THE ROLE OF THE PHILADELPHIA TRANSLOCATION IN CHRONIC MYELOID LEUKEMIA

PROEFSCHRIFT

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ADRIANUS HENRICUS MARIA GEURTS VAN KESSEL
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CO-REFERENTEN : Prof.Dr. J. Abels
Prof.Dr. P.L. Pearson

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Aan Francine

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ABBREVIATIONS

ACO1	soluble aconitase
ACO2	mitochondrial aconitase
AK1	adenylate kinase-1
ALL	acute lymphocytic leukemia
AML	acute myeloid leukemia
A-MuLV	Abelson murine leukemia virus
αNA	alpha-napthyl acetate esterase
ANLL	acute non-lymphocytic leukemia
AP	acid phosphatase
APL	acute promyelocytic leukemia
ARSA	arylsulfatase-A
AT	ataxia telangiectasia
BL	Burkitt lymphoma
BS	Bloom's syndrome
CAE	naphtol-AS-D-chloroacetate esterase
c-ALL	common acute lymphocytic leukemia
SCE	sister chromatid exchange
cλIg	immunoglobulin lambda light chain constant region
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
c-onc	cellular oncogene
del	deletion
DM	double minute chromosome
DMSO	dimethyl sulfoxide
ENO1	enolase-1
EL	erythroid leukemia
DNA	deoxyribonucleic acid
FA	Fanconi's anemia
FAB	French-American-British classification
FH	fumarate hydratase
G-banding	trypsin-Giemsa banding
GLA	α-galactosidase
G6PD	glucose-6-phosphate dehydrogenase
HAT$^-$	hypoxanthine-aminopterin-thymidine
HPRT$^-$	hypoxanthine phosphoribosyl transferase deficient
HSR	homogeneously staining region
HTLV	human T cell leukemia virus
Igλ	immunoglobulin lambda light chain
ISCN	international system for cytogenetic nomenclature
ISGN	international system for human gene nomenclature
kb	kilo base
kd	kilo dalton
LDH	lactate dehydrogenase
LTR	long terminal repeat
MPD	myeloproliferative disease
MuLV	murine leukemia virus
NAGA	N-acetyl-α-D-galactosaminidase
PAGE	polyacrylamide gel electrophoresis
PAS	periodic acid Schiff

PEG	polyethylene glycol
PGD	6-phosphogluconate dehydrogenase
PGK	phosphoglycerate kinase
PGM1	phosphoglucomutase-1
Ph^1	Philadelphia chromosome
PV	polycythemia vera
Q-banding	quinacrine-mustard banding
R-banding	reverse banding
RNA	ribonucleic acid
SB	Sudan black
SDS	sodium dodecyl sulphate
SRO	shortest region of overlap
SSC	standard saline citrate
SSV	Simian sarcoma virus
TK	thymidine kinase deficient
UMPK	uridine monophosphate kinase
v-onc	viral oncogene
XP	xeroderma pigmentosum

PREFACE - VOORWOORD

Iedereen die heeft meegewerkt aan het tot stand komen van dit proefschrift zou ik willen bedanken voor de konstante of variabele, lichte of zware, samenbindende of juist tot diversiteit leidende bijdragen. Een aantal namen wil ik er uit lichten. Op de eerste plaats mijn promotor Prof. Bootsma. Beste Dick, jouw niet aflatende belangstelling, enthousiasme en steun tijdens het gehele verloop van dit onderzoek heb ik bovenmate gewaardeerd. Tot in de kleinste details was jij steeds op de hoogte. Jij stuwde daarmee niet alleen mij, maar de hele leukemie-groep vooruit. Dr. Hagemeijer, beste Anne, nadat jij mij de beginselen der somatische cel genetica had bijgebracht, bleef ik vrijwel dagelijks met jou belangrijke en minder belangrijke zaken doorspreken, steeds in een prettige sfeer en vol belangstelling, bedankt. Prof. Pearson en Prof. Abels, de discussies die ik met jullie als coreferenten heb gehad naar aanleiding van dit proefschrift heb ik als aangenaam en zeer leerzaam ervaren.
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AD

1. INTRODUCTION

1.1 General aspects of chromosomal abnormalities in neoplasias

Karyotypic abnormalities have been known to occur in neoplastic cells since the end of the last century (Hansemann, 1890; Boveri, 1914). Significant progress in the interpretation of these aberrations was made after the introduction and continuous improvement of various chromosome banding techniques, which allowed the precise identification of each individual chromosome or chromosomal region (Caspersson, 1970; Seabright, 1970; Hagemeijer et al., 1979). Through the years that followed, it became apparent that the malignancy associated aberrations are clustered to specific chromosomes, both in experimental animals and in man (Lawler, 1977; Bloch-Stachner and Sachs, 1977; Sandberg, 1980; Mitelman and Levan, 1981; Spira et al., 1981; Hagemeijer et al., 1982a; Rowley, 1982). Three major types of chromosomal changes that occur in malignant cells, either alone or in combination, can be distinguished: a) numerical changes, due to gain or loss of whole chromosomes, b) structural changes, due to translocation or deletion of specific chromosomal regions and, c) double minutes (DM's) or homogeneously staining regions (HSR's). DM's and HSR's are attributed to gene amplification (Haber and Schimke, 1981). The nature of chromosomal aberrations associated with neoplasia is usually acquired, but may also be congenital or inherited. Examples of Mendelian inherited diseases that are known to be closely associated with the occurrence of neoplasia are Fanconi's anemia (FA; Hecht and Mc Caw, 1977), Bloom's syndrome (BS; Hecht and Mc Caw, 1977), ataxia

telangiectasia (AT; Harnden, 1974) and xeroderma pigmentosum (XP; Robbins et al., 1974). In FA and BS patients an increase in spontaneous chromosome breakage and sister chromatid exchanges (SCE's) has been observed (Chaganti et al., 1974; Fujiwara et al., 1977), whereas XP cells are known to be defective in a DNA repair mechanism (Cleaver, 1969; Bootsma, 1977). AT is accompanied by immunological deficiency (Mc Farlin et al., 1972) and the patients usually develop lymphoid malignancies. These lymphoid malignancies are frequently associated with chromosomal translocations involving band q12 and/or band q32 of chromosome 14 (Mc Caw et al., 1975; for nomenclature see ISCN, 1978). Specific congenital chromosomal deletions have been observed in retinoblastoma (deletion 13q14) and Wilm's tumor (deletion 11p13) (Bonaiti-Pellie et al., 1976; Riccardi et al., 1978; Rüdiger, 1978). Acquired chromosomal abnormalities have been found in several solid tumors including benign mesenchymal and epithelial tumors, malignant sarcomas, carcinomas and melanomas (review Sandberg, 1980). In most meningiomas (benign mesenchymal) with an abnormal karyotype, the aberration specifically involves chromosome 22, i.e. a complete loss or a partial deletion of the q arm of one chromosome 22 homolog (Zang and Singer, 1967; Mark, 1974). Other solid tumors usually show very complex karyotypic rearrangements with sometimes a 'common' specific change as e.g. deletion 3p14-p23 in small cell carcinoma of the lung (Whang-Peng et al., 1982).

Acquired chromosomal aberrations have been extensively studied in leukemia, preleukemia and other myeloproliferative and lymphoproliferative malignant disorders. This is due, in part, to the relative

ease in which tumor cells, which are present in blood, bone marrow or lymph nodes, can be obtained. Human acute non-lymphocytic leukemias (ANLL) can be classified in subtypes (M1-M6) on basis of morphological criteria (FAB classification, Bennet et al., 1976). Translocation (8;21)(q22;q22) is an acquired chromosomal anomaly specific for acute myeloblastic leukemia (M2; Rowley, 1973b), whereas translocation (15;17)(q22;q12) is specific for acute promyelocytic leukemia (M3; Rowley et al., 1977). The chromosomal region 11q23-q25 is specifically involved in acute myelomonocytic leukemia (M4; Berger et al., 1980; Yunis et al., 1981) and, recently, a specific translocation (9;11)(p12;q23) has been observed in patients with acute monoblastic leukemia (M5; Hagemeijer et al., 1982b). It has been stated by Yunis et al. (1981) that all ANLL patients may have a chromosomal anomaly in the leukemic cells which can be visualized by high resolution chromosome banding techniques. In human malignant lymphomas and acute or chronic lymphocytic leukemias chromosome 14 is often affected. A specific type of aberration, a 14q+ marker, is the most common abnormality in this group of disorders. In Burkitt lymphoma (Zech et al., 1976; Mc Caw et al., 1977) the 14q+ marker results from a specific translocation with chromosome 8: t(8;14)(q24;q32). Recently, two other specific translocations in Burkitt lymphomas involving chromosomes 8 and 2, t(2;8)(p12;q24) and chromosomes 8 and 22, t(8;22)(q24;q11) have been reported (Miyoshi et al., 1979; Van Den Berghe et al., 1979; Berger et al., 1979; Miyoshi et al., 1981; Rowley et al., 1981). The region q13-q22 of chromosome 12 has been shown to be specifically involved in chronic B lymphocytic leukemia (CLL; Gahrton et al., 1982).

In analogy, non-random involvement of particular chromosomes or chromosomal regions have been described for spontaneous and induced neoplasias in experimental animals. In the mouse, duplication of one chromosome 15 homolog is closely associated with lymphoid, erythroid and myeloid leukemias (Wiener, 1978; Hagemeijer et al., 1982a). Specific translocations between chromosome 15 and chromosomes 6 or 12 have been reported for murine plasmacytomas (Wiener et al., 1976; Ohno et al., 1979). The latter is of particular interest since the immunoglobulin heavy chain and κ light chain gene clusters have been localized on mouse chromosomes 12 and 6, respectively (Hengartner et al., 1978; Meo et al., 1980). The heavy chain locus is actually involved in the 12;15 translocation (Calame et al., 1982). Similarly, in human Burkitt lymphomas, the chromosomal regions involved in the specific translocations (see above) carry the genes for the immunoglobulin κ light chain (2p12- cen; Malcolm et al., 1982), λ light chain (22; Erikson et al., 1981) and the heavy chain (14q32; Croce et al., 1979; Cox et al., 1982; Kirsch et al., 1982). In t(8;14) the chromosomal break has been shown (Erikson et al., 1982) to be within the heavy chain gene cluster, whereas in t(8;22) λ constant region gene sequences are translocated to chromosome 8 (De la Chapelle et al., 1983). These latter results suggest that chromosomal regions involved in the expression of differentiated functions of particular cells may be the targets for malignancy associated chromosomal aberrations.

The occurrence of the Philadelphia (Ph^1) translocation (9;22) (q34;q11) in patients with chronic myeloid leukemia is one of the most outstanding and best documented examples of a specific chromoso-

mal aberration in human neoplasia (Rowley, 1973a; 1980). Progression of the disease into an accelerated phase and finally, a blast crisis is often accompanied by the acquisition of extra chromosomal anomalies by the malignant cells, several of which are again non-random. An extra Ph^1 chromosome, trisomy 8, trisomy 19 and an isochromosome 17, are frequently encountered (Rowley, 1980; Hagemeijer et al., 1980). The latter aberration often precedes and accompanies the final blast crisis (Prigogina and Fleischman, 1975).

1.2 The Philadelphia translocation in chronic myeloid leukemia

In 1960, Nowell and Hungerford described for the first time an abnormal small chromosome in the leukemic cells of two patients with chronic myeloid leukemia (CML). They considered the chromosome involved to be a Y. Baikie et al. (1960) established that the abnormality involved one of the small acrocentric autosomes and Tough et al. (1961) applied the term Philadelphia (Ph^1) chromosome to it, according to the location of the laboratory of discovery. Until 1970 the Ph^1 chromosome was generally assumed to be derived from chromosome 21. Prieto et al. (1970) combined morphological and autoradiographical information and concluded that chromosome 22 was the source of the Ph^1 chromosome. Fluorescence banding techniques confirmed this finding shortly thereafter (Caspersson et al., 1970; O'Riordan et al., 1971). In 1973 Rowley showed that the deleted portion of chromosome 22 was translocated onto the long arm of chromosome 9. Consequently the translocation now reads: t(9;22)(q34;q11)(ISCN, 1978; Fig. 1 and 5). At present, Ph^1 variants have been described in which an array of translocation sites

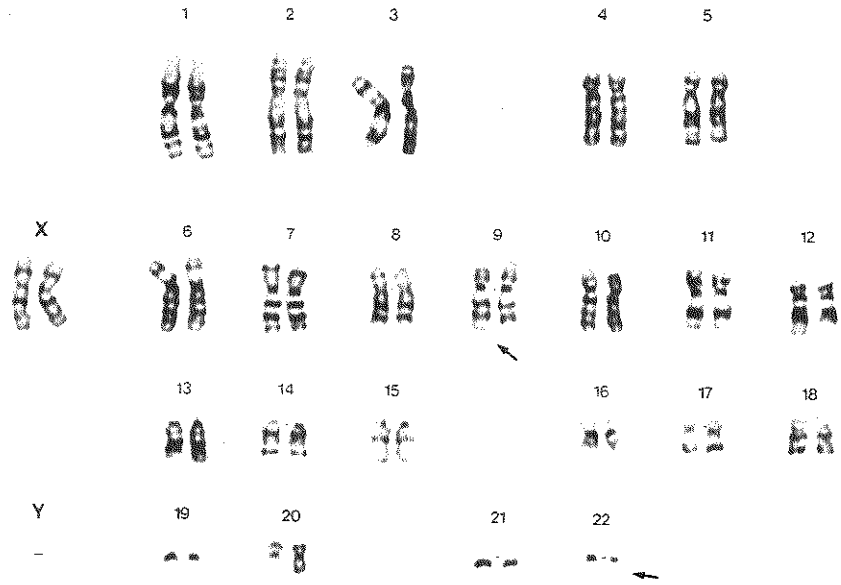

Fig. 1 Karyogram of a CML cell showing the Ph^1 translocation: t(9;22)(q34;q11).

other than 9q have been observed, but chromosome 22 is always involved (review Sandberg, 1980). Complex Ph^1 variants, involving chromosome 9, 22 and one or two other chromosomes, have been reported by various investigators (Nowell et al., 1975; Berger et al., 1976; Sandberg, 1980; Borgström, 1981). Indications that the Ph^1 chromosome is the result of a reciprocal translocation were found by Mayall et al. (1977), Watt and Page (1978) and Wayne and Sharp (1982) using various chromosome banding and cytophotometric techniques. The first unequivocal evidence for reciprocity of the translocation has recently been obtained in our laboratory (appendix, Paper VI) by showing that in Ph^1

positive CML cells a gene, known to be located on chromosome 9, is translocated to the 22q- derivative of the translocation (Fig. 5). In case of complex Ph^1 variants, material from chromosome 22 is usually translocated onto a third chromosome, whereas material from this third chromosome is translocated to chromosome 9. Simultaneously, material from chromosome 9 would be expected to be translocated onto chromosome 22. Again, evidence for the latter has recently been obtained in our laboratory (Bartram, 1983). Rarely, a reverse pattern has been observed in which material from the third chromosome is translocated to chromosome 22, resulting in a so called 'masked' Ph^1 chromosome (Tanzer et al., 1977; Lessard et al., 1981; Marinello et al., 1981; Oshimura et al., 1981).

Commonly, CML is considered to be a clonal stem cell disease (Till and Mc Culloch, 1980) in which only the myeloid differentiation pathway is affected by the presence of the Ph^1 translocation (Fig. 2). The clonal origin of the disease has been demonstrated repeatedly (Fialkow et al., 1967; Fitzgerald et al., 1971; Moore et al., 1974; Hayata et al., 1974; Gahrton et al., 1974a; Hossfeld, 1975; Fialkow et al., 1980). Studies showing the presence of the Ph^1 translocation not only in the granulocytic lineage of bone marrow from CML patients, but also in the erythroid (Whang et al., 1963; Rastrick et al., 1969; Andersson et al., 1979), the monocyte-macrophage (Golde et al., 1977) and the B-lymphoid (Van Den Berghe et al., 1979; Minowada et al., 1979; Martin et al., 1980; Bernheim et al., 1981; Karpas et al., 1982) compartments, strengthen the stem cell hypothesis. T cells are usually not involved (Bernheim et al., 1981).

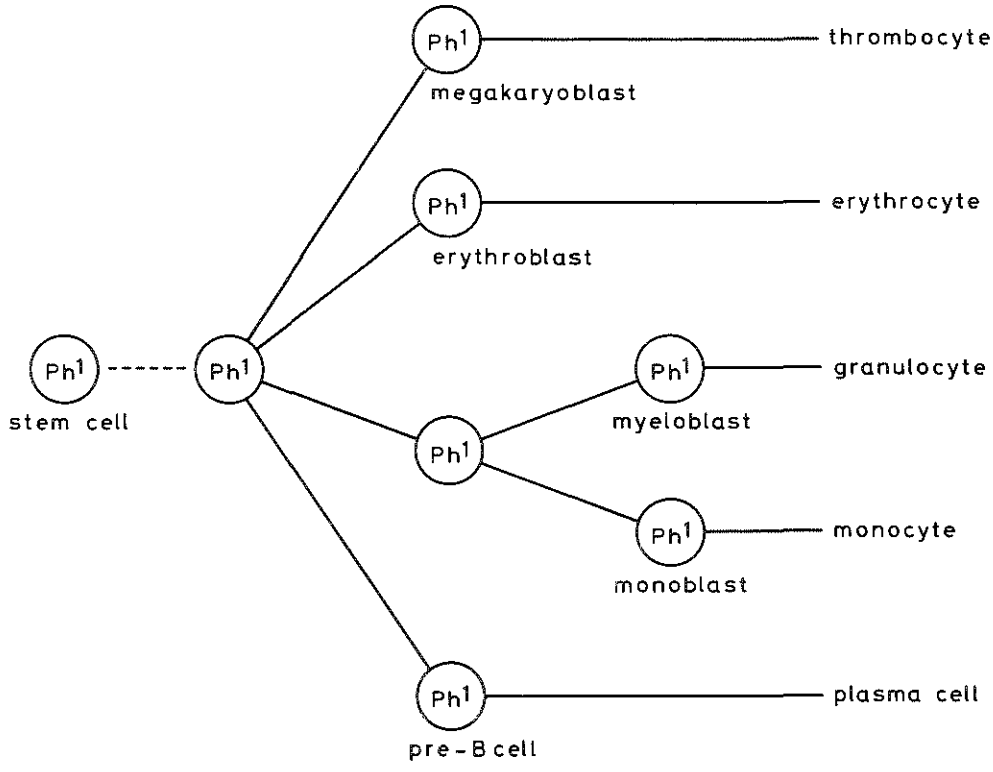

Fig. 2 A simplified scheme of hemopoiesis and the involvement of the Ph^1 translocation in CML. Ph^1 positive stem cells have overgrown all normal bone marrow stem cells which results in the presence of Ph^1 in all progenitor cells. The commitment of the various precursor cells to a specific cell lineage is probably brought about by micro-environmental signals within the bone marrow. The commitment of T cells is thought to be regulated by e.g. thymic epithelial cells.

The Ph^1 translocation is typically an acquired chromosomal anomaly and has occasionally been found also in patients with acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), erythroid leukemia

(EL), polycythemia vera (PV), thrombocythemia, osteomyelofibrosis, osteosclerosis and multiple myeloma (Philip et al., 1976; Janossy et al., 1978; review Sandberg, 1980). Except for ALL, these diseases are classified as myeloproliferative disorders. Ph^1 positive ALL evolves sometimes, after a successful induction of remission, into classical CML (Rowley, 1980). In CML, in contrast to the above malignancies, the bone marrow is already largely or completely replaced by Ph^1 positive cells at the time of diagnosis. Consequently, a complete elimination of cells carrying the Ph^1 translocation is often impossible. In acute leukemias, however, therapeutically induced remissions are characterized by the presence of karyotypically normal stem cells and the eradication of leukemic cells with chromosomal abnormalities.

About 10% of the CML patients is Ph^1 negative (Rowley, 1980) and, occasionally, conversion of Ph^1 positive CML into Ph^1 negative CML is observed (Hagemeijer et al., 1979b; Sharp et al., 1979). A few CML cases have been described (Gahrton, 1974b; Lisker et al., 1980) in which Ph^1 was absent in the bone marrow at diagnosis, but was found to be present in all bone marrow cells 42-90 days after the initial analysis.

The Ph^1 translocation has never been reported as a constitutional anomaly, whereas other translocations involving 22q11 as e.g. t(11;22)(q23;q11) are frequently encountered as such (Fraccaro et al., 1980). This indicates that the Ph^1 translocation is a chromosomal aberration which characteristically differs from balanced constitutional chromosomal translocations in that it is incompatible with the normal development of at least myeloid cells. The exact role played by this highly

specific chromosomal aberration in the pathogenesis of CML, however, remains to be established.

1.3 Scope of the thesis

Since the discovery of the Philadelphia (Ph^1) chromosome in chronic myeloid leukemia (CML) (Nowell and Hungerford, 1960), there has been much speculation about the role of such a non-random acquired chromosomal abnormality in the development of the disease. It has been suggested that loss of specific loci predisposes the cell to malignant transformation. This hypothesis was based on evidence that malignancy behaves as a recessive trait (Harris et al., 1969) and as such is likely to be expressed when chromosomal material is deleted. The discovery that the Ph^1 chromosome results from a translocation and not a simple deletion (Rowley, 1973) has had its implications for this interpretation. The recent discovery that chromosomal translocations in B cell lymphomas in both human and mouse occur predominantly at sites within the genome containing sequences encoding the immunoglobulin heavy and light chains, (review Rowley, 1982) has led investigators to propose (Klein, 1981; Dalla-Favera et al., 1982b) that the translocation of specific genes to a transcriptionally active region may lead to an enhanced expression of these translocated genes. The presence of disproportionate amounts of such gene products in the cell could ultimately lead to malignancy (Hayward et al., 1981; Collins and Groudine, 1982; Cooper, 1982; Chang et al., 1982; Kirsch et al., 1982). This hypothesis would be compatible with one interpretation of the results obtained by transfection experiments showing that DNA fragments of normal tissue culture cells can induce neoplastic

transformation of NIH-3T3 cells in vitro with low efficiency. High molecular weight DNA isolated from the transformed cells causes transformation at high efficiency when used in a secondary transfection experiment (Cooper et al., 1980). These results suggest transformation via dissociation of certain genes from their normal regulators and a possible activation of these genes by the subsequent integration nearby a cellular promotor, leading to abnormal gene expression (Cooper, 1982). Such experiments would predict that abnormally expressed transforming genes, when present in spontaneous or induced tumors, can also be transmitted via transfection. This has indeed been found in a number of tumor cell types in diverse species, including man (Weinberg, 1982a).

Retroviruses, that cause acute transformation in vertebrate cells contain a genome that is considered to be a recombinant between viral (replication) and cellular (transformation) derived sequences. When integrated into the host cell genome, these sequences are transcriptionally active (Hayward, et al., 1981). Malignant transformation of cells by chronic RNA tumor viruses, devoid of cellular derived oncogenic sequences, is apparently brought about by the integration of viral promotor sequences adjacent to potentially oncogenic cellular sequences: oncogenesis by promotor insertion (Neel et al., 1981; Dalla-Favera et al., 1982c; Nusse and Varmus, 1982). These latter observations on RNA tumor viruses are compatible with the hypotheses that have emerged from DNA transfection experiments. Recently, Tabin et al. (1982), Papageorge et al. (1982) and Reddy et al. (1982) have shown that a single point mutation within a specific DNA sequence may

be responsible for acquisition of transforming activity by this sequence. In this case an aberrant gene product is supposed to be responsible for inducing the malignant state of the cell.

Previous experiments that were aimed at a better understanding of the role played by the Ph^1 anomaly in the etiology of CML have been hampered by several technical limitations. The generally poor morphology of leukemic metaphase chromosomes has long been the main obstacle towards a better characterization of the Ph^1 translocation. In this thesis the application of somatic cell hybridization and gene segregation analyses to this latter problem is described. Recent advancements in DNA technology were used to investigate whether one or more of the above described etiologic mechanisms for the acquisition of malignancy, with or without tumor virus involvement, are applicable to the Ph^1 translocation and CML. Moreover, attempts were made to generate myeloid intralineage cell hybrids which would permit the identification of the genes involved in the expression of human myeloid associated functions. These genes are likely to be involved in the disregulation of myeloid differentiation in CML and, like the immunoglobulin genes in B cell lymphomas, they may be involved in specific chromosomal aberrations.

2. EXPERIMENTAL WORK AND DISCUSSION

2.1 Expression and segregation of genes in somatic cell hybrids

The introduction of somatic cell hybridization techniques by Barski et al. (1960) has greatly facilitated the study of genetic factors involved in the expression of phenotypic characteristics in various types of living cells. Genetic analysis of the malignant phenotype of tumor cells was one of the first objectives of these studies (Scaletta and Ephrussi, 1965; Silagi, 1967; Harris et al., 1969; Croce and Koprowski, 1974). Introduction of the HAT system for hybrid selection (Littlefield, 1964) and of Sendai virus (Okada, 1962; Harris and Watkins, 1965) and polyethylene glycol (Pontecorvo, 1975) as fusogens has improved the cell hybridization technique (Fig. 3) considerably. Interspecific hybrid cells (Weiss and Ephrussi, 1966) that segregate chromosomes of one of the fusion partners have shown to be useful for gene localization studies (Santachiari et al., 1970; Ruddle et al., 1970; Westerveld et al., 1971; Geurts van Kessel et al., 1983a; van de Rijn et al., 1983; Versnel et al., 1983). Chromosomes and phenotypes of both fusion partners can usually be distinguished in such a system and expression of a phenotype of interest can be correlated with the presence or absence of specific chromosomes (Fig. 3). During the last two decades, over 450 genetic loci have been assigned to specific human chromosomes which has resulted in a rather detailed gene map (Mc Kusick, 1980; Human Gene Mapping Conference 6 (HGM6), 1981). Whereas constitutive ('household') phenotypes of both fusion partners tend to be expressed coordinately, irrespective of the histiogenic origin of the fused cells, differentiation associated characteristics

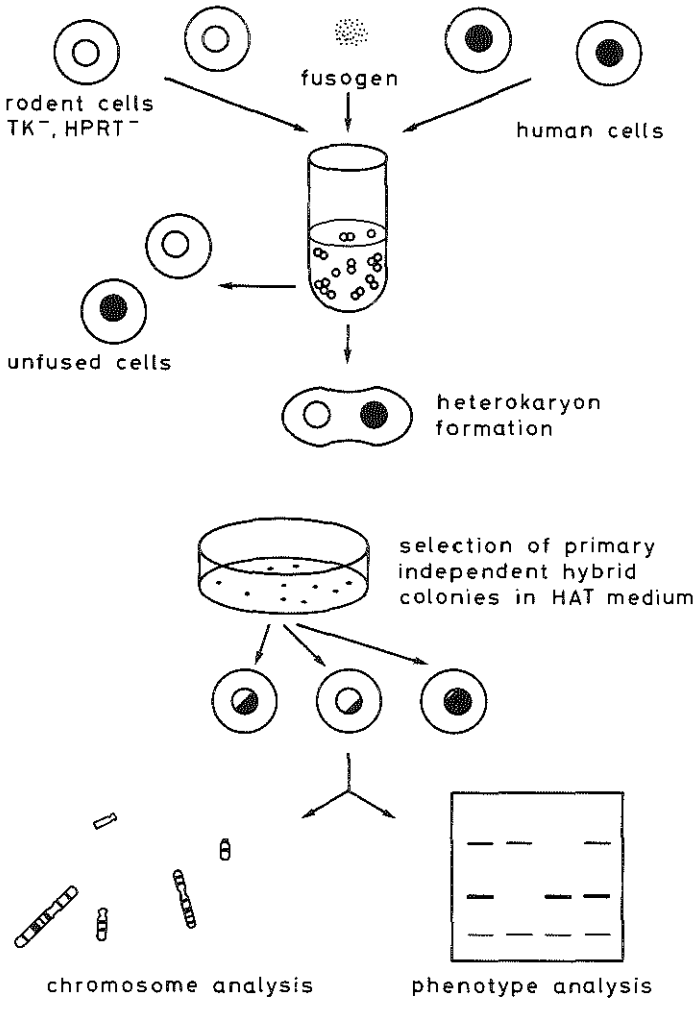

Fig. 3 Schematic illustration of the somatic cell hybridization technique used throughout this thesis. Rodent established cell lines, deficient for the enzymes thymidine kinase (TK^-) or hypoxanthine phosphoribosyltransferase ($HPRT^-$), were mixed with human (CML) cells having a limited life span. Fusion of these cells was induced by using a fusogen (Sendai virus, polyethylene glycol). Hybrid clones were selected in culture medium supplmented with hypoxanthine, aminopterin and thymidine (HAT).
Primary, independent hybrid colonies were propagated further and analysed for their chromosomal constitution and for the expression of phenotypic traits or the presence of gene specific DNA sequences.

usually exclude each other and expression of such traits is, therefore, repressed when fusions are carried out between cells of different differentiation lineages (Davis and Adelberg, 1973; Fougère and Weiss, 1978; Klein et al., 1980; Koeffler et al., 1981). This problem can be overcome by fusion of cells within a specific differentiation lineage (intralineage). The latter hybrids generally allow the expression of differentiation associated traits of the fusion partners (Köhler and Milstein, 1975; Hämmerling, 1977; Deisseroth and Hendrick, 1978; Croce et al., 1979; Laskov et al., 1979; Suomalainen et al., 1980; Quinn et al., 1981; Kaufmann et al., 1981). Assays for the presence or absence of genes can also be carried out via molecularly cloned gene-specific DNA sequences. In this case identification of the genes does not depend on the presence of gene products. Via molecular hybridization the presence of the genes can be demonstrated by using the cloned fragments as probes (Scott et al., 1979; Malcolm et al., 1982; Nusse et al., 1983).

In this thesis the construction of somatic cell hybrids, by fusion of rodent cells with human CML cells containing the Ph^1 translocation, is described. These hybrids were used for studies aimed at resolving a number of questions concerning the nature and the role of the Ph^1 translocation in CML. Specifically, the questions of whether the Ph^1 translocation is reciprocal or not, whether chromosomal breakpoints are at identical sites in different patients, whether chromosomal material is deleted during translocation and whether the Ph^1 translocation is clonal in origin, have been studied. Several techniques, based on e.g. chromosome banding and DNA measurement, have in the past

been applied to some of these questions, but results concerning reciprocity of the Ph^1 translocation and differences in chromosomal breakpoints in different patients remained contradictory (review Rowley, 1980; Sandberg, 1980). The segregation of marker genes, known to be located on the chromosomes involved in the translocation, was studied in the somatic cell hybrids in conjunction with the segregation of Ph^1 translocation chromosomes. Such an analysis at the single gene level has, in principle, a much higher resolution than chromosome banding and cytophotometric techniques, provided that appropriate marker genes in the breakage regions are available. Furthermore, the segregation of two cellular genes (oncogenes) homologous to the transforming sequences of a murine leukemia virus (c-abl; for nomenclature see Coffin et al., 1981) and a simian sarcoma virus (c-sis), known to be located on the chromosomes involved in the Ph^1 translocation, (Heisterkamp et al., 1982; Swan et al., 1982; Dalla-Favera et al., 1982a), has been studied. Involvement of these oncogenes in the Ph^1 translocation, i.e. transposition, would be suggestive for their role in the pathogenesis of CML. Another series of experiments was aimed at unraveling the genetic control over the expression of myeloid associated differentiation functions. It would be of particular interest to find out whether the chromosomes involved in the Ph^1 translocation carry genes that play a role in the expression of such characteristics, as has been found in the lymphoma associated translocations described in chapter 1.1 (Erikson et al., 1981; Kirsch et al., 1982; Malcolm et al., 1982; Erikson et al., 1982).

2.2 *Characterization of the Philadelphia translocation by gene segregation analysis*

2.2.1 Regional gene maps of chromosome 9 and 22

For the characterization of the Ph^1 translocation, genes known to be located in the chromosomal regions involved, were used as markers. In the past, a rather detailed gene map of chromosome 9 has been constructed (Ferguson-Smith and Aitken, 1976; Westerveld et al., 1978; Carritt and Povey, 1978; Mohandas et al., 1981). The gene coding for the enzyme adenylate kinase-1 (AK1) has been localized on the most distal band of 9q (band q34), the band involved in the Ph^1 translocation. This marker was, therefore, particularly useful for our study. A detailed gene map was not available for chromosome 22. Experiments directed at the construction of such a map are described in paper I and VII. Chinese hamster-human hybrid cell lines, derived from fusion of leucocytes from donors carrying reciprocal translocations involving different regions of chromosome 22, were used: t(X;22)(q21;q11) and t(1;22)(q42;q13). These hybrids appeared to segregate human chromosomes, including the translocation derivatives. Chromosome analysis of the hybrids was carried out in conjunction with assays for the presence or absence of the chromosome 22 markers mitochondrial aconitase (ACO2; Sparkes et al., 1978; Meera Khan et al., 1978), arylsulfatase-A (ARSA; Bruns et al., 1978) and N-acetyl-α-D-galactosaminidase (NAGA; de Groot et al., 1978), immunoglobulin λ light chain constant region sequences (cλIg; Erikson et al., 1981) and the cellular homolog (see chapter 2.3) of the simian sarcoma virus transforming gene (c-sis; Swan et al., 1982; Dalla-Favera et al., 1982a). Figure 4 shows in a

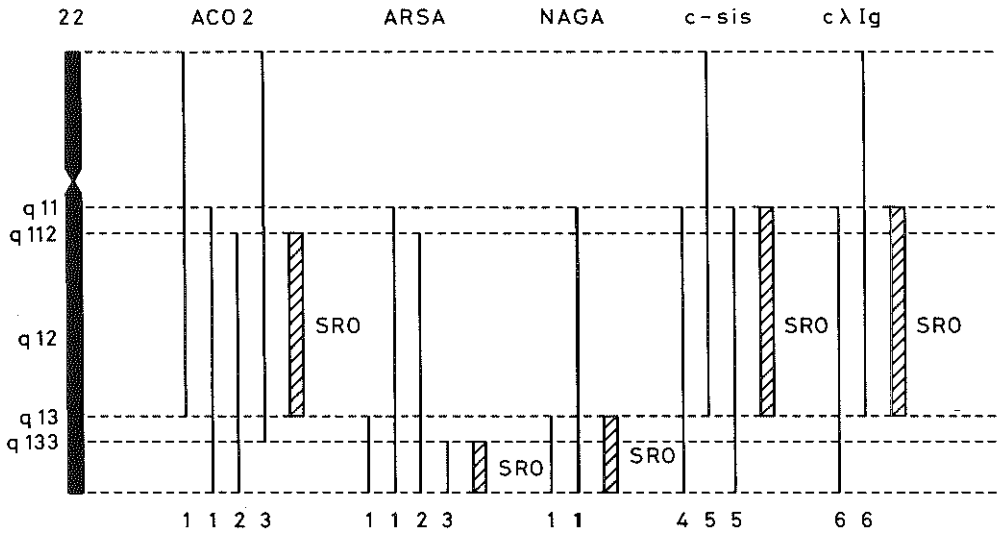

Fig. 4 Diagram showing the regional localization of ACO2, ARSA, NAGA, cλIg and c-sis on human chromosome 22. SRO: shortest region of overlap.

1. Geurts van Kessel et al. (1980)(Paper I)
2. Hors-Cayla et al. (1980)
3. Francke et al. (1981)
4. Dalla-Favera et al. (1982a)
5. Young and Geurts van Kessel (unpublished results)
6. Young et al. (in press)(Paper VII)

diagram the regional gene map of chromosome 22 thus obtained.

Our results indicate that ACO2, c-sis and cλIg are in the region q11-q13 and that ARSA and NAGA are in the region q13-qter. Subsequent results, obtained by other investigators, appeared to be in full agreement with those described in paper I and VII and provide, in some cases, an additional refinement of the map. At present, the shortest regions of overlap (SRO's), indicated in Figure 4, locate ACO2 in the region q112-q133, cλIg and c-sis in the region q11-q13, ARSA in the region q133-qter and NAGA in the region q13-qter.

2.2.2 Segregation of genes on chromosome 9 and 22 in the Philadelphia translocation.

The regional gene maps of chromosome 9 and 22 were used in the study of segregation of genes in the Ph^1 translocation. The results obtained with c-sis will be discussed later (chapter 2.3). The CML patients used for this analysis carried either the classical Ph^1 translocation (paper II, IV, VII), or a complex Ph^1 variant involving chromosome 1, 9 and 22 (paper III). In the classical Ph^1 translocations studied, the genes coding for the chromosome 22 enzyme markers AC02, ARSA and NAGA, were consistently translocated onto chromosome 9, whereas the Igλ constant region sequences studied were retained by the 22q- derivative (Fig. 5). The two chromosome 9 encoded enzymes studied (AC01 and AK1) were expressed when the 9q+ derivative was present and hence their loci were not translocated. In case of the complex Ph^1 variant, the three chromosome 22 encoded enzyme markers were translocated to 1p in a similar manner as to 9q in the classical cases (Fig. 6). The two chromosome 9 markers were again retained by the 9q+ derivative. Three markers of chromosome 1 (ENO1, PGD, UMPK) were translocated to chromosome 9 distal to the AK1 locus as observed for the chromosome 22 fragment in the 9;22 translocations. Taken together, no differences in breakpoints were observed in these CML patients, which is in support of the hypothesis that specific chromosomal regions (loci) may be involved in specific chromosomal aberrations associated with malignancy (Rowley, 1982). No evidence was obtained for loss of chromosomal material since the markers studied were not deleted during translocation. These gene segregation studies did not reveal

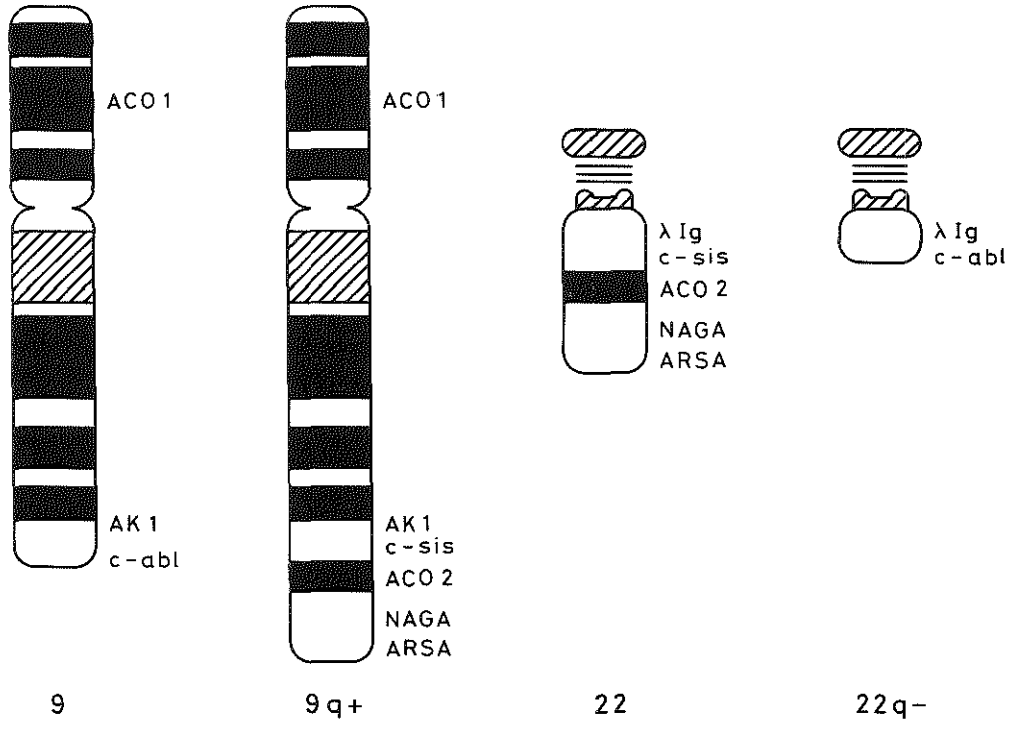

Fig. 5 Diagram showing the Ph^1 translocation (9;22)(q34;q11) and the location of marker genes relative to the breakpoints.

evidence for reciprocity of the Ph^1 translocation. Results obtained via cloned DNA fragments described in paper VI and discussed in chapter 2.3, however, unequivocally demonstrate reciprocity of the Ph^1 translocation. Since a consistent segregation pattern of the gene markers was observed in hybrids containing Ph^1 translocation products, the obtained results may provide some additional information concerning the regional localization of these markers on the chromosomes involved (Fig. 5, Fig. 6). ACO2 appears to be distal, and the Igλ constant region sequences studied proximal to the breakpoint in 22q11. AK1 is proximal to the breakpoint in 9q34.

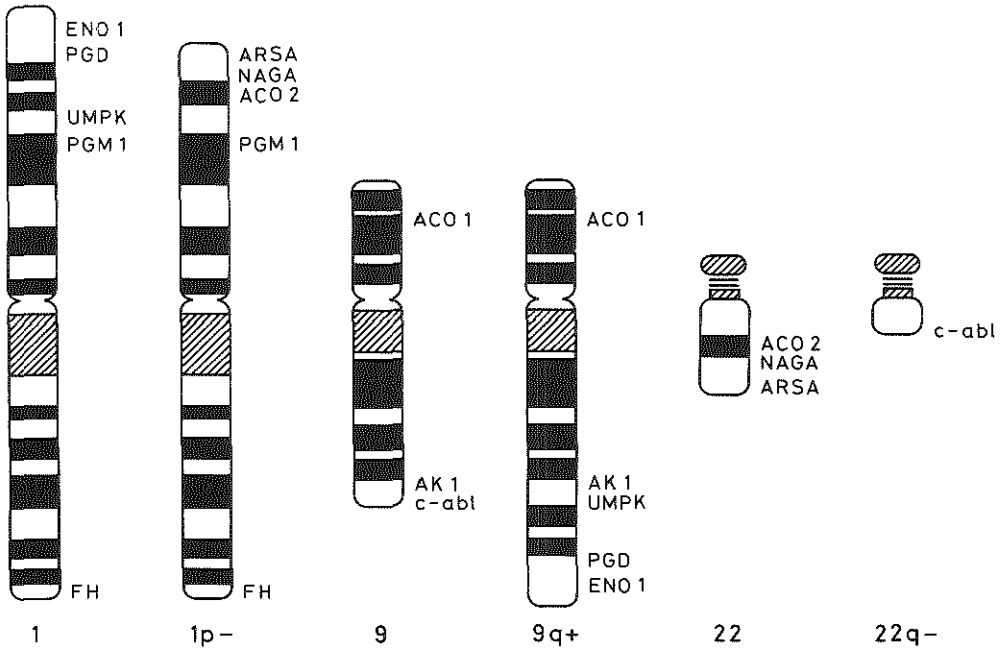

Fig. 6 Diagram illustrating the location of marker genes relative to the breakpoints in a variant Ph^1 translocation (1p-;9q+;22q-).

The Igλ locus has been reported (De la Chapelle et al., 1983) to be involved in the human Burkitt lymphoma associated translocation (8;22)(q24;q11). In this latter study a DNA probe for a Igλ constant region sequence was used in conjunction with in situ hybridization techniques. Our results, which were obtained by using another Igλ constant region genomic probe, did not show involvement of the Igλ locus in the Ph^1 translocation in four CML patients studied (Paper VII). These experiments suggest that in the Burkitt lymphoma associated translocation (8;22) the breakpoint in chromosome 22 differs from that observed in the CML associated translocation (9;22). It should be

kept in mind, however, that different genomic probes were used for these studies. Since distinct Igλ constant region genes do exist (Elliott et al., 1982) further experiments are required to solve this problem definetely.

2.2.3 Clonal origin of the Philadelphia translocation

The first evidence for a clonal origin of CML was obtained by Fialkow et al. (1967) using a glucose-6-phosphate dehydrogenase (G6PD) polymorphism. A single form ($G6PD^{x}A$) of the X-chromosome encoded enzyme was observed in the Ph^{1} chromosome positive hemopoietic cell population of a female patient, whereas in her skin fibroblasts both A- and B-type G6PD were present. This result was confirmed by other investigators using either the same enzyme polymorphism or chromosome polymorphisms (Graham et al., 1974; Hayata et al., 1974; Hossfeld, 1975; Lawler, 1976; Fialkow et al., 1977; 1980; Koeffler et al., 1980). Interpretation of G6PD data, however, should be handled with caution since small subpopulations of cells with an enzyme pattern different from that of the bulk of cells, e.g. a small $G6PD^{x}B$ positive population besides a major population with $G6PD^{x}A$ enzyme activity, may remain undetected (Adamson, 1978).

In this thesis, somatic cell hybridization and gene segregation analyses, using an adenylate kinase-1 (AK1) polymorphism (Bowman et al., 1967), were applied to this problem (paper IV). In the hybrid clones, obtained by fusion of CML cells with rodent cells, a consistent segregation pattern of the chromosome 9 encoded polymorphic AK1 isoenzymes was observed. $AK1^{x}1$ was formed when the normal chromosome 9 was present whereas the $AK1^{x}2$ allele was expressed in all clones

which had retained the 9q+ derivative. These results are compatible with a single cell origin of the Ph^1 translocation in CML. Since the somatic cell hybrids are obtained by fusion of rodent cells with single cells derived from the leukemic cell population, the problem of overlooking underrepresentated subpopulations can, in principle, be overcome by this system. Admittedly, more hybrid clones will be needed to definitely prove this point.

2.3 Involvement of oncogenes in the Philadelphia translocation

2.3.1 Viral and cellular oncogenes in malignant transformation.

It has been well established that in several animal species malignant transformation can be induced by RNA or DNA virus infection (Rous, 1911; Temin and Rubin, 1958; Gross, 1974; Stephenson, 1980). In man, association of Epstein-Barr virus with Burkitt's lymphoma and nasopharyngeal carcinoma (Epstein et al., 1964; Klein, 1972), of hepatitis-B virus with hepatoma (Chakraborty et al., 1980) and of papilloma virus with urogenital carcinoma (Green et al., 1982) have been found. However, there is no clear evidence for an etiological role of the viruses in these diseases. Recently, Poiesz et al. (1980) discovered a, so far, unknown retrovirus (HTLV) in some T cell malignancies. At about the same time, Hinuma et al. (1981) and Yamamoto et al. (1982a,b) found a similar association between the presence of such a virus and an endemic form of T cell leukemia in Japan. Involvement of viruses in human neoplasias in general, however, could not be established in spite of more than ten years of extensive research (Nooter et al., 1975; Chan et al., 1976; Lewin, 1981).

In 1969 Huebner and Todaro proposed a model which could explain the induction of cancer by many different agents in different species, including man. They suggested that the transforming sequences of retroviruses are a part of the genetic bagage of all cells. These transforming genes (oncogenes) would be innocuous as long as they remain quiescent. When stimulated into activity by a carcinogenic agent, however, they would convert cells to cancerous growth. At present there is ample evidence that viral oncogenes show a high degree of homology with DNA of uninfected cells (Stehelin, 1978) and it is assumed (Temin, 1974) that retroviral oncogenes have been derived from the host cell genome after integration of the RNA virus via a DNA intermediate (Oskarsson et al., 1980; Eva et al., 1982; Dalla-Favera et al., 1982c). The Moloney murine sarcoma virus and the Abelson murine leukemia virus genomes, for example, appear to be recombinants between the original Moloney murine leukemia virus and host cell sequences with transforming activity (Witte et al., 1980; Oskarsson et al., 1980). In contrast to fast transforming (acute) RNA tumor viruses, slow transforming (chronic) RNA tumor viruses do not contain oncogenes. Malignant transformation via slow RNA tumor viruses is apparently brought about by integration of the viral genome adjacent to a cellular oncogene (Fung et al., 1981; Noori-Daloii et al., 1981; Cooper, 1982). It has recently been shown (Hayward et al., 1981; Temin, 1982) that proviruses contain a sequence, the so-called long terminal repeat (LTR; Fig. 7), which includes a promotor that can activate adjacent cellular genes (e.g. oncogenes). Induction of malignancy via such a mechanism has been called 'oncogenesis by promotor insertion' (Neel et al., 1981; Payne et al., 1982).

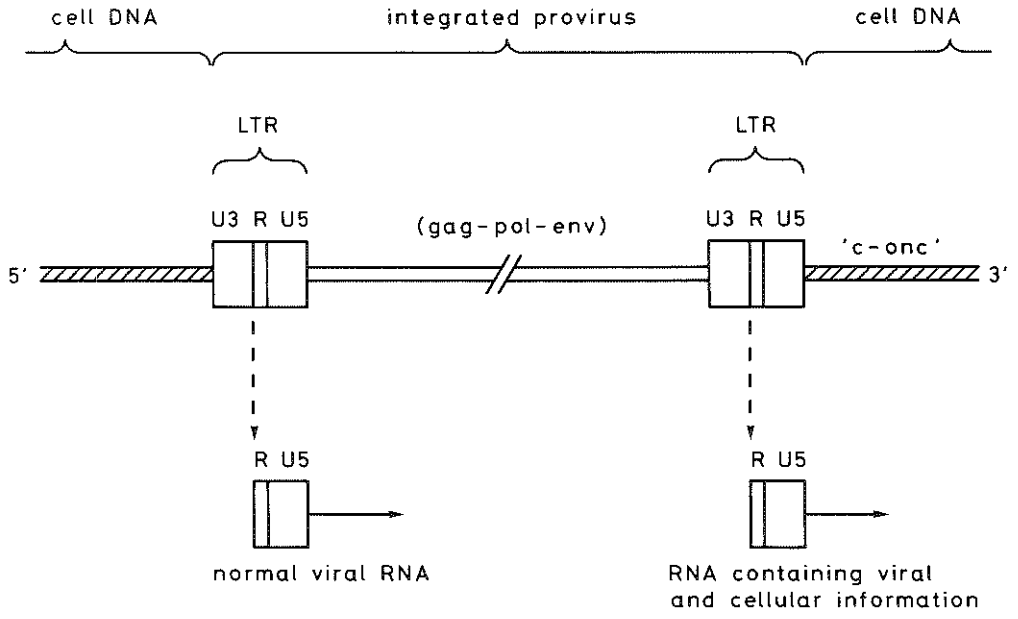

Fig. 7 Schematic representation of structure and transcriptional products of an integrated chronic RNA tumor virus. The provirus is flanked by sequences termed long terminal repeats (LTR's). Synthesis of viral RNA initiates within the left LTR. Initiation within the right LTR would generate a molecule containing viral sequences (R+U5) plus information encoded by the adjacent cellular DNA. If the provirus is integrated upstream from a potentially oncogenic cellular gene (c-onc) initiation within the right LTR could cause elevated expression of the c-onc gene (from Hayward et al., 1981).

Malignant transformation can be induced in non tumorigenic murine fibroblasts (NIH-3T3) in vitro via transfection using DNA fragments of normal cells (Cooper et al., 1980). The efficiency of such a transformation is greatly increased when the recipient murine fibroblasts are preinfected with non transforming leukemia viruses (Krump-Konvalinkova and van den Berg, 1980). It was postulated that certain cellular genes (oncogenes) may cause malignant transformation when integrated adjacent to a cellular promotor (Cooper, 1982) and that pre-

infection of the cells with leukemia virus might provide additional promotor sites. Recently, it has been shown that a human cellular gene (c-ras^H), homologous to the ras oncogene of Harvey murine sarcoma virus, induces oncogenic transformation of NIH-3T3 cells with high efficiency when ligated to a LTR sequence from a murine or feline retrovirus (Chang et al., 1982). The latter experiments suggest that also in human malignancies oncogene activation may be involved. Elevated levels of oncogene transcripts have indeed been observed in a variety of human tumor cell lines (Eva et al., 1982; Collins and Groudine, 1982; Westin et al., 1982; Ozanne et al., 1982; Dalla-Favera et al., 1982c; Weinberg, 1982b).

In contrast to the quantitative changes in oncogene products described above, Tabin et al. (1982), Papageorge et al. (1982) and Reddy et al. (1982) found that in human bladder carcinoma activation of the c-ras^H gene is associated with a single nucleotide change (point mutation) within this gene. This change had no effect on the level of expression of the oncogene but rather affected the structure of the oncogene encoded protein, which could ultimately, as postulated, lead to malignant transformation.

Oncogenes are highly conserved during evolution (Duesberg, 1980) which indicates that they may play an important role in normal cellular functions (Cooper, 1982). The strict regulation of the expression of c-sis (Westin et al., 1982), c-fos and c-abl sequences (Müller et al., 1982) during cellular differentiation and morphogenesis is indicative for such a role.

In general, c-onc transcripts are tyrosine specific protein

kinases that may affect a variety of cellular functions (review Bishop, 1982). Cell surface proteins are amongst the targets of these kinases (Courtneidge et al., 1980). Such surface structures may play a role in cellular communication or may function as receptors for growth regulating factors e.g. hormones (Simantov and Sachs, 1978) or colony stimulating factors (Metcalf, 1980). Disregulation of membrane bound functions through phosphorylation may impair the normal regulation of cellular proliferation and the normal restraints to invasive growth.

2.3.2 *Transposition of cellular oncogenes as a result of the Philadelphia translocation*

Due to the homology between viral oncogenes (v-onc) and human cellular oncogenes (c-onc), several of the latter genes could be identified and localized on specific human chromosomes (Table 1) by using cloned v-onc and c-onc sequences as probes. It is of particular interest that some of these c-onc genes have been assigned to chromosomes that are frequently involved in specific cytogenetic abnormalities associated with certain types of neoplasia (see chapter 1.1). With regard to the Ph^1 translocation, c-sis and c-abl on chromosome 22 and 9, respectively, are of particular interest. Using hybrid cell lines containing Ph^1 translocation products we investigated whether these oncogenes are involved in this specific chromosomal translocation in CML. The results (paper V, VI, VII) unequivocally establish that both c-sis and c-abl are indeed involved in this translocation. The c-abl sequences studied were absent when the 9q+ derivative of the translocation was present, whereas such sequences were present when the 22q- deriva-

Table 1

Cellular oncogenes, their chromosomal localization and the involvement of these chromosomes in karyotypic changes in certain neoplastic diseases

Oncogene	Virus strain	Human chromosome localization	Chromosome aberration	Disease[xx]	References
c-myb	Avian myeloblastosis virus	6(q22-q24)	6q-	ALL	1.2
c-mos	Moloney murine sarcoma virus	8(q22)	t(8;21)(q22;q22)	AML	3.4
c-myc	Avian myelocytomatosis virus	8(q24)	t(8;14)(q24;q23)	BL	3.5.6
c-abl	Abelson murine leukemia virus	9(q34)	t(9;22)(q34;q11)	CML	7.8
c-ras^{H1}[x]	Harvey murine sarcoma virus	11(p11-pter)	del 11p13	Wilm's	9.10
c-ras^{K2}	Kirsten murine sarcoma virus	12	+12	CLL	11
c-fes	Snyder-Thielen feline sarcoma virus	15(q24-q25)	t(15;17)(q22;q12)	APL	1.2.7
c-src	Rous sarcoma virus	20	20q-	MPD	12.
c-sis	Simian sarcoma virus	22(q11-q13)	t(9;22)(q34;q11)	CML	13.14.15.16

[x] The genes designated ras constitute a multigene family (Ellis et al., 1981). Note: very recently we found (Geurts van Kessel and Nusse, 1983c) that the c-ras^{H1} oncogene is not included in the Wilm's tumor associated deletion of chromosome 11.

[xx] See chapter 1 and Rowley (1983)

1. Dalla-Favera et al. (1982b)
2. Harper et al. (in press)
3. Neel et al. (1982)
4. Prakash et al (1982)
5. Dalla-Favera et al. (1982d)
6. Taub et al. (1982)
7. Heisterkamp et al. (1982)
8. de Klein et al. (1982)(Paper V)
9. Mc Bride et al. (1982)
10. De Martinville et al. (1983)
11. O'Brien et al. (1983)
12. Sakaguchi et al. (in press)
13. Swan et al. (1982)
14. Dalla-Favera et al. (1982a)
15. Groffen et al. (in press)(Paper VI)
16. Young et al. (submitted)(Paper VII)

tive was retained by the hybrid cells. In reverse, c-sis sequences were present when 9q+ was retained and absent when 22q- was present (Fig. 5). Leucocytes from different CML patients were used for these analyses and identical results were obtained throughout the experiments. This indicates that the observed translocations are likely to be gene-

ral phenoma in CML. In addition, a similar translocation of c-abl from chromosome 9 to chromosome 22 was observed in a CML patient (patient DY in paper II and VIII) carrying a complex Ph^1 variant (Fig. 6) involving chromosomes 1, 9 and 22 (Bartram, 1983).

Although in some primary leukemias and lymphomas and in some derived permanent cell lines, a moderate to high expression of oncogenes is observed (Collins and Groudine, 1982; Dalla-Favera et al., 1982c; Westin et al., 1982; Eva et al., 1982; Ozanne et al., 1982), an increase in c-sis and c-abl transcripts could not yet be established in the leukemic cell populations of CML patients or derived cell lines (Swan et al., 1982; Grosveld and Groffen, 1983). The involvement of c-abl and c-sis in the Ph^1 translocation, however, remains suggestive of their role in the pathogenesis of CML, especially when seen in the light of very recent reports demonstrating that the oncogene c-myc is involved in the Burkitt lymphoma associated translocation (8;14) (Taub et al., 1982; Dalla-Favera et al., 1982d) and in the murine plasmacytoma associated translocation (12;15) (Harris et al., 1982; Crews et al., 1982). It has been shown by these investigators that c-myc was translocated directly into restriction fragments that encode immunoglobulin chains. They suggested that, due to such a translocation, the c-myc gene may be activated as has been observed in B cell malignancy in chickens (Hayward et al., 1981; Payne et al., 1981). Alternatively, it has been suggested (Dalla-Favera et al., 1982d) that translocation of the c-myc oncogene may have resulted in failure of the mechanism which controls repression of this gene. In analogy with c-abl and c-sis in CML, c-myc related RNA levels in Burkitt lymphoma cell lines were not

significant above the levels found in other human normal or neoplastic tissues (Westin et al., 1981). It should be noted, however, that populations of normal control cells and malignant cells, which represent specific stages of differentiation were not compared, so far, in these lymphoid and myeloid neoplasias. The latter is of import since it has been shown that the expression of a c-onc gene can be switched off during differentiation, also in malignant cells (Westin et al., 1981).

The c-abl sequence is hypo-methylated in a human leukemia cell line (SMS-SB), derived from a patient with pre-B ALL. RNA isolates from these cells, which are homologous to v-abl, are able to transform NIH-3T3 cells in a transfection assay (Ozanne et al., 1982). Attempts to transform NIH-3T3 cells with c-abl sequences have failed so far (Heisterkamp et al., 1982) but these results are inconclusive since only incomplete oncogene sequences were present in the DNA used for transfection (Srinivasan et al., 1982).

2.4 Somatic cell hybrid analysis of myeloid differentiation

2.4.1 Monoclonal antibodies against differentiation antigens

In the past several attempts have been made to isolate antibodies which react with determinants which were thought to be specifically present on surfaces of human leukemic cells (Garb et al., 1962; Metzgar et al., 1972; Baker and Taub, 1973; Harris, 1973; Lozzio et al., 1977; Baker et al., 1979). Evidence for the actual existence of such tumor specific (neo-)antigens, however, is scarce (Faldt and Ankerst, 1980; Clavell et al., 1981; Ashall et al., 1982; Brown et al., 1982). It is

now generally believed that the antigenic make up of malignant cells is not a unique property of these cells (Greaves and Janossy, 1978), but represents markers normally present on immature cells (Bradstock et al., 1980). Antibodies directed against such differentiation associated determinants can be used in the study of normal differentiation and its disregulation in malignant disorders (Roberts and Greaves, 1978; Mulder et al., 1981). In addition they may, occasionally, serve as therapeutic agents (Lanier et al., 1980; Hamblin et al., 1980; Bernstein et al., 1980; Miller et al., 1981; Ritz and Schlossman, 1982).

The use of conventional (hetero-) antisera is hampered by the limited amounts available, low titers and the presence of contaminating antibodies. Absorption with cells of various tissues is required to render such antisera specific. With the introduction of the hybridoma technique by Köhler and Milstein (1975), the major drawbacks of conventional antisera have been overcome. Briefly, this technique includes the following steps: spleen cells from e.g. mice, immunized with an antigen of interest, are fused (Fig. 3) with an established myeloma cell line and the hybrid cells (hybridomas) are cloned and screened for antibody production. Positive clones of interest are propagated further in continuous cell culture or via serial transplantation in mice. Since each independent hybridoma results from the fusion of a single splenic B cell with a myeloma cell, the antibodies produced by such a hybridoma clone are directed at a single antigenic determinant (epitope). The so-called monoclonal antibodies, obtained from culture supernatants or ascites fluids can, in principle, be produced

in large quantities by the hybridomas which have an unlimited life span. The development of a battery of T cell specific monoclonal antibodies (OKT series; Kung et al., 1979; Reinhertz and Schlossman, 1980), several of which identify subsets of human thymocytes and circulating T cells, is well known and the antibodies are widely used. Similarly, large series of monoclonal antibodies reactive with B-lymphocytic, erythroid, monocytic, megakaryocytic and myeloid cells have been isolated (First International Workshop on Leucocyte Differentiation Antigens, Paris 1982).

Tissue specific cell surface determinants are thought to serve as recognition structures that define the biological activities of the cells bearing them. Changes in myeloid surface determinants that occur during malignant transformation (Taub et al., 1980) can be identified by using antibodies as probes. Analysis of these changes in conjunction with the occurrence of genetic (chromosomal) alterations (see chapter I) may provide insight into the mechanism leading to malignancy. The chromosomal localization of the genes controlling the expression of myeloid associated characteristics could indicate whether their loci are involved in chromosomal abnormalities observed after malignant transformation (Forman and Rowley, 1982).

2.4.2 Chromosomal localization of genes controlling the expression of myeloid differentiation characteristics.

The genetic control over the expression of human myeloid associated surface antigens was analyzed using intralineage human-mouse myeloid cell hybrids which segregate human chromosomes. The requirement of intralineage hybrid cells for the study of differentiation associa-

ted characteristics and the first succesful attempt to generate such hybrids within the myeloid differentiation pathway has been outlined in chapter 2.1 and is described in paper VIII. For the production of these hybrids, human CML cells were fused with an established mouse myeloid cell line (WEHI-TG) using Sendai virus as fusogen. The WEHI-TG line is a hypoxanthine phosphorybosyl transferase deficient ($HPRT^-$) mutant that we have isolated from WEHI-3B, a murine myeloid leukemia cell line, originally obtained by Metcalf et al. (1969). Chromosomal, immunological and functional markers that have been described for the WEHI-3B cell line (Greenberger et al., 1978; Bornstein and Mc Morrow, 1980; van Loveren et al., 1982) were found to be present in the WEHI-TG cells, which confirms that the mutant cell line is a WEHI-3B derivative. M1 and RMB are two other well documented (Ichikawa, 1969; de Both et al., 1981) established murine myeloid cell lines. From these lines we isolated $HPRT^-$ mutants as well, but by using these mutant cells, we failed to obtain hybrid cell lines in spite of various fusion and hybrid isolation protocols tested. Since the human leukemic leucocytes used for the cell fusion experiments with WEHI-TG were usually mixed with karyotypically normal lymphoid cells, Ph^1 translocation products were used as markers to indicate that indeed human CML cells were fused. It is of interest, that these leukemia associated aberrant chromosomes were recovered at a high frequency in the hybrid clones. It is not clear at present whether these aberrant chromosomes are actively retained by the hybrid cells or whether hybrid cells containing such chromosomes have an in vitro growth advantage. Cytochemical, morphological and immunological studies further confirmed the myeloi-

Table 2

Sixteen monoclonal antibodies, their isotype and specificity, the molecular weight of the glycoprotein carrier molecules and the chromosomal localization of the gene(s) involved in the expression of the antigens recognized.

Monoclonal antibody	Specificity	Molecular weight[x]	Isotype	Human Chromosome[xx]	References
B4.3	Myeloid	105-150 K	IgM	11	1.
MI/N1	Myeloid	105-150 K	IgM	11	2.
UJ-308	Myeloid	105-150 K	IgM	11	2.
VIM-D5	Myeloid	105-150 K	IgM	11	3.
FMC-10	Myeloid	105-150 K	IgM	11	4.
RIB-19	Myeloid	105-145 K	IgM	11	5.
S4.7.13	Myeloid/Mono./Lymph	150 K	IgM	11	6.
B.37.4	Myeloid	ND	IgM	11	6.
1G10	Myeloid/Mono.	150 K	IgM	11	7.
IH4	Myeloid	ND	IgM	11	8.
3B9	Myeloid	105-150 K	IgM	11	8.
6G5	Myeloid	105-150 K	IgM	11	8.
4C10	Myeloid	105-150 K	IgM	11	8.
VIM-2	Myeloid/Mono.	ND	IgM	11	9.
VIM-7	Myeloid	ND	IgM	11	9.
VIM-8	Myeloid	ND	IgM	11	9.

[x]Molecular weights of antigens detected by B4.3, MI/N1, UJ-308, VIM-D5, FMC-10, 3B9, 6G5 and 4C10 determined by Tetteroo and Visser, of antigens detected by RIB-19 and S4.7.13 determined by Rovera and of antigen detected by 1G10 determined by Urdal.

[xx]Chromosomal assignments: see Geurts van Kessel et al. (1983b)

ND: not determined

1. Tetteroo et al. (1983a)
2. Kemshead et al. (1981)
3. Majdic et al.(1981)
4. Zola et al. (1981)
5. Perussia et al. (1982)
6. Ferrero et al. (1983)
7. Urdal et al. (1983)
8. Lansdorp, Tetteroo and Bos (1983)
9. Knapp (unpublished results)

nature of the hybrids.

Several monoclonal antibodies, most of which were known to recognize antigens specifically present on human myeloid cell surfaces (Table 2), appeared to be reactive with the hybrid cells. This indicates that this intralineage human-mouse hybrid cell system allows the

expression of differentiation associated antigens recognized by the antibodies used and that, therefore, this hybrid cell system is suitable for the present study. In contrast, human CML x Chinese hamster fibroblast hybrids tested so far (Paper VIII), failed to react with myeloid specific antibodies. Chromosome analysis of the intralineage myeloid hybrid clones showed that one or more genes, responsible for the expression of the antigen(s) detected by the sixteen monoclonal antibodies listed in Table 2, are located on human chromosome 11. The antibodies are all of the IgM class. At least five of these monoclonal antibodies (B4.3, MI/N1, UJ-308, VIM-D5, FMC-10), obtained from different laboratories, appeared to be reactive with the same human myeloid associated antigen present on the hybrid cells (Paper VIII). This finding was also based on other studies (Tetteroo et al., 1983a) which showed that all five antibodies precipitated similar glycoproteins with apparent molecular weights of 105.000 and 150.000 daltons present on normal human granulocytes. Furthermore, these studies showed that preincubation of granulocytes with four of the antibodies tested inhibited the binding of labeled B4.3 antibody in a very similar way. Identical competition binding and immunoprecipitation results were recently obtained by Perussia et al. (1982) and Skubits et al. (1983), respectively, using newly isolated monoclonal antibodies against myeloid antigens. These results indicate that either only a limited number of antigenic structures are exposed on myeloid cells or that certain components on the surface are far more antigenic than others. Most monoclonal antibodies with specificity for myeloid cells are reactive with carbohydrate surface determinants (Urdal et al., 1983), which makes these

structures the main sites of recognition (Rovera et al., 1983) in this cell type. Indeed, the antibodies used in our study have recently been shown (Tetteroo, 1983b) to be directed against carbohydrate determinants on myeloid cells as well. Since such carbohydrates may be present on different carrier molecules, differences that we observed in immunoprecipitation patterns of normal granulocytes and hybrid cells (Paper VIII), may easily be explained by the presence of different (mouse or mouse-human heteropolymeric) proteins, carrying the antigenic determinant in the hybrids. Alternatively, differences in glycosylation, occurring e.g. during myeloid differentiation, may result in different molecular weights of antigenic molecules in an SDS-PAGE assay (Breitman et al., 1980). None of our hybrids had completely maturated to polymorphonuclear granulocytes. The relationship between the carbohydrate determinants detected by the above monoclonal antibodies and the fucose containing oligosaccharides studied by gel filtration (van Beek et al., 1975; Smets, 1980; Geurts van Kessel et al., 1981a) remains to be established.

In the past, several investigators have found an association between the presence of human surface antigens and chromosome 11 (Nabholz et al., 1969; Puck et al., 1971; Buck and Bodmer, 1974; Barnstable et al., 1978; Goodfellow et al., 1982; Haynes et al., 1982). These antigens appear to have tissue distributions different from those detected by the myeloid specific antibodies described in this thesis (Paper VIII) and summarized in Table 2. It has been shown, that at least in some of the cases reported by other investigators, the antigenic determinant recognized resides in a carbohydrate structure as well (Jones et al.,

1979). The interrelationship between these various antigens, i.e. the glycosyltransferases involved in the biosynthesis of the antigenic carbohydrate structures on myeloid and other cell types, remains to be elucidated. Regional localization of the involved gene(s) on chromosome 11 could indicate whether different genes are responsible for the expression of the antigens in question. Further characterization of the antigenic determinants recognized by the antibodies could give more information about the nature of the gene(s) governing the antigenic make up of myeloid and other cell types and about how the expression of such genes may be (dis)regulated in malignancy (see also chapter 2.3).

Sofar, no differentiation functions of human myeloid cells could be associated with chromosomes involved in the Ph^1 aberration, but it seems clear from the above studies, that the newly isolated intralineage myeloid hybrids could be of great value for future studies in this field. Such studies could be directed at raising antibodies against hybrid cells containing only human chromosomes of interest (Ph^1,9,22). Spleen cells of BALB/c mice immunized with such hybrids (the WEHI-TG parental cell line also having a BALB/c background) could be used to make hybridomas producing monoclonal antibodies directed at determinants whose loci are e.g. on the Ph^1 chromosome.

3. HYPOTHESIS

Based on the experimental work described in this thesis the following hypothesis concerning the role of the Ph^1 translocation in CML is postulated: as a result of the Ph^1 translocation (classical or complex variant) in CML a particular oncogene (c-abl or c-sis) may be dissociated from its normal cis- or trans-acting regulator(s). Activation of this oncogene may result from its transposition (via translocation) to a genomic region that is actively transcribed in myeloid cells. Loss of normal regulation and subsequent activation of the oncogene could lead, e.g. via an elevated level of oncogene encoded protein, to malignant transformation. Such a mechanism does not exclude the possible presence of an aberrant oncogene product in CML cells. The latter might result from gene mutation and/or abnormal processing of the protein involved. Other bone marrow progenitors (erythroblasts etc.) are not affected by transposition of the oncogene. This could either be due to lack of oncogene activation in these cells (the region of translocation is only actively transcribed during myeloid differentiation), or to the specific impairment of myeloid associated cellular functions (e.g. receptors for myeloid growth regulating factors) as a result of oncogene activation.

Several Ph^1 variants have been described in which involvement of chromosome 9 could not be established, at least not at the microscopic level. Preliminary indications are now available (Bartram, 1983) suggesting that also in these variant cases c-abl is translocated to the Ph^1 chromosome, whereas c-sis is translocated to various other sites within the genome. If this appears to be a general phenomenon then,

of these two, c-abl is most likely to be directly involved in the neoplastic process leading to CML. The latter would be in agreement with the postulate of Pasquali et al. (1979) suggesting that translocation of the distal part of 9q to the Ph^1 chromosome is the most crucial event in the development of CML. It still remains to be established whether c-abl is similarly translocated in cases with a so-called 'masked' Ph^1 chromosome (see chapter 1.2).

It has long been recognized (Hakama, 1971; Boutwell, 1974) that carcinogenesis is a multi-stage process. The occurrence of particular chromosomal abnormalities in neoplastic cells and the subsequent appearance of populations with additional karyotypic changes are closely associated with step-wise changes in the malignant character of these cells (Klein, 1979). Lane et al. (1982) postulated that in mouse tumors, induced by the Abelson murine leukemia virus, v-abl may induce early events in the neoplastic process, but that secondary activation of a distinct cellular transforming gene may be involved in the progression of neoplasia (see also Ozanne et al., 1982; Cooper, 1982). The acquisition of secondary chromosomal abnormalities during progression of the disease in CML patients (see chapter 1) may be associated with the involvement of different oncogenes during these subsequent stages as well.

In two well documented patients, conversion of Ph^1 positive CML into Ph^1 negative CML has been observed (Hagemeijer et al., 1979b; Sharp et al., 1979). These observations may either be explained by an initial eradication, through treatment, of the Ph^1 positive leukemic cell population and the subsequent development of a new myeloid

leukemia in the same patient, or by supposing that there is at least one more step in the development of CML prior to the acquisition of the Ph^1 translocation by the leukemic cells. Since 'contaminating' Ph^1 negative cells are usually not observed in unstimulated blood or bone marrow cultures of CML patients, one must assume that such a (pre-) leukemic population, if it exists, is immediately replaced by Ph^1 positive cells in the bone marrow of most patients. About 10% of the CML patients remains Ph^1 negative (see chapter 1.2). The prognosis for these patients, however, is significantly poorer than for Ph^1 positive CML patients (Rowley, 1980) and it is now generally accepted that Ph^1 negative CML should be considered as a separate clinical entity. Recently, it has been demonstrated in our laboratory that neither c-abl nor c-sis are translocated in leucocytes from Ph^1 negative CML patients (Bartram, 1983).

The above hypothesis is in agreement with various previously made postulates and with experimental data recently obtained in related fields of cancer research. In 1969 Huebner and Todaro suggested that normal cells contain transforming genes in an inactive state and that activation of these genes could lead to the formation of tumor cells. These transforming genes might, according to Huebner and Todaro, be homologous to oncogenes of RNA tumor viruses. At present this latter postulate has been established firmly (review Bishop, 1982). Comings (1973) suggested that cellular transforming genes are under the influence of normal regulator genes and that transforming genes may escape from their normal regulation when the regulator genes are inactivated by e.g. mutation or position effect after translocation.

Trosko and Chang (1978) postulated that such transforming genes may serve an important role in normal cellular development. Specific transforming genes were thought to be activated during restricted phases of particular differentiation pathways. The recent finding that cellular oncogenes are strictly regulated during normal differentiation (Müller et al., 1982) is in support of this latter notion.

Recent results based on DNA transfection experiments are also compatible with the above hypothesis: Cooper et al. (1980) found that non tumorigenic cells can be transformed with DNA fragments from normal cells, which suggests that certain genes in normal cells (oncogenes) can bring about malignant transformation when taken out of their normal environment (dissociated from their normal regulators) and when subsequently integrated into an appropriate site in the genome of a non tumorigenic cell. This process was enhanced considerably when RNA tumor viruses were included in the transfection (Krump-Konvalinkova and van den Berg, 1980). Such proviruses are known to contain long terminal repeat sequences (LTR) which may serve as activators or promotors of cellular oncogenes (Neel et al., 1982). Promotor sequences involved in the expression of normal cellular functions, e.g. myeloid specific functions or immunoglobulin production in plasma cells, may serve as activators of oncogenes as well (Klein, 1981).

Since Harris et al. (1969) showed that the malignant character of somatic cells can be suppressed by fusing them with non malignant cells, many authors have confirmed this finding in a variety of tumor cell systems (review Klinger, 1982). It could be argued that introduction, via cell fusion of trans-acting regulator genes in the ma-

lignant cells results in reversion to a non malignant state and that malignancy is re-expressed when these regulator genes are lost due to chromosome segregation (Harris et al., 1969). When we fused tumorigenic rodent cells with human CML cells or tumorigenic mouse myeloid leukemia cells, the obtained hybrids appeared to be reduced in their tumorigenic capacity as well (Geurts van Kessel et al., 1981, 1982). These results suggest that tumorigenic cells can complement each other in the hybrid cell system. This would be compatible with the idea that in different types of tumor cells, or even tumor cells in different stages of progression of the disease, different loci (oncogenes) may be involved.

At present, one can only speculate about the question of what determines the specificity of the Ph^1 translocation. Is it one chromosomal aberration, out of a pool of many randomly occurring anomalies, which happens to impose a growth advantage on myeloid cells, as outlined above? Are the involved regions on chromosome 9 and 22 closely associated in interphase nuclei of myeloid cells, which increases the chance of this specific recombination to occur, or are the translocation sites subject to increased genome rearrangement, e.g. the λ light chain region(s) on chromosome 22? It is obvious that these questions, and several others concerning the Ph^1 translocation in CML, remain to be elucidated.

SUMMARY

During the last two decades evidence for a close association between the presence of specific chromosomal abnormalities and the occurrence of several types of cancers and leukemias has accumulated. The Philadelphia (Ph^1) translocation, present in about 90% of the patients with chronic myeloid leukemia (CML), is one of the most typical and best documented examples of such an aberration. Usually this translocation involves chromosome 9 and 22: t(9;22)(q34;q11). The translocation products are designated 9q+ and 22q-. Variant translocations involving an array of translocation sites different from 9 have been described as well, but chromosome 22 is always involved. So far, no clear indications were found for the possible role played by this highly specific chromosomal aberration in the etiology of CML. Moreover, results concerning the exact nature of the Ph^1 translocation, obtained by different investigators using different techniques, appeared to be contradictory.

In this thesis the application of somatic cell hybridization and gene segregation analyses to these questions has been described. Rodent cells (fibroblasts) were fused with human Ph^1 positive leucocytes and, subsequently, hybrid cell lines were isolated. These hybrids appeared to segregate human chromosomes, including the Ph^1 translocation products. The segregation of genes, previously assigned to the regions of the chromosomal breakpoints, was studied together with the segregation of the relevant human (translocation) chromosomes. Several genes on chromosome 22 were found to be translocated to the 9q+ chromosome which confirmed, on a molecular level, the trans-

location of chromosome 22 material to chromosome 9. Another gene on chromosome 22 (immunoglobulin λ light chain) stayed on the Ph^1 chromosome (22q-). One gene on chromosome 9 (c-abl) appeared to be translocated to 22q-. This latter result provided unequivocal evidence for reciprocity of the Ph^1 translocation. No apparent differences in chromosomal breakpoints could be revealed in the different CML patients used for analysis and no evidence was found for loss of chromosomal material (genes) as a result of the Ph^1 translocation. The clonal origin of the Ph^1 translocation in CML was confirmed using a chromosome 9 encoded polymorphic enzyme (AK1).

Through the demonstration that two cellular oncogenes (c-abl and c-sis), homologous to transforming sequences of acute RNA tumor viruses, were transposed as a result of the Ph^1 translocation in different CML patients, indications were found for a possible role of these oncogenes in the etiology of CML. A similar result was recently obtained by other investigators for the c-myc oncogene in relation to the Burkitt lymphoma associated translocation 8;14. It is proposed that, due to transposition, a cellular oncogene may be disregulated and/or activated which, in turn, could lead to malignant growth.

Via the successful production of human-mouse myeloid cell hybrids, the human myeloid compartment has been disclosed for genetic analysis. Genes involved in the expression of human myeloid specific antigen(s) were assigned to human chromosome 11, using such hybrids. These myeloid hybrid lines may be useful tools for a further genetic analysis of human myeloid differentiation functions and of the disregulation of the involved genes in human myeloid leukemias.

SAMENVATTING

Gedurende de laatste twee decennia heeft zich het bewijsmateriaal voor een nauw verband tussen de aanwezigheid van specifieke chromosomale afwijkingen en het voorkomen van verschillende typen kankers en leukemieën opgestapeld. De Philadelphia (Ph^1) translokatie, aanwezig in ongeveer 90% van de patienten met chronische myeloide leukemie (CML), is een van de meest typische en best gedokumenteerde voorbeelden van zo'n afwijking. Gewoonlijk zijn bij deze translokatie chromosoom 9 en 22 betrokken: t(9;22)(q34;q11). De translokatie produkten worden aangeduid met 9q+ en 22q-. Variante translokaties met een serie uiteenlopende translokatie plaatsen buiten chromosoom 9 zijn tevens beschreven, maar chromosoom 22 is er altijd bij betrokken. Tot dusver werden geen duidelijke aanwijzingen gevonden voor de mogelijke rol welke deze zeer specifieke chromosomale afwijking zou spelen in het ontstaan van CML. Bovendien bleken de resultaten met betrekking tot de juiste aard van de Ph^1 translokatie, verkregen door verschillende onderzoekers met behulp van verschillende technieken, tegenstrijdig te zijn.

In dit proefschrift is de toepassing van somatische cel hybridisatie en gen segregatie technieken op deze vraagstukken beschreven. Knaagdiercellen (fibroblasten) werden gefuseerd met menselijke Ph^1 positieve leukocyten en vervolgens werden hybride cellijnen geisoleerd. Deze hybriden bleken menselijke chromosomen te segregeren met inbegrip van de Ph^1 translokatie produkten. De segregatie van genen, voordien gelokaliseerd in de chromosomale breukpunt-regio, werd bestudeerd samen met de segregatie van de relevante humane (translokatie)

chromosomen. Voor verschillende genen op chromosoom 22 werd aangetoond dat deze getranslokeerd zijn naar het 9q+ chromosoom hetgeen, op moleculair niveau, de translokatie van chromosoom 22 materiaal naar chromosoom 9 bevestigde. Een ander gen op chromosoom 22 (immunoglobuline λ lichte keten) bleef op het Ph^1 chromosoom (22q-). Een gen op chromosoom 9 (c-abl) bleek te zijn getranslokeerd naar 22q-.
Dit laatste resultaat leverde het onweerlegbare bewijs op voor het reciproke karakter van de Ph^1 translokatie. Geen duidelijke verschillen in chromosomale breukpunten konden worden aangetoond in de verschillende CML patienten welke gebruikt werden voor de analyses en er werd geen bewijs gevonden voor verlies van chromosomaal materiaal (genen) als gevolg van de Ph^1 translokatie. De klonale oorsprong van de Ph^1 translokatie in CML werd bevestigd met behulp van een door chromosoom 9 gekodeerd polymorf enzym (AK1).

Door aan te tonen dat twee cellulaire oncogenen (c-abl en c-sis), homoloog aan transformerende sequenties van akute RNA tumor virussen, verplaatsen tengevolge van de Ph^1 translokatie in verschillende CML patienten, werden aanwijzingen gevonden voor een mogelijke rol van deze oncogenen in het ontstaan van CML. Een zelfde resultaat werd onlangs door andere onderzoekers verkregen voor het c-myc oncogen met betrekking tot de Burkitt lymfoom geassocieerde translokatie 8;14. Voorgesteld wordt dat, als gevolg van transpositie, een cellulair oncogen mogelijkerwijs ontregeld en/of geaktiveerd kan worden hetgeen, op zijn beurt, zou kunnen leiden tot maligne groei.

Via de succesvolle produktie van mens-muis myeloide cel hybriden werd het menselijke myeloide kompartiment ontsloten voor genetische

analyse. Genen, betrokken bij de expressie van humane myeloid-specifieke antigen(en), werden gelokaliseerd op chromosoom 11 met gebruikmaking van zulke hybriden. Deze myeloide hybride lijnen zouden een nuttig gereedschap kunnen vormen voor een verdere genetische analyse van humane myeloide differentiatie funkties en van de ontregeling van de betrokken genen in humane myeloide leukemieën.

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CURRICULUM VITAE

3 februari 1951	- geboorte te Schaijk
4 juni 1969	- eindexamen HBS-B aan het Titus Brandsma Lyceum te Oss.
9 januari 1973	- kandidaatsexamen Biologie (BIw) aan de Katholieke Universiteit te Nijmegen.
7 september 1976	- doktoraalexamen Biologie met als hoofdvak Embryologie (Zoölogisch Laboratorium, Katholieke Universiteit Nijmegen) en als bijvakken Genetica (Genetisch Laboratorium, Katholieke Universiteit, Nijmegen) en Botanie (Department of Biological Sciences, Loyola University, New Orleans, U.S.A.), plus onderwijsbevoegdheid Biologie.
13 juni 1977	- begin met het in dit proefschrift beschreven onderzoek op de afdeling Celbiologie en Genetica, Erasmus Universiteit, Rotterdam onder leiding van Prof.Dr. D. Bootsma en Dr. A. Hagemeijer; met financiële steun van het Koningin Wilhelmina Fonds.

APPENDIX PAPER I

Regional localization of the genes coding for human ACO2, ARSA, and NAGA on chromosome 22

A.H.M. GEURTS VAN KESSEL,[1] A. WESTERVELD,[1] P.G. DE GROOT,[2] P. MEERA KHAN,[3] and A. HAGEMEIJER[1]

[1]Department of Cell Biology and Genetics, Erasmus University, Rotterdam,
[2]Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, and
[3]Department of Human Genetics, University of Leiden

Abstract. The segregation of the chromosome 22 markers ACO2, ARSA, and NAGA was studied in somatic cell hybrid clones. These hybrids were isolated following fusion of Chinese hamster (E36 or a3) cells with leucocytes of donors carrying an (X;22) or (1;22) translocation. The results suggest the assignment of *ARSA* and *NAGA* to the region 22q13→22qter and of *ACO2* to the region 22q11→22q13.

Recently, genes coding for mitochondrial aconitase (ACO2), arylsulfatase-A (ARSA) and *N*-acetyl-α-D-galactosaminidase-A (NAGA) (for nomenclature, see ISGN, 1979) have been located on chromosome 22 (BRUNS et al., 1978; DE GROOT et al., 1978; SPARKES et al., 1978). The assignments of the first two have been confirmed (MEERA KHAN et al., 1978; HORSCAYLA et al., 1979), while that of *NAGA* is still provisional. A regional map of markers known to be located on 22 can be of particular value in, e.g., determining the exact breakpoint in the Philadelphia chromosome. This report deals with the regional localization of these markers on 22 using primary somatic cell hybrid clones derived from fusions of human cells carrying reciprocal translocations (X;22) or (1;22) with Chinese hamster cells.

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Request reprints from: A.H.M. GEURTS VAN KESSEL, Department of Cell Biology and Genetics, Erasmus University, Postbus 1738, 3000 DR Rotterdam (The Netherlands).

Materials and methods

Twelve human-rodent hybrid clones were studied for human chromosome content and enzyme activities. Five primary clones resulted from fusion of Chinese hamster E36 cells with human leucocytes carrying a reciprocal translocation (X;22), also known as the X/22 Breda (DE WIT et al., 1977). Seven primary clones were isolated after fusion of Chinese hamster a3 cells with human leucocytes carrying a reciprocal translocation (1;22),

also known as the 1/22 Amsterdam (JONGSMA and BURGERHOUT, 1977).

The following enzymes were analyzed by cellulose acetate gel (cellogel) electrophoresis (MEERA KHAN, 1971): the chromosome 1 markers fumarate hydratase (FH; E.C. 4.2.1.2), 6-phosphogluconate dehydrogenase (PGD; E.C. 1.1.1.44) and enolase-1 (ENO1; E.C. 4.2.1.11); the X chromosome markers glucose-6-phosphate dehydrogenase (G6PD; E.C. 1.1.1.49), phosphoglycerate kinase (PGK; E.C. 2.7.2.3) and α-galactosidase (GLA; E.C. 3.2.1.22) and the chromosome 22 marker mitochondrial aconitase (ACO2; E.C. 4.2.1.3). For the determination of the chromosome 22 marker arylsulfatase-A (ARSA; E.C. 3.1.6.1) polyacrylamide gel electrophoresis (DUBOIS et al., 1974) was used. The chromosome 22 marker N-acetyl-α-D-galactosaminidase (NAGA; E.C. 3.2.1.49) was assayed by an immunochemical method (DE GROOT et al., 1978).

Chromosome studies were done on air dried preparations using different banding techniques. R-banding with acridine orange after heat denaturation was particularly useful for the study of 22.

Results and discussion

X/22 Breda × E36 hybrids

Characterization of the parental translocation by R-banding revealed that the previously published breakpoints using Q-banding (DE WIT et al., 1977) must be revised. The karyotype is: t(X;22)(q21;q11) (fig. 1). The breakpoint in 22 is quite near the centromere, and the larger part of 22q can be recognized on the X/22 derivative. Previous studies had shown the normal X of this female patient to be late-replicating and therefore inactive (PEARSON et al., 1974).

The segregation of the 22 markers NAGA, ARSA, and ACO2 and the X-chromosome markers G6PD, GLA, and PGK is shown in table I. Three hybrid clones expressed G6PD and GLA, but not PGK or any of the No. 22 markers tested. Chromosome analysis revealed the presence of the 22/X derivative and in one clone an inactive normal X chromosome was also observed. The remaining two clones were positive for all the markers studied. They contained both derivatives of the translocation, and in one clone an inactive normal X chromosome was also present. From these results it can be concluded that the loci for ACO2, ARSA, and NAGA are located on the X/22 chromosome and therefore are located distal to the breakpoint in 22q11.

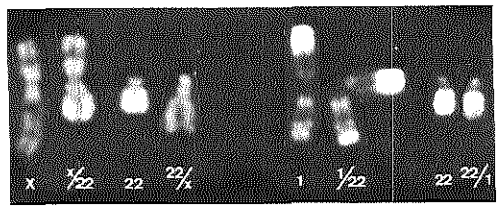

Fig. 1. Chromosomes X, 22 and the X/22 and 22/X derivatives of t(X;22)(q21;q11) and chromosomes 1, 22, and the 1/22 and 22/1 derivatives of t(1;22)(q42;q13). R-banding with acridine orange.

1/22 Amsterdam × a3 hybrids

Using R-banding it was shown that the breakpoint in 22 previously reported to be in band 22q12 (JONGSMA and BURGERHOUT, 1977) is distal to this band (fig. 1). Band 22q12 can still be seen in the derivative chromosome 22/1. Therefore the breakpoint is probably located in the proximal part of band 22q13. The breakpoint in 1 is in 1q42.

The segregation data of the No. 22 markers NAGA, ARSA, and ACO2 and the No. 1 markers PGD, ENO1, and FH are presented in table II. ACO2 segregated independently of NAGA and ARSA in five clones. Three clones were positive for ACO2 and FH and contained the 22/1

Table I. Presence of material from human chromosomes X and 22 and the relevant enzyme markers in five hybrid clones obtained by fusion of X/22 Breda × E36 Chinese hamster cells

Hybrid cell line	Chromosomes				Enzyme markers					
					X chromosome			Chromosome 22		
	X[1]	X/22	22/X	22	PGK	GLA	G6PD	ACO2	NAGA	ARSA
33-2	−	+	+	−	+	+	+	+	+	+
33-5	+	+	+	−	+	+	+	+	+	+
33-9	−	−	+	−	−	+	+	−	−	−
33-15	+	−	+	−	−	+	+	−	−	−
E16-8a	−	−	+	−	−	+	+	−	−	−

[1] Normal X lyonized.

Table II. Presence of material from human chromosomes 1 and 22 and the relevant enzyme markers in seven hybrid clones obtained by fusion of 1/22 Amsterdam × a3 Chinese hamster cells

Hybrid cell line	Chromosomes				Enzyme markers					
					Chromosome 1			Chromosome 22		
	1	1/22	22/1	22	ENO1	PGD	FH	ACO2	NAGA	ARSA
AM-3	+	−	−	−	+	+	+	−	−	−
AM-6	−	+	−	−	+	+	−	−	+	+
AM-21	−	+	−	−	+	+	−	−	+	+
AM-11	−	−	+	−	−	−	+	+	−	−
AM-27	−	−	+	−	−	−	+	+	−	−
AM-44	−	−	+	−	−	−	+	+	−	−
AM-43	−	−	+	+	−	−	+	+	+	+

chromosome. In two clones ACO2 and FH were absent, but NAGA, ARSA, ENO1, and PGD were present, as well as the 1/22 derivative. This segregation suggests that *NAGA* and *ARSA* are situated distal to the breakpoint in 22q13 whereas *ACO2* is located proximally to this breakpoint as evidenced by its expression together with FH.

Segregation of the enzyme markers in the hybrid clones obtained by fusing leucocytes carrying the (X;22) and the (1;22) translocations is summarized in fig. 2. *NAGA* and *ARSA* are located distal to the breakpoint in 22q13 involved in the translocation 1/22 AM. *ACO2* is located between the breakpoints in 22q11 and 22q13.

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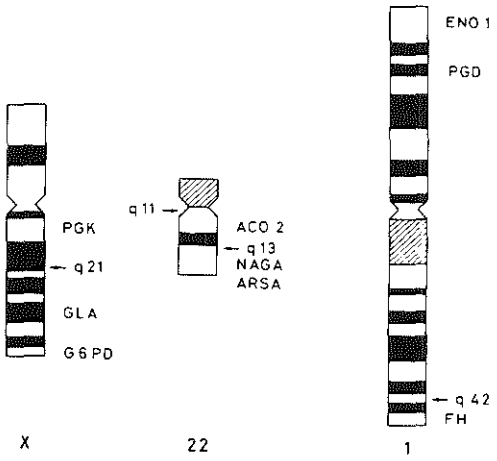

Fig. 2. Diagram illustrating the regional localization of the enzyme markers observed in hybrids containing translocation chromosomes 1/22 Amsterdam and X/22 Breda.

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APPENDIX PAPER II

Characterization of the Philadelphia chromosome by gene mapping

A.H.M. Geurts van Kessel,[1] H. ten Brinke,[2] W.A.M. Boere,[1] W.C. den Boer,[1] P.G. de Groot,[3] A. Hagemeijer,[1] P. Meera Khan,[2] and P.L. Pearson[2]

[1]Department of Cell Biology and Genetics, Erasmus University, Rotterdam; [2]Department of Human Genetics, University of Leiden, and [3]Laboratory of Biochemistry, BCP Jansen Institute, University of Amsterdam

Abstract. Chinese hamster×human and mouse×human somatic cell hybrid lines were obtained using circulating leucocytes from six chronic myeloid leukemia patients. All six patients carried the Ph[1] translocation, t(9q+;22q−), characteristic of chronic myeloid leukemia, in their dividing immature granulocytes. Analysis of independent hybrid clones yielded the following results: 1. The chromosome 9 markers, soluble aconitase and adenylate kinase-1, segregated with the 9q+ derivative. The latter marker has previously been localized to 9q34. 2. The chromosome 22 markers, mitochondrial aconitase, N-acetyl-α-D-galactosaminidase, and arylsulfatase-A, also segregated with the 9q+ derivative. Mitochondrial aconitase has recently been assigned to 22q11→22q13. No evidence was obtained either for reciprocity of the translocation or for variations in breakpoints in different patients. The results reported in this paper provisionally assign the gene for mitochondrial aconitase to a region distal to the breakpoint in 22q11.

The Philadelphia chromosome (Ph[1]) is the acquired cytogenetic abnormality most consistently found in the bone marrow and circulating, dividing leucocytes of patients suffering from chronic myeloid leukemia (CML). Since its discovery (Nowell and Hungerford, 1960), there has been much speculation about the relationship between the presence of this chromosome abnormality and the etiology of the disease. The later discovery that the deletion always involved 22 and that the deleted segment was translocated to the long arm of 9 in the majority of cases (Rowley, 1973) has reinforced interest in the role of such a specific acquired chromosome abnormality in CML. Specifically, the questions of whether the breakpoints in the chromosomes involved are identical in all patients and of whether the

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Request reprints from: A.H.M. Geurts van Kessel, Department of Cell Biology and Genetics, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam (The Netherlands).

translocation is reciprocal have been a matter of dispute (MULDAL et al., 1975; MAYALL et al., 1977; WATT et al., 1977; MITELMAN and LEVAN, 1978; VERMA and DOSIK, 1980).

We report the application of gene mapping methods to these problems. The segregation of enzyme markers coded by genes known to be located in the regions of the breaks was studied using somatic cell hybrids derived from fusion of rodent cells and leucocytes from six CML patients. Two of these genes are located on 9. Adenylate kinase-1 *(AK1)* has recently been mapped to band 9q34 (FERGUSON-SMITH et al., 1976), a band believed to be involved in the translocation with 22 in CML (WATT and PAGE, 1978). Soluble aconitase *(ACO1)* is located on the short arm of 9 (MOHANDAS et al., 1979). Three enzyme markers of 22, namely mitochondrial aconitase *(ACO2)*, N-acetyl-α-D-galactosaminidase *(NAGA)*, and arylsulfatase-A *(ARSA)* were studied. *ACO2* has recently been assigned to the region 22q11→22q13; *ARSA* and *NAGA* have been localized in the region 22q13→22qter (GEURTS VAN KESSEL et al., 1980). Band 22q11 is thought to be involved in the translocation with 9 in CML (WATT and PAGE, 1978).

Our results confirm the translocation of chromosome 22 material to 9. No evidence was obtained either for the reciprocity of the translocation or for variation in the position of the breakpoints in 9 and 22.

Materials and methods

Patients. Peripheral leucocytes of six patients (A through F) with chronic myeloid leukemia (CML) and the Ph^1 translocation (9q+;22q−) were used for the cell fusion experiments. Differential blood counts showed that the majority of cells belonged to the myeloid-monocytic lineage which is Ph^1 positive, and only a minority (3% to 11%) were lymphocytes which are Ph^1 negative. Patients A, B, and E were males; C, D, and F females. Patients A and B were in a blast phase; the other four patients in the chronic phase of the disease. The karyotypes of patients B and D showed abnormalities in addition to t(9q+;22q−). They are reported by HAGEMEIJER et al. (1980; patients No. 25 and 14, respectively).

Rodent cell lines and somatic cell hybridization. Interspecific hybrid cell lines were obtained by fusion of thymidine kinase deficient (TK$^-$) or hypoxanthine phosphoribosyltransferase deficient (HPRT$^-$) rodent cells with peripheral leucocytes of the CML patients using standard techniques. A3 Chinese hamster cells (TK$^-$) were fused with cells of A and C; 3T3 mouse cells (TK$^-$) with cells of B, D, and F; Pg19 mouse cells (HPRT$^-$) with cells of D and E; a23 Chinese hamster cells (TK$^-$), and E36 Chinese hamster cells (HPRT$^-$) with cells of F. Inactivated Sendai virus (HARRIS and WATKINS, 1965) was used as the fusogen in all fusions except one (a3×C). Polyethylene glycol (PEG) (PONTECORVO, 1975) was used in this fusion. Independent hybrid clones, selected in F10 medium supplemented with HAT (LITTLEFIELD, 1964) and 10% fetal calf serum (Flow), were grown in F10 medium with 10% newborn calf serum (Flow).

Cytogenetic and enzyme analysis. Chromosome analysis was done by G (trypsin-Giemsa) or R (with acridine orange) banding; at least ten metaphases per hybrid cell line were studied. The same populations of cells were used for analysis of enzyme markers. The lysates for all the assays were prepared using the lysis buffer and sonication procedure described earlier (MEERA KHAN, 1971).

The hybrid cells, parental rodent cells, parental human cells, and various human tissue culture lines were screened for the enzyme markers. Adenylate kinase-1 (E.C. 2.7.4.3; AK1), soluble aconitase (E.C. 4.2.1.3; ACO1), and mitochondrial aconitase (E.C. 4.2.1.3; ACO2) were analysed by electrophoresis on cellulose acetate gel (cellogel) (MEERA KHAN, 1971). To determine human N-acetyl-α-D-galactosaminidase (E.C.3.2.1.49; NAGA) activity, the enzyme was absorbed from the cell lysate with anti-human NAGA serum covalently coupled to Sepharose 4B. The activity was mea-

sured by incubating the Sepharose-antiserum-NAGA complex with p-nitrophenyl-N-acetyl-α-D-galactosaminide (DE GROOT et al., 1978). Aryl-sulfatase-A (E.C. 3.1.6.1; ARSA) was analysed by polyacrylamide gel electrophoresis (HORS-CAYLA et al., 1979).

Results

Cytogenetic analysis of bone marrow and dividing peripheral leucocytes showed no obvious differences among the 9;22 translocations of the different patients. The breakpoints were consistent with those usually described, namely 9q34 and 22q11 (fig. 1). In fig. 2*a* and *b* examples of hybrid cells containing the translocation chromosomes are shown.

Since one of the objectives of this study was to investigate whether possible differences in the t(9;22) of different patients can be detected by somatic cell genetic methodology, the results of the cytogenetic and enzyme analyses are given for the hybrid cells prepared from each patient separately (tables I and II). Only the chromosome and enzyme data on chromosomes 9, 9q+, 22, and 22q− are presented. Other human chromosomes present in most of the hybrids are not included. ACO1, AK1 and ACO2 analyses were performed on all the hybrids used. ARSA and NAGA can be determined only in Chinese hamster×human hybrids. The anti-NAGA serum cross-reacted with the mouse enzyme. Leucocytes from patients A, C, and F were fused with Chinese hamster cells (table I); leucocytes from patients B, D, E, and F were fused with mouse cells (table II).

Sixty-seven primary hybrid lines were studied. In all six patients we found evidence for a similar segregation pattern of the enzyme markers: the No. 9 markers ACO1 and AK1 segregated with 9q+ as did the markers of 22, ACO2, NAGA, and ARSA. None of the markers studied were expressed in the clones in which only 22q− was present, with the exception of one clone from patient F which expressed ARSA and AK1, indicating that chromosome breakage or a rearrangement may have occurred after fusion. Table III gives the pooled data of the informative clones.

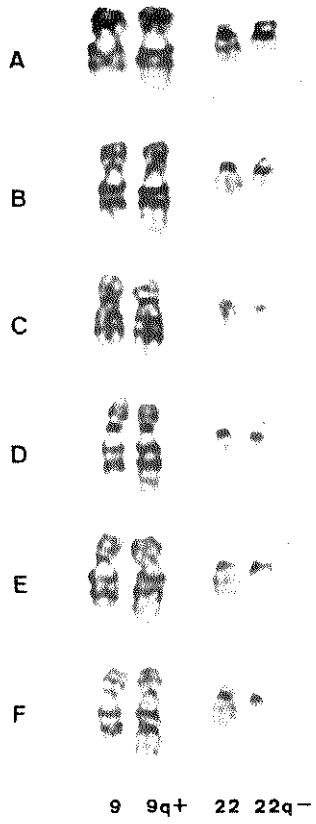

Fig. 1. Trypsin-Giemsa preparations from blood cultures of patient A, B, C, D, E, and F, showing chromosome 9, 9q+, 22, and 22q−.

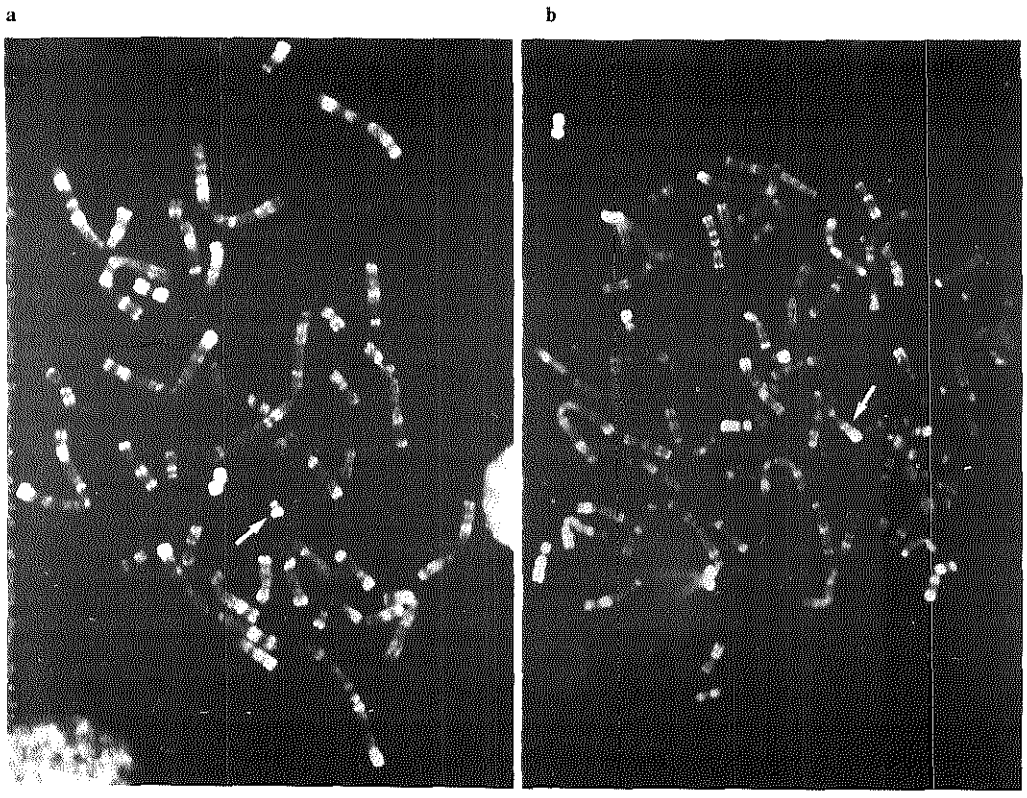

Fig. 2. Two metaphases of a3 × patient A hybrids containing several human chromosomes. Arrows indicate **(a)** the 22q− derivative and **(b)** the 9q+ derivative. R banding with acridine orange.

In three exceptional hybrid clones, derived from fusion of leucocytes from patient F, AK1 was not expressed, but 9q+ was present. In one exceptional clone, derived from patient C, human ACO2 activity was found in the absence of both 9q+, 22 and 22q−.

Discussion

Our results show that in hybrid clones obtained by fusion of leucocytes from six CML patients very similar segregation patterns of the several enzyme markers tested occur, irrespective of the status of the patient (i.e. chronic phase or blast phase), of secondary chromosomal changes, or of the rodent cells used (i.e. a3, a23, E36, Pg19, 3T3). In four clones there is no complete concordance between the expression of some enzyme markers and the presence of the relevant chromosomes. These observations are probably due to undetected secondary chromosomal changes which frequently occur in hybrid cell lines. The absence of human AK1 activity in several hybrid cell lines

Table I. Presence of the human 9 and 22, their translocation products, and the relevant human enzyme markers in hybrid clones obtained by fusion of leucocytes of patients A, C, and F with Chinese hamster cells

No. of clones	Chromosomes				Enzyme markers for chromosomes				
	9	9q+	22	22q−	9		22		
					ACO1	AK1	ACO2	ARSA	NAGA
Patient A×a3									
1	−	+	+	−	+	+	+	+	+
1	−	+	−	+	+	+	+	+	+
2	−	−	−	+	−	−	−	−	−
1	+	−	−	−	+	+	−	−	ND
1	−	−	+	+	−	−	+	+	+
1	+	+	+	−	+	+	+	+	+
Patient C×a3									
3	−	+	−	−	+	+	+	+	+
1	+	+	−	−	+	+	+	+	+
1	+	−	−	+	+	+	−	ND	−
1	+	−	−	+	+	+	−	−	−
1	+	−	+	−	+	+	+	+	+
1	+	+	+	+	+	+	+	+	+
1	+	+	+	−	+	+	+	+	+
1	+	−	−	−	+	+	−	−	−
1	−	−	−	−	−	−	−	ND	−
1	−	−	−	−	−	−	+	−	−
Patient F×E36 or a23									
1	−	+	−	−	+	+	+	+	+
1	−	+	−	−	+	−	+	+	+
1	−	−	−	+	−	+	−	+	−
1	−	−	+	−	−	−	+	+	+
2	+	+	−	+	+	+	+	+	+
1	+	−	+	+	+	−	+	+	+
1	−	+	+	+	+	+	+	+	+
2	−	+	+	−	+	−	+	+	+
1	−[1]	−	−	+	−	+	−	−	−
1	+	+	+	−	+	+	+	+	+
2	−	−	−	−	−	−	−	−	−

ND: not done.

[1] In this clone a translocation product between a Chinese hamster chromosome and 9q was observed.

Table II. Presence of the human 9 and 22, their translocation products, and the relevant human enzyme markers in hybrid clones obtained by fusion of leucocytes from patients B, D, E, and F with mouse cells

No. of clones	Chromosomes				Enzyme markers for chromosomes		
	9	9q+	22	22q−	9		22
					ACO1	AK1	ACO2
Patient B×3T3							
8	−	−	−	+	−	−	−
1	−	−	−	−	−	−	−
Patient D×3T3 or Pg19							
1	−	+	+	−	+	+	+
1	−	+	−	−	+	+	+
1	+	+	−	−	+	+	+
1	+	−	−	+	+	+	−
3	−	−	−	+	−	−	−
1	+	−	−	−	+	−	−
1	+	−	+	+	+	−	+
2	−	−	+	+	−	−	+
Patient E×Pg19							
2	−	+	−	+	+	+	+
2	+	+	−	−	+	+	+
1	+	−	−	−	+	+	−
1	+	+	+	−	+	+	+
2	+	+	+	+	+	+	+
1	−	−	+	−	−	−	+
1	−	−	−	−	−	−	−
Patient F×3T3							
1	+	+	−	−	+	+	+
2	−	−	−	+	−	−	−
1	−	−	−	−	−	−	−

derived from fusions in which leucocytes from patient F were used could be due to the presence of a regulator mechanism effecting the expression of *AK1* as proposed by POVEY et al. (1978). This phenomenon is presently being investigated in more detail.

In general it appears that the breakpoints involved in the 9;22 translocation are in bands 9q34 and 22q11, respectively (WATT et al., 1977; MITELMAN and LEVAN, 1978). Other rearrangements have been suggested including an interstitial insertion and deletion (PRAVTCHEVA and MANOLOV, 1974). Despite several studies, it remains unclear whether the breakpoints are identical in all cases (WATT et al., 1977; VERMA and DOSIK, 1980). Scanning cytophotometric measurements have not revealed significant differ-

Table III. Occurrence of the translocation chromosomes 9q+ or 22q− and the enzyme markers of human origin in the absence of the normal 9 or 22, respectively. Data from six patients are summarized

Chromosomes		Enzyme markers for chromosomes									
		9				22					
		ACO1		AK1		ACO2		ARSA		NAGA	
		+	−	+	−	+	−	+	−	+	−
9q+	+	14	0	11	3	16	0	9	0	9	0
	−	0	28	1	27	1	30	1	9	0	11
22q−	+	4	19	5	18	5	20	4	4	3	6
	−	10	9	7	12	12	10	6	5	6	5

ences in the DNA content of the Ph[1] chromosomes from several patients (MAYALL et al., 1977; GERAEDTS and VAN DER PLOEG, 1980). A complicating factor in such studies is the generally poor morphology of metaphase chromosomes of leukemic cells. It seems clear that if size differences among Ph[1] chromosomes of different patients do exist, they are small and possibly at the limit of resolution of the light microscope.

Regional localization studies have shown *ACO2* to be in the region 22q11→22q13, the region involved in the Philadelphia translocation. The No. 22 markers, ARSA and NAGA, have been mapped to band 22q13 (GEURTS VAN KESSEL et al., 1980). The assignment of *NAGA* to band 22q11 (GEURTS VAN KESSEL et al., 1979) was based on mouse×human hybrids, but the technical limitations noted previously imposed restrictions on resolution of localization. Segregation of *ACO2*, *ARSA*, and *NAGA* with 9q+ agrees with the cytogenetic observation that the breakpoint is in 22q11 and with the regional localization of the markers on 22. These results, however, do not further clarify the position of the break in the six CML patients studied. Resolution of this problem must await the discovery of new markers in the region of the break or the discovery of patients with a break distal to *ACO2*.

WATT and PAGE (1978) used banding profiles to suggest that the translocation is reciprocal. In our approach to this problem AK1, a marker for 9q34, the most distal band of the long arm of 9, was used. This marker consistently segregates with the 9q+ derivative. If reciprocity of the chromosomal exchanges occurs, then the material involved must be distal to the *AK1* locus in band 9q34. Both family and somatic cell studies have led to the conclusion that *AK1* may be in the proximal region of band 9q34 (COOK et al., 1978; FERGUSON-SMITH and AITKEN, 1978). Again determination of whether the translocation is reciprocal requires new markers.

Although the 9;22 translocation occurs in the majority of CML patients, other translocations giving rise to a Ph[1] chromosome have been described (MULDAL et al., 1975; ROWLEY, 1980). It would be of interest to determine if the same amount of 22 material is translocated in these cases.

The role of acquired chromosomal aberrations in neoplasia has been the subject of

considerable speculation. It has been suggested that the loss of specific loci predisposes the cell to malignant transformation. This hypothesis is based on evidence that malignancy behaves like a recessive trait and as such is likely to be expressed when a chromosome is lost or deleted (HARRIS et al., 1969). The discovery that the Ph^1 chromosome is not the result of a simple deletion, but that a translocation is involved, has implications in this interpretation of the etiology of neoplasia in CML patients. Since we are unable to determine if the formation of the Ph^1 chromosome via translocation involves loss of material, the question remains unresolved.

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APPENDIX PAPER III

Characterization of a Complex Philadelphia Translocation (1p−;9q+;22q−) by Gene Mapping

A. H. M. Geurts van Kessel[1], A. J. van Agthoven[1], P. G. de Groot[2], and A. Hagemeijer[1]

[1] Department of Cell Biology and Genetics, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands
[2] Laboratory of Biochemistry, BCP Jansen Institute, University of Amsterdam, Amsterdam, The Netherlands

Summary. Human-Chinese hamster somatic cell hybrids were obtained using circulating leucocytes from a chronic myeloid leukaemia (CML) patient carrying a complex Philadelphia (Ph^1) translocation (1p−; 9q+; 22q−). Hybrid clones which showed segregation of the translocation chromosomes were studied. The chromosome 22 markers ACO2, ARSA, and NAGA segregated with the 1p− derivative; and the chromosome 1 markers UMPK, PGD, and ENO1 segregated with the 9q+ derivative. Hence, molecular evidence has been obtained for the translocation of the distal part of 22q to chromosome 1 and for the translocation of the distal part of 1p to chromosome 9. No conclusions could be drawn either about translocation of chromosome 9 material or about a possible difference in breakpoint in chromosome 22 when compared with six cases of 9;22 translocations similarly studied and previously reported. In addition, a more precise mapping of PGM1 was obtained, the gene being proximal to UMPK and the breakpoint in 1p32.

Introduction

About 85% of all the patients suffering from chronic myeloid leukaemia (CML) carry the Philadelphia chromosome (Ph^1) in their bone marrow and dividing peripheral leucocytes (Rowley 1980). In most cases Ph^1 results from a translocation between chromosomes 22 and 9 but several variants have been described (for reviews see Mitelman and Levan 1978; Rowley 1980). Chromosome 22 is always involved. Whether the breakpoint in chromosome 22 is identical in all cases and whether Ph^1 translocations are reciprocal are still matters of dispute (Rowley 1980). Recently, we described an investigation in which gene mapping methods were applied to these problems (Geurts van Kessel et al. to be published). The segregation of marker genes, known to be located in the regions of breakage, was studied in somatic cell hybrids derived from fusion of rodent cells with leucocytes from six CML patients carrying the 9;22 translocation.

We report here the results of an investigation in which these methods were applied to a complex Ph^1 translocation (1p−;9q+; 22q−). The cytogenetic picture, suggesting translocation of chromosome 22 material to chromosome 1 and that of chromosome 1 material to chromosome 9 was confirmed and the breakpoints were defined relative to the enzyme markers studied.

Offprint requests to: A. H. M. Geurts van Kessel

Materials and Methods

Production of Hybrid Cell Lines

Interspecific hybrid cell lines were produced by fusion of thymidine kinase deficient (TK^-) Chinese hamster a3 or a23 cells with leukocytes from a chronic myeloid leukaemia (CML) patient carrying a complex Philadelphia (Ph^1) translocation (1p−;9q+;22q−). Blood of this patient was obtained during the chronic phase of the disease and specific therapy was interrupted one week before sampling. For a more complete description of this patient see Hagemeijer et al. (1980). The Sendai virus mediated fusion experiments were carried out under standard conditions (Harris and Watkins 1965). Hybrid clones were selected in F10 medium (Ham 1965) supplemented with hypoxanthine (10^{-4} M), aminopterin (10^{-7} M), and thymidine (1.6×10^{-5} M) (Littlefield 1964), glutamine (2 mM), 10% foetal or newborn calf serum (Flow), penicillin (100 U/ml), and streptomycin (100 µg/ml).

Cytogenetic and Enzyme Analysis

Air dried chromosome preparations were analysed using R-banding. At least 16 metaphases of each hybrid clone were studied. Cell lysates, derived from the same passage, were used for enzyme marker analysis. The hybrid and parental cells were screened for the following isoenzymes by cellulose acetate (cellogel) electrophoresis (Meera Khan 1971): the chromosome 1 markers fumarate hydratase (FH; E.C. 4.2.1.2), phosphoglucomutase-1 (PGM1; E.C.2.7.5.1), 6-phosphogluconate dehydrogenase (PGD; E.C. 1.1.1.44), enolase-1 (ENO1; E.C. 4.2.1.11), and uridine monophosphate kinase (UMPK; E.C. 2.7.4.4); the chromosome 9 markers soluble aconitase (ACO1; E.C. 4.2.1.3) and adenylate kinase-1 (AK1; 2.7.4.3); and the chromosome 22 marker mitochondrial aconitase (ACO2; E.C. 4.2.1.3). For the determination of the chromosome 22 marker N-acetyl-α-D-galactosaminidase (NAGA; E.C. 3.2.1.49) an immunochemical method was applied (de Groot et al. 1978) and for the chromosome 22 marker arylsulfatase-A (ARSA; E.C. 3.1.6.1) polyacrylamide gel electrophoresis was used (Hors-Cayla et al. 1979).

Results and Discussion

Figure 1 represents the karyogram of a bone marrow cell of the patient studied, showing the complex Ph^1 translocation (1p−; 9q+;22q−). The breakpoints in chromosome 9 and 22 appeared to be similar to those usually found in Ph^1 positive CML, namely in bands q34 and q11, respectively. The breakpoint in chromosome 1 was in band p32.

Primary and secondary hybrid cell lines were obtained after fusion of peripheral leucocytes from this patient with a3 Chinese hamster cells (11 clones) or a23 Chinese hamster cells (10 clones). Detailed chromosome and enzyme data concerning only chromosomes 1, 9, 22, and the derivatives 1p−, 9q+, and 22q−

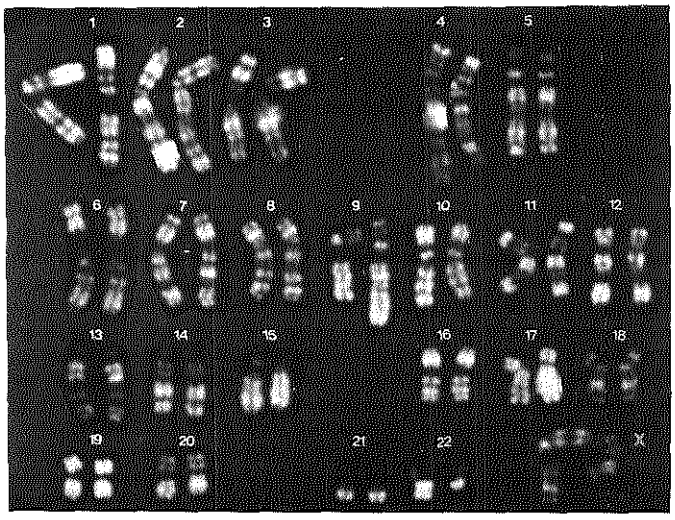

Fig. 1. Karyogram of a bone marrow cell showing the complex Ph^1 translocation: (1p−; 9q+; 22q−)

Table 1. Presence of human chromosomes 1, 9, and 22; their translocation products; and the relevant enzyme markers of human origin in 21 hybrid clones

No. hybrid cell lines	Chromosomes						Enzyme markers									
							Chromosome 1					Chromosome 9		Chromosome 22		
	1	1p−	9	9q+	22	22q−	ENO1	PGD	UMPK	PGM1	FH	ACO1	AK1	ACO2	NAGA	ARSA
1	+		+		+		+	+	+	+	+	+	+	+	+	+
1	+	+			+		+	+	+	+	+	−	−	+	−	+
1	+	+	+			+	+	+	+	+	+	+	+	+	+	+
1	+	+		+		+	+	+	+	+	+	+	+	+	ND	ND
1[a]	+			+			+	+	+	+	+	+	+	−	−	ND
2		+	+				−	−	−	+	+	+	+	+	+	+
1		+			+		−	−	−	+	+	−	−	+	+	+
1		+					−	−	−	+	+	+	−	+	−	+
1		+		+[b]	+[b]	+	+	−	−	+	+	−	−	+	+	+
1			+	+	+	+	+	+	+	−	−	+	+	+	+	+
2			+	+	+		+	+	+	−	−	+	+	+	+	+
1[a]				+	+		+	−	+	−	−	−	+	+	+	ND
1			+	+		+	+	−	+	−	−	+	−	−	−	−
2			+		+	+	−	−	−	−	−	−	+	+	+	+
1			+		+		−	−	−	−	−	+	+	+	+	ND
1			+				−	−	−	−	−	+	−	−	−	ND
1					+		−	−	−	−	−	−	−	+	+	+

[a] Secondary clones
[b] Chromosome present in a low frequency (1/32)
ND = not determined

are shown in Table 1. A summary of the informative clones in which the presence or absence of enzyme markers are evaluated in the absence of the normal chromosomes which carry these markers, is given in Table 2.

It appeared that the chromosome 1 markers ENO1, PGD, and UMPK segregated with the 9q+ derivative (three clones) and not with 1p− (five clones) or 22q− (two clones). In one line in which the frequency of 9q+ was low (1/32 metaphases), ENO1 was expressed but not PGD and UMPK. The other two chromosome 1 markers tested, FH and PGM1, segregated with the 1p− derivative (five clones) and not with either 9q+ (three clones), 22q− (two clones), or both 9q+ and 22q− (one clone).

The chromosome 9 markers ACO1 and AK1 segregated with the 9q+ derivative (two clones) and not with the 1p− derivative

Table 2. Summary of hybrid clones containing the translocation chromosomes 1p−, 9q+, and 22q− and the relevant enzyme markers of human origin

Chromosomes	Enzyme markers																			
	Chromosome 1[a]										Chromosome 9[b]				Chromosome 22[c]					
	ENO1		PGD		UMPK		PGM1		FH		ACO1		AK1		ACO2		NAGA		ARSA	
	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−
1p− +	1	5	0	6	0	6	6	0	6	0	3	3	1	5	6	0	5	0	5	0
−	5	5	5	5	5	5	0	10	0	10	2	1	2	1	0	3	0	3	0	1
9q+ +	6	0	5	1	5	1	1	5	1	5	4	0	3	1	1	2	0	2	0	1
−	0	10	0	10	0	10	5	5	5	5	1	4	0	5	5	1	5	1	5	0
22q− +	3	2	2	3	2	3	1	4	1	4	2	0	1	1	2	1	1	1	1	1
−	3	8	3	8	3	8	5	6	5	6	3	4	2	5	4	2	4	2	4	0

[a] In absence of the normal chromosome 1
[b] In absence of the normal chromosome 9
[c] In absence of the normal chromosome 22
+ = present
− = absent

(four clones). Two clones were exceptional: one expressed ACO1 in the presence of 1p− only and another did not express AK1 in the presence of 9q+. The latter is probably due to the low frequency of the 9q+ chromosome while the former could be due to a secondary chromosomal change involving 9p.

The chromosome 22 markers ACO2, NAGA, and ARSA segregated with the 1p− derivative (three clones) and not with 9q+ (one clone) or both 9q+ and 22q− (one clone). One clone containing 1p− and 22 did not express NAGA, which remains unexplained. None of the enzyme markers tested segregated concordantly with the 22q− derivative.

This segregation is in agreement with the cytogenetic observation that the breakpoint in chromosome 22 is in q11, in chromosome 1 in p32, and in chromosome 9 in q34, and with the regional localization of these markers on chromosome 22 (Geurts van Kessel et al. 1980; Junien personal communication), on chromosome 9 (Ferguson-Smith et al. 1976; Mohandas et al. 1979), and on chromosome 1 (Burgerhout et al. 1977; Cook and Burgerhout 1978). The regional assignment of PGM1 has so far yielded three conflicting shortest regions of overlap (SRO): 1p33→1p34, 1p32 and 1p22↑→1p31↑ (Cook and Burgerhout 1978). Our data indicate that PGM1 is proximal to both the breakpoint in 1p32 and to UMPK, a marker for the latter band.

In six previously studied CML patients carrying the 9;22 translocation (Geurts van Kessel et al. to be published), the chromosome 22 markers ACO2, NAGA, and ARSA and the chromosome 9 markers ACO1 and AK1 segregated consistently with the 9q− derivative. In this study the chromosome 22 markers were translocated to 1p in a similar manner as to 9q in the classical cases. The translocation of the terminal part of 1p occurred onto chromosome 9 distal to the AK1 locus again as observed for the chromosome 22 fragment in the 9;22 translocations. No evidence was obtained for translocation of chromosome 9 material to either chromosome 22 or 1. Resolving the questions of whether the breakpoint in chromosome 22 is identical in all cases and of whether Ph[1] translocations are reciprocal with current techniques, must either await the localization of new markers in the regions of breakage or of patient material with breakpoints distal to ACO2 on chromosome 22 or proximal to AK1 on chromosome 9. Verma and Dosik (1977) have described one other (1;9;22) variant Ph[1] translocation. The breakpoints reported were in 1q32, 9q22, and 22q12, with loss of chromosome 9 material distal to q22. Application of the techniques used here to such a case would be of particular interest.

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APPENDIX PAPER IV

Clonal Origin of the Philadelphia Translocation in Chronic Myeloid Leukemia Demonstrated in Somatic Cell Hybrids Using an Adenylate Kinase-1 Polymorphism

A. H. M. Geurts van Kessel, A. J. van Agthoven, and A. Hagemeijer

ABSTRACT: *Hybrid cell lines were derived from fusion of rodent cells with leukocytes from a t(9q+; 22q−)-positive chronic myeloid leukemia (CML) patient carrying a chromosome No. 9-linked adenylate kinase-1 (AK1) polymorphism (AK1 1–2). The AK1*2 allele was consistently expressed when 9q+ was present, whereas the AK1*1-coded isozyme was formed when the normal chromosome No. 9 was present. These results provide additional data confirming the clonal origin of the Ph¹ translocation in CML.*

INTRODUCTION

Evidence for the clonal origin of chronic myeloid leukemia (CML) was first obtained by Fialkow et al. [1] using the X-linked enzyme glucose-6-phosphate dehydrogenase (G6PD). They demonstrated a single enzyme pattern (G6PD*A) in the Philadelphia chromosome (Ph¹)-positive hemopoietic cell population, whereas in the skin fibroblasts both A- and B-type G6PD were present. Since then, confirmatory data have been obtained by several investigators using the same enzyme polymorphism. Further evidence for the clonal origin of CML was obtained by the investigation of two patients with mosaic 46,XY/47,XXY Klinefelter's syndrome [2, 3], in which only cells with the 46,XY karyotype showed the Ph¹ chromosome, and by the investigation of patients that showed a polymorphism for either satellites of chromosome No. 22 or secondary constrictions in chromosome No. 9 [4–7]. The investigation reported here deals with a CML patient carrying the Ph¹ abnormality, t(9q+;22q−), and a chromosome No. 9-linked adenylate kinase-1 (AK1, EC 2.7.4.3) polymorphism (AK1 1–2). This heterozygote patient provided us with the opportunity to test further the clonal origin of the Ph¹ translocation by gene-mapping analysis. Hybrid cell lines, obtained after fusion of the leukemic leukocytes with rodent cells, were assayed simultaneously for the presence of chromosome No. 9, 9q+, and 22q− material and for the AK1 phenotypes 1 and 2 [8]. Soluble aconitase (ACO1, EC 4.2.1.3) was used as an independent chromosome No. 9 marker [9].

From the Department of Cell Biology and Genetics, Erasmus University, Rotterdam.

Address requests for reprints to A. H. M. Geurts van Kessel, Department of Cell Biology and Genetics, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.

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MATERIAL AND METHODS

Leukocytes, derived from peripheral heparinized blood of a CML patient, were fused with hypoxanthine phosphoribosyltransferase-deficient ($HPRT^-$) E36 Chinese hamster cells or with thymidine kinase-deficient (TK^-) 3T3 mouse cells using Sendai virus. Specific therapy of the patient was interrupted 1 week before fusion. Hybrid cell lines were isolated in hypoxanthine–aminopterin–thymidine (HAT) medium as described elsewhere [10], and care was taken that each clone originated from a single fusion event. For the analysis of AK1 and ACO1 enzymes, cellulose acetate gel (Cellogel) electrophoresis was used [11]. Cell lysates were prepared using a lysis buffer and a sonication procedure.

Simultaneously, chromosome studies were done on air-dried preparations using R-banding with acridine orange after heat denaturation. At least 16 metaphases of each hybrid cell line were analyzed.

RESULTS AND DISCUSSION

All 39 bone marrow metaphases of the patient studied at the time of the fusion experiment showed the presence of the CML-specific t(9q+;22q−), and in 28 of these cells an i(17q) was also observed, resulting in the following karyotype: 46,XX,t(9q+; 22q−) (30%)/46,XX,t(9q+;22q−),i(17q) (70%).

Fourteen hybrid cell lines segregating chromosome No. 9, 9q+, and 22q− were assayed for AK1 and ACO1 enzyme activity (Table 1, Fig. 1). The presence of at least one of the derivative chromosomes of the translocation in the hybrid cell lines confirmed that indeed a leukemic cell had been fused with a rodent cell and not an eventual lymphocyte present at a low percentage in the blood sample used. ACO1 was expressed in all clones containing chromosome No. 9 material. Two hybrids containing the normal chromosome No. 9 and the 22q− derivative expressed AK1*1. Five hybrids that had retained both No. 9 and 9q+ (and 22q− in three cases) showed AK1*1 and AK1*2 activity, whereas six clones containing 9q+ (and 22q− in two cases) expressed AK1*2. The segregation of AK1 with 9q+ (and not 22q−) is consistent with earlier published results [10] from six other CML patients studied similarly.

Since each hybrid cell line resulted from the fusion of one CML cell with a rodent cell, this study reflects an analysis of AK1 isozyme patterns of single cells within the original leukemic cell population. All CML cells studied this way showed the same pattern, the gene coding for AK1*1 being located on the unaffected chromosome No. 9 and the gene coding for AK1*2 being on the 9q+ deriv-

TABLE 1 Presence or absence of human chromosomes No. 9, 9q+, and 22q− and AK1 or ACO1 enzyme activity in human CML × rodent hybrid cell lines

No. of hybrid cell lines	Chromosomes			Enzymes		
	9	9q+	22q−	AK1*1	AK1*2	ACO1
2	+	−	+	+	−	+
2	+	+	−	+	+	+
3	+	+	+	+	+	+
4	−	+	−	−	+	+
2	−	+	+	−	+	+
1	−	−	−	−	−	−

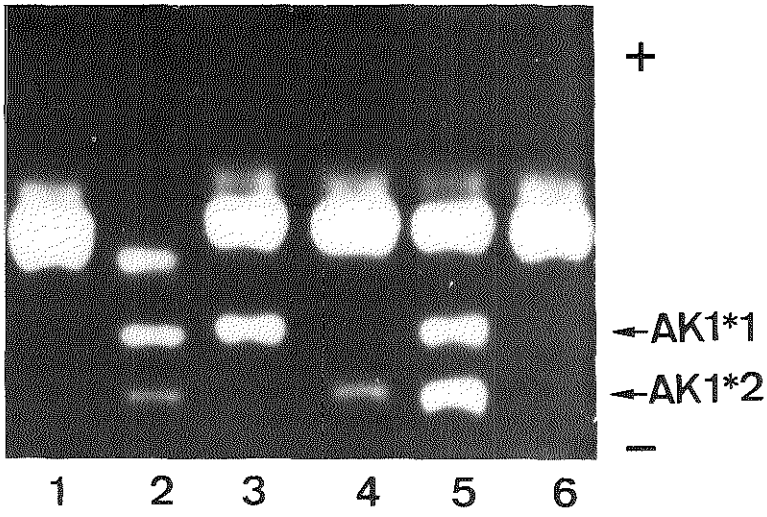

Fig. 1. Electrophoretic patterns of adenylate kinase (AK) in the parental Chinese hamster E36 cells (channel 1), in human CML cells from a AK1 1-2 heterozygote (channel 2), and in four hybrid cell lines expressing AK1*1 (channel 3), AK1*2 (channel 4), both AK1*1 and AK1*2 (channel 5), and no human AK1 activity (channel 6). Electrophoresis was performed according to van Someren et al. [12]. Zones of AK activity are seen as white bands against a dark background [8]. The most anodal band in channel 2 represents human AK2 activity. The AK2 locus has been assigned to chromosome No. 1 [13].

ative. No clones were found in which either AK1*1 segregated with 9q+ or AK1*2 segregated with the normal chromosome No. 9. The latter would be expected if the translocation had occurred simultaneously in more than one bone marrow cell, provided that the two chromosome No. 9 homologs were randomly involved. Therefore, these results are compatible with the hypothesis of a single-cell origin of the Ph^1 translocation and further substantiate the evidence for a clonal origin of CML [1–7].

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APPENDIX PAPER V

A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukaemia

Annelies de Klein*, Ad Geurts van Kessel*, Gerard Grosveld*, Claus R. Bartram*, Anne Hagemeijer*, Dirk Bootsma*, Nigel K. Spurr†, Nora Heisterkamp‡, John Groffen‡ & John R. Stephenson‡

* Department of Cell Biology and Genetics, Erasmus University, PO Box 1738 – 3000 DR Rotterdam, The Netherlands
† Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, UK
‡ Laboratory of Viral Carcinogenesis, National Cancer Institute-FCRF, Frederick, Maryland 21701, USA

The transforming genes of oncogenic retroviruses are homologous to a group of evolutionary conserved cellular *onc* genes[1]. The human cellular homologue (c-*abl*) of the transforming sequence of Abelson murine leukaemia virus (A-MuLV) was recently shown[2] to be located on chromosome 9. The long arm of this chromosome is involved in a specific translocation with chromosome 22, the Philadelphia translocation (Ph[1]), t(9; 22) (q34, q11), which occurs in patients with chronic myelocytic leukaemia (CML)[3–5]. Here we investigate whether the c-*abl* gene is included in this translocation. Using c-*abl* and v-*abl* hybridization probes on blots of somatic cell hybrids, positive hybridization is found when the 22q$^-$ (the Philadelphia chromosome), and not the 9q$^+$ derivative of the translocation, is present in the cell hybrids. From this we conclude that in CML, c-*abl* sequences are translocated from chromosome 9 to chromosome 22q$^-$. This finding is a direct demonstration of a reciprocal exchange between the two chromosomes[6] and suggests a role for the c-*abl* gene in the generation of CML.

The human c-*abl* sequences represent a cellular homologue of the transforming component of A-MuLV. This retrovirus is a recombinant between Moloney MuLV and mouse cellular c-*abl* sequences[7] and induces lymphoid tumours on *in vivo* inoculation of the mouse[8,9]. The major A-MuLV translational product has been identified as a poly-protein, P120$^{gag-abl}$, consisting of amino-terminal structural proteins encoded by the M-MuLV *gag* gene, linked to an acquired cellular sequence encoded carboxy-terminal component[10,11]. This protein is one of several virus-encoded transforming proteins with tyrosine-specific protein kinase activity[12–15]. Similar oncogenic sequences of Harvey and Kirsten sarcoma virus are homologous to transforming sequences (c-Ha-*ras*, c-Ka-*ras*) isolated from human bladder and lung carcinoma cell lines[16–18]. Both these sequences induce transformation of mouse NIH 3T3 cells after transfection, establishing that the human genes have potential transforming activity. Recently, the human c-*abl* gene has been cloned in cosmids[19]. Using v-*abl* DNA as a probe, several clones containing overlapping sequences representing the entire c-*abl* gene were isolated from a human lung carcinoma cosmid library. The restriction enzyme map of the human v-*abl* cellular homologue, presented in Fig. 1, identifies areas of the gene which hybridize to v-*abl* sequences. The gene is distributed over a region of 40 kilobases (kb) of human DNA and contains multiple intervening sequences. On transfection of Rat-2 cells with the c-*abl* cosmids, no transforming activity was detected, not unexpectedly, as none of the cosmid clones tested contained the entire c-*abl* gene[19].

By Southern blot analysis of a series of somatic cell hybrids, the human c-*abl* gene has been localized on chromosome 9[2]. This finding is of interest because of the involvement of the long arm of chromosome 22 (band 22q11) in a specific translocation with the long arm of chromosome 9 (band 9q34), the Philadelphia translocation (Ph[1]), occurring in human CML[3,4]. The abnormal chromosomes are designated 9q$^+$ and 22q$^-$; of these, the 22q$^-$ chromosome is observed in 92% of CML cases[5]. We investigated the chromosomal location of the human c-*abl* gene in cases of CML, where the Philadelphia translocation is present. Southern blot analyses with c-*abl* and v-*abl* probes were performed on *Eco*RI-digested DNAs from somatic cell hybrids segregating the 9q$^+$ and 22q$^-$ chromosomes.

The cell hybrids used here contain a full complement of mouse or Chinese hamster chromosomes and a limited number of human chromosomes. The hybrid cell lines have been obtained by fusion of cells from mouse (Pg 19 and WEHI-3B) or Chinese hamster (E36 and a3) origin with leukocytes from different CML patients and from a normal donor[6,20]. The human chromosome content of these cells is summarized in Table 1 and is based on chromosome analysis. In addition, the hybrid cells were tested for the expression of human adenylate kinase-1 (AK1) enzyme activity, a marker localized proximal in band

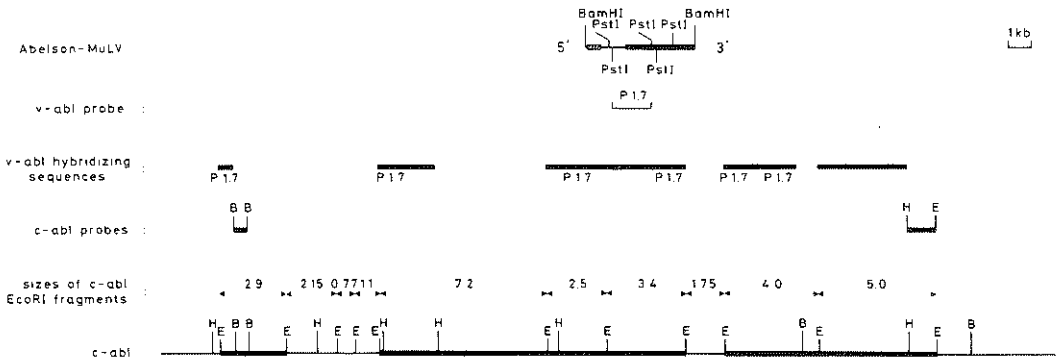

Fig. 1 Restriction enzyme map of the human c-*abl* region[19]. The upper line of the figure shows the *Bam*HI subclone of A-MuLV; the hatched box presents the long terminal repeat, the solid bar the acquired cellular sequences. Directly beneath the A-MuLV genome, a subgenomic *Pst* 1.7-kb fragment, used as a probe in this study, is shown. Human c-*abl* DNA restriction fragments homologous to v-*abl* sequences are indicated as black boxes and those that show homology to the 1.7-kb *Pst* v-*abl* fragments are designated by P 1.7. The third line shows the human c-*abl* 0.6-kb *Bam*HI and 2.2-kb *Hin*dIII–*Eco*RI probes, which hybridize to 5' and 3' c-*abl* *Eco*RI fragments, respectively. The sizes of all *Eco*RI c-*abl* fragments are indicated on the fourth line. The bottom line represents the restriction enzyme map of the human c-*abl* gene. Restriction enzymes include *Bam*HI (B), *Hin*dIII (H) and *Eco*RI (E). A more detailed characterization of the human c-*abl* locus will be published elsewhere[19].

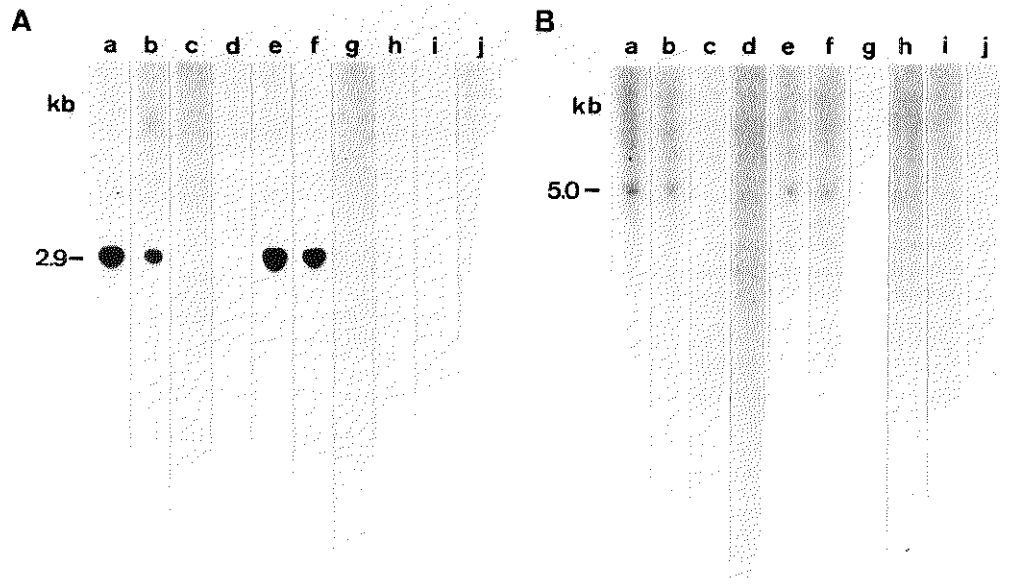

Fig. 2 Localization of human c-*abl* sequences on the Philadelphia chromosome, using hybrid cell lines and human c-*abl* probes. *A*, detection of the human 5′ end 2.9-kb *Eco*RI c-*abl* fragment in DNA from *a*, human placenta; *b*, 10CB-23B (chromosome 9); *c*, PgMe-25NU (chromosome 22q); *d*, 14CB-21A (chromosome $9q^+$); *e*, 1CN-17aNU (chromosome $22q^-$); *f*, WESP-2A (chromosome $22q^-$); *g*, mouse Pg19; *h*, Chinese hamster E36; *i*, mouse WEHI-3B; *j*, Chinese hamster a3. *B*, detection of the 3′-end 5.0-kb *Eco*RI c-*abl* fragment in DNAs as indicated in *A* (*a–j*). The derivations of all these cell lines and their complements of human chromosomes are summarized in Table 1.
Methods: All cell lines used in this experiment were grown in large batches (10^7–10^8 cells) and DNA was prepared as described by Jeffreys and Flavell[26]. *Eco*RI-restricted DNAs (10 μg per lane) from human placenta, hybrid cell lines, mouse and Chinese hamster fusion partners were electrophoresed on 0.7% agarose gels. *Hin*dIII and *Hin*dIII–*Eco*RI-digested λ DNAs were included as molecular weight markers (not shown). After blotting to nitrocellulose, the filters were hybridized to the 0.6-kb *Bam*HI c-*abl* (*A*) or 1.1-kb *Hin*dIII–*Eco*RI c-*abl* (*B*) restriction fragments described in Fig. 1. Hybridization and washing procedures (to 0.1 × SSC at 65 °C) were carried out according to the method of Bernards and Flavell[23].

9q34 (ref. 21). This latter test was necessary to exclude the possibility of hidden (broken or rearranged) chromosome 9 fragments in the 22 and $22q^-$ cell lines.

Detection of the human c-*abl* restriction fragments in hybrid cell DNAs is often inconclusive using v-*abl* probes, because the human sequences are present in submolar amounts (20–50%) and also because many of the human c-*abl* restriction fragments electrophorese in close proximity with strongly hybridizing mouse or Chinese hamster fragments. To obtain molecular probes with specificity for human c-*abl* sequences, two restriction fragments were isolated from subclones of c-*abl*-containing cosmids, with homology to the presumptive 5′ and 3′ proximal *Eco*RI fragments of c-*abl*. These are 2.9 and 5.0 kb, respectively, in size (Fig. 1). After hybridization and washing to high stringency (0.1 × SSC)[12], the 5′-terminal 0.6-kb *Bam*HI probe and the 3′-terminal 1.1-kb *Hin*dIII–*Eco*RI probe cross-hybridize to a very low extent with mouse or hamster c-*abl* sequences. Figure 2*A* shows an example of a hybridization experiment with the 0.6-kb *Bam*HI probe. This Southern blot illustrates hybridization of *Eco*RI-restricted DNAs of hybrid cell lines containing chromosomes 22, 9, $9q^-$ or $22q^-$. As controls, hybridization of the probe with human placenta DNA and DNA from the mouse and Chinese hamster fusion partners is shown. It is clear that the 2.9-kb *Eco*RI fragment, detected in human placenta DNA, is also present in the lanes containing DNA from the hybrid cell lines 10 CB-23B (chromosome 9), 1CB–17a NU and WESP-2A (both containing chromosome $22q^-$). The band is not detected in lanes containing DNA from

Table 1 Human chromosome content of human–mouse and human–Chinese hamster somatic cell hybrids

Hybrid	Human chromosomes																									Human isoenzyme AKl	Ref.
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y	$9q^+$	$22q^-$		
PgMe-25NU	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	26
10CB-23B	–	–	–	–	+	–	–	–	+	–	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	+	6
14CB-21A	–	–	–	+	–	–	+	+	–	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–	+	–	+	20
1CB-17a NU	–	–	–	–	–	–	–	+	–	–	–	+	–	+	+	–	–	–	+	–	+	–	–	–	–	+	–	6
WESP-2A	–	–	–	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	+	–	–	+	–	×

The origin and details of the initial characterization of the somatic cell hybrids are described in the references listed in the last column. PgMe-25NU and WESP-2A are hybrids obtained from fusions with mouse Pg19 and WEHI-3B cells, respectively. Chinese hamster cell line E36 was used to produce hybrid clones 10CB-23B and 14CB-21A, while Chinese hamster cell line a3 was used to obtain 1CB-17aNU. Chromosome analysis was done using reverse (R) banding with acridine orange, after heat denaturation. At least 16 metaphases were analysed per cell line. The presence of human AKl activity was assayed by cellulose acetate (Cellogel) electrophoresis[27]. This test is inconclusive for the WESP-2A cell line (×), because the expression of AKl was found to be repressed in hybrids derived from fusion with WEHI-3B cells (A.H.M., G.v.K., unpublished results). Chromosome and isoenzyme analyses[22] were performed on the same batches of hybrid cells that were used for the isolation of DNA.

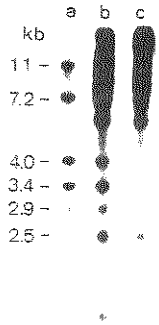

Fig. 3 Localization of human c-*abl* sequences on the Philadelphia chromosome, using a 22q⁻ somatic cell hybrid and a v-*abl* probe. *Eco*RI-digested DNAs (10 μg) from human placenta (*a*), hybrid WESP-2A (*b*) and mouse WEHI-3B cells (*c*) were hybridized with the 1.7-kb *Pst* v-*abl* fragment (Fig. 1), as described in Fig. 2 legend. After hybridization, the filters were washed to 1 × SSC at 65 °C. Molecular weights of human c-*abl* fragments were deduced from co-electrophoresed *Hin*dIII and *Hin*dIII–*Eco*RI-digested λ DNA markers.

PgMe-25Nu (chromosome 22), 14CB-21A (chromosome 9q⁺), Pg19 and WEHI-3B (mouse controls) or E36 and a3 (Chinese hamster controls). Analogous results are obtained when the same *Eco*RI-digested DNAs are hybridized to the 3'-terminal 1.1-kb *Hin*dIII–*Eco*RI probe (Fig. 2*B*). The 5.0-kb *Eco*RI fragment is detected only in DNA from human placenta and from hybrid cell lines containing chromosome 22q⁻ or 9.

The above results show that both the 5' and 3' ends of the c-*abl* gene are translocated to chromosome 22q⁻. Because all other c-*abl* *Eco*RI fragments, which hybridize to v-*abl* sequences, are flanked by the 2.9-kb and 5.0-kb *Eco*RI fragments, it seems highly probable that these fragments are also included in the translocation to the Philadelphia chromosome. To test this possibility directly, hybridization was performed using a 1.7-kb *Pst* v-*abl* probe (Fig. 1). Because of the problems with v-*abl* probes indicated above, only WESP-2A, the hybrid containing the most 22q⁻ sequences (50% of the molar amount), was tested. As shown in Fig. 3, the viral probe detects human *Eco*RI c-*abl* fragments of 11, 7.2, 4.0, 3.4, 2.9 and 2.5 kb (weakly). Of these fragments, the 11-kb band has been shown to map outside the main human c-*abl* locus[19] and will not be considered here. The human 2.9-, 3.4-, and 4.0-kb c-*abl* fragments are readily detected in the WESP-2A DNA. In contrast, the 7.2-kb *Eco*RI fragment can only be seen in a short exposure of this filter (not shown), due to spill-over of radiation from strongly hybridizing mouse c-*abl* fragments in this area. The 2.5-kb *Eco*RI human c-*abl* fragment co-migrates with a mouse fragment of similar size and thus cannot be identified in this analysis.

The hybrid cell lines containing the 9q⁺ and 22q⁻ chromosomes examined in the present study, were obtained from fusion experiments with CML cells from three different individuals. Therefore, we conclude that in the Philadelphia translocation a fragment of chromosome 9 is translocated to chromosome 22q⁻ and that this fragment includes the human c-*abl* sequences. This finding establishes that the translocation is reciprocal, a general assumption which is now demonstrated unequivocally. Moreover, the data map the human c-*abl* sequences distal to AK1 (not translocated to 22q⁻, 6, 20) on chromosome 9. The most interesting aspect is that it raises the possibility of involvement of the human c-*abl* gene in the generation of CML.

In principle, the chromosomal translocation associated with CML could lead to elevated levels of c-*abl* expression which, by analogy to the c-Ha-*ras* gene in bladder carcinoma, would induce malignant transformation[24]. Elevated levels of c-*abl* expression could be the result of coupling of the gene to an enhancer sequence present on chromosome 22 or, alternatively, the gene could be linked to a strong promoter of another gene. To test these possibilities, we have initiated studies to clone the c-*abl* gene from the 22q⁻ chromosome using WESP-2A DNA and a cosmid vector system. Finally, it is of interest that in some CML patients variant Ph¹ translocations are observed, in which the participation of chromosome 9 cannot be detected by classical cytogenetic analysis[3,5]. In another group of CML patients the Ph¹ translocation appears to be completely absent[25]. We are now investigating whether the c-*abl* gene is translocated to chromosome 22 in these cases also.

These studies were initiated as part of a collaborative effort with W. F. Bodmer; his helpful discussions throughout the work are greatly appreciated. We also thank F. Grosveld for important contributions to this study, R. A. Flavell for useful suggestions, Ton van Agthoven, Gail T. Blennerhassett and Pam Hansen for technical assistance and Ad Konings and Rita Boucke for help with the preparation of the manuscript. The work was supported by the Netherlands Cancer Society (Koningin Wilhelmina Fonds) and under NCI PHS contract NOI-CO-75380. C.R.B. is a recipient of a fellowship from the Deutsche Forschungsgemeinschaft.

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APPENDIX PAPER VI

c-*sis* IS TRANSLOCATED FROM CHROMOSOME 22 TO CHROMOSOME 9 IN CHRONIC MYELOCYTIC LEUKEMIA*

BY JOHN GROFFEN, NORA HEISTERKAMP, JOHN R. STEPHENSON, AD GEURTS VAN KESSEL, ANNELIES DE KLEIN, GERARD GROSVELD, AND DIRK BOOTSMA

From the Laboratory of Viral Carcinogenesis, National Cancer Institute, Frederick, Maryland 21701; and the Department of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands

Several relatively specific chromosomal translocations are known to be associated with particular human cancers (1–3). One of these, the Philadelphia translocation, t(9;22) (q34;q11) is observed in over 90% of chronic myelocytic leukemias (CML)[1] (3). Translocation of the q11 to qter segment of chromosome 22 to chromosome 9 results in a deleted form of chromosome 22, referred to as the Philadelphia (Ph') chromosome (4). Recently, we have localized a human oncogene, c-*abl*, on chromosome 9 (q34 to qter) (5) and demonstrated its translocation to chromosome 22q− (the Ph' chromosome) in CML (6). Because of the small size of the segment of chromosome 9 that translocates to chromosome 22 (6) and the localization of immunoglobulin λ light chain sequences on chromosome 22 (7), c-*abl* appears to map in close proximity to λ sequences in the Ph' chromosome.

Another acute transforming retrovirus, the Simian sarcoma virus (SSV), is a genetic recombinant between a nontransforming retrovirus and cellular sequences of woolly monkey origin (8, 9). The SSV transforming gene, v-*sis*, and its human cellular homologue, c-*sis*, have been molecularly cloned (8–10), and c-*sis* has been localized on the q arm of chromosome 22 (11, 12). In the present study, we report the localization of c-*sis* on the q11 to qter segment of chromosome 22 and its translocation from chromosome 22 to chromosome 9 in CML.

Materials and Methods

Cells. Cell lines, propagated in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, included NIH/3T3 mouse cells and a human cell line, A673 (5). Somatic cell hybrids containing full complements of either mouse or Chinese hamster chromosomes and a limited number of human chromosomes were derived by fusion of either mouse or Chinese hamster cells with leukocytes from different CML patients or from normal donors (Table I); details concerning their origin and initial characterization have been previously reported (13, 14).

Preparation of a Human c-sis Probe (c-sis $B_{1.7}$). A cosmid clone with a cellular insert of ~30 kb containing v-*sis*-homologous sequences was isolated from a library of human lung

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[1] *Abbreviations used in this paper:* CML, chronic myelocytic leukemia; Ph', Philadelphia chromosome; SSV, Simian sarcoma virus.

carcinoma DNA (15) using, as a probe, a 1.2 kb PstI v-*sis* restriction fragment in pBR322 (8), generously provided by K. Robbins and S. A. Aaronson. Isolation of a 1.7 kb BamHI v-*sis*-homologous restriction fragment from the cosmid clone was performed according to previously described methods (15).

Molecular Hybridization. Restriction enzymes were purchased from New England Biolabs, Beverly, MA and Bethesda Research Laboratories, Rockville, MD and were used according to the suppliers' specifications. DNA were digested with restriction enzymes, subjected to electrophoresis through 0.75% agarose gels, and transferred to nitrocellulose essentially as described by Southern (16). Nick translation of probes and filter hybridization were as described (15). Specific activity of the probes was $2–5 \times 10^8$ cpm/μg. After hybridization, filters were washed under high stringency conditions (10% standard saline citrate, 65°C) and exposed to XAR-2 film (Eastman Kodak Co., Rochester, NY) for up to 5 d at −70°C with Dupont Lightning Plus intensifying screens (Dupont Instruments, Wilmington, DE).

Results

To prepare a probe suitable for identification of somatic cell hybrids containing human c-*sis* sequences, a previously described (15) cosmid library of human lung carcinoma DNA was screened for clones containing sequences homologous to the 1.2 kb PstI v-*sis* probe. As shown in Fig. 1, a single cosmid clone was obtained containing a 30 kb cellular insert with v-*sis*-homologous cellular sequences. By restriction endonuclease analysis this clone was shown to correspond to a previously described v-*sis*-homologous human sequence, designated c-*sis* (12). For generation of a c-*sis*-specific probe, a single 1.7 kb BamHI fragment, possessing strong homology to v-*sis* (c-*sis* $B_{1.7}$), was isolated from the cosmid cellular DNA insert.

Human and mouse control cellular DNA were digested with Sst-I and analyzed for homology to the above described c-*sis* $B_{1.7}$ probe. As shown in Fig. 1, a single mouse cellular restriction fragment of around 10.0 kb (lane B) is detected, while the only human c-*sis* $B_{1.7}$ cross-reactive Sst-I restriction fragment is 3.6 kb in length (lane C). The size of the latter restriction fragment corresponds to that predicted on the basis of the human c-*sis* restriction map shown in Fig. 1. Cellular DNA from a mouse × human somatic cell hybrid, PgMe-25NU, previously shown to have chromosome 22 as its only human component (14), contains the 3.6 kb human c-*sis* $B_{1.7}$ cross-reactive Sst-I restriction fragment (lane A), thus confirming the mapping of c-*sis* on chromosome 22. Localization of c-*sis* to the region of chromosome 22 (q11 to qter) which is translocated to chromosome 9 in CML, is established by the absence of c-*sis*-homologous sequences from hybrid WESP-2A (lane D), which contains chromosome 22q− (the Ph′ chromosome) but lacks detectable amounts of chromosomes 9, 22, or 9q+ (5).

To independently show the localization of c-*sis* on chromosome 22 (q11 to qter) and demonstrate its translocation to chromosome 9 in CML, a series of Chinese hamster × human somatic cell hybrids were analyzed for human c-*sis* sequences. As shown in Fig. 1, lanes E and F, the only c-*sis* $B_{1.7}$ cross-hybridizing Sst-I restriction fragment in Chinese hamster cellular DNA is around 2.7 kb in size and thus clearly resolved from the 3.6 kb human Sst-I fragment. Hybrid 1CB-17aNU, which contains chromosome 22q−, lacks detectable human c-*sis* sequences (lane F), while a second hybrid, 14CB-21A, containing chromosome 9q+ but not chromosome 9 or 22 (13), is positive for human c-*sis* sequences (lane

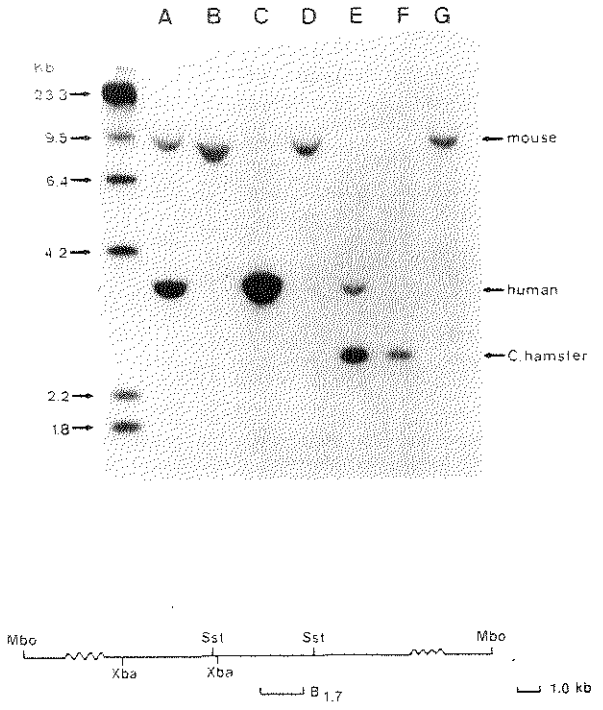

FIGURE 1. Localization of human c-*sis* on chromosome 22 (q11 to qter) and its translocation to chromosome 9 in CML. SstI-digested cellular DNA (10 μg/lane) were electrophoresed on 0.7% agarose gels, blotted to nitrocellulose, and hybridized to the c-*sis* $B_{1.7}$ probe shown in the lower portion of the figure. Cell lines analyzed are described in Table I and include PgMe25Nu (*A*), NIH/3T3 (*B*), A673 (*C*), WESP 2A (*D*), 14CB21A (*E*), 1CB17ANu (*F*). The positions of single mouse, human, and Chinese hamster c-*sis* $B_{1.7}$ homologous SstI restriction fragments are shown. Hind III-digested DNA, included as a molecular weight marker, is shown on the left side of the figure. In the lower portion of the figure, the restriction map of the 30 kb cellular insert from the v-*sis* cross-reactive cosmid clone of MboI-digested human lung carcinoma DNA is shown. MboI (Mbo) sites indicate the ends of the insert; the positions of XbaI (Xba) and SstI (Sst) restriction sites are shown for purposes of orientation of this clone with the more detailed previously published restriction maps of c-*sis* (10). The position of a single 3.6 kb human SstI restriction fragment overlapping with the 1.7 kb BamHI restriction fragment ($B_{1.7}$), used as a c-*sis*-specific probe for analysis of somatic cell hybrids, is also shown.

E). Finally, hybrid 10CB-23B, which contains chromosome 9 in the absence of detectable 22, 9q+, or 21q−, lacks human c-*sis* (Table I). As internal controls, each of the above hybrids were also analyzed for c-*abl*, a marker for the portion of chromosome 9 translocated to chromosome 22 in CML (6), and for AK1, which maps near the breakpoint but within the nontranslocated portion of chromosome 9 (Table I).

Discussion

Several of the human cellular homologues of viral oncogenes studied to date including c-*abl* (5, 6), c-*sis* (11, 12), c-*fes* (5, 17), and c-*mos* (18, 19) have been localized on human chromosomes frequently involved in translocations associated with specific human cancers. One of these, c-*myc*, is translocated from chromo-

TABLE I

Translocation of c-sis from Chromosome 22 to Chromosome 9 in Chronic Myelocytic Leukemia

Cell line	AK1	Human chromosomes				Oncogenes	
		9	22	9q+	22q−	*c-abl*	*c-sis*
Mouse NIH/3T3	NT	−	−	−	−	−	−
Human A673	NT	+	+	+	+	+	+
Mouse × human hybrid							
PgMe-25NU	−	−	+	−	−	−	+
WESP-2A	−	−	−	−	+	+	−
Chinese hamster × human hybrid							
10CB-23B	+	+	−	−	−	+	−
14CB-21A	+	−	−	+	−	−	+
1CB-17a NU	−	−	−	−	+	+	−

PgMe-25NU cells contain chromosome 22 as their only human component, while each of the other five hybrid clones contain a few human chromosomes in addition to those relevant to the t(9;22)(q34;q11) translocation (13, 14). Identification of hybrid clones containing *c-abl* sequences (6) and analysis of human adenylate kinase (AK1) enzymatic activity (6) have been previously reported. Cells were analyzed for human *c-sis*-specific sequences as described in Fig. 1.

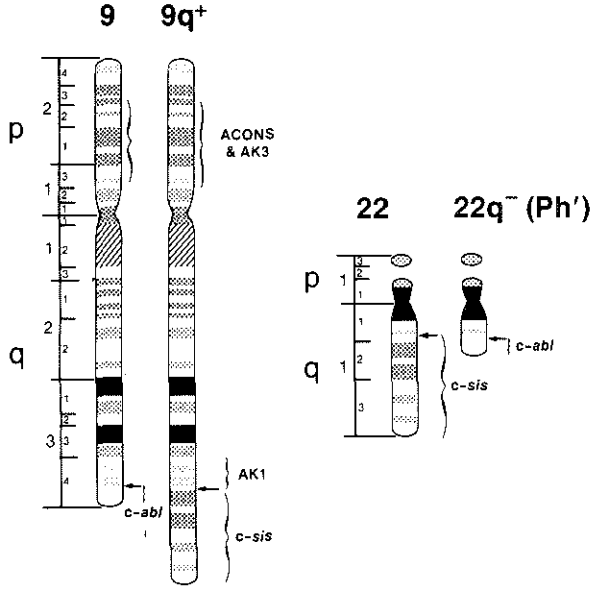

FIGURE 2. Diagrammatic representation of the involvement of c-*abl* and c-*sis* in the Ph' translocation. Chromosome banding patterns are as previously shown by Yunis (3); map positions of ACONS, AK3, and AK1 are as previously reported (24, 25). Localization of c-*abl* within the terminal portion of chromosome 9 (q34), which is translocated to chromosome 22 in CML, is as described by de Klein et al. (6) while localization of *c-sis* in the region of chromosome 22 (q11 to qter) translocated to chromosome 9 is based on the results of the present study.

some 8 to chromosomes 14, 2, or 22, each of which contain immunoglobulin sequences, in Burkitt's lymphoma (1, 19–21). Similarly, c-*abl* maps on the region of chromosome 9 which translocates to chromosome 22 in CML (5, 6). Other translocations, such as the t(15;17) reciprocal translocation associated with acute promyelocytic leukemia (3), involve regions to which human cellular oncogenes (in this case c-*fes*) have been mapped (22), but appear to be independent of immunoglobulin sequences. The present demonstration that c-*sis* is translocated from chromosome 22 to chromosome 9 in CML raises the possibility that c-*sis* rather than c-*abl* may be involved in CML. Resolution of these alternatives will require a determination of the proximity of these genes to the breakpoints in chromosomes 22 and 9, respectively, and analysis of the expression of their transcriptional and translational products in CML cells.

In addition to possible implications regarding the cause of CML and the significance of the associated t(9;22) (q34;q11) translocation, the localization of c-*sis* within the translocated region of chromosome 22 (Fig. 2) provides a unique molecular marker for studies of the more complex translocations associated with minority populations of CML patients. These can involve translocation of the q11 to qter region of chromosome 22 to chromosomes other than chromosome 9, or can in some instances involve more complex rearrangements including three or occasionally even four or five chromosomes (23). Analysis of these translocations using c-*abl* and c-*sis* probes should allow a determination of the critical translocation event resulting in the generation of CML.

Summary

By analysis of a series of somatic cell hybrids derived by fusion of either mouse or Chinese hamster cells with leukocytes from different chronic myelocytic leukemia (CML) patients or from normal donors, we have localized the human oncogene, c-*sis*, on the q11 to qter segment of chromosome 22 and demonstrated its translocation from chromosome 22 to chromosome 9 (q34) in CML.

We thank P. Hansen and G. T. Blennerhassett for excellent technical assistance.

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APPENDIX PAPER VII

THE HUMAN IMMUNOGLOBULIN LAMBDA LIGHT CHAIN IS ON THE PHILADELPHIA CHROMOSOME (22q11) IN CHRONIC MYELOID LEUKEMIA

B.D. Young, M. Goyns, A. Geurts van Kessel, A. de Klein, G. Grosveld, C.R. Bartram, D. Bootsma and T. Rabbitts.

During the last two decades a number of acquired chromosomal abnormalities have been found to be specifically associated with particular human cancers[1-3]. The occurrence of the Philadelphia (Ph^1) translocation (9;22)(q34;q11) in patients with chronic myeloid leukemia (CML) is one of the best documented examples of such an aberration[3]. The q11-qter region of chromosome 22 is translocated to chromosome 9[4], which results in 9q+, and 22q- derivative chromosomes. The latter is also referred to as Ph^1 chromosome. A cellular oncogene c-sis, known to be located on chromosome 22, is included in the translocation to chromosome 9[5] (Table 1). The reciprocity of this translocation has recently been confirmed by the demonstration[6] that the human cellular homologue (c-abl) of the transforming sequence of the Abelson murine leukemia virus (A-MuLV) is translocated from chromosome 9 to chromosome 22q- (Table 1). Specific chromosomal rearrangements have also been reported in Burkitt's lymphoma and other B-cell neoplasms[7-10]. The most common form is a translocation between chromosomes 8 and 14, (t(8;14)(q24;q32) with variant forms between 8 and 2, (t(2;8)(p12;q24) and between 8 and 22, (t(8;22)(q24;q11) occurring less frequently. Since chromosomes 14, 2 and 22 bear the genes for the immunoglobulin heavy chain, and kappa and lambda light chains, respectively[11-14], Klein[8]

Table 1

Segregation of human c-abl and c-sis oncogenes and immunoglobulin lambda light chain constant region sequences in somatic cell hybrids containing Ph[1] translocation chromosomes

Hybrid cell line	Human chromosomes 9	22	9q+	22q-	Oncogenes c-abl[x]	c-sis[xx]	Lambda light chain 22 - λ5
PgMe-25NU	-	+	-	-	-	+	+
10CB-23B	+	-	-	-	+	-	NT
WESP-2A	-	-	-	+	+	-	+
1CB-17ANU	-	-	-	+	+	-	+
14CB-5A	+	-	-	+	+	-	+
14CB-21A	-	-	+	-	-	+	-
PgMo-22	-	-	+	-	-	+	-

x de Klein et al. (1982) and unpublished results.
xx Groffen et al. (in press) and B.D. Young (unpublished results).
NT: not tested.

and Rowley[9] have proposed that a cellular oncogene may be activated by juxta-position of part of an active immunoglobulin locus. The localization of the heavy chain[15-17] (14q32) and the kappa light chain[14] (2 cen-2p13) to the regions involved in the chromosomal breakpoints has reinforced this hypothesis. We report here the assignment of the lambda light chain (constant region) to 22q11, the region in which breakpoints occur for both the 8;22 translocation of Burkitt's lymphoma and the 9;22 translocation in chronic myeloid leukemia. Furthermore, our results indicate that the constant region of the lambda

locus remains on the Ph^1 chromosome in the 9;22 translocation.

Our evidence has been obtained using a DNA probe for the lambda constant region and cell hybrids bearing different translocated parts of chromosome 22. The human lambda DNA fragment (22 - λ5) was obtained by screening a phage library of chromosome 22[18] with mouse lambda constant region probes and consists of an 8KB EcoRI fragment in λgt WES which includes 2 constant region structural genes ($Kern^-Oz^-$, $Kern^-Oz^+$)[19]. The identity of this clone was confirmed by restriction site mapping and by subcloning and sequencing of a part of one of its coding regions. The isolation and initial characterization of the somatic cell hybrids used in this study have been reported previously[4,20,24]. They were obtained by fusion of rodent cells with either human leucocytes carrying balanced reciprocal translocations (X;22)(q21;q11) and (1;22)(q42;q13) or human CML cells carrying the Ph^1 translocation (9;22)(q34;q11). For the present study hybrids derived from four different CML patients were used. DNA was prepared from the various cell hybrids, the parental cell lines and human placenta or peripheral blood, digested with EcoRI and analysed by the Southern[21] blotting technique with nick translated 22 - λ5 DNA as probe. It can be seen from Fig. 1 that 22 - λ5 hybridizes to normal human DNA with a band at 8 Kb and that this band is present only in certain hybrid cell lines (Table 1). An exception to this observation is the 15 Kb hybridizing band in the 22q- containing cell line 1CB-17ANU. This 15 Kb hybridizing band is due to an EcoRI polymorphism as was checked by HindIII, BglII and BamHI DNA digests of this cell line (not shown). EcoRI polymorphism in the 22 - λ5 probe area have previously been observed by Hieter el al.[19]. Clearly, there

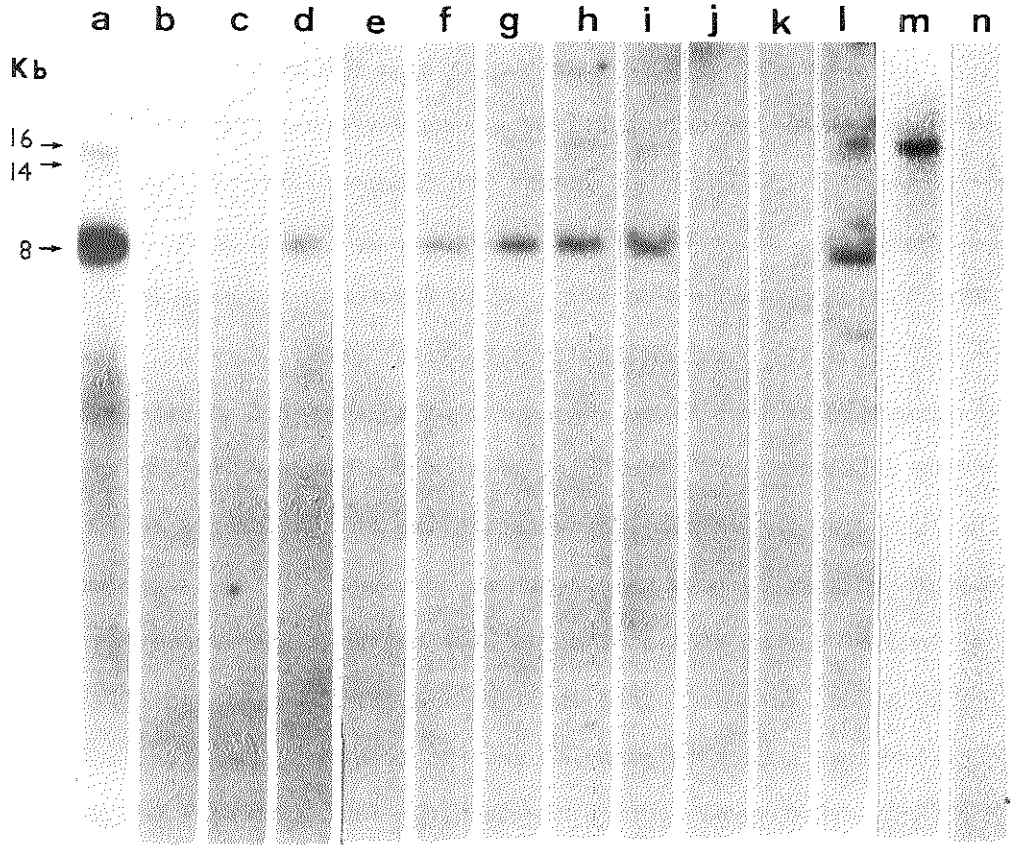

Fig. 1 Segregation of the constant region of the lambda immunoglobulin locus in rodent-human hybrids. DNA from the hybrids was digested with EcoRI restriction enzyme, fractionated on a 1.0% agarose gel, blotted to nitrocellulose filters and hybridized with nick-translated DNA from the Chr 22 λ 5 clone. After hybridization, filters were washed to a stringency of 0.1xSSC at 65°C. a) normal human fibroblast; b) a3 (hamster parent cell); c) AM21; d) AM27; e) E36 (hamster parent cell); f) 33-11; g) 33-11TG; h) WESP2A; i) 14CB-5A; j) 14CB-21A; k) PgMo-22; l) PgMe-25NU; m) ICB-17ANU; n) WEHI-3B. 1/22 AM and 33-11 hybrid clones were derived from fusion of human leucocytes carrying translocations 1;22 and X;22 with Chinese hamster a3 and E36 cells, respectively. Hybrid cell lines PgMe-25NU and PgMo-22 were obtained after fusion of mouse Pg19 cells with leucocytes from a normal human donor and a CML donor, respectively. WESP-2A results from fusion of mouse WEHI-3B cells with leucocytes from a second CML patient. Leucocytes from a third CML patient were fused with Chinese hamster E36 cells to produce both 14CB clones and Chinese hamster cell line a3 was used to obtain ICB-17ANU (fourth CML patient).

is no hybridization to the Chinese hamster or mouse parental cell DNA under these conditions. Figure 2 shows in a diagram the regions of chromosome 22 that have been retained by the different hybrid cell lines used. Hybrid clone PgMe-25NU has a normal chromosome 22 as its only human component, while each of the other hybrids contains a few human chromosomes in addition to those relevant to the translocations. Positive hybridization of 22 - λ5 to clone PgMe-25 NU (Fig. 2, lane 1) confirms the localization of the lambda light chain locus to chromosome 22. It is also evident that the constant part of the lambda locus lies within band 22q11 in a region (Fig.2: SRO) bounded by the chromosomal breakpoints producing the X;22 and 9;22 translocations. The breakpoint on chromosome 22 has been reported[20] to be just below the centromere in the X;22 translocation and, therefore, appears to be proximal to that observed in the 9;22 translocation. Our present analysis is in agreement with that. Since for this analysis hybrid cell lines derived from four different leukemia patients were used, we conclude that the observed localization of lambda constant region sequences is most probably a general phenomenon in the Ph^1 translocation in CML. The human lambda constant region locus contains 6 structural genes, two of which are present in the 22 - λ5 clone used in our experiments (Fig. 1). The other 4 structural genes are on 14 and 16 Kb EcoRI fragments[15]. These bands may be seen faintly in lane 1 of Fig. 1 and can also be seen in certain other lanes. This indicates that the entire constant region locus (50 Kb) is present in germline form within a given cell hybrid and, therefore, the transloction breakpoints do not lie within the constant region locus.

Of significance may be the recent demonstration that the human cellular homologue (c-abl) of the transforming sequence of Abelson murine leukemia virus is translocated to 22q- in the 9;22 translocation in CML. Thus the c-abl gene and the lambda light chain gene are brought into close proximity on the Philadelphia chromosome. So far, it has not been shown that the restriction patterns of either the lambda constant region or the c-abl gene are altered as a result of the Ph^1 translocation. This would be expected if the genes were brought into close proximity to each other in a manner analogous to that recently described by Croce[22] and Taub and Leder[23] for the 8;14 translocation in Burkitt lymphoma in which the c-myc oncogene was translocated directly into restriction fragments that encode immunoglobulin heavy chain regions. Recently, it has been found[25], using in situ hybridization techniques, that human lambda constant region sequences are translocated to chromosome 8 in the Burkitt lymphoma associated translocation (8;22)(q24;q11). Translocation of lambda constant region sequences was not observed in the CML patients studied here, which indicates that the breakpoint in chromosome 22 in the Ph^1 translocation is distal to that observed in the Burkitt lymphoma associated translocation 8;22.

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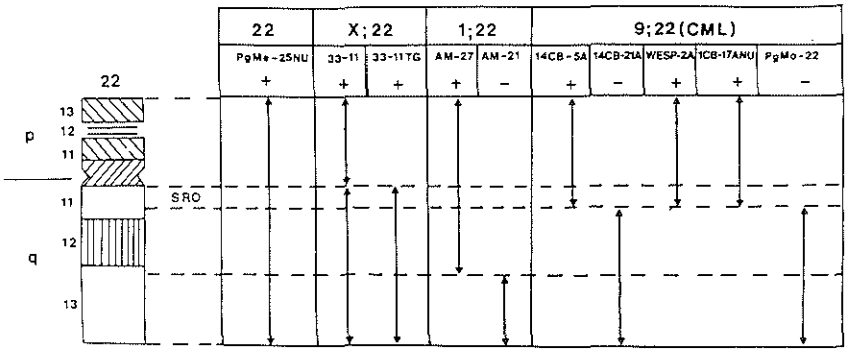

Fig. 2 Schematic representation of the distribution of translocated parts of chromosome 22 in the somatic cell hybrids. Chromosome analysis was done using reverse (R) banding with acridine orange, after heat denaturation. At least 16 metaphases were analyzed per cell line. Enzyme marker analysis of the hybrids has been reported previously[4,5,24]. + indicates hybridization of 22 λ 5 probe. SRO indicates the shortest region of overlap: the region of chromosome 22 that contains the lambda light chain constant region sequences.

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APPENDIX PAPER VIII

Expression of human myeloid-associated surface antigens in human–mouse myeloid cell hybrids

(chronic myeloid leukemia/Philadelphia chromosome/monoclonal antibodies/chromosome 11)

A. H. M. GEURTS VAN KESSEL*, P. A. T. TETTEROO†, A. E. G. Kr. VON DEM BORNE†‡, A. HAGEMEIJER*, AND D. BOOTSMA*

*Department of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands; †Department of Immunohaematology, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands; and ‡Department of Haematology, Academic Medical Centre, Amsterdam, The Netherlands

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ABSTRACT Hybrid cell lines were obtained after fusion of mouse myeloid cells (WEHI-TG) with leukocytes from two patients with chronic myeloid leukemia. A third fusion was carried out with leukocytes from a patient with acute lymphocytic leukemia. All three patients carried the Philadelphia chromosome (Ph^1) in the leukemia cell population. Cytochemical analysis confirmed the myelo-monocytic nature of the hybrid cell lines. The presence of Ph^1 translocation products could be established in most hybrids derived from the two chronic myeloid leukemic patients, which confirms that indeed human myeloid cells were fused. Several of these hybrid lines showed reactivity with monoclonal antibodies known to be specific for human myeloid cells, whereas interlineage Chinese hamster fibroblast–human chronic myeloid leukemia hybrids failed to react with these antibodies. Five independently obtained monoclonal antibodies—MI/N1, UJ-308, VIM-D5, FMC-10, and B4.3—showed very similar reactivity patterns when tested on the hybrid clones. This result substantiates the evidence obtained from other studies, that these five antibodies are directed against the same myeloid-associated antigen. The gene(s) for expression of the latter antigen could be assigned to human chromosome 11.

Hybrid cell lines, derived from fusion of cells within the hemopoietic system, have been isolated by several investigators (1–7). In such hybrids the genetic control of expression of specific blood cell characteristics can, in principle, be studied. It has been shown that (at least) some of the differentiation programs of different types of blood cells are mutually exclusive (1, 2). Fusion of cells of a specific state of differentiation generally results in the expression of the differentiation-associated traits of both fusion partners in the hybrids. The generation of antibody-producing hybrids by Köhler and Milstein (3) is a well-known example of the latter. Recently, secretion of human Ig heavy and light chains in intralineage human–rodent cell hybrids has been found (4, 5); also, expression of the human α-globin gene (6) and the expression of the erythrocyte-rosette receptor (7) have been reported. So far, the production of intralineage human–rodent hybrid cell lines expressing human myeloid characteristics has not been published.

We report here the isolation of proliferating human myeloid–mouse myeloid cell hybrids, in which human chromosomes segregate and which express characteristics specific of human myeloid cells. A human myeloid-associated antigen, detected by five monoclonal antibodies, could be assigned to human chromosome 11, when using these hybrids.

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MATERIAL AND METHODS

Patient Material and Production of Hybrid Cell Lines. Leukocytes from three patients (S.P., D.Y., and F.) with chronic myeloid leukemia (CML) and one patient (R.O.) with acute lymphocytic leukemia (ALL) were used for the cell fusion experiments. When leukocytes were obtained, patient S.P. was in the terminal blastic phase of the disease, marked by additional karyotypic changes besides the Philadelphia (Ph^1) translocation: 48,XY,+8,t(9q+;22q−),i(17q),+22q−. Patients D.Y. and F. were in a chronic phase of the disease. F. carried the classical Ph^1 translocation, whereas D.Y. carried a complex Ph^1 translocation in the immature dividing leukocytes: 46,XX, t(1p−;9q+;22q−). The leukemia cells of patient R.O. carried the Ph^1 translocation as well: 46,XY,t(9q+;22q−).

From the *in vitro* established mouse myeloid cell line WEHI-3B (8), a hypoxanthine phosphoribosyltransferase-deficient ($HPRT^-$) mutant was obtained after UV irradiation (10 J/m^2), followed by culture in medium containing 10 μg of 6-thioguanine per ml. The drug-resistant line (WEHI-TG) failed to incorporate [^{3}H]hypoxanthine. Isolation of the thymidine kinase-deficient (TK^-) Chinese hamster fibroblast cell line a23 has been reported previously (9). Fusions between patient-derived leukocytes and WEHI-TG cells were carried out according to standard procedures. Inactivated Sendai virus was the fusogen and hybrid selection was carried out in hypoxanthine/aminopterin/thymidine (10) medium. After fusion, cells were either seeded in methylcellulose-supplemented (1.2%) medium in dishes with a 0.5% agar base or in T30 flasks (Falcon) without the addition of methylcellulose or agar. Subcloning experiments were carried out in methylcellulose-supplemented cultures. The isolation of hybrids derived from fusion of Chinese hamster fibroblasts (a23) with CML cells has been described earlier: 9CB hybrids were obtained by using leukocytes from patient F. (11), whereas for the isolation of the 12CB hybrids leukocytes from patient D.Y. were used again (12). Cells were grown in F10 or RPMI 1640 medium with 10–15% fetal calf serum/2 mM glutamine/penicillin at 100 units/ml/streptomycin at 100 μg/ml.

Immunoassays and Antisera. The indirect immunofluorescence test described by Verheugt *et al.* (13) was used throughout this study, whereas the immunoperoxidase technique described by Mason *et al.* (14) was used to demonstrate the presence of intracytoplasmic antigen. Immunoprecipitations were carried out as described by Borst *et al.* (15), after ^{125}I labeling, according to Fraker's method (16). The precipitates were ana-

Abbreviations: CML, chronic myeloid leukemia; ALL, acute lymphocytic leukemia; HPRT, hypoxanthine phosphoribosyltransferase; TK, thymidine kinase.

lyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis in 10% acrylamide slab gels (17).

Monoclonal antibody MI/N1 was raised against neuroblastoma cells (18) and monoclonal antibody UJ-308 was raised against human fetal brain cells (J. T. Kemshead, personal communication). B4.3 and B13.9 are monoclonal antibodies obtained after immunization of a mouse with peripheral human leukocytes (19) and monoclonal antibody VIM-D5 was raised against human cell line K562 (20). Immunization of mice with human granulocytes yielded monoclonal antibodies FMC-10, -11, -12, and -13 (21). Within the hemopoietic system, all of these monoclonal antibodies react specifically with myeloid cells. Conventional antisera were used to detect the c-ALL antigen (22) and the Ia antigen (anti-SB), whereas monoclonal antibody 3A1 (23) was used to detect T-cell determinants.

Chromosome and Isoenzyme Analysis. Air-dried chromosome spreads were R-banded with acridine orange after heat denaturation. At least 16 metaphases of each hybrid line were analyzed. Lactate dehydrogenase (EC 1.1.1.27) isoenzymes were assayed by cellulose acetate gel (Cellogel) electrophoresis (24). The same populations of cells were used for immunologic, chromosome, and isoenzyme analyses.

Cytochemistry. Cytospin preparations were assayed for α-naphthyl acetate esterase, Sudan black, naphthol AS-D chloroacetate esterase, acid phosphatase, and periodic acid–Schiff by using standard procedures (25).

Table 1. Presence or absence of human leukemia-associated chromosomal abnormalities and five cytochemical reactions in WEHI-TG parental cells and 31 derived hybrid cell lines

Cells	Human leukemia-associated chromosome markers	Cytochemical reactions				
		SB	NCAE	α-NAE	AP	PAS
WEHI-TG		+	−	+	−	−
WESP-1	22q−	(+)	+	(−)	−	+ (10%)
WESP-2	22q−; i(17q)	ND	ND	ND	ND	ND
WESP-5	22q−; i(17q)	−	+	(+)	+	−
WESP-6	22q−	(+)	+	(+)	−	+
WESP-11	22q−	(+)	+	(+)	−	+ (<10%)
WEDY-1	1p−; 22q−	−	−	+	−	−
WEDY-3	—	(+)	−	+	−	+
WEDY-5	—	−	−	+	−	+
WEDY-7	9q+	+	−	+	S+	−
WEDY-8	22q−	ND	ND	ND	ND	ND
WEDY-9	22q−	(+)	−	+	ND	+ (<10%)
WEDY-10	1p−; 9q+; 22q−	+	S+	+	S+	+
WEDY-11	1p−; 22q−	−	−	+	−	+ (<10%)
WEDY-12	9q+	−	−	+	−	+
WEDY-13	1p−; 22q−	+	+	+	−	−
WEDY-14	22q−	+	−	+	−	+ (<10%)
WEDY-15	1p−; 22q−	+	+	+	−	+
WEDY-16	22q−	+	−	+	−	−
WEDY-17	22q−	−	−	+	−	+
WEDY-18	1p−; 22q−	−	−	+	−	S+
WERO-1	—	+	−	+	S+	+
WERO-2	—	+	+	+	−	+
WERO-3	—	+	+	+	S+	+
WERO-4	—	(+)	−	+	S+	S+
WERO-5	—	+	−	+	S+	+
WERO-6	—	ND	ND	ND	ND	ND
WERO-8	—	+	S+	+	+	+
WERO-9	—	+	−	+	+	+
WERO-10	—	+	+	+	−	+
WERO-11	—	+	−	+	+	S+
WERO-12	—	−	−	+	−	+

SB, Sudan black; NCAE, naphthol AS-D chloroacetate esterase; α-NAE, α-naphthyl acetate esterase; AP, acid phosphatase; and PAS, periodic acid–Schiff. (+), Weak expression. S+, <1% positive. ND, not determined.

RESULTS

Independent hybrid cell lines were obtained after fusion of WEHI-TG cells with leukocytes from S.P. (WESP), D.Y. (WEDY), and R.O. (WERO). These hybrid lines grow in suspension. Fusion of Chinese hamster a23 fibroblasts with leukocytes from F. and D.Y. provided the 9CB and 12CB hybrids, respectively. These hybrids grow in monolayer as do the parental a23 cells.

CML-associated aberrant human chromosomes were found in all but two WESP and WEDY clones (Table 1 and Fig. 1), indicating that human myeloid cells were fused. Ph^1 translocation-derived chromosomes were observed in the two 9CB and in five of seven 12CB hybrids as well (data not shown). In contrast, WERO hybrids lacked Ph^1 translocation products.

All WESP, WEDY, and WERO hybrids tested were positive (Table 1) for α-naphthyl acetate esterase, whereas several hybrid lines were positive for Sudan black or naphthol AS-D chloroacetate esterase, or both. These results confirm the myelomonocytic nature of these hybrids. In addition, some of the clones were positive for acid phosphatase or periodic acid–Schiff.

Immunologic characterization of the parental cells and the different hybrid lines is shown in Table 2. Monoclonal antibodies MI/N1, UJ-308, VIM-D5, B4.3, and FMC-10 reacted with 95% of the peripheral leukocytes of patient D.Y. Per-

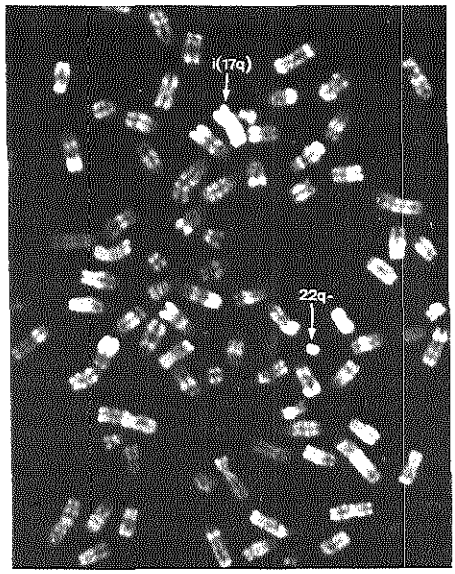

FIG. 1. Metaphase of hybrid cell line WESP-2 showing, in addition to the WEHI-TG mouse chromosomes, a variety of human chromosomes. The CML-specific Ph^1 chromosome (22q−) and i(17q) chromosome are indicated. The air-dried spread was heat-denatured and stained with acridine orange (R-banding).

Table 2. Reactivity patterns of 11 antibodies with parental cells and WEHI-TG or a23-derived hybrid cell lines

Cells	Antibodies						
	*	FMC-11	FMC-12	FMC-13	B13.9	c-ALL	3A1
a23	–	ND	ND	ND	–	–	ND
WEHI-TG	–	–	–	–	–	–	–
SP	ND	ND	ND	ND	ND	ND	ND
WESP-1	–	–	–	–	–	–	–
WESP-2	+ (30%)	ND	ND	ND	–	–	–
WESP-5	+ (50%)	+ (4%)	+ (8%)	+ (14%)	–	–	–
WESP-6	+ (10%)	+ (2%)	+ (4%)	–	–	–	–
WESP-11	–	–	–	–	–	–	–
DY	+ (95%)	+ (94%)	+ (89%)	+ (100%)	+ (83%)	–	–
WEDY-1	–	–	–	–	–	ND	–
WEDY-3	+ (30%)	–	+ (1%)	+ (2%)	–	–	–
WEDY-5	–	–	–	–	–	–	–
WEDY-7	–	–	–	–	–	ND	–
WEDY-8	–	–	–	–	–	–	–
WEDY-9	–	–	–	–	–	–	–
WEDY-10	+ (20%)	+ (6%)	+ (5%)	+ (15%)	–	–	–
WEDY-11	–	–	–	–	–	–	–
WEDY-12	–	–	–	–	–	–	–
WEDY-13	–	–	–	–	–	–	–
WEDY-14	–	–	–	–	–	–	–
WEDY-15	–	–	–	–	–	–	–
WEDY-16	–	–	–	–	–	–	ND
WEDY-17	–	–	–	–	–	–	–
WEDY-18	–	–	–	–	–	–	–
RO	+ (10%)†	ND	ND	ND	ND	+ (50%)	ND
WERO-1	+ (30%)	+ (1%)	+ (1%)	+ (4%)	–	–	–
WERO-2	+ (20%)	+ (8 %)	+ (5%)	+ (11%)	–	–	–
WERO-3	–	–	–	–	–	–	–
WERO-4	–	–	–	–	–	–	–
WERO-5	–	–	–	–	–	–	–
WERO-6	+ (15%)	+ (5%)	+ (3%)	+ (4%)	–	–	–
WERO-8	+ (30%)	+ (2%)	+ (2%)	+ (15%)	–	–	–
WERO-9	+ (70%)	+ (15%)	+ (15%)	+ (12%)	–	–	–
WERO-10	+ (50%)	+ (11%)	+ (13%)	+ (26%)	–	–	–
WERO-11	+ (45%)	+ (15%)	+ (15%)	+ (20%)	–	–	–
WERO-12	+ (30%)	+ (13%)	+ (6%)	+ (16%)	–	–	–
9CB-4*	–				–		–
9CB-14	–				–		–
12CB-4A	–				–		–
12CB-4B	–				–		–
12CB-14B	–				–		–
12CB-17B	–				–		–
12CB-20B	–				–		–
12CB-24D	–				–		–
12CB-27B	–				–		–

Identical results obtained with MI/N1, UJ-308, VIM-D5, FMC-10, and B4.3 are combined. Scores are based on indirect immunofluorescence tests. ND, not determined. +, In 9CB and 12CB series, FMC-11, -12, and -13 and c-ALL were not tested.
*MI/N1; UJ-308; VIM-D5; FMC-10; B4.3.
†Only B4.3 tested.

centages of FMC-11, -12, and -13 reactive cells ranged from 89% to 100%, whereas 83% were positive with B13.9. No c-ALL- or 3A1-positive cells were found. Only a minority (10%) of the bone marrow cells of patient R.O. in the ring fraction obtained by Ficoll-Isopaque centrifugation were positive with monoclonal antibody B4.3, whereas 50% of these blast cells were c-ALL antigen-positive, and 70% were Ia antigen-positive (data not shown). None of the antisera used reacted with WEHI-TG cells, dimethyl sulfoxide-stimulated WEHI-TG cells, or normal BALB/c granulocytes. Similarly, a23 cells failed to react with the human myeloid specific antisera. Cells of patients S.P. and F. were not tested for the presence of the antigens in question.

In the indirect immunofluorescence assay 13 hybrids reacted with monoclonal antibodies MI/N1, UJ-308, VIM-D5, B4.3, and FMC-10, -11, -12, and -13 (Table 2). Identical patterns of reactivity were obtained with the former five antibodies, whereas

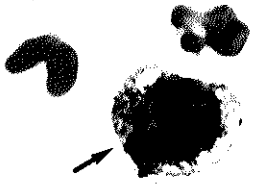

FIG. 2. Intracytoplasmic presence of human myeloid-associated antigen in a cell (arrow) of hybrid WESP-5. The antigen was visualized by using an immunoperoxidase technique and monoclonal antibody B4.3. Morphology of the nuclei is characteristic of myeloid cells.

for FMC-11, -12, and -13 lower frequencies of positive-reacting cells were found. The 9CB and 12CB hybrids did not react with MI/N1, UJ-308, VIM-D5, B4.3, or FMC-10. None of the hybrids tested was positive with B13.9, c-ALL, or 3A1. Presence of intracytoplasmic antigen was demonstrated in WESP-2 and WESP-5 with monoclonal antibodies B4.3 and FMC-10 by using an immunoperoxidase technique (Fig. 2). The latter was also observed in immature and mature human myeloid cells. The frequencies of positive-reacting hybrid cells were similar

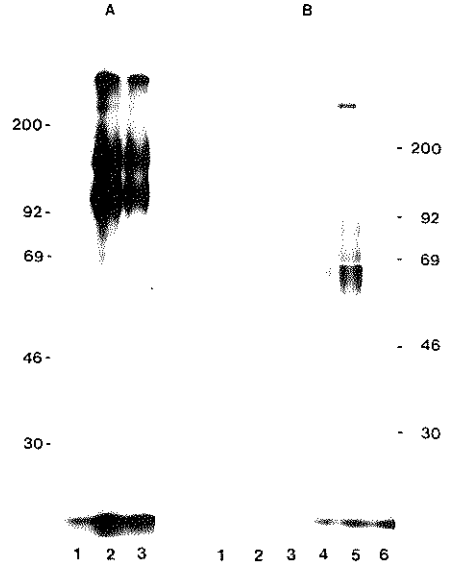

FIG. 3. (*A*) Immunoprecipitation patterns of human granulocytes with normal mouse serum (control) (lane 1), monoclonal antibody B4.3 (lane 2), and FMC-10 (lane 3). (*B*) Precipitation patterns of WEHI-TG cells with normal mouse serum (lane 1), B4.3 (lane 2), and FMC-10 (lane 3) and of hybrid cell line WESP-5 with B4.3 (lane 4), FMC-10 (lane 5), and normal mouse serum (lane 6). Two major bands with apparent M_rs of 105,000 and 150,000 are precipitated from granulocytes, whereas two major bands with apparent M_rs of 60,000 and 80,000 are precipitated from hybrid WESP-5. M_rs are shown as $M_r \times 10^{-3}$.

Table 3. Relationship between the human myeloid-associated antigen detected by MI/N1, UJ-308, VIM-D5, FMC-10, and B4.3 and human chromosomes in 64 primary and secondary hybrid cell lines

	Chromosome/antigen, no. of clones			
Chromosome	+/+	+/−	−/+	−/−
1	11	27	4	22
2	13	20	2	29
3	11	9	4	40
4	13	19	2	30
5	7	19	8	30
6	11	42	4	7
7	14	24	1	25
8	12	20	3	29
9	6	2	9	47
10	14	40	1	9
11	13	0	2	49
12	14	17	1	32
13	6	13	9	36
14	9	24	6	25
15	6	7	9	42
16	12	16	3	33
17	5	17	10	32
18	4	16	11	33
19	11	19	4	30
20	10	30	5	19
21	15	34	0	15
22	13	12	2	37
X	12	45	3	4
Y	4	0	11	49
1p−*	0	7	15	42
9q+*	2	1	13	48
i(17q)*	4	4	11	45
22q−*	4	40	11	9

*Leukemia-associated chromosomal abnormalities.

to those obtained with the indirect immunofluorescence assay.

From the primary hybrid cell lines WESP-1, -2, -5, -6, and -11 and WEDY-1, 33 subclones were isolated. All of the 64 hybrid lines (primary and secondary) were tested with monoclonal antibodies MI/N1, UJ-308, VIM-D5, B4.3, and FMC-10 and the same batches of cells were assayed for the human chromosome content (Table 3). A close correlation was observed between reactivity with the five monoclonal antibodies and human chromosome 11 only. Moreover, the percentages of positive cells in the indirect immunofluorescence tests correlated closely with the frequencies of chromosome 11 scored in these populations (data not shown). In two exceptional clones (WEDY-3 and WERO-2) an intact chromosome 11 could not be identified, whereas antibody-reactive cells were found. Screening of the total panel of 64 hybrids for the presence of the human chromosome 11 marker lactate dehydrogenase A gave 100% concordance. Also WEDY-3 and WERO-2 were positive for lactate dehydrogenase A. All human chromosomes were represented at least once among the a23 × CML hybrid cell lines. Three of these lines contained human chromosome 11 and, in addition, Ph^1 translocated products, but they failed to react with MI/N1, UJ-308, VIM-D5, B4.3, and FMC-10.

Immunoprecipitation (Fig. 3) of the antigens detected by monoclonal antibodies B4.3 and FMC-10 on the surface of hybrid cell lines WESP-2 and WESP-5 and the subsequent Na-DodSO$_4$/polyacrylamide gel electrophoretic analysis revealed two major bands with apparent M_rs of 60,000 and 80,000, whereas no such antigens were found on WEHI-TG cells. Similarly obtained immunoprecipitation patterns from normal human granulocytes revealed two bands with apparent M_rs of 105,000 and 150,000.

DISCUSSION

Proliferating myeloid hybrids were obtained after fusion of an established mouse myeloid cell line (WEHI-TG) with leukocytes from two Ph^1-positive CML patients and one Ph^1-positive ALL patient. Morphological, cytochemical, and immunological studies confirmed the myeloid character of the hybrids. In contrast to rodent fibroblast–human CML hybrids, they appeared to be suitable for the study of myeloid differentiation markers. Despite the fact that we failed to isolate Ph^1-positive hybrids in the fusion with ALL cells (WERO), our results seem to indicate that the mouse parental cell line determines the myeloid character of the hybrids.

Some clones reacted with monoclonal antibodies detecting human myeloid-associated antigens both on the surface and in the cytoplasm of the cells. Recently, we found by immunoprecipitation and competition binding experiments that monoclonal antibodies MI/N1, UJ-308, VIM-D5, B4.3, and FMC-10 react with the same antigen present on human myeloid cells (19). This observation is supported by the identical reaction patterns exhibited by the hybrid panels tested in our present study. According to Zola *et al.* (21) monoclonal antibodies FMC-10, -11, -12 and -13 react with distinct surface antigens. This is also substantiated by the differences observed in frequencies of positive-reacting cells. None of the hybrids tested reacted with monoclonal antibody B13.9. This antibody is known to react with mature granulocytes only (19) and none of the hybrids showed complete morphologic maturation.

A close correlation was observed between reactivity of hybrid cells with monoclonal antibodies MI/N1, UJ-308, VIM-D5, B4.3, and FMC-10 and the presence of chromosome 11, or its marker, lactate dehydrogenase A. This concordance suggests the provisional localization on chromosome 11 of one or more genes responsible for the expression of the human myeloid-associated antigen detected by these five antibodies. Although expressed in a much lower percentage of cells, FMC-11, -12, and -13 had similar segregation patterns, which suggests that chromosome 11 may also be involved in the expression of the antigens recognized by these antibodies. In the past, several antibodies have been described that appeared to be directed against human membrane determinants coded by genes located on chromosome 11 (26–30). These antigens appear to be different from the antigen detected by MI/N1, UJ-308, VIM-D5, B4.3, and FMC-10, because they show different tissue distributions. In contrast to the myeloid-associated antigen described here, several of these chromosome 11-encoded antigens are expressed in lymphocytes and erythrocytes and in human–rodent fibroblast hybrids. The antigen detected by monoclonal antibody F10.44.2 (30) is also expressed in human–mouse B-cell hybrids, whereas the myeloid-specific antibodies used in this study do not react with such hybrids (data not shown). Moreover, the antigen detected by monoclonal antibody W6/45 (29) differs in apparent molecular weight with the antigen studied here (M_r 16,000 versus M_rs 105,000 and 150,000).

The human chromosome 11-encoded antigen studied by Jones *et al.* (31) resides in a glycolipid, the biosynthesis of which requires participation of specific glycosyl transferases. Further characterization of the antigenic determinants recognized by the antibodies used in our study must be undertaken to determine whether glycolipids or carbohydrates, or both, are involved as well. Such human antigenic carbohydrate determinants may be present on mouse or mouse–human heteropolymeric carrier molecules in the hybrid cells, which, in turn, could explain the differences observed in immunoprecipitation patterns from hybrid cells and human granulocytes.

The hybrids reported here have shown to be useful for the chromosomal localization of myeloid-associated antigens. They may also be helpful in studying the genetic control over various other properties of normal or malignant myeloid cells, such as the capacity to form colonies in semisolid media or to express transformation-related (oncogene) products.

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