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Quantitative comparison of histone proteins in healthy and crown gal-infected vicia faba stem tissue.

Donald Louis Gantz
University of Massachusetts Amherst

QUANTITATIVE COMPARISON OF HISTONE PROTEINS IN HEALTHY AND CROWN GALL-INFECTED *VICIA FABA* STEM TISSUE

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Donald Louis Gantz

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Donald Louis Gantz

Approved as to style and content by:

Mark S. Mount

Dr. Mark S. Mount, Chairman of Committee

Richard A. Rohde

Dr. Richard A. Rohde, Member

Henry M. Hagedorn

Dr. Henry M. Hagedorn, Member

Richard A. Rohde

Dr. Richard A. Rohde, Department Head

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ABSTRACT

QUANTITATIVE COMPARISON OF HISTONE PROTEINS
IN HEALTHY AND CROWN GALL-INFECTED
VICIA FABA STEM TISSUE

by Donald Louis Gantz

The objective of this research was to compare the amounts of each species of histone protein in healthy and crown gall-infected *Vicia faba* (broad bean) stem tissue in order to obtain insight into the mechanism (tumor-inducing principle) responsible for conversion of healthy cells to tumor cells. Healthy tissue from uninoculated, unwounded broad bean stems and crown gall tissue from inoculated stems were harvested at 6 weeks of age. Chromatin from healthy and diseased tissues was isolated and purified by differential centrifugation and sucrose density gradient centrifugation. Histones were extracted with 1.0N H_2SO_4 and precipitated in ice cold ethanol. Histones were fractionated by discontinuous gel electrophoresis at pH 4.3 on 15% polyacrylamide gels containing 8M urea. Histone bands were stained with 0.7% amido schwarz in 7% acetic acid.

Nine histone bands were resolved in both healthy and diseased stem tissue. Gels were scanned at 525 nm on a microdensitometer to quantitate band percentages. The percentages of corresponding bands in healthy and diseased tissue were compared by a student-t distribution at the 5% level of significance. Significant

differences existed between three bands, all of which were tentatively-identified as subfractions of lysine-rich histone based on their migration ratios. The gels containing histone from crown gall tissue exhibited a "smearing effect" which was not visible in gels containing histone from healthy stem tissue.

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CHAPTER I

INTRODUCTION

Agrobacterium tumefaciens is the causal agent of Crown Gall, a disease capable of effecting severe losses on nursery stock of fruit trees, grapes, and woody ornamentals. In the field, roots and crowns are the most commonly-infected plant parts. The characteristic symptom is a gall which may crush phloem and xylem tissue as it enlarges, leading to yellowing, wilting, and death of the plant.

Two to three days after inoculation of the bacterium into a healthy plant, healthy cells in the area of the wound site become converted into tumorous cells. The exact mechanism of cell conversion is as yet unknown and is referred to in the literature as the "tumor-inducing principle". This conversion process requires the conditioning of healthy cells by wounding to make them vulnerable to transformation. The tumor cell undergoes alteration in biosynthetic pathways, leading to increased production of nucleic acids with associated proteins (8, 23, 28, 29, 32, 35) and growth regulators. Tumor cells are considered permanently altered and will not revert back to controlled-growth cells. All daughter cells produced by a tumor cell will also be tumorous. These permanent traits of a crown gall-infected plant cell suggest that an alteration has occurred at the genetic level. Braun has provided discussions of all aspects of the Crown Gall Disease (8, 9, 10).

Histones are low molecular weight basic proteins that are ionically attached to the DNA (deoxyribonucleic acid) of eukaryotic organisms (3, 12, 14, 16, 26, 34). Histone and non-histone chromosomal proteins, DNA, and a small amount of RNA (ribonucleic acid) compose chromatin, the genetic material of a plant or animal cell. It has been well-established that histones are involved in the regulation of messenger RNA synthesis apparently by blocking (repression) or allowing (derepression) the transcription of DNA (1, 4, 12, 13, 14, 21, 22, 26, 34, 39). Direct or indirect action by the tumor-inducing principle could alter the histone-chromatin configuration of a normal cell, leading to its conversion to a tumor cell.

It was the intent of this study to compare the amounts of each species of histone protein in healthy and crown gall-infected stem tissue in order to obtain some insight into the mechanism effecting conversion of healthy cells into tumor cells.

CHAPTER II

LITERATURE REVIEW

Investigations into the identity of the tumor-inducing principle seem to have been concentrated in two theoretical areas. The first theory is that a portion of the genome of Agrobacterium tumefaciens has become integrated into the host cell genome. Schilperoort, et al. (33) were able to form complexes between DNA isolated from crown gall tumors of Nicotiana tabacum, var. White Burley and RNA that was complementary to DNA from A. tumefaciens (strain A6). In addition, hybridization occurred between DNA from tumors on tobacco induced by A. tumefaciens strain A6 (as above) and RNA from A. tumefaciens (strain B6).

Similarly, Quétier, et al. (27) noted common sequences between DNA of strain B6 of the bacterium and fully transformed, bacterial-free N. tabacum cell DNA. Stroun, et al. (40) found RNA of A. tumefaciens, strain B6, in non-tumorous tomato cells after plants had been dipped in a bacterial suspension. This implied to the researchers that bacterial RNA synthesis had apparently occurred in the host cells.

Other hybridization experiments were carried out by Srivastava (36). Working with A. tumefaciens strain 4-32 and N. tabacum L. var. Wisconsin 38, he observed that the homology between bacterial and tobacco tumor DNA was twice as great as that between bacterial and normal tissue DNA.

The second theory, and the one to which this researcher has addressed himself, attributes the transformation of a normal cell into a tumor cell to persistent derepression of a portion of the host genome. Although much of the work with histones has been done with animal tissues, plant histones are believed to be structurally and functionally homologous to histones in animal cells (4, 17).

Stedman and Stedman (39) made a comparison of the amounts of histones in normal and tumorous animal tissues. They found less histone in carcinomas than in healthy tissue. Using microphotometry studies of Broad Bean (Vicia faba) and lily nuclei, Rasch and Woodward (29) observed that, while total histone content of tumor tissue increased over normal tissue, DNA/histone ratios were essentially constant. However, the concentration of histones in diseased tissue was reduced because the average volume of nuclei of tumor cells increased compared to nuclei of healthy cells (28).

Fellenberg (19) was able to achieve significant inhibition of tumor formation in stems of Kalanchöe daigremontiana by adding calf thymus histone at wound sites four days after inoculation with A. tumefaciens.

A comparison of chromatin in normal and crown gall tumor tissue cultures of Nicotiana tabacum var. Wisconsin 38 was made by Srivastava (37). Analysis of histone fractions from a carboxymethyl cellulose column revealed that tumor tissue contained

twice as much of histone fraction F2 (moderately lysine-rich histone) than did healthy tissue. However, amino acid compositions of histone fractions, melting points, RNA polymerase activity, and template activity of chromatins in both healthy and tumor tissue were very similar.

Description of Histones. Histones are basic chromosomal proteins found only in eukaryotic organisms; prokaryotes such as A. tumefaciens do not have histones. There are five generally-accepted classes or fractions of histone: 1) very lysine-rich (VLR) or f1; 2) slightly lysine-rich (SLR) or f2b; 3) arginine-lysine-rich (AL) or f2a2; 4) arginine-rich (AR) or f3; and 5) glycine-arginine-rich (GAR) or f2a1. The molecular weights of these fractions range from 21,000 to 11,000 daltons, respectively. Each fraction may have 1 to 5 sub-fractions depending upon the tissue type. More than 20% of the amino acids composing each class of histone protein are lysine and arginine, both basic amino acids (26).

Modifications of the amino acids contained in histones by acetylation, methylation, and phosphorylation (the most common) give some specificity to histones but it is believed that enough unique species of histones do not exist to account for complete gene regulation (1, 18). It is currently suspected that non-histone chromosomal proteins play an important role in the control of gene transcription (26). (See Appendix B for detailed information on non-histone chromosomal proteins.)

The site of histone synthesis in the cell has yet to be resolved. Olson, et al. (26) suggested that histone synthesis is probably totally or predominately in the cytoplasm. Other researchers (12, 30) feel that histones are probably made within the nucleus near DNA sites. Appendix A contains additional information on histone modification, synthesis, and roles.

CHAPTER III

MATERIALS AND METHODS

Culturing of A. tumefaciens. Virulent strain 806 of Agrobacterium tumefaciens, obtained from Dr. T. T. Stonier, Manhattan College, N. Y., was maintained on slants of N agar (N broth from Difco; agar from Sigma) supplemented with 0.5% glucose (Anhydrous Grade III, Sigma). Transfers were made each month to fresh, sterile slants.

Cultures of A. tumefaciens for inoculation purposes were grown in N Broth supplemented with 0.5% glucose and placed on a shaker for 48-60 hr at 150 rpm.

Vicia faba (broad bean) was chosen as the plant system since it can be rapidly grown from seed and has thick stems for easy inoculation. Although not a natural host of A. tumefaciens, it is extremely susceptible when artificially inoculated. Plants were grown in the greenhouse in wooden flats containing a mixture of peat moss, soil, and sand (1:2:2). Plants were fertilized with Start'n Gro 16:32:16 on a monthly basis. Temperature range in the greenhouse was 15 - 25 C. Captain-treated seeds were obtained from Harris Seed Company, Rochester, N. Y.

Method of Inoculation. Crown gall inoculations were made in stems when plants were 5-10 cm high using disposable hypodermic needles. Several punctures were made along the length of each stem. The needle tip was inserted into the pith and liquid was injected until exudation was observed at the wound site.

Uninoculated, unwounded broad bean plants and crown gall-infected plants were harvested at age 6 weeks. Sacrificed material was placed on ice as soon as possible and kept at 4 C throughout subsequent operations. Leaves, buds, and flowers were cut from stems of healthy plants; stems were minced into short pieces and weighed in cold beakers. The weight of minced stems was approximately 700 gm. Galls were excised from stems with as little healthy tissue as possible, weighed, and minced into small pieces in preparation for chromatin isolation. The minced gall tissue weighed about 100 grams.

Isolation of Chromatin. Chromatin isolation and purification were based on methods by Huang and Bonner (22). Minced stem or gall tissue was homogenized in a 2-speed Waring Blender (Model 5011S) at 4 C with homogenizing medium in a ratio of 1:1:5 (w/v). The medium, isotonic with the stem tissue, was composed of 0.05M Tris-HCl pH 8.0, 0.25 M sucrose, and 0.001 M $MgCl_2$. After thorough homogenizing for 90 sec at high speed, the homogenate was filtered through 4 layers of cheesecloth to remove the bulk of the cell wall debris. Additional cell wall material was eliminated by filtration through 2 layers of Miracloth (Chicopee Mills, Inc. N. Y.). The filtrate was placed in polypropylene centrifuge tubes (250 ml capacity) and centrifuged in a GSA rotor at 4,000 x g for 30 min in a Sorvall RC2-B Centrifuge. After removal of the supernatant, the green, gelatinous portion of each pellet was scraped from the underlying starch and resuspended in 300 ml of homogenizing medium.

This suspension was centrifuged at 10,000 x g for 10 min and the pellet was scraped as before and resuspended in 300 ml of 0.05 M Tris-HCl buffer, pH 8.0. After centrifugation at 10,000 x g for 10 min, the entire pellet was resuspended in 300 ml of Tris-HCl buffer. Two additional cycles of centrifugation and resuspension followed, with the entire pellet being suspended in 100 ml and 15 ml of buffer, respectively. The 15 ml suspension was thoroughly mixed with 20 strokes in a Ten Broeck Homogenizer. The suspension was stirred for 1 hr to ensure the solubilization of contaminating materials.

Sucrose density gradient techniques were utilized to remove remaining starch, chlorophyll, and chloroplasts. Two and 1/2 ml of crude chromatin solution were layered on 11 ml of 1.7 M sucrose in each of 6 cellulose nitrate centrifuge tubes. The upper one-third of each tube was gently stirred to create a gradient. The six tubes were placed in an SW-40 Swinging Bucket Rotor and centrifuged at 22,000 rpm for 105 min in an L2-65B Beckman Preparative Ultracentrifuge.

The supernatants along with suspended material were discarded. Each of the six pellets was rinsed once with 0.05 M Tris-HCl buffer and suspended in 2 ml of the same buffer. The 12 ml chromatin solution was dialyzed overnight in 100 volumes of 0.05 M Tris-HCl to remove remaining sucrose. Chromatin yields per gram of stem tissue were calculated using Diphenylamine Test (11)

based on a calf thymus DNA standard. Absorbance at 595 nm was read on a Baush and Lomb Spectronic 20 and compared to a 0.05 M Tris-HCl buffer control.

Extraction of Histone. Histones were extracted from previously-isolated chromatin using the method of Bonner, et al. (5). One quarter volume of 1.0 N H_2SO_4 was added to the chromatin solution (chromatin concentration had been adjusted to less than 400 ug DNA/ml) and stirred at 4 C for 30 min. The DNA was pelleted by centrifugation at 17,000 x g for 20 min and the supernatant was saved. Two additional cycles of extraction were carried out with an amount of 0.4 N H_2SO_4 equal to 1/2 the final volume of the first extract followed by centrifugations as before. To the combined supernatants was added four volumes of ice-cold ethanol to precipitate the histone sulfate. The solution was stored at -20 C for at least 24 hr and then centrifuged at 2500 x g for 25 min to pellet histone sulfate. The pellet was rinsed twice in cold ethanol and centrifuged at 10,000 x g for 15 min. The final pellet was dissolved in 2 ml of 0.05 M Tris-HCl, pH 8.0, frozen, and dried on a Thermovac FD-2a vacuum dessicator. Micrograms of histone per milligram of histone sulfate was determined by using the method of Lowry (24). Absorbance at 500 nm of histone sulfate dissolved in 0.01 M Tris-HCl buffer on a Baush and Lomb Spectronic 20 was compared to the absorbance of a 0.01 M Tris-HCl control.

Fractionation of Histone. Histone fractions were separated by discontinuous gel electrophoresis on polyacrylamide gels based on a modification by Bonner, et al. (3) of the method of Reisfield et al. (31).

Glass electrophoresis tubes, 7.5 cm x 5 mm I. D. were soaked overnight in sulfuric acid chromerge and rinsed well with distilled water. When dry, the tubes were coated with a 1:1 dilution of Photoflo 200 (Kodak) in distilled water and allowed to air dry.

In the bottom of each stoppered electrophoresis tube was placed 1.1 ml of separating gel, pH 4.3, containing fresh 8 M urea in 15% acrylamide. The gel was carefully overlaid with 0.1 ml of 3 M urea. A flat interface between gel and water resulted during the 45 min polymerization period. After gel hardening, the 3 M urea was removed by gently shaking the inverted tube. Next, 0.15 ml of stacking gel, pH 6.7 and 2.5% acrylamide, were pipetted on top of the separating gel. The stacking gel was layered with 0.1 ml of distilled water and polymerized for 30 min using a polymerizing light source (Canalco). Each tube was then placed in a Pharmacia Electrophoresis Apparatus with sample end on top.

The 200 ul sample solution, which was layered on the stacking gel, contained 1 part each of glycerol and 8 M urea, 0.03 parts of 0.5 N HCl (20), and 32 ug to 50 ug of histone. A small amount of methyl green was added to each sample solution to serve as a marker dye. Next, the upper electrode buffer was layered on each

histone sample until the tube was completely filled. Upper and lower electrode buffers (31.2 gm B-alanine and 8 ml glacial acetic acid to one liter with distilled water) were adjusted to pH 5.0 with 1 N NaOH.

The electrodes were connected to a Canalco 300B Electrophoresis Constant Rate Source such that the lower electrode was the cathode. Electrophoresis was carried out at 4 ma per tube for approximately 6 hr, the exact time depending upon the rate of migration of the methyl green marker band.

At the completion of electrophoresis, the gels were loosened from the glass tubes by spraying distilled water between gel and glass with a blunt hypodermic needle while gently rimming each gel. Gels were removed as quickly as possible to reduce diffusion of the unfixed protein. Gels were stained in 0.7% amido schwarz in 7% acetic acid, incubated at 90 C for 45 min, rinsed twice with 7% acetic acid, and electrically destained in a Pharmacia GD-4 Destainer at 12.5 ma per gel rod. The destaining solvent was 7% acetic acid. After destaining, gels were stored in 7.5 cm x 9 mm glass culture tubes containing 7% acetic acid.

Quantitation of Histone Fractions. Each gel was scanned at 525 nm using a Quick Scan, Jr. Microdensitometer (Helena Laboratories). The relative percentage of each histone fraction was calculated based on the automatically-plotted optical density and integration curves. A student-t distribution at the 5% level of significance was computed for each of the nine histone

bands in healthy and crown gall tissue.

Gels were photographed with a Retina Reflex III Camera (Kodak) and type R - 32 mm close-up lens (Kodak).

CHAPTER IV

RESULTS

The electrophoretic patterns of histones isolated from healthy and crown gall-infected broad bean stem tissue are shown in figure 1. Gels had been stained with 0.7% amido schwarz in 7% acetic acid. Nine histone bands were visible in both healthy and diseased tissue. The first three bands (closest to the anode) in healthy and the first four bands in diseased tissue appeared grey to blue-grey while the remaining histone bands appeared blue to blue-black when viewed with a fluorescent light background. Gray (20) characterized histones from healthy Vicia faba shoot tissue and noted the grey color of the five slow-moving bands whereas the remaining four rapidly-moving histone bands were blue to blue-black. Gray had stained gels with 0.5% amido schwarz in 7% acetic acid. Easton and Chalkley (15), in their electrophoretic studies of histones from embryos and sperm of sea urchin, observed a distinct blue-grey color in the lysine-rich and slightly lysine-rich fractions stained with amido schwarz.

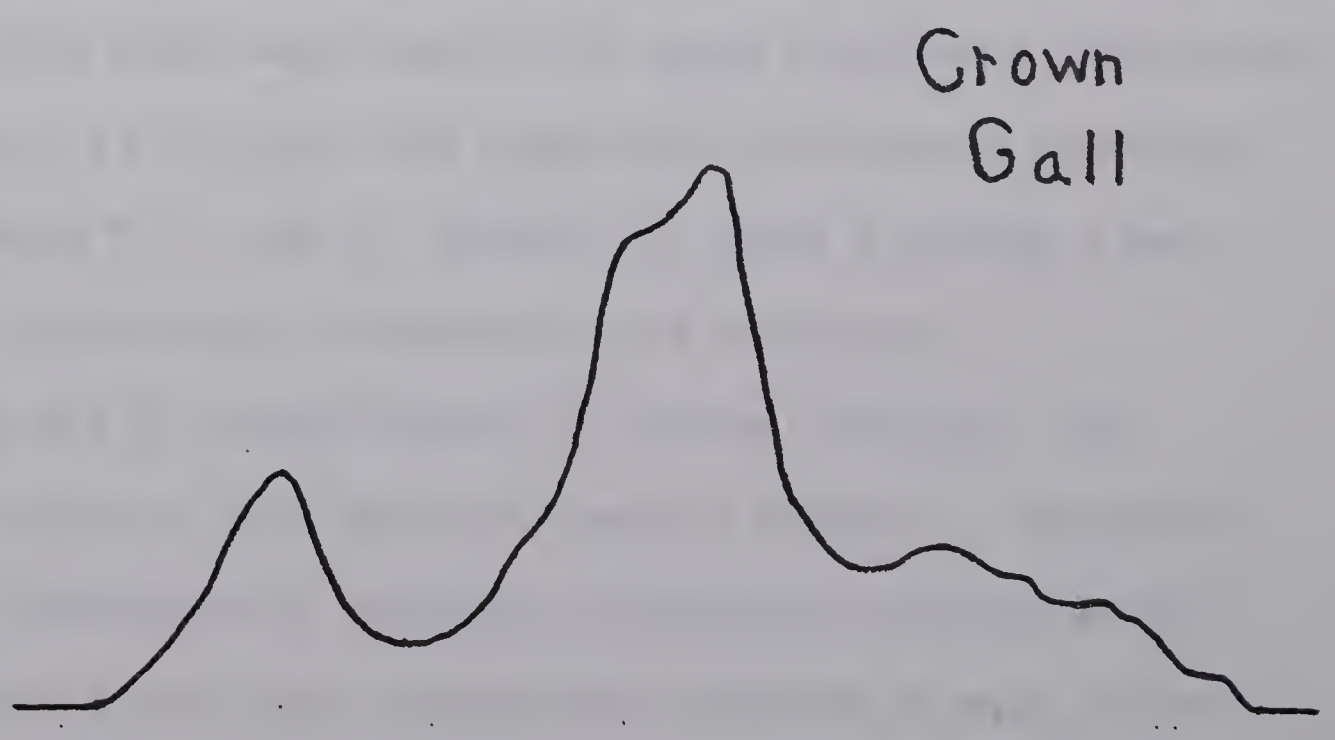
Visual inspection of the gels suggested differences among the five slower-moving bands near the anode. To quantitate these differences, microdensitometer readings were taken at 525 nm and tracings of these readings are shown in figure 2. The peaks of the healthy shoot histone bands were much sharper than

Fig. 1. Histones from healthy (a) and crown gall-infected (b) Vicia faba (broad bean) stem tissue. Electrophoresis was carried out at 4 ma/tube for 6 hr. Gels were loaded with 32 - 50 ug of histone and stained with 0.7% amido schwarz in 7% acetic acid.


1
2
3
4
5
6
7
8
9

Fig. 2. Microdensitometer tracings of histone bands from healthy and crown gall-infected Vicia faba stem tissue. Band number 1 is at far right; band number 9 is at far left. Marker pen gain on Quick Scan, Jr. (Helena Laboratories) was set at 4; chart paper was on low speed.


Healthy
⊖
⊕


Crown
Gall

those of the crown gall histone bands. This "smearing effect" was only evident in gels of histone from diseased tissue. It cannot be attributable to diffusion of protein within the gel since the gels were placed in stain within a few minutes after termination of electrophoresis. Nor can it be due to incomplete destaining, as all gels were destained for the same time interval.

As an additional aid in the determination of differences among the five slower-moving bands, expanded microdensitometer readings were taken by increasing the "gain" of the marker pen and by increasing the speed of the chart paper (Figure 3).

The percentage of each of the 9 histone bands was calculated from the microdensitometer readings as indicated in Materials and Methods. An average of the percentage of each band from healthy and diseased tissue appears in Table 1. Comparisons of corresponding bands were carried out using a Student-t distribution ($t < -t_{0.025}$; $t > +t_{0.025}$) with significant differences occurring between bands 3, 4, and 5. However, if bands 1 through 5 were compared collectively, differences were negligible.

As an aid in identification of histone fractions, the migration ratio of each band was computed (Table 2). Migration ratio was determined by dividing the migration distance of the fastest-moving band into the migration distance of each of the other bands. The distance from the top of the separating gel to the center of each band was considered the migration distance.

Tentative band identification was made based on migration

Fig. 3. Expanded microdensitometer tracings of histone bands from healthy and crown gall-infected Vicia faba stem tissue. Band number 1 is at far right; band number 5 is at far left. Marker pen gain on Quick Scan, Jr. (Helena Laboratories) was set at 10; chart paper was on high speed.

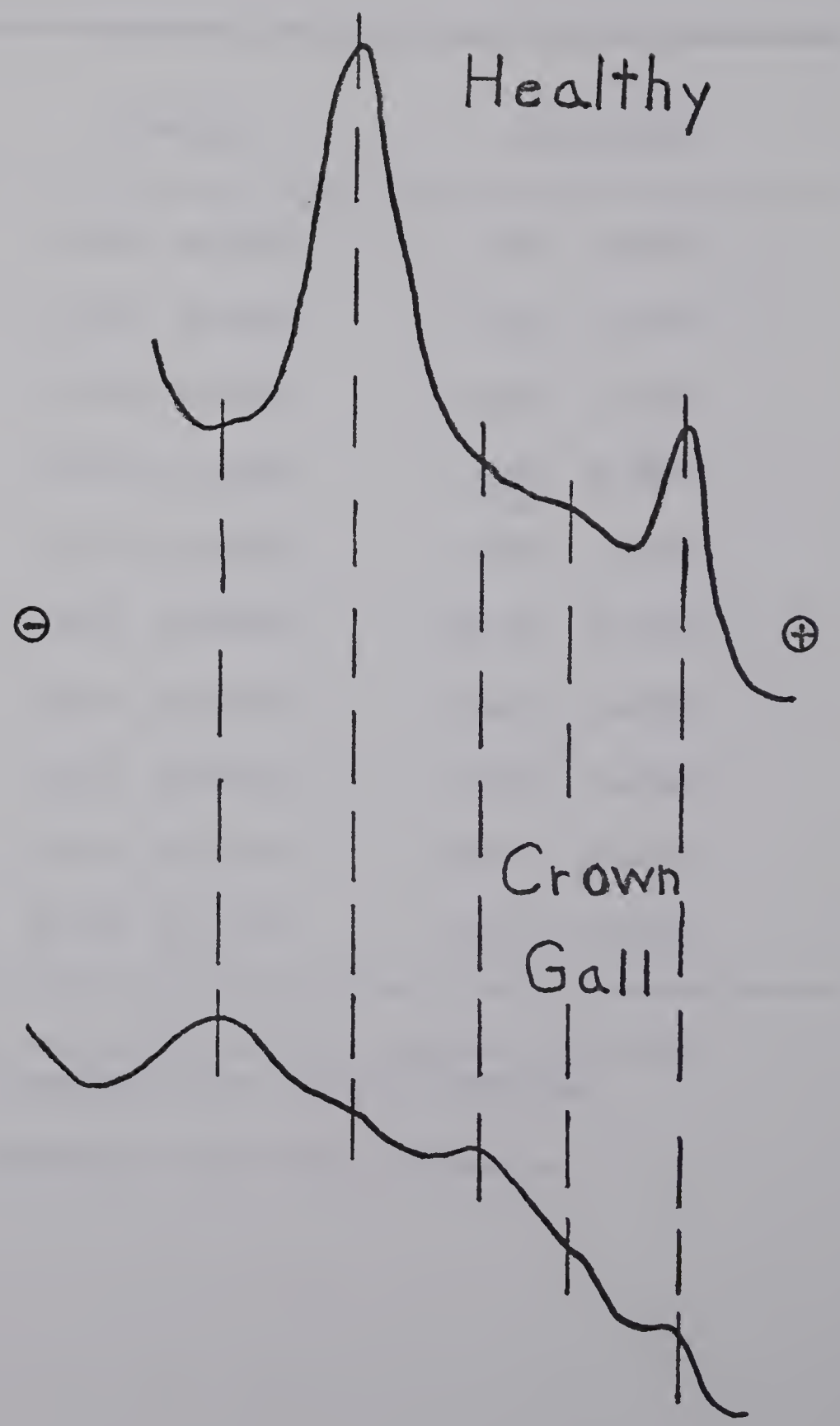

Healthy
⊖
⊕
Crown
Gall

TABLE 1. Comparison of histone band percentages from healthy and crown gall-infected Vicia faba stem tissue on polyacrylamide gels.[a]

Band Number	Healthy	Crown Gall
1	2.35 ±0.354	1.95 ±0.071
2	1.90 ±0.424	2.85 ±1.485
3[b]	1.80 ±0.849	4.90 ±0.566
4[b]	9.70 ±2.550	3.60 ±1.560
5[b]	1.95 ±0.636	8.90 ±0.707
6	35.75 ±5.020	36.10 ±9.620
7	15.45 ±4.740	15.65 ±1.060
8	9.05 ±0.354	7.75 ±4.600
9	22.00 ±1.130	18.30 ±3.820
1-5	20.58 ±3.258	22.92 ±0.731

[a]Electrophoresis was performed at 4 ma/tube for 6 hr. Each 7 cm gel contained 32 - 50 ug of histone.

[b]Significant differences with 95% confidence.

TABLE 2. Migration ratios of histone bands from healthy and crown gall-infected Vicia faba stem tissue relative to the fastest-moving band on polyacrylamide gels.[a]

Band Number	Healthy		Crown Gall	
1	0.524	0.471	0.447	0.407
2	0.561	0.518	0.489	0.453
3	0.610	0.541	0.521	0.488
4	0.646	0.588	0.574	0.558
5	0.707	0.635	0.617	0.593
6	0.780	0.765	0.745	0.721
7	0.817	0.812	0.798	0.779
8	0.878	0.859	0.851	0.860
9	1.000	1.000	1.000	1.000

[a]Electrophoresis was carried out at 4 ma/tube for 6 hr on 7 cm gels.

ratios. Referring to Table 2, the fast-moving band, number 9, is most likely f2a1, a glycine-arginine-rich histone band of low molecular weight. Band number 8, was considered band f3, an arginine-rich band. No specific identification could be suggested for bands 6 and 7. However, Nadeau, et al. (25), in their electrophoretic comparison of plant and animal histones referred to the histone bands intermediate between f3 and f1 bands as "plant histones". In animal tissue, they found two histone bands, f2a2 and f2b, between f2a1 and f3 but found no bands between f3 and f1 histones. In the present study, bands 6 and 7 were considered to be plant histones.

The difference in migration ratios between bands 5 and 6 was considerably larger (0.130) than the difference between bands 4 and 5 (range of 0.037-0.058) or between 6 and 7 (0.043-0.059). Thus, bands 1 through 5 may be considered sub-fractions of fraction f1, a lysine-rich histone of higher molecular weight.

CHAPTER V

DISCUSSION

Current information points to phosphorylation as the primary mechanism of histone modification. Attachment of a negatively-charged phosphate group on a positively-charged histone would change the conformation of the protein and its binding affinity to DNA. As a result, particular genes normally repressed by histones could be made available for transcription; histones could be replaced by another protein type; or chromatin structure could be modified in preparation for DNA synthesis and cell division (26).

Recent evidence suggests that the very lysine-rich histone (f1) is apparently phosphorylated during DNA synthesis. Bradbury, et al. (6, 7) proposed that f1 phosphorylation is the initiation step for mitosis by triggering chromosomal condensation. They felt that histones are not in control of specific gene transcription but rather are involved in maintenance and control of chromosome structure.

The variation in the amounts of lysine-rich bands obtained in this study seem to support the idea that an alteration in charge and/or size of some histone molecules does result from the conversion of a healthy cell to a tumor cell. Since the altered histone molecules were of the lysine-rich type, phosphorylation could account for the alteration. It is feasible that

during conversion, lysine-rich histones could be replaced by modified lysine-rich histones or by other protein types. The similarity in percentages of histone bands 1 through 5 in healthy and tumor tissue suggests that replacement of lysine-rich histones with other protein types did not occur. However, whether lysine-rich histones were modified by phosphorylation while still attached to DNA or were replaced by previously modified lysine-rich histones cannot be determined from this datum.

Srivastava found twice as much FII histone in crown gall tobacco tissue cultures than in healthy tissue cultures (37). FII histone was defined as a moderately lysine-rich fraction (16% Lys), being intermediate to the fractions FI (21% Lys) and FIII (13% Lys). All fractions were eluted from a carboxymethyl-cellulose column. Because of differences in fractionation procedures it is difficult to accurately compare Srivastava's results to those in the present study.

Clubroot of Crucifers, caused by Plasmodiophora brassicae, is characterized by cell hypertrophy and hyperplasia. As in crown gall disease, the exact nature of the "growth stimulus" is unknown but appears to diffuse in advance of the pathogen. Williams et al. (41) made a comparison of the amounts of chromosomal protein and DNA in healthy and clubroot-infected cabbage root hair cells. Although the DNA content was similar in healthy and diseased, there was a 10 - 15% reduction in the histone content of infected cells. The lysine-rich fraction of histone declined by 25% in

diseased tissue; the arginine-rich fraction remained the same. On the other hand, infected tissue contained higher amounts of RNA and non-histone chromosomal protein and enlarged nucleoli. The data suggested that the fungal pathogen may be altering the normal transcriptional process in an infected root hair cell. Williams, et al. postulated that the "stimulus" could be effecting a derepression of the host genome by indirectly or directly causing modification and subsecuent removal of lysine-rich histone from the DNA.

In the present crown gall study, the percentages of total lysine-rich histones (bands 1 through 5) did not significantly vary between healthy and diseased tissue. This suggests that there was no net loss of lysine-rich histone from DNA of crown gall tissue compared to DNA of healthy tissue.

Increased peroxidase activity in diseased tissue may play a role in the alteration of histone charge and/or size. Stahmann and Demorest (38) determined that an oxidation product, 3-methyleneoxindole, resulting from incubation of indole-3-acetic acid with horseradish peroxidase, conjugated with the sulfydryl groups of arginine-rich calf thymus histone. When crude calf thymus histone was incubated with horseradish peroxidase, catechol, and H_2O_2, and then subjected to electrophoresis, a shift in histone bands toward lower mobility was observed. The latter incubation mixture apparently oxidized the ϵ-amino groups of lysine molecules in calf thymus histone. The shift toward lower mobility could be

explained by partial neutralization of positive charges and by slight increases in size of histone molecules. These data suggested that peroxidase systems could alter the physical and chemical properties of histone. A modification of ϵ-amino groups could reduce ionic bonding between lysine-rich histone and DNA, perhaps leading to changes in transcription.

The "smearing effect" in gels containing histone from crown gall-infected tissue is difficult to explain. As previously mentioned, protein diffusion prior to staining and incomplete destaining are unlikely. Modifications such as phosphorylation, acetylation, and methylation could account for minor changes in the molecular weight of histones; however, it is doubtful that these modifications are responsible for the extensive size range in histone that the smearing effect seems to suggest. Perhaps new species of basic proteins are synthesized in crown gall tissue as a consequence of derepression of the host genome.

Proteolytic degradation of histone could produce a wide range of polypeptide residues. Amido schwarz stain is amphoteric and would bind to amino or carboxyl groups in the residues. If partially-degraded histone residues were present in purified histone, these residues would have been produced during the isolation of chromatin or have already been present on DNA of crown gall cells. Cold temperature during chromatin isolation should have minimized histone degradation; protease activity

would be highly unlikely during acid extraction of histone. It is feasible that crown gall tissue could produce higher amounts of histone proteases than healthy tissue, perhaps as a result of transcriptional alterations. The pressure on cells created by hypertrophy and hyperplasia of a gall might force the release of proteases which are normally compartmentalized.

Bartley and Chalkley (2) have observed a higher turnover rate of histone in actively-dividing calf thymus cells than in non-dividing cells. They suggested that the increased turnover rate might be due to higher protease activity. Their experiments also indicated that lysine-rich histone was the most vulnerable to proteolysis of all histone fractions in intact calf thymus nucleohistone.

If a high level of protease activity exists in crown gall tissue, then there would likely be partially-degraded histone molecules attached to DNA. These molecules could remain attached throughout chromatin isolation and be extracted along with the non-degraded histones.

SUMMARY

The percentages of each histone species in healthy and crown gall-infected Vicia faba stem tissue have been compared. Healthy tissue from uninoculated, unwounded broad bean stems and crown gall tissue from inoculated stems were harvested at age 6 weeks. Healthy and diseased stem tissue was minced and then homogenized in an isotonic medium. Chromatin was isolated and purified by differential centrifugation and by sucrose density gradient centrifugation. Histones were extracted from chromatin with 1.0N H_2SO_4; histone sulfate was precipitated in ice cold ethanol. Fractionation of histones was accomplished by discontinuous gel electrophoresis at pH 4.3 on 15% polyacrylamide gels containing 8M urea. Gels were stained with 0.7% amido schwarz in 7% acetic acid and electrically destained.

Nine histone bands were resolved in healthy and diseased stem tissue. Histone bands were scanned at 525 nm on a microdensitometer to quantitate band percentages. The percentages of corresponding bands in healthy and crown gall tissue were compared using a student-t distribution at the 5% level of significance. Significant differences existed between three bands, all of which were tentatively identified as subfractions of lysine-rich histone based on their migration ratios. The modification of histone by phosphorylation could account for the quantitative differences among these histone bands. Variation in histone bands between

healthy and tumor tissue would seem to be an effect of the conversion process rather than the tumor-inducing principle.

The gels containing histone from crown gall histone exhibited a "smearing effect" which was not visible in gels of histone from healthy stem tissue. It has been speculated that the smearing effect could be the result of high histone protease activity in crown gall tissue.

BIBLIOGRAPHY

1. Allfrey, V. G., R. Faulkner, and A. E. Mirsky. 1964. Acetylation and Methylation of Histones and Their Possible Role in the Regulation of RNA Synthesis. Proc. Nat. Acad. Sci. 51:786-794.

2. Bartley, James and Roger Chalkley. 1970. Further Studies of a Thymus Nucleohistone-associated Protease. J. Biol. Chem. 245:4286-4292.

3. Bonner, James. 1965. The Molecular Biology of Development. The Clarendon Press, Oxford. 155p.

4. Bonner, James. 1967. The Role of Histones in the Regulation of RNA Synthesis. In Regulation of Nucleic Acid and Protein Synthesis. V. V. Koningsberger and L. Bosch, eds. Elsevier Publishing Company, Amsterdam. p. 211-224.

5. Bonner, James, G. Roger Chalkley, Michael Dahmus, Douglas Fambrough, Frank Fujimura, Ru-chih C. Huang, Joel Huberman, Ronald Jensen, Keiji Marushige, Heiko Ohlenbusch, Baldomero Olivera, and Jack Widholm. 1968. Isolation and Characterization of Chromosomal Nucleoproteins. In Methods in Enzymology. Nucleic Acids. L. Grossman and K. Moldave, eds. Academic Press, New York. Vol. XII, pt. B, p. 3-65.

6. Bradbury, E. M., P. D. Cary, C. Crane-Robinson, and H. W. E. Rattle. 1973. Conformations and Interactions of Histones and Their Role in Chromosome Structure. Annals N. Y. Acad. Sci. 222:266-289.

7. Bradbury, E. M., R. J. Inglis and H. P. Matthews. 1974. Control of Cell Division by Very Lysine-Rich Histone (F1) Phosphorylation. Nature 247:257-261.

8. Braun, Armin C. 1962. Tumor Inception and Development in the Crown Gall Disease. Annual Review Plant Physiology 13:533-558.

9. Braun, Armin C. 1972. The Relevance of Plant Tumor Systems to an Understanding of the Basic Cellular Mechanisms Underlying Tumorigenesis. In Progress in Experimental Tumor Research - Plant Tumor Research. F. Homburger and A. C. Braun, eds. Karger Press, Basel. Vol. 15, p. 165-187.

10. Braun, Armin C. and Henry N. Wood. 1961. The Plant Tumor Problem. Advances in Cancer Research 6:81-109.

11. Burton, K. 1956. A Study of the Conditions and Mechanism of the Diphenylamine Reaction for the Colorimetric Estimation of Deoxyribonucleic Acid. Biochemical Journal 62:315-323.

12. Butler, J. A. V., E. W. Johns, and D. M. P. Phillips. 1968. Recent Investigations on Histones and Their Functions. In Progress in Biophysics and Molecular Biology. Butler and Noble, eds. Pergamon Press, Oxford. p. 209-244.

13. Dahmus, Michael E. and James Bonner. 1970. Nucleoproteins in Regulation of Gene Function. Federation Proceedings 29:1255-1260.

14. DeLange, Robert J. and Emil L. Smith. 1971. Histones: Structure and Function. Annual Review of Biochemistry 40:279-314.

15. Easton D. and R. Chalkley. 1972. High-resolution Electrophoretic Analysis of the Histones from Embryos and Sperm of Arbacia punctulata (sea urchin). Exp. Cell Research 72:502-508.

16. Elgin, Sarah C. R., Stanley C. Froehner, John E. Smart and James Bonner. 1971. The Biology and Chemistry of Chromosomal Proteins. In Advances in Cell and Molecular Biology. E. J. DuPraw, ed. Academic Press, New York. p. 2-29.

17. Fambrough, Douglas M. and James Bonner. 1966. On The Similarity of Plant and Animal Histones. Biochemistry 5:2563-2569.

18. Fambrough, Douglas M. and James Bonner. 1969. Limited Molecular Heterogeneity of Plant Histones. Biochim. Biophys. Acta. 175:113-122.

19. Fellenberg, Gunter. 1967. Versuche zur Hemmung pflanzlicher Tumoren mit Histon. Zeitschrift Fur Pflanzenphysiologie 56:446-452. (abstr.)

20. Gray, Robert Howard. 1967. Characterization of Labeled Histones of Vicia faba by Gel Electrophoresis. Ph. D. Thesis, University of Illinois. 120p.

21. Hnilica, Lubomir S. 1967. Proteins of the Cell Nucleus. In Progress in Nucleic Acid Research and Molecular Biology. Davidson and Cohn, eds. Academic Press, New York. p. 25-106.

22. Huang, Ru-chih C. and James Bonner. 1962. Histone, A Suppressor of Chromosomal RNA Synthesis. Proc. Nat. Acad. Sci. 48:1216-1222.

23. Klein, Richard M., Ellen M. Rasch, and Hewson Swift. 1953. Nucleic Acids and Tumor Genesis in Broad Bean. Cancer Research 13:499-502.

24. Lowry, Oliver H., Nira J. Rosebrough, A. Lewis Farr, and Rose J. Randall. 1951. Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem. 193:265-275.

25. Nadeau, Paul, Dominick Pallotta, and Jean-G. LaFontaine. 1974. Electrophoretic Study of Plant Histones: Comparison with Vertebrate Histones. Arch. Biochem. Biophys. 161:171-177.

26. Olson, Mark O. J., Wesley C. Starbuck, and Harris Busch. 1974. Nuclear Proteins. In The Molecular Biology of Cancer. Harris Busch, ed. Academic Press, New York. p. 309-353.

27. Guétier, Francis, Thierry Huguet et Etienne Guillé. 1969. Induction of Crown Gall: Partial Homology between Tumor-cell DNA, Bacterial DNA, and the G & C-rich DNA of stressed normal cells. Biochem. Biophys. Res. Commun. 34:128-133.

28. Rasch, Ellen, Hewson Swift, and Richard M. Klein. 1959. Nucleoprotein Changes in Plant Tumor Growth. J. Cell Biology 6:11-34.

29. Rasch, Ellen and John W. Woodward. 1959. Basic Proteins of Plant Nuclei during Normal and Pathological Cell Growth. J. Cell Biology 6:263-276.

30. Reid B. R. and R. David Cole. 1964. Biosynthesis of a Lysine-Rich Histone in Isolated Calf Thymus Nuclei. Proc. Nat. Acad. Sci. 51:1044-1050.

31. Reisfield, R. A., U. J. Lewis, and D. E. Williams. 1962. Disk Electrophoresis of Basic Proteins and Peptides on Polyacrylamide Gels. Nature 195:281-283.

32. Robson, Hope H., Matthew A. Budd, and Henry T. Yost, Jr. 1959. Comparison of the Nucleic Acid, Total Nitrogen, and Protein Nitrogen Levels of Normal and Tumor Tissue under Similar Growth Conditions. Plant Physiology 34:435-440.

33. Schilperoort, R. A., H. Veldstra, S. O. Warnaar, G. Mulder, and J. S. Cohen. 1967. Formation of Complexes between DNA isolated from tobacco crown gall tumors and RNA complementary to Agrobacterium tumefaciens DNA. Biochim. Biophys. Acta. 145:523-525.

34. Smith, Emil L., Robert J. Delange, and James Bonner. 1970. Chemistry and Biology of the Histones. Physiological Reviews 50:159-170.

35. Srivastava, B. I. Sahai. 1968. Patterns of Nucleic Acids Synthesis in Normal and Crown Gall Tumor Tissue Cultures of Tobacco. Arch. Biochem. Biophys. 125:817-823.

36. Srivastava, B. I. Sahai. 1970. DNA-DNA Hybridization Studies between Bacterial DNA, Crown Gall Tumor Cell DNA, and the Normal Cell DNA. Life Science 9:889-892.

37. Srivastava, B. I. Sahai. 1971. Studies of Chromatin from Normal and Crown Gall Tumor Tissue Cultures of Tobacco. Physiological Plant Pathology 1:421-433.

38. Stahmann, M. A. and D. M. Demorest. 1973. Changes in Enzymes of Host and Pathogen with Special Reference to Peroxidase Interaction. In Fungal Pathogenicity and the Plant's Response. Byrde, R. J. W. and C. V. Cutting, eds. Academic Press, New York. p. 405-422.

39. Stedman E. and Ellen Stedman. 1943. Probable Function of Histone As a Regulator of Mitosis. Nature 152:556-557.

40. Stroun, Maurice, Peter Gahan, and Sara Sarid. 1969. Agrobacterium tumefaciens RNA in Non-tumorous Tomato Cells. Biochem. Biophys. Res. Commun. 37:652-657.

41. Williams, P. H., J. R. Aist and P. K. Bhattacharya. 1973. Host-Parasite Relations in Cabbage Clubroot. In Fungal Pathogenicity and the Plant's Response. Byrde, R. J. W. and C. V. Cutting, eds. Academic Press, New York. p. 144-158.

APPENDIX A

HISTONES

General Characteristics. The histones, basic proteins associated ionically with DNA (deoxyribonucleic acid), are present in eukaryotes but not in prokaryotes. Twenty percent or more of the amino acids in a histone molecule are the basic amino acids histidine, lysine, and arginine; but the latter two predominate. In addition, histones contain no tryptophan and very small amounts of cysteine.

The species of histones have been enumerated and characterized by polyacrylamide gel electrophoresis. It is generally accepted that there are 5 histone fractions in vertebrates containing approximately 10-12 sub-fractions (36). Each fraction is distinctive in its lysine and arginine content and molecular weight. Calf thymus histone will be discussed here because its histone band pattern on acrylamide gels is typical of other vertebrate histones characterized to date (34, 36). Histone fractions will be described in order of the fastest-moving to the slowest-moving fraction.

The most rapidly-moving fraction during electrophoresis is a glycine-arginine rich fraction (GAR) containing 9.8% lysine, 13.7% arginine, and a molecular weight of about 11,000. This histone is also known as f2a1 or IV and may have one or two sub-fractions.

The next three fractions band closely together because of their similarity in size. The fastest-moving of these is an

arginine-lysine rich fraction known as AL, f2a2, and IIb1. It has approximately equal amounts of lysine and arginine (9-11%), a molecular weight of about 14,000, and one sub-fraction.

The second of this group is a slightly lysine-rich fraction containing one sub-fraction (SLR, f2b, IIb2). Its molecular weight is approximately 13,800; it contains 16% lysine and 6.5% arginine.

The third fraction of the cluster of 3 is arginine-rich containing up to 3 sub-fractions. It contains 9.6% lysine and 13.3% arginine and has a molecular weight of 15,000. It is symbolized as AR, f3, or III.

The slowest-moving fraction of the 5 fractions (VLR, f1, I) has 26.8% lysine, 2% arginine, and 3-4 sub-fractions. Its molecular weight ranges from 19,500 to 21,000.

Electrophoretic comparisons of histones from plant and vertebrate tissues have revealed differences (32, 35). Nadeau, et al. (35) studied electrophoretic mobilities and molecular weights of histone from barley, leek, onion, pea, radish, rye, wheat, calf thymus, and rat liver on polyacrylamide-urea and polyacrylamide-SDS gels. Fractions f2a1 (GAR) and f3 (AR) were very similar in the plant and animal tissues. Sub-fractions of fraction f1 (VLR) in plants were higher in number and in molecular weight (22,000-25,000). Fractions f2a2 (AL) and f2b (SLR), normally present between fractions f2a1 and f3 in animal tissues, were not present in the plant tissues. Instead, two to three bands

appeared between fractions f3 and f1. These bands were referred to by the researchers as "plant histones". Additional work is required to determine whether these histone bands are modified forms of f2a2 and f2b or a different class of histone unique to plants.

Fambrough and Bonner (14) obtained 8 fractions or species of peabud histone by discontinuous gel electrophoresis. Properties such as amino acid composition, number of tryptic peptides, electrophoretic mobility, and order of elution on gel-filtration chromatography indicated very limited heterogeneity of the histones.

The amino acid sequences of all histone fractions have been determined. There are two major segments in each histone sequence (6). One segment contains a large amount of basic residues (+ charge) and helix destabilizing residues (i.e. proline, glycine). The other segment is dominated by low basicity, apolar (leucine, valine) and acidic residues. Specific segments of the histones rich in basic residues are apparently involved in interactions with DNA while the apolar segments are involved in conformational changes and interhistone interactions.

The manner and location of attachment of a histone molecule to DNA has yet to be determined. Evidence so far indicates that the histone molecules probably bind at the major groove of DNA although this investigation is beyond the means of current fiber X-ray diffraction and electron microscopy techniques (4). Olins (33) suggested that f1 histone probably binds within the large

groove of DNA based on evidence from dye-binding, glucosylation, and actinomycin D-binding experiments. Sung and Dixon (55) proposed a model of the binding of f2a1 (GAR) to DNA. The N terminal portion of the GAR histone (strongly basic) would fit into the major groove of DNA and bind to some of the phosphate groups. A slight lapse of a-helicity would be required to allow the histone to follow the helical pattern. The less basic half of a histone molecule could also lie in the major groove and bind additional phosphates.

Apparently 50-60% of the phosphate groups in DNA are bound when DNA is saturated with histone molecules. The other phosphates behave as free phosphates, being accessible to dyes and nucleases. It may be that more than 50-60% of DNA is covered by histones.

Synthesis Location and Turnover. Periods of DNA and histone synthesis prior to cell division were observed to closely coincide in onion root meristem (3). Histones were marked with tritium-labelled lysine and arginine; DNA with tritium-labelled thymidine. Synthesis comparisons were made by determining times required for cells with marked chromosomes to begin and terminate division stages. An additional period of chromosomal protein synthesis occurred late in interphase. Robbins and Borun (42) also noted a correlation between initiation of histone and DNA synthesis in HeLa cells.

There is considerable evidence to support a cytoplasmic location for histone synthesis. Based on pulse-chase labelling

of cells in G1 and S phases and on examination of cytoplasmic polysomes labelled with ^{14}C tryptophan and ^{3}H lysine, histones were apparently made in the cytoplasm on small polysomes.

Additional evidence for cytoplasmic synthesis of histone was provided by Gallwitz and Mueller (16) in their work with HeLa cells. Three messenger RNA's (ribonucleic acid) found only on microsomes which were actively synthesizing histone were resolved on polyacrylamide gels with 0.5% agarose. Their molecular sizes corresponded to the size of a messenger RNA needed to code for proteins of the histone class.

Nuclei as a site of histone synthesis cannot be ruled out. Reid and Cole (40) felt that the calf thymus nucleus was the site of synthesis of lysine-rich histone. The lack of sensitivity of this histone to RNase suggested that synthesis was not occurring on cytoplasmic microsomes attached to the nuclear membrane. Lysine-rich histone synthesis was shown to require Na^{+} ion; previous work had indicated that Na^{+} ion was specifically required for amino acid transport across the nuclear membrane.

In studies of onion root meristem by Block, et al. (3), histone proteins appeared to be synthesized in the nucleus or to migrate there shortly after synthesis (within one hour after uptake of ^{3}H lysine and arginine).

Experiments by Gurley, et al. (17) suggested that histone f1 could be made in cytoplasm and in nuclei. Histone f1 was extracted from chromatin, nucleoplasm, and polysomes of Chinese hamster cells.

Pulse-labelling experiments demonstrated that the f1 found in chromatin was the most newly-synthesized, f1 in nucleoplasm was intermediate in age, and f1 in polysomes was the oldest. The f1 associated with polysomes had much less phosphorylation than did chromatin f1. These differences suggested that polysomal f1 was a distinct fraction of total cellular f1 (about 10%) and might constitute a pool of non-chromatin-bound f1.

Turnover rates of histones and DNA in normal and tumorous rat tissues were studied by Byvoet (8). Histones were labelled with ^{14}C lysine and DNA with ^{125}I (iodo-2'-deoxyuridine). Histone: DNA ratios remained near unity in all types of normal and tumor tissue. Data indicated that the biological breakdown of histones and DNA may be linked after they are combined to form deoxyribonucleohistone.

Whatever the turnover rate of histones, a mechanism must exist to remove histones from DNA. One method of removal could be competition from a stronger cation (i.e. a slightly modified histone). This occurs in the replacement of histone f1 by protamine in trout spermatogenesis. Another possibility for removal is competition by a strong anion (i.e. NHCP). A third method could be by proteolytic degradation. Such degradation has been observed in trout spermatogenesis but it was preceded by phosphorylation of the histone. The phosphorylation did not seem to affect binding to DNA but may have been a signal for histone protease action (12).

DNA Template Regulation. One of the most common arguments

for assigning a role of repressor-derepressor to histones has been their ability to regulate RNA synthesis *in vitro*. Dahmus and Bonner (9) studied the template activity of chromatins from rat liver, rat spleen, and calf thymus during the removal of chromosomal proteins. As the chromosomal proteins were removed by increasing the ionic strength of the solution with sodium perchlorate, template activity increased. The increase in activity closely paralleled the removal of protein from the DNA. If rat liver chromatin was treated with acid to remove only histone proteins, the template activity of the DNA-nonhistone protein complex was almost identical to that of completely deproteinized DNA. It appeared that histones were responsible for RNA synthesis control.

In contrast, Johns and Hoare (20) felt that the increase in transcription was probably a matter of accessibility or solubility of the DNA complex. They observed that when the histone:DNA ratio was raised with the addition of f1 and f3 histone, inhibition of RNA synthesis occurred. However, DNA complex precipitated as histone was added. Peak transcription inhibition was present when f1 histone:DNA ratio was 0.8 and f3 histone:DNA ratio was 1.0-1.5. At higher ratios the trend reversed and the DNA complex apparently became more soluble allowing an increased rate of RNA synthesis.

Differences of opinion exist as to whether the various histone fractions show specificity in template activity control. Spelsberg and Hnilica (48) selectively removed histone fractions indicating that not all histones were involved in genetic restriction.

Removal of lysine-rich histones (f1) yielded no significant change in available template of rat thymus chromatin. Dissociation of slightly lysine-rich or arginine-rich histone increased available template; however, more derepression resulted from removal of slightly lysine-rich histone. After complete dehistonization, some residual restriction remained as compared to naked DNA. These residual repressors later proved to be non-histone chromosomal proteins.

Shih and Bonner (47) found that arginine-rich and lysine-rich histone were equally effective in preventing transcription of calf thymus DNA.

Langan, et al. (29) separated two lysine-rich histone fractions from rabbit thymus by ion-exchange chromatography. The two fractions differed in their ability to be phosphorylated with a liver histone kinase. The readily-phosphorylated fraction contained serine in a location shown to be a major site of phosphorylation. The other fraction contained alanine in this position and did not show phosphorylation. This difference in primary structure indicated that these histone fractions were distinct species and might have specificity of function.

Histone Modifications. Phosphorylation has been the most commonly observed modification and is probably the most important. All histone fractions are able to undergo phosphorylation but f1 histone has been the most frequently-studied. Phosphates are esterified to hydroxyl groups of serine and threonine. Placement

of a negatively-charged phosphate moiety on a positively-charged histone protein would be expected to change the protein conformation and binding affinity to DNA. These changes could: (1) activate specific genes which are normally repressed by histones; (2) modify chromatin structure in preparation for DNA synthesis and cell division; or (3) aid in removal of histones from DNA to allow replacement by another protein type (10).

A positive correlation has been noted between the growth rate of Morris hepatoma and the degree of phosphorylation of one sub-fraction of f1 histone (34). The sub-fraction was apparently only phosphorylated during DNA synthesis. Since the phosphorylation was largely present in rapidly replicating tissues and essentially absent in stationary tissues, cell division seemed related to this modification.

Bradbury, et al. (7) performed measurements of the net phosphorylating activity of nuclei of Physarum polycephalum (a slime mold), isolated at different stages of the mitotic cycle, and acting on added calf thymus f1 histone. They concluded that f1 phosphorylation initiated chromosome condensation. It was proposed that f1 phosphorylation is the initiation step for mitosis and this step is triggered or controlled by the net f1 phosphorylating enzyme activity. They felt that it may be possible to stimulate mitosis with purified phosphokinase and to inhibit mitosis with f1 histone phosphatase.

DeLange and Smith (10) felt that phosphorylation of histone

f1 may be related to DNA synthesis while phosphorylation of other histones may correlate to processes of RNA synthesis.

Histone modification by acetylation has been observed in all histones except very lysine-rich histones. Acetylation is known to occur at two locations: (1) amino terminal serine residues and (2) ϵ-amino groups of the amino acid lysine. Acetyl groups of the ϵ-amines of lysine seem to be metabolically active and turnover at varying rates. No important physiological role can currently be assigned to acetylation (34).

Methylation, a third type of histone modification, is considered to be relatively unimportant because of the small size of a methyl group. Methylation tends to occur late in the cell cycle while phosphorylation and acetylation usually occur prior to or during DNA synthesis (34).

Histone modifications seem to play a role in chromosome metabolism, perhaps in the transport of histones into the nucleus or in their binding to and removal from DNA (12). However, the precise functions of the modifications have yet to be determined.

Histone Roles. It is known that histones are composed of relatively few unique polypeptides. In a differentiated eukaryotic cell, 80-90% of the genome is normally repressed (4). Histones would seem to be "general" gene suppressors, probably involved in maintaining and controlling chromosome structure (6). Other more specific mechanisms are implicated in the maintenance of the 10-20% of active genome.

According to Johns (19), there is no real evidence *in vivo* to show that histones need to be removed from DNA to enable it to serve as a template for RNA synthesis. Therefore, the structure of chromatin must be important. When chromatin is condensed during mitosis, it is inactive. During interphase, the chromatin is more diffuse, allowing parts to be copied and parts to be rendered inactive. X-ray studies have indicated that the nucleoprotein molecule has straight-chained and supercoiled portions. although there is presently no means to determine the relative amounts of each. It seems feasible that the histones cause supercoiling. A large molecule such as RNA polymerase would have difficulty negotiating supercoiled chromatin. Other macromolecules (perhaps non-histone chromosomal proteins) having the necessary specificity to recognize a particular gene could cause derepression by breaking the weak bonds of the supercoil.

A hypothesis to explain supercoiling has been suggested by Bonner and Garrard (4). It is known that a histone molecule contains a high concentration of basic amino acids at one end and fewer basic amino acids at the other. The attraction between the highly basic end of a histone and the negative charges of the DNA phosphates could result in a tight complex causing that region of the DNA double helix to slightly shorten compared to the native DNA configuration. On the other hand, the less basic end of a histone molecule would not bind as tightly, resulting in less shortening at that point. A series of unequal bindings might cause

a shift in the direction of the long axis of the DNA double helix resulting in supercoiling.

Another structural role for histones was proposed by Littau, et al. (31). They showed that removal of lysine-rich histone from condensed chromatin in calf thymus lymphocyte nuclei caused the chromatin to dissociate into a diffuse network of fibrils. If lysine-rich histone was restored, clumps of condensed chromatin were again present. Removal and replacement of arginine-rich histones did not affect this change. Observations were made under the electron microscope. These results indicated to the investigators that lysine-rich molecules had combined with phosphate groups of DNA to cross-link DNA double helices while the arginine-rich histone molecules combined with phosphate groups along a double helix. The ratios of arginine to lysine-rich histone were similar in condensed and diffuse chromatin. This suggested that although the lysine-rich histone was not cross-linking diffuse chromatin, it was still in contact with DNA.

Histones may also be involved in the formation of metaphase chromosomes (37). Histone f3 (AR) in organisms as highly evolved as rodents contains one cysteine residue while f3 in higher mammals contains two cysteine residues. In interphase chromosomes, the cysteine residues all contain sulfhydryl groups (reduced form). In metaphase chromosomes, many of the cysteines are present as disulfides because of covalent bonding with other molecules in the supercoil. These disulfide bonds may serve to stabilize the

the superstructure of metaphase chromosomes.

In addition, histones probably stabilize the DNA double helix against thermal denaturation and assist in protecting it from radiation damage. Hnilica (18) provided an overview of the interaction of the four macromolecules contained in chromatin. If histones are genetic repressors, then non-histone chromosomal proteins are associated with the less permanent kind of repression. RNA would provide the site of interaction with a specific genetic locus on DNA. NHCP would protect DNA from close association with histones in the area of the DNA-RNA interaction site, allowing transcription. All remaining DNA loci not protected by NHCP would be associated with histones, rendering them inactive. The need for specificity of histones associated with DNA would be small. Derepression could be achieved by chemical modifications of histones (phosphorylation, etc.) resulting in exposure of a particular part of DNA to interaction with NHCP.

No role in chromatin structure or function can be assigned to the histones with certainty at the present time. However, it seems more probable that histones are general suppressors than specific ones.

APPENDIX B

NON-HISTONE CHROMOSOMAL PROTEINS

General Characteristics. Non-histone proteins include common enzymes of nucleic acid and histone metabolism, structural proteins and non-histone chromosomal proteins. Non-histone chromosomal proteins (NHCP) or hertones* (12) are rich in glutamic and aspartic acids or their amides; thus they tend to be acidic in nature. The precise number of species of NHCP are unknown for any tissue due to limited methodology. However, two-dimensional polyacrylamide gel electrophoresis will prove useful in future work.

The range of molecular weights is wide. In HeLa cells, estimated weights by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was 15,000 - 180,000 with 85% of the NHCP over 40,000 (2). Weights of rat liver NHCP ranged from 15,000 - 100,000 (13). Although isolation and fractionation of NHCP have been hampered by their tendency to aggregate with DNA, histones, and one another, it is fairly certain that non-histone chromosomal proteins are more heterogeneous than histones.

Work performed by Farber, et al. (15) suggested that the chromatin proteins regulating transcription are located in the major groove of DNA. A reporter molecule, which binds exclusively to the minor DNA groove, was shown not to interfere with transcription

*Term suggested by R. D. Cole based on the idea that this class of proteins may be involved in more delicate aspects of gene regulation than histones.

of chromatin from HeLa S3 cells by exogenous E. coli RNA polymerase.

Synthesis and Turnover of NHCP. The location of the synthesis of non-histone chromosomal proteins is probably in the cytoplasm. Kawashima, et al. (23) utilized pulse chase studies of ^{14}C labelled amino acids to observe protein transfer in mouse ascite tumor cells. Radioactivity was seen to increase greatly in nucleoli and to a lesser extent in extranucleolar nuclear fractions. At the same time, a corresponding decrease of radioactivity occurred in the cytoplasmic fraction. These results strongly suggested to the researchers that the majority of protein was synthesized on the outside of the nucleoli, probably in the cytoplasmic polysomes. Similar results were obtained by Stein and Baserga (51), who concluded that more than 90% of the nuclear proteins including acidic chromosomal proteins were synthesized in the cytoplasm and transferred to the nucleus. These studies were based on pulse labelling of HeLa S3 cells with ^{3}H leucine.

Evidence seems to suggest that non-histone proteins are synthesized and turned-over much more rapidly than histones. Determinations of synthesis and turnover rates have been carried out during the four cell cycle phases (pre-replication (G1), DNA replication (S), post-DNA synthesis (G2), and mitosis) using radioactive amino acid incorporation by Rovera and Baserga (43). Resting WI-38 human fibroblast cells were stimulated to grow by changing the growing medium. Incorporation rates increased during G1 with a maximum just prior to S, reflecting an increase in rates

of NHCP synthesis. Acidic proteins that were synthesized during the first hour after stimulation had a turnover time of less than four hours while those proteins in non-stimulated cells were stable for at least 12 hours. In a similar study, Levy, et al. (30) observed an increase in non-histone chromatin protein as early as 1 to 3 hours after the addition of phytohemagglutinin to guinea pig lymphocytes. No increase in histone synthesis occurred.

Work by Borun and Stein (5), using pulse-chase labelling techniques in HeLa S3 cells indicated that the amount of protein synthesized, transported, and retained in the acidic residual chromosomal protein fraction was higher immediately after mitosis and later in G1 than in S or G2 phases. During S phase, 25% of the protein entering the acidic chromosomal protein fraction had turned-over after two hours, while up to 40% entering the fraction in mitosis, G1 and G2 had left within two hours.

The increase in synthesis of the nuclear acidic proteins after cell stimulation is apparently limited to particular classes of protein as revealed in polyacrylamide gel profiles of human and mouse fibroblasts (56).

When actinomycin D, an inhibitor of DNA-dependent RNA synthesis, was added to dividing cells, it did not reduce the increased rate of NHCP synthesis normally occurring early in G1. Instead, NHCP synthesis during later phases was reduced. This implied that initial synthesis may take place on preformed templates. When actinomycin D was applied to HeLa S3 cells during late G2, the synthesis of

several classes of NHCP during the subsequent G1 period was not suppressed. Apparently, then, unaffected NHCP species are synthesized on stable species of mRNA, transcribed prior to mitosis (when cessation of mRNA synthesis and disaggregation of polyribosomes normally occurs), and reactivated during G1 phase (54).

While histone synthesis appears to be coupled to DNA replication, NHCP synthesis is apparently not. In both continuously-dividing cells and stimulated quiescent cells, NHCP synthesis has been shown to continue throughout the cell cycle (1, 5, 30, 43, 58).

Effects of NHCP on Chromatin Template Activities. Reconstitution experiments seem to be the predominant method of investigation into the control of template activity by non-histone chromosomal proteins. Template activity was found higher in HeLa S3 cell chromatin reconstituted with S phase NHCP than with mitotic NHCP (50). Also, template activity was higher in WI-38 human fibroblast chromatin reconstituted with NHCP from human fibroblast cells one hour after proliferation stimulation than in chromatin reconstituted with NHCP from non-dividing fibroblast cells (53). However, in both stimulated and non-stimulated fibroblast cells, chromatin reconstituted with histones isolated from various cell cycle stages did not show template activity differences.

It is known that histones are more tenaciously-bound to DNA during mitosis than during S phase. In addition, differences have been observed in histone binding between chromatin reconstitution with mitotic NHCP and with S phase NHCP. Since template

activity of chromatin reconstituted with mitotic phase NHCP is lower than with S phase NHCP, one could speculate that NHCP are involved with mediation of the binding of histones to DNA (54).

Spelsberg, et al. (49) found that the residual repressors remaining on DNA after removal of histones by salt extraction were non-histone chromosomal proteins. Using artificial chromatins that were rat liver and rat thymus hybrids, they demonstrated that the NHCP remaining on DNA determined the pattern of DNA restriction and were essential for tissue-specific restriction.

Template specificity of NHCP was demonstrated in experiments involving hybridization of NHCP and chromatin from normal and Walker tumor rat liver tissues (27). When Walker tumor non-histone chromosomal protein was allowed to activate normal rat liver chromatin, the RNA produced was similar to that produced by Walker tumor chromatin. Similarly, when normal rat liver NHCP activated Walker tumor chromatin, the RNA produced was similar to normal rat liver RNA.

Data by Kamiyama and Wang (21) suggested the activation of the genome by the addition of NHCP. RNA transcribed from chromatin stimulated by NHCP had a different nucleotide composition and hybridized more with homologous DNA than RNA transcribed from non-stimulated chromatin. Transcribed RNA from activated chromatin coded different polypeptides which had longer average chain lengths than polypeptides coded by RNA from control chromatin. Latter study based on ribosomal amino acid incorporation (in vitro).

Further evidence pointing toward the involvement of non-histone chromosomal proteins in a repressor-derepressor function is that active chromatin has a higher content of NHCP than inactive chromatin (34, 52).

Specific NHCP changes have been noted from interaction with carcinogens, drugs, plant and mammalian hormones. In regard to the role of NHCP in the mechanism of hormone action, it is suspected that certain non-histone chromosomal proteins of target chromatins serve as acceptors for hormone-receptor complexes while others are synthesized in response to hormonal stimulation (34).

Non-Histone Chromosomal Phosphoproteins. An important modification to non-histone chromosomal proteins that has been commonly observed is phosphorylation. According to Langan (28), over 90% of the nuclear protein-bound phosphorus is associated with NHCP, mainly in the form of phosphoserine which composes about 5% of amino acid residues.

Nuclear phosphoproteins demonstrate species specificity in patterns of phosphorylation, electrophoretic mobilities, and DNA binding within the same species. The non-histone chromatin phosphoproteins from bovine thymus, liver and brain were shown to be highly heterogeneous based on mobilities on 10% SDS- acrylamide gels and on (^{32}P) radioactivity profiles (39).

Teng, et al. (57) found that patterns of phosphorylation of individual acidic proteins varied from one tissue to another. Further, that phosphorylated acidic proteins prepared from rat

liver and kidney nuclei formed complexes with rat liver DNA. Little or no binding occurred between rat liver phosphoproteins and DNA from calf thymus, human placenta, dog liver, or bacteria.

The phosphorylation of non-histone chromatin proteins in HeLa S3 cells was investigated by Platz, et al. (38) during G1, early and late S, G2, and mitosis. Phosphorylation rates were at a maximum in G1 and G2, somewhat less during S phase, and 90% lower during mitosis. Acrylamide gel analysis indicated that particular species were phosphorylated during particular phases of the cell cycle.

In a 1966 study by Kleinsmith et al. (25), it was shown that ^{32}P-labelled orthophosphate could be incorporated into proteins of calf thymus nuclei and that phosphorylation was independent of protein synthesis. The uptake of phosphorus required energy from ATP. Once incorporated, the phosphate groups turned over rapidly; this turnover was also energy dependent.

Considerable evidence exists for the involvement of phosphorylated non-histone proteins in gene activation:

1) There is a correlation between phosphorylation and gene activity. Kleinsmith, et al. (26) observed an increase in the rate of phosphorylation and dephosphorylation of nuclear proteins within a few minutes after gene activation of human lymphocytes was induced by phytohemagglutinin (PHA).

2) Stimulation RNA synthesis results from the addition of non-histone phosphoproteins in vitro (28, 41, 57). When Shea and

Kleinsmith (46) added phosphorylated non-histone chromatin proteins from rat liver to a cell-free system containing rat liver RNA polymerase and rat DNA, stimulation occurred. If other DNA species were used (i.e. E. coli, calf, salmon), no stimulation occurred, implying specific recognition of DNA sites by the NHPP.

3) Removal of phosphate groups from NHPP abolished the RNA synthesis in vitro. In the latter study, Shea and Kleinsmith (46) utilized alkaline phosphatase to remove 20-30% of the protein-bound phosphate groups.

As yet, however, it is not known definitely whether NHPP have the capacity to control gene read-out. DeMorales, et al. (11) felt that phosphorylation of NHCP was probably involved in the initial events leading to cellular proliferation. In their experiments with baby hamster kidney cells, the peak in phosphorylation of total proteins coincided with the maximum in DNA synthesis. However, the highest peak in phosphorylation of non-histone chromosomal protein was six hours earlier than the maximum of DNA synthesis. A second lower peak in NHCP phosphorylation did correspond to the DNA and RNA synthesis maximums.

The regulation of phosphorylation requires kinases (to catalyze the addition of phosphate groups), phosphatases (to catalyze the removal of phosphate groups) and ATP (to yield phosphate groups and supply energy). It has been determined that specific kinases are required in the phosphorylation of specific non-histone chromosomal proteins.

Kish and Kleinsmith (24) found 11 distinct regions of kinase activity in non-histone chromatin phosphoproteins from bovine liver. The addition of cyclic AMP, which is known to mediate the effects of hormones and other agents on cell activity, caused stimulation in 5 of the 11 kinase regions and inhibition in the other 6. Two protein kinases that catalyze the phosphorylation of specific seryl and threonyl residues of nuclear non-histone proteins were found to be associated with rat liver chromatin by Takedo, et al. (56). The two enzymes could be differentiated by substrate specificities. In contrast, Ruddon and Anderson (44) obtained four kinase activities in the acidic nuclear protein fraction extracted from rat liver nuclei, each distinct on the basis of pH optimum and substrate specificity.

Kaplowitz, et al. (22) obtained a 5 to 10 fold stimulation in phosphorylation of calf thymus non-histone chromosomal protein in vitro if they added calf thymus histone. The most effective stimulator of phosphorylation was f1 histone, although all other histone fractions were capable of stimulation. The histones seemed to be acting in some way to make more phosphorylation sites available.

A current theory to explain control of transcription suggests an interaction between non-histone chromosomal proteins and histones. The histones may stimulate phosphorylation of NHCP. This increase in phosphorylation would increase negative charges on NHCP and strengthen ionic bonding between non-histone phosphoproteins and histones. This additional attraction might also displace histone

from the DNA double helix, allowing gene transcription to increase (54).

This theory would seem to require specificity of phosphorylation of NHCP attached to DNA or perhaps pre-phosphorylated NHCP binding to specific sites on the DNA. As yet, specificity of NHPP binding to DNA covered with histones has not been demonstrated.

Baserga and Stein (1) have put forth a hypothesis concerning the overall control of cell division in mammalian cells. A stimulus reacts with the cell membrane causing a change which results in the loss of certain components of the cytoplasm. Among the lost components is a postulated macromolecule which inhibits translation of a pre-existing RNA template (i.e. interferon inhibits viral RNA translation). Thus, the pre-existing RNA template is translated and an acidic protein is synthesized independent of gene activation. The acidic protein is transferred to the nucleus where it binds at the major groove of DNA and activates a segment of the genome resulting in rounds of RNA and protein synthesis leading to the onset of DNA synthesis and cell division.

Although the precise roles of non-histone chromosomal proteins have not been established, it does seem likely that they play an extremely important role in gene activation and regulation in the cell.

LITERATURE CITED

1. Baserga, Renato and Gary Stein. 1971. Nuclear Acidic Proteins and Cell Proliferation. Federation Proceedings 30:1752-1759.

2. Bhorjee, Jaswant S. and Thoru Pederson. 1972. Nonhistone Chromosomal Proteins in Synchronized HeLa Cells. Proc. Nat. Acad Sci. 69:3345-3349.

3. Bloch, David P., R. A. MacQuigg, S. D. Brack, and Jung-Rung Wu. 1967. The Synthesis of Deoxyribonucleic Acid and Histone in the Onion Root Meristem. J. Cell Biol. 33:451-467.

4. Bonner, James and William Taft Garrard. 1974. Biology of the Histones. Life Sciences 14:209-221.

5. Borun, T. W. and G. S. Stein. 1972. The Synthesis of Acidic Chromosomal Proteins During the Cell Cycle of HeLa S-3 Cells. II. The Kinetics of Residual Protein Synthesis and Transport. J. Cell Biol. 52:308-315.

6. Bradbury, E. M., P. D. Cary, C. Crane-Robinson, and H. W. E. Rattle. 1973. Conformations and Interactions of Histones and Their Role in Chromosome Structure. Annals N. Y. Acad Sci. 222:266-289.

7. Bradbury, E. M., R. J. Inglis, and H. R. Matthews. 1974. Control of Cell Division by Very Lysine-Rich Histone (F1) Phosphorylation. Nature 247:257-261.

8. Byvoet, Paul. 1966. Metabolic Integrity of Deoxyribonucleohistones. J. Mol. Biol. 17:311-318.

9. Dahmus, Michael E. and James Bonner. 1970. Nucleoproteins in Regulation of Gene Function. Federation Proceedings 29:1255-1260.

10. DeLange, Robert J. and Emil L. Smith. 1971. Histones: Structure and Function. Annual Review of Biochemistry 40:279-314.

11. DeMorales, M. Marty, C. Blat, and L. Harel. 1974. Changes in the Phosphorylation of NHCP in Relationship to DNA and RNA Synthesis in $BHK_{21}C_{13}$ Cells. Exp. Cell Research 86:111-119.

12. Elgin, Sarah, C. R. and James Bonner. 1972. Isolated Chromatin in the Study of Gene Expression. In The Biochemistry of Gene Expression in Higher Organisms. J. K. Pollak and J. Wilson Lee, eds. Australia and New Zealand Book Company, Syndney. p. 142-163.

13. Elgin, Sarah C. R. and James Bonner. 1972. Partial Fractionation and Chemical Characterization of the Major Nonhistone Chromosomal Proteins. Biochemistry 11:772-781.

14. Fambrough, Douglas M. and James Bonner. 1969. Limited Molecular Heterogeneity of Plant Histones. Biochim. Biophys. Acta. 175:113-122.

15. Farber, John, Renato Baserga, and Edmond J. Gabbay. 1971. The Effect of a Reporter Molecule on Chromatin Template Activity. Biochem. Biophys. Res. Commun. 43:675-681.

16. Gallwitz, Dieter and Gerald C. Mueller. 1970. RNA From HeLa Cell Microsomes with Properties of Histone Messenger. FEBS Letters 6:83-85.

17. Gurley, L.R., M. D. Enger, and R. A. Walters. 1973. The Nature of Histone F1 Isolated from Polysomes. Biochemistry 12:237-245.

18. Hnilica, Lubomir S. 1967. Proteins of the Cell Nucleus. In Progress in Nucleic Acid Research and Molecular Biology. J. N. Davidson and Waldo E. Cohn, eds. Academic Press, New York. p. 25-106.

19. Johns, E. W. 1969. The Histones, Their Interactions with DNA, and some Aspects of Gene Control. In Ciba Foundation Symposium on Homeostatic Regulators. G. E. W. Wolstenholme and Julie Knight, eds. J. and A. Churchill, Ltd., London. p. 128-143.

20. Johns, E. W. and Tanya A. Hoare. 1970. Histones and Gene Control. Nature 226:650-651.

21. Kamiyama, M. and T. Y. Wang. 1971. Activated Transcription from Rat Liver Chromatin by Non-Histone Proteins. Biochim. Biophys. Acta. 228:563-576.

22. Kaplowitz, Paul B., Robert D. Platz, and Lewis J. Kleinsmith. 1971. Nuclear Phosphoproteins: III. Increase in Phsophorylation During Histone-Phosphoprotein Interaction. Biochim. Biophys. Acta. 229:739-748.

23. Kawashima, Kazuko, Mitsuo Izawa, and Shizuo Sato. 1971. Studies on the Origin of Proteins in the Nucleoli of Mouse Ascites Tumor Cells: A Test for Protein Transfer to the Nucleoli. Biochim. Biophys. Acta. 232:192-206.

24. Kish, Valerie M. and Lewis J. Kleinsmith. 1972. Heterogeneity of Nuclear Protein Kinases Associated with Non-histone Chromatin Proteins. J. Cell Biology 55:138a.

25. Kleinsmith, Lewis L., Vincent G. Allfrey, and Alfred E. Mirsky. 1966. Phosphoprotein Metabolism in Isolated Lymphocyte Nuclei. Proc. Nat. Acad. Sci. 55:1182-1189.

26. Kleinsmith, Lewis J., Vincent G. Allfrey, and Alfred E. Mirsky. 1966. Phosphorylation of Nuclear Protein Early in the Course of Gene Activation in Lymphocytes. Science 154:780-781.

27. Kostraba, Nina, C. and T. Y. Wang. 1972. Transcriptional Transformation of Walker Tumor Chromatin by Non-histone Proteins. Cancer Research 32:2348-2352.

28. Langan, Thomas A. 1967. A Phosphoprotein Preparation from Liver Nuclei and its Effect on the Inhibition of RNA Synthesis by Histones. In Regulation of Nucleic Acid and Protein Biosynthesis. V. V. Koningsberger and L. Bosch, eds. Elsevier Publishing Company, Amsterdam. p. 233-242.

29. Langan, Thomas A., S. C. Rall, and R. David Cole. 1971. Variation in Primary Structure at a Phosphorylation Site in Lysine-rich Histones. J. Biol. Chem. 246:1942-1948.

30. Levy, Ronald, Shoshana Levy, Steven A. Rosenberg, and Robert T. Simpson. 1973. Selective Stimulation of Nonhistone Chromatin Protein Synthesis in Lymphoid Cells by Phytohemagglutinin. Biochemistry 12:224-228.

31. Littau, V. C., C. J. Burdick, V. G. Allfrey, and A. E. Mirsky. 1965. The Role of Histones in the Maintenance of Chromatin Structure. Proc. Nat. Acad. Sci. 54: 1204-1212.

32. Nadeau, Paul, Dominick Pallotta, and Jean-G. LaFontaine. 1974. Electrophoretic Study of Plant Histones: Comparison with Vertebrate Histones. Arch. Biochem. Biophys. 161:171-177.

33. Olins, Donald E. 1969. Interaction of Lysine-rich Histones and DNA. J. Mol. Biol. 43:439-460.

34. Olson, Mark O. J., Wesley C. Starbuck, and Harris Busch. 1974. Nuclear Proteins. In The Molecular Biology of Cancer. Harris Busch, ed. Academic Press, New York. p. 309-353.

35. Panyim, Sakol and Roger Chalkley. 1969. High Resolution Acrylamide Gel Electrophoresis of Histones. Arch. Biochem. Biophys. 130:337-346.

36. Panyim, Sakol, David Bilek, and Roger Chalkley. 1971. An Electrophoretic Comparison of Vertebrate Histones. J. Biol. Chem. 246:4206-4215.

37. Panyim, Sakol, K. R. Sommer and Roger Chalkley. 1971. Oxidation of the Cysteine-Containing Histone F3. Detection of an Evolutionary Mutation in a Conservative Histone. Biochemistry 10:3911-3917.

38. Platz, Robert D., Gary S. Stein, and Lewis J. Kleinsmith. 1973. Changes in the Phosphorylation of Non-histone Chromatin Proteins during the Cell Cycle of HeLa S3 Cells. Biochem. Biophys. Res. Commun. 51:735-740.

39. Platz, Robert D., Valerie M. Kish, and Lewis J. Kleinsmith. 1970. Tissue Specificity of Non-histone Chromatin Phosphoproteins. FEBS Letters 12:38-40.

40. Reid, B. R. and R. David Cole. 1964. Biosynthesis of a Lysine-rich Histone in Isolated Calf Thymus Nuclei. Proc. Nat. Acad. Sci. 51:1044-1050.

41. Rickwood, David, G. Threlfall, A. J. MacGillivray, J. Paul, and P. Riches. 1972. Studies on the Phosphorylation of Chromatin Non-Histone Proteins and their Effect on Deoxyribonucleic Acid Transcription. Biochem. J. 129: 50-51P.

42. Robbins, Elliott and Thaddeus W. Borun. 1967. The Cytoplasmic Synthesis of Histones in HeLa Cells and Its Temporal Relationship to DNA Replication. Proc. Nat. Acad. Sci. 57:409-416.

43. Rovera, Giovanni and Renato Baserga. 1971. Early Changes in the Synthesis of Acidic Nuclear Proteins in Human Diploid Fibroblasts Stimulated to Synthesize DNA by Changing the Medium. J. Cell Physiology 77:201-212.

44. Ruddon, Raymond W. and Sharon L. Anderson. 1972. Presence of Multiple Protein Kinase Activities in Rat Liver Nuclei. Biochem. Biophys. Res. Commun. 46:1499-1508.

45. Sadgopal, Anil and James Bonner. 1970. Proteins of Interphase and Metaphase Chromosomes Compared. Biochim. Biophys. Acta. 207:227-239.

46. Shea, Michael and Lewis J. Kleinsmith. 1973. Template-Specific Stimulation of RNA Synthesis by Phosphorylated Non-histone Chromatin Proteins. Biochem. Biophys Res. Commun. 50:473-477.

47. Shih, Thomas Y. and James Bonner. 1970. Template Properties of DNA-Polypeptide Complexes. J. Mol. Biol. 50:333-344.

48. Spelsberg, Thomas C. and L. S. Hnilica. 1971. Proteins of Chromatin in Template Restriction. II. Specificity of RNA Synthesis. Biochim. Biophys. Acta. 228:212-222.

49. Spelsberg, Thomas C., L. S. Hnilica, and A. T. Ansevin. 1971. Proteins of Chromatin in Template Restriction. III. The Macromolecules in Specific Restriction of the Chromatin DNA. Biochim. Biophys. Acta. 228:550-562.

50. Stein, Gary and John Farber. 1972. Role of Nonhistone Chromosomal Proteins in the Restriction of Mitotic Chromatin Template Activity. Proc. Nat. Acad. Sci. 69:2918-2921.

51. Stein, Gary and Renato Baserga. 1971. Cytoplasmic Synthesis of Acidic Chromosomal Proteins. Biochem. Biophys. Res. Commun. 44:218-223.

52. Stein, Gary and Renato Baserga. 1972. Nuclear Proteins and the Cell Cycle. Advances in Cancer Research 15: 287-330.

53. Stein, Gary, Sukhen Chaudhuri, and Renato Baserga. 1972. Gene Activation in WI-38 Fibroblasts Stimulated to Proliferate. J. Biol. Chem. 247:3918-3922.

54. Stein, Gary, Thomas C. Spelsberg, and Lewis J. Kleinsmith. 1974. Nonhistone Chromosomal Proteins and Gene Regulation. Science 183:817-824.

55. Sung, Michael T. and Gordon H. Dixon. 1970. Modification of Histones during Spermiogenesis in Trout: A Molecular Mechanism for Altering Histone Binding to DNA. Proc. Nat. Acad. Sci. 67:1616-1623.

56. Takeda, Masao, Hirohei Yamamura, and Yuzo Ohga. 1971. Phosphoprotein Kinases Associated with Rat Liver Chromatin. Biochem. Biophys. Res. Commun. 42:103-110.

57. Teng, C. S., C. T. Teng, and V. G. Allfrey. 1971. Studies on Nuclear Acidic Proteins. Evidence for Their Phosphorylation, Tissue Specificity, Selective Binding to DNA, and Stimulatory Effects on Transcription. J. Biol. Chem. 246:3597-3609.

58. Tsuboi, Atsushi and Renato Baserga. 1972. Synthesis of Nuclear Acidic Proteins in Density-inhibited Fibroblasts Stimulated to Proliferate. J. Cell Physiol. 80:107-118.