THE GENETIC BASIS OF CHRONIC MYELOGENOUS AND ACUTE LYMPHOBLASTIC LEUKEMIA

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STELLINGEN

1. Analyse van het genoom met behulp van specifieke DNA probes kan een belangrijke bijdrage leveren aan de diagnose van chronische myeloide en acute lymfoblastische leukemie.
(Dit proefschrift)

2. Twardzik en medewerkers concluderen ten onrechte, dat de aanwezigheid van "transforming growth factor α" (TGFα) in de urine van kankerpatienten een goede tumormarker is.
(Twardzik et al., Cancer Research 45, 1934-1939, 1985)

3. De conclusie van Bolen en medewerkers, dat in dikke darm carcinomen de pp60 c-<u>src</u> proteine kinase activiteit is verhoogd, wordt niet voldoende ondersteund door hun waarnemingen.
(Bolen et al., P.N.A.S. 84, 2251-2255, 1987)

4. Wanneer met <u>in vitro</u> analyse van kernextracten binding van eiwitten aan DNA aangetoond kan worden, betekent dit nog geenszins dat die binding ook bij genregulatie <u>in vivo</u> van belang is.
(Becker et al., Cell 51, 435-443, 1987)

5. Het lijkt hoogst onwaarschijnlijk, dat de blastcrisis van CML patienten gepaard gaat met een verandering van het breukpunt in het bcr gebied van het Philadelphia chromosoom, zoals gesuggereerd wordt door Schaefer-Rego en medewerkers.
(Schaefer-Rego et al., Blood 70, 448-455, 1987)

6. In tegenstelling tot wat gesuggereerd wordt door Carpenter en medewerkers, lijkt de tyrosine kinase activiteit van de "epidermal growth factor (EGF) receptor" geen rol te spelen bij de stimulering van de celgroei.
(Carpenter et al., Cell 37, 357-358, 1984; Prywes et al., The EMBO Journal 5, 2179-2190, 1986)

7. Het rapport waarin gesteld wordt dat aspirine de kans op een hartaanval kan verkleinen, zou niet door de farmaceutische bedrijven in advertenties gebruikt mogen worden.
   (The Steering Commitee of the Physicians' Health Study Research Group, N.Engl.J.Med. 318, 262-264, 1988)

8. Een land dat bekend is om zijn "hollanditis" zou geen bom moeten gebruiken om de eindronde van het Europees voetbalkampioenschap te bereiken.

Stellingen behorende bij het proefschrift: The genetic basis of chronic myelogenous and acute lymphoblastic leukemia.

Kees Stam, Wageningen, 15 april 1988.

aan mijn ouders

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Preface/Voorwoord

I would like to express my gratitude to all who have contributed to this thesis. First of all I would like to thank Nora Heisterkamp and John Groffen. Their help and guidance have been very important for the work presented here. I thank John Stephenson for hiring a dutch student and for giving him the time and financial support necessary to work on the CML project. I am endebted to my two promotors Prof.Dr. Ab van Kammen en Prof.Dr. Dick Bootsma for giving me the opportunity to get a dutch Ph.D degree and for their intellectual input and suggestions during the preparation of this thesis. I also wish to thank my colleagues and co-workers Dr. Gerard Grosveld, Dr. Fred Reynolds, Gail Blennerhassett and Pamela Hansen. Finally, I am grateful to the secretaries for typing of the manuscript: Sue Marcus, Karen Lang and Pam Lederer.

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CHAPTER 1

SCOPE OF THE INVESTIGATION

Scope of the Investigation

The Ph′ chromosome is a cardinal feature of chronic myelogenous leukemia (CML). Originally described by Nowell and Hungerford (1960) as a small chromosome 22 it was later demonstrated by de Klein and co-workers(1982) to typically result from a reciprocal translocation t(9;22)(q34.1;q11.2). In approximately 5 percent of patients, the Ph′ chromosome results from anomalous complex translocation. Detailed studies using in situ hybridization have demonstrated that chromosomes 9 and 22 are usually involved in such translocations. Translocations between chromosome 22 and a chromosome other than 9 rarely occur.

The human c-*abl* sequences represent the cellular homologue of the transforming gene of Abelson Murine Leukemia Virus (A-MuLV). This retrovirus is a recombinant between Moloney Murine Leukemia virus and mouse cellular c-*abl* sequences (Goff et al., 1980). A-MuLV induces lymphoid tumors following *in vivo* inoculation of the mouse (Potter, 1983; Prekumar et al., 1975). Southern blot analysis of a series of somatic cell hybrids demonstrated that the human c-*abl* gene is localized on chromosome 9 (Heisterkamp et al., 1982). The finding that a small region of chromosome 9 is translocated to chromosome 22 in CML prompted studies to elucidate whether the *abl* gene was involved in this disease (de Klein et al., 1982). Bartram and colleagues (1983) first reported that the c-*abl* gene is translocated in Ph′ positive but not in Ph′ negative patients while Heisterkamp and co-workers (1983) reported a CML patient with a breakpoint 14 kb 5′ of the c-*abl*

sequences homologous to v-abl. This data suggested a role for c-abl in CML, a theory supported by the presence of an abnormally sized abl messenger RNA (Collins et al., 1984; Gale and Canaani, 1984) and abl protein in the CML cell line K562 (Konopka et al., 1984).

The region of chromosome 22 involved in the translocation has also been identified. Cloning of a chimeric breakpoint fragment from a CML patient (Heisterkamp et al., 1983) enabled the use of a chromosome 22 specific probe. Subsequent Southern blot analysis of the DNA of a number of CML patients showed that chromosome 22 breakpoints map to a stretch of 5.8 kb of DNA (Groffen, et al., 1984). This area on chromosome 22 was designated breakpoint cluster region or bcr.

The investigation described in this thesis was undertaken to gain further insight into the genetic organization of the c-abl gene on the Ph′ chromosome and the consequence of the Ph′ translocation at the transcription and translation level. Chapter 2 is an extensive review of the genetic basis of CML and acute lymphoblastic leukemia (ALL). Discussed is the cytogenetic and molecular aspects of CML and ALL and the activation of the c-abl protein kinases as a consequence of the Ph′ translocation. In Chapter 3, we show that the breakpoint cluster region on chromosome 22 is part of a gene, called phl. Molecular characterization demonstrates that phl is a large gene oriented with its 5′ end towards the centromere and with its 3′ end toward the telomere of chromosome 22. As a consequence of the Ph′ translocation the 3′ end of this gene is translocated to chromosome 9, whereas the 5′ sequences remain on the Ph′ chromosome. Following translocation, the remaining phl sequences become fused to the c-abl gene in a head to tail fashion on chromosome 22. We hypothesized that this genomic

configuration could result in the transcription of a chimeric mRNA consisting of 5′ phl and 3′ abl sequences. In the study presented in Chapter 4, we analyzed the RNA of CML patients and found strong evidence for this model. Direct proof for the hypothesis was provided by cloning of a chimeric phl/c-abl cDNA in the CML cell line K562, described in Chapter 5. The experiments described herein have also contributed to more information about the genomic organization of the phl and c-abl genes on the Ph′ chromosome.

Reports by Konopka et al. (1985) showing the presence of a larger 210K c-abl protein in the leukemic cells of CML patients initiated the studies described in Chapter 6. We demonstrate that this abnormal c-abl protein is a fusion protein containing amino terminal phl and carboxy terminal abl sequences. This phl/c-abl protein has an elevated tyrosine kinase activity when compared to the normal c-abl protein. In addition, we show in this chapter that the normal phl gene encodes for a 160 K phosphoprotein exhibiting an associated kinase activity.

A better understanding of the Ph′ chromosome at the molecular level has allowed us to develop a probe for the detection of the Ph′ translocation. In Chapter 7, we report the results of the clinical trials done at seven medical centers involving more than 400 clinical samples. In this report we demonstrate that the use of DNA probe analysis in the CML diagnostics has several advantages over cytogenetic methods.

CHAPTER 2

THE GENETIC BASIS OF CHRONIC MYELOGENOUS AND ACUTE LYMPHOBLASTIC LEUKEMIA

The Genetic Basis of Chronic Myelogenous and Acute Lymphoblastic Leukemia

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I. Introduction

In 1890, David Hansemann first drew attention to the frequent occurrence of mitotic irregularities in malignant tissues. He associated them with the origin and development of malignancy and suggested that such nuclear abnormalities could be used as a criterion for diagnosis of the malignant state. Such notions formed the basis of the mutation theory of cancer, presented in 1914 by Theodore Boveri in his book "Zur Frage der Entstehung Maligner Tumoren", in which chromosomal aberrations were suggested as the cause of the change from normal to malignant growth. This long remained a theoretical idea because of the technical difficulties in chromosome preparation throughout the first half of the century.

During the 1950s and 1960s, the application of methods developed for the study of chromosomes in plants and insects, coupled to the availability of mammalian tissue culture made it possible to work out detailed karyotypes. Clear-cut correlations were established between specific chromosome abnormalities and a number of disease states, laying a firm foundation for modern cancer cytogenetics. The results obtained, clearly demonstrated that the chromosome changes observed were an integral part of tumor evolution. However, most of this early work employed metastatic tumor cell populations, in which the chromosomal picture can be assumed to have changed considerably since the primary oncogenic process. Few, if any, conclusions, therefore, could be drawn as to the role of chromosome changes in etiology of neoplasia. The early chromosome studies on malignant cells have been summarized in detail previously (Atkin, 1976; Levan and Mittelman,

1977; Hsu, 1979; Sandberg, 1980).

In 1960 Nowell and Hungerford (1960) reported the first consistent chromosome abnormality in a human cancer; they observed an unusually small G group chromosome in leukemic cells from patients with chronic myelogenous leukemia (CML). This chromosome, which appeared to have lost about one half of its long arm, was named the Philadelphia (Ph′) chromosome in honor of its city of discovery. The question of whether the deleted portion of the long arm was missing from the cell or whether it was translocated to another chromosome could not be answered at that time because it was impossible to identify each human chromosome precisely with the techniques available. The discovery of the Ph′ chromosome in CML seemed to be the conclusive verification of Boveri's idea: a malignant disorder strictly correlated to a specific chromosomal change. However, results obtained after a search for similar abnormalities closely associated with other types of malignant hematologic diseases were quite disappointing. Although the abnormalities seemed to be consistent in any particular patient, the patterns varied greatly from one patient to another. Moreover about one-half of the patients with acute leukemia of both the myeloid and lymphoid types appeared to have a normal karyotype in their leukemic cells (Rowley and Testa, 1982; Mitelman and Levan, 1981; Sandberg, 1980). Thus, the accepted notion was that the Ph′ chromosome was a unique example of a consistent karyotypic abnormality, and the general rule was one of marked variability in karyotype. This in turn led most investigators to assume that chromosome changes were a secondary phenomenon, rather than being fundamentally involved with the process of malignant transformation.

Evidence obtained during the 1980s showed that these assumptions were incorrect. The introduction and consistent refinement of chromosome banding techniques (Hagemeijer et al., 1979; Yunis, 1981; Testa, 1984) made it possible to identify and define tumor specific chromosomal aberrations. Combination of these sophisticated cytogenetic techniques with cytological, cytochemical and immunological studies revealed that the malignant cells of most human tumors have a clonal karyotypic defect (Yunis et al., 1982; Yunis, 1983; Berger and Flandrin, 1984). A major breakthrough in cancer research came with the development of recombinant DNA techniques which made it possible to localize, clone and characterize the individual genes involved in tumorigenesis.

It is well known that cancer has myriad causes, but many of these may act in a common way by damaging DNA, resulting in the activation, mutation or loss of distinct genes. At least three major functional groups of such genes can be discerned. The oncogenes represent the first group (Bishop, 1983, 1985, 1987; Duesberg, 1983, 1985; Slamon et al., 1984; Weinberg, 1985; Land et al., 1983). They seem to act dominantly in the sense that they can induce transformation when transferred to cultured cells. Oncogenes are present in the human genome as normal cellular genes (proto-oncogenes) frequently involved with the control of normal cell growth; so far more than 50 proto-oncogenes have been identified.

A second group of genes are called "tumor suppressors" or "anti-oncogenes". These genes act recessively in the sense that both alleles need to be deleted or functionally incapacitated before a tumor can arise. The most thoroughly analyzed example of such a gene is rb-1,

both alleles of which are lost in retinoblastoma tumor cells (Cavenee et al., 1983; Murphree and Benedict, 1984; Benedict et al., 1983). It has been suggested that the rb-1 gene may have a normal regulatory function, possibly in the induction of an essential step in the terminal differentiation of this tissue (Murphee and Benedict, 1984; Knudson, 1971; Knudson and Strong, 1972; Comings, 1973; Robertson, 1984). Certain other neoplasms such as Wilms' tumor, familial renal cell carcinoma, neuroblastoma and small-cell carcinoma of the lung (SCLC), may also arise from the loss of both alleles of a single gene. This evidence for recessive anti-oncogenes is consistent with much earlier reports of the suppression of tumorigenic behavior in hybrids derived from the fusion of malignant with normal cells (Harris, et al., 1969; Klein et al., 1981; Wiener et al., 1973; Stanbridge et al., 1976). Cytogenetic analysis of such hybrids and their malignant segregants suggests the existence of different suppressor genes on different chromosomes (Jonasson et al., 1977; Spira et al., 1981; Evans et al., 1982; Klinger and Shows, 1983). There is now evidence that the transforming effect of certain retroviral oncogenes can be counteracted by different suppressor genes. For instance, revertants of _in vitro_ Kirsten-murine sarcoma virus-transformed fibroblasts have been found to suppress _ras_, _fes_, and _sis_, mediated transformation after somatic hybridization (Noda et al., 1983). Eventually, the suppressor genes may turn out to be as diverse as the oncogenes.

The third group of genes that influence neoplastic behavior are designated modulators. They do not, on their own, transform normal into neoplastic cells, but they modify their spread in the organism. They probably constitute a large and heterogenous group. For example,

metastatic spread may be influenced by the genes of the major histocompatibility complex (MHC) (Katzav et al., 1983; Sandersson and Beverley, 1983) and invasiveness by those genes controlling proteolytic and homing mechanisms (Nicholson, 1982; Dano et al., 1985). Cellular resistance to immune rejection (Dalianis et al., 1979; Fenyö and Klein, 1976) may also play a part.

Data concerning the molecular basis of the Ph′ translocation in CML have accumulated rapidly over the past few years. The Ph′ chromosome is observed in high frequency in CML (approximately 95 percent of patients with typical CML have this marker [Sandberg, 1980; Rowley, 1973, Heim et al., 1985]). In addition, the Ph′ chromosome is also found in both acute lymphoblastic leukemia (ALL) and, more rarely, in acute nonlymphocytic leukemia (ANLL) (Le Beau and Rowley, 1984; Chessells et al., 1979; Priest et al., 1980; Abe and Sandberg, 1979; Yunis et al., 1984). It offers one of the most clearly documented examples of a translocation which leads to the activation of a human cellular oncogene. The fusion of the c-abl and phl gene result in the production of an activated abl protein (Ben Neriah et al., 1986b; Stam et al., 1987; Hermans et al., 1987), which is probably directly involved with the malignant process. In the following review I will discuss the genetic basis of chronic myelogenous and acute lymphoblastic leukemia.

II. Chronic Myelogenous and Acute Lymphoblastic Leukemia: Disease and Treatment

a) Leukemias

Cancer is the leading cause of death in the Western world, after heart disease. In the United States alone, 500,000 people die per year of cancer (source Surveillance, Epidemiology and End Results (SEER) from the National Cancer Institute). Lung cancer is responsible for most of the cancer deaths, with 36 percent in man and 20 percent in women (Table I). Leukemias and lymphomas are responsible for 10 percent of the cancer deaths. The annual mortality of leukemia varies from three to seven per 100,000 in different countries. In the United States the estimated new cases of leukemias for 1987 is 26,000.

Leukemia is not a single disease entity but refers to a heterogenous group of neoplastic disorders involving the cells of the blood-forming organs. The leukemias are usually classified as acute or chronic, based on length of survival and degree of maturation of the cells. They are further subclassed according to the predominant cell line involved. This classification broadly divides them into lymphocytic and myelogenous forms. Acute leukemias are characterized by a predominance of immature myeloid or lymphoid precursors (blasts or blast forms). The blasts progressively replace normal bone marrow, migrate and invade other tissues. In the acute state, production of normal erythrocytes, granulocytes (neutrophils, eosinophils and basophils), and platelets is diminished leading to anemia, infection and hemorrhage. If untreated, the acute leukemias are fatal within a period of twelve months.

Table 1. 1987 estimated cancer incidence and death by site and sex.

Source National Cancer Institutes Surveillance, Epidemiology and End Results (SEER) program.
Normelanoma skincancer and carcinoma in situ have not been included.

	INCIDENCE		DEATH	
SITE	MALE	FEMALE	MALE	FEMALE
Skin	3 %	3 %	2 %	1 %
Oral	4 %	2 %	2 %	1 %
Breast		27 %		18 %
Lung	20 %	11 %	36 %	20 %
Pancreas	3 %	3 %	5 %	5 %
Colon and Rectum	14 %	16 %	11 %	14 %
Ovary		4 %		5 %
Uterus		10 %		4 %
Prostate	20 %		10 %	
Urinary	10 %	4 %	5 %	3 %
Leukemia and Lymphomas	8 %	7 %	9 %	9 %
All other	18 %	13 %	20 %	20 %

The acute leukemias are divided into two major types called acute lymphoblastic leukemia (ALL) and acute nonlymphoblastic leukemia (ANLL). The latter is divided into several subtypes depending on the predominant cell types present. The different subtypes, erythroleukemia, acute monocytic leukemia, acute myelomonocytic leukemia, acute promyelocytic leukemia (APL), acute megakaryoblastic leukemia, acute eosinophilic and acute basophilic leukemia are collectively called acute myelogenous leukemia (AML).

Chronic leukemias are hematologic malignancies in which the predominant leukemic cell will differentiate normally. The chronic leukemias include chronic lymphocytic leukemia (CLL) and chronic myelogenous leukemia (CML). CLL is a malignant hematologic disorder characterized by a persistent, absolute increase in morphologically mature lymphocytes in the peripheral blood and bone marrow. It is a rare disorder in persons less than 30 years of age and gradually increases in incidence with each decade. The median age of CLL patients is 60 years. The median survival of CLL patients varies from 24 months for patients with anemia and thrombocytopenia to eight to ten years for patients whose primary symptoms are lymphocytosis and lymphadenopathy.

CML is a clonal myeloproliferative disorder arising from neoplastic transformation at the level of the pluripotent stem cell. It is characterized by a greatly elevated leukocyte count with all stages of granulocytic development in both peripheral blood and bone marrow. Within a mean of three years after diagnosis, the relatively benign chronic phase of CML gives way to a phase known as blast crisis. CML accounts for 20 to 30 percent of cases of leukemia and can occur at

any age. It is rare in childhood and peaks in the middle 40's.

b) Chronic myelogenous leukemia

Clinical characterization

CML, originally described by Craigie (1854), Bennett (1845) and Virchow (1845), is a hematologic malignancy characterized by excessive growth of myeloid cells and their progenitors (Koeffler and Golde, 1981; Goldman and Lei, 1982). The hallmark of CML is the Philadelphia (Ph') chromosome. This is an acquired chromosomal defect and is recognized by a shortening of the long arm of chromosome 22. Cytogenetic studies and analysis of glucose-6-phosphate dehydrogenase (G-6PD) isoenzyme analysis (Beutler et al., 1962; Lyon, 1962) demonstrated that CML is a clonal disorder of pluripotent hematopoietic stem cells. The Ph' chromosome, which is present in the leukemic cells of over 95 percent of the CML patients (Rowley, 1973; Sandberg, 1980; Heim et al., 1985), has been identified in neutrophils, monocytes, macrophages, erythrocytes, megakaryocytes, eosinophils, basophils and their committed progenitors (for review, Champlin and Golde, 1985; Greaves et al., 1982; Koeffler and Golde, 1981). Reports by Hernandez et al. (1982) and Griffen et al. (1983) of cases with a T lymphocyte blast crisis suggest that the disease may involve a pluripotent cell capable of differentiating to T lymphocytes as well as to other myeloid and lymphoid cells. The Ph' chromosome is not present in bone marrow fibroblast or other mesenchymal tissues (Maniatis et al., 1969).

The clinical features of CML are usually limited to excessive granulocytosis, although in many patient's blood levels of platelets,

monocytes and even lymphocytes are also increased. Untreated patients have a high white blood cell count (WBC) generally peaking at 100 to 400 x 10^9 per liter blood. The red blood cell production is often decreased, resulting in anemia. The patient's principal complaint is fatigue. Abnormal physical findings usually are limited to pallor, splenomegaly and sternal tenderness.

Increase of myelopoiesis appears not to be due to an accelerated proliferative rate (Chervenick and Boggs, 1968), but rather the disease is characterized by massive expansion of pools of committed myeloid progenitors which have a growth advantage over normal bone marrow progenitors (Galbraith and Abu-Zahra, 1972; Moore et al., 1973; Goldman et al; 1980). The proportion of Ph' chromosome-positive cells progressively increases over time to ultimately representing more than 99 percent of dividing bone marrow cells. Philadelphia chromosome-negative cells persist in the bone marrow but this growth is eventually suppressed by the malignant clone (Gupta et al., 1984; Dube et al., 1984).

Myeloid cells mature normally during the chronic phase of CML. There are subtle abnormalities of granulocyte (Broxmeyer et al., 1977; Olofsson et al., 1976) and platelet (Schafer, 1984) function, but these rarely lead to symptomatic complications. The mature cells have usually a decreased leukocyte alkaline phosphatase (LAP). The malignant cells are minimally invasive in the chronic phase and generally remain retracted to the hematopoietic tissues (the marrow and spleen). Release of immature cells to the blood often indicates evolution to the acute phase (Theologides, 1972; Barton et al., 1979).

The chronic phase of CML is unstable. At some point, the disease

undergoes transformation to an aggressive leukemia. Transformation may be clinically manifest as in acute leukemia (acute phase or blast crisis) or by progression of symptoms and resistance to chemotherapy (termed accelerated phase) (Karanas and Silver, 1968). Features associated with transformation include systemic symptoms (fever, sweats, or weight loss) increasing organomegaly, or extramedullary leukemias. The granulocyte and/or platelet count typically becomes less responsive to chemotherapy while the proportion of blasts and promyelocytes increases, often associated with the development of anemia and/or thrombocytopenia. In more than 75 percent of patients, transformation is accompanied by karyotypic evolution with development of abnormalities superimposed upon the Ph' chromosome, most commonly trisomy 8 or, isochromosome 17 or duplication of the Ph' chromosome (Spiers and Baikie, 1968; Rowley, 1975).

Most patients ultimately develop acute phase (blast crisis), in which the disease resembles acute leukemia. The cells no longer differentiate to mature granulocytes; maturation arrest occurs at the level of the blast or promyelocyte (Golde et al., 1974). This phase can be divided into two general forms: lymphoid and myeloid (Rosenthal et al., 1977; Boggs, 1974; Janossy et al., 1979). Lymphoid blast crisis develops in approximately 30 percent of the patients (Catovski, 1979). In this variant, the blast cells are phenotypically similar to the common form of acute lymphoblastic leukemia. Myeloid blast crisis is heterogenous. The blasts appear to be morphologically similar to myeloblasts and express myeloid antigens and cytoplasmic enzymes. Erythroid and megakaryocytic variants of blast crisis also occurs (Bain et al., 1977; Rosenthal et al., 1977).

Several factors present at the time of diagnosis are associated with early transformation to blast crisis, including a high WBC count, a large proportion of immature cells, large spleen or liver size and large number of eosinophils or basophils. Patients with an apparently normal karyotype generally have an extremely poor prognosis compared to patients with the typical Ph′ chromosome abnormality. Patients presenting with other chromosome abnormalities may also have a worse prognosis (Ezdinli et al., 1970; Whang-Peng et al., 1968). After the first year, there is a relatively constant risk of transformation to blast crisis; approximately 25 percent of patients surviving at any point will evolve into blast crisis over the ensuing year. Medium survival is three to four years. Less than 30 percent of patients survive five years (Fialkow et al., 1981).

Treatment

Several different strategies have been developed for the treatment of CML. The treatment of CML is mainly palliative (Koeffler and Golde, 1981; Goldman and Lei, 1982; Wiernik, 1984; Priesler and Raza, 1982), being hampered by the lack of differential sensitivity to chemotherapy between the malignant cells and their normal hematopoietic counter parts (Goto et al., 1982). Treatment with an alkylating agent like busulfan or an anti-metabolite (DNA-synthesis inhibitor) like hydroxyurea is generally effective in controlling the granulocytosis and thrombocytosis. Other therapeutic approaches have included intensive leukapheresis, splenic irradiation and radioactive phosphorus (Reinhard et al., 1959).

Such methods usually suppress the growth of the malignant clone

but fail to eradicate the disease. Patients who achieve "clinical remission", i.e., normal blood cell counts, continue to have predominantly Ph' chromosome-positive cells in the bone marrow. Although chemotherapy will relieve the symptoms of the disease, there is no evidence that treatment with any chemotherapeutic agent delays the development of blast crisis or prolongs patient survival (Minot et al., 1924; Medical Research Council, 1968).

For CML patients in the early benign phase of the disease, recombinant human interferon alpha-A has recently been shown to induce hematologic remission (as reviewed by normalization of the WBC count, normalization of differential cell counts with no immature forms and the disappearance of all clinical symptoms)(Talpaz et al., 1986). Therapy with interferon also appears to result in the suppression of the Ph' chromosome in some of these patients. Yoffe et al. (1987) reported 2 cases where interferon therapy resulted in the restoration of normal bone marrow, whith apparently complete remission monitored, to date, for 6 months in one case and 9 months in the other. Molecular analysis of these patients after treatment, showed (with a probe sensitivity of 5 percent Ph' positive cells) that no Ph' positive cells were present. However, interferon has sometimes strong side effects of fever and malaise. Further studies are needed to assess the effect of interferon on the course of Ph' positive CML and to determine the effect of combining it with other chemotherapeutic and biologic agents that may have antitumor activity in this disease (Kurzrock et al., 1985).

The most encouraging results have been obtained with high dose combination chemo-radiotherapy, followed by syngeneic or allogeneic

bone marrow transplantation. This is the only treatment so far capable of eradicating the leukemic clone. Fever et al. (1982) initially reported prolonged disease-free survival in patients with CML receiving high-dose cyclophosphamide, total body irradiation and syngeneic bone marrow transplants. Approximately 65 percent of patients receiving syngeneic transplants in chronic phase and 20 percent of those treated while in blast crisis have achieved complete remission free of Ph′ chromosome-positive cells and have survived for over five years free of disease.

Similar results have been obtained with allogeneic marrow transplantation with marrow from HLA-identical sibling donors (Goldman et al., 1986). The actuarial two year survival among patients given transplants in the chronic phase of their disease was 72 percent, a figure that contrasts with an actuarial survival of 18 percent among those treated in later phases of the disease. The actuarial risk of relapse at two years in these two categories of patients was 7 and 42 percent, respectively. Unrelated donor bone marrow transplantation is possible when a suitable matched sibling donor is not available and when performed before the onset of advanced disease (Ganesan et al., 1987). Unfortunately, only a minority of patients are currently candidates for bone marrow transplantation as most patients either lack an HLA-identical sibling donor or are too old to be considered.

c) Acute lymphoblastic leukemia

Clinical characterization

Acute lymphoblastic leukemia (ALL) is a hematologic malignant

disease characterized by an uncontrolled proliferation of immature lymphocytes and their progenitors. The fundamental defect appears to be an unregulated proliferation of early precursor cells that have lost their capacity to differentiate. Studies with glucose-6 phosphate dehydrogenase and chromosome markers provided evidence that ALL is a disease in which normal and malignant cells coexist and compete for ascendancy within the bone marrow. This is in contrast to CML, in which only few normal hematopoietic progenitors are detectable at the time of diagnosis. Although ALL is most common in children, a substantial proportion of cases occur in adolescents and adults. The clinical symptoms of ALL relate to decreased numbers of hematopoietic cells. The signs and symptoms may have been present for several weeks to months before diagnosis and are usually nonspecific, consisting of mild fatigue, malaise and anorexia. With the development of anemia, the symptoms generally progress to more marked fatigue and pallor.

Classification

The complex array of hematopoietic differentiation suggests that the acute leukemias are extraordinarily heterogenous. Both morphological and immunological features are used to classify patients with ALL. A French-American-British (FAB) morphologic classification of ALL has been developed that recognizes three types of lymphoblasts termed L1, L2 and L3 (Bennett et al, 1976). The FAB classification is based on a spectrum of cell properties, such as the ratio of nucleus to cytoplasm, the number and size of nucleoli, and the degree of cytoplasmic basophilia. In childhood ALL, 85 percent of patients have L1 morphology. In contrast, adults with ALL have a predominance of the

L2 morphology. The L3 morphology, resembling that in Burkitt's lymphoma is occasionally present in adults with ALL.

A second approach to the classification of ALL is based on the immune features of the leukemic cells, in this system subtypes are termed common, T, B or null phenotypes, based on the detection on the cell surface of the common ALL antigen (a polypeptide with a molecular weight of 100,000), receptors for ovine red blood cells, T-cell antigens, or immunoglobulin molecules, respectively. Null cells have none of these surface features. In children, approximately 75 percent of cases are of the common ALL phenotype which may belong to the B-lymphocyte lineage (Korsmeyer et al., 1983). The remainder consist of T-cell or null cell phenotypes: B-cell ALL accounts for less than 1 to 2 percent of cases. In adults, the distribution of immunophenotypes is significantly different. Only 50 percent of cases are the common phenotype. Null cell ALL accounts for up to 40 percent in some studies, while the incidence of B-cell ALL also increases (Greaves and Lister, 1981). The increased frequency of phenotypically undifferentiated or null-cell ALL in adults is of possible prognostic importance. Recent data suggest that in some cases of null-cell ALL the cells may represent myeloid or hybrid leukemias rather than typical ALL (Desforges, 1981).

Chromosomal abnormalities

Data indicate that up to two thirds of adults with ALL have chromosomal abnormalities, including hypodiploidy, pseudodiploidy, and hyperdiploidy, as well as specific chromosome translocations. Approximately 17 to 25 percent of adults with ALL (LeBeau and Rowley,

1984) and 2 to 6 percent of children with ALL (Chessels et al, 1979; Priest et al, 1980) have the Ph' chromosome. Patients with Ph' positive ALL are heterogenous; up to 20 percent have a disease best characterized as lymphoid transformation of CML (Pittman et al, 1979). These patients may have a remission after treatment with vincristine and prednisone. They, nevertheless, retain the Ph' chromosome while in remission (as do patients with typical CML) and usually relapse within a short time with either lymphoid-blast or, less typically, myeloid-blast crises (Catovsky, 1979). The second group of Ph'-positive patients with ALL are more typically of high-risk ALL. If these patients respond to chemotherapy, the bone marrow in remission is cytogenetically normal. Remission tends to be brief, with bone marrow or central nervous system relapse; myeloid transformation does not occur. Patients with ALL and the Ph' chromosome tend to be younger and have a lower white-cell count than those with lymphoid transformation of CML (Raza et al, 1982). Patients with L3 or the B-cell form of ALL, which is related to Burkitt's lymphoma or leukemia, have a high frequency of the t(8;14) translocation (Zech et al, 1976). Less commonly, translocations involving chromosome 8 and either 2 or 22 are found (t(2;8) and t(8;22)) (Van den Berghe et al, 1979). Other even less frequent aberrations found in ALL are t(4;11), a deletion of the long arm of chromosome 6(6q-) and trisomy of chromosome 12(12+) (Jacobs and Gale, 1984). The result of cytogenetic studies suggest that ALL patients with chromosomal abnormalities have a worse prognosis. For example, over 70 percent of adults with a normal karyotype have remission with conventional chemotherapy, as compared with only 45 percent of those with chromosomal abnormalities (Third International

Workshop, 1983). Abnormalities associated with a poor prognosis include t(9;22), t(4;11) and t(8;14). In contrast, hyperdiploidy and 6q- are both associated with a relatively good prognosis.

Treatment

The treatment of ALL is generally divided into two phases, called induction and continuation. The latter is subdivided into consolidation or intensification, and maintenance. The objective of induction chemotherapy is to achieve remission, i.e., the eradication of leukemia that is detectable by conventional techniques. Consolidation or intensification refers to the use of high-dose chemotherapy in patients who are in remission. These are aimed at completely eliminating residual clinically undetectable leukemia and preventing relapse and the emergence of drug resistant cells. Maintenance usually involves less intensive chemotherapy. Its purpose is to reduce the leukemia cell burden further.

Approximately 50 percent of patients less than 15 years of age with ALL are experiencing long-term leukemia-free survival. This success has evolved during the past two decades by improving the rate of induction of complete remission, establishing the necessity for continued combination chemotherapy during remission with maximally tolerated doses of drugs, and prophylactically treating the central nervous system.

The combination of vincristine and prednisone induces complete remission in more than 90 percent of pediatric patients. These drugs result in minimal suppression of normal regenerating bone marrow and allow for rapid induction of remission with minimal morbidity.

Patients more than 15 years of age and rare individuals with morphologically undifferentiated leukemia remain relatively resistant to this induction therapy. The addition of L-asparaginase or an anthracycline, such as daunomycin or adriamycin, may increase the proportion of patients who enter remission and may also result in lower relapse rates.

Methotrexate and 6-MP have been the most effective agents for prolonging hematologic remission in ALL. Treatment in remission with drug combinations different from those employed in induction has become a standard part of the complete program. Combined with central nervous system prophylaxis it has, in the last decade, produced apparent cures in about 50 percent of children with ALL. Duration of maintenance therapy has varied from 2 1/2 to 5 years.

Recent advances in understanding the biology and therapy of ALL have had a considerable effect on survival in this heterogeneous group of diseases; long term disease free survival rates of 50 to 75 percent are now observed in patients who receive chemotherapy (George et al., 1979; Nesbit et al., 1983; Niemeyer et al., 1985; Hoelzer et al., 1984). Some patients who have a first remission lasting more than 18 months will have a prolonged second remission by chemotherapy alone (Rivera et al., 19861; Chessels et al., 1986). Chemotherapy followed by bone marrow transplantation from matched sibling donors has been used successfully in several centers to treat patients with leukemia in whom chemotherapy fails or who are at very high risk for relapse after primary therapy (Chessels, 1986; Johnson et al., 1981; Dinsmore et al., 1983; Blume et al., 1980). In the 60 to 70 percent of patients that do not have a matched allogeneic marrow donor, autologous marrow

transplantation has been used (Ritz et al., 1982; Ramsay et al., 1985). The reports by Kersey et al. (1987) are encouraging, suggesting that in high risk group patients with a first remission lasting less than 18 months, long-term survival can be obtained.

III. Oncogenes, Mechanism of Activation and Biochemical Function

a) Oncogenes

The search for genetic damage in neoplastic cells now occupies a central place in cancer research. The belief that genetic damage might be responsible for cancer is based on a number of different findings: the recognition of hereditary predispositions to cancer (Lynch, 1976; Schimke, 1978; Ponder, 1980), the detection of damaged chromosomes in cancer cells (Rowley, 1983, 1984; Yunis, 1983), the apparent connection between susceptibility to cancer and impaired ability of cells to repair damaged DNA (Lehmann, 1982; Hanawalt and Sarasin, 1986), and evidence that relates the mutagenic potential of substances to their carcinogenicity (Ames, 1979). The discovery of cellular genes (proto-oncogenes) that in another form (oncogenes) can be clearly demonstrated to induce neoplastic growth implicated DNA in the genesis of human tumors.

To date more than 50 (proto) oncogenes (Table 2, Figure 1) have been identified. Several of the oncogenes have been associated with specific forms of human cancer (Bishop, 1983, 1985). They are believed to act individually or in concert; the interaction of oncogene-encoded proteins possibly accounts for the multi-stage nature of cancer.

Table 2. Proto-oncogenes.

PROT ONC is proto-oncogenes; MODE DISCOV is mode of discovery; V is homologue of v-onc, I is target of proviral or transposon like element insertion, C is involved in chromosomal translocation, A is as amplified DNA, T is a gene in DNA mediated gene transfer; CHROM LOC is chromosome location; BIO FUNC is putative biological function, 1 is tyrosine specific protein kinase, 2 is serine/threonine specific protein kinase, 3 is protein involved in control of gene expression and/or participation in the replication of DNA, 4 is G or N like protein, 5 is growth factor, 6 is receptor, 7 is protein with unknown function.

PROT ONC	MODE DISCOV	CHROM LOC	BIO FUNC	PROT ONC	MODE DISCOV	CHROM LOC	BIO FUNC
abl	V C A	9 (q34)	1	c-myc	V I C A	8(q24)	3
akt	V	14 (q32)	7	L-myc	A	1(p32)	3
arg	V	1 (q24-q25)	1	N-myc	A	2(p23-p24)	3
bcl-1	C	11 (q13)	7	p53	I	17(p13)	3
bcl-2	C	18 (q21)	7	phl	C	22(q11)	2
B-lym	T	1 (p32)	7	pim-1	I	6(p21)	2
dbl	T		7	pvt-1 (mis-1)	I C		7
erbA	V	17 (q11-q12)	3	raf-1 (mil,mht)	V I T	3(p25)	2
erbB-1	V I	7 (p12-p14)	1	raf-2 (ψ)	V	4	2
erbB-2(neu)	V A T	17 (q12-q22)	1	A-raf-1(c-pks)	V	X(p11.4)	2
ets-1	V	11 (q23)	3	A-raf-2	V	7(p14-q21)	2
ets-2	V	21 (q22)	3	H-ras-1	V I T	11(p15)	4
fgr (src-2)	V	1 (p36)	1	H-ras-2 (ψ)	V	X	4
fms	V	5 (q33)	1	K-ras-1(ψ)	V	6(p23-q12)	4
fos	V	14 (q21-q31)	3	K-ras-2	V I A T	12(p12.1)	4
fps (fes)	V	15(q26)	1	N-ras	A T	1(p22/p11-p12)	4
fyn	V		1	rel	V	2(p11-p12)	2
hck	V		1	ret	T		1
hst	T		5	ras (mcf-3)	V T	6(q16-q22)	1
int-1	I	12(pter-q11)	5	sea	V		1
int-2	I	11(q13)	5	sis	V	22 (q13.1)	5
int-3	I		5	ski	V	1 (q22-qter)	3
jun (AP-1)	V		3	src-1	V	20 (q13.3)	1
kit	V	4	1	tck	I		7
lck (p56)	V		1	tcl-1	C	14(q32.3)	7
lyn	V		1	tcl-2	C	11 (p13)	7
mas	T		6	αTGF		2 (p13)	5
mcf-2	T	X (q27)	7	tkns	C		7
mel	T	19	7	T-lym	T		7
met	T	7 (q21-q31)	1	trk (onc D)	T	1(q32)	1
mlvi-3	I		7	tx1-4	T		7
mos	V I T	8(q22/q11)	2	yes-1	V	6	1
myb	V I A	6(q22-q23)	3	yes-2	V	18(q21)	1

Oncogenes are present in the human genome as normal cellular genes (proto-oncogenes) with normal cellular functions. Proto-oncogenes have been highly conserved throughout vertebrate evolution suggesting that they play a critical role in cellular metabolism or at particular developmental stages.

Activation to oncogene activity can result from either qualitative or quantitative changes in the protein product encoded by an altered proto-oncogene. The activation could be caused by the expression of a normal proto-oncogene product in the wrong tissue or at the inappropriate development stage. The cellular processes affected by the conversion of a proto-oncogene into a transforming activated oncogene are not fully understood.

b) Mode of discovery

Viral transduction

Cellular oncogenes were first identified as the homologues (c-onc) of the transforming genes of acute RNA tumor viruses (Bishop, 1983; Varmus, 1982, 1983). In contrast to the slowly transforming RNA tumor viruses, these acute transforming RNA tumor viruses were found to contain extra host cell derived sequences, responsible for the acute form of transformation. Typically, the viral oncogenes were found to represent truncated and/or mutated forms of normal cellular genes (Bishop, 1983; Duesberg, 1983). The genesis of retroviral oncogenes from cellular proto-oncogenes has been called transduction.

Two different possibilities have been proposed to explain why the transduced oncogenes of retroviruses are pathogenic, even though they derive from seemingly harmless cellular genes. First, expression of

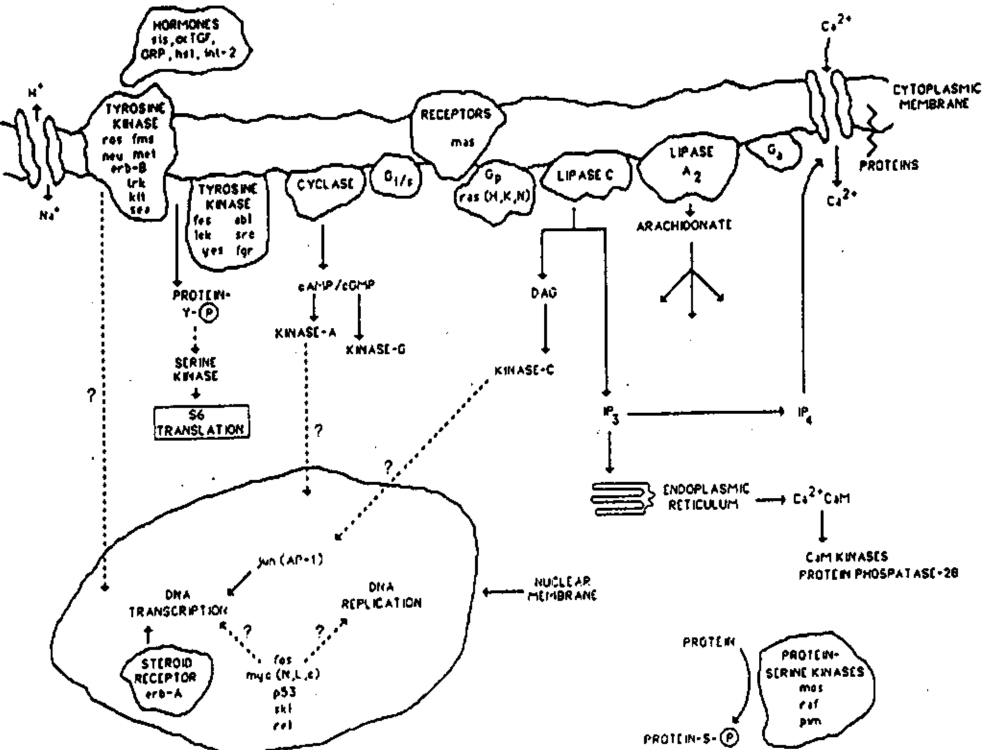

Figure 1. Functional characterization of cellular oncogenes.

the transduced genes is driven by potent viral regulatory elements. Thus sustained and abundant expression of an otherwise normal gene might cause neoplastic growth. Efforts to test this possibility have given ambiguous results and remain incomplete. When expressed at high levels by experimental means, most of the proto-oncogenes tested to date can transform established lines of cells but not primary explants of normal tissues. There may be exceptions in both regards: the proto-oncogene c-src has not elicited a completely neoplastic phenotype in established cells (Iba, et al, 1984; Parker et al., 1984; Johnson et al., 1985), whereas over expression of the normal c-myc and c-H-ras proto-oncogenes transform primary as well as established cultures (Baumbach et al., 1986; Martin et al., 1986; Patchinsky et al., 1986; Cichutek and Duesberg, 1986).

Second, transduced genes generally acquire mutations while en route from proto-oncogene to oncogene (Bishop, 1983; Varmus, 1984). Comparisons of retroviral oncogenes with their cellular progenitors have uncovered point mutations, deletions and genetic substitutions in the viral alleles. In two instances, this genetic damage appears to release the biochemical activities of the gene products from allosteric controls. Firstly, the mutations in v-src (Coussens et al., 1985; Iba et al., 1985; Cooper et al., 1986, Levy et al., 1986) and v-erb B (Gilmore et al., 1985; Kris et al., 1985; Lax et al., 1985) confer higher constitutive activity on the protein-tyrosine kinases encoded by these genes (they might also alter substrate specificities of the enzymes, but there is no evidence for this as yet). Secondly, mutations present in the oncogenic versions of c-ras reduce the GTPase (GTP is guanosine 5′ triphosphate) activity compared to the activity of the

normal c-ras protein. Since the GTP-ras complex is the active version, this could imply that the oncogenic version may transmit a continuous signal rather than a regulated, transient one (Gibbs et al., 1984; McGrath et al., 1984; Sweet et al., 1984; Manne et al., 1985; Colby et al., 1986; Levinson, 1986). Transformation by these retroviral oncogenes may therefore result from sustained levels of otherwise normal biochemical activities.

Insertional mutagenesis

A second mechanism of activation is called insertional mutagenesis. Integration of viral DNA into genomic DNA can influence expression of cellular genes by bringing them under control of powerful regulatory elements in the viral genome (Varmus, 1982; Nusse, 1986) or damage them directly (Bishop, 1983; Varmus 1982; Varmus, 1983). Besides previously identified cellular oncogenes like c-myc (Hayward et al., 1981; Payne et al., 1982; Corcoran et al., 1984), c-mos (Rechavi et al., 1982; Cohen et al., 1983, Gattoni-Celli et al., 1983); c-myb (Shen-Ong et al., 1984; Rosson and Reddy, 1986) and c-erbB (Fung et al., 1983; Raines et al., 1985), other cellular genes can serve as targets for activation by proviral or transposon-like integration, like int-1 (Nusse and Varmus, 1982; Nusse et al., 1984), pim-1 (Cuypers et al., 1984; Selten et al., 1985) or Mvli-1 (Tsichlis et al., 1983).

There is as yet no direct evidence that activation of proto oncogenes by insertional mutagenesis is tumorigenic, but the argument nevertheless has considerable logical force. First, several of the genes attached by integration are known to be progenitors of retroviral oncogenes. Second, integration of retroviral DNA displays little (if

any) specificity within the cellular genome, yet integration in the tumors affects specific proto-oncogenes, presumably the consequence of selection for cells that have undergone neoplastic transformation.

Third, retroviral vectors have been used to demonstrate that such mutant proto-oncogenes have transforming activity. Examples include c-myc and c-erbB (Baumbach et al., 1986; Martin et al., 1986; Patschinsky et al., 1986; Keath et al., 1984); and the proto-oncogene int-1, first identified at the integration site of MMTV (Brown et al., 1986).

Translocation

Cancer cells have provided clues to oncogenes in the form of microscopically visible damage to chromosomes. Three types of damage can be recognized: translocations between (or inversion within) chromosomes, deletions affecting discrete portions of chromosomes, and abnormal amplifications of large domains within chromosomes. Deletions on occasion may signal the existence of a different type of genetic element the so called "anti-oncogenes".

Specific translocations have been shown to affect known c-oncogenes such as c-myc and c-abl in the Burkitt's lymphoma and chronic myelogenous leukemia specific translocations respectively, or lead to the identification of new putative c-oncogenes as bcl-1 and bcl-2 (Nowell et al., 1984; Cleary and Sklar, 1985) or pvt-1 (Webb et al., 1984; Cory et al., 1985). Like c-myc these latter genes are joined to the immunoglobulin genes following translocation.

Although karyotypic instability of cancer cells is commonplace, and could represent effect rather than cause, several observations suggest that the translocation of proto-oncogenes plays a crucial role

in tumorigenesis. Some translocations occur with great consistency in particular tumors (Rowley, 1983; 1984; Yunis, 1983) and can affect the same proto-oncogene in different species, for example, c-myc in B cell tumors (Varmus, 1984). Three of the proto-oncogenes first recognized during the study of retroviral oncogenes (c-abl, c-ets and c-myb) have now been implicated in translocations that exemplify various forms of malignancy (Klein, 1983; Leder et al., 1983; Nowell et al., 1985; Shtivelman et al., 1985; Bartram et al., 1983; Groffen et al., 1984; Stam et al., 1985; Grosveld et al., 1986; Rovigatti et al., 1986; Sacchi et al., 1986). Translocation of a proto-oncogene can damage both the structure and the function of the gene in ways that echo those found in the transduced and overtly oncogenic version of the same gene (Davis et al., 1985). Finally, mice carrying a translocated c-myc gene in their germinal DNA develop lymphoid tumors (Adams et al., 1985).

Amplification

Amplification of proto-oncogenes has been shown in two patterns: as an occasional feature of diverse tumors (Varmus, 1984; Alitalo and Schwab, 1986) and as a recurrent abnormality of specific proto-oncogenes in particular tumors (Kohl et al., 1983; Little et al., 1983; Schwab et al., 1983b, 1984, 1985a; Schwab, 1985; Nau et al., 1985, 1986; Escot et al., 1986; Wong et al., 1986; Libermann et al., 1985; Yamamoto et al., 1986). Investigation of amplified DNA sequences in tumor cells showed the involvement of previously identified oncogenes: c-myc (Alitalo et al., 1983) and K-ras (Schwab et al. 1983a) and has also lead to the discovery of new, related oncogenes: N-myc (Schwab et al., 1983b) or L-myc (Nau et al., 1985).

There are three reasons why amplification may be of significance in cancer cells. First because amplification frequently affects known proto-oncogenes (Varmus, 1984; Schwab et al., 1985b; Yancopoulos et al. 1985; Alitalo and Schwab, 1986), secondly, the amplification of a proto-oncogene sometimes correlates with a particular feature of cancer cells, as if one were cause and the other effect (Winter and Perucho, 1986), and thirdly, because amplified DNA persists in mammalian cells only if it provides a selective advantage to the cells (Stark and Wahl, 1984; Schimke, 1984), it must be necessary in the cancer cells, where it has survived countless rounds of cell division.

Transfection

DNA-mediated gene transfer, led to the characterization of transforming genes present in tumor cells, competent to transform appropriate recipient cultured cells, e.g. the mouse fibroblast cell line NIH-3T3 (Cooper et al., 1980). Approximately 20 percent of the DNA's extracted from primary human tissue or human tumor cell lines were able to induce focus formation in cultures of this immortalized, rodent cell line. The isolated transforming genes are generally mutated members of the _ras_ gene family: H-_ras_, K-_ras_ or N-_ras_ (Cooper, 1982; Der et al., 1982; Shimizu et al., 1983; Hall et al., 1983; Cooper and Lane, 1984). Despite obvious limitations, probably due to the recipient cell line used, occasionally new and often truncated transforming genes have been isolated like _met_ (Cooper et al., 1984; Dean et al., 1985; Park et al., 1986), _trk_ (Martin-Zanca et al., 1986), _B_- and _T-lym_ (Lane et al., 1982) or _neu_ (Schechter et al., 1984; 1985; Bargmann et al., 1986a, 1986b).

c) Biochemical function

Although a variety of proto-oncogenes and oncogenes have been discovered, only four biochemical mechanisms have been identified by which this rich diversity of proteins may act; control of gene expression by influencing the biogenesis of mRNA (Kingston et al., 1984, 1985; Setoyama et al., 1986); participation in the replication of DNA (Studzinski, 1986); metabolic regulation by proteins that bind GTP in the manner of the familiar G or N proteins (Hurley et al., 1984); and protein phosphorylation with either tyrosine, serine or threonine as substrate amino acids (Hunter and Cooper, 1985). For a more detailed review of the different mechanisms see Weinberg (1985), Bishop and Varmus (1985) and Bishop (1985).

The oncogene products of fos, myc, myb, ski, p53, erb-A and jun are all found in the nucleus of the cell (Klempnauer et al., 1984; Verma et al., 1984; Bishop, 1985; Weinberger, et al, 1986; Struhl, 1987) and are implicated in control of gene expression and replication of DNA. The exact mechanisms by which these proteins induce transformation is not known.

Activation of ras oncogenes in human tumors is most commonly due to point mutations in the ras coding sequence (Bos et al., 1985; Fasano et al., 1984; Reddy et al., 1982; Tabin et al. 1982). Activated ras oncogenes are found in 10-20% of human tumors and have been detected in carcinomas, sarcomas, and hematopoetic malignancies (Der et al., 1982; Eva et al., 1983; Pulciani et al., 1982). Mammalian ras gene products are associated with the inner face of the plasma membrane, bind GTP, and exhibit a low GTPase activity (Sefton et al., 1982; Sweet et al.,

1984). On the basis of sequence homology and biochemical properties, it has been suggested that _ras_ proteins may be members of the G protein family that serve to transduce information from cell surface receptors to internal effector molecules in a variety of systems (Gibbs et al., 1984; Hurley et al., 1984), e.g. hormonal regulation of adenylate cyclase (Gilman, 1984) and phosphoinositide turnover (Berridge and Irvine, 1984). Although there are few data concerning the normal function of p21 _ras_ in mammalian cells, in yeast, one role of the _ras_ protein may be to integrate nutritional information with intracellular cAMP levels via the adenylate cyclase system (Fraenkel, 1985; Toda et al., 1985). The GTPase activity of the oncogenic versions of p21 _ras_ is considerably reduced compared to the normal product (Gibbs et al., 1984; Sweet et al., 1984). Since the GTP-G protein complex is the active version of the G protein family, this could imply that the oncogenic version of p21 may transmit a continuous signal rather than a regulated, transient one. In the case of adenylate cyclase, this would lead in to activition of the cAMP-dependent protein kinase and would thereby increase the phosphorylation state of its protein substrates.

IV. Protein Kinases

For nearly thirty years phosphorylation has been recognized as a means of reversibly modulating the function of proteins. Over this period many protein kinases specific for serine and threonine residues in their substrates have been identified and characterized. The first protein kinase to be purified was phosphorylase kinase in 1959 (Krebs

et al., 1959) followed in 1968 by the cAMP-dependent protein kinase (Walsh et al., 1968). Only recently have enzymes with specificity for tyrosine been reported. Tyrosine phosphorylating activity was originally detected in partially purified preparations of two viral transforming proteins (Eckhart et al., 1979; Witte et al., 1980). Since that time it has become clear that there is a large family of protein tyrosine kinases (Table 3). The group of oncogenic protein kinases account for approximately one third of all known oncogene products (Bishop, 1985, 1987, Table 2). Eight such cellular enzymes were first recognized in altered guises as parts of the transforming proteins of a series of acutely oncogenic retroviruses; these are v-src, v-yes, v-fgr, v-fps/fes, v-abl, v-ras, v-erbB, and v-fms. Some tyrosine kinases are growth factor receptors. The proto-oncogenes c-mos, c-raf, A-raf, pks and pim-1 show homology with protein serine/threonine kinases.

All protein kinases have a striking sequence similarity in their catalytic domains. The first demonstration of this similarity came when the cAMP-dependent protein kinase subunit was shown to be related to pp60v-src over a region of about 300 amino acids (Barker and Dayhoff, 1982) an unexpected finding given the different amino acid specificities of these enzymes. This relatedness implies that the cAMP-dependent protein kinase and pp60c-src are descended from the same ancestral protein kinase. Based on sequence similarities, it appears that most if not all eukaryotic protein-serine/threonine and protein-tyrosine kinase genes arose from a single archetypal gene.

A comparison of a large number of catalytic domains reveals a series of short sequence motifs that are highly conserved. Starting

Table 3. Protein kinases.

PROTEIN-SERINE/THREONINE KINASES	PROTEIN-TYROSINE KINASES
Cyclic nucleotide - regulated:	src gene family:
cAMP-dependent protein kinases (Cα, Cβ)	c-src (fibroblast, neuronal forms)
cGMP-dependent protein kinase	c-yes, lck
Calmodulin-regulated:	fgr, hck, fyn, lyn proteins
Phosphorylase kinase	abl gene family:
Myosin light chain kinases (skeletal, smooth muscle)	c-abl (Type 1A and 1B N terminus)
Type II - calmodulin dependent protein kinase	arg-protein
(brain α, β, β' subunits; liver α, α' subunits; muscle β, β' subunits)	fps gene family:
Calmodulin-dependent protein kinases I and II	c-fps
Diacylglycerol - regulated:	NCP 94
Protein kinase Cs (α, β and β', γ, δ)	c-fps related protein (TKR 11 and TKR 16)
Others:	EGF receptor family:
Casein kinases I and II	EGF receptor (c-erbB protein)
Nuclear protein kinases N1 and N2	neu protein (erbB2 protein)
Protease - activated kinases I and II	Insulin receptor family
Glycogen synthase kinases 3 and 4	Insulin receptor
Heme - regulated protein kinase	IGF-1 receptor
Double-stranded RNA regulated protein kinase	c-ros, met, trk proteins
Double-stranded DNA regulated protein kinase	PDGF receptor family:
S6 kinase	PDGF receptor
β - adrenergic receptor kinase	CSF-1 receptor (c-fms protein)
Rhodopsin kinase	c-kit protein
Histone H1 kinase	c-sea, ret proteins
Hydroxymethylglutaryl - CoA reductase kinase	Others: p 75 (liver)
Pyruvate dehydrogenase kinase	p 120 (brain)
Branched chain ketoacid dehydrogenase kinase	
Polypeptide - dependent protein kinase	
Polyamine-stimulated protein kinase	
c-mos, c-raf, A-raf, pks, pim-1 proteins	
CDC-R (PSK-J3), CDC2Hs, PSK H1, PSK-C3	

from the N-terminus, these include Gly-X-Gly-X-X-Gly followed by a Lys 15-20 residues downstream both of which form part of the ATP binding site. At a distance varying from 80-180 residues further C-terminal, a second conserved region is observed, consisting of a sixty amino acid region which contains the sequences Arg-Asp-Leu, Asp-Phe-Gly and Ala-Pro-Glu (Hunter and Cooper, 1985, 1986). Although the protein-serine/threonine kinases and protein-tyrosine kinases are closely related, they are distinguished from one another by a number of primary sequence features in their catalytic domains. Among these is the region between the Asp-Phe-Gly and Ala-Pro-Glu elements, which contains the major autophosphorylation site. The existence in this region of a Tyr surrounded by acidic amino acids is, so far, a perfect prediction that a protein will be a protein-tyrosine kinase (Hunter and Cooper, 1986).

The screening of cDNA and genomic libraries with different probing techniques revealed new protein kinases in the src gene family (Semba et al., 1986; Kawakami et al., 1986, Quintrell et al., 1987; Yamanashi et al., 1987; Ziegler et al., 1987), abl and fps gene family (Kruh et al. 1986; Foster et al., 1986) and new members of the protein serine/threonine family (PSK-J3, PSK-HI, PSK-C3, Hanks, 1987). In addition, well characterized protein kinases that were thought to be single catalytic entities proved to be encoded by multiple genes.

Four mammalian protein kinase C genes have been identified, one of which gives rise to two alternately spliced mRNAs encoding proteins with different C-termini; this places at least five distinct protein kinase isozymes in this subfamily (Coussens et al., 1986; Knopf et al., 1986; Ono et al., 1986a, 1986b; Parker et al. 1986; Housey et al.,

1987; Ohno et al., 1987). Likewise, the cAMP dependent protein kinase, although known to have multiple regulatory subunits, was believed to have a single type of catalytic subunit. Now it has emerged that there are at least two genes encoding different, although highly related, catalytic subunits (Uhler et al., 1986; Showers and Maurer, 1986). The generation of multiple protein kinases from a single gene by alternate splicing, exemplified by protein kinase C, is also found with the c-abl gene, whose products can have one of two different N-termini (Ben-Neriah et al., 1986a; Shtivelman et al., 1986a), as well as the src gene, which encodes two forms of pp60 c-src differing by the presence or absence of an internal amino acid exon (Martinez et al., 1987).

Although protein-serine kinase activity has been shown in prokaryotes, protein-tyrosine kinase activity has not yet been demonstrated. Protein phosphorylation seems to have become common in parallel with the increase in complexity of eukaryotic organisms. In yeast a total of 14 serine/threonine kinases but no tyrosine-kinases have been discovered. This is in contrast to Drosophila were at least 9 protein tyrosine-kinases have been found (Hunter, 1987b). Probably, the first protein kinase originated from a nucleotide binding protein and had specificity for serine and/or threonine, subsequently a protein-tyrosine kinase evolved. The expansion in the number of protein tyrosine kinase genes may have coincided with the evolution of multicellular differentiated organisms. Many protein tyrosine kinases serve as receptors and signal transducers for circulatory peptide hormones and growth factors, a function that is largely unnecessary in unicellular organisms. The existence in mammals and Drosophila of so many growth factor receptor protein tyrosine kinases implies that

tyrosine phosphorylation plays an important role in mitogenic response pathways. However, the fact that both in mammals and in Drosophila several of the protein-tyrosine kinases are expressed at the highest levels in terminally differentiated neural tissue suggest that specific types of protein phosphorylation are essential for the functioning of differentiated as well as growing cells.

Hunter (1987b) compares protein kinases with sophisticated transistors in electronic circuits. Protein kinases can be envisaged as components of biological feedback and amplification pathways, as well as switching or signalling systems, serving as major components of the essential regulatory circuitry of the cell. Their activity can be regulated positively or negatively by several types of input such as specific ligands or by phosphorylation of the protein kinases themselves. Most protein kinases have multiple substrates as outputs.

For the cell to use protein phosphorylation as a regulatory mechanism, this modification must be reversible. Dephosphorylation is brought about by protein phosphatases. It has yet to be shown whether the protein phosphatases are as diverse as the protein kinases or whether fewer phospatases with broad specificity will suffice. To date, little is known about phosphotyrosyl-protein phosphatases, but there appears to be multiple forms, all of which are distinct from the better characterized phosphoseryl-phosphothreonyl phosphatases (Foulkes, 1983).

The discovery of a protein kinase activity associated with pp60 v-src (Collett and Erikson, 1978), the product of the Rous sarcoma virus (RSV) src gene, suggested that transformation might be due to aberrant protein phosphorylation events which would modulate the functions of

critical cellular proteins. Abnormal patterns of tyrosine phosphorylation are manifest in cells transformed by the viral protein-tyrosine kinases. However, little real progress in identifying cellular proteins phosphorylated on tyrosine have been made. First it has been difficult to demonstrate that cellular proteins phosphorylated on tyrosine in transformed cells are direct substrates of the kinase. Second, it has not yet been possible to firmly establish a causal relationship between tyrosine phosphorylation of any particular cellular protein and cell transformation. This raises the possibility that many of the known or suspected cellular substrates could be the result of simply adventitious phosphorylation with no physiological relevance.

Although the exact mechanism through which phosphorylation is involved in cell transformation is unknown, the correlation between protein-tyrosine kinase activity of certain oncogene products and their ability to transform cells is significant. Mutation experiments with v-abl and v-src/c-src have shown that tyrosine kinase activity is necessary for transformation (Prywes et al., 1983, 1985; Coussens et al., 1985; Hanafusa et al., 1984; Levy et al., 1986; Jove and Hanafusa, 1987). Overexpression of c-erbB-2 has been reported in approximately 30 percent of human breast carcinomas (Slamon et al., 1987; Venter et al., 1987; Kraus et al., 1987) and activated c-abl proteins are present in the leukemic cells of Ph' positive CML and ALL patients.

V. The Ph′ Chromosome

The Ph′ chromosome or $22q^-$ is a cytogenetic abnormality usually resulting from a reciprocal translocation t(9;22) (q34; q11). This marker chromosome is found in the leukemic cells of more than 95 percent of patients with chronic myelogenous leukemia (Rowley, 1973; Heim et al., 1985). A Ph′ chromosome indistinguishable by cytogenetics is observed in the leukemic cells of about 17 to 25 percent of adults with a suspected diagnosis of ALL (Le Beau and Rowley, 1984). A lower incidence has been reported for childhood ALL (2 to 6 percent) (Chessels et al., 1979; Priest et al., 1980) and AML (less than 1 percent, Abe and Sandberg, 1979; Yunis et al., 1984). The presence of the Ph′ chromosome in CML usually means a better prognosis than when the Ph′ chromosome is absent (Ezdinli et al., 1970; Pugh et al., 1985; Travis et al., 1986). However, Ph′-positive ALL patients have a worse prognosis than the Ph′-negative ALL patients (Bloomfield et al., 1986; Secker Walker, 1984).

The Ph′ chromosome is the result of either a standard t(9;22) translocation (90 percent of the CML cases) (Rowley, 1973) or a variant translocation (3 to 8 percent, Heim et al., 1985). A minority of the CML cases (3 to 7 percent) are without a Ph′ chromosome (Ph′-negative CML, Rowley and Testa, 1982). The standard Ph′ translocation is the result of reciprocal exchange between chromosome 9 and 22, shown by de Klein et al. (1982) and Bartram et al. (1983) with the help of _in situ_ hybridization. The translocation juxtaposes c-_abl_ proto-oncogene sequences on chromosome 9 (Heisterkamp et al., 1982, 1983b; de Klein et al., 1982) with a gene of unknown function denoted _phl_, on chromosome

22 (Groffen et al., 1984; Heisterkamp et al., 1985). The translocation breakpoints are within a 5.8 kilobase (kb) region of phl, designated bcr (breakpoint cluster region) (Groffen et al., 1984). Transcription of the phl/c-abl gene gives rise to a spliced 8.5 kb mRNA (Stam et al., 1985; Shtivelman et al., 1985, Heisterkamp et al., 1985; Grosveld et al., 1986) encoding a fusion protein of relative molecular mass 210,000 called P210 phl/c-abl (Ben-Neriah et al., 1986b; Stam et al., 1987).

Molecular analysis of Ph'-positive ALL reveals two classes of rearrangements involving c-abl. One group (designated Ph'-positive, bcr-negative) comprises phl/c-abl translocations indistinguishable from those found in CML, as shown by chromosomal breakpoints within bcr and synthesis of the P210 phl/c-abl fusion protein. The second group (designated Ph'-positive, bcr-negative) has breakpoints lying outside of the bcr region (Rodenhuis et al., 1985; Erikson et al., 1986; de Klein et al., 1986b) but still in the phl gene (Hermans et al., 1987). The Ph'-positive, bcr-negative ALLs are associated with the transcription of a 7.0 kb chimeric mRNA (Hermans et al., 1987) and expression of a P190 c-abl protein (Clark et al., 1987; Chan et al., 1987; Kurzrock et al., 1987; Hermans et al., 1987). Sequential immunoprecipitation with antisera against abl and phl peptides confirmed that the P190 is a fusion protein consisting of phl and abl sequences (Walker et al., 1987). Very occasionally, CML patients positive for the Ph' chromosome but negative for bcr have also been reported (Leibowitz et al., 1985; A. Hermans, personal communication). It is not known whether these patients have a breakpoint in the phl gene.

Variant translocations have been found in CML, ALL and in AML,

although the incidence of variant translocations in ALL is higher (Whang-Peng and Knutsen, 1982; de Klein et al., 1986b). Three forms of cytogenetic variants have been reported in CML: firstly; simple translocations between chromosome 22 and another chromosome, without visible involvement of chromosome 9, secondly; complex translocations involving 9, 22 and at least one other chromosome (in this case the third chromosome is recipient of the deleted part of $22q^-$, while 9 is recipient of the deleted part of the third chromosome). Thirdly; a rare type of variant called masked Ph′ chromosome where the Ph′ chromosome is the recipient of a part of another chromosome (de Klein and Hagemeyer, 1984). Studies showed that the c-abl oncogene is consistently translocated to the proximal (5′) phl sequences on the Ph′ chromosome. This is also the case in patients with a simple translocation variant where there was no visible alteration of chromosome 9 (Bartram et al., 1983; Hagemeyer et al., 1984; Hagemeyer et al., 1985). Simple variant translocations may be complex variant translocations affecting the terminal bands of the chromosomes (Mittelman and Levan, 1981; Heim and Mittelman, 1987). The small fragments involved masks the 9q34 involvement.

A consistent fusion of c-abl and phl sequences is not only found in variant translocations but also in the leukemic cells of some Ph′-negative CML patients (Bartram et al., 1985; Morris et al., 1986; Ganesan et al., 1986; Kurzrock et al., 1986). Clinical and hematological features of Ph′-negative CML patients are indistinguishable from Ph′-positive CML patients. This is in contrast to the Ph′-negative CML patients who lack the phl/c-abl juxtaposition and probably represent myelodysplasia and reactive myeloproliferative

conditions other than CML, with an overall poorer prognosis (Pugh et al., 1985; Travis et al., 1986). Applying a strict set of clinical and hematological criteria, Wiedemann et al. (submitted) defined bcr positive CML as chronic granulocytic leukemia (CGL). Other myeloproliferative disorders including atypical CML (aCML), chronic myelomonocytic leukemia (CMML), juvenile CML, (JuCML) and chronic neutrophilic leukemia (CNL) do not express elevated levels of abl protein tyrosine kinase activity and therefore must rely on other mechanisms of leukemogenesis.

Approximately 75 to 80 percent of chronic phase CML patients develop additional aberrations when entering the acute, blastic phase (Alimena et al., 1979; First International Workshop, 1978; Lawler, 1982; Mittelman et al., 1976; Rowley, 1980; Sandberg, 1980a, 1980b). Chromosomes 8, 17 and 22 are those most often involved in secondary changes, with the frequent occurrence of trisomy 8, isochromosome 17, an extra Ph' chromosome, and less frequent trisomy 19 (Mittelman et al., 1976; Heim and Mittelman, 1987). The extra Ph' chromosome is the result of a second translocation in a different hematopoietic stem cell as described for a patient by Shtalrid et al. (submitted), or is the result of a duplication of the primary Ph' chromosome (Collins, 1986; Selden et al., 1983; Grosveld et al., 1986). The pattern of secondary changes can be found both in the CML patients with standard and variant translocations. In CML patients without the Ph' chromosome trisomy 8 and isochromosome 17 are the most frequent cytogenetic hallmarks. Trisomy 19 is less frequent, while rearrangements of chromosome 3 and monosomy 7 seem to be common changes in Ph'-negative CML (Heim and Mittelman, 1987). The latter feature is reminiscent of the chromosome

7 involvement in acute t(9;22) associated leukemias (Heim and Mittelman, 1986). The absence of trisomy 8 and 19 and a second Ph' chromosome in acute leukemia will help to distinguish between the acute phase of CML and Ph'-positive ALL.

The fact that the t(9;22) abnormality is such a specific and regular feature of CML argues forcefully that it is an important step in leukemogenesis. Likewise, the finding of Ph'-positive bone marrow cells preceding the leukemia (Canellos and Whang-Peng, 1972) supports this interpretation. However, in spite of the attractiveness of the t(9;22) oncogene rearrangement hypothesis, the evidence favoring this model is still circumstantial and it remains possible that earlier submicroscopic changes elsewhere in the genome are crucial. For example, Fialkow et al. (1978) showed that long term bone marrow cultures containing blood lymphocytes arising from Ph'-positive CML clones, were Ph'-negative. This led to the hypothesis that at least two steps were involved in the leukemogenesis; one causing growth of a clone of pluripotent hematopoietic cells, the other inducing Ph' in descendants of these progenitors (Fialkow, 1984; Fialkow et al., 1978). Compatible with this, are reports of six CML patients who developed the Ph' chromosome only late in their disease (Hayata et al., 1975; Lisker et al, 1982, 1980; Tanzer, 1977). Heim and Mittelman (1987) suggest a three step scheme for the pathogenesis of CML. Step 1 involves a clonal proliferation of pluripotent Ph'-negative bone marrow cells. These are genetically unstable but have a growth advantage over normal stem cells. This step, which would involve an, as yet, unidentified primary event, might be equivalent to initation in experimental tumorigenesis. Step 2 would involve the acquisition of the Ph'

chromosome (or a functionally equivalent cytogenetic rearrangement), in a suitable primed cell, presumably a pluripotent stem cell, for CML to evolve. The key effect in molecular terms of the translocation would be a deranged c-abl protein. This stage might be analogous to tumor promotion in experimental systems. Step 3 is the blast crisis. The increased genetic instability now results in additional chromosomal abnormalities, thereby enhancing the malignant potential of the abnormal clone. This stage might cover the same development which in experimental carcinogenesis is termed tumor progression.

VI. Involvement of the c-abl and phl gene in CML and ALL

a) The c-abl gene

The cellular gene c-abl represents the progenitor of the transforming gene (v-abl) within the genome of Abelson leukemia virus (Goff et al., 1980). This genome was formed by a process of recombination between a Moloney murine leukemia virus and the DNA of the host cell. The v-abl gene confers to this virus the capacity to rapidly transform a broad range of hematopoietic cell types such as B-lymphoid cells, plasma cells, macrophages, and promyelocytes as well as NIH 3T3 fibroblasts (for review, see Konopka and Witte, 1985a). Transformation is mediated by a protein tyrosine kinase activity encoded by the v-abl polypeptide attached at its N-terminus to a viral gag polypeptide.

c-abl is a highly conserved gene and is present not only in vertebrates but also in Drosophila (Hoffman et al., 1983). The human

gene is localized on chromosome 9 (Heisterkamp et al., 1982) and spans more than 230 kb of DNA (Bernards et al., 1987, Heisterkamp et al., 1983, Figure 2). It is transcribed into two major mRNAs of 6 and 7 kb (Canaani et al., 1984; Gale and Canaani, 1984; Stam et al., 1985), each initiated by independent promotors (Shtivelman et al., 1986a; Bernards et al., 1987). Cloning of human c-abl cDNA (Shtivelman et al., 1986a) showed the presence of two alternative first exons (1A and 1B) each spliced to a common set of ten 3′ exons. Exon 1A, homologous to the mouse first exon type I, is present in the 6 kb mRNA, while exon 1B, equivalent with mouse first exon type IV, is part of the 7 kb c-abl transcript. The region of homology with v-abl starts within the third exon and extends to the 5′ part of the last exon (exon 11). The most 3′ 2 kb of exon 11 contains untranslated sequences. Exons 2 through 11 cover approximately 48 kb of genomic DNA (Groffen et al., 1986, 1987) preceded by the two alternative exons 1A and 1B at a distance of 19 and 200 kb, respectively (Shtivelman, 1985, 1986a; Bernards et al., 1987). It is not known whether in the human genome more N-terminal coding exons are present. In the mouse, 4 such exons exist (Ben-Neriah et al., 1986a) of which the expression may be restricted to specific cell types or developmental stages.

Alternative splicing can affect the level of gene expression by selection of different promotors (Young et al., 1981; Benyajati et al., 1983) or can generate different proteins (Early et al., 1980; Breitbart et al., 1985; Basi et al., 1984; Rozek and Davidson, 1983; Nabeshima et al., 1984; Amara et al., 1982). In the case of c-abl, selection is at the level of the transcriptional promotor. Since the coding regions begin within the first exon, selection of different exons will result

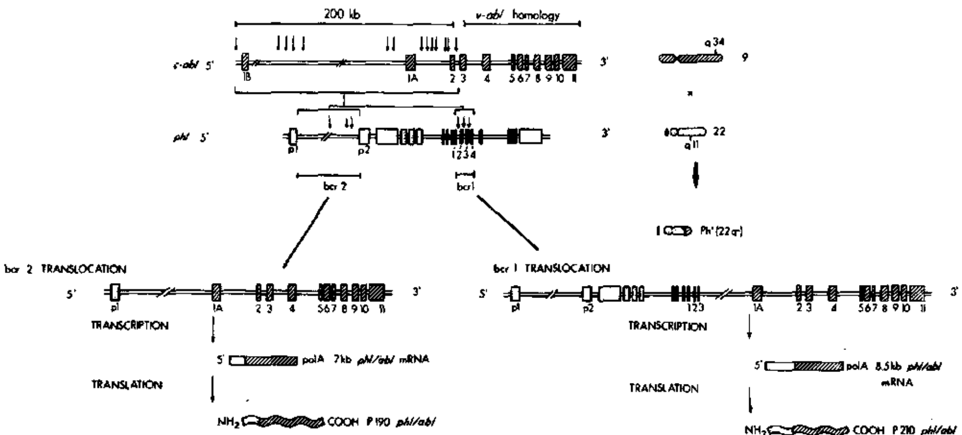

Figure 2. Molecular basis of the Ph' translocation.

Generation of the Ph' chromosome in CML and ALL by recombination between the abl locus at band q34 of chromosome 9 and the phl locus at q11 of chromosome 22. The reciprocal t(9;22) translocation is depicted on the right and the corresponding schematic gene structures on the left. The 9q+ chromosome is not shown. Arrows indicate known positions of translocation breakpoints (individual cases for c-abl, representative for phl). Breakpoints in bcr-1 result in 8.5 kb mRNA and P210 phl/c-abl protein. An example is shown with the breakpoint between c-abl exons 1B and 1A and bcr-1 exons 3 and 4. Breakpoints in the putative bcr-2 region result in a 7 kb mRNA and P190 phl/c-abl protein. p1 is phl exon 1, p2 is phl exon 2.

in different protein products. Both c-abl mRNAs are detected in all tissues analyzed (Wang and Baltimore, 1983; Westin et al., 1982); differential expression of either mRNA has not been observed. The low expression of c-abl in many tissues is common with some genes considered "housekeeping genes" such as hypoxanthine phosphoribosyl transferase (HPRT), 3-hydroxy-3 methylglutaryl coenzyme A (HMGCoA), reductase, and adenosine deaminase (ADA). Like these genes (Melton et al., 1986; Reynolds et al., 1984; Valerio et al., 1985), c-abl lacks TATA boxes, its two promotor regions are G+C rich and contain multiple GGGCGG repeats (Shtivelman et al. 1986a). This repeat was identified as the core within the consensus binding site of the Spl factor, which has been shown to regulate transcription *in vitro* (Dynan et al., 1986). RNase protection experiments showed four transcription initiation sites in the exon 1B promotor region and two in the exon 1A promotor region (Shtivelman et al. 1986a). The presence of CAAT boxes and a sequence resembling the CATAAAAGG element, which functions as a TATA box in the rabbit beta-globin gene (Dierks et al., 1983), have been demonstrated in the 1B promotor region (Bernards, 1987).

In addition to the 6 and 7 kb c-abl mRNA a 4.5 kb c-abl transcript was shown to be present in haploid spermatoids in the testis of the mouse (Ponzetto and Wolgemuth, 1985). Kretser (1986) described additional mRNAs of 2.5 and 10 kb in human leukemic lymphoblasts. In addition, at least one abl related gene, termed arg, has been reported (Kruh et al., 1986). This gene was identified in human placenta and mapped on chromosome 1q 24-25. A 12 kb mRNA is transcribed from this gene and is present both in normal and tumor cells.

b) The phl gene

Analysis of the Ph' translocation identified a 5.8 kb "hot spot" region on chromosome 22 containing the translocation breakpoints. This region was designated the breakpoint cluster region or bcr (Groffen et al., 1984). The isolation of cDNA clones with bcr probes demonstrated that bcr is part of a gene called phl (Figure 2, Heisterkamp et al., 1985; Mes-Masson et al., 1987; Hariharan et al., 1987). The phl gene encompasses approximately 90 kb and is oriented with its 5' end towards the centromere of chromosome 22 (Heisterkamp et al., 1985, unpublished information). Southern hybridization identified a minimum of 18 exons of which 9 have been mapped and sequenced (Heisterkamp, et al., 1985; Hermans et al., 1987; unpublished information). Exon 1 is 1.7 kb which is relatively large in comparison to the 4 exons which are present in bcr, which vary in size from 76 to 140 bp. The intron between exon 1 and 2 is at least 30 kb (Hermans et al., 1987).

Two messengers of 4.5 and 7 kb are transcribed from the phl gene (Stam et al., 1985). Transcription occurs at low level in different cells and tissue type (Stam et al., 1985; Heisterkamp et al., 1985, Shtivelman et al., 1985; Grosveld et al., 1986; M. von Lindern personal communication). The relationship of the 4.5 kb and 7 kb transcripts is not clear. Hariharan et al. (1987) showed that sequences present in the 4.5 kb transcript are also present in the 7 kb mRNA. Additional sequences in the 7 kb transcript might occur at its 5' end, internally or at its 3' end. The 7 kb mRNA may be generated by a more distal polyadenylation signal or as alternative splice in the 3' region. Promotor regions in the phl gene have not yet been identified.

Although no sequence homology with other known genes has been

found (Heisterkamp et al., 1985; Mes-Masson et al., 1987; Hariharan et al., 1987) a few phl related genes have been reported (Croce et al., 1987; Heisterkamp et al., unpublished). Three genes homologous to the 3' end of the phl gene have been mapped on chromosome 22, both 5' and 3' of the phl gene. A fourth related gene showing homology with the exons in bcr is located on chromosome 17. From this gene, cDNAs have been isolated, and approximately 25 kb of genomic DNA has been cloned and mapped for restriction enzyme sites (Heisterkamp et al., unpublished). Present in the phl related gene are two so called variable regions of approximately 1.5 kb containing repeats of 43 bp. The variable regions are prone to deletions resulting in heterozygosy for this region. Currently this gene is being screened for translocations or rearrangements specifically associated with various neoplastic and hereditary disorders. Screening of a variety of 60 different tumors (including two acute promyelocytic leukemia samples with t(15;17), Stam et al., unpublished) did not show translocations in this gene. It might still be worthwhile, however, to determine the exact location of this phl related gene on chromosome 17, making it possible to screen disorders with known specific translocations in this region.

c) The Fusion of phl and abl on the Ph' chromosome

The discovery of a breakpoint 14 kb 5' of the v-abl homologous c-abl exons in a CML patient (Heisterkamp et al., 1983) indicated the possibly active involvement of the c-abl gene in CML. To date, the breakpoints of 6 patients have been mapped between c-abl exon 1A and 2 (15 percent of total amount of samples studied)(Figure 2)(Collins,

1986; Leibowitz et al., 1985; Heisterkamp et al., 1983), and the breakpoint of 4 patients (Heisterkamp et al., unpublished) and the CML cell lines K562 and BV173 (Grosveld et al., 1986; Bernards et al., 1987; Westbrook, personal communications) between exon 1B and 1A. From one patient the breakpoint lies 5' of exon 1B (Bernards et al., 1987) while from another patient the break occurs between exon 2 and 3 (Grosveld, Berlin Human Genetics meeting 1986, Proc. of International Congress Human Genetics Springer Verlag, 428-434). It is expected that the majority of the breaks occur in the 200 kb intron between exon 1B and 1A. Summarizing, the amount of c-abl sequences that are translocated can vary from the whole c-abl locus (patient with breakpoint 5' of exon 1B) to only the v-abl homologous part (patient with breakpoint between exon 2 and 3). However, in all cases of CML studied to date, the protein-tyrosine kinase domain of c-abl is translocated to the Ph' chromosome.

All breakpoints in the phl gene are clustered in bcr (Figure 2) (Groffen et al., 1984). Of more than 400 reported CML patients positive for the Ph' chromosome (Groffen et al., 1984, 1986, 1987; Collins, 1986; Chan et al., submitted; Wiedeman et al., submitted; Blennerhassett et al., submitted; Stahlrid et al., submitted) only 4 have a breakpoint outside bcr (Leibowitz et al., 1985; A. Hermans pers. comm.). Breakpoints are always in an intron primarily between bcr exons 2 and 3 or 3 and 4 (Collins, 1986; Heisterkamp et al., 1985). Some cases have been described where the break occurs between bcr exon 4 and 5 (Shtalrid et al. submitted) or between exon 1 and 2 (Schaefer-Rego et al., 1987). Schaefer-Rego and co-workers (1987) found a correlation between a 3' bcr breakpoint and blast crisis. However,

this observation is in contrast to results obtained by Shtalrid and colleagues (submitted) who found the distribution of the breakpoints in bcr to be similar in blast and chronic phase patients.

In a group of Ph' positive ALL patients the break occurs in the phl gene, but outside bcr. Hermans et al. (1987) showed that in these patients the break is in an intron between phl exon 1 and 2. Although the exact size of this intron (minimum of 30 kb) and location of the breakpoints have not been determined, it is possible that there is a second breakpoint cluster region, in the phl gene.

In most cases of CML, the 3' end of the phl gene is translocated to chromosome 9 and forms part of the 9q+ chromosome. Several studies have demonstrated that 3' phl deletions occur in the sequences on 9q+. Popenoe et al. (1986) found large deletions in 4 of 14 Ph' positive CML patients and suggested that more proximal deletions occurred in 4 other patients. In the CML cell line K562, 3' phl sequences are also deleted (Grosveld et al., 1986). In contrast, de Klein et al. (1986b) found only small deletions in Ph' positive CML patients. Large deletions may occur in 10 to 20 percent of patients and are independent of the size of breaks within bcr (Shtalrid et al. submitted); no unique features are associated with these Ph' positive CML patients. Incidence of 3' phl deletions is higher in Ph'-positive ALL patients (de Klein et al. 1986b). Deletions probably occur at the time of translocation and may be involved in the mechanism of Ph' chromosome recombination (Showe and Groce, 1986).

Comparison of DNA sequences of several breakpoints showed that there is no apparent homology between the chromosome 9 and 22 sequences, making homologous recombination unlikely (Heisterkamp et al.

1985, de Klein et al. 1986a). However, since most of the breakpoints occur within Alu-repeats (Schmid and Jelinek, 1982) or Alu-repeatlike sequences (Rogers et al. 1985; Groffen et al. 1986, 1987; de Klein et al. 1986a), it is possible that Alu repetitive sequences are hot spots of recombination.

The fusion of genomic phl and c-abl sequences results in the expression of an abnormal c-abl gene product. In the leukemic cells of CML patients and CML-derived cell lines an abnormal 8.5 kb mRNA is present (Canaani et al., 1984; Gale and Canaani, 1984; Collins et al., 1984; Blick et al., 1984). Stam et al. (1985) and Grosveld et al. (1986) showed that this mRNA is a chimeric mRNA consisting of 5′ phl (3.2 kb) and 3′ c-abl (5.3 kb) sequences. This 8.5 kb mRNA is specific for Ph′ positive cells, (Gale and Canaani, 1984; Romero et al. 1986; Grosveld et al. 1986) and its expression has been reported in both the myeloid and lymphoid acute phase of CML (Romero et al., 1986), in erythroleukemia or lymphoid CML derived cell lines (Collins et al., 1984; Konopka et al., 1986), and in somatic cell hybrids retaining the Ph′ chromosome (Kozbar et al., 1986). Direct proof for the presence of a chimeric mRNA was provided with the cloning of chimeric cDNA (Shtivelman et al., 1985, Grosveld et al., 1986; Mes-Masson et al., 1986).

The mature 8.5 kb mRNA lacks the first exon of c-abl. It is produced by splicing of the precursor RNA, in which the versatility of the splicing system accommodates for the large variation in intron size present between the phl and c-abl exons in different patients. The breakpoint on chromosome 22 defines whether bcr exon 2 or 3 will be spliced to the first available c-abl exon, usually exon 2 (Figure 2).

In either case, however, this results in a hybrid transcript with one long open reading frame coding for a protein of 902 or 927 amino acids from the phl gene plus 1104 amino acids from c-abl (Figure 3). Shtivelman et al. (1987) reported the presence of hybrid transcripts with the bcr exon 3/abl exon 2 junction but also with bcr exon 2/abl exon 2 junction in the leukemic cells of a few CML patients with a breakpoint between bcr exon 3 and 4. Alternative splicing may be responsible for this finding. In the few rare cases where breakpoints occur between bcr exon 1 and 2 or the one reported case with a breakpoint between abl exon 2 and 3 result in a long phl/c-abl open reading frame as well. The bcr1/abl2 and bcr2/abl3 junction transcripts code for polypeptides of 1971 and 1948 amino acids, respectively (bcr exon 2 codes for 35 amino acids, abl exon 2 for 58 amino acids). However, junctions between bcr exon 4 and abl exon 2 are not in frame. Ben-Neriah et al. (1986b) and Stam et al. (1987) demonstrated that the 8.5 kb hybrid transcripts translate into a 210 K phl/c-abl fusion protein.

In Ph′ positive, bcr negative ALL patients, the fusion of the phl and c-abl genes result in a 7 kb hybrid transcript (Hermans et al., 1987). This transcript, which is the result of a junction between phl exon 1 and c-abl exon 2 and contains 1.7 kb of phl and 5.3 kb of c-abl sequences, codes for 1530 amino acids (Figure 3). The P190 c-abl protein found in the leukemic cells in the Ph′ positive, bcr negative ALL patients has been shown to be the translational product of the 7 kb phl/c-abl mRNA (Walker, et al., 1987; Clark et al. 1987; Kurzrock et al.; 1987; Chan et al., 1987).

VII. Activation of c-abl Protein Kinase

a) The phl/c-abl aberrant proteins

The human c-abl gene encodes a 145 K protein as shown by immunoprecipitation (Konopka et al., 1984). Both the human P145 c-abl protein and its mouse counterpart P150 have been shown to possess a low protein tyrosine kinase activity (Konopka and Witte, 1985b). In addition, the c-abl protein is phosphorylated *in vivo* on serine but not on tyrosine (Konopka et al., 1984). cDNA cloning of the 6 and 7 kb human c-abl transcripts (Shtivelman et al., 1986a) showed that although 2 protein subtypes exist (Figure 3), neither contains a transmembrane region nor a signal peptide, indicating that the abl protein is not a classical membrane receptor for extracellular factors and is therefore distinct from a number of protein-tyrosine kinases such as the EGF receptor (Ullrich et al., 1984), the insulin receptor (Ebina et al., 1985) and CSF-1/fms (Sherr et al., 1985). Of the two predicted c-abl proteins, the one encoded by the 7 kb mRNA (1148 amino acids versus 1130 amino acids encoded by the 6 kb mRNA) has the sequence Met-Gly at the N-terminus. It is possible that the methionine will be removed in processing. If so, this is significant because the N-terminal Gly of murine gag protein (Henderson et al., 1983), the catalytic subunit of cyclic AMP dependent protein kinase (Carr et al., 1982), protein phosphatase 2B (Aitkin, 1982) and v-src and c-src (Buss and Sefton, 1985) are linked to myristic acid, which is thought to stabilize the interaction of proteins with the lipid bilayer of cell membranes. Myristyllation is required for membrane association of p60 src and for its capacity to induce cell transformation (Cross et al., 1984; Kamps

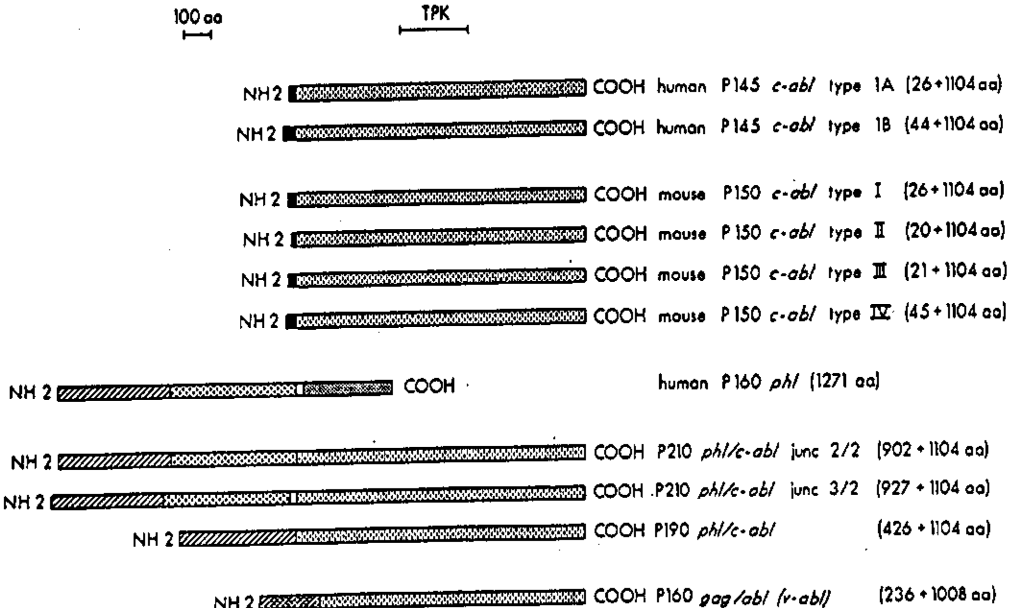

Figure 3. Aberrant abl proteins

TPK phosphotyrosine kinase domain; ▬ alternative c-abl exon 1; ▒ c-abl body exons; ▨ phl exon 1; ▒ phl exon 2--bcr exon 2; ☐ bcr exon 3; ▬ bcr exon 4--last phl exon; ▬ gag; NH2 is aminoterminal end; COOH is carboxy terminal end; aa is amino acid.

et al., 1986). The extreme conservation between the human and mouse polypeptides coded by exons 1A (100 percent homology with the 26 amino acids of mouse type I) and 1B (90 percent homology, with 42 amino acids out of 44 amino acids of mouse type IV) makes it likely that the two abl proteins have distinct functions, depending on their myristillation status, and their targets could be found in both the cytoplasm and in the membrane. Human homologues of the mouse type II (20 amino acids) and type III (21 amino acids) peptides have not been described.

Computer analysis of the predicted amino acids sequences showed four potential asparagine-linked glycosylation sites (Asn x Ser/Thr) in position 146, 478, 770 and 812 of the protein. However, there is no evidence for the glycosylation of either mouse or human abl protein. The tyrosine kinase domain spans amino acids 235-485 (Feng et al., 1985) and includes the ATP binding site (Kamps et al., 1984) composed of the conserved 9 amino acid peptide at position 248-256 and lysine at position 27. The major tyrosine phosphorylation site *in vitro* (Konopka and Witte 1985b) is position 393. There are 13 cysteine residues in the molecule with no obvious clustering. The region spanning amino acids 800-1000 is proline rich (17.5 percent), while the rest of the protein averages 5.5 percent proline.

Comparison of the predicted amino acid sequence of the human c-abl kinase with the mouse enzyme shows an overall homology of 85 percent (Shtivelman et al., 1986a; Lee et al., 1985; Ben-Neriah et al., 1986a). Remarkably the 500 amino acids at the N-terminus and 130 amino acids at the C-terminus show homologies of 99 percent and 96 percent, respectively. Although the extreme conservation of the catalytic domain is not surprising, the conservation of the two termini of the

protein is unexpected and suggest that these regions also play an essential role in the function of c-abl protein, perhaps via interactions with other cell proteins. Comparison of c-abl protein with other tyrosine kinases shows most similarity with the src and yes proteins (Ben-Neriah et al., 1986a). However, the c-abl protein is distinct from phosphotyrosine kinases described to date in that the catalytic domain is in the amino terminal domain of the protein.

In addition to the normal P145 c-abl protein, leukemic cells from CML patients contain, a large c-abl protein of 210 K (Kloetzer et al., 1985, Konopka et al., 1984, 1985, 1986, Konopka and Witte 1985a&b; Davis et al., 1985). This c-abl related protein was shown to be the translational product of the 8.5 kb hybrid phl/c-abl transcript (Ben-Neriah et al. 1986b, Stam et al., 1987). The P210 phl/c-abl protein contains 902/927 amino acids from phl (depending on presence of 25 amino acids of bcr exon 3) and 1104 amino acids derived from c-abl (exon 2 through 11).

The predicted molecular weight of the phl polypeptide based on the sequence of the cDNA of the 4.5 kb transcript is 143 K (Hariharan et al., 1987). This polypeptide consists of 1271 amino acids, and represents a relatively hydrophilic protein, with no obvious regions of marked hydrophobicity that are characteristic of transmembrane domains. Several investigators demonstrated by immunoprecipitation, with phl antiserum, the presence of 190K (Ben-Neriah et al., 1986b) and 160 K (Stam et al., 1987, O. Draezen pers. comm.) phosphoproteins. These proteins exhibited an associated protein-serine kinase activity. However, it has not been demonstrated whether this kinase activity is intrinsic to the phl gene product or whether it derives from a protein

coprecipitated in a complex with the phl protein. A detailed comparison of the phl amino acid sequences with other protein kinases, fails to reveal any sequence homology (Hariharan et al., 1987, Heisterkamp et al., 1985).

The replacement of N-terminal c-abl sequences with phl sequences in the P210 fusion protein is reminiscent to the replacement of N-terminal c-abl sequences by gag sequences following viral transduction of the abl oncogene in A-MuLV. The resulting P160 gag-abl protein contains 236 amino acids of gag fused with 1008 amino acids of the mouse c-abl protein (encoded by part of exon 3 through 11, Figure 3). There is no sequence homology between gag and phl (Heisterkamp et al., 1985, Hariharan et al., 1987, Mes-Masson et al., 1987). However, both P160 and P210 exhibited elevated tyrosine kinase activity compared to P145 (Konopka et al. 1984; Davis et al. 1985). In addition, in contrast to P145 c-abl, both proteins are phosphorylated on tyrosine in vivo (Davis et al., 1985; Stam et al., 1987; Konopka et al., 1984).

In the subgroup of Ph'positive, bcr negative ALL, a P190 c-abl protein is expressed (Clark et al., 1987; Chan et al., 1987; Kurzrock et al., 1987; Hermans et al., 1987; Walker, et al., 1987). This protein is the translational product of the 7 kb phl/abl hybrid transcript, and contains 1530 amino acids, 426 from phl and 1104 from c-abl. P190 is mainly phosphorylated on tyrosine (> 95 percent of total phosphate), with very little Pi on serine, in the immunocomplex assay and has like P210 elevated tyrosine kinase activity. Phosphoamino acid analysis of the different c-abl proteins showed different patterns of in vivo and in vitro tyrosine phosphorylation (Konopka and Witte, 1985b; Clark et al., 1987). The P145 and P150 c-

abl proteins are predominantly phosphorylated on a single site corresponding to the human c-abl amino acid 393 (v-abl residue 514)(Shtivelman et al., 1986a). P190 phosphotyrosine was found on a single major site similar to the site in P145 and P150, and on three minor sites. P210 and P160 are each phosphorylated at several major sites, distinct from the major c-abl and P190 phosphorylation site. A P160 minor site corresponds with the P150 and P145 major site while the major sites have been mapped to the amino terminal gag sequences (Konopka and Witte, 1985b; Clark, et al., 1987). The exact location of the P210 phosphorylation sites have not yet been determined. However, its pattern of in vitro phosphorylation is distinct from the P160 pattern. In vivo studies demonstrated that P145 and P150 are not phosphorylated on tyrosine in vivo. P160 is phosphorylated on tyrosine 514 (major site) and on tyrosine 385 (minor site). The P210 phosphorylation site has not been localized, but is apparently different from v-abl tyrosine 514 (Konopka and Witte, 1985b). In vivo studies have not been reported for P190.

The different manner in which the abl proteins utilize themselves as substrates for phosphorylation in vitro suggest that they may interact differently with substrates in vivo. This idea is supported by the fact that P150 and P145 are not detected by phosphorylation on tyrosine in vivo but P160 and P210 are. Quantitative and qualitative changes, like altered substrate specificity, of the P210 kinase may be important in the leukemogenesis of Ph' positive bcr positive CML and ALL. A more similar phosphorylation pattern between P190 and P145 suggest that a quantitative change in abl kinase activity may be important for the pathogenesis in Ph' positive bcr-negative ALL. Of

interest is the report of enhanced tyrosine kinase activity or elevated levels of P145 in a Ph' negative ALL patient (Chan et al., 1987). Different mechanisms of c-abl activation could result in clinically distinctive leukemias.

b) Regulation of kinase activity and transformation

To address the question of how the c-abl kinase activity could be affected by the viral transduction or translocation, it is important to understand the mechanisms of which protein kinases are regulated. The precise mechanisms involved in regulating protein kinase activity are quite diverse. Protein kinases are often multisubunit enzymes, either of homo or hetero-oligomeric nature. Such oligomeric structures allow for cooperative and highly sensitive responses to regulatory inputs. Monomeric protein kinases commonly have discrete regulatory domains, which may contain pseudo substrate sequences involved in negative regulation (Hunter, 1987b). In general it appears that protein kinases exist in the cell in the off-state. Many protein kinases are positively regulated by ligands, which bind to regulatory domains or subunits. For instance, growth factor receptor protein tyrosine kinases are stimulated by binding their cognate peptide growth factor. Other protein kinases are activated by second messengers. The activity of cyclic nucleotide regulated protein kinases is increased through elevation of cyclic nucleotide levels generated by hormone-dependent membrane-associated cyclases, whereas the protein kinase C family is stimulated by diacylglycerol turnover of phosphoinositides. There are also protein kinases regulated by changes in Ca^{2+} concentration through the agency of calmodulin.

Kinase activity can also be regulated through a positive or negative mechanism of autologous or heterologous phosphorylation. Autophosphorylation, generally, results in activation as is the case with the insulin (Rosen et al., 1983) and probably the EGF receptor (Bertics and Gill, 1985). Autophosphorylation could also provide a negative feedback mechanism to down regulate the phosphorylation signal. Regulation by heterologous phosphorylation can occur both between protein-tyrosine and protein-serine kinases or within one class. For example, the activity of the EGF receptor protein-tyrosine kinase is decreased upon phosphorylation by protein kinase C (Cochet et al., 1984; Friedman et al., 1985). The activity of the protein-tyrosine kinase pp60c-src is suppressed by a tyrosine phosphorylation event, which appears to be mediated by another protein-tyrosine kinase (Cooper et al., 1986; Cooper and King, 1986; Jove et al., 1987). An example of positive regulation is the increase in activity of phosphorylase kinase following phosphorylation by cAMP-dependent protein kinase (Walsh et al., 1968).

Several cellular protein tyrosine kinases are subject to tight negative regulation by noncatalytic domains. A good example is the regulation of pp60v-src and pp60c-src. Comparison of the predicted amino acid sequences of v-src with that of c-src shows that the residues 1-514 differ only in a few amino acids but starting at residue 518, the proteins diverge completely due to a deletion of the coding sequences for the C-terminal nineteen amino acids of pp60c-src (Takeya and Hanafusa, 1983). pp60v-src has a novel C-terminus of twelve amino acids derived from a sequence believed to originate from the 3' untranslated region of the c-src gene. The protein kinase domain of

pp60v-src has been localized to its C-terminal half, approximately between residues 260 and 516 (Yacuik and Shalloway 1986; Wilkerson et al., 1985).

Courtneidge et al. (1985) demonstrated that dephosphorylation of Tyr 527 in pp60c-src increases the protein kinase activity by 5-10 fold. Tyr 527 is deleted in pp60v-src explaining the increased tyrosine kinase activity of v-src. pp60c-src is phosphorylated in the cell on Tyr 527 and can be activated in vitro to nearly the pp60v-src level by phosphatase treatment or by an antibody directed against a synthetic peptide corresponding to the C-terminus of pp60c-src (Cooper and King, 1986).

Phosphorylated Tyr 527 could inhibit pp60c-src by acting as product analog or in some allosteric fashion. Little is known about how phosphorylation of Tyr 527 is regulated; it does not appear to be an effective autophosphorylation site. The absence of the C-terminal domain in pp60v-src, however, prevent negative regulation of the kinase leading to unscheduled tyrosine phosphorylation and transformation.

Phosphorylation of Tyr 527 can play a role in transformation as shown by different groups. It has been found that for polyoma transformation it is necessary that the polyoma middle T antigen binds to pp60c-src (Bolen et al. 1984). The binding of polyoma middle T prevents phosphorylation of Tyr 527 and therefore activates the kinase activity. Other groups (Piwnica-Worms et al., 1987; Cartwright et al. 1987; Kmiecek and Shalloway, 1987) demonstrated that mutations preventing phosphorylation of Tyr 527 in pp60c-src (Tyr ---> Phe) is sufficient to activate pp60c-src oncogenically. Although the mutated proteins transform NIH-3T3 cells, the transforming ability is weaker

than that of pp60v-src. Additional activating mutations acquired by pp60v-src may be necessary to increase the tumorigenic potential of the mutated c-src proteins.

Other protein kinases especially those from the c-src gene family (Table 3) also have a highly conserved C-terminal extension beyond the catalytic domain including a Tyr equivalent to Tyr 527. The deletions of these C-terminal sequences in v-fes and v-fgr could lead to oncogenic activation. The v-fms protein lacks the C-terminal fourty residues of c-fms (alias the receptor for colony stimulation factor CSF-1) which include a Tyr 969. Restoration of the normal c-fms C-terminus of the v-fms gene reduces its transforming activity 20 fold implying that this region has a negative regulatory role (Hunter, 1987a; Roussel et al., pers. comm.).

The elimination of negative regulatory elements could also be responsible for the activation of the different aberrant c-abl kinases (Figure 3). As a consequence of viral transduction or translocation new sequences are juxtaposed to c-abl sequences. Additionally, these events result in a loss of c-abl sequences at the 5′ end. It is possible that deletion of these sequences result in activation of the c-abl kinase activity. Wang et al. (pers. commun.) expressed a full length cDNA of human c-abl in E. coli cells. This protein had low kinase activity. Deletions of 5′ sequences of the cDNA resulted in the expression of a protein with elevated tyrosine kinase activity.

Deregulation of phosphorylation may not be sufficient for transformation. For example, there is no direct correlation between in vitro tyrosine kinase activity of the different abl proteins and their transforming capacity in the cell. Although, the tyrosine kinase

activity of P210 is very similar to P160, expression under several promotors (SV40, MMuLV-LTR, bcr promotor) of the 8.5 kb full length phl/c-abl cDNA fails to transform NIH 3T3 fibroblast, while the MMuLV-LTR driven v-abl gene does induce transformation (Daley et al., 1987; Hermans et al.,1987) P210 phl/c-abl may not transform fibroblasts in culture because of an inability to bind to cellular membranes. The gag portion of v-abl, like v-src, contains an NH_2-terminal myristic acid moiety thought to determine membrane association (Schultz and Oroszlan, 1985; Sefton et al., 1982). NH_2-terminal mutants of v-abl and v-src that lack a myristylation site no longer localize to membranes and are transformation-defective (Garber et al., 1983, Cross et al., 1984; Pellman et al., 1985). It is significant therefore, that spontaneous recombinants in which NH_2-terminal viral gag sequences have fused to the phl/c-abl protein, result in a gag/phl/c-abl protein that readily transforms NIH 3T3 cells (Daley et al., 1987). Daley and co-workers have demonstrated that for fibroblast transformation not only membrane localization is important but deletion of abl sequences as well. Deletions in the normal c-abl type 1B that remove sequences near the NH_2-terminus, but preserve the glycine at position 2 (a putative myrystilation site) produce transforming c-abl proteins. Although P210 phl/c-abl appears not to transform NIH 3T3 fibroblasts, data from Whitlock/Witte culture systems suggest that the P210 phl/c-abl protein can stimulate growth of cells of hematopoietic lineage but is not sufficient for full oncogenic behavior (McLaughlin et al., 1987)

Questions as what is the role of P210 and P190 in CML, and what defines their specificity have to be answered in the future. For v-abl it has been shown that deletions in the P15 region of gag can

specifically abolish lymphoid transforming activity (Prywes et al., 1985a, 1985b). The P15 deleted proteins demonstrated a marked instability in lymphoid cells. _phl_ sequences present in P210 but removed in P190 may be involved in controlling the action of the c-_abl_ moiety and/or be responsible for host cell specificity resulting in different clinical manifestations.

What are the molecular events that connect the kinase activity of the aberrant kinases with the myriad and complex changes collectively termed transformation? The answer will undoubtedly be found at many different levels. A critical step is identification of primary cellular substrates involved in transformation. To date this quest has met with little success, although phosphorylated proteins specific for CML cells have been found (Huhn et al., 1987). However, it has yet to be established whether they are specific _abl_ substrates. Phosphorylation of some substrates may directly trigger gross phenotypic alterations. Other phosphorylated substrates may represent an initial step in a cascade or a series of reactions that are involved in signal transduction and amplification. The observation that a putative phosphatidylinositol kinase represents a substrate for both the PDGF receptor and polyoma middle T/pp60 c-_src_ tyrosine kinase activities (Kaplan et al. 1987; Courtneidge and Heber, 1987) is significant in this regard. This links protein tyrosine kinases to the phosphoinositide pathway with its important secondary messengers diacylglycerol and inositoltriphosphate.

VIII. Conclusion

Since the days of Hansemann and Boveri, chromosomal aberrations have been associated with malignant growth. The Philadelphia (Ph') chromosome is the cytogenetic hallmark of CML and is the product of a specific translocation between chromosomes 9 and 22. The c-abl oncogene on chromosome 9 is translocated into a 5.8 kb breakpoint cluster region (bcr) in the phl gene on chromosome 22. Although the breakpoints on both chromosomes are variable, RNA splicing generates a unique 8.5 kb phl/c-abl chimeric message. This chimeric message encodes a phosphoprotein (P210 phl/c-abl) that contains phl encoded NH_2-terminal sequences and a c-abl derived COOH-terminal segment. The P210 phl/c-abl protein has, in comparison to the normal P145 c-abl protein, elevated tyrosine kinase activity.

A Ph' chromosome, cytogenetically indistinguishable from the Ph' chromosome in CML, is present in a percentage of acute lymphoblastic leukemia (ALL) patients. Molecular analysis reveals two classes of rearrangements. The first of these has a breakpoint in bcr resulting in the expression of P210 phl/c-abl. In the second class, the breakpoint lies outside bcr, in an intron between phl exon 1 and 2. Transcription results in a 7 kb phl/c-abl message that translates into a P190 phl/c-abl fusion protein with, like P210, an elevated tyrosine kinase activity.

Although the evidence is circumstantial, the translocation of the c-abl oncogene to the phl sequences appears to represent a critical event in the pathogenesis of CML and ALL. The Ph' chromosome, or better, the expression of P210 phl/c-abl or P190 phl/c-abl, are

important diagnostic and prognostic markers. It has been shown that CML patients with the phl/c-abl juxtaposition have identical clinical and hematological features. This is in contrast to the real Ph′ negative CML patients who are considered to represent myelodysplasia and reactive myeloproliferative conditions other than CML with an overall poorer prognosis.

The presence of the Ph′ chromosome in ALL is associated with extremely poor prognosis. Patients expressing the P210 phl/c-abl protein could represent a lymphoid blast crisis of CML, evolving from a clinically silence, or short chronic phase. ALL patients expressing the P190 phl/c-abl are more typical of high-risk ALL. However, the assumptions made are based on the partial analysis of leukemic cells from a limited number of Ph′ positive ALL patients. A more extensive study will be required in order to corroborate the clinical and/or biological importance of these molecular differences among the Ph′ positive leukemia patients.

The Ph′ translocation results in different activated c-abl protein kinases. Although implicated in CML and ALL, the exact role of the aberrant abl kinases is not known. The next step will require the identification of crucial substrates for the phosphotyrosine kinases and the demonstration of functional effects of tyrosine phosphorylation. In the last few years, we have come a long way. The understanding of the genome of the leukemic cell helps to acquire new devices for the prevention, diagnosis and therapy of chronic myelogenous and acute lymphoblastic leukemia.

IX. References

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CHAPTER 3

STRUCTURAL ORGANIZATION OF THE BCR GENE AND ITS ROLE IN DE PH' TRANSLOCATION

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Structural organization of the *bcr* gene and its role in the Ph′ translocation

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The Philadelphia (Ph′) chromosome, an abnormal chromosome 22 (ref. 1), is one of the best-known examples of a specific human chromosomal abnormality strongly associated with one form of human leukaemia, chronic myelocytic leukaemia (CML). The finding[2] that a small region of chromosome 9 which includes the c-*abl* oncogene is translocated to chromosome 22 prompted studies to elucidate the molecular mechanisms involved in this disease. We have demonstrated previously that the chromosome 9 of one patient with CML contains a breakpoint 14 kilobases (kb) 5′ of the most 5′ v-*abl*-homologous exon[3]. These data suggest a role for c-*abl* in CML, a theory supported by the presence of an abnormally sized *abl* messenger RNA[4,5] and protein[6] in the CML cell line K562. The region involved in the translocation on chromosome 22 has also been identified: all Ph′-positive patients examined to date have a breakpoint within a 5.8-kb region, for which we have proposed the name 'breakpoint cluster region' (*bcr*)[7]. To determine whether *bcr* contains protein-encoding regions, probes from *bcr* were tested for their ability to hybridize to complementary DNA sequences. A 0.6-kb *Hin*dIII/*Bam*HI *bcr* restriction enzyme fragment proved suitable for isolating several cDNA clones from a human fibroblast cDNA library[8]. Using *bcr* cDNA sequences, we obtained data strongly suggesting the presence of a chimaeric *bcr/abl* mRNA in the leukaemic cells of Ph′-positive CML patients. The recent isolation of cDNA clones containing *bcr* and *abl* sequences confirms this finding[12]. Because the *bcr* part of the chimaeric mRNA could be required to induce the transforming activity of the human c-*abl* oncogene, we have now initiated studies to characterize the normal '*bcr* gene' and to determine the effect of a translocation within its coding domain. We demonstrate that as a result of the Ph′ translocation, a variable number of *bcr* exons are included in the chimaeric *bcr/abl* mRNA. The *bcr* gene sequences in this mRNA could be responsible for the transition of the *abl* cellular proto-oncogene into an oncogene.

The largest cDNA, V1-3, containing an insert of 2.2 kb, was characterized in detail by restriction enzyme mapping (Fig. 1*a*) and sequence analysis (Fig. 1*b*). The cDNA contains one long open reading frame, starting at the poly(G) tail at the 5′ end and continuing to nucleotide 1,770, where a stop codon is encountered. All other reading frames have many stop codons within the entire region. The long open reading frame has the coding capacity for 589 amino-acid residues, corresponding to a protein of relative molecular mass (M_r) ~65,000; at the 3′ end, a polyadenylation signal occurs at nucleotide 2,182 followed by a poly(A) tail beginning at base 2,208 indicating that the cDNA contains the complete 3′ end of the gene. Although translational start sequences are encountered at the 5′ end, it is unlikely that this cDNA contains a complete copy of the mRNA, as Northern blot hybridizations indicate the presence of *bcr* mRNAs of ~4.0 and ~6.5 kb. Computer searches of newly isolated protein sequences derived directly from proteins or deduced from cDNA nucleotide sequences frequently result in the identification of proteins with partial homology. Such information is valuable, frequently allowing the assignment of a preliminary function to an unknown protein. Therefore, the PIR FASTP program[10] was used to search for *bcr*-homologous proteins; no proteins with significant homology were found, indicating that the *bcr* protein exhibits an as yet unidentified cellular function.

To determine the orientation of the *bcr* gene on chromosome 22, 5′ and 3′ probes were prepared from the V1-3 cDNA and hybridized to cosmids[7] containing human chromosome 22 sequences. This established that the 5′ end of the *bcr* gene is towards the centromere of chromosome 22 and is retained after the Ph′ translocation; the 3′ end of the *bcr* gene lies in the direction of the telomere and is translocated to chromosome 9 in the t(9; 22) translocation. The cDNA hybridizes to restriction enzyme fragments distributed over a region of up to 45 kb of chromosome 22 DNA (Fig. 2*a*). Within this region, a minimum of 13 exons are present. To determine the exact position and number of exons within the breakpoint cluster region, all hybridizing regions in *bcr* were sequenced and compared with the V1-3 cDNA. Four relatively small exons, designated 1–4, were present within *bcr*, varying in size from 76 to 105 base pairs (bp) (Fig. 2*b*); in the cDNA, these exons correspond to nucleotides 483–836 (Fig. 1*b*). As *bcr* was defined as the area on chromosome 22 in which the Ph′ breakpoints are found, we conclude that the breakpoints occur within a gene.

Having determined the position of the exons within *bcr*, we investigated whether the breakpoints occur in exon or intron regions. For CML DNA such as that of CML patient C481, this was readily determined. We had previously demonstrated[7] a breakpoint within a 1.2-kb H/Bg *bcr* fragment in several CML DNA samples, including that of patient C481. As no coding sequences are located within this region (see Fig. 2), patients such as C481 must have a chromosomal breakpoint in the intron between the exons designated 3 and 4.

A less simple situation was encountered in the DNA of patients 0311068 and 7701C. Nonetheless, cloning of $9q^+$ breakpoint fragments from these DNAs (data not shown) and restriction enzyme analysis followed by Southern hybridization enabled us to locate the breakpoints between exons 2 and 3 (see Fig. 2). The breakpoints in the previously cloned[3,7] $9q^+$ breakpoint fragments of patients 0319129 and 02120185 were analysed by DNA sequencing. In addition, we cloned the $22q^-$ breakpoint fragments of patient 0319129 (unpublished results) and of the cell line K562. DNA sequence analysis of the breakpoint regions of these fragments and that of the sequence of the corresponding chromosome 22 regions enabled us to define the point of translocation for these DNAs (see Fig. 2). None of the breakpoints analysed here could be located within an exon, indicating that in the Ph′ translocation, breakpoints occur within intron regions of *bcr*. For four of the six DNA samples analysed, the translocation would result in the transcription of a mRNA that includes *bcr* exons 1 and 2 (see Fig. 2); in two CML DNAs the third exon would be additionally incorporated in the chimaeric *bcr*/c-*abl* mRNA. Exon 3 encodes only 25 amino acids, not substantially increasing the size of the chimaeric protein.

A knowledge of the DNA sequence of the translocation junction point may provide additional information about the mechanism of chromosomal translocation. Figure 3*a* shows the sequence of the region containing the crossover point for translocation in the DNA of patient 0319129 and compares this with normal chromosome 22 and 9 DNA sequences. In 0319129 DNA, the chromosomal break has occurred in a rather 'precise' manner, leading to the generation of a $22q^-$ and $9q^+$ sequence exactly reflecting the sequence of the normal chromosome 22 and 9 DNA sequences. However, at the breakpoint, a C nucleotide is found in both the $9q^+$ and $22q^-$ sequences, whereas the chromosome 22 sequence contains a G at that position. Note that both chromosomes 9 and 22 contain limited stretches of homology near the break (Fig. 3*a* underlined); a DNA search revealed homology of this region to human *Alu* repetitive sequences.

No such similar stretches of homologous sequences between chromosomes 9 and 22 occur at the breakpoint in the DNA of patient 0212015 (Fig. 3*b*) or in that of K562 DNA (data not shown). In the $9q^+$ DNA of patient 0212015, sequences are

Fig. 1 *bcr* cDNA V1-3. *a*, Restriction enzyme map of V1-3. The black bar indicates the open reading frame sequences, the thin line non-translated sequences. The 3′ end of the cDNA containing the poly(A) tail is depicted as A_n. Restriction enzymes include: A, *Ava*I; Ap, *Apa*I; B, *Bgl*II; Bs, *Bst*EII; H, *Hin*dIII; Hf, *Hin*fI; P, *Pst*I; PII, *Pvu*II; S, *Sau*3A. *b*, DNA sequence and translation of V1-3 sequence. Amino-acid residues are indicated with a one-letter symbol below the sequence. The exact position of exons designated 1–6 (see Fig. 2*a*) within V1-3 *bcr* cDNA is shown by the numbers above the sequence. DNA sequencing was performed using the dideoxy-chain termination method[14] on restriction enzyme fragments of the cDNA subcloned into M13 phage[15]. All regions were sequenced on both strands.

Fig. 2 Genomic organization of the *bcr* gene. *a*, Restriction enzyme map of chromosome 22 sequences encompassing *bcr*. Exons are indicated by black boxes below the restriction enzyme map. The position of the numbered exons has been determined by sequencing; all other exons were located by hybridization to the V1-3 cDNA. The asterisk indicates a polymorphic *Bgl*II restriction enzyme site. *b*, Restriction enzyme map of the breakpoint cluster region, with the exons as indicated in *a*. Below the map, the approximate positions of the breakpoints in different CML DNAs are indicated by horizontal or vertical arrows. Restriction enzymes include: A, *Ava*I; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI, H, *Hin*dIII; P, *Pst*I; S, *Sma*I; Ss, *Sst*I; X, *Xho*I. The breakpoint of the $22q^-$ chromosome from DNA 0319129 was cloned as a 9.5-kb *Bgl*II fragment in Charon 30 according to previously described methods[16]. $9q^+$ fragments were isolated by cloning a 7.2-kb *Bam*HI fragment and a 7.7-kb *Eco*RI fragment into Charon 30 and λgtwes, respectively.

found between the breakpoints on chromosomes 9 and 22 (Fig. 3*b*) which are not present in the sequenced region of the normal chromosomes 9 and 22. This suggests that in the $9q^+$ chromosome a secondary event has taken place; it is possible that after translocation of chromosome 22 sequences to chromosome 9, a deletion affecting chromosome 9 sequences occurred. The arrow indicating the breakpoint on chromosome 9 (see Fig. 3*b*) would then represent the point of deletion. In addition, compared with the normal chromosome 9 sequences, the chromosome 9 sequences of the $9q^+$ fragment contain 13 nucleotide changes within an 81-bp stretch. These changes may reflect differences between individuals (the control chromosome 9 sequences were isolated from a human lung carcinoma cosmid library); however, the number of nucleotide substitutions would be very high in such an event. Moreover, the chromosome 22 part of the $9q^+$ fragment contains no nucleotide changes in either intron or exon sequences, favouring the explanation of inefficient DNA repair on deletion of chromosome 9 sequences.

These results indicate that *bcr* is part of a gene oriented with its 5' end towards the centromere of chromosome 22. In the Philadelphia translocation, a break occurs within *bcr*, and sequences from chromosome 9 which contain the human c-*abl* oncogene in all Ph'-positive cases examined[7,9] are translocated to the 3' of the truncated *bcr* gene. The joining of *bcr* and c-*abl* sequences is highly specific for CML, as this configuration has been found in complex translocations[9] and even in the leukaemic cells of one CML patient cytogenetically lacking the Ph' chromosome[11]. Because the orientation of c-*abl* on chromosome 9 is also centromere-5'-3'-telomere, *bcr* and c-*abl* are joined in a head-to-tail fashion on the Ph' chromosome. Although the distance between the most 5' v-*abl* homologous to v-*abl* and the physical breakpoint may vary from 14 kb (patient 0319129) to >100 kb (K562), the effect of the translocation on the expression of the *bcr* gene and c-*abl* seems to be very similar in different patients: in K562 and in Ph'-positive CML patients, abnormal RNA transcripts of ~8.5 kb are detected, which hybridize to both c-*abl* and 5' *bcr* exon probes. The molecular cloning of a chimaeric cDNA from K562 cells has provided definitive proof for the existence of chimaeric mRNA[12]. The chimaeric mRNAs must be the result of transcription initiating at the promoter of the *bcr* gene; depending on the exact location of the breakpoint, the transcript will include all 5' exons in addition to either exons 1 and 2 or exons 1, 2 and 3 of *bcr*. Recent sequence analysis of K562 *bcr*/*abl* cDNA confirms the variable presence of the third exon. In K562 we found that, as predicted, the third exon is present in the cDNA, immediately preceding c-*abl* sequences. In contrast, Southern blot analysis of the molecularly cloned $22q^-$ genomic DNA fragment of, for example, patient 0319129 unambiguously demonstrates that in this patient exon 3 has been removed from the Ph' chromosome and will not be included in a chimaeric *bcr*/*abl* transcript. Transcription continues into the c-*abl* oncogene, including, as a minimum, the most 5' v-*abl*-homologous exon and all exons 3' of it, including the phosphotyrosine acceptor site[13]. We do not know whether the inclusion of exon 3 in the chimaeric mRNA has an effect on the progression of the disease.

Chromosomal aberrations may be generated by specific events involving recombination-prone DNA sequences. Alternatively, such recombination events could occur almost at random. In either case, a very limited number of translocations will result in gene alterations leading to the disruption of normal growth and differentiation. In the Ph' translocation, we have found that breakpoints on chromosome 9 are spread over a region of up to 100 kb. The breakpoints on chomosome 22 occur within a smaller region of around 5.0 kb. Nonetheless, no sequence homology can be found between breakpoint regions of different CML patients or coding regions of c-*abl* and *bcr* genes. Therefore, we may conclude that the processes underlying the Ph' translocation are random recombination events. Once such recombinations result in a genomic configuration that allows the transcription of chimaeric *bcr*/*abl* mRNA, malignant proliferation of specific cell types may occur. It seems highly likely that this chimaeric mRNA is translated into protein because an abnormally sized 210,000-M_r c-*abl* protein was detected in K562 cells. In contrast to the normal c-*abl* protein, the P210 has tyrosine kinase activity[6].

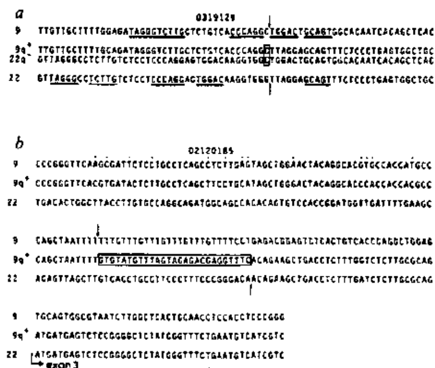

Fig. 3 Breakpoint sequences of the DNAs of two CML patients. *a*, Sequence of 0319129 DNA; the sequences are in a 5'-3' orientation. Normal chromosome 9 sequences (first line) are from non-CML DNA; the $9q^+$ and $22q^-$ sequences (second and third lines) are from DNA of patient 0319129. Normal chromosome 22 sequences (fourth line) are from non-CML DNA. An arrow indicates the breakpoint on chromosomes 9 and 22; the nucleotide C found in both the $9q^+$ and $22q^-$ sequences at the breakpoint is boxed. Limited regions of homology between the normal chromosome 9 and 22 sequences are underlined. *b*, Breakpoint sequence of 02120185 DNA; normal chromosome 9 and 22 sequences (first and third lines in each set) were from non-CML DNA. The $9q^+$ sequence on the second line contains an area boxed to indicate that it does not originate from the normal chromosomes 9 or 22 sequenced in the present experiments. Dots above the chromosome 9 sequences indicate nucleotide differences at those positions from the $9q^+$ chromosome. The beginning of exon 3 (see Fig. 2*b*) in the $9q^+$ and 22 sequence is indicated in the figure. Small restriction enzyme fragments containing the breakpoints were chosen for sequence analysis, based on restriction enzyme mapping data and comparison with normal chromosome 9 and 22 maps.

It is tempting to speculate that the *bcr* moiety of the fusion protein is responsible for this effect. However, although the consequences of the Ph' translocation on a molecular level are becoming evident, it remains to be established whether this phenomenon is actually the cause or merely one of the steps that eventually result in CML.

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CHAPTER 4

EVIDENCE OF A NEW CHIMERIC BCR/C-ABL MRNA IN PATIENTS WITH CHRONIC MYELOCYTIC LEUKEMIA AND THE PH' CHROMOSOOM

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EVIDENCE OF A NEW CHIMERIC *bcr/c-abl* mRNA IN PATIENTS WITH CHRONIC MYELOCYTIC LEUKEMIA AND THE PHILADELPHIA CHROMOSOME

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Abstract The hallmark of chronic myelocytic leukemia is the presence of the Philadelphia chromosome (Ph[1]). In recent studies, we obtained data that strongly suggested the involvement of an oncogene, c-*abl*, in this type of leukemia. This oncogene, normally located on chromosome 9, is translocated to chromosome 22 as a result of the Ph[1] translocation. In addition, we identified a region on chromosome 22, the breakpoint cluster region *(bcr)*, which contains the chromosomal breakpoint in all patients with chronic myelocytic leukemia who are positive for Ph[1]. Recent studies have suggested that the *bcr* is part of a gene that is truncated as a consequence of the Ph[1] translocation. The deleted part of this gene could be replaced by c-*abl* sequences; to test this hypothesis we analyzed the RNA of five patients with chronic myelocytic leukemia. All five had chimeric *bcr/c-abl* messenger RNA, suggesting that the deleterious effects of this disease can be associated with an abnormal chimeric protein encoded by the *bcr* and the c-*abl* oncogene. (N Engl J Med 1985; 313:1429-33.)

RECENT studies indicate a close association between chronic myelocytic leukemia (CML) and the activation of an oncogene, human c-*abl*. The hallmark of CML is the presence of an abnormal chromosome, the Philadelphia (Ph[1]) chromosome, found in the leukemic cells of over 95 per cent of all patients. This chromosome is the result of a reciprocal translocation involving chromosome 9 band q34 and chromosome 22 band q11.[1] Since only a small region of chromosome 9, on which the oncogene c-*abl* is located, is translocated to chromosome 22,[2] the abnormal, shortened chromosome 22 ($22q^-$, or Ph[1]) can be easily identified cytogenetically. In some patients with CML the abnormal chromosome 9 ($9q^+$) is not detectable; nonetheless, we have found that in these patients, c-*abl* is also translocated to chromosome 22.[3]

We have previously identified a region about 5.8 kb long on chromosome 22, in which the breakpoint occurred in the DNA of all of more than 30 patients with CML who were positive for Ph[1].[4] We proposed the name "breakpoint cluster region," or *bcr*, for this relatively small region. In recent experiments we have shown that the *bcr* is part of a gene, the *bcr* gene[5]; coding regions of this gene are spread over at least 66 kb of genomic DNA including the *bcr* and exons 5′ and 3′ to it. As a consequence of the Ph[1] translocation part of this gene, the 3′ end is translocated to chromosome 9, whereas the 5′ part remains on the Ph[1] chromosome.

The remaining *bcr* sequences act as an acceptor of a chromosome 9 gene, the c-*abl* oncogene: the c-*abl* oncogene is fused in head-to-tail fashion to the chromosome 22 sequences. This genomic configuration could result in the transcription of a novel chimeric mRNA (messenger RNA) consisting of 5′ *bcr* sequences and 3′ c-*abl* oncogene sequences.

In the present studies we analyzed the RNA of five patients with CML and found strong evidence for this model. We demonstrated the presence of a chimeric *bcr*/c-*abl* transcript in RNA specimens isolated directly from the bone marrow of all patients studied.

Methods

Patients

We studied five patients with Ph[1]-positive CML. Four had the usual Ph[1] translocation, and one had a complex translocation. Patient 2128 was a 37-year-old Asian (East Indian) man, in whom CML was diagnosed on June 17, 1982. He had a complex t(9;12),t(12;22) translocation.[6] Chromosome studies revealed a 46,XY,Ph[1] karyotype. His disease is still in the chronic phase, and his peripheral blood is normal.

Patient 2252 was a 45-year-old white man. He presented with CML on January 26, 1983 and had the usual t(9;22) translocation. His karyotype (46,XY,Ph[1]) has remained unchanged, and his peripheral blood is normal. Patient 2397 was a 43-year-old white woman with a 46,XX,t(9;22),Ph[1] karyotype. CML was first diagnosed on October 17, 1983. She has been evaluated regularly, and her karyotype has not changed; her peripheral blood is normal.

Patient 1708 was a white woman who presented with CML on

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July 24, 1980. All 40 cells examined had the 46,XX,t(9;22),Ph[1] karyotype. At the latest bone marrow aspiration (July 9, 1984), the 40 cells examined showed similar cytogenetic findings. Her disease is in the chronic phase. During the past six years cytogenetic evaluation has been performed serially. Stimulation of peripheral-blood cells with phytohemagglutinin has given normal results.

Patient 2172 was a 52-year-old white man, in whom CML was originally diagnosed on September 22, 1982. Cytogenetic analyses have been performed every three months. His karyotype remains 46,XY,t(9;22),Ph[1], and phytohemagglutinin-stimulated peripheral blood is normal.

Cytogenetic Techniques

Initially, 20 metaphases were banded by G banding each time a blood sample was drawn. Afterward, 20 cells were banded by R banding as described by Verma and Lubs.[7] Metaphases were photographed in color but printed in black and white. Cytogenetic evaluation was performed by projecting the color slides.

RNA Isolation and Electrophoresis

Total RNA was isolated according to the lithium chloride–urea method.[8] Fresh bone marrow samples (1 to 2.5 ml) were homogenized in 25 ml of 3 M lithium chloride and 6 M urea on ice in a polytron homogenizer, two bursts for 30 seconds each. The homogenate was kept on ice for three hours. The precipitate was pelleted at $10{,}000\times g$ for one hour and dissolved in 3.2 ml of a 1:1 mixture of buffer (10 mM TRIS-HCl [pH 7.5], 5 mM EDTA, and 0.2 per cent sodium dodecyl sulfate) and phenol:chloroform:isoamylalcohol (25:24:1). The water phase was extracted two times with phenol:chloroform:isoamylalcohol (25:24:1), and the RNA precipitated overnight at −20°C with 2.5 volumes of absolute ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2). The RNA was rinsed three times in 70 per cent ethanol and stored at −70°C. Poly A RNA was obtained after one passage of the RNA over oligo-dT-cellulose; 10 μg was subjected to electrophoresis on a 1 per cent agarose gel in the presence of formaldehyde.[9] Blotting to nitrocellulose (Schleicher and Schuell filters, PH 79) was performed essentially as described elsewhere.[9]

Hybridization

^{32}P-labeled RNA probes were generated by transcription labeling according to the pSP riboprobe method (Promega Biotech), essentially as previously described[10]; 1 to 5×10^8 cpm of [^{32}P]RNA was used per hybridization, corresponding to 0.5 to 2.5×10^8 cpm per microgram of input DNA.

Blots were first prehybridized in 100 ml of 50 per cent formamide, 50 mM sodium phosphate buffer (pH 6.5), 5×SSC (1×SSC = 0.15 M sodium chloride and 0.15 M sodium citrate [pH 7.0]), 1 mM EDTA, 0.1 per cent sodium dodecyl sulfate, 2.5-fold-concentrated Denhardt's solution, 250 μg of salmon-sperm DNA (per milliliter), 250 μg of calf-liver RNA (per milliliter), and 10 μg of poly A (per milliliter) for four hours at 55°C. Hybridization was performed in 35 ml of hybridization mix (four parts prehybridization mix and one part 50 per cent dextran sulfate) overnight at 55°C. The filters were washed in 20 mM sodium phosphate buffer (pH 6.5), 1 mM EDTA, 0.1 per cent sodium dodecyl sulfate, and 2.5× SSC, twice (20 minutes each time) at 55°C. They were then washed twice for 20 minutes in 20 mM sodium phosphate buffer (pH 6.5), 1 mM EDTA, 0.1 per cent sodium dodecyl sulfate, and 0.3× SSC at 65°C, and similarly, in the same solution but with 0.1× and 0.03× SSC, consecutively, at 65°C. Filter washings were stopped when no radioactivity could be detected in the washing solutions. Filters were exposed to XAR-2 film (Kodak) at −70°C with intensifying screens (Dupont Lightning Plus).

Results

Northern Blot Analysis

To demonstrate the presence of chimeric *bcr*/c-*abl* mRNAs in the bone marrow cells of the patients, a number of probes were required to perform Northern blot analysis.

We recently isolated a *bcr* cDNA clone from a library of normal human-fibroblast cDNA (complementary DNA).[11] Upon hybridization of this clone with previously isolated cosmid clones encompassing the *bcr*, we demonstrated that the *bcr* is part of a large gene, the *bcr* gene, consisting of at least 12 exons located 5′ and 3′ of the *bcr*.[5] Because the 5′ and 3′ ends of the *bcr* cDNA are known,[12] the orientation of the *bcr* gene on chromosome 22 could be determined; its 5′ end is pointed to the centromere, and its 3′ end to the telomere of chromosome 22. As a consequence of Ph[1] translocation, the 5′ part of the *bcr* gene remains on chromosome 22 while the 3′ part is translocated to chromosome 9 in the t(9;22).[5] In previous studies, the majority of all Ph[1] breakpoints have been shown to occur in the intron sequences between the exons denoted as II and III or in the intron between exons III and IV (Fig. 1A).[4,5] This implies that the part of the cDNA corresponding to probe A (Fig. 1B) remains on chromosome 22, whereas the cDNA sequences corresponding to probe B are translocated to chromosome 9 in the Ph[1] translocation. Both probes were inserted in reversed orientation in pSP vectors, permitting isolation of [^{32}P]RNA complementary (anti-sense) to normal *bcr* mRNA. Labeled probes were generated by means of this vector construct, since previous experiments[11] had demonstrated that such probes enhance the signal by at least fivefold in Northern hybridiza-

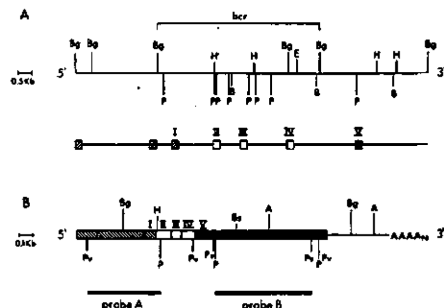

Figure 1. Exon–Intron Organization within the *bcr*.

Panel A is a restriction-enzyme map of genomic *bcr*; the 5.8-kb *bcr*, in which all breakpoints occur on chromosome 22 in the Ph[1] translocation, is indicated above the map. The location of exons immediately 5′,3′ and within the *bcr* are shown (not drawn to scale) beneath the map. The numbered exons are referred to in the text.

Panel B is a restriction-enzyme map of *bcr* cDNA; the numbers and shading of regions in the cDNA correspond to the exons similarly labeled in Panel A. The *bcr* cDNA probes used in this study are shown beneath the map. The following abbreviations denote the restriction enzymes used in mapping: A represents *Ava*I, B *Bam*HI, Bg *Bgl*II, Bs *Bst*EII, E *Eco*RI, H *Hin*dIII, P *Pst*I, and Pv *Pvu*II. The AAA$_N$ at the 3′ end of the cDNA indicates the poly A tail.

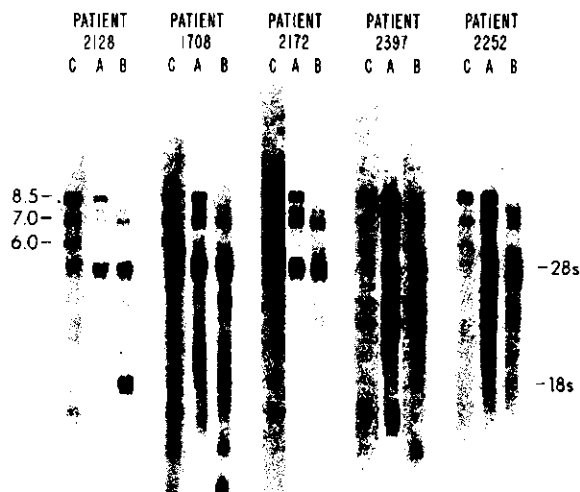

Figure 2. Northern Blot Analysis of RNA from Five Patients with CML.
The lanes show results with the following probes: A denotes the 5′ *bcr* probe (see Fig. 1B); B, the 3′ *bcr* probe (Fig. 1B); and C, the c-*abl* probe. The molecular weight of c-*abl*-hybridizing RNA (in kilobases; 1 kb = 3.3×10^5 daltons) is indicated at the left; the position of ribosomal 28s and 18s RNA is shown at the right.

tions, as compared with conventional, nick-translated DNA probes. Similarly, a human c-*abl* fragment encompassing the most 5′ v-*abl* homologous exon (probe C) was inserted into a pSP vector.[13]

Abnormal *bcr*/c-*abl* mRNA in Leukemic Cells

If a chimeric *bcr*/*abl* mRNA exists in the leukemic cells of patients with CML, we would expect to detect an abnormally sized *abl* transcript; the same abnormal mRNA should hybridize with the 5′ *bcr* probe (probe A) but not with the 3′ *bcr* probe (probe B), since the genomic sequences corresponding to the latter probe are translocated to chromosome 9 in the t(9;22). The data from our experiment with these probes are shown in Fig. 2.

All patients had an 8.5-kb transcript that hybridized both with the c-*abl* and the 5′-specific *bcr* probe (Fig. 2). The 3′-specific *bcr* probe did not hybridize with this transcript, thus excluding it as a normal *bcr* mRNA. The 8.5-kb transcript seems to be characteristic of Ph^1-positive cells, since it is not found in either normal control cells or cells of other types of leukemia.[14,15] Three bands around 7.5, 7.0, and 4.5 kb were detected with both the 5′ and the 3′ *bcr* probes, indicating that these bands may represent normal *bcr* mRNAs. The transcripts of 7.5, 7.0, and 4.5 kb are also present in other human cell lines and tissues, including several myeloid and lymphoid cell lines, erythroid precursor cells, skin, gut, kidney, and spleen. None of the samples tested contained the 8.5-kb *bcr*-related RNA.[11]

The bone marrow cells from all the patients contained a 6.0-kb and a 7.0-kb c-*abl* transcript (Fig. 2). These transcripts are also present in normal cells[14] and represent normal c-*abl* mRNAs. Presumably, both transcripts are initiated at the same promotor but terminate differently at the 3′ end of the *abl* oncogene. In addition, an 8.5-kb c-*abl* hybridizing mRNA was clearly demonstrated in all patients. Although all five patients had both the 8.5-kb chimeric and the normal *abl* transcripts, the chimeric transcript was expressed at a higher level.

Patient 2128

Cytogenetic analysis of Patient 2128 revealed a complex [t(9;12),t(12;22),Ph^1] translocation. In a patient with an analogous karyotype,[16] both *bcr* and c-*abl* sequences were found on chromosome 12 by means of in situ hybridization techniques. This suggests that even in complex Ph^1 translocations, *bcr* and c-*abl* are fused, producing a chimeric *bcr*/*abl* mRNA. Northern blot analysis of Patient 2128 provided confirmative evidence: both *bcr* and c-*abl* hybridized with the same 8.5-kb mRNA (Fig. 2). Interestingly, probe B strongly hybridized with an mRNA of approximately 2.0 kb (18s) in this patient. This suggests that as a result of the complex Ph^1 translocation, the 3′ part of the *bcr* gene fuses to a currently unknown gene that directs expression of the 3′, translocated part of the *bcr* gene.

Discussion

Cells from patients with Ph^1-positive CML and from different CML cell lines contain an abnormal c-*abl*-related transcript of approximately 8.5 kb.[14,15,17] In the present studies, we demonstrated that this abnormal mRNA was present in RNA from each of five patients with CML and that it represents a chimeric *bcr*/c-*abl* transcript.

The specific presence of this novel transcript in leukemic cells of patients with CML supports the view that both c-*abl* and *bcr* are involved in this disorder and that the fusion of *bcr* and c-*abl* sequences is a crucial event in the disease. The recent demonstration[16] of a breakpoint within the *bcr*, and the location of genomic *bcr* and c-*abl* sequences on chromosome 12 in a patient with a t(9;12) but without a cytogenetically detectable Ph^1 chromosome, further substantiates this model.

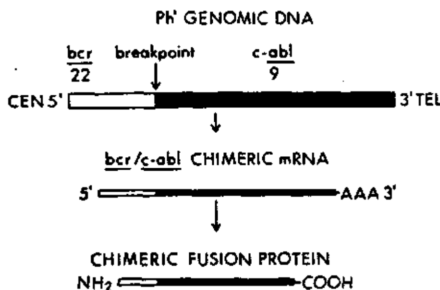

Figure 3. Results of Ph[1] Translocation at the Molecular Level. During the Ph[1] translocation, a break occurs within an intron of the *bcr* gene on chromosome 22 and within the c-*abl* oncogene on chromosome 9; these DNA sequences are physically joined on the Ph[1] chromosome in a head-to-tail fashion, with *bcr* (open part of bar) centromeric (CEN) and c-*abl* (solid part of bar) telomeric (TEL). This configuration allows transcription of a chimeric mRNA, consisting of 5′ *bcr* exons fused to 3′ c-*abl* coding sequences, including the region coding for phosphotyrosine kinase activity. After splicing, this mRNA is translated into a chimeric fusion protein with a *bcr* amino terminus and a c-*abl* carboxy terminus.

In approximately 15 to 25 per cent of patients with acute lymphoblastic leukemia, the Ph[1] chromosome is found in the leukemic cells.[18] Preliminary experiments indicate that these patients also have a breakpoint within the *bcr*, suggesting that a chimeric *bcr*/c-*abl* mRNA will be transcribed in the leukemic cells. Translation of a chimeric mRNA, as found in our five patients, would result in a *bcr*/*abl* fusion protein (Fig. 3). Evidence for the existence of such a protein has been recently found in K562, a cell line derived from the leukemic cells of a patient with CML in blast crisis.[19] In this cell line a similar *bcr*/c-*abl* transcript could be demonstrated.[11] In addition, a c-*abl* protein of abnormal size was detected (P210; 210,000 daltons),[20] suggesting that this protein is the translational product of the chimeric *bcr*/*abl* mRNA. Although the tumorigenic potential of this protein is unknown, it involves the c-*abl* oncogene; v-*abl* — the result of a recombination of murine leukemia virus and the mouse c-*abl* oncogene[21] — induces rapid B-cell lymphoma in mice.[22,23] As yet, the normal cellular function of c-*abl* has not been defined; however, this gene belongs to a broader family of genes, some of which may encode cellular receptors. Recently, the insulin receptor was molecularly cloned and shown to have a marked degree of homology to v-*ros* and other members of the tyrosine-specific protein kinase family, such as v-*abl*[24]; the receptor for epidermal growth factor was found to be homologous to the v-*erbB* oncogene, another member of this family of oncogenes.[25]

How the *bcr* part of the abnormal *bcr*/c-*abl* fusion protein could contribute to tumorigenic activity is unknown at the moment. However, studies with the CML cell line K562 may provide insight into how the protein is altered: the abnormal P210 protein has tyrosine kinase activity, in contrast to the normal, P145 c-*abl* protein.[20,26] The *bcr* moiety of this chimeric molecule could therefore have altered the structure of the c-*abl* protein and so unmasked its associated tyrosine kinase activity. It remains an interesting question, though, whether the *bcr* would be sufficient or necessary for this effect. On analogy to the v-*abl* polyprotein, which is also a tyrosine kinase,[27,28] the CML-specific *bcr*/c-*abl* protein might have transforming activity and could play an essential part in the causation or persistence (or both) of CML.

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CHAPTER 5

THE CHRONIC MYELOCYTIC CELL LINE K562 CONTAINS A BREAKPOINT IN BCR AND PRODUCES A CHIMERIC BCR/C-ABL TRANSCRIPT

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The Chronic Myelocytic Cell Line K562 Contains a Breakpoint in *bcr* and Produces a Chimeric *bcr*/c-*abl* Transcript

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In the DNAs of all Ph[1]-positive chronic myelocytic leukemia patients studied to date, a breakpoint on chromosome 22 (the Ph[1] chromosome) can be demonstrated with a probe from the *bcr* (breakpoint cluster region). Although the K562 cell line was established from cells of a chronic myelocytic leukemia patient, we have been unable to detect the Ph[1] chromosome by cytogenetic means. Employing a probe from the 5' region of *bcr*, we have cloned an amplified Ph[1] breakpoint fragment from K562. This demonstrates that K562 contains multiple remnants of a Ph[1] chromosome with a breakpoint within *bcr* and thus may serve as a model system for the study of Ph[1]-positive chronic myelocytic leukemia at a molecular level. The isolation of *bcr* cDNA sequences shows that parts of *bcr* encode a protein. Employing K562, we demonstrate the presence of an abnormally sized mRNA species hybridizing to c-*abl* and to a *bcr* cDNA probe, indicating the possible consequence of the Ph[1] translocation on a transcriptional level in chronic myelocytic leukemia. The isolation and sequencing of a cDNA containing the breakpoint area of this mRNA provide further evidence for its chimeric structure. Cloning of large stretches of chromosomal DNA flanking *bcr* and c-*abl* sequences in K562 and identification of the exons participating in the formation of the chimeric mRNA shows that a splice of at least 99 kilobases is made to fuse the 3' *bcr* exon to the 5' c-*abl* exon. Furthermore two chimeric cDNAs were isolated containing chromosome 9 sequences that map 43.5 kilobases downstream from the K562 breakpoint. These chromosome 9 sequences neither hybridize to the 8.5-kilobase chimeric c-*abl* mRNA nor to normal c-*abl* mRNAs in Hela cells and probably represent incorrect splicing products present in the K562 cell line.

Chronic myelocytic leukemia (CML) is a pluripotent stem cell disease characterized by the presence of the Philadelphia (Ph[1]) chromosome in the leukemic cells of 96% of all CML patients. The Ph[1] chromosome is the result of a translocation between chromosomes 22 and 9 (31). The human c-*abl* oncogene (17) has been mapped to the long (q) arm of chromosome 9 (18). By analysis of somatic cell hybrids, we have shown that this oncogene is translocated to the Ph[1] (22q−) chromosome in Ph[1]-positive CML, demonstrating that c-*abl* is involved in the translocation between chromosomes 9 and 22 (8). The location of the c-*abl* oncogene adjacent to the translocation breakpoint in CML was shown by the isolation of a DNA fragment from the 9q$^+$ chromosome of a CML patient; this fragment contained sequences of both chromosomes 9 and 22. The breakpoint had occurred 14 kilobases (kb) immediately 5' of the v-*abl* homologous sequences and resulted in a 9q$^+$ chromosome in which the tip of chromosome 9, including the v-*abl* homologous sequences, was replaced by sequences of chromosome 22 (20). The isolated chromosome 22 sequences of this chimeric DNA fragment enabled us to study their role in the Ph[1] translocation in greater detail. A breakpoint cluster region (*bcr*) was identified on chromosome 22; the DNAs of all (over 30) Ph[1]-positive CML patients examined to date have breakpoints in this region of up to 5.8 kb. As a result, c-*abl* is linked to the same chromosome 22 sequences on the Ph[1] chromosome in all patients, oriented with its 5' end toward and its 3' end away from *bcr* (14).

In 1975, Lozzio and Lozzio (25) reported the isolation of a cell line, K562, from the pleural effusion of an adult patient with CML. This cell line expresses phenotypic markers of eythroid lineage and displays induced and spontaneous globin synthesis (23). We and others (5, 20, 33) have shown that the c-*abl* oncogene and the λ immunoglobulin light chain constant region (Cλ) are amplified at least fourfold in this cell line. In contrast, another human oncogene, c-*sis*, is not amplified (20) and is normally located on chromosome 22 but transposed to chromosome 9 in the Ph[1] translocation (13). These data suggest that K562 contains part of a Ph[1] chromosome which is at least fourfold amplified. However, cytogenetic data are not confirmative, because we cannot detect a Ph[1] chromosome in different pedigrees of this cell line and others (25) have suggested the presence of a single Ph[1] chromosome. Such findings leave the question unresolved as to whether K562 cells may serve as a model system for the study of Ph[1]-positive CML at the molecular level. In the present study we demonstrate the presence of a Ph[1] chromosomal breakpoint in the DNA of K562. The breakpoint has occurred in *bcr*, confirming our previous results that a breakpoint in *bcr* is highly specific for CML. In addition, we have established that *bcr* is part of a protein-encoding region (19). Shtivelman et al. (34) have described the existence of a chimeric *bcr*/c-*abl* mRNA in the CML derived cell lines K562 and EM-2, by cloning partial cDNAs for these molecules. From sequence analysis of the chimeric cDNAs the authors show that the mRNA can code for a *bcr*/c-*abl* fusion protein. In the present paper we provide independent confirmation for the presence of the chimeric *bcr*/c-*abl* mRNA in K562. We identify and sequence the chromosomal exons of *bcr* and c-*abl* that participate in the formation of the chimeric mRNA and show that they are at minimum 97 kb apart in K562. Furthermore we provide

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evidence that probably alternative splicing products can be formed from the *bcr/c-abl* precursor mRNA in K562.

MATERIALS AND METHODS

Southern blotting and hybridization. High-molecular-weight DNAs were isolated as described previously (22), digested with restriction enzymes, and electrophoresed on agarose gels. Blotting was as described by Southern (35) on nitrocellulose (Schleicher & Schuell Co.; ph 79). Nick translation of probes and filter hybridizations were as described previously (2, 10). The specific activity of the probes was 2×10^8 to 5×10^8 cpm/μg. Filters were exposed to XAR-2 film (Eastman Kodak Co.) at −70°C with Du Pont Lightning Plus intensifying screens.

Isolation of probes. DNA probes were prepared by digestion with appropriate restriction enzymes, followed by electrophoresis through low-melting-point agarose gels. Desired bands were cut from the gel and brought into solution by heating at 65°C for 30 min. Agarose was removed by two extractions with phenol equilibrated with 0.3 M sodium acetate (pH 5.0) and one extraction with phenol-chloroform-isoamyl alcohol (25:24:1). DNA was precipitated with ethanol and 0.2 M sodium acetate (pH 5.6) in the presence of 20 μg of Dextran T-500 per ml as a carrier. Restriction enzymes and low-melting-point agarose were purchased from Bethesda Research Laboratories, Inc., and were used according to the supplier's specifications.

Molecular cloning. A cosmid library was constructed of size-fractionated K562 DNA partially digested with *Mbo*I by previously published procedures (12) and screened with the 0.6-kb *Hin*dIII-*Bam*HI *bcr* probe (see Fig. 2) by the method of Grosveld et al. (15). Three positive cosmid clones were isolated and mapped independently by digestion of individual restriction enzyme fragments isolated from low-melting-point agarose gels.

Construction of k562 cDNA libraries. Total polyadenylated [poly(A)$^+$] RNA (50 μg) was denatured with 1 mM methylmercuric hydroxide before cDNA synthesis (26). First-strand synthesis was primed with oligo(dT) or 120 ng of c-*abl* 27-mer primer. cDNA synthesis was performed as described by Gubler and Hoffman (16). The double-stranded cDNA was treated with 10 U of T4 polymerase (Bethesda Research Laboratories) for 10 min at 37°C before *Eco*RI methylation and *Eco*RI linker addition. After *Eco*RI digestion, excess linkers were removed by passage of the cDNA over Sepharose 2B-CL (Pharmacia Fine Chemicals). The cDNA was ligated to λgt10 DNA cut with *Eco*RI, essentially as described by Huynh et al. (21). For the total library 4×10^6 plaques were screened, and for the c-*abl* primed library 2×10^5 plaques were screened. *Eco*RI inserts from positive plaques were subcloned into the *Eco*RI site of pUC18 or pUC19.

RNA analysis. Total RNA was isolated by the LiCl-urea method (1). Poly(A)$^+$ RNA was obtained after two passages of the RNA over oligo(dT)-cellulose, and 20 μg of poly(A)$^+$ RNA of K562 was electrophoresed on a 1% agarose gel in the presence of formaldehyde (26). After blotting, nitrocellulose filters were hybridized to the probes indicated in the legends to Fig. 3 and Fig. 8.

RESULTS

Identification of K562 chimeric DNA fragments. In the DNAs of all Ph[1]-positive CML patients examined to date, the presence of a breakpoint on chromosome 22 can be demonstrated for the majority of the DNAs by using a 1.2-kb *Hin*dIII-*Bgl*II probe (14) (Fig. 1A); for example, abnormal *Eco*RI restriction enzyme fragments are clearly present in the DNAs of CML patients 02120185, 0319129, and 0311068 (Fig. 2A). Restriction enzyme fragments containing these breakpoints have been molecularly cloned (14, 20; unpublished results) and shown to represent 9q$^+$ fragments. In the K562 cell line abnormal fragments could not be detected either with *Eco*RI (Fig. 2A, lane 4) or with each of several other restriction enzymes tested (data not shown) after hybridization to the 1.2-kb *Hin*dIII-*Bgl*II probe. This could indicate that K562 does not contain a breakpoint on chromosome 22 within *bcr*. To examine this more thoroughly, a probe more to the 5' (0.6-kb *Hin*dIII-*Bam*HI probe; Fig. 1A) within *bcr* was prepared and hybridized to the DNA of K562 digested with different enzymes. This probe detects, in addition to the normal 5.0-kb *Bgl*II fragment (Fig. 2B, lanes 3 and 4), abnormal *Bgl*II fragments in K562 DNA (Fig. 2B, lane 2). Moreover, one of these fragments is amplified at least fourfold. Abnormal amplified restriction enzyme fragments in K562 could also be detected by using other enzymes (Fig. 2B, lane 1). Since the 0.6-kb *Hin*dIII-*Bam*HI probe has detected 22q$^-$ fragments in the DNAs of a number of CML patients (14), it seemed likely that the abnormal amplified fragments in K562 represent amplified sequences on the 22q$^-$ chromosome.

Molecular cloning of the K562 22q$^-$ breakpoint fragment. To analyze the abnormal amplified fragments in more detail, a cosmid library was constructed from K562 DNA partially digested with *Mbo*I (12, 15). Numerous colonies of the approximately 100,000 recombinants hybridized with the 0.6-kb *Hin*dIII-*Bam*HI probe; three such positive colonies containing overlapping portions of the same region were selected for further restriction enzyme analysis (Fig. 1D). It is evident from a comparison of the detailed restriction enzyme maps of normal chromosome 22 sequences (Fig. 1B) and K562 DNA (Fig. 1C) that the homology between the two terminates 3' to the most 5' *Ava*I site.

A 1.0-kb *Eco*RI probe prepared from K562 DNA immediately 3' to the breakpoint (Fig. 1D) hybridizes to DNA isolated from somatic cell hybrids containing human chromosome 9 in the absence of chromosome 22, but not to DNA isolated from hybrids containing chromosome 22 (data not shown). This indicates that the sequences isolated from K562 DNA are chimeric and contain the breakpoint of the 22q$^-$ chromosome. The entire region is amplified at least fourfold; the chromosome 9-specific sequences are also amplified, as can be demonstrated by the strong hybridization of the 1.0-kb *Eco*RI probe to K562 DNA in comparison with control DNA (Fig. 2C). Thus the amplification of chromosome 9 sequences begins at the point where the breakpoint has occurred on chromosome 9 in the Ph[1] translocation and extends in the direction of the telomere of the chromosome, including the c-*abl* oncogene. The amplified region may be relatively large, since the distance between the breakpoint on chromosome 9 and the most 5' v-*abl* homologous exon is, at minimum 99 kb (this paper).

***bcr* is part of a gene.** These results indicate that all Ph[1]-positive CML DNAs, including that from the cell line K562 established almost a decade ago and propagated in tissue culture, contain a common genetic defect, a break on chromosome 22 within a very narrowly defined region. This region was found to code for part of a gene of unknown function on chromosome 22 (19). Normal *bcr* cDNA clones isolated with the 0.6-kb *Hin*dIII-*Bam*HI probe (Fig. 1A), such as pV1-3, were characterized by restriction enzyme mapping (Fig. 3) and hybridization to the genomic DNA of

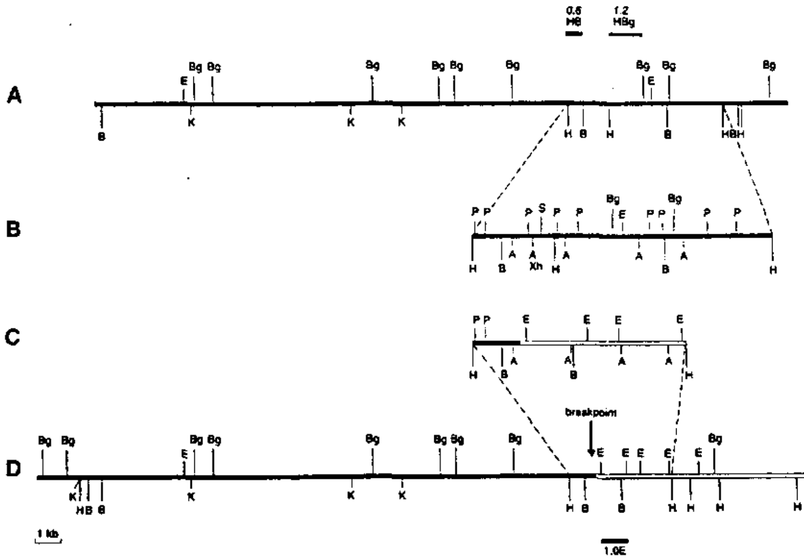


FIG. 1. Restriction enzyme map of the K562 breakpoint region on chromosome 22. A. Restriction enzyme map of human chromosome 22 sequences; the 5.8-kb *Bgl*II-*Bam*HI (*bcr*) region encompasses the 0.6-kb *Hin*dIII-*Bam*HI and 1.2-kb *Hin*dIII-*Bgl*II probes. D. Restriction enzyme map of the Ph' chromosome in K562; the Ph' chromosomal breakpoint is indicated with an arrow. B and C represent more detailed restriction enzyme maps of the indicated regions of A and D, respectively. The solid bars represent chromosome 22 sequences, whereas the open bars indicate sequences originating from chromosome 9. Probes used in the study are shown above A and below D. Abbreviations: A, *Ava*I; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sst*I; Xh, *Xho*I.

chromosome 22. DNA sequence analysis of the cDNA and homologous genomic *bcr* sequences showed exact concordance (19).

Since the cDNA cloning procedure orients the cDNA in the vector (29), the transcriptional orientation of the *bcr* gene could be established; it points toward the telomere of chromosome 22. This implies that *bcr* and the linked c-*abl* gene are transcribed in the same direction on the Ph[1] chromosome (5' end centromeric, 3' end telomeric).

Consequence of the Ph' translocation on *bcr* and c-*abl* expression. Since the *bcr* gene is oriented in the same transcriptional direction as the c-*abl* oncogene, we next examined the influence of the Ph[1] translocation on the transcription of these genes in K562.

For the detection of c-*abl* mRNA we used a human c-*abl* riboprobe containing a 0.6-kb *Eco*RI-*Bam*HI fragment in pSP64 (28). This fragment contains the most 5' human v-*abl* hybridizing exon as identified by DNA sequencing (see Fig. 5 and 6). This exon is homologous to the mouse c-*abl* exon containing the v-*abl–gag-abl* junction identified and sequenced by Wang et al. (37). To examine the effect of the amplification and translocation of the human c-*abl* oncogene on its expression, poly(A)$^+$ RNA was isolated from K562 and control Hela cells. In concordance with results obtained by others (6, 11), the c-*abl*-specific probe detects 6.0- and 7.0-kb mRNAs both in Hela cells and in K562. In addition, a novel c-*abl* homologous mRNA of 8.5 kb is detected in K562 (Fig. 3, left lane). An extra band of approximately 11 kb is also visible and may represent c-*abl* precursor RNA, which has not been studied in further detail. The abnormally sized mRNA seems to be characteristic of Ph'-positive cells, as it is not detected in either normal control cells or cells of other types of leukemia (4, 11).

For experiments involving *bcr* gene expression, the following two probes were subcloned from the normal *bcr* cDNA pV1-3: probe A, *Pvu*II-*Pst*I, containing sequences 5' of the K562 breakpoint, and probe B, *Pst*I-*Pvu*II, 3' of the breakpoint (Fig. 3). The relative position of the breakpoint within the cDNA was determined by Southern hybridization: cDNA sequences 5' of the breakpoint hybridize with the K562 and normal chromosome 22 cosmid clones, whereas sequences 3' of it only hybridize with normal cloned chromosome 22 DNA sequences (Fig. 1). Both probes were cloned in reversed orientation in pSP64 and pSP65, respectively. Hybridization of the Northern blot with *bcr* probe A shows a strongly hybridizing band of 8.5 kb that migrates at the same position as the 8.5-kb c-*abl*-hybridizing RNA (Fig. 4). Vague bands of around 7.0 and 4.5 kb are also detected and could represent the normal *bcr* transcripts; however the nature and structure of these transcripts has not been analyzed in detail (35a). Because the c-*abl* and *bcr* probes do not cross-hybridize, the nature of the 8.5-kb RNA can be explained in two ways: either this new RNA is a hybrid RNA consisting in part of *bcr* and in part of c-*abl* sequences, or alternatively K562 contains a new *abl* and a *bcr* RNA that happen to be of the same size. When the K562 poly(A)$^+$

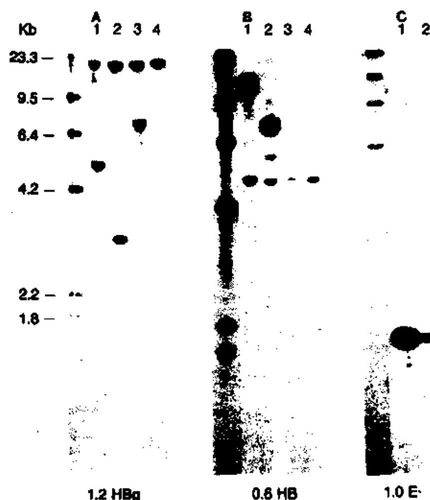


FIG. 2. Ph[1] chromosomal breakpoint in K562. A, *Eco*RI digest of 10 μg of DNA from CML patients 02120185 (lane 1), 0319129 (lane 2), and 0311068 (lane 3) and from the cell line K562 (lane 4). B, K562 DNA digested with *Sst*I (lane 1) and *Bgl*II (lane 2); DNAs of human cell lines AG1732 (lane 3) and AG2655 (lane 4) digested with *Bgl*II. C, *Eco*RI digest of K562 DNA (lane 1) and DNA of human cell line GM3344 (lane 2). A, B, and C were hybridized with different molecular probes as shown at the bottom of the figures; the origin of the probes is as indicated in Fig. 1. ^{32}P-labeled *Hin*dIII-digested λ DNA is included in the left lane of each panel as a molecular weight marker.

RNA blot is hybridized to probe B (3' of the K562 breakpoint) no hybridization to the 8.5-kb RNA can be detected (Fig. 4). In longer exposures of this Northern blot, only faint hybridization can be seen with the normal *bcr* RNAs. This experiment suggests that the 8.5-kb mRNA is indeed a chimera and contains, in addition to c-*abl* sequences, the 5' region of the *bcr* gene.

Cloning of a cDNA containing the chimeric part of the 8.5-kb mRNA. To investigate the structure of the 8.5-kb mRNA in more detail, a K562 cDNA library was constructed in λgt10 (21) by using a 27-mer c-*abl* oligonucleotide to prime the first-strand cDNA synthesis. The primer was derived from the 5' side of the human v-*abl* homologous region (designated exon a2 in Fig. 5), and its sequence is shown in Fig. 6A. After screening of this library with a combined c-*abl* 0.3-kb *Eco*RI-*Kpn*I probe (specific for the 5' side of exon a2, Fig. 5) and *bcr* probe A (Fig. 3) several hybridizing plaques were found; one, ba 4.1, was subcloned in pUC19 and analyzed in detail. This cDNA contains a insert of 468 bp flanked by synthetic *Eco*RI linkers. The restriction map of ba 4.1 is given in Fig. 5. Hybridization of the ba 4.1 insert to a Southern blot containing *Eco*RI-digested K562 DNA and non-CML DNA (GM3344) results in the detection of three amplified *Eco*RI fragments of 16.0, 7.0, and 2.9 kb in K562 and single-copy fragments of 17.0 kb in K562 and 17.0, 7.0, and 2.9 kb in GM3344 (Fig. 7). These bands could be identified by hybridization of the cDNA probe to cloned

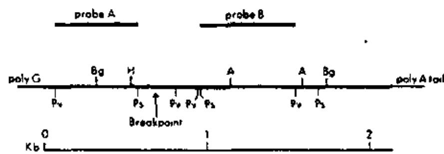


FIG. 3. Restriction enzyme map of the normal *bcr* cDNA, pV1-3. Three normal *bcr* cDNA clones were isolated from a fibroblast cDNA library (29) after screening with the 0.6-kb *Hin*dIII-*Bam*HI probe (Fig. 1A). Of the largest cDNA, pV1-3, only the 2.2-kb cDNA insert is shown flanked by poly(G) and poly(A) tails. A restriction enzyme map was deduced with the following enzymes: Pv, *Pvu*II; Bg, *Bgl*II; H, *Hin*dIII; Ps, *Pst*I; and A, *Ava*I. The arrow indicates the position of the *bcr* breakpoint in the cell line K562. Sequences to the left are linked to c-*abl* and are amplified in K562; sequences to the right are not amplified. Probe A represents a 0.5-kb *Pvu*II-*Pst*I fragment, and probe B a 0.56-kb *Pst*I-*Pvu*II fragment, both cloned in reversed orientation into pSP65 and pSP65, respectively.

c-*abl* and *bcr* sequences (data not shown). The 17.0-kb single-copy fragment in both DNAs represents the normal *Eco*RI fragment on chromosome 22, whereas the amplified 16.0-kb band represents the *bcr* gene containing the Ph[1] breakpoint (Fig. 1D). The 2.9-kb band contains c-*abl* exon a2 (Fig. 5), and the 7.0-kb band could be mapped directly 5' to the 2.9-kb c-*abl* fragment. The exon sequences present in

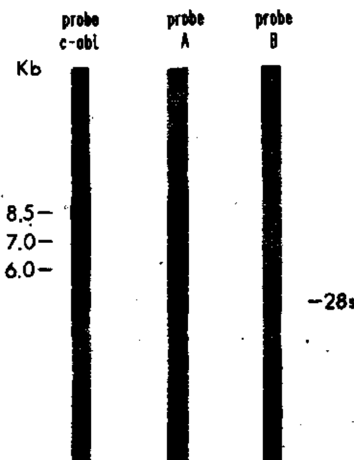


FIG. 4. Hybridization of K562 Northern blot with c-*abl* and *bcr* probes. Poly(A)$^+$ RNA (20 μg) of K562 was run on a 1% agarose gel and transferred to nitrocellulose. As probes we used a 0.6-kb *Eco*RI-*Bam*HI c-*abl* fragment (8), a 0.5-kb *Pvu*II-*Pst*I 5' *bcr* fragment (probe A) and a 0.56-kb *Pst*I-*Pvu*II 3' *bcr* fragment (probe B) cloned in pSP64, pSP65, and pSP64, respectively. The probes were labeled and hybridized as described in the supplier's manual (Promega Biotec). After hybridization the filters were washed under stringent conditions (0.03X SSC [1X SSC in 0.15 M NaCl plus 0.015 M sodium citrate], 65°C).

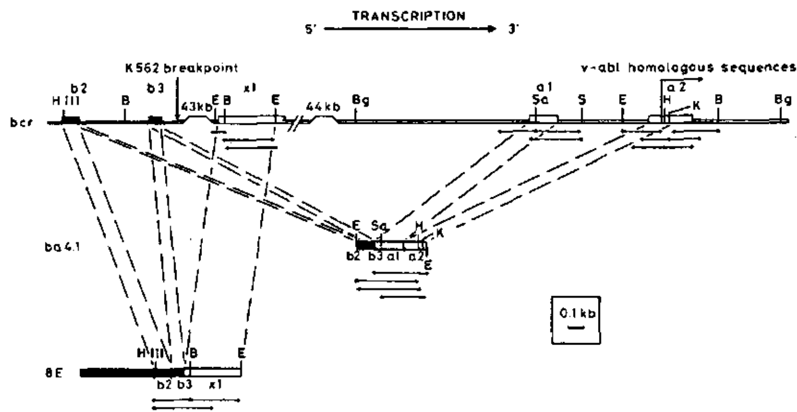
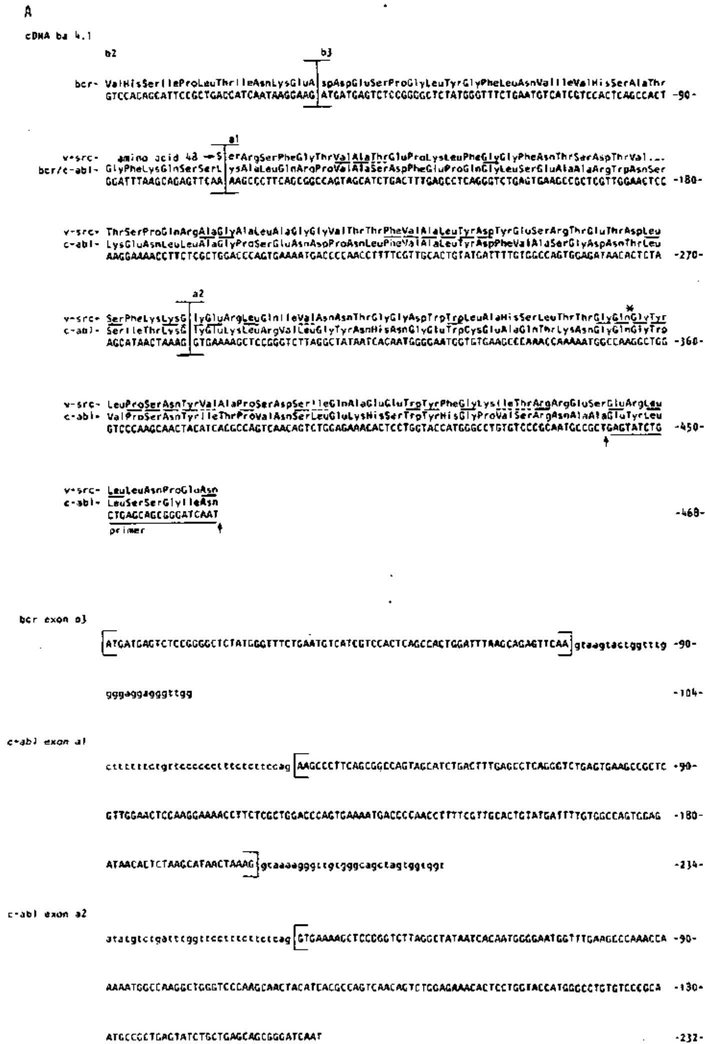


FIG. 5. Restriction enzyme maps of two chimeric cDNAs and the positions of the exon sequences in the K562 breakpoint region. The upper line represents the restriction map of the Ph[1] chromosome in K562. The chromosomal breakpoint is indicated by an arrow above the map, as is the region of c-*abl* that is homologous to v-*abl* sequences. Blocks represent exon sequences of which b2 and b3 are *bcr* exons and a1 and a2 c-*abl* exons. x1 represents a chromosome 9-specific exon not homologous to c-*abl*. Symbols: ⌒, mapped and cloned chromosome 9 sequences (at the 3' side of exon x1 another 10 kb of chromosome 9 sequences have been cloned and mapped, but this has been omitted from the figure); -//-, gap of unknown size. The middle line represents the restriction map of the chimeric K562 cDNA ba 4.1. The relative positions of the ba 4.1 exon sequences on K562 chromosomal DNA are indicated by the dashed lines. The lower line represents the restriction map of chimeric K562 cDNA 8E. The relative positions of the 8E exon sequences on K562 chromosomal DNA are again indicated by dashed lines. Only the *bcr* exons of interest in 8E have been indicated. The arrows under both cDNAs and chromosomal exon sequences indicate the sequence strategies. All sequences were done by the method of Maxam and Gilbert (27). Dots indicate the positions of the end labels. The arrows indicate directions and lengths of the sequences; an exception on this was the chromosomal exon x1, of which the 1.6-kb *Eco*RI-*Bam*HI and 0.3-kb *Bam*HI-*Eco*RI fragments were cloned in M13 and sequenced by the dideoxy method (32). All sequences were determined from both strands, except for the 1.6-kb chromosomal *Eco*RI-*Bam*HI fragments of exon x1, which was sequenced from one strand. Solid bars represent chromosome 22 sequences, whereas open bars indicate sequences from chromosome 9. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; HIII, *Hin*dIII; K, *Kpn*I; S, *Sal*I; Sa, *Sau*I.

these fragments are located within one 2.7-kb *Bgl*II fragment, indicated in Fig. 5. From these two experiments we concluded that cDNA ba 4.1 contains previously identified *bcr* and c-*abl* coding sequences complemented with as yet unidentified c-*abl* hybridizing DNA. To determine the exact nature of ba 4.1, the cDNA was sequenced (27) as designated in Fig. 5; the sequence is shown in Fig. 6A. Comparison of the 5' side of the clone to the pV1-3 *bcr* cDNA sequence revealed exact concordance with the 3' part of *bcr* exon 2 and the entire *bcr* exon 3 (here designated b2 and b3; Fig. 5) (19). The *bcr* sequence in ba 4.1 is immediately flanked at the 3' side by an unknown non-*bcr* sequence. The 3' end of the clone is identical to the sequence of c-*abl* exon a2 (Fig. 6A) and ends with the sequence of the 27-mer c-*abl* primer. The unknown middle part of ba 4.1 contains an *Sau*I site (position 147; Fig. 6A) that also seemed to be present in the genomic 2.7-kb *Bgl*II c-*abl* fragment (Fig. 5). Sequencing of the genomic DNA around the *Sau*I site (Fig. 5) identified the c-*abl* exon a1 coding for the unknown ba 4.1 cDNA sequence (Fig. 6A). Mapping of the *Sau*I site localizes c-*abl* exon a1 0.56 kb upstream from c-*abl* exon a2. The genomic 0.3-kb *Sau*I-*Sal*I fragment containing exon a1 (Fig. 5) detects the c-*abl* mRNAs of 6.0 and 7.0 kb in HeLa cells and of 6.0, 7.0, and 8.5 kb in K562 cells (data not shown). This proves that exon a1 is part of the c-*abl* gene. Comparison of the ba 4.1 cDNA sequence to the sequence of Shtivelman et al. (34) shows exact concordance.

The sequence of the genomic *bcr*-exon b3, as defined by the *bcr* cDNA sequence, is followed by a splice donor site (3) (Fig. 6A); the K562 breakpoint has been localized to 3' of this splice site in the intron between *bcr* exons b3 and b4 (19). The genomic sequence of c-*abl* exon a1 is immediately preceded by a splice acceptor and followed by a splice donor (Fig. 6A), and c-*abl* exon a2 again is preceded by a splice acceptor. Therefore in cDNA ba 4.1 these three exons are linked following the GT-AG rule, resulting in the open reading frame (Fig. 6A). This frame is in phase with the predicted *bcr* (19), v-*abl*, and mouse c-*abl* (37) reading frames.

Minimal distance between *bcr* exon b3 and c-*abl* exon a1 in K562. By chromosomal walking experiments with genomic K562 cosmid and λ libraries, we cloned and mapped 54 kb of chromosome 9 DNA downstream of *bcr* exon b3 and 45 kb of DNA upstream of c-*abl* exon a1 as indicated in Fig. 5. Still, the overlap between the two has not been found. This implies that in K562 a splice of at minimum 99 kb has to be performed to link *bcr* exon b3 and c-*abl* exon a1. An obvious question is how the splice system deals with an intron of this size and whether alternative splices are made. Screening with *bcr* probe A (Fig. 3) of an oligo(dT)-primed K562 cDNA library in λgt10 produced two clones that contained *bcr* sequences linked to DNA that did not cross-hybridize to cDNA ba 4.1. One of these clones, 8E, is shown in Fig. 5. Due to incomplete *Eco*RI methylation of the cDNA during



the construction of the library, both clones have been truncated at their 3′ end at an endogenous *Eco*RI site. The 0.3-kb *Bam*HI-*Eco*RI fragment of 8E (Fig. 5) is amplified in K562 DNA (data not shown) and hybridizes to cloned genomic DNA sequences mapping 43.5 kb downstream from the K562 Ph[1] breakpoint (Fig. 5). DNA sequence analysis of the chimeric 3′ part of the 8E cDNA (strategy indicated in Fig. 5) and the homologous genomic exon (designated x1; Fig. 5) shows exact splicing of *bcr* exon b3 to exon x1 following the GT-AG rule, because x1 is preceded by a splice acceptor site (Fig. 6B). However this mRNA cannot code for a chimeric *bcr/c-abl* protein since exon x1 contains transla-

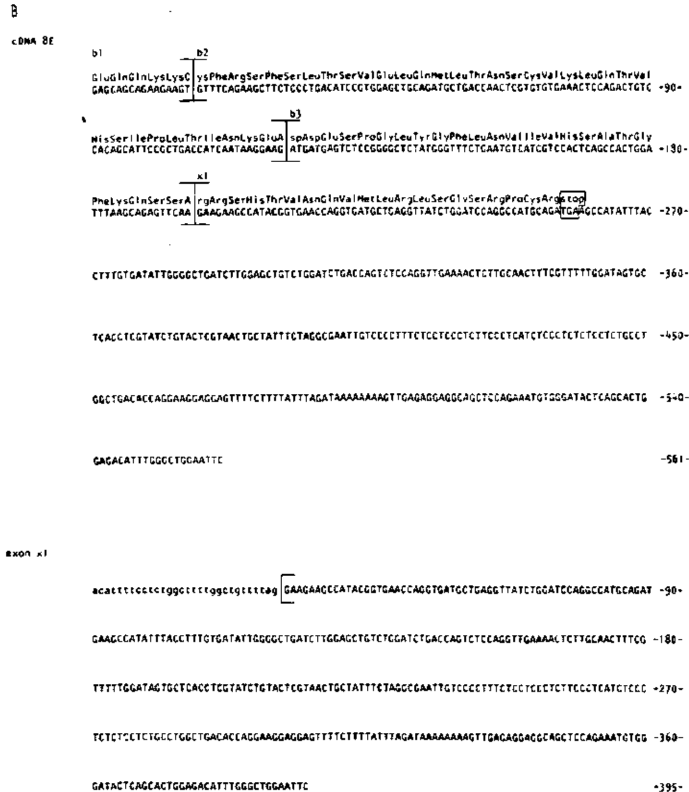


FIG. 6. DNA sequence analysis of cDNA ba 4.1 and cDNA 8E and the corresponding chromosomal exons. Sequence strategies and methods are as indicated in the legend to Fig. 4. A, (i) The nucleotide sequence of the 468-base-pair *Eco*RI fragment of cDNA ba 4.1: b2, b3, a1, and a2 indicate the *bcr* and c-*abl* exons included in ba 4.1; brackets mark the boundaries of the exons. The synthetic c-*abl* primer used for the cDNA synthesis is underlined and indicated by arrows. The amino acids encoded by the cDNA sequence are shown. The v-*abl* homologous sequences start at position 373. The amino acid sequence of c-*abl* is aligned with that of v-*src* amino acids (36). Symbols: *, the position and number of amino acids deleted from v-*src*; —, gap in v-*src* to align the sequences. A solid underline indicates an exact match between v-*src* and c-*abl* amino acids, a dashed underline indicates a match between v-*src* and c-*abl* amino acids with similar structure or identical charge. (ii) The nucleotide sequence of *bcr* exon b3 (19), followed by the 3' intron sequence. Brackets indicate the boundaries of the exon, as defined by the cDNA sequence. (iii) The nucleotide sequence of c-*abl* exon a1, preceded and followed by intron sequences. Brackets indicate the boundaries of the exon as defined by the ba 4.1 cDNA sequence. (iv) The nucleotide sequence of the 5' half of c-*abl* exon a2, preceded by intron sequences. Bracket indicates the boundary of the exon as defined by the ba 4.1 cDNA sequence. B: (i) The nucleotide sequence of the chimeric 3' end of cDNA 8E: b1, b2, b3, and x1 indicate the *bcr* and chromosome 9 exons included in 8E. Brackes mark exon boundaries. The amino acid sequence encoded by this cDNA is shown; 21 codons 3' of the breakpoint a stopcodon are encountered. (ii) The nucleotide sequence of the exon x1 preceded by the intron sequence. Bracket indicates the 5' exon boundary as defined by the 8E cDNA sequence.

tion termination signals in all reading frames including the frame predicted by the *bcr* sequence (Fig. 6B). Hybridization of the exon x1 BE probe to Northern blots containing poly(A)$^+$ RNA of K562 and Hela cells showed two hybridizing bands of 9.5 and 5.5 kb in K562 RNA (Fig. 8A), whereas no signal could be detected in HeLa RNA (Fig. 8B). Since the sizes of the RNAs detected by this 0.3-kb *Bam*HI-*Eco*RI probe in K562 differ from those of the normal c-*abl* transcripts, we conclude that 8E represents the product of an alternatively spliced precursor mRNA, resulting in a chimeric nonfunctional mRNA that contains chromosome 9 sequences not belonging to the c-*abl* gene.

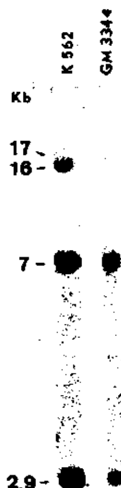


FIG. 7. Hybridization of cDNA ba 4.1 to chromosomal DNA of K562 and GM3344 cells. Southern blot of *Eco*RI digests of 10 μg of DNA from the cell line K562 and cell line GM3344. Both lanes were hybridized to the ^{32}P-labeled *Eco*RI insert of cDNA ba 4.1. Molecular masses are indicated at the side of the gel.

DISCUSSION

The present findings demonstrate that the CML cell line K562, like all Ph^1-positive CML patient material examined to date, contains a breakpoint on chromosome 22 within the breakpoint cluster region on chromosome 22. However, in contrast with the leukemic cells of patients, the K562 cell line contains amplified remnants of the Ph^1 chromosome, including Cλ, a part of *bcr*, and c-*abl*. These amplified regions do not represent multiple copies of intact Ph^1 chromosomes, but rather are present on one acrocentric marker chromosome (33). Most probably, the regions originate from a multiplication of a large region of DNA from the original Ph^1 chromosome: all copies of the *bcr* breakpoint region contain identically sized breakpoint fragments with the restriction enzymes tested.

K562 cells also differ from other CML cells in that the 9q$^+$ chromosome cannot be detected by Southern blot analysis, in concordance with results of cytogenetical analysis in which the 9q$^+$ chromosome was found to be absent from K562 (33); the absence of the 9q$^+$ chromosome strengthens the hypothesis that the 22q$^-$ chromosome is critical to the malignant proliferation of these leukemic cells.

The breakpoint cluster region on chromosome 22 was found to be part of a gene; in K562, the chromosomal break leading to the formation of the Ph^1 chromosome has occurred within an intron of this gene; exons 5' of this point remain on the Ph^1 chromosome, whereas exons to the 3' side most probably were translocated to the 9q$^+$ chromosome in the original recombination event in the patient from whose cells K562 was established.

K562 contains an increased level of the abnormal 8.5-kb c-*abl* mRNA as compared with other CML cell lines (6). Since c-*abl* is amplified in K562, it is likely that this higher expression is caused by the higher copy number of the oncogene. In addition, 5' *bcr*, located on the same amplification unit and present in approximately the same copy number, exhibits high expression of an abnormal 8.5-kb mRNA.

Direct proof that both probes hybridize to the same molecule was given by Shtivelman et al. (34) and by cloning of cDNA ba 4.1 containing the chimeric portion of the *bcr*/c-*abl* mRNA.

Translation of the chimeric cDNA sequence of ba 4.1 into protein shows one open reading frame (Fig. 6A) that is compatible with both the predicted *bcr* and the known v-*abl* reading frames (37). Comparison of the human c-*abl* amino acid sequence of ba 4.1 with the protein sequence of homologous regions in v-*abl* and mouse c-*abl* shows complete amino acid conservation between exon a2 and v-*abl* and one difference with mouse c-*abl* (Fig. 6A). Amino acid 117 of ba 4.1 is a tyrosine, and in mouse c-*abl* it is a cystine. Extensive amino acid sequence homology has been described between v-*src* (36), v-*abl*, and mouse c-*abl* (37). The homology between v-*src* and human c-*abl* does not stop at the 5' side of

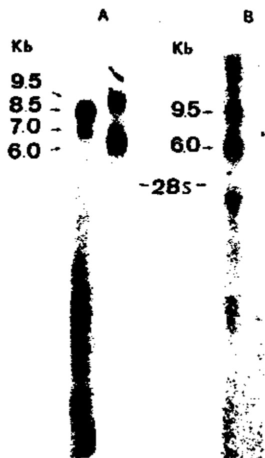


FIG. 8. Hybridization of exon x1 probe to Northern blots of K562 and HeLa poly(A)$^+$ RNA. Northern blots with K562 and HeLa poly(A)$^+$ RNA were prepared as described in the legend of Fig. 3. A, In the left lane, K562 RNA was hybridized with a 0.6-kb *Eco*RI-*Bam*HI c-*abl* probe (Fig. 5); in the right lane, K562 RNA was hybridized with a 0.3-kb *Bam*HI-*Eco*RI exon x1 probe (Fig. 5). B, K562 RNA (left lane) and HeLa RNA (right lane) were hybridized with the 0.3-kb *Bam*HI-*Eco*RI x1 probe. The probe was labeled with ^{32}P by the oligo-labeling method of Feinberg and Vogelstein (9). The molecular masses and the position of the 28S rRNA are indicated at the side of the gel.

c-*abl* exon a2 (Wang et al. [37] have compared the homologous mouse c-*abl* exon to v-*src*), but extends further 5' into c-*abl* exon a1 (Fig. 6A); 15 of the 57 amino acids of a1 are homologous with v-*src*. Although exon a1 is absent in the v-*abl* gene it apparently belongs to part of the protein that is as conserved between v-*src* and c-*abl* as the 3' adjoining kinase domains (as defined by v-*abl* [30]). These results further underline the supposition that c-*src* and c-*abl* have a common ancestor.

From the ba 4.1 sequence (Fig. 6A), it is clear that *bcr* exon b3 and c-*abl* exon a1 are compatible, although the splice takes place within a codon. Mapping experiments and sequencing of several Ph[1] breakpoint clones from different patients indicated that breakpoints occur in introns between *bcr* exons b2 and b3 or b3 and b4 (19; unpublished results). Since these two exons have the 3' splice donor site after the first nucleotide of the codon, both can be spliced to c-*abl* exon a1, resulting in mRNAs that can differ in the presence or absence of one *bcr* exon, i.e., 75 nucleotides in size, dependent on the position of the Ph[1] breakpoint in *bcr*. Whether the proteins encoded by these two mRNAs will have different characteristics remains to be elucidated. This splicing pattern would also explain why the same 8.5-kb mRNA is detected in different patients (4, 11, 35a) although the distance between the Ph[1] breakpoint and v-*abl* hybridizing sequences varies from 14 to at least 101 kb.

As shown by Shtivelman et al. (34), we have strong evidence that exon a1 does not represent the 5' end of the c-*abl* gene. Moreover we have also isolated a normal c-*abl* cDNA clone that contains sequences further 5' of c-*abl* exon a1 (unpublished results). So at least one or more c-*abl* exons must be located further 5' of exon a1. Whether these exons can also be included in the K562 splicing event is unknown and leaves the possibility that ba 4.1 represents only one of several translatable chimeric mRNAs that could be produced in K562. However, if this indeed is the case then it seems unlikely that the 5' end of the c-*abl* gene can be included in a protein-coding chimeric mRNA, since no splice acceptor site will be available and frameshift mutations and stop codons are probably introduced by the nontranslated region of the 5' end of the gene.

Splices over large and varied distances such as those found between *bcr* and c-*abl* on the Ph[1] chromosome open the possibility that exons from other genes not belonging to c-*abl* could be included in the pre-mRNA and therefore end up in a mature mRNA. In fact, cDNA 8E represents an example of this possibility. Although the chromosome 9-specific exon x1 was spliced to *bcr* exon b3 following the GT-AG rule (Fig. 6B), it does not contain an open reading frame. Employing the *bcr* reading frame, a protein termination signal in 8E is encountered at 21 codons 3' of the breakpoint. Hybridization of exon x1 to Northern blots of K562 and HeLa RNA results in the detection of two RNAs of different size than the c-*abl* RNAs for K562, whereas in HeLa cells no RNA at all could be detected. This strongly suggests that exon x1 does not belong to the c-*abl* gene. Because 8E is clearly a chimeric cDNA, hybridization of *bcr* probe A (Fig. 3) to K562 Northern blots should show up the same 9.5 and 5.5 kb RNAs as probe x1. For reasons we do not understand, this does not seem to be the case, but we are confident that 8E represents an aberrant chimeric RNA because a second, independently picked cDNA clone has the same chimeric structure. Unfortunately, both clones have been truncated at their 3'-end *Eco*RI site, so no information is available concerning the nature of the 3' region of these mRNAs. The possibility remains that x1 is spliced at its 3' side to c-*abl* sequences, although hybridization of c-*abl* probes to K562 Northern blots does not show up the 9.5- and 5.5-kb mRNAs. Why this differential splice occurs in the K562 *bcr*/c-*abl* pre-mRNA remains an open question.

The implications of the translocation of c-*abl* to the 3' side of *bcr* on the Ph[1] chromosome are as follows. Transcription is likely to be initiated from the *bcr* promoter and probably stops at the 3' end of the c-*abl* gene. By splicing of the precursor RNA the 8.5-kb mRNA is produced, in which the versatility of the splicing system accomodates for the large variation in intron size that links *bcr* and c-*abl* in different patients. From the data now available, we know that the variation in this chimeric 8.5-kb mRNA can comprise one *bcr* exon, i.e., 75 nucleotides (19). Translation of the 8.5-kb mRNA into protein seems almost certain, since the chimeric part of the molecule contains an open reading frame that links the predicted *bcr* and c-*abl* reading frames. A likely candidate for such a protein is the abnormally sized 210-kilodalton c-*abl* protein found in K562 cells, which in contrast to normal c-*abl* has in vitro tyrosine kinase activity (24), very similar to that of the v-*abl* gene product (7). The *bcr* moiety of this hybrid molecule could unmask the c-*abl* tyrosine kinase activity, raising the question whether *bcr* is sufficient or necessary for this effect. The *bcr*/c-*abl* hybrid protein may have transforming activity; since the chimeric 8.5-kb mRNA is also found in Ph[1]-positive CML patients (35a), the protein is likely to be present in all cases of Ph[1]-positive CML, but it remains to be established whether this plays an essential role in the generation or maintenance of CML.

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CHAPTER 6

EVIDENCE THAT THE PHL GENE ENCODES A 160,000 DALTON PHOSPHOPROTEIN WITH ASSOCIATED KINASE ACTIVITY

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NOTES

Evidence that the *phl* Gene Encodes a 160,000-Dalton Phosphoprotein with Associated Kinase Activity

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In chronic myelocytic leukemia, the human c-*abl* oncogene is translocated from chromosome 9 to a region on chromosome 22 designated as the breakpoint cluster region (bcr) (A. de Klein, A. Geurts van Kessel, G. Grosveld, C. R. Bartram, A. Hagemeyer, D. Bootsma, N. K. Spurr, N. Heisterkamp, J. Groffen, and J. R. Stephenson, Nature (London) 300:765–767, 1982; J. Groffen, J. R. Stephenson, N. Heisterkamp, A. de Klein, C. R. Bartram, and G. Grosveld, Cell 36:93–99.) Abnormal c-*abl* homologous mRNA and protein have been detected in the leukemic cells of patients with chronic myelocytic leukemia (E. Canaani, D. Stein-Saltz, E. Aghai, R. P. Gale, A. Berrebi, and E. Januszewicz, Lancet 1:593–595, 1984; S. J. Collins and M. T. Groudine, Proc. Natl. Acad. Sci. USA 80:4813–4817, 1983; R. P. Gale and E. Canaani, Proc. Natl. Acad. Sci. USA 81:5648–5652, 1984; J. B. Konopka, S. M. Watanabe, J. W. Singer, S. J. Collins, and O. N. Witte, Proc. Natl. Acad. Sci. USA 82:1810–1814, 1985). The abnormal mRNA represents a chimeric transcript consisting of 5' bcr and 3' c-*abl* sequences (G. Grosveld, J. Verwoerd, T. van Agthoven, A. de Klein, K. L. Ramachandran, N. Heisterkamp, K. Stam, and J. Groffen, Mol. Cell. Biol. 6:607–616, 1986; E. Shtivelman, B. Lifshitz, R. B. Gale, and E. Canaani, Nature (London) 315:550–554, 1985; K. Stam, N. Heisterkamp, G. Grosveld, A. de Klein, R. S. Verma, M. Coleman, H. Dosik, and J. Groffen, N. Engl. J. Med. 313:1429–1433, 1985). In the present study, we demonstrated that the abnormal c-*abl* protein is a fusion protein. In addition, the normal gene encompassing bcr sequences was shown to encode a 160,000-dalton phosphoprotein with an associated serine or threonine kinase activity. We propose that this gene be designated *phl*, reserving the term bcr for the region within the *phl* gene encompassing the Ph' translocation breakpoints.

Chronic myelocytic leukemia (CML) is a malignancy of the pluripotent hematopoietic stem cell. A clonal expansion of the stem cell resulting in an increased proliferation of myeloid and B lymphoid lineages is characteristic of the chronic phase of the disease. In a later stage, the blast crisis, cells lose their ability to differentiate, resulting in malignant proliferation of myeloid or lymphoid cells. The leukemic cells undergo a specific chromosomal translocation (the Philadelphia [Ph'] translocation) affecting the long arms of chromosomes 9 and 22 (20). The resulting $22q^-$ or Ph' chromosome characteristic of this type of neoplasia is found in over 95% of all CML patients. We have previously localized the human c-*abl* oncogene to chromosome 9 (10) and shown that the tip of the long arm of chromosome 9, including c-*abl*, is consistently translocated to the breakpoint cluster region (bcr) within the *phl* gene on chromosome 22 (6, 8). The *phl* gene is oriented with its 5' end toward the centromere of chromosome 22. As a result of the Ph' translocation, 3' *phl* exons are physically removed, whereas 5' exons remain on chromosome 22 (11). A chromosomal breakpoint was previously localized within the c-*abl* gene in one CML patient (12). Since the genomic sequences of the *phl* and *abl* genes are fused in a head-to-tail fashion, we hypothesized that an abnormal c-*abl* gene product could be expressed in Ph'-positive leukemia cells (9). Indeed, an abnormally sized *abl* mRNA of 8.5 kilobases (2, 3, and 7) and an *abl* protein of 210,000 molecular weight (P210) have been demonstrated in leukemic cells of CML patients (15). In subsequent experiments, the mRNA was found to be chimeric, consisting of 5' *phl* exons fused to 3' c-*abl* sequences (9, 21, 23). Apart from the fact that it is specifically involved in the Ph' translocation, little is known regarding the *phl* gene and its translational product. Moreover, sequence analysis of a *phl* cDNA clone did not reveal homology to previously identified genes (11). The present studies were initiated in an attempt to establish the chimeric character of the P210 protein and to identify and characterize the normal *phl* gene product.

To raise antisera against the *phl* gene product, we selected two different regions of the *phl* cDNA V1-3. These were a 486-base-pair *Pvu*II-*Hin*dIII fragment, which is retained on the Ph' chromosome in the CML cell line K562 (9) and thus is included in the chimeric *phl*/c-*abl* mRNA, and a 691-base-pair *Pvu*II fragment originating from the 3' translated region of the cDNA (Fig. 1A). These latter sequences are absent from the chimeric mRNA detected in the leukemic cells of CML patients and in the CML cell line K562 (9, 23). Both segments were inserted into appropriate *trpE* expression vectors (22), and *trpE* fusion proteins were synthesized in *Escherichia coli*. Antisera (designated 486 for the *Pvu*II-*Hin*dIII fragment and 691 for the *Pvu*II fragment) were

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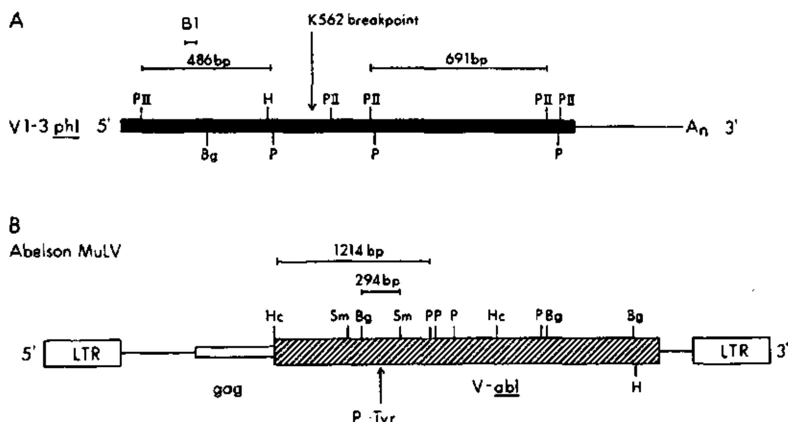

FIG. 1. Schematic representation of the regions of *phl* and *abl* used to raise antisera. (A) *phl* cDNA V1-3. The translated region of the *phl* cDNA V1-3 is indicated by a solid bar; 3' untranslated regions are indicated by a single line. Regions of the cDNA inserted into expression vectors are indicated above the restriction enzyme map of the cDNA by horizontal lines; the approximate location of the synthetic peptide B1 is also shown. A vertical arrow points to the chromosomal breakpoint in the CML cell line K562; all sequences 5' of this breakpoint remain on the Ph' chromosome and are included in the chimeric *phl/c-abl* mRNA. (B) Schematic representation of Abelson murine leukemia virus (MuLV). The acquired cellular sequences (v-*abl*) of Abelson murine leukemia virus are indicated by the cross-hatched box; the long terminal repeats, *gag* portion, and putative phosphotyrosine acceptor region are also shown. Fragments inserted into the expression vectors are indicated above the restriction enzyme map. Restriction enzymes include: Bg, *Bgl*II; Hc, *Hinc*II; H, *Hind*III; P, *Pst*I; PII, *Pvu*II, Sm, *Sma*I. Abbreviations: An, poly(A) tail; P-tyr, putative phosphotyrosine acceptor region; bp, base pair; LTR, long terminal repeat.

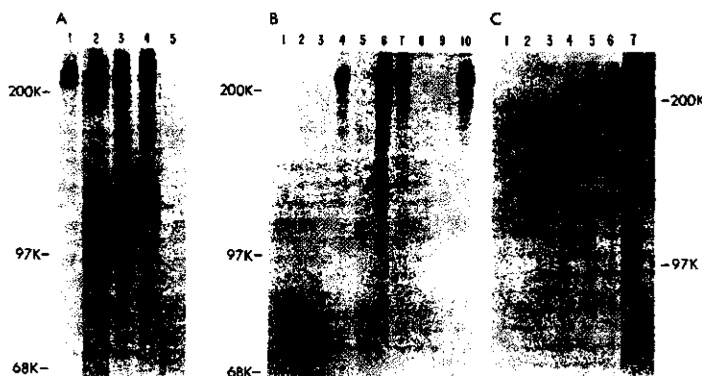

FIG. 2. In vivo and in vitro labeling of P210 with ^{32}P and sequential immunoprecipitation of P210 in K562 cells. (A) Labeling of K562 CML cells in vivo. Immunoprecipitations include sera BgS *abl* (lane 1), HcPs *abl* (lane 2), 486 5' *phl* (lane 3), B1 5' *phl* (lane 4), and normal mouse serum (lane 5). (B) In vitro phosphorylation. Cell extracts were prepared from either K562 cells (lanes 2, 4, 6, 7, and 10) or A498 cells (lanes 1, 3, 5, 8, and 9) with normal mouse serum (lanes 1 and 2), 5' *phl* 486 antiserum (lanes 3 and 4), 5' *phl* B1 antiserum (lanes 5 and 6), *abl* BgS antiserum (lanes 7 and 8) or *abl* HcPs antiserum (lanes 9 and 10). (C) Double immunoprecipitation of the P210 in K562 cells; proteins were precipitated with a first antiserum, labeled in vitro, eluted from the immune complex, and precipitated with a second antibody. The following antisera were used: normal mouse serum and normal mouse serum (lane 1); 5' *phl* 486 antiserum and normal mouse serum (lane 2); *abl* HcPs antiserum and normal mouse serum (lane 3); 5' phl 486 and *abl* HcPs antisera (lane 4); *abl* HcPs and 5' *phl* 486 antisera (lane 5); *abl* HcPs and *abl* HcPs antisera (lane 6); and 5' *phl* and 5' *phl* antisera (lane 7). The immunoprecipitated proteins were analyzed on a 7.5% polyacrylamide gel. The phosphorylated proteins were detected by autoradiography. Exposure time was 4 h. The molecular masses of three protein standards (in kilodaltons) are indicated to the left or right of each panel.

obtained from immunized mice. In addition, a synthetic peptide originating from the 5' region of the *phl* cDNA which is included within the *Pvu*II-*Hin*dIII fragment was used to immunize a rabbit (B1 antiserum; Fig. 1A). Two different bacterially expressed regions of v-*abl*, a 1,214-base-pair *Hin*cII-*Pst*I fragment and a 294-base-pair *Bgl*II-*Sma*I fragment, were also used as immunogens; these antisera were designated HcPs and BgS, respectively.

K562 cells were labeled in vivo with $^{32}P_i$, and analyzed for immunoprecipitation with 5' *phl*-specific antisera 486 and B1. Cells (10^8) were labeled with $^{32}P_i$ (12 mCi) for 4 h and disrupted in 10 ml of RIPA buffer (19). Cell extracts were clarified by centrifugation (13), and a 1-ml extract was incubated overnight at 4°C with 5 μl of antiserum. Protein A agarose (Bethesda Research Laboratories) was used as an immunoadsorbant. The immune complexes were washed, suspended in loading buffer, and analyzed on a 7.5% polyacrylamide gel. The phosphorylated proteins were detected by autoradiography. Both sera immunoprecipitated a ^{32}P-labeled phosphoprotein of a molecular mass of approximately 210 kilodaltons (210K phosphoprotein) (Fig. 2A, lanes 3 and 4). A ^{32}P-labeled phosphoprotein of the same size was similarly detected by two independent *abl* antisera, BgS and HcPs (Fig. 2A, lanes 1 and 2). In contrast, normal mouse serum did not immunoprecipitate detectable amounts of phosphoprotein in K562 (Fig. 2A, lane 5).

To confirm that the above-described 210K protein (P210) represents the abnormal c-*abl* gene product found in K562 cells (16), extracts were subjected to an immune complex kinase assay. The immune complexes collected with protein A agarose were washed and suspended in 20 mM PIPES [piperazine-*N*,*N'*-bis(2-ethanesulfonic acid)] (pH 7.0)–20 mM $MnCl_2$. The reactions were initiated by addition of 10 μCi of [γ-^{32}P]ATP (5,000 Ci/mmol). Reactions (10 min at 30°C) were terminated by the addition of cold RIPA buffer and analyzed as described above. Both *abl* antisera BgS and HcPs immunoprecipitated P210, which autophosphorylated upon addition of [γ-^{32}P]ATP in vitro (Fig. 2B, lanes 7 and 10). Our results with the BgS antiserum are in contrast to results of others, who used a *Bgl*II-*Sst*I fragment of v-*abl* (encompassing the *Bgl*II-*Sma*I fragment) to produce protein in bacteria and to raise antiserum (14). The *Bgl*II-*Sst*I antiserum inhibited the in vitro kinase activity of P210 (5). P210 precipitated with both *phl* 5'-specific antisera 486 and B1 and also exhibited autophosphorylation activity in vitro (Fig. 2B, lanes 4 and 6). Morever, P210 precipitated by *abl*, as well as 5' *phl* antisera, phosphorylated exogenously added substrate, enolase, in vitro. Upon treatment of the gel containing ^{32}P-labeled enolase with KOH, the enolase remained labeled, demonstrating that it contains phosphotyrosine or phosphothreonine (4; results not shown).

The above findings demonstrate the recognition by both 5' *phl* and *abl* antisera of a 210K phosphoprotein with both protein kinase and autophosphorylation activity in vitro. To demonstrate the chimeric nature of P210, we performed sequential (double) immunoprecipitations. Protein was immunoprecipitated from K562 cell extracts with an antiserum, labeled in vitro with ^{32}P as described above, eluted from the immune complex with sodium dodecyl sulfate sample buffer by incubating at 80°C for 5 min, and precipitated with a second antiserum after a 30-fold dilution in RIPA buffer. To ascertain that the first antibody was completely inactivated before addition of the second antibody, we did control experiments with either normal mouse, 5' *phl* 486, or *abl* HcPs antiserum first and normal mouse serum subsequently. The inactivation treatment was effective in that P210 was not detected in these controls (Fig. 2C, lanes 2 and 3). Subsequently, P210 was immunoprecipitated with 5' *phl* antiserum 486 and labeled in vitro. The first antiserum was inactivated, and *abl* HcPs antiserum was added. Conversely, P210 was precipitated with *abl* HcPs antiserum and labeled in vitro; the antiserum was inactivated, and the proteins were incubated with 5' *phl* 486 antiserum. In both experiments, the presence of P210 could be demonstrated unambiguously (Fig. 2C, lanes 4 and 5). Thus, the P210 protein recognized by both antisera is the same molecule, a *phl*/c-*abl* fusion protein.

Since K562 also contains a normal, nonrearranged *phl* allele which is transcribed, albeit at low levels (9), normal *phl* gene products should be present in this cell line. However, even P210 is difficult to detect in [^{35}S]methionine-labeled extracts of K562 cells (data not shown). To find a more abundant source of the *phl* gene product, we prepared poly(A) RNA from different human cell lines and subjected them to Northern blotting and hybridization with a *phl* cDNA probe (23). Of the cell lines tested, a kidney carcinoma cell line, A498, was found to express the highest levels of the approximately 7.0- and 4.5-kilobase (7, 8) *phl* mRNAs (data not shown). However, *phl* gene protein products were not detectable with *phl* 486 antiserum in [^{35}S]methionine-labeled A498 cell extracts (data not shown).

Upon reexamination of proteins immunoprecipitated from $^{32}P_i$-labeled K562 cells, it was evident that a phosphoprotein of around 160,000 molecular weight (P160) is immunoprecipitated with the 5' *phl* antisera 486 and B1 but not with *abl* or normal mouse antiserum (Fig. 2A). As this protein perhaps represents the normal *phl* gene product, A498 and K562 cells were labeled in vivo with $^{32}P_i$. The 5' *phl* B1 antiserum specifically precipitated the P160 in A498 cells (Fig. 3A, lane 2). In K562 cells, P210 was detected in addition to the P160 (Fig. 3A, lane 1). To further demonstrate that P160 is the *phl* gene product, a second, independent antiserum was utilized. This antiserum was raised against a *trpE* fusion protein containing the 3' region of the *phl* cDNA expressed in *E. coli* (Fig. 1A; 691 antiserum). As expected, this antiserum did not detect P210 in K562 (Fig. 3A, lane 3), as the region against which it is directed is not included in the chimeric *phl*/c-*abl* mRNA (9). However, it did immunoprecipitate P160 both in K562 (Fig. 3A, lane 3) and in A498 cells (Fig. 3A, lane 4) labeled in vivo with ^{32}P. These findings establish that the *phl* gene product is a phosphoprotein with a molecular mass of 160,000 daltons.

Since P160 is a phosphoprotein, the possibility was considered that it might exhibit kinase activity. To test this, the *phl* gene product was immunoprecipitated with 691 antiserum from extracts prepared from K562 and A498 cells. Upon addition of [γ-^{32}P]ATP and $MnCl_2$ in vitro, P160 was labeled in complexes from both cell extracts (data not shown). In a second experiment, the substrate enolase was added to the immune complex in vitro to test whether it could be labeled by the P160-associated kinase activity. Both P160 and enolase were labeled in K562 and A498 immune complexes (Fig. 3B, lanes 7 and 8). This demonstrates that the P160-associated kinase exhibits an autophosphorylation activity and in addition has the ability to phosphorylate on exogenously added substrate.

The phosphorylation reaction associated with P160 was not easily detected in immune complexes of P160 with 5' *phl* 486 or B1 antiserum (Fig. 2B, lanes 3 through 6). Phosphorylation of P160 precipitated with 3' *phl* antiserum was inhibited after incubation of the immune complex with B1 antiserum. Preincubation of B1 antiserum with the peptide

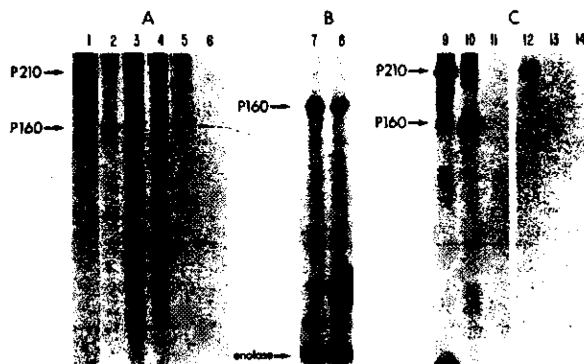

FIG. 3. *phl* gene product. (A) Immunoprecipitation of P160 labeled in vivo with ^{32}P. ^{32}P-labeled cellular extracts are from K562 (10^7 cells; lanes 1, 3, and 5) or A498 (4×10^6 cells; lanes 2, 4, and 6). Antisera included 5' *phl* B1 (lanes 1 and 2), 3' *phl* 691 (lanes 3 and 4), and normal mouse serum (lanes 5 and 6). Exposure time was 8 h for lane 1 and 16 h for lanes 2 through 6. (B) Kinase activity associated with the P160. 3' *phl* 691 antiserum was added to A498 (lane 7) or K562 (lane 8) cell extracts and phosphorylation reactions in vitro were performed in the presence of enolase. (C) KOH treatment of gels containing in vitro ^{32}P-labeled P210 and P160. K562 cellular extracts were incubated with 5' *phl* B1 (lanes 9 and 12), 3' *phl* 691 (lanes 10 and 13), or normal mouse serum (lanes 11 and 14). The segment containing lanes 12 through 14 was fixed, incubated for 2 h at 55°C in 1 N KOH, and refixed before exposure. The positions of P210, P160, and enolase are indicated to the left in each panel. Labeling was performed essentially as described in the text. Before its use as a substrate, the rabbit skeletal muscle enolase (Sigma Chemical Co.) was activated by incubation of a 5-μg/μl solution in the presence of an equal volume of 50 mM acetic acid for 5 min at 30°C (14). For the in vitro phosphorylation, 5 μg of activated enolase was added to the reaction mixture.

B1 neutralized this effect (results not shown). Thus binding of immunoglobulin to a specific domain of P160 may interfere with its associated kinase activity in vitro.

To characterize the P160-associated kinase activity further, a different substrate was added to the immune complex of P160 and 3' *phl* 691 antiserum in vitro. Although enolase was a suitable substrate, we could not detect incorporation of ^{32}P into angiotensin I (data not shown). This is in marked contrast to the protein kinase activity of the chimeric *phl*/c-*abl* P210, which phosphorylates both enolase and angiotensin I (5; results not shown). Angiotensin I is a small molecule containing only tyrosine as a putative phosphate acceptor (14), whereas enolase has tyrosine, serine, and threonine residues. Therefore, the P160-associated kinase activity could exhibit specificity for serine or threonine. In accordance with this, the phosphoamino acids present in P160 were hydrolizable with KOH, indicating that they are phosphoserine or phosphothreonine (4) (Fig. 3C, cf. lanes 10 and 13). The phosphoamino acids in the P210 (phosphotyrosine and 15 to 50% phosphoserine) (5) were less affected by this treatment (Fig. 3C, cf. lanes 9 and 12). Final confirmation was obtained by phosphoamino acid analysis (17). P210 and P160 phosphorylated in the immune complex kinase assay were eluted from the gel and hydrolyzed for 1.5 h at 110°C in 6 N HCl, as described by Davis et al. (5). Approximately 1,000 cpm of *phl* p160 and 1,500 cpm of *phl*/c-*abl* P210 hydrolysates were applied to the thin-layer cellulose plates. Our results show that *phl* P160 (Fig. 4, lane 1) was phosphorylated on serine (±40%) and threonine (±60%), whereas no labeling of tyrosine could be detected. Phosphoamino acid analysis of P160-phosphorylated enolase showed a similar phosphoamino acid composition (data not shown). The *phl*/c-*abl* P210 (Fig. 4, lane 2) was phosphorylated on serine and threonine, as well as on tyrosine. Preliminary results (data not shown) suggest that the phosphoamino acid composition of in vivo labeled *phl* P160 was similar to that of P210 in that the P160 is also phosphorylated on tyrosine (±40%).

The juxtaposition of *phl* gene sequences to those of the

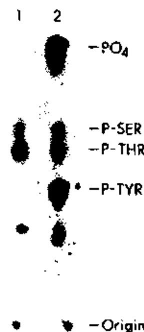

FIG. 4. Phosphoamino acid analysis of in vitro phosphorylated *phl*/c-*abl* P210 (lane 2) and *phl* P160 protein (lane 1). Hydrolysates were applied to thin-layer cellulose plates (10 by 10 cm) and electrophoresed toward the anode at the top for 1.5 h at 400 V in TLE buffer (pyridine-acetic acid-water 5:50:945). Phosphoamino acid standards indicated to the left were detected by ninhydrin staining. Phosphoamino acid composition of the hydrolysates was analyzed by autoradiography. Exposure with intensifying screen was 8 h for lane 1, and 16 h for lane 2. Abbreviations: P-SER, phosphoserine; P-THR, phosphothreonine; P-TYR, phosphotyrosine.

c-*abl* oncogene in CML has a marked influence on the activity of the c-*abl* protein. Whereas normal human c-*abl* P145 lacks high levels of phosphorylating activity in vitro under the extraction and reaction conditions suitable for *phl*/c-*abl* P210 (17), the latter protein has a pronounced tyrosine-specific protein kinase activity in vitro (13, 16). Moreover, *phl*/c-*abl* P210 is phosphorylated on tyrosine in vivo, whereas P145 is not (17). The normal mouse c-*abl* protein has properties similar to the normal human P145 protein (17). However, in Abelson murine leukemia virus, the 5' end of the murine c-*abl* has been removed and replaced by *gag* sequences of the virus; the *gag*/*abl* fusion protein (P160) exhibits a modified *abl* function (v-*abl*) similar to that of P210 (5).

Since the properties of human *phl*/c-*abl* P210 and v-*abl* P160 seem similar (5), the specific contribution of the *phl* or *gag* moieties of these fusion proteins (apart from furnishing promoter sequences and a novel 5' end) to the in vitro protein kinase activity is unclear. Interestingly, the deletion of *gag* sequences in Abelson murine leukemia virus abolishes its lymphoid transforming activity (18). The Ph' chromosome and its novel protein product, *phl*/c-*abl* P210, is found in a lymphoproliferative disease, CML. Quite unexpectedly, the normal *phl* gene product is a phosphoprotein with an associated protein kinase activity, making it entirely dissimilar to *gag*. We have not demonstrated whether this kinase activity is intrinsic to the *phl* gene product or that it is coprecipitated in a complex with *phl* P160. The fact that the 5' *phl* antisera B1 and 486 inhibit the in vitro kinase activity does not necessarily indicate that the kinase activity is intrinsic to P160. The failure to detect protein kinase activity with these antisera could be explained if these antisera cannot recognize a subpopulation of *phl* P160 which is associated with the putative protein kinase. If the protein kinase is distinct from, yet tightly associated with *phl* P160, it would be of interest to examine its interaction with *phl*/c-*abl* P210. Phosphoamino acids detected upon labeling of v-*abl* in vitro are predominantly phosphotyrosine; P210 is found to contain 15 to 50% phosphoserine in addition to phosphotyrosine under the same reaction conditions (5; our results). The *phl*/c-*abl* P210 protein could combine an intrinsic tyrosine kinase and an associated serine kinase activity. In this case, the *phl*/c-*abl* fusion protein would exhibit two protein kinase activities which possibly interact with each other; in addition, the normal cellular substrates of both the *phl* and the c-*abl* proteins could be affected by *phl*/c-*abl* P210.

phl-specific mRNAs of approximately 4.5 and 7.0 kilobases are found in normal cells (21, 23). Since the apparent molecular mass of the *phl* protein is 160,000 daltons, either or both of the mRNAs could encode this protein. We occasionally have observed a protein with a molecular mass of approximately 180,000 daltons immunoprecipitated by *phl* antiserum. This protein might represent the translational product of the larger mRNA; alternatively, both mRNAs might be translated in the 160-000-dalton *phl* protein. Since the sequence of the partial *phl* cDNA bears no homology to the region conserved among the members of the tyrosine-specific protein kinase family, we did not expect the *phl* gene product to belong to this family. In fact, despite an extensive search, we could find no significant homology of the *phl* cDNA sequence to any other previously identified gene product (11). Therefore, its protein kinase activity was unanticipated. If the chimeric *phl*/c-*abl* P210 protein is involved in, or is contributing to CML, the influence of the *phl* moiety must now be considered in concert with that of the modified c-*abl* tyrosine kinase activity. However, it seems clear that the *phl* gene product, apart from its possible involvement in CML, may be interesting in its own right as a phosphoprotein with associated kinase activity.

After completion of this manuscript, Y. Ben-Neriah et al. (1) reported that P210 represents a fusion protein. In addition, they identified a 190-kilodalton phosphoprotein that might be a candidate for the normal *phl* gene product. Our data establish that P160 is the *phl* gene product; P190 could be identical to the P180 protein we observed in some experiments.

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CHAPTER 7

EVALUATION OF THE CLINICAL USEFULNESS OF A DNA PROBE ASSAY FOR THE DETECTION OF THE PH' TRANSLOCATION IN CHRONIC MYELOGENOUS LEUKEMIA

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ABSTRACT

We report the evaluation of the clinical usefulness of a DNA probe assay for the characteristic genetic marker of human chronic myelogenous leukemia (CML), observed by cytogenetics and designated the "Philadelphia chromosome" (Ph'). This translocation activates the c-abl oncogene, normally located on chromosome 9, by fusion to the phl gene[8] on chromosome 22. The assay uses "Southern blot" hybridization of DNA, extracted from peripheral blood or bone marrow, with a probe, designated phl/bcr-210, spanning a breakpoint cluster region (bcr) within phl. Rearrangements are revealed by the presence of one or two novel junction fragments.

Clinical specimens were compared by cytogenetic and DNA probe analysis at seven centers in the United States and Europe. The probe assay identified the phl rearrangement in 201 of 203 cases of Ph'-positive CML, as well as in 12 of 29 confirmed CML specimens lacking a visible Ph' chromosome. DNA rearrangements also were seen in two of six cases of Ph'-positive acute lymphoblastic leukemia. No false positive results were obtained among 93 non-leukemic controls.

Mixing experiments showed that the probe assay can detect cellular populations of one percent carrying the phl/abl gene fusion. The test revealed residual leukemic cells, often not found by cytogenetic analysis, in a significant fraction of CML patients in clinical remission after allogeneic bone marrow transplantation.

NOTE

8. The *phl* gene is often referred to as *bcr*. However, for clarity we prefer to reserve the designation "bcr" for the regions within the *phl* gene in which translocation breakpoints have been found to occur. We also find it useful to distinguish between two such regions in *phl*, bcr-210 and bcr-190, named after the 210 kDa and 190 kDa *phl*/*abl* fusion proteins resulting from translocations with breakpoints in the respective regions. We refer to the corresponding chromosomal translocations as Ph'(bcr-210) and Ph'(bcr-190).

INTRODUCTION

The activation of cellular oncogenes plays a crucial role in neoplastic disease (1,2). Substantial evidence shows that specific chromosomal abnormalities, often involving the sites of known proto-oncogenes, are associated with various forms of leukemia (3). Chromosomal translocations can cause oncogenic activation both by altering the control of gene expression and by causing protein structural changes which lead to the deregulation of the enzymatic activities of oncogene-encoded proteins (1,2). These mutations may result in profound abnormalities in the control of proliferation and differentiation in a given cell lineage.

The Philadelphia (Ph′) chromosome offers one of the most clearly documented examples of a translocation which leads to the activation of a human cellular oncogene. The characteristic cytogenetic abnormality is the $22q^-$ chromosome, usually resulting from a reciprocal translocation t(9;22)(q34;q11) (4). This marker chromosome is found in the leukemic cells of more than 95 percent of patients with chronic myelogenous leukemia (CML) (4,5). A Ph′ chromosome indistinguishable by cytogenetics from that found in CML is also observed in the leukemic cells of about 17 to 25 percent of adults with acute lymphoblastic leukemia (ALL) (6,7,8). A lower incidence has been reported for childhood ALL (about five percent) (9,10) and acute non-lymphoblastic leukemia (ANLL) (less than one percent) (11,12).

The Ph′ chromosome can be used as a prognostic indicator. CML patients with this marker generally show increased survival compared to patients with Ph′-negative CML (13,14,15). By contrast, the presence of the Ph′ chromosome in the leukemic cells of ALL patients appears to

correlate with decreased survival (10,16).

The critical molecular consequence of the Ph′ translocation is a specific gene fusion (Fig. 1). A segment of the c-abl proto-oncogene, located at chromosome 9 band q34, becomes joined to a segment of the phl gene, located at chromosome 22 band q11 (17,18,19,20). In Ph′-positive CML the translocation breakpoint almost invariably lies within a 5.8 kb "breakpoint cluster region" of phl, designated here as bcr-210 (21). Detailed mapping has revealed that translocation breakpoints in c-abl occur within intervening sequences (introns) located in the 5′ region of the gene, almost always upstream of c-abl exon 2. The breakpoints within the bcr-210 region of phl occur in any of three introns separating the four small coding exons (numbered 1 to 4) in this region. The Ph′(bcr-210) translocation diagrammed in Fig. 1 depicts a hybrid gene with a junction between c-abl, in the intron bounded by exons 1B and 1A, and phl, in the intron bounded by exons 3 and 4 of the bcr-210 cluster region. Transcription of such a fused phl/c-abl gene, followed by RNA splicing, gives rise to an 8.5 kb polyadenylated RNA, with phl sequences at the 5′ end and abl sequences (almost invariably beginning with exon 2) at the 3′ end (22,23,24). This mRNA encodes a fusion protein of approximately 210 kiloDalton (kDa), designated P210 phl/abl (25,26). Compared to the normal c-abl gene product, a polypeptide of 145 kDa, the hybrid P210 phl/abl protein exhibits elevated, constitutive tyrosine protein kinase activity (27,28,29).

Molecular analysis of Ph′-positive ALL reveals two classes of rearrangements, both involving phl and c-abl. One group exhibits Ph′(bcr-210) translocations indistinguishable from those found in CML,

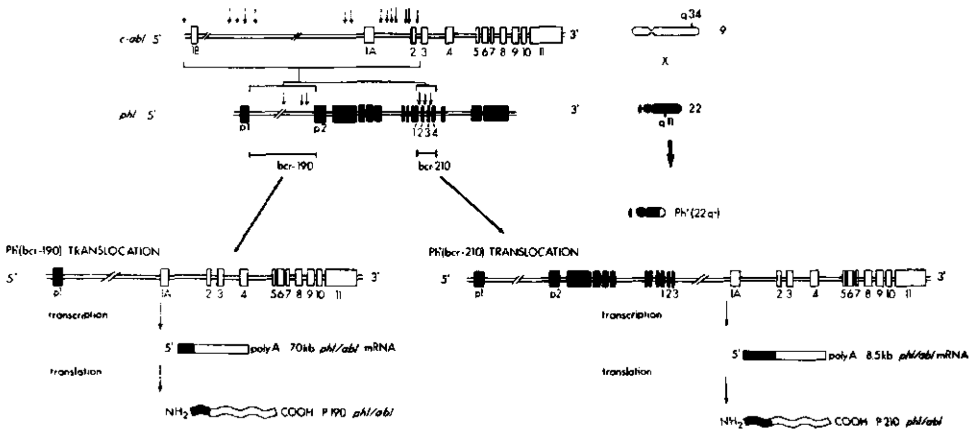

Figure 1. Molecular Basis of the Ph' Translocations.

Generation of Ph' (22q⁻) chromosomes by recombination between phl and c-abl, and structures of spliced mRNA and of protein products. Chromosomal localization of genes and translocation event are depicted at right, and schematic gene structures at left. Arrows indicate known positions of translocation breakpoints (individual cases for c-abl, representative examples for phl). Alternative regions in phl gene introns in which translocation breakpoints are found are labeled bcr-190 and bcr-210. Exons indicated by boxes, introns by double lines. 5' and 3' refer to chemical polarity of mRNA or sense strand of DNA. Wavy boxes: protein products (amino-terminal and carboxyl-terminal ends indicated). Open symbols: c-abl sequences. Closed symbols: phl sequences.

as shown by chromosomal breakpoints within bcr-210, and synthesis of 8.5 kb mRNAs and P210 phl/abl fusion proteins. The second group, here designated Ph′(bcr-190), has translocation breakpoints lying nearer to the 5′ end of the phl gene, within the large intron located between exons p1 and p2 (Fig. 1) (30). The Ph′(bcr-190) translocations are associated with the expression of a 7 kb chimeric phl/c-abl mRNA (30), encoding a 190 kDa phl/abl fusion protein (P190) with elevated tyrosine protein kinase activity (31,32,33,34). The P190 and P210 fusion proteins differ structurally only in the amount of phl-derived sequence at the amino-terminal end of the polypeptide.

The precise role of the phl/abl fusion proteins in leukemogenesis is not known. However, the consistent and specific presence of the P210 and P190 proteins in leukemic cells argues forcefully that their expression is an important step in the pathogenesis of Ph′-positive CML and ALL.

The molecular dissection of the Ph′ translocation offers novel approaches to the differential diagnosis of human leukemias. In particular, because of the relatively small size of the bcr-210 breakpoint cluster region in the phl gene, it is possible to utilize hybridization with a single DNA probe to detect any rearrangement of genomic DNA in this region. We report here the results of the clinical evaluation of such a DNA probe assay. Specimens obtained from CML patients, patients with other leukemias, and non-leukemic controls were assessed for the presence of a Ph′ translocation by both cytogenetic and molecular hybridization analysis. The results show that the DNA probe assay provides a highly specific and sensitive clinical diagnostic test for Ph′(bcr-210) translocations.

METHODS

Sample source

Bone marrow and/or peripheral blood cell specimens obtained from leukemic individuals and non-leukemic (NL) controls were evaluated at seven independent clinical trial sites by both DNA probe and cytogenetic (karyotype) analysis. The participating trial sites and the total number of patient specimens studied per site are as follows: Erasmus University, 25 (I); Leukaemia Research Fund Centre, 53 (II); Vanderbilt University Medical School, 59 (III); M.D. Anderson Hospital and Tumor Institute, 91 (IV); Memorial Sloan-Kettering Cancer Center, 111 (V); University of Rochester, 24 (VI); Oncogene Science, Inc., 65 (VII).

The diagnosis of CML, ALL, or ANLL was based upon clinical and hematological criteria. Samples used in both karyotype and DNA probe procedures were obtained from patients on the same day. 230 samples were obtained from 218 CML patients with active disease. In 12 cases both blood and marrow specimens were obtained simultaneously from the same patient and tested by DNA probe analysis. In 14 additional cases, samples were obtained from patients after extensive chemotherapeutic treatment immediately prior to bone marrow transplantation. 141 of the CML specimens were from peripheral blood and 103 were from bone marrow aspirates. An additional 34 bone marrow specimens were obtained from CML patients in clinical remission after allogeneic bone marrow transplantation.

Cytogenetics

Bone marrow samples were cultured overnight, in the absence of mitogenic stimulation, in Ham's F10 media supplemented with 10% fetal calf serum. Standard cytogenetic procedures were used. Slide preparations were pre-treated with trypsin and either stained with Giemsa to yield G-banded chromosomes, or stained with quinacrine dihydrochloride to yield Q-banded chromosomes. At least 25 metaphase spreads were analyzed from each sample (35). The karyotype was reported according to the International System for Human Cytogenetic Nomenclature (36).

DNA probe construction

A DNA probe, designated _phl_/bcr-210, was designed to detect all Ph'(bcr-210) translocations (37, Fig. 2). The probe consists of genomic sequences which span the entire 5.8 kb bcr-210 region of the _phl_ gene, as well as an additional 5' flanking segment, but lacks an internal _Hin_d III restriction fragment of 1.6 kb containing repetitive sequences. The probe was assembled using DNA fragments obtained from two genomic clones. One clone contained an 11.0 kb _Hin_d III fragment including the 5' portion of the bcr-210 region, while the second contained a 5.2 kb _Hin_d III fragment including the 3' portion of this region. A 2.3 kb _Sal_ I - _Hin_d III fragment and a 2.2 kb _Hin_d III-_Bam_H I fragment (Fig. 2) were excised by restriction endonuclease digestion from the 5' and 3' clones, respectively, and these fragments were purified by gel electrophoresis in low melting point agarose. The plasmid pSP65 (Promega) was digested with restriction endonucleases _Sal_ I and _Bam_H I, and the linearized vector DNA was

mixed together with the two purified phl fragments described above. The DNA was incubated with T4 DNA ligase under conditions described by the supplier (BRL) (38). The ligated preparation was introduced into *E. coli* K12 strain HB101 (39), and transformants were selected by growth on ampicillin plates. Plasmid DNA was isolated from individual transformed clones, selected randomly, and analyzed for the presence of the desired 4.5 kb phl insert (resulting from joining of the 2.3 kb *Sal* I - *Hin*d III fragment to the 2.2 kb *Hin*d III - *Bam*H I fragment) by digestion with *Sal* I, *Bam*H I, *Hin*d III, and combinations of these restriction endonucleases.

A clone with the structure shown in Fig. 2, designated T47-1-8, was identified and used in all subsequent studies. For purification of the phl/bcr-210 probe, circular plasmid DNA was digested with *Sal* I and *Bam*H I, and fragments were separated by electrophoresis on a 1% low melting point agarose gel. The 4.5 kb phl/bcr-210 fragment was excised from the gel and the agarose was melted by heating at 65° C for 30 minutes. DNA was extracted twice with phenol, equilibrated with 0.3 M sodium acetate (pH 5.0), then once with phenol/chloroform/isoamyl alcohol (25:24:1). The extracted DNA fragment was precipitated with ethanol (70% final v/v) and 0.2 M sodium acetate (pH 5.6) in the presence of 20 g/ml Dextran T-500 as carrier. The precipitated DNA was rinsed two times with 70% ethanol, and dissolved in Tris-EDTA buffer.

Isolation of cellular DNA

High molecular weight DNA was isolated from cells of peripheral blood or bone marrow aspirates. Nucleated cells were obtained from

peripheral blood by centrifugation on Ficoll-Hypaque (Pharmacia) according to manufacturer's recommendations. White blood cells were lysed with sodium dodecyl sulfate (38), and DNA was obtained by extraction with phenol/chloroform (1:1) and precipitation with two volumes of ethanol. RNA and protein were removed from the DNA by digestion with RNAse A (final concentration 20 g/ml) for 30 minutes at 37° C followed by proteinase K (final concentration 100 g/ml) for at least 30 minutes at 37° C. The DNA was extracted again with phenol/chloroform (1:1), followed by ethanol precipitation as described (39). The yield of DNA was determined by absorbance at 260 nm.

Hybridization analysis

The purified cellular DNA was digested with Bgl II or another restriction endonuclease, and the resulting fragments were separated by electrophoresis on an agarose gel (0.7 to 0.8% w/v), and transferred to a nitrocellulose or nylon filter by the method of Southern, with minor modifications (39,40). The phl/bcr-210 DNA probe was labelled with ^{32}P to a specific activity of 2×10^8 to 1×10^9 cpm/ g by either nick translation (41) or the random primer method of Feinberg and Vogelstein (42,43). Filter hybridization with at least 2.5×10^6 cpm/ml of labeled probe was performed overnight at 65° C in a solution containing 10 percent dextran sulphate as described elsewhere (44). Filters were subjected to several consecutive washing steps for 30 minutes at 65° C under increasingly stringent conditions (2.5-strength SSC to 0.1-strength SSC; SSC = 0.15 M NaCl, 0.015 M sodium citrate). After the final wash filters were dried, and hybridized probe was

detected by autoradiography using XAR-2 film (Kodak) at -70° C with intensifying screens (Dupont Lightning Plus) for four hours or longer.

RESULTS

The phl/bcr-210 probe test

As a consequence of a reciprocal Ph′ translocation, two novel junctions should be present in genomic DNA, corresponding to the $22q^-$ and $9q^+$ chromosomes, respectively. If the breakpoint in phl lies within the bcr-210 region (Fig. 1), then molecular hybridization with a DNA probe spanning this region should reveal two rearranged fragments in genomic DNA digested with an appropriate restriction endonuclease. The phl/bcr-210 probe (Fig. 2 and Methods) encompasses the entire bcr-210 region, with the exception of an internal 1.6 kb Hind III fragment found to contain repetitive sequences. Figure 2 indicates the human genomic DNA fragments, generated by several restriction endonucleases, that hybridize with this probe. For example, digestion with Bgl II normally yields three detectable fragments of 4.8, 2.3 and 1.1 kb (Fig. 2). A translocation involving bcr-210 would disrupt one of these fragments and generate two new fragments. Because only one copy of chromosome 22 is generally rearranged in Ph′-positive leukemic cells, DNA from such cells would be expected to yield up to five DNA fragments that can hybridize with the phl/bcr-210 probe, i.e. the three germ line DNA fragments and two junction fragments.

We have found Bgl II to be a suitable restriction enzyme for the identification of rearranged phl bcr-210 regions using the phl/bcr-210 probe (37), because of the excellent electrophoretic resolution of the three hybridizable fragments generated by this enzyme, and the apparent absence of polymorphism of the four relevant Bgl II target sites in human genomic DNA. BamH I, Xba I, and EcoR I can be used as well. A

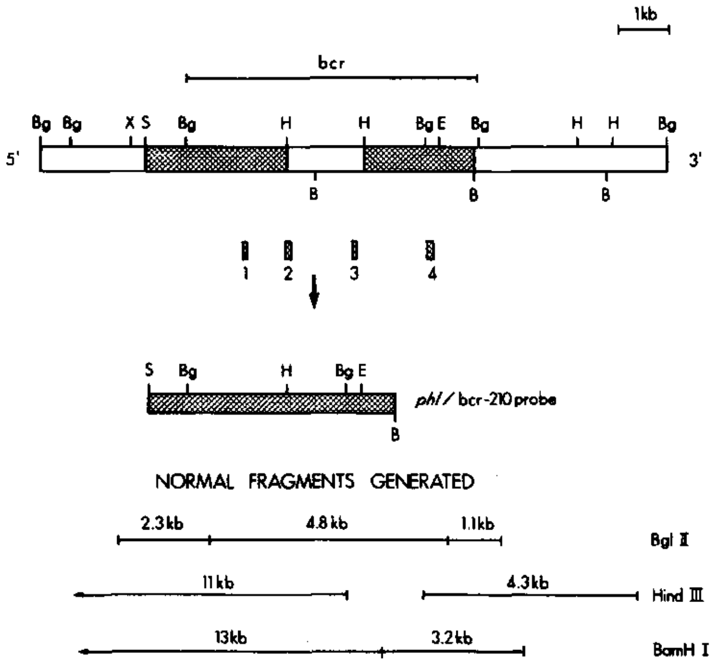

Figure 2. **Restriction Map of phl and Construction of the phl/bcr-210 Probe.**

The position of the bcr-210 breakpoint cluster region (labeled bcr), and regions of genomic DNA present in the phl/bcr-210 probe (cross-hatched boxes) are indicated. Small boxes labeled 1-4 indicate positions of coding exons in bcr-210 region of phl gene. Fragments in normal genomic DNA generated by digestion with several restriction endonucleases, and detectable by the probe are indicated (size in kb) at bottom. Restriction endonuclease cleavage sites: E = EcoR I, B = BamH I, Bg = Bgl II, X = Xba I, S = Sal I, H = Hind III.

rare polymorphism has been observed affecting one of the BamH I sites, which may complicate the analysis using this enzyme. Hind III is a poor choice of enzyme for the identification of Ph′(bcr-210) translocations using the phl/bcr-210 probe, because breakpoints lying within the genomic 1.6 kb Hind III fragment, absent from the probe, will not be detected (Fig. 2).

The results of DNA probe analysis to detect Ph′(bcr-210) rearrangements in the K562 cell line and in cells of five representative Ph′-positive CML patients are shown in Figure 3. The HL-60 cell line, derived from a human promyelocytic leukemia with no Ph′ chromosome, and bone marrow cells of a Ph′-negative CML patient served as controls lacking bcr-210 rearragements. Genomic DNA was digested with Bgl II, and Southern blot hybridization with the phl/bcr-210 probe was carried out as described (Methods). The three germ line fragments (4.8, 2.3 and 1.1 kb) are present in every case. No additional DNA fragments can be detected in DNA from either HL-60 cells (lane 1) or from leukemic cells of the Ph′-negative CML patient (lane 3). In DNA from the CML cell line K562, one novel band is observed (lane 2). This presumably represents a $22q^-$ fragment, since cytogenetic analysis revealed no $9q^+$ chromosome in these cells (23). The intensity of the rearranged band confirms the finding that the chimeric phl/c-abl gene is amplified in K562 (23). In three of the samples from Ph′-positive CML patients, two novel bands can be seen, corresponding to the junctions present in the $22q^-$ and $9q^+$ chromosomes (lanes 4,6,8). Another CML patient sample (lane 5) also appears to have two rearranged DNA fragments, but one co-migrates with the 2.3 kb germ line fragment, as judged by the intensity of the corresponding

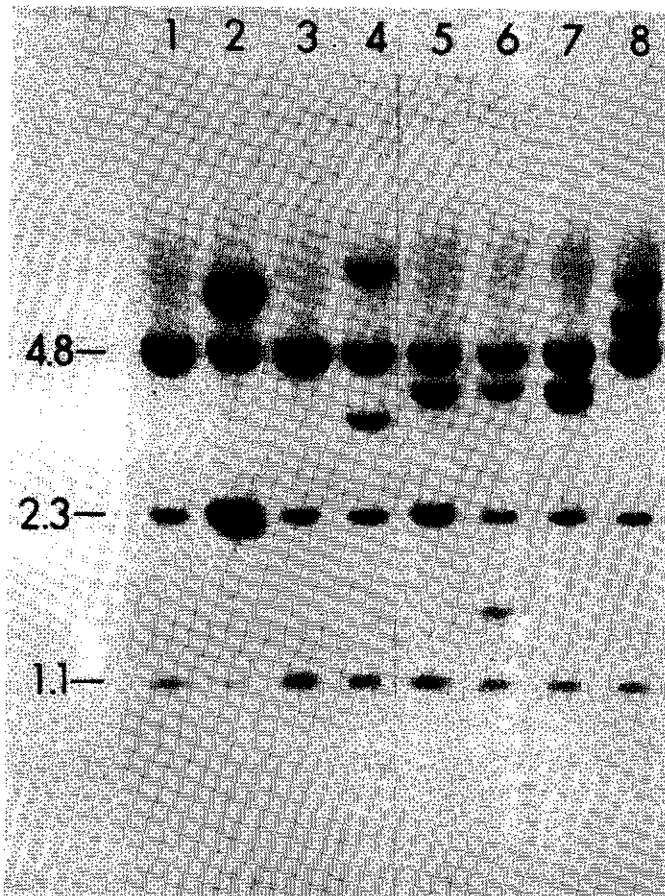

Figure 3. **Assay for Ph′(bcr-210) Translocations by Hybridization with phl/bcr-210 Probe**

DNA from control cell lines or patient specimens was digested with Bgl II and analyzed for the Ph′(bcr-210) translocation by DNA probe assay as described (Methods). Sources of DNA: (1) HL-60 cell line (human acute promyelocytic leukemia); (2) K562 cell line (human CML); (3) Ph′-negative CML patient specimen; (4 - 8) Ph′-positive CML patient specimens. Positions of germ line bands (sizes in kb) are indicated at left.

autoradiographic band. In the analysis of one of the Ph'-positive leukemic DNA samples, only a single extra DNA fragment is apparent (lane 7). It is possible that a small rearranged fragment ran off the gel or that a $9q^+$ fragment is not present in these cells.

Assay sensitivity

In order to determine the minimal fraction of leukemic cells detectable by the probe test, we performed mixing experiments using DNA samples obtained from peripheral blood of a CML patient and from a non-leukemic individual. By cytogenetic analysis, all dividing bone marrow cells analyzed from the CML patient were found to contain a Ph' chromosome. Normal and leukemic DNA were mixed in varying ratios, the DNA was digested with Bgl II restriction endonuclease, and a total of 15 g DNA per slot was separated by gel electrophoresis and analyzed by Southern hybridization with ^{32}P-labeled phl/bcr-210 probe. The results of this experiment are shown in Figure 4. The anticipated germ line fragments (4.8, 2.3, 1.1 kb) are present in both the normal and leukemic DNA samples. A single novel junction fragment (labeled Ph') of approximately 4 kb is observed in the CML patient DNA. The intensity of the corresponding band is roughly proportional to the fraction of leukemic DNA in the input sample, a conclusion supported by densitometric scanning, and ten percent Ph'-positive DNA is readily detectable in a short autoradiographic exposure (Fig. 4A). We repeated the analysis using smaller percentages of leukemic DNA, and a longer exposure time (Fig. 4B). The results of this and comparable experiments indicate that the phl/bcr-210 probe assay can routinely detect less than five percent leukemic DNA, and that as little as one

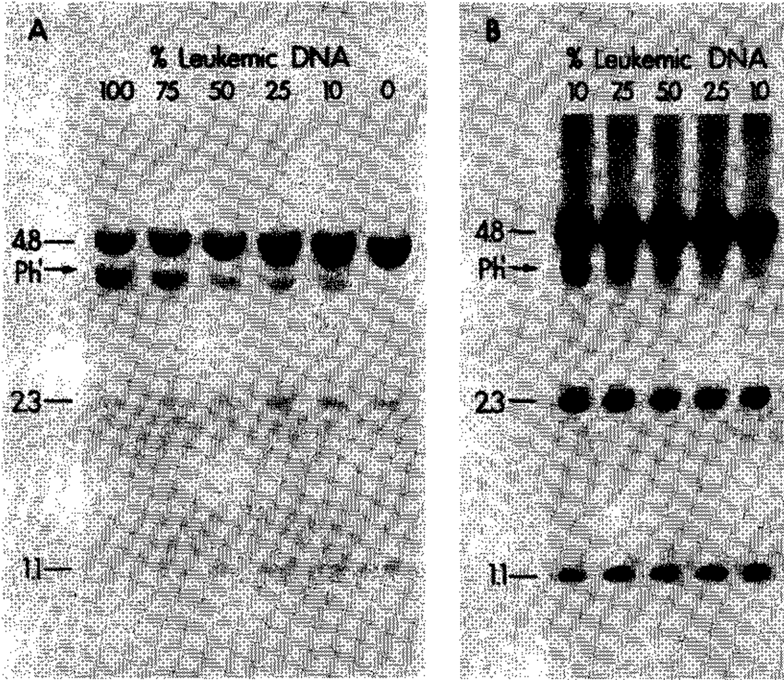

Figure 4. Sensitivity of phl/bcr-210 DNA Probe Analysis.

DNA from a CML patient and a normal donor were mixed and analyzed by digestion with Bgl II and blot hybridization with the phl/bcr-210 probe, by the method of Southern, as described in the text. (A) and (B) show independent experiments using DNA from the same individuals. Percentage of leukemic DNA is denoted above each lane. Positions of normal germ line DNA fragments (sizes in kb) are indicated at left. Arrow labeled Ph' indicates position of the rearranged DNA fragment from the CML patient. Autoradiographic exposure times: (A) 4 hours; (B) 20 hours.

percent leukemic DNA can be observed under favorable conditions. Reconstruction experiments in which leukemic and normal cells were mixed in varying ratios prior to DNA isolation demonstrated a similar assay sensitivity (data not shown).

The sensitivity of the probe assay would be expected to show some dependence on the size of the rearranged DNA fragments, since smaller fragments give a less intense hybridization signal. However, to date, all Ph'(bcr-210) samples have shown at least one rearranged *Bgl* II restriction fragment larger than 2.3 kb. Thus, the reconstruction experiment of Fig. 4 should accurately represent the situation in the vast majority of clinical specimens.

Clinical application

The DNA probe assay was compared with cytogenetic analysis in the diagnosis of Ph'-positive leukemia in over 400 leukemic patients and control individuals tested at seven trial sites. Karyotyping was carried out on cells obtained from bone marrow aspirates, while the probe analysis was carried out on DNA extracted from peripheral blood or bone marrow. Most specimens for the two assays were obtained at the time of initial presentation at a clinic. In all cases *Bgl* II restriction endonuclease was used to digest genomic DNA. If the results obtained with this enzyme were ambiguous, or in cases of apparent discrepancy between cytogenetic observations and the DNA probe assay, the probe analysis was repeated using DNA digested with at least one other restriction endonuclease (usually *Bam*H I). The results of the study are summarized in Table 1.

Both the cytogenetic and DNA hybridization assays revealed Ph'

translocations only in samples from patients with leukemia, and not in any of 93 non-leukemic controls. This confirms the specificity of the probe test.

Among 232 cases of clinically diagnosed CML, 203 were Ph′-positive by cytogenetic analysis. 218 of the patients had clinically active disease, while 14 were in apparent clinical remission induced prior to bone marrow transplantation. Among the latter group, karyotype analysis revealed a population of residual Ph′-positive cells in 12.

Hybridization with the _phl_/bcr-210 probe showed definitive evidence of DNA rearrangement in 201 of the 203 Ph′-positive CML patients (99%). In neither of the two apparently discrepant cases (both tested at site V) was the probe analysis confirmed using a restriction endonuclease other than _Bgl_ II, so it remains to be determined whether these represent examples of rearrangements occurring within or outside of the bcr-210 region. Furthermore, in one case in which the probe analysis failed to confirm a positive karyotype, the patient was sampled during induced clinical remission immediately prior to transplantation.

The DNA probe assay revealed novel _phl_ junction fragments in an additional 12 clinically CML cases in which karyotype analysis failed to detect a Ph′ chromosome. In two of these cases, cytogenetic analysis showed the presence of a complex rearrangement, while in 10 cases a normal diploid karyotype was reported.

Among six cases of cytogenetically Ph′-positive ALL, only two showed bcr-210 rearrangements using the DNA probe test. It seems very likely that in each of the four remaining cases the breakpoint on chromosome 22 was located within the bcr-190 region of _phl_ (Fig. 1), and therefore could not be detected by hybridization with the _phl_/bcr-210 probe. In

Table 1. **Comparison of _phl_/bcr-210 DNA Probe Assay and Cytogenetic Analysis in Clinical Diagnosis of Ph′-positive Leukemia**

DIAGNOSIS (NUMBER)	KARYOTYPE PH′	DNA PROBE POSITIVE / NUMBER TESTED CELL SOURCE PB	BM	TOTAL
CML (232)	+	121 / 121#	80 / 82	201 / 203*
	-	4 / 8	8 / 21	12 / 29
ALL (24)	+	2 / 4	0 / 2	2 / 6
	-	0 / 6	1 / 12	1 / 18
ANLL (33)	+	0 / 1		0 / 1
	-	1 / 21	0 / 11	1 / 32
NL (93)	-	0 / 70	0 / 23	0 / 93

PB = peripheral blood; BM = bone marrow; CML = chronic myelogenous leukemia; ALL = acute lymphocytic leukemia; ANLL = acute non-lymphocytic leukemia; NL = non-leukemic.

#From 12 Ph′-positive CML patients DNA was obtained from both PB and BM with consistent probe test results. For these 12 patients only the BM data are included in the Table.

*In the two apparently discrepant cases, probe analysis was carried out only on DNA digested with _Bgl_ II.

one additional case the probe test disclosed a Ph'(bcr-210) rearrangement that was not detected by cytogenetics.

Only one example of a visible Ph' chromosome was reported among 33 cases of clinically diagnosed ANLL, and this specimen was negative by DNA probe analysis. Conversely, in another case the probe test revealed a bcr-210 rearrangement in the absence of an obvious Ph' karyotypic abnormality.

A diagnostic test for the Ph'(bcr-210) rearrangement has potential utility in monitoring patients during the course of therapy. At two test sites (III and V) specimens were analyzed from CML patients in clinical remission, immediately prior to or several months after undergoing allogeneic bone marrow transplantation. In 11 of 12 samples obtained prior to transplantation (site V), residual Ph'-positive leukemic cells could be detected by both cytogenetic and DNA probe analysis, while in one specimen a cytogenetically observed Ph' chromosome was not confirmed by the DNA probe test.

A total of 34 specimens from 31 different patients were sampled from post-transplant CML patients in clinical remission (Table 2). In about one-half of these cases, both cytogenetic and DNA probe analysis were negative. However, in the remaining cases either one or both assays revealed a Ph' translocation, indicative of residual leukemic cells. The DNA probe test was more successful than cytogenetic analysis in detecting these Ph'-positive cells (15 _versus_ 10 positive specimens), but there were three cases in which hybridization with the _phl_/bcr-210 probe did not corroborate positive karyotypic results.

Table 2. Detection of Ph′ Translocation in CML Patients in Clinical Remission after Bone Marrow Transplantation

TEST RESULTS		NUMBER OF SAMPLES
KARYOTYPE	DNA PROBE	
-	-	16
+	+	7
+	-	3
-	+	8

During this study a few CML patients were tested sequentially at intervals before and after allogeneic bone marrow transplantation. Two cases proved of particular interest.

From one patient at site V an initial specimen of splenic tissue was obtained just prior to transplantation, and the presence of a Ph′(bcr-210) chromosome was verified by both karyotype and DNA probe analysis. Eight months after allogeneic transplantation the patient's disease was in remission, and both cytogenetic and probe assays were negative. A specimen taken at fifteen months after transplantation still showed a normal diploid karyotype, and the patient remained in clinical remission. However, DNA probe analysis carried out at this time revealed a rearranged bcr-210 DNA fragment, suggesting the presence of a population of Ph′-positive leukemic cells. This patient

is still being followed.

In the second case (studied at site III) the diagnosis of Ph'-positive CML was confirmed at presentation by both cytogenetic and DNA probe assays. The patient was brought into remission and received a bone marrow transplant. Several months later the patient was clinically disease-free, and the karyotype appeared normal, but the phl/bcr-210 probe test revealed a rearranged DNA fragment. Densitometric analysis suggested that this represented a population of no more than five percent cells with a Ph' translocation. Several months later the patient developed a clinical relapse resembling an acute lymphoblastic crisis. At this time cytogenetic analysis was attempted, but adequate chromosome spreads were not obtained. However, the DNA probe test showed a high level of a rearranged bcr-210 DNA fragment in bone marrow cells. Comparison of the mobility of the junction fragments obtained by restriction endonuclease digestion with Bgl II showed that the same rearrangement was present in DNA obtained during the chronic phase of CML, during apparent remission after transplantation, and at the time of the clinical relapse. This implies that the lymphoblastic cells observed at relapse were derived from the same leukemic clone that was present in the patient during the chronic phase of CML prior to transplantation. The patient has subsequently died, and no additional karyotyping could be done for confirmation.

DISCUSSION

Elucidation of the molecular structure of the Ph′ translocation has resulted in the first oncogene-based assay for the diagnosis of a specific form of human cancer, CML. We have constructed an improved DNA probe, phl/bcr-210, to detect rearrangements in the bcr-210 segment of the phl gene which are associated with activation of the c-abl oncogene. The assay entails Southern blot hybridization of the probe to genomic DNA fragments generated by digestion with Bgl II or another restriction endonuclease. In the clinical study described here the probe test correctly identified 99 percent of confirmed cases of Ph′-positive CML. Furthermore, no false positive results were obtained among 93 samples from non-leukemic individuals. This indicates that the four Bgl II restriction sites critical to the assay are highly conserved and that extraneous Bgl II sites in this region occur rarely, if at all, in the human population.

The DNA probe test also revealed rearrangements of the characteristic breakpoint cluster region of the phl gene (bcr-210) in 12 out of 29 cases (41%) of karyotypically Ph′-negative CML, only two of which showed complex chromosomal rearrangements. In agreement with this observation, others have demonstrated both a bcr-210 rearrangement and the expression of a P210 phl/abl tyrosine protein kinase in some CML cases lacking a marker chromosome (45-49). Wiedemann and colleagues (45) recently reported that in Ph′-negative CML the presence or absence of the DNA rearrangement correlates with clinical and morphological features. Among 12 such cases reviewed in their study, five were clinically indistinguishable from Ph′-positive CML, and these were marked by molecular rearrangements of bcr-210 and, where tested,

expression of P210 phl/abl. The remaining seven cases involved no bcr rearrangement detectable by the phl/bcr-210 probe test. Upon independent review, these leukemias were reclassified on the basis of cellular morphology and clinical features as either atypical CML (aCML; 6 cases) or chronic myelomonocytic leukemia (CMML; 1 case). Thus, irrespective of the presence of a visible Ph' chromosome, CML involving a breakpoint in the bcr-210 region appears to define a distinct clinical entity.

Among 203 patients with Ph'-positive CML, as judged by karyotype, only two did not appear to have a DNA rearrangement in bcr-210. One of these probe-negative specimens was obtained from a patient after radical chemotherapeutic treatment, shortly before bone marrow transplantation, and might have contained very few leukemic cells. Neither discrepant case was confirmed by analysis of DNA using a second restriction endonuclease, and it remains possible that a breakpoint was present in the bcr-210 region, but was not detected. Alternatively, although almost all translocation breakpoints in Ph'-positive CML lie within bcr-210 (21,37,50,51), a small minority may lie outside of this cluster region (52,53; our data would indicate a frequency of one percent or less).

A somewhat higher frequency (3/34) of specimens from patients in clinical remission after allogeneic bone marrow transplantation were Ph'-positive by cytogenetic analysis, but showed no evidence for a bcr-210 rearrangement by DNA probe test with a single restriction endonuclease (Bgl II). A partial explanation for this observation may be that leukemic cells were infrequent in these samples, so that both cytogenetic and DNA probe assays were pushed near to their thresholds

of sensitivity, leading to some discrepant results due to sampling variation. For example, in some cases Ph'-positive cells might have grown out selectively during in vitro culture for cytogenetic analysis. It is also possible that secondary deletions or entirely new translocations are selected preferentially in this group of patients. However, in the one case in which specimens from a single patient were studied sequentially during chronic phase CML, during a post-transplant clinical remission, and at the time of a lymphoblastic relapse, the same bcr-210 rearrangement was observed at each point. Similarly, Ganesan et al. reported four CML cases cases in which leukemic cells present before allogeneic bone marrow transplantation and after relapse were derived from the same clone, as judged by identity of the bcr-210 rearrangement (54).

The chromosomal rearrangements in Ph'-positive ALL recently have come under intense scrutiny. Two classes of phl/c-abl gene fusions are found. One group has molecular characteristics indistinguishable from those in Ph'-positive CML, and may represent cases in which clinically silent CML progresses to a lymphoblastic crisis. For this group the phl/bcr-210 probe can be used to diagnose the presence of the Ph'(bcr-210) translocation. The second group is distinguished by rearrangements with breakpoints in a phl gene intron located upstream of those typically involved in CML, defining a region we call bcr-190. The Ph'(bcr-190) translocations cannot be identified utilizing the assay described here, although the phl/bcr-210 probe would detect such rearrangements if sufficiently large DNA restriction endonuclease fragments were generated and resolved, for example by pulsed field gel electrophoresis (55,56). In ALL the Ph' chromosome is

generally associated with extremely poor prognosis (10,16,57). It will be of interest to determine the particular clinical implications of the Ph'(bcr-210) and Ph'(bcr-190) rearrangements, which give rise to similar but distinct _phl_/c-_abl_ fusion proteins (P210 and P190, respectively) with elevated tyrosine protein kinase activity.

The same molecular heterogeneity in the precise location of breakpoints may be involved in Ph'-positive ANLL as in Ph'-positive ALL (58,59). In our study only two of 33 ANLL specimens showed evidence for a Ph' translocation. In one sample cytogenetic analysis established the presence of a Ph' chromosome, but no rearrangement of bcr-210 could be found by DNA probe assay. Assays for activated _abl_ kinase might suggest whether such cases involve rearrangements in bcr-190, or elsewhere on chromosome 22. In the second example the probe test revealed a bcr-210 rearrangement, although a Ph' chromosome was not apparent in the karyotype. As in Ph'(bcr-210)-positive ALL, such cases of ANLL may progress from clinically unrecognized CML.

In summary, the _phl_/bcr-210 DNA probe assay compares very favorably with karyotype analysis as a specific diagnostic test for CML. The probe assay can be carried out on peripheral blood specimens as well as bone marrow aspirates, identifies the translocation in at least 99 percent of cases of cytogenetically Ph'-positive CML, and reveals a significant number of _phl_/c-_abl_ translocations not detected by karyotype analysis. Furthermore, the test offers useful information, that cannot be obtained from cytogenetics, in the differential diagnosis of atypical CML, CMML, and those cases of Ph'-positive ALL and ANNL involving bcr-210 rearrangements. As treatment of the leukemias becomes increasingly sophisticated, it seems highly probable

that the precise diagnosis of these diseases, at the molecular level, will be accompanied by the development of distinct therapeutic strategies for each identifiable class.

The DNA probe test is likely to find a second major use in monitoring CML patients, particularly to determine the response to therapy. A key consideration for this application is assay sensitivity. Reconstruction experiments indicate that Southern hybridization with the phl/bcr-210 probe test can reveal leukemic cells present at one percent in a peripheral blood or bone marrow cellular population. The threshold for detection of Ph′-positive cells by karyotype analysis depends on the number of metaphase spreads studied per sample, but is usually in the range of ten percent of the cell population. Thus, under routine laboratory conditions the probe assay appears several to ten-fold more sensitive than conventional cytogenetic analysis.

Recent reports demonstrate that hematological remission of CML can be induced by recombinant human interferon alpha-A, and possibly interferon-gamma (60,61,62). In some patients the fraction of bone marrow cells containing the Ph′ chromosome drops significantly. This has been demonstrated most convincingly by molecular hybridization analysis (63,64). Similarly, the DNA probe assay should have utility in monitoring the response of CML patients to other biological response modifiers, such as granulocyte colony-stimulating factor (65).

Recent advances in bone marrow transplantation suggest that this approach will play an increasingly important role in the treatment of CML (54,66-69). Although the systematic study of allogeneic transplant recipients was outside the main focus of the present work, we found

that the phl/bcr-210 DNA probe assay readily revealed residual leukemic cell populations in nearly half of such patients sampled during clinical remission after transplantation (Table 2). The superior sensitivity of the probe test, and its precision in the identification of Ph'(bcr-210) translocations clearly make it a powerful tool to supplement clinical observation and cytogenetic analysis in following the complex biology of bone marrow transplantation.

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CHAPTER 8

CONCLUDING REMARKS

Concluding remarks

The experiments described in this thesis show that as a consequence of the Ph′ translocation, a new P210 _phl_/c-_abl_ fusion-protein is expressed in the leukemic cells of Ph′ chromosome positive CML patients. The results demonstrate that translocation of a cellular oncogene c-_abl_, can result in the activation of its gene product. The _phl_/c-_abl_ protein has an increased tyrosine specific protein kinase activity.

The specific contribution of the _phl_ moiety to the P210 _phl_/c-_abl_ protein kinase activity is unclear. No homology of _phl_ to sequences of known proteins have been found. Although experiments have suggested that the loss of amino terminal _abl_ sequences can result in activation of the _abl_ kinase, a change in protein configuration, or phosphorylation of the _phl_ domain may affect the kinase activity as well. It has been demonstrated that the _in vitro_ phosphorylation patterns of the P145 c-_abl_, P210 _phl_/c-_abl_ and P190 _phl_/c-_abl_ differ. This different manner in which the _abl_ proteins utilize themselves as substrates _in vitro_ suggest that they may interact differently with substrate _in vivo_; they may have different substrate specificities. Deletion of _phl_ amino acids in P190 _phl_/c-_abl_ compared to P210 _phl_/c-_abl_ removes a part of the _phl_ protein, which may be involved in controlling the action of the c-_abl_ moiety of the protein. This may explain why the presence of P190 is associated with an acute leukemia, in which there is autonomous

growth of immature invasive lymphoid blast cells, while P210 in CML is present in an initially non-aggressive tumor with an excess of apparently normal myeloid cells of all differentiation stages. It is not clear whether the presence of the phl domain has an effect on the location of the abl protein. The NH_2 terminal phl sequences do not provide a myristilation site for association to the membrane. This site is indispensable for the transforming capacity of P160 gag/abl.

Apart from the fact that the phl gene is involved in the Ph' translocation little is known regarding its translational product. The identification of the normal phl gene product is of importance, since it may give us insight into whether the part of the phl protein appended to the truncated c-abl protein plays any role in the properties of P210 phl/c-abl. In this thesis we present evidence that the phl gene encodes an 160 K phosphoprotein with an associated kinase activity. However, it is not shown whether this activity is intrinsic. The evidence does not distinguish whether this activity is inherent or due to a protein kinase activity tightly associated with P160 phl.
Many questions as to what is the primary event that causes CML and ALL, what is responsible for initiation of the blastcrisis in CML, and what is the role of P210 and P190 in tumorigenesis still remain to be elucidated. Introduction of full length 7 kb and 8.5 kb phl/c-abl cDNAs in in vitro hematopoietic culture systems and in transgenic mice will be necessary to define the precise role of these proteins and hence of the Ph' translocation in the pathogenesis of CML and ALL.

Molecular analysis of the Ph' chromosome demonstrated the possibility of the use of DNA probe analysis in diagnostics. A probe test specific for translocations in bcr has been developed and has been

shown to overcome several of the limitations of karyotypic detection of the Ph' chromosome in CML. The present DNA based test requires the isolation of DNA from the patients leukemic cells, restriction enzyme digestion, electrophoresis and Southern blot analysis with a ^{32}P labeled probe. However, the probe does not allow the detection of the c-abl translocation in the Ph'-positive, bcr negative ALL patients. It will be necessary to identify the breakpoint of a larger number of Ph'-positive, bcr negative ALL patients to establish the possibility of another breakpoint cluster region in the phl gene. The presence of such a region will initiate efforts to develop a probe specific for translocation in this region. Specific probe tests for Ph' positive CML and ALL would contribute to the accurate diagnosis of these diseases. As treatment of the leukemias becomes increasingly sophisticated, it is highly probable that the differential diagnosis of ALL at the molecular level will be accompanied by the development of distinct therapeutic strategies for each identifiable class.

The use of DNA probes to diagnose specific chromosomal rearrangements associated with human cancer presents several general advantages, which are exemplified by studies of the Ph' translocations. The tests should be more accurate than cytogenetic analysis, particularly because complex rearrangements are frequently difficult to resolve by chromosome banding techniques. The tests may also be more sensitive than karyotyping with respect to the detection of a small percentage of abnormal cells, and thus should have particular utility in the monitoring of patients during disease remission for early indication of relapse. As noted above, DNA probes can discriminate between related forms of cancer which have minor differences at the

molecular level, and which cannot be distinguished by cytogenetics or histopathology. In the case of CML and other leukemia, DNA tests can be performed on peripheral blood cells, obviating the need for bone marrow biopsy. Finally, the probe tests should prove considerably less expensive than karyotyping, and, especially if non-isotopic methodology can de developed, should be easier to implement in typical clinical laboratories.

Although DNA probe analysis is faster than cytogenetic methods, the procedure is still relatively complex and takes five days to perform. Since the Ph' chromosome is, besides a diagnostic marker, a prognostic marker as well, early diagnosis is required. The development of immunological assays could allow a rapid, convenient and accurate diagnosis of Ph' positive CML and ALL. Antisera specific for abl and phl sequences or antisera made against the junction domain of the P210 and P190 proteins could be used for the detection of the aberrant abl proteins in patients sera, plasma or lysates of peripheral blood.

Early diagnosis plays an important role in leukemia research. However, the ultimate goal is to find ways to cure patients with this disease. CML is in most cases incurable. The presence of an activated tyrosine protein kinase implicated in CML could be a target for drugs inhibiting its activity. Another equally valid approach is to find drugs activating the tyrosine specific protein phosphatases. Establishment of normal phosphorylation levels in the cell may be a novel approach with regard to cancer chemotherapy.

CHAPTER 9

SAMENVATTING

Samenvatting

Cytogenetisch onderzoek heeft aangetoond dat een aanzienlijk aantal humane tumoren, vooral leukemieen en lymfomen geassocieerd is met consistente specifieke chromosomale afwijkingen. Het Philadelphia (Ph') chromosoom, aanwezig in meer dan 95 procent van de patienten met chronische myeloide leukemie (CML), is een van de meest karakteristieke en best gedokumenteerde voorbeeld van zo'n afwijking. Dit chromosoom is gewoonlijk het resultaat van een reciproke translokatie tussen chromosoom 9 en 22 (t(9;22)(q34;q11)). Hierbij ontstaat een kleiner chromosoom 22 (22q- of Ph') en een groter chromosoom 9 (9q+). Met behulp van somatische celhybriden en _in situ_ hybridisatie werd aangetoond dat het oncogen c-_abl_, gelegen op chromosoom 9, verhuist naar het Ph' chromosoom. _abl_ blijkt ook in de gevallen waar chromosoom 9 niet zichtbaar betrokken is in de translokatie, naar chromosoom 22 te verhuizen. Deze gegevens en de vinding dat in een CML patient het breukpunt 14 kb van de v-_abl_ homologe c-_abl_ sequenties gelegen is, suggereren een rol voor c-_abl_ in CML. Dit idee wordt gesteund door de ontdekking dat in de CML cellijn K562 een groter c-_abl_ mRNA en eiwit aanwezig is.

De isolatie van het breukpunt fragment van een CML patient maakte het mogelijk om een chromosoom 22 specifieke DNA probe te construeren. Met deze probe kan worden aangetoond dat op chromosoom 22 een gebied aanwezig is waarin de breukpunten van de verschillende CML patienten geklusterd voorkomen. Dit gebied wordt breakpoint cluster region (bcr) genoemd. bcr bleek deel uit te maken van een gen, genaamd _phl_

(Hoofdstuk 3). Dit _phl_ gen wordt overgeschreven in een 4,5 en 7 kb mRNA molecuul (Hoofdstuk 4,5). In immunoprecipitaties met _phl_ antiserum werd een 160K fosfo-proteine geprecipiteerd waarmee kinase activiteit geassocieerd is (Hoofdstuk 6). Niet aangetoond kon worden echter of deze activiteit intrinsiek is. De cellulaire functie van het _phl_ eiwit is tot nu toe onbekend.

Het _phl_ gen is gelegen met het 5' uiteinde gericht naar de centromeer en met het 3' uiteinde naar de telomeer van chromosoom 22 (Hoofdstuk 3). Als gevolg van de Ph' translokatie gaat een deel van het _phl_ gen, het 3' uiteinde, naar chromosoom 9 terwijl het 5' uiteinde op het Ph'chromosoom blijft. De achterblijvende _phl_ sequenties fuseren met het c-_abl_ gen. Northern blot analyse van het RNA uit de leukemie cellen van CML patienten laat zien dat deze genomische configuratie resulteert in de transcriptie in een 8,5 kb hybride RNA waarvan het 5' uiteinde afkomstig is van het _phl_ gen en het 3' uiteinde van het c-_abl_ gen (Hoofdstuk 4). Dit RNA komt ook voor in de CML cellijn K562. Een direct bewijs hiervoor word geleverd door de klonering van een cDNA overeenkomend met het chimaere RNA (Hoofdstuk 5). Aangezien in K562 en in alle Ph' chromosoom positieve CML patienten het 8,5 kb chimere transcript wordt aangetroffen ondanks dat de breukpunten op chromosoom 9 verspreid zijn over een gebied dat varieert van 5 kb tot meer dan 175 kb, lijkt het splicing systeem erg flexibel te zijn en geen moeite te hebben met de grote variatie in intron grootte tussen de _phl_ en c-_abl_ genen.

Translatie van het 8,5 kb transcript resulteert in de synthese van een 210K fusie-eiwit met een amino terminaal _phl_ en een carboxy terminaal c-_abl_ uiteinde (Hoofdstuk 6). Dit eiwit heeft in vergelijking

met het normale c-abl eiwit hogere tyrosine kinase activiteit en is *in vivo* gefosforyleerd op tyrosine. Het is nog niet duidelijk wat de preciese rol van dit eiwit is. Thans wordt verder onderzocht met behulp van transfectie experimenten of dit eiwit transformerende eigenschappen heeft, en indien dit het geval is welke rol de tyrosine kinase activiteit daarbij speelt.

De kennis van de genetische basis van CML kan gebruikt worden in de diagnostiek. Naast detectie van het Ph' chromosoom door middel van cytogenetische methoden is het nu ook mogelijk deze translokatie te detecteren door hybridiseren van Southern blots van genomisch DNA met een geschikte DNA probe. Deze toets heeft het voordeel boven karyotypering dat het niet noodzakelijk is uit te gaan van actief delende cellen. Bovendien kan in plaats van beenmergcellen ook bloed worden gebruikt. Het klinische onderzoek naar de toepassingsmogelijkheden van deze methode is uitgevoerd in 7 verschillende onderzoeks centra. Aangetoond word dat er in CML patienten met het Ph' chromosoom de correlatie tussen karyotypering en probe analyse 99% is (Hoofdstuk 7). Met de probe kunnen meer complexe translokaties worden aangetoond die met karyotypering niet of zeer moeilijk te detecteren zijn. Daarbij is ook de detectiegrens, waarbij de aanwezigheid van leukemie cellen nog kan worden aangetoond bij DNA probe analyse lager dan bij karyotypering, respectievelijk 1 en 10 procent. Gevoeligheid van de detectie methode is van belang voor het volgen van de patient tijdens de remissie en voor de vroege detectie van relapse.

Curriculum Vitae

Kees Stam werd geboren op 20 april 1959 te Purmerend. Hij groeide op in de Beemster en behaalde in 1977 het diploma VWO-B aan het St Michaels College te Zaandijk. In hetzelfde jaar werd begonnen met de studie Planteziektenkunde aan de Landbouw Universiteit te Wageningen. In 1980 werd het kandidaatsdiploma in deze studierichting behaald en in november 1984 slaagde hij cum laude voor het doctoraalexamen. Uit de drie gekozen hoofdvakken, Erfelijkheidsleer (Prof. van der Veen), Virologie (Prof. van der Want) en Moleculaire Biologie (Prof. van Kammen) blijkt zijn interesse voor fundamenteel biologisch onderzoek.

Zijn interesse in het kankeronderzoek bracht hem overzee waar hij stage liep bij het amerikaanse biotechnologie bedrijf Oncogene Science Inc., gevestigd in New York. Vanaf november 1984 tot heden is hij in dienst als stafwetenschapper bij dit bedrijf. Oncogene Science is opgericht door een groep wetenschappers van het amerikaanse National Cancer Institute (NCI) en is gespecialiseerd in het onderzoek aan oncogenen en groei- factoren. De resultaten beschreven in dit proefschrift zijn verricht in het laboratorium van Oncogene Science in samenwerking met Gerard Grosveld, Anne Hagemeijer en medewerkers van de afdeling Celbiologie en Genetica aan de Erasmus Universiteit in Rotterdam.