing, rather than analyzing their structure.

In an effort that aims toward this eventual goal, we have begun to make a systematic high-resolution map of the fission yeast genome. This involves creating an ordered set of cosmid clones that can then be used to map the genomic location of any gene of interest very rapidly. The genome mapping work also involves development of computational tools for record keeping and display of the many clones used in mapping.

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PRE-mRNA PROCESSING AND snRNA SYNTHESIS IN *SCHIZOSACCHAROMYCES POMBE*

D. Frendewey D. Kim
M. Gillespie
M. Ghee

Our research interests are split between two related areas: the processing of messenger RNA precursors (pre-mRNAs) and the synthesis of small nuclear RNAs (snRNAs). These two lines of research overlap, in general, by the fact that snRNAs participate in the catalysis of a wide variety of RNA-processing reactions. Most notably, the U1, U2, U4, U5, and U6 RNAs are required for pre-mRNA splicing and are components of the spliceosome, the large ribonucleoprotein structure that catalyzes the splicing of pre-mRNAs. The system in which we investigate these phenomena is the fission yeast *Schizosaccharomyces pombe*. Pre-mRNA splicing and snRNA synthesis are even more closely associated in *S. pombe* than in most other organisms, because the fission yeast U6 RNA is produced by the splicing of a precursor (pre-U6 RNA) that contains an intron whose structure is typical of those found in pre-mRNAs. This peculiarity of the fission yeast system presents some novel and interesting problems in snRNA synthesis and pre-mRNA splicing and, in addition, affords certain analytical advantages that will be explained below.

Four years ago, we began a three-stage approach to the study of pre-mRNA splicing in *S. pombe* that also led us into an investigation of snRNA synthesis. The first step was to identify genes required for pre-mRNA splicing or snRNA synthesis by isolating mutants that are defective in these processes. The isolation and characterization of our original pre-mRNA splicing and snRNA synthesis mutants were described in previous Annual Reports. These projects are an ongoing part of our research. Some new mutants isolated in the last year will be described in this report. Our second objective was to isolate and characterize the genes effected in the mutants by cloning and sequencing them. Progress on the cloning of three genes is presented below. Finally, our long-term goal is to dissect the biochemical mechanisms of pre-mRNA splicing and snRNA synthesis and to determine the functions of individual gene products in these processes. In this year's report, we summarize our efforts to biochemically characterize pre-mRNA splicing components and analyze directly U6 RNA transcription in *S. pombe*.

Pre-mRNA Splicing Mutants

D. Kim, D. Frendewey

The isolation of our first three pre-mRNA splicing mutants, *prp1*, *prp2*, and *prp3* (Potashkin et al., *EMBO J. 8:* 551 [1989]), was accomplished by screening a bank of temperature-sensitive (ts^-) *S. pombe* strains for mutants that accumulate unspliced pre-mRNAs. This procedure involved the isolation of whole-cell RNA from each ts^- mutant, fractionation of the RNA by gel electrophoresis, blotting to nylon membranes, and probing with oligodeoxynucleotides (oligos) directed against a pre-mRNA intron. Besides being slow and laborious, there are several drawbacks to this method of screening. mRNAs, and especially pre-mRNAs, are very susceptible to nucleolytic degradation during isolation, and most mRNAs are not very abundant, making detection of pre-mRNAs by Northern blotting of whole-cell RNA dif-

ficult. Since pre-mRNAs are large, they must be fractionated on formaldehyde agarose gels, which run slowly and have poor resolution, and subsequently blotted by diffusion, usually requiring 12–16 hours.

To overcome these difficulties, we have taken advantage of our observation (Potashkin and Frendewey, *Nucleic Acids Res. 17:* 7821 [1989]) that splicing of pre-U6 RNA is impaired in the *prp⁻* mutants. We produced approximately 200 new *ts⁻* strains and screened them by the Northern assay for accumulation of pre-U6 RNA. Since pre-U6 RNA is small (150 nucleotides), the whole-cell RNA was rapidly electrophoresed on short polyacrylamide gels and transferred to nylon membranes by electroblotting, which can be completed in 4 hours. The abundance and stability of the U6 RNA made detection of the precursor very much simpler and improved the sensitivity.

Six new splicing mutants were identified in a screen of 180 *ts⁻* strains (Fig. 1). Three different molecular phenotypes were observed among the new mutants: (1) four mutants (ts363, ts377, ts426, and ts481) accumulated pre-U6 RNA at 37°C (restrictive) but not 23°C (permissive); (2) one mutant (ts370) exhibited an equal accumulation of pre-U6 RNA at both 37°C and 23°C; and (3) one mutant (ts369) accumulated pre-U6 RNA and two other intron-containing U6 RNAs at 37°C but not at 23°C. The latter two phenotypes were not observed in our previous searches for pre-mRNA splicing mutants. As expected from our previous results with *prp1*, *prp2*, and *prp3*, all of the new mutants also accumulate β-tubulin pre-mRNA at 37°C; ts370 shows a pre-mRNA splicing defect at 23°C as well. The pre-mRNA signal on Northern blots of ts369 RNA appeared to be a smear of poorly resolved bands, some running slower than pre-mRNA by comparison with the other mutants. This result could be evidence for the accumulation of other intron-containing RNAs as was seen for the pre-U6 RNA analysis. Thus, the pre-U6 RNA and pre-mRNA splicing defects are equivalent in the six new mutants.

The ts369 mutant is particularly interesting because it is the first *S. pombe* mutant that accumulates possible intermediates of the splicing reaction. In addition to pre-U6 RNA, two other RNAs of about 120 and 50 nucleotides are detected on Northern blots of ts369 RNA when probed with two different U6 intron probes (Fig. 2). The 120-nucleotide RNA hybridizes to probes against the intron and exon 2, whereas the 50-nucleotide RNA is recognized by intron probes but not those to exon 2. Both RNAs exhibit slightly retarded mobilities relative to the pre-U6 and mature U6 RNAs on higher percentage acrylamide gels. These results suggest that the 120-nucleotide RNA is the intron-exon 2 lariat intermediate product and the

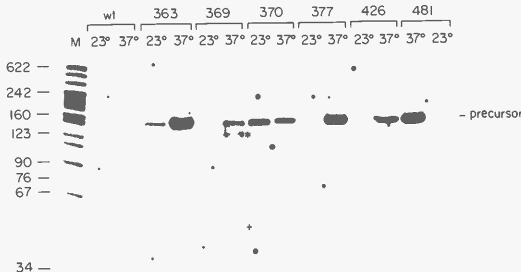

FIGURE 1 Northern blot analysis of pre-U6 RNA splicing in the wild-type (wt) and six *ts⁻* mutants (indicated by their numbers above the lanes). RNA was prepared from wild-type and mutant cells grown to mid-log stage at 23°C and after a shift to 37°C for 5 hr The RNA was fractionated by polyacrylamide gel electrophoresis and electroblotted to a nylon membrane The blot was probed with an oligonucleotide that hybridizes to the intron of the pre-U6 RNA. The position of the pre-U6 RNA is indicated on the right (precursor). The asterisk and plus symbols identify intron-containing RNAs that accumulate in ts369. Lane M contains pBR322 *MspI* fragments (sizes in nucleotides given at left).

50-nucleotide RNA is the intron lariat final product (theoretical size = 50 nucleotides). Further analysis is required to confirm these assignments.

In vitro studies in the human and *Saccharomyces cerevisiae* systems have shown that after the completion of a normal splicing reaction, the spliceosome disassembles, releasing the mature RNA product. The intron is released as a short-lived complex with some of the spliceosomal components that include the U2, U5, and U6 RNAs. When this association breaks up, the free intron is linearized by a debranching enzyme and degraded. The mutation in ts369 might affect the disassociation of the spliceosome and release of the spliced products required for the recycling of the spliceosome. In many enzymatic reactions, a block in the release of products reduces the overall rate of catalysis. Such a mechanism could explain the accumulation of the intermediate and the precursor substrate in ts369. We are continuing our analysis of the splicing defect in ts369 by investigating the splicing of pre-mRNAs in this mutant in greater detail.

Genetic characterization of the new mutants is under way and is nearly complete for ts426 and ts481. Linkage analysis indicates that ts426 is an allele of *prp3*, and we have tentatively assigned it the name *prp3-2*. (The original *prp3* allele will be *prp3-1*.) Experiments supporting this result are presented below. ts481 is not linked to *prp1*, *prp2*, or *prp3* or to a new mutant, *prp4*, isolated in Norbert Käufer's laboratory at Drexel University. We have therefore given ts481 the formal name *prp5*. When the genetic analysis is complete, we will attempt to clone the wild-type genes that are affected in one or more of the mutants by complementation with an *S. pombe* genomic DNA library.

Cloning of the *prp3*$^{+}$ Gene

D. Frendewey

Our original splicing mutants, *prp1*, *prp2*, and *prp3*, were isolated by Judy Potashkin, a former postdoctoral fellow in the laboratory. Judy started her own laboratory at the Chicago Medical School in January 1990, where she plans to characterize the *prp1*$^{+}$ and *prp2*$^{+}$ genes. This year, I attempted to clone the *prp3*$^{+}$ gene. The *prp3* mutant accumulates pre-mRNA at 37°C, but unlike *prp1* and *prp2*, it produces some mRNA at the restrictive temperature. Consistent with this molecular phenotype, *prp3* continues to grow slowly at 37°C. However, the difference between the growth rate of *prp3* and that of the wild

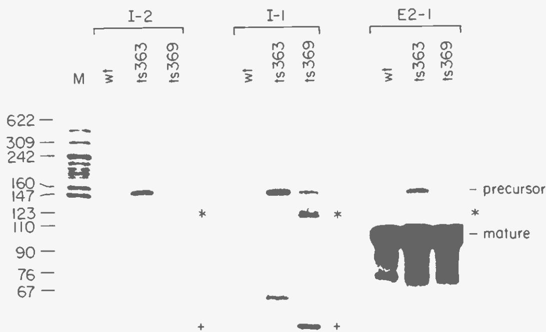

FIGURE 2 Northern blot analysis of U6 RNA transcripts that accumulate at 37°C in ts369. RNA from 37°C cultures of wild type, ts363, and ts369 was prepared, fractionated, and blotted as described in Fig. 1. The same blot was sequentially hybridized with two different intron probes (I-1 and I-2) and one exon 2 probe (E2-1), as indicated above the lane groupings. The position of spliced U6 RNA (mature) is shown to the right of the E2-1 panel. All other symbols and designations are the same as those in Fig. 1.

type at 37°C is sufficient to enable cloning of the *prp3⁺* gene by complementation of the *ts⁻* growth phenotype.

The *prp3* mutant was transformed with an *S. pombe* genomic DNA library (a gift from Paul Young, Queens University, Canada), and rapidly growing transformants (*ts⁺*) were selected at 37°C. Of the 14 *ts⁺* transformants obtained, six grew consistently well after a second challenge at 37°C and were dependent on the transforming plasmid for growth at the restrictive temperature. Plasmid DNA was isolated from the six *ts⁺* yeast clones and used to transform *Escherichia coli*. Bacterial transformants were obtained for four of the six *ts⁺* clones. All of the plasmids recovered from the bacterial transformants had an identical 8.7-kilobase-pair (kb) *Hin*dIII insert. One of these plasmids was characterized further.

The plasmid clone was reintroduced into *prp3* to confirm that it cures the *ts⁻* growth defect. Three fragments of the 8.7-kb clone were subcloned into yeast vectors and used to transform *prp3*. None of these subclones cured the *ts⁻* defect, indicating that the fragments tested did not contain the complete gene. I am currently constructing new overlapping subclones, which will be tested in *prp3*. As mentioned in the previous section, one of our new mutants, ts426, appears to be an allele of *prp3* (*prp3-2*). Consistent with this result, the *prp3⁺* clone is able to cure the *ts⁻* growth defect in *prp3-2*. The *prp3-2* mutation causes a more severe impairment to growth and splicing than the original *prp3-1* mutation. In the future, in addition to characterizing the *prp3⁺* gene, we would like to sequence the *prp3⁻* genes of *prp3-1* and *prp3-2* to determine the mutations in these alleles. This information may give us an insight into the function of the *prp⁺* gene product.

A Mutant Defective in mRNA 3′-end Formation

D. Frendewey [in collaboration with T. Humphrey and N. Proudfoot, Oxford University]

All mRNAs, with the exception of certain histone mRNAs of higher eukaryotes, have a stretch of adenosines at their 3′ends that is essential for the stability of the mRNAs and may be a signal for transport out of the nucleus. The polyadenosine (poly[A]) tract is not encoded in the DNA but is added post-transcriptionally. In mammals, the mechanism of mRNA 3′-end formation has been described with a fair degree of biochemical detail. Although the connection between 3′processing and termination of transcription is poorly understood, it is clear that the nascent pre-mRNA (perhaps still tethered to the transcription complex) is cleaved a short distance downstream from a nearly invariant AAUAAA signal and immediately polyadenylated. The AAUAAA is essential for both cleavage and polyadenylation. A second conserved feature in mammalian pre-mRNAs is a stretch of RNA rich in G and U immediately downstream from the poly(A) site. The GU stretch is not required for 3′-end formation in vitro but improves efficiency in vivo. A factor that binds to the AAUAAA and functions in both cleavage and polyadenylation has been identified. Additional proteins that participate in the cleavage reaction and the poly(A) polymerase itself have been purified from mammalian nuclear extracts. Although the U11 snRNP has been implicated in mRNA 3′-end formation, solid evidence in support of snRNP involvement has not yet been obtained.

Very little is known about mRNA 3′-end formation in yeast. Poly(A) tails are found on the 3′ ends of *Saccharomyces cerevisiae* and *S. pombe* mRNAs, but the mechanism by which this polyadenylation is achieved is poorly understood. Sequences required for mRNA 3′-end formation have been identified for some *S. cerevisiae* pre-mRNAs; however, no clear signal is apparent. If conserved primary sequence elements are involved in the polyadenylation of yeast pre-mRNAs, then their features are much more degenerate than those of mammals. The AAUAAA sequence is often found near the 3′ends of yeast mRNAs, but there is no evidence that it is required for polyadenylation. Essentially nothing is known about the cellular factors that participate in mRNA 3′ maturation in yeast.

Our interest in mRNA 3′-end formation originates with an observation we made while screening for pre-mRNA splicing mutants. One of the strains in our *ts⁻* collection (ts39) produced an apparently normal β-tubulin mRNA at the restrictive temperature, but it also accumulated a β-tubulin transcript that was larger than the unspliced pre-mRNA (Potashkin et al., *EMBO J. 8:* 551 [1989]). The ts39 mutant showed no pre-mRNA splicing defect, so the aberrant transcript was probably extended at either its 5′end or its 3′end. In collaboration with Tim Humphrey, a student in Nick Proudfoot's laboratory at Oxford, we have recently begun to reinvestigate ts39. To confirm the original observation, a Northern blot analysis was performed for the *ura4⁺* mRNA.

This RNA is transcribed from an intronless gene. As we observed for β-tubulin mRNA, an aberrantly large *ura4*$^+$ transcript was seen at 37°C; only normal size *ura4*$^+$ mRNA was detected at 23°C. A closer analysis of the 3′ end of the *ura4*$^+$ mRNA in ts39 indicated that RNA with the correct 3′ end was formed at 37°C, but there was a significant accumulation of RNA that was extended beyond the normal poly(A) site. We are currently attempting to map the precise 3′ end of the aberrant *ura4*$^+$ transcript produced in ts39. Primer extension experiments reveal no difference in the 5′ end of the *ura4*$^+$ mRNA between 23°C and 37°C. These results strongly suggest that ts39 suffers from a partial impairment in mRNA 3′-end formation at 37°C. This is the first mutant of this type in *S. pombe*. Several mutants that exhibit defects in mRNA 3′-end formation have been described for *S. cerevisiae*, but their characterization is not yet complete.

We have transformed ts39 with an *S. pombe* genomic library and selected transformants that grow at 37°C. We isolated six *ts*$^+$ transformants that were dependent on the transforming plasmid for growth at the restrictive temperature. So far, we have been able to recover plasmid in *E. coli* from one of the *ts*$^+$ transformants. This plasmid has a 5.8-kb insert and is able to cure the *ts*$^-$ defect upon reintroduction into ts39. Since none of the factors identified as part of the pre-mRNA cleavage and polyadenylation apparatus have been completely characterized, the cloning and sequencing of the gene effected in ts39 would be an important advance in our understanding of the types of gene products that are required for mRNA 3′-end formation.

S. Pombe snRNP Proteins

M. Ghee, D. Frendewey [in collaboration with
D. Spector and A. Krainer, Cold Spring
Harbor Laboratory]

One of our long-term goals is the biochemical characterization of the cellular components involved in pre-mRNA splicing in fission yeast. In the absence of a homologous in vitro pre-mRNA splicing system for *S. pombe*, we have resorted to the genetic strategy described in the previous sections to identify and characterize gene products essential for pre-mRNA splicing. However, one set of known splicing factors is available for direct biochemical analysis: the small nuclear ribonucleoprotein particles (snRNPs). The U1, U2, U4/6, and U5 snRNPs are constituents of the spliceosome and are required for pre-mRNA splicing. Each snRNP consists of an snRNA (two in the case of the U4/6 snRNP) complexed with a set of proteins. Six core proteins are common to all of the spliceosomal snRNPs, and, in addition, one or more proteins specific to each of the individual snRNPs have been identified. Due to their great abundance in vertebrate nuclei and the availability of powerful immunochemical reagents, a large amount of information has been gathered about vertebrate snRNP proteins. The snRNPs have been purified, and their protein components have been cataloged. The genes for several of the snRNP proteins have been cloned and sequenced, and a crystal structure for one has been determined.

In contrast, very little is known about snRNP proteins and snRNP structure in yeast. The major snRNAs have been identified and sequenced, but only a few snRNP-specific proteins have been characterized. Yeast homologs of the core snRNP proteins have not yet been found. The main impediment has been that in *S. cerevisiae* (the yeast in which pre-mRNA splicing has been most thoroughly studied), the snRNPs are more than 1000-fold less abundant than in higher eukaryotic cells. An additional technical difficulty has been that most of the antibodies that were used so effectively for the study of vertebrate snRNPs do not react with the *S. cerevisiae* counterparts. Both of these drawbacks are partially overcome in the fission yeast system. First, the cellular snRNP concentration in *S. pombe* is 10–100 times higher than that in *S. cerevisiae*. Second, some of the common human snRNP antisera efficiently select the homologous snRNPs in *S. pombe*. We have therefore begun a biochemical investigation of the snRNP proteins in fission yeast.

Patients suffering from connective tissue diseases often produce antibodies to snRNPs. One of the most common of these antisera, anti-Sm, reacts with epitopes on the core snRNP proteins; thus, anti-Sm antibodies recognize all of the spliceosomal snRNPs. We obtained a potent anti-Sm serum from E. DeRobertis (University of California, Los Angeles) and used it to immunoprecipitate snRNPs from *S. pombe* whole-cell extracts. The protein was removed from the immunoprecipitates, and the RNA was fractionated on an acrylamide gel and blotted to a nylon membrane. The Northern blot was then probed with oligonucleotides complementary to the major snRNAs. This experiment showed that the human anti-Sm antibodies recognized the U1, U2, U4/6, and

U5 snRNPs from *S. pombe*. No signals were observed with probes for U3 RNA, a nucleolar snRNA whose snRNP does not have the Sm-reactive core proteins, or K RNA, the RNA subunit of the nucleoplasmic tRNA processing enzyme RNase P. Judy Potashkin and David Spector (Molecular Genetics of Eukaryotic Cells Section) had previously obtained evidence by immunoelectron microscopy that another patient antisera known as anti-A protein recognizes snRNPs in *S. pombe* nuclei. Anti-A protein sera react with the U1 snRNP-specific A protein and usually also recognize the closely related B′protein of the U2 snRNP. In collaboration with David Spector, we performed an immunoprecipitation experiment with a number of anti-A protein sera. Most of these sera efficiently selected the *S. pombe* U1 snRNP and one also reacted weakly with the U2 snRNP. The U3, U4, U5, U6, and K RNAs were not detected on the blot of the anti-A protein immunoprecipitate. The results of the immunoprecipitation with the anti-Sm and anti-A protein sera indicate that *S. pombe* has homologs of the core proteins and the U1 A protein or the U2 B′ protein that are antigenically similar to their vertebrate counterparts. We may now be able to use the human antisera to characterize further the structure and function of the *S. pombe* snRNPs and to eventually clone the genes that encode these proteins in fission yeast.

All of the U snRNAs, with the exception of U6, have an unusual and characteristic trimethylguanosine (m_3G) 5′"cap." Adrian Krainer (Tumor Viruses Section) has produced a monoclonal antibody against this cap structure, which we previously showed could select the U1, U2, U3, U4/6, and U5 snRNPs from *S. pombe*. To look more directly at the fission yeast snRNP proteins, we immunoselected snRNPs from an *S. pombe* whole-cell extract with the anti-m_3G antibody. For comparison, the selection was also performed on an equivalent amount of *S. cerevisiae* whole-cell extract and a small amount of HeLa nuclear extract. The proteins that bound to the antibody were analyzed by SDS-polyacrylamide gel electrophoresis, followed by silver staining. We obtained a simple pattern of 10–15 protein bands for *S. pombe* that was remarkably like the human snRNP protein set. For example, bands similar in size to the human D, E, F, and G core proteins were seen in *S. pombe*. We have preliminary evidence from Western blotting experiments that a single protein from an *S. pombe* whole-cell extract reacts with anti-A protein antibodies. This protein is slightly larger than the human A protein control on the same blot and may correspond to a prominent band at the same position in the *S. pombe* sample from the anti-m_3G immunoselection experiment. We have not absolutely identified any of the *S. pombe* proteins selected by the anti-m_3G antibody, but the resemblance to the human pattern and Western blotting results are encouraging. To illustrate the advantage of fission yeast for this type of investigation, the immunoselection of the *S. cerevisiae* snRNPs produced a protein pattern that was too faint to be interpreted.

As mentioned above, the U6 RNA does not have an m_3G cap. Its 5′end has a simple methylesterification of the gamma phosphate on the 5′- terminal guanosine triphosphate residue (MeGTP). Ram Reddy's group at the Baylor College of Medicine has generated an antibody against the MeGTP cap and sent it to us to determine if *S. pombe* U6 RNA has the same 5′terminus. The anti-MeGTP antibody immunoprecipitated only a small percentage of the total U6 RNA in an *S. pombe* whole-cell extract. The antibody is equally ineffective against mammalian nuclear extracts. The reason for this poor performance could be the antibody's low titer or the MeGTP cap might be masked in the U4/6 snRNP. Our results indicate that the latter explanation may account, at least in part, for the low immunoprecipitation efficiency. When anti-MeGTP immunoprecipitates from *S. pombe* extracts were assayed by Northern blotting, we observed a weak U6 signal, but, surprisingly, U6's snRNP partner, U4 RNA, was not detected. An even more unexpected result was that U2 RNA was immunoprecipitated by anti-MeGTP in amounts approximately equal to that seen for U6 RNA. Alan Weiner's group at Yale has recently demonstrated a base-paired interaction between the 5′end of U2 and the 3′end of U6 in HeLa nuclear extracts. If our immunoprecipitations detected such an interaction in *S. pombe* extracts, then the MeGTP cap must be more accessible to the antibody in the U2/U6 complex than in the U4/6 snRNP. At the completion of the pre-mRNA splicing reaction, the intron lariat is released in an RNP with the U2, U6, and U5 RNAs. The U2/U6 interaction may be a remnant of this complex and represent an intermediate in spliceosome recycling.

Cloning of the *snm1*$^+$ Gene

M. Gillespie, D. Frendewey

In previous Annual Reports, we described the isolation and characterization of an interesting *ts*$^-$ mutant,

which we named *snm1* because it expresses a defect in *sn*RNA *m*aintenance. A single recessive mutation in *snm1* causes a two- to tenfold reduction in the steady-state levels of the U1, U2, U4, U5, and U6 snRNAs and the RNase P RNA subunit. In addition, 3′-extended U2 and U4 RNAs and unspliced U6 RNA accumulate at the restrictive temperature. This mutant phenotype implies that the *snm1*$^+$ gene product plays a central role in the correct and efficient synthesis of a family of snRNAs. In last year's Annual Report, we briefly described our initial attempts to clone the *snm1*$^+$ gene. This project has continued to be a major part of our effort, and our progress in the last year is outlined below.

The *snm1* mutant exhibits a "leaky" *ts*$^-$ growth phenotype; it continues to grow at about one half the wild-type rate after a shift from 23°C to 37°C. For this reason, when we transformed *snm1* with an *S. pombe* genomic library and selected for growth at 37°C, many transformants appeared on the plates; 200 transformants were transferred to a fresh plate and immediately challenged again at 37°C. Of these 200, 2 grew after this second selection. To confirm these results, *snm1* was transformed with the genomic library again in a second independent experiment. This time, 4 *ts*$^+$ clones out of 200 original transformants were obtained after the second selection. The six *ts*$^+$ transformants all required the transforming plasmid for growth at 37°C. Plasmids were isolated from the yeast clones and recovered in *E. coli*.

Five classes of bacterial clones were obtained, having yeast DNA inserts ranging in size from 3.6 kb to 10.3 kb. One class appeared to represent a rearrangement of the plasmid in that the intact vector was not seen. Four of the five classes of clones (including the rearranged plasmids) contained 3.2- and 0.4-kb *Hin*dIII fragments, suggesting that the *snm1*$^+$ gene was on one or both of these pieces of DNA. Two of the classes of clones that contained the common fragments were used to transform *snm1* and were shown to cure the *ts*$^-$ defect. Subsequently, a 4.3-kb *Pst*I fragment from one of the larger clones was shown to complement the *snm1*$^-$ mutation. The *Pst*I subclone contains a 3.2-kb *Hin*dIII fragment. Further analysis has demonstrated that a 2.2-kb *Hin*dIII-*Nde*I subclone is sufficient to transform *snm1* to rapid growth at 37°C. The sequencing of this clone is nearly complete, but we have not yet assigned an open reading frame. Genetic and molecular examination of this clone is continuing in order to confirm that it does contain the *snm1*$^+$ gene.

U6 RNA Transcription in *S. pombe*

M. Gillespie, D. Frendewey

In last year's Annual Report, we presented the results of our analysis of the U6 RNA genes from the three known species of *Schizosaccharomyces* and their variants (Frendewey et al., *Nucleic Acids Res. 18:* 2025 [1990]). All of the fission yeast U6 genes were found to have introns. The sequences of the introns were different in each of the species, except for the bases at the 5′and 3′splice sites and putative branchpoints. However, we also noticed an unexpected region of sequence conservation just upstream of the expected branchpoint. The 11 conserved bases fit the consensus for the B-box RNA polymerase III (pol III) promoter element found in tRNA genes. This surprising result suggested that conventional pol III promoter elements might serve as transcription signals for U6 RNA synthesis in fission yeast. It is now known that transcription of the *S. cerevisiae* U6 RNA requires a B box at an unusual position in the 3′flank (Brow and Guthrie, *Genes Dev. 4:* 1345 [1990]). During the past year, we have begun experiments to define the sequences required for U6 transcription in *S. pombe* and, specifically, to test whether the conserved B-box similarity is essential.

Our initial strategy to investigate U6 transcription was to create a series of deletions in the U6 gene and then to test their expression in vivo and in vitro. To facilitate identification in vivo, each construct was "marked" with a short stretch of foreign DNA placed in the middle of exon 2. This was accomplished by amplifying two fragments of the *S. pombe* U6 gene by the polymerase chain reaction (PCR) and then cloning them into the pBSM13 vector in such a way that the sequences between the *Bam*HI and *Sac*I sites of the plasmid's polylinker interrupted exon 2. One of the U6 fragments included 102 bp of 5′flank (−102), exon 1, the intron, and about half of exon 2; the second consisted of the 3′half of exon 2 and 155 bp of 3′flank. This marked U6 gene (SpU6FL+) was subcloned into a yeast vector and used to transform wild-type *S. pombe*. Primer extension analysis of RNA from the transformants showed that SpU6FL+ was efficiently expressed, with transcription initiating at the wild-type start site. Both pre-U6 RNA and spliced product were detected in an apparently wild-type ratio. These results demonstrate that as little as 102 bp of 5′flank is sufficient for accurate and efficient expression of the U6 gene in vivo. We have also

made a marked wild-type U6 gene (SpU6-2+) having 1500 bp of 5′ flank (a gift from T. Tani and Y. Ohshima, Kyushu University, Japan). However, we have based our mutant constructs on SpU6FL+. We made a series of five deletions from −102 in the 5′ flank to position 22 in exon 1. We also deleted the intron from SpU6FL+ and from some of the 5′ deletion mutants. The structures of the mutant genes were confirmed by sequencing.

We first tested the U6 deletion mutations in vitro. Since our attempts to make an *S. pombe* extract that accurately and efficiently transcribes pol III genes were unsuccessful, we resorted to two heterologous pol III systems. It had been reported that the *S. pombe* U6 gene is transcribed in an extract from HeLa cells (Kleinschmidt et al., *J. Mol. Biol. 211:* 7 [1990]). However, when we tested SpU6-2+ and SpU6FL+ in a HeLa nuclear extract (a gift from S. Lobo and N. Hernandez, Cold Spring Harbor Laboratory), our results indicated that transcription initiated incorrectly at several sites greater than 50 bp upstream of the normal starting point. We therefore switched to an *S. cerevisiae* whole-cell extract (a gift from I. Willis, Albert Einstein College of Medicine). Both SpU6-2+ and SpU6FL+ were accurately transcribed in this extract. We then assayed the transcription of each of the mutant U6 constructs. A deletion that left 78 bp of 5′ flank was transcribed as efficiently as SpU6-2+ and SpU6FL+ (1500 bp and 102 bp of 5′ flank, respectively). The transcription efficiency for a mutant that retains 52 bp of 5′ flank was slightly lower than for SpU6-2+ and SpU6FL+. However, when the presumptive TATA box at −25 was replaced with the vector sequence, transcription efficiency was reduced and a new start site was detected a few bases upstream of the true initiation point. Deletion/replacement of the complete 5′ flank (SpU6Δ4+) reduced the amount of transcription further but appeared to restore the normal start site. A deletion of the 5′ flank and the first 21 bp of exon 1 (SpU6Δ5+) abolished transcription.

The in vivo expression of the Δ1+ and Δ2+ constructs (78 and 52 bp of 5′ flank, respectively) mimicked the in vitro results. However, deletion of the TATA box (Δ3+, 21 bp of 5′ flank) was more deleterious in vivo than in vitro. The Δ3+ mutant produced only a very small amount of incorrectly initiated transcript in vivo. The Δ4+ and Δ5+ deletions were not expressed at all in *S. pombe* cells. The SpU6Δ5+ deletion/replacement creates three point mutations in a sequence of 11 bases that matches the consensus for the A-box pol III promoter element. If these same three base changes are introduced into SpU6FL+, they have as severe an effect on transcription as the SpU6Δ5+ deletion. This result implicates the A box as an essential promoter element in the *S. pombe* U6 gene. Removal of the intron from SpU6FL+ resulted in a severe reduction in transcription efficiency; a very small amount of correctly initiated transcript was detected. This result is consistent with the hypothesis that the intron contains a B-box promoter element. However, a point mutation in the B box has little or no effect on transcription efficiency in vitro. The same mutation in the B box of a tRNA gene prevents transcription.

We have subcloned our U6 mutants into a yeast vector and tested their expression in vivo following transformation of a wild-type *S. pombe* strain. As stated above, SpU6FL+ (102 bp of 5′ flank) was very efficiently expressed in vivo. Removal of the intron and the point mutation in the B box, in contrast to the in vitro results, had a similarly negative effect in vivo: both of these mutant templates produced barely detectable amounts of U6 transcript.

Our experiments with the U6 gene deletions have pointed to a requirement for sequences in the 5′ flank, the first exon, and the intron for accurate and efficient transcription. The results to date allow us to make the following preliminary conclusions: (1) 78 bp of 5′ flank is sufficient for accurate and efficient transcription; (2) an element between −78 and −52 is required for optimal expression; (3) the TATA box at −25 appears to be important for precise initiation; (4) the A-box similarity in exon 1 appears to be essential; and (5) the intron contains a sequence, most likely the B-box similarity, that is required for transcription. These results indicate that U6 RNA transcription in *S. pombe* is significantly different from the situation in vertebrates, where transcription is controlled by elements that lie solely in the 5′-flanking sequence.

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PLANT GENETICS

V. Sundaresan	S. Allen	A. Duseff	A. Jahrsdoerfer
R. Martienssen	P. Athma	E. Grotewold	Z.-Y. Zhao
T. Peterson	J. Colasanti	C.-D. Han	

Identification of Transposition Factors of the Robertson's Mutator System

Z.-Y Zhao, V Sundaresan

The family of transposable elements known as the *Mu* transposable elements constitute the Robertson's Mutator system, an unusually active transposon system in maize that generates new mutations at frequencies of 50-fold over background. The mechanism of transposition of these elements is believed to be replicative, but the elements or genes encoding the transcription factors or transposase are presently unknown. We have identified specific binding sites for maize nuclear proteins on the termini of the *Mu1* element. One of the binding proteins is positively correlated with *Mu* activity, and the other is at the same level in both active and inactive lines. In the latter case, the binding site is a hexamer sequence motif (CGGT/GAA) that is also repeated in the essential subterminal region of elements of the *Ac/Ds* family. These binding proteins are candidates for transposition factors required for *Mu* transposition: The first factor is *Mu*-specific and the other factor is a more general host factor that may be involved in the transposition of other elements as well. Recently, evidence that the same factor also binds to the ends of the *Ac* element has been obtained in Dr. Starlinger's laboratory at Koln (P. Starlinger, pers. comm.). Nothing is known about host factors that are required for transposition of plant transposons, and our identification of such factors in nuclear protein extracts should lead to their eventual cloning and characterization.

Splicing of the *Mu1* Transposable Element Generates a Functional *Bronze* Gene with a Novel Intron

V. Sundaresan, A. Doseff

We have previously isolated several "changes in state" of a *Mu1* insertion at the *Bronze* (*Bz*) locus (CSHL Annual Report 1989). Changes in state (originally observed by Dr. B. McClintock) are heritable changes in patterns of ex-pression of a transposable element at a locus. The Mu1 insertion at Bronze that we have used in our studies is called *bzMum9* and was originally isolated by Robertson. This insertion results in kernels that are colored bronze, with purple spots. The background bronze color is due to inactivation of the *Bz* gene by the *Mu1* element, and the purple spots are due to excisions of the *Mu1* element that restore *Bz* function. One of the changes in state that we have isolated leads to normal phenotypic expression of the *Bz* gene, i.e., kernels that are completely purple in color. We have found that this change in state is associated with the deletion of half of the *Mu1* element. Analysis of the RNA from these kernels shows that the remainder of the *Mu1* element is spliced out from the *Bz* transcript using donor and acceptor sites that are present within *Mu1*. Therefore, this change in state results in a functional *Bz* gene with a novel intron. Interestingly, the *Mu1* element itself is not normally transcribed at detectable levels, so that the intron is probably not utilized unless inserted into a gene. Thus, the *Mu1* transposable element acts as a portable intron that participates in the evolution of the transcription units of the maize genome, in this case by generating a new *Bz* gene with two introns.

Somatic Excision Activity of the *Mu1* Element

A Doseff, V Sundaresan

Mu elements differ from other transposons in that there is no apparent correlation between excision and forward transposition, i.e., the rate of new integrations is very high, whereas germinal excision events are very rare. It is presumed that unlike other plant transposons, *Mu* elements transpose replicatively. Although the rates of germinal excision of the *Mu* elements is very low, the rates of somatic excision are

quite high. It is therefore of interest to see if the empty sites left by excision of *Mu* somatically resemble that of the animal transposable elements such as the P elements or Tc1, whose genetic behavior is closer to that of *Mu*. We have studied the somatic excision of a *Mu1* element at *Bz* by analyzing DNA from somatic tissues and sectors and find that it leaves the same types of footprints that are characteristic of other plant transposons and not those of the animal transposons. This result suggests that a common mechanism is involved in the excision of all plant transposons. Interestingly, we also found that the *Mu1* element also promotes intrachromosomal recombination, i.e., crossovers and conversions of short stretches of DNA near the excised site. Such footprints have not been previously observed with plant transposons, but we think that they might be quite general. Interchromosomal gene conversion of extensive stretches of DNA promoted by transposable element excision has been observed with the P elements of *Drosophila* (Engels et al., *Cell 62:* 515 [1990]), but intrachromosomal conversion events were not examined in their study. It is possible that the latter types of conversion events promoted by *Mu* transposable elements might not be limited to the *Mu* system, but might be of general importance to the evolution of complex genomes.

Control of Cell Division in Higher Plants

J. Colasanti, V. Sundaresan

This research is aimed toward elucidation of the mechanisms of cell division in plants. The control of cell division in animals and yeasts has been shown to be regulated by a complex of proteins, with the central role played by a protein kinase that is a homolog of the *cdc2* gene of *Schizosaccharomyces pombe* (and the *cdc28* gene of *Saccharomyces cerevisiae*). We have recently cloned and characterized maize homologs of the *cdc2* kinase genes. We find, by the isolation of two different but closely related cDNA clones, *cdc2ZmA* and *cdc2ZmB*, that there are at least two *cdc2* genes in maize. Southern blot hybridization using maize DNA suggests the possibility that there may be up to four maize *cdc2* genes. The expression of these genes in different tissues is correlated with the state of proliferation of the tissue, as well as the amount of *cdc2*-specific histone H1 kinase activity measured using $p13^{suc1}$ beads. One of the maize *cdc2* genes, when introduced into the yeast *S. pombe*, caused severe inhibition in growth of the yeast cells, suggesting that the maize *cdc2* gene interacts improperly with some of the other cell-division proteins in *S. pombe* (e.g., cyclins) and that there might be important differences in the plant *cdc2* protein that are responsible for this result (see below). At the amino acid level, the maize *cdc2* protein is 65% identical to both yeast and animal proteins. We have been able to demonstrate functionality of the maize protein in *S. cerevisiae* by its ability to complement a *cdc28* mutation in this yeast. We have also expressed the maize gene in vitro and have shown that we can synthesize a 34-kD protein that interacts specifically with the yeast *suc1* protein. More recently, we have generated antibodies specific to the maize *cdc2* protein, and we will use these to study the intracellular localization of the kinase during cell division in mitotic and meiotic cells.

Effects of the Maize *cdc2* Protein in Yeast Cells

J. Colasanti, V. Sundaresan

Definitive proof that a maize *cdc2* cDNA clone encodes a functional $p34^{cdc2}$ kinase lies in its ability to complement either the *cdc2* mutation of *S. pombe* or the *cdc28* mutation of *S. cerevisiae*. Expression of the maize *cdc2ZmA* clone under the control of the constitutive ADH promoter of *S. pombe* yielded microcolonies that could not be propagated. Viable transformants were obtained when the *cdc2* gene was cloned under the control of the inducible *thiA* promoter of *S. pombe* under conditions where expression was repressed. Expression of the maize *cdc2* protein, however, resulted in cell death after approximately six cell doublings. Lethality of maize *cdc2* expression could be partially alleviated in *S. pombe* cells in which the endogenous *cdc2* protein was overexpressed. This suggests that the maize $p34^{cdc2}$ kinase may interfere specifically with the yeast cell cycle machinery, possibly by interacting and sequestering other components required for cell division.

Expression of maize *cdc2* protein was not lethal in *S. cerevisiae*. In three *cdc28* alleles tested, only

one, 28-1N, was complemented by expression of the maize *cdc2* protein. This allele is defective at the G_2/M transition of the cell cycle, whereas the others are defective at the G_1/S transition referred to as "START." These preliminary results suggest that the maize protein encoded by *cdc2ZmA* can interact successfully with the cell cycle components required for the initiation of mitosis, but not for traversion of the "START" point of the cell cycle. One possibility is that the latter function may be provided by a separate maize *cdc2* gene. Experiments are under way to characterize this phenomenon further.

Transposon Tagging of Gibberellin Biosynthesis Genes in Maize

J. Colasanti, V. Sundaresan

The gibberellins are plant growth substances that mediate various aspects of plant growth and development. Mutants of maize that are defective at different points in the gibberellin biosynthesis pathway are characterized by short stature (dwarfs) and aberrant floral development. We have taken advantage of the well-characterized transposable element system of maize to tag GA biosynthesis genes to facilitate their eventual cloning and characterization.

We reported last year the isolation of a new *Mu*-induced suppressible dwarf allele. In this case, a *Mu* transposable element appears to have inserted itself into a gene that catalyzes synthesis of gibberellic acid (GA), resulting in a dwarf phenotype. This allele is unique, however, in that the appearance of the dwarf phenotype is correlated with the activity of the *Mu* element: i.e., when *Mu* is "active," the plants are dwarf and when *Mu* is inactive, the plants appear to be wild type. Activity of *Mu* is correlated with the state of methylation as determined by *Hin*fI digestion. This type of suppressible *Mu*-induced phenotype was first reported for the *hcf106* gene by Martienssen et al. (*EMBO J. 8:* 1633 [1989]). Plants that are homozygous for the *Mu*-induced lesion all appear as dwarfs initially; however, as they grow, some attain larger stature and eventually appear normal, whereas others remain as dwarfs or are intermediate in height. We interpret this observation to be the result of somatic inactivation of the *Mu* element during plant development. This would result in sectors of plant tissue in which the cells are able to produce the wild-type enzyme and therefore synthesize the diffusible GA that would mediate normal growth. Plants that have large somatic sectors, due to *Mu* inactivation early in development, would produce more GA and appear nearly wild type in height and flower development, whereas plants with very small sectors of GA-producing tissues will remain as dwarfs. We are further characterizing this allele with the aim of eventually isolating the gene tagged by *Mu*.

The anther ear (*an1*) allele is GA-defective mutation in maize that is characterized by aberrant floral development, i.e., male anthers appear on the female flower (the ear) of the plant. In addition, the plants are semi-dwarf and grow to approximately 50% of normal height. The lesion has been characterized biochemically as a defect early in the GA biosynthesis pathway. The *An1* gene is closely linked to the *Bz2* gene; the latter is required for anthocyanin pigment production in the aleurone of the kernel and the plant. An allele of *Bz2*, *bz2-m2*, contains a *Ds* element, a defective form of the Activator (*Ac*) element first characterized by Barbara McClintock. In the presence of *Ac*, the *Ds* element is able to transpose from its location in the *Bz2* gene and move elsewhere in the genome. This transposition results in a mutable phenotype characterized by purple spots on the kernels and occasional fully purple revertant kernels. The *Ac*/*Ds* elements have a predisposition to move to linked sites in the genome, therefore increasing the chances of the *Ds* element at *Bz2* inserting into the *An1* gene. We have taken advantage of this proximity to isolate a new *an1* mutation that resulted from the insertion of the *Ds* element into the *An1* gene, thus facilitating the eventual isolation of this gene by transposon tagging.

Molecular Analysis of Nuclear Mutations Affecting Chloroplast Development

R. Martienssen, C.-D. Han, A. Jahrsdoerfer

Chloroplast biogenesis is a complex process in higher plants, requiring the coordination of plastid and cellular differentiation during leaf development. Although structural genes have been characterized for many of the more abundant chloroplast proteins (both nucleus- and plastid-encoded), genes that regulate chloroplast development have not been widely

studied at the molecular level. We are taking a genetic approach to understanding the role of nuclear genes in chloroplast and leaf development by studying the maize mutants *hcf106* and *iojap*.

HCF106: A MUTATION AFFECTING CHLOROPLAST MEMBRANE ORGANIZATION

The thylakoid membrane system is one of the best-studied morphological features of the mature chloroplast. This internal membrane system has a highly organized structure comprising stacked and unstacked lamellae, with each accommodating a characteristic set of membrane proteins. However, the mechanisms by which these membranes are organized and the protein complexes assembled are poorly understood, particularly in higher plants. As we described in last year's Annual Report, the nonphotosynthetic, seedling lethal mutant of maize, *hcf106*, has a recessive nuclear mutation that prevents the normal assembly of photosynthetic electron transport complexes and causes aberrant thylakoid membrane organization. Consequent reductions in chloroplast pigment levels result in pale green leaves that fluoresce under UV illumination in the dark (h*igh* c*hlorophyll* f*luorescence*). This mutation arose in a Robertson's *Mutator* line, and a molecular clone of the locus was obtained using the transposon *Mu1* as a tag. A 1.2-kb transcript that hybridized to the cloned locus was identified on Northern blots, and genetic analysis showed that it encoded the *hcf106* gene product (Martienssen et al., *EMBO J. 8:* 1633 [1989]).

We have now cloned and sequenced full-length cDNA clones of this transcript, and a 243-amino-acid open reading frame corresponding to the *hcf106* gene product has been identified. This encodes a protein without global homology with any protein sequence from the Genbank database, but the sequence does contain a putative ATP-binding motif, a potential chloroplast transit peptide, and a membrane spanning domain. We have expressed *TrpE::hcf106* fusion proteins in *Escherichia coli*, and antibodies have been raised. In preliminary experiments, these antibodies have been used on Western blots of leaf proteins from membrane and soluble fractions. A membrane protein of approximately 30 kD is detected with our antibody in wild-type leaves, but not in mutant leaves. We are currently trying to obtain higher-affinity antibodies for immunocytochemistry.

The 1.2-kb *hcf106* transcript accumulates in the light to levels five- to tenfold higher than in the dark, reaching a peak at around 6 hours after light exposure. However, it is also found at low levels in the root and cob, so that the *hcf106* gene product may have a general role in the biogenesis of both amyloplasts and chloroplasts. The RACE protocol for the rapid amplification of cDNA ends was used to isolate full-length cDNA clones, but additional clones made by conventional methods were also obtained from A. Barkan (University of California, Berkeley). These clones include a second, distantly related sequence that appears to be the product of a low-stringency cognate gene. We have found that the *hcf106* mutation is nonlethal in some inbred backgrounds of maize, and sequences specific to the cognate gene appear to cosegregate with the nonlethality factor. It is thus possible that some alleles of the cognate gene can provide a level of duplicate function in *hcf106* mutants. Recombinant inbred lines have been used to map the *hcf106* gene to chromosome 2, close to the endosperm marker *floury*. This marker will be used to follow the mutant *hcf106* allele for subsequent genetic analysis.

IOJAP: A VARIEGATED MUTANT OF MAIZE

Maize plants homozygous for the recessive mutation *iojap* have variegated leaves and transmit defective plastids through their female gametes. Since the work of Jenkins (*J. Hered. 25:* 467 [1924]) and of Rhoades (*Proc. Natl. Acad. Sci. 29:* 327 [1943]), the *iojap* gene has been thought to have a fundamental role in the interaction of nuclear and plastid compartments during the development of the maize leaf. In particular, the pattern of leaf variegation in mutant plants suggests that defective plastids may have failed to respond to positional signals that coordinate cellular and plastid development in the leaf. Embryos with maternally inherited defective plastids also arise at specific positions on the ear, rather than in clonal groups.

A transposon-tagged allele of *iojap* was obtained by C.-D. Han and E.H. Coe at the University of Missouri using the transposable element Robertson's *Mutator*, and a *Mu1* element was found to cosegregate with the new *ij-mum1* allele (Han and Coe, *Maize Newsletter 64:* 46 [1990]). In collaboration with Coe, we have now obtained a molecular clone of this *Mu1* element. DNA flanking the *Mu1* element hybridizes to a 1.3-kb transcript found in wild-type seedlings, but reduced in abundance and altered in mobility in seedlings homozygous for the original

iojap allele. Two rare germinal revertants of this allele, isolated by Coe, have suffered genomic rearrangements that can be detected by Southern hybridization using this probe. We conclude that this clone is likely to be a part of the *iojap* locus. In the coming year, we hope to characterize the molecular role of the *iojap* gene product in coordinating chloroplast biogenesis with leaf development.

Suppression of *Mu*-induced Mutations

R. Martienssen, A. Jahrsdoerfer

As we reported last year, the *hcf106* mutation is phenotypically suppressed when *Mu* transposons in the genome lose activity; i.e., *hcf106* homozygotes adopt a wild-type phenotype despite the continued presence of the *Mu1* transposon at the locus. Loss of *Mu* activity and phenotypic suppression of *hcf106* are both reversible and are associated with DNA methylation of the *Mu* transposon family. Phenotypic suppression of *hcf106*, then, provides a marker for DNA modification of *Mu* transposable elements. Suppression of the mutant phenotype is accompanied by accumulation of mRNA transcripts from the *hcf106* locus, despite the insertion of a *Mu1* element into the untranslated leader of the *hcf106* gene, close to the start of transcription. In contrast, mutant seedlings have no detectable transcript (Martienssen et al. 1989). In collaboration with A. Barkan (University of California at Berkeley), we showed that suppression of the *hcf106* phenotype is mediated by an outward reading promoter near the end of *Mu1* that substitutes for the normal *hcf106* promoter when *Mu* is inactive (A. Barkan and R. Martienssen, *Proc. Natl. Acad. Sci.* [1991] in press). In the last year, the phenotypes caused by several other *Mu1*-induced mutations have been shown to respond to *Mu* activity in a similar fashion. Those that have been cloned have a *Mu1* element close to the start of transcription, as is the case with *hcf106* (P. Chomet, S. Hake, D. McCarty, pers. comm.).

In some families, *hcf106* homozygotes were observed that had large, phenotypically wild-type sectors superimposed on pale green, mutant leaves. DNA isolated from these sectors contained modified *Mu1* elements, including the one at the *hcf106* locus (Martienssen et al., *Genes Dev. 4:* 331 [1990]). The pattern of stripes suggested that clonal switching events associated with DNA modification had occurred in cells near the tip of the apical meristem, so that more, and larger, sectors were observed in successive leaves until, in the upper leaves, the entire plant was phenotypically wild type. This resulted in a gradient of transposon inactivity (and methylation) up the plant. We have now shown that a second suppressible mutation, a dominant *Lesion-mimic*, is coordinately regulated in these sectors; i.e., plants that are doubly mutant have sectors that are suppressed for both phenotypes together. This suggests that a factor influencing suppression must operate in *trans* to suppress both unlinked mutations. In crosses between sectored and unsectored plants, the "sectored" phenotype appears to segregate among the progeny, so that a single dominant factor may be responsible for this somatic variegation. In collaboration with V. Sundaresan (Cold Spring Harbor Laboratory), we are pursuing the hypothesis that this factor might encode a protein that binds *Mu* termini (Z.-Y. Zhao and V. Sundaresan, CSHL Annual Report 1990). We are also in the process of constructing double and triple mutants for seedling, plant, and kernel markers whose phenotypes depend on *Mu* activity. This should help to reveal the developmental and genetic basis of the sectored patterns observed.

Molecular and Genetic Analysis of the Maize *P* Locus

S. Allan, P. Athma, E. Grotewold, T. Peterson

Our research concerns the question of how gene expression is controlled during development in higher eukaryotes. Toward this end, we are continuing our analysis of the maize *P* gene, which is required for flavonoid pigmentation of specific floral parts in maize. The *P* gene is an excellent model system for this study, in part because of its potential for incisive genetic analysis. We have made significant progress in the past year in defining the structure and function of *P*.

ELUCIDATING THE COMPLEX TRANSCRIPTIONAL PATTERN OF *P*

Since one major aim of our work is to understand how *P* expression is controlled, we needed exact in-

formation on the gene structure, especially the location of the 5′ start sites. Previous attempts to isolate full-length cDNA clones from conventional cDNA libraries were unsuccessful, probably due to a region of strong secondary structure in the *P* message that inhibited the progression of reverse transcriptase. Therefore, we analyzed the *P* gene transcripts by primer extension, RNase protection, and S1 mapping experiments, as well as conventional and polymerase chain reaction (PCR)-derived cDNA cloning. The results showed that *P* produces at least two alternatively spliced messages and that all of the messages are initiated at the same point (Fig. 1) (Grotewold et al., *Proc. Natl. Acad. Sci.* [1991] in press).

REGULATORY FUNCTION OF THE *P* GENE

The sequence of the *P* transcripts showed an amino-terminal region that has significant homology (40%) with the DNA-binding domain of the vertebrate Myb oncoprotein. Myb is a DNA-binding protein that can activate transcription from promoters containing the Myb-binding site. In mammalian cells, Myb is a regulator of cell growth and differentiation, primarily in hematopoietic cells. Our results indicate that studies of *P* function could provide an important complement to research on Myb in mammalian systems. One of the *P* transcripts encodes a protein with a negatively charged domain that has the structural features of a transcriptional activating domain. This finding supports our previous hypothesis that *P* is a transcriptional regulator. A functional test of this hypothesis came from Northern blot analysis of RNA from structural genes in the pigment biosynthetic pathway. RNA transcripts from the maize genes *C2* and *A1*, which encode chalcone synthase and flavonol/flavonoid reductase, respectively, are present in tissues that have a functional *P* gene but absent from plants in which *P* is deleted. Additionally, we have isolated cDNA clones encoding maize chalcone isomerase, which catalyzes another step in pigment biosynthesis. Like *C2* and *A1*, the chalcone isomerase message is also dependent on a functional *P* allele. Thus, the hypothesis that *P* is a transcriptional regulator is supported not only by sequence homology, but also by direct measurement of transcript accumulation from three target genes.

ANALYSIS OF THE CHANGE IN STATE ALLELE *P-OVOV*

The *P-ovov* allele, which produces orange variegated pericarp and cob glumes, resulted from a short-range transposition of *Ac* to a new site in the *P* locus. It has been suspected for some time that new alleles of genes carrying transposon insertions could arise from intragenic transpositions, but this is the first published example confirmed at the molecular level. This paper was published recently in *Genetics* (Peterson 1990). Since the *Ac* element preferentially transposes to nearby sites, the *P-ovov* allele has been an excellent source of new mutant alleles carrying *Ac* at diverse sites in the *P* locus.

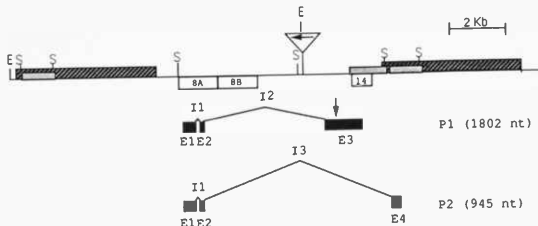

FIGURE 1 Structure of *P* gene transcripts. (*Top*) Map of the *P* locus, with restrictions sites for *Eco*RI (E) and *Sal*I (S) indicated. The cross-hatched boxes indicate a 5.2-kb direct repeat, and the stippled boxes indicate a 1.2-kb direct repeat sequence. The triangle containing the arrow marks the site of insertion and transcriptional orientation of the transposable element *Ac* in the *P-ovov-1114* allele; in *P-vv*, the *Ac* element is inserted 161 bp 3′ of the site in *P-ovov-1114*, in the opposite orientation.

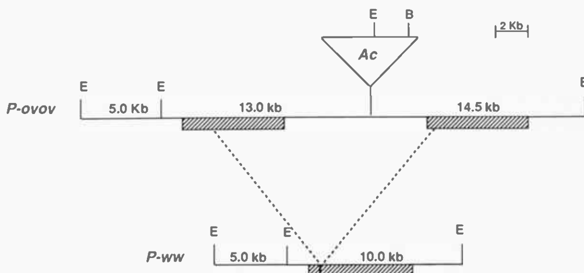

FIGURE 2 Structures of the *P-ovov-1114* and *P-ww-d* alleles. The map at the top shows the *P-ovov-1114* genomic region with *Eco*RI sites. The two hatched boxes indicate the two 5.2-kb direct repeats on either side of *Ac*; the smaller repeats are not shown. The map at the bottom shows the structure of a typical *P-ww-d* allele with a 17-kb deletion, including 4.5 kb of *Ac* and 12.5 kb of the *P* locus.

DIRECT REPEAT RECOMBINATION INDUCED BY THE TRANSPOSON *AC*

The *P* gene has the unusual feature that the transcribed region is flanked by two long repeats of 5.2 kb (Fig. 1); the function of these repeats, if any, is unknown. During the course of our genetic experiments, we found that *P* alleles that carry the transposable element *Ac* inserted between the repeat sequences produce a high frequency of null alleles. We analyzed the structure of 51 null alleles and found that all but 4 carried a large common deletion that removed the *Ac* element, the *P* transcribed region, and one copy of the flanking direct repeat sequences (Fig. 2). Since *P* alleles that do not have *Ac* inserted are very stable, we conclude that the *Ac* insertion induces recombination between the repeats. This represents a previously unknown activity of *Ac* (Athma and Peterson, *Genetics* [1991] in press).

We thank Michael Persans for assistance in various aspects of this work.

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G_1 AND G_2 CELL CYCLE CONTROL IN *SACCHAROMYCES CEREVISIAE*

B. Futcher B. Elliott M. Linskens G. Tokiwa
I. Fitch N. Nash M. Tyers

Our main interest continues to be the regulation of START in *Saccharomyces cerevisiae*. In particular, we are interested in the mechanism that tethers division to growth. To this end, we have studied two mutations that provide a Wee phenotype and alter the normal relationship between division and growth. One of these mutations, *WHI1-1*, was in a cyclin-like gene now known as *CLN3*. The encoded protein was the first member of a growing set of cyclin-like proteins that regulate the G_1/S transition. In the past year, we have found that Cln3 has a very short half-life, is somehow affected by a ubiquitin-conjugating enzyme, and is associated with a protein kinase activity. The other mutation, *whi3*, appears to be in a gene encoding an RNA-binding protein of unknown function.

Our interest in *CLN3* has led us to examine in a more general way the role of various cyclins. In the last year, we have found four B-type mitotic cyclins in *S. cerevisiae* and possibly an A-type cyclin. When added to the three previously known G_1 cyclins (Cln1, 2, and 3), this gives *S. cerevisiae* by far the largest repertoire of known cyclins of any organism. These different cyclins may confer different properties on their respective kinase complexes; work in this area is continuing. There is preliminary evidence that other organisms have a similarly wide array of different cyclins doing different jobs.

Biochemistry of Cln3

M. Tyers, G Tokiwa, B. Futcher

The Cln1, Cln2, and Cln3 proteins of *S. cerevisiae* help regulate START, the G_1/S transition. Overexpression or hyperactivation of these proteins advances START, shortening G_1 and decreasing cell size, whereas deletion of one or two of the genes delays START. Deletion of all three genes causes a lethal G_1 arrest (Richardson et al., *Cell 59*: 1127 [1989]). These three proteins are related to each other and distantly related to the mitotic cyclins. We believe that Cln1, 2, and 3 are "G_1 cyclins" and regulate START by a mechanism similar to that by which the mitotic cyclins regulate mitosis.

CLN3 mRNA has a very short half-life of just a few minutes. The protein has a similar very short half-life (Fig. 1). Whereas mitotic cyclins are completely destroyed at one point in the cell cycle, but are otherwise stable, the Cln3 protein appears to be continuously turned over. The original *WHI1-1* (also known as *CLN3-1*) mutation identifying the gene is dominant. The molecular lesion is a stop codon that removes the last third of the protein; this third contains PEST regions, which may be signals for proteolysis. The truncated, Cln3-1 protein is relatively abundant, and it turns over very slowly (Fig. 1), suggesting that the indestructibility of this protein causes the mutant phenotype.

If *CLN3-1* is transcribed from the *GAL1* promoter, and then transcription (and, in fact, all Cln3-1 synthesis) is stopped by the addition of glucose, the cell's store of Cln3-1 protein is sufficient to drive it through several cell cycles. In this situation, the Cln3-1 protein is clearly not oscillating in abundance through the cycle (it is still not clear whether the abundance of the wild-type protein oscillates). Surprisingly, a cell that is deleted for *CLN1* and *CLN2*, and lives only because it contains the *CLN3-1* gene, is reasonably healthy (although Wee and otherwise abnormal). Thus, oscillation of a G_1 cyclin is not essential for viability.

As expected for a cyclin-like molecule, Cln3 is associated with a protein kinase activity (Fig. 2A). The protein kinase activity will phosphorylate histone H1, Cln3, and an unidentified protein of 45 kD that is coprecipitated by anti-Cln3 antibodies. The Cdc28 polypeptide is present in anti-Cln3 immunoprecipitates, and thus we believe that Cdc28 is responsible for at least some of the kinase activity. However, only an extremely tiny fraction of the Cdc28 protein in the cell is Cln3-associated. *CLN3-1* mutant cells have 50–100 times as much Cln3-1-associated kinase activity as do *CLN3* cells.

If the Cln3-associated kinase complex is treated with a phosphatase, and then the phosphatase is

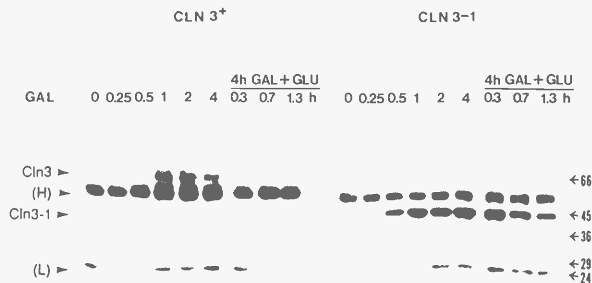

FIGURE 1 Induction and decay of Cln3. Cells containing a Lerner-epitope-tagged version of Cln3 or Cln3-1 expressed from the *GAL1* promoter were grown in YEP + raffinose and then induced with galactose. The number of hours of galactose induction is shown above each lane. To examine turnover, Cln3 or Cln3-1 expression was induced for 4 hr, glucose was then added to 2% to stop transcription, and samples were harvested after 0.3, 0.7, or 1.3 hr. Lysates from 2 x 10^9 cells were immunoprecipitated with 0.5 μl of 12CA5 ascites fluid, electrophoresed, and transferred to nitrocellulose. The filter was probed with a 1:2000 dilution of 12CA5 ascites fluid followed by ^{125}I-labeled goat anti-mouse IgG, and then autoradiographed. Cln3 and Cln3-1 are indicated, as well as the IgG heavy (H) and light (L) chains. Molecular mass (kD) is shown to the right.

washed away, the kinase activity is destroyed. This is also true of the Cln3-1-associated kinase. In contrast, when the same experiment is done with the mitotic (Clb-associated) form of the Cdc28 kinase, the kinase activity is increased. We believe that some protein in the Cln3 kinase complex must be phosphorylated for the kinase to be active. We cannot tell whether the relevant phosphate(s) is on Cln3, on Cdc28, or elsewhere. Cln3 and Cln3-1 appear to have only one major phosphopeptide in common (containing phosphothreonine); this peptide is a good candidate for the region where the regulatory phosphorylation occurs.

Cln3 metabolism is dramatically affected by the *cdc34* mutation. *cdc34* mutants are temperature-sensitive lethal *cdc* mutants. At 36°C, they arrest with buds (i.e., after START), but before initiation of DNA synthesis. Cdc34 is a ubiquitin-conjugating enzyme, and so is presumably involved in protein degradation. In a *cdc34* mutant at the *permissive* temperature, most of the Cln protein is in a highly phosphorylated form, whereas only a few percent of the Cln3 protein in a *CDC34* strain is in this form. However, the total amount of Cln3 protein does not change very much (perhaps twofold). Strikingly, the Cln3-associated kinase activity goes up 50–100-fold in the *cdc34* background at the permissive temperature (Fig. 2B). Therefore, we speculate that the Cdc34 protein is somehow involved in degradation of the phosphorylated form of Cln3, and it is this phosphorylated form that is the active form. Consistent with this hypothesis, the *cdc34* mutation does not affect the kinase activity associated with the indestructible Cln3-1 mutant protein (Fig. 2B). The results of phosphatase experiments discussed above are also consistent with this hypothesis.

CLN3-1 mutants are resistant to α-factor arrest. We imagined that α-factor caused arrest by inducing degradation of the wild-type protein but that the truncated, mutant protein would be resistant to degradation. Surprisingly, we find that α-factor-*arrested* wild-type cells still contain normal levels of the Cln3 protein. Even more surprisingly, these arrested cells still contain normal levels of Cln3-associated histone H1 kinase activity. There is strong genetic evidence that α-factor does somehow inactivate Cln3, but this inactivation does not seem to be caused by degradation or by destroying the kinase activity. Perhaps the kinase activity in α-factor-arrested cells has been qualitatively changed.

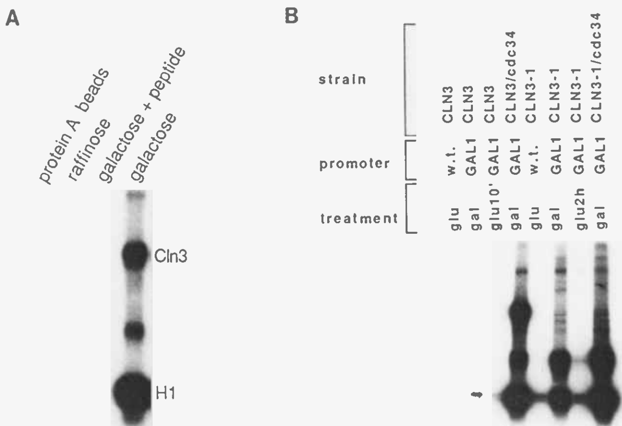

FIGURE 2 Cln3 has an associated kinase activity. (*A*) Cells containing a Lerner-epitope-tagged version of Cln3 expressed from the *GAL1* promoter were grown in YEP + raffinose and then either induced (1st, 3rd, and 4th lanes) or not induced (2nd lane, "raffinose") with galactose. Material was precipitated from cell lysates with protein-A beads alone (lane *1*), or 12CA5 ascites fluid (lane *2*), or 12CA5 ascites fluid plus an excess of a competing epitope peptide (lane *3*), or 12CA5 ascites fluid (lane *4*). Immunoprecipitates were incubated with histone H1 and [γ-^{32}P]ATP in kinase buffer, and the reaction products were electrophoresed and autoradiographed. (*B*) *cdc34* mutation increases Cln3-associated kinase activity. In a *cdc34* mutant strain at the permissive temperature, the Cln3-associated kinase is about 50-fold more active than in a *CDC34* wild-type strain (compare lanes *2* and *4*). In contrast, the Cln3-1-associated kinase is about equally high in both *cdc34* and *CDC34* strains (compare lanes *6* and *8*).

G_1 and Other Cyclins of *Schizosaccharomyces pombe* and Humans

T. Connolly, I. Fitch, M. Caligiuri, K. Galactionov, B. Futcher, D. Beach, Y. Xiong

Cyclin-like molecules functioning in G_1 phase have so far been identified only in *S. cerevisiae*. However, they may occur in other organisms as well. To find such molecules, we have constructed a strain deleted for *cln1* and *cln2* and that has *CLN3* transcribed from the *GAL1* promoter. This strain is viable in the presence of galactose, but dead in the absence of galactose or in the presence of glucose. L. Guarente and co-workers and M. Wigler, J. Colicelli, and co-workers have supplied us with *Schizosaccharomyces pombe* and human cDNA expression libraries in *S. cerevisiae* vectors. We have transformed the *cln1 cln2 GAL1-CLN3* strain with these libraries and have selected for growth in the presence of glucose. Several dozen transformants have been recovered. In each case, the transforming vector contained a cyclin homolog. From *S. pombe*, we obtained the *cdc13*$^+$-encoded B-type mitotic cyclin and two previously unidentified *cdc13*$^+$ homologs. In addition, we have recovered a good homolog of *S. cerevisiae CLN3*, and we hope that this may be an *S. pombe* G_1 cyclin. From human cells, we have recovered two B-type mitotic cyclins, B1 and B2, and also an interesting cyclin-like molecule that does not seem to be a member of any of the previously defined classes of cyclins. As yet, there is no evidence that these new *CLN* homologs function in G_1 phase in *S. pombe* or human cells.

Identification of Four Mitotic Cyclins of *S. cerevisiae*

I. Fitch, B. Futcher [in collaboration with U. Surana, H Robitsch, C. Price, T Schuster, and Kim Nasmyth, IMP, Vienna]

If Cln1, Cln2, and Cln3 really represent a new class of "G_1 cyclins" (as opposed to being "misplaced" mitotic cyclins), then *S. cerevisiae* should possess one or more mitotic cyclins as well as its G_1 cyclins. We have therefore searched for mitotic cyclins by the polymerase chain reaction (PCR). Three such cyclin homologs were identified, which we named *CLB1*, *CLB3*, and *CLB4* (for CycLin B-type). While the PCR experiments were under way, our collaborators in Vienna identified seven different high-copy-number suppressors of an exceptional allele of *cdc28* called *cdc28-1N*. *cdc28-1N* is defective only in a G_2/M function, whereas most alleles of *cdc28* are defective in both a G_1 function and a G_2/M function. Since it seemed possible that high-copy-number mitotic cyclins might suppress *cdc28-1N*, the *CLB1*, *CLB3*, and *CLB4* PCR fragments were hybridized to each of the seven groups of high-copy-number suppressors. *CLB1* and *CLB4* were found among the suppressors. In addition, *CLB1* hybridized weakly to a third suppressor. Sequencing showed that this third suppressor was also a B-cyclin homolog, which we now call *CLB2* (Fig. 3).

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CDC13 MTTRRLTRQHLLANTLGNNDENHPSNHIARAKSSLHSSENSLVNGKKATVSSTNVPKKRHALDDV
CLB1  MSRSLLV-ENSRTINSNEEKGVNESQYILQKRNVPRTILGNVTNNANILQEISMNRKIGMKNFSK
CLB2  MSNPIENTENSQNTSSSRFLRNVQRLALNNVTNTTFQKSNANNPALTNFKSTLNSVKKEGSRIPQ

CDC13 SNFHN---------------KE-GVPLASKNTNVRHTTASVSTRR--------ALEEKSIIP-ATDD
CLB1  LNNF--------------------FPLKDDVSRADDFTSSFNDSRQ-GVKQEVLNNKENIPEYGYS
CLB2  FTRESVSRSTAAQEEKRTLKENGIQLP-KNNLLDDKENQDPSSQQFGALTSIKEGRAELP-ANIS

CDC13 EPASKKRRQPSVFN-------SSVPSLPQHLSTKSHSVSTHGVDAFHKDQATIPKKLKKDVDERV
CLB1  EQEKQQCSNDDSFHTN-----STALSCNRLIYSENKSISTQMEWQKKIMREDSKKKRPISTLVEQ
CLB2  LQESSSAKEIIQHDPLKGVGSSTEVVHNSVENEKLHPARSQLQVRNTESETDSGKKRPISTIVEQ

CDC13 VSKDIPKLHRDSVESP--ESQDWDDLDAEDWADPLMVSEYVVDIFEYLNELEIETMPSPTYMDRQ
CLB1  DDQKKFKLHELTTEEEVLEEYEWDDLDEEDCDDPLMVSEEVNDIFDYLHHLEIITLPNKANLYKH
CLB2  ELPKKFKVCDENGK----EEYEWEDLDAEDVNDPFMVSEYVNDIFEYLHQLEVITLPKKEDLYQH

CDC13 KELAWKMRGILTDWLIEVHSRFRLLPETLFLAVNIIDRFLSLRVCSLMKLQLVGIAALFIASKYE
CLB1  KNIKQN-RDILVNWIIKIHNKFGLLPETLYLAINIMDRFLCEEVVQLNRLQLVGTSCLFIASKYE
CLB2  RNIHQN-RDILVNWLVKIHNKFGLLPETLYLAINIMDRFLGKELVQLDKLQLVGTSCLFIASKYE
CLB3  PELRWSFRSTLIDWIVQVHEKFQLLPETLYLCINIIDRYLCKEVVPVNKFQLVGAASLFIAAKYE
CLB4  VELTWPFRRTMIDWLVQLHFRFRLLPETLYLTINIVDRFLSKKTVTLNRFQLVGVSALFIAAKFE

CDC13 EVMCPSVQNFVYMADGGYDEEEILQAERYILRVLEFNLAYPNPMNFLRRISKADFYDIQTRTVAK
CLB1  EIYSPSIKHFAYETDGACSVEDIKEGERFILEKLDFQISFANPMNFLRRISKADDYDIQSRTLAK
CLB2  EVYSPSIKHFASETDGACTEDEIKEGEKFILKTLKFNLNYPNPMNFLRRISKADDYDIQSRTLAK
CLB3  EINCPTIKDFVYMSENCYSRNDLLDAERTILNGLEFELGWPGPMSFLRRISKADDYEHDTRTLAK
CLB4  EINCPTLDDLVYMLENTYTRSDIIRAEQYMTDTLEFEIGWPGPMPFLRRISKADDYDFEPRTLAK

CDC13 YLVEIGLLDHKLLPYPPSQQCAAAMYLAREMLGRGPWNRNLVHYSG-YEEYQLISVVKKMINYLQ
CLB1  FLMEISIVDFKFIGILPSLCASAAMFLSRKMLGKGTWDGNLIHYSGGYTKAKLYPVCQLLMDYLV
CLB2  FLLEISLVDFRFIGILPSLCAAAAMFMSRKMLGKGKWDGNLIHYSGGYTKEELAPVCHMIMDYLV
CLB3  YLLESTIMDHRLVSAQPSWLAAGAYFLSKIILGQNQWSLAHVYYSN-YTQEQILPLATIIL
CLB4  YLLETTIVEPKLVAAAPSWLAAGAYFLSRTILGSNDWSLKHVFYSG-YTSSQIIPLASLIL

CDC13 KPVQHEAFFKKYASKKFNKASLFVRDWYKKNSIPLGDDADEDYTFHKQKRIQHDNKDEEW
CLB1  GSTIHDEFLKKYQSRRFLKASIISIEWALKVRKNGYDIMTLHE
CLB2  SPIVHDEFHRKYQSRRFMKASIISVQWALKVRKNGYDIMTLHE
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FIGURE 3 Alignment of the *S. pombe cdc13*$^+$-encoded cyclin with the complete amino acid sequence of *CLB1* and *CLB2*, and part of *CLB3* and *CLB4*. Residues shown in bold are identical in two of three cyclins, or three of five cyclins, as appropriate. Underlined residues distinguish the *CLB1/CLB2* pair from the *CLB3/CLB4* pair. Also underlined is the "destruction box," a region found in the amino-terminal region of most cyclins, and thought to be a signal for ubiquitin-mediated degradation.

CLB1 and *CLB2* are a very closely related pair of genes, and *CLB3* and *CLB4* form a second pair (Fig. 3). Disruptions of *CLB1* or *CLB2* alone have very little phenotype, but a double *clb1 clb2* disruption is lethal, and dies *in* mitosis (at a stage where spindles have formed, but have not elongated) at about the same stage as *cdc28-1N* mutants arrest. As yet, *CLB3* and *CLB4* have not been disrupted. *CLB1* and *CLB2* transcription is cell-cycle-regulated and peaks in G_2/M (Fig. 4). Several lines of evidence suggest that *CLB1* and *CLB2* perform a qualitatively different function than *CLB3* and *CLB4*.

The *whi3* Mutation

R Nash

Like *CLN3-1*, *whi3* causes cells to divide at abnormally small sizes. Unlike *CLN3-1*, *whi3* is recessive, suggesting that *WHI3* inhibits or delays commitment to division. The mutation was induced using a $G418^R$-marked Ty transposable element. The mutant gene and flanking DNA were then cloned using the $G418^R$ marker, and the wild-type gene was cloned shortly thereafter. Sequencing showed that *WHI3* has an open reading frame of 661 amino acids. The predicted protein includes a domain found in a family of RNA-binding proteins. Some preliminary evidence suggests that *whi3* may affect expression of *CLN3* posttranscriptionally. Sequencing also showed that the original mutation was indeed caused by insertion of a Ty element into the open reading frame.

Targeted disruption of the *WHI3* locus produced the same Wee phenotype as the original Ty-induced mutation. Integration of one copy of *WHI3* at the *ura3* locus in a *whi3* strain reverts the mutant phenotype. Several copies of *WHI3* appear to cause a very large cell size and make the strain very sick. Thus, as for *CLN3*, the effect of *WHI3* on the critical size for commitment may be dose-dependent. In summary, we think that Whi3 is an RNA-binding protein that acts at START to inhibit commitment to division. It could do this by, for instance, down-regulating translation of Cln1, Cln2, and/or Cln3.

The Stress and Quiescence Response of *S. cerevisiae*

B. Elliott

In *S. cerevisiae*, the *RAS*/cAMP pathway can regulate the physiological state of the cell in response to nutri-

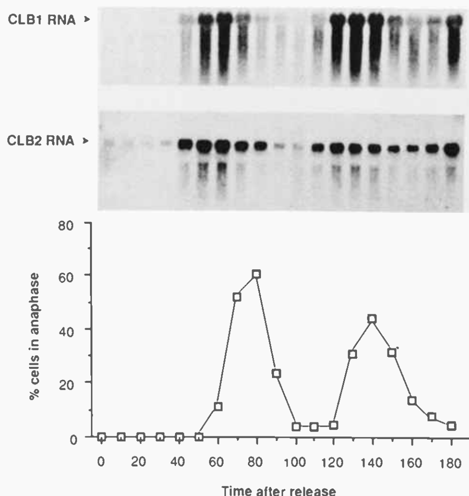

FIGURE 4 *CLB1* and *CLB2* are transcriptionally cell-cycle-regulated. Cells were arrested in G_1 with α-factor and then released, and samples were withdrawn at a 10-min interval. Northern blots were analyzed with *CLB1*- and *CLB2*-specific probes. The graph (*bottom*) shows the percentage of cells with fully formed mitotic spindles following release.

tional cues. However, when the *RAS*/cAMP signaling pathway is destroyed, cells still respond appropriately to nutritional cues (Cameron et al., *Cell 53*: 555 [1988]). Thus, there is at least one signaling pathway in addition to the *RAS*/cAMP pathway. To identify this pathway, we have used a strain lacking a *RAS*/cAMP pathway and looked for mutations that prevent appropriate nutritional responses. Several such mutations have been found. Different mutations affect different aspects of the normal nutritional response. These genes are in the process of being identified.

Effects of SV40 T-antigen Expression in *S. cerevisiae*

M. Linskens

Eukaryotic cells have a control ensuring that DNA is replicated once and once only. The mechanism of this control is wholly unknown. However, the mechanism can be overcome: In CV-1 monkey cells, SV40 T antigen can cause multiple rounds of initiation of DNA synthesis from an SV40 origin. We are interested in the mechanism of once and once-only control, and since yeast is a powerful model system, we asked whether T antigen could cause SV40 origin overreplication in yeast.

Yeast were transformed with a 2-micron circle-based plasmid carrying SV40 T antigen under the control of the *GAL1* promoter, and an *ARS* centromere plasmid carrying the SV40 origin. When the T antigen was turned on, there was at best a very slight increase in the copy number of the origin plasmid, so it appears that overreplication did not occur. However, expression of T antigen caused some unexpected but striking effects. A small proportion (2–5%) of the cells in an expressing population permanently arrested with extremely long buds.

Different *cdc* mutant strains were transformed with the T-antigen expression plasmid. *cdc34* mutant cells were very strongly affected even at the permissive temperature. The T-antigen-induced phenotype was seen in about 25% of the cells. Expression of the protein was assayed using an anti-T-antigen antibody provided by B. Stillman (Tumor Viruses Section). Wild-type cells expressed only very small amounts of T antigen, perhaps accounting for the low penetrance. This T antigen appears to have a short half-life. *cdc34* cells at the permissive temperature contained much larger amounts of T antigen. As noted above, *CDC34* encodes a ubiquitin-conjugating enzyme, and so is presumably involved in protein turnover. We have no idea why T antigen is toxic, but we believe that the Cdc34 protein participates in the degradation of T antigen. When Cdc34 activity is reduced, T antigen accumulates to toxic levels in a greater proportion of the cells.

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TRANSCRIPTION AND CELL CYCLE REGULATION IN YEAST

K.T. Arndt C. Devlin, C. DiComo, A. Doseff, M.J. Fernandez-Sarabia, F. Lin, M. Luke, A. Sutton, T. Zhong

In almost all aspects of basic cellular processes, the yeast *Saccharomyces cerevisiae* has been found to be very similar to higher eukaryotic cells. For this reason, and the ease of manipulation and the powerful genetic approaches available with yeast, we are using yeast in our laboratory to study transcriptional regulation and cell cycle control.

Transcriptional Regulation of the *HIS4* Gene

C. Devlin

Our ultimate goal is to understand completely the regulation of the yeast *HIS4* gene and to use *HIS4*

regulation as a model for the transcriptional regulation of other systems. The *HIS4* gene is under complex transcriptional control. Two independent systems activate *HIS4* transcription: basal control and general amino acid control. In general amino acid control, starvation for any one or more amino acids causes an increase in the levels of the GCN4 protein. The GCN4 protein binds to the sequence TGACTC, repeated five times in the *HIS4* promoter, to activate *HIS4* transcription. GCN4 binds one of these *HIS4* elements, repeat sequence C, much more tightly than the others, and most (>80%) of the GCN4-dependent activation of *HIS4* transcription is due to repeat sequence C. We are using defective GCN4 derivatives to obtain mutations in the general transcription machinery (see below).

The basal level control activates *HIS4* transcription in the absence of amino acid starvation. In addition, either phosphate or adenine starvation will cause a further increase in the already high basal levels of *HIS4* transcription. Activation of the basal level transcription of *HIS4* requires two *trans*-acting proteins, encoded by the *BAS1* and *BAS2* genes. Mutations in *BAS2* cause a phosphate requirement (*BAS2* is the same gene as *PHO2*), and mutations in either *BAS1* or *BAS2* cause an adenine requirement. Thus, in yeast, the phosphate, purine, and histidine pathways are coregulated. Analysis of the metabolites in these pathways shows the biological rational for the coregulation.

BAS1 contains an amino-terminal region similar to that of Myb proteins, and BAS2 contains a homeo box. The Myb domain is the DNA-binding domain for BAS1, and the homeo box region is the DNA-binding domain for BAS2. The Myb motif was first identified in the v-*myb* gene of avian myeloblastosis virus. Cellular homologs of v-*myb* were then found in all vertebrates. In addition, Myb-type proteins have been found in *Drosophila* and corn (C1). The Myb motif contains three repeats of a sequence whose most striking feature is three regularly spaced tryptophans. The binding sites of these factors to the *HIS4* promoter are shown in Figure 1.

Basel level transcription of the *HIS4* gene requires both BAS1 and BAS2. Either protein alone, even when overexpressed, can only poorly activate *HIS4* transcription (at 1/100th the level of when both BAS1 and BAS2 are present). Since BAS1 and BAS2 do not bind cooperatively to the *HIS4* promoter, the nature of the requirement for both BAS1 and BAS2 for basal level *HIS4* transcription remains to be determined. In addition to activation of *HIS4* transcription, BAS2 is required to activate the transcription of the secreted acid phosphatases (the major secreted acid phosphatase is encoded by the *PH05* gene). However, BAS1 has no role in the activation of the transcription of the secreted acid phosphatases. In the next year, we would like to determine if BAS2 requires a second DNA-binding protein (to take the place of BAS1) for activation of the secreted acid phosphatases.

BAS1, BAS2, and GCN4 are the three activation proteins for *HIS4* transcription. In strains deleted for all three proteins, *HIS4* transcription is extremely low and the strains are His⁻. However, as measured by gel-shift assays using total yeast extracts, these three activator proteins represent only a very small fraction of the binding activity to the *HIS4* promoter. The major *HIS4* promoter-binding activity is due to the RAP1 protein, which binds to the *HIS4* promoter as shown in Figure 1. In the absence of GCN4, BAS1, and BAS2, YNF1 is not able to activate *HIS4* transcription. In addition, the binding sites for YNF1 and GCN4 overlap; GCN4 binds much tighter to the middle repeat element than to the other four repeat elements, and most GCN4-dependent transcription oc-

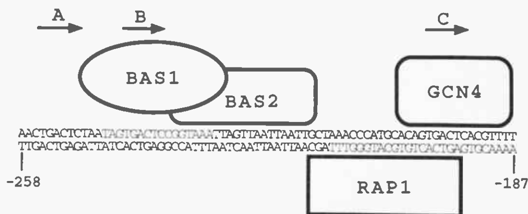

FIGURE 1 The *HIS4* promoter and its binding factors.

curs through the middle repeat sequence. In vitro DNA-binding studies show that GCN4 and YNF1 bind completely to their respective sites.

We have determined that YNF1 is the same protein as RAP1. RAP1 (which is probably the same as GRF1-TUF) is an abundant DNA-binding protein in yeast that binds to silencers, telomeres, and the promoters of many of the genes encoding ribosomal proteins and glycolytic enzymes. In addition, most of the RAP1 protein is found associated with the nuclear matrix or scaffold. The binding of RAP1 to the *HIS4* promoter is somewhat unexpected, since it was believed that RAP1 *by itself* is a transcriptional activation protein. In the context of the *HIS4* promoter, RAP1 *cannot* activate transcription by itself, since a strain lacking GCN4, BAS1, and BAS2 has extremely low levels of *HIS4* transcription. We have prepared a set of isogenic strains that differ only by point mutations in the chromosomal *HIS4* promoter. These mutations eliminate building of RAP1, GCN4, or both proteins. Using these strains, we have found that RAP1 is required for BAS1/BAS2-dependent activation of *HIS4* basel level transcription. The RAP1-binding site overlaps with the single high affinity *HIS4* GCN4-binding site. Even though RAP1 and GCN4 bind competitively, RAP1 is required for (1) the normal steady-state levels of GCN4-dependent *HIS4* transcription under nonstarvation conditions and (2) the rapid increases in GCN4-dependent steady-state *HIS4* mRNA levels following amino acid starvation. The presence of the RAP1-binding site in the *HIS4* promoter causes a dramatic increase in the micrococcal nuclease sensitivity of two adjacent regions within *HIS4* chromatin: one region contains the high-affinity GCN4-binding site and the other region contains the BAS1- and BAS2-binding sites. These results suggest that RAP1 functions at *HIS4* by increasing the accessibility of GCN4, BAS1, and BAS2 to their respective binding sites when these sites are present within chromatin.

A Genetic Selection for General Transcription Factors

C. Devlin

When the levels of *HIS4* transcription are quantitated by assaying the levels of β-galactosidase from a *HIS4-lacZ* fusion, a wild-type strain gives about 400 units of activity, whereas a strain containing deletions of the *GCN4*, *BAS1*, and *BAS2* genes gives less than one unit of activity. As a result, a strain deleted for the three activators of *HIS4* transcription is His$^-$. We have reverted this strain to His$^+$ with the assumption that mutations in genes encoding general transcriptional factors could result in increased *HIS4* transcription. This reversion analysis has identified five suppressor genes that permit *HIS4* transcription in the absence of GCN4, BAS1, and BAS2. We have termed these suppressors *sit* genes for *s*uppressors of *i*nitiation of *t*ranscription. These suppressor genes encode factors that affect the transcription of many diverse genes. Two of the suppressors, *SIT1* and *SIT2*, are encoded by *RPB1* and *RPB2*, the genes for the two largest subunits of RNA polymerase II. All strains containing suppressor mutations in *RPB1* and *RPB2* have reduced transcription of the *INO1* gene and an inositol requirement. Mutations in *SIT3* or high-copy-number *SIT3* increase *HIS4* transcription in the absence of GCN4, BAS1, and BAS2. *SIT3* is the only suppressor that suppresses when the wild-type gene is present in high copy number. The increase in *HIS4* transcription by high-copy-number *SIT3* or by *sit3* alleles is largely independent of the *HIS4* TATA sequence. We sequenced the *SIT3* gene and found that it is identical to *GCR1*, a gene previously identified as being required for high-level transcription of almost all glycolytic enzymes (whose combined mRNAs constitute over 50% of the mRNA in a yeast cell).

The SIT4 protein is over 50% identical to the catalytic subunit of bovine type-2A protein phosphatase. The analysis of SIT4 is discussed below. We have recently identified a new *sit* complementation group that we call *sit5*. DNA sequence analysis of the *SIT5* clone and genetic analysis have shown that *sit5* mutations are mutations in the histone $H2A_1$ gene. Experiments with the *sit5* mutations show the following: (1) At permissive temperatures, the *sit5* mutations cause a slow growth defect (doubling time of about 5 hr). This was surprising because *deletion* of the histone $H2A_1$ gene causes only a slight to moderate growth defect. (2) The *sit5* mutations cause a rapid (within 2 hr) and reversible temperature-sensitive growth defect at 38.4°C. At the nonpermissive temperature, the cells arrest with about 50% large budded cells. Again, this result is surprising since *deletion* of the histone $H2A_1$ gene does not result in temperature sensitivity. (3) Deletion or overproduction of the histone 2A-2B dimer does not promote *HIS4* transcription in the absence of GCN4, BAS1, and BAS2. This lack of suppression of the ab-

sence of GCN4, BAS1, and BAS2 is in contrast to the ability of altered histone 2A and 2B gene dosage to suppress Ty element insertion into the *HIS4* promoter. (4) That the *sit5* mutants require RAP1 to promote transcription of *HIS4* suggests that $H2A_1$ may interact with RAP1. In the future, we will determine if *sit5* mutations alter the nucleosome positioning at the *HIS4* promoter and at other chromosomal locations. We will also determine the sequence alteration(s) in the two *sit5* alleles to see if both alleles alter a specific region of the $H2A_1$ protein. This information may be useful for experiments to investigate specific interactions between RAP1 and $H2A_1$.

Progression from G_1 to S Phase Requires the SIT4 Protein Phosphatase

A. Sutton

Regulation of the cell cycle, most importantly the decision of whether or not to initiate a new cycle, is a major determinant of cell proliferation. For the yeast *Saccharomyces*, cells that are nutritionally starved (such as by limiting an essential nutrient in the growth medium) or cells in a saturated culture arrest in G_1. Cells that have arrested at this point in G_1 are in a physiological state distinct from G_1 cells in actively growing cultures. In general, cells initiate a new cycle only when they will be able to complete the entire cycle.

Much of what is known about regulation of the cell cycle comes from strains containing conditional mutations that arrest in G_1 at the nonpermissive temperature. Strains containing *cdc19*, *cdc25*, or *cdc35* (adenylate cyclase) arrest in G_1 at what seems to be close to the nutritional arrest point (unbudded uninucleate G_1 cells with no spindle pole satellites). Conditional mutations in the *CDC28* gene, which encodes a protein kinase, cause arrest in G_1 as unbudded uninucleate G_1 cells containing spindle poles with a satellite structure. This G_1 arrest point in *cdc28* mutants has been operationally defined as START. Supposedly, if a normal cell passes START, it is committed to completion of the cycle.

The SIT4 protein phosphatase was originally identified in *S. cerevisiae* by mutations (*sit4*) that restore transcription to the *HIS4* gene in the absence of GCN4, BAS1, and BAS2. GCN4, BAS1, and BAS2 are *trans*-acting DNA-binding factors that are normally required for *HIS4* transcription. These *sit4* mutations cause alterations in the transcription of many diverse genes in addition to *HIS4*. Strains containing transcriptional suppressor *sit4* mutations grow very slowly, are temperature-sensitive for growth, and do not grow on nonfermentable carbon sources. The *SIT4* gene encodes a predicted protein of 35.5 kD that is 55% identical to the catalytic subunit of mammalian type-2A PPases and 40% identical to the catalytic subunit of mammalian type-1 PPases.

When strains containing the *sit4* mutations are shifted to the nonpermissive temperature, 85–90% of the cells give a first cycle arrest as large unbudded uninucleate cells with a single microtubule organizing center. Arrested *sit4* cells have a 1n DNA content as determined by flow cytometry analysis. Electron microscopic analysis of serial sections of these arrested cells shows a single unduplicated spindle pole body. Although we could not determine if the single spindle pole body had formed a satellite structure, reciprocal shift experiments showed that strains containing temperature-sensitive mutations in *sit4* arrest late in G_1 at or very close to START.

SIT4 associates with two high-molecular-weight phosphoproteins. For immunoprecipitation analysis, the SIT4 protein was tagged with a nine-amino-acid epitope for which a high-affinity monoclonal antibody is available (12CA5). Two proteins, with apparent molecular weights of 155,000 and 190,000, specifically coimmunoprecipitate with SIT4. Immunoprecipitation of extracts prepared from cells labeled with [^{32}P]orthophosphate shows that both p155 and p190 are phosphorylated in vivo. In contrast, SIT4 is not detectably phosphorylated in vivo. Western analysis of extracts prepared from cells synchronized by either α-factor or centrifugal elutriation shows that the steady-state levels of SIT4 do not vary in the cell cycle. In contrast, the association of SIT4 with p155 and p190 is regulated in the cell cycle. To obtain uniformly labeled cells from different stages of the cell cycle, yeast cells containing the epitope-tagged SIT4 protein were grown in the presence of [^{35}S]methionine and then subjected to centrifugal elutriation. Extracts were prepared from fractions containing cells at different stages of the cell cycle. Immunoprecipitation of SIT4 from extracts prepared from cells in the later stages of the cell cycle contain both p155 and p190. In contrast, p155 and p190 are not detectable when SIT4 is immunoprecipitated from extracts prepared from G_1 cells. At about the time of the G_1 to S phase transition, SIT4 abruptly as-

sociates with p155 and p190. We are currently using a variety of genetic approaches to identify the genes that encode p155 and p190 (see below).

SSD1: A Polymorphic Gene That in Some Forms Can Permit Strains with a Deletion of *SIT4* to Live

A. Sutton

A deletion of the *SIT4* gene in laboratory strain W303 is lethal, and it is in this strain background that our analysis of the role of *SIT4* in the cell cycle was carried out. Surprisingly, we have found that a deletion of *SIT4* in about one half of our laboratory strains is not lethal, although the cells grow extremely slowly. Genetic analysis showed that the ability to grow very slowly in the absence of *SIT4* segregates as a single genetic locus, unlinked to *SIT4*. We have cloned the gene that allows life in the absence of *SIT4* and term this gene *SSD1* (*s*uppressor of *SIT4* *d*eletion). Although the function of *SSD1* is not currently known, a variety of data implicate *SSD1* in G_1 control. First, the *SSD1* gene has been independently isolated in two other laboratories, one as a suppressor of mutations that result in increased kinase activity of the *RAS*/cAMP pathway and another as a suppressor of an as yet unidentified (not *sit4*) mutation that causes a block in the G_1 to S transition. Second, we have found that strains containing a deletion of *SSD1* have a Whi phenotype and are hypersensitive to caffeine. Interestingly, *SSD1* has significant homology with the *S. pombe dis3* gene. Cold-sensitive mutations in *dis3* cause mitotic arrest and result in similar phenotypes to mutations in *dis2*. The *dis2* gene encodes a type-1 phosphatase. One model for the function of *SSD1* is that it is a positive regulatory factor for SIT4 or a related phosphatase that targets the phosphatase to its substrate at a precise point in the cell cycle. The possibility that SSD1 coimmunoprecipitates with SIT4 is currently under investigation.

Isolation and Characterization of Suppressors of Mutations in *SIT4*

A. Sutton

We are also investigating the role of SIT4 in progression from G_1 into S phase using genetic approaches. Hopefully, these approaches will identify substrates of SIT4 or regulators of SIT4 activity. One approach to SIT4 function is to obtain second-site suppressors of the temperature-sensitive-arrest phenotype of *sit4* strains. This analysis has identified three different complementation groups, which we call *sts1* through *sts3*. At permissive temperatures, *sts1* mutants (in a wild-type *SIT4* background) have an unusual morphology; cells are rod-shaped rather than round and form multiple buds. Furthermore, like strains containing certain mutations in the *RAS*/cAMP pathway, diploid strains containing the *sts1* mutation sporulate on rich medium. Although the *sts1* mutation results in temperature resistance in *sit4* strains, strains containing *sts1* in a wild-type *SIT4* background are temperature-sensitive. Like *sts1* mutants, strains containing *sts2* mutations grow slowly; however, the morphology of these cells appears to be normal. Interestingly, the presence of the *sts2* mutations results in the failure to germinate. The *STS1* gene has recently been cloned and sequence analysis is in progress. Hopefully, the *STS* genes have identified important G_1 control proteins that relate to SIT4 function. This possibility will be determined by biochemical and genetic analyses.

Serine/Threonine Protein Phosphatases in *S. cerevisiae*

F. Lin

Recent studies have shown that protein phosphatases, like protein kinases, play important and specific roles in cellular regulation, including cell cycle control. Four types of serine/threonine protein phosphatases, PP1, PP2A, PP2B, and PP2C, are known from biochemical analysis of mammalian cells. These phosphatases were grouped into these classes by substrate specificities, sensitivity to various inhibitors, and cation requirements. The catalytic subunits of PP1- and PP2A-type phosphatases are highly similar and share about 50% sequence identity. *S. cerevisiae* has a single PP1 phosphatase, two PP2A phosphatases, and the SIT4 phosphatase. Genetic analysis shows that the two PP2A phosphatases have largely overlapping functions. Deletion of either one of the *PP2A* genes is viable but deletion of both genes causes lethality. In addition, the PP1 and SIT4 phosphatases each provides essential functions for yeast. A number of experiments implicate PP1

phosphatase in mitosis and glycogen regulation, SIT4 in transcription and G_1/S phase control, and PP2A in regulation of activation of the *CDC28/cdc2* kinase. However, each of these phosphatases probably has many functions, some of which have yet to be identified.

Our focus is to investigate how these protein phosphatases function in cellular regulation, especially on cell cycle control. Immunoprecipitation analysis shows that PP1 interacts with at least eight proteins. Experiments using mammalian cells show that a major active form of PP1 is particulate and that the association of the catalytic subunit with different targeting subunits determines the in vivo specificity of PP1. It is possible that PP1 of *S. cerevisiae* has similar characteristics. Several different approaches are in progress to define those PP1-targeting subunits that are required for chromosome disjoining in metaphase and regulation of glycogen synthesis. Similar experiments are in progress with the two yeast PP2A phosphatases. In addition, we are preparing hybrid proteins among PP1, SIT4, and PP2A catalytic subunits to define which regions of each of these very similar proteins confer uniqueness and subunit association.

In Press, Submitted, and In Preparation

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ARABIDOPSIS SIGNAL TRANSDUCTION AND FLOWER DEVELOPMENT

H. Ma H. Huang
A. Tagle

The research in our laboratory includes two areas of plant biology, both of which are based on the work that one us (H.M.) did with Dr. Martin Yanofsky, when both were postdoctoral fellows in Dr. Elliot Meyerowitz's laboratory at the California Institute of Technology. The first area of research is a study of G protein functions in plant signal transduction, starting with isolated gene(s) coding for G protein subunits. The second area is an analysis of genes involved in flower morphogenesis; specifically, genes potentially encoding transcription factors were isolated, and their functions are now being tested using different approaches.

Plant cells respond to a large number of external and internal stimuli, such as light, gravity, microbes, and hormones. Little is known about the molecular mechanisms of plant signal transduction pathways for these responses. One approach to the understanding of plant signaling processes is to study homologs of proteins known to play important roles in signal transduction in animals and simple eukaryotes, such as G proteins. G proteins are members of a specific family of guanine-nucleotide-binding regulatory proteins that participate in a variety of eukaryotic signaling processes, from yeasts to humans. These heterotrimeric proteins (α, β, and γ subunits) are associated with the cytoplasmic side of cell membranes and transmit signals from transmembrane receptors to effector proteins. These effector proteins in turn produce, often through a cascade of reactions, changes in cellular metabolism. G protein α subunits bind guanine nucleotides and have GTPase activity.

We believe that G proteins play important roles in the signal transduction processes of plants, as they do in other eukaryotic organisms. As a first step in testing this hypothesis, we set out to isolate genes encoding G proteins from *Arabidopsis thaliana*. To begin with, we chose the genes coding for the α-subunits because of their highly conserved short stretches of amino acid sequences. Using polymerase chain reaction (PCR) amplification and degenerate oligonucleotides, a gene (*GPA1*) was isolated from *A. thaliana* that encodes a G protein α subunit (Ma et al., *Proc. Natl. Acad. Sci. 87:* 3821 [1990]). We are currently characterizing *GPA1* in several ways, attempting to establish association of *GPA1* with particular plant signaling pathway(s). In addition, we are continuing to look for other G protein genes in *Arabidopsis*.

Expression of *GPA1* Under Heterologous Promoters

H Ma, A Tagle

Many G protein genes are known to be expressed in specific cell types. Although *GPA1* is expressed in several organs, flowers, stems, and leaves, all of these share common cell types, for example, photosynthetic cells. It is possible that *GPA1* expression is not constitutive, but rather in a subset of cells in these organs. Although we will test this directly by performing more detailed analysis of the *GPA1* expression pattern, we would also like to mis-express *GPA1* under the control of heterologous promoters, e.g., the cauliflower mosaic virus (CaMV) 35S and heat-shock promoters (HSPs), to obtain clues about its function. The constitutive CaMV 35S promoter will direct increased *GPA1* expression in many cell types. The elevated level of the *GPA1* gene product may produce visible phenotypes. To achieve inducible expression, we would like to use HSPs. Constructs fusing 35S and HSP to *GPA1* cDNA were made, and they have been used in *Agrobacterium*-mediated plant DNA transformations.

We are in the process of generating transgenic plants, which will then first be analyzed for visible phenotypes.

Construction and Analysis of Mutant Alleles of *GPA1*

H. Huang, H. Ma

To test the function of *GPA1*, it would be very useful to obtain mutations in the *GPA1* gene. It is technically impractical to obtain chromosomal recessive mutations in *Arabidopsis*, starting with a cloned gene. However, for the G protein α-subunit, conserved residues exist that can be altered and thus can lead to dominant mutations. We have designed mutant oligonucleotides and have generated four mutations using PCR amplification and a *GPA1* cDNA. These mutant *gpa1* alleles have now been fused to 35S and HSP and are in the process of being fused to the native *GPA1* promoter. We will soon introduce these constructs into plants by transformation, and the phenotypes of the transgenic plants will then be examined.

In Search of New G Protein Genes

H Huang, H Ma

We believe that *Arabidopsis*, like most other characterized eukaryotes, has more than one gene coding for the G protein α-subunit. We are using two approaches to isolate additional G protein genes. PCR amplification was chosen as the main approach in order to isolate as diverse a group of sequences as possible. We have performed a preliminary experiment, and the sequences amplified include both *GPA1* and additional sequences, some resembling G protein sequences. We will repeat the PCR experiment, and then use new G-protein-like sequences as probes to isolate cDNA and genomic clones. Low-stringency hybridization was chosen as the second approach in order to isolate sequences similar to *GPA1*. This approach is better for genes that are more homologous to *GPA1*.

Floral Genes Potentially Encoding Transcription Factors

H. Ma, A. Tagle

Flower morphogenesis is a complex developmental process. Although flower development has been studied for many years, very little is known about the molecular machinery that controls the cellular differentiation in developing flowers. In recent years, *A. thaliana* has been used increasingly for plant molecular and genetic studies, and a number of *Arabidopsis* floral homeotic mutants have been characterized. Phenotypes of several homeotic mutants indicate that they alter floral organ identities. One example is *AGAMOUS* (*AG*). Previous genetic studies (Bowman et al., *Plant Cell 1:* 37 [1989]) indicate that homozygous *ag* mutant plants produce double flowers. Similar to wild-type flowers, *ag* mutant flowers have four sepals, and four normal petals develop in the outer two whorls (sets of similar floral organs); on the other hand, *ag* mutant flowers have six additional petals occupying the third whorl, which are stamens in the wild type. In addition, a new flower appears in the position occupied in wild type by the ovary. The pattern of four sepals surrounding ten (4 + 6) petals repeats until the whole flower has about 70 organs. The *AG* gene has been recently cloned

(Yanofsky et al., *Nature 346:* 35 [1990]), and DNA sequence analysis indicates that it encodes a protein that shares striking similarity in its amino-terminal portion with the DNA-binding domains of transcription factors from humans (SRF) and yeast (MCM1), suggesting that the AG protein is a transcription factor.

To coordinate the formation of floral organs at the proper time and location, the process of flower development is likely to require more regulatory genes than those dozen or so genes defined by mutations. In an effort to gain further understanding of *Arabidopsis* flower development, six new genes were isolated that share substantial sequence similarity with AG, including the DNA-binding motif and additional amino acid sequences (Ma et al., *Genes Dev. 5:* 484 [1991]). They were designated *AGL1* through *AGL6* for *AG-Like*. Five of the new genes were found to be preferentially expressed in flowers, and one of them is also expressed in stems and leaves. In situ RNA hybridizations showed that *AGL1* and *AGL2* are expressed in specific floral organs. We are continuing the characterization of the *AG* and *AGL* genes.

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INITIATION AND TERMINATION OF TRANSCRIPTION IN HUMAN snRNA GENES AND HIV-1

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SMALL NUCLEAR RNA GENES

The human small nuclear RNA (snRNA) genes U2 and U6 are part of a growing family of genes that share two characteristics: They have similar transcriptional control elements, and they encode short RNA molecules whose roles, in the cases where they are known, are in the processing of other RNA molecules. Therefore, by analogy to messenger (m)RNAs and transfer (t)RNAs, whose names refer to their function, we refer to the RNAs encoded by this gene family as "processor RNAs" or "pRNAs," and to the genes as the "pRNA genes." Table 1 lists the vertebrate pRNA genes that have been cloned to date. Surprisingly, as indicated in Table 1, some of the pRNA genes (e.g., the U2 snRNA gene) are transcribed by RNA polymerase II, whereas others (e.g., the U6 snRNA gene) are transcribed by RNA polymerase III. As a result, the RNA polymerase II and III pRNA promoters are more similar to each other than to either the RNA polymerase II mRNA-type promoters or the gene internal RNA polymerase III promoters of tRNA and 5S genes.

The structures of the RNA polymerase II and III pRNA promoters are shown in Figure 1. The RNA polymerase II pRNA promoters consist of a proximal element and an enhancer characterized by the presence of an octamer motif. In addition, a "3′ box" located 3′ of the gene directs termination of transcription. Remarkably, the 3′ box is recognized as a termination signal only when transcription is initiated at RNA polymerase II pRNA promoters, suggesting that these promoters direct the formation of transcription complexes that are different from those directed by mRNA promoters. The RNA polymerase III pRNA promoters are nearly identical to the RNA polymerase II pRNA promoters. They also contain a

TABLE 1 Cloned pRNA Genes with an Octamer/PSE Promoter Structure

Genes	RNA polymerase	Organism[a]	Function of the RNP	RNA length[b]
U1	II	H, M, R, C, X	mRNA splicing	164
U2	II	H, M, R, C, X	mRNA splicing	187–188
U3	II	H, R	rRNA processing	214–217
U4	II	H, C	mRNA splicing	141–145
U5	II	X	mRNA splicing	116
U11	II?	H	polyadenylation?	135
HSUR1	II?	Hs	unknown	143
HSUR2	II?	Hs	unknown	115
HSUR3	II?	Hs	unknown	76
HSUR4	II?	Hs	unknown	106
HSUR5	II?	Hs	unknown	115
U6	III	H, M, X	mRNA splicing	106–107
7SK	III	H	unknown	330–332
H1	III	H	tRNA 5' processing	341–344
MRP/Th	III	H, M	RNA primer cleavag in mitochondrial DNA synthesis	265–275
hY1	III	H	unknown	111–112
hY3	III	H	unknown	100–101

[a] H, human, M, mouse; R, rat, C, chicken; X, *Xenopus*; Hs, herpesvirus saimiri.
[b] The lengths indicated do not include the cap.

proximal element and an enhancer with an octamer motif. In fact, both of these elements have been shown to be interchangeable between RNA polymerase II and III pRNA promoters. However, the RNA polymerase III pRNA promoters also contain an A/T box, which is reminiscent of the TATA box of mRNA promoters. Surprisingly, in the human U6 gene, the A/T box is a dominant element that determines the RNA polymerase III specificity of the promoter. When the A/T box is mutated, the U6 promoter switches to RNA polymerase II, whereas when the A/T box is introduced into the U2 promoter, the U2 promoter becomes a predominantly RNA polymerase III promoter. Thus, the U2 and U6 genes constitute an ideal model system to study the mechanisms for RNA polymerase selection by promoter se-

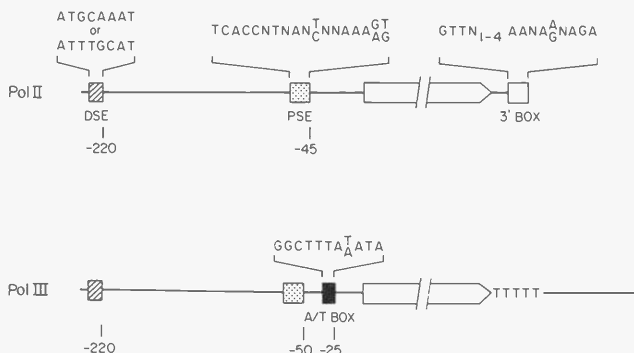

FIGURE 1 Structures of the RNA polymerase II and III pRNA promoters.

quences. In addition, the RNA polymerase II pRNA genes represent rare examples where the site of termination by RNA polymerase II is known and where the *cis*-acting elements required for the reaction have been characterized in detail. Both of these characteristics make the RNA polymerase II and III pRNA genes attractive transcription units to study transcriptional mechanisms.

HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

Expression of the human immunodeficiency virus type-1 (HIV-1) promoter is regulated by a complex combination of cellular and viral factors. These include Tat, a potent viral *trans*-activator that functions at least in part by increasing the amounts of HIV-1 mRNAs at the level of transcription. Tat affects both initiation and elongation of transcription, although some assays detect only the effect on elongation. Tat is unusual in that it acts through an RNA element, the *trans*-activation response (TAR) element, encoded by sequences downstream from the HIV-1 initiation site, between nucleotides +18 and +44. The TAR element corresponds to the upper half of a buldged stem-and-loop structure that extends from nucleotide +1 to +59.

In transfection experiments, constructs that contain the HIV-1 promoter and downstream sequences to position +80 direct the synthesis of a large amount of short transcripts that encompass the secondary structure from +1 to +59. In the presence of Tat, the number of short transcripts diminishes, and the number of long transcripts extending through the entire HIV-1 transcription unit increases. A few years ago, this observation prompted the suggestion that Tat functions as an antiterminator that overcomes a block to transcription elongation located around position +59. However, in other experimental systems, the number of short transcripts derived from the HIV-1 promoter in the absence of Tat is low, and coexpression of Tat does not decrease the number of short transcripts but only increases the number of RNA molecules extending through the entire transcription unit, suggesting that in this case, Tat acts mainly on initiation rather than elongation of transcription. In addition, there is clearly no universal termination site around position +60, because deletion of this region or its insertion into other transcription units does not have any positive or negative effect on gene expression as measured by chloramphenicol acetyltransferase (CAT) assays. We are interested in determining the mechanisms of short transcript formation, the role of the short transcripts, and the link, if any, between short transcript formation and *trans*-activation by Tat.

Factors Involved in Transcription of the Human U6 Gene

S. Lobo, M. Cockerill, S. Ifill, M. Sullivan,
N. Hernandez

In the past few years, the *cis*-acting elements responsible for transcription of the human U2 gene by RNA polymerase II and the human U6 gene by RNA polymerase III have been precisely defined. We are now in the process of identifying and purifying the *trans*-acting factors involved in transcription of the U6 gene by RNA polymerase III. A crude nuclear Dignam extract was fractionated over a P11 column into four fractions: TFIIIA (flow-through), TFIIIB (350 mM KCl), TFIIIC (600 mM KCl), and TFIID (1 M KCl). The TFIIIA fraction contains a factor that binds to the internal promoter element of the 5S gene, the TFIIIB fraction is required for transcription of every RNA polymerase III gene tested to date, and the TFIIIC factors bind to the internal B and A boxes of tRNA-type genes, as well as to the TFIIIA-5S gene complex. The TFIID fraction is required for transcription of mRNA encoding genes by RNA polymerase II and contains a 38-kD TATA-box-binding factor referred to as TFIID. In yeast, TFIIIB is a key transcription factor for 5S and tRNA gene transcription by RNA polymerase III. TFIIB is thought to allow promoter recognition by RNA polymerase III, whereas TFIIIA and TFIIIC are assembly factors whose only role is to promote binding of TFIIIB to the DNA.

We found that the TFIIIA fraction stimulated, but was not essential for, U6 transcription. U6 transcription could be reconstituted by a combination of either the TFIIIB and TFIIIC fractions or the TFIIIB and TFIID fractions. Consequently, in later experiments, we eluted the column with 350 mM KCl and with 1 M KCl to obtain a TFIIIB fraction and a TFIIIC/TFIID fraction. The activity required for U6 transcription in the TFIIIC/TFIID fraction seemed to consist of a single factor, because it eluted in a single peak from several chromatography columns. We therefore tested whether it might correspond to the cloned 38-kD TATA-box-binding factor TFIID by substituting human TFIID made in *Escherichia coli* for the TFIIIC/TFIID fraction. Whereas the TFIIIB fraction

alone did not sustain U6 transcription, addition of increasing amounts of bacterially produced human TFIID resulted in increasing levels of U6 transcription. This transcription was dependent on the A/T box, was directed by RNA polymerase III, and was initiated at precisely the same site as U6 transcription in vivo. These results demonstrate that the cloned TFIID polypeptide is capable of directing in vitro transcription of the U6 gene by RNA polymerase III and suggest that it may play the same role in vivo.

Figure 2 shows a hypothetical cartoon of the human U2 and U6 transcription complexes. We imagine that the minimal U2 transcription complex consists of Oct-1, one or several factors binding to the proximal element, and RNA polymerase II, which may contact the U2 promoter downstream from the proximal element with relaxed sequence specificity. In the wild-type promoter, Sp1 also binds to the enhancer, but the Sp1-binding site can be replaced by a binding site for another factor, indicating that Sp1 per se is not an essential component of the U2 transcription complex. The minimal U6 transcription complex may contain similar if not identical enhancer and proximal element binding proteins. However, downstream from the proximal element, the A/T box binds the TFIID 38-kD protein. TFIID is shown interacting with the proximal element binding factor, because the distance between the proximal element and the A/T box is strictly conserved in the human RNA polymerase III pRNA genes. Furthermore, increasing the spacing between the proximal element and the A/T box is detrimental for RNA polymerase III transcription both in the *Xenopus* U6 promoter and in plant snRNA promoters. Perhaps in combination, the TFIID protein and the proximal element binding factor(s) create a surface that can bind TFIIIB and mediate its interaction with the DNA, thus defining the U6 promoter as an RNA polymerase III promoter. As illustrated in the last panel of Figure 2, in other RNA polymerase III promoters such as tRNA promoters, this role may be played by the TFIIIC factors, which bind the B and A internal promoter elements.

The observation that TFIID can direct U6 transcription by RNA polymerase III raises the question of whether it is also involved in transcription of other RNA polymerase III genes. Several RNA polymerase III genes contain A/T-rich regions in their 5′-flanking sequences that are required for efficient transcription. In addition, we have shown by Western blots that the TFIIIC fraction contains the TFIID 38-kD polypeptide. The TFIIIC fraction can be further divided into two fractions, TFIIIC1 and TFIIIC2, that are both required for transcription of tRNA-type genes by RNA polymerase III. TFIIIC2 has been purified to five polypetides that may all be required for activity, but the active components of the TFIIIC1 fraction have not been identified and may well include TFIID.

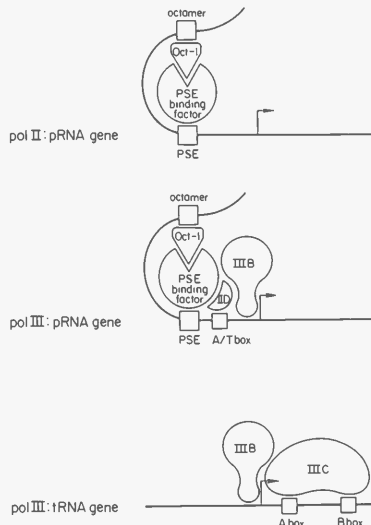

FIGURE 2 Hypothetical cartoon of the human U2 and U6 transcription complexes.

We will now pursue several questions. We will determine whether the TFIID domains required for RNA polymerase III transcription are identical to those required for RNA polymerase II transcription. We are also pursuing the purification of the factors required for U6 transcription present in the TFIIIB fraction.

Formation of Short RNAs by the HIV-1 LTR

R. Ratnasabapathy, M. Sheldon, L. Johal, N. Hernandez

In the human RNA polymerase II snRNA genes, termination of transcription is dependent on transcription initiating at an snRNA promoter. To

determine the sequence requirements for formation of short transcripts, we generated several constructs in which either the HIV-1 promoter sequences or the sequences downstream from the transcriptional start site were replaced by foreign sequences. Formation of short transcripts was monitored in transient transfection experiments.

PROMOTER SEQUENCES ARE NOT REQUIRED FOR THE FORMATION OF SHORT TRANSCRIPTS NOR FOR TAT *TRANS*-ACTIVATION

To determine the mechanisms of short transcript formation in the HIV-1 long terminal repeat (LTR), we first examined whether short transcript formation was dependent on promoter sequences. We exchanged the HIV-1 promoter for a variety of other promoters, including two promoters from mRNA encoding genes, the U2 promoter and the U6 promoter. All of these constructs directed the synthesis of a large amount of short transcripts in the absence of Tat. In the presence of Tat, the number of short transcripts was diminished, whereas the number of long transcripts was greatly increased. The sole exception was the construct containing the U6 promoter, whose expression was not affected by Tat. These experiments showed that several different RNA polymerase II promoters can be *trans*-activated by Tat and that short transcript formation is not dependent on promoter sequences.

THE HIV-1 SEQUENCES FROM –5 to +82 CONTAIN AN INDUCER OF SHORT TRANSCRIPTS

We then replaced the HIV-1 sequences downstream from the HIV-1 promoter (from –5 to +82) by foreign sequences. In these constructs, no short transcripts were formed as expected, but surprisingly, the number of long transcripts was not increased. Thus, in the absence of the HIV-1 sequences from –5 to +82, the total number of transcripts, short and long, derived from the HIV-1 promoter and other promoters was much lower than that in the presence of the –5 to +82 HIV-1 sequences. This suggested that these sequences contained an activator of transcription. To demonstrate this directly, we performed run-on experiments and showed that indeed this region could activate transcription from different RNA polymerase II promoters and even from the U6 RNA polymerase III promoter. Thus, the –5 to +82 HIV-1 sequences contain an unusual element: In its presence, the total transcriptional activity of different promoters is greatly increased, but all of the additional RNA molecules resulting from this increase in transcription are short. We therefore refer to this element as IST, for inducer of short transcripts.

TAT CAN CHANGE ELONGATION PROPERTIES OF AN ELONGATION COMPLEX

As mentioned in the introduction, the U2 snRNA promoter directs the formation of peculiar transcription complexes that are incapable of efficient elongation and terminate transcription at "3′boxes." To determine whether Tat could affect elongation of transcription, we tested the effects of Tat on transcription from the U2 promoter. We generated a construct that contained the U2 promoter followed by HIV-1 sequences from –5 to +82, which include the sequences encoding the TAR element. Just downstream from the HIV-1 sequences, we inserted a 3′box and further downstream, a polyadenylation site. Thus, this construct contained three 3′-end formation signals in a row: the putative signal for formation of short transcripts, a 3′box, and a polyadenylation site. In the absence of Tat, this construct generated short transcripts as well as transcripts that terminated at the 3′box. In the presence of Tat, the number of short transcripts was reduced as expected, but the number of transcripts ending at the 3′box was not increased. Instead, a large number of RNA molecules polyadenylated at the downstream site were generated. Thus, in the presence of Tat, the transcription complexes derived from the U2 promoter were capable of reading through a 3′box. This suggests that Tat modifies the transcription complexes derived from the U2 promoter and renders them equivalent to transcription complexes derived from mRNA promoters.

The discovery of the IST element and the observation that Tat can modify the elongation properties of the U2 transcription complex suggest that the HIV-1 promoter is capable of directing the formation of two types of transcription complexes: one dependent on IST and incapable of efficient elongation, and the other dependent on TAR and Tat and capable of efficient elongation. Perhaps because the total number of initiation events that can occur at the promoter is limited, the effect of Tat can be perceived as an effect on transcription elongation or transcription initiation, depending on the levels of IST activity: If IST activity in the absence of Tat is high, Tat *trans*-activation results in elongation-efficient transcription complexes displacing some of the IST-directed transcription complexes at the promoter, thus an apparent

effect on transcription elongation. If IST activity in the absence of Tat is low, Tat *trans*-activation results in an increase in the total number of transcription complexes fired by the HIV-1 promoter, thus an effect on transcription initiation.

What is the role of IST, and what is the role of short transcripts in HIV-1-infected cells? Because Tat acts through an RNA target (TAR) encoded downstream from the HIV-1 promoter, the HIV-1 promoter must be transcriptionally active to generate TAR and be responsive to Tat. Perhaps the function of IST is to generate the target for Tat *trans*-activation in the absence of any viral gene expression. If this were the case, the ability of the HIV-1 LTR to generate short transcripts would allow an otherwise silent and perhaps defective (Tat$^-$) provirus to respond to Tat provided by another provirus. Alternatively, very high expression of short transcripts might down-regulate Tat *trans*-activation. Indeed, it has been shown that overexpression of TAR sequences renders cells resistant to HIV-1 replication. Thus, the short transcripts may be involved in regulating the switch from latent to productive infection. The production of short transcripts may thus define an intermediate stage between a completely silent provirus and an actively expressed provirus.

Precise Mapping of the IST Element in the HIV-1 LTR

M. Sheldon, R Ratnasabapathy, N Hernandez

IST is located between positions –5 and +82 relative to the start site of transcription in the HIV-1 LTR. IST can be described as having two activities: On the one hand, it activates transcription and, on the other hand, it directs premature 3′-end formation of the RNA molecules resulting from this activation of transcription. The simplest model is that IST consists of a single sequence element that activates the formation of transcription complexes that, like the U1 and U2 transcription complexes, are not capable of efficient elongation. The short transcripts would then represent the final products of random termination, followed by exonucleolytic degradation to the base of the stable stem-and-loop structure that folds the first 59 nucleotides of the RNA. However, it is also possible that IST consists of two distinct sequence elements, an activator of transcription and a 3′-end formation signal. These could be two DNA elements, a DNA element and an RNA element, or even two RNA elements. Localizing the IST element is complicated by the fact that mutations downstream from the start site of transcription alter the sequence of the RNA and thus may change its stability. In particular, the stem-and-loop structure that folds the first 60 nucleotides of the HIV-1 mRNAs may be required for stabilization of the short transcripts.

To determine the precise location of the IST element, we have generated a series of mutations in the –5 to +82 region that maintain the base-paring in the RNA secondary structure. We are in the process of analyzing both steady-state RNA and nascent RNA derived from these mutated constructs. This analysis should define the exact nature and location of the IST element.

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In Press, Submitted, and In Preparation

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TELOMERASE AND TELOMERE LENGTH

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L.A. Harrington K.R. Prowse

We are studying the enzyme telomerase from *Tetrahymena* in order to understand how telomeres replicate and maintain their length. Telomerase recognizes the 3′end of telomeric G-strand sequences and adds tandem repeats of the *Tetrahymena* telomeric sequence, $d(TTGGGG)_n$, in an apparently template-independent manner. Thus, net lengthening of telomeres allows telomere length to be maintained since conventional replication would lead to loss of sequences from chromosome ends at each round of replication. Telomerase is a ribonucleoprotein complex (RNP). We and other investigators have shown that the essential RNA component provides the template for the $d(TTGGGG)_n$ repeats that the enzyme synthesizes.

TELOMERASE IS PROCESSIVE

We are pursuing the biochemical characterization of this unusual DNA polymerase. Using primer challenge experiments, we have shown that telomerase acts processively; i.e., once telomerase is bound to a primer, many base pairs of d(TTGGGG) will be synthesized before the enzyme dissociates. Telomerase was reacted in the presence of excess primer under standard reaction conditions (1 μM [^{32}P]dGTP, 100 μM dTTP, 1 μM Mg^{++}, 10 μM Tris, pH 8.0) for 5 minutes, and the reaction was then diluted 30-fold into unlabeled dGTP, 100 μM dTTP, and a large excess of competitor primer $d(TTGGGG)_3$ oligonucleotide. The reaction was then allowed to proceed for 5, 10, 15, 20, 30, 40, 60, or 90 minutes, and the labeled products were run on an alkaline agarose gel (Fig. 1). The increase in length of the products, even after dilution into excess competitor oligonucleotide, indicates that telomerase is processive.

Processivity is the probability that the enzyme will dissociate from a given primer that is being elongated. We define the processivity of telomerase as the number of nucleotides synthesized when half of the telomerase bound to primers has dissociated. This value can be determined directly from the distribution of labeled products in the alkaline agarose gel (Fig. 1). We quantitated the distribution of counts in the gel using the Phosphor-Imager (Molecular Dynamics) and found that telomerase will synthesize 520 bp before half of the enzyme has dissociated. The average rate of primer elongation is about 80 nucleotides per minute. When compared to other "conventional" polymerases, telomerase is a slow but very processive enzyme.

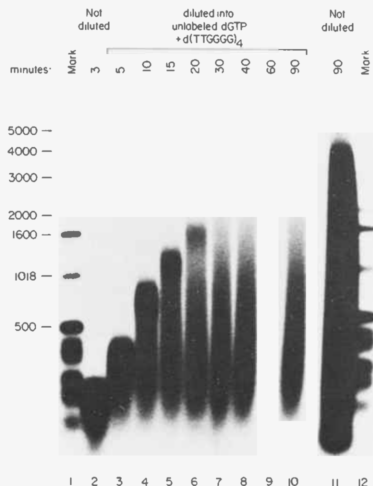

FIGURE 1 How processive is telomerase? Telomerase was reacted for 3 min in the presence of [^{32}P]dGTP, dTTP, and excess primer $d(TTGGGG)_4$ oligonucleotide. A portion was stopped to determine the length obtained in the initial reaction (lane *2*), and a portion was diluted 30-fold into a mixture containing unlabeled dGTP, dTTP, and excess primer $d(TTGGGG)_4$ oligonucleotide The reaction was allowed to proceed, and aliquots were removed after 5, 10, 15, 20, 30, 40, 60, and 90 min total reaction time (lanes *3–10*). A portion of the initial reaction was allowed to proceed for 90 min without dilution (lane *11*). The markers are the 1-kb ladder from BRL (lanes *1* and *12*). The 60-min sample was lost in loading the gel (lane *9*). The products were electrophoresed on an alkaline agarose gel.

Since the *Tetrahymena* telomerase acts processively to synthesize, on average, 520 bp before dissociating, and the RNA template contains only 9 bp of CAACCCCAA, there must be a translocation step in the elongation mechanism. Telomerase reaction products show a characteristic "pausing" pattern on sequencing gels. To define at what position within the repeat the translocation occurs, the banding pattern produced by telomerase in vitro was analyzed. Since the concentration of dTTP affects the pattern produced, a titration of dTTP was carried out. To assign sequence to the in vitro pattern, $d(GGGGTT)_3$ was elongated in the presence of $[^{35}S]$dGTP and dideoxyTTP (ddTTP). Since ddTTP will be incorporated and cause chain termination, this experiment defined the position of the first four added dGs and a single ddT. Using this reaction as a reference, the sequence added in the reactions using $[^{35}S]$dGTP and dTTP could be assigned (Fig. 2). The strong pause in the banding pattern that appears every six bases occurs after addition of the first G residue within the d(TTGGGG) repeat. The pause at this position suggests that the entire 5′-CAACCCCAA-3′ RNA template is copied to the 5′-most C residue and then translocation occurs. The pause after the addition of the first G residue would result if translocation were a slow step.

TELOMERASE PRIMER RECOGNITION

To gain a detailed understanding of the telomerase enzyme mechanism, we asked whether $d(TTGGGG)_n$ repeats are required at the 3′ end of primer oligonucleotides for recognition and elongation by telo-

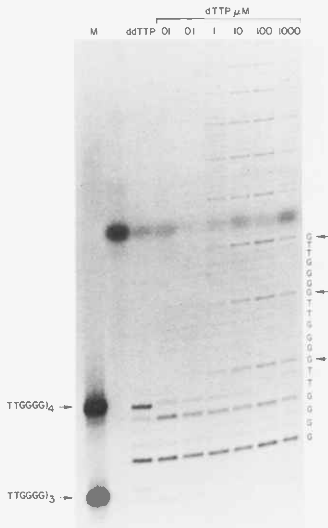

FIGURE 2 Pausing pattern. Telomerase was reacted under standard conditions using $[^{35}S]$dGTP, $d(GGGGTT)_3$, and various concentrations of unlabeled dTTP. The concentration of dTTP is shown above each lane. In lane *2*, only ddTTP was added to determine the sequence of the pausing pattern. In lane *1*, ^{32}P-labeled 18-base $d(TTGGGG)_3$ and 24-base $d(TTGGGG)_4$ were run as markers.

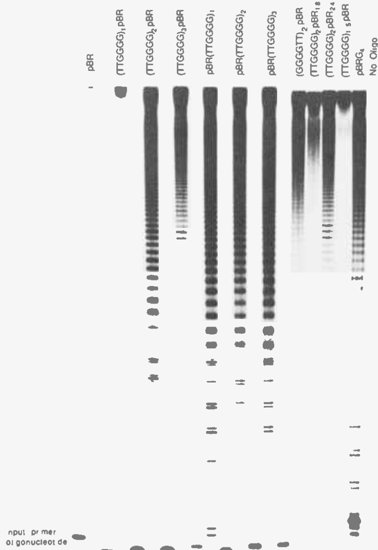

FIGURE 3 Telomerase elongation of telomeric and nontelomeric olignucleotide substrates Test oligonucleotides are indicated above their respective two lanes. The left lane in each set indicates the gel-purified, 5′ ^{32}P-end-labeled oligonucleotide as a size marker for input primer. The right lane represents oligonucleotide that has been incubated with telomerase for 1 hr under standard reaction conditions. The sequences of the oligonucleotides are shown in Fig. 4.

omerase. Earlier experiments showed that telomeric sequence oligonucleotides are elongated and nontelomeric oligonucleotides are not. Various oligonucleotides containing different lengths of nontelomeric sequence (pBR322 plasmid sequence) were synthesized and tested for the ability to prime d(TTGGGG)$_n$ addition in vitro. The results are shown in Figure 3 and summarized in Figure 4. At least two repeats of d(TTGGGG) are required at the 5′end of the primer oligonucleotide for recognition and elongation by telomerase. However, the sequence d(TTGGGGTTGGGG) can be followed by up to 24 bases of nontelomeric sequence on the 3′end and still be elongated.

The results in Figures 3 and 4 also show that oligonucleotides that have d(TTGGGG)$_1$ or d(GGGG) at their 3′end can be elongated. Since there is only one short stretch of dG residues, these oligonucleotides cannot form intramolecular G-G base-paired structures. Structures containing G-G Hoogsteen base pairs, or "G quartet" interactions, have been postulated to play a role in recognition by telomerase. Substitution of dG by deoxyinosine in telomeric oligonucleotides renders them unable to form any intramolecular structures but does not affect their ability to be elongated by telomerase (Henderson et al., *Biochemistry 29:* 732 [1990]). These results, in conjunction with our finding that telomerase can efficiently elongate oligonucleotides with only one or less than one d(TTGGGG) repeat at the 3′end, suggest that such secondary structures are not required for telomerase recognition.

It is possible that elongation of oligonucleotides with nontelomeric 3′ends was due to exonucleolytic degradation of telomeric sequences and subsequent elongation. The results in Figure 3 suggested that this was not the case, since the telomerase banding pattern began above the position of the input primer oligonucleotide. As a further control to show that nontelomeric sequences were indeed retained on substrate oligonucleotides, the primers in Figure 4 were reacted with telomerase in the presence of radioactively labeled dTTP and dideoxy GTP (ddGTP). These end-labeled oligonucleotides were then subjected to the dC-specific Maxam-Gilbert sequencing reaction; 3′-end-labeled cleavage products of the expected lengths were observed, indicating that the nontelomeric sequences were retained at the 3′end

Name	Oligonucleotide	Elongation
(TTGGGG)$_4$	TTGGGGTTGGGGTTGGGGTTGGGG	+
pBR	AGCCACTATCGACTACGCGATCAT	-
pBRG$_4$	AGCCACTATCGACTACGCACGGGG	+
pBR(TTGGGG)$_1$	AGCCACTATCGACTACGCTTGGGG	+
pBR(TTGGGG)$_2$	AGCCACTATCGATTGGGGTTGGGG	+
pBR(TTGGGG)$_3$	AGCCACTTGGGGTTGGGGTTGGGG	+
(TTGGGG)$_1$ pBR	TTGGGGTATCGACTACGCGATCAT	-
(TTGGGG)$_{1.5}$pBR	TTGGGGTTGCAGCTACGCGATCAT	+ \ -
(TTGGGG)$_2$ pBR	TTGGGGTTGGGGCTACGCGATCAT	+
(GGGGTT)$_2$ pBR	GGGGTTGGGGTTCTACGCGATCAT	+
(TTGGGG)$_3$pBR	TTGGGGTTGGGGTTGGGGGATCAT	+
(TTGGGG)$_2$ pBR$_{18}$	TTGGGGTTGGGGTATCGACTACGCGATCAT	+
(TTGGGG)$_2$ pBR$_{24}$	TTGGGGTTGGGGAGCCACTATCGACTACGCGATCAT	+

FIGURE 4 Sequences and names of telomeric and nontelomeric oligonucleotides.

after telomerase elongation. These results confirmed that telomerase was able to recognize and elongate oligonucleotides that contained up to 24 bases of nontelomeric sequence at the 3′ end. Thus, hybridization of the telomerase RNA to the 3′ sequence of a telomeric oligonucleotide is not required for primer elongation. The primer recognition site of telomerase may be functionally distinct from the site of addition of d(TTGGGG). The ability of telomerase to elongate nontelomeric sequence substrates suggests how this enzyme might function in healing broken chromosomes and in new telomere formation in ciliates.

TELOMERES AND AGING

In addition to telomerase biochemistry, we are interested in how telomere length is maintained in vivo. In a collaborative effort, we have found that as primary human fibroblasts divide and undergo senescence in culture, telomeric sequences are lost from chromosome ends. The limited replicative capacity of primary fibroblasts was well documented by Hayflick and others in the 1960s (Hayflick et al., *Exp. Cell. Res. 25:* 585 [1961]); however, the mechanism that limits cellular life span is not yet known. Our data suggest that telomere shortening may play some role in cellular senescence. We have recently shown that telomere shortening occurs in vivo as well as in vitro. Primary fibroblasts from older individuals have shorter telomeres than those from younger individuals. Human sperm telomeres are several kilobases longer than somatic cell telomeres (Cooke et al., *Cold Spring Harbor Symp. Quant. Biol. 51:* 213 [1986]). This, together with the telomere reduction seen with age, suggests a simple model for telomere shortening: Human telomerase is active only in the germ line where telomeres are lengthened, and then telomerase is shut off in the soma. The net loss of telomeric sequences may then occur in somatic tissue through incomplete replication at each round of division. Experiments are currently under way to determine whether telomerase activity is present in the primary fibroblasts where telomere shortening has been well documented.

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In Press, Submitted, and In Preparation

Greider, C.W. 1991 Telomerase is processive. (Submitted.)

STRUCTURE AND COMPUTATION

This section includes five laboratories interested in the detailed structural properties of proteins and computational biology. It includes the laboratories of Drs. Jim Pflugrath and John Anderson, who are interested in macromolecular crystallography, and Dr. Rich Roberts, whose group has had a long-standing interest in restriction enzymes and their associated methylases. Dr. Jeff Kuret studies protein kinases, and his group has established a close collaboration with Dr. Pflugrath's laboratory. The most recent addition to this section is Dr. Tom Marr, who joined us in 1989 having been the lead designer of the GENBANK Database at Los Alamos National Laboratory. Dr. Marr is building a group devoted to computational biology, with particular emphasis on database applications. He is currently interacting extensively with Dr. David Beach's group to provide database support for the *Schizosaccharomyces pombe* genome mapping project.

NUCLEIC ACID CHEMISTRY

R.J. Roberts	A. Dubey	D. Macelis	G. Otto
	J. Earl-Hughes	C. Marcincuk	J. Posfai
	S. Klein	S. Mi	D. Roberts
	S. Klimasauskas	M. Miyahara	M. Wallace
	C.-L. Lin		

Purification and Characterization of the *Msp*I Methylase

A. Dubey, M Miyahara

The *Msp*I restriction-modification system recognizes the sequence 5′-CCGG-3′. The complete system has previously been cloned and sequenced (Lin et al., *Nucleic Acids Res. 17:* 3001 [1989]). The methylase gene has been further subcloned, placed downstream from the pTac promoter in a pUC119 derivative, and overexpressed in *Escherichia coli* strain RRI (B. Mollet and R.J. Roberts, unpubl.). This recombinant strain overproduces the *Msp*I methylase to high levels, causing aggregation of the protein to form inclusion bodies. These inclusion bodies are temperature-sensitive. They are formed when the cells are grown at 37°C but are not produced at 30°C. The extent of soluble protein present does not seem to vary significantly at either temperature.

Abundant proteins with apparent molecular masses of 40 kD and 51 kD are present in the overexpressing strain. Amino-terminal sequence analysis of these two proteins shows that the 40-kD protein is a truncated form of the *Msp*I methylase, missing exactly 40 amino acids from the amino terminus. The new amino terminus is a methionine residue corresponding to the second AUG in the reading frame. It is not known whether the carboxyl terminus is also truncated. As confirmed by immuno-Western blot analysis, it is the 51-kD species that is the bona fide methylase, since only this form is present in the original *Moraxella* species from which the gene was cloned. Interestingly, polyclonal antibodies raised against the *Msp*I methylase cross-react with m5C-methylases of several other restriction-modification systems. This is consistent with the existence of a fundamental structural similarity among this family of proteins.

A scheme has been developed to purify the *Msp*I methylase to homogeneity from this overexpression

system. This purification scheme includes treatment of the cell lysate with protamine sulfate to remove nucleic acids, followed by chromatography on phosphocellulose and FPLC columns: Mono S and Mono Q. Approximately 0.8 mg of pure protein per gram of cells (wet weight) could be recovered.

While characterizing *Msp*I methylase–DNA interactions, it was found that the enzyme can form a stable complex with DNA, as judged from mobility shift assays, either in the presence or in the absence of the cofactor *S*-adenosylmethionine or its analog sinefungin. A detailed functional and structural characterization of this enzyme is under way. Crystallization is being attempted in collaboration with Drs. X. Cheng and J. Pflugrath (Macromolecular Crystallography).

Additional studies have been aimed at preparing mutants of the *Msp*I methylase that will be useful in delineating the functions of the conserved regions within all m5C-cytosine methylases. The main goal is to find temperature-sensitive mutants that are deficient in their ability to release from the DNA after initial covalent bond formation. Such mutants would be invaluable if our crystallization attempts are successful.

Comparative Methods of Sequence Analysis

G Otto

We are continuing our work on probabilistic methods of comparative sequence analysis. The major focus is on the comparison of protein sequences sharing a common function. The goal of these comparisons is to define significant sequence patterns that correlate with and are predictive of function. In this context, functions are taken to be specific ligand-binding activities. The elements of the derived patterns are often structurally relevant, tending to be those amino acids in close contact with the bound ligand. There is a large element of phylogenetic analysis in these methods. It has become clear that the best representation of sequence similarities predictive of function is actually a phylogenetic tree of related sequence patterns. A single, maximally inclusive pattern is just the root of this larger tree. Beyond the pragmatic goal of predicting protein function, it will be interesting to examine the set of these pattern trees for similarities that might reflect the evolution of protein diversity.

Our work on comparative methods involves a number of mathematical issues. We departed from standard comparative techniques by defining the measure of similarity as the probability that an optimal alignment of sequences is due just to the random matching between unrelated sequences. We proposed a basic, empirical test that any probabilistic measure must satisfy. An estimator of these probabilities for pairwise comparisons was developed that satisfied the test quite well. This measure has been incorporated into a program for the comparison of one sequence with a database. This measure has been generalized to the case of multiple sequence comparisons, providing an estimate of the probability of a given multiple alignment occurring among a random set of proteins.

We have also developed a probabilistic measure for the matching of consensus patterns with protein sequences and have written a program that searches a database for pattern matches of varying qualities. However, many new developments are required for this approach to achieve its full power.

The sheer volume of sequence data and the additional structure imposed on these data by biological theory and experiments has generated considerable interest among mathematicians and computer scientists. It is also recognized that the measure of sequence similarity arises in many fields and is of general scientific interest. We are beginning collaborations with a number of these mathematical scientists with the aim of developing a coherent interdisciplinary effort in the New York area.

Architecture of Cytosine-m5C Methylases

S. Klimasauskas

One goal of our work is to identify the protein domain(s) responsible for sequence-specific recognition of DNA by m5C-methylases. The experimental approach is to construct hybrids between two methylases that recognize different sequences. These experiments employ the *Hpa*II (recognition sequence: 5′-C^{me}CGG-3′) and *Hha*I (recognition sequence: 5′-G^{me}CGC-3′) DNA methylases that form part of their respective restriction-modification systems. A plasmid was constructed with the two methylase genes in tandem and arranged in the same orientation

relative to transcription. The polymerase chain reaction (PCR) was used to construct exact deletions from the plasmid containing both methylase genes such that the amino terminus of one methylase becomes joined to the corresponding carboxyl terminus of the second methylase. These constructions take advantage of the conserved sequence motifs that are present within the cytosine methylases so that the boundaries in the hybrids are defined by the sequence motifs. In each case, the overall architecture of the methylase is maintained.

We have tested several constructs for their ability to methylate plasmid DNAs in vivo as well as their ability to induce an *Mcr* response using appropriate tester strains. The majority of the hybrids tested were able to induce an *Mcr* response in vivo, and yet no methylation was detectable as judged from the sensitivity of the plasmid DNAs to the corresponding restriction enzymes. However, one hybrid that contains the amino-terminal sequence of the *Hpa*II methylase up to the amino-terminal boundary of the variable region, joined to a complementary fragment containing the complete variable region and carboxyl terminus of the *Hha*I methylase, is active in vivo. This hybrid induces a strong *Mcr* response and leads to partial methylation of the plasmid DNA. The methylation induced by this hybrid gives low but noticeable partial protection against the action of the *Hha*I restriction enzyme but is completely sensitive to cleavage by the *Hpa*II restriction enzyme. The plasmid encoding the hybrid methylase, like that encoding the parent *Hha*I methylase, is restricted by *Mcr*B and is insensitive to *Mcr*A. These results show that the new hybrid methylase has the specificity of *Hha*I, as expected if the variable region encodes the DNA recognition domain.

To increase the level of DNA methylation in vivo and to obtain manageable amounts of proteins for in vitro studies, we have overproduced the hybrid proteins by subcloning corresponding constructs into a pUHE25-2 vector that utilizes a T7-promoter-driven *lacI*-repressible expression system. Upon induction, it leads to the synthesis of the hybrid proteins in amounts above 10% of total soluble proteins in the cell. In the case of the active hybrid methylase described above, overexpression results in an almost complete protection of the plasmid DNA in vivo against the action of R·*Hha*I and no detectable protection against R·*Hpa*II. We have also prepared a correponding overexpressing clone carrying the gene for the wild-type *Hpa*II methylase. This appears to be a promising source of the methylase for large-scale purification and protein crystallization experiments.

These experiments demonstrate that the region responsible for sequence specificity in the monospecific methylases lies in the carboxy-terminal half of the molecule. To define the DNA recognition domain more precisely as well as to obtain insight into the flexibility with which active hybrids can be obtained, we have prepared a series of reciprocal constructs using the amino terminus of the *Hha*I methylase and the carboxyl terminus of the *Hpa*II methylase. We have also prepared hybrids in which the variable region, between conserved motifs VIII and IX, from one methylase has been replaced exactly by the variable region from a second methylase. These derivatives have been made by using a different approach. PCR-generated fragments containing the appropriate portions of the genes to be fused are made in vitro such that small overlaps are present that allow fusion during the PCR. The resulting gene is cloned into pUHE25-2 through appropriate restriction enzyme sites. The level and specificity of methylase activity of these hybrids are now under investigation using the above techniques.

A New Method for the Analysis of Global Sequence Similarities

J. Posfai, R J Roberts

Similarity between protein sequences may reveal structural, functional, or evolutionary relationships. We have developed a new computer tool that helps to discover and analyze similarities among multiple sequences. Most of the commonly used methods of sequence comparisons yield some numerical values (such as the percentage of identities of aligned sequences or distances between sequences embedded into a sequence space) that are intended to characterize the relationship between sequences. These values are very limited in their scope. They cannot fully describe complex sequence relationships, nor can they easily reveal how similarity changes along the sequences. The visualization of similarities by a dot matrix is a method that can give a more full picture of sequence relationships, but the method is not applicable for the comparison of multiple sequences. The concept of our new approach is similar to that of the dot matrix method in that common sequence fea-

tures are presented to a human investigator who can then manipulate visual representations of sequences.

The method is based on algorithms that we have developed previously to detect regions of local similarity among families of proteins. First, we explore the common elements of the sequence family under analysis. We usually determine the set of quasi-common amino acid triplets present in the family of proteins under consideration. However, other common sequence features, such as dipeptides or segments with Dayhoff scores over a predetermined threshold, can also be used in the analysis. We count the number of common elements tied to every sequence position. The distribution of these common elements, along the sequences, are then displayed on the computer screen using bar graphs or histograms. Horizontal lines represent the sequences and the vertical bars show the number of common elements at each position within the sequence (Fig. 1). Peaks within the distribution represent the sites where conserved regions are likely to be located. Segments that lack common elements are likely to represent the unique regions of each sequence. Sequences that show few common elements are probably not true members of the sequence family being investigated and can be excluded from the further phases of analysis.

Sequence segments where the dominant peaks of the graphs occur can be delineated by the user, and unique colors can be assigned to the sequence ele-

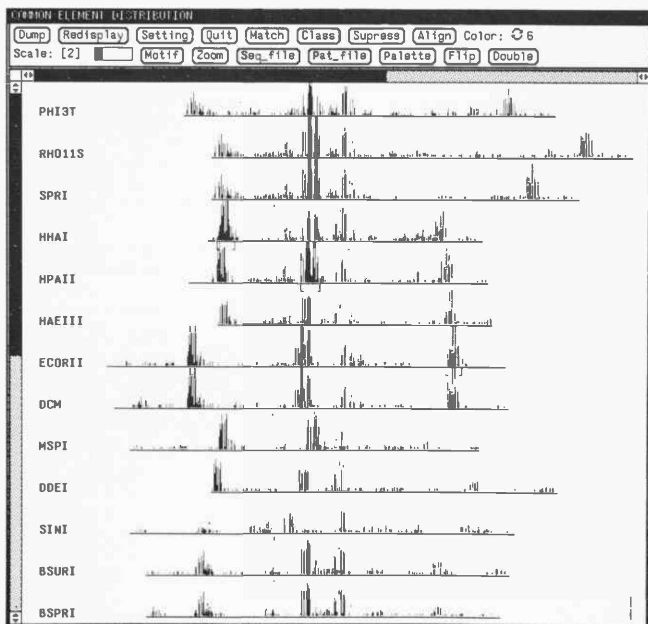

FIGURE 1 Sequences of several m5C-methylases are represented by horizontal lines, and vertical bars indicate the number of common sequence elements present at each position within the sequence. The header contains the tools for manipulating the image. Six conserved blocks are identified here. The order of the blocks is the same in all the sequences, and the distances between the blocks have obvious regularity. A variable region in the carboxy-terminal half of the sequences is apparent, and unique amino-terminal arms can be seen in some of the sequences.

ments (triplets) represented. All sequence elements contained within them are then repainted with the assigned color. This includes every occurrence of the element throughout the sequence. If, for example, a particular position matches to three common elements, two of which occur in a region assigned red, and one in blue, the corresponding bar is painted red up to the two thirds of its height, and the remaining part will be painted blue. Identically colored segments indicate conserved regions. The heights of the histograms give quantitative information about the level of sequence conservation. Missing or swapped colored segments point to deletions or to shuffled domains. Elements that appear as common by chance are effectively filtered out, because they either remain noncolored or occur in segments where many other colors also appear. The resulting image is easily interpreted and can allow the investigator to gain deep insight into the organization of the analyzed sequences. Importantly, the investigator can gain an understanding of the common elements present within the whole family of sequences by a single glance at the screen. This is very difficult to accomplish by most conventional methods for multiple sequence comparison.

An extensive set of image-manipulation tools are integrated into the program to facilitate the creation of color images and to boost their descriptive value. Mouse-controlled buttons, menus, scrollbars, sliders, etc., initiate the actions required by the investigator. Preset palettes, color cycling, and foreground/background color flipping can help in the creation of bright images. Subwindows allow the user to examine individual sequence blocks that are aligned on selected colors and allow the display of the actual common elements that led to the original peak.

This sequence analysis tool has been developed for Sun workstations that have color monitors. The programs are written in C, and the user interface (buttons, sliders, menus, mouse, etc.) and screen manipulation take advantage of the SunView library. Several print options are available for Postscript printers.

Isolation of Mutants of Integration Host Factor

D. Roberts

Integration host factor (IHF) is a site-specific DNA-binding protein in *E. coli* that has been shown to play a role both in regulation of gene expression and in site-specific recombination. It is a heterodimer composed of two homologous subunits, IHF-α and IHF-β, each about 100 amino acids long. IHF binds DNA by contacts in the minor groove (Craig and Nash, *Cell 39:* 707 [1984]; Yang and Nash, *Cell 57:* 869 [1989]). Binding of IHF to DNA causes the DNA to bend (Robertson and Nash, *J. Biol. Chem. 263:* 3554 [1988]; Prentki et al., *EMBO J. 6:* 2479 [1987]; Stenzel et al., *Cell 49:* 709 [1987]). We are interested in learning more about the structure and function of IHF, and as a first step, we have isolated new mutants of IHF.

After mutagenesis of plasmids containing either IHF-α or IHF-β, we isolated about 80 mutants of IHF. These mutants were tested for IHF function in five different genetic assays, involving five different IHF-binding sites. Since IHF is homologous to the bacterial histone-like protein HU, and the crystal structure of HU has been determined (Tanaka et al., *Nature 310:* 376 [1984]; White et al., *Proteins 5:* 281 [1989]), the effects of the mutations can be interpreted with reference to the structure of HU. Of particular interest were 13 mutations that were defective in some in vivo assays and functional in others. Five of these mutations mapped to the putative DNA-binding arm, and three of the mutations mapped to the third α-helix. It has been proposed that the third α-helix could make additional contacts with DNA to facilitate or stabilize DNA bending, although this has not been proven. Currently, work is continuing with the mutants to measure directly the effects on DNA-binding and site specificity. In addition, more mutants are being generated. Three types of changes are being made by site-specific mutagenesis. First, mutations were found in the DNA-binding arm of IHF-α but not IHF-β. The same amino acid changes are being made in IHF-β to see if a similar phenotype is observed. Second, additional mutations are being made in the third α-helix to try to determine the role of this region. Finally, since only the heterodimer functions in vivo, hybrids are being made between IHF-α and IHF-β to map the essential regions and to understand the possible differential role of each subunit.

Restriction Endonucleases

J. Earle-Hughes, S. Klein, D. Macelis, R.J. Roberts

The collection of type II restriction endonucleases continues to grow, and more than 1700 enzymes have

now been characterized; 172 different specificities are known. During the last year, we have characterized 25 new enzymes as part of a continuing collaboration with I. Schildkraut and D. Comb (New England BioLabs). Among these are several valuable new specificities, including *Pac*I from *Pseudomonas alcaligenes*, which recognizes the octanucleotide sequence TTAATTAA. This brings the total number of enzymes recognizing octanucleotide sequences to six. These enzymes are especially useful for genome mapping.

During the last year, much effort has gone into the maintenance of the restriction enzyme database. The ORACLE database management system is proving inadequate for many of our purposes, and a new implementation under SYBASE is being written. This will greatly increase the scope and utility of the database and is expected to be on-line during the first half of 1991. We have continued to expand our service role to the molecular biology community by providing specialized reports from the database that will feed various computer programs. The user community continues to grow, and their needs will be more adequately met from the SYBASE version of the database.

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MACROMOLECULAR CRYSTALLOGRAPHY

J. Anderson	E. Chang	R. Hsu	T. Malone
J.W. Pflugrath	X. Cheng	C.-S. Hung	D. Milano
	C.K. Cheung	J. Keller	T. Sellati
	F.-C. Chuan	C.-L. Lin	

The primary goal of the Macromolecular Crystallography group—to determine the structures of biologically important proteins and nucleic acids to atomic resolution to better understand their functions—drew closer to realization in 1990. The year saw significant progress in two projects: one on the cAMP-dependent protein kinase from yeast and another on the *Pvu*II restriction endonuclease. More details can be found in the individual sections below. These developments have helped us to expand the scientific personnel in our group significantly. Several new researchers joined the laboratory in 1990, and several more are expected in 1991. We therefore expect that in the coming months and years, the group will become increasingly productive in our investigations of the structures of kinases, DNA-binding proteins, oncogene products, and other macromolecules.

Our studies are designed to benefit from and to complement the genetic and biochemical experiments performed by our own group and by other groups at the laboratory and elsewhere. Together with these data, the crystallographic structures we determine will help us to understand the roles the macromolecules play in the normal day-to-day lives of cells, as well as in afflictions such as cancer, AIDS, and Alzheimer's disease.

We require well-formed crystals of the macromolecule being investigated. To grow crystals of a useful size (>0.3 mm on a side), we need several milligrams of the highly purified macromolecule. This does not guarantee that crystals can be produced, but

it is the minimal prerequisite before attempting to crystallize the molecule. A major part of our laboratory and time is devoted to producing and purifying to crystallographic homogeneity the proteins we are studying. When a suitable crystal is obtained, it is placed in an intense X-ray beam smaller than a pencil lead. The X-ray beams diffracted by the crystal are measured on our area detector. We must quantitate tens of thousands of these diffracted beams from several dozen different crystals. Fortunately, these experiments are computer controlled, with much of the software written in-house. Only after all the data are collected can we begin to construct a three-dimensional model of the macromolecule—all the atoms and bonds—with the aid of computer graphic tools.

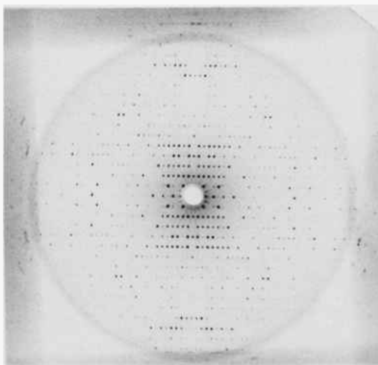

FIGURE 2 $\mu = 15^{o}$ precession photograph of the *h0l* plane of the diffraction pattern of R·*Pvu*II-DNA cocrystals. The resolution at the edge of the circular region is 3.0 Å. For this picture, CuK_{α} radiation of wavelength 1.5418 Å was used. Exposure was for 20 hr at 18°C. The crystal to film distance was 100 mm.

Structural Studies of *Pvu*II Endonuclease and Its Interaction with DNA

J. Anderson, F.-C. Chuan, J. Keller [in collaboration with I. Schildkraut, New England BioLabs, and R.M. Blumenthal, Medical College of Ohio]

We have cocrystallized the restriction endonuclease from *Proteus vulgaris*, R·*Pvu*II, with oligonucleotides carrying its recognition sequence 5′-CAGCTG-3′. The best crystals form with the 13-base-pair double-stranded oligonucleotide shown in Figure 1. These crystals are space group $P2_12_12_1$ with unit cell dimensions a = 95 Å, b = 85 Å, c = 48 Å, and they diffract to at least 2.8-Å resolution. A screened precession photograph of one plane of the diffraction pattern is shown in Figure 2.

We have collected data sets from native cocrystals and from isomorphous cocrystals grown with heavy atom derivative-1 oligo (see Fig. 1). Isomorphous derivative crystals are necessary in order to compute phases for the data. We are currently characterizing cocrystals grown with derivative-2 oligo; the preliminary indication is that these are also isomorphous. The heavy atoms for both derivatives are introduced by covalent modification of the oligo, substituting an iodine atom for the methyl group of thymine at certain positions in the sequence (see Fig. 1). This simplifies the task of locating the iodine atoms, and hence of calculating the phases, because we expect the conformation of the DNA in the crystal to be similar to the well-known structure of B-DNA. Once phases are determined, we will calculate an electron density map and begin building a model of the complex.

```
TGACCAGCTGGTC          native
 CTGGTCGACCAGT

TGACCAGCTGGUC          derivative 1
 CUGGTCGACCAGT

UGACCAGCTGGTC          derivative 2
 CTGGTCGACCAGU
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FIGURE 1 Sequences of the native and two derivative oligonucleotides used in R·*Pvu*II cocrystallization. The recognition sequence is underlined. The single-base overhangs serve to stabilize the interactions between adjacent protein-DNA complexes in the crystals. In the two derivative sequences, U = 5-iododeoxyuridine.

A more complete understanding of the enzymology of R·*Pvu*II will require the structure of the unbound protein. Although we are now concentrating on the crystals of the protein-DNA complex, we intend to pursue the structure of free R·*Pvu*II as well. We have crystals of R·*Pvu*II in the absence of DNA that diffract to better than 2.5 Å. We will prepare heavy atom derivatives of these crystals in the conventional way by soaking the crystals in various heavy atom solutions. If the structure of the complex

proceeds quickly enough, we may be able to use a model of R·*Pvu*II derived from it to calculate phases for the unbound protein data, eliminating the need for derivatization. Once structures for the complex and the free protein are in hand, it should be relatively easy to determine corresponding structures for mutant proteins and DNAs given to us by our collaborators, using difference Fourier analysis. This set of structures will provide much information about the structure and enzymatic activity of R·*Pvu*II.

Structural Studies of AP-1 Proteins

C.K. Cheung, F.-C. Chuan, J. Keller, E. Chang,
J. Anderson [in collaboration with T. Curran,
Roche Institute]

The protein products of the proto-oncogenes c-*fos*, c-*jun*, and a number of related genes form a major component of eukaryotic transcription factor AP-1 activity. Heterodimers of Fos-related and Jun-related proteins, and homodimers of Jun-related proteins, bind to DNA at AP-1 sites (5′-TGACTCA-3′) in the promoters of many genes. Once bound, they can activate or repress transcription, depending on the composition of the dimer and the context within which it binds DNA. We have overexpressed Jun- and Fos-related antigen FRA1 proteins from rat in *Escherichia coli* in order to carry out X-ray crystallographic studies of them and their complexes with DNA.

We have purified the Jun protein to 98% homogeneity by using the following protocol. Full-length Jun is found in the pellet of the bacterial lysate. The pellet is dissolved in 6 M urea, and chromatography on heparin-Sepharose is carried out in the presence of 6 M urea. After concentration of the protein in 6 M urea to about 10 mg/ml, the urea concentration is lowered to 0.6 M by diluting 0.1 ml into 1 ml of a binding buffer containing an oligonucleotide bearing an AP-1 site. Under these conditions, the protein renatures and forms complexes with the oligo. This mixture is then subjected to native polyacrylamide gel electrophoresis. Jun-oligo complexes are located by UV shadowing over fluorescent thin-layer chromatography plates and then excised from the gel. Elution from the gel slice yields approximately 1 mg of pure Jun. In analytical gel-shift assays, this purified Jun binds DNA containing AP-1 sites, including sites from the metallothionein promoter and from the human immunodeficiency virus type-1 (HIV-1) long terminal repeat.

Crystallization trials have been carried out with purified Jun, so far without success. One reason for this may be that the solubility of the protein is limited to 1–2 mg/ml in the absence of urea; much higher concentrations are usually required for crystallization. It is also possible that the conformation of the free protein is unstable or improperly renatured. Jun homodimers undergo a conformational change in the presence of DNA containing AP-1 sites (Patel et al., *Nature 347:* 572 [1990]), and the resulting conformation may be more stable. Consistent with this, we have found that renaturation and concentration of Jun in the presence of plasmid DNA carrying AP-1 sites permits protein concentrations of 10 mg/ml or higher to be attained, compared to 1–2 mg/ml without DNA. We believe that similar results will be achieved using AP-1-site-containing oligos, a prediction that we are about to test. If so, we will immediately set up cocrystallization attempts with the Jun-oligo complexes.

We are developing a purification procedure for the bacterially produced FRA1 protein and will attempt to prepare and characterize heterodimers of FRA1 and Jun and complexes of the heterodimers and AP-1 oligos. We will then attempt to crystallize the complexes. Determination of these heterodimer structures and Jun homodimer structures will help us to understand the role that these proteins play in normal cellular regulation and the role that altered versions of them play in oncogenesis.

Yeast cAMP-dependent Protein Kinase

J. W. Pflugrath, T. Malone, X. Cheng, J. Kuret,
A. O'Connor

Protein kinases are important and conserved components of many regulatory pathways because they can integrate several input signals and coordinate a response through the phosphorylation of a limited number of substrate proteins. The three-dimensional crystal structure of the cAMP-dependent protein kinase will allow us to define the structural basis of enzyme/substrate recognition and to assess the implications of this information for the protein kinase family in general.

We have crystallized the cAMP-dependent protein kinase catalytic subunit from *Saccharomyces cerevisiae*. The kinase catalytic subunit crystallizes in space group $P6_522$ with cell dimensions $a = b =$

61 Å, c = 320 Å. We have grown crystals as large as 0.6 x 0.6 x 1.5 mm that have diffracted to 2.2-Å resolution at the National Synchrotron Light Source.

We have collected partial X-ray diffraction data in our laboratory with a FAST area detector and rotating anode generator by rotating the crystals for 40° around the a axis with c^* initially oriented toward the beam at a crystal-to-detector distance of 70 mm. Initial screening in this way yielded a data set from p-chloromercuriphenyl sulfonate (PCMBS)-soaked crystals that showed a single mercury site in both isomorphous difference Patterson maps and anomalous Patterson maps. This derivative with its anomalous scattering constitutes the basis of our approach to solving the phase problem. To obtain complete data sets with reflections separated along l when c^* is oriented near the plane of the detector, we would need to use better optics with a relatively long crystal-to-detector distance of 320 mm with CuK_α radiation.

To overcome the limitations of our laboratory X-ray source, we used the facilities at beamline X12C at the Brookhaven National Laboratory National Synchrotron Light Source with the assistance of Dr. Robert Sweet. The optics and intense tunable radiation of X12C allowed us to use a wavelength of 0.95 Å and a crystal-to-detector distance of 360 mm to separate the reflections, minimize air absorption, and enhance the anomalous signal from the mercury atoms. More than 37,000 measurements from four kinase crystals soaked in PCMBS resulted in 9,613 unique reflections between 20 Å and 2.5 Å with an overall Rmerge of 6.1%. The data are 92% complete between 20 Å and 2.8 Å, and 93.5% complete in the 3.0–2.8-Å shell. Representative Harker sections of the anomalous Patterson map shown in Figure 3 clearly reveal the mercury site that had been found in a previous isomorphous difference Patterson map.

Single isomorphous replacement with anomalous scattering refinement gave us a trial set of phases that were then used to calculate a 3.2-Å resolution electron density map, a portion of which is shown in Figure 4. Regions of the map could be interpreted as α helices. Experiments are under way to improve the interpretability of the map.

Structural Studies of S100β

R. Hsu, T. Malone, J Kuret, J W Pflugrath
[in collaboration with D. Marshak, Cold Spring Harbor Laboratory]

Predominant among water-soluble brain proteins is S100β, an acidic 10.5-kD protein that contains the sequence requirements necessary to form two EF hand calcium-binding loops. Besides postulated calcium signal mediation function, S100β binds zinc very tightly and has been implicated as a neurite extension factor. In both Alzheimer's disease and Down's syndrome, elevated levels of S100β have been detected [Marshak et al., *Neurobiol. Aging* [1991] in press). We are expending considerable effort to determine the three-dimensional structure of S100β so that we can better understand the relationships among the many functions of this protein.

S100β was initially purified from bovine brain extracts that typically yielded 50 mg of protein in 5 days. Rough purity estimates from silver-stained SDS-PAGE revealed both monomeric S100β migrating as expected at about 10 kD and dimeric S100β as a 1% contaminant. Crystals that diffracted X-rays to 3.5-Å resolution were grown in the tetragonal space group $P4_12_12$ (or its enantiomorph) with unit cell a = b = 64 Å, c = 49 Å via the hanging drop microvapor

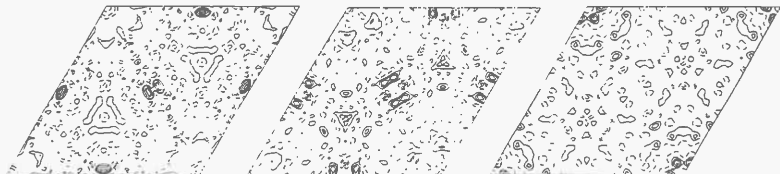

FIGURE 3 Harker sections (w = 1/6, w = 1/3, w = 1/2; u down 0 to 1, v across 0 to 1) contoured in intervals of standard deviations except the zero-contour level Solid lines denote positive levels, and dashed lines denote negative levels

diffusion technique in the presence of calcium and polyethylene glycol 400. When first set up in the hanging drops, the protein precipitated; crystals appeared only after 5–7 days.

Crystal analysis of bovine S100β was not pursued. First, crystallization experiments did not yield routinely reproducible crystals. Second, bovine brain extracts were oftentimes unwieldy and the purification was labor-intensive. Third, and most important, sequence analysis of the purified S100β revealed significant contamination by the associated protein S100α. Because of similarities in the properties (molecular weight, amino acid sequence, function) of the two proteins, we found it very difficult to purify them separately from brain extracts.

To overcome the above problems with bovine-brain-derived protein, we decided to overexpress S100β in bacteria where other S100 proteins were not present. Beginning with the cDNA for rat S100β (supplied by D. Marshak), we used the polymerase chain reaction to obtain the S100β gene flanked by useful restriction sites; the resulting fragment was ligated to a T7 expression vector in *E. coli*. When a late log phase bacterial culture growing at 37°C is induced with isopropyl-β-D-thiogalactoside, harvested, and lysed, S100β amounts to about 40% of the total soluble protein. The purification scheme consists of ammonium sulfate precipitation, a 2-chloro-10-(3-aminopropyl)phenothiazine affinity column, and a MonoQ ion-exchange column. We now obtain 15–20 mg of pure S100β from 1 liter of cells in 3 days free from S100α contamination—a major improvement over the brain preparations. Amino-terminal sequence analysis confirmed the sequence as that of rat S100β, and the removal of the initiator methionine in 25% of the S100β. We await activity analyses to ascertain the effect of overexpression in a bacterial system and the lack of amino-terminal acetylation, which is normally present in mammalian S100β.

Crystallization trials of the recombinant S100β showed that any combination of polyethylene glycol and calcium resulted in rapid and excessive precipitation of the protein. Although crystals could be obtained under conditions similar to those used for bovine S100β, the excessive precipitation led us to consider other conditions. Further experiments eventually provided crystals that grow as three-dimensional rectangular columns 0.6 to 0.8 mm long in about 1 day, without any hint of our previous precipitation problems. These crystals do not diffract very well, and thus we will continue to experiment with the crystallization conditions.

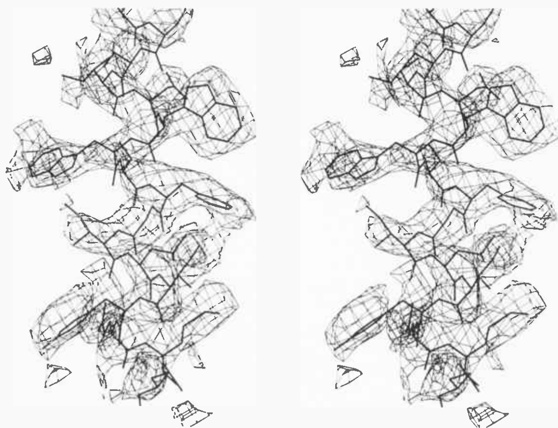

FIGURE 4 Stereoplot of a portion of the electron density map calculated from 3 2-Å resolution SIR-AS phases showing residues 182–197 of TPK1Δ with sequence KSIDWWSF-GILIYEML

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PROTEIN KINASE STRUCTURE AND FUNCTION

J. Kuret D. Adelman T. Mitcheson
A. Desai A. O'Connor

During 1990, this laboratory saw the arrival of three talented young investigators: Arshad Desai from California State University at Hayward, Thomas Mitcheson from Eton College in England, and David Adelman from Commack High School right here on Long Island. After joining the "firm" of Kuret & O'Connor, all three began work on protein kinases to learn more about this important family of regulatory molecules. In an effort to determine the common three-dimensional structure that kinases adopt, and the implications of this structure for selective recognition of protein substrates and catalysis of the phosphotransferase reaction, Arshad applied his energy (mostly nocturnal) to the study of TPK1, the *Saccharomyces cerevisiae* homolog of the cAMP-dependent protein kinase (cAMPdPK) catalytic subunit. This kinase, along with its isozymes, regulates the ability of that organism to grow. Meanwhile, David, as part of Cold Spring Harbor Laboratory's Partners for the Future program, addressed the biological function of casein kinase I, an enzyme found in all eukaryotic cells in which it has been sought. It is unrelated to casein kinase II, which is described elsewhere in this volume. Late in the year, Tom also began work on this enzyme, adding enthusiasm and a keen understanding of tea and its uses. Our progress in combining the techniques of X-ray crystallography and molecular genetics in the study of protein kinases is described below.

Yeast Casein Kinase I

J. Kuret, D. Adelman, T. Mitcheson, A. O'Connor

As the name implies, casein kinases are enzymes that phosphorylate casein (an acidic, heavily phosphorylated family of proteins found normally in milk) efficiently. Although casein is an unlikely target for casein kinases in living cells, it is a useful and surprisingly specific tool in the isolation, assay, and characterization of these enzymes because few of the many protein kinases cloned or purified are capable of phosphorylating it. Two classes of casein kinases termed casein kinase I (CK-I) and casein kinase II (CK-II) are recognized in eukaryotic cells. Although progress is being made on CK-II (described by Dan Marshak in the Tumor Viruses Section), the structure, intracellular location, and biological function of CK-I have been a complete mystery. Our goal is to discover the biological role of CK-I using the yeast *S. cerevisiae* as a model system.

Last year, we purified a novel form of CK-I from yeast using classical methods and described some of its biochemical properties in solution. Like the enzyme isolated from mammalian and other sources, the yeast enzyme is a monomer with a very alkaline isoelectric point. After purifying large quantities of yeast CK-I, we partially proteolyzed the enzyme with trypsin and chymotrypsin, separated the resulting

TABLE 1 Comparison among Carboxy-terminal Regions of Yeast Casein Kinase-I and Low-molecular-weight GTP-binding Proteins

Locus	Species	Sequence
YPT1	Sc	TGGGCC
SEC4 (*YPT2*)	Sc	SKSNCC
ypt3	Sp	SSSQCC
SAS1	Dd	KKKACC
SAS2	Dd	KKNTCC
CKI1	Sc	SKLGCC

Sc = *Saccharomyces cerevisiae*. Sp = *Schizosaccharomyces pombe*. Dd = *Dictyostelium discoideum*

peptide fragments by gel electrophoresis, isolated the products by blotting them onto membranes, and finally subjected them to automated Edman degradation (performed by the Protein Chemistry Core facility). The internal amino acid sequence information we obtained as a result was used to design an oligonucleotide probe suitable for screening a yeast cDNA library we had prepared earlier in bacteriophage λ. Thus, we obtained the full-length clone of yeast CK-I.

Examination of yeast CK-I primary structure reveals some interesting features. The sequence begins with about 80 hydrophilic amino acid residues, followed by a canonical protein kinase domain. This is followed by a long stretch of hydrophilic residues that are predicted to be highly flexible in the folded protein. Finally, the protein chain terminates with a short, hydrophobic sequence. Closer inspection of the hydrophobic carboxyl terminus reveals a striking homology with members of the low-molecular-weight GTP-binding protein family (Table 1). In these proteins, the sequence is associated with lipid modification and intracellular membrane localization. Thus, we predict that a hydrophobic, lipoprotein "tail" anchors the CK-I protein kinase domain to intracellular membranes via a hydrophilic and flexible "tether" (Fig. 1). This organization is undoubtedly important for facilitating access of CK-I to physiological substrate proteins. In the coming year, we hope to test this hypothesis by identifying the nature of the posttranslational lipid modification and the consequences of that modification for CK-I localization. In addition, we plan to employ the genetic tools available in yeast to obtain the first indications of the biological function of CK-I.

Yeast cAMP-dependent Protein Kinase

J. Kuret, A. Desai, A. O'Connor [in collaboration with J. Pflugrath and X. Cheng, Cold Spring Harbor Laboratory]

Despite clear amino acid sequence homology among members of the eukaryotic family of protein kinases,

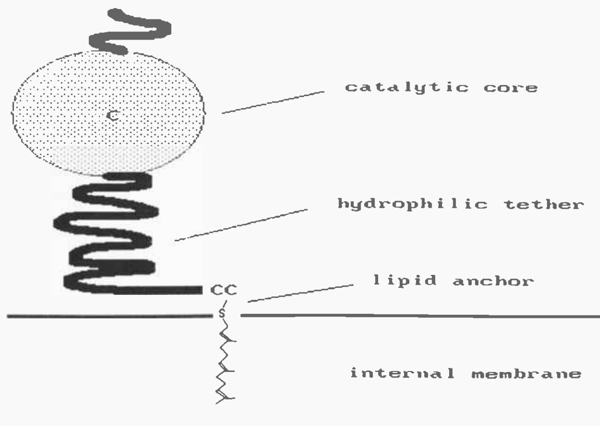

FIGURE 1 Proposed model of yeast CK-I structure.

and the probable existence of a common enzymatic mechanism of phosphoryl transfer, each protein kinase possesses a unique (although potentially overlapping) recognition selectivity for protein substrates. For the handful of kinases studied in detail, the ability of a protein to serve as a substrate is determined by the amino acid sequence surrounding its phosphorylatable hydroxy amino acid (i.e., serine, threonine, or tyrosine), with the location and spacing of charged amino acids being especially important. Because we wish to understand the overall folding pattern of eukaryotic protein kinases and to identify the structural features of kinases that are involved in catalysis, regulation, and substrate selectivity, we initiated a crystallographic study of the well-characterized cAMPdPK catalytic subunit from yeast, TPK1. We chose this enzyme for study because (1) its gene has been cloned (by Mike Wigler's laboratory), (2) mutant proteins with altered catalytic properties can be obtained through genetic screens in yeast, (3) it can be overexpressed in a soluble form in yeast, and (4) it retains the well-characterized structural and kinetic features of the mammalian enzyme. Thus, the yeast system allows us to study protein kinase enzymology with diverse techniques.

Last year, we reported the overexpression, purification, and crystallization of a truncated version of TPK1, termed TPK1Δ. The crystals grow to more than 1 mm in length, are hexagonal dipyramids in shape (Fig. 2), and diffract beyond 2.8-Å resolution. Our efforts this year have been directed toward obtaining heavy atom derivatives of our crystals so that we can solve the structure of TPK1Δ by multiple isomorphous replacement. The method involves harvesting a fresh crystal, soaking it in a dilute solution of heavy metal ions such as mercury or platinum, and assaying for their incorporation by inspection of the soaked crystal's X-ray diffraction pattern. A Patterson map showing the successful derivitization of TPK1Δ with a mercury compound can be found in the preceding section of this report (Macromolecular Crystallography). The map is dominated by families of concentric circles marking the position of electron-dense mercury atoms.

We are pursuing two principal strategies to obtain the additional derivatives needed to solve the structure of TPK1Δ. The first is to continue soaking crystals in different heavy atom solutions in the hope of finding a new compound that binds to a novel site. The second is to reengineer TPK1Δ to provide additional heavy-atom-binding sites. This is accomplished by modifying the cysteine content of the protein through site-directed mutagenesis, crystallizing the resulting mutant, and soaking it in heavy atom solutions as described above. Because the bond between mercury and the sulfhydryl group of cysteine is essentially covalent, cysteines are efficient heavy atom binders. By the end of 1990, we had prepared more than one dozen such mutants. In the coming year, we plan to analyze these mutants and to create still more to obtain our necessary derivatives.

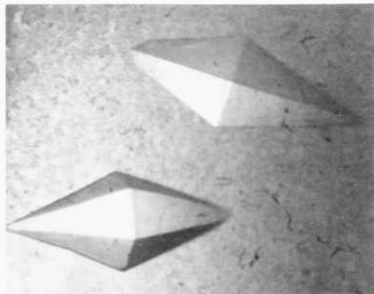

FIGURE 2 Crystals of TPK1Δ.

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COMPUTATIONAL MOLECULAR BIOLOGY

T. Marr D. Lombardi

This is a new discipline in molecular biology that merges the arts and crafts of scientific computing with the arts and crafts of molecular biology. Computational Molecular Biology is not unlike other disciplines that arose quite naturally in molecular biology, such as nucleic acid chemistry and the biophysics of protein crystallography. The two primary goals of our group are (1) to establish a strong program in basic research in computational molecular biology, with an initial focus on the development of mathematical methods for constructing molecular maps of DNA and new methods for the retrieval, analysis, display, and manipulation of molecular sequence data, and (2) to enhance and develop computer technologies that help the Laboratory maintain its research excellence.

A Comprehensive Computing Environment for Molecular Biology and Genetics

T. Marr

In collaboration with Andrew Reiner, a computer scientist and consultant to the Jackson Laboratory, design work has progressed on a new computer system architecture for retrieval, analysis, and display of molecular biology and genetic data. It is based on a new type of software technology known as object-oriented technology. Funding for this effort begins in May of 1991, at which time Andy will be joining the computational biology group as a research computer scientist. Our goals are listed below.

1. Design and implement a system architecture for the management, analysis, and display of molecular genetics information and augment this environment to support the creation of new management, analysis, and display techniques, and the enhancement or retargeting of existing ones.
2. Develop a database environment that is community-driven and investigator-maintained. In other words, this environment will support the innovative investigator who is not fortunate enough to have institutional access to the expensive resources (i.e., qualified people) that traditionally have been necessary to develop a readily accessible research resource.
3. Develop an infrastructure to support the transfer of research findings from mathematicians and computational scientists into mainstream biological research.

A Computerized Laboratory Information Management System for Molecular Biologists

D. Lombardi, T. Marr [in collaboration with D. Beach, Cold Spring Harbor Laboratory]

This project is aimed at computerization of most of the day-to-day operations of information collection and management of the laboratory of David Beach (Genetics Section). We expect to use this system as a general model for many experimental molecular biology laboratories. Our first goal is to get a fully functional system going in the Beach laboratory, and then we will expand the system to include other laboratory operations at Cold Spring Harbor and elsewhere, for example, with collaborators at other locations. This system includes an intuitive graphical interface for data entry and retrieval. The system is now at the prototype and testing phase and includes provision for managing the fission yeast strain collection, clone tracking, probe tracking, monoclonal and polyclonal antibodies, proteins, and molecular sequences. The system runs in client-server mode over the Laboratory's local area network and is implemented in a relational database management system, Sybase, running on a Sun/Unix server, with Macintosh computers running the Claris/Hypercard and Sy-base/-DBlib software. This way, users can use the highly "user-friendly" Macintosh as the interface into the system, with the complexity of powerful, but "user-unfriendly" Unix workstations "hidden" from them. The central database can be accessed simultaneously from several locations with the Beach laboratory.

Committees and Workshops

T. Marr

This was a busy year in terms of national scientific research service activities. I was invited by the Director of the National Institutes of Health (NIH) to be a member of the first chartered research review committee for the National Center for Human Genome Research. This committee is responsible for the scientific review of research and training grant proposals for the National Institutes of Health Human Genome activities. The first year of review of proposed National Institutes of Health Genome Centers was particularly demanding. I site-visited and reported on five of the nine proposed Centers, as well as serving on the Genome Center Parent Committee and the Large-Scale DNA Sequencing study section. It is important, for long-term success of this emerging major national scientific effort, to get limited dollars into the hands of the groups qualified best to tackle the new-to-biology multidisciplinary nature of the human genome program.

I also served on the National Academy of Science committee "DNA Technology in the Forensic Sciences." This committee examined and has written a report on the underlying scientific principles and the societal impact of the use of "DNA fingerprinting" in court cases involving violent crimes against members of our society. This report is scheduled for release in the spring of 1991.

At the Banbury Center, I was coorganizer of a workshop entitled "Issues in Training Computational and Mathematical Biologists." This workshop was sponsored by the National Science Foundation and was aimed at examining how to meet the current and future need for computational and mathematical biologists. These scientists are needed to work on new classes of problems in biology that require substantial cross-disciplinary skills, such as global warming, human genome, neuroscience, and structural biology.

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NEUROSCIENCE

Neuroscience at Cold Spring Habor Laboratory has focused on the role of growth factors in the differentiation and aging of neuronal cells. For some years, the research has primarily centered on a glial-derived protein, S100β, that acts as a neurotrophic factor for embryonic cerebral cortical neurons. Abnormal levels of this protein have been seen in Alzheimer's disease, in proximity to the amyloid plaques that are characteristic of this degeneration. We are also pursuing the role of this factor in Down's syndrome, a developmental neurological disease involving chromosome-21 abnormalities. This year, our research has taken us into the realm of protein kinases, as we probe the mechanisms of action of growth factors of the nervous system, and as we try to understand the molecular basis of neuronal differentiation. As we look forward to the new Neuroscience Center at Cold Spring Harbor, our studies continue to probe the fundamentals of growth and differentiation of neurons, particularly pertaining to human neurological diseases.

NEURONAL GROWTH AND DIFFERENTIATION

D.R. Marshak E. Azmitia, Y.-S. Bae, N. Chester, S. Pesce, A. Wilson

During the last 5 years, the work in our laboratory related to neuroscience has centered on the outgrowth of processes, or neurites, from embryonic neurons in cell culture. This system serves as a model for the development of neurons in vivo, by allowing us to isolate and characterize a neurite extension factor. We have characterized the structure and function of the factor, and we have developed reagents to permit analysis of brain tissue taken on autopsy from patients with several neurological diseases. Newer studies involve other growth factors of the nervous system, including a substrate-attachment factor that is homologous to the family of fibroblast growth factors (FGFs). Our long-term projects aim at discovering the mechanisms of action of neuronal growth factors, including changes in signal transduction pathways, such as protein kinases, that stem from neuronal growth.

Role of S100β in Neurological Diseases

D.R. Marshak, S. Pesce [in collaboration with W.S.T. Griffin, University of Arkansas]

A significant segment of the population over 65 years of age is afflicted with Alzheimer's disease (AD), a neurodegenerative process that results in memory loss and progressive dementia. The hallmarks of AD neuropathology are prevalent in temporal lobe structures, such as the hippocampal formation, that have been associated with learning and memory. These neuropathological symptoms include abnormal numbers of neurons that contain neurofibrillary tangles, extracellular plaques with β-amyloid cores, and reactive astrocytes. A puzzling feature of plaque pathology is the presence of enlarged neurites encircling the

β-amyloid deposits. The presence of soluble neurotrophic factors could give rise to inappropriate growth of neurites. In support of this idea, we have shown that the levels of a neurotrophic factor, the protein S100β, and its encoding mRNA are elevated in AD, but not control, brain temporal lobe. Moreover, AD temporal lobe extracts had more S100β-specific neurotrophic activity than similar extracts from age-matched controls. We have suggested that elevated levels of S100β contribute to the cellular neuropathology in AD.

Down's syndrome (DS) is a developmental disorder arising from extra copies of all or part of human chromosome 21, either by trisomy, partial duplication, or translocation. In addition to mental retardation, DS patients that live to middle age invariably develop a degenerative neurological disorder that is indistinguishable from AD. This is further supported by the findings that genes related to AD reside on chromosome 21, although distinct from the DS regions. Because the gene for S100β is located on chromosome 21 near the telomere of the long arm, we tested samples of DS autopsy brain for S100β levels. It appears that immunoreactive levels of S100β are elevated in DS early in life, reaching fivefold over normal levels at 3 months and decreasing to normal levels by 9 months. This correlation holds for both frontal and parietal cortex in 21 normal and DS samples. We are trying to obtain tissue from DS patients of other ages, including middle age, elderly, and fetal, in an attempt to identify the critical time of S100β elevation in human brain development.

Molecular Cloning and Expression of S100β

D.R. Marshak, A. Wilson, N. Chester

The coding region of the cDNA clone was isolated as a 916-bp *Bam*HI fragment and subcloned into pBS+. Restriction maps of this construct indicated that the insert was oriented with the 5′end of the complementary strand under control of the T3 promoter. Synthesis of RNA probes using the T3 polymerase resulted in positive slot blots and Northern blots of brain RNA. These RNA probes have been very useful in quantitating mRNA levels in AD and DS autopsy brain samples. In addition, A. Wilson has probed primary human fibroblast extracts of RNA to determine if the age of the cells in culture was related to S100β expression. C. Greider (Genetics Section) and colleagues previously described the phenomenon of telomere shortening in aging fibroblasts. Because the gene for S100β is very close to the telomere of the long arm of chromosome 21, we considered that the telomeric shortening might affect S100β gene expression. In preliminary experiments, little S100β could be detected in fibroblasts at either early or late stages in culture. However, this project is ongoing and promises to provide unique information concerning the relationship between a telomere and a nearby single-copy gene in the human genome.

Transgenic mice are being constructed through a collaboration with Dr. Charles Epstein (University of California, San Francisco). These mice may provide an animal model for DS. We are also investigating trisomy-10 mice (with Dr. Charles Epstein, UCSF) that have an extra copy of the S100β gene and severe neurological defects. Present experiments revolve around the construction of expression plasmids that utilize tissue-specific promoters for specific populations of cells in the brain.

The cDNA probe has also been used in collaboration with Dr. Kathleen Gardiner (Eleanor Roosevelt Institute, Denver) to confirm the localization of the gene and continue fine-structure mapping of chromosome 21. Further mapping has been done in collaboration with J. Korenberg (Cedars Sinai Medical Center, Los Angeles). Patients have been studied that have selected symptoms of DS, but who have a circular fragment of additional chromosome 21, rather than a full trisomy 21. We are attempting to investigate the question of whether these circular chromosomes still contain the S100β gene or if recombination has occurred proximal to the gene. In either case, these results will allow us to study the contribution of the S100β gene dosage to DS in humans.

Role of S100b In Vivo

E. Azmitia, D.R Marshak, S Pesce [in collaboration with P. Whitaker, SUNY Stony Brook]

We have begun to examine the role of S100β in vivo using the rat model system. The findings of Drs. Whitaker and Azmitia indicate that serotonergic neurons of the brain stem that innervate the hippocampus appear to respond to S100β as a neurotrophic factor. This, combined with our findings of increased S100β in temporal lobe areas including

the hippocampus, suggests that S100β may play a key role in the normal development of contacts between serotonergic neurons of the Raphe nuclei and neurons of the hippocampus. Abnormal levels of S100β either during development (as in DS) or in aging (as in AD) may lead to errors or lesions in these connections. We have demonstrated that serotonergic agonists can specifically release S100β from glial cells that surround the neurons of the hippocampus, indicating that the neurotransmitter compound itself might regulate the relase of the neurotrophic factor in the developing brain. Further studies indicate that chemical lesion of the connections between the brain stem and the hippocampus results in up-regulation of serotonin receptors and sensitivity to S100β release by serotonergic agonists. Injection of radioactively labeled S100β into the hippocampus indicates that much of the S100β released is associated with hippocampal neurons. Further studies are in progress to map the pathways and pharmacology of S100β action in vivo that may reveal some of the characteristics of S100β function in normal brains and in diseased states.

Structural Analysis of Neurite Extension Factor

D.R. Marshak [in collaboration with J. Pflugrath, Cold Spring Harbor Laboratory]

Further analyses of the three-dimensional structure of the S100β monomer and dimer are being accomplished by solving the X-ray crystal structure. In collaboration with Dr. Jim Pflugrath (Structure Section), we have grown crystals of S100β suitable for X-ray analysis. These crystals diffract to 2.5 Å and have the P_{21} space group. These crystals were difficult to reproduce consistently, so we turned to recombinant S100β for large quantities of protein. The cDNA clone was inserted into a T7 expression system for large-scale expression in *Escherichia coli*. Material expressed in these bacteria can be purified to apparent homogeneity in a few days, and crystals have been grown from this fresh, recombinant material. Dimensions of the crystals are larger than those from the bovine brain material, and the space group and unit cell appear to be different. These crystals will be used to solve the complete structure of S100β in the coming year.

We have also succeeded in synthesizing the protein chemically using solid-phase methods on an automated instrument. The protein has 91 amino acids and took approximately 4 weeks to synthesize after several initial failures. Purification and characterization of the product are now under way. Preliminary results indicate that the synthetic product is biologically active and can be promoted to form dimers in vitro. We would like to compare the three-dimensional structures of the native, synthetic, and dimerized forms of S100β.

Regulation of Brain Protein Kinases

Y.-S. Bae, D R. Marshak

The action of growth factors in the nervous system has been implicated in the onset of symptoms of neurological disease, such as AD. We have begun to study the regulation of the signal transduction systems that are stimulated by growth factors in the brain. In particular, we have purified casein kinase II (CK-II) from bovine brain to examine the stimulation and repression of the activity. Brain CK-II appears to have an endogenous inhibitor as well as compounds that can activate the enzyme. Our current research focuses on the isolation and characterization of molecules that regulate this kinase. CK-II is important to transcriptional control, and it is possible that alterations in CK-II levels or activity is associated with degeneration of neurons. In other studies, we have analyzed the protein kinase $p34^{cdc2}$ in cells that can become neurons in culture, rat PC12 cells. Upon treatment with the nerve growth factor (NGF), these cells differentiate into sympathetic, peripheral neurons in culture. This appears to be accompanied by changes in the expression of $p34^{cdc2}$ and in its activity as a protein kinase. One potential target of phosphorylation, tyrosine hydroxylase, has been studied. In vitro, $p34^{cdc2}$ can phosphorylate tyrosine hydroxylase, and the site and consequences of this phosphorylation are under investigation.

Analysis of a Neurotrophic Heparin Binding Growth Factor

D.R. Marshak [in collaboration with W.H. Burgess, American Red Cross Research Center, Maryland]

During the past year, we have been fortunate to be involved in the analysis of several proteins related to

growth in human physiology. In collaboration with a group at the American Red Cross Research Center in Maryland, we characterized a new type of neurotrophic factor, HB-GAM. This small protein is homologous to the family of fibroblast growth factors (FGFs), but contains a high proportion of lysyl residues. This highly basic protein was isolated from bovine brain at neutral pH, and the complete sequence of the protein was determined by chemical analysis and by plasma desorption mass spectrometry. In biological assays, the protein does not appear to be active as a fibroblast or endothelial cell mitogen, but it is highly active in neurotrophic assays. In particular, when HB-GAM is coated onto the surface of cell culture matrix, the factor promotes neurite outgrowth from chick cortical neurons. As a soluble factor, HB-GAM does not significantly promote extension above that done as an attachment factor. These results suggest that this FGF family member may act as a neurite outgrowth factor by substratum attachment, working in concert with soluble growth factors in the brain.

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CSH LABORATORY JUNIOR FELLOWS

In 1986, Cold Spring Harbor Laboratory began a new program to encourage independent research by outstanding young scientists who, during their graduate studies, displayed exceptional promise of becoming leading scientists of the future. The purpose of this program is to provide an opportunity for each Fellow to work independently at the Laboratory for a period of up to 3 years on projects of his/her choice. The Fellow is provided with a salary, research support, and technical assistance so that they can accomplish their goals free from other distractions. The interaction among research groups at the Laboratory and the program of courses and meetings on diverse topics in molecular biology contribute to a research environment that is ideal for innovative science by these Fellows.

Two previous Cold Spring Harbor Fellows, Dr. Adrian Krainer (1987) and Dr. Carol Greider (1988), are currently members of the scientific staff at the Laboratory. The most recent Fellow, Dr. Eric Richards, joined us in 1989 from Fred Ausubel's laboratory at the Massachusetts General Hospital. Dr. Richards is studying the molecular biology of the centromeres and telomeres of the plant *Arabidopsis thaliana.*

E.J. Richards

Molecular Chromosome Studies in *Arabidopsis thaliana*

E.J. Richards, A. Vongs, S. Chao

We are currently pursuing three related projects by investigating different aspects of plant molecular chromosome structure and genome organization using *Arabidopsis thaliana* as a model system. The continuing projects concern the characterization of genomic DNA organization at centromeric and telomeric regions of plant chromosomes. In a recently initiated project, we are looking at a different aspect of genome organization: the role of DNA methylation.

CHARACTERIZATION OF TELOMERIC DNA FROM *A. THALIANA*

Plants possess G-rich simple-sequence repeats at their chromosome ends similar to those originally described in unicellular eukaryotes (Richards and Ausubel, *Cell 53:* 127 [1988]). Since the establishment of the ubiquity of telomeric repeats, much of the focus in the telomere field has turned to the genomic regions that lie just proximal to the G-rich repeats. Such telomere-associated sequences are commonly composed of repeated DNA sequences that exhibit variability in terms of DNA structure and chromosome distribution (see, e.g., Horowitz et al., *Mol. Cell. Biol. 4:* 2509 [1984]; Brown et al., *Cell 63:* 119 [1990]). We are currently characterizing telomere-associated sequences present on eight *A. thaliana* telomere clones recently isolated by selection for plant DNA fragments that function as telomeres on yeast artificial chromosome (YAC) vectors.

We have analyzed one of these clones in detail. As expected, the clone contains a block of $[TTTAGGG]_n$ corresponding to the most distal chromosome end. Numerous variant repeats are also found, particularly TTAAGGG, at the centromere-proximal edge of the terminal simple-sequence block, indicating that telomeric repeats diverge as one moves away from the chromosome end. The region adjacent to the telomeric repeat block corresponds to single-copy DNA in the Landsberg strain of *A. thaliana* from which the clone was derived. A domain of the characterized flanking sequence also

cross-hybridizes to an additional telomere-flanking region in a different *A. thaliana* strain, Columbia. As noted above, such variable distribution of telomere-associated sequences is also found in fungi and mammals and is thought to arise by recombination between nonhomologous chromosome ends.

Through analyses such as these, we hope to complete the molecular description of the chromosome ends and provide useful landmarks for the chromosome ends on the physical and genetic map.

CHARACTERIZATION OF CENTROMERIC DNA FROM *A. THALIANA*

We are interested in identifying and characterizing the DNA sequences required for centromere function as a first step toward understanding how centromeres work. At present, functional centromeric DNA sequences have been identified in only two systems, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. The picture that emerges from centromere studies in the yeasts is rather confusing; the budding yeast centromeres are quite small (120 bp) and do not resemble the large (50–100 kb) arrays of repetitive DNAs that make up *S. pombe* centromere regions (Clarke, *Trends Genet. 6:* 150 [1990]). Although it is generally thought that the *S. pombe* centromeres may provide a simple model for the larger, more structurally complex centromeres of higher eukaryotes, we have decided to begin characterizing a higher eukaryotic centromere directly.

Our approach to isolating *A. thaliana* centromeric DNA is similar to that first used to clone yeast centromeres: begin with molecular markers that flank a centromere and walk across the gap. The centromere of *A. thaliana* chromosome 1 is the best target, since it is the most precisely mapped, and several genetic and restriction-fragment-length polymorphism (RFLP) markers are closely linked to this centromere.

Our current picture of the *cen1* region, shown at the bottom of Figure 1, is based on RFLP analysis of

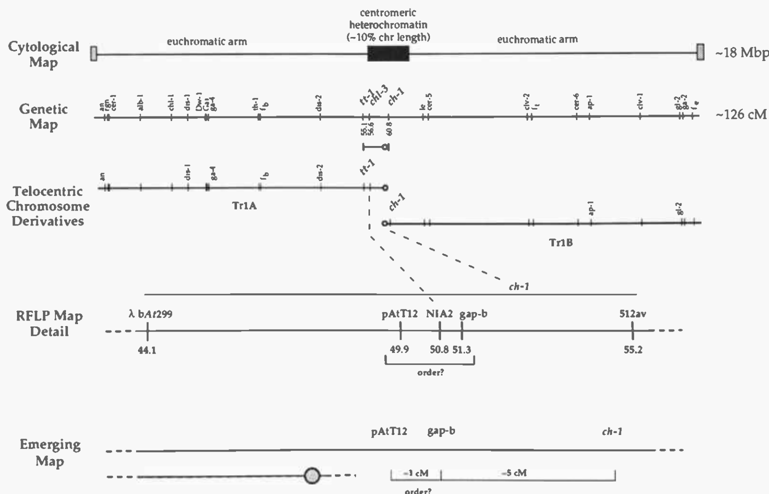


FIGURE 1 Cytological (Schweizer et al., *Arabid. Inf. Serv. 25:* 27 [1987]) and genetic (Koornneef, *Genetic Maps*, Cold Spring Harbor Laboratory Press [1978]) maps of *A. thaliana* chromosome 1. The approximate position of *cen1* relative to classical genetic markers was determined by Koornneef (*Genetica 62:* 33 [1983]) by analyzing crosses between marked strains and telotrisomic plants carrying the telocentric chromosome derivatives. The correlation between the classical genetic map and the RFLP map (Nam et al., *The Plant Cell 1:* 699 [1989]) is indicated by the dashed lines. Our present refinement of the map is shown at the bottom of the figure.

segregating F_2 populations of an interstrain cross, where one parent carries an additional telocentric chromosome 1 derivative (Tr1A Landsberg x disomic Columbia). Distortion of RFLP allele ratios in F_2 indicates that the marker in question is located on the A arm of the chromosome and, consequently, to the "left" of the centromere as drawn in Figure 1. At present, we have placed the gap-b and pAtT12 RFLP markers to the "right" of the centromere on the B arm, since alleles of these loci segregated normally. We are currently determining the position of the other known markers in the region in order to find a useful molecular probe that flanks *cen1* on the A arm.

Flanking markers will then be used to construct large-scale primary restriction maps of the region, providing a low-resolution picture of the size and DNA organization at the centromere. Such information will supply a foundation for the next phase: isolation of centromeric sequences in YAC vectors and functional analysis of these sequences.

DNA HYPOMETHYLATION MUTANTS

In parallel with its small genome size, *A. thaliana* has extremely low levels of methylcytosine (approximately 5%) for an angiosperm (Leutwiler, *Mol. Gen. Genet. 194:* 15 [1984]). Most of the methylated residues are sequestered into the highly repeated DNA fraction (e.g., rDNA and satellite repeats) thought to be localized in heterochromatic regions of the chromosomes. To assess the role DNA methylation plays in chromosome structure and mechanics (e.g., heterochromatization and segregation), we have begun a collaboration with Rob Martienssen (Genetics Section) to characterize *A. thaliana* mutants with reduced levels of cytosine methylation.

At present, three hypomethylation mutants have been recovered from M2 populations of EMS-mutagenized seed by screening for plants that have satellite arrays susceptible to digestion by methylation-sensitive endonucleases. Both satellite arrays and rDNA are hypomethylated in the mutants, but methylation at some single-copy sequences is unaffected. Supporting this observation, biochemical analysis of global methylcytosine levels indicates that the mutants still contain appreciable quantities of methylcytosine (20% of wild type in the most hypomethylated mutant). No morphological phenotype has yet been associated with hypomethylation.

We are currently investigating whether the hypomethylation mutants have any defects in chromosome ultrastructure or behavior. In addition, we plan to look for possible effects of hypomethylation on rDNA transcription, expression of "silenced" ectopic insertions, and transposition in order to evaluate the role DNA methylation plays in these general processes.

In Press, Submitted, and In Preparation

Richards, E.J., S. Chao, and A. Vongs. 1991. Isolation and characterization of telomere-associated sequences from *Arabidopsis thaliana*. (In preparation.)

Richards, E.J., W.D.B. Loos, J. Giraudat, H.M. Goodman, and F.M. Ausubel. 1991. The centromere region of *Arabidopsis thaliana* chromosome 1 contains telomere-similar sequences. (Submitted.)

COLD SPRING HARBOR MEETINGS

55th COLD SPRING HARBOR SYMPOSIUM ON QUANTITATIVE BIOLOGY

The Brain

May 30–June 6, 1990

ARRANGED BY

James D. Watson, Cold Spring Harbor Laboratory
Eric D. Kandel, Columbia University College of Physicians & Surgeons
Charles Stevens, Yale University
Terrance Sejnowski, Salk Institute

281 participants

Over 280 outstanding scientists from research centers in Europe, the Far East, and the United States gathered at Cold Spring Harbor to present their latest ideas and data on the central question in neurobiology: How does the brain work? The program included 105 speakers who explored such fundamental processes as vision, olfaction, learning, memory, and motor control. These scientists, acknowledged leaders from such diverse areas in neurobiology as molecular neurobiology, systems neurobiology, and brain function modeling are attempting to solve similar problems with different approaches. The meeting provided an opportunity to illustrate how these different approaches complement and reinforce each other.

J.D. Watson, E. Watson, L.A. Hazen, F. Crick

Opening presentations by Shosaku Numa, Martin Raff, Sten Grillner, Will Newsome, and Marcus Raichle outlined the themes of the symposium. One of the highlights of the meeting was the Dorcas Cummings Lecture "How Do We See Things? " given by Francis Crick of the Salk Institute. An excellent summary talk of the meeting was given by Michael Stryker of the University of California, San Francisco. The conference resulted in productive scientific exchange and was a fine way to begin the congressionally proclaimed "Decade of the Brain."

H. Damasio, T.J. Sejnowski, C. Gilbert

J. Byrne, C.F. Stevens

C Shatz, F. Bonhoeffer, C. Blakemore, M Raff, P. Patterson

The meeting was supported in part by the National Institute of Mental Health (an Institute of the Alcohol, Drug Abuse, and Mental Health Administration); the National Cancer Institute, the National Institute of Child Health and Human Development, and the National Institute of Neurological Disorders and Stroke (all divisions of the National Institutes of Health); the U.S. Department of Energy; and the National Science Foundation.

PROGRAM

Welcome: J.D. Watson

Opening Remarks
Chairman: W.M. Cowan, *Howard Hughes Medical Institute, Bethesda*

Synaptic Mechanisms
Chairman: P. Andersen, *University of Oslo*

Vision
Chairmen: D.H. Hubel, *Harvard Medical School*
T.N. Wiesel, *Rockefeller University*

Learning and Memory
Chairman: S. Benzer, *California Institute of Technology*

Ion Channels
Chairman: L. Jan, *University of California, San Francisco*

Cognitive Neuroscience: Attention
Chairman: F. Crick, *Salk Institute*

Cell Adhesion and Recognition
Chairman: G.M. Edelman, *Rockefeller University*

Motion Detection, Stereopsis, and Neural Codes
Chairman: R.H. Wurtz, *National Eye Institute, National Institutes of Health*

Cognitive Neuroscience: Memory
Chairman: P.S. Goldman-Rakic, *Yale University School of Medicine*

Synaptic Plasticity
Chairman: T.V.P. Bliss, *National Institutes for Medical Research*

Dorcas Cummings Lecture
Speaker: F. Crick, *Salk Institute*

Motor Control
Chairman: E. Bizzi, *Massachusetts Institute of Technology*

Snyapse Formation and Fine Tuning
Chairman: J.P. Changeux, *Institut Pasteur*

Remodelling
Chairman: C.J. Shatz, *Stanford University School of Medicine*

Control of Cell Identity
Chairman: T.M. Jessell, *Columbia University College of Physicians & Surgeons*

Sensory Processing
Chairman: H.R. Horvitz, *Massachusetts Institute of Technology*

Cognitive Neuroscience: Perception
Chairman: V.B. Mountcastle, *Johns Hopkins University School of Medicine*

Summary: M.P. Stryker, *University of California, San Francisco*

Genome Mapping and Sequencing

May 2–May 6, 1990

ARRANGED BY

Charles Cantor, Human Genome Center, Lawrence Berkeley Laboratory
Maynard Olson, Washington University School of Medicine
Richard Roberts, Cold Spring Harbor Laboratory

300 participants

The 1990 meeting on Genome Mapping and Sequencing was the third in what appears destined to become a continuing series. The meeting focused on the current techniques for genome mapping and sequencing and highlighted some of the systems in which great progress is being made. Throughout both the formal presentations and the posters sessions, it was clear that the polymerase chain reaction (PCR) is playing a dominant role in almost all aspects of the field. The use of sequenced tagged sites (STSs) as landmarks for physical mapping studies is becoming popular and will greatly facilitate information exchange between mapping laboratories. Yeast artificial chromosomes (YACs) are proving very popular as vectors for the isolation and characterization of large pieces of DNA. Nowhere was this more apparent than in the mapping of the *Caenorhabditis elegans* genome, which is currently the most advanced of all mapping projects. The map progresses at great pace, and plans are already being made for an assault on its complete sequence.

K. Isono, M. Kroger

R. Roberts, C. Cantor, M. Olson

Although no dramatic new methods for DNA sequencing have appeared, several technical advances were announced. Magnetic beads, in combination with the biotin/streptavidin system, can be used to separate DNA strands after PCR and can facilitate template preparation. This method is particularly well-suited for automation. The need for increasingly automated procedures was emphasized during a whole session devoted to the subject. The need is especially

obvious for DNA sequencing, and it was gratifying to learn of improvements in the current sequencing machines and their associated computer software. Finally, a new strategy for reducing the cost of large-scale sequencing, based upon libraries of short oligonucleotide primers, was proposed and is being tested.

Two clear lessons emerged from the meeting. The first relates to the paramount importance of oligonucleotides as reagents for both mapping and sequencing. If easier and cheaper methods for their production can be devised, this could have a dramatic impact on the cost of the human genome project. Second, if PCR, or some alternative technique, could be extended to allow the routine amplification of 50- to 100-kb stretches of DNA, novel strategies for mapping and sequencing will become possible. These, in turn, could markedly affect both the time-scale and the cost of many genome projects.

The meeting was supported in part by the National Center for Human Genome Research, a division of the National Institutes of Health.

PROGRAM

Genome Organization
Chairman: K. Davies, *John Radcliffe Hospital, Oxford*
Large DNA Cloning Methods
Chairman: W. Bodmer, *Imperial Cancer Research Fund*
ALU-PCR and Other Methods
Chairman: M. Olson, *Washington University School of Medicine*
Informatics
Chairman: C. Cantor, *Human Genome Center, Lawrence Berkeley Laboratory*
Polymorphisms and Linkage Maps
Chairman: A.-M. Frischauf, *Imperial Cancer Research Fund*
Sequencing
Chairman: K. Isono, *Kobe University*
Automation
Chairman: R. Roberts, *Cold Spring Harbor Laboratory*
Large-scale Mapping Projects
Chairman: S. Naylor, *University of Texas, San Antonio*

C. Petit, A. Ballabio

Function and Evolution of RAS Proteins

May 9–May 13, 1990

ARRANGED BY

David Botstein, Genentech, Inc.
James Feramisco, University of California, San Diego
Michael Wigler, Cold Spring Harbor Laboratory

162 participants

A meeting was held on the subject of the family of low-molecular-weight GTP/GDP-binding proteins that are among the most highly conserved proteins in eukaryotes. Their biological role, biochemical functions, regulation, structure, and evolution were discussed. The known members of this superfamily have grown in the past decade, from the two oncogenic *RAS* genes first discovered in

retroviruses, to over thirty members. The charter members of the family, i.e., the genes most homologous to the oncogenic *RAS* genes, are involved in signal transduction pathways that control cell growth, proliferation, and differentiation. More diverse members of the family are involved in disparate cellular functions, such as vesicular trafficking, organization of the cytoskeleton, and determination of cellular polarity. Several general themes are emerging. These proteins are acted on by families of proteins that regulate guanine nucleotide exchange, and there may be proteins that block guanine nucleotide exchange. Families of proteins that specifically enhance guanine nucleotide hydrolysis, known as GAPs, have been identified. Evidence was presented that GAPs both regulate and mediate RAS action. Novel forms of carboxy-terminal covalent modifications target these proteins to specific subcellular compartments, and considerable progress has been made in elucidating these pathways. The function of the oncogenic RAS proteins still remains a mystery. This meeting was supported in part by the National Science Foundation and the National Cancer Institute, a division of the National Institutes of Health.

D. Stacey, T. Pawson

D. Gallwitz, C. Der, E. Racker

M. Wigler, J. Feramisco

PROGRAM

Mediators of RAS Activity
Chairman: F. McCormick, *Cetus Corporation*
Control of RAS. I
Chairman: D.W. Stacey, *Cleveland Clinic Foundation*
RAS Superfamily Structure and Function. I
Chairman: D. Botstein, *Genentech, Inc.*
Processing of RAS
Chairman: D. Gallwitz, *Max-Planck Institut*
Control of RAS. II
Chairman: S. Powers, *Robert Wood Johnson Medical School*
Cell Biology of RAS
Chairman: M. Perucho, *California Institute of Biological Research*
RAS Superfamily-Structure and Function. II
Chairman: P. Novick, *Yale University School of Medicine*

RNA Processing

May 16–May 20, 1990

ARRANGED BY

Anita Hopper, Pennsylvania State University
James Manley, Columbia University
Olke Uhlenbeck, University of Colorado, Boulder

445 participants

The field of RNA processing concerns all the cellular mechanisms that convert the primary transcripts into functional RNAs and their subsequent degradation. This field has undergone explosive growth in the past several years, resulting in a very large meeting and a long waiting list. The award of the 1989 Nobel Prize in Chemistry to two long-standing participants of this meeting added to this interest. The 5-day meeting includes seven sessions of ten, short presentations each and more than 240 posters in three poster sessions. A very broad range of disciplines were represented, including structural biologists, biochemists, and molecular and cellular biologists.

A major focus of the meeting was RNA splicing. Considerable data was presented defining the more than 50 basic components of the reaction by genetic and biochemical methods. Through the use of mutants and partial reactions, a preliminary definition of the reaction pathway has been achieved. A good deal of attention has focused on the large number of ways that different organisms have adapted the splicing pathway either to prepare special RNAs, as in *trans*-splicing, or as a means to regulate gene expression, as in alternate splicing. Another area that received a lot of attention was the relation of RNA structure to its function. This theme pervaded many sessions and underscored the continued difficulties of understanding RNA structure. Several papers on catalytic mechanisms of enzymatic reactions involving RNA provided a view of the future. A final session was devoted to the newly emerging field of RNA editing. The major puzzle of determining the origin of the information for this reaction appears to be solved and work can now focus on establishing a mechanism.

The meeting was supported in part by the National Institute of General Medical Sciences (a division of the National Institutes of Health) and the National Science Foundation.

E. Brody, M. Rosbash, M. Wickens, J. Abelson, C. Guthrie

O. Uhlenbeck, A. Hopper, J. Manley

PROGRAM

Mechanism of Splicing
Chairman: M. Rosbash, *Brandeis University*

Catalytic RNAs
Chairman: T. Cech, *University of Colorado, Boulder*

Ribonucleoprotein Particles
Chairman: C. Guthrie, *University of California, San Francisco*

Stable RNAs
Chairman: C. Greer, *University of California, Irvine*

3′ Processing and Nuclear Export
Chairman: M. Edmonds, *University of Pittsburgh*

Regulation of mRNA Splicing
Chairman: B. Nadal-Ginard, *Harvard Medical School*

Editing, Modification, and Turnover
Chairman: K. Stuart, *Seattle Biomedical Research Institute*

RNA Tumor Viruses

May 23–May 27, 1990

ARRANGED BY

Paul Jolicoeur, Clinical Research Institute of Montreal
Maxine Linial, Fred Hutchinson Cancer Research Center

435 participants

Papers were presented at the 1990 RNA Tumor Virus meeting that covered all aspects of retrovirus replication and pathogenesis. As in the previous few years, about half of the meeting involved discussion of human immunodeficiency

viruses (HIV), human T-cell leukemia viruses (HTLV), and animal models for these. Some of the highlights included isolation and characterization of several retroviral receptors, a new model for the function of the HIV TAT protein, and new insights into virus assembly. The Abstract bookcover highlighted a novel mutant of D-type Mason-Pfizer monkey virus that assembles like a C-type virus. Progress continues to be made in the study of retroviral integration. This year an in vitro integration system using only purified murine leukemia virus integrase was reported. Much attention was focused on the pathogenesis of lentiviruses, including results from the simian and feline animal models.

Reverse transcriptase took center stage in a special session that commemorated the 20th anniversary of the discovery of this remarkable enzyme. Both Drs. Temin and Baltimore were on hand to present overviews of the discovery and importance of reverse transcriptase as well as recent data from their laboratories.

D. Baltimore

M. Linial

P. Jolicoeur, H. Fan, N. Rosenberg

H. Temin

PROGRAM

Receptors/Glycoproteins
Chairmen: E. Hunter, *University of Alabama, Birmingham*
J. Sodroski, *Dana-Farber Cancer Institute*

Integration/Endogenous Viruses
Chairmen: A.M. Skalka, *Fox Chase Cancer Center*
R. Craigie, *National Institutes of Health*

Reverse Transcription
Chairmen: J. Coffin, *Tufts University School of Medicine*
S. Goff, *Columbia University*

I. Dotau, R. Craigie

M. Kotler, A. Skalka

Celebration of the 20th Anniversary of the Discovery of Reverse Transcriptase
Speakers: H. Temin, *McArdle Laboratory for Cancer Research, University of Wisconsin*
D. Baltimore, *Whitehead Institute for Biomedical Research*

Transcription/*trans*-Activation (TAT)
Chairmen: G. Pavlakis, *NCI Frederick Cancer Research Facility*
H.J. Kung, *Case Western Reserve University*

Pathogenesis/Latency
Chairmen: H. Fan, *University of California, Irvine*
N. Rosenberg, *Tufts University Medical School*

Structural Proteins and Processing/Assembly
Chairmen: V. Vogt, *Cornell University*
R. Swanstrom, *University of North Carolina*

Transformation/Proliferation
Chairmen: H. Robinson, *University of Massachusetts Medical Center*
A. Rein, *NCI Frederick Cancer Research Facility*

Transcription (TAX)/Posttranscriptional Regulation
Chairmen: C. Rosen, *Roche Institute of Molecular Biology*
K. Beemon, *Johns Hopkins University*

SV40, Polyoma, and Adenoviruses

August 15–August 19, 1990

ARRANGED BY

Michael Botchan, University of California, Berkeley
Terri Grodzicker, Cold Spring Harbor Laboratory
David Livingston, Dana-Farber Cancer Institute

323 participants

The Tumor Virus meeting on SV40 virus, polyoma virus, and adenoviruses was attended by over 300 scientists who met to discuss their latest work. The DNA tumor viruses continue to serve as model systems to study mechanisms of replication, transformation, transcription, and RNA processing.

Much of the emphasis in the meeting was placed on the interaction of cellular and virus-encoded proteins as they affect key points of cell and gene regulation.

Thus, investigations on the interaction of adenovirus E1 proteins and/or SV40 large T antigen with the retinoblastoma (RB) susceptibility gene product, p53 protein(s), and cyclin A were discussed. The role of protein modifications of viral regulatory proteins by different kinases and phosphatases was also the subject of some attention. The analysis of interactions of polyoma middle T antigen with cellular tyrosine kinases and the role of such complexes in the transformation process were also discussed. Much research continues to focus on the purification and analysis of cellular proteins that are concerned with viral DNA replication and the use of in vitro replication systems to dissect different steps in the replication process. The analysis of transcriptional regulation of viral promoters and enhancers continued to produce a variety of interesting results. Several cellular transcription factors have been purified, the corresponding genes have been

T. Grodzicker, C. Prives, D. Livingston

M. Carey, M. Botchan, A. Stenlund

M. Green, C. Cole

S. Courtneidge, T. Roberts

cloned, and the interactions of the factors with viral regulatory proteins have been studied, using in vivo and in vitro transcription systems. Talks were also given on RNA splicing, control of poly(A)-site usage, translational control, the role of viral proteins in cell-cycle regulation, cell-cycle-specific protein modifications, and the role of different viral proteins in the host's immune response.

PROGRAM

Transcription. I: Adenoviruses
Chairman: A. Berk, *University of California, Los Angeles*

Protein Complexes
Chairman: S. Courtneidge, *European Molecular Biology Laboratory, Heidelberg*

Replication. I
Chairman: B. Stillman, *Cold Spring Harbor Laboratory*

Transcription. II: SV40 and Polyoma
Chairman: M. Green, *Harvard University*

Transformation. I: Adenoviruses
Chairman: P. Hearing, *State University of New York, Stony Brook*

Posttranscriptional Regulation
Chairman: J. Nevins, *Duke University Medical Center*

Replication. II.
Chairman: E. Fanning, *Institute for Biochemistry, Munich*

Transformation. II: SV40 and Polyoma
Chairman: D. Livingston, *Dana-Farber Cancer Institute*

Molecular Genetics of Bacteria and Phages

August 21–August 26, 1990

ARRANGED BY

Lucia Rothman-Denes, University of Chicago
Miriam Susskind, University of Southern California
Andrew Wright, Tufts Medical School

301 participants

The 1990 meeting on Molecular Genetics of Bacteria and Phages attracted over 300 participants, who presented 220 talks and posters. A common thread was the critical involvement of specific protein-DNA, protein-protein, protein-RNA, and RNA-RNA interactions in a variety of fundamental genetic processes, including gene regulation, DNA replication and packaging, recombination, and the mobility of transposons and introns. A spectacular presentation was the direct visualiza-

M. Susskind, J. Hu, D. Daniels

A. Wright, I. Molineux

N. Hackett, G. Mosig

J. Ketter, S. Adhya

tion of DNA loops formed between RNA polymerase bound to a nitrogen-regulated promoter and an activator protein bound to an enhancer sequence 390 bp upstream. A session was devoted to the role of small, abundant DNA-binding proteins that are involved in an ever-increasing variety of cellular processes. The session on protein structure included several demonstrations of the mysterious and amazing ability of certain heat-shock ("chaperonin") proteins to help other proteins perform and an elegant study of the structural requirements for zipping a leucine zipper.

PROGRAM

Role of Abundant, Small DNA-binding Proteins
Chairman: N. Craig, *University of California, San Francisco*
DNA Replication and Packaging
Chairman: G. Mosig, *Vanderbilt University*
RNA Polymerase and Promoter Interactions
Chairman: G. Gussin, *University of Iowa*
Transcription Activation
Chairman: S. Adhya, *National Institutes of Health*
Transcription Antitermination Global Control
Chairman: J. Roberts, *Cornell University*
Posttranscriptional Control
Chairman: D. Wulff, *State University of New York, Albany*
Hard Times and Development
Chairman: P. Youngman, *University of Pennsylvania*
Membranes: Transport, Secretion, and Signaling
Chairman: S. Gottesman, *National Institutes of Health*
Protein Structure and Chaperonins
Chairman: R. Sauer, *Massachusetts Institute of Technology*
Recombination
Chairman: R. Wiesberg, *National Institutes of Health*

Mouse Molecular Genetics

August 29–September 2, 1990

ARRANGED BY

Douglas Hanahan, University of California, San Francisco
Richard Palmiter, University of Washington, Seattle
Erwin Wagner, Institute of Molecular Pathology, Vienna

D. Hanahan, R. Palmiter, E. Wagner

D. Solter, S. Waelsch

381 participants

The third annual Mouse Molecular Genetics meeting represented a milestone in the development of the field. The organizers invited only eight senior scientists to serve as session chairpersons, and yet more than 400 people chose to attend the meeting, resulting in over 100 short talks and 50 posters, which were presented during eight sessions and two poster sessions. Whereas the first two meetings justifiably emphasized the diverse biological possibilities afforded by transgenic mice produced via embryo microinjection, this meeting revealed wider horizons. One of these was the undeniable arrival of embryonic stem cells as a practical vehicle for introducing site-directed integrations that alter or simply knock out the function of endogenous chromosomal genes. Another was the remarkable convergence of classical mouse genetics with the new genetics of cloned genes and transgenic technology. There was also continuing progress with the candidate gene approach toward the control of development, using both traditional mice and their transgenic compatriots. In addition, transgenic mice continue to bear fruit in studies on control of gene regulation and on mechanisms of tumorigenesis, as well as in generating models of disease conditions.

Although it is impossible to review this remarkably diverse meeting succinctly, a few highlights bear mention. The search for the testes-determining gene has now reached fruition in the identification of a gene *(Sry)* encoding a putative DNA-binding protein that is transiently expressed during male embryogenesis and deleted in certain XY female mice. Classical genetics and molecular cloning provided access to the *Sry* gene, and it is clear that transgenic mice will now allow a convincing test of this gene as the necessary and sufficient component of the Y chromosome for the determination of male sexuality.

The capability to knock out specific endogenous genes in embryonic stem cells was demonstrated in a number of talks reporting mice homozygous for genes disrupted by homologous integration. Eleven homozygous knockouts were described, with another half dozen clearly in the pipeline. The knockout of the *GATA-1* erythroid-specific transcription factor resulted in the failure of erythroid cells to develop, and the lack of an intact *Wint-1* gene produced mice with major disruptions in their cerebellum. Remarkably, the absence of an intact c-*src* gene did not obviate prenatal development, but rather produced viable pups with defects in bone development and immune function, similar to a disease condition called osteopetrosis. Taken together with the knockout of β_2-microglobulin, which results in mice lacking class I major histocompatibility complex and $CD8^+$ T cells, it becomes evident that this technology will have a major impact in testing the biological roles (and necessity) of specific genes whose

functions have been postulated from indirect experiments, such as their expression patterns during development.

It was gratifying to see the continuing convergence of classical and molecular genetics. Several mouse mutants have now been identified as cloned genes. The most long awaited was the *steel* mutation, which has been found to be in a gene encoding a mast cell growth factor that is the ligand for the c-*kit* receptor that is encoded by a gene disrupted in a mutation called *W*. Another spontaneous mouse mutant showing both immunological and bone defects is the osteopetrotic mouse, and the *op* mutation has now been found to reside in the gene that encodes the macrophage growth factor M-CSF. It is provocative that the *op/op* mutation, which results in an absence of the secreted M-CSF, has a phenotype remarkably similar to that arising in mice lacking the *c-src* protein kinase, suggesting that both play important and possibly interconnected roles in bone development. In another causality proof, the Snell *dwarf* mutation, which is characterized by a failure to develop pituitary somatotrophs and lactotrophs, has been shown to result from a deletion of the gene encoding the Pit-1 pituitary-specific transcription factor, confirming that a cell-type-specific DNA-binding protein identified by biochemical techniques is necessary for the function of these cell types.

These and other examples throughout this meeting illustrate the breadth and biological possibilities afforded in both the traditional and the new transgenic techniques, as well as their convergence into an experimental mammalian system that will prove to be the genetic system of the 1990s.

The meeting was supported in part by the National Institute of Child Health and Human Development, the National Institute of Neurological Disorders and Stroke, and the National Institute of Diabetes, Digestive and Kidney Diseases (all divisions of the National Institutes of Health) and the National Science Foundation (Genetics Program).

J. DeMayo, A. McLaren

V. Price, L. Pevny, F. Conlon, T. DeChiara

PROGRAM

Gene Expression during Development and Germ Cells
Chairman: A. McLaren, *University College, London*

Functional Analysis of Development (Dominant Transgenes)
Chairman: K. Paigen, *Jackson Laboratory*

Functional Analysis of Development (Dominant Transgenes: Tumorigenesis)
Chairman: R Brinster, *University of Pennsylvania, School of Veterinary Medicine*

Functional Analysis of Development (Gain-of-function: Disease Models)
Chairman: A. Berns, *The Netherlands Cancer Institute*

Developmental Mutants (Loss-of-function: Homologous Recombination)
Chairman: N Jenkins, *ABL-Basic Research Program, Frederick, Maryland*

Developmental Mutants (Loss-of-function: Insertional Mutations and Imprinting)
Chairman: D. Solter, *Wistar Institute*

Developmental Mutants (Loss-of-function: Spontaneously or Environmentally Induced)
Chairman: S Waelsch, *Albert Einstein College of Medicine*

Regulation of Gene Expression (*cis*-Acting Elements)
Chairman: V. Chapman, *Roswell Park Memorial Institute*

Origins of Human Cancer

September 4–10, 1990

ARRANGED BY

Joan Brugge, University of Pennsylvania School of Medicine
Thomas Curran, Roche Institute of Molecular Biology
Ed Harlow, Cold Spring Harbor Laboratory
Frank McCormick, Cetus Corporation

317 participants

The second meeting on Origins of Human Cancer, which took place 14 years after the original landmark meeting, was a testament to the power of molecular genetics in probing the mechanisms involved in carcinogenesis. Since the first meeting on this subject, there has been a virtual explosion of information relating to the identification and characterization of the oncogenes associated with known tumorigenic viruses, and the identification of these oncogenes as the homologs of normal chromosomal genes (proto-oncogenes). Several of these proto-oncogenes have been shown to be mutated or rearranged in human tumors, and

H. Bourne, A. Mahowald, J. Brugge

A. Levine, F. McCormick

- R. Rose, I. Herskowitz, P. Sharp, J. Minna, J. Bolen

F. Li, E. Harlow

B. Ames

C. Herrmann, H. Ozer, J. Butel

the natural functions of some of the proto-oncogene products have been identified. Yet other genes have been found to suppress the development of tumors (suppressor genes); loss or mutation of the suppressor genes or inactivation of the function of their protein products by association with viral transforming proteins represents an important stage in the etiology of this disease.

The meeting included a mixture of sessions dealing with mechanisms underlying normal cellular growth control and oncogenic transformation (including comparisons of the biological and biochemical activities of the proteins encoded by proto-oncogenes and their oncogenic counterparts) with sessions that dealt with model tumor paradigms (i.e., leukemia, breast, colon, lung, cervix, and liver). In each tumor system, there was a discussion of the epidemiology of the disease, the associated chromosomal abnormalities, and the multistage processes involved in the development of the neoplasia. Throughout all the sessions, significant new findings were presented, and certainly the density of new information introduced at this meeting was a reflection of the impressive pace at which this research is moving.

The meeting was supported in part by the National Cancer Institute, a division of the National Institutes of Health.

PROGRAM

Welcome: J.D. Watson

Growth Control
Chairman: I. Herskowitz, *University of California, San Francisco*

Oncogenes. I: RAS/GAP
Chairman: H. Bourne, *University of California, San Francisco*
Carcinogenesis and Mutagenesis
Chairman: C. Barrett, *National Institute of Environmental Health Sciences*
Epidemiology
Chairman: J. Fraumeni, *National Institutes of Health*
Tumor Suppressor Genes
Chairman: E. Stanbridge, *University of California, Irvine*
Carcinomas. I: Colon
Chairman: B. Vogelstein, *Johns Hopkins University*
Oncogenes. II: Signal Transduction
Chairman: J. Brugge, *University of Pennsylvania School of Medicine*
Carcinomas. II: Breast
Chairman: A. Harris, *Imperial Cancer Research Fund, Oxford*
Oncogenes. III: Transcription
Chairman: P. Sharp, *Massachusetts Institute of Technology*
Carcinomas. III: Cervix and Liver
Chairman: H. zur Hausen, *Deutsches Krebsforschungszentrum, Heidelberg*
Regulation of Hematopoiesis
Chairman: D. Baltimore, *Rockefeller University*
Carcinomas. IV: Lung
Chairman: J. Minna, *National Institutes of Health*
Leukemia
Chairman: P. Nowell, *University of Pennsylvania*
Tumor Development
Chairman: P. Leder, *Harvard Medical School*
New Approaches to Therapy
Chairman: E. Frei, *Dana-Farber Cancer Institute*

Modern Approaches to New Vaccines including Prevention of AIDS

September 12–16, 1990

ARRANGED BY

Fred Brown, Wellcome Biotechnology, Ltd.
Robert Chanock, NIAID, National Institutes of Health
Harold Ginsberg, Columbia University College of Physicians & Surgeons
Richard Lerner, Research Institute of Scripps Clinic

198 participants

The eighth annual meeting on Modern Approaches to New Vaccines provided an opportunity for basic scientists and clinical investigators to exchange their latest research observations and broaden their perspective of the many interactive components of experimental immunoprophylaxis, such as immunology, virology, bacteriology, parasitology, epidemiology, molecular pathogenesis, clinical infectious diseases, and clinical trials.

Many important advances in various interrelated fields of investigation were reported. For example, cloning of viral-antigen (influenza A virus hemagglutinin)-specific murine immunoglobulin Fab fragments from a combinatorial expression

H. Ginsberg, R.M. Chanock

T. Miyamura, H. Takahashi

M. Kaczorek

R. A. Lerner

library was described for the first time. Noteworthy also was the report of successful intracellular immunization using a parvovirus (adeno-associated virus)-based antisense vector to transduce intracellular resistance to human immunodeficiency virus (HIV) or human herpes virus. Despite a number of previous failures to induce a neutralizing antibody response with picornavirus outer capsid proteins expressed by a recombinant vector, success was reported for hepatitis A virus capsid proteins expressed by a baculovirus recombinant. In addition, two examples were presented of a new strategy to increase the immunogenicity of a viral antigen by altering its intracellular pathway and ultimate site of localization. Rotavirus VP7 outer capsid protein and dengue virus envelope glycoprotein that were modified so that they were displayed on the surface of infected cells, rather than being retained intracellularly, each exhibited a striking increase in immunogenicity.

This meeting was supported in part by the Rockefeller Foundation.

PROGRAM

AIDS. I
Chairman: H. Ginsberg, *Columbia University College of Physicians & Surgeons*
Immunology. I
Chairman: R. Lerner, *Research Institute of Scripps Clinic*

Chairman: R. Chanock, *National Institutes of Health*

Parasitology and Bacteriology

Chairman: J. Young, *Molecular Vaccines, Inc.*

Virology. II

Chairman: F. Brown, *Wellcome Biotechnology, Ltd.*

AIDS. II

Chairman: P. Nara, *NCI, Frederick Cancer Research Facility*

AIDS. III

Chairman: F. Ennis, *University of Massachusetts Medical School*

Virology. III

Chairman: A. Kapikian, *National Institutes of Health*

AIDS. IV and Virology. IV

Chairman: E. Norrby, *Karolinska Institutet*

Summary: E. Norrby, *Karolinska Institutet*

F. Ennis

B. Murphy, J. Esposito

Evolution: Molecules to Culture

September 24–27, 1990

ARRANGED BY

Richard Dawkins, Oxford University
Jared Diamond, University of California School of Medicine, Los Angeles

119 participants

The problem of similar structures arises in many fields, as when molecular biologists compare structures of two proteins or when historical linguists compare languages. The meeting on Evolution: Molecules to Culture considered the relative contributions to similarity from common ancestry, convergence, borrowing, and chance in six fields whose practitioners rarely communicate with each other. The fields included molecular biology, functional morphology, animal be-

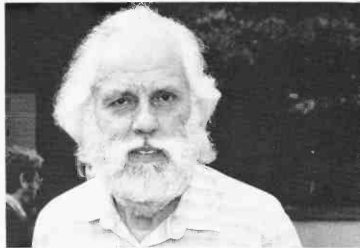
R. Dawkins, L. Cavalli-Sforza

J. Diamond

J. Bradbury, L. Partridge, N. Davies

J. Witkowski

J. Maynard Smith, P. Jenkins, R. Hickson, D. Labuda


V. Shevoroshkin

H. Wright, P. Kirch

R. Sakal, G. Lauder, J. Kiu

P. Harvey, M. Anderson

W. Sterrer, A. Manaster-Ramer

D. Hull, S. Herring

havior, studies of sexual evolution, cultural anthropology, and linguistics. Among the many highlights of the meeting was the realization that there is now an emerging science of the comparative method to solve the problems that arise in determining ancestral states and phylogenetic trees. Another highlight was the recognition of the exciting prospect of the marriage between historical linguistics and other fields, whereby statistical and mathematical models derived from other areas could be applied to linguistic problems and whereby also human history could be inferred from the degree of concordance between molecular and linguistic characteristics of human populations.

The meeting was supported in part by the Alfred P. Sloan Foundation.

PROGRAM

Introduction: J. Diamond, *University of California School of Medicine, Los Angeles*

Molecular Evolution
Chairman: R. Doolittle, *University of California, San Diego*

Functional Morphology
Chairman· G.V Lauder, *University of California, Irvine*

Comparative Method
Chairman: P. Harvey, *Oxford University*

Animal Behavior
Chairman: N B Davies, *University of Cambridge*

Sex and Sexual Selection
Chairman: L. Partridge, *University of Edinburgh*

Human Society
Chairman: P.V. Kirch, *University of California, Berkeley*

Linguistics
Chairman: A Manaster Ramer, *IBM Research and Wayne State University*

Overview
Chairman: J Diamond, *University of California School of Medicine, Los Angeles*
Speakers: J. Maynard Smith, *University of Sussex*
L.L. Cavalli-Sforza, *Stanford University*
E. Mayr, *Harvard University*
D. Hull, *Northwestern University*
R. Dawkins, *University of Oxford*

Cell and Molecular Neurobiology of *Aplysia*

October 3–October 7, 1990

ARRANGED BY

Eric Kandel, Columbia University College of Physicians & Surgeons
Hersch Gerschenfeld, Ecole Normale Supérieure
Leonard Kaczmarek, Yale University Medical School
Richard Scheller, Stanford University
Micha Spira, Hebrew University, Israel

84 participants

The second international meeting on Cell and Molecular Biology of *Aplysia* was attended by 90 people from the United States, the Union of the Soviet Socialist Republics, Europe, the Middle East, Canada, and Japan. Participants included a number of investigators who work on related gastropod preparations, including *Bulla, Clione, Helix, Helisoma, Hermissenda, Lymnaea, Planorbis,* and *Tritonia*. All of these species share the property that originally made *Aplysia* so attractive—neurons with giant cell bodies. This feature, which allows neurons to be identified as unique individuals that can be identified across preparations, was originally valued because it allows one to work out neuronal wiring diagrams in cellular detail. More recently, the large size of these neurons has also been important in facilitating biochemical and molecular biological studies.

There were 40 platform presentations and 33 posters. The presentations were of high quality and included the first molecular characterization in *Aplysia* of K^+ channels, a novel mechanism for processing peptides to target different peptides to different terminals of the neuron, and the first analysis of operant conditioning in *Aplysia*. A number of papers focused on the molecular mechanisms that underlie the behavioral plasticity, defined as a change in the response of an animal to a repeated, unchanging stimulus: those due to changes in motivational state (e.g., hunger) and those due to experience (learning). Presentations addressed the biochemical mechanisms that generate the structural changes that contribute to long-term behavioral plasticity; their relationship to mechanisms that operate during development, the interactions of second messenger systems that mediate behavioral plasticity at the cellular level, the functional implications of a neuron having more than one synaptic transmitter, and the molecular mechanisms that cause changes in activity produced by a circadian rhythm-generating neural network. The meeting concluded with a demonstration by Paul Forscher of a new imaging method for studying growth-cone movement and a summary of the proceedings by Irwin Levitan. The meeting proved a highly profitable forum for the discussion of both new methods and findings emerging from studies related to the cellular and molecular biology of *Aplysia*.

The meeting was supported in part by the fidia Research Foundation.

PROGRAM

Welcome: H. Gerschenfeld, *Ecole Normale Supérieure*
G.N. Orlovsky, *Moscow State University*

Cell Imaging and Neuronal Growth
Chairman: I. Levitan, *Brandeis University*

E. Kandel, J. Kehoe

I. Levitan, J. Jacklet, T. Carew

L. Kaczmarek, P. Pfaffinger

B. Peretz, G. Orlovsky

L. Tauc

R. Sheller, L. Kaczmarek, K. Lukowiak

Ion Channels and Channel Modulation
Chairman: J.S. Kehoe, *Ecole Normale Supérieure*

Peptides, Small Molecule Transmitters, and Synaptic Transmission
Chairman: L. Tauc, *CNRS, Gif-sur-Yvette*

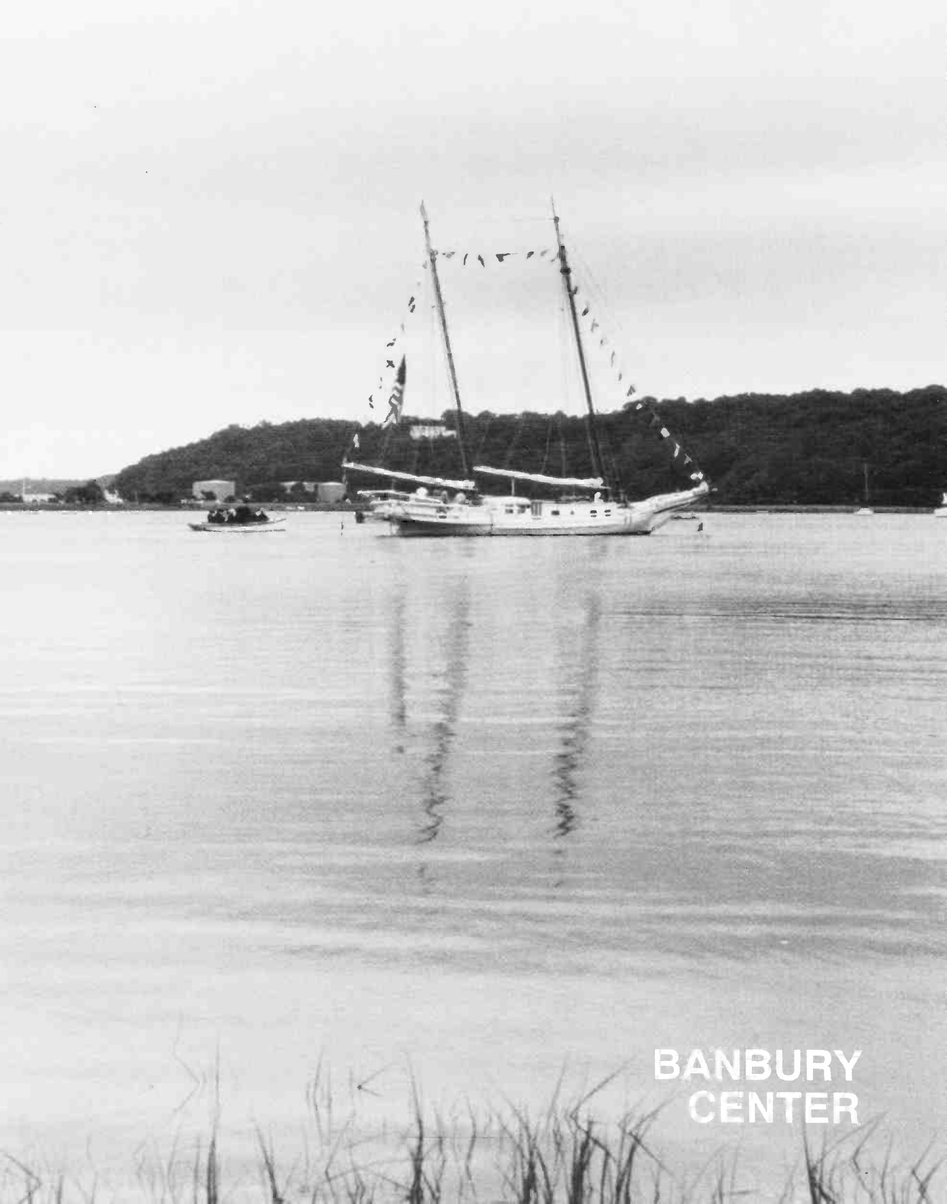
Circadian Rhythm and Rhythmic Behavior
Chairman: J. Jacklet, *State University of New York, Albany*

Neural Circuitry and Behavior
Chairman: I. Kupfermann, *Columbia University*

Nonassociative Learning
Chairman: C.H. Bailey, *Columbia University*

Associative Learning
Chairman: T.J. Carew, *Yale University*

Demonstration and Summary
Chairman: L. Kaczmarek, *Yale University*
Speakers: P. Forscher, *Yale University*
I. Levitan, *Brandeis University*


BANBURY
CENTER

BANBURY CENTER DIRECTOR'S REPORT

Robertson House provides housing and dining accommodations at Banbury Center

The Banbury Center continues to play a special part in the Laboratory's meetings program. Fourteen workshop-style meetings were held here in 1990, and over 500 scientists attended them. In addition, there were three special meetings for science journalists, congressional staff, and senior executives involved in biotechnology. Bea Toliver and Ellie Sidorenko in the Center's office and Katya Davey at Robertson House once again coped wonderfully with the pressures that always arise when dealing with a program of this nature—cancellations, unusual requests, and meetings arranged at the last minute. The 1990 program at the Banbury Center seemed to be even more varied than usual, so it is even more difficult to group the meetings into categories.

Molecular Genetics

Banbury continues to be a leading center for discussion meetings on human molecular genetics, and there were four meetings on this theme in 1990. The meetings ranged from discussions of methodology, through applications to particular diseases, to the societal implications of the powers of a genetic approach to the ills that afflict human beings.

Although electrophoresis is used in every molecular biology laboratory to separate DNA molecules, the technique as originally developed could not be used for very large DNA molecules. In recent years, further developments have led to systems that separate DNA fragments as large as intact yeast chromosomes. Electrophoresis of Large DNA Molecules was concerned with the theory and the practice of these techniques. In addition, the participants dis-

cussed some of the novel techniques being developed for the physical analysis of DNA. The most remarkable of these uses scanning tunneling microscopy to "see" a DNA molecule directly.

The furthest extreme from analyzing individual DNA molecules is the study of human genetics as revealed by the inheritance of genes in families. This has proved to be an extremely powerful approach, as shown by the cloning of human disease genes such as that for cystic fibrosis. Genetic analysis of human disorders that may result from the effects of several genes, acting independently or together, and in which there is a strong environmental influence is much more difficult. This was the subject of the meeting on the Genetics and Molecular Biology of Complex Diseases. These diseases include autoimmune diseases such as arthritis, alcoholism, and schizophrenia. The participants discussed general approaches to the genetic analysis of these disorders and the progress, or lack of it, in specific disorders.

Neurofibromatosis was one of the successes of cloning human disease genes in 1990, and the Banbury Center meeting on Neurofibromatosis was held at just the right time, in October. The gene for one form of neurofibromatosis had been cloned in the Spring of 1990, and its similarity to genes known to be involved in cancer was recognized just a few weeks prior to the meeting. The success of research on neurofibromatosis is an example of what can be achieved through a judicious combination of cooperation and competition and by the efforts of a small foundation.

Increasingly, the results of the research laboratories' cloning of human disease genes are translated into practical, diagnostic applications. However, it is becoming clear that DNA-based diagnosis may not be an unalloyed success. The Impact of Human Molecular Genetics on Society considered the difficulties that may arise when DNA diagnosis becomes possible for common, complex genetic disorders. The issues discussed included how to decide when population screening programs should be implemented and how an individual's access to health care and insurance may be affected. The comments of the European geneticists, working in countries with national health care systems, were particularly interesting, and there appear to be strong parallels with these same issues in relation to AIDS.

A final meeting in this area dealt with Mapping the Genomes of Agriculturally Important Animals. Although concerted efforts are being made to map and analyze the genomes of human beings, the fruit fly, a nematode worm, a bacterium, and yeast, the same resources have yet to be applied to the genomes of domesticated animals. The bovine genetic map is perhaps the best developed, but even there the numbers of genes assigned to particular chromosomes is very small. This meeting was intended as a planning meeting to discuss what should be done to rectify this situation.

Sammis Hall, guest house

Plant Molecular Biology

Despite the obvious practical importance of studying the molecular biology of plants, it has not been easy to find funding for meetings on this topic. So in 1990, a meeting on Recognition in Plant-Pathogen Interactions was included in Banbury's Corporate Sponsor series of meetings. Plants have devised a variety of means to deal with pathogens, but little is known of the molecular specificity that determines the interaction between a plant and potential pathogens. This

meeting reviewed some of the best understood plant-pathogen interactions and dealt particularly with progress toward cloning the genes involved. The ability to manipulate the genes responsible for the resistance of plants to bacteria and fungi could have far-reaching effects for agriculture.

Topics in Basic Research

Two meetings dealt with technical developments in molecular biology. It might seem at first sight to be easy to derive the three-dimensional structure of a protein from its amino acid sequence. In fact, it is still not possible to do this, and participants in the meeting Computational Aspects of Protein Folding critically reviewed the approaches that have been devised. Among the topics discussed were methods based on free-energy calculations, molecular dynamics studies, and knowledge-based approaches. The importance of the subject was underscored by the large attendance of scientists from the Laboratory's corporate sponsors.

Monoclonal antibodies are among the most important tools available to molecular biologists. Because of their specificity, monoclonal antibodies allow very precise detection and purification of proteins. These antibodies are produced by cells growing in tissue culture, but over the past 2 years, methods have been developed for using recombinant DNA techniques to produce antibodies in bacteria. Vectors for Cloning the Immune Response reviewed the present state of development of this field and looked forward to new developments in vectors and techniques that will speed up the move to creating antibodies in vitro.

Banbury Meeting House

Cell death is usually thought of as the ultimate response of a cell to trauma,

but so-called programmed cell death is a part of normal cell and tissue functioning. The phenomenon has been studied in a variety of cells, tissues, and organisms and involves an interesting set of morphological and molecular changes. Our meeting on Programmed Cell Death: Concepts and Mechanisms was a first attempt at drawing these different interests together to determine the degree to which common mechanisms may be involved.

One factor contributing to cell death may be the generation of free-oxygen radicals. These are highly reactive and toxic to cells, and cells have evolved methods for neutralizing these free radicals before they can do harm. The meeting on Molecular Biology of Free-radical Scavenging Systems dealt with the molecular mechanisms by which cells cope with oxidative stress. In addition, there were discussions of some of the biomedical applications that might be developed for protecting cells.

Environmental Hazards

The primary focus of the first meetings at the Banbury Center was on "biological risk assessments, especially of agents thought to act at the genetic level." The first of the two 1990 meetings on environmental hazards continued this theme. Molecular Mechanisms of Fiber Cytotoxicity and Carcinogenesis reviewed the current research on the biological effects of asbestos and the extent to which extrapolations can be made to the possible risks associated with man-made substitutes for asbestos. The meeting was notable for the range of mechanisms examined, including the disruption of cell division by fibers, their effects on oxyradicals, and their role in inducing genetic changes.

The second of the year's meetings in this area was The Biological Basis for Risk Assessment of Dioxins and Related Compounds. A meeting dealing with the biological effects of dioxin was held at the Banbury Center in 1984. As its title indicates, the 1990 meeting dealt with the impact of the results of biological and epidemiological research on risk assessment and on regulations governing environmental levels. There was much discussion about the biological basis for the models that are used to estimate human exposure to dioxin. This is a highly controversial subject, and it is to be hoped that the discussions that went on at this meeting will lead to a resolution of some of the problems involved.

Sloan Foundation Workshops

The findings of biological research are having, or should have, an increasing relevance to many aspects of society. The importance of a proper interpretation and implementation of those findings cannot be overemphasized. The aim of these workshops is to provide two influential groups, congressional staff and science journalists, with an opportunity to learn at first hand about some of this research. The subject of the congressional staff meeting was Addiction. The workshop covered an enormous range of topics, including an historical survey of drug addiction and alcoholism in the United States, the pharmacology of addiction, the relationship (if any) between "psychological" addiction (e.g., gambling) and drug addiction. The meeting finished with a session debating whether controlled legalization of drugs would alleviate or exacerbate the drug epidemic.

The science journalists' workshop dealt with an epidemic of another kind.

Cancer rates are increasing in women, and the meeting on Women and Cancer surveyed the latest research on the genetics of breast, cervical, and lung cancer and how this research is being translated into diagnostic tests and treatments. Some of this work is controversial, as for example, studies of the effectiveness of screening tests for breast cancer. A particularly disturbing presentation showed how the increase in women smoking parallels the increasing incidence of lung cancer in women and how women are special targets for advertising.

The Baring Brothers/Cold Spring Harbor Laboratory Meeting

Cancer is one area where genetic analysis at the molecular level has led to revolutionary new insights, and it seemed to be just the right topic for our 1990 senior executives meeting. It was an outstanding meeting. A series of talks on fundamental research on cancer dealt with oncogenes and the activities of their protein products, control of the cell cycle, and genetic prognosis. These were followed by presentations on metastasis, drug-resistance genes, and new therapies for breast cancer. Cold Spring Harbor Laboratory has had a long involvement with cancer research, and it was appropriate that the participants spent an afternoon discussing research with some of the Laboratory's scientists.

Other Meetings

As in previous years, the Banbury Center has been used as a meeting center by a small number of outside groups. The Esther A. and Joseph Klingenstein Fund held their annual meeting of Klingenstein research fellows at the Center in April. A participant in this year's meeting was Walter Gilbert, who speculated on what the genome projects might mean for neurobiology research. All research in biol-

ogy is coming to depend increasingly on mathematics and computers, not just for analysis, but also for modeling. The National Science Foundation sponsored a small workshop that discussed what might be done to develop training in mathematical and computational skills for biologists. The Huntington Hospital Board of Trustees and the Psychiatry Department of Mt. Sinai Medical School also came to the Center.

Banbury Meeting House, rear view

Funding

Acknowledgments for financial support of meetings at the Banbury Center must always begin with the members of the Laboratory's Corporate Sponsor Program. In 1990, five meetings at the Banbury Center were supported by this program. It is no exaggeration to say that the number and diversity of our meetings could not be maintained without our Corporate Sponsors. Companies were also generous in their support of other meetings. Five companies interested in electrophoresis techniques contributed to the Electrophoresis of Large DNA Molecules meeting, and SmithKline Beecham sponsored the meeting on Vectors for Cloning the Immune Response. The Chlorine Institute contributed to the meeting on The Biological Basis for Risk Assessment of Dioxins and Related Compounds. The Environmental Protection Agency helped fund two meetings, and funding from the United States Department of Agriculture, the National Science Foundation, and Granada BioSciences helped fund the meeting on Mapping the Genomes of Agriculturally Important Animals. The Alfred P. Sloan Foundation continues to fund the science journalists' and congressional staff workshops. The Pew Charitable Trusts supported the meeting on The Impact of Human Molecular Genetics on Society. A full listing of Banbury Center funds can be found with the financial statements for the Laboratory.

We have received two major grants for meetings beginning in 1991. The Wil-

liam Stamps Farish Fund has made the Center a three-year award for meetings on the genetics of common polygenic diseases. This is undoubtedly where the next major advances in human genetics will take place. The Banbury Center and the DNA Learning Center have received a joint grant from the Department of Energy for a series of workshops on genetics. The aim of the workshops will be to provide information on human genetics for nonscientists involved in human genome projects. We hope that the participants in the workshops will make use of the information in their own special areas.

Banbury Center Books

Cold Spring Harbor Laboratory Press continues to produce an excellent series of publications based on Banbury Center meetings. There were two further books in the *Banbury Report* series, from meetings held in 1989. These were *Genetics and Biology of Alcoholism* and *Biology of Mammalian Germ Cell Mutagenesis*. The first of the new series of *Current Communications in Cell & Molecular Biology* was published in early 1991. These books will still be based on Banbury meetings but will now contain about ten selected reviews instead of short abstracts of all presentations. They have more figures and references, and the new format should result in books of lasting value. *Electrophoresis of Large DNA Molecules* is the first in the new series, and it will be followed by books based on other 1990 meetings. The Press also published a report of the meeting on *Mapping the Genomes of Agriculturally Important Animals*.

Looking Forward to 1991

As is usual at the time this report is written, the program for the current year is still being developed. However, it promises to be as stimulating and exciting as in previous years. There will be meetings on basic research (adhesion molecule receptors, receptors for viruses, and membrane proteins), molecular genetics (Marfan disease, breast cancer, *Escherichia coli* genome, and AIDS), and social issues in biological research. One of the most exciting developments, referred to above, will be the first of the genetics workshops for individuals involved in genome projects. In his 1981 Director's Report, Dr. Watson described the Banbury Center program as being a "most intellectually diverse smorgasbord" of meetings. It is this variety of subjects and the outstanding quality of the participants that makes the Banbury Center one of the most exciting meetings centers in the world.

Jan A. Witkowski

Publications

Witkowski, J.A. 1990. [correspondence]. Carrel's Cultures. *Science* **247**: 1385–1386.

Witkowski, J.A. 1990. The 51 most-cited articles in the *Cold Spring Harbor Symposia on Quantitative Biology*. *Current Contents* **28**, July 9 1990: 7–17.

Witkowski, J.A. 1990. The inherited character of cancer *Cancer Cells* **2**: 229–257.

Witkowski, J.A. 1990. Milestones in the development of DNA technology. In *Forensic DNA Technology* (ed. M.A. Farley and J.J Harrington), pp. 1–23. Lewis Publishers, Inc., Michigan.

Sloan Foundation Congressional Workshop on Addiction

January 25–January 27

ARRANGED BY

J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York

SESSION 1

M.J. Kreek, Rockefeller University, New York, New York: Pharmacology and physiology of opiate addiction.

N. Grunberg, Uniformed Services University of the Health Sciences, Bethesda, Maryland: Tobacco as an addictive substance.

M. Eckardt, National Institute on Alcohol Abuse and Alcoholism, Bethesda, Maryland: Alcohol as an addictive substance.

S. Blume, South Oaks Hospital, Amityville, New York: Gambling as an addiction.

SESSION 2

T.R. Kosten, Yale University, New Haven, Connecticut: Treatments for drug addiction.

W. Comer, Bristol-Myers Squibb Company, New York, New York: Response of the pharmaceutical industry.

SESSION 3

D. Courtwright, University of North Florida, Jacksonville: Social and legislative origins of narcotic control.

P. Reuter, RAND Corporation, Washington, D.C.: Legalization issues: The current debate.

A. Hamid, John Jay College of Criminal Justice, New York, New York: Legalization: Its potential impact on the streets.

E.A. Nadelmann, Princeton University, New Jersey: Comments on legalization.

A. Goldstein, Stanford University, California: Comments on legalization

Mapping the Genomes of Agriculturally Important Animals

February 25–February 28

ARRANGED BY

C.J. Arntzen, Texas A&M University, College Station
N.L. First, University of Wisconsin, Madison
J.E. Womack, Texas A&M University, College Station

SESSION 1: Mammalian Gene Mapping: A Comparative Approach

Chairperson: C.J. Arntzen, Texas A&M University, College Station

D.E. Housman, Massachusetts Institute of Technology, Cambridge: The human gene map: An overview of strategies, status, and application.
D.L. Nelson, Baylor College of Medicine, Texas: Molecular dissection of human X chromosome.
J.E. Womack, Texas A&M University, College Station: Genomic conservation in humans, mice, and cattle.
L.C. Skow, Texas A&M University, College Station: The mouse gene map: Implications for the genomes of domestic animals.
V. McKusick, Johns Hopkins University, Baltimore, Maryland: Discussion: Potential interrelationships with human genome initiative.

SESSION 2: Potential Benefits of Animal Gene Maps to Agriculture

Chairperson: J. Lunney, Agricultural Research Service, USDA, Beltsville, Maryland

A.H. Paterson, E.I. du Pont de Nemours & Co., Inc., Wilmington, Delaware: Mapping QTL in tomato using genetic linkage to DNA markers.
M. Soller, The Hebrew University of Jerusalem, Israel: Experimental designs and statistical analyses for mapping QTL in animal populations.
A. Teale, ILRAD, Nairobi, Kenya: Genes controlling disease resistance as targets of bovine genome research.
L.A. Schuler, University of Wisconsin, Madison: The prolactin gene family in cattle.

SESSION 3: Approaches to the Development of Animal Gene Maps

Chairperson: J.E. Womack, Texas A&M University, College Station

M.S. Georges, Genmark, Inc., Salt Lake City, Utah: Characterization of highly polymorphic bovine markers.
H. Lewin, University of Illinois, Urbana: UFO-PCR: A strategy for animal genome mapping.
R.L. White, Howard Hughes Medical Institute, University of Utah Medical Center, Salt Lake City: New and emerging technologies in linkage marker development: Parallel application in humans and animals.
J. Hetzel, CSIRO, Queensland, Australia: International collaboration: Use of pedigreed reference families.

J. Hetzel, J. Womack, C. Arntzen

M. Soller

A. Teale, V. McKusick

SESSION 4: The Current Status of Gene Maps in Animals

Chairperson: H. Lewin, University of Illinois, Urbana

J.E. Womack, Texas A&M University, College Station: Cow.
L.B. Schook, University of Illinois, Urbana: Pig.
L.C. Skow, Texas A&M University, College Station: Horse.
J. Hetzel, CSIRO, Queensland, Australia: Sheep and goat.
R.M. Shuman, North Carolina State University, Raleigh: Poultry.

SESSION 5: Discussion of Programs and Strategies

Chairperson: N.L. First, University of Wisconsin, Madison

J.E. Womack, Texas A&M University, College Station: USDA Joint Committee on Animal Genome Mapping.
L.B. Schook, University of Illinois, Urbana: NC-150 Committee on the Application of Cellular and Molecular Biology to Animal Science Research.
N.P. Clarke, Texas A&M University, College Station: National initiatives in animal agriculture.
J. Hetzel, CSIRO, Queensland, Australia: International programs.
H. Mussman, US Department of Agriculture, Washington, D.C.: Summary.

Electrophoresis of Large DNA Molecules: Theory, Practice, and Future

March 5–March 8

ARRANGED BY

B. Birren, California Institute of Technology, Pasadena
E. Lai, University of North Carolina at Chapel Hill

SESSION 1: DNA Molecules and Gels

Chairperson: P. Serwer, University of Texas Health Science Center at San Antonio

F.H. Kirkpatrick, FMC BioProducts, Rockland, Maine: Overview of agarose gel properties.
B. Akerman, Chalmers University of Technology, Gothenburg, Sweden: Reorientational dynamics and mobility of DNA during pulsed-field gel electrophoresis.
C. Bustamante, University of New Mexico, Albuquerque: Observations of kinked configurations in DNA molecules undergoing orthogonal field alternating gel electrophoresis.
J.A. Schellman, University of Oregon, Eugene: Orientation response and relaxation of DNA in agarose gels.
S. Smith, University of New Mexico, Albuquerque: Computer simulation of individual DNA molecular motion during pulsed-field gel electrophoresis.
J.M. Deutsch, University of California, Santa Cruz: Prediction of electrophoresis experiments using computer simulations.
G. Holzwarth, Wake Forest University, Winston-Salem, North

C.R. Cantor, J.A. Witkowski, E. Lai

C. Bustamante

E. Lai, L. Lerman

Carolina: Acceleration of DNA during PFGE.
L.S. Lerman, Massachusetts Institute of Technology, Cambridge: The interaction between DNA melting and its electrophoretic mobility in polyacrylamide gels.

SESSION 2: Instrumentation

Chairperson: E. Lai, University of North Carolina at Chapel Hill

J. Noolandi, Xerox Research Center of Canada, Ontario: The Xerox CAGE (computer-assisted gel electrophoresis) system for pulsed-field gel electrophoresis of DNA.
J.R. Fassett, Beckman Instruments, Inc., Fullerton, California: The transverse alternating field system: New designs.
S. Ferris, Bio-Rad Laboratories, Hercules, California: Algorithm-optimized, multistate CHEF electrophoresis.
R. Blakesley, GIBCO/BRL, Life Technologies, Inc., Gaithersburg, Maryland: Small DNA separations with a PACE apparatus.
K. Kolble, University of Oxford, United Kingdom: ST/RIDE–An angle-variable 3D-PFGE system.
G.-J.B. van Ommen, Sylvius Laboratories, Leiden, The Netherlands: Design and use of a simple space-saving CHEF system driving four independent time-ramp programs.

SESSION 3: Electrophoresis

Chairperson: L.S. Lerman, Massachusetts Institute of Technology, Cambridge

G.F. Carle, University of Nice, France: Field inversion gel electrophoresis.
G. Chu, Stanford University Medical Center, California: Separation of very large DNA with a variable angle CHEF device.
P. Serwer, University of Texas Health Science Center at San Antonio: New modes and effects of pulsed-field gel electrophoresis.
E. Lai, University of North Carolina at Chapel Hill: Studies of DNA migration made with the PACE system.
B. Birren, California Institute of Technology, Pasadena: Factors influencing pulsed-field migration of DNA.
C. Heller, University of Constance, Germany: Field inversion gel electrophoresis with different pulse-time ramps.
S. Beverely, Harvard Medical School, Boston, Massachusetts: Circular DNA and pulsed-field gel electrophoresis.
C.R. Cantor, Lawrence Berkeley Laboratory, California: Accelerating PFGE separations.

SESSION 4: Applications

Chairperson: C.R. Cantor, Lawrence Berkeley Laboratory, California

G.-J.B. van Ommen, Sylvius Laboratories, Leiden, The Netherlands: Applications of PFGE and CHEF analysis to disease study and YAC analysis.
C.L. Smith, University of Berkeley, California: Optimizing applications of PFGE electrophoresis.
H. Lehrach, Imperial Cancer Research Fund, London, United Kingdom: Experimental approaches and results in mapping large regions of mammalian genomes.

SESSION 5: The Future

Chairperson: B. Birren, California Institute of Technology, Pasadena

S. Williams, Cold Spring Harbor Laboratory, New York: Image analysis of gel patterns.

A.S. Cohen, Northeastern University, Boston, Massachusetts: Restriction fragments and DNA sequencing using open and gel high-performance capillary electrophoresis (HPCE).

W. Efcavitch, Applied Biosystems, Inc , Foster City, California: Capillary gel electrophoresis.

C. Bustamante, University of New Mexico, Albuquerque: STM studies of DNA.

Genetics and Molecular Biology of Complex Diseases

March 12–March 15

ARRANGED BY

A. Chakravarti, University of Pittsburgh, Pennsylvania
E.R. Lander, Whitehead Institute for Biomedical Research, Cambridge, Massachusetts
J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York

SESSION 1: Genetics I: The Complexity of Inheritance

Chairperson: E.R. Lander, Whitehead Institute for Biomedical Research, Cambridge, Massachusetts

J.V. Neel, University of Michigan, Ann Arbor: The "bottom up" approach to multifactorial inheritance: Some problems.

J.-M. Lalouel, University of Utah School of Medicine, Salt Lake City: Genotypic versus phenotypic inference in complex inheritance.

M.-C. King, University of California, Berkeley: Gene mapping of complex diseases, especially cancer (inherited vs somatic alterations, choice of models, heterogeneity, etc.).

C. Sapienza, Ludwig Institute for Cancer Research, Montreal, Canada: Genetic models for penetrance and expressivity.

E.H. Leiter, The Jackson Laboratory, Bar Harbor, Maine: The role of environment in modulating the penetrance of diabetogenic susceptibility genes in nonobese diabetic (NOD) mice.

SESSION 2: Complex Diseases I: Cardiovascular and Psychiatric Diseases

Chairperson: A.G. Motulsky, University of Washington School of Medicine, Seattle

H.H. Hobbs, University of Texas Southwestern Medical Center at Dallas: Evidence for an LDL-lowering gene in a family with familial hypercholesterolemia.

S. Humphries, Charing Cross Sunley Research Center, London, United Kingdom: Strategies to identify functionally important common polymorphisms in candidate genes involved in atherosclerosis, thrombosis, and coronary artery disease risk.

M. Baron, New York State Psychiatric Institute, New York, New York: Genetic mapping of mental illness: Opportunities and pitfalls.

I.I. Gottesman, University of Virginia, Charlottesville: Phenotypic confusion in the classification of schizophrenics and their relatives for genetic analyses.

J.D. Rine, University of California, Berkeley: A possible contribution of dogs to behavioral genetics of mammals.

A. Chakravarti

J.V. Neel, S. Beall

SESSION 3: Genetics II: Genetic Tools

Chairperson: A. Chakravarti, University of Pittsburgh, Pennsylvania

G.J. Thomson, University of California, Berkeley: Mathematical techniques for molecular mapping of complex human genetic diseases.

N.J. Risch, Yale University School of Medicine, New Haven, Connecticut: Linkage strategies for genetically complex traits.

E.R. Lander, Whitehead Institute for Biomedical Research, Cambridge, Massachusetts: Quantitative analysis of polygenic traits.

M. Lathrop, CEPH, Paris, France: Non-HLA genes in type I diabetes.

J.H. Edwards, University of Oxford, United Kingdom: Nucleus and cytoplasm. Two maps or one.

SESSION 4: Complex Diseases II: Cancer and Autoimmune Disorders

Chairperson: M.-C. King, University of California, Berkeley

A.G. Knudson, Institute for Cancer Research, Philadelphia, Pennsylvania: Oncodemes and pathodemes.

M. Trucco, University of Pittsburgh, Pennsylvania: Susceptibility markers for IDDM.

S. Beall, California Institute of Technology, Pasadena: Strategies for determining the candidate genes involved in etiology of multiple sclerosis.

SESSION 5: Complex Diseases III: Congenital Defects

Chairperson: M.-C. King, University of California, Berkeley

D.H. Ledbetter, Baylor College of Medicine, Houston, Texas: Molecular studies of contiguous gene deletion syndromes.

A.A. Schinzel, Zurich Medical School, Switzerland: From the visible chromosome aberration to the mutant gene: Analysis of complex diseases with combined cytogenetic and molecular genetic techniques.

SESSION 6: Genetics III

Chairperson: J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York

F. Smith, Mount Sinai School of Medicine, New York, New York: Analysis of the acid β-glucosidase gene by PCR: Heterogeneity of mutations in Gaucher's Disease.

C. Venter, National Institute of Neurological Disorders and Stroke, Bethesda, Maryland: Large-scale automated DNA sequencing of chromosome regions associated with genetic diseases.

G.A. Evans, Salk Institute, San Diego, California: Structure and micropathology of human chromosome.

D.R. Cox, University of California, San Francisco: New approaches for mapping bipolar affective disease genes.

A.G. Motulsky, University of Washington School of Medicine, Seattle: Summation.

Molecular Mechanism of Fiber Cytotoxicity and Carcinogenesis

March 19–March 22

ARRANGED BY

W.R. Brinkley, University of Alabama at Birmingham
C.Harris, National Cancer Institute, Bethesda, Maryland
J.F. Lechner, National Cancer Institute, Bethesda, Maryland

SESSION 1: Background

W. Rom, New York University School of Medicine, New York, New York: Epidemiology and chemoprevention.
S. Knuutila, University of Helsinki, Finland: Cytogenetics of human mesothelioma.
J.E. Craighead, University of Vermont, Burlington: Animal models.
J.F. Lechner, National Cancer Institute, Bethesda, Maryland: In vitro models and effects of growth factors.
T.W. Hesterberg, Manville Corporation, Denver, Colorado: Cytotoxic and cytogenetic effects of asbestos on human bronchial epithelial cells: Chronic inhalation study with refractile ceramic fibers in rats and hamsters—18-month interim results.

SESSION 2: Growth Factors, Signal Transduction, and Cytoskeleton

W.R. Brinkley, University of Alabama at Birmingham: Overview.
M. Gilman, Cold Spring Harbor Laboratory, New York: Intracellular mediators of *c-fos* induction.
C.J. Molloy, National Cancer Institute, Bethesda, Maryland: Oncogenes and signal transduction in malignancy.
D. Beach, Cold Spring Harbor Laboratory, New York: Genetic control of the cell cycle.
J.G. Rheinwald, Dana-Farber Cancer Institute, Boston, Massachusetts: The involvement of EGF, FGF, and a novel autocrine mitogen in human mesothelial differentiation and transformation.

SESSION 3: Mitotic Spindle Control and Aberrations

W.R. Brinkley, University of Alabama at Birmingham: The centromere mitotic apparatus and aneuploidy.
C.L. Rieder, The Wadsworth Center, Albany, New York: Mitotic spindle assembly and chromosome movement.
G. Sluder, Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts: Biology of the centrisome: Formation, function and reproduction.
J.C. Barrett, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina: Induction of chromosomal aberrations in rodent cells.

SESSION 4: Molecular Mechanisms

C. Harris, National Cancer Institute, Bethesda, Maryland: Overview.

B.T. Mossman, University of Vermont, Burlington: Role of oxy-radicals in rodent cells.

J.D. Brain, Harvard University, Boston, Massachusetts: Role of nonalveolar macrophages in lung injury.

M.-C. Jaurand, CHU Henri Mondor, France: Neoplastic transformation of rodent cells by fibers.

T.F. Hei, Columbia University, New York, New York: Interactive effects of fibers and radon in neoplastic transformation.

E.M. Johnson, Mount Sinai School of Medicine, New York, New York: DNA transfection by fibers.

R.R. Reddel, Children's Medical Research Foundation, Camperdown, Australia: Neoplastic transformation of human mesothelial cells by activated proto-oncogenes.

C. Walker, Chemical Industry Institute of Toxicology, Research Triangle Park, North Carolina: Growth factor gene expression in *rat* mesothelioma.

B.I. Gerwin, National Cancer Institute, Bethesda, Maryland: Role of growth factors, oncogenes, and tumor suppressor genes in human mesothelial cell carcinogenesis.

SESSION 5: Implications for Risk Assessment

R.O. McClellan, Chemical Industry Institute of Toxicology, Research Triangle Park, North Carolina: Overview.

E. McConnell, Raleigh, North Carolina: Discussion leader.

Recognition in Plant-Pathogen Interactions

April 9–April 12

ARRANGED BY

S.P. Briggs, Pioneer Hi-Bred International, Inc., Johnston, Iowa
R.W. Michelmore, University of California, Davis
B. Staskawicz, University of California, Berkeley

SESSION 1: Interactions between Viruses and Plants

Chairperson: B. Staskawicz, University of California, Berkeley

D. Baulcombe, John Innes Institute, Norwich, United Kingdom: Identification of viral components that trigger responses in the host plant: Examples from PVX, TRV, and CMV.

R.N. Beachy, Washington University, St. Louis, Missouri: Role of the TMV movement protein in host susceptibility.

J. Culver, University of California, Riverside: The role of the tobacco mosaic virus coat protein in the induction of the hypersensitive reaction.

SESSION 2: Interactions between Bacteria and Plants

Chairperson: B. Staskawicz, University of California, Berkeley

J. Leach, Kansas State University, Manhattan: Avirulence genes from *Xanthomonas campestris* pv. *oryzae*, the bacterial blight of rice pathogens.

N. Keen, University of California, Riverside: Characterization of avirulence genes from *Pseudomonas syringae* pv. *tomato*.

B. Staskawicz, University of California, Berkeley: Gene-for-gene interactions specifying disease resistance in plant-bacterial interactions.

J. Dangl, Max-Delbrück-Laboratorium in der MPG, Koln, Germany: *Arabidopsis thaliana* and *Pseudomonas syringae:* Toward a simple pathosystem.

F. Ausubel, Massachusetts General Hospital, Boston: *Pseudomonas* induction of *Arabidopsis thaliana* phenylalanine ammonia lyase and B-1,3-glucanase genes and the use of "deletion cloning" to identify *Pseudomonas* avirulence genes.

M.J. Daniels, John Innes Institute, Norwich, United Kingdom: Specificity in *Xanthomonas-Arabidopsis* interactions.

SESSION 3: Interactions between Fungi and Plants: Potential Gene-for-Gene Interactions

Chairpersons: R.W. Michelmore, University of California, Davis, and **S.P. Briggs,** Pioneer Hi-Bred International, Inc., Johnston, Iowa

B. Tyler, University of California, Davis: Molecular genetics of *Phytophthora megasperma*.

P.J.G.M. de Wit, Wageningen Agricultural University, The Netherlands: Cloning of a gene encoding a race-specific elicitor from the tomato pathogen *Cladosporium fulvum*.

N.J. Talbot, University of East Anglia, Norwich, United Kingdom: Molecular genetic analysis of *Cladosporium fulvum* race specificity.

J. Ellis, CSIRO, Canberra, Australia: Can *Ac* be used to tag rust-resistance genes in flax?

J. Jones, John Innes Institute, Norwich, United Kingdom: Strategies for the isolation of tomato genes for resistance to leaf mold.

R.W. Michelmore, University of California, Davis: Molecular markers in the analysis of lettuce downy mildew.

B. Valent, E.I. du Pont de Nemours & Company, Wilmington, Delaware: Genes for cultivar specificity in the Rice Blast fungus, *Magnaporthe grisea*.

J.E. Hamer, Purdue University, West Lafayette, Indiana: Genome evolution, genetic mapping, and race-specific interactions in Rice Blast disease.

S. Leong, University of Wisconsin, Madison: Toward the cloning of genes controlling cultivar specificity in *Magnaporthe grisea*.

J. Bennetzen, Purdue University, West Lafayette, Indiana: Fine-structure analysis of a maize disease-resistance gene.

T. Pryor, CSIRO, Canberra, Australia: The *Rp1* gene complex specifying rust resistance in maize.

S.P. Briggs, Pioneer Hi-Bred International, Inc., Johnston, Iowa: Transposon-tagging the *Hm1* locus in maize.

S. Briggs, R. Michelmore, A. Ellingboe

B. Staskawicz

SESSION 4: Interactions between Fungi and Plants Involving Toxins

Chairperson: S.P. Briggs, Pioneer Hi-Bred International, Inc., Johnston, Iowa

C.A. Bronson, Iowa State University, Ames: The genetics of T-toxin synthesis in *Cochliobolus heterostrophus*.

O.C. Yoder, Cornell University, Ithaca, New York: Specificity in fungi-plant interactions.

J. Walton, Michigan State University, East Lansing: The biochemistry of HC-toxin synthesis.

V. Macko, Cornell University, Ithaca, New York: Host- selective toxins as molecular determinants of plant disease.

SESSION 5: Signal Transduction in Plants

Chairperson: S.P. Briggs, Pioneer Hi-Bred International, Inc., Johnston, Iowa

M.A. Lawton, Salk Institute for Biological Studies, San Diego, California: Molecular analysis of plant protein kinase genes.

C.J. Lamb, Salk Institute for Biological Studies, San Diego, California: Integration of pathways for developmental and environmental regulation of plant genes.

Programmed Cell Death: Concepts and Mechanisms

April 16–April 19

ARRANGED BY

F.O. Cope, Ross Laboratories, Columbus, Ohio
D. Goldgaber, State University of New York at Stony Brook
L.D. Tomei, Ohio State University, Columbus

Definition and Incidence of Apoptosis: An Historical Perspective
J.F.R. Kerr, University of Queensland, Australia

SESSION 1: Radiobiology and Carcinogenesis

Chairperson: L.D. Tomei, Ohio State University, Columbus

R. Schulte-Hermann, Vienna University, Austria: Role of apoptosis during carcinogenesis by nongenotoxic carcinogens.
T.R. Tritton, University of Vermont, Burlington: Cell death by chemotherapy.
S.R. Umansky, USSR Academy of Sciences, Pushcino, USSR: Radiation and glucocorticoid induced death of lymphoid cells.
A. Columbano, Universita di Cagliari, Italy: Cell proliferation and cell death in multistage chemical hepatocarcinogenesis.
K. Valerie, Virginia Commonwealth University, Richmond: Involvement of chromatin structure on radiation-induced gene expression in human cells.
J. Hully, McArdle Laboratory for Cancer Research, Madison, Wisconsin: Gap junctions and programmed cell death.
F.O. Cope, Ross Laboratories, Columbus, Ohio: Retinoid receptor cell death in carcinogenesis.

SESSION 2: Developmental Biology

Chairperson: R.A. Lockshin, St. John's University, Jamaica, New York

R.A. Lockshin, St. John's University, Jamaica, New York: Proteins synthesized during programmed cell death.
K.E. Alley, Ohio State University, Columbus: Cell death: A developmental strategy for all stages.
A. Alles, University of North Carolina, Chapel Hill: Retinoic-acid-induced cell death: Relationship to regions of programmed cell death in embryos.
J. Yuan, Massachusetts Institute of Technology, Cambridge: Mechanisms of cell death in the nematode *Caenorhabditis elegans*.

SESSION 3: Impact of Programmed Cell Death on Development of Clinical and Applied Concepts

Chairperson: F.O. Cope, Ross Laboratories, Columbus, Ohio

B. Szende, Semmelweis University Medical School, Budapest, Hungary: The role of apoptosis in the regression of experimental mammary and pancreatic tumors treated with luteinizing-hormone-releasing hormone (LHRH) and somatostatin analogs.
J.T. Isaacs, Johns Hopkins Oncology Center, Baltimore, Maryland: Programmed cell death of normal and malignant prostatic cells.
R. Buttyan, Columbia University, New York, New York: The regressing prostate gland as a model to elucidate the molecular pathway leading to programmed cell death.
L.E. Gerschenson, University of Colorado, Denver: Hormones and growth factors regulation of programmed cell death in rabbit uterine epithelium: Whole animal and cell-culture studies.
P.H. Krammer, German Cancer Research Center, Heidelberg: A monoclonal-antibody-induced tumor regression by induction of apoptosis.

SESSION 4: Immunology and Transplantation Biology

Chairperson: R.C. Duke, University of Colorado School of Medicine, Denver

D.J. McConkey, Dana-Farber Cancer Institute, Boston, Massachusetts: Cellular signaling in thymocyte apoptosis.

R.C. Duke, University of Colorado School of Medicine, Denver: Killer T cells possess the key to programmed cell death.

D.R. Green, University of Alberta, Edmonton, Canada: Activation-induced cell death in developing T cells and T-cell hybridomas.

E.A. Copelan, Ohio State University Hospital, Columbus: Selective eradication of malignant lymphoid cells from marrow using deoxycoformycin to initiate programmed cell death.

L. Fesus, University Medical School of Debrecen, Hungary: Transglutaminase-catalyzed cross-linking of proteins in the program of physiological cell death.

Open discussion: Role of programmed cell death in immune function.

Plenary discussion: Formation of an international organizing committee for an *International Conference on Apoptosis in Biology and Experimental Medicine, 1991,* and a committee on terminology.

SESSION 5: Biology of Differentiation and Aging

Chairperson: D. Goldgaber, State University of New York at Stony Brook

J.C. Barrett, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina: Tumor suppressor genes: Role of cellular senescence and differentiation.

D. Goldgaber, State University of New York at Stony Brook: Homeobox genes expression and aging.

Open discussion: How does apoptosis alter the prevailing view of aging?

SESSION 6: Neurobiology

Chairperson: D. Goldgaber, State University of New York at Stony Brook

D.P. Martin, Washington University School of Medicine, St. Louis: Neuronal cell death caused by trophic factor deprivation.

P. Davies, Albert Einstein College of Medicine, New York, New York: Cell death in the developing human brain may share features with Alzheimer's Disease.

P.B. Farel, University of North Carolina, Chapel Hill: Role of the periphery in regulating naturally occurring death among spinal motoneurons.

Open discussion: How has the concept of programmed cell death influenced neuropathology?

Vectors for Cloning the Immune Response

April 23–April 26

ARRANGED BY

R. Lerner, The Research Institute of Scripps Clinic, La Jolla, California

SESSION 1

Chairperson: W. Szybalski, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison

N.R. Klinman, Scripps Clinic and Research Foundation, La Jolla, California: The rules that govern V_H diversification.

M.G. Weigert, Fox Chase Cancer Center, Philadelphia, Pennsylvania: Antibody repertoires in autoimmune mice.

N.R. Klinman, Scripps Clinic and Research Foundation, La Jolla, California: Repertoire expression in the memory B-cell lineage.

G. Smith, G.P. Moore, M. Klinman

M. Gefter, M. Scharff

SESSION 2

Chairperson: H. Wigzell, Karolinska Institute, Stockholm, Sweden

J.A. Sorge, Stratagene, La Jolla, California: New technologies for expressing the immune repertoire.

W. Huse and A.S. Kang, The Research Institute of Scripps Clinic, La Jolla, California: Generation of immunoglobulin libraries in phage lambda.

D. Burton, Scripps Clinic and Research Foundation, La Jolla, California: Expression of a human antibody repertoire in *Escherichia coli* using phage lambda.

M. Schulman, University of Toronto, Canada: Homologous recombination between transferred and chromosomal immunoglobulin genes: A low-tech method of genetic engineering.

SESSION 3

Chairperson: H. Wigzell, Karolinska Institute, Stockholm, Sweden

W. Szybalski, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison: Bacteriophage lambda vectors decorated with fusion protein.

A. Pluckthun, University of Munich, Martinsried, Germany: Improving F_V fragments by engineering for selection in *E. coli*.

D. J. Henner, Genentech, Inc., South San Francisco, California: Expression of Fab fragments in *E. coli*.

D. Filpula, Genex Corporation, Gaithersburg, Maryland: Production of anti-fluorescein antigen-binding proteins.

SESSION 4

Chairperson: E.A. Kabat, Columbia University, New York, New York

E. A. Kabat, Columbia University, New York, New York: The repertoire of anti-α (1-6) dextrans.

K. Nickerson, Columbia University, New York, New York: Cloning the antidextran repertoire.

G. Smith, University of Missouri, Columbia: Filamentous fusion phage as vectors for antibody libraries.

F.D. Finkelman, Uniformed Services University of Health Sciences School of Medicine, Bethesda, Maryland: Polyclonal B-lymphocyte activation by anti-IgD antibody and other methods.

V. Chaudhary, National Institutes of Health, Bethesda, Maryland: Cloning of functional antibody variable domains as single-chain immunotoxins.

Sloan Foundation Science Journalists Workshop on Women and Cancer

April 29–May 1

ARRANGED BY

J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York

C Prives, Columbia University, New York, New York: Oncogenes and anti-oncogenes.
J. Cairns, Harvard School of Public Health, Boston, Massachusetts: Epidemiology of cancer, 1990.
H.S. Smith, Brush Cancer Research Institute, San Francisco, California: Oncogenes, chromosomal abnormalities, and diagnosis in breast cancer.
A.B. Miller, University of Toronto, Ontario, Canada: Controversial issues in breast cancer screening.
S. Swain, Lombardi Cancer Center, Washington, D.C.: Controversial issues in treatment of breast cancer.
D. Micklos and M Bloom, DNA Learning Center, Cold Spring Harbor Laboratory: Laboratory experiment: Bacterial transformation using an antibiotic-resistance gene.
S. Naylor, University of Texas Health Science Center, San Antonio: Oncogenes, chromosomal abnormalities, and diagnosis in lung cancer.
V.L. Ernster, University of California School of Medicine, San Francisco: Lung cancer in women: Smoking and cigarette advertising.
P. Howley, National Cancer Institute, Bethesda, Maryland: Viruses and cervical cancer.
D. Spiegel, Stanford University School of Medicine, California: Psychosocial intervention in treatment of cancer.

Neurofibromatosis

October 10–October 12

ARRANGED BY

F.S. Collins, University of Michigan Medical School, Ann Arbor
B.A. Ponder, Cambridge University, United Kingdom
B.R. Seizinger, Harvard Medical School, Boston, Massachusetts

SESSION 1: Introduction to the Clinical and Cell Biological Features of NF1/NF2

Chairperson: B.A. Ponder, Cambridge University, United Kingdom

S. Huson, Oxford University, United Kingdom: Clinical characteristics of NF1 and NF2.
R. Martuza, Harvard Medical School, Boston, Massachusetts: Overview of cell types affected by NF1 and NF2.

SESSION 2: Cloning the NF1 Gene

Chairperson: F.S. Collins, University of Michigan Medical School, Ann Arbor

M.R. Wallace, University of Michigan Medical School, Ann Arbor: Identification and characterization of the NF1 gene.
P. O'Connell, University of Utah Medical Center, Salt Lake City: Identification of the neurofibromatosis type-1 gene: Characterization of a gene within gene complex.

SESSION 3: NF1, GAP, and IRA

Chairperson: D.E. Housman, Massachusetts Institute of Technology, Cambridge

K. Tanaka, University of Chicago, Illinois: Negative regulation of *RAS* activity by a yeast homolog of GAP, *IRA*.
F. McCormick, Cetus Corporation, Emeryville, California: The biochemical properties of the NF1 protein.
R.L. White, Howard Hughes Medical Institute, University of Utah Medical Center, Salt Lake City: Mutation potential of the NF1 gene.

SESSION 4: Recent Progress in Cloning the NF2 Gene

Chairperson: B.R. Seizinger, Harvard Medical School, Boston, Massachusetts

J. Gusella, Massachusetts General Hospital East, Charlestown: Neurofibromatosis 2.
D.R. Cox, University of California, San Francisco: Fine-structure mapping of the NF2 region of human chromosome 22 by using radiation hybrids.
M. Nordenskjold, Karolinska Hospital, Stockholm, Sweden: Deletion mapping of the tentative meningioma locus on chromosome 22.

SESSION 5: Developmental Aspects of Neural Crest and Schwann Cells

Chairperson: D. Pleasure, Children's Hospital, Philadelphia, Pennsylvania

G.S. Ciment, Oregon Health Science University, Portland: The melanocyte/Schwann cell progenitor: Effects of growth factors on commitment.
G. Lemke, The Salk Institute, San Diego, California: Transcriptional regulation of Schwann cell development.
D. Pleasure, Children's Hospital, Philadelphia, Pennsylvania: Effects of protein growth factors on the development of neural-crest-derived cells.
M. Noble, Ludwig Institute, London, United Kingdom: Cellular biological studies on glial division and differentiation.

SESSION 6: Round Table Discussion of NF1/NF2 Tumorigenesis

SESSION 7: Models for the Road Ahead

Chairperson: D.R. Cox, University of California, San Francisco

F.S. Collins, University of Michigan Medical School, Ann Arbor: Cystic fibrosis.
E.P. Hoffman, University of Pittsburgh, Pennsylvania: Life after cloning: Recent advances in the muscular dystrophies.
J.M. Horowitz, Duke University Medical Center, Durham, North Carolina: Function of the retinoblastoma protein.
T. Doetschman, University of Cincinnati, Ohio: Targeted gene modification in the mouse germline.
D.E. Housman, Massachusetts Institute of Technology, Cambridge: Discussion: Where do we go from here?

Biological Basis for Risk Assessment of Dioxins and Related Compounds

October 21–October 24

ARRANGED BY

M.A. Gallo, UMDNJ-Robert Wood Johnson Medical School, Piscataway, New Jersey
R.J. Scheuplein, Food and Drug Administration, Washington, D.C.
C.A. van der Heijden, National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands

Introduction and Goals of the Second Conference on Dioxins
J. A. Moore, Institute for Evaluating Health Risks, Irvine, California and **A. Poland,** McArdle Laboratory for Cancer Research, University of Wisconsin, Madison

SESSION 1: Overview of the Adverse Health Effects of PCDDs and PCDFs

Chairperson: J.A. Moore, Institute for Evaluating Health Risks, Irvine, California

Animal Studies

R. Kociba, Dow Chemical Company, Midland, Michigan: Summary and interpretation of chronic rodent bioassays of TCDD.

W. Ray Brown, Research Pathology Services, Inc., New Britan, Pennsylvania: Implications following the reexamination of the slides from the chronic rat bioassay of TCDD.

D. Neubert, Free University of Berlin, Germany: Reproductive toxicity of PCDDs and PCDFs in animal models.

L. Birnbaum, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina: Developmental toxicity of TCDD and related compounds: Species sensitivities and differences.

J.G. Vos, National Institute for Public Health and Environmental Protection, Bilthoven, The Netherlands: Immunotoxicity of dioxin: Immune function and host resistance.

Human Studies

P. Mocarelli, University Hospital, Milan, Italy: Effects of acute and chronic exposure of humans to dioxins: A 9-year follow-up to Seveso.

M.A. Fingerhut, National Institute for Occupational Safety and Health, Cincinnati, Ohio: Studies of health effects from occupational exposures to dioxins.

L.L. Needham, Toxicology Branch, Centers for Disease Control, Atlanta, Georgia: Levels of TCDD in selected human populations.

Panel Discussion: Relevance of the animal studies to humans. Current state of the evidence supporting human toxicity (carcinogenicity, reproductive and developmental effects, and immunotoxicity).

SESSION 2: Sources of Exposure to Dioxins and Tissue Levels in Animals and Humans

Chairperson: C. Rappe, University of Umeå, Sweden

Environmental Sources of Exposure

C Rappe, University of Umeå, Sweden: Primary sources of PCDDs and PCDFs and human exposure via air and water.

H. Beck, Max von Pettenkofer Institute, Berlin, Germany: PCDD/PCDF levels in human milk and the problem of breastfeeding.

P. Furst, Federal State Control Laboratory for Food and Environmental Chemistry of North Rhine Westphalia, Munich, Federal Republic of Germany: Body burden with PCDD and PCDF from food.

P.M. Cook, U.S. Environmental Protection Agency, Duluth, Minnesota: Bioaccumulation and toxicity of PCDDs, PCDFs, and PCBs in aquatic ecosystems.

A. Schecter, State University of New York, Binghamton, New York: Levels of PCDDs and related compounds in ancient and modern human tissues: Exposed and general populations.

Human Body Burden and Distribution in Tissue

C. Schlatter, Institute for Toxicology Technical High School and University, Zurich, Switzerland: Pharmacokinetics of PCDDs in humans.

V. Houk, Centers for Disease Control, Atlanta, Georgia: Health effects of service in Viet Nam.

R.M.C. Theelen, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands: Modeling of human exposure from relevant sources of exposure.

M.E. Andersen, Chemical Industry Institute of Toxicology, Research Triangle Park, North Carolina: Biological determinants of the disposition of TCDD and related compounds.

SESSION 3: Mechanism of Action

Chairperson: A. Poland, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison

Receptor Biochemistry

J.-A. Gustafsson, Huddinge University Hospital, Sweden: Molecular biology and physiology of TCDD receptor–DNA interactions.

T.A. Gasiewicz, University of Rochester School of Medicine, New York: Different forms of the Ah receptor.

M.S. Denison, Michigan State University, East Lansing: Species variation in Ah receptor transformation and DNA binding.

Regulation of Gene Expression

J.P. Whitlock, Jr., Stanford University School of Medicine, California: Genetic and molecular aspects of TCDD action.

S.H. Safe, College of Veterinary Medicine, Texas A&M University, College Station: Regulation of growth factor and hormone receptors.

W.F. Greenlee, Chemical Industry Institute of Toxicology, Research Triangle Park, North Carolina: Isolation of novel dioxin-responsive genes: Implications for toxicity/carcinogenicity.

G. Lucier, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina: Tumor promotion in liver.

S. Flodstrom, Karolinska Institute, Stockholm, Sweden: Tumor-promoting activity of TCDD and related compounds.

SESSION 4: Implications of Receptor-mediated Toxicity to Carcinogenic Risk Assessment

Chairperson: M.A. Gallo, UMDNJ-Robert Wood Johnson Medical School, Piscataway, New Jersey

A. Poland, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison: TCDD and related aromatic hydrocarbons: Reexamination of the mechanism of toxicity and carcinogenesis.

S.H. Safe, L.S. Birnbaum, T.A. Gasiewicz: Short reports from the NIEHS Conference on Dioxin Risk Assessment.
(1) Survey of thresholdable dose response curves involving Ah-receptor-mediated responses.
(2) Survey of three papers on nuclear receptor response.
(3) Discussion of animal versus human sensitivity.
(4) Discussion of animal and human blood levels versus response.

M.A. Gallo, UMDNJ-Robert Wood Johnson Medical School, Piscataway, New Jersey: Can a threshold be credibly associated with the Ah receptor mechanism.

E. Silbergeld, University of Maryland Program in Toxicology, Baltimore: Receptor-based models for risk assessment: Application to TCDD.

Panel Discussion: Integration of mechanistic/dosimetry data into human risk assessment.

The Impact of Human Molecular Genetics on Society

November 5–November 8

ARRANGED BY

C.T. Caskey, Baylor College of Medicine, Houston, Texas
P. Reilly, Shriver Center for Mental Retardation, Waltham, Massachusetts
J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York

SESSION 1: Genetics and Society: Past, Present, and Future

Chairperson: C.T. Caskey, Baylor College of Medicine, Houston, Texas

P. Reilly, Shriver Center for Mental Retardation, Waltham, Massachusetts: Lessons from past experiences of society's involvement with genetics.

C.T. Caskey, Baylor College of Medicine, Houston, Texas: Current studies of molecular diagnosis.

D. Botstein, Stanford University, California: Present and future prospects for genetic testing of predisposition to polygenic disease.

M.W. Shaw, Evansville, Indiana: The human genome: Private property or public domain?

Case study and discussion: Newborn and prenatal screening.

SESSION 2: Issues in Genetic Screening

Chairperson: J. Beckwith, Harvard Medical School, Boston, Massachusetts

F. Greenberg, Baylor College of Medicine, Houston, Texas: Humanitarian issues raised by prenatal and neonatal screening and diagnosis.

M. Angastiniotis, Archbishop Makarios Memorial Hospital, Nicosia, Cyprus: Social or community responses to a national control program for Thalassemia.

A. Chakravarti, University of Pittsburgh, Pennsylvania: Strategies for screening large populations: CF as a model.

N. Fost, University of Wisconsin Hospital, Madison: Ethical issues in newborn and carrier screening for CF.

Case study and discussion: Carrier testing and screening.

SESSION 3: Genetic Testing: Health Care and Insurance

Chairpersons: J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York, and **L.M. Russell,** Committee on Energy and Commerce, Washington, D.C.

T.H. Murray, Case Western Reserve University School of Medicine, Cleveland, Ohio: Genetic testing in insurance: Ethical issues.

J.M. Stein, The Prudential, Newark, New Jersey: Factors determining the implementation of genetic testing for insurance purposes.

M.A. Rothstein, University of Houston, Texas: Genetic screening in the workplace.

P. Harper, University of Wales College of Medicine, Cardiff, United Kingdom: Huntington's disease: A model for ethical problems in testing children for late onset genetic disorders.

R.E. Pyeritz, The Johns Hopkins Hospital, Baltimore, Maryland: Health-care and insurance problems of families with genetic disease.

J. Levi, Washington, D.C.: Early experience of HIV testing as a model.

R. Bachman, Kaiser-Permante Medical Center, Oakland, California: High-tech health care: Hard choices in an HMO.

Case study and discussion: Disclosure of genetic risk.

SESSION 4: Data Banks and DNA Banking

Chairperson: A. Chakravarti, University of Pittsburgh, Pennsylvania

G.J. Annas, Boston University Schools of Medicine & Public Health, Massachusetts: Legal issues in DNA banking.

G.F. Vovis, Collaborative Research, Inc., Bedford, Massachusetts: Commercial DNA banking.

P.R. Billings, Pacific Presbyterian Medical Center, San Francisco, California: Privacy issues and the human genome projects.

T. Marr, Cold Spring Harbor Laboratory, New York, and P. Reilly, Shriver Center for Mental Retardation, Waltham, Massachusetts: Data banks, DNA banking, and forensic science.

Case study and discussion: DNA forensic/genetic data banks.

Molecular Biology of Free-radical Scavenging Systems

November 11–November 14

ARRANGED BY

I. Fridovich, Duke University Medical Center, Durham, North Carolina
J.G. Scandalios, North Carolina State University, Raleigh

Introductory Remarks
J.G. Scandalios, North Carolina State University, Raleigh

SESSION 1: Prokaryotic Gene Regulation

Chairperson: K. Asada, Kyoto University, Japan

D. Touati, University of Paris, France: A double lock, implying two global regulatory systems, *fur* (ferric uptake regulation) and *arc* (aerobic respiration control) shut off anaerobic expression of MnSOD in *Escherichia coli* K12.

J.A. Fee, Los Alamos National Laboratory, New Mexico: Role of the iron uptake locus (*fur*) in the regulation of bacterial *Sod* genes.

H.M. Hassan, North Carolina State University, Raleigh: Regulation of MnSOD in *Escherichia coli:* Role of DNA topology.

P.C. Loewen, University of Manitoba, Winnipeg, Canada: Regulation of *katE* and *katF* transcription in *Escherichia coli.*

C. Foyer, National Institute of Agronomic Research, Versailles, France: Effects of variations of the activity of glutathione reductase on cellular glutathione contents and metabolism in *Escherichia coli* and tobacco.

SESSION 2: Eukaryotic Gene Regulation

Chairperson: I. Fridovich, Duke University Medical Center, Durham, North Carolina

J.G. Scandalios, North Carolina State University, Raleigh: The antioxidant defense genes *Cat* and *Sod* of maize: Structure and regulation.

T. Asahi, Nagoya University, Japan: Catalase genes in the castor bean: Structure and regulation.

J. P. Phillips, University of Guelph, Ontario, Canada: Genetic analysis of free-radical scavenging systems in *Drosophila.*

H. Ruis, University of Vienna, Austria: Differential control of synthesis of peroxisomal and a cytosolic catalase of *Saccharomyces cerevisiae* suggests different functions.

R.W. Skadsen, University of Wisconsin, Madison: Molecular mechanisms regulating *Cat2* gene expression in the acutellum of maize seedlings.

A.P. Autor, University of British Columbia, Vancouver, Canada: Regulation of MnSOD in eukaryotes.

SESSION 3: Biomedical Approaches

Chairperson: S. Linn, University of California, Berkeley

L.W. Oberley, University of Iowa, Iowa City: Role of antioxidant enzymes in cancer.

G.H.W. Wong, Genentech, Inc., South San Francisco, California: Relative protective effects of MnSOD, Cu/Zn SOD, Ec-SOD against various cellular insults.

P.A. Cerutti, Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland: Genetic modulation of antioxidant enzymes in mammalian cells.

J.V. Bannister, Cranfield Institute of Technology, Bedfordshire, United Kingdom: Biomedical aspects of the application of recombinant SOD.

K. Matsushima, Frederick Cancer Research Facility, Maryland: MnSOD modulates the sensitivity of tumor cells to cytokine, radiation, and chemotherapy.

SESSION 4: Targets and Targeting

Chairperson: J.G. Scandalios, North Carolina State University, Raleigh

I. Fridovich, Duke University Medical Center, Durham, North Carolina: Enzymatic targets for the superoxide radical.

P.B. Lazarow, Mount Sinai School of Medicine, New York: Peroxisome biogenesis.

S. Linn, University of California, Berkeley: Mechanisms of damage to DNA by hydrogen peroxide.

B. Halliwell, Kings College, London, United Kingdom: Fingerprints of oxidative damage to DNA.

M. Nishimura, National Institute for Basic Biology, Okazaki, Japan: Biosynthesis, transport, and degradation of plant microbody enzymes.

R.S. Sohal, Southern Methodist University, Dallas, Texas: Relationship between generation of prooxidants and the aging process.

SESSION 5: Structure, Function, and Evolution

Chairperson: H. Hassan, North Carolina State University, Raleigh

K. Asada, Kyoto University, Japan: Molecular mechanisms of production and scavenging of active oxygen in chloroplasts.

E.A. Havir, Connecticut Agricultural Experimental Station, New Haven: Characterization of catalase isozymes with enhanced peroxidatic activity in plants.

W. Stallings, The Monsanto Company, St. Louis, Missouri: Fe and Mn SODs: Catalytic inferences from crystal structures.

R.E. Cannon, North Carolina State University, Raleigh: Structure and expression of the maize Cu/Zn *Sod4* and *Sod4A* cytosolic isozyme genes.

J. Kwiatowski, University of California, Irvine: Structure and evolution of the Cu/Zn SOD in Diptera.

Computational Aspects of Protein Folding

December 3–December 6

ARRANGED BY

R.L. Jernigan, National Cancer Institute, Bethesda, Maryland
H.A. Scheraga, Cornell University, Ithaca, New York

SESSION 1: Potentials, Free Energies, and Methods

Chairperson: D.L. Beveridge, Wesleyan University, Middletown, Connecticut

B. Honig, Columbia University, New York, New York: An evaluation of energetic contributions to protein stability.

A.A. Rashin, Biosym Technologies Inc., Parsippany, New Jersey: Electrostatics and the energetics of hydration in proteins.

G.M. Crippen, University of Michigan, Ann Arbor: Protein folding is a combinatorial contest between geometry and energy.

P.A. Kollman, University of California, San Francisco: Free-energy calculations on macromolecules.

K.D. Gibson, Cornell University, Ithaca, New York: Recent approaches to the multiple minimum problem in protein folding.

H.A. Scheraga, Cornell University, Ithaca, New York: Multiple-minima problem.

SESSION 2: Molecular Dynamics Simulations

Chairperson: P.A. Kollman, University of California, San Francisco

M. Karplus, Harvard University, Cambridge, Massachusetts: Aspects of protein folding/stability.

W.L. Jorgensen, Yale University, New Haven, Connecticut: Molecular dynamics simulations of peptide and protein unfolding.

J. Hermans, University of North Carolina at Chapel Hill: Molecular dynamics studies of peptide conformational equilibria.

D.L. Beveridge, Wesleyan University, Middletown, Connecticut: Aspects of protein-folding accessible to molecular dynamics simulation.

N. Go, Kyoto University, Japan: Description of protein dynamics in terms of normal mode variables and its application in the refinement of protein X-ray crystallography.

A. Brunger, Howard Hughes Medical Institute, Yale University, New Haven, Connecticut: Simulation of helix-helix interactions: Applications to "leucine zippers."

SESSION 3: Models of Folding

Chairperson: H.A. Scheraga, Cornell University, Ithaca, New York

F.M. Richards, Yale University, New Haven, Connecticut: On packing and cavities.

K.A. Dill, University of California, San Francisco: On the origins of structure in globular proteins.

F.E. Cohen, University of California, San Francisco: The utility of simplified models in understanding protein structure.

R.L. Jernigan, National Cancer Institute, Bethesda, Maryland: Characteristics of compact conformations.

J. Skolnick, Scripps Clinic and Research Foundation, La Jolla, California: Computer simulations of the folding of plastocyanin.

J. Moult, Center for Advanced Research in Biotechnology, Rockville, Maryland: Analysis of protein-folding pathways.

B. Robson, Proteus Molecular Design Limited, Cheshire, United Kingdom: Folding proteins by the creation of new conservation laws.

SESSION 4: Crystal Gazing and New Approaches

Chairperson: R.L. Jernigan, National Cancer Institute, Bethesda, Maryland

G.D. Fasman, Brandeis University, Waltham, Massachusetts: Prediction of protein conformation: Why have prediction methods failed to give the correct structure?

S. Rackovsky, University of Rochester, New York: Quantitative classification of protein structures.

C.H. Chothia, M.R.C. Laboratory of Molecular Biology, Cambridge, United Kingdom: Sequence determinants of protein folds.

J.J. Wendoloski, E.I. du Pont de Nemours & Company, Wilmington, Delaware: Rebuilding proteins from limited structural data.

S.J. Wodak, Free University of Brussels, Belgium: Knowledge-based structure predictions: What are the limitations?

S.H. Kim, University of California, Berkeley: Prediction of structural features by neural network.

SESSION 5: Model Building and Design

Chairperson: C.H. Chothia, M.R.C. Laboratory of Molecular Biology, Cambridge, United Kingdom

V.S. Madison, Hoffmann-La Roche, Inc., Nutley, New Jersey: A beta-barrel model for ECGF.

O. Teleman, Lund University, Sweden: Peptide folding: An attempt at a peptide catalyst, and the amino-terminal EGF-homologous peptide from blood coagulation factor X.

M.R. Pincus, State University of New York Health Science Center, Syracuse: The chain build-up procedure in protein folding: Peptide determinants of protein structure.

G. Némethy, Mount Sinai School of Medicine, New York, New York: Interactions of local structures in protein folding.

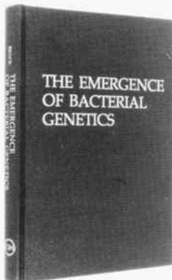
F.R. Salemme, E.I. du Pont de Nemours & Company, Wilmington, Delaware: Statistical approaches to protein reconstruction using substructure libraries.

H.R. Guy, National Cancer Institute, Bethesda, Maryland: Modeling the structure of membrane channel proteins.

C. Sander, EMBL, Heidelberg, Germany: Protein design: Theory and experiment.

COLD SPRING HARBOR
LABORATORY PRESS

1990 PUBLICATIONS



General Books

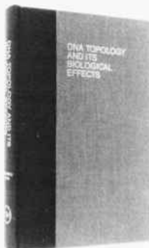
Immunological Recognition
Symposia on Quantitative Biology 54

Vaccines 90
F. Brown, R.M Chanock, H.S. Ginsberg, and R.A Lerner (eds.)

Genetic Maps (5th edition)
S.J. O'Brien (ed.)

The Emergence of Bacterial Genetics
T.D. Brock

Mapping the Genomes of Agriculturally Important Animals
J.E. Womack (ed.)



CSHL Monograph Series

Oncogenes and the Molecular Origins of Cancer
R.A. Weinberg (ed.)

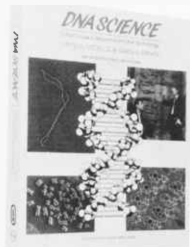
Stress Proteins in Biology and Medicine
R.I. Morimoto, A. Tissières, and C. Georgopoulos (eds.)

DNA Topology and Its Biological Effects
N.R. Cozzarelli and J.C.Wang (eds.)

Banbury Report Series

Genetics and Biology of Alcoholism (Banbury Report 33)
C.R. Cloninger and H. Begleiter (eds.)

Biology of Mammalian Germ Cell Mutagenesis (Banbury Report 34)
J.W. Allen, B.A. Bridges, M.F. Lyon, M.J. Moses, and L.B. Russell (eds.)



Manuals

DNA Science: A First Course in Recombinant DNA Technology
D.A. Micklos and G.A. Freyer

Methods in Yeast Genetics: A Laboratory Course Manual
M.D. Rose, F. Winston, and P. Hieter

Journals

Genes & Development (Volume 4, numbers 1–12B)

Cancer Cells: A Monthly Review (Volume 2, numbers 1–12)

Other

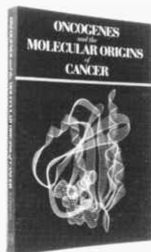
CSHL Annual Report 1989

Abstract/program books for 12 CSHL meetings

COLD SPRING HARBOR LABORATORY PRESS

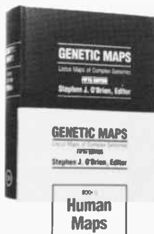
To our great satisfaction, 1990, like its predecessor, was another notable year for publishing at Cold Spring Harbor Laboratory. We ended the year with total revenues in excess of $4.19 million, giving an operating margin before depreciation of over $480,000. Three factors contributed to this outcome: sustained sales from our best-selling books, with *Molecular Cloning* being the most outstanding; solid sales from the new 1990 titles, which were assisted by complimentary reviews in prominent journals; and, for the first time, a major contribution to revenues from our journal program.

Reviving a Respected Series and Breaking New Ground



In 1990, 12 books were published. This was a smaller number than in recent years, but each was a substantial work. We have now reduced to a satisfactory minimum our former dependence on the proceedings of meetings, particularly those at the Banbury Center, whose extended abstracts accounted for well over half of our output in the past five years. Three of the 1990 titles, on oncogenes, stress (heat-shock) proteins, and DNA topoisomerases, revived the series of Cold Spring Harbor Monographs begun in 1970. *Oncogenes and the Molecular Origins of Cancer* was particularly popular, with sales of well over 1500 copies in the year. Its editor, Robert Weinberg, clearly succeeded in his aim of producing a book with broad appeal.

Space prevents individual mention of each title, but reference should be made to the success of the fifth edition of *Genetic Maps*, edited by Stephen O'Brien and produced for the first time in two editions, a cloth-bound compendium and a series of six inexpensive paperbacks. The current interest in genome sequencing helped to drive sales in 1990 past 2600 copies. It was gratifying to see strong demand and admiring reviews for Michael Ashburner's huge work, *Drosophila*, published in two parts at the end of 1989. We welcomed also the appearance of *DNA Science* by David Micklos and Greg Freyer, a textbook and lab manual aimed at students taking their first courses in molecular biology, based largely on courses taught at the Laboratory's DNA Learning Center. A handsome, two-color book, it triumphantly broke new ground for our production staff. We and our copublishers, the Carolina Biological Supply Company, were delighted when the first printing of 5000 copies rapidly sold out.



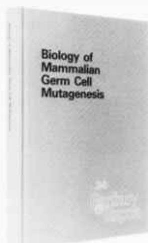
Underpinning our sales returns were our best-selling laboratory manuals. In its second full year of publication, *Antibodies* surpassed 20,000 copies, and we welcomed its authors Ed Harlow and David Lane back to Cold Spring Harbor in November to celebrate that event. *Molecular Cloning*, by Joe Sambrook, Ed Fritsch, and Tom Maniatis added 15,000 sales in 1990 to achieve in one year of publication an extraordinary total of 50,000 copies. Its reputation as the bible of the field seems still to be secure.

As usual, the book editorial and production staff, led by Nancy Ford, made exceptional efforts to ensure that the design and accuracy of these books matched both the authors' aspirations and their own customary high standards. New technology provided invaluable assistance: 6 of the 12 books published

Staff of the Editorial Production Department outside Urey Cottage. From right to left: Pat Barker, Elaine Gaveglia, Lee Martin, Maryliz Dickerson, Marie Sullivan, Liz Ritcey, Dorothy Brown, Nancy Ford, Jim Suddaby, Inez Sialiano, Annette Kirk, Mary Cozza, Pauline Tanenholz, Joan Ebert, Ralph Battey, Christy Kuret.

were typeset in-house, and the optical scanner bought last year quickly became a mainstay. The equipment's success gave us the confidence to commit to completing our investment in independent typesetting next year, at a level of sophistication adequate for all our needs, from books to journals to internal publications such as this *Annual Report*.

Establishing a New Voice



Our monthly review journal, *Cancer Cells*, completed its first full year of publication in September. It has had to jostle in a crowded marketplace, appearing just as four other new journals were launched in cancer research, all with somewhat similar aims. The field is in an optimistic phase, buoyed up by discoveries in genetics and molecular biology in recent years, and the proliferation of new journals demonstrates a desire in the scientific community for new sources of the best and most exciting work. This proliferation justifies the existence of *Cancer Cells*, which recognizes the information overload and attempts to explain and interpret advances for readers who lack either the time or the technical expertise to keep up with the primary sources. In its first year, *Cancer Cells* established a unique voice, a tribute to the editorial skills of Paula Kiberstis, who ensured that the journal's coverage was broad and interesting and enhanced the submitted commissioned manuscripts to high standards of clarity. In this task, she was greatly helped by the appointment in October of Catriona Simpson as the journal's Assistant Editor. The journal's special qualities were acknowledged in a prominent review of the year's new publications. Several editorial features proved popular, particularly a digest of information about new oncogenes and a historical article, written by Jan Witkowski for the August/September issue, on the con-

cept of cancer as a genetic disease. During the year, the circulation reached a satisfactory level, and having established the journal's voice, the challenge now is to ensure that it is loudly and widely heard.

Combining Excellence with Financial Success

In 1990, *Genes & Development* had another excellent year: 583 manuscripts were offered for publication, and 204 (35%) were published in 13 issues. The editorial standard remained strikingly high, which is a credit to its editors Terri Grodzicker and Nicholas Hastie and to the uncompromising advice they received from editorial board members and other reviewers. The issues were notable for their editorial accuracy and production standards, and in mid-year, the journal was awarded a printing industry prize, the Neographic Gold Award, in recognition of the graphic excellence of its design and its covers designed by Judy Cuddihy, the Managing Editor. The journal staff did an outstanding job under high pressure throughout the year and was greatly assisted by the return to our ranks of an experienced technical editor, Nadine Dumser.

Our sense that *Genes & Development* had advanced rapidly into the small, select group of top-class journals was underlined by citation analysis, which showed that even in 1988, its second year of publication and the most recent year for which data are available, the journal was being cited at a rate higher than any other journal of genetics or developmental biology. This impact was reflected in an overall 22% increase in paid subscribers in 1990. Growth was particularly strong among libraries, which gave a healthy stimulus to the journal's revenues. Income was boosted further by substantial increases in secondary sources such as advertising, which rose by over 30% under the guidance of our

Staff of the Acquisition and Marketing Departments outside Carnegie Library. From left to right: Catriona Simpson, Nancy Kuhle, John Inglis, Paula Kiberstis, Connie Hallaran, Nadine Dumser, Terri Grodzicker, Lisa Sweeney, Judy Cuddihy.

Advertising Manager, Nancy Kuhle, who joined us in February. Rising income and prudent financial management ensured that, as predicted, the journal ended the year with a surplus for the first time, bringing to an end the four-year period of cumulative investment required from the Press and its partner, the Genetical Society of Great Britain. For a journal with a professional staff, dedicated to rapid publication and to quality, this rate of progress to profitability has been highly satisfactory.

Improving Marketing and Customer Service

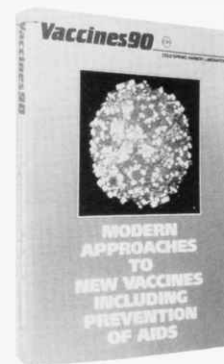
Our marketing activities remain centered on direct mail, display advertisements, and sales at the larger scientific conferences. A concerted effort was made to track down smaller meetings worldwide and send brochures which were often created in-house. These activities, the production of three issues of our newsletter the *Notebook*, the creation of the catalog and two short variations on it, and the management of our bookstore in Grace Auditorium demanded much of the marketing department staff, led by Charlaine Apsel. Our ever-expanding stock of active titles continued to press the need for new and larger warehouse space. The persistence of Guy Keyes, our Warehouse Manager, led eventually to the acquisition of suitable premises in Plainview, about five miles from Cold Spring Harbor, in which the whole Customer Service department could be housed. Our entire inventory, consisting of over 68,000 volumes plus a variety of other materials, was moved to Plainview in September.

A further step in the improvement of our customer service was the purchase in December of a new computer system for maintaining sales records, fulfilling orders, and analyzing our customer data base. Breaking our former reliance on a rented, shared system, the new computer's software was tailored to our specific requirements. Its installation and the mounting of existing sales records required extraordinary efforts from the staff involved. Nevertheless, all concerned recognize that once it is functional and familiar, the new system will be quicker, more cost-effective, and a powerful marketing tool.

Staff of the Business Operations Department outside the offices and warehouse at Plainview. From left to right: Elaine Gaveglia, Ann Felten, Jackie Matura, Guy Keyes, Charlaine Apsel, Barbara Terry, Bill Dickerson, Penny Sheppard, John Flynn.

Launching Three New Series and a Third Journal

A considerable variety and number of new publishing projects were initiated in 1990, most of which require time to mature. Four projects, however, will bear fruit next year. In September, we completed negotiations with the Imperial Cancer Research Fund for the rights to publish on its behalf the prestigious review series *Cancer Surveys*. Three 300-page volumes, each containing invited reviews on a single topic, will appear in 1991. Another new series for 1991 will be *Current Communications in Cell & Molecular Biology*. These books range widely in subject and in some cases their preparation will be assisted by a Banbury Center meeting on the topic. Jan Witkowski's participation with me in editing the series has been invaluable. A third book series, *Genome Analysis,* edited by Kay Davies and Shirley Tilghman, will begin early in 1991.



In December, we completed plans for the launch of a third journal in the coming year. The polymerase chain reaction (PCR) has recently revolutionized biology with its elegance and seemingly limitless application to DNA detection. Our journal, *PCR Methods and Applications*, edited by Judy Cuddihy in association with four academic scientists, will publish peer-reviewed research papers and commissioned reviews on the principles and practice of PCR and other amplification methods. The journal will be quarterly at first, appearing for the first time in July. We have been fortunate to receive from Perkin-Elmer Cetus, the leading PCR company, generous support for the journal in the form of a bulk subscription purchase, guaranteed advertising and access to its customer database. Although it is very early days for this project, the journal promises to be highly regarded by a scientific community anxious to harness the power of this remarkable technique.

Restructuring and Rationalizing Resources

During the year, it became clear that our investments in new technology and new premises off the Laboratory grounds would permit us to rationalize resources and restructure the management of the Press. This process took place in December, and we prepared to enter the New Year reconfigured in four departments: Editorial/Production, based in Urey Cottage and headed by Nancy Ford as Managing Editor of the Press, responsible for both the book and journal programs; Business Operations, based at Plainview and managed by Charlaine Apsel, responsible for customer service, sales, and financial reporting; a newly independent Marketing Department, based in Carnegie Library, for which a new head is needed and which is responsible for marketing strategy and execution; and Acquisition, based in Carnegie and headed by me, which is responsible for the development of new publishing projects and the existing journals. The restructuring affected a substantial proportion of our staff, who accepted the changes with grace and adapted willingly. I am particularly grateful to the senior staff of the Press, without whose support the restructuring would not have been possible.

We ended 1990, therefore, on a note of real optimism, with satisfactory financial results, the immediate prospect of major benefits from new equipment and improved organization, and the promise of publishing over 20 new books and further expanding our journal program in 1991.

John R. Inglis

DNA LEARNING CENTER

DNA LEARNING CENTER

David A. Micklos, Director
Mark V. Bloom, Assistant Director
Susan M. Lauter, Designer
Margaret E. Henderson, Education Manager
Sandra H. Ordway, Administrative Assistant

Periodic studies conducted since 1959 show that general scientific illiteracy is a persistent problem in the United States. Recent studies sponsored by the National Science Foundation (NSF) suggest that only about 6% of Americans can be considered functionally literate in scientific matters and that college graduates are only marginally more literate.

Clearly, Americans have had enough difficulty understanding developments in a postmodern world clouded by questions of nuclear fission/fusion and increasingly controlled by devices crammed with computer microprocessors. Now, the proliferation of gene-manipulation technology adds a new dimension to our national science confusion. Indeed, a 1987 Office of Technology Assessment survey found that 63% of Americans claim to have heard or read relatively little or almost nothing about "genetic engineering" and 44% cannot give any meaningful definition of the word. The NSF-sponsored studies show that only 10% of the American public can explain what a molecule is, and only 22% can define DNA correctly.

At the same time, we have a national research agenda to identify all of the approximately 100,000 different human genes, the Human Genome Project. The impact of this work is already being felt. In September 1990, a 4-year-old girl suffering from a severe immune deficiency received an infusion of genetically altered cells, in the first attempt to replace a defective gene with a normal one. Gene therapy such as this promises the ultimate cure for the thousands of known genetic diseases, and the molecular tracings of DNA fingerprints provide the ultimate evidence in rape, murder, and paternity cases nationwide. These activities also throw into sharp relief the potential risks of gene manipulation, the difficulties of fairly allocating resources for biological research, and the issues of proper uses of and access to personal genetic profiles.

The good news is that 57% of Americans appear able to understand a simple problem dealing with the inheritance of a genetic illness, suggesting that they find this topic relevant to their personal lives. We also have a self-professed "education president," and some two dozen bills to encourage science, math, and engineering education were considered by Congress in 1990. In the context of this renewed sense of urgency, the social importance of the Human Genome Project offers an opportunity to reorganize substantially biology and health education to include new emphasis on human molecular genetics.

Ideal and Reality of Biology Education

Public education has long been viewed as a key enabler of democratic pluralism, providing individuals with access to elements of cultural, political, and scientific

literacy. If we take stock in this Madisonian concept of an informed citizenry that participates in public decision making, DNA literacy must be considered an essential element of precollege public education. In this view, science teachers, who interface with a cross section of America's youth, constitute a major conduit through which understanding of the uses and misuses of genetic technology can flow broadly to society.

One assumes that children begin life with curiosity for the natural world. Indeed, data from the 1986 National Assessment of Educational Progress (NAEP) study show that the vast majority of American third graders both like their science lessons (67%) and find them interesting (78%). However, there are many indicators that this enthusiasm for science erodes dramatically with increased exposure to formal science during junior and senior high school. The NAEP study shows that whereas 67% of third graders believe that things learned in science class will be useful in everyday life, only 54% of seventh graders and 49% of eleventh graders see the practicality of their science studies. A recent study by the International Association for the Evaluation of Educational Achievement found that American fifth graders rank eighth in science achievement among their peers in 14 developed and developing nations; however, advanced high school seniors rank last in biology achievement among their international peers.

It is not that we don't have the chance to influence high school students to pursue any native interest they may have in living things. Biology is the most frequently studied science in high school and is taken by 90% of all American students. In spite of, or perhaps because of, this exposure, only 6% of high school students take an advanced course in biology, and only 3.7% of college freshmen intend to major in the biological sciences.

All this suggests that it is the manner in which science is taught that causes young people to become so disinterested in science. The NAEP study paints a disturbing picture of the American precollege science classroom, where the "preponderance of class time [is] spent listening to teachers' lectures" and where

DNA Learning Center staff: (*standing*) David Micklos, Sandy Ordway, Margaret Henderson, Mark Bloom, (*seated*) Susan Lauter, Amy Phillips

there are "few opportunities to explore natural phenomena directly or engage in discussions about the limited experiences that they did have." The scientific content in these classes "appears to be largely textbook- and workbook-driven, reflecting little—or not at all—the recent technological advances in the domain of science." Students interviewed in the NAEP study gave the following report on their eleventh grade science classes:

- 79% listen to a lecture daily or several times per week.
- 54% read their textbooks daily or several times per week.
- 47% do not participate in experiments on at least a weekly basis.
- 41% had never written up experiments.
- 52% had never done an oral or written report.
- 39% had never read articles on science.
- 86% had never been on a field trip.

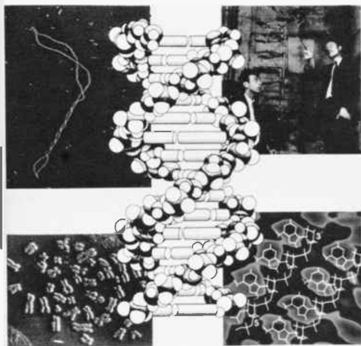
According to the NAEP study, more than one-third of eleventh grade science teachers do not have access to a general purpose or specialized science laboratory. Our conversations with high school biology teachers in a number of states indicate that science departments typically have annual supply budgets calculated at just several dollars per student and that capital equipment purchases are limited to replacement of existing items. Many older high schools are functioning with essentially the same basic set of equipment purchased with the bond issue with which they were constructed during the "baby boom" of the 1950s–1960s.

Although the nature and practice of biology is forward looking, the burden of evidence shows that teaching high school biology is reactionary. In many ways, biology education has changed little from the days of our grandparents. Hands-on laboratories are the exception, and rote memorization is the norm. This is in large part because high school biology curricula have evolved in the twentieth century essentially by cramming in more and more facts. Faced with the specter of a standardized test that covers a vocabulary-driven syllabus, the teacher is often constrained to spend his or her time lecturing on science facts. Meager equipment/supply budgets and a lack of teaching laboratories preclude any but the most simplistic laboratories, which reflect primarily the observational traditions of biology. Missing are meaningful experiments that reflect the quantitative and biochemical basis of modern molecular genetics.

Publication of *DNA Science*

Since 1985, Cold Spring Harbor Laboratory has expended $2.5 million to infuse genetic literacy through the public education system. The publication of *DNA Science: A First Course in Recombinant DNA Technology* marked the culmination of our effort to influence biology curricula at the advanced high school and beginning college levels. The combined lab and text was coauthored by Dave Micklos and Greg Freyer (now at Columbia University), with computer-generated illustrations by Sue Lauter. We believe it is the first integrated *learning program* to introduce novice biology students to the theory, practice, and applications of DNA manipulation.

Unlike "super texts" that can only be sampled by even the most ambitious instructor or student, the 477-page *DNA Science* is designed to be read from cover to cover. The eight text chapters are written in a semi-journalistic style and



DNA Science was released in October 1990, and by year's end, plans were already made for a second printing.

adopt an historical perspective to explain where DNA science has come from and where it is going. Combining the unique perspectives of a research biologist and a science writer, the topical treatment integrates up-to-the-minute examples drawn directly from research literature. For these reasons, we believe the text is suitable for introducing recombinant DNA in science and society courses.

Extensively tested by thousands of biology instructors nationwide, the ten laboratory experiments cover the basic techniques of gene isolation and analysis. The experiments engender systematic repetition to build student confidence and mastery of techniques. Extensive preliminary notes for each experiment explain how to schedule and prepare them, and flow charts and icons make the protocols easy to follow. A discussion section at the end of each experiment reviews the laboratory in a rhetorical style, analyzing controls and showing both ideal and "less-than-ideal" results. A final section suggests simple research projects that extend the techniques learned in the laboratory and require few, if any, additional reagents.

Truly a first course in recombinant DNA technology, the laboratory sequence presupposes no prior experience on the part of instructor or student. Structured to follow directly from an introduction to principles of biology, the experiments are equally appropriate for the advanced high school student and the beginning college student. It can be used as the first course in a molecular biology sequence, integrated as a genetics/DNA structure component of a general biology course, or used as a unit within a microbiology or genetics course. Laboratories 3 and 5, which illustrate methods to analyze DNA and to introduce genes into bacteria, are recommended by the Educational Testing Service as part of the laboratory curriculum for Advanced Placement (AP) students who take a college level biology course.

DNA Science is a copublication of Cold Spring Harbor Laboratory Press and Carolina Biological Supply Company, which is one of the nation's oldest purveyors of laboratory supplies and curriculum materials for biology teachers. The laboratory course is completely supported by quality-assured Carolina products to satisfy a range of teaching applications, from bulk reagents to reusable reagent systems to single-use kits. Kits designed to introduce teachers to the AP laboratories have quickly become among the company's largest sellers. Also completed in 1990 was "Easy Gene Splicer," which is a simple kit that allows students to construct safely and screen for small recombinant DNA molecules.

Renewed NSF Support for *DNA Science* Workshops

The 5-day *DNA Science* Workshop is our most well-known effort to update biology teaching faculty. Through 1990, the DNA Learning Center staff have instructed 1,400 high school and college educators at 66 workshops in 27 states and Canada, and allied programs in California, North Carolina, Wisconsin, and Florida have used the curriculum as the basis for training an additional 700 teachers at 30 workshops.

The continued influence of the *DNA Science* Workshop was assured with the renewal in 1990 of our core grant from the NSF. This three-year support will allow us to teach 15 workshops, extending our coverage to the midwestern states in 1990 and to the deep south and southwestern states in 1991. Implementing workshops nationwide is a complex task, involving liaison through numerous local organizers, coordinating the travels of two *Vector* vans and six staff, and shipping perishable reagents. However, this task has become sufficiently routine that we intend to target the Rocky Mountain region for workshops in summer 1992.

Recognizing the educational value of the workshop experience, the State University of New York at Stony Brook offers a credit option to *DNA Science* Workshop participants nationwide. Teachers who complete both a workshop and a follow-up are eligible for three graduate credits from the Continuing Education Department. The University's Center for Biotechnology has sponsored a *DNA Science* Workshop at Stony Brook each year since 1987. Travel support is also provided for workshops held at distant sites.

We were honored when Howard Hughes Medical Institute (HHMI) Vice-President Joe Perpich asked us to help establish a model genetics program in its home area of Montgomery County, Maryland. The HHMI grant provides $46,500 to the DNA Learning Center over three years to support teacher/student training workshops and curriculum-enrichment activities. A coordinate grant was awarded to the Montgomery County Public Schools Educational Foundation, Inc., for the implementation of the new curriculum. HHMI is respected as the largest nongovernment contributor to medical research, and the Montgomery County program is considered a test for wider support of precollege and public science education in the next several years.

Measures of Success

Statistics derived from our student field-trip program and our hectic schedule of teacher-enhancement activities (see list at back) give evidence that we have been busy at our work to stimulate widespread DNA literacy. In 1990, DNA

Learning Center staff logged 11,000 person-hours of contact with precollege students and 25,000 person-hours of contact with teaching faculty.

However, funding agencies, particularly the NSF, have found that hard data gauging the effects of faculty enhancement programs are difficult to attain. Therefore, educators greeted with enthusiasm the results of a long-term study of 390 biology teachers trained at 22 *DNA Science* Workshops in 1987 through 1988. The report, "Retooling Biology Education for the Gene Age," was coauthored by Dave Micklos and John Kruper.

Formerly a summer intern with the *Vector* Workshop program and now a postdoctoral fellow at the University of Chicago, John was responsible for the sophisticated computer analysis that included several types of multivariate statistics. The data set for each teacher consisted of answers to surveys administered at three time points: a 60-item presurvey completed at the beginning of the summer workshop, a 95-item postsurvey completed at the end of the workshop, and an 87-item follow-up mail survey that was completed approximately 17 months after the workshop.

The analysis confirmed that participants in the *DNA Science* Workshop are a rather select and highly professional group. Of ten, eight taught AP, honors biology, or advanced biology electives. Seasoned teaching professionals, most had taught at least 6 to 20 years, achieved a master's degree, and carried a full teaching certification in biology or science. The typical respondent was likely a member of several professional organizations and actively participated in professional-development and curriculum-development activities. Many reported regular participation in student-enrichment activities, including science field trips (58%); science fairs and competitions (45%); after-school student research (34%); and joint activities with research scientists (25%).

A majority of teachers appear to have made substantive changes in their classroom behavior during the year after participation in the workshop. Two-thirds or more of all respondents affirmed that they had in several ways integrated concepts from the workshop into their teaching. This included relating a personal account of the making of a recombinant DNA molecule, giving new examples for topics already included on their teaching syllabi, and presenting new topics not yet on their syllabi.

Significant numbers of teachers had attempted new laboratory techniques in their classrooms in the school year after the workshop. Two-thirds had tried experiments in bacteriology, which can be considered the entry into molecular genetics. Thirty-five percent had implemented an AP-type lab on DNA transformation, which requires no specialized equipment. Fewer (25%) had implemented an AP-type lab on DNA restriction analysis, which requires an investment in electrophoresis equipment. Based on a conservative estimate of 25 students per lab (the median reported class size), the teachers in this study were responsible for 5,775 student exposures to the AP labs on DNA transformation and restriction analysis. Furthermore, a number had performed relatively sophisticated experiments on DNA recombination (18%) and plasmid DNA isolation (13%) that require a major investment in equipment and preparation time.

A number of respondents carried on networking activities to educate other teaching professionals. More than one-third had made presentations about their workshop experience to local teachers or school officials, while 7% had made a presentation at a professional meeting. Most important, 18% had conducted a laboratory demonstration for other educators, and 9% had led a teacher-training workshop. Based on the assumption that each reported lab activity involved five

Margaret Henderson discusses results of a DNA restriction analysis with high school students in the *Bio2000* Laboratory.

other teachers, we estimate conservatively that workshop participants demonstrated or taught new lab techniques to 520 additional educators.

Half of all respondents attempted to secure funds with which to implement new labs, typically approaching one or two sources. Fewer (29%) attempted to secure donations of equipment or reagents from various sources, including universities, hospitals, and industry. Of those who tried, 64% were successful in securing monetary or equipment gifts. Respondents reported receiving a total of $507,000 in funding, including monetary donations of $381,000 and reagent donations valued at $126,000. This was equivalent to $1,144 per participant, which more than matched the average per-person training cost of $928. We believe this level of fund-raising success is unprecedented in the history of American biology education.

We judged that about 70% of teachers, although progressive, were primarily *adapting* their new knowledge within the framework of the existing educational system. In contrast to these "adaptors," true innovators had substantially revamped their teaching style to make room for a considerable amount of new experimentation in molecular genetics, including the suggested AP labs on bacterial transformation and DNA restriction analysis.

We used several statistical measures to highlight key attitudinal and behavioral determinants that distinguish adaptors from innovators. Taken together, the analysis emphasized that the innovative teacher is not a "lone wolf," but, rather, is an active professional who operates within a social system. A teacher's involvement with and positive evaluation of the "relevant others" within his/her work environment (students, parents, administrators, and other teachers) belongs to a set of core evaluations and attitudes that are highly resistant to change and appear to be reliable predictors of innovative behavior. This "silent" background of positive attitudes empowers the innovator to seek means to overcome difficult infrastructure constraints to innovation: insufficient lab time, space, and equipment. A decreased reliance on textbooks and an ability to seek out information from primary sources allows the innovator to overcome the curriculum constraints of poor texts and an overly ambitious syllabus.

Ironic Situation in Freshman Biology

Five years ago, high school teachers had neither the training nor equipment for hands-on experimentation with DNA. This situation has changed dramatically, thanks to teacher-training institutes sponsored primarily by NSF, the incorporation of molecular genetics labs into AP biology, the unprecedented fund-raising success of precollege biology teachers, and the availability of suitable teaching kits from science suppliers. Extrapolating from the results of our study and estimated sales of teaching kits, plus the actual numbers of students taught by ourselves and others, we estimate from 50,000 to 70,000 student exposures per year to the AP experiments on DNA transformation and restriction analysis in American high schools.

This figure points up an ironic situation in American biology education. Advanced high school students perform DNA manipulation labs to fulfill a college level biology curriculum; however, these labs are absent from the vast majority of introductory college biology courses. Even at the largest and best of our universities, laboratory instruction on gene manipulation is confined mainly to upper-level courses for biology majors. Thus, beginning college students who had been excited by doing DNA experiments at the high school level are now frustrated to learn that they will not likely work again with DNA until their junior or senior year.

One assumes, prima facie, that college biology professors are up-to-date on advances in research biology; however, many American students are introduced to biology by *nonresearch teaching faculty*. As with their high school counterparts, most of these individuals do not specialize in molecular biology and have been out of school for a number of years. Molecular techniques have not effectively filtered down through the collegiate education system, in part because of a psychological schism between *researchers* and *teachers* that thwarts the flow of laboratory innovation. This is a remnant of separation previously achieved by a bicameral system of research universities and teacher-training colleges. Teaching faculty may be reluctant to seek advice from research colleagues for fear of appearing less "professional." The problem is especially acute at small institutions or state colleges lacking active research programs.

Our experience suggests that college teaching faculty are eager to learn and incorporate new laboratory teaching methods in molecular biology. A preliminary study of college faculty we have trained at *DNA Science* Workshops showed that they implement new laboratories at a significantly higher rate than do high school teachers and that they carry on more networking activities. College biology departments have infrastructures conducive to implementing new labs, and faculty have easier access to funds for new equipment. College instructors reported obtaining an average of $4,700 in start-up funding for new labs, compared with $1,200 per high school trainee.

The Forgotten Biology Teacher

The lack of laboratory teaching in molecular genetics in freshman biology is, in part, another sorry legacy of Reaganomics. Precollege and college faculty alike have long relied on faculty-enhancement programs funded by the NSF to keep up with advances in research science and for instruction on implementing new student labs. NSF funding for precollege and undergraduate programs was greatly expanded after the launch of Sputnik and the attendant perception that the United States was falling behind in the technology race. After peaking at $89

million in 1968, NSF support for precollege and undergraduate programs (funded through its Directorate for Science and Engineering Education [SEE]) declined in the 1970s.

However, President Reagan added insult to injury during his first term in office. Budget cuts during the early 1980s essentially dismantled the SEE Directorate, when *total* precollege and undergraduate funding fell to a pre-Sputnik level of $3.82 million in 1982. This was a national disgrace and shows that even the best efforts of this lead agency are not immune to the vagaries of our legislative process.

College biology teaching suffered most during this period, and funding for undergraduate faculty enhancement programs was eliminated from 1982 to 1987. This coincided precisely with the isolation of the first human cancer genes, the explosive development of the biotechnology industry, and the development of key methods for analyzing human genes. It was a period during which researchers themselves had difficulty keeping apace with the flurry of discovery. Thus, the lack of NSF enhancement programs during this critical time in effect put undergraduate faculty nearly a decade behind in the meaningful transfer of genetic technology to the college teaching laboratory.

Only in 1989 did NSF undergraduate funding regain a pre-Reagan funding level of $28 million, with precollege funding reaching a record high of $104 million. This new heyday of NSF funding for precollege science education appears to be spurred by a growing perception that the United States is now losing the technology race with Japan. The looming shadow of Gramm-Rudman-Hollings notwithstanding, recent history suggests that we can't afford to entrust the retraining of our biology teaching resource entirely with the federal government.

NSF Funding for a *DNA Science II* College Workshop

Against this backdrop, we were happy to receive word in December that the NSF had approved funding for a two-year enhancement program for college faculty to begin in 1991. The program will be administered by Assistant Director Mark

Dr. Greg Freyer, coauthor of *DNA Science*, assists college workshop participants with a DNA hybridization experiment.

Bloom, who developed a second series of 11 laboratories that articulate with and extend the concepts in *DNA Science*. A bacterial system (*Escherchia coli*, plasmids, and bacteriophage λ) is used as a simple model to illustrate techniques for constructing and screening gene libraries, including the techniques of non-radioactive hybridization and polymerase chain reaction.

The NSF-sponsored program will consist of a 10-day workshop and 2-day follow-up program conducted at urban sites: Atlanta and San Francisco in 1991 and Boston and Baltimore in 1992. The labs will be further refined in manuscript during the 1991 workshops. After incorporating feedback from the 1991 workshops, we hope to speed production work so that the laboratories can be formally published early in 1992 as *DNA Science II: An Introduction to Methods of Genome Analysis*.

The success of the NSF proposal was, in large part, due to core support from the Josiah Macy, Jr., Foundation, the J.M. Foundation, the Richard Lounsbery Foundation, and the Banbury Fund, which allowed Mark and the high school interns to do extensive preliminary lab work. Core support also allowed Mark to field test the new lab program at a workshop held in July, which was attended by 24 college faculty from 19 metro-area institutions.

NSF Funding for the *Exploring Human Genetics* Workshop

Advanced high school teachers in the study mentioned above rated genetics along with ecology as the biology topics "most important in preparing students for adult life." Considering this perceived social and personal relevance of human genetics, it is beginning to occur to many educators that introduction to human genetics should, in fact, begin at the middle school level (grades 5–8). Making best use of this window of opportunity may have two important effects. First, it can inculcate basic tenets of scientific literacy that are essential for all children as they grow into adulthood. Second, it may light an academic spark for science that will survive into the college years.

Although human health is a prevalent middle school science topic, many teachers emphasize drug and sex awareness rather than principles of science. Human genetics at once not only builds on this traditional health emphasis of middle school science, but also offers an entree to pure science. Genetics emphasizes science as a problem-solving venture involving the collecting, sharing, and analyzing of data. It is also consistent with the across-curriculum approach that incorporates science, math, and social studies and emphasizes the impact of science and its practical applications.

Thus, we were happy to receive funding in the spring from the National Science Foundation for a model program to train 336 middle school instructors in New York and Maryland to teach a 15–20-hour unit, *Exploring Human Genetics*. The process began in summer 1990 with a 4-day workshop to train 12 pairs of lead teachers from school districts throughout New York State. In 1991, we will have the difficult task of supporting and coordinating the activities of these teachers as they lead "second-round" workshops for 12 pairs of additional teachers in each of their local areas. The entire schedule of lead and second-round workshops will be repeated in the state of Maryland, hopefully proving the suitability of the program as a model for similar programs nationwide.

The workshop presupposes no specific experience in genetics, and it is aimed at both 5th and 6th grade classroom teachers, as well as 7th and 8th

David Micklos discusses a corn cross with middle school teachers in a pretest of the *Exploring Human Genetics* Workshop.

grade life science teachers. Each participant is provided with a teaching kit containing text and all necessary lab materials needed to initiate an experience-based program at his or her school. Regular follow-up activities will further encourage implementation and networking between participants.

Continued Popularity of Local Programs

The Laboratory's current involvement in public education began with the establishment, in 1985, of a consortium of eight local school districts. This consortium, the Curriculum Study, has grown over the years, reaching a new high of 27 districts with the addition in 1990 of Garden City, Kings Park, and Roslyn. Curriculum Study schools have served as a proving ground for both the *DNA Science* and *Exploring Human Genetics* curricula, and their teachers frequently form ad hoc committees to advise us on future projects. It is a tribute to the enthusiasm with which local teachers adopted our ideas that, despite the lack of a major biotechnology industry, the metropolitan New York area ranks along with San Francisco as a national leader in advanced biology teaching at the high school level.

Curriculum Study schools receive reduced admission fees to DNA Learning Center programs, spaces in student/teacher workshops, and equipment purchase options. Students and teachers receive an insider's view of current biological research through the "Great Moments in DNA Science" Lecture Series held each spring. The 1990 lecturers and topics were the following:

William Gergits, Lifecodes Corporation. *From Antelopes to Zebra Finches: The A to Zs of Animal DNA Fingerprinting.*

Carolyn Truncer, Health Science Center at the State University of New York, Stony Brook. *Clinical Applications of Genetic Technologies.*

Robert Franza, Cold Spring Harbor Laboratory. *DNA-binding Proteins and Gene Function.*

David Helfman, Cold Spring Harbor Laboratory. *Protein Diversity through Alternative RNA Splicing.*

The laboratory field-trip program conducted in the *Bio2000* Laboratory continues to operate at capacity, drawing both Curriculum Study members and schools from throughout metropolitan New York. In 1990, 3,758 students and 270 teachers (180 classes) conducted the AP laboratories on DNA restriction analysis and bacterial transformation. The field-trip program is subsidized by core grants from the Esther A. and Joseph Klingenstein Fund, the Josiah Macy, Jr., Foundation, the J.M. Foundation, Boehringer Mannheim Biochemicals, and United States Biochemical Corporation.

Since the opening in September 1988 of the DNA Learning Center, 22,000 visitors have toured the exhibits housed here. *The Search for Life: Genetic Technology in the Twentieth Century*, on loan from the National Museum of American History of the Smithsonian Institution, chronicles the study of heredity from Darwin to DNA and confronts the visitor with the promise and concern of genetic technology. *DNA Detective*, which opened in September 1989, examines the uses of DNA fingerprinting in forensic and paternity law.

Exploring the Uses of Multimedia in Biology Education

During the last several decades, we have witnessed the virtual perfection of several audiovisual technologies: television, video, computers, and random-access laser disks. Taken alone, none has lived up to its potential as a teaching tool. However, "multimedia" or "hypermedia" programs now offer the potential to link these technologies into a flexible system that allows individuals to structure their own learning experiences. With the help of our new Laboratory trustee Owen Smith and senate majority leader Ralph Marino, we received a grant of $66,300 from the New York State Legislature to develop multimedia learning experiences in human genetics.

Thus, at the end of 1990, Sue Lauter began to equip a multimedia development laboratory. Her system is centered around a Macintosh IIci computer, which has adequate memory and processing speed to display various types of analog and digital information, including video, photographs, artwork, sound, and text. The information is accessed from CD-ROM and laser video disks, which have the capability to store high-resolution images that are input from a scanner and video camera/recorder. Authoring software, including a digital "paintbox," Hypercard, and Macromind Director, allows Sue to merge the various media into a seamless, interactive system. Our objective is to create open-ended learning experiences,

in which students can actively explore a complex information field, that access multiple analog and digital sources according to their preferences of information use.

After pretesting with students from Curriculum Study schools, the multimedia experiences will be offered as student field trips and will be incorporated into a new public exhibit, *Exploring the Human Genome*. Because of its location and specialization, the DNA Learning Center can never expect to draw significant numbers of off-the-street visitors. However, it can influence widespread science literacy by producing high-quality multimedia exhibitry for the use of other museums. Our in-house expertise in science interpretation and easy access to the major figures in the world of molecular biology make the DNA Learning Center an ideal interface between genetic science and society.

With assistance from the Albert and Mary Lasker Foundation, Sue is developing a new exhibit, *Eye on the Prizes: Milestones in Molecular Genetics and Genetic Medicine*, highlighting recipients of the Nobel Prize and the Albert Lasker Medical Research Award. Through a time line of prizewinners, the exhibit will chronicle the development of modern genetics. We already have a small collection of Nobel Prizes, and the Lasker Foundation is donating one of its award statuettes of the Winged Victory of Samothrace.

Doing Our Part for Soviet Glasnost

Steven Malloy, one of our student interns, combined his interests in Russian studies and DNA to spark a collaboration between the DNA Learning Center and the Shemyakin Institute of Bioorganic Chemistry (part of the of Soviet Academy of Sciences). Through the New York State/Moscow Telecommunication Project, sponsored by the Copen Family Foundation, Steve contacted Moscow student Nikita Skryabin, whose father is Vice-Director of the Academy's Engelhardt In-

Mark Bloom (*left*) and David Micklos (*right*) with Dimitri Debabov from the Engelhardt Institute in front of Moscow State University during their visit to the Soviet Union in May.

Laboratory Director James Watson (*center*) speaks with Soviet scientists (*left to right*) Leonid Barsukov, Irina Severtsova, Nikolay Zvonok, and Dimitri Debabov during their visit to the DNA Learning Center in August.

stitute of Molecular Biology. Discussion centered on the notion of creating a *DNA Science* education program in Moscow modeled after the DNA Learning Center.

During a visit to the Soviet Union in May, Dave Micklos and Mark Bloom reached an agreement with their counterparts from the Soviet Academy of Sciences to initiate a joint education program. At that time, it was agreed that the immediate objective was to set up a Moscow DNA Learning Center within the Science-Education Center at the Shemyakin Institute. The Moscow DNA Learning Center will adapt our successful *DNA Science* laboratories to suit the Soviet educational system. The DNA Learning Center agreed to seek donations from American and European manufacturers to equip and supply the Moscow Center. The participating institutions further agreed to seek funding for a van to carry equipment and reagents to schools in Moscow modeled after our *Vector* Mobile DNA Laboratory.

Our trip to Moscow and Leningrad was a real eye-opener and pointed up the potential impact of the proposed collaboration. In spite of a history of achievement and strong public support, Soviet biologists are suffering under perestroika. Lack of hard currency makes it very difficult to pay for imported equipment and reagents. All orders for supplies must go through the central bureaucracy, whose already slow distribution system has been further stymied. If it is difficult to obtain meat in Moscow, imagine the difficulty of obtaining a centrifuge. Even biologists employed by the prestigious institutes of the Academy of Sciences have grown used to waiting a full year for the arrival of even the most trivial reagent.

The shortfall of hard currency and supplies makes it difficult for researchers to plan their experiments, but it makes it virtually impossible for undergraduate students to obtain any practical experience with the tools of molecular biology. There simply are not enough supplies and equipment to "waste" on students. The science laboratories of the monolithic Moscow State University, the nation's finest, look unchanged from the days of Stalin (except for the dust and the missing squares of parquet flooring). Few high schools appear to have anything comparable to a teaching laboratory. Clearly, rote learning reigns in the Soviet Union.

Our experiences in the Soviet Union made us realize that the implementation of a DNA Learning Center-style program to put micropipets in the hands of Soviet high school students would be truly revolutionary. We saw the real possibility that

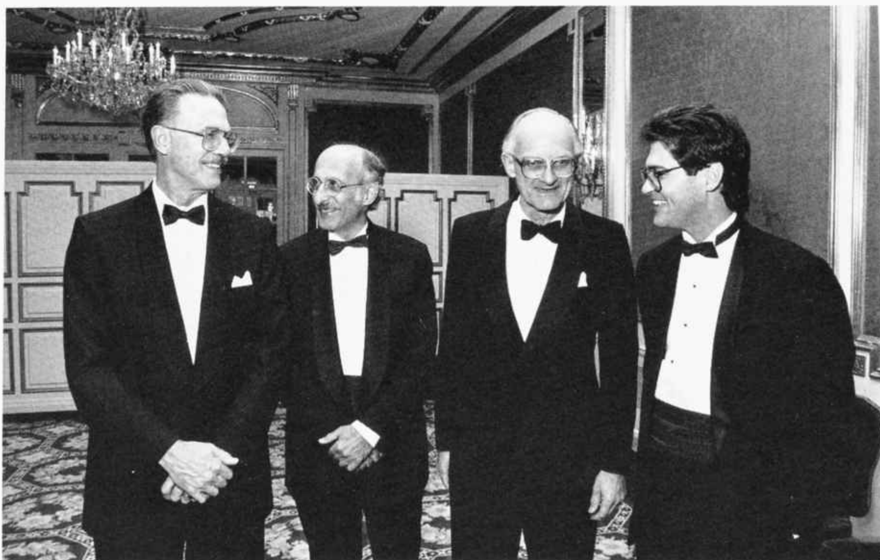
our ongoing collaboration might stimulate a general reform of biology education in the Soviet Union similar to that which is occurring at the AP/college level in the United States.

A delegation of four Soviet scientists from the Shemyakin and Engelhardt Institutes visited New York in August to participate in a *DNA Science* Workshop at the DNA Learning Center. During their visit, they met with American educators, observed our teaching methods, and performed the *DNA Science* laboratories. We traveled with the delegation to Washington, where we met with NSF Assistant Directors Luther Williams and Mary Clutter to discuss our plans for collaboration. We also finalized plans to conduct jointly a *DNA Science* Workshop at the Shemyakin Institute in early 1991 to open the Moscow DNA Learning Center.

The Soviet scientists returned home carrying with them enough supplies and equipment to begin implementing student experiments. The DNA Learning Center had coordinated donations valued at $14,000 for the Moscow DNA Learning Center: digital micropipets and a microcentrifuge from Eppendorf-Netheler-Hinz GmgH of Germany (through arrangements by Brinkmann Instruments of Westbury, New York); electrophoresis chambers, power supplies, and laboratory manuals from Carolina Biological Supply Company of Burlington, North Carolina; and a transilluminator/Polaroid camera system from Fotodyne, Inc., of New Berlin, Wisconsin. Publications, reagents, and expendable supplies were provided by Cold Spring Harbor Laboratory Press and the DNA Learning Center.

Charles A. Dana Award for Dave Micklos

On November 8th, director Dave Micklos was one of four individuals honored by the Charles A. Dana Foundation for pioneering achievements in health and education. The Dana Awards, given annually since 1986, are the only ones made exclusively to recognize innovative ideas in health and disease prevention and are among the largest awards for innovations in education. Recipients of the $50,000



David Micklos (*far right*) with other 1990 Dana Award winners (*from left*) John W. Farquhar, M.D., Norbert Hirschhorn, M.D., and David P. Billington at the presentation banquet at the Plaza Hotel.

award are selected from nationwide nominations by a jury of leaders in health and education.

David Mahoney, chairman of the Charles A. Dana Foundation, presented the award to Dave "for conceiving and directing a national scientific center's pioneering mission to carry the concepts and applications of the modern biological revolution—and the protean potential of recombinant DNA technology—to future biological scientists and all future citizens in the nation's schools."

David P. Billington, Professor of Civil Engineering and Operations Research at Princeton University, received the other award in education; the recipients of the health award were Norbert Hirschhorn, Vice-President of John Snow, Inc., and a lecturer at Harvard Medical School, and John W. Farquhar, Professor of Disease Prevention at Stanford University and Director of Stanford's Center for Research in Disease Prevention. Past honorees have included Donald Henderson, for his work in the eradication of smallpox, and F. Sherwood Rowland, for his research and advocacy to prevent further depletion of the earth's ozone layer.

Staff Changes

Sandy Ordway ably stepped up to the hectic responsibilities as administrative assistant, following the departure of Anne Zollo in June. Sandy needed no instruction on her new position. She had served as a volunteer to prepare for the opening of the DNA Learning Center and had worked part-time on our evaluation study and organizing special summer programs. Poised and polished, Sandy is a wonder of organization. We all feel confident in her ability to orchestrate with aplomb our many programs and collaborations.

After the departure of John LeGuyader for a position in the biotechnology department of the U.S. Patent Office, Margaret Henderson assumed the responsibilities of Education Manager. A native of London, Ontario, Margaret has an undergraduate degree in biology and a graduate degree in library science; her husband Scott is a postdoctoral fellow in electron microscopy on the main

Sandy Ordway guides students through the *Search for Life* exhibit.

Laboratory campus. She gamely stepped up to the demands of teaching six student labs per week, getting rave reviews on her patience and providing an excellent role model for male and female students alike. Her library skills will be put to use in a collaborative program with Biosis, the publisher of *Biological Abstracts*, and in designing a proposed student research library.

Kelly Flynn continued to share some of the teaching duties in the *Bio2000* Laboratory and also acted as editorial and photo researcher for *DNA Science*. After contributing to the summer workshop program, Kelly entered the master's program in genetic counseling at Sarah Lawrence University. While completing her master of fine arts degree at Long Island University, Carrie Abel assisted Sue Lauter with artwork for *DNA Science*, continuing on to a career in graphic design.

Interns, ranging from high school sophomores to graduate students, provide critical assistance to our teaching staff. Amy Phillips, a senior at Huntington High School, joined the staff in the spring and traveled with the *Vector* van this summer. Amy helped to develop two new educational kits to be marketed by Carolina Biological Supply Company and supervised newer interns Mark Staudinger and Richard Chiang. Chai "Sol" Chen graduated from Rensselaer Polytechnic Institute in June and reliably assisted the senior workshop instructors throughout the summer. He continues to keep us informed of his travels.

Publications

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Micklos, D. DNA Science and Education. *Carolina Tips* 53, 1990.

Micklos, D. "Preparing for the Gene Age." Essay for the Fifth Annual Charles A. Dana Awards for Pioneering Achievements in Health and Education, New York, 1990.

Micklos, D. and J. Kruper. Retooling Biology Education for the Gene Age: A Profile of Innovative High School Science Teachers. Submitted.

Curriculum Study Membership 1989–1990

Cold Spring Harbor Central School District*
Commack Union Free School District
East Williston Union Free School District*
Garden City Union Free School District
Great Neck Public Schools*
Half Hollow Hills Central School District
Harborfields Central School District
Herricks Union Free School District*
Huntington Union Free School District
Island Trees Union Free School District
Jericho Union Free School District*
Kings Park Central School District
Lawrence Public Schools
Lindenhurst Public Schools
Locust Valley Central School District
Manhasset Public Schools
Northport-East Northport Union Free School District*
North Shore Central School District
Oyster Bay-East Norwich Central School District*
Plainedge Public Schools
Plainview-Old Bethpage Central School District
Portledge School
Port Washington Union Free School District
Roslyn Union Free School District
Sachem Central School District at Holbrook
South Huntington Union Free School District
Syosset Central School District*

*Founding members

Sites of Major 4–8 Day Workshops 1985-1990

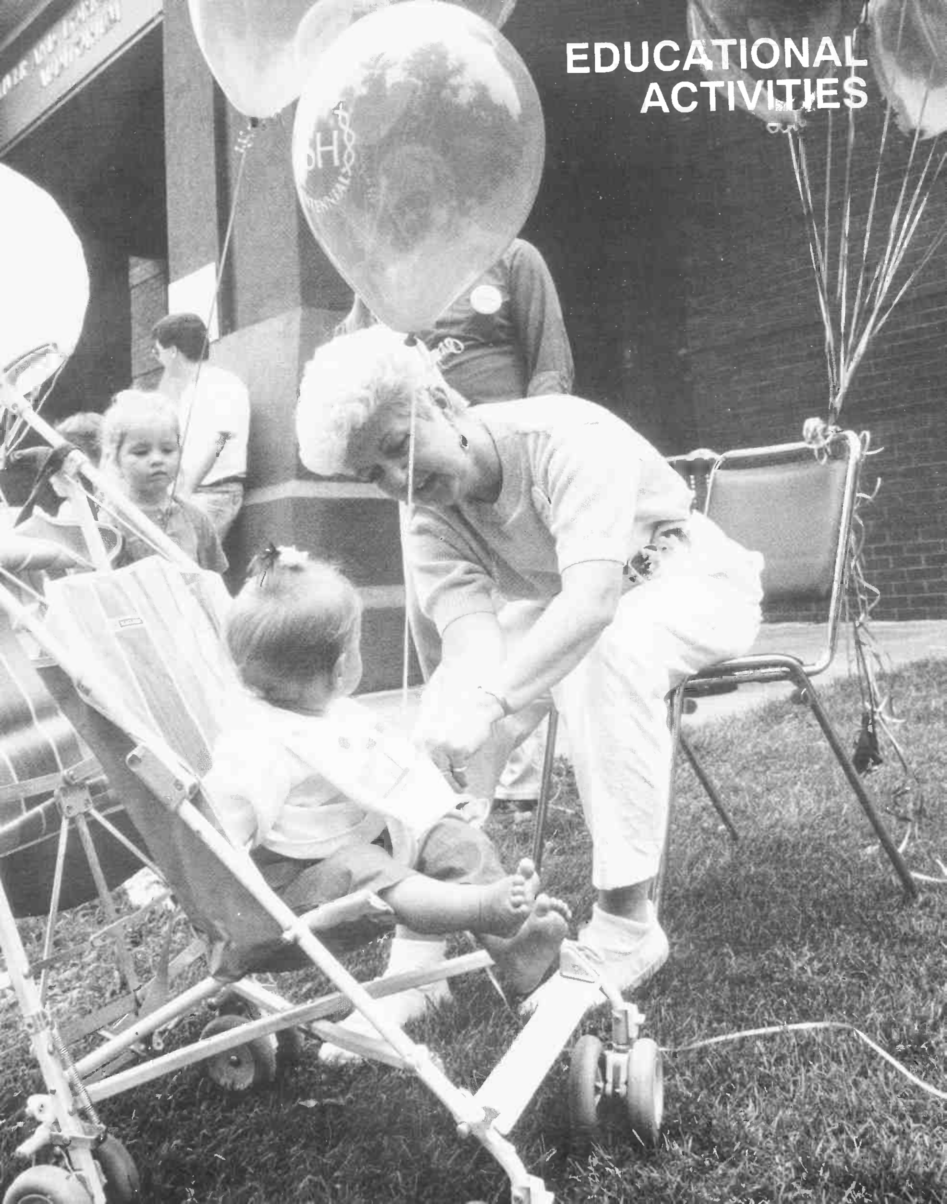
ALABAMA	University of Alabama, Tuscaloosa	1987, 1988, 1989, 1990
ARIZONA	Tuba City High School	1988
CALIFORNIA	University of California, Davis	1986
CONNECTICUT	Choate Rosemary Hall, Wallingford	1987
GEORGIA	Fernbank, Inc., Atlanta	1989
HAWAII	Kamehameha Secondary School, Honolulu	1990
ILLINOIS	Argonne National Laboratory, Chicago	1986, 1987
INDIANA	Butler University, Indianapolis	1987
IOWA	Drake University, Des Moines	1987
KENTUCKY	Murray State University	1988
LOUISIANA	Jefferson Parish Public School, Harvey	1990
MANITOBA	Red River Community College, Winnipeg	1989
MARYLAND	Annapolis Senior High School	1989
	McDonogh School, Baltimore	1988
	Montgomery County Public Schools	1990
MASSACHUSETTS	Beverly High School	1986
	Dover-Sherborn High School, Dover	1989
	Randolph High School	1988
	Winsor School, Boston	1987
MICHIGAN	Athens High School, Troy	1989
MISSISSIPPI	Mississippi School for Math & Science, Columbus	1990
MISSOURI	Washington University, St. Louis	1989
NEW HAMPSHIRE	St. Paul's School, Concord	1986, 1987
NEW YORK	Albany High School	1987
	Bronx High School of Science	1987
	Cold Spring Harbor High School	1985, 1987
	DNA Learning Center, high school workshops	1988(3), 1989(2), 1990(2)
	DNA Learning Center, college workshop	1990
	DNA Learning Center, middle school workshop	1990
	Huntington High School	1986
	Irvington High School	1986
	State University at Purchase	1989
	State University at Stony Brook	1987, 1988, 1989, 1990
	Wheatley School, Old Westbury	1985
NORTH CAROLINA	North Carolina School of Science, Durham	1987
OHIO	Case Western Reserve University, Cleveland	1990
	Cleveland Clinic	1987(2)
	North Westerville High School, Westerville	1990
PENNSYLVANIA	Duquesne University, Pittsburgh	1988
	Germantown Academy, Fort Washington	1988
SOUTH CAROLINA	Medical University of South Carolina, Charleston	1988
	University of South Carolina, Columbia	1988
TEXAS	J.J. Pearce High School, Richardson	1990
VERMONT	University of Vermont, Burlington	1989
VIRGINIA	Jefferson School of Science, Alexandria	1987
	Mathematics and Science Center, Richmond	1990
WEST VIRGINIA	Bethany College	1989
WISCONSIN	Marquette University, Milwaukee	1986, 1987
	University of Wisconsin, Madison	1988, 1989

1990 Workshops, Meetings, and Collaborations

January 6–7	Follow-up Workshop, National Science Foundation Athens High School, Troy, Michigan
January 13–14	Follow-up Workshop, National Science Foundation State University of New York at Purchase
January 17–18	Workshop, Cornell University, Ithaca, New York
January 20–21	Follow-up Workshop, National Science Foundation Fernbank Science Center, Atlanta, Georgia
January 26	Banbury Workshop for Congressional Aides DNA Learning Center, Cold Spring Harbor, New York
January 27–28	Follow-up Workshop, Curriculum Study DNA Learning Center, Cold Spring Harbor, New York
February 17–18	Follow-up Workshop, Red River Community College, Manitoba, Canada
March 3	Workshop, New York City Biology Teachers DNA Learning Center, Cold Spring Harbor, New York
March 10	Workshop, Long Island Biological Association DNA Learning Center, Cold Spring Harbor, New York
March 14	Follow-up Workshop, Curriculum Study DNA Learning Center, Cold Spring Harbor, New York
March 24	Follow-up Workshop, Curriculum Study DNA Learning Center, Cold Spring Harbor, New York Workshop, New York Microscopical Society DNA Learning Center, Cold Spring Harbor, New York
March 16–17	Workshop, Smith College, Northampton, Massachusetts
March 20–21	Conference, National Academy of Sciences, Washington, D.C.
April 6	Workshop, National Science Teachers Association Meeting, Atlanta, Georgia
May 5	Board of Trustees Meeting, Lunch DNA Learning Center, Cold Spring Harbor, New York
May 5–19	Meetings, Soviet Academy of Sciences, Moscow and Leningrad, Soviet Union
May 12	Workshop, Long Island Biological Association DNA Learning Center, Cold Spring Harbor, New York
May 17–18	Workshop, Macy Foundation A. Philip Randolph High School, New York, New York
May 21–26	Workshop, Case Western Reserve, Cleveland, Ohio
June 11–15	Workshop, Macy Foundation, University of Alabama, Tuscaloosa, Alabama
June 13–15	Workshop, Pretest for Middle School Program DNA Learning Center, Cold Spring Harbor, New York
June 18–22	Workshop, National Science Foundation Mississippi School for Math and Science, Columbus, Mississippi Workshop, National Science Foundation Jefferson Parish Public Schools, Harvey, Louisiana Workshop, Kamehameha Secondary School Honolulu, Hawaii Workshop, Republic of Singapore
June 25–29	Workshop, National Science Foundation J.J. Pearce High School, Richardson, Texas
June 27	Workshop, Seminole Community College, Sanford, Florida
July 9–13	Workshop, Howard Hughes Medical Institute Wheaton High School, Wheaton, Maryland
July 16–20	Workshop, National Science Foundation Mathematics and Science Center, Richmond, Virginia
July 18–28	Workshop, College Faculty DNA Learning Center, Cold Spring Harbor, New York
July 29–30	Workshop, Curriculum Study DNA Learning Center, Cold Spring Harbor, New York

July 31	Workshop and Seminar, Florida Department of Education Honors Science Symposium, Orlando, Florida
August 6–10	Workshop, National Science Foundation Westerville North High School, Westerville, Ohio
August 13–19	Workshop, Curriculum Study DNA Learning Center, Cold Spring Harbor, New York
August 13–15	Conference, UNESCO University of Maryland, College Park, Maryland
August 20–24	Workshop State University of New York at Stony Brook
August 21	National Science Foundation Assistant Directors Meeting Washington, D.C.
August 27–31	Workshop, National Science Foundation Middle School Program DNA Learning Center, Cold Spring Harbor, New York
September 29–30	Follow-up Workshop, National Science Foundation Mississippi School for Math and Science, Columbus, Mississippi
October 6	Workshop, Ohio College Biology Teachers Columbus, Ohio
October 10–11	Workshop, Board of Cooperative Education Services State University of New York at Binghamton
October 13–14	Follow-up Workshop, Howard Hughes Medical Institute Wheaton High School, Wheaton, Maryland
October 27–28	Follow-up Workshop, National Science Foundation Mathematics and Science Center, Richmond, Virginia
November 1–3	Workshop, National Science Teachers Association Meeting Long Beach, California
November 3–4	Follow-up Workshop, National Science Foundation J.J. Pearce High School, Richardson, Texas
November 7–11	Seminar, National Association of Biology Teachers Meeting Houston, Texas
November 14–17	Workshop, United States Biochemical Cleveland, Ohio
November 29	Workshop, National Science Teachers Association Meeting San Juan, Puerto Rico
November 30–December 1	Follow-up Workshop Case Western Reserve, Cleveland, Ohio
December 8–9	Follow-up Workshop, State University of New York at Stony Brook
	Follow-up Workshop, National Science Foundation Westerville North High School, Westerville, Ohio
December 10	Workshop, New York Association of Independent Schools DNA Learning Center, Cold Spring Harbor, New York
December 14	Workshop, National Science Teachers Association Meeting Washington, D.C.
December 15–16	Follow-up Workshop, Curriculum Study DNA Learning Center, Cold Spring Harbor, New York

EDUCATIONAL ACTIVITIES



The academic program at Cold Spring Harbor Laboratory now extends from the beginning of April through the beginning of November, with hundreds of visiting scientists taking part in the courses as students, instructors, and lecturers. The first summer course given at the Laboratory was the famous "Phage Course," which began in 1945. This year, the Advanced Bacterial Genetics course, its successor, celebrated their joint 45th anniversary. The Advanced Bacterial Genetics course continues to be oversubscribed as are, in fact, all of the Lab's course offerings. Two new courses were offered this summer: Advanced *Drosophila* Genetics, taught by Gerry Rubin and Michael Ashburner, and Computational Neuroscience: Learning and Memory, taught by Michael Jordan and Terrence Sejnowski.

Nine 3-week laboratory courses in Molecular Genetics and Neurobiology (instructors and students are listed in the following pages) were held during the summer in James, Jones, and Delbrück Laboratories. As is usually the case, the course labs were open from nine in the morning until after midnight seven days a week. Five 1- to 2-week lecture courses were held at the Banbury Center. In addition to the new lecture courses mentioned above, courses were offered in Developmental Neurobiology, Genetic Approaches to Human Disease Using DNA Markers, and Molecular Genetic Analysis of Diseases of the Nervous System.

The spring and fall courses, which started with a single course on X-ray Crystallography in 1988, now seem settled into the Laboratory landscape. Four 2-week courses cover Protein Purification and Characterization, Cloning and Analysis of Large DNA Molecules, Molecular Genetics of Fission Yeast, and Macromolecular Crystallography and take place with the same mixture of intensity and informality as the summer courses.

The courses are supported by, and would not be possible without, a series of grants from federal and private sources. The summer Molecular Genetics courses have been supported for many years by grants from the National Institutes of Health and the National Science Foundation, and a grant from the National Institute of Mental Health has supported several of the Neurobiology courses. However, it has been a large education grant from the Howard Hughes Medical Institute (HHMI) that has provided stable support for the neurobiology program. Funds from HHMI to support new courses also allowed the Laboratory to begin the series of spring and fall courses. As has been the case for several years, the Grass Foundation provided funds for scholarships for students in neurobiology courses.

The logistics of arranging for and dealing with the large number of scientists who participate in the courses is handled with enormous goodwill and efficiency by the staff in the Meetings Office: Barbara Ward, Maureen Berejka, Diane Tighe, Karen Otto, Micki McBride, Marge Stellabotte, and Nancy Weeks.

While the various courses brought graduate students, postdoctoral fellows, and faculty to the Laboratory, the Undergraduate Research Program (URP) continued to allow college students to spend the summer doing research in the laboratories of staff scientists. This program, headed by Winship Herr, a Senior Scientist at the Lab, allowed 18 students to work at Cold Spring Harbor this summer. Funds essential for the support of the URP Program were provided by the Burroughs Wellcome Fund, Baring Brothers, Miles Inc./Bayer AG, the Cold Spring Harbor Laboratory Robert H.P. Olney Fund, and Bio-Rad.

Postgraduate Courses

The summer program of Postgraduate Courses at Cold Spring Harbor Laboratory is aimed at meeting the special need for training in interdisciplinary subjects that are either so new or so specialized that they are not adequately treated by universities. Our aim is to provide intensive study in the most recent developments and techniques in these subjects and to prepare students to enter directly into research in a particular area. To ensure up-to-date coverage of current research work, we bring together a workshop staff from many laboratories around the world and supplement this staff with a series of seminar speakers.

Cloning and Analysis of Large DNA Molecules

April 17–April 30

INSTRUCTORS

Gemmill, Robert, Ph.D., Eleanor Roosevelt Institute, Denver, Colorado
Klapholz, Sue, Ph.D., Stanford University, California
Sternberg, Nat, Ph.D., E.I. du Pont de Nemours & Company, Inc., Wilmington, Delaware

ASSISTANTS

Mendez, Michael, Eleanor Roosevelt Institute, Denver, Colorado
McGuigan, Terri, E.I. du Pont de Nemours & Company, Inc., Wilmington, Delaware
Williams, Reggie, Eleanor Roosevelt Institute, Denver, Colorado

This course covered the theory and practice of manipulating and cloning high-molecular-weight DNA. Lectures and laboratory work dealt with the use of bacteriophage P1 and yeast artificial chromosome (YAC) cloning systems, the isolation and manipulation of high-molecular-weight DNA from mammalian cells for cloning (including the size selection of 200–300-kb DNA fragments), and the analysis of high-molecular-weight DNA by pulsed-field gel (PFG) separation techniques. P1 and YAC recombinant DNA molecules were produced, introduced into cells (*Escherichia coli* and yeast, respectively), and reisolated after appropriate clone selection and colony-screening procedures. Comparison of DNA separation capabilities was made between the five major PFG techniques commonly in use. All aspects of Southern blot analysis of DNA were covered, includ-

ing sample digestion with restriction enzymes, separation on pulsed-field gels, Southern transfer, hybridization, and interpretation of results. Students gained hands-on experience through participation in ongoing efforts to prepare physical maps of portions of human chromosomes 3 and 12. Lectures by outside speakers on topics of current interest supplemented the laboratory work.

PARTICIPANTS

Durkin, A., B.S., American Type Culture Collection, Rockville, Maryland
Fey, G., Ph.D., Research Institute of Scripps Clinic, La Jolla, California
Frey, B., Ph.D., Boehringer Mannheim GmbH, Penzberg, Federal Republic of Germany
Hampikian, G., Ph.D., University of Connecticut, Storrs
Horowitz, M., Ph.D., Weizmann Institute, Israel
Kimura, S., Ph.D., Memorial Sloan-Kettering Cancer Center, New York
King, T., Ph.D., Jackson Laboratory, Woods Hole, Massachusetts
Kitamura, Y., Ph.D., University of Tokyo, Japan
Mackie, S., Ph.D., University of Sheffield, England
Negrutiu, I., Ph.D., Institute of Molecular Biology, Belgium
Nimmo, E., M.S., St. Mary's Hospital, London, England
Pauletti, G., Ph.D., California Institute of Technology, Pasadena
Rao, N., Ph.D., Eli Lilly & Company, Indianapolis, Indiana
Rijnkels, M., M.S., Gorleus Laboratory, Leiden, The Netherlands
Tribioli, C., Ph.D., Istituto di Genetica, Italy
Tsui, H., Ph.D., Toronto Western Hospital, Canada

SEMINARS

Jesse, J., Bethesda Research Laboratories. High-efficiency transformation of *E. coli* by electroporation
Smith, S., University of New Mexico. Observation of DNA molecular motions during electrophoresis.
Garza, D., Stanford University. YAC cloning the *Drosophila* genome
Burke, D., Princeton University. YAC cloning
Hieter, P., Johns Hopkins University. Manipulation of large DNA as yeast artificial chromosomes.
Roberts, R., Cold Spring Harbor Laboratory. Restriction endonucleases
Gardiner, K., Eleanor Roosevelt Institute. Physical mapping and human chromosome 21.
McClelland, M., California Institute of Biological Research. Rare cleavage specificities generated by methylation.

Protein Purification and Characterization

April 16–April 30

INSTRUCTORS

Marshak, Dan, Ph.D., Cold Spring Harbor Laboratory, New York
Erickson, Bruce, Ph.D., University of North Carolina, Chapel Hill
Kadonaga, James, Ph.D., University of California, San Diego
Smith, John, M.D., Ph.D., Harvard Medical School, Boston, Massachusetts

ASSISTANTS

Vasquez, Greg, University of North Carolina, Chapel Hill
Kerrigan, Leslie, University of California, San Diego
Lee, Fang-Jen S., Massachusetts General Hospital, Boston

This course was intended for scientists who are not familiar with the techniques of protein isolation and characterization. Students learned the major techniques in protein purification by actually performing four separate isolations: (1) a regulatory protein from muscle tissue; (2) a fusion protein from *Escherichia coli*; (3) a DNA-binding protein from nuclei of tissue culture cells; and (4) a chemically synthesized peptide. A variety of chromatographic, electrophoretic, and bulk

fractionation techniques were employed including the following: ion exchange, gel filtration, hydrophobic interaction, affinity-based adsorption, and immunoaffinity chromatography; polyacrylamide gel and two-dimensional gel electrophoresis and electroblotting; precipitation by salt and pH; and high-performance liquid chromatography analysis. Methods of protein characterization were discussed, including amino acid analysis, protein sequencing, and mass spectrometry. Emphasis was placed on methods of protein purification rather than automated instrumental analysis. Guest lecturers discussed protein structure, modifications of proteins, and methodologies for protein purification. Applications of protein biochemistry to various areas of research in molecular biology were discussed.

PARTICIPANTS

Bastiani, C., B.A., University of California, Davis
Bode, A., Ph.D., University of North Dakota, Grand Forks
Gahlmann, R., Ph.D., University of Southern California, Los Angeles
Helm, B., Ph.D., University of Sheffield, England
Hocke, G., Ph.D., Research Institute of Scripps Clinic, La Jolla, California
Khursheed, B., Ph.D., University of Chicago, Illinois
Lee, S.-K., M.S., University of Notre Dame, Indiana
Lissemore, J., Ph.D., Syracuse University, New York
Lockhart, A., B.S., Birkbeck College, London, England
Luini, A., M.D., Mario Negri Institute, Italy
Macaulay, C., Ph.D., University of California, San Diego
Nepveu, A., Ph.D., Ludwig Institute, Montreal
Pintel, D., Ph.D., University of Missouri, Columbia
Samuelsson, E., M.S., Royal Institute of Technology, Sweden
Stewart, V., Ph.D., Cornell University, Ithaca
Von Kalm, L., Ph.D., University of California, Berkeley

SEMINARS

Rose, G., Hershey Medical, Pennsylvania State University. Fundamentals of protein structure
Smith, J., Harvard Medical School. Protein purification—Basic methods
Gierasch, L., University of Texas Southwestern Medical Center. Spectroscopic techniques
Pace, N., Texas A&M. Thermodynamics of protein folding.
Graves, D., Iowa State University. Modification of proteins
Moremen, K., Massachusetts Institute of Technology. Glycosylation of proteins
Marshak, D., Cold Spring Harbor Laboratory. Mass spectrometry of proteins
Aebersold, R., University of British Columbia. Methods of microanalysis and transfer.
Dunbar, B., Baylor College of Medicine. Two-dimensional gel electrophoresis
Wilson, K., Applied Biosystems. High-performance separation methods.
Heckendorf, A., The Nest Group. High-performance liquid chromatography column materials
Erickson, B., University of North Carolina. Peptide synthesis
Paterson, Y., University of Pennsylvania. Designing synthetic antigens.

Advanced Bacterial Genetics

June 8–June 28

INSTRUCTORS

Berget, Peter, Ph.D., Carnegie Mellon University, Pittsburgh, Pennsylvania
Maurer, Russell, Ph.D., Case Western Reserve University, Cleveland, Ohio
Weinstock, George, Ph.D., University of Texas, Houston

ASSISTANTS

Bianco, Piero, University of Texas, Houston
Heath, Joe Don, University of Texas, Houston
Slater, Steven, Case Western Reserve University, Cleveland, Ohio

AIDE

Karen, Marcia, Carnegie Mellon University, Pittsburgh, Pennsylvania

This laboratory course demonstrated genetic approaches that can be used to analyze biological processes and their regulation, as well as detailed structure/function relationships of genes. Techniques that were covered included the following: isolation, complementation, and mapping of mutations; use of transposable genetic elements; construction of gene fusions; cloning of DNA; restriction enzyme mapping; Southern blotting; macrorestriction mapping of genomes by pulsed-field gel electrophoresis; and DNA sequencing. The course consisted of a set of experiments incorporating most of these techniques and was supplemented with lectures and discussions. The aim was to develop in students the ability to design a successful genetic approach to any biological problem.

PARTICIPANTS

Bandopadhyay, A., M.S., Albert Einstein College of Medicine, Bronx, New York
Barz, W., M.S., Max-Planck Institut, Martinsried, Federal Republic of Germany
Cohen-Fix, O., M.S., Weizmann Institute, Israel
Fenwick, B., Ph.D., Kansas State University, Manhattan
Kohler, S., M.A., University of Wurzburg, Federal Republic of Germany
Lantz, M., Ph.D., University of Alabama, Birmingham
Maslow, J., Ph.D., Boston Veteran's Administration Medical Center, Massachusetts
Munoz, R., M.S., Rockefeller University, New York, New York

Resnekov, O., Ph.D., Karolinska Institute, Sweden
Rubin, L., M.D., Long Island Jewish Medical Center, New York
Schaefer, M., Ph.D., Texas A&M University, College Station
Sirois, M., M.S., Laval University, Canada
Spormann, A., Ph.D., University of Minnesota, Minneapolis
Trucksis, M., Ph.D., Massachusetts General Hospital, Boston
Versalovic, J., B.A., Baylor College of Medicine, Houston, Texas
Wegrzyn, G., M.S., University of Gdansk, Poland

SEMINARS

Kaplan, S., University of Texas Medical School. The wonders of photosynthetic bacteria.
Roth, J., University of Utah. Chromosome rearrangements.
Stewart, V., Cornell University. Nitrate regulation of anaerobic respiration in *Escherichia coli*.
Taylor, R., University of Tennessee. Molecular genetics of toxin coregulated genes in *Vibrio cholera*.
Maloy, S., University of Illinois. Genetic analysis of proline transport in *Salmonella*
Susskind, M., University of Southern California. How RNA polymerase recognized promoters.

Molecular Embryology of the Mouse

June 8–June 28

INSTRUCTORS

Lovell-Badge, Robin, Ph.D., National Institute for Medical Research, London, England
McMahon, Andy, Ph.D., Roche Institute of Molecular Biology, Nutley, New Jersey

COINSTRUCTORS

Parada, Luis, National Cancer Institute, Frederick, Maryland
Rastan, Sohaila, Clinical Research Centre, Middlesex, England

ASSISTANT

Nichols, Jenny, ICRF Developmental Biology Unit, Oxford, England

This course is designed for molecular biologists, biochemists, and cell biologists interested in applying their expertise to the study of mouse embryonic development. Lecture and laboratory components of the course emphasized both the practical aspects of working with mouse embryos and the conceptual basis of current embryonic research. The following procedures and their potential applications were described: isolation and culture of germ cells and preimplantation and postimplantation embryos, embryo transfer, establishment and genetic manipulation of embryo-derived stem cell lines, germ-layer separation, chimera formation, nuclear transplantation, microinjection of DNA into eggs, retroviral infection of embryos, microinjection of cell lineage tracers, in situ hybridization, and immunohistochemistry. Guest lecturers discussed current research in the field.

PARTICIPANTS

Albano, R., M.S., National Institute for Medical Research, London, England
Basler, K., Ph.D., University of Zurich, Switzerland
Charnay, P., Ph.D., Ecole Normale Superieure, Paris, France
Dhawan, J., M.S., Boston University, Massachusetts
Elias-Arnanz, M., Ph.D., University of Murcia, Spain
Estreicher, A., Ph.D , State University of New York, Stony Brook
Goate, A., Ph.D., St. Mary's Hospital, London, England
Himmelbauer, H., M.S., ICRF, London, England
Lanahan, A., Ph.D., Johns Hopkins Medical School, Baltimore, Maryland
Ott, M.-O., Ph.D., Institute Pasteur, Paris, France
Rey-Campos, J., Ph.D., Institute Pasteur, Paris, France
Roelink, H., M.S., Stanford University, California
Sutherland, H., B.S., Western General Hospital, Edinburgh, Scotland
Vasicek, T., Ph.D., Harvard Medical School, Boston, Massachusetts

SEMINARS

Ziomek, C., Worcester Foundation for Experimental Biology. Preimplantation developments.
Beddington, R., ICRF Developmental Biology Unit Postimplantation development.
McLaren, A., MRC Mammalian Development Unit. Germ cells.
———. Sex determination I.
Rastan, S., MRC Clinical Research Centre. X-inactivation.
Lovell-Badge, R., National Institutes for Medical Research. Sex determination II.
Hogan, B , Vanderbilt University Extraembryonic membranes.
———. Role of TGF-β-related genes in organogenesis and pattern formation in the mouse
Lovell-Badge, R., National Institutes for Medical Research; and
Robertson, L., Columbia University College of Physicians & Surgeons. EC and ES cells
____ ES cells/gene disruption.
Wassarman, P., Roche Institute of Molecular Biology. Fertilization.
Solter, D , Wistar Institute Imprinting
Rossant, J., Mount Sinai Hospital. Strategies for identifying and mutating developmentally important genes.
Krumlauf, R , MRC National Institute for Medical Research. Homeobox genes I (structure/regulation).
Wilkinson, D., MRC National Institute for Medical Research. Segmentation in the nervous system.
Noden, D., New York State College of Veterinary Medicine. Cell migration and tissue assembly during craniofacial development.
Krumlauf, R., MRC National Institute for Medical Research. Homeobox genes II and discussion on segmentation in vertebrates.
Rinchik, G , Oak Ridge National Laboratory. Genetic resources.
Papaioannou, G., Tufts University. Chimeras in development.
McMahon, A., Roche Institute of Molecular Biology. *int* genes and cell interactions.
Parada, L., National Cancer Institute. Oncogenes and development.
Bradley, A , Baylor College of Medicine. Homologous recombination in ES cells.
Gearhart, J., Johns Hopkins School of Medicine. I. Molecular analysis of postimplantation development; II. YACs and development.
Strickland, S., State University of New York, Stony Brook. Maternal RNA and the mouse cycle.
DePamphilis, M., Roche Institute of Molecular Biology. DNA replication and gene expression in mouse oocytes and preimplantation embryos.
Jessell, T., Columbia University College of Physicians & Surgeons Regional induction and axial formation in the vertebrate nervous system.
McKay, R., Massachusetts Institute of Technology. Regulation of cell number and type in the mammalian central nervous system.
Bodine, D., National Institutes of Health. Hematopoiesis I.
Williams, D., Children's Hospital. Hematopoiesis II.
More, K., Frederick Cancer Research Facility. The analysis of coat color mutations in the mouse.

Molecular Approaches to Ion Channel Function and Expression

June 8–June 28

INSTRUCTORS

Goldin, Al, M.D., Ph.D., University of California, Irvine
Snutch, Terry, Ph.D., University of British Columbia, Vancouver, Canada
White, Michael, Ph.D., University of Pennsylvania, Philadelphia

ASSISTANT

Gilbert, Mary, University of British Columbia, Vancouver, Canada

Application of the techniques of molecular biology has provided novel approaches and a new level of sophistication in the examination of many neurobiological problems. This intensive laboratory/lecture course is designed to introduce students to the application of these techniques to the study of ion channels. Students concentrated initially on basic aspects of this approach and then proceeded to more integrated studies. The course dealt with the following topics: mRNA isolation and handling; preparation of in vitro transcripts for expression and use as hybridization probes; *Xenopus* oocytes as an expression system; characterization of newly expressed channels in oocytes using ligand binding, affinity chromatography, voltage- and patch-clamping techniques; design and implementation of recording equipment; and monitoring levels of and changes in channel expression using northern blot analysis.

PARTICIPANTS

Bassnett, S., Ph.D., Uniformed Services University, Bethesda, Maryland
Duguay, F., B.S., Hospital for Sick Children, Toronto, Canada
Lievano, A., Ph.D., Roche Institute of Molecular Biology, Nutley, New Jersey
Lummis, S., Ph.D., University of Cambridge, England
Li, W., M.S., Harvard University, Boston, Massachusetts
Maguire, G., Ph.D., University of California, Berkeley
Rossie, S., Ph.D., University of Arizona, Tucson
Ruben, P., Ph.D., University of Hawaii, Honolulu
Wheeler, S., B.S., Southampton University, England
Zupan, J., M.D., University of California, San Diego

SEMINARS

White, M., University of Pennsylvania. Biosynthesis and assembly of the acetylcholine receptor.
Snutch, T., University of British Columbia. Ca^{++} channels—It's a bit more complicated than T, N, and L.
Enyeart, J., Ohio State University. Ca^{++} channels in secretory cells: Excitation-secretion coupling.
Goldin, A., University of California, Irvine Structure/function studies of Na^{+} channels.
Margiotta, J., Mt. Sinai School of Medicine. Regulation of neuronal acetylcholine receptor expression
White, M., University of Pennsylvania Cloning around with neuropeptide receptors.
Claudio, T., Yale University. bS about the acetylcholine receptor.
Fluharty, S., University of Pennsylvania. Molecular characterization of neuronal angiotensin receptors.
MacKinnon, R., Harvard University. Searching for the conductance pose in K^{+} channels.
Schwarz, T., Stanford University. Why waste your time with *Drosophila?* What's where in K^{+} channels.
Hess, P., Harvard University. Gating of cloned K^{+} channels.
Mandel, G., State University of New York, Stony Brook. Control of Na^{+} channel gene expression.
Levinson, R., University of Colorado. Role of nonprotein domains in Na^{+} channel function.
Dunn, R , McGill University. More on Na^{+} channel structure/function relationships.

Developmental Neurobiology

June 8–June 21

INSTRUCTORS

Goodman, Corey, Ph.D., University of California, Berkeley
Patterson, Paul, Ph. D., California Institute of Technology, Pasadena

This lecture course reviewed established principles and recent advances in developmental neurobiology. Major topics considered were: proliferation, migration, and aggregation of nerve cells; factors influencing the differentiation of neurons; trophic interactions in neural development; patterns, gradients, and compartments; genetic programs for development; the guidance of axons to targets; and the formation of synaptic connections. Particular emphasis was given to synapse formation and to mechanisms underlying the specificity of this process. Finally, the operation of developmental principles was examined in the context of the mammalian visual system and in the development of learning and behavior.

PARTICIPANTS

Coles, H., B.A., Columbia University, New York, New York
Collazo, D., B.A., Harvard Medical School, Boston, Massachusetts
Furley, A., Ph.D., Columbia University, New York, New York
Galli-Resta, L., Ph D., Istituto di Neurofisiologia, Italy
Goda, Y., B S, Stanford University, California
Groves, A, B.A, Ludwig Institute, London, England
Harper, S., B S., St. George's Hospital, London, England
Hassinger, T., B.S., Colorado State University, Fort Collins
Hohn, A, M.A, Max-Planck Institute, Martinsried, Federal Republic of Germany
Hume, C., Ph.D, Columbia University, New York, New York
Kasper, E., B.S., University of Oxford, England
Lans, D., B A., University of California, Berkeley
Lee, R., M A., University of Colorado, Boulder
Montgomery, J., B.A., California Institute of Technology, Pasadena
Nelson, B., Ph D, University of Connecticut, Storrs
Okamoto, H., Ph.D, University of Michigan, Ann Arbor
Ramirez, R., B.S., University of Puerto Rico, Rio Piedras
Ramon-Cueto, A., M.D., Instituto Cajal, Madrid, Spain
Ruiz i Altaba, A., Ph.D., Columbia University, New York, New York
Schiffman, J., B.A., Thomas Jefferson University, Philadelphia, Pennsylvania
Seaver, E., B S., University of Utah, Salt Lake City
Sehgal, R, B A., University of California, San Francisco

SEMINARS

McConnell, S, Stanford University. Neurogenesis and migration in the vertebrate central nervous system.
Sternberg, P., California Institute of Technology. Nematode development.
Anderson, D., California Institute of Technology. Neural crest differentiation
Jessell, T., Columbia University. Axon guidance.
Landmesser, L., University of Connecticut Neuromuscular specificity.
Fraser, S., University of California, Irvine Neuronal specificity.
Sanes, J, Washington University Synapse formation.
Frank, E., University of Pittsburgh. Synaptic specificity and rearrangement.
Shatz, C., Stanford University. Central nervous system connectivity and plasticity.
Breedlove, M., University of California, Berkeley. Hormones and plasticity.
Kandel, E., Columbia University. Plasticity and learning.

Advanced *Drosophila* Genetics

June 23–July 6

INSTRUCTORS

Ashburner, Michael, Ph.D., University of Cambridge, England
Rubin, Gerald, Ph.D., University of California, Berkeley

This intensive seminar course provided an introduction to the theory and practice of methods that are used to manipulate the *Drosophila* genome. It is suitable for graduate students and researchers with some experience with *Drosophila* who are interested in expanding their knowledge of the wide range of genetic techniques now available for use with this organism. Topics covered included chromosome mechanics, the design and execution of genetic screens, and using transposable elements as genetic tools.

PARTICIPANTS

Cagan, R., Ph.D., University of California, Los Angeles
Carmena, M., M.S., Universidad Autonoma de Madrid, Spain
Clark, H., B.S., University of California, Berkeley
Cook, K., M.S., University of Iowa, Iowa City
Grossniklaus, U., B.S., University of Basel, Switzerland
Harrison, S., Ph.D., MRC Laboratory of Molecular Biology, England
Hart, M., M.S., St. Louis University, Missouri
Heck, M., Ph.D., Carnegie Institution of Washington
Huang, J., M.S., Brandeis University, Waltham, Massachusetts
Hultmark, D., Ph.D., University of Stockholm, Sweden
Knust, E., Ph.D., University of Koln, Germany
Louvi, A., B.S., University of Athens, Greece
Michelson, A., Ph.D., Harvard University, Cambridge, Massachusetts
Nakano, Y., Ph.D., Imperial Cancer Research Fund, England
Nichols, R., Ph.D., University of Michigan, Ann Arbor
Parry, D., B.S., University of California, San Francisco
Rio, D., Ph.D., Whitehead Institute, Cambridge, Massachusetts
Ronchi, E.,Ph.D.,Rockefeller University, New York, New York
Wolk, A., M.S., Max-Planck Institute, Martinsried, Federal Republic of Germany
Yee, G., B.A., Massachusetts Institute of Technology, Cambridge

SEMINARS

Ashburner, M., University of Cambridge. Introduction to *Drosophila*: lab practice; biology; phylogeny.
———. Chromosomes; cytogenetics; genetic behavior of chromosomal aberrations; translocations, inversions, deletions, duplications, etc.; balancers.
———. Screens; mutagenesis; genetic and cytogenetic mapping; genetic characterization of mutations.
Hall, J., Brandeis University. Methods for mosaic analysis; genetic analysis of behavior.
Greenspan, R., Roche Institute of Molecular Biology. Interpretation of mosaic data; interpretation of epistatic interactions (using neurogenesis as examples).
Lehmann, R., Whitehead Institute, Massachusetts Institute of Technology. Genetic screens for mutations affecting embryogenesis (including maternal affect mutations); methods for their analysis including injection and transplantation experiments.
Belote, J., Syracuse University. Genetic screens as emplified by screens for loci affecting sex determination; genes and pathways.
Hawley, S., Albert Einstein College of Medicine. Genetics of meiosis—Methods of study and exploitation; distributive pairing; predictive value of distributive models.
Glover, D., University of Dundee, Scotland. Genetic control of cell cycle; meiosis.
Rubin, G., University of California, Berkeley. Genetic screens for suppressors and enhancers.
Gelbart, W., Harvard University. Hobo elements—Discovery, characterization, and use.
Engels, W., University of Wisconsin, Madison. Genetics of PM hybrid dysgenesis and its control; genetic behavior of P elements.
Spradling, A., Carnegie Institution of Washington. P elements as genetic tools—Single P-element mutagenesis, including enhancer trap screens; P-element-mediated transformation.

Molecular and Developmental Biology of Plants

July 2–July 22

INSTRUCTORS

Gruissem, Wilhelm, Ph.D., University of California, Berkeley
Maliga, Pal, Ph.D., Waksman Institute, Rutgers University, Piscataway, New Jersey
Varner, Joseph, Ph.D., Washington University, St. Louis, Missouri

ASSISTANTS

Benner, Michael, Ph.D., Waksman Institute, Rutgers University, Piscataway, New Jersey
Gibson, Susan, Ph D., Michigan State University, East Lansing
Loos, William, Ph.D., Massachusetts General Hospital, Boston
Manzara, Thianda, Ph.D , University of California, Berkeley
Simmonds, Daina, Ph.D , Plant Research Center, Agriculture Canada, Ottawa

This course provided an intensive overview of current topics and techniques in plant biology, with an emphasis on molecular and developmental biology and genetics. It is designed for scientists with experience in molecular techniques who are working with, or wish to work with, plant systems. The course consisted of a rigorous lecture series, a hands-on laboratory, and informal discussions. Guest speakers provided both an in-depth discussion of their work and an overview of their specialty. The laboratory covered established and novel techniques in plant biology, including cell and tissue culture techniques, gene transfer techniques, assays for transient gene expression, protein targeting, analysis of transcription factors, in situ detection of RNA and protein, restriction-fragment-length polymorphism mapping, genome mapping using yeast artificial chromosomes, genetics of *Arabidopsis thaliana* and cytogenetics of *Zea mays*.

PARTICIPANTS

Ach, R., Ph.D., Yale University, New Haven, Connecticut
Bird, D , Ph D , University of California, Riverside
Bouchez, D., Ph D , Institut National de la Recherche Agronomique, France
Feiler, H., M.A., University of Illinois, Chicago
Hershkovitz, M.S , Hebrew University of Jerusalem
Hobbie, L., Ph.D., Massachusetts Institute of Technology, Cambridge
Loza-Tavera, H., M.S., Universidad Nacional Autonoma de Mexico
Metzger, J., Ph.D , U.S. Dept. of Agriculture, Fargo, North Dakota
Mishra, K., M.S., Pioneer Hi-Bred International, Johnston, Iowa
Norelli, J , Ph.D , Cornell University, Ithaca, New York
Pla, M., M.S., Consell Superior d'Investigacions Cientifiques, Barcelona, Spain
Riechmann, J., M.S., Universidad Autonoma de Madrid, Spain
Shen, W.-H., Ph.D., Friedrich Miescher Institute, Basel, Switzerland
Singh, A., Ph D., Pennsylvania State University, Philadelphia
Stein, D , Ph.D., Mount Holyoke College, South Hadley, Massachusetts
Vigil, E., Ph.D., U.S. Dept. of Agriculture, Beltsville, Maryland

SEMINARS

Nester, E., University of Washington. *Agrobacterium* biology and applications.

Bizaro, D., Ohio State University. Molecular biology of geminiviruses and geminivirus vectors.

Poethig, S., University of Pennsylvania. Developmental patterns in plants.

Kao, T.-H., Pennsylvania State University. Self-incompatibility during reproduction.

Klessig, D., Waksman Institute, Rutgers University. Pathogenesis-related proteins in plants.

Walbot, V., Stanford University. The Mu transposable element in maize.

Boynton, J., Duke University. *Chlamydomonas* organelle genetics.

Vierstra, R., University of Wisconsin. Control of the level of phytochrome.

Kondorosi, A., CNRS Institut des Sciences Vegetales. *Rhizobium*-plant symbiotic interactions.

Hanson, M., Cornell University. Molecular biology of plant mitochondria—Cytoplasmic male sterility.

Nelson, T., Yale University. Ontogeny of C3 and C4 leaves.

Phillips, R., University of Minnesota. Genomic rearrangements induced by tissue culture.

Beachy, R., Washington University. Plant RNA viruses—Molecular biology and applications.

Ho, D., Washington University. Hormonal control of gene expression in barley.

Somerville, C., Michigan State University. Biochemical genetics in *Arabidopsis*.

Starlinger, P., University of Koln. Ac transposable element.

Hauge, B., Massachusetts General Hospital. The *Arabidopsis* genome.

Keegstra, K., University of Wisconsin. Protein targeting in plants.

Chua, N.-H., Rockefeller University. Control of tissue-specific gene expression in plants.

Meyerowitz, E., California Institute of Technology. Genes regulating flower development in *Arabidopsis*.

Molecular Cloning of Neural Genes

July 2–July 22

INSTRUCTORS

Chao, Moses, Ph.D., Cornell University Medical College, New York

Eberwine, Jim, Ph.D., University of Pennsylvania, Philadelphia

COINSTRUCTOR

Inman, Irene, Ph.D., Stanford University, California

ASSISTANTS

Patel, Nila, Cornell University Medical College, New York

This intensive laboratory/lecture course was intended to provide scientists with a basic knowledge of the use of the techniques of molecular biology in examining questions of neurobiological significance. Particular emphasis was placed on using and discussing methods for circumventing the special problems posed in the study of the nervous system; for example, examination of low-abundance mRNAs in extremely heterogeneous cell populations.

The laboratory work included mRNA quantitation methods (e.g., nuclease protection), preparation of hybridization probes, library construction (λ ZAP and IST procedure), plaque-screening techniques (probe hybridization and antibody interaction), DNA sequencing, polymerase chain reaction amplification, RNA amplification, and DNA-mediated gene transfer. A major portion of the course was devoted to in situ hybridization and in situ transcription technologies. The lecture series, presented by invited speakers, focused on emerging techniques and how they may be applied to the study of the nervous system.

PARTICIPANTS

Barres, B., M D., University College, London, England
Berman, H., Ph.D., State University of New York, Buffalo
Bottenstein, J., Ph.D., Marine Biomedical Institute, Galveston, Texas
Danoff, S., M.S., Johns Hopkins University, Baltimore, Maryland
Gallagher, D., Ph.D., Yale University, New Haven, Connecticut
Hall, A., Ph.D., Case Western Reserve University, Cleveland, Ohio
Kahoun, J., B.A., University of Wisconsin, Madison
McMorris, A., Ph.D , Wistar Institute, Philadelphia, Pennsylvania
Milovanovic, S., M.D., Cornell University Medical College, Ithaca, New York
Perrone Capano, C., Ph.D., Universita Degli Studi di Napoli, Italy
Peters, L., Ph.D., Jackson Laboratory, Woods Hole, Massachusetts
Pierce, M., Ph.D., Northwestern University, Evanston, Illinois
Pinto, L., Ph.D., INSERM, Paris, France
Ruberg, M., Ph.D., INSERM, Paris, France
Schinstine, M., Ph.D., University of California, San Diego
Serbedzija, G., B.S., University of California, Irvine

SEMINARS

Lemke, G., Salk Institute. Schwann cell gene expression.
Curran, T., Roche Institute. Oncogenes in the brain: *fos*, *jun*, and the AP-1 binding site.
Hahn, B., University of Colorado. Functional validation of genes expressed in the brain in perspective with developmental and evolutionary aspects.
Potter, H., Harvard Medical School. Molecular biology of Alzheimer's Disease.
Dixon, R., Merck. Mechanisms of desensitization of G-protein-coupled receptors.
Amara, S., Yale University. Mechanisms of alternative splicing for hnRNAs.
Cepko, C., Harvard University. Retroviruses in neurobiology.
Parada, L., Frederickson Cancer Center. *trk* oncogene expression in the nervous system.
Young, M., Rockefeller University. Molecular and cellular studies of neurogenic genes in *Drosophila*.

Neurobiology of *Drosophila*

July 2–July 22

INSTRUCTORS

Aldrich, Richard, Ph.D., Stanford University, California
Hartenstein, Volker, Ph.D., University of California, San Diego
Ready, Donald, Ph.D., Purdue University, West Lafayette, Indiana

ASSISTANT

Wolff, Tanya, Purdue University, West Lafayette, Indiana

This laboratory/lecture course, intended for researchers at all levels who may want to use *Drosophila* as an experimental system for studying neurobiology, provided an introduction to current research in neuronal function and development in *Drosophila*. The course began with a crash course in *Drosophila* genetics and other techniques that make *Drosophila* research distinctive, such as cytogenetics and DNA transformation. The main emphasis, however, was on studies of the nervous system. The course covered basic electrophysiological techniques, as applied to mutants with altered ion channels or sensory physiology. In the development section, processes of neurogenesis and pathfinding were examined. The course familiarized students with various preparations, including the embryonic nervous system, imaginal discs, and adult nervous system. It also reviewed the different approaches being used in attempts to unravel the molecular basis of neural development.

PARTICIPANTS

Abrams, J., Ph.D., Massachusetts Institute of Technology, Cambridge
Broadie, K., B.S., University of Cambridge, England
Crew, J., B.S., Carnegie Mellon University, Pittsburgh, Pennsylvania
Cui, X., M.A., University of Illinois, Chicago
Hacohen, N., B.A., Stanford Medical School, California
Incardona, J., B.S., Case Western Reserve University, Cleveland, Ohio
Pulido, D., Ph.D., Squibb Institute for Medical Research, Princeton, New Jersey
Schweisguth, F., Ph.D., Institut Jacques Monod, Paris, France
Shibanaka, Y., Ph.D., Ciba-Geigy (Japan) Ltd.
Treisman, J., B.A., Rockefeller University, New York, New York
Zhong, Y., M.S., University of Iowa, Iowa City

SEMINARS

Dickson, M., Roche Institute of Molecular Biology. Physiology of campaniform sensilla and the control of flight.
Schwarz, T., Stanford University. Physiology and molecular biology of *Shaker*.
Ganetzky, B., University of Wisconsin. Genetics and molecular biology of ion channels.
Bate, M., University of Cambridge. Mesoderm and muscle development.
Bodmer, R., University of California, San Francisco. Embryonic development of the peripheral nervous system.
Thomas, J., Salk Institute Neural pathfinding in the embryonic central nervous system.
Taghert, P., Washington University. Development of the enteric nervous system.
Doe, C., University of Illinois. Neurogenesis and the specification of neural fates in the embryo.
Rubin, G., University of California, Berkeley. Molecular biology of eye development.
Meinertzhagen, I., Dalhousie University. Visual pathways in *Drosophila*.
Carlson, J., Yale University. Genetics of olfaction.
Tully, T., Brandeis University. Learning and memory in *Drosophila*.
Campos-Ortega, J., Universität zu Köln. Neurogenic genes.
Truman, J., University of Washington. Postembryonic development of the *Drosophila* nervous system.

Computational Neuroscience: Learning and Memory

July 14–July 27

INSTRUCTORS

Jordan, Michael, Ph.D., Massachusetts Institute of Technology, Cambridge
Sejnowski, Terrence, Ph.D., Salk Institute, San Diego, California

ASSISTANTS

Bachrach, Jonathan, University of Massachusetts, Amherst
Viola, Paul, Massachusetts Institute of Technology, Cambridge
Wathey, Jack, Salk Institute, San Diego, California

This was an intensive laboratory and lecture course that examined computational approaches to problems in learning and memory. Problems and techniques from both neuroscience and cognitive science were covered, including learning procedures that have been developed recently for neural network models.

This course included a computer-based laboratory so that students could actively explore computational issues. Students were able to interact informally with some of the leading researchers in the field. A strong grounding in mathematics is important and previous exposure to neurobiology is essential for students.

PARTICIPANTS

Ahissar, E., B S., Hadassah Medical School, Israel
Brotchie, P., Ph.D., Massachusetts Institute of Technology, Cambridge
Buxbaum, J., Ph.D., Rockefeller University, New York, New York
Elliot, D., B.S , Massachusetts Institute of Technology, Cambridge
Foldiak, P., M S , University of Cambridge, England
Gullapalli, V., M S., University of Massachusetts, Amherst
Hegde, V., M.S., University of Rochester, New York
Kapur, A., M.S., University of Wisconsin, Madison
Ringo, J., Ph.D., University of Rochester, New York
Rosen, D., B.A., Stanford University, California
Rubin, N., M.S., Hebrew University, Israel
Stern, J., B.S., Massachuselts Institute of Technology, Cambridge
Worth, A., M.S., Boston University, Massachusetts
Ye, H., Ph.D., Institut de la communication Parlee, France

SEMINARS

Sejnowski, T., Salk Institute. Neuroscience overview.
Jordan, M., Massachusetts Institute of Technology. Computation overview.
Byrne, J., University of Texas Medical School. Introduction to invertebrate learning.
Brown, T., Yale University. Introduction to vertebrate learning.
Byrne, J., University of Texas Medical School. Synaptic plasticity in *Aplysia*.
Lisberger, S., University of California School of Medicine. Introduction to the oculomotor system.
Brown, T., Yale University. Synaptic plasticity in the hippocampus.
Lisberger, S., University of California School of Medicine. Learning in the VOR.
Donegan, N., Yale University. Introduction to conditioning.
Tesauro, G., IBM T.J. Watson Laboratories. Introduction to models of conditioning
Donegan, N., Yale University. Integrating biological and psychological models of conditioning.
Sutton, R., GTE Laboratories. Temporal difference learning.
Linsker, R., IBM T.J.Watson Research Center. Introduction to the visual system.
Durbin, R., Stanford University. Optimization and cortical maps.
Linsker, R., IBM T.J.Watson Research Center. Infomax and receptive fields.
Durbin, R., Stanford University. Directed unsupervised learning.
Moody, J., Yale University. Development of modularity.
Rumelhart, D., Stanford University. Introduction to supervised learning.
Sutton, R , GTE Laboratories. Reinforcement learning and planning.
Rumelhart, D., Stanford University. Cognition and learning systems.
LeCun, Y., AT&T Bell Laboratories. Learning as optimization.
Tesauro, G., IBM T.J. Watson Laboratories Introduction to motor learning.
Atkeson, C., Massachusetts Institute of Technology. Introduction to motor learning.
———. Associative motor memory.
Jordon, M., Massachusetts Institute of Technology. Learning dynamical systems.

LeCun, Y., AT&T Bell Laboratories. Learning on real-world problems.
Poggio, T., Massachusetts Institute of Technology. Theoretical framework for learning.
Moody, J., Yale University. Strategies for efficient learning.
Poggio, T., Massachusetts Institute of Technology. Regularization and radial basis functions: Applications.
Kearns, M., Massachusetts Institute of Technology. Introduction to learning theory and learning and natural problems.
Sejnowski, T., Salk Institute. General discussion.

Yeast Genetics

July 24–August 13

INSTRUCTORS

Hieter, Phil, Ph.D., Johns Hopkins University, Baltimore, Maryland
Mitchell, Aaron, Ph. D., Columbia University, New York, New York
Rose, Mark, Ph.D., Princeton University, New Jersey
Winston, Fred, Ph.D., Harvard Medical School, Boston, Massachusetts

ASSISTANTS

Floy, Kim, Johns Hopkins University, Baltimore, Maryland
Gansheroff, Lisa, Harvard Medical School, Boston, Massachusetts
Scidmore, Marci, Princeton University, New Jersey

The major laboratory techniques used in the genetic analysis of yeast were studied, including the isolation and characterization of mutants, tetrad analysis, complementation, and mitotic recombination. Micromanipulation used in tetrad analysis were carried out by all students. Molecular genetic techniques, including yeast transformation gene replacement, analysis of gene fusions, and electrofluorescence experiments were done to identify the nucleus, microtubules, and other cellular components. Lectures on fundamental aspects of yeast genetics were presented along with seminars given by outside speakers on topics of current interest.

PARTICIPANTS

Altschuler, M., Ph.D., Williams College, Williamstown, Massachusetts
Aspenstrom, P., B.S., University of Uppsala, Sweden
Blanc, A., B.S., McGill University, Montreal, Canada
Brodsky, J., Ph.D., Harvard University, Cambridge, Massachusetts

Chang, A., Ph.D., Yale University, New Haven, Connecticut
Flaggs, G., B.S., Stanford Medical School, California
Greenleaf, A., Ph.D., Duke University, Durham, North Carolina
Hwung, Y.-P., Ph.D., Baylor College of Medicine, Houston, Texas
Kirkegaard, K., Ph.D., University of Colorado, Boulder
Nacht, M., B.S., University of Pennsylvania, Philadelphia
Padilla, C., B.S., University of New Mexico, Albuquerque
Pfaffle, P., Ph.D., University of Texas, Dallas
Rhoads, D., Ph.D., Kansas State University, Manhattan
Roth, S., Ph.D., National Institutes of Health, Bethesda, Maryland
Stuart, F., B.S., University of Edinburgh, Scotland
Zhang, J., M.S., Mt. Sinai Medical Center, New York, New York

SEMINARS

Mitchell, A., Columbia University. Control of meiosis in yeast.
Hieter, P., Johns Hopkins Medical School. Chromosome transmission fidelity in yeast.
Sherman, F., University of Rochester. Mutations that stabilize yeast iso-1-cytochrome *c*.
Schekman, R., University of California, Berkeley. Control of protein transport in yeast.
Rose, M., Princeton University. Microtubules, motors, membranes, mating, and mitosis.
Johnson, S., University of California, San Francisco. Cell-type control and transcriptional repression in yeast.
Fink, G., Whitehead Institute. Unusual genetic events in *Saccharomyces*.
Carlson, M., Columbia University. Glucose repression in yeast.
Guarente, L., Massachusetts Institute of Technology. Eukaryotic transcription: A conserved process from yeast to man.
Sternglanz, R., State University of New York, Stony Brook. DNA topoisomerases.
Winston, F., Harvard Medical School. Analysis of transcription mutants in yeast.
Petes, T., University of North Carolina. Homologous and nonhomologous recombination in yeast.
Wigler, M., Cold Spring Harbor Laboratory. *RAS* control pathways in yeast.
Botstein, D., Stanford University Medical School. Genetics of cell biology in yeast.
Hinnebusch, A., National Institutes of Health. Translational control of the transcriptional activator protein GCN4.
Broach, J., Princeton University. Switching and silencing.
Weinert, T., University of Arizona. Cell-cycle control and checkpoints.
Silver, P., Princeton University. Import of proteins into the yeast nucleus.

Advanced Molecular Cloning and Expression of Eukaryotic Genes

July 24–August 13

INSTRUCTORS

Kingston, Robert, Ph.D , Harvard Medical School, Boston, Massachusetts
Myers, Richard, Ph.D., University of California, San Francisco
Rio, Donald, Ph.D., Whitehead Institute, Cambridge, Massachusetts
Robbins, Alan, Ph D., E.I. du Pont de Nemours & Company, Inc., Wilmington, Delaware

ASSISTANTS

Hartzog, Grant, University of California, San Francisco
Louvi, Angeliki, University of Athens

This course focused on how to manipulate cloned eukaryotic genes to probe questions on their structure, expression, and function. As a model system, we examined *cis*- and *trans*-acting components involved in the regulation of eukaryotic gene expression. Students learned the theoretical and practical aspects of constructing genomic and cDNA libraries. Expression libraries from various organisms were screened with recognition-site probes for specific DNA-binding proteins. A variety of transfection techniques were used to introduce cloned DNA molecules that have been manipulated in vitro into *Drosophila*.

PARTICIPANTS

Bates, G., Ph.D., Imperial Cancer Research Fund, London, England

Blanchard, K., Ph.D., Brigham & Women's Hospital, Boston, Massachusetts

Buxton, J., B.S., St. Mary's Hospital, London, England

Clifford, J., B.S., M.D. Anderson Cancer Center, Houston, Texas

Collinge, J., B.S., Clinical Research Center, England

Dmitrovsky, E., M.D., Memorial Sloan-Kettering Cancer Center, New York, New York

Ho, S.-M., Ph.D., Tufts University, Medford, Massachusetts

Joost, H., Ph.D., Institute of Pharmacology, Göttingen, Federal Republic of Germany

Legay, C., Ph.D., Ecole Normale Superieure, Paris, France

Lu, M., Ph.D., New York University, New York

Ma, A., M.D., Johns Hopkins University, Baltimore, Maryland

Poon, D., B.A., Vanderbilt University, Nashville, Tennessee

Shaw, L., B.S., Harvard Medical School, Boston, Massachusetts

Sirois, M., M.S., Laval University, Canada

Sokolowski, M., Ph.D., York University, Canada

Venter, H., M.S., University of Witwatersrand, South Africa

SEMINARS

Kingston, R., Massachusetts General Hospital. Transcription factor interactions in chromatin.

Robbins, A., E.I. du Pont de Nemours & Company, Inc. Effect of brefeldin A on alphaherpesvirus membrane protein glycosylation and virus egress.

Yamamoto, K., University of California, San Francisco. Positive and negative transcriptional regulation by the glucocorticoid receptor.

Tjian, R., University of California, Berkeley. Species-specific coactivators mediate transcriptional activation by eukaryotic upstream regulators.

Myers, R., University of California, San Francisco. Regulation of mammalian β-globin gene expression.

Treisman, R., Imperial Cancer Research Fund, London. SRF and the serum response element.

Johnson, S., University of California, San Francisco. Cell-type control and transcriptional repression in yeast.

Stillman, B., Cold Spring Harbor Laboratory. Mechanism and regulation of DNA regulation in human and yeast cells.

Lehmann, R., Whitehead Institute, Massachusetts Institute of Technology. Maternal control of pattern formation in *Drosophila*.

Guarente, L., Massachusetts Institute of Technology. Eukaryotic transcription: A conserved process from yeast to man.

Levine, M., Columbia University. Transcriptional control of *Drosophila* embryogenesis.

Baker, T., National Institutes of Health. Initiation of *Escherichia coli* chromosomal replication: Role of transcription and other factors that influence DNA structure.

Maniatis, T., Harvard University. Regulation of human β-interferon gene expression

Rio, D., Whitehead Institute, Massachusetts Institute of Technology. Multiprotein complexes involved in transcription by RNA polymerase II.

McKnight, S., Carnegie Institution of Washington. Mammalian transcription factors.

Botchan, M., University of California, Berkeley. Roles of a transcription factor in viral DNA replication.

Molecular Probes of the Nervous System

July 24–August 13

INSTRUCTORS

Carlson, Steve, Ph.D., University of Washington, Seattle
Evans, Christopher, Ph.D., Stanford University, California
Levitt, Pat, Ph.D., Medical College of Pennsylvania, Philadelphia

ASSISTANT

Hallden, Gunnell, University of California, Berkeley

This course was designed for scientists from various disciplines who are interested in understanding the power and pitfalls of antibodies and nucleotide probes as biochemical and anatomical reagents. A series of evening lectures addressed basic and advanced immunology concepts and the use of molecular probes to investigate current issues in neurobiology. The primary emphasis of the course was to acquire practical laboratory experience through daily exercises using an extensive number of techniques, including generation and characterization of monoclonal antibodies to synthetic peptides and complex neural antigens, immunocytochemistry, in situ hybridization, immunoassays (enzyme-linked immunoadsorbent assay and radioimmune assay), affinity chromatography, and western blot hybridization. Approaches that combine the use of molecular probes were highlighted by performing expression library screening, oligonucleotide synthesis, probe labeling, and exercises employing double-labeling strategies. Participants also designed biological assays in tissue culture to demonstrate functional relevance of specific molecules.

PARTICIPANTS

Diaz, R., M.S , Wisconsin Regional Primate Center, Madison
Durand, G., M.S., Albert Einstein College of Medicine, Bronx, New York
Giddings, C., Ph.D., University of Texas, Dallas
Henson, J., M.D., Memorial Sloan-Kettering Cancer Center, New York, New York
Koester, S., B.A., Washington University School of Medicine, St. Louis, Missouri

Krajcik, R., B.S., Wright State University, Dayton, Ohio
Kruger, C., B.S., University of Bremen, Federal Republic of Germany
Matise, M., B.S., University of Pittsburgh, Pennsylvania
Pinkus, L., Ph.D., A.H Robins Company, Richmond, Virginia
Redondo, J., Ph.D., Universidad Nacional Autonoma de Mexico
Sholl, S., Ph.D., Wisconsin Regional Primate Center, Madison
Wu, W., B.S., University of Minnesota, Minneapolis

SEMINARS

Fleischman, J., Washington University Medical School. Immunoglobulins and superfamily.
Kurt-Jones, E., Brigham & Women's Hospital. T cells.
Wortis, H., Tufts University School of Medicine. B cells and networks.
Alt, F., Columbia University College of Physicians & Surgeons. Immunoglobulin gene rearrangement.
Helfman, D., Cold Spring Harbor Laboratory. Gene cloning and differential RNA processing.
Pintar, J., Columbia University College of Physicians & Surgeons. Gene expression in development.
Keller, F., Genentech, Inc. Adhesion molecules in invert.
Hockfield, S., Yale University School of Medicine. Activity-dependent molecular expression.
Bothwell, M., University of Washington. Growth factor receptors in development.
Levitt, P., Pennsylvania Medical College. Specification of cortex in development.
Pittman, R., University of Pennsylvania. Growth factor receptors in development.
Evans, C., Stanford Medical School. Peptide processing and expression.
Carlson, S., University of Washington. Proteoglycans as synaptic components.
Maidment, N., Stanford University School of Medicine. In vivo dialysis and brain function.

Genetic Approaches to Human Disease Using DNA Markers

July 30–August 5

INSTRUCTORS

Lander, Eric, Ph.D., Whitehead Institute and Massachusetts Institute of Technology, Cambridge
Page, David, M.D., Whitehead Institute and Massachusetts Institute of Technology, Cambridge

ASSISTANT

Jacob, Howard, Whitehead Institute, Cambridge, Massachusetts

It has recently become feasible to map genes underlying some human diseases and traits, even when the molecular basis is unknown, by studying individuals from natural populations and using DNA markers (such as restriction-fragment-length polymorphisms). This intensive lecture course explored the possibilities and difficulties of applying these methods to the study of various human diseases and traits, including simply inherited disorders (e.g., cystic fibrosis), complex disorders (e.g., heart disease or psychiatric conditions), inherited and spontaneous cancers, and developmental abnormalities (e.g., sex determination). Understanding such biological systems requires an interplay among transmission genetics and cytogenetics in mammals, human population genetics, and molecular biological techniques for analyzing large genomes. Accordingly, the course concentrated on ways to employ genetics to dissect complex biological problems in natural populations. The course is intended for students with backgrounds in either molecular biology, molecular genetics, or medical genetics interested in undertaking research on particular diseases, traits, or physiological systems.

PARTICIPANTS

Brink, P.A., M.D., Baylor College of Medicine, Houston, Texas
Dermody, J., Ph.D., New Jersey Medical School, Newark
Eisenbarth, G., Ph.D., Joslin Diabetes Center, Boston, Massachusetts
Fechner, P., M.D., Johns Hopkins Hospital, Baltimore, Maryland
Fidani, L., B.S., St. Mary's Hospital, London, England
Gnatt, A., M.S., Hebrew University of Jerusalem
Gore-Langton, R., Ph.D., University of Western Ontario, Canada
Green, E, Ph D, Washington University Medical School, St Louis, Missouri
Hastbacka, J., M.D., University of Helsinki, Finland
Kavuru, M., B.A., National Institutes of Health, Bethesda, Maryland
Lanser, M., M.D., University of California, San Francisco
Lehesjoki, A.-E., M.D., University of Helsinki, Finland
Lupski, J., Ph.D., Baylor College of Medicine, Houston, Texas
Mirow, A., M.D., University of California, San Diego
Nevin, D., M.D., University of Wisconsin, Madison
Ngan, B., Ph.D., University of Toronto, Canada
Nimgaonkar, V., Ph.D., University of Pittsburgh, Pennsylvania
Printz, M., Ph.D., University of California, San Diego
Romero, J., B.S., Boston University School of Medicine, Massachusetts
Schalling, M., Ph.D., Karolinska Institute, Sweden
Whitehouse, W., Ph.D., John Radcliffe Hospital, Oxford, England

SEMINARS

Gilliam, C., Columbia University. Mapping neurological and muscular disorders.
Tanzi, R., Harvard Medical School. Alzheimer's disease.
Housman, D., Massachusetts Institute of Technology. Cancer and recessive oncogenes.
King, M.-C., University of California, Berkeley. Breast cancer: Segregation and linkage analysis.
Nussbaum, R., University of Pennsylvania. Fragile X syndrome.
Puck, J., University of Pennsylvania. X inactivation and mapping of X-linked immunodeficiencies.
Nicholls, R., University of Florida. Genomic imprinting.
Haines, J., Harvard Medical School. Linkage analysis methods (two lectures).
Chakravarti, A., University of Pittsburgh. 1. Epidemiology; 2. Down's syndrome and centromere mapping.
Lincoln, S., Whitehead Institute. Linkage analysis.
Hobbs, H., University of Texas Southwestern Medical School. Heart disease.
Page, D., Whitehead Institute and Massachusetts Institute of Technology. Chromosomes.

Molecular Genetic Analysis of Diseases of the Nervous System

August 7–August 13

INSTRUCTORS

Breakefield, Xandra, Ph.D., Massachusetts General Hospital and Harvard Medical School, Boston
Evans, Glen, Ph.D., Salk Institute, San Diego, California
Gusella, James, Ph.D., Massachusetts General Hospital and Harvard Medical School, Boston

This lecture course explored new techniques of molecular biology and neuroscience being used to elucidate the molecular and cellular basis of neurologic and psychiatric diseases in humans and in animal models. Many recently developed genetic methodologies provided the means to elucidate the action of disease genes and to manipulate and explore normal neural development and functions. Areas covered included: linkage analysis and identification of disease genes; action of *onc* genes in development and tumor formation; gene transfer into the nervous system using viral vectors; creation of animal models by

homologous recombination and transgenes; specificity of neurotropic viruses; gene families encoding receptors and ion channels; genes active in nerve regeneration and cell death.

PARTICIPANTS

Brice, A., M.D., INSERM, Paris, France
Buxbaum, J., Ph.D., Rockefeller University, New York, New York
Crawford, F., B.S., St. Mary's Hospital, London, England
Fotaki, M., Ph.D., Montreal General Hospital, Canada
Gasser, T., M.D., Ludwig Maximilian's University, Munich, Federal Republic of Germany
Gates, H., Ph.D., University of Bristol, England
Gaughan, G., Ph.D., Oxford University, England
Hautamaa, D., Ph.D., University of Minnesota, Minneapolis
Kaye, E., M.D., Massachusetts General Hospital East, Charlestown
Kowallis, G., M.D., St. Vincent's Hospital, New York
Lehesjoki, A.-E., M.S., University of Helsinki, Finland
Loring, J., Ph.D., GenPharm International, Mountain View, California
Mathews, K., M.D., University of Iowa, Iowa City
McClatchey, A., Massachusetts General Hospital, Boston
McKee, M.A., M.D., Columbia Presbyterian Hospital, New York, New York
Montag, D., M.D., Research Institute of Scripps Clinic, La Jolla, California
Montal, M., Ph.D., University of California, San Diego
Orr-Urtreger, A., M.D., Weizmann Institute, Israel
Schalling, Ph.D., Karolinska Institute, Sweden
Teng, S., Ph.D., Massachusetts General Hospital, Boston
Waltman, S., M.A., Brown University, Providence, Rhode Island

SEMINARS

Evans, G., Salk Institute. Strategies for scanning the human genome: Human Genome Project.
Moyzis, R., Los Alamos National Laboratory. Repeat elements.
Gusella, J., Massachusetts General Hospital and Harvard Medical School. Finding disease genes I: Linkage analysis in Huntington's disease.
O'Connell, P., University of Utah Medical Center. Finding disease genes I: Linkage analysis in neurofibromatosis type I.
Brownstein, B., Washington University School of Medicine. Finding disease genes II: Yeast artificial chromosomes as cloning vehicles.
Ledbetter, D., Baylor College of Medicine Contiguous gene syndromes.
Hoffman, E., Children's Hospital. Characterization of disease genes: Using genetics to define a disease—Duchenne/Becker muscular dystrophy.
MacLennan, D., University of Toronto Malignant hyperthermia.
Gravel, R., McGill University. Mutational analysis: Lysosomal storage diseases.
Wallace, D., Emory University. Mitochondrial defects.
Nussbaum, R., University of Pennsylvania Medical School. Fragile X syndrome.
Breakefield, X., Massachusetts General Hospital and Harvard Medical School. MAO and its role in disease.
Jolicoeur, P., Institut de Recherches, Canada. Retroviral neurotropism.
Benowitz, L., McLean Hospital. Genes in regeneration: GAP43.
Brugge, J.S., University of Pennsylvania Medical School. *onc* genes in development and tumors: *src* and tyrosine kinases in neuronal development.
Lee, W.-H., University of California School of Medicine, San Diego. Role of tumor suppression genes in the development of neoplasia.
Heineman, S., Salk Institute. Receptors: Glutamate receptor family—Structure and function.
Oppenheim, R., Wake Forest University. Cell death in the

nervous system.

Sutcliffe, G., Research Institute of Scripps Clinic. Spontaneous mouse mutants—Identifying mouse disease genes.

Milner, R., Research Institute of Scripps Clinic. Myelin mutants.

Prusiner, S., University of California, San Francisco. Prions.

Mallet, J., Centre National de la Recherche Scientifique, France. Catecholamine biosynthetic enzymes and their regulation.

Beyreuther, K., Universitat Heidelberg, Federal Republic of Germany. Amyloid transcriptions in Alzheimer's disease.

Isacson, O., McLean Hospital. Grafting genetically modified cells.

Rossant, J., Mt. Sinai Hospital Research Institute. Creating mouse mutants: Homologous recombination.

Evans, G., Salk Institute. Transgenics.

Civelli, O., Oregon Health Science University. Dopaminergic receptors.

Caron, M., Duke University Center. Adrenergic receptors.

Stevens, J., University of California, Los Angeles. Gene transfer with latent herpesvirus simplex.

Herrup, K., E.K. Shriver Center. Genetic modification to study linkage in the developing nervous system.

Macromolecular Crystallography

October 11–October 24

INSTRUCTORS

Pflugrath, J.W., Ph.D., Cold Spring Harbor Laboratory, New York
McPherson, A., Ph.D., University of California, Riverside
Gilliland, G., Ph.D., Center for Advanced Research in Biotechnology, Rockville, Maryland
Furey, W., Ph.D., Veterans Administration Medical Center, Pittsburgh, Pennsylvania

ASSISTANT

Pechik, I., Center for Advanced Research in Biotechnology, Rockville, Maryland

Crystallography and X-ray diffraction yield a wealth of structural information unobtainable through other methods. This intensive laboratory/computational course focused on the major techniques used to determine the three-dimensional structures of macromolecules. It was designed for scientists with a working knowledge of protein structure and function, but who were new to macromolecular crystallography. Topics covered included: protein purification, crystal-

lization, crystal characterization, data collection (area detector methods), data reduction, anomalous dispersion, phase determination, multiwavelength anomalous diffraction, lane diffraction, molecular replacement and averaging, electron density interpretation, structure refinement, molecular graphics, and molecular dynamics. Participants learned through extensive hands-on experiments, informal discussions, and lectures on current applications of these related procedures given by outside speakers.

PARTICIPANTS

Air, G., Ph.D., University of Alabama, Birmingham
Berger, S., Ph.D., University of British Columbia, Canada
Clawson, D., M.S., Eli Lilly & Co., Indianapolis, Indiana
Cox, M., M.S., Burroughs Wellcome Company, Research Triangle Park, North Carolina
Dencher, N., Ph.D., Hahn-Meitner Institut, Berlin, Federal Republic of Germany
Diaz, D., M.S., Case Western Reserve University, Cleveland, Ohio
Djebli, A., Ph.D., Case Western Reserve University, Cleveland, Ohio
Griffith, O.H., Ph.D., University of Oregon, Eugene
Hallows, W., Ph.D., University of Rochester, New York
Kuo, C.-F., Ph.D., Research Institute of Scripps Clinic, La Jolla, California
Lima, C., B.A., Northwestern University, Evanston, Illinois
Morais Cabral, J., B.S., University of Edinburgh, Scotland
Nunn, R., M.S., University of Sheffield, England
Sinclair, A., Ph.D., University of Leeds, England
Thorn, J., Ph.D., Northwestern University, Evanston, Illinois
Young, A., M.A., New York University, New York

SEMINARS

Kuret, J., Cold Spring Harbor Laboratory. Protein purification and characterization.
McPherson, A., University of California, Riverside. Crystallization of macromolecules I.
Sweet, R., Brookhaven National Laboratory. Introduction to crystallography.
———. X-ray sources and optics.
McPherson, A., University of California, Riverside. Crystallization of macromolecules II.
Gilliland, G., National Institute of Standards and Technology. Biological macromolecule crystallization database: A tool for the development of crystallization strategies.
McPherson, A., University of California, Riverside. Introduction to Bragg's Law.
Aggarwal, A., Columbia University. Crystallization of nucleic acids with proteins.
McPherson, A., University of California, Riverside. Preliminary crystal characterization.
———. Crystallographic symmetry and unit cells.
———. Precession photography.
Pflugrath, J., Cold Spring Harbor Laboratory. Data collection: Area detector madness.
Herzberg, O., University of Maryland. Structure and function of β-lactamase.
McPherson, A., University of California, Riverside. Difference pattersons.
Furey, W., Veteran's Administration Medical Center, Pittsburgh. Multiple isomorphous replacement phase refinement.
Sacchettini, J., Albert Einstein College of Medicine. Structure of rat intestinal fatty acid binding protein at 1.3 Å resolution.
Jones, T.A., University of Uppsala, Sweden. Map interpretation.
Furey, W., Veteran's Administration Medical Center, Pittsburgh. Solvent flattening/phase combination.
Petsko, G., Brandeis University. Laue diffraction—Principles and practice.
———. Time-resolved studies of enzymes by Laue diffraction.
Hendrickson, W., Columbia University. Crystal structure of ribonuclease H.
Horton, J., Columbia University. Multiwavelength anomalous dispersion: Theory and practice.
Tronrud, D., University of Oregon. Structure refinement with TNT.
Remington, S.J., University of Oregon. Molecular replacement methods I.
———. Molecular replacement methods II.
Gilliland, G., University of California, Riverside. Three-dimensional structure of recombinant bovine chymosin at 2.3 Å resolution.
Weis, W., Columbia University. X-PLOR: Macromolecular structure refinement by simulated annealing.
Hogle, J., Research Institute of Scripps Clinic. Virus crystallography.
Gilliland, G., National Institute of Standards and Technology. Conformational flexibility of surface residues of bovine RNAse A at 1 Å resolution.
Clore, G.M., National Institutes of Health. Determination of protein structure by two-dimensional, three-dimensional, and four-dimensional nuclear magnetic resonance.

Molecular Genetics of Fission Yeast

October 29–November 5

INSTRUCTORS

Fantes, Peter, Ph.D., University of Edinburgh, Scotland
Hyams, J., Ph.D., University College, London
McLeod, M., Ph.D., State University of New York, Brooklyn

ASSISTANTS

Alfa, Caroline, University College, London, England
Devoti, Jamie, State University of New York, Brooklyn
Warbrick, Emma, University of Edinburgh, Scotland

The fission yeast *Schizosaccharomyces pombe* is increasingly being used as a model organism for the study of basic aspects of cell biology. This course introduced the students to all aspects of fission yeast biology but with particular emphasis on genetic manipulation (both classical and with recombinant DNA) and the use of the organism for the study of cell biology. Topics covered included chemical mutagenesis and mutant analysis, transformation and gene replacement techniques, isolation of nuclei, preparation of nuclear DNA, plasmid recovery from yeast into bacteria, cell-cycle methods, cytology, and immunocytochemical techniques. In addition to hands-on experience, participants had the opportunity to learn through informal group discussions and a lecture series designed to complement the experimental section.

PARTICIPANTS

Alahari, S., M.S., Drexel University, Philadelphia, Pennsylvania
Andrisani, O., Ph.D., Purdue University, West Lafayette, Indiana
Basi, G., M.S., EMBL, Heidelberg, Federal Republic of Germany
Beck, P., Ph.D., University of Texas Southwestern Medical Center, Dallas
Dass, S., Ph.D., Michigan State University, East Lansing
Daya, M., Ph.D., University of British Columbia, Canada
Dillon, P., Ph.D., Roche Institute of Molecular Biology, Nutley, New Jersey
Grallert, B., Ph.D., L.K. University, Hungary
Lim, J., B.S., UMDNJ-Robert Wood Johnson Medical School, New Jersey
Merrill, G., Ph.D., Oregon State University, Corvallis
Ottilie, S., Ph.D., Harvard University, Cambridge, Massachusetts
Robinson, J., B.A., California Institute of Technology, Pasadena
Rowley, R., B.A., University of Utah, Salt Lake City
Toyamama, R., Ph.D., National Cancer Institute, National Institutes of Health, Bethesda, Maryland
Wagner, P., Ph.D., Marie Curie Institute
Weil, T., Ph.D., Vanderbilt University, Nashville, Tennessee

SEMINARS

Yamamoto, M., University of Tokyo. Signal transduction during sexual development in *S. pombe*.
Young, P., Queen's University, Ontario. Genetics of ion transport in *S. pombe*.
Wigler, M., Cold Spring Harbor Laboratory. Signal transduction pathways.
Klar, A., National Cancer Institute. Yeast yeast.
Chappell, T., Duke University Medical Center. The Golgi apparatus of *S. pombe*.

Seminars

Cold Spring Harbor Seminars were initiated to provide a semiformal avenue for communication between the various research groups at the Laboratory. They are particularly useful for research personnel who have joined the Laboratory during the summer. The seminars also afford a necessary opportunity for the graduate students and postgraduate staff to develop their skills in defending, organizing, and presenting their research. In addition to those listed below, seminars were given by many others involved in research at this laboratory.

1990

January

David Goldfarb, University of Rochester, New York. Pathways in nuclear transport.

Alexander Titomirov, Institute of Molecular Biology, Union of Soviet Socialist Republics Academy of Sciences, Moscow. In vivo electroporation and stable transformation of skin cells of mice.

William Earnshaw, Johns Hopkins School of Medicine, Baltimore, Maryland. Structural domains of the vertebrate centromere.

Heinz Saedler, Max-Planck Institute, Koln, Federal Republic of Germany. En/Spm transposable element system of maize.

Charles Sherr, St. Jude's Children's Research Hospital, Memphis, Tennessee. c-*FMS* (CSF-1 receptor) proto-oncogene.

Jack Szostak, Massachusetts General Hospital, Boston. Toward the design of a simple cell.

February

David Barford, Oxford University, England. Stereochemical basis for the allosteric mechanism of glycogen phosphorylase.

Kanna Visvanathan, ICRF, London, England. Introduction of β-interferon expression. Reduced Oct-1 binding to the TATA box and the adjacent negative element and NF-kB activation.

Jonathan Gold, Memorial Sloan-Kettering Cancer Center, New York, New York. AIDS: Epidemiological and clinical update.

Aria Baniahmad, Max-Planck Institut, Martinsried, Federal Republic of Germany. Are silencers negative enhancers?

March

Jonathan Pines, Salk Institute, San Diego, California. Mammalian cell-cycle cycling from B to A.

Gerry Klaus, National Institute for Medical Research, London, England. Control of surface immunoglobulin receptor signaling by G proteins.

Ryuji Kobayashi, Nagasaki University, Japan. Structural and functional analysis of bacterial proteases.

Michael Green, Harvard University, Cambridge, Massachusetts. Mechanisms of viral and cellular transcription activators.

April

Satoru Uzawa, Kyoto University, Japan. Fission yeast *cut1*$^+$ gene couples chromosome disjunction with other cell cycle events and has homology with budding yeast *ESP1*.

Jim Manley, Columbia University, New York, New York. Analysis of transcription factors that control embryonic development in *Drosophila*.

Jennifer Pietenpol, Vanderbilt University, Nashville, Tennesee. TGF-β growth inhibitory pathway in keratinocytes.

Zachary Pitluk, Yale University, New Haven, Connecticut. Regulation of the murine minute virus P4 promoter.

Julio Celis, University of Aarhus, Denmark. Comprehensive two-dimensional gel human protein databases offer a global approach to the study of gene regulation.

Paul Tempst, Harvard Medical School, Boston, Massachusetts. Protein microsequence analysis in modern biology: Challenges and strategies.

Gisela Storz, Harvard Medical School and Massachusetts General Hospital, Boston. Transcriptional regulator of oxidative stress-inducible genes: Direct activation.

May

Ed Ziff, New York University School of Medicine, New York, New York. Gene regulation by growth factors.

Robert Knowles, Memorial Sloan-Kettering Cancer Center, New York, New York. More complexity in the HLA system.

Elizabeth Robertson, Columbia University, New York, New York. Using embryonic skin cells to transfer mutations into the mouse germ line.

Claude Desplan, Rockefeller University, New York, New York. Transcriptional control during early *Drosophila* development.

June

Louis Mahadevan, Oxford University, England. Chromatin-associated signaling and induction and superinduction of proto-oncogenes.

Alan Harris, Yale University, New Haven, Connecticut. Calpain I and calmodulin coordinately regulate fodrin, a multifunctional protein of the actin-based cytoskeleton.

E.H. Coe, University of Missouri, Columbia. Toolkit of maize.

Adrian Bird, Institute of Molecular Pathology, Vienna, Austria. Effect of DNA methylation on chromatin structure and gene expression.

Ivan Raska, Institute of Experimental Medicine, Czechoslovak Academy of Sciences, Prague. Autoantibodies as probes to study the ultrastructure of the cell nucleus.

August

Mike Palazzolo, California Institute of Technology, Pasadena. Reverse *Drosophila* neurogenics.

Caroline Dean, John Innes Institute, U.K. Chromosome walking and transposon tagging in *Aribidopsis* to clone genes involved in floral induction.

September

Eric Fearon, Johns Hopkins Oncology Center, Baltimore, Maryland. Genetic alterations in colorectal tumor progression.

Scott Patterson, University of Queensland Equine Blood Typing Research Laboratory, Australia. Methods of protein identification following two-dimensional electrophoresis.

October

Harold Vaessin, University of California, San Francisco. Neuronal precursor formation in *Drosophila*.

Jim Howe, Yale University, New Haven, Connecticut. Glutamate-receptor ion channels in rat cerebellar granule cells.

William Tansey, University of Sydney, Australia. Analysis of promoter elements in the human growth hormone gene family.

Richard Carthew, University of California, Berkeley. *seven in absentia*, a gene required for specification of R7 cell fate in the *Drosophila* eye.

Maynard Olson, Washington University, St. Louis, Missouri. Physical mapping of whole human chromosomes: What is the best path forward?

Andrew Darrow, State University of New York, Stony Brook. Tissue plasminogen activator (TPA) gene regulation upon differentiation of F9 EC cells.

Hiroyuki Nawa, Kyoto University, Japan. How does the neuron decide which neurotransmitter to express?

November

Jim Van Etten, University of Nebraska, Lincoln. Virus-infected eukaryotic green alga is the new source of modification and restriction enzymes.

Alex Toker, National Institute of Medical Research, Mill Hill, London, England. Characterization of protein kinase C inhibitor proteins.

Pablo Scolnick, E.I. du Pont de Nemours and Co., Inc., Wilmington, Delaware. du Pont *Arabidopsis* project.

Richard Kramer, Columbia University College of Physicians & Surgeons, New York, New York. New approaches to studying intracellular signaling.

Roger Papke, Salk Institute, San Diego, California. Effects of subunit composition on the single-channel properties of rat neuronal nicotinic acetylcholine receptors.

Ron Davis, Baylor College of Medicine, Houston, Texas. *Dunce* and other learning and memory mutants in *Drosophila*.

Agiris Efstratiadis, Columbia University, New York, New York. Parental imprinting in the insulin-like growth factor II gene locus.

Rod MacKinnon, Harvard Medical School, Cambridge, Massachusetts. Molecular physiology of potassium channels.

December

Robert Goldberg, University of California, Los Angeles. Cell-specific gene regulation in higher plants and its application to agriculture.

Conley Rieder, New York State Dept. of Health, Wadsworth Center for Laboratories and Research, Albany, New York. How chromosomes attach to the vertebrate spindle: Implications for force production.

Masami Horikoshi, Rockefeller Foundation, New York, New York. Analysis of the structure and function of the TATA box binding factor TFIID.

Simon Bright, ICI Pharmaceuticals, England. Biotechnology research at ICI/GARST seed company.

James Huettner, Harvard Medical School, Cambridge, Massachusetts. Glutamate receptor channels in central and peripheral neurons.

Richard Hynes, Massachusetts Institute of Technology, Cambridge, Massachusetts. Genetic analyses of integrins.

Undergraduate Research

An important aspect of the summer program at the Laboratory is the participation of college undergraduate students in active research projects under the supervision of full-time laboratory staff members. The program was initiated in 1959. Since that year, 335 students have completed the course, and many have gone on to creative careers in biological science.

The objectives of the program are to provide (1) a greater understanding of the fundamental principles of biology; (2) an increased awareness of major problem areas under investigation; (3) a better understanding of the physical and intellectual tools for modern research and the pertinence of this information to future training; and (4) a personal acquaintance with research workers and centers for study.

The following students, selected from over 145 applicants, took part in the program, which was supported by the Burroughs Wellcome Fund, Baring Brothers, Miles Inc./Bayer AG, Cold Spring Harbor Laboratory Robert H.P. Olney Fund, and BioRad.

Benjamin Abella, Washington University
Research Advisor: **Bruce Futcher/Carol Greider**
Telomere structure in aging fibroblasts.

Luis Alvarez, University of California, Los Angeles
Research Advisor: **Venkatesan Sundaresan**
Mu-induced gene expression in maize.

Clare Baker, University of Cambridge
Research Advisor: **Bruce Stillman**
Investigation of a 13-kD single-stranded DNA-binding protein from *Saccharomyces cerevisiae.*

Steven Chao, Harvard University
Research Advisor: **Eric Richards**
Cloning of TAS sequences of *Arapidopsis thaliana* by complementation in YACs.

Matthew Cockerill, University of Cambridge
Research Advisor: **Nouria Hernandez**
A sensitive assay for U1/U2 snRNA gene transcription in vitro.

Arshad Desai, California State University, Los Angeles
Research Advisor: **Jeff Kuret**
Solution of the three-dimensional structure of a protein kinase using site-specific mutagenesis to create sites for isomorphous replacement.

Medeva Ghee, North Carolina State University
Research Advisor: **David Frendewey**
Analysis of snRNAs and snRNPs in fission yeast.

Gilbert Henry, University of California, Los Angeles
Research Advisor: **David Helfman**
Isolation of the cDNAs encoding a putative *Schizosaccharomyces pombe* tropomyosin and a novel actin-like protein.

Chia-Suei Hung, Beloit College
Research Advisors: **James Pflugrath/Thomas Marr**
Purification and crystallization of proliferating cell nuclear antigen.

David Immanuel, Wesleyan University
Research Advisor: **Kim Arndt**
Cloning of *SDS1*, a gene that suppresses a deletion of *SIT4*.

Michele Lozeron, University of Wisconsin, Madison
Research Advisor: **Thomas Peterson**
Restriction mapping of the *P Mosaic* allele of maize.

Monn Monn Myat, Mount Holyoke College
Reseach Advisor: **Adrian Krainer**
Purification and characterization of U2 snRNP auxiliary factor.

Stanford Peng, Stanford University
Research Advisor: **David Spector**
Eukaryotic RNA levels after heat shock.

Urmas Saarma, Tartu University, Estonia, USSR
Research Advisor: **Arne Stenlund**
Generation of an expression vector for the replication protein E1 from bovine papillomavirus.

Angela Wilson, University of Wisconsin, Madison
Research Advisor: **Daniel Marshak**
Molecular analysis of the expression of neurotrophic factor S100β.

John Yates, Glasgow University
Research Advisor: **Dafna Bar-Sagi**
Sequencing of PLA2 from muscle and liver, using polymerase chain reaction and subcloning techniques.

Ann Yonetani, University of Pennsylvania
Research Advisor: **Gilbert Morris/Michael Mathews**
Characterization of an *Alu* sequence transcribed from the human proliferating cell nuclear antigen promoter.

Karen Zito, Indiana University
Research Advisor: **Masafumi Tanaka/Winship Herr**
Study of transcriptional activation by Oct-2, a lymphoid octamer-binding protein.

Nature Study Program

The Nature Study Program gives elementary and secondary school students the opportunity to acquire a greater knowledge and understanding of their environment. Through a series of specialized field courses, students can engage in such introductory programs as Nature Detectives, Seashore Life, and Pebble Pups, as well as more advanced programs such as Marine Biology and Nature Photography.

During the summer of 1990 a total of 480 students participated in the Nature Study Program. Most classes were held outdoors, when weather permitted, or at the Uplands Farm Preserve, the headquarters of the Long Island Chapter of the Nature Conservancy. The Laboratory has equipped and maintains classrooms/laboratories as well as a darkroom at Uplands Farm. This facility is used as a base for the student's exploration of the local environment. Field classes are held on Laboratory grounds, St. John's Preserve, Fire Island National Seashore, Caumsett State Park, the Cold Spring Harbor Fish Hatchery and Aquarium, as well as other local preserves and sanctuaries. Students in the Marine Biology class participated in a whale watch aboard the Finback II, operated by the Okeanos Ocean Research Foundation, Inc., Hampton Bays, New York.

In addition to the 4-week courses, the 2-day Adventure Education class took students on an 18-mile bike hike to Caumsett State Park and a 12-mile canoe trip on the Nissequogue River.

PROGRAM DIRECTOR

William M. Payoski, M.A., Adjunct Professor,
Nassau Community College

INSTRUCTORS

Cheryl Littman, B.A., science teacher, Northport School District
Michael Manfredonia, teaching candidate, Marist College
Maria Onfora, B.A., science teacher, West Babylon High School
Linda Payoski, B.A., science teacher, Uniondale High School
Marjorie Pizza, B.A., science teacher, Glen Cove School District
Donna Stokes, teaching candidate
Edward Tronolone, B.A., M.S., Principal, Locust Valley School District

COURSES

Nature Bugs
Nature Detectives
Advanced Nature Study
Ecology Explorers
Frogs, Flippers, and Fins
Pebble Pups
Bird Study
Fresh Water Life
Seashore Life
Marine Biology
Nature Photography
Adventure Education

FINANCIAL
STATEMENT

FINANCIAL STATEMENT

BALANCE SHEET
year ended December 31, 1990
with comparative figures for year ended December 31, 1989

ASSETS

	Operating Funds						
	Unrestricted			Endowment &	Land, Building, &	Total All Funds	
	Undesignated	*Designated*	*Restricted*	*Similar Funds*	*Equipment Funds*	*1990*	*1989*
Cash and cash equivalents	$2,710,250	650,000	804,086	11,854,697	7,294,160	23,313,193	30,717,964
Marketable securities	14,355	–	–	5,928,296	100,125	6,042,776	6,045,676
Accounts receivable:							
Publications (less allowance for doubtful accounts of $50,000 in 1990 and $95,000 in 1989)	729,472	–	–	–	–	729,472	1,884,531
Other	113,782	–	15,048	–	–	128,830	528,905
Grants receivable	–	–	1,301,854	–	–	1,301,854	2,024,479
Accrued interest receivable	16,982	–	–	256,444	–	273,426	153,978
Publications inventory	900,727	–	–	–	–	900,727	617,542
Other assets, principally prepaid expenses	279,843	–	–	–	557,122	836,965	937,415
Investment in employee residences	–	–	–	–	1,084,424	1,084,424	853,524
Land, buildings, and equipment:							
Land and improvements	–	–	–	–	4,535,715	4,535,715	3,248,451
Buildings	–	–	–	–	23,692,813	23,692,813	23,045,909
Furniture, fixtures, and equipment	–	–	–	–	2,278,950	2,278,950	2,013,732
Laboratory equipment	–	–	–	–	5,044,457	5,044,457	4,752,761
Library books and periodicals	–	–	–	–	365,630	365,630	365,630
	–	–	–	–	35,917,565	35,917,565	33,426,483
Less accumulated depreciation and amortization	–	–	–	–	12,610,734	12,610,734	11,172,671
Land, buildings, and equipment, net	–	–	–	–	23,306,831	23,306,831	22,253,812
Construction in progress	–	–	–	–	20,166,508	20,166,508	7,222,851
Total assets	$4,765,411	650,000	2,120,988	18,039,437	52,509,170	78,085,006	73,240,677

LIABILITIES AND FUND BALANCES

	Operating Funds						
	Unrestricted				*Land,*	*Total All Funds*	
	Undesignated	*Designated*	*Restricted*	*Endowment & Similar Funds*	*Building, & Equipment Funds*	*1990*	*1989*
Liabilities:							
Accounts payable and accrued expenses	$2,415,394	–	–	–	776,642	3,192,036	3,002,980
Notes payable	–	–	–	–	964,869	964,869	1,063,857
Bonds payable	–	–	–	–	20,000,000	20,000,000	20,000,000
Deferred revenue	697,470	–	2,120,988	–	–	2,818,458	3,153,137
Total liabilities	3,112,864	–	2,120,988	–	21,741,511	26,975,363	27,219,974
Fund balances:							
Unrestricted–undesignated	1,652,547	–	–	–	–	1,652,547	1,238,405
Unrestricted–designated	–	650,000	–	–	–	650,000	400,000
Endowment and similar funds	–	–	–	18,039,437	–	18,039,437	15,263,933
Land, buildings, and equipment:							
Expended	–	–	–	–	28,222,713	28,222,713	17,424,570
Unexpended–Donor restricted	–	–	–	–	1,460,522	1,460,522	10,843,739
Unexpended–Board authorized	–	–	–	–	1,084,424	1,084,424	850,056
Total fund balances	1,652,547	650,000		18,039,437	30,767,659	51,109,643	46,020,703
Total liabilities and fund balances	$4,765,411	650,000	2,120,988	18,039,437	52,509,170	78,085,006	73,240,677

STATEMENT OF SUPPORT, REVENUE AND EXPENSES AND CHANGES IN FUND BALANCES
year ended December 31, 1990
with comparative figures for year ended December 31, 1989

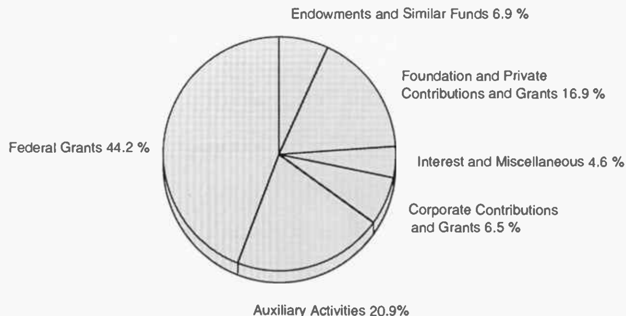
	Operating Funds				*Land,*		
	Unrestricted			*Endowment &*	*Building, &*	*Total All Funds*	
	Undesignated	*Designated*	*Restricted*	*Similar Funds*	*Equipment Funds*	*1990*	*1989*
Support and revenue:							
Public support	$1,244,697	–	3,359,572	2,324,069	629,592	7,557,930	11,959,169
Government grant awards	–	–	10,250,777	–	–	10,250,777	9,412,256
Indirect cost allowances	6,633,895	–	–	–	–	6,633,895	6,456,143
	7,878,592	–	13,610,349	2,324,069	629,592	24,442,602	27,827,568
Other revenue:							
Program fees	1,123,793	–	–	–	–	1,123,793	1,298,031
Rental income	248,333	–	–	–	–	248,333	93,036
Publications	4,222,694	–	–	–	–	4,222,694	4,449,690
Dining services	1,154,033	–	–	–	–	1,154,033	1,336,698
Rooms and apartments	795,995	–	–	–	–	795,995	774,769
Distribution from Robertson Funds	450,000	–	700,000	–	–	1,150,000	976,800
Investment income	333,083	–	–	1,363,202	1,157,630	2,853,915	2,572,913
Miscellaneous	169,564	–	–	–	–	169,564	262,109
Total other revenue	8,497,495	–	700,000	1,363,202	1,157,630	11,718,327	11,764,046
Total support and revenue	16,376,087	–	14,310,349	3,687,271	1,787,222	36,160,929	39,591,614
Expenses:							
Program services:							
Research	–	–	11,348,636	–	–	11,348,636	11,049,896
Summer programs	865,847	–	2,186,278	–	–	3,052,125	2,858,253
Publications	3,708,062	–	–	–	–	3,708,062	3,934,216
Banbury Center conferences	178,424	–	321,518	–	–	499,942	416,681
DNA Education Center programs	32,616	–	290,603	–	–	323,219	349,586
Total program services	4,784,949	–	14,147,035	–	–	18,931,984	18,608,632
Supporting services:							
Direct research support	609,502	–	–	–	–	609,502	495,853
Library	388,221	–	–	–	–	388,221	367,448
Operation and maintenance of plant	4,070,385	–	–	–	–	4,070,385	3,706,217
General and administrative	2,832,903	–	–	44,557	–	2,877,460	3,122,091
Dining services	1,296,009	–	–	–	–	1,296,009	1,377,351
Interest	–	–	–	–	1,413,344	1,413,344	1,149,302
Total supporting services	9,197,020	–	–	44,557	1,413,344	10,654,921	10,218,262

Depreciation	–	–	–	–	1,485,084	1,485,084	1,399,409
Total expenses	13,981,969	–	14,147,035	44,557	2,898,428	31,071,989	30,226,303
Excess (deficiency) of support and revenue over expenses before designation	$2,394,118	–	163,314	3,642,714	(1,111,206)	5,088,940	9,365,311
Designation:							
Funds designated for neuroscience program	(250,000)	250,000	–	–	–	–	–
Excess (deficiency) of support and revenue over expenses and designation	2,144,118	250,000	163,314	3,642,714	(1,111,206)	5,088,940	9,365,311
Other changes in fund balances:							
Capital expenditures	(1,098,586)	–	(235,914)	(1,426,000)	2,760,500	–	–
Transfer to restricted funds	–	–	72,600	(72,600)	–	–	–
Transfer to endowment funds	(631,390)	–	–	631,390	–	–	–
Net increase in fund balances	414,142	250,000	–	2,775,504	1,649,294	5,088,940	9,365,311
Fund balances at beginning of year	1,238,405	400,000	–	15,263,933	29,118,365	46,020,703	36,655,392
Fund balances at end of year	$1,652,547	650,000	–	18,039,437	30,767,659	51,109,643	46,020,703

Copies of our complete, audited financial statements, certified by the independent auditing firm of KPMG, Peat, Marwick & Co., are available upon request form the Comptroller, Cold Spring Harbor Laboratory.

SOURCES OF REVENUE

YEAR ENDED DECEMBER 31, 1990



COMPARATIVE OPERATING HISTORY
1986-1990
(Dollars in Thousands)

	1986	1987	1988	1989	1990
Income:					
Main lab:					
Grants & contracts	$9,439	10,409	10,799	13,062	13,535
Indirect cost reimbursement	4,533	4,779	5,707	6,412	6,558
Other	2,572	2,727	3,205	4,034	3,976
CSH Press	1,357	1,556	1,641	4,450	4,223
Banbury Center	963	982	976	1,012	1,120
DNA Learning Center	-	349	660	622	585
Total income	18,864	20,802	22,988	29,592	29,997
Expenses:					
Main lab:					
Grants & contracts	9,439	10,409	10,799	13,062	13,535
Operation & maintenance of plant	2,442	2,791	3,010	3,412	3,759
General & administrative	1,889	1,975	2,102	2,377	2,414
Other	2,124	2,633	3,049	3,165	2,973
CSH Press	1,060	1,311	1,719	3,934	3,708
Banbury Center	868	947	910	1,038	1,125
DNA Learning Center	-	260	590	635	615
Total expenses	17,822	20,326	22,179	27,623	28,129
Excess before depreciation and designation of funds	1,042	476	809	1,969	1,868
Depreciation	(1,093)	(1,127)	(1,286)	(1,399)	(1,485)
Designation of funds	-	-	-	(400)	(250)(2)
Net operating excess (deficit)	$ (51)	(651)	(477)	170	133

(1) The above amounts are presented on a combined basis for all funds for which Cold Spring Harbor prepares operating budgets.

(2) Funds designated to underwrite future direct and indirect expenses of the neuroscience program.

FINANCIAL SUPPORT OF THE LABORATORY

SOURCES OF SUPPORT

Cold Spring Harbor Laboratory is a nonprofit research and educational institution chartered by the University of the State of New York. Less than half of its annual support is derived from Federal grants and contracts. Contributions from the private sector are tax exempt under the provisions of Section 501(c)(3) of the Internal Revenue Code. In addition, the Laboratory has been designated a "public charity" and, therefore, may receive funds resulting from the termination of "private foundations."

Over the years, the Laboratory has earned a reputation for innovative research and high-level science education. By training young scientists in the latest experimental techniques, it has helped spur the development of many research fields, including tumor virology, cancer genes, gene regulation, movable genetic elements, yeast genetics, and molecular neurobiology. This continual development of new research programs and training courses requires substantial support from private sources.

Because its endowment is small and because government support is highly competitive and the uses of research grants are restricted, the Laboratory depends on **annual** contributions from the private sector; foundations, corporations, and individuals for its central institutional needs.

The Second Century Campaign seeks to raise $44M in **capital** funds by December 1991 for construction of new facilities, renovation of existing facilities, and for staff and student endowment. This is the Laboratory's first public capital campaign and it marks its Centennial.

METHODS OF CONTRIBUTING TO COLD SPRING HARBOR LABORATORY

Gifts of money can be made directly to Cold Spring Harbor Laboratory.

Securities You can generally deduct the full amount of the gift on your income tax return, and, of course, you need pay no capital gains tax on the stock's appreciation.
We recommend any of the following methods:
(1) Have your broker sell the stock and remit the proceeds to Cold Spring Harbor Laboratory.
(2) Deliver the stock certificates to your broker with instructions to him to open an account for Cold Spring Harbor Laboratory and hold the securities in that account pending instructions from Cold Spring Harbor Laboratory.
(3) Send the *unendorsed* stock certificates directly to the Laboratory: Comptroller, Cold Spring Harbor Laboratory, Box 100, Cold Spring Harbor, New York 11724. In a separate envelope send an *executed* stock power.

Pooled Income Funds Combine gifts from a number of donors in a pool for attractive investment and tax purposes.

Appreciated real estate or personal property Sizable tax benefits can result from such donations; the Laboratory can use some in its program and can sell others.

Life insurance and charitable remainder trusts can be structured to suit the donor's specific desires as to extent, timing, and tax needs.

Bequests Most wills probably need to be updated. Designating Cold Spring Harbor Laboratory as beneficiary ensures that a bequest will be utilized as specified for continuing good.

Conversion of private foundation to "public" status on termination This may be done by creating a separate fund within Cold Spring Harbor Laboratory whereby the assets of the private foundation are accounted for as a separate fund and used for the purposes specified by the donor. Alternatively, the private foundation can be established as a "supporting organization of Cold Spring Harbor Laboratory."

For additional information, please contact the Director of Development, Cold Spring Harbor Laboratory, Box 100, Cold Spring Harbor, NY 11724, or call 516-367-8840.

GRANTS

January 1, 1990–December 31, 1990

COLD SPRING HARBOR LABORATORY

Grantor	Program/Principal Investigator	Duration of Grant	Total Award
FEDERAL GRANTS			
NATIONAL INSTITUTES OF HEALTH			
Program Projects	Cancer Research Center, Dr. Mathews	1/87–12/91	$16,787,416
	Cancer Center Support, Dr. Roberts	8/87–7/95	8,823,360
	PEBRA-HIV Grant, Dr. Herr	9/88–8/91	3,672,816
	Oncogene Program Project, Dr. Wigler	3/88–2/93	4,869,923
Research Support	Dr. Anderson	4/90–3/93	176,000*
	Dr. Arndt	4/88–3/93	1,288,964
	Dr. Beach	12/84–1/93	1,481,482
	Dr. Beach	9/86–8/94	3,032,957
	Dr. Beach	7/88–6/93	1,488,740
	Dr. Beach	8/90–7/93	1,287,507*
	Dr. L. Field	4/87–3/92	568,521
	Dr. Franza	9/85–11/94	1,497,483
	Dr. Frendeway	4/87–3/92	775,814
	Dr. Futcher	4/88–3/93	1,115,434
	Dr. Garrels	1/85–12/92	5,016,584
	Dr. Gilman	9/87–8/92	608,291
	Dr. Greider	12/89–11/94	1,130,019
	Dr. Helfman	9/85–3/94	2,109,264
	Dr. Hernandez	7/87–6/92	1,293,754
	Dr. Krainer	7/89–6/94	1,298,402
	Dr. Kuret	7/89–6/94	1,312,808
	Dr. Marr	9/89–9/90	126,428
	Dr. Moran	3/88–2/93	547,214
	Dr. Peterson	4/88–3/93	704,475
	Dr. Pflugrath	4/88–3/91	573,503
	Dr. Rice	9/87–8/90	528,037
	Dr. Richards	7/90–6/93	424,932*
	Dr. Roberts	7/88–6/93	1,598,876
	Dr. Roberts	9/88–8/91	446,241
	Dr. Spector	4/90–3/95	1,441,475*
	Dr. Stillman	7/83–6/92	1,869,678
	Dr. Wigler	7/85–6/92	8,426,929
	Dr. Wigler	8/90–7/93	640,923*
Equipment Support	Dr. Roberts	8/90–7/91	37,583*
	Dr. Spector	4/90–4/91	180,000*
Fellowships	Dr. Ballester	2/89–1/92	63,996
	Dr. Brill	8/89–7/92	46,667
	Dr. Connolly	1/90–1/93	69,000*
	Dr. Grueneberg	10/90–9/93	69,000*
	Dr. Kessler	8/88–7/91	93,996
	Dr. Otto	9/90–8/93	100,500*
	Dr. Steinhelper	3/89–2/92	77,254
	Dr. Stern	11/88–11/91	63,996
	Dr. Szymanski	11/90–11/92	49,000*

* New Grants Awarded in 1990

Grantor	Program/Principal Investigator	Duration of Grant	Total Award
Training Support	Institutional, Dr. Grodzicker	7/78–4/94	2,364,146
Course Support	Advanced Bacterial Genetics, Dr. Grodzicker	5/80–4/93	559,540
	Cancer Research Center Workshops, Dr. Grodzicker	1/83–3/92	1,010,057
	Neurobiology Short-term Training, Dr. Hockfield	5/82–4/91	818,188
Meeting Support	Cancer Cells—Origins of Human Cancer	1990	5,000*
	Genome Mapping and Sequencing Conference	1990–1993	78,040*
	Mouse Molecular Genetics	1990	6,000*
	Function and Evolution of RAS Protein	1990	2,000*
	RNA Processing	1988–1991	9,500
	Symposium	1990	25,000*
NATIONAL SCIENCE FOUNDATION			
Research Support	Dr. Anderson	9/90–8/93	315,000*
	Dr. Herr	6/88–5/91	240,000
	Dr. Marr	7/90–6/91	49,290*
	Dr. Marshak	7/87–12/90	190,000
	Dr. Martienssen	7/89–6/92	300,000
	Dr. Richards	7/89–6/90	95,000
	Dr. Roberts	1/83–5/90	640,000
	Dr. Roberts	8/87–7/92	63,106
	Dr. Roberts	2/90–2/93	210,000*
	Dr. Sundaresan	5/87–4/91	360,000
Training Support	Undergraduate Research Program, Dr. Herr	6/89–5/90	42,000
Course Support	Plant Molecular Biology, Dr. Grodzicker	8/86–1/90	137,490
Meeting Support	Molecular Neurobiology of *Aplysia*	1990	7,192*
	Mouse Molecular Genetics	1990	5,000*
	Function and Evolution of RAS Protein	1990	5,000*
	Symposium	1990	10,000*
	RNA Processing Conference	4/88–3/91	30,000
DEPARTMENT OF ENERGY			
Meeting Support	Symposium	1990	10,000*

* New Grants Awarded in 1990

Grantor	Program/Principal Investigator	Duration of Grant	Total Award
NONFEDERAL GRANTS			
Research Support			
Aaron Diamond Foundation	Dr. Anderson	12/88–11/91	200,000
American Cancer Society	Dr. Bar-Sagi	7/89–6/91	194,000
	Dr. Moran	1/88–12/90	90,500
	Dr. Spector	7/87–6/91	363,000
	Drs. Stillman, Gluzman, Welch, Gilman, Institutional Award	7/82–6/90	300,000
	Dr. Wigler	4/90–3/91	10,000 *
	Dr. Wigler, Professorship	1986–2012	1,333,333
	Dr. Gilman, Institutional Award	7/90–6/91	50,000 *
Amersham International plc	Dr. Harlow	11/86–10/91	799,635
Istituto Farmaco-terapico Italiano (IFI)	Dr. Harlow	5/90–4/91	100,000 *
Howard Hughes Medical Institute	Neurobiology Support	1987–1990	1,000,000
Japan Health Science Foundation	Dr. Roberts	12/89–2/90	31,700
J.N. Pew Jr. Charitable Trust	Plant Group Support	4/87–4/90	260,000
Lucille P. Markey Charitable Trust	Neurobiology Support	7/90–6/96	
	(Neuroscience)		2,000,000 *
	(Building Fund)		2,000,000 *
Muscular Dystrophy Association	Dr. Mathews	1/87–6/90	100,718
Mellam Family Foundation	Dr. Franza	12/88–11/90	150,000
Richard Meltzer Fund Fund for Cancer Research (World Business Council)	Dr. Harlow	3/90–2/91	16,667 *
Merck Sharp & Dohme Research Laboratories	Dr. Harlow	3/90–2/91	50,000 *
National Downs Syndrome Society	Dr. Marshak	7/90–6/92	25,000 *
New England Biolabs	Dr. Roberts	1990	10,000 *
Pew Memorial Trust	Dr. Greider	7/90–6/94	200,000 *
Pfizer, Inc.	Dr. Wigler	1985–1990	500,000
Pioneer Hi-Bred International, Inc.	Cooperative Research	8/85–4/91	2,500,000
Rita Allen Foundation	Dr. Hernandez	10/89–9/94	150,000
Samuel Freeman Charitable Trust	Freeman Laboratory of Cancer Cell Biology	7/89–6/94	1,000,000
Equipmnent Support			
Fannie E. Rippel	Neurobiology Support	10/89–7/91	250,000
Fellowships			
American Cancer Society	Dr. Conway	10/88–9/91	63,000
	Dr. Ryan	7/88–6/90	90,000
	Dr. Nefsky	7/90–6/93	69,000 *
American Foundation for Aids Research	Dr. Laspia	7/89–6/92	112,200
Bristol-Myers Company	Fellowship Support	6/86–5/91	500,000
Bioseeds International	Plant Fellowship Support	7/88–6/90	75,000
Damon Runyon-Walter Winchell Cancer Fund	Dr. Michaeli	3/87–2/90	69,000
	Dr. Melendy	1/89–12/91	69,000
	Dr. Walworth	9/90–8/93	84,000 *
	Dr. Su	1/90–12/90	22,000 *

* New Grants Awarded in 1990

Grantor	Program/Principal Investigator	Duration of Grant	Total Award
Anna Fuller Fund	Dr. Bauer	1/90–12/90	21,000 *
Government of Canada	Dr. Tyers	9/89–8/90	1,082
	Dr. Tyers	9/90–8/91	1,099 *
	Dr. Labrie	10/90–9/91	1,300 *
Helen Hay Whitney Foundation	Dr. Ma	4/90–6/90	5,750 *
Irvington Institute for Medical Research	Dr. Giordano	7/90–6/93	78,000 *
Esther A. and Joseph Klingenstein Fund, Inc.	Dr. Marshak	7/87–6/90	100,000
Ladies Auxiliary to the VFW	Dr. Yaciuk	7/90–6/91	20,000 *
The Leukemia Society of America	Dr. Das	7/89–6/92	76,140
	Dr. Riabowol	7/90–6/93	93,960 *
LIBA	Four Fellowships a Year	1987–1990	300,000
Life Science Research	Dr. D. Roberts	9/89–8/93	105,000
Merck Sharp & Dohme Research Laboratories	Graduate Student Support	12/88–11/90	30,000
Muscular Dystrophy Association	Dr. Lees-Miller	7/88–6/89	51,000
	Dr. Kazzaz	1/90–12/90	25,000 *
Andrew Seligson Memorial Fellowships	Two Postdoctoral Fellows (Cancer Research Support)	9/90–8/91	70,000 *
Weizmann Institute of Science	Dr. Gerst	9/89–8/91	57,000
Training Support			
Baring Brothers & Co., Ltd.	Undergraduate Research Program	1990	26,625 *
Burroughs Wellcome Foundation	Undergraduate Research Program	1987–1990	50,960
Bio-Rad Laboratories	CSHL Summer Undergraduate	1990	1,000 *
CSHL Centennial Fund	Partners in the Future	10/90–6/91	27,500 *
Grass Foundation	Neurobiology Scholarship Support	1980–1990	166,670
Miles, Inc.	CSHL Summer Undergraduate	1990	4,803 *
Robert H.P. Olney Memorial Fund	Undergraduate Research Program	1989–1990	2,400
Course Support			
Howard Hughes Medical Institute	Neurobiology Courses	1987–1990	1,000,000
Meeting Support			
Council for Tobacco Research USA, Inc.	Role of Isoform Diversity in Cytoskeletal Function	7/90–6/91	1,000 *
FIDIA Research Foundation	Molecular Neurobiology of *Aplysia*	8/90–12/90	5,000 *
Fondazione Sigma-Tau, Foundation	Molecular Neurobiology of *Aplysia*	8/90–12/90	5,000 *
Rockefeller Foundation	Modern Approach to New Vaccines (Publication Support)	9/90–8/91	20,000 *
Rockefeller Foundation	Vaccines Conference	1986–1990	45,000
Alfred P. Sloan Foundation	Evolution: From Molecules to Culture	3/90–2/91	30,000 *
Carl Zeiss, Inc.	Role of Isoform Diversity in Cytoskeletal Function	7/90–6/91	1,000 *

* New Grants Awarded in 1990

BANBURY CENTER

Grantor	Program/Principal Investigator	Duration of Grant	Total Award
FEDERAL GRANTS			
ENVIRONMENTAL PROTECTION AGENCY			
Course Support	Molecular Mechanisms of Fiber Cytotoxicity and Carcinogenesis	1990	20,000*
	Dioxin Meeting	1990	39,180*
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES			
Meeting Support	Control of HIV Gene Expression	1990	25,432*
	Immunological Aspects of AIDS	1990	21,268*
NATIONAL SCIENCE FOUNDATION			
Meeting Support	Mapping the Genomes of Domestic Animals	1990	5,000*
OFFICE OF NAVAL RESEARCH			
Course Support	Computational Eye Movement Workshop	7/88-6/91	87,125
U.S. DEPARTMENT OF AGRICULTURE			
Course Support	Mapping the Genomes of Domestic Animals	1990	5,000*
NONFEDERAL SUPPORT			
Meeting Support			
Abbott Laboratories	Programmed Cell Death—Concepts and Mechanisms	1990	500*
Alfred P. Sloan Foundation	Journalist and Congressional Workshops	1990-1992	150,000*
Alfred P. Sloan Foundation	Molecular Clocks of Evolution	1990	3,000*
Beckman Instruments, Inc.	Electrophoresis of Large DNA Molecules	1990	5,000*
Chlorine Institute	Dioxin Meeting	1990	15,000*
CNS Research	Programmed Cell Death—Concepts and Mechanisms	1990	1,000*
FMC Corporation	Electrophoresis of Large DNA Molecules	1990	5,000*
Granada Biosciences, Inc.	Mapping the Genomes of Domestic Animals	1990	3,000*
Hoefer Scientific Instruments	Electrophoresis of Large DNA Molecules	1990	2,500*
Metropolitan Life Foundation	Programmed Cell Death—Concepts and Mechanisms	1990	10,000*
Ohio State University	Programmed Cell Death—Concepts and Mechanisms	1990	7,000*
Pew Charitable Trust	New Human Molecular Genetics—Impact on Society	1990	35,000*
Pharmacia	Electrophoresis of Large DNA Molecules	1990	4,000*
Risk Science Institute	Molecular Mechanisms of Fiber Cytotoxicity and Carcinogenesis	1990	3,000*
Ross Laboratories	Programmed Cell Death—Concepts and Mechanisms	1990	2,500*
SmithKline Beecham Pharmaceuticals	Cloning the Antibody Repertoire	1990	35,000*
Thermal Insulation Manufacturers Association	Molecular Mechanisms of Fiber Cytotoxicity and Carcinogenesis	1990	15,000*
William Stamps Farish Fund	Meeting on Complex Genetic Diseases	1991-1993	150,000*

* New Grants Awarded in 1990

DNA LEARNING CENTER

Grantor	Program/Principal Investigator	Duration of Grant	Total Award
FEDERAL GRANTS			
NATIONAL SCIENCE FOUNDATION	Teacher Enhancement Program	6/87–11/90	415,928
	Teacher Enhancement Program	3/90–4/93	474,036 *
	Teacher Enhancement Program	9/90–6/93	252,614 *
NONFEDERAL GRANTS			
Howard Hughes Medical Institute	DNA Science Workshop Program	5/90–4/93	46,500 *
Josiah Macy, Jr., Foundation	DNA Literacy Program	7/87–6/90	490,850
Josiah Macy, Jr., Foundation	DNA Literacy Program	7/90–6/91	98,905 *
The Banbury Fund	Core Support	1990	30,000 *
Brinkman Instruments, Inc.	Core Support	1990	2,500 *
The Esther and Joseph A. Klingenstein Fund, Inc.	Core Support	1990	25,000
North Carolina Biological Supply Co.	Core Support	1990 3,125	*
Nancy VanVranken	Core Support	1990	500 *
Case Western Reserve University	Vector Workshop	1990	11,075 *
Center for Biotechnology, SUNY Stony Brook	Vector Workshop	1990	10,500 *
Cornell University	Vector Workshop	1990	1,550 *
Jefferson Parish School District Louisiana	Vector Workshop	1990	2,000 *
Kamehameha Schools, Hawaii	Vector Workshop	1990	12,000 *
Richmond Mathematics & Science Center	Vector Workshop	1990	2,000 *
Mississippi School for Math and Science	Vector Workshop	1990	1,550 *
J.J. Pearce High School, Richardson, Texas	Vector Workshop	1990	2,000 *
Ohio Academy of Science	Vector Workshop	1990	2,000 *
Smith College	Vector Workshop	1990	1,550 *
Commack School District	Curriculum Study	1990	500
East Williston School District	Curriculum Study	1990	500
Garden City School District	Curriculum Study	1990	2,000 *
Great Neck Public Schools	Curriculum Study	1990	500
Half Hollow Hills School District	Curriculum Study	1990	500
Harborfields School District	Curriculum Study	1990	500
Herricks School District	Curriculum Study	1990	500
Huntington School District	Curriculum Study	1990	500
Island Trees School District	Curriculum Study	1990	1,500
Jericho School District	Curriculum Study	1990	500
Kings Park School District	Curriculum Study	1990	2,000 *
Lawrence School District	Curriculum Study	1990	500
Lindenhurst School District	Curriculum Study	1990	1,500
Locust Valley School District	Curriculum Study	1990	500
Manhasset School District	Curriculum Study	1990	500
Northport-East Northport School District	Curriculum Study	1990	500
North Shore School District	Curriculum Study	1990	500
Oyster Bay-East Norwich School District	Curriculum Study	1990	500

* New Grants Awarded in 1990

Grantor	Program/Principal Investigator	Duration of Grant	Total Award
Plainview-Old Bethpage School District	Curriculum Study	1990	500
Plainedge School District	Curriculum Study	1990	1,500
Portledge School	Curriculum Study	1990	500
Port Washington School District	Curriculum Study	1990	500
Rosyln School District	Curriculum Study	1990	2,000 *
Sachem School District	Curriculum Study	1990	500
South Huntington School District	Curriculum Study	1990	1,500
Syosset School District	Curriculum Study	1990	500

* New Grants Awarded in 1990

ANNUAL CONTRIBUTIONS

Unrestricted

Long Island Biological Association (LIBA)

The Long Island Biological Association is the oldest supporting organization for Cold Spring Harbor Laboratory. Over the years a most unique and productive partnership has developed wherein LIBA has become our "Friends of the Laboratory" and accounts for the largest amount of unrestricted annual giving for the Laboratory. (The detailed report of their activities appears later in this Annual Report.)

Memorial Gifts

American Management Systems, Inc.
Mr. & Mrs. Lionel Bal
Baltic Linen Company, Inc.
Mrs. Eric Belmont
The Bibb Company
Mr. & Mrs. Richard Blick
Mr. & Mrs. Bruce R. Blovsky
Ms. Joan B. Brady
Ms. Lisa D. Casentini
Mr. & Mrs. Richard Charkow
Mr. William H. Chisholm
Mr. & Mrs. P. Chisum, Jr.
Mr. Lawrence M. Clum
Mr. & Mrs. Robert E. Cooper
Mr. Jim Cronin
Mr. & Mrs. Wayne A. Davidson
Mr. & Mrs. Kurt E. Dreyfuss
Dr. & Mrs. Lester Dubnick
Dr. & Mrs. Dennis Eryou
Mr. & Mrs. Joel M. Fairman
Mr. & Mrs. Raymond E. Fluddy
Mr. Richard L. Gelb
Mr. & Mrs. Eugene M. Geddes
Mrs. J. Bardin Goodman
Ms. Ruth S. Greenberg
Mr. & Mrs. Howard Gross
Mr. & Mrs. Homer Guinan
Mr. & Mrs. Raymond L. Hallaran
Harken, Inc.
Mr. & Mrs. Stanley Himmelstein
Mrs. Frank Hohner
Mr. & Mrs. Richard Hollander
Mr. Thomas Chase Homan
Mr. Jim Houlihan
Mr. Tony Hoyt
Mr. Joseph T. Jones
Mr. & Mrs. Melvin P. Klein
Mr. Robert B. Kraus
Ms. Timothea S. Larr
Mr. & Mrs. Richard B. Leather
Mr. & Mrs. William E. Mahoney
Marine Midland Bank
Mr. & Mrs. Markidis
Mr. Konrad Matthaei
Mr. Robert R. McMillan
Mr. & Mrs. Stephen Miller
Mr. George B. Munroe
Nassau County Division of Data Processing
Dr. & Mrs. James R. Nemeth
Mr. Denis E. O'Connor
Ms. Dixie Pasciucco
Mr. & Mrs. Julius L. Pericola
Phelps Dodge Corporation
Ms. Nancy L. Robinson
Mr. & Mrs. Seymour Schlaefer
Mr. & Mrs. Gary Schweon
Ms. Carole Smith
Mrs. R. Brinkley Smithers
Dr. Abramo Virgilio
Ms. Ronni Wald

In memory of .....

Agnes Alesi
Vincent Battista
Cathy Susan Bird
Mamie Botwick
Oliver T. Carpenter
Margaret Carroll
Garner Cline
Janet Cook Cotter
Patricia Crowe
Sophie E. Diaman
Clifford Fronapfel
Prudence Gaveglia
Nick Giammalvo
Arthur Greenberg
Herbert Hallaran
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Summary of Annual Contributions

Unrestricted Annual Contributions

CSHL Associates (1/1/90–12/31/90)	$200,137	
LIBA Members (1/1/90–12/31/90)	64,225	
General	17,951	
Memorials	4,278	
		$286,591

Restricted Annual Contributions

Miscellaneous	55,782	
		$ 55,782

Total Annual Contributions $342,373

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CORPORATE SPONSOR PROGRAM

Restricted

Once again, Cold Spring Harbor Laboratory is very pleased to acknowledge the generosity of members of the Corporate Sponsor program. These companies, with a special interest in biotechnology and a concern for fostering research in molecular biology and genetics, contribute $18,500 each to underwrite the costs of meetings held at Grace Auditorium and the Banbury Center. This support is now essential for what we believe to be the most comprehensive series of meetings on molecular biology available anywhere.

There were 12 large meetings held in Grace Auditorium, including the 55th Cold Spring Harbor Laboratory *Symposium on Quantitative Biology* on "The Brain," and five special meetings held at the Banbury Center. The latter included "Electrophoresis of Large DNA Molecules: Theory, Practice and Future;" "Genetics and Molecular Biology of Complex Diseases;" "Recognition in Plant-Pathogen Interactions; Molecular Biology of Free Radical Scavenging Systems;" and "Computational Predictions of Protein Structure." The benefits to sponsoring companies include the waiver of all fees for eight representatives at our meetings. Three of these meetings may be at the Banbury Center, where attendance is otherwise only by invitation of the organizers. In addition, sponsors receive gratis Cold Spring Harbor Laboratory and Banbury Center publications, including the Laboratory's journals, *Genes & Development* and *Cancer Cells*.

We list 28 companies as members of the Corporate Sponsor program in 1990. This is a remarkable number, especially in the current economic climate. It reflects the genuine contribution made by our meetings program to the research activities of the scientists of member companies. However, the principal beneficiaries are the over 5000 scientists from around the world who were able to review data, generate ideas, and exchange gossip on some of the most exciting topics in modern biology. We join with them in thanking our Corporate Sponsors.

Jan A. Witkowski

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SECOND CENTURY CAMPAIGN

January 1, 1986–December 31, 1990

Unrestricted Contributions $7,260,186

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Anonymous 3
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Restricted Contributions $33,133,674

Endowment $ 5,162,226

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Page Laboratory of Plant Genetics

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Total Second Century Campaign Contributions **$40,393,860**

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THE LONG ISLAND BIOLOGICAL ASSOCIATION (LIBA) and Its Relation to the Cold Spring Harbor Laboratory

Biological research at Cold Spring Harbor began in 1890 when the Brooklyn Institute of Arts and Sciences was looking for a place to set up a summer laboratory as the headquarters of its Department of Zoology. Representatives of the Institute were invited by Eugene S. Blackford, at that time the Fish Commissioner of the State of New York, to inspect a site on Bungtown Road across Northern Boulevard (25A) from the Fish Hatchery. The site was found to be ideal, and so the original Laboratory was organized with Mr. Blackford as president of the Board of Trustees. The land was leased from Mr. John D. Jones, whose family since 1819 had operated various industries including shipbuilding, coopering, and textile manufacture (later whaling, also) at the head of Cold Spring Harbor. Bungtown Road, which runs through the Lab property, got its name from the factory that specialized in making bungs–or stoppers–for barrels.

In 1892 the Laboratory's land was leased for a dollar a year from the Wawepex Society, which Mr. Jones had organized as a corporation for holding real estate and for investing funds for the propagation of fishes and for scientific research. In 1904 the Wawepex Society leased additional land to the Carnegie Institution of Washington, which wanted to locate a Department of Experimental Evolution in the Cold Spring Harbor area. Charies B. Davenport, who had been directing the Laboratory since 1896, assumed the additional duties of director of the Carnegie Institution's experimental station. Until 1934, Mr. Davenport lived in the large Victorian house that still stands at the corner of Bungtown Road and 25A. Built in 1882 by John D. Jones, the house was renovated and repainted in its original colors in 1979-80, when it was renamed Davenport House. Since 1934 it has served as a dormitory for Laboratory scientists.

The Long Island Biological Association was established in 1924 when the Brooklyn Institute decided to discontinue its research at Cold Spring Harbor and offered its laboratory to two universities. Fortunately, a local group of interested neighbors decided to assume responsibility for the Lab, and thus LIBA came into being. For 38 years LIBA actually operated the Laboratory in conjunction with the Carnegie Institute, but in 1962 it seemed advisable for the Laboratory to be reorganized as an independent unit. Therefore, the property on which it now stands was conveyed to it by LIBA, which, however, still retains reversionary rights. Today LIBA is one of eleven institutions participating in the support of the Laboratory, each institution being represented on the Laboratory's Board of Trustees.

LIBA has become an expanding group of "Friends of the Laboratory" who help support it through annual contributions.

A large part of the Laboratory's resources is obtained from governmental, corporate, and foundation sources as a result of grant applications which are submitted by the individual scientists. Years ago, 85% of the funding came from governmental agencies, but presently less than 50% comes from these sources. Therefore the scientists must rely on an assortment of foundations, corporations,

Membership in LIBA requires a minimum annual contribution (tax deductible) of $25 to the Cold Spring Harbor Laboratory. Further information can be obtained from the Long Island Biological Association, Box 100, Cold Spring Harbor, N.Y 11724, or by telephoning the Laboratory at (516) 367-8840.

and individuals for an increasing share of their support. The researchers compete for grants in their specific areas of study. If an award is made, a portion of the award is returned to the Laboratory in the form of indirect costs for overhead. It is important to remember that these grants are highly competitive, and even if a grant is given an outstanding score by scientific peers, the funding may not be available.

LIBA sponsors the Laboratory's Annual Giving Program, which is its largest source of unrestricted annual gifts. These gifts enable the Laboratory to respond quickly to urgent or unexpected needs. Also, primarily through LIBA Fellowships and funds to start up new laboratories, LIBA helps ensure that the Cold Spring Harbor Laboratory continues to attract the best and brightest young scientists.

The affairs of LIBA are handled by a board of 28 directors who are elected to office by the membership at an annual meeting. LIBA members are invited to bring their friends to lectures and open houses at the Laboratory.

CHAIRMAN'S REPORT

The Long Island Biological Association's sponsorship of Annual Giving for the Cold Spring Harbor Laboratory has completed its fourth year, and the unrestricted funds that are provided are more important than ever before. Because of the federal deficit, funding for biomedical research has become more difficult to obtain, and even grants previously awarded to scientists have been cut 10–20%. The scientists here rely on LIBA members (contributors of $25 or more), the Cold Spring Harbor Laboratory Associates (donors of $1000 and more), and other private sources for a substantial portion of their support. Annual Giving enables the Laboratory to respond more quickly to meet its challenges and particularly to fund bright young incoming scientists. As part of the appreciation for their support, donors are afforded opportunities to meet many of the scientists they help support and to increase their understanding of today's biology through special LIBA events.

Events Sponsored by LIBA

Winter '90

Glasnost and dramatic political events during the Fall of 1989 prompted a panel discussion by four of our visiting Soviet scientists and their wives at the LIBA Annual Meeting on January 21. Drs. Konstantin Galactionov, Salius Klimasauskas, Mart and Ene Ustav, and Grigori and Natasha Yenikolopov discussed scientific research, science education, and the daily routine in the Soviet Union. Of particular interest was the effect of all of the recent political changes on the availability (or shortage) of supplies and laboratory equipment. Also of note, the Soviet Union appears to have the same difficulty as the United States in attracting young people into science. In both countries, a truck driver can earn more than a scientist with a PhD. Underlying the discussions was a feeling that the objectives of the republics were not necessarily acceptable to the central government, a situation that became more and more apparent in the ensuing months.

"Getting Involved: An Artist's Reaction to Environmental Issues" was the title of a centennial lecture by renowned photographer Robert G. Ketchum. Mr. Ketchum is the curator of photography for the National Parks Foundation and the author of several books, including *The Tongass: Alaska's Vanishing Rain Forest*. The North Country, South Side, and Three Harbors Garden Clubs sponsored the event on February 25 that inspired some of us to take a more active role in environmental concerns.

On March 4, LIBA members and friends were entertained by the New York Virtuosi String Quartet featuring bassoonist Daniel Smith and harpsichordist Elaine Comparone in a program entitled *Music from Bach to Joplin*. We are grateful for the generous support of our patrons: Dr. and Mrs. Neil Battinelli, Drs. John and Elaine Broome, Mrs. Joan E. Flint, Drs. Peter and Bernardita Luke, Dr. and Mrs. Walter Meier, Mrs. Henry M. Minton, Mr. and Mrs. James J. Pirtle, Jr., Mr. and Mrs. James E. Swiggett, and Mrs. George G. Walker.

John LeGuyader, education manager at the DNA Learning Center, instructs LIBA members during a hands-on workshop with DNA experiments

Spring '90

In March and May, the Associates Program sponsored two DNA workshops at the DNA Learning Center in Cold Spring Harbor on a new technique called

polymerase chain reaction (PCR). This procedure enables scientists to amplify a small sample of DNA one million times in less than two hours. PCR is becoming widely used in DNA fingerprinting, mapping the human genome, genetics research and diagnostics, and forensics.

Drug legalization was the subject of an Associates lecture given by United States District Court Judge Robert W. Sweet on April 20 in Bush Lecture Hall. His lecture entitled "Legalization of Certain Controlled Substances" stirred much debate and these discussions continued during cocktails and supper in Blackford Hall immediately afterward.

55th Symposium

"The Brain" was the topic of Cold Spring Harbor Laboratory's 55th Symposium on Quantitative Biology at the end of May. As in the past, the Dorcas Cummings Memorial Lecture was held at the midpoint of the week-long meeting on Sunday, June 3. LIBA members witnessed a reunion of the codiscoverers of the double-helical structure of DNA when Dr. Watson introduced Dr. Francis H.C. Crick; Drs. Watson and Crick shared the Nobel prize in 1962 for their discovery. Dr. Crick's

Francis Crick greets LIBA members after speaking at the Dorcas Cummings Memorial Lecture

lecture entitled "How Do We See Things?" was delivered before a capacity crowd in Grace Auditorium and was carried via closed-circuit TV to another "full house" in Bush Lecture Hall across the street. Dr. Crick fascinated those present as he related the latest efforts of scientists to understand how the brain recognizes and interprets vision. Afterward, nearly 140 Symposium speakers and senior scientists from the laboratory were entertained at 21 dinner parties held throughout the community at the homes of LIBA members. This delightful tradition has been carried on continuously for the past 36 years, and we owe its success during the past few years to the tireless efforts of Joan Pesek and Edward Pulling. We are grateful to this year's hosts:

LIBA hosts assemble outside Grace Auditorium to gather Symposium speakers into dinner party groups

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Summer '90: Centennial Celebrations

Tall ships, sea chantey singers, Grucci fireworks, brass bands, bagpipers, a re-enactment of the first biology class on July 6, 1890, and an enormous barbecue and birthday cake were some of the highlights of the birthday bash the Laboratory held on July 14 to celebrate its Centennial. Over 1500 friends and staff were present to celebrate 100 years of outstanding scientific achievements and to usher in the Laboratory's second century of excellence.

In September, eminent evolutionist Dr. Richard Dawkins from the University of Oxford spoke to LIBA members and community residents on *Darwinizing with a Vengeance*. Dr. Dawkins has won numerous awards and honors for his books *The Blind Watchmaker* and *The Selfish Gene*, which have been published in numerous foreign translations, and for his work in evolutionary biology and animal behavior. He stirred much debate in his stimulating and sometimes controversial lecture.

Fall '90

On October 7, a reception was held for Associates on the terrace beside Bush Lecture Hall. Attendees were able to meet many of the LIBA Fellows and to view the plans for the renovation of Blackford Hall. Built in 1907 to house the kitchen and dining facilities, it is one of the earliest examples of reinforced concrete construction. With a capacity of 150, Blackford has been unable to meet the dining needs of the Laboratory for many years. Only with the annual addition of tents on the lawn, staggered dining shifts, and truly inventive food preparation by Jim Hope and his staff has the Laboratory been able to feed its visiting scientists and staff during the meetings season. The expansion and renovation planned over the next three years will almost triple Blackford's capacity.

The final event of the Fall was a panel discussion on the Life Science Industry. The panelists were Douglas E. Rogers of Baring Brothers, David Bonagura of Ernst and Young, and John R. Drexel IV of Concord Capital Management; John

Reese moderated. Topics covered included some of the problems facing small biotech companies and the effect that the "log jam" in the patent office was having on United States competition with other countries.

LIBA Directors

At the Annual Meeting in January 1990, Jim Eisenman retired as Treasurer of LIBA after 27 years of loyal and most effective service. Also retiring as Directors were Arthur Crocker, Penny Chenery, and Lon Chaiken.

The Nominating Committee, comprised of Larry Davis, Chairman, and Directors Phyllis Weekes and John Cleary, nominated Jack Evans, Missy Geddes, Alan Kisner, and Alec Thayer to succeed the outgoing Directors.

LIBA's Support

During the LIBA fiscal year ending September 30, 1990, Annual Giving contributions were received from 671 contributors totaling $284,214. Included in this total were 139 Cold Spring Harbor Associates (contributors of $1000 or more).

This year's report reflects a far reaching and many faceted development effort, which would not have been possible without our small but most effective staff of Konrad Matthaei, Joan Pesek, Claire Fairman, and Debra Mullen. The staff relies on volunteer committees to expand the public's support and awareness of the Laboratory.

For the past four years, LIBA has provided funds for postdoctoral fellows who work under the guidance of senior laboratory scientists. This year, six awards were made. The 1990 fellowship recipients are Young-Seuk Bae, Steven Bell, Saulius Klimasauskas, Jacqueline Lees, Akila Mayeda, and Toshifumi Tsukahara. In addition to the fellowship program, LIBA provides a New Investigator Start-up Fund to a new staff member to purchase equipment or supplies or for salary support for an assistant. This year's recipient is Nicholas Tonks, who comes to us from the Department of Biochemistry of the University of Washington. Dr. Tonks received his PhD from the University of Dundee in 1984.

The Future

On July 14, 1990, Cold Spring Harbor Laboratory celebrated its Centennial and ushered in its second century. We can look back on its many accomplishments with pride. The Laboratory is now in an important transition stage. Gone is the summer laboratory on the shore of Cold Spring Harbor and coming is a year-round teaching institute. The first steps have been taken. The Arnold and Mabel Beckman Neuroscience Center is nearing completion. The first scientists will move into the building in March, and the dedication will take place in May. The facility will include two teaching laboratories and six neuroscience laboratories to enable year-round research and teaching.

What the future will hold will depend on many factors. However, as the Laboratory enters its second century, there are many common links with its past.

- Excellence in Leadership . . . from Charles B. Davenport to James D. Watson, Cold Spring Harbor is simply unmatched.
- Education . . . the mission to stimulate scientific inquiry among students of all ages.

- Basic Research . . . the highest caliber of thinkers assembled in an environment that fosters the exchange of ideas.
- Community Support . . . awareness of the importance of the Laboratory to us all, and a willingness to step forward to answer its call.

It is this community support that has led to the impending success of the Second Century Campaign that has given the Laboratory the resources to enter its next 100 years. It is also the continuance of this support through LIBA's sponsorship of Annual Giving that will play an increasingly important role in the successes of the future. Therefore, let us not rest too long to reflect on past success, but broaden our support to ensure continued excellence.

George W. Cutting, Jr., Chairman

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