CHIMERIC GENES IN ACUTE LEUKEMIAS

CHIMERE GENEN BIJ ACUTE LEUKEMIEEN

PROEFSCHRIFT

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When the storm-damaged ship
reaches harbour safely,
it can desire nothing more.
Thus my heart, amid distress and tears,
has now found comfort,
my soul is filled with joy once more.

Cleopatra's Aria in "Giulio Cesare" by Georg Friedrich Händel

Voor Marco en mijn ouders

Contents

Chapter I

GENERAL INTRODUCTION

One of the major causes of human mortality is cancer (Parkin et al, 1988). Cancer is a disease in which regulatory systems of normal cell-growth and -differentiation are disturbed thereby causing cells to multiply in a disorganized fashion. Depending on the type of cells which proliferate, various tumors can be found in man. Solid tumors represent 93% of all human neoplasms while the remaining 7% is accounted for by leukemias and lymphomas which arise from blood cells and lymph nodes respectively.

The way in which normal cell-growth and -differentiation are controlled has been an enigma for ages. Even more so, the events taking place during neoplastic transformation have been largly unknown. Yet, around the turn of the century Van Hansemann and Boveri already postulated that the chromosomes in the cell-nucleus might play a role in tumorigenesis (Van Hansemann, 1890, Boveri, 1914). However, it would take more than half a century before a specific association between chromosomes and cancer was discovered.

1. Abnormal chromosomes are found in human tumors.

The foundation of human cancer genetics was laid in 1960 when Nowell and Hungerford detected an abnormal small chromosome 21 or 22 in bone marrow cells from patients with chronic myeloid leukemia (CML) (Nowell and Hungerford, 1960). They called this abnormal chromosome the Philadelphia (Ph) chromosome after the city of Philadelphia. At that time, the identification of each individual chromosome out of the 46 human chromosomes was still impossible and chromosomes were categorized according to size in 7 different groups. However, karyotyping took a flight in the following decades due to the development of "banding" techniques and this enabled cytogeneticists to recognize each chromosome separately by size and structure (Caspersson et al, 1970, Yunis, 1981). In fact, using these refined methods Rowley discovered in 1973 that the Ph chromosome was an abnormal chromosome 22, which had lost part of the long arm in a translocation to the long arm of chromosome 9 (Rowley, 1973). Since that discovery an increasing number of human neoplasms has been associated with non-random chromosomal abnormalities. These abnormalities can be subdivided into two main groups (Mitelman and Levan, 1981, Trent et al, 1989):

I. Structural abnormalities.
Four categories of structural abnormalities in chromosomes occur in malignancies:
a. Deletions: part of a chromosome is missing. This phenomenon is mainly seen in solid tumors, like small-cell lung cancer where deletion of the short arm of chromosome 3 may occur, or in neuroblastoma in which deletion of part of the short arm of chromosome 1 is found (Whang-Peng et al, 1982, Brodeur et al, 1977).
b. Amplifications: part of the genetic material is present in more than its normal copy number. The karyotypic abnormalities representing this phenomenon are Double Minutes (DM's) or chromosomes with Homogeneously Staining Regions (HSR's). These aberrati-

ons are found in a wide variety of human solid tumors (Barker, 1982, Kovacs, 1979).
c. Translocations: part of a chromosome moves to another chromosome. Usually, exchange of material occurs between two chromosomes which is called a "reciprocal" translocation. This type of aberration is mostly found in leukemias and lymphomas. Well-known examples are the t(8;14) in Burkitt's lymphoma (BL) and the t(9;22) in CML (Manolova et al, 1979, Rowley, 1973).
d. Inversions: part of a chromosome is dislodged and reinserts at the same position in reverse orientation. For example, in a specific morphologic subtype of acute myeloid leukemia (AML) an inversion of the centromeric part of chromosome 16 is often found (Le Beau et al, 1983).

II. Numerical abnormalities.
Chromosomes are either missing ("monosomies") such as -22 in meningioma or present in more than two copies, e.g. trisomy 12 in chronic lymphocytic leukemia (CLL) (Mark et al, 1972, Knuutila et al, 1986).

While cytogenetic investigation of leukemias and lymphomas proved to be quite successful from the beginning, karyotyping of solid tumors was difficult (for review, see Sandberg and Turc-Carel, 1987). Research on solid tumors lagged behind for some years but due to adjusted culture- and banding techniques a growing number of specific chromosomal aberrations was found in a variety of these tumors (for review, see Teyssier, 1989).
In Table 1 a representation is given of the cytogenetic abnormalities which are found in human solid tumors, leukemias and lymphomas.

2. Cytogenetic abnormalities are the symptoms of gene-deregulation.

The involvement of specific chromosomes, or more precisely of specific chromosomal regions, in cytogenetic abnormalities led to the concept that in these areas genetic information was located which played a crucial role in tumorigenesis (Rowley, 1983, Yunis, 1983, Heim and Mitelman, 1987). An example of such a region is band q11 of chromosome 22. This area is involved in translocations which characterize various leukemias and lymphomas (t(9;22)(q34;q11) in CML, acute lymphoblastic leukemia (ALL), AML; t(8;22)(q24;q11) in Burkitt's lymphoma) and in deletions in tumors like meningioma (del(22)(q11)). Moreover, the existence of hereditary forms of cancer and the tumorigenic potency of certain agents which damage deoxyribonucleic acid (DNA) convinced scientists that genes must play a major role in the origin of cancer (Heath and Moloney, 1965, Papadrianos et al, 1967, Fraumeni and Thomas, 1967, Lynch et al, 1967, Li and Fraumeni, 1969, McCann et al, 1975, McCann and Ames, 1976).
While cytogenetic investigations expanded fast, several lines of research in the developing scientific field of molecular biology provided strong evidence in favor of this theory (for review, see Bishop, 1988). Molecular techniques allowed analysis of genes at the DNA-level which meant a totally new approach of the cancer-problem. Using these techniques two types of genes were discovered to be involved in tumorigenesis.

Table 1.

Cytogenetic Abnormalities in Human Solid Tumors, Leukemias and Lymphomas

Carcinomas	
bladder	i(5p),+7,-9/9q-,11p-,+13
breast	-8,-13
colon	-17,17(q11),-18,12q-,+7,+8,+12
kidney	del(3)(p21)
lung (small cell)	del(3)(p14p23)
ovary	6q-,t(6;14)(q21;q24),del(3)(p14p21),-X
prostate	del(10)(q24)
uterus	1q-
Germ cell tumors	i(12p)
Glioblastoma	9p24 - pter (rearrangements),+7,-10
Melanoma	del(6)(q11q27),i(6p),del(1)(p11p23),t(1;19)(q12;q13)
Meningioma	-22,22q-
Neuroblastoma (progressive)	del(1)(p32p36)
Retinoblastoma	del(13)(q14)
Sarcomas	
liposarcoma (myxoid)	t(12;16)(q13;p11)
synovial	t(X;18)(p11;q11)
rhabdomyosarcoma	t(2;13)(q37;q14)
Leukemias	
chronic myelocytic (CML)	t(9;22)(q34;q11)
acute myeloblastic (AML)	inv(3)(q21q25-27),t(3;5)(q26;q22),t(8;21)(q22;q22) t(15;17)(q22;q11),inv(16)(p13;q22),16q-(q22) t(9;11)(p22;q23),t(9;22)(q34;q11),t(6;9)(p23;q34) trisomy 4, trisomy 8
Secondary AML[a)]	del 5q/-5, del 7q/-7
Chronic lymphocytic (CLL)	trisomy 12, t(11;14)(q13;q32)
Acute lymphoblastic (ALL)	t(9;22)(q34;q11),t(4;11)(q21;q23),t(8;14)(q24;q32) t(1;19)(q21-23;p13),t(11;14)(p13;q13) t(2;8)(p11-13;q24),t(8;22)(q24;q11),del(6)(q21q25),9p-,12p-, 14q-,14q+,+21
Myelodysplastic syndrome (MDS)	t(1;3)(p36;q21),t(1;7)(p11;p11),t(2;11)(p11;q23)
Myeloproliferative disorder (MPD)	del(9)(q13q22),del(20)(q12q13),5q-,7q-,-7,12p-,+8,t(3;17) (q26;q22),t(11;21)(q22;q21),21q-,9q-,20q-
Polycythemia Vera	del(20)(q11q13)
Multiple myeloma	t(11;14)(q13;q32)
Lymphomas	
Burkitt's, non-Burkitt's	t(8;14)(q24;q32)
follicular	t(14;18)(q32;q21)
small cell lymphocytic	trisomy 12

[a)]Secondary to mutagen/carcinogen exposure

References Yunis, 1983
Sandberg, 1986
Sandberg and Turc-Carel, 1987
Teyssier, 1989

I. Tumor-suppressor genes.

The normal function of these genes is probably to hold cellular growth in check in specific types of tissue. The history of the gene involved in the development of retinoblastoma (an eye tumor) can stand model as the classic example of the discovery of a tumor-suppressor gene (for review, see Weinberg, 1988).

Until the first half of the 19th century children with a malignant tumor of the retina or retinoblastoma died. However, since the invention of the ophthalmoscope the diagnosis of this disease was possible and treatment existed of enucleation of the affected eye. Children of these ex-patients suffered from retinoblastomas in a high number of cases and often both their eyes were affected. Besides this form of hereditary retinoblastoma, cases were known without a family history: the so-called "sporadic" cases. Here, only one eye was affected and the disease was not transmitted to the off-spring of the patient. A hypothesis for the genetic mechanism underlying this disease was postulated by Knudson in 1970 (Knudson, 1971). He proposed that according to its Mendelian behaviour a recessive gene must be involved which had a protective function against tumor-formation. In the hereditary form of retinoblastoma, the child inherits a non-functioning allele from the affected parent and is heterozygous for this gene in all the cells of the body. Only one mutation in the other allele in one of the retinal cells is necessary to abolish its protective function thereby allowing growth of the tumor. In sporadic cases of retinoblastoma both loci in one retinal cell must be knocked out by mutations to allow formation of this tumor in that particular cell. However, in this case the disease is non-inheritable, since the germ cells of the patient are not affected.

Meanwhile, cytogenetic analysis of retinoblastoma cells had shown a deletion of band 14 of the long arm of chromosome 13 which was thought to contain the protective gene or retinoblastoma (RB) gene (Yunis and Ramsay, 1978). Probes derived from the deleted region became available. Southern blot analysis showed frequent homozygous deletions of some of these probes in retinoblastoma tumors (Dryja et al, 1984). In 1987 the whole RB gene was isolated and identified (Lee et al, 1987). Abnormalities in the structure of the RB-gene are not only found in retinoblastomas but also in osteosarcomas, breastcarcinomas and small-cell lung cancer (Friend et al, 1986, Lee et al, 1988, Harbour et al, 1988). These findings are in concordance with the clinical observation that many patients with retinoblastoma suffer from osteosarcoma in later life and that a higher incidence of breast cancer is found in female relatives of patients with osteosarcomas. It seems that the inheritance of an affected RB-allel increases the probability of tumor formation in various types of tissue.

The search for other tumor suppressor genes intensified and was partly guided by the work done on the RB-gene. In fast succession several tumor suppressor genes were isolated such as the WT-1 gene (Wilms' tumor), the NF-1 gene (neurofibromatosis), the gene encoding the p53 protein (osteosarcomas, brain tumors, leukemias, carcinomas of breast and lung, Li-Fraumeni syndrome) and the DCC-gene (colorectal cancer) (Call et al, 1990, Haber et al, 1990, Gessler et al, 1990, Viskochil et al, 1990, Cawthon et al, 1990, Malkin et al, 1990, Srivastava et al, 1990, Chen et al, 1990, Fearon et al, 1990). All these genes are similar in that abolishment of their normal function allows tumor growth (Table 2). Due to the 'recessive' behaviour at the cellular level of the tumor-suppressor genes, their detection is more complicated than that of the second type of gene which will be discussed, the oncogene.

Table 2.

Genes incriminated in human tumors

Tumor suppressor genes	Chromosomal localization	Tumor
RB1	13q14	Retinoblastoma, osteosarcoma cancer of breast/bladder/lung
p53	17q12-13	Astrocytoma, osteosarcoma cancer of breast/lung
WT1	11p13	Wilms' tumor
DCC (deleted in colorectal carcinoma)	18q21	Colon cancer
NF1	17q11	Neurofibromatosis type 1
FAP (familial adenomatous polyposis)	5q21 - 22	Colon carcinoma
Oncogenes		
abl	9q34	CML, ALL, AML
erbb-2 (neu)	17q11 - q12	Adenocarcinoma of breast/ ovary/stomach
myc	8q24	Burkitt's lymphoma, cancer of lung/breast/cervix
L-myc	1p32	Carcinoma of lung
N-myc	2p24	Neuroblastoma, small cell lung carcinoma
H-ras	11p15	Cancer of colon/lung/ pancreas, melanoma
K-ras	6p11 - p12	AML, ALL, thyroid cancer melanoma
N-ras	1p22 or p13	Cancer of genitourinary tract and thyroid, melanoma

References Verma, 1986
Bishop, 1991

II. Oncogenes.
The study of oncogenes is mainly based on two lines of research.

a. Onco-viruses
The discovery in 1910 by Peyton Rous that the Rous Sarcoma Virus is responsible for tumor formation in chicken is one of the pillars of oncogene research (Rous, 1911). The validity of Rous's discovery was confirmed by the growing number of animal tumors which were found to be the result of viral infection (Jarret et al, 1964, Snyder and Theilen, 1969, Rickard et al, 1969). However, it would take more than 50 years before viruses could even be visualized via the electron microscope and before their genomic organization was unraveled (Coffin and Billeter, 1976).
In 1969, Huebner and Todaro proposed a model for the mode in which viruses can cause tumors (Huebner and Todaro, 1969). Sero-epidemiological studies and cell culture studies had given evidence for the presence of viral genes in the genome of many vertebrates. The viral genetic sequences contained genes which supposedly transformed normal cells into tumor cells, hence these genes were called "oncogenes". Huebner and Todaro postulated that the viral genes, which were integrated into the host cell genome, were transmitted in a vertical fashion (i.e. from animal to progeny animal and from cell to progeny cell). Due to modifying factors in the host cell environment, the viral genes stayed in a functionally "repressed" form. Factors such as carcinogens, irradiation or the aging process might overcome the control exerted by the host cell. Under the influence of mutagens viral genes, especially oncogenes, were expressed and tumorigenesis was initiated. However, one problem still had to be solved in this hypothetical model. At that time, it was generally assumed that the only way in which genetic information could be transmitted was from DNA in the cell-nucleus to RNA in the cytoplasm. However, the genome of most of the acutely transforming viruses found in animal tumors consisted of RNA. The discovery of the enzyme reverse transcriptase by Baltimore and Temin in 1970 made clear that genetic information could also flow from RNA to DNA (Baltimore, 1970, Temin and Mizutani, 1970). So, if an RNA-virus infects a cell, reverse transcriptase transcribes viral genes into DNA that integrates into the host cell genome. Via the host cell systems the viral genes are expressed (Varmus, 1983). Ever since the discovery of reverse transcriptase, onco-viruses were accepted as model systems to study tumor formation (Coffin et al, 1981, Besmer et al, 1983).
One of the most surprising findings was that viral oncogenes were shown to have normal homologues in non-tumorigenic cells, not only in animals but also in humans (Varmus et al, 1972, Stehelin et al, 1976, Oskarsson et al, 1980). These were called "proto-oncogenes". The hypothesis for the origin of oncogenes is that during evolution viruses integrated into a host cell genome and copied normal cellular genes of the host cell during their life cycle. In this process the normal cellular genes or proto-oncogenes were mutated and became oncogenes (Bishop, 1983).
Proto-oncogenes encode proteins which have a function during normal cell-growth and differentiation. So far, three types of proteins encoded for by proto-oncogenes were found:
1) Proteins with phosphorylating capacities
2) Proteins that use GTP-GDP for signal transduction
3) Proteins that control transcription of DNA (Bishop,1991).

For the discussion of the role of these proteins in normal cell-growth and differentiation and the role of tumor proteins in carcinogenesis, I refer to Chapter IV.

b. Transfection assays.
Another line of research generated additional evidence for the existence of tumorigenic genes. This work was based on experiments by Hill and Hillova (1972). Tumors were induced in mice by chemical substances or oncogenic viruses. DNA was isolated from these tumors. When the isolated DNA was introduced into a non-tumorigenic cell line, the cells acquired a malignant phenotype (Shih et al, 1979, Cooper et al, 1980). The conclusion was that the introduced DNA contained tumorigenic properties. Later experiments using DNA that was directly isolated from human tumors showed the same results (Krontiris and Cooper, 1981).

Both the study of onco-viruses and transfection assays largly contributed to the basic understanding of tumor formation by oncogenes. One of the first human oncogenes which was cloned was derived from a human bladder carcinoma cell line (Pulciani et al, 1982, Shih and Weinberg, 1982). Structural analysis revealed that the gene was homologous to the retroviral ras oncogene and that it was a slightly altered version of a normal cellular gene (Parada et al, 1982, Santos et al, 1982). The final proof that proto-oncogenes can turn into oncogenes was given by a functional assay: a normal human proto-oncogene was linked to viral sequences which caused overexpression of the human gene. Introduction of the construct gene into non-tumorigenic cells caused transformation (Chang et al, 1982).

The link between oncogenes and chromosomal aberrations in man was made when translocation breakpoints were found to be located close to or in proto-oncogenes (Taub et al, 1982, Dalla-Favera et al, 1982 and 1983, De Klein et al, 1982, Heisterkamp et al, 1983). The first two genes which were discovered to be deregulated by chromosomal translocation were the myc gene, involved in the t(8;14), and the abl gene, disrupted by the t(9;22). Since mutation of one allel in a somatic cell is sufficient for activation of oncogenes, they are regarded as dominant in their behaviour as opposed to the tumor suppressor genes (Table 2).

3. Gene damage is found in many human tumors.

Many proteins encoded for by the normal homologues of tumor suppressor genes and oncogenes are probably components of a transducing system which conducts signals for growth and differentiation from outside the cell to the nucleus. Inactivation of tumor-suppressor genes or activation of oncogenes generates quantitatively or qualitatively abnormal gene products. These abnormal proteins deregulate processes involved in normal cell-growth and -differentiation (for review, see Nishimura and Sekiya, 1987). The study of human tumors at the genetic level indicates that abnormalities of both tumor-suppressor genes and oncogenes show a restricted number of structural changes. The main groups are: deletions, amplifications, point mutations and rearrangements.

a. Deletion: the complete gene or part of it is removed from the genome. This type of damage is often found in tumor-suppressor genes. In fact, the isolation of the RB-gene and other similar genes was possible by studying patients having large deletions in these genes (Friend et al, 1986, Gessler et al, 1990).
b. Amplification: the gene is present in more than its usual copy number. A well-known example is the amplified myc gene, resulting in its increased expression. This aberration

is found in neuroblastomas and small-cell lung cancer for example (Schwab et al, 1983, Nau et al, 1986). Amplification of myc in neuroblastomas indicates an increased malignant state of the tumor (Seeger et al, 1985).
c. Point mutation: one of the four bases in the DNA-molecule is replaced by any of the other three bases. Point mutations can either inactivate tumor-suppressor genes or activate oncogenes. The most extensively studied oncogene which is altered by point mutation is the ras oncogene (for review, see Lowy and Willumsen, 1986). Point mutations in ras have been found in many human tumors and leukemias (Lyons et al, 1988, Vogelstein et al, 1988, Bos, 1989, Radich et al, 1990, Hirsch-Ginsberg et al, 1990, Lübbert et al, 1990).
d. Rearrangement: part of the gene is juxtaposed to a different area in the genome. This type of gene disturbance is mostly found in leukemias and lymphomas. An example is Burkitt's lymphoma which is a B cell neoplasm. In 85% of such tumors a t(8;14)(q24;q32) is found. The translocation moves the c-myc gene on chromosome 8 to the immunoglobulin heavy chain locus on chromosome 14. Either intact c-myc or a 5' truncated c-myc is joined to one of the heavy chain constant region genes, usually $C\mu$. Since translation of myc starts in the second exon of the gene, no difference exists between MYC proteins from an intact or 5' truncated c-myc gene. However, the translocation causes loss of control at the transcriptional level resulting in increased expression of the c-myc gene (for review, see Croce and Nowell, 1985).
Part of this thesis deals with the t(9;22) which is found in the leukemic cells of patients with CML and ALL. This translocation moves the abl-gene from chromosome 9 to chromosome 22 where it fuses with the bcr-gene (De Klein et al, 1982, Heisterkamp et al, 1983, Groffen et al, 1984, for review, see Kurzrock et al, 1988). The structure of the chimeric bcr-abl gene will be discussed in Chapters II.2 and II.3. Furthermore, implications of the translocation for the function of the ABL protein are exposed in Chapter IV.

Abnormalities at the genetic level in neoplasms are non-random. Specific changes are associated with specific tumors or subtypes of leukemia in humans. Besides a diagnostic value, several of these gene abnormalities have a prognostic significance in predicting the outcome of certain diseases. Amplification of c-myc is found in advanced tumors or aggressive subtypes of colorectal cancer (Yokota et al, 1986, Heerdt et al, 1991). Deletions of H-ras or c-myb are a sign of progression in carcinomas and sarcomas (Yokota et al, 1986). Amplification of the HER-2/neu oncogene indicates proneness to relapse and shorter survival in patients with breast cancer (Slamon et al, 1987, 1989). K-ras point mutation is found in a subgroup of patients with adenocarcinomas of the lung associated with a poor prognosis and short survival (Slebos et al, 1990).
The exact role of the abnormal proteins in the origin or progression of human tumors is not completely clear. Study of the structure and function of altered genes and their protein products is essential to understand the origin and development of cancer (See further Chapter IV).

4. Scope of the thesis.

In this thesis the genetic analysis of two types of acute leukemia characterized by translocations will be described. Both translocations generate chimeric genes which are specific for these subtypes of leukemia. The non-random changes that occur at the molecular level can be used in addition to karyotyping to diagnose the presence of tumor cells.
The linkage of specific subtypes of acute myeloid leukemia with t(6;9) to a consistent rearrangement involving the dek-gene on chromosome 6 and the can-gene on chromosome 9 will be shown (Chapters III.2 and III.3). This is of clinical importance since a correct diagnosis and subclassification indicates the prognosis of the disease and helps to choose the right therapy, thereby improving the prognosis for a patient.
The second type of acute leukemia that will be discussed is the acute lymphoblastic leukemia associated with the t(9;22). Here, rearrangement of genes results in a chimeric bcr-abl gene on the Philadelphia chromosome. A variant of this chimeric gene was found and analyzed (Chapter II.3). The study of altered genes in leukemia provides us with a multitude of questions concerning their function and role in the origin of the tumor. However, unusual variants of tumor-specific products may help to solve parts of the mystery of tumor formation.

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CHAPTER II

ACUTE LYMPHOBLASTIC LEUKEMIA

Chapter II.1

AN INTRODUCTION TO THE DISEASE ACUTE LYMPHOBLASTIC LEUKEMIA (ALL).

Acute lymphoblastic leukemia (ALL) is characterized by the uninhibited proliferation of cells derived from the lymphoid compartment of the blood. Normally, these cells defend the body against attacks by foreign microorganisms or entrance by non-self material. B-cells produce immunoglobulines which are mediators of the humoral defense response while T-cells are the effectors of the cell-mediated immunity (Roitt, 1980).

ALL is a disease of childhood and among malignancies in children of the Western world it is the main cause of death (Breslow and Langholz, 1983). In adults this type of leukemia accounts for 20% of the acute leukemias. Forty years ago the diagnosis of acute leukemias was mainly based on clinical characteristics and morphology of the blood. The sudden onset of the disease which was often accompanied by fever was one of the striking features hence the name "acute" leukemia. The clinical features of ALL result from the effects of bone marrow failure (bleeding, infection, fatigue) and from the accumulation of leukemic cells (enlarged lymph nodes, hepatosplenomegaly, central nervous system involvement) (Macleod, 1981).

In the past the prognosis for a patient with this disease was gloomy and survival was short. However, examination of groups of patients with acute leukemia showed that parameters such as age and white blood cell count (WBC) were important indicators of prognosis. Development and use of a wide array of diagnostic methods revealed that diagnosis 'acute leukemia' covers a large number of subgroups, each showing its own response to therapy. Nowadays, patients with acute lymphoblastic leukemia are subclassified according to a combination of parameters. Current efforts are directed toward more individual therapy, which depends partly on the subgroup in which the patient is classified.

Classification of ALL.

In 1986 a group of physicians devised a mode for classifying ALL using morphologic, immunologic and cytogenetic findings (First MIC Cooperative Study Group, 1986). Nowadays, their stratification scheme is widely used and is based on the following phenotypes of the leukemic cells:

Hematomorphologic phenotype.
The joint efforts of hematologists from several countries resulted in the so-called FAB (French-American-British)-classification of leukemias which is based on hematomorphologic criteria (Bennett et al, 1976). This classification separated the acute leukemias of lymphoid origin from those of myeloid origin. Acute lymphoblastic leukemia is subdivided into three groups (L1, L2 and L3) according to the FAB-classification. In table 1 the main characteristics of each subgroup are given.

Table 1. Hematomorphologic phenotype of ALL according to the FAB-classification

FAB	Blasts	Age-distribution
L1	Small uniform lymphoblasts	Most common subtype in children
L2	Large pleomorphic lymphoblasts	More common in adults
L3	Large uniform lymphoblasts one or more cytoplasmic vacuoles (resembles Burkitt's lymphoma)	Children or young adults

Ref. Bennett et al., 1976.

L1 and L3 mainly occur in children while L2 is mostly found in adults. The FAB-classification per se is a minor contributor to the prediction of the prognosis of ALL but is essential for diagnosis in combination with other parameters (see below).

Immunophenotype.
A second system of subclassification of ALL is based on immunologic criteria. Here, the blasts are characterized via various antigens which are mainly expressed on the cell surface (Foon and Todd, 1986). Specific patterns of antigen expression indicate the B- or T-cell lineage as the origin of the blast. Moreover, analysis of immunoglobulin-genes and T-cell receptor genes at the DNA-level defines specific stages of B- or T-cell development in which leukemic derangement has occurred (Arnold et al, 1983, Waldmann et al, 1985). In table 2 a simplified scheme is given for the subclassification of ALL, based on immunologic criteria. 80% of cases of ALL are derived from the B-cell lineage and are mostly seen in children, while T-cell ALL is mainly found in adults. Some ALLs neither have B- nor T-cell features and are called null-ALLs.
Due to increasingly refined techniques quite a number of ALLs are found to express both myeloid and lymphoid markers on their cell surface (Smith et al, 1983, Mirro et al, 1985, Sobol et al, 1987). These are called the hybrid or biphenotypic leukemias. This specific subgroup is associated with a poorer prognosis than the exclusively lymphoid ALL (Pui et al, 1987a). Moreover, prognostic adverse translocations such as t(9;22) or those involving 11q23 are found in this subgroup (Stong et al, 1985, Mirro et al, 1986, Berger et al, 1990).

Karyotype.
Since ALL is a disease mainly occurring in children, large scale cytogenetic research was focused on this age group. Clonal abnormalities, both numerical and structural, are found in 75 to 90% of childhood-ALL cases (Stewart et al, 1986, Heinonen et al, 1988). However, similar studies on adult patients are reported with increased frequency (Bloomfield et al, 1989, Secker-Walker, 1990). These indicate that the same numerical and structural chromosomal abnormalities may be found in this age group albeit in different frequencies from those found in children.

Table 2. Subclassification of ALL based on immunologic criteria

	lineage	blast cells		immunophenotype		prognosis
ALL	80 % B cell	majority CALLA (= CD10) + (mostly children)	80 % cytoplasmic Ig - =	early pre B		favourable
		B cell differentiation antigens +	20 % cytoplasmic Ig + = (= cIg + = CD19 +)	pre B		worse than early pre B
		Ig genes rearranged				
		surface Ig -				
		< 5 %: express surface antigens of mature B cells (including surface Ig) often found with: high WBC, CNS involvement, translocations (± 100 %), FAB - L_3		B cell		poor
	10-20 % T cell	E-rosette receptor +		early		child: poor
	more in adults	T cell differentiation antigens +		intermediate	thymocyte types	
	44 % + translocations	CALLA -		mature		adult: better with intensified chemotherapy
		T cell receptor genes rearranged				

CALLA: common ALL antigen
Ig: immunoglobulin
WBC: white blood cell count
CNS: central nervous system

ref.: Champlin and Gale, 1989; Crist et al, 1989

In fact, the difference in frequency of the Ph chromosome in both groups is one of the important reasons for the adverse prognosis of ALL in adults in contrast to the rather good prognosis of ALL in children.
Cytogenetic findings are an independent prognostic factor in children with ALL while in adults it largly contributes to prognosis in combination with other parameters (Bloomfield et al, 1989). Karyotypic abnormalities can be divided into abnormal chromosome number or structural abnormalities.

1. Numerical abnormalities:
According to chromosome number three main groups are discerned: hyperdiploid (more than 46 chromosomes), pseudodiploid (46 chromosomes but structural abnormalities present), and hypodiploid (less than 46 chromosomes). A representation of these groups is given in tabel 3.
Especially in the hyperdiploid group, the subgroup with more than 50 chromosomes per leukemic cell but no structural abnormalities carries a favourable prognosis (Secker-Walker et al, 1978, Bloomfield et al, 1989, Pui et al, 1989). The majority of patients in this specific group show other favorable features such as low WBC and a cALL immunophenotype (Kaneko et al, 1982). Although the over-all long term event free survival in this group is 75% a problem may be late relapse (Bloomfield et al, 1989, Secker-Walker et al, 1989).

Table 3. **Chromosomal ploidy in childhood ALL**

Ploidy			Frequency	Prognosis
Near haploid			< 1%	Poor
Hypodiploid	(< 46 chromosomes)		± 7%	Poor
Peudodiploid	(46 chromosomes, + abnormalities)		41.5%	Poor
Normal diploid	(46 chromosomes, - abnormalities)		8%	Intermediate
Hyperdiploid	(> 46 chromosomes)	47 - 50 chr.	15.5%	Intermediate
		> 50 chr.	27%	Good
Near triploid/tetraploid			< 2%	Unknown

Ref. Pui et al., 1990[b].

Table 4. Non-random chromosomal abnormalities in ALL

	Involved Genes		Frequency		Prognosis
			Child	Adult	
Pre-B/Early pre-B					
t(9;22)(q34;q11)	ABL	BCR	3 - 5%	17 - 25%	Poor
t(1;19)(q23;p13)	PRL	E2A	5 - 6%		Modest - Poor
t(11:V)(q23;V)			6%		Poor (especially t(4;11))
t(12;V)(p12-p13;V)			5%		
dic(9;12)(p11 - p12; p12)			1 - 2%		
dic(7;9)(p13;p11)			< 1%		
Mature B-cells					
t(8;14)(q24;q32.3)	MYC	IgH	2%		Poor
t(8;22)(q24;q11)	MYC	IgL	0.3%		Poor
t(2;8)(p11 - p12; q24)	IgK	MYC	Rare		Poor
t(14;18)(q21;q32)	BCL2	IgH			
T-cells					
t(11;14)(p13;q11)	TCL2	TCRα/δ	1%		Variable
t(11;14)(p15;q11)	TCL1	TCRα/δ	Rare		
t(10;14)(q24;q11)	TCL3	TCRα/δ	< 1%		
inv(14)(q11;q32.3)	TCRα/δ		< 1%		
t(1;14)(p32 - p34; q11)	TAL1	TCRα/δ	Rare		
t(8;14)(q24;q11)	MYC	TCRα/δ	Rare		Poor
t(7;V)(q34 - q36; V)	TCRß		2%		
t(7;19)(q35;p13)	TCRß	LYL1	Rare		
6q-, 9p-					

V = variable chromosome, variable region

Ref. Champlin and Gale, 1989
Secker-Walker, 1990
Pui et al., 1990[b]

2. *Structural abnormalities:*
Structural aberrations are mainly seen in the pseudodiploid and hypodiploid groups and are mostly translocations. They usually herald a deteriorated prognosis for an individual patient since they are associated with an increased risk for early treatment failure (Third International Workshop on Chromosomes in Leukemia, 1981, Williams et al, 1986, Pui et al, 1990b). Some translocations are particularly associated with a poor prognosis such as t(9;22), t(8;14) or t(4;11). So far, several oncogenes involved in translocations have been isolated. Their exact role in the origin of the leukemia is still unknown. However, the general assumption is that most play a crucial role in this proces. In table 4 the subdivision of ALL according to cytogenetic characteristics is given.

Subdivision of ALL into risk-groups.

Prognosis for each individual patient depends on various interrelated factors. These are: age, sex, race, WBC, karyotype, immunophenotype, involvement of the central nervous system (CNS) in the disease, extent of lymphadenopathy and response to therapy (Bennett et al, 1976, Sather et al, 1981, Kalwinsky et al, 1985, Pui et al, 1987a, Hoelzer et al, 1988). The stratification of ALL into different risk-groups is essential for predicting the chance of cure for a patient and for choosing the appropriate therapy regimen in each individual case (reviewed by Champlin and Gale, 1989). Coincidentally, this subdivision follows the parameter age.

Infants 0-1 year: this group in general is annotated as a high-risk group and carries a poor prognosis (Crist et al, 1986). ALL in infants shows aggressive behaviour (high WBC, CNS-involvement, hepatosplenomegaly) and poor response to modern chemotherapeutic regimen. The unfavourable prognosis in this specific age group is mainly due to increased incidence of hybrid leukemias and overrepresentation of translocations involving 11q23 (Reaman et al, 1985, Pui et al, 1987b, Ludwig et al, 1989, Raimondi et al, 1989).

Children 2-10 years: this is the low to average risk-group. Most patients with ALL are found here. The majority carries a good prognosis and responds very well to the chemotherapy courses (Champlin and Gale, 1989, Pui et al, 1990b). This is mainly due to the presence of most cases of hyperdiploidy with more than 50 chromosomes, the subtype of ALL which is curable, in this age group. However, as soon as a translocation is found the prognosis declines and is defined by the structural aberration (Williams et al, 1990).

Adolescents 11-21 years: this group was recently separated from the children since their sole age showed to have an adverse effect on prognosis (Crist et al, 1988, Santana et al, 1990). Research so far indicates that adolescents of 11 to 16 years tend to show some favorable prognostic features as found in children while adolescents 16 to 21 years resemble adult ALL-patients. However, more research on a larger number of adolescent patients is needed before any defined conclusions can be given concerning prognosis and treatment outcome in this particular age group.

Adults over 21 years: this age group forms a minority in ALLs and prognosis is clearly inferior to that in children. Most adults with ALL are less than 30 years of age. Increasing age predicts lower remission rates and shorter remissions after chemotherapy, both

indicators of a poor outcome (Hoelzer et al, 1988). T-cell and null-cell ALLs are relatively common in this age group.

Modern chemotherapy, using vincristine and prednisone as induction treatment followed by maintenance therapy, can cure 60% of children and 30% of adults suffering from ALL (Jacobs and Gale, 1984, Pinkel, 1987, Champlin and Gale, 1989, Rivera et al, 1990). Indispensable for this success rate was the recognition of the various risk-groups in ALL, each with its own clinical and biologic characteristics. The practical consequence of this subdivision was that groups with poorer prognoses were treated with intensified chemotherapeutic regimen. As a result, several prognostic factors lost their predictive value especially for a dire outcome of the disease (Gaynon et al, 1988, Fletcher et al, 1989, Secker-Walker et al, 1989, Pui et al, 1990a). However, many high-risk groups remain, e.g. with hypodiploidy or the Ph translocation, in which even the contemporary chemotherapeutic courses seem to have little or no effect (Bloomfield et al, 1990). Probably, in these specific patients alternative ways of therapy should be tried such as bone marrow transplantation (Forman et al, 1987, Crist et al, 1990).

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Chapter II.2

THE PHILADELPHIA TRANSLOCATION IN CML AND ALL: RECENT INVESTIGATIONS, NEW DETECTION METHODS

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Summary
As a consequence of the Ph translocation, a bcr-abl fusion gene is formed that encodes chimeric BCR-ABL proteins. The latter are probably causally involved in leukemogenesis, although the mechanism is not yet known. Meanwhile, the heterogeneous aspects of the Ph translocation have been explored in an effort to disclose their eventual clinical significance. Innovative and useful methods have been devised for the detection of the Ph translocation products e.g. the PCR technique or the produc-tion of specific antibodies that undoubtedly will prove to be of great help to patients and clinicians in future.

Introduction
The Philadelphia (Ph) chromosome is the hallmark of chronic myeloid leukemia (CML), where it is found in more than 95% of the cases. Cytogenetically, the standard Ph results from a reciprocal translocation t(9;22)(q34;q11). Variant translocations involving at least one additional chromosome are seen in about 5% of the cases (1). Molecularly, a truncated c-abl oncogene is transferred from 9q34 to the bcr gene on chromosome 22q11. A recombinant gene consisting of 5' bcr sequences and 3' c-abl sequences is formed. This gene encodes a hybrid mRNA, which is translated into chimeric BCR-ABL proteins exhibiting enhanced tyrosine kinase activity (2). The fusion proteins presumably play a key role in the oncogenesis of Ph+ leukemia. But, so far, the crucial question regarding the mechanism by which the activated abl oncogene participates in leukemic transformation is not yet answered.

The heterogeneity of the Ph chromosome can be analyzed using molecular techniques, that demonstrate variation in chromosomal breakpoints or in bcr-abl gene products. In recent years, clinically distinct groups of patients with Ph+ leukemias, have been investigated (for review see 3).

Ph+ Leukemias: Clinical association.
The Ph chromosome is found most consistently in CML at diagnosis and throughout the chronic phase. When acceleration or blast crisis occurs, a second Ph is often observed, together with other non-random additional chromosomal changes. The Ph is also found in other leukemias, particularly in 15 to 20% of adult ALL cases and in 1 to 2% of AML. It

is occasionally present in childhood leukemia (AML, ALL or adult type CML), in myelodysplastic syndrome, essential thrombocythemia or other myeloproliferative disorders.
In CML, the Ph indicates a more favorable outcome of the disease than in Philadelphia negative cases (Ph-), in contrast with acute leukemia where the Ph is associated with poor response to treatment and shorter survival.

Breakpoint in c-abl in CML and ALL.
The c-abl gene has two first exons (1A and 1B) that can be spliced to a set of c-abl common exons of which the first is termed a2 (2). The breakpoints in the Ph translocation are localized 5' of a2 and can be scattered over more than 200 kb of DNA. Whatever the localization of the breakpoint in c-abl, the exon a2 serves as splice acceptor for the bcr exon that is juxtaposed to it by the Ph translocation. In three exceptional cases, two Ph+ ALLs and one Ph+ CML, the abl exon a3 has been found to be spliced to bcr exons. (4,5).

Breakpoint in bcr gene in CML and ALL.
In CML, the breaks on chromosome 22 are clustered within a 5.8 kb BglII restriction fragment, termed M-BCR-1 for major Breakpoint Cluster Region (6). The M-BCR-1 is situated in the central part of the bcr gene and contains 4 small exons designated b1 to b4 (Fig. 1). Breakpoints cluster precisely in the 2 introns between b2 and b3
and between b3 and b4. After translocation of c-abl sequences, splicing of either b2 or b3 to the abl a2 exon takes place during pre-mRNA processing, resulting in 8.5 kb chimeric mRNA with specific b2a2 or b3a2 bcr-abl joining regions. As a result of this heterogeneity, two different $p210^{bcr\text{-}abl}$ fusion proteins are encoded that differ by only 25 amino-acids (encoded by exon b3). In Ph+ ALL, the breakpoint in bcr may take place much further upstream of M-BCR-1 in the first intron of the bcr gene, designated m-BCR (Minor Breakpoint Cluster Region).

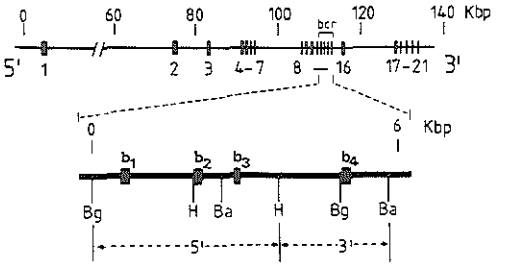

Fig.1 Schematic representation of the bcr gene according to Heisterkamp et al. (8). Exons are indicated by vertical barre. The M-BCR-1 region is enlarged, showing the exons b1 to b4, the restriction sites and the arbitrary division in 5' and 3' segments. Bg: BglII, H: HindIII, Ba: BamHI.

Translocation of c-abl to this position of bcr results in transcription of a 7 kb bcr-abl fusion mRNA in which the first exon of bcr (e1) is spliced to abl exon a2, producing an in frame e1-a2 junction (7). This mRNA encodes a chimeric protein of 190 kD ($P190^{bcr-abl}$) with enhanced phosphotyrosine kinase activity. The first intron of bcr measures 68 kb (8) and detection of chromosomal breakpoints by Southern blotting is not very practical (9). Alternative methods have been used to demonstrate the occurrence of breakpoints in first bcr intron i.e. 1) long range mapping by pulsed-field gel electrophoresis (10); 2) amplification by PCR of cDNA fragments containing the e1-a2 junction; 3) demonstration of the $P190^{bcr-abl}$ protein by immunoprecipitation technique. Approximately half of the cases of Ph+ ALL are M-BCR-1+ (with CML specific breakpoint); the other half is Ph+, M-BCR-1- and in the majority of these cases a breakpoint is found in the first intron of the bcr gene (m-BCR). Actually, in a small number of ALL patients with (variant) Ph translocation, neither type of breakpoint could be demonstrated. However, few, if any of these cases have been analyzed carefully enough to jump to the conclusion that an alternative oncogenic c-abl activation may exist, not involving bcr.

Mapping of BCR breakpoint in CML.

Southern blot analysis of bcr has been performed in a large number of CML patients. Because of polymorphism in restriction sites and comigration of restriction fragments, several probes and enzymes should be used to allow a solid mapping of the breakpoint within M-BCR-1 (Fig. 1). In 1987, Schaeffer-Rego et al., reported a positive correlation between a 3' localization of M-BCR-1 breakpoint and early occurrence of blast crisis (11). These results were not confirmed by a number of other investigators (12-14). A methodological difficulty in these studies is that the restriction analysis used does not define the position of the breakpoints accurately enough. Therefore, the area designated as 5' may have a breakpoint at either 5' or 3' sides of exon b3. Also, recruitment and treatment of patients in different series is too heterogeneous for valid comparison.

In blast crisis of CML, the bcr rearrangements remain the same as in chronic phase, although secondary changes are observed in approximatively 10% of the cases. But, so far, the number of patients sequentially studied is still very limited.

Ph negative CML.

A small percentage ($\leq$ 5%) of patients with a presumptive diagnosis of CML do not have the Ph chromosome (3,15). The karyotype is normal in 2/3 of the cases. A variety of chromosomal abnormalities are found in the remaining cases, among which translocation of 9q34 with another chromosome than 22 was observed. Currently patients of earlier reports have been reclassified as MDS (mostly CMML and Juvenile CML) on morphological ground (16). A number of patients with typical CML clinical features and evolution shows molecular changes identical to the normal Ph translocation, and in situ hybridization studies have mapped bcr and c-abl sequences at unusual chromosomal positions (15). These cases are clinically indistinguishable from the patients with Ph+ CML. A small group of patients remains that appears to have rather typical CML disease at presentation, but fails to show a Ph chromosome both cytogenetically and molecularly. Follow-up information on these cases is too scarce for clinical evaluation. Larger prospective studies are needed to assess the clinical significance of these negative findings and to explore the possibility of completely novel molecular events participating in leukemogenesis in these patients.

PCR studies of the Ph translocation.
The polymerase chain reaction (PCR) method allows enzymatic amplification of short genomic DNA segments in vitro. However, the technique is not immediately applicable to the detection of the t(9;22) on genomic DNA in CML because of the wide variation of chromosomal breakpoints. The PCR technique has been modified in order to amplify the cDNA copied from the hybrid portion of the specific bcr-abl mRNA transcripts, i.e. the e1-a2, b2-a2 or b3-a2 junctions. Adequate choice of primers will allow amplification of the junction specific cDNAs and of normal c-abl cDNA as internal control. This is particularly useful to demonstrate a breakpoint in the first intron of bcr (m-BCR) (7) although it is also used to discriminate between b2-a2 and b3-a2 mRNA junctions in Ph+, M-BCR-1+ leukemia. Dilution experiments have shown that one Ph positive cell/10^5 to 10^6 normal cells can be detected. Thus, the PCR method is used to diagnose residual disease in autografts and for follow-up of patients that became Ph- by cytogenetic analysis (and Southern blotting) after bone marrow transplantation or interferon treatment (17). But, because of the extreme sensitivity of the PCR method, the danger of finding false positive signals due to DNA contamination is quite high. Extreme care should be taken carrying out these experiments, and negative controls should always be included.

Immunodiagnosis of CML.
$P210^{bcr\text{-}abl}$ and $P190^{bcr\text{-}abl}$ are expressed exclusively in Ph+ leukemias and are thus by definition tumor specific proteins. However, they are hybrid proteins composed in part of BCR and ABL sequences which themselves are normal cellular proteins, expressed in most cells. The tumor specific determinant of the fusion proteins is found in the unique amino-acid composition found at the joining of BCR and ABL sequences. Three types of junction are currently found at the mRNA level i.e. e1-a2, b2-a2 and b3-a2 (7). Oligopeptides corresponding to the predicted amino-acid sequences of the different BCR-ABL junctions have been synthesized and used as immunogens. Van Denderen et al. have isolated a polyclonal antiserum "BP-ALL" that specifically and uniquely recognizes e1-a2 $P190^{bcr\text{-}abl}$ and not $P210^{bcr\text{-}abl}$ (18). Another antiserum "BP-1" recognizing the b2-a2 junction sequences of $P210^{bcr\text{-}abl}$ had been isolated previously (19). Attempts to isolate an antiserum specific for the b3-a2 joining in $P210^{bcr\text{-}abl}$ have not yet been successful. BP-1 and BP-ALL are used to determine the specific BCR-ABL protein expressed by the cells using immuno-precipitation experiments combined with autophosphorylation. Therefore this approach is applicable to the study of acute leukemia, blast crisis of CML or otherwise purified blast cell populations. Methodological difficulties still have to be overcome to make this antisera directly applicable and perform immunocytochemical detection of BCR-ABL fusion proteins.
So far, the demonstration of antigenic specificity of the fusion protein opens perspectives for future immunodiagnosis of CML and ALL, a development of great interest to clinicians.

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Chapter II.3

A NOVEL VARIANT OF THE BCR-ABL FUSION PRODUCT IN PHILADELPHIA CHROMOSOME-POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA.

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Summary

Two patients with Philadelphia chromosome-positive acute lymphoblastic leukemia showed novel variants of the chimeric bcr-abl mRNA. The bcr-abl breakpoint region on cDNA derived from the chimeric mRNA was amplified, using the polymerase chain reaction (PCR). Sequence analysis of the breakpoint-containing fragment showed that in both patients exon a2 of the abl gene was deleted, giving rise to an in-frame joining at the mRNA level of 5' bcr-sequences to the abl exon a3. These findings were confirmed by Southern blot analysis and cloning of chromosomal DNA. Protein studies showed a BCR-ABL protein with heightened tyrosine kinase activity in blast cells of both patients: one of the P190 type, the other of the P210 type. The significance of these findings and the role of this new type of translocation in the deregulation of the abl gene are discussed.

Introduction

In 95% of the patients with chronic myeloid leukemia (CML) the Philadelphia (Ph) chromosome is found, which is the result of a reciprocal translocation between chromosomes 9(q34) and 22(q11) (1). Due to this event the abl oncogene is translocated to the major breakpoint cluster region (M-BCR-1) of the bcr gene on chromosome 22 (2,3). So far, the breakpoint in the abl gene on chromosome 9 seems to occur in a 200-kilobase (kb) intron, always 5' of abl exon a2 (4). Most breakpoints in the M-BCR-1 region on chromosome 22 are located between exons b2 and b3 or b3 and b4. The bcr-abl fusion gene on the Ph chromosome is transcribed into an 8.5-kb chimeric bcr-abl mRNA that shows either a b2a2 or a b3a2 joining at the mRNA level (5-8). The 8.5-kb bcr-abl mRNA is translated into a 210-kilodalton (kD) BCR-ABL fusion protein showing enhanced in vitro tyrosine kinase activity when compared to the normal 145-kD ABL protein (9).

The Ph chromosome does not solely occur in CML but can also be found in approximately 20% of adult patients with acute lymphoblastic leukemia (ALL) (10). Roughly 50% of these cases show a bcr-abl joining at the mRNA level similar to CML

(11). The remaining 50% have a breakpoint 5' upstream from the M-BCR-1 region, i.e. in the first intron of the bcr gene (minor breakpoint cluster region, m-BCR-1) (12). In the latter case the bcr-abl fusion gene on chromosome 22 gives rise to a 7.0-kb mRNA. Here the first exon of the bcr gene (e1) is spliced to the second exon of the c-abl gene (a2), resulting in an e1a2 joining (13). The corresponding hybrid protein has a molecular weight of 190 kD and also shows enhanced tyrosine kinase activity (14-17, for review see Ref. 18).

The elucidation of the molecular structure of the bcr-abl chimeric product and the development of the highly specific and sensitive polymerase chain reaction (PCR) allows us to diagnose Ph positivity in patients with leukemia. Therefore, patients with a Ph chromosome are routinely screened in our laboratory by PCR analysis to determine breakpoint joinings at the mRNA-level, i.e. e1a2, b2a2 and b3a2 (19). Surprisingly two patients with ALL were found who showed an amplified bcr-abl fragment that did not hybridize to any of the available breakpoint probes. Sequence analysis revealed a deletion of abl exon a2 in the bcr-abl mRNA, which was confirmed by DNA-studies. Both patients expressed a BCR-ABL protein, one of the P190 type, the other of the P210 type. To our knowledge, these two patients are the first reported to have such a deletion in association with a Ph-positive leukemia.

Materials and methods.

Patients.

Both patients were diagnosed and treated in Rotterdam, the Netherlands: patient 1 at the University Hospital Dijkzigt, patient 2 at the Dr. Daniel den Hoed Cancer Center. The diagnosis ALL was based on the clinical and hematologic data, bone marrow morphology and immunophenotyping of the blast cells (Table 1).

At the time of diagnosis patient 1, a 39-year-old female, presented with a history of bleeding and infections. On clinical examination there were no signs of lymphadenopathy or hepatosplenomegaly. The white blood cell count (WBC) was 8.4×10^9/liter showing 60% lymphoblasts. A bone marrow aspirate showed hypercellularity with 75% lymphoblasts. Cytochemically, 70% of the blasts were characterized by positivity for ANA esterase with resistance to NaF; 30% of the blasts were PAS-positive. Peroxidase and acid phosphatase staining were negative. Immunophenotypical analysis of the lymphoblasts showed reactivity with antibodies for CD10, CD19, CD24, HLA-DR and TdT.

Table 1.

HEMATOLOGIC AND CYTOGENETIC DATA OF CELLS USED FOR THE MOLECULAR INVESTIGATION OF PATIENT 1 AND 2.

Patient	Age/Sex (Yrs)	Clinical Phase	WBC $x10^9$/l	% Blasts BM	% Blasts PB	Immuno-phenotype	Karyotype
1	39,F	relapse	40.0	89	82	pre-pre-B	46,XX(70%)/46,XX,t(9;22) + other aberrations (30%)
2	61,F	diagn.	209.0	79	53	pre-B	46,XX(44%)/46,XX, t(9;11;22)(56%).

Partial positivity for CD20 and CD34 existed. From this pattern it was concluded that the patient had pre-pre-B acute lymphoblastic leukemia. Remission-induction treatment consisted of daunorubicin, vincristin, L-asparaginase, and prednisone. In addition, CNS prophylaxis was given (methotrexate and dexamethasone). Complete remission was attained 2.5 months after diagnosis. Despite consolidation-maintenance therapy, 3 months later a florid relapse occurred with more than 80% blast cells in bone marrow and peripheral blood. The patient died 9 months after diagnosis.

Data of patient 2 have been published previously (ref.11: patient R6, 20). Briefly, at the time of diagnosis this 61-year-old female presented with a 3-month history of fatigue and fever. Clinical examination showed a hepatosplenomegaly and a leucocytosis (WBC $209x10^9$/liter). The differential count showed 53% blast cells, 1% myelocytes, 5% metamyelocytes, 20% neutrophils, 9% bands, 4% monocytes, 1% eosinophils, 6% lymphocytes. The bone marrow was hypercellular with 79% blast cells, which were Sudan Black negative and PAS positive. The blasts were of the immunologic pre-B phenotype (TdT+, CD10+, CIg+). The diagnosis ALL was made although a CML in blast crisis at presentation could not be ruled out. Remission-induction treatment (daunorubicin, vincristin and prednisolone) and CNS prophylaxis were given, and subsequently a complete remission was achieved. Notably, although cytogenetic study of the bone marrow showed persistence of the Ph chromosome in 70% of the metaphases, there were no hematologic features during remission to suggest a chronic phase of CML; 10.5 months later this patient suffered from a relapse of the ALL and died after a total survival time of 14 months.

Samples.
Bone marrow aspirates and blood samples were part of diagnostic and clinical follow-up studies. Data of specimen used for the molecular investigations are presented in Table 1. Sterile samples were collected in heparinized tubes. After isolation of blast cells by Dextran or a Ficoll-Hypaque gradient, cells were frozen and stored in liquid Nitrogen until used.

Karyotyping.
Cytogenetic studies were performed using standard procedures. Chromosomes were identified by G, Q and R banding techniques and classified according to the ISCN (1985) (21). In both patients PHA-stimulated blood cultures were used to determine the constitutional karyotype.

Polymerase Chain Reaction (PCR).
The RNA-cDNA preparation was performed as described by Hermans et al. (19) with the following modifications for the PCR: Half of the cDNA preparation was used for amplification of a control abl fragment; the other half was used for amplification of the bcr-abl fragment. The PCR was performed in a volume of 55 μl. Changes in the composition of the Taq polymerase buffer were as follows: the molarity of $MgCl_2$ was raised from 6 mM to 9 mM, and 40 mM KCl was added. The molarity of the dNTPs was raised from 2.5 mM to 5 mM each. Amplification of the bcr-abl fragment was performed by adding 0.25 μg of primer 2 and 3 per PCR-reaction (Fig.1). As a positive control for the PCR experiment, a 450-bp abl fragment was amplified, which was present both in healthy individuals and in patients with leukemia. This was done by combining primer 1 with a 25-mer sense oligonucleotide located in exon IB of the human abl gene

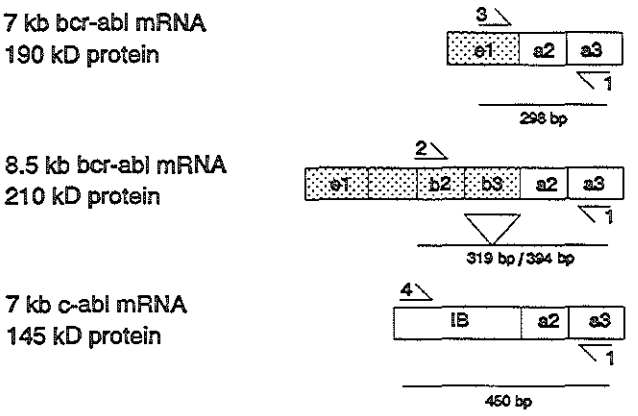


Figure 1. The various mRNA-molecules studied by cDNA preparation and the PCR are represented by bars. Exons derived from the bcr gene are hatched, and exons from the abl gene are open. Primers for the PCR are depicted as arrows and numbered according to the text. Our primers 1, 2 and 3 are the same as c-abl a3, mcr b2, and bcr gene 1st exon e1, respectively, in Hermans et al (19). The size of the fragments generated by the PCR is given underneath the corresponding fragments. The triangle indicates that exon b3 may be present or absent in the 8.5 kb bcr-abl mRNA.

(ATGCAGCGAATGTGAAATCCCACGT, primer 4 in Fig.1). To exclude contamination during the PCR by bcr-abl-containing fragments, RNA from a healthy individual was reverse transcribed and amplified as a control in all the experiments. One unit of Taq polymerase (Cetus Corp., Emeryville, CA.) was added per PCR-reaction, and the reaction mixture was covered by a layer of paraffin oil. Denaturation of the sample was allowed for 3 min. at 93°C, and annealing of the primers was allowed for 2 min. at 55°C. This was followed by 24 cycles of extension (5 min., 70°C), denaturation (1 min., 93°C), and annealing (1 min., 55°C). Ten percent of the reaction was used for another 24 cycles after adding new reagents. Ethanol precipitation was followed by electrophoresis of 30% of the reaction mixture after 48 cycles of amplification through a 2% agarose gel. Four identical Southern blots on nylon filter (Zeta probe, Biorad Lab., Richmond, CA.) were prepared from this gel. Each filter was hybridized separately to ^{32}P end-labeled breakpoint oligonucleotides specific for the b2/b3-a2 and e1a2 junctions at 65°C (probes described in Ref. 19). A 25-mer oligonucleotide (TTGAACCCTCTTCTGGAAAGGGGTA), situated 43 nucleotides 3' of primer 4 in the IB exon of the human abl gene, was hybridized at 42°C to detect the normal abl fragment.

Construction and Screening of λ EMBL-3 Library of Patient 2.
High molecular weight DNA of patient 2 was partially digested with MboI and cloned into the BamHI site of λ EMBL-3 as described by Frischauf et al (22); 10^6 independent plaques were screened according to the method of Benton and Davis (23), using a genomic 2-kb 5' M-BCR BglII-HindIII fragment. Four hybridizing plaques were rescreened with the 0.9-kb SauI-KpnI genomic abl fragment. One of the four plaques appeared to hybridize to the abl probe and contained a 13.5-kb insert. The 3.5-kb bcr-abl chimeric BglII fragment was subcloned into the BamHI site of PUC 19.

Sequence Analysis.
After amplification of the bcr-abl fragment of patient 1 by the PCR, the reaction-mixture was loaded on a 2% low-melting point agarose gel (BRL, Gaithersburg, MD.), and the band of interest was cut out from the gel. DNA was isolated using phenol/chloroform extraction, and the amplified fragment was cloned into a Bluescript vector (Stratagene, La Jolla, CA.). Nucleotide sequences were obtained by the dideoxy chain termination method (24) on double stranded DNA (25). In the case of patient 2, separation of the amplified bcr-abl fragment from the PCR amplification primers was obtained by filtration over a Centricon S-100 device (Amicon, Danvers, MA.) (26). Direct sequencing of the purified fragment was performed according to Winship (27).
For sequence analysis of chromosomal DNA fragments of patient 2, a 0.5-kb M-BCR PstI-SmaI genomic fragment that contains the M-BCR 5' BamHI site was subcloned into PUC 19. The DNA sequence of both strands was determined according to the method of Maxam and Gilbert (28) using PstI, BamHI and SmaI as labeling sites. The sequence strategy for the genomic SauI-KpnI abl fragment containing parts of abl exons a2 and a3 has been published by Grosveld et al (6). The sequence of the chimeric portion of the 3.5-kb BglII bcr-abl fragment of patient 2 was determined by the method of Maxam and Gilbert (28) using the BamHI site in M-BCR and the SalI site in abl as labeling sites.

Southern Blot Analysis of Patient 1.
High molecular weight DNA was extracted by the usual techniques and blotted, after digestion with the restriction enzymes BglII, BamHI or KpnI (29). As a probe for the detection of a breakpoint between abl exon a2 and a3, a 0.9-kb SauI-KpnI genomic fragment was used that covers the intron between both exons (6).

Protein Studies.
Chimeric proteins from blast cells of both patients were analyzed by immunoprecipitation and followed by autophosphorylation as described previously (30). BCR-ABL proteins were precipitated with anti-ABL and anti-BCR polyclonal antisera. The anti-ABL antiserum was raised against a synthetic peptide with proven antigenecity comprising the amino acids: CSISDEVEKELGK (31).

The polyclonal anti-BCR antiserum was raised in rabbits against the NH2-terminus of the BCR protein as described previously (30). Blast cells of an ALL patient with e1a2 $P190^{BCR\text{-}ABL}$ were precipitated as a control for patient 1. In the case of patient 2, blast cells of a CML patient and BV173 cells, both containing $b2a2^{BCR\text{-}ABL}$ (32), were precipitated as controls.

Results

At the time of diagnosis only five metaphases from peripheral blood of patient 1 could be analysed. They showed a 46, XX normal karyotype. At the time of relapse, cytogenetic analyses were performed twice on bone marrow and blood, mounting to a total of 110 cells karyotyped. These showed a mosaicism of normal and Ph positive cells with additional changes of the following type: 46, XX(70%)/ 46, XX, t(9;22)(q34;q11)(2%)/ 46, XX, t(9;22), inv(1)(p36;q42), del(15)(q22)(10%)/ 46, XX, t(9;22), inv(1), del(15), t(4;6;17)(q26;q16;q24)(18%) (Fig. 2A).

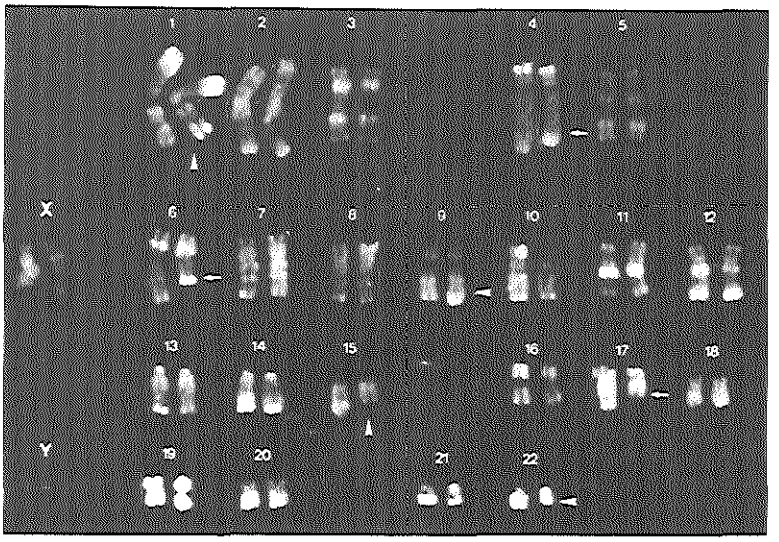


Figure 2A. Karyogram of patient 1 showing t(9;22), inv(1), del(15), t(4;6;17). R-banding with acridine orange.

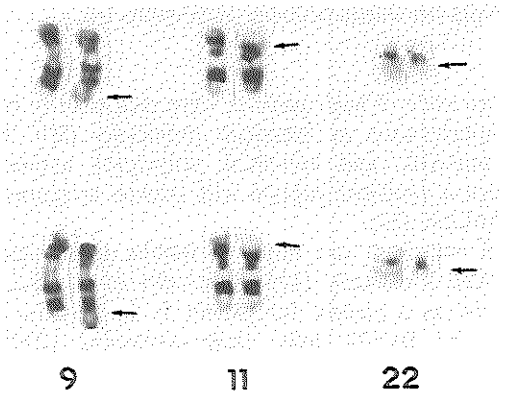


Figure 2B. Partial karyotype of patient 2 showing t(9;11;22). G-banding with Trypsin-Giemsa staining.

Patient 2 was previously reported (11) and showed a variant of the Ph translocation at diagnosis, i.e t(9;11;22) in 56% of the metaphases (Fig.2B). Seventy percent of the karyotypes remained Ph-positive during clinical and hematologic remission, and additional chromosome abnormalities heralded progression and relapse of the disease.
Since patient 1 showed a Ph chromosome in metaphases of blood and bone marrow cells, PCR analysis was performed on cDNA preparations of RNA of this patient in order to determine the type of bcr-abl joining at the mRNA level. None of the available oligonucleotide probes complementary to the already known breakpoint joining regions that occur in Ph-positive leukemia (i.e., e1a2, b2a2, or b3a2) hybridized to any bcr-abl fragment in the PCR mixture of this patient (Fig. 3, sensitivity of the PCR $1:10^4$-10^5, D. Soekarman, unpublished results). However, the PCR generated a fragment of approximately 120 bp, which was clearly visible on the agarose gel stained with ethidium bromide (Fig. 3, patient 1). We hypothesized that a fragment of such size could be produced if abl exon a2, spanning 174 bp, missed from the 298-bp fragment normally detected after amplification of the e1a2 bcr-abl joining in ALL patients (19).
We described one other patient with Ph-positive leukemia previously, analyzed by Northern blotting in our laboratory, whose bcr-abl mRNA apparently lacked abl exon a2 (20). We proposed that in this chimeric RNA, joining had occurred of bcr exon b2 to abl exon a3. To prove this hypothesis, PCR was performed on cDNA derived from this patient using primers 1, 2 and 3.

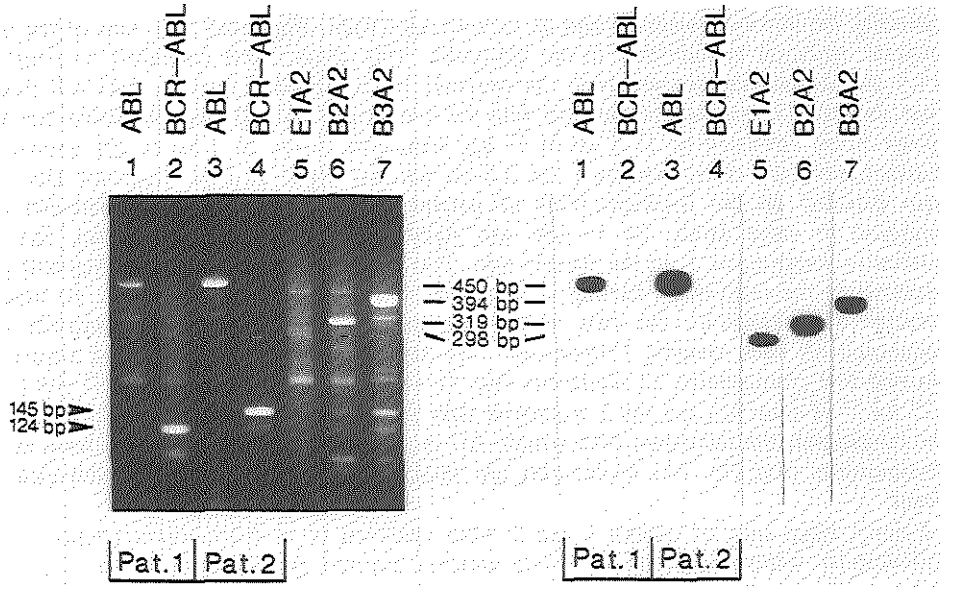


Figure 3. On the left side of the figure the results of the PCR experiment are shown on gel; on the right side the corresponding autoradiogram is depicted. Lanes 1 and 3 contain the PCR-mixture of patient 1 and 2 after amplification of the 450-bp abl fragment. Lanes 2 and 4 contain the PCR-mixture of patient 1 and 2 after amplification of the bcr-abl fragment. Lanes 5 to 7 contain the result of amplification of the bcr-abl fragment of 3 patients with Ph' positive leukemia who show, respectively, an e1a2, b2a2 and b3a2 joining. Sizes of the PCR-fragments usually found in our experiments are given in the middle of the figure. Arrowheads indicate the aberrant bcr-abl fragments of patient 1 and 2 on gel.

A fragment of approximately 150-bp length was generated (Fig. 3, patient 2) that matched the size of a fragment covering the b2a2 junction missing the 174 bp of abl exon a2 (319-174=145 bp).
Sequence analysis of the amplified bcr-abl fragments of both patients confirmed our suppositions: the joining in patient 1 consisted of the first exon of the bcr gene to abl exon a3, giving rise to an e1a3 bcr-abl mRNA, while in patient 2 a b2a3 joining was found (data not shown).
These data were confirmed by Southern blot analysis. DNA of patient 1, digested with restriction enzymes BamHI, BglII and KpnI, shows two extra bands in all digests after hybridization with a SauI-KpnI fragment that covers the intron between abl exon a2 and a3 (Fig. 4). Since no aberrant bands are detected in corresponding digests of normal human thymus DNA, we conclude that the breakpoint of patient 1 in the abl gene is located between abl exon a2 and a3.

Southern blot analysis of patient 2 (R6 in Ref. 11) showed that 5' M-BCR probes hybridized to a 3.5-kb rearranged BglII M-BCR fragment on the Ph chromosome. To investigate the molecular structure, this fragment was cloned in E. Coli in order to sequence the bcr-abl junction. From the DNA of the leukemic cells of this patient, an MboI-partial library was constructed in λ EMBL-3. Screening of the library with a 5' M-BCR probe (2-kb BglII-HindIII fragment) yielded four independent hybridizing phage-plaques. Since Northern blot analysis indicated that the breakpoint in the abl gene would map between abl exons a2 and a3 (20), the phage-plaques were rescreened with a probe covering the intron between a2 and a3 (0.9-kb SauI-KpnI, Fig.4 and 5A). One of the four plaques appeared to hybridize to the abl-probe. This phage clone contained an insert of 13.5 kb, which was mapped with the enzymes BglII, HindIII, BamHI, SalI and EcoRI (not shown). Comparison of the map with the known bcr and abl maps indicated that this phage insert consisted of 11.5 kb of 5' bcr sequences linked to 2 kb of abl sequences. Furthermore, the clone contained the 3.5-kb aberrant BglII M-BCR fragment that was also detected on the Southern blots of patient 2. This fragment was subcloned and analyzed in more detail. Its 5' side was identical to 5' M-BCR sequences, but the similarity stopped just 3' of the BamHI side. The 3' part of the 3.5-kb BglII fragment was identical to the c-abl-map, starting in or just 3' of exon a2 of abl (Fig. 5A). To exactly localize the breakpoint, the area of the breakpoint of the 3.5-kb BglII fragment was sequenced on both strands. Comparison with the corresponding bcr and abl sequences showed that chromosome 22 sequences stop 11-14 bp 3' of the M-BCR BamHI site (i.e., 175 bp 5' of exon b3) and the 9 sequences start 21-24 bp 3' of abl exon a2. At the site of the breakpoint, the redundant dinucleotide AG is present that could be derived from either chromosome (Fig.5B). No nucleotides are inserted or deleted during the translocation event.

Finally, blast cells of patient 1 and 2 were analyzed in an immunoprecipitation assay followed by autophosphorylation. Using antisera directed against abl-sequences and bcr-sequences, a protein of approximately 190 kD was precipitated from blast cells of patient 1. This "P190-like" protein exhibits similar tyrosine kinase activity as e1a2 $P190^{BCR-ABL}$ in blast cells of a Ph-positive ALL patient, as equivalent amounts of cells were taken (Fig. 6A). From blast cells isolated from patient 2, a protein was precipitated both by anti-ABL and anti-BCR antisera with a molecular weight of 210 kD. The tyrosine kinase activity of this "P210-like" protein is comparable with the activity of the b2a2 $P210^{BCR-ABL}$ molecule, derived from a comparable number of blast cells from a Ph-positive CML patient and BV173 cells (Fig.6B).

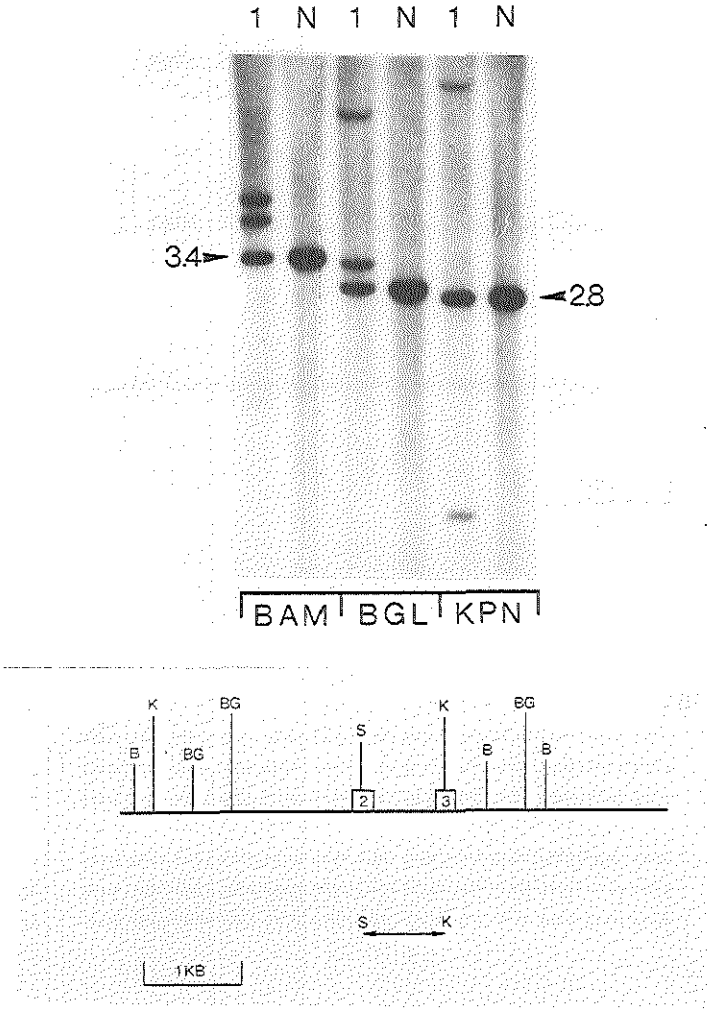
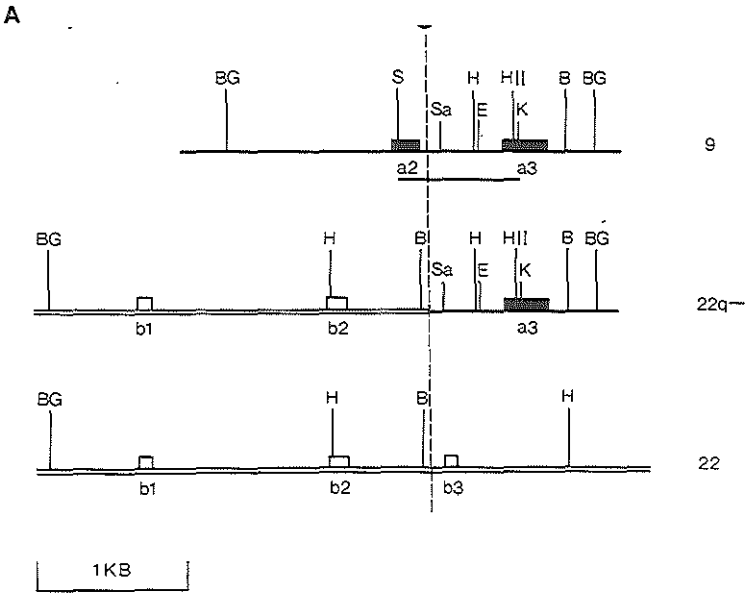


Figure 4. Southern blot analysis of patient 1. DNA of patient 1 was digested with restriction enzymes BamHI, BglII and KpnI. Normal human thymus DNA (N) was used as a control. A 0.9-kb SauI-KpnI fragment was used as a probe. Germline bands are indicated by arrowheads, and the size of the fragments is given on the left for the BamHI digest, and on the right for the BglII and KpnI digests. Below the Southern blot a simplified restriction map of the region of interest is given. B=BamHI, BG=BglII, K=KpnI, S=SauI. Abl exons a2 and a3 are depicted as boxes and numbered 2 and 3, accordingly. The localization of the SauI-KpnI fragment used as a probe is given.



B

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TTTGT GGCCA GTGGA GATAA CACTC TAAGC ATAAC TAAAG GTAAA ACGGT TGTGC GCAGC TACTC    9
ATTAC ACTTC GAGTC ACTGG TTTGC GTGTA TTGCT AAACC AAGTC GATCC TGAGA TCCCC AAGTC    22q-
ATTAC ACTTC GAGTC ACTGG TTTGC GTGTA TTGTC AAACC AGCTC GATCC TGAGA TCCCC AACAC    22

GTGGT TGCAC GAGAT AGAAA TGTGC GAATT GCGGT TTGAC GTACC ACGCT TTGCT CGTTA AAGGA    9
CTGGT TGCAC GAGAT AGAAA TGTGC GAATT GCGGT TTGAC GTACC ACGCT TTGCT CGTTA AAGGA    22q-
AGAAA TCATC ATGAC TATGT TTTTC GCCCA TGACA CTGGC TTACC TTGTC CCACC CAGAT GGCAG    22
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Figure 5

A. Restriction map of part of the abl gene on chromosome 9 (upper line), the 3.5-kb Bgl fragment containing the bcr-abl joining of patient 2 (middle line) and part of the bcr gene on chromosome 22 (lower line). Chromosome 9 sequences are depicted as black lines, chromosome 22 as open lines. Exons of the abl gene are depicted as black boxes and of the bcr gene as open boxes. Exons are numbered according to the text. The arrowhead and the dashed line indicate the breakpoint localization of patient 2. The 0.9-kb SauI-KpnI probe is depicted as a black bar underneath the restriction map of part of the abl gene. BG=Bgl II, S=SauI, HII=HincII, K=KpnI, H=HindIII, B=BamHI, Sa=SalI, E=EcoRI.

B. Corresponding sequence analysis of part of the fragments depicted in Figure 5A. The arrow indicates the end of exon a2 of abl. The arrowhead points at the AG dinucleotide at the transition of the bcr to the abl sequences. The BamHI site in the bcr sequence is underlined.

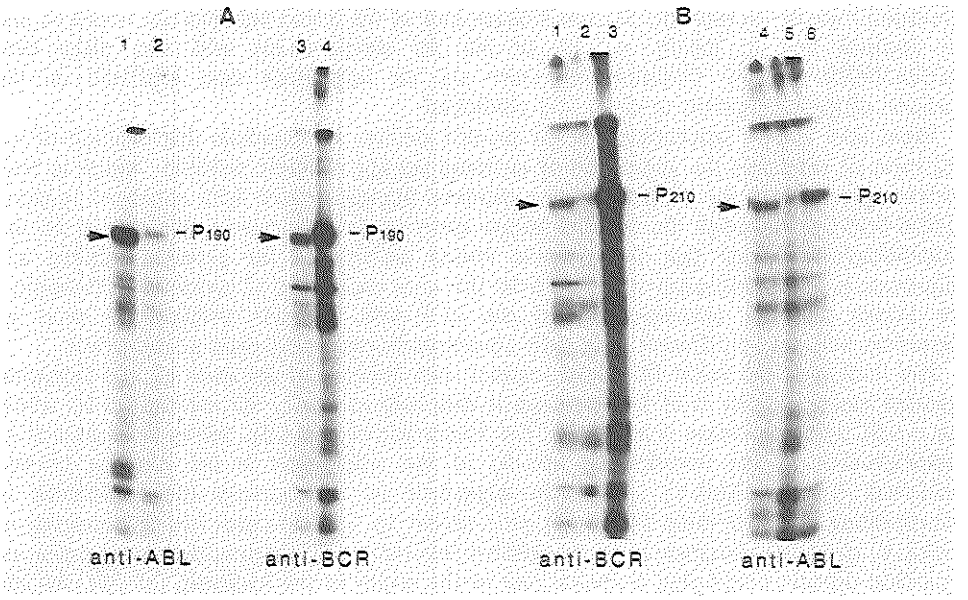

Figure 6

A. Immunoprecipitation analysis followed by autophosphorylation of blast cells isolated from patient 1; $1x10^7$ blast cells were isolated from peripheral blood and precipitated with 50 μl Na_2SO_4 precipitated anti-ABL antiserum (lane 1) and anti-BCR antiserum (lane 3). As a positive control, $1x10^7$ blast cells from an ALL patient with an e1a2$^{BCR-ABL}$ junction were precipitated with anti-ABL antiserum (lane 2) and anti-BCR antiserum (lane 4).

B. Immunoprecipitation analysis followed by autophosphorylation of blast cells isolated from patient 2; $1x10^7$ blast cells were isolated from peripheral blood and precipitated with 50 μl Na_2SO_4 precipitated anti-BCR antiserum (lane 1) and anti-ABL antiserum (lane 4). As positive controls, $1x10^7$ blast cells from a CML patient with a b2a2$^{BCR-ABL}$ junction (lane 2 and 5) and $1x10^7$ BV173 cells (lane 3 and 6) were precipitated, respectively.

Discussion.

In this article we present the clinical, cytogenetic, and molecular data of two patients with Ph-positive ALL. We investigated the bcr-abl rearrangements in both patients using the polymerase chain reaction (PCR) on cDNA. Sequence analysis of the amplified bcr-abl fragments showed that in both patients a deletion of exon a2 of the abl gene had occurred in the chimeric bcr-abl mRNA. Consequently, the joining-configuration in patient 1 was e1a3 and in patient 2 b2a3. This is a remarkable finding since all patients with Ph-positive leukemia molecularly investigated so far always seem to include abl exon a2 in their chimeric bcr-abl mRNA. In addition, Southern blotting data from patient 1 (Fig.4) and

chromosomal cloning and sequencing data from patient 2 (Fig.5, A and B) showed that the deletion of abl exon a2 from the chimeric bcr-abl mRNA was due to the position of the breakpoint in the abl gene on chromosome 9. In both cases the breakpoint maps in the 0.6-kb intron, separating abl exons a2 and a3. Since breakpoints in abl can be scattered over an area of more than 200 kb (4,6,33), and assuming that the position of the breakpoint would be random, the small target size of the intron between abl exons a2 and a3 predicts that in theory $<0.3\%$ of the Ph translocations will take place here (0.6kb:200kb=0.003). Unfortunately there are no large-scale data available on the position of abl breakpoints to check this prediction.

From both cDNA sequencing and the protein studies, we conclude that deletion of the abl exon a2 results in the generation of in-frame bcr-abl chimeric mRNAs, which in both patients give rise to the expression of a chimeric bcr-abl protein with enhanced tyrosine kinase activity. Although the tyrosine kinase assay is not quantitative, the signals on the autoradiogram suggest that the tyrosine kinase activity of the proteins missing a2-sequences is not significantly different from the ones containing a2 sequences. Therefore, we expect that the tumorigenicity of the BCR-ABL proteins without abl exon a2 sequences will not be different from their counterparts containing exon a2 sequences.

The finding of the novel BCR-ABL variant with deletion of abl exon a2 and increased tyrosine kinase activity is of interest since it may help to clarify the mechanism by which the c-ABL tyrosine kinase is activated. The kinase activity of ABL seems to be crucial in tumorigenesis, as the transforming activity of the v-abl gene in the Abelson Murine Leukemia Virus (AMuLV) is directly dependent on this activity (34). Evidence is accumulating that disruption of the abl gene (either by viral transduction as occurring in AMuLV, or by translocation as in Ph-positive leukemia) and concomittant deregulation of its kinase domain could play a pivotal role in the origin of the leukemia (35-38). As has been published, the kinase domain of the abl gene shows strong homology with the kinase domain of the c-src oncogene and is called the src-homology 1 region (SH1) (39). Amino-terminal from this region, two additional homology regions have been described in the abl gene: the SH2 and the SH3 region (Fig.7).

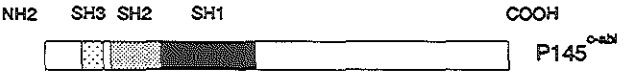

Figure 7. The orientation of the various SH-regions in the c-ABL protein is given. Src-homology 1 region (SH1) contains the kinase domain of c-abl. SH2 is a positive regulator and SH3 is a negative regulator of SH1.

The SH2-region is suggested to be a positive regulatory region immediately N-terminal of the kinase domain (39), and this region is needed by the v-ABL kinase for its transforming activity (37). The SH3-region is located directly N-terminal of SH2 and comprises 50 amino acids (40). Deletions in this particular region of the c-SRC (41,42) as well as deletions in the homologous domains encoding for mouse ABL type IV proteins activate the tyrosine kinase (43,44).

The mutant proteins show transforming abilities in NIH3T3 cells. Therefore, a negative regulatory influence on the kinase domain of the abl gene has been proposed for the SH3-region (43,44). Jackson and Baltimore (43) suggested that the SH3 inhibiting function on the catalytic domain may be modulated by interaction of the SH3 region with an as yet unidentified cellular factor. Deletions in SH3 would prevent the interaction with this factor, resulting in the loss of the blocking function and concomittant unleashing of the tyrosine kinase activity. Supporting evidence for this mechanism already exists in vivo since in the v-ABL protein, which has heightened tyrosine kinase activity, the SH3-region has been deleted.

The two patients described in this article also show a deletion in the SH3-region, since the SH3-region is partly encoded by abl exon a2 which is missing in both patients. Due to this deletion, 17 N-terminal amino acids of the SH3-region are removed from the BCR-ABL fusion proteins in both patients. This includes amino acids, which upon deletion potentiate the c-SRC transforming activity and in this way may give rise to a BCR-ABL transforming protein with enhanced tyrosine kinase activity. However, in published data of patients with Ph-positive leukemia, so far an e1a2, b2a2 or b3a2 joining has been found at the mRNA level. This implicates that abl exon a2 is present and that the SH3-region is still intact in the BCR-ABL fusion proteins of these patients. Since these proteins show enhanced tyrosine kinase activity, we assume that the inhibiting function of the SH3-region is impaired by the BCR-moiety of the proteins by an unknown mechanism. Probably the 5' BCR-sequences downregulate SH3-function either by steric hindrance or by binding to the cellular factor, which regulates SH3. Thus, negative regulation of the kinase domain of ABL can be disturbed either by deletion of the SH3-region (as in v-ABL or in both patients described in this article) or by interfering 5' sequences that impair the function of SH3 (as in most patients with Ph-positive leukemia). In this respect no difference exists between the two patients described in this article and the majority of patients with Ph-positive leukemia.

Understanding of the regulation of the kinase domain of ABL and identification of substrate proteins for the tyrosine kinase activity will elucidate its significance for the origin and development of Ph-positive leukemias. Patients as described in this paper may help to unravel this issue.

This paper is dedicated to the memory of André Hermans, M.D.

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CHAPTER III

ACUTE MYELOID LEUKEMIA

Chapter III.1

AN INTRODUCTION TO THE DISEASE ACUTE MYELOID LEUKEMIA (AML).

While ALL is a childhood disease, acute myeloid leukemia (AML) occurs mainly in adults with a preference for the age group older than 50 years (Copplestone and Prentice, 1988). Its incidence increases with age. Symptoms and signs of this disease may have been present from a few days to several months and are often directly related to bone marrow failure (Macleod, 1981). Acute onset of fever, malaise and anemia can be found accompanied by an increased bleeding tendency, mouth ulcers, leukemic infiltrations (e.g. of the skin) and hepatosplenomegaly. While normal hematopoiesis results via maturation of bone marrow stem cells in the population of the periferal blood by normal leucocytes, erythrocytes and thrombocytes, this proces is disturbed in AML. Here, failure of maturation in a developmental stage of a myeloid stem cell occurs and stage specific blast cells are generated (Figure 1). Therefore, examination of the blood and bone marrow is essential for a correct diagnosis of AML.

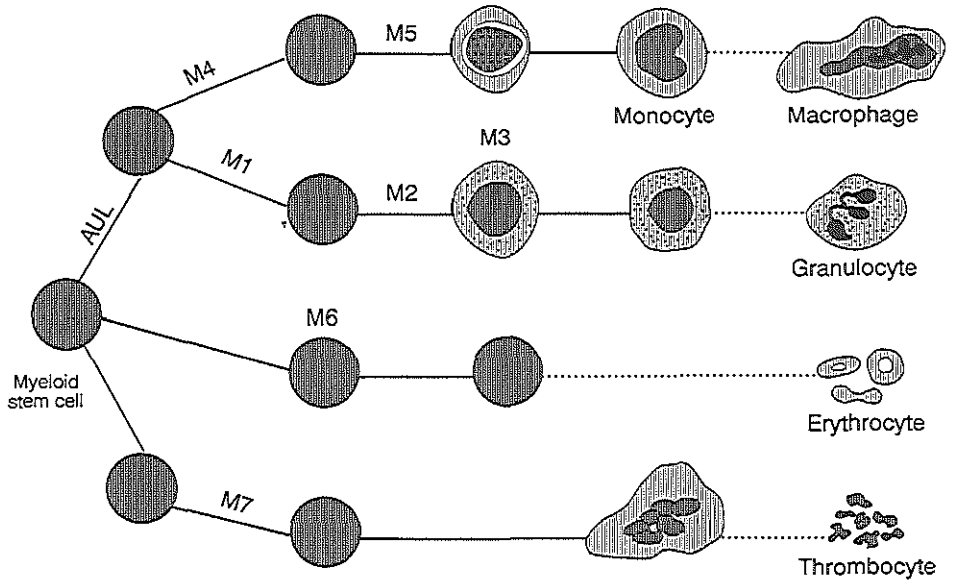

Figure 1. Schematic representation of the differentiation-pathways of a myeloid stem cell into mature blood cells. The various subtypes of AML according to the FAB-classification are indicated at the developmental stage they belong to.

Table 1 Cytogenetic-Morphologic Subclassification of AML[1)]

FAB definition		Frequency of FAB subtypes in AML[1)]	Cytogenetic Abn. often found	Frequency of the karyotypic change in de novo AML	Prognosis
AUL	acute undifferentiated leukemia	< 1%			
M1	acute myelogenous leukemia without maturation	15%			
M2	acute myelogenous leukemia with maturation	34%	t(8;21)	7-12%	intermediate - good
M3	acute promyelocytic leukemia	9%	t(15;17)	6-10%	intermediate
M4	acute myelomonocytic leuk. +/- eosinophilia	23%	inv(16)	2-5%	good
M5	acute monocytic leukemia	14%	abn.11q23	4-6%	intermediate - poor
a	poorly differentiated (monoblastic)				
b	differentiated				
M6	acute erythroleukemia	4%			
M7	acute megakaryoblastic leukemia	< 1%			

[1)]de novo AML = no exposure to cytotoxic agent in the past.
Ref. Schiffer et al., 1989; Second MIC Cooperative Study Group, 1988; Arthur et al., 1989.

The diagnosis of AML.

As in ALL, the examination of blood and bone marrow of an AML-patient is performed using various diagnostic methods. Each contributes in varying degrees to the eventual diagnosis. Nowadays the three main techniques are: morphologic examination (Romanowsky- or Wright's stain) using the FAB-classification, immunologic investigation and chromosomal studies.

The FAB-classification of AML.

The FAB-classification of AML is based on the morphologic phenotype of the blast cells, the degree of maturation along one or more cell lines and the degree of maturation of the cells which are generated by a myeloid stem cell (Bennett et al, 1985, Figure 1, Table 1). This classification is used to separate the myeloid from the lymphoid leukemias. It also distinguishes AML from another disorder with characteristics similar to those of AML: the myelodysplastic syndrome (MDS). MDS occurs in patients older than those with AML. Its onset is insidious and its course is mitigated in the majority of cases. However, 30% of MDSs evolve to AML. Both MDS and AML are characterized by proliferation of blast cells and ineffective maturation of progenitor cells. MDS presents with multiple cytopenias in the blood with a normo- or hypercellular marrow showing evidence of maturation arrest and dysplasia. The absolute amount of blast cells in the bone marrow and periferal blood is lower than in AML which is in fact one of the criteria by which the FAB-classification separates MDSs from AMLs (Bennett et al, 1982). The FAB-classification, using precise quantitative and qualitative criteria of bone marrow smears and peripheral blood, established five subtypes of MDS (Table 2). The separation of MDSs and leukemias of lymphoid origin from AML is crucial since these disorders require different therapeutic approaches.

Table 2

	FAB-Classification of MDS[1]
RA	Refractory anemia
RARS	Refractory anemia with ring sideroblasts
RAEB[2]	Refractory anemia with excess of blasts
CMML	Chronic myelomonocytic leukemia
RAEB-t[2]	Refractory anemia with excess of blasts in transformation.

[1] MDS = less than 30% blasts in the bone marrow (while AML = more than 30% blasts in the bone marrow)

[2] Highest risk for evolving into AML

Ref. Bennett et al., 1982

Immunophenotyping of AML.
Certain cell surface markers are characteristic for various stages of stem cell development and these are used to study AML-blasts. Immunophenotyping demonstrates the myeloid origin of the blast cell and is especially helpful in cases of FAB-M0 or acute undifferentiated leukemia (AUL) (Second MIC Cooperative Study Group, 1988, Bennett et al, 1991). This FAB-subtype originates from a very early stage of development and blasts show almost no differentiation which do cause problems with morphologic classification.

Cytogenetic investigation of AML.
Chromosomal aberrations are found in the majority of patients with AML (Yunis et al, 1981). These abnormalities have an impact on prognosis, independently of other factors (Golomb et al, 1978, Fourth International Workshop on Chromosomes in Leukemia, 1984, Arthur et al, 1989, Keating et al, 1987). Various subclassifications of cytogenetic abnormalities are used. Sakurai and Sandberg reported on the significance of the presence of abnormal metaphases for prediction of prognosis (Sakurai and Sandberg, 1973). Patients with only normal metaphases clearly had a better prognosis than those with partly or only abnormal metaphases. Another subdivision is based on the complexity of the abnormal karyotype and this shows a deterioration of prognosis with increasing complexity (Lawler and Swansbury, 1987). However, intensified therapy regimen in the past few years showed that a third subdivision or the Chicago-classification retained its predictive value for prognosis. This classification is based on specific chromosomal abnormalities which are often found in specific FAB-subtypes (First International Workshop on Chromosomes in Leukaemia, 1978, Second International Workshop on Chromosomes in Leukemia, 1980, Second MIC Cooperative Study Group, 1988, Bitter et al, 1987, Keating et al, 1987, table 1).

Cytogenetic-morphologic classification of AML (Chicago-classification) subdivides this disease in distinct prognostic groups.

T(8;21)(q22;q22) in AML-M2: this translocation is found in 40% of AMLs with FAB-classification M2 and represents 7 to 12% of all AMLs. In 80% an additional karyotypic abnormality is found, mostly loss of a sex chromosome or an interstitial deletion of 9q21-31. The median age of the patients is 30 years and the disease is rare in patients over 50 years of age. This translocation is associated with a good prognosis since most patients obtain a complete remission (CR) using standard AML therapy. However, a recent study reported a survival rate similar to that found in AML in general (Groupe Français de Cytogénétique Hématologique, 1990).

T(15;17)(q22;q12) in AML-M3: in 70 to 90% of acute promyelocytic leukemia (APL) or AML-M3 a t(15;17) is found. This chromosomal translocation is also found in younger patients with a median age of 30 years and is associated with an intermediate to good prognosis (Keating et al, 1987). A remarkable finding in these patients is the frequent occurrence of a disturbance of normal blood coagulation in the form of a disseminated intravascular coagulation (DIC). Contrary to the usual therapy in AML, patients with APL respond very well to therapy with all-trans retinoic acid which may effect a complete remission in the majority of these patients (Castaigne et al, 1990).

Recently, several groups isolated the gene on chromosome 17 which is involved in the translocation (Borrow et al, 1990, Chomienne et al, 1990a, de Thé et al, 1990, Alcalay et al, 1991). This gene encodes the retinoic acid receptor α (RARα). The translocation cleavage of this gene in its first intron is followed by juxtaposition to the pml gene on chromosome 15, which has an as yet unknown function.
Retinoic acid is a derivative of Vitamin A and induces differentiation of leukemic cells of a patient with APL into mature granulocytes in vitro (Chomienne et al, 1990b). Although the general assumption exists that the translocated RARα plays a crucial role in APL with t(15;17), the exact mode in which it participates in the proces of leukemogenesis is still an enigma.

T(16;16)(p13;q22), inv(16)(p13;q22) or del(16)(q22) in AML-M4 with eosinophilia: abnormalities involving chromosome 16 are found in 20% of AML-M4. Patients are young and morphologic examination of the bone marrow reveals an increase in abnormal eosinophilic cells. This specific cytogenetic-morphologic subgroup carries a favourable prognosis since CR-rate and survival are reportedly better than in AML-M4 without involvement of chromosome 16 (Le Beau et al, 1983). However, this is disputed by others who stress the fact that involvement of the central nervous system at diagnosis and relapse is responsible for a deteriorated prognosis (Bernard et al, 1989).

Translocations or deletions with involvement of 11q23: these are found in 35% of AML-M5 and most of these patients are infants (Kaneko et al, 1982, Köller et al, 1989, Raimondi et al, 1989). A frequent translocation in this subtype of AML with a monocytic component is t(9;11)(p22;q23). The prognosis for a patient with AML and 11q23-abnormality is poor and the disease is characterized by a short duration of remission after conventional chemotherapy.

Nonrandom cytogenetic abnormalities which are not restricted to a specific FAB-subtype: the frequency of most of these aberrations is low and their prognosis is poor (Table 3). They include aberrations such as:
-inversions and translocations involving band 3q21 and 3q26 which are found in AML-M1, 2, 4 and 7. Many patients show an increase in abnormal megakaryocytes in the bone marrow and thrombocytosis in the periferal blood.
-trisomy 8. This is the most common cytogenetic abnormality in AML. As a sole phenomenon it is found in 8% of AMLs, mostly M1, M4 and M5. In combination with other karyotypic changes it is found in 11% of AMLs, mostly M3.
-the t(9;22). As in ALL, this translocation heralds an unfavourable disease course in AML characterized by therapy-resistance. The genetic organization of the t(9;22) is extensively described in this thesis in Chapters II.2 and II.3.
-the t(6;9). The clinical and molecular aspects of this translocation are reported in Chapters III.2 and III.3 of this thesis.

Monosomy or abnormality of the long arm of chromosome 5 or 7 (Mitelman et al, 1978 and 1981): these karyotypic abnormalities are mainly found in the so-called secondary AMLs and are not associated with a specific FAB-subtype (Table 3). Breakpoints on the q-arm of chromosome 5 mostly involve bands 5q13-15 and 5q33 while on 7q this is 7q21-22 and 7q32-33 or q34-35 (Bitter et al, 1987, Second MIC Cooperative Study Group, 1988).

Table 3

Cytogenetic Abnormalities Not Restricted to a Specific FAB-subtype

	Frequency in AML	Mostly found in	Prognosis
inv(3)	1%	FAB-M1	Poor
t(9;22)	1 - 3%	FAB-M1	Poor
t(6;9)	0.5-4%	FAB-M2, M4	Poor
tris 8 (as a sole abn.)	4 - 8%	FAB-M1, M4, M5	
-Y	1%		
+21	1%		
-5/5q-, -7/7q-	12%	Secondary AML (up to 47% of the cases)	Poor

Ref. Schiffer et al., 1989.
Second MIC Cooperative Study Group, 1988.
Arthur et al., 1989.

Contrary to de novo AML, in secondary AMLs an exposure to a toxic factor in the past is found which might contribute to the origin of the leukemia. Usually, these patients suffered from cancer in the past for which they were treated with radio- or chemotherapy. This type of AML is mainly found in patients over 50 and carries a poor prognosis, since it responds poorly to therapy and patients show short survival in general (Fourth International Workshop on Chromosomes in Leukemia, 1984, Hoyle et al, 1989).
Without therapy a patient with AML usually dies within two months. Using chemotherapy consisting of daunorubicin, cytosine arabinoside and thioguanin an overall first complete remission (CR) is achieved in 70% of adult patients with AML (Gale, 1990). However, the major complication in curing this disease is the frequent relapse after first CR which occurs also in prognostically favourable subgroups such as those with a t(8;21) or inv(16) (Fenaux et al, 1989, Preisler et al, 1989, Schiffer et al, 1989). An indication of poor survival is the fact that only 1 in 6 patients will be alive 5 to 8 years after AML was diagnosed (Bandini et al, 1991). Therefore, postremission therapy is necessary and new approaches such as high dose chemotherapy or bone marrow transplantation after first CR are currently investigated on their value to increase the number of long term survivors (Reiffers et al, 1989, Geller et al, 1990, McMillan et al, 1990).

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Chapter III.2

THE TRANSLOCATION (6;9)(P23;Q43) SHOWS CONSISTENT REARRANGEMENT OF TWO GENES AND DEFINES A MYELOPROLIFERATIVE DISORDER WITH SPECIFIC CLINICAL FEATURES.

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SUMMARY

Translocation (6;9)(p23;q34) is a cytogenetic aberration which can be found in specific subtypes of both acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). This translocation is associated with an unfavorable prognosis. Recently, the genes involved in the t(6;9) were isolated and characterized. Breakpoints in both the dek-gene on chromosome 6 and the can-gene on chromosome 9 appear to occur in defined regions which allows us to diagnose this type of leukemia at the molecular level. Moreover, due to the translocation a chimeric dek-can mRNA is formed which, as we show here, is an additional target for diagnosis via cDNA-preparation and the polymerase chain reaction (PCR). We studied 17 patients whose blood- and/or bone marrow cells showed a t(6;9) with karyotypic analysis (Table 1). Fourteen patients suffered from AML, one patient had a refractory anemia with excess of blasts in transformation (RAEBt), one patient had an acute myelofibrosis (AMF) and one patient a chronic myeloid leukemia (CML). In 9 cases studies at the DNA- and RNA-level were possible while in 7 cases only the DNA could be analyzed. In one case only RNA was available. Conventional Southern blot analysis showed the presence of rearrangements of both the dek-gene and the can-gene. In both genes, breakpoints cluster in one intron in the patients investigated. The presence of a consistent chimeric dek-can product after cDNA-preparation followed by the PCR was demonstrated. We conclude from our data that the t(6;9) is found in myeloproliferative disorders with typical clinical characteristics. This translocation results in highly consistent abnormalities at the molecular level.

INTRODUCTION

Since the discovery in 1960 of the Philadelphia chromosome in cases of chronic myeloid leukemia (CML) by Nowell and Hungerford, a large number of leukemias has been associated with specific chromosomal translocations (1,2). The development of new techniques enabled molecular biologists to isolate and characterize a number of genes

Table 1 CLINICAL AND HEMATOLOGIC DATA OF 17 PATIENTS WITH A TRANSLOCATION (6;9)(p23;q34)

Patient	Age/ Sex[c]	FAB	Clinical phase[d]	Karyotypic abnorm.	% Abn. metaph.	Baso-philia[e]	WBC 10^9/l	% Blasts BM	% Blasts PB	Southern blot[f]	PCR[g]	Response to therapy[h]	Survival after diagnosis
1[a]	13/F	RAEB M4	diagn. relapse	t(6;9) t(6;9) + add.abn.	100	no	1,66			 +	 RNA n.a.	PR	± 18 months
2[a]	63/F	M4	diagn.	t(6;9)	93		87	47	30	+	no ampl.	PR	
3[a]	38/M	AMF AMF	diagn. relapse	NM t(6;9)	 30	no	5,2	9	5	 +	 no ampl.	PR	29 months
4[a]	17/F	M4	diagn.	t(6;9)	97	no	21,2	59	18	+	+	no therapy	3/4 months
5[b]	13/F	M4	diagn. relapse	t(6;9) 6p- +add.abn.	87 91	no	29,4	73	58	DNA n.a.	+	CR	30 months
6	35/M	M2	diagn.	t(6;9) t(6;9), +8	80 20	yes	12,6	58	35			PR	18 months
			relapse	6p-	100		92		95	+	no ampl.		
7	28/F	M4	relapse	t(6;9)	91	no	5,1	92	20	+	+	CR	17 months
8	18/M	M2	diagn. after ther.	t(6;9), inv.1 ND	35	no	10	25 10	< 1	+ DNA n.a.	RNA n.a. BM: + PB: -	CR	> 24 months
9	19/F	M2	relapse	t(6;9)	100	yes	100	87	94	+	+	CR	14 months
10	14/F	M4	diagn.	t(6;9)				30		+	+	CR	> 36 months
11	6/F	M4	diagn.	t(6;9)		no	262	80	45	+	+	NR	2 months

12	28/M	M1	diagn.	t(6;9)	100		65,9		79	+	+		
13	24/F	M1	diagn.	t(6;9), del.7q t(6;9)	40 60		54,8		95	+	RNA n.a.		
14	10/M	M2	diagn.	t(6;9)	100	no	47,5	52	37	+	no ampl.	PR	20 months
15	53/M	M4	relapse	t(6;9)	91	yes	100	41	31	+	+	PR	> 4 months
16	54/F	CML Ph⁻, bcr⁻	diagn.	t(6;9)	100	no	92		9	+	RNA n.a.		11 months
17	53/F	RAEBt	diagn. after ther.	t(6;9) ND	100	no	2,5	22	3	+	+	no chemoth.	15 months

a) Published previously by Von Lindern et al., ref. 19
b) Published previously by Adriaansen et al., ref. 21
c) Age in years at diagnosis Sex: M = male, F = female
d) diagn. = at diagnosis
after ther. = after therapy: patient 8 - chemotherapy, patient 17 - IL2 therapy
e) > 0.25% basophils in the bone marrow
f) + = Southern blot analysis shows rearrangement of the dek- and the can- gene
DNA n.a = DNA was not available.
g) + = PCR analysis shows amplification of a chimeric dek-can fragment
- = no amplification of dek-can while can is amplified.
RNA n.a. = RNA was not available
no ampl. = despite intact RNA no amplification by PCR
h) CR = complete remission, PR = partial remission, NR = no remission

NM = no mitosis
ND = not done
WBC = white blood cell count
BM = bone marrow
PB = peripheral blood

involved in reciprocal chromosome translocations. Well-known examples are the t(8;14) in Burkitt's lymphoma (BL) in which the myc-gene on chromosome 8 is linked to the IgH-chain locus on chromosome 14 and t(9;22) in CML in which breakpoints occur in the abl-gene on chromosome 9 and the bcr-gene on chromosome 22 (3,4,5).
In a specific subgroup of AML a t(6;9)(p23;q34) can be found (6-10). Patients with this type of leukemia are usually quite young and their prognosis is poor. Blast-cells are mostly classified as FAB-M2 or M4 (90%) and in a minority as M1(10%). At the time of diagnosis the t(6;9) is usually the sole cytogenetic aberration. Additional karyotypic abnormalities are rare but may occur during progression of the disease (9-18). Recently, the genes located at the chromosomal breakpoints of this translocation were isolated and characterized (19). The gene on chromosome 6 which participates in the reciprocal exchange is called dek and encompasses 40 kb (von Lindern et al, manuscript in preparation). Southern blot analysis of 4 patients with t(6;9) indicated that breakpoints are located in one intron of 9 kb which is called 'intron containing breakpoints on chromosome 6' or icb-6. The can-gene on chromosome 9 is more than 130 kb in length. Here, breakpoints occur in one intron of 7.5 kb (icb-9) which is located in the middle of the gene. The can-gene is transcribed into a 6.6 kb mRNA. Due to the translocation the 3' part of the can-gene is fused to the 5' part of dek, resulting in a chimeric dek-can gene on the 6p-derivative (von Lindern et al, manuscript in preparation). This chimeric gene is transcribed into an aberrant 5.5 kb mRNA. The functions of the normal dek and can gene products are as yet unknown and it is equally unclear in which way the hybrid product may be involved in leukemogenesis.
In this study we analyzed 14 patients with AML, one patient with RAEBt, one patient with AMF and one patient with CML whose blood or bone marrow cells carried a t(6;9). Investigation of the leukemic cells at the DNA- and RNA-level confirms the highly consistent involvement of both the dek- and can-genes in this translocation. The myeloproliferative disorder marked by a t(6;9) appears as a distinct clinical entity which, as we show here, can now be diagnosed and monitored at the molecular level.

Patients.
Clinical and hematologic data of the patients are given in table 1. In the case of patients 1 to 5 various data were published previously (13,19,20,21). Patients 6 to 17 were newly admitted cases and fresh or frozen samples were sent to us for molecular investigations by the following centers: Regional Cancer Center Marseille, France (patients 6 and 17), Universitätsklinik Ulm, Germany (patients 7 and 16), University Hospital Groningen, The Netherlands (patient 8), Centre Regional de Transfusion Sanguine et de Génétique Humaine Bois-Guillaume, France (patient 9), Free University Hospital Amsterdam, The Netherlands and Stichting Nederlandse Werkgroep Leukemie bij Kinderen The Hague, The Netherlands (patient 10), Medical Center of the University of Amsterdam, The Netherlands (patients 11 and 15), Imperial Cancer Research Fund, Saint Bartholomew's Hospital, London, United Kingdom (patients 12 and 13), Children's Cancer Research Institute Vienna, Austria (patient 14).

MATERIALS AND METHODS

Samples.
Bone marrow aspirates and blood samples were collected in heparinized tubes. After isolation of the white fraction by Dextran or a Ficoll-Hypaque gradient, cells were frozen and stored in liquid Nitrogen until used.

Conventional Southern Blot Analysis.
DNA was isolated from blood or bone marrow cells according to standard procedures (22) or high molecular weight DNA was prepared in agarose plugs as described previously (23). The following restriction enzymes were used for digestion of DNA: EcoRV, BamHI, HindIII and/or BglII. DNA-fragments were separated on a 0.7% agarose gel and blotted onto nylon filters (Zeta-probe, Biorad Lab., Richmond, CA) according to the manufacturers instructions. Probes used for hybridization, hybridization- and washing-conditions were described previously (19). In short, rearrangement of the dek-gene is detectable using radio-labeled probes MF1E.5 (a 500 bp EcoRI-EcoRI fragment) and MF2BH (an 800 bp BglII-HindIII fragment). Probes AL1F4EP (a 200 bp EcoRI-PstI fragment) and AL1F6E.3 (a 300 bp EcoRI-EcoRI fragment) are used for the detection of breakpoints in the can-gene. In figures 1A and 1B a schema-tic representation is given of a simplified restriction-map of both icb-6 and icb-9 and the localization of the probes used is indicated.

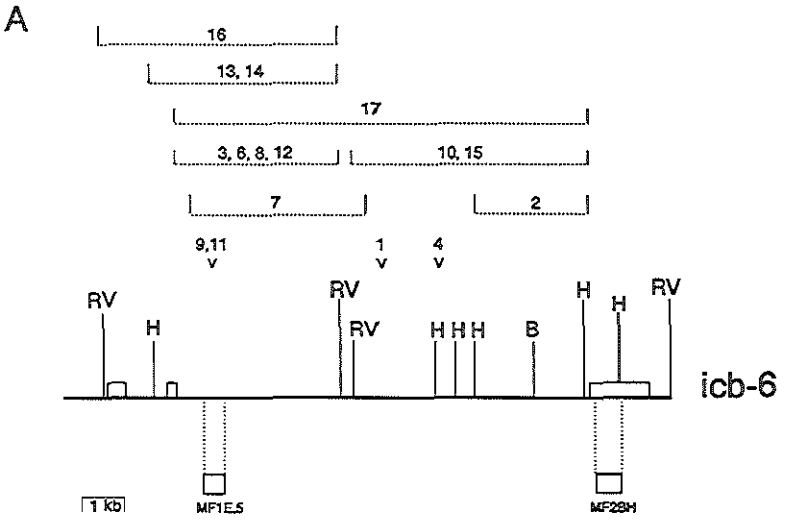

Figure 1A. Restriction map of the icb-6. Exons are depicted as open boxes. The localization of probes MF1E.5 and MF2BH are indicated as open boxes beneath the map. Stippled lines or arrowheads indicate localization of the breakpoint in the dek-gene of the patients. The patients are numbered according to table 1. The breakpoint-containing fragments of patients 1 and 4 were cloned and the precise breakpoints were localized (Ref. 19). The breakpoints of patients 9 and 11 fall into probe MF1E.5. RV=EcoRV, H=HindIII, B=BamHI.

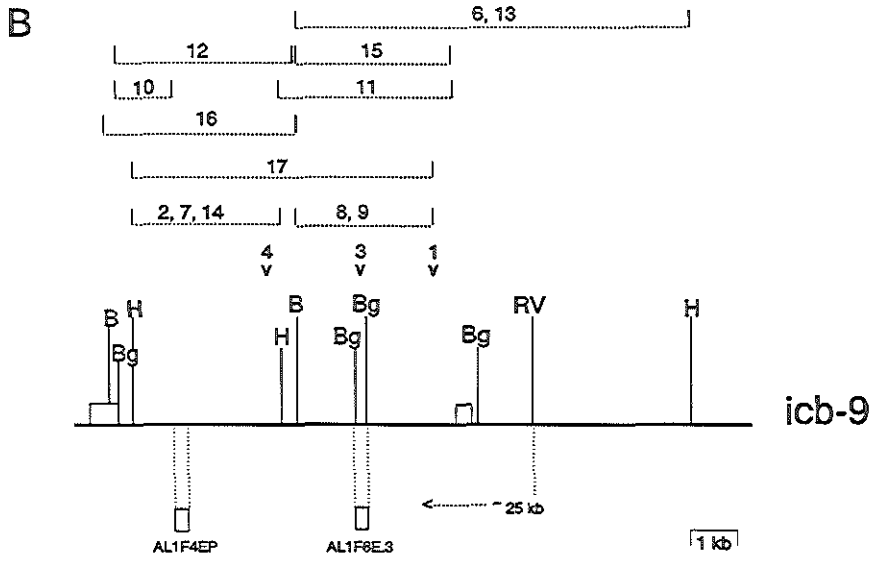

Figure 1B. Restriction map of icb-9. Exons are indicated as open boxes. Probes AL1F4EP and AL1F6E.3 are indicated beneath the map as open boxes. Stippled lines or arrowheads indicate localization of the breakpoint in the can-gene of the patients which are numbered according to table 1. The breakpoint-containing fragments of patients 1 and 4 were cloned and the precise breakpoints were localized (Ref. 19). The breakpoint of patient 3 falls into probe AL1F6E.3. RV=EcoRV, H=HindIII, B=BamHI, Bg=BglII.

Polymerase Chain Reaction (PCR).

RNA-isolation, cDNA-preparation and PCR-conditions were described previously (24,25). As a control for the cDNA-synthesis and PCR-reaction not only the chimeric dek-can cDNA was amplified from the patient RNA but also the normal can cDNA. 1 unit Taq polymerase was added per reaction (Cetus Corp., Emeryville, CA, USA or BRL, Gibco Lab., Life Technol. Inc., NY, USA). Primers used for cDNA-synthesis and PCR-amplification consisted of either of the two following sets of sequences:

Primer-set I
3'primer in can = 5' ACCAGGTGATTCAGCCT 3'
5'primer in can = 5' CTGAAAACAACTTTACTTGA 3'
5'primer in dek = 5' CCTACAGATGAAGAGTTAA 3'

or:

Primer-set II
3'primer in can = 5' GTGTCTCTCGCTCTGG 3'
5'primer in can = 5' AAGAGACCACAGAGTCG 3'
5'primer in dek = 5' GGCCAGTGCTAACTTGG 3'

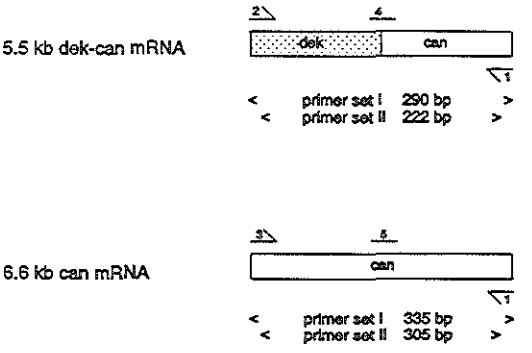

Figure 2. Schematic representation of the reversed PCR of dek-can and can mRNA. Dek is represented as a stippled bar while can is shown as an open bar. A 3' primer in can (1) is used to make cDNA while the PCR is performed with this primer in combination with a 5' primer either in dek (2) or in can (3). This results in amplification of a dek-can or can fragment which can be detected by the specific oligo-probes 4 and 5. The size of the fragments generated by the various primer-sets are indicated beneath each drawing.

The anneal-temperature for these primers was chosen at 45°C. The PCR was performed using 24 cycles of denaturation (1 min. 30 sec. at 92°C), annealing (1 min. at 45°C) and extension (5 min. at 72°C). Analysis of residual disease was performed by serial 10-fold dilutions of 1 μg of total patient RNA into 10 μg of yeast RNA. Procedures for cDNA-synthesis and PCR were as described above. Although we do not know yet the exact copy number of the can- and dek-can mRNA per leukemic cell, we assume that both show comparable levels of expression based on data obtained by Northern blot analysis (2 to 3 fold more dek-can mRNA than can mRNA, ref.19). Amplification by PCR of the can- and dek-can fragment occurs with the same efficiency. We compare PCR-results for can with dek-can. If 100% of cells with can mRNA also express dek-can mRNA all dilution samples will be positive for both can and dek-can. If 10% of cells carry a t(6;9) no dek-can signal is present in the highest dilution while this still contains can signal.
For the sequence-specific detection of amplified fragments two oligomers were designed:
detection of normal can-cDNA : 5' GTTATCTGCATTTGCT 3'
detection of dek-can fusion-cDNA: 5' GCAAAAAGGAAATTCG 3'
Both ^{32}P end-labeled probes were hybridized at 39°C for 3 hours and filters were washed in 1xSSPE for 1 hour at the same temperature. A schematic representation of the PCR-procedure is given in figure 2.

RESULTS

Karyotyping of blood or bone marrow cells of patients was performed in the centers that sent samples for molecular analysis. In metaphases of all patients a t(6;9)(p23;q34) had been found. Patients 1 to 4 were analyzed previously with Southern blot (table 1, ref. 19). Additionally, we obtained DNA from 12 newly admitted cases (patients 6-17, table 1). DNA of each patient was digested using a minimum of three different restriction enzymes. In figures 3 and 4 some results are shown of conventional Southern blot analysis in various patients. A combination of three different restriction enzymes and four probes enabled us to localize breakpoints in the dek- and the can-gene in all 16 patients. Moreover, digestion with an additional restriction enzyme (BglII or EcoRI) (or results of PCR-experiments, see further) narrowed down the breakpoint localization in most patients to the icb-6 and icb-9.

An example is patient 10: exact localization of the breakpoint in the can-gene was impossible using Southern blot alone since only the EcoRV-digest generated an aberrant fragment with probes AL1F4EP and AL1F6E.3 (figure 4), while the HindIII- and BamHI-digests showed germline bands (data not shown). However, a PCR-experiment generated a chimeric dek-can fragment (see below), indicating that in frame joining of dek and can had occurred.

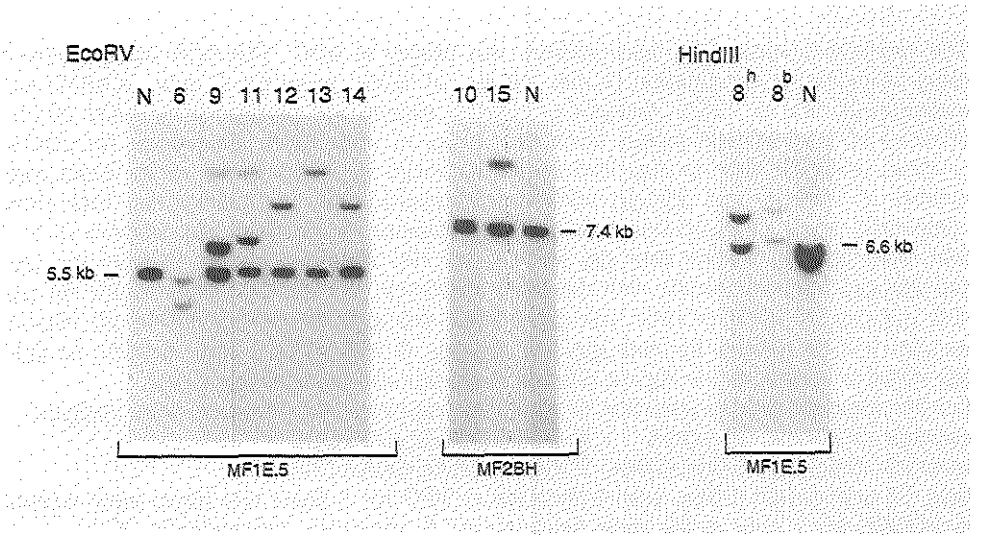

Figure 3. Detection of breakpoints in the dek-gene using EcoRV (patients 6,9,11,12,13,14,10,15) or HindIII (patient 8) as restriction enzymes in combination with probes MF1E.5 and MF2BH. The patients are numbered according to table 1. The size of the germline bands is indicated in kilobases (kb's). Aberrant fragments have the following sizes: patient 6: 4.4kb, patient 9: 7.5kb and >23kb, patient 11: 8kb and >23kb, patient 12: 16kb, patient 13: >23kb, patient 14: 16kb, patient 10: 7.5kb, patient 15: >23kb, patient 8: 9.5kb. 8h=DNA of periferal blood cells from patient 8, 8b=DNA of bone marrow cells from patient 8. N=DNA of thymus or white blood cells from a non-leukemic individual.

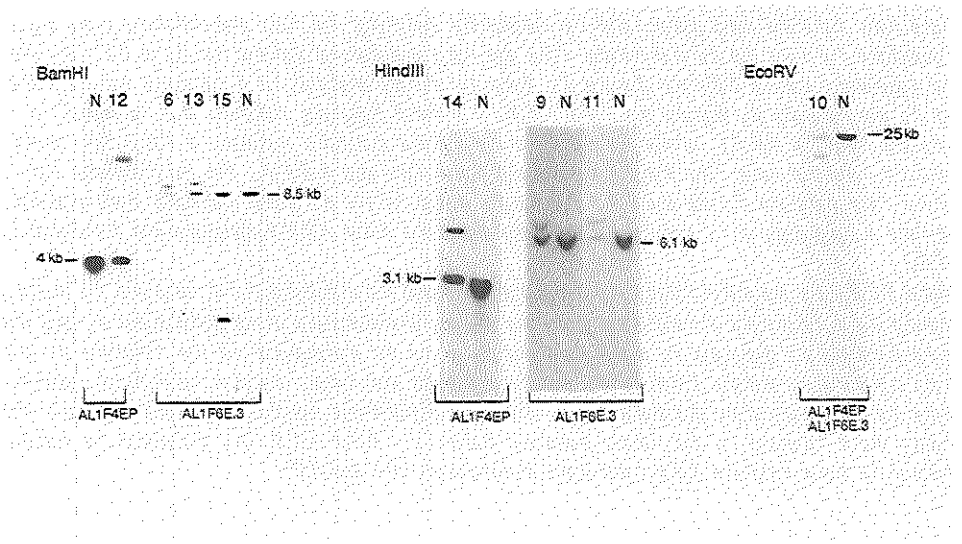

Figure 4. Detection of breakpoints in the can-gene of 8 patients using restriction-enzymes BamHI, HindIII or EcoRV in combination with probes AL1F4EP and AL1F6E.3. The patients are numbered according to table 1. The size of the germline fragments is indicated in kilobases (kb's). Aberrant fragments have the following sizes: patient 12: >23kb, patient 6: 9.5kb, patient 13: 10kb, patient 15: 2.4kb, patient 14: 5kb, patient 9: 6.5kb, patient 11: 8kb, patient 10: 11kb. N=DNA of thymus or white blood cells from a non-leukemic individual.

In the EcoRV-digest of this patient, 3' dek probe MF2BH detects an aberrant band of 7.5 kb (figure 3). However, a much larger fragment should be expected since the first EcoRV site 5' of icb-9 in can is located 16 kb upstream of this intron (Figure 1B). Therefore, we assume that patient 10 has a large deletion of the 5' can-gene which has not been mapped more precisely. The most accurate prediction for the position of the icb-9 breakpoint in this patient is between the 5' end of icb-9 and probe AL1F4EP (results not shown).
Patient 8 was diagnosed in a preleukemic phase preceding overt AML. His bone marrow contained 25% blasts while in the periferal blood only 1% blasts was observed (table 1). He suffered from paraneoplastic neutrophilic dermatosis also called variant Sweet's syndrome (26). Blood differential counts indicated that more than 95% of his leucocytes were granulocytes. The marrow also contained variably high numbers of granulocytes which diluted the blasts and therefore it was difficult to diagnose AML on purely morphological grounds. We determined whether the periferal blood granulocytes carried the t(6;9). Indeed, Southern blot analysis of both blood and bone marrow generated germ line and aberrant fragments of equal intensity indicating the presence of the t(6;9) in the vast majority of the marrow and blood cells and implying that the granulocytes carried the t(6;9) as well (figure 3).

In 14 cases (patients 2-12, 14, 15, 17, table 1) sufficient material was available for RNA-isolation and subsequent PCR-amplification of chimeric dek-can cDNA. As shown in figure 2, primer-set I generates a fragment of 290 bp when a chimeric dek-can mRNA is present and a 335 bp fragment from the can mRNA-template. However, these primers

were not optimal and gave rise to aspecific priming on the can-mRNA (see fig.6). Therefore, primer set II was made. The dek-can product of primer set II is 222 bp while the can-fragment is 305 bp (figure 2). Results of the analysis of 4 patients using primer-set II are shown in figure 5A. Via PCR a hybrid dek-can mRNA was detected in 10 patients with a t(6;9), while RNA from leukemia patients without a t(6;9) or healthy controls yielded only the fragment derived from the can mRNA. No conclusions can be drawn for patients 2,3,6 and 14. Although intact RNA of these patients was available, neither a dek-can or a can-fragment was detected after cDNA-preparation followed by PCR.
Since PCR is a sensitive diagnostic method we used this technique for analysis of material containing a minority of cells carrying the t(6;9). Comparison of amplification of can with amplification of dek-can in a dilution series gives an estimation of the fraction of cells with t(6;9) in a sample from a patient. Both blood and bone marrow cells from patient 8 were investigated after chemotherapy. Hematomorphologic studies indicated that still 10% blasts were present in the bone marrow (table 1). With PCR the presence of residual disease in bone marrow cells was seen in dilutions 10^{-1} and 10^{0}.

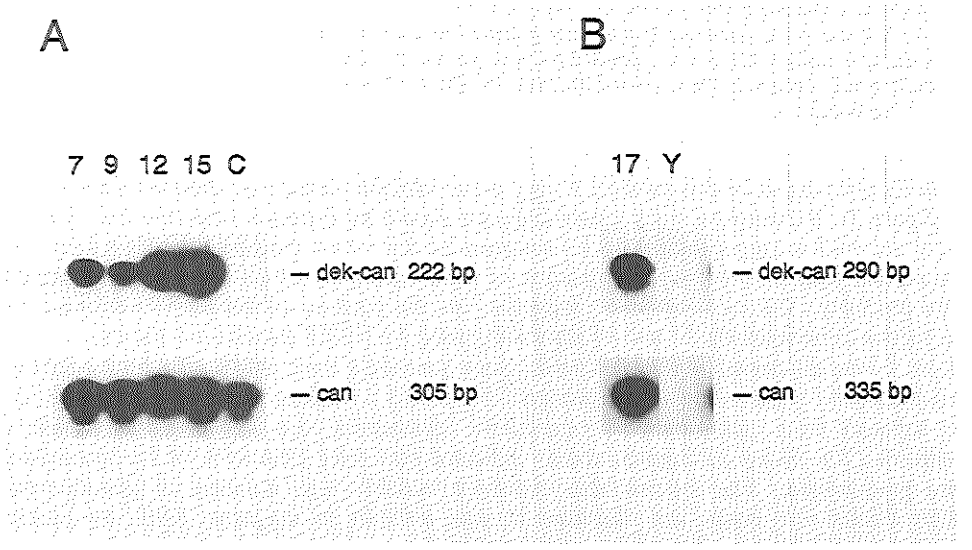

Figure 5. PCR detection of can and dek-can transcripts.
A. Results of a PCR-experiment of four patients using primer set II. C=control material from an ALL patient with a normal karyotype. Primers in can generate a 305 basepair can fragment in all five individuals while a primer in can in combination with a primer in dek generates a 222 basepair dek-can fragment only in the four patients with a t(6;9).
B. Results of the PCR-experiment of patient 17 using primer set I. Y=yeast RNA, used as a negative control. Primers in can generate a 335 bp fragment while a primer in can in combination with a primer in dek generate a 290 bp dek-can fragment in the patient. Y=Yeast stays negative for both can and dek-can.

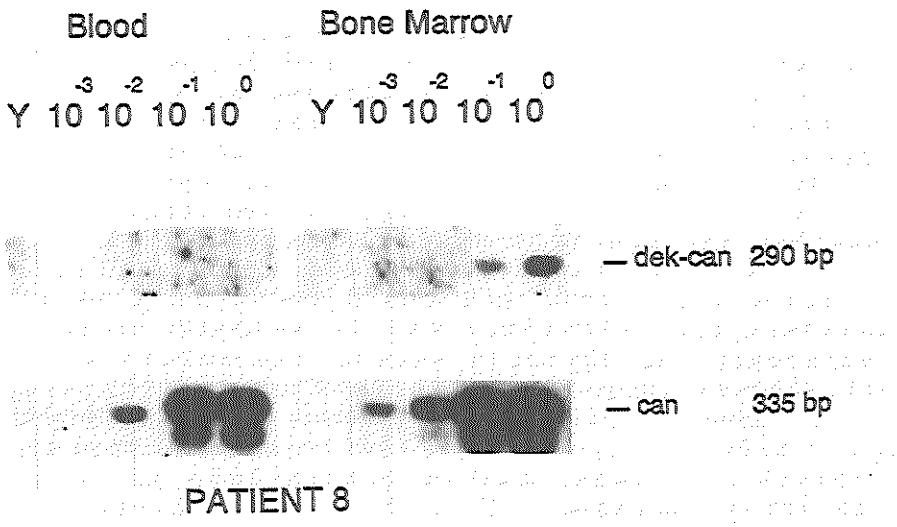

Figure 6. Results of the PCR in patient 8. RNA was isolated from blood and bone marrow after a second chemotherapy course. 1 μg total RNA of patient 8 was serially 10-fold diluted in 10 μg yeast RNA. $10^0=1$ μg patient RNA in 10 μg yeast RNA, $10^{-1}=0.1$ μg patient RNA in 10 μg yeast RNA, etc. cDNA-preparation and PCR were performed according to the protocol using primer set I. The 335 basepair can fragment which is generated by the PCR is detectable both in the blood and the bone marrow of this patient. A 290 bp chimeric fragment is visible in the first two dilution steps of the bone marrow sample indicating that 1/10x1/10=1/100 cells contain the t(6;9). Y=10 μg of yeast RNA which is used as a negative control.

The signal of dek-can in the 10^0 dilution is comparable with the can signal in the 10^{-2} dilution, indicating that roughly 1/100 cells carry the t(6;9) (see Materials and Methods). In the blood no t(6;9) carrying cells could be detected (figure 6).

Patient 17 had been diagnosed as an RAEBt and in diagnostic phase all metaphases showed a t(6;9). Blood cells were analyzed by us from a period after 4 days of IL-2 therapy which was given for activation of the T-cells. The karyotype of this sample was unknown but 3% blasts had been observed in the periferal blood. Since this low amount of blasts may be difficult to detect by Southern blotting we decided to use PCR. Clearly, a chimeric dek-can fragment was generated, indicating persistence of the translocation (figure 5B). Despite the low number of blast cells, Southern blot analysis confirmed this result (data not shown).

DISCUSSION

The entity of acute myeloid leukemia (AML) consists of a heterogeneous group of diseases. Subclassification according to the French-American-British Cooperative Group (FAB) facilitates diagnosis and enables physicians from different hematologic centers to exchange and compare data (27). Moreover, the prognosis for a patient depends on the FAB-subtype which is found. Additional independent prognostic factors are the various chromosomal abnormalities which are linked to specific FAB-subtypes (28,29). A t(6;9)(p23;q34) can be found in 0.5 to 4% of patients with AML (6-8,11,14,30-32). To date, 34 patients have been reported in the literature whose karyotype showed a t(6;9) (6-12,14-18,30-36). When the 17 patients are added who were analyzed by us at the molecular level, some general features emerge for this group of 51 patients. Diagnosis of this disease is usually made in the second or third decade of life in contrast to AML as a whole group in which the median age is above 60 years. No striking male or female preponderance is found. FAB-classification of the AML was frequently reported as M2 or M4 with a minority of M1. However, the translocation is not restricted to these subtypes of AML. Cuneo et al (20) pointed out that a number of patients with t(6;9) was reported who were diagnosed as a refractory anemia with excess of blasts (RAEB) which is a subtype of myelodysplastic syndrome (MDS). Close scrutiny of the literature confirmed this observation and we estimated RAEB with t(6;9) to be third in rank after AML-M2 and M4 with this translocation (11,13,14,16,30,35). Patient 3 presented with acute myelofibrosis (AMF) which eventually evolved into AML-M4. One of our 17 patients was diagnosed as a Ph-, BCR- CML. CML with t(6;9) was reported once before by Fleischman (31). However, in some cases differentiation between CML and MDS/AML may be difficult. Whether t(6;9) truly may occur in CML or whether the hematologic subclassification is incorrect, is not clear (37). The molecular data are strongly in favour of the second possibility.

Pearson et al proposed that a correlation might exist between the t(6;9) and basophilia, though this has been contradicted by others (10,11,15). In our patient-group this phenomenon was reported in two patients with AML-M2 and in one with AML-M4. However, to establish the real incidence of basophilia in this specific patient-group, standardized prospective studies are needed since detection of this feature involves careful examination of marrow morphology.

To date, the immunophenotype of blast cells has only been studied in two patients and this showed HLA-DR/TdT/CD13-positivity in both cases (21). It would be interesting to investigate a larger group of patients with t(6;9) for TdT-positivity since its presence in leukemias of myeloid origin indicates an unfavorable prognosis.

One of our patients (patient 8) presented with a variant of Sweet's syndrome, a paraneoplastic syndrome characterized by high fever, cutaneous lesions and granulocytosis (26). At diagnosis, his blood contained mainly mature granulocytes. Southern blot analysis of the blood and bone marrow cells of this patient showed germ line and aberrant fragments of similar intensity (Figure 3, patient 8). This indicated that the dek-can rearrangement was present in the majority of cells investigated. Therefore, we assume that cells which appear as "mature" granulocytes in the blood of this patient at diagnosis carry the dek-can rearrangement. This observation gives support to the finding of others that leukemic cells can differentiate into polymorphonuclear leucocytes (38,39).

Karyotyping usually shows a simple t(6;9)(p23;q34) at the time of diagnosis. It is noteworthy that in patients 5 and 6 a 6p- derivative was seen in relapse while the 9q+ was lost. This is a cytogenetic indication that the 6p- derivative carrying the dek-can

fusion gene is important in this type of leukemia. Additional aberrations are rare but may occur, especially during progression of the leukemia (9-18). The most frequently observed extra cytogenetic abnormalities are trisomy 8 and trisomy 13. Trisomy 8 is often seen during progression of myeloid leukemias and is found in many cases of AML and CML in accelerated or blastic phase (40). Trisomy 13 is a rare event in leukemias but has been reported to occur as a sole aberration in AUL, AML and RAEB (41-44). The finding of a trisomy 8 or a trisomy 13 in any leukemia is an ominous sign and heralds an unfavorable outcome of the disease. One of the patients studied here (patient 13) showed deletion of the long arm of chromosome 7 in addition to a t(6;9). This abnormality is associated with the so called secondary AML or MDS and also predicts an unfavorable prognosis (45). To what extent all these secondary cytogenetic changes contribute to the overall prognosis of patients with a t(6;9) is unknown at present.

The discovery of a translocation (6;9) in metaphases of a patient with leukemia is alarming since such patients respond poorly to therapy: in our group of patients only half of the recorded cases achieved a complete remission after therapy which is in concordance with the data in the literature. This forms a sharp contrast to a comparable age-group of patients with AML in whom a 77% complete remission rate can be achieved with chemotherapy (46,47). Mostly, survival does not exceed 3 years and in the group we analyzed only one case, patient 10, survived 3 years after a bone marrow transplantation and is still in remission. Correct diagnosis of t(6;9) is of utmost importance in trying to improve the prognosis for these patients in future. Since the reciprocal translocation involves small chromosomal fragments of similar morphology, cytogenetic diagnosis of this disease can be difficult. Initially, von Lindern et al (19) showed that four out of four t(6;9) patients contained breakpoints in both icb-6 and icb-9. Since icb-6 and icb-9 represent introns in the dek- and can-genes respectively, the translocation apparently fuses the same dek 'donor'-exon to the same can 'acceptor'-exon, resulting in the formation of uniform dek-can fusion genes on the 6p- chromosome of these patients. The data presented in this paper corroborate and further extend the initial observation: the translocation is amazingly precise and highly consistent in 17 t(6;9) patients analyzed at the molecular level. Since the total target size for the translocation per haploid genome amounts to less than 20 kb of DNA (icb-6 and icb-9), this may well explain the low incidence of the translocation in AML (0.5%-4%). Standardized Southern blot analysis, using restriction enzymes EcoRV, HindIII and BamHI in combination with the limited number of four probes, is a reliable method for diagnosis of t(6;9) and is therefore clinically applicable.

Due to the limited amount of material, intact RNA could only be analyzed in 14 cases (table 1). In 10 out of 14 cases the PCR generated a dek-can chimeric fragment using one primer set and confirmed the results obtained by Southern blot analysis. Intact RNA of 4 patients neither yielded can- nor dek-can fragments after amplification. Probably this is due to technical insufficiency, since inhibition of the Taq polymerase by substances in blood has been described (48). (Moreover, Northern blot analysis of patient 2 showed a dek-can mRNA, see ref. 19). We infer from our results that the chimeric gene is expressed in the leukemic cells of all the t(6;9)-patients that could be analyzed. Moreover, the chimeric fragment is detected in all these patients using the same dek-can oligomer, indicating that it originates from the same exon fusion product in these 10 cases.

The uniform findings at the DNA- and RNA-level in 17 patients with t(6;9) indicate that this translocation is highly suitable for molecular detection by Southern blotting and reversed PCR. The latter technique allows sensitive detection of residual leukemic cells

after chemotherapy and after bone marrow transplantation through monitoring the presence of the dek-can mRNA. The consistent finding of the chimeric product in patients with a t(6;9) also strongly argues for a distinct causative role of the dek-can fusion gene in this myeloproliferative disorder. Occurrence of this translocation in various subtypes of AML (M1,2,4,AMF) and the observations in patient 8 indicate that the dek-can gene product does not cause maturation block. Whether once taken place the translocation invariably leads to leukemia remains to be established.

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Chapter III.3

DEK-CAN REARRANGEMENT IS RESTRICTED TO THE TRANSLOCATION (6;9)(p23;q34).

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SUMMARY

The translocation (6;9)(p23;q34) is mainly found in specific subtypes of Acute Myeloid Leukemia (AML) and Myelodysplastic Syndrome (MDS). The diagnosis of this translocation is not easy since the cytogenetic change is quite subtle. However, recently the two genes involved in this translocation were isolated and diagnosis at the DNA-level became an additional option. Both the dek-gene on chromosome 6 and the can-gene on chromosome 9 contain one specific intron where breakpoints of t(6;9) patients were found to cluster. The translocation results in a consistent chimeric dek-can mRNA which is generated from the 6p- derivative.

Five centers participated in a study to estimate the incidence of t(6;9) in leukemic patients using conventional Southern blot analysis. Patients (n=320) with either acute undifferentiated leukemia (AUL), AML, MDS or acute lymphoblastic leukemia (ALL) were screened for rearrangement of the genes involved in this translocation. Four of these 320 patients showed rearrangement of the can-gene on chromosome 9 of which one had also a rearranged dek-gene on chromosome 6. Moreover, 20 additional patients were studied with karyotypic aberrations in which either the short arm of chromosome 6 or the long arm of chromosome 9 were specifically involved. Both conventional Southern blot analysis and contour-clamped homogenous electric field (CHEF) analysis failed to show dek-can rearrangement in any of these patients. The results of our study indicate that the incidence of the t(6;9) is as low as reported based on cytogenetic data and that rearrangement of the dek- and can-genes is mainly restricted to this specific translocation.

INTRODUCTION

Research in the past twenty years has clearly proven the diagnostic value of cytogenetics in neoplasms since defined karyotypic abnormalities are found in specific tumors and subtypes of leukemia and lymphoma (1-4). Moreover, these aberrations are helpful in predicting the outcome of the disease in quite a number of cases (5,6). The translocation (6;9)(p23;q34) is mainly found in acute myeloid leukemia (AML) with FAB-classification M1, M2 or M4 and refractory anemia with excess of blasts (RAEB) (7-13). This chromosome change predicts a poor prognosis for a patient-group which is characterized by its young age. Recently, the genes were isolated which are disrupted by the translocation (14). The dek-gene in band 6p23 is 40 kb large while the can-gene in band 9q34 is more than 130 kb. Analysis of 17 patients with a t(6;9) showed that breakpoints cluster in one specific 9 kb intron in the dek-gene ('intron containing breakpoints on chromosome 6' or icb-6) and a 7.5 kb intron in the can-gene (icb-9) (13,14). The precise definition of the t(6;9) at the DNA-level enables us to use Southern blot analysis for diagnosis of this disease in addition to karyotyping. We report on the molecular analysis of 340 patients with various types of leukemia which were studied by Southern blot analysis to detect rearrangement of the dek- and the can-gene.

Patients.

Patient material originated mainly from five sources: the Cytogenetic Laboratory, Department of Cell Biology and Genetics of the Erasmus University Rotterdam, The Netherlands, the University Hospital in Modena, Italy, Children's Hospital of the University of Ulm, Ulm, Germany, Department of Hematology, Cliniques Universitairs St. Luc, UCL, Brussels, Belgium, and the Department of Haematology, University of Wales College of Medicine, Cardiff, United Kingdom. A total of 320 patients were screened by conventional Southern blot analysis and this patient-group is represented in table 1. The following diagnoses were made: 50 AULs, 102 AMLs, 60 MDSs, 1 Polycythemia Vera and 107 ALLs.

In addition, 20 patients with karyotypic abnormalities involving 6p or 9q were analyzed by Southern blot and, if enough material was available, by contour-clamped homogenous electric field (CHEF) analysis. Data of these patients are given in table 2. The clinical diagnoses in these 20 patients were: 8 AMLs, 4 MDSs, 6 ALLs and 2 CMLs.

The molecular analysis of the patients was performed at the place of origin except for the patients from Modena, Italy and the 20 patients with specific involvement of the short arm of chromosome 6 or the long arm of chromosome 9 who were analyzed in Rotterdam.

MATERIALS AND METHODS

Karyotyping.

Karyotyping was performed at the place of origin. The results of cytogenetic analysis were available in 111 cases: 4 cases of AUL, 54 cases of AML, 44 cases of MDS, 6 cases of ALL, 2 cases of chronic myeloid leukemia (CML) and 1 case of polycythemia vera (tables 1 and 2). Although karyograms showed a variable pattern from normal karyotype to very complex abnormalities, no classic t(6;9)(p23;q34) had been observed

Table 1. **320 Patients screened by conventional Southern blot analysis for *dek-can* rearrangement.**

Source	Diagnosis	Karyotype	*can*-gene[a)]	*dek*-gene[a)]
Rotterdam	2 AUL	known: no t(6;9)	-	-
	12 AML	known in 10 cases: no t(6;9)	-	-
Modena	13 AML	known in 7 cases: no t(6;9)	-	-
	10 MDS	known in 6 cases; no t(6;9)	-	-
	1 polycythemia vera	known: no t(6;9)	-	-
Ulm	46 AUL	unknown	- (n = 46)	- (n = 14)
	48 AML	unknown	- (n = 46) + (n = 2, case A and case B)	- (n = 29) case A: not tested case B: +
	43 pre-T/T-ALL	unknown	-	not tested
	64 c-ALL	unknown	- (n = 63) + (n = 1, case C)	- (n = 24) -
	14 MDS	unknown	- (n = 13) + (n = 1, case D)	not tested case D: -
Brussels	2 AUL	normal karyotype	-	not tested
	29 AML	normal karyotype	-	not tested
	5 MDS	normal karyotype	-	not tested
Cardiff	31 MDS	known in 29 cases: no t(6;9)	- (n = 26)	- (n = 27)

[a)] - : not rearranged
+: rearranged

Table 2. 20 Patients with karyotypic abnormalities involving 6p or 9q.

Patient	Diagnosis	Age	Sex[a)]		Karyotype
1[b)]	AML	67	F	16%	46,XX,del(13)(q13q2100)
				25%	46,XX,-6,+ring(6p)
				59%	47,XX,-6,+8,+ring(6p)
2[b)]	AML-M2	39	M	13%	46,XY
				23%	46,XY,inv(16)(p13q22)
				64%	46,XY,t(1;18)(q21;q21),inv(2),t(2;8)(q37;q21),t(2;11)(p22;p15),t(3;20)(p26;q12),t(6;17)(p22;q11),inv(16)
3[b)]	ALL relapse after BMT	6	M	45%	46,XX
				55%	53,Xq-,+Xq+,Y,+Y,6p- and multiple numerical and structural abnormalities.
4[b)]	AML	52	F	65%	47-50,XX,t(3;22)(p12;p11),5q-,6p+,(6qter→p23::?),11q+,13p+,+13p+,15p+,-17,-21,+ a variable number of unidentified markers
				35%	49-51 idem, +8
5[b)]	Secondary AML	63	F	61%	44,XX,inv(3),der(5) t(5;12)(q13;q13),-12,der(16),t(16;17)(q13;q11),-17, and 21q+
				25%	44,XX,inv(3),der(5) t(5;12)(q13;q13),-12,der(16),t(16;17)(q13;q11),-17, and 6p+
				14%	44,XX,inv(3),der(5) t(5;12)(q13;q13),-12,der(16),t(16;17)(q13;q11),-17, and i(8q)
6[b)]	AML	59	M	54%	42-43,XY,-5,-7-,17-,19,-21,6p-[der(6)t(6;?)(p22;?)],der(12),t(12;17)(p11;q11),22p+,22q+,+2 markers
				46%	42,XY,idem,-3,der(18),t(3;18)(q22;q23)
7[c)]	CML	68	M		46,XY,t(6;9;22)(p24;q34;q11)
8[c)]	Myelofibrosis	59	M		46,XY,6p+,t(7;13),13q-
9[c)]	Myelofibrosis	64	M		44,XY,-3,-5,-6,-12,-21, 6p+, +mar 1, +mar 2, +mar 3
10[d)]	Secondary MDS	40	M		t(3;6)(p11;p23)

11[e)]	T-cell ALL	26	M		t(3;6)(q13;p23)
12[d)]	CML relapse after BMT				t(9;12;12)(q34;p13;q15)
13[b)]	cALL	24	M	38%	46,XY
				62%	45,XY,4p+,6q-,9p+,del(9)(q21q33),t(12;21)(p13;q22),13q-,t(13;15)(q12;q24),-20
14[b)]	cALL	17	M	16%	46,XY
				8%	45,XY,+Y,-7,-20
				15%	idem, del(1)(p21p31)
				61%	idem, 9q+,11q-,15q-,17q-
15[b)]	cALL	4	F	40%	46,XX
				60%	46,XX,der(9),t(9;?)(q34;?),del(12)(p12),inv(14)(q21?q32),18q+
16[b)]	cALL	2	M	25%	46,XY
				75%	45,XY,-7,-9,1p+,16q-,19q+,20p-,+mar[7qter → p11::9q11 → q34::?]
17[b)]	AML-M6	80	M	22%	46,XY
				28%	46,XY,t(1;22)(p11;q12)
				50%	46,XY,t(1;22)(p11;q12),t(9;17)(q34;q12)
18[b)]	AML relapse after BMT	26	M	6%	46,XY
				94%	48,X,t(Y;18)(p11;q22),+14,+21,t(1;20)(p35;q13),t(9;12)(q3400;q34),13q-,14p+
19[b)]	MDS III after M. Hodgkin	57	F	3%	45,X,-X
				50%	43,X,-X,-3,-5,7q-,12p-,13p+,15q- and der(9),t(9;?)(q31;?)
				17%	86,XX,-X,-X,etc. (tetraploid)
				30%	44-45, idem and variable markers
20[b)]	AML-M5	67	M	33%	46,XY
				67%	45,XY,+X,-8,-15,dup(1)(q21q31),del(5)(q12q34),t(6;9)(q23;q34),der(9)(p+),del(14)(q21q31)

a) M = male, F = female

Cytogenetic Diagnosis b) Rotterdam, c) Modena, d) Leuven, e) Torino, f) Glasgow.

in any of these cases. Twenty of these patients showed specific involvement of either 6p or 9q. Their karyotype is described in table 2.

Conventional Southern blot analysis.
High molecular weight DNA was extracted from blood or bone marrow cells by standard techniques or trapped in agarose plugs as described (15,16). The following restriction enzymes were used for digestion of the DNA: HindIII, BamHI, BglII and/or EcoRV. Probes used for hybridization were: a 500 bp EcoRI-EcoRI fragment (MF1E.5) and an 800 bp BglII-HindIII fragment (MF2BH) which both detect breakpoints in the icb-6 and a 200 bp EcoRI-PstI fragment (AL1F4EP) and a 300 bp EcoRI-EcoRI fragment (AL1F6E-.3) which do the same for the icb-9. Hybridization- and washing conditions were described previously (14). A simplified restriction-map of part of the dek- and can-genes containing icb-6 and icb-9 is given in figure 1 and the location of the probes is indicated.

Contour-clamped Homogenous Electric Field electrophoresis (CHEF).
High molecular weight DNA from blood or bone marrow cells was isolated in agarose plugs as described (16). The rare cutting enzyme BsshII was used for digestion of the DNA since this generates fragments which contain the whole dek-gene and the whole can-gene (figure 1, Ref. 14). On chromosome 6 the dek-gene is contained in a 150 kb BsshII fragment and if a t(6;9) occurs, an abnormal 180 kb fragment is generated. For the can-

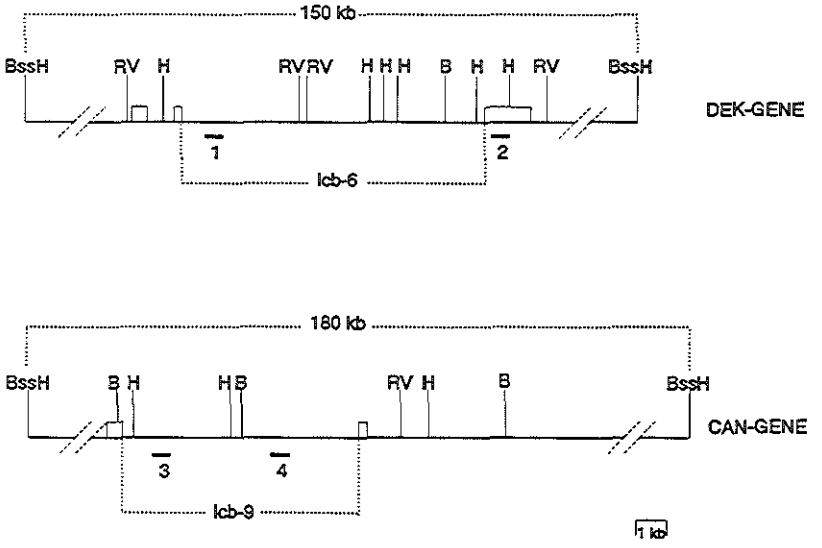

Figure 1. Simplified restriction map of part of the dek-gene on chromosome 6 and the can-gene on chromosome 9. The introns in the genes which contain the breakpoints in patients with a t(6;9) are indicated as icb-6 and icb-9. The rare cutting enzyme BssHII generates a 150 kb fragment containing the whole dek-gene while this is a 180 kb fragment for the can-gene. Restriction sites: BssH=BssHII, RV=EcoRV, B=BamHI, H=HindIII. Probes: 1=MF1E.5, 2=MF2BH, 3=AL1F4EP, 4=AL1F6E.3

gene the normal fragment is 180 kb while the aberrant fragment is 150 kb (14). Separation of fragments on a 1% agarose gel was performed by a contour-clamped homogenous electric field apparatus (CHEF) at 140 Volts with 30 sec. pulse-time for 18 hours (17). Phage lambda oligomers and yeast chromosomes were used as size markers. The four probes described above or cDNA-probes of dek or can were used for hybridization. Hybridization- and washing conditions were identical to a standard Southern blot procedure (14).

RESULTS AND DISCUSSION

The translocation (6;9)(p23;q34) is a relatively rare event since reports indicate an incidence of 0.5 to 4% in patients with AML (13). Cytogenetic detection of the translocation is difficult due to the exchange of chromosomal parts of almost similar length. To exclude misdiagnosis of patients with a t(6;9) due to masked translocations (as has been described for Philadelphia-negative-BCR-positive leukemias), it was decided to screen a large group of patients with AML using Southern blot analysis (18). Although the first reports of the t(6;9) mostly involved patients suffering from AML, we and others discovered that this translocation is not only restricted to AML (13). Therefore, we decided to analyse not only AMLs but also MDSs, AULs and ALLs.

A total of 320 patients originating from 5 centers were analyzed with conventional Southern blotting for rearrangement of the dek- and the can-gene, more precisely; for the occurrence of breakpoints in icb-6 or icb-9. In some cases only one of these two genes could be analyzed due to restrictive amounts of material (table 1). The screening revealed four patients with a rearranged can-gene of which one of the three tested had also a rearranged dek-gene. Data of these four patients are given in more detail in table 3. It should be stressed that the karyotypes of these four patients were not known. The patient with a dek-can rearrangement (case B in table 3) is 39 years old and has an AML-M4 which are both features fitting the classic t(6;9) patient (13). In case A analysis of the dek-gene was not performed while in cases C and D Southern blotting using restriction enzymes EcoRV, BglII and HindIII in combination with probe MF1E.5 and restriction enzyme EcoRV in combination with probe MF2BH showed germline dek-bands.

Patients C and D may belong to a separate group of patients in which the can-gene translocates to another, as yet unknown gene. It is noteworthy that patient C is a cALL, a phenotype discordant with the leukemic subgroup associated with t(6;9). In fact, von Lindern et al analyzed a patient with AUL and normal karyotype in whom the can-gene was fused to a new and unknown gene (von Lindern et al, manuscript in preparation). Causal relationship of the can rearrangement and the leukemia in patients C and D remains to be established. However, the data suggest that domains in the C-terminus of can are involved in the leukemic process and can be activated by linkage to different N-terminal protein sequences.

Since the remaining 316 patients showed only germline bands in various digests (data not shown) we decided to focus on patients with involvement of the short arm of chromosome 6 and the long arm of chromosome 9 in all kinds of cytogenetic abnormalities. Involvement of 6p seems to occur predominantly in Non-Hodgkin Lymphoma (19-21). Involvement of the short arm of chromosome 6 is only rarely found in leukemias of myeloid origin (22-25). Recently, strong association of 6p-aberrations with lipoma was reported (26,27).

Table 3 Four patients with rearrangement of the can-gene

Patient	Age (yrs)	Sex	Diagnosis	Karyotype	WBC 10^9/liter	Blasts (%) BM	Blasts (%) PB	*can*-gene[2]	*dek*-gene[3]
A	59	F	RAEB	unknown	24		17	+	not tested
B	39	M	AML-M4	unknown	42.5		52	+	+
C	46	M	c-ALL	unknown	29	92	64	+	-
D	64	F	RAEB	unknown	1.8	14	3	+	-

[2] - : not arranged / + : rearranged

Involvement of 6p was reported in an endometrial polyp and other benign uterine tumors (28). Besides these main groups no other neoplasms have been described to be associated with specific aberrations involving the short arm of chromosome 6. The patients we collected mostly suffered from leukemias of myeloid origin. Their karyotype is described in table 2 and partial karyotypes of some are shown in figure 2.

Cases with involvement of band 9q34 are obviously numerous since this region is participating in the Philadelphia translocation. However, involvement in other cytogenetic abnormalities is very rare. Only few associations between specific malignancies and karyotypic aberrations involving 9q34 were described such as t(2;9)(q33;q34), t(7;9)(q34;q34), t(9;14)(q34;q11), t(9;17)(q34;q23), mostly in Non-Hodgkin Lymphoma and t(8;9)(p11;q34) in Ph-negative CML (29-36). The ten patients with involvement of band 9q34 showed leukemias of lymphoid or myeloid origin (table 2).

It was investigated whether

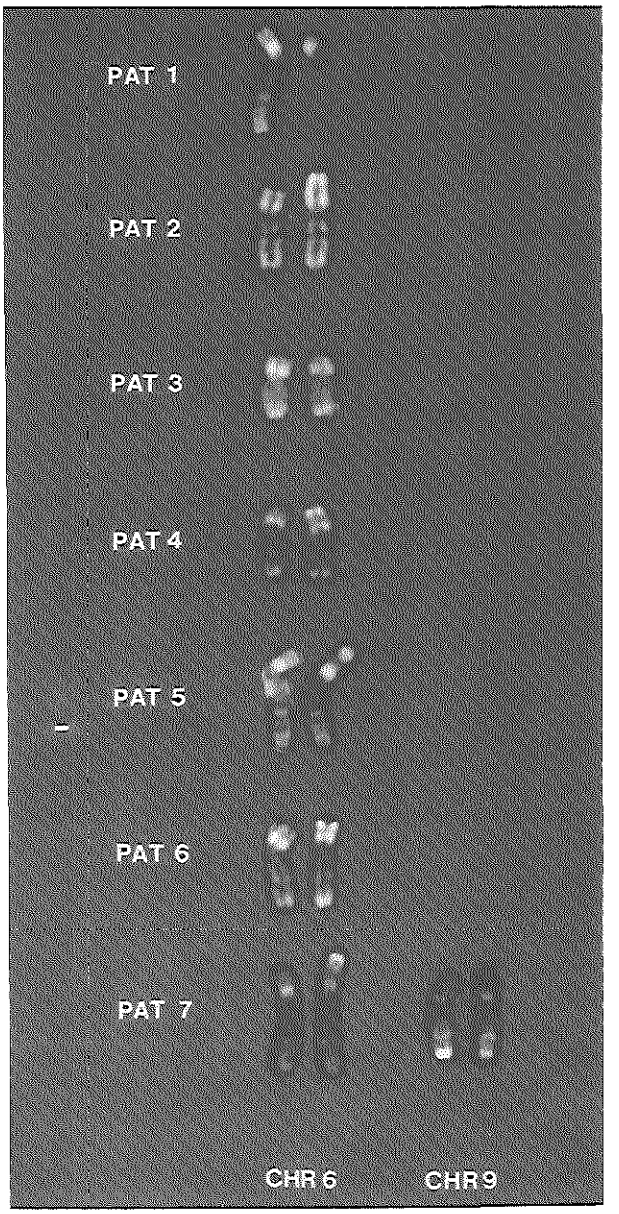

Figure 2. Partial karyotype of patients 1 to 7 whose data are given in table 2. Chr 6=chromosome 6 pair, chr 9=chromosome 9 pair. The left chromosome of each pair is normal, while the one on the right represents the abnormal derivative.

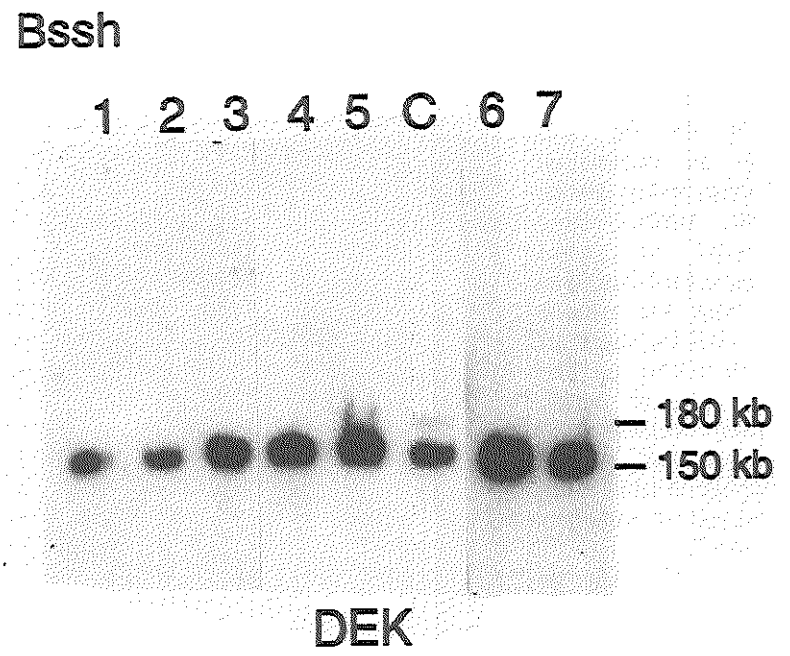

Figure 3. Results of the CHEF-experiments of patients 1 to 7 whose data are represented in table 2 and a t(6;9) patient as a positive control (C). The restriction enzyme used is BsshII and the filter was hybridized with a probe which covers almost the entire dek cDNA (DK9). A wild type 150 kb fragment containing the whole dek-gene is seen in all 8 cases while an additional 180 kb BsshII-fragment can be seen in lane C.

involvement of band 6p23 or 9q34 at the cytogenetic level would be due to participation of the dek- and/or can-gene in the cytogenetic aberrations. Most patients show a complex karyogram in which the contribution of a 9q34 or 6p23 aberration to the leukemic process is highly questionable. However, in patient 7 with CML a t(6;9;22) was seen as a single translocation, suggesting a direct causal relationship with the disease (Table 2). Southern blot analysis in this case had already shown a bcr-abl translocation.

In all 20 patients with aberrations of 6p or 9q conventional Southern blot analysis using various digests in combination with the four probes only showed germ line bands, indicating that no breakpoints occurred in icb-6 or icb-9 (data not shown).

To screen for breakpoints outside icb-6 and icb-9 but still within the dek- and/or can-gene, we used CHEF-analysis in cases where enough material was available. As an example results of the CHEF-experiments of patients 1 to 7 are represented in figure 3. Clinical data and karyograms of these patients are shown in table 2. Also with this method, no rearrangement of dek or can could be found in any of the patients.

Clinical findings in this specific group of 20 patients with abnormal 6p or 9q chromosomes indicate that they are dissimilar to t(6;9) AML patients (table 2):

1) They do not show a consistent diagnosis of AML-M1,2, 4 or RAEB
2) Cases with AML or MDS are relatively old (between 50 to 70 years)
3) Most patients show very complex karyotypes.

These findings sharply contrast to the 'classic' t(6;9) patient who is young (20 to 30 years) and shows FAB-classification restricted to AML M1, M2, M4 or RAEB. Moreover, leukemic cells in these patients have a simple karyotype with t(6;9) often as the sole

aberration.
Our results confirm the rare occurrence of this translocation and show that despite its difficult cytogenetic diagnosis this abnormality is seldomly missed. In fact, in the collection of 340 patients studied here, one case of t(6;9) AML was found with Southern blot analysis that had not been identified before. However, no cytogenetics had been performed in this case. Molecular analysis of patients with karyotypic abnormalities involving the short arm of chromosome 6 or the long arm of chromosome 9 indicate that dek-can rearrangement is mostly restricted to the t(6;9).

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Chapter IV

GENERAL CONSIDERATIONS.

A number of cytogenetic translocations in human tumors give rise to chimeric genes which code for abnormal proteins. Chimeric genes and their protein products can be used as tumor-specific markers. The analysis of the structure and function of oncogene products is essential to discover the mode in which a particular tumor is formed. The first part of this chapter deals with the basic relay system which transmits signals for growth and differentiation from outside the cell via the cytoplasm to the nucleus. Current knowledge of the signal transducing system for cell growth and differentiation will be illustrated by examples. In the second part of this chapter, the results of the experimental work on Ph+ ALLs and myeloproliferative disorder with a t(6;9) are discussed.

1. The derangement of a signal-transducing pathway for growth and differentiation may occur at various cellular levels.

Normal growth and differentiation of a cell are regulated by many different protein factors which participate in intricate networks of control. The relay system on which these networks are based is schematically shown in Figure 1 (Bishop, 1991).
In this system, the binding of a ligand to a specific receptor on the cell surface membrane triggers processes in the cytoplasm which eventually result in activation and modification of nuclear factors. These nuclear factors regulate the expression of genes which encode proteins involved in growth and differentiation processes. Disturbance at any level of the signal-transducing pathway results in the deregulation of normal growth and differentiation.
The model of the regulatory pathway is mainly based on studies of viral oncogenes. However, evidence is accumulating that protein products of oncogenes and altered tumorsuppressor genes may disturb similar regulatory pathways in human tumors (Sawyers et al, 1991).
The proteins which participate in transduction of signals for growth and differentiation can be divided in three main groups:
a) Cell surface receptors
b) Factors in the cytoplasm
c) Nuclear factors

a. Cell-surface receptors.
Many growth- and differentiation factors bind to receptors which are protein tyrosine kinases (PTKs). Receptor PTKs contain three domains: an extra-cellular binding region, a hydrophobic transmembrane domain and an intracellularly located tyrosine kinase domain (Figure 1). Functional studies of this group of proteins started with the Platelet Derived Growth Factor (PDGF) and the Epidermal Growth Factor (EGF) and their receptors (Heldin and Westermark, 1990, Ullrich and Schlessinger, 1990, Cantley et al, 1991). A growth factor binds extracellularly to a specific receptor.

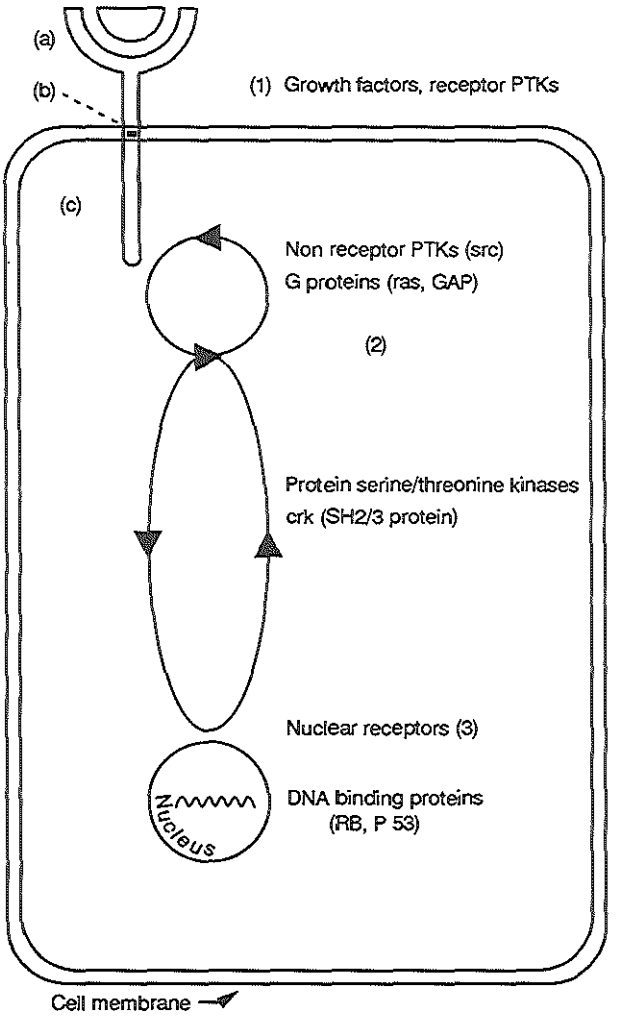

Figure 1. Receptors for growth factors and other regulatory proteins exist of an extracellular ligand-binding domain (a), a transmembrane domain (b), and a cytoplasmic domain (c). Ligand-binding induces a conformational change in the receptor and activation of the cytoplasmic domain. Thereupon, cytoplasmic machineries involving many circuitries and proteins (PTKs, serine/threonine kinases, GTPases) generate signals (2). These are transferred to the nucleus via nuclear receptors and DNA-binding proteins will regulate gene expression of proteins involved in growth and differentiation (3).

Factor-binding causes a conformational change of the receptor and concomittant activation of the intracellular kinase domain of the receptor (Ullrich and Schlessinger 1990, Cross and Dexter, 1991). Next, various cytoplasmic systems are activated such as those involving the G-proteins and GTPase Activating Proteins (GAPs) (see further).

Another example of a PTK-receptor which binds a growth factor is the human CSF-1 (colony stimulating factor 1) receptor. CSF-1 is a hemopoietic growth factor which stimulates growth and expansion of the monocyte-macrophage compartment in the blood and bone marrow. Binding of this growth factor causes auto-phosphorylation of the CSF-1 receptor. As a result the receptor is internalized rapidly by the cell and is degraded. The CSF-1 receptor is encoded for by the c-fms proto-oncogene. Transfection assays with mutants of the CSF-1 receptor showed that a point mutation in the extracellular domain of the receptor caused transformation of cells while a point mutation in the intracytoplasmic C-terminal part resulted in enhanced transformation (Roussel et al, 1988). Moreover, the fms-gene had been assigned to chromosome 5q32, a region that is frequently altered in MDS-patients. Based on these observations it was thought that mutations in the CSF-1 receptor might play a role in certain human leukemias. However, screening of a large number of patients with AML and MDS showed that these specific mutations occurred only in about 10% of such patients (Tobal et al, 1990, Ridge et al, 1990). Probably, additional abnormalities, either of the receptor or of regulatory cellular factors play a role in the origin of AML and MDS.

b. Factors in the cytoplasm.
So far, most experimental work was performed on two types of proteins which act in the cytoplasm: 1) Proteins of the src-family. Contrary to the receptor PTKs such as the CSF-1 receptor, these are non-receptor PTKs. 2) The GTPases including the ras-family.

1) The src family.
The SRC protein can phosphorylate proteins on tyrosine residues. Its structure has been discussed in Chapter II.3. The protein contains several functional domains (Figure 2). An amino-terminal myristylation site is necessary for membrane association of the protein (Cross et al, 1985, Buss and Sefton, 1985, Kamps et al, 1985). Association of SRC with the cell membrane is probably required to function properly. Three important domains can be discerned in SRC: the src-homology 3 (SH3) region, the SH2-region and the SH1-region. The SH1-region codes for the kinase function of the protein. The SH3-region has a negative regulatory influence on the kinase function of the protein while the SH2-region plays a crucial role in the positive regulation of the SRC-protein. The following model has been proposed for control of the function of SRC. A tyrosine residue at the C-terminal part of the protein is phosphorylated by a distinct cytoplasmic PTK (Figure 2(1), Okada and Nakagawa, 1989). Probably the phosphorylated tyrosine interacts with a niche in the SH2-region of SRC (Cooper et al, 1986, Schuh and Brugge, 1988). This causes folding of the protein and thereby inactivation of the kinase domain (Matsuda et al, 1990). Deregulation and inappropriate kinase activation may occur if an oncogene product such as v-crk interacts with the phosphorylated tyrosine (Figure 2). The protein is unfolded and the kinase domain stays in permanent activated condition. Hence, the SRC protein is withdrawn from normal control mechanisms (Cantley et al, 1991).

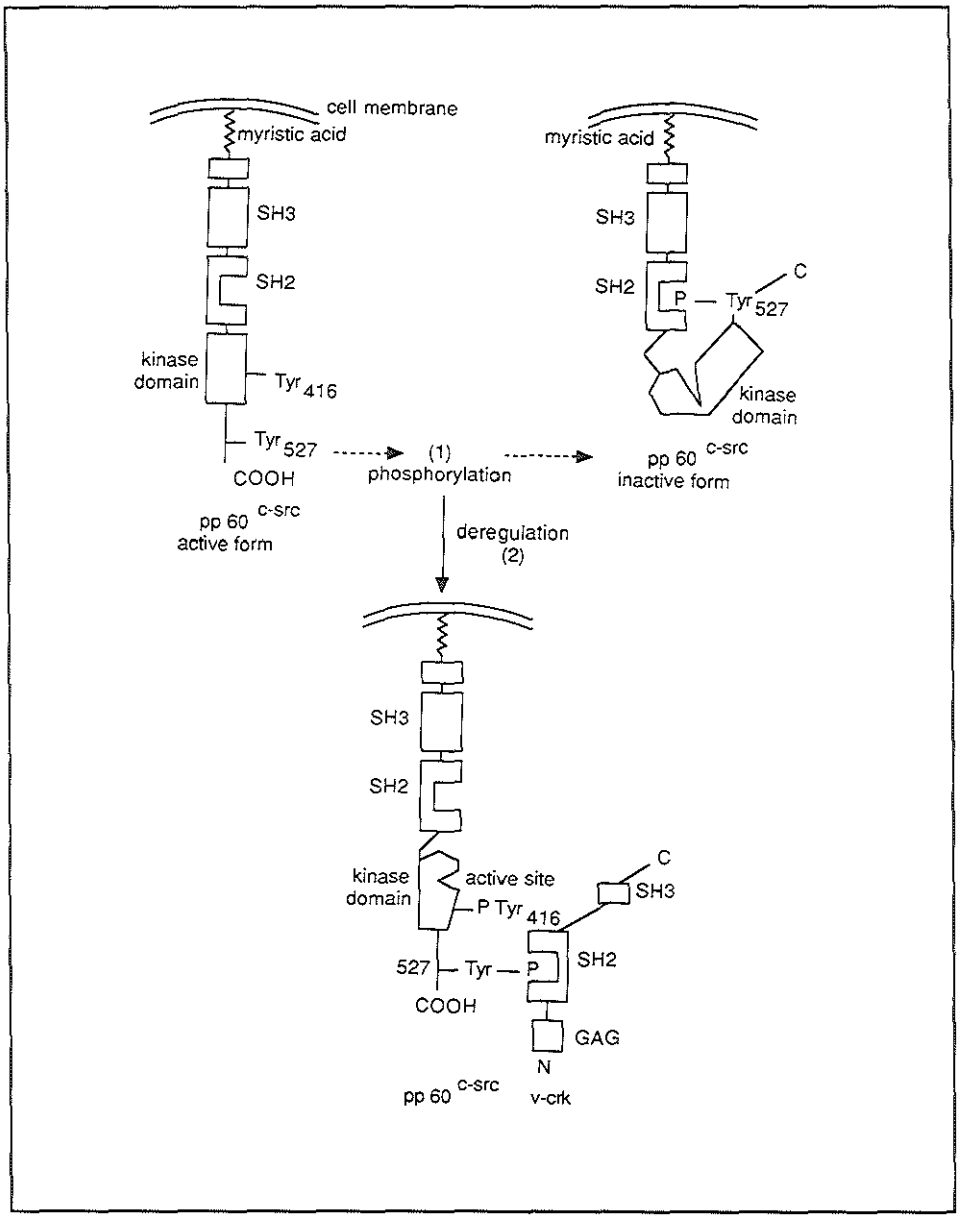

Figure 2. Control of activation of pp60 c-src. Normally, control of pp60 c-src occurs via phosphorylation of the C-terminal Tyr-527 residue and folding of the molecule (1). Deregulation occurs when this phosphorylated residue interacts with the SH2-region of an oncogene product such as v-crk (2). Reference Cantley et al, 1991.

The elucidation of the structure and function of SRC is important to understand how the function of the ABL-protein is disturbed by the t(9;22) which is found in patients with CML and ALL. The ABL-protein is also a cytoplasmic PTK and shows a striking similarity to the SRC-protein. It contains a kinase domain (the SH1 region) which is regulated by two 5' domains: the SH2-region which has a positive regulatory function on the kinase domain and the SH3-region. Experiments, in which ABL-variants with deleted or mutated SH3-regions were studied, showed that the SH3-region is required for the inhibition of the ABL-kinase function (Franz et al, 1989, Jackson and Baltimore, 1989). However, in most cases of Ph+ leukemia the SH3 region remains intact in the BCR-ABL fusion protein while this protein also shows an increased kinase activity (Konopka et al, 1984). Moreover, two patients with Ph+ leukemia were described whose BCR-ABL fusion proteins had a partly deleted SH3-region while the kinase activity of these BCR-ABL variants did not show difference with the kinase activity of BCR-ABL proteins with intact SH3-region (Chapter II.3, this thesis). These findings support the hypothesis that in the Ph-translocation, deregulation of the kinase function of the abnormal ABL protein is exerted via the 5' BCR-sequences (Franz et al, 1989). BCR-ABL constructs with varying deletions in the BCR part showed, that the aminoterminal BCR-sequences are important for increased tyrosine kinase activity of the BCR-ABL protein (McWhirter and Wang, 1991). Specifically the BCR first exon sequences seem to play a major part in the transformation capacity and tyrosine kinase activation of BCR-ABL proteins with intact SH3-region (Muller et al, 1991). Pendergast et al (1991) found that the BCR first exon sequences contain at least two SH2-binding sites which specifically interact with the SH2-domain of ABL and which are essential for BCR-ABL mediated transformation. These findings support a model for deregulation of the ABL protein by the t(9;22) which is similar to deregulation of SRC (Figure 2). Normal regulation of the ABL protein by intramolecular folding and shielding of the kinase domain is impossible because of the presence of 5' BCR sequences. Binding of the 5'BCR-sequences to the SH2-region of ABL results in an 'open' structure of the protein thereby allowing continuous exertion of the kinase function (McLaughlin et al, 1987 and 1989, Lugo et al, 1990, Cantley et al, 1991).

Another oncogene product and its family which has been studied intensively is the RAS-protein. This protein binds GTP and GDP and has intrinsic GTPase activity (Lowy and Willumsen, 1986). Abnormalities of this protein and its family members are often found in a broad range of human tumors and are mainly point mutations (Bos, 1989). The hypothesis is that point mutations in RAS prevent the hydrolysis of a GTP thereby causing the RAS protein to remain in a permanently activated form (Barbacid, 1987, Bourne, 1990a&b). The activated RAS proteins provide continuous signals to depending circuitries which might eventually result in activation of nuclear factors (Figure 1). The GTPase activating protein (GAP) is probably of major importance in the control of the function of RAS (McCormick, 1989, Wigler, 1990, Hall, 1990, Bourne et al, 1990a&b). RAS itself has only weak intrinsic GTPase activity. GAP stimulates the intrinsic GTPase activity of RAS, which keeps the protein in GDP-bound (inactive) state. Of interest is that GAP contains two domains homologous to the src-homology 2 (SH2) region. The SH2-region forms a link between the proteins of the tyrosine kinase group (receptors and non-receptors) and proteins of the RAS family (Ellis et al, 1990, Kaplan et al, 1990, Anderson et al, 1990, Muller et al, 1991).
Proteins of the SRC and RAS families play prominent roles in cytoplasmic transduction of

signals for growth and differentiation. Moreover, the pathways they use are not separated. Interaction between proteins of both families do occur.

c. Nuclear factors.
Translocations in human tumors may generate chimeric gene products which disturb the signal-transducing pathway for growth and differentiation at the nuclear level.
An example is the t(1;19) which is found in part of childhood acute pre-B leukemias. Study of cell lines with the translocation showed that part of the E2A gene, containing the transcriptional activating domain, is joined to a homeobox gene on chromosome 1, prl (pre-B cell leukemia)(Kamps et al, 1990, Nourse et al, 1990). The fusion protein has the features of a chimeric transcription factor. The hypothesis is that this chimeric product activates transcription of prl target genes that normally are not transcribed in pre-B cells.
Another protein product that exerts its function at the nuclear level and that is disrupted by a translocation is the RARα receptor (see Chapter III.1). The t(15;17) in APL joins part of a new gene, pml, to part of the RARα gene. Normally, ligand-binding by the RARα receptor induces expression of particular target genes in the cell nucleus. The receptor-ligand complex binds to specific response elements in promoter/enhancer sequences of the nuclear target genes. The t(15;17) might render the RARα receptor nonresponsive to normal levels of RA. In that case expression of RAR target genes is obstructed and normal cell differentiation is prevented (Lewin, 1991).
Both chimeric gene products illustrate that expression of certain genes at appropriate times of cell development is important for normal growth and differentiation.

Many tumor suppressor genes of which altered forms are found in human tumors contain regions homologous to DNA-binding regions of nuclear proteins (Marshall, 1991).
For example, the retinoblastoma gene product (RB) is located in the nucleus and shows DNA-binding properties (Lee et al, 1987). Phosphorylation of this protein, probably via a cytoplasmic kinase, attenuates the binding to DNA. This event is associated with the beginning of the S phase and cell proliferation (Shenoy et al, 1989, Ludlow et al, 1990). Hypophosphorylation of the protein is thought to play a role in the tumor suppressive function of the RB protein (Mihara et al, 1989, Stein et al, 1990).

Many products of oncogenes or tumorsuppressor genes contain domains characteristic for proteins which play a role at the nuclear level of the signal-transducing pathway for growth and differentiation. In many cases more study of structure and function of these proteins is needed before their exact role in growth and differentiation can be established. So far, the supposition seems true that mutated genes generate abnormal proteins involved in tumorigenesis.

2. Cancer is the result of a multistep proces.

Slowly, parts of regulatory relays controlling growth and differentiation come into focus. Results of in vitro and in vivo studies confirm that most types of tumors have a multifactorial origin and transgress several phases prior to a full blown neoplasm (Nowell, 1986, Hunter, 1991). Although in the case of tumors such as retinoblastoma and Ph-positive leukemia one step was studied extensively, research on other tumors such as colon

carcinoma indicate complex multistep processes in tumor formation (Fearon and Vogelstein, 1990). To study these processes an in vitro model is required. Cooperation of oncogenes can be studied in cell culture systems and many synergisms have been revealed so far using these systems (Land et al, 1983a&b, Ruley, 1983, Graf, 1988, reviewed in Weinberg, 1989, Ruley, 1990).
More sophisticated are transgenic mice (Hanahan, 1989). Introduction of a gene into the germ line of a mouse and the generation of inbred offspring enables one to study the function of the gene in a whole organism. The first experimental models already show that oncogenes or abnormal tumor suppressor genes can cause diseases in transgenic mice. For example, the retinoblastoma gene, the p53-gene and the bcl-2 gene were studied using this system (Windle et al, 1990, Lavigeur et al, 1989, McDonnell and Korsmeyer, 1991).
The bcl-2-Ig transgene of the t(14;18) causes a disease in mice similar to human follicular lymphoma (McDonnell et al, 1989). Autopsy of the transgenic mice shows indolent follicular hyperplasia in lymph nodes and spleen. At the cellular level polyclonal expansion of mature B-cells is found. Ten percent of the bcl-2-Ig transgenic mice develop aggressive monoclonal large cell lymphomas. Analysis of the tumors shows rearrangement of the c-myc gene in 50% (McDonnell and Korsmeyer, 1991). Double transgenic mice, that are the result of crossing Igh enhancer Eμ-bcl-2 (Eμ-bcl-2) transgenics with Eμ-myc transgenics, die of disseminated malignant lymphomas (Strasser, 1990). The Eμ-bcl-2-myc gene causing aggressive malignant lymphomas in transgenic mice illustrates a type of tumor formation in which multiple factors play a role.

Attempts to generate transgenic mice with bcr-abl genes driven by the bcr-promoter have not succeeded so far. Bone marrow cells infected with bcr-abl expressing retrovirus can repopulate the bone marrow of aplastic mice and cause myeloid, macrophage or lymphoid leukemias (Daley et al, 1990, Elefanty et al, 1990, Kelliher et al, 1990). A bcr-abl transgene under the control of the metallothionein promoter results in lymphoid and myeloid leukemias in transgenic mice (Heisterkamp et al, 1990). A bcr-v-abl transgene related to the p210 causes pre-B and T lymphomas if an Immunoglobulin Heavy chain enhancer and promoter are used (Hariharan et al, 1989). However, the bcr-abl gene driven by the bcr-promoter might be too toxic to generate offspring (Dr. G. Grosveld, personal communication).

3. The bcr-abl and dek-can chimeric genes in human acute leukemias: significance for diagnosis

The aim of the work described in this thesis was to compare the value and practical use of molecular techniques to those of karyotyping in the diagnosis of acute leukemias with specific translocations. Characterization of specific subtypes of leukemia at the cytogenetic and molecular level is essential since defined groups carry their own prognosis and require appropriate therapy strategies (reviewed in Chapters II.1 and III.1).
The experimental work in this thesis concerns Ph+ ALLs and AML with t(6;9).
a) Ph+ ALL
The observation of a Ph chromosome indicates a poor prognosis for both adults and children with ALL (Fletcher et al, 1991, Secker-Walker et al, 1991). Studies of the

leukemic cells at the morphologic, immunologic, cytogenetic or genetic level point to a multipotent stem cell or an early myeloid or lymphoid progenitor as the site of origin of the leukemia (Tachibana et al, 1987, Kalousek et al, 1988, Secker-Walker et al, 1988, Turhan et al, 1988, Craig et al, 1990). Analysis of the t(9;22) at the DNA-level shows that breakpoints in the abl-gene on chromosome 9 occur 5' of its tyrosine kinase domain, in a 200 kb intron. Two breakpoint regions are found in the bcr-gene on chromosome 22: one is the bcr-region. This region in the bcr-gene contains most breakpoints of Ph-positive CML cases and half of the breakpoints of adult cases with Ph+ ALL. The other half of adults with Ph+ ALL show a breakpoint in the first intron of the bcr-gene, 5' of the bcr-region (Hermans et al, 1987). The chimeric bcr-abl gene is transcribed into a mRNA with exon joinings b2a2, b3a2 or e1a2. While the b2-b3/a2 junction is found in Ph-positive CML and in 50% of adults with Ph-positive ALL, the e1a2 joining is almost exclusively found in Ph-positive ALL (50% of adults with Ph-positive ALL, almost all children with Ph-positive ALL) (Heisterkamp et al, 1989).
In murine bone marrow cells infected by retroviruses containing either e1a2 or b2-b3/a2 bcr-abl gene constructs the e1a2 protein had a higher tyrosine kinase activity and greater transforming capacity than the b2-b3/a2 protein (McLaughlin et al, 1989). According to some authors this difference might indicate that in humans two diseases exist: Ph+, e1a2+ 'true' de novo ALL and Ph+, b2-b3/a2 ALL. In fact, the latter would be a blast crisis of CML of which the chronic phase was not recognized in the past (Gale and Butturini, 1990). The distinction between CML and ALL is of clinical importance since Ph+ ALL has a variable course after treatment, while lymphoid blast crisis of CML may revert to a second chronic phase with appropriate therapy. However, reports of patients with Ph+ ALL give no clear evidence for differences in clinical course and prognosis between those with e1a2 or those with b2-b3/a2 breakpoint joinings (Secker-Walker et al, 1991). Moreover, the e1a2 junction has been found in clinically 'classic' CML (Selleri et al, 1990). More research is needed to prove that indeed two distinct diseases exist.
Since in 50% of adults and in almost all children with Ph-positive ALL breakpoints occur in the first intron of bcr, analysis at the DNA-level is cumbersome because the intron measures at least 68 kb (Heisterkamp et al, 1988). A solution might be reversed PCR: with this method the mRNA is analyzed. At RNA-level bcr-sequences are linked to abl sequences in a small defined region. We started studying a group of patients with ALL. Karyotyping of blood and bone marrow of the patients was performed and showed normal, abnormal but no Ph or abnormal with Ph configuration. In all patients studied so far, a bcr-abl chimeric fragment was consistently found with reversed PCR if a Ph was present. Absence of a Ph chromosome correlated with reversed PCR which generated abl control fragments, but no bcr-abl chimeric fragments. Surprisingly, in two patients with a Ph chromosome we could not detect a bcr-abl chimeric fragment with the standard bcr-abl oligoprobes we use after reversed PCR. Further investigation with Southern blot, sequence studies and protein analysis revealed that these two patients had bcr-abl variants in which abl exon a2 was deleted (Chapter II.3). This variant is not restricted to acute leukemias, since it is also found in a case of CML (van der Plas et al, 1991). Theoretically, the expected frequency of this unusual variant is 0.3% in all patients with bcr-abl rearrangement (0.6 kb(=intron between exon a2 and a3) divided by 200 kb(=intron where breakpoints are usually found in abl) is 0.003), assuming that breakpoints in abl occur at random. So far, 3 patients were found with the bcr-abl variant lacking abl exon a2, confirming its rare occurrence (Chapter II.3 and Appendix of this thesis). However, reports of Ph+ leukemia patients with breakpoints in bcr, but no bcr-

abl fragment amplifiable by PCR, indicate that in such cases a variant junction might be found (Hooberman et al, 1989). For such cases the use of a 3' primer in abl exon a3 is recommended for the PCR.
In conclusion, most Ph+ ALL patients show either one of three bcr-abl transitions at the mRNA level (i.e. e1a2, b2a2 or b3a2). In very few cases deletion of abl exon a2 is found at the RNA-level. These variants may be missed if only reversed PCR is used for diagnosing bcr-abl positive leukemia. To detect such variants, different techniques such as karyotyping, Southern blot or sequence analysis are required. In the case of deletion of abl exon a2, detection by reversed PCR is possible if a 3' primer in abl exon a3 is used. Deletion of abl exon a2 has no functional implications for the BCR-ABL protein. Studies at the protein level show no significant difference between bcr-abl proteins with and without abl exon a2 sequences (Chapter II.3 of this thesis).

Nowadays, clinicians favour high dose chemotherapy in combination with bone marrow transplantation (BMT) as therapy of choice for patients with Ph+ ALL (Fletcher et al, 1991). Monitoring of the disease course under therapy requires a specific and sensitive method which is provided by the above mentioned PCR-technique. In our hands this method reaches a sensitivity of $1:10^5$, which means that 1 bcr-abl positive cell in a population of 10^5 cells can be detected (Chapter II.2). Therefore, it is the ideal method to diagnose minimal residual disease after therapy. However, the high sensitivity of the PCR is at the same time its drawback. Contamination with bcr-abl cDNAs might give false-positive results and utmost precautions should be taken to prevent such contaminations.

Not much is known about the mechanisms which are involved in leukemic relapse after BMT. Since PCR-techniques became widely available, the course after BMT could be studied with a more sensitive technique than karyotyping or Southern blot analysis (Lawler et al, 1989). Cytogenetic studies of Ph+ CML had already shown that the presence of Ph- donor cells and Ph+ acceptor cells in the post-transplantation bone marrow ('mixed chimerism') occurred relatively frequently (Arthur et al, 1988). Analysis by PCR of bone marrow (BM) status after transplantation confirmed that patients may still be bcr-abl positive after marrow-ablative treatment and BMT. Despite this observation some patients stay in clinical remission (Roth et al, 1989, Lange et al, 1989, Delfau et al, 1990, Pignon et al, 1990). One explanation for this phenomenon might be that acceptor cells with the chimeric gene are still present in the bone marrow after marrow-ablative therapy. The cells are in a non-proliferative or "silent" state, but they still posses the capacity to multiply. So far, it is clear that persistent finding of the bcr-abl product with PCR after BMT precedes cytogenetic and clinical relapse (Sawyers et al, 1990). For Ph+CML two main periods can be discerned in the post transplantation period. In the first 6 months after BMT transient expression of bcr-abl may be found in patients who might still stay in long-term remission. However, recurrence of the leukemia can be expected if the bcr-abl product is persistently found beyond the first 6 months after BMT (Hughes et al, 1991).

b) T(6;9) in AML
While research on bcr-abl has relatively progressed, the study of the dek-can chimeric gene which is the product of the t(6;9) started recently (von Lindern et al, 1990). The dek-can chimeric gene is found in various subtypes of AML and MDS. Patients with the translocation are young and respond poorly to therapy. In many, an MDS-phase was

reported to precede overt AML (Horsman and Kalousek, 1987). These characteristics point to a multipotent stemcell as the origin of the leukemia.
The dek-can chimeric gene is specifically found in all patients studied with a t(6;9) (see Chapter III.2). Diagnosis of this subtype of leukemia is possible now using conventional Southern blot analysis, since breakpoints both in the dek-gene on chromosome 6 and the can-gene on chromosome 9 consistently occur in one relatively small intron in each of the genes (less than 10 kb). Moreover, the dek-can chimeric gene is transcribed into one unique chimeric dek-can mRNA which forms an ideal target for reversed PCR. This sensitive technique enables us to monitor minimal residual disease in patients after chemotherapy or BMT (Chapter III.2). Analysis of a large group of patients with acute myeloid leukemia, acute lymphoblastic leukemia, or myelodysplastic syndrome confirmed that the dek-can chimeric gene is restricted to the myeloproliferative disorder with a t(6;9) (Chapter III.3). Despite the subtle change at the cytogenetic level, the molecular studies indicate that the translocation is seldom missed with karyotyping.
In conclusion, a new subtype of leukemia is characterized at the molecular level. In addition to karyotyping, Southern blot analysis and reversed PCR are appropriate methods for the diagnosis and follow up of the myeloproliferative disorder with a t(6;9).

4. Gene therapy.

An increasing number of genes involved in tumorigenesis is identified and their concomittant abnormality at the molecular level is elucidated. Molecular techniques enable manipulation and modification of such genes in vitro and make introduction of genetic material into cells possible. These developments raise hope for the application of somatic-cell gene therapy in treatment of human malignancies in the future. In cancer of the hematopoietic system the target cell for such therapy is the pluripotent hematopoietic stem cell. This is an ideal vehicle for the introduction of correcting genes since it produces offspring which can repopulate the blood and bone marrow of an individual for a prolonged period. Many technical problems still have to be solved before gene therapy may be an alternative in the treatment of human malignancies. However, results of ongoing research are encouraging (Apperley and Williams, 1990, Cournoyer and Caskey, 1990, Weatherall, 1991).

5. Concluding remarks.

It is clear that in the beginning of cancer research, many scientists hoped to find one universal cause of this dire disease. Soon it became evident that a simple explanation of the cancer problem did not exist. Although in the past few years many genes and their tumor-associated defects became known, their actual function and position in the cascades regulating growth and differentiation were a mystery. Recent progress in this field is hopefull and indicates that all along using relatively simple theoretical models we are on the right track.

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SUMMARY.

Many subtypes of leukemia are characterized by specific cytogenetic translocations. The karyotyping of blood or bone marrow is one of the methods which is used nowadays to establish a diagnosis of leukemia and to predict the outcome of the disease in an individual patient. In an increasing number of translocations which are found in human leukemias, the genes at the translocation breakpoints are isolated. The possibility to study translocations at the genetic level provides additional methods of diagnosis such as Southern blot analysis or the Polymerase Chain Reaction (PCR) (Chapter II.2). The latter technique is a major breakthrough. Its high sensitivity allows the monitoring of residual leukemia after bone marrow transplantation or chemotherapy more accurately than with any other method so far.

The t(9;22) or Philadelphia translocation is found in 20% of adult ALL and in 5% of childhood ALL. This karyotypic abnormality is associated with a poor prognosis within the group of ALLs. Diagnosis at the genetic level is possible since breakpoints occur in the abl-gene on chromosome 9 and the bcr-gene on chromosome 22. Due to the translocation a BCR-ABL protein is formed with an increased kinase activity as compared to the normal ABL protein. Using various diagnostic methods we detected a variant bcr-abl product in two ALL-patients with a classic t(9;22) (Chapter II.3). In most Ph+-patients a bcr-abl chimeric gene is found in which the region, regulating the abl-kinase domain, stays intact. The two patients we analyzed showed bcr-abl variants in which this regulatory domain was partly deleted. However, protein studies showed no difference in kinase activity between the BCR-ABL variants and the BCR-ABL proteins which are usually found in Ph+-leukemias. These findings give additional support to a hypothesis which proposes that in the bcr-abl translocation the 5' bcr-sequences are important for deregulation of the ABL kinase.

The t(6;9) was reported to occur in AML M1, M2 and M4. The translocation is associated with a poor prognosis. Most patients with this translocation are young. A survey of the literature showed that the t(6;9) can also be found in a subtype of MDS. Recent cloning of the genes involved in the translocation provided additional means to diagnosis this subtype of malignancy. We observe that rearrangement of the dek-gene on chromosome 6 and the can-gene on chromosome 9 consistently occur in patients with a t(6;9) (Chapter III.2). Breakpoints in both genes are located in an intron of less than 10 kb. A combination of three different restriction enzymes with four different probes suffice to diagnose the translocation at the genetic level. Moreover, an additional method of diagnosis is the reversed PCR since a dek-can mRNA is formed with a unique breakpoint junction. The screening of a large group of patients with various types of leukemia and MDS indicates that the dek-can chimeric product is restricted to the translocation (Chapter III.3).

Characterization of subtypes of leukemia at the genetic level provides additional methods of diagnosis besides karyotyping, hematomorphology or immunophenotyping. Subdivision of leukemia in prognostic groups is important for further therapeutic approach in an individual case. The finding of molecular variants in patients with specific translocations may shed light on the basic proces which underlies a specific subtype of leukemia.

SAMENVATTING.

In veel subtypen van leukemie worden specifieke cytogenetische translocaties gevonden. Karyotypering van bloed of beenmerg is dan ook één van de methoden die tegenwoordig gebruikt wordt om tot een diagnose te komen. Het vinden van bepaalde cytogenetische afwijkingen kan van belang zijn om de prognose voor een individuele patient te bepalen. In toenemende mate worden de genen geisoleerd, die gelegen zijn op de translocatie-breekpunten van diverse leukemieën. Hierdoor is het mogelijk deze ziekten te bestuderen op genetisch niveau. Diagnose van specifieke subtypen van leukemie wordt nu gedaan met behulp van de Southern blot techniek of de Polymerase ketting reactie (PCR) naast karyotypering (Hoofdstuk II.2). Vooral de Polymerase ketting reactie is een aanwinst voor de diagnostiek, omdat de zeer hoge sensitiviteit gebruikt kan worden om kleine aantallen leukemische cellen te detecteren na beenmerg transplantatie of chemotherapie.

De t(9;22) of Philadelphia translocatie wordt gevonden in 20% van de volwassenen met acute lymfatische leukemie (ALL) en in 5% van de kinderen met ALL. Deze translocatie is geassocieerd met een slechte prognose binnen de groep van ALLs. De diagnose op genetisch niveau is mogelijk omdat breuken optreden in het abl-gen op chromosoom 9 en het bcr-gen op chromosoom 22. Door de translocatie ontstaat er een BCR-ABL eiwit dat een verhoogde kinase activiteit vertoond in vergelijking met het normale ABL eiwit. Met behulp van verschillende diagnostische technieken vonden we een variant van het bcr-abl product bij twee patienten met Ph' positieve ALL (Hoofdstuk II.3). Bijna alle patienten met een Ph' chromosoom hebben een bcr-abl chimeer gen waarbij het domein dat de kinase activiteit van ABL reguleert intact blijft. De twee patienten die wij bestudeerden hadden variante bcr-abl genen waarbij dit regulerende domein gedeeltelijk gedeleteerd was. Onderzoek van het eiwit toonde echter aan dat er geen quantitatief verschil was tussen de kinase activiteit van de variante BCR-ABL eiwitten en de BCR-ABL eiwitten die in het merendeel der gevallen bij Ph' positieve patienten gevonden worden. Deze bevindingen ondersteunen de hypothese dat binnen het abnormale BCR-ABL product de 5' BCR sequenties van belang zijn voor de deregulatie van ABL.

De t(6;9) wordt gevonden in acute myeloide leukemie (AML) M1, M2 en M4. De translocatie is geassocieerd met een slechte prognose. Dit subtype van leukemie wordt gevonden bij jong volwassenen. Literatuuronderzoek wees uit dat deze translocatie ook gevonden wordt bij een subtype van MDS. Door de recente klonering van de genen die betrokken zijn bij deze translocatie kan dit subtype van leukemie nu ook op genetisch niveau gediagnosticeerd worden. Ons onderzoek toonde aan dat zowel het dek-gen op chromosoom 6 als het can-gen op chromosoom 9 betrokken zijn bij de t(6;9) (Hoofdstuk III.2). De breukpunten in beide genen worden gevonden in een intron van minder dan 10 kb. Voor de diagnose van de translocatie op genetisch niveau is het voldoende drie verschillende restrictie enzymen te gebruiken in combinatie met vier verschillende probes. Omdat op mRNA-niveau een unieke dek-can overgang optreedt kan men de Polymerase ketting reactie ook gebruiken als diagnosticum. We bekeken een grote groep patienten met uiteenlopende typen leukemie en MDS. Dit onderzoek gaf aan dat het dek-can chimere product alleen gevonden wordt bij de t(6;9) (Hoofdstuk III.3).

Onderzoek van subtypen van leukemie op genetisch niveau kan gebruikt worden naast karyotypering, celmorfologisch onderzoek of immunologisch onderzoek om tot een

diagnose te komen. Onderverdeling van leukemieën in groepen met een eigen prognose is belangrijk voor het therapeutisch beleid in een individueel geval. Het vinden van variante producten bij patienten met specifieke translocaties kan helpen bij het vinden van het basale mechanisme wat ten grondslag ligt aan een specifiek subtype van leukemie.

Curriculum Vitae.

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Abbreviations.

ALL	acute lymphoblastic leukemia
AMF	acute myelofibrosis
AML	acute myeloid leukemia
ANLL	acute non-lymphoblastic leukemia
APL	acute promyelocytic leukemia
AUL	acute undifferentiated leukemia
BCR	5.8 kb breakpoint cluster region=M-BCR-1
bcr-gene	breakpoint cluster region gene
BL	Burkitt's lymphoma
BM	bone marrow
BMT	bone marrow transplantation
bp	basepair
c-abl gene	cellular abl gene
CALLA	common ALL antigen
cDNA	complementary deoxyribonucleic acid
CHEF	contour-clamped homogenous electric field
CLL	chronic lymphocytic leukemia
CMML	chronic myelomonocytic leukemia
CML	chronic myeloid leukemia
CNS	central nervous system
CR	complete remission
DIC	disseminated intravascular coagulation
DCC-gene	deleted in colorectal carcinoma gene
DM	double minutes
FAB	French-American-British (classification)
GAP	GTPase activating protein
GDP,GTP	guanosine diphosphate, guanosine triphosphate
HSR	homogeneously staining regions
kb	kilobase
kD	kilodalton
MIC	morphologic-immunologic-cytogenetic (classification)
M-BCR-1	major breakpoint cluster region=CML breakpoint cluster region=BCR
m-BCR	minor breakpoint cluster region=ALL breakpoint cluster region
MDS	myelodysplastic syndrome
MPD	myeloproliferative disorder
mRNA	messenger ribonucleic acid
NF-1 gene	neurofibromatosis-1 gene
PCR	polymerase chain reaction
Ph	Philadelphia chromosome
PR	partial remission
PTK	protein tyrosine kinase
RAEB(-t)	refractory anemia with excess of blasts (in transformation)
RARα	retinoic acid receptor α
RB gene	retinoblastoma gene
SH region	src-homology region

WBC	white blood cell count
WT-1 gene	Wilms' tumor-1 gene

APPENDIX

Cytogenetic and Molecular Analysis in Philadelphia Negative CML

By D.C. van der Plas, A.B.C. Hermans, D. Soekarman, E.M.E. Smit, A. de Klein, N. Smadja, G. Alimena, R. Goudsmit, G. Grosveld, and A. Hagemeijer

We studied the clinical, hematologic, cytogenetic and molecular biologic features in four patients with Philadelphia (Ph) negative chronic myeloid leukemia (CML). In all four cases the clinical and hematologic characteristics were indistinguishable from Ph positive CML. Cytogenetic analysis showed a normal karyotype in two patients and chromosomal translocations apparently not affecting chromosome 22 in the other two cases. Southern blot analysis using probes of the *bcr* region, demonstrated a *bcr* breakpoint in all four patients. In situ hybridization with *bcr*, *c-abl*, and *c-sis* probes showed unusual hybridization sites for 5'-*bcr* and *c-abl* indicating complex chromosomal rearrangements affecting three different chromosomes in the four patients investigated. Using polymerase chain reaction (PCR) followed by hybridization to oligonucleotide probes specific for the *bcr-abl* fusion region, the expression of a chimeric *bcr-abl* mRNA was detected. In these patients we demonstrated that (*a*) CML with a breakpoint in the *bcr* region without cytogenetically detectable Ph chromosome is characterized by the same genomic recombination of 5'-*bcr* and *c-abl* as CML with standard Ph translocation and (*b*) unusual localization of 5'-*bcr* and *c-abl* sequences caused by complex Ph translocation does not interfere with transcription of the *bcr-abl* fusion gene.

THE PHILADELPHIA CHROMOSOME (Ph) is found in 94% of chronic myeloid leukemia (CML) patients. In the majority of cases it originates from the standard Ph translocation t(9;22)(q34;q11).[1] As a result of this translocation the c-*abl* oncogene is translocated to a specific site in a gene on chromosome 22 band q11 called breakpoint cluster region (*bcr* region).[2] The part of chromosome 22 distal to the breakpoint is translocated to chromosome 9 band q34.

The 5'*bcr-abl* DNA on the Ph chromosome is transcribed into an 8.5 kilobase (kb) chimeric *bcr-abl* mRNA. Depending on the position of the breakpoint in *bcr* either *bcr* exon b2 or b3 are spliced to *abl* exon a2, resulting in either b2a2 or b3a2 chimeric mRNA.[3-10] This 8.5 kb *bcr-abl* mRNA encodes an *abl* protein of 210 kilodalton (kD), which has in vitro enhanced tyrosine kinase activity compared with the normal *abl* protein.[9-11] Approximately 5% of the Ph-positive CML patients have cytogenetic variants of the Ph translocation.[12,13] Southern blot and in situ hybridization studies have shown that in variant Ph translocation molecular recombination of 5'-*bcr* and c-*abl* takes place in exactly the same way as in standard Ph-translocation.[14-20] The remaining 6% of CML patients show no Ph chromosome and are classified as Ph-negative. Ph-negative CML patients have different clinical and hematologic features, ie, older median age (>65 years), monocytosis, thrombopenia, poor response to chemotherapy, rapid transformation to acute leukemia, and shorter survival than Ph-positive CML.[12,15,21,22] Nevertheless a few Ph-negative CML patients present with clinical and hematologic features that are indistinguishable from Ph-positive CML, ie, median age <60 years, higher WBC with basophilia but without monocytosis, no dysplastic changes in bone marrow cells and same survival as in Ph-positive CML.[23]

We report in this article detailed cytogenetic and molecular analysis in four patients with a disease indistinguishable from Ph-positive CML, with either a normal karyotype (two cases) or chromosomal translocations apparently not affecting chromosome 22 (two cases). Southern blot analysis and in situ hybridization studies demonstrated genomic recombination of 5'-*bcr* and c-*abl* sequences caused by complex Ph translocations. cDNA analysis using the polymerase chain reaction (PCR) showed expression of *bcr-abl* mRNA. These findings are identical to the findings in Ph-positive CML.

CASE REPORTS

Patients were diagnosed and treated in three different European centers: patient no. 1 in Rome, patient no. 2 and 3 in Paris, and patient no. 4 in Amsterdam. A summary of clinical and hematologic data at diagnosis is given in Table 1.

Patient no. 1. In September 1984 CML was diagnosed that responded to hydroxyurea (2.5 g/d): splenomegaly regressed and hematologic findings returned to normal values. Hematologic remission was maintained until January 1986 when thrombocytosis appeared increasing to 1,000 × 10^9/L, but responded well to busulfan (2 mg/d). In December 1987, the patient developed a nonlymphoid blast crisis and died in June 1988 due to septic complications.

Patient no. 2. CML was diagnosed in February 1983. Clinical and hematologic remission were obtained with hydroxyurea and lasted 3 years. In April 1986 accelerated phase occurred with 20% myeloblasts in bone marrow (BM). 6-Mercaptopurine was added to hydroxyurea, and again hematologic stabilization was obtained. In October 1986 a splenectomy was performed. The spleen showed important myeloid metaplasia. In April 1987 engraftment with allogeneic BM was realized with success.

Patient no. 3. In May 1977 CML diagnosis was made and successfully treated with busulfan. Chronic phase lasted 8½ years. In January 1986 the first signs of accelerated phase appeared, characterized by bone pains, asthenia, blast cells in BM (10%) and

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Table 1. Clinical and Hematologic Data

	Patient No. 1	Patient No. 2	Patient No. 3	Patient No. 4
Sex/age at diagnosis	M, 40	F, 33	M, 55	F, 64
Organomegaly	Spleno- and hepatomegaly	Splenomegaly	Hematomegaly	None
Hb (mmol/L)	9.0	8.0	8.0	6.8
WBC (10^9/L)	128	80.6	20	121
Myeloblasts	1%	5%	Normal differential count	—
Promyelocytes	2%	4%		5%
Myelocytes	20%	6%		10%
Metamyelocytes	14%	—		5%
Neutrophils	53%	71%		65%
Eosinophils	1%	2%		1.5%
Basophils	1%	2%		2.5%
Lymphocytes	8%	9%		8%
Monocytes	—	1%		3%
Platelets (10^9/L)	470	510	450	218
Bone marrow	Hypercellular granulocytic + megakaryocytic hyperplasia	Hypercellular typical CML no myelofibrosis	Hypercellular myeloid hyperplasia normal maturation	Hypercellular myeloid + megakaryocytic hyperplasia
LAP	4	4	Unknown	9
(normal value)	(20–100)	(20–80)		(25–100)
Duration of chronic phase (yr)	3.25+	3	8.5	7
Survival (yr)	3.75+	5+	9.3	7.3

peripheral blood (10%) and thrombopenia (72×10^9/L). In September 1986, blastic transformation occurred with 55% of undifferentiated blast cells in bone marrow aspirate. The patient was treated with vincristine, daunorubicine, novantrone, and aracytidine. No remission was obtained and the patient died in October 1986 of aplasia, cardiac and renal failure, and hemorrhage.

Patient No. 4. CML was diagnosed in the beginning of 1980, following incidental discovery of granulocytosis without other symptoms. The patient reacted favorably to repeated courses of busulfan. In February 1987 a steady increase of myeloblasts in the peripheral blood was seen, followed within a month by a full blown myeloblastic crisis. Among other drugs treatment with alpha-2-interferon had no success. The patient died 2 months later.

MATERIALS AND METHODS

Samples. Bone marrow aspirates and blood samples were sent to Rotterdam for molecular investigations. The samples were sterile and heparinized and reached the laboratory within 24 hours after aspiration. All sampling was part of diagnostic and clinical follow-up procedures and obtained only after informed consent of the patients.

Cytogenetics. The karyotype of leukemic cells was investigated at diagnosis using standard cytogenetic procedures. Chromosomes were identified using G and/or R banding techniques and classified according to ISCN (1985).[24] Because of unusual findings the cytogenetic analyses were repeated several times locally and in Rotterdam at the time of the molecular investigation. The constitutional karyotype of each patient was determined and found normal using PHA stimulated blood cultures.

DNA probes. The following probes were used in Southern blot analysis and in situ hybridization[2]: c-*abl*, 0.6 kb *Bam*HI + 1.1 kb *Hin* dIII-*Eco*R1 fragment; c-*sis*, 1.7 kb *Bam*HI fragment; 5′-*bcr*, 2 kb *Bgl*II-*Hin* dIII fragment; 3′-*bcr*, 1.2 kb *Hin* dIII-*Bgl*II fragment. In standard Ph translocations the 5′-*bcr* probe recognizes the 22q- derivative and the 3′-*bcr* probe recognizes the 9q+ derivative.

Southern blotting was performed following standard techniques.[2,25] In situ hybridization was performed as reported previously.[26] The probes were ^{3}H-labeled, using the method of Feinberg and Vogelstein[27] to a specific activity of 10^8 cpm/μg DNA. After hybridization and autoradiography the labeled sites were scored on R or Q banded metaphases and assigned to a chromosomal band or region. Grain distribution in the in situ hybridization experiments was tested on its statistical significance using the Poisson distribution followed by the binomial method.

cDNA preparation and amplification with the PCR were performed as described by Hermans et al.[28] Oligonucleotides[28] 2 and 3 (Fig 1) were used as primers to amplify a fragment covering the *bcr-abl* region. As internal positive control in the PCR, an *abl* fragment was amplified, using oligonucleotides 1 and 3, that is always present, irrespective of the Ph translocation. Two 40 mer oligonucleotides specific for the breakpoint junctions b2a2 and b3a2 were used as probes.

RESULTS

Cytogenetics

Patient no. 1. At diagnosis 75 metaphases were analyzed after Giemsa staining and GTG banding technique without evidence of a recognizable Ph chromosome. However, one of

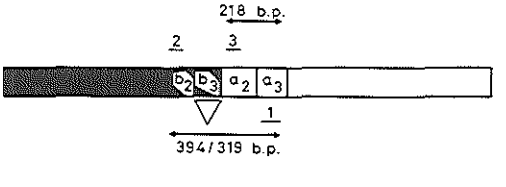

Fig 1. Schematic representation of 8.5 kb *bcr-abl* mRNA. Localization of primers used in PCR experiment is given. ▽ indicates that exon b3 may be absent or present in 8.5 kb *bcr-abl* mRNA. The size and localization of the two fragments amplified in the PCR experiments are presented.

the two chromosomes 22 appeared slightly shorter, one chromosome 19 appeared smaller and sub-metacentric and one chromosome 9 appeared identical to the 9q+ derivative in the standard t(9;22). R-banding studies confirmed these findings (Fig 2). A complex chromosomal translocation t(9;19;22) resulting in a masked Ph was retained as probable interpretation of the karyotype, but routine karyotyping showed two apparently normal chromosomes 22, which was the reason for inclusion of the case in this study.

Patient no. 2. At diagnosis 26 metaphases were analyzed and 80 metaphases in subsequent studies. All metaphases examined showed the same karyotype that was equivocal in R and G bands. There were two possible interpretations: either a simple translocation t(9;14) or a complex Ph translocation t(9;14;22) (Fig 2).

Patient no. 3. The BM karyotype of patient no. 3 was studied twice during the chronic phase of the disease. All 50 metaphases showed a 46,XY normal male karyotype (Fig 2). Molecular investigations were performed at the time of acceleration 8 years and 10 months after diagnosis. At that time the BM karyotypes were 46,XY in 90% and 46,XY, t(1;21)(p21;q22) in 10% of the metaphases. Six months later, in blast crisis 45 metaphases were analyzed, showing additional abnormal clones: 46,XY(16%)/46,XY,t(1;21)(13%)/46,XY,del (6)(p22)(13%)/49,XY,+10,+21,+22 (58%).

Patient no. 4. The BM karyotype of patient no. 4 was found to be normal: 46,XX in repeated investigations of blood and BM cells during the chronic phase of the disease (Fig 2). In January 1987 hematologic and clinical data indicated progression of CML, 20% of the metaphases were abnormal: 47,XX,+8,i(17q). Remarkably, the type of aberrations is the same as often described in progression of Ph positive CML.

Southern Blot Analysis

DNA from bone marrow and/or blood cells of the four patients was digested with *Bgl*II, *Bam*HI, and *Hin* dIII and hybridized to 5'- and 3'-*bcr* probes. All patients showed extra bands with more than one probe and more than one restriction enzyme indicating the existence of a breakpoint in the *bcr* region of chromosome 22 (Fig 3). The breakpoint was found in the *Hin* dIII-*Bgl*II fragment of the *bcr* region in patient no. 1, 2, and 4 (ie, between *bcr* region exon 3 and 4) and in the *Hin* dIII-*Bam*HI fragment in patient no. 3 (ie, between *bcr* region exon 2 and 3). These results are similar to our observations in more than 50 CML patients with standard t(9;22).

In Situ Hybridization Studies

The absence of a cytogenetically recognizable Ph chromosome in the presence of a *bcr* breakpoint prompted investigation of the chromosomal localization of the various genes of interest using in situ hybridization. To this aim metaphase spreads of the four patients were hybridized to four different probes: (1) c-*abl*, (2) 5'-*bcr* mapping proximal to the breakpoint, (3) 3'-*bcr* mapping distal to the breakpoint, and (4)

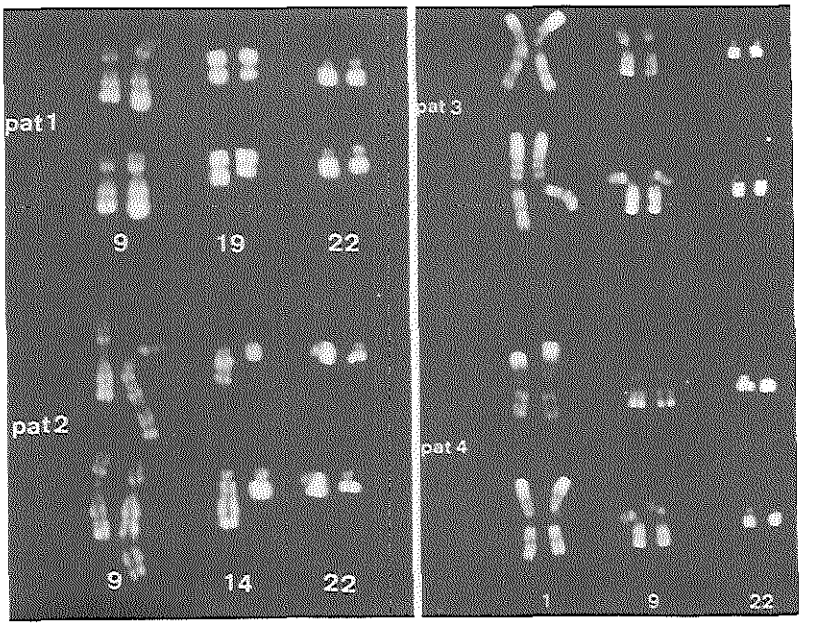

Fig 2. Partial karyotype of patients no. 1, 2, 3, and 4. R-bands with acridine orange. Patient no. 1 shows t(9;19;22), patient no. 2 t(9;14;22), and patients no. 3 and 4 have normal karyotype.

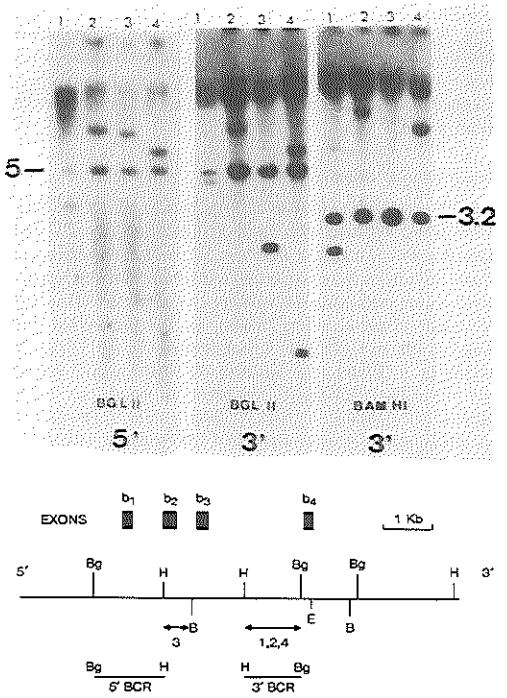

Fig 3. Southern blot showing genomic DNA from Ph-negative CML patients no. 1, 2, 3, and 4 digested with *Bgl*II and *Bam*HI. The filter with *Bgl*II digests was probed with 3'- and 5'-*bcr* probe. The filter with *Bam*HI digests was probed with 3'-*bcr*. Size of germline bands is marked. Extra bands are found in all lanes except in *Bam*HI digest of patient no. 3 probed with 3'-*bcr*, indicating the presence of a breakpoint in the *bcr* region in all four patients. Below the Southern blot a simple restriction map of the *bcr* region is given.[2] The 5'-*bcr* and 3'-*bcr* probes are indicated. Bg, *Bgl*II; H, *Hin* dIII; B, *Bam*HI; E, *Eco*RI; b1-b4, *bcr* region exon 1-4. Arrows marked 1, 2, 3, and 4 indicate the fragment in which the breakpoint on chromosome 22 is mapped in patients no. 1 through 4.

c-*sis* as an indicator of the distal part of chromosome 22. Results are detailed in Table 2 and summarized in Fig. 4.

Patient no. 1. In patient no. 1, with presumably a complex t(9;19;22) (Fig 2), it appeared that 5'-*bcr* and c-*abl* probes hybridized to the shortened q arm of the 19q− while 3'-*bcr* and c-*sis* hybridized to the 9q+ as in classical t(9;22). It is obscure whether the centromere of the chromosome designated as 19q− belongs to chromosome 19 or 22.

Patient no. 2. In patient no. 2 specific hybridization indicated a complex t(9;14;22). Indeed, 5'-*bcr* and c-*abl* probes strongly hybridized to the smallest chromosome therefore identifying itself as Ph or 22q−. 3'-*bcr* and c-*sis* only hybridized to chromosome 22 and to the 14q− that are cytogenetically indistinguishable in this case.

Patients no. 3 and 4. c-*abl* and 5'-*bcr* showed an abnormal localization on the distal part of 1p in patients no. 3 and 4. This part of chromosome 1 showed the same staining properties as the distal part of chromosome 22. In both cases c-*sis* only maps to chromosome 22, while 3'-*bcr* maps on chromosome 22 and in patient no. 3 also on 9q34. In patient no. 3 in the minority of cells with t(1;21) specific hybridization of c-*abl* and 5'-*bcr* occurred on the normal chromosome 1, not involved in the translocation t(1;21). These results indicated that in both patients a complex rearrangement has occurred between chromosomes 1p36, 9q34, and 22q11, resulting in the presence of hybridization sites for c-*abl* and 5'-*bcr* probes on 1p36.

Statistics Used in the In Situ Hybridization Experiments

Grain distribution in the in situ hybridization experiments was tested on its statistical significance using the Poisson distribution followed by binomial method in the case of chromosomes 9 and 9q+. In the case of chromosomes 14q−, 19q−, 22, and 22q− only the Poisson distribution was applied, since the contribution of these chromosomes to the DNA-content of a metaphase is relatively small.[29] Binomial distribution was tested in the case of chromosome 1, since this chromosome contributes to a relatively large part of the genomic DNA. The P value for both Poisson and binomial distribution was determined at 10^{-3}.

All experiments were statistically significant for the probes used with the exception of the 3'-*bcr* probe, which showed no statistical significance according to the Poisson distribution in patient no. 1 on chromosomes 9q+ and 22 and in patients no. 3 and 4 on chromosome 9. Though, in the case of patients no. 1 and 3, experiments with the 3'-*bcr* probe could be demonstrated to be statistically significant for the chromosomes 9q+ and 9, respectively, when the binomial distribution alone was tested.

Amplification of cDNA by the PCR

The results of amplification of cDNA followed by hybridization to breakpoint specific oligonucleotides are shown in Fig 5. A 319 base pairs fragment (bp) corresponding to b2a2 joining in the *bcr-abl* mRNA has been found in patient no. 3 a control CML patient with standard Ph translocation and cell line BV173. A 394 bp fragment corresponding to b3a2 joining has been found in patients no. 1 and 4 and cell line K562. As expected neither b2a2 nor b3a2 joining have been detected in leukocytes of a healthy control.

DISCUSSION

In this article we report clinical, cytogenetic and molecular data obtained in four patients with CML and absence of Ph chromosome in bone marrow metaphases. In these patients clinical features at presentation and long survival (3.75 to 9.3 years) contrasted with the atypical symptomatology and rapid transformation to blast crisis usually associated with Ph-negative CML. Reclassification of these cases as myelodysplastic (CMML) or myeloproliferative syndrome[21,30] does not apply to these four patients who had a characteristics of classic CML except for the Ph chromosome.

Southern blotting demonstrated a *bcr* breakpoint in the four cases, similar to our findings in Ph-positive CML

Table 2. In Situ Hybridization Studies

Patient and Karyotype	Probes	No. of Metaphases Analyzed	No. of Labeled Sites on Chromosomes and Specific Bands*								
			Total	9	(q33-34)	9q+	(q-distal)	22, der (22)	22q−	Other Chromosomes Involved	No. of Background Grains
Patient No. 1	c-*abl*	30	96	11	(10)	3		2†		19/19q−:1/8 (8)‡	46
46, XY,	c-*sis*	192	346	10		32	(22)	24		19/19q−:6/5	103
t (9;19;22)	5′-*bcr*	30	97	2		3		10		19/19q−:1/7 (7)‡	42
	3′*bcr*	32	86	2		8	(7)	6		19/19q−:1/1	50
Patient No. 2	c-*abl*	50	165	20	(18)	8		3§	11	no. 14:3	95
46, XX	c-*sis*	50	158	9		10		24	2	no. 14:3	120
t (9;14;22)	5′-*bcr*	50	156	5		4		16	13	no. 14:3	61
	3′-*bcr*	50	157	4		6		24	1	no. 14:3	94
Patient No. 3	c-*abl*	63	230	27	(19)			4		no. 1:28 (20)‖	150
46, XY	c-*sis*	50	187	9				22		no. 1:22	97
	5′-*bcr*	65	210	10				18		no. 1:25 (15)‖	113
	3′-*bcr*	66	214	21	(14)¶			27		no. 1:19	153
Patient No. 4	c-*abl*	100	208	36	(29)			4		no. 1:26 (17)‖	106
46, XX	c-*sis*	63	182	9				22		no. 1:15	103
	5′-*bcr*	50	146	7				19		no. 1:19 (12)‖	75
	3′-*bcr*	56	174	12	(5)#			21		no. 1:17	118

*The number of labeled sites on specific parts of chromosomes or specific bands is written in parentheses.

†In patient 1 chromosome 22 and der(22) were indistinguishable in mitoses studied after in situ hybridization.

‡Number of labeled sites on the deleted arm of 19q−.

§In patient 2 chromosome 22 and 14q− are indistinguishable by cytogenetics only.

‖Number of labeled sites on chromosome 1 (p34-p36).

¶Statistically significant using the binomial distribution alone.

#Statistically not significant according to the Poisson and binomial distribution.

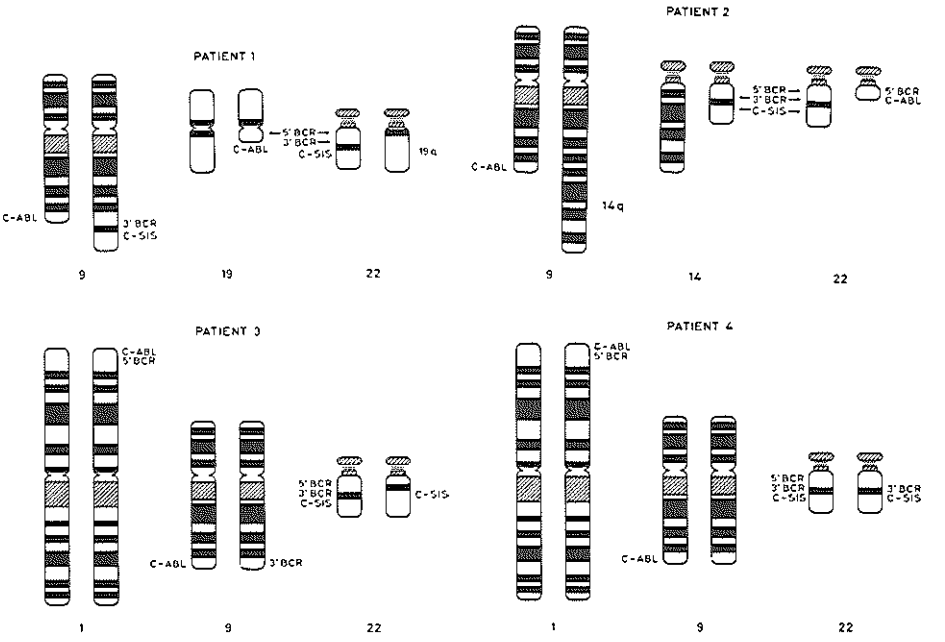

Fig 4. Summary of results of in situ hybridization with 5′-*bcr*, 3′-*bcr*, c-*sis*, and c-*abl* probes in Ph-negative CML patients no. 1, 2, 3, and 4. Normal chromosomes on the left. Localization of the different probes by in situ hybridization is presented.

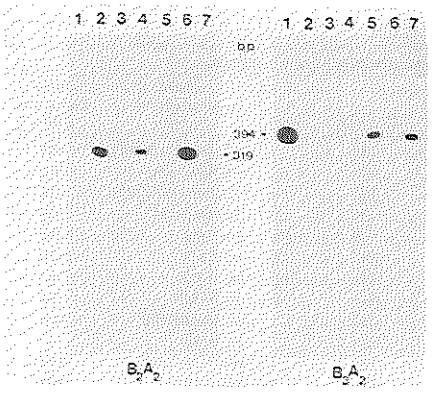

Fig 5. Autoradiogram of the PCR experiment: Using *bcr* and *abl* primers (Fig 1) a cDNA fragment containing the *bcr-abl* fusion region was amplified. After gel electrophoresis and blotting onto nylon membranes hybridization was performed to breakpoint specific oligonucleotide probes b2a2 and b3a2. A 319 bp fragment corresponding to a b2a2 joining has been found in lane 2 (BV173 cell line), lane 4 (Ph positive CML), and lane 6 (patient no. 3). A 394 bp fragment corresponding to a b3a2 joining has been found in lane 1 (cell line K562), lane 5 (patient no. 1), and lane 7 (patient no. 4). As expected no *bcr-abl* joining has been found in lane 3 (leukocytes of a healthy control).

patients with standard t(9;22). Cytogenetically patients no. 1 and 2 showed chromosomal rearrangements involving 9q34 and chromosomes 19 and 14 respectively, while involvement of chromosome 22 was dubious. In situ hybridization studies demonstrated that complex translocations t(9;19;22) and t(9;14;22) had occurred. As a result of these translocations 5'-*bcr* and c-*abl* probes hybridized to the same chromosomal region, ie, 19q− in patient no. 1 and 22q− in patient no. 2. Similar results were reported by Bartram et al[31] in a Ph-negative CML with t(9;12), and 1 year later by Kurzrock et al[32] in a Ph-negative, *bcr*-positive CML patient with t(9;11). In a CML patient with complex t(9;13;15) and two normal chromosomes 22 we also found a *bcr* breakpoint by Southern blot analysis (case referred to us by D. Riviere [Brest, France], unpublished observation, 1985). These data strongly suggest that in apparently Ph-negative CML, with chromosomal rearrangement of 9q34, molecular evidence for a *bcr* breakpoint and/or *bcr-abl* recombination will usually be found and that these cases constitute a rare type of variant Ph translocation.

A normal bone marrow karyotype was found in patients no. 3 and 4. In situ hybridization studies unexpectedly showed hybridization of c-*abl* and 5-*bcr* probes to the chromosomal region 1p35-36 in addition to 9q34 (c-*abl*) and 22q11 (5'-*bcr*). c-*Sis* hybridized to chromosome 22 as expected. In patient no. 3, 3'-*bcr* hybridized to chromosome 22 and also to 9q34, which is an indication that t(9;22) almost certainly was the first step in a two-step rearrangement in this patient. In situ hybridization studies have been reported in four cases of CML, Ph-negative, *bcr*-positive, and a normal karyotype.[19,33] In all four cases c-*abl* was found to hybridize to 22q11 and the rearrangement was interpreted as an insertion of c-*abl* in the *bcr* gene. In patients no. 3 and 4, the exact mechanism of chromosomal rearrangements is not completely elucidated, but they are to the best of our knowledge the first cases of Ph-negative CML with an apparently normal karyotype and translocations or insertions of 5'-*bcr* and c-*abl* on 1p35-36.

The PCR experiments described here are of crucial importance because they demonstrate the presence of *bcr-abl* mRNA in the three cases investigated (patients no. 1, 3, and 4). The b3a2 joining detected in patients no. 1 and 4 and the b2a2 joining in patient no. 3 corresponded to the mapping of the breakpoint on chromosome 22 by Southern blot analysis. The results of Southern blot analysis and the PCR experiment indicated that in these Ph-negative CML patients *bcr-abl* fusion has taken place analogous to Ph-positive CML patients and that transcription was not influenced by the unusual chromosomal localization of 5'-*bcr* and c-*abl* in these patients. The occurrence of *bcr-abl* rearrangements in Ph-negative CML and the clinical significance of such findings have been studied by others.[19,23,31-37]

From the 50 cases with normal karyotypes that have been investigated by Southern blotting, including our own patients, 20 showed a breakpoint in the *bcr* region.[19,32-36] In ten cases RNA or protein analysis demonstrated transcription or translation[19,36] of the chimeric *bcr-abl* gene. The clinical criteria for CML diagnosis, the frequency of molecular rearrangements, and the interpretation of data are different in each study. Nevertheless most investigators[31-37] concluded from their studies that in Ph-negative CML the finding of *bcr-abl* molecular rearrangements indicated an undetected Ph translocation and therefore classify these patients to the group of Ph-positive CML with the prognostic and therapeutic consequences attached to this diagnosis. The remaining cases (Ph negative, *bcr* negative) constitute the group of Ph-negative CML with atypical (ie, myelodysplastic) hematologic features and a relatively short survival. Dreazen et al[19] challenged this hypothesis and claimed the presence of the *bcr* rearrangement in the majority of their Ph-negative CML patients and the finding of atypical features.

Our data support the former observations and indicate that in Ph-negative CML, the finding of molecular evidence for the presence of a *bcr-abl* fusion gene is diagnostic for classical CML. Larger prospective studies are needed to clarify the clinical relevance of molecular investigations in CML.

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bcr-abl mRNA Lacking *abl* Exon A2 Detected by Polymerase Chain Reaction in a Chronic Myelogeneous Leukemia Patient

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Using the polymerase chain reaction and Southern blot analysis the expression was detected of a *bcr-abl* mRNA lacking *abl* exon a2. This was due to a corresponding unusual localization of the breakpoint in the *c-abl* gene and was seen in a patient with Philadelphia (Ph) chromosome positive chronic myelogeneous leukemia in chronic phase. This type of mRNA has been described only once before in two Ph-positive acute lymphoblastic leukemia patients, by Soekarman *et al.* (1). The *abl* exon a2 sequences, which are missing in the three reported patients, code for a part of the SH3 region of the abl protein, which is supposed to be involved in negative regulation of the kinase domain. The clinical significance of this finding is discussed.

INTRODUCTION

Chronic myelogenous leukemia (CML) is cytogenetically characterized by the presence of the Philadelphia (Ph) chromosome, which originates from the Ph translocation t(9;22) (q34;q11) (2).

On chromosome 22 the breakpoint is located in band q11 in the *bcr* gene. In CML the breakpoints in the *bcr* gene are clustered in a region of 5.8 kb, the breakpoint cluster region (BCR) (3). The BCR region is part of a gene, the *bcr* gene, and contains four exons (b1–b4) (4). In nearly all CML patients the chromosome 22 breakpoint is located between exons b2 and b3, or between b3 and b4.

On chromosome 9 the breakpoints are scattered over a region of more than 200 kb in the c–*abl* gene, either between exons 1B and 1A, or between 1A and the second exon of c-*abl*, which is referred to as *abl* exon a2 in this paper (Figure 1) (5). However, the breakpoint is always located 5′ of c-*abl* exon a2. The *bcr-abl* fusion gene on the Ph chromosome is transcribed into a *bcr-abl* mRNA, in which either BCR exon b2 or b3 is fused to *abl* exon a2 (6,7). These chimeric mRNAs encode bcr-abl fusion proteins of 210 kD (p210), which have enhanced *in vitro* tyrosine kinase activity as compared to the normal abl protein (8) and have transforming activity. In Abelson murine leukemia virus (AMLV) the transforming potential of v–abl is dependent on the tyrosine kinase activity (9). Therefore regulation of the tyrosine kinase activity of p210 bcr-abl is thought to play an important role in the leukemogenesis of CML.

The Ph translocation is also observed in acute lymphoblastic leukemia (ALL) and acute myeloblastic leukemia (AML), although less frequently than in CML. The breakpoint in the *abl* gene always occurs at the same position as in CML, i.e. 5′ of *abl* exon a2. In 50% of the Ph-positive acute leukemias the breakpoint in *bcr* is also identical to that observed in CML, resulting in the same *bcr-abl* products as are observed in CML. In the remaining 50% the breakpoint in the *bcr* gene is located more 5′, i.e. in the first intron. In the latter cases an mRNA is transcribed, in which the first exon of the *bcr* gene (e1) is spliced to *abl* exon a2, resulting in an e1a2 junction (10). This mRNA is translated into a bcr-abl fusion protein of 190 kD (p190), that also shows enhanced tyrosine kinase activity and transforming potential (8).

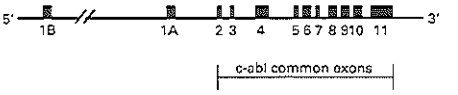

Figure 1. Schematic representation of the normal *abl* gene. The exons are depicted as black boxes and numbered according to Groffen *et al.* (3).

Recently Soekarman *et al.* (1) reported an aberrant localization of the chromosome 9 breakpoint in the *abl* gene in two Ph-positive ALL patients. This new breakpoint was located in the intron between *abl* exons a2 and a3, resulting in a *bcr-abl* mRNA lacking *abl* exon a2. In their first patient, *bcr-abl* fusion in the mRNA occurred between exons b2 and a3, resulting in a 210 kD fusion protein, whereas in the second patient *bcr* exon e1 appeared to be fused to *abl* exon a3, resulting in a fusion protein of 190 kD. Both proteins showed enhanced tyrosine kinase activity in autophosphorylation assays as compared to the normal abl protein, but in the same range as observed in other ALL patients.

In the novel bcr-abl fusion protein 58 amino acids are missing, which are encoded by *abl* exon a2. Interestingly, the last 17 amino acids encoded by a2 are part of a stretch of 50 amino acids that form the src-homology 3 region (SH3) of the abl protein (11). This is one of the regions that is highly homologous in abl and scr. The SH3 region is thought to have a negative regulatory effect on the kinase domain, termed SH1 (12,13). Therefore the finding of partial deletion of the SH3 region in the two ALL patients indicates that this regulatory region is inactive in the bcr-abl fusion proteins.

In this paper Southern blot and polymerase chain reaction (PCR) analysis are reported of bone marrow cells of a Ph-positive CML patient in chronic phase, with a b2a3 chimeric mRNA and a chromosome 9 breakpoint between *abl* exons a2 and a3, much the same as in the two ALL patients reported by Soekarman *et al.* (1). The significance of this finding is discussed. A PCR and

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LEUKEMIA

Southern blot strategy is proposed, which can be used for screening patients for the presence of this new *bcr-abl* rearrangement.

MATERIALS AND METHODS

Patient

A 59-year-old man was first seen in December 1988 with complaints of tiredness. Physical examination showed splenomegaly. Laboratory investigations revealed Hb 6.0 mmol/l, leucocyte count 254×10^9/l and thrombocyte count 180×10^9/l. The peripheral blood smear contained 42% myelocytes, 10% promyelocytes, 5% metamyelocytes, 3% normoblasts, 14% bands, 24% segmented neutrophils, and 2% eosinophils. Blood urea, creatinine, and electrolytes were within the normal range. Plasma lactate dehydrogenase was elevated (1153 U/l). The bone marrow smear showed increased myelopoiesis. Based on these findings, a diagnosis of CML was made.

The patient was treated with hydroxyurea. During the next 2 years he remained in chronic phase CML. In December 1990 acceleration of the disease occurred. The bone marrow biopsy showed myelofibrosis. To control the serious anemia the patient frequently received blood transfusions.

Samples

Sterile bone marrow aspirates and blood samples were taken and heparinized. Sampling was part of diagnostic and clinical follow-up procedures and was obtained only after informed consent of the patient. After Dextran separation, leucocytes were frozen and stored in liquid nitrogen until DNA and RNA analysis was performed.

Cytogenetics

The karyotype of blood and bone marrow cells was investigated at diagnosis and during the next 2 years at 6 month intervals using standard cytogenetic procedures (14). Chromosomes were identified using the R-banding technique, and classified according to ISCN (1985) (15). The constitutional karyotype was determined and found to be normal using PHA stimulated blood cultures.

DNA Probes

The following DNA probes were used: 5'-BCR, 2 kb BglII–HindIII fragment; 3'-BCR, 1.2 kb HindIII–BglII fragment; abl, 0.9 kb SaulI–KpnI fragment, spanning the 0.6 kb intron between exons a2 and a3.

Southern Blotting

This was performed using standard techniques (16).

Reversed PCR Analysis

cDNA synthesis and amplification using the PCR technique were performed as described by Hermans *et al.* (17). The localization of the primers used in the PCR reaction is shown schematically in Figure 2. In brief: oligonucleotides 1, 2 and 3 were used as primers to amplify the fragment covering the *bcr-abl* region. As an internal positive control in the PCR reaction, an *abl* fragment was amplified, using oligonucleotides 1 and 4. This *abl* fragment is always present, irrespective of the Ph translocation. Four oligonucleotides specific for the breakpoint junctions e1a2, b2a2, b3a2, b2a3, and two oligonucleotides specific for b2 and a2 sequences were used as probes. The first three oligonucleotides mentioned are the same as described by Hermans *et al.* (17). The sequences of the remaining oligonucleotides are: b2a3, 5' GCTGACCATCAATAAGGAAGGTGAAAAGCTCCGGGTCTTA 3'; b2, 5' GTGAAACTCCAGACTGTCCACAGCA 3'; and a2, 5' TCCACTGGCCACAAAATCATACAGT 3'.

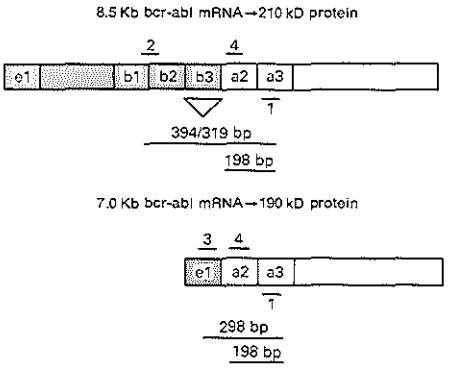

Figure 2 Schematic representation of the 8.5 and 7.0 kb *bcr-abl* mRNA. The localization of *bcr* and *abl* primers is shown. Using primer 1 and 2, a fragment of 394 or 319 bp is amplified, depending on the exon b3 in the mRNA. These fragments have a b3a2 and a b2a2 junction, respectively. Using primer 1 and 3 a fragment of 298 bp is amplified with an e1a2 junction. Primers 1 and 4 are used as an internal control to amplify an *abl* fragment of 198 bp, which should always be present irrespective of the presence of the Ph translocation.

RESULTS AND DISCUSSION

At diagnosis the karyotype of all 32 metaphases analysed from blood and bone marrow was 46,XY,t(9;22)(q34;q11), showing a standard Ph translocation. Treatment was started with hydroxyurea. For the next 2 years, cytogenetic analysis was performed every 6 months and showed persistence of the Ph translocation in all metaphases examined, without additional cytogenetic aberrations. Both clinically and cytogenetically the patient was in chronic phase of CML for these 2 years.

Molecular analysis was performed on the bone marrow sample obtained at diagnosis. Using Southern blot analysis the breakpoint in the BCR region was determined (Figure 3). A rearranged band was detected in the BglII, but not in the BamHI digested DNA after hybridization with the 5'-BCR probe. Hybridization with the 3'-BCR probe showed no rearranged bands. This can be explained by: (1) a breakpoint in the 5' part of BCR in combination with a deletion in the 3' part of BCR; (2) a breakpoint in the 5' part of BCR, resulting in the BamH1 digested DNA in an aberrant BamH1 *bcr-abl* fragment of the same size as the germline BamH1 *bcr* fragment; or (3) by a BglII polymorphism without a BCR breakpoint.

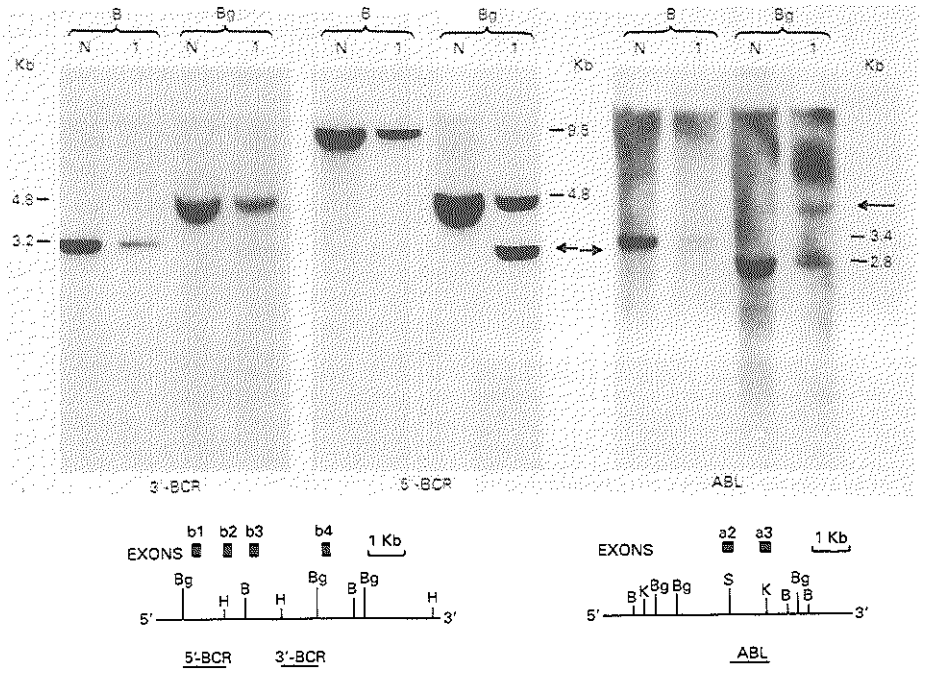

Figure 3. Upper panel: Southern blot analysis of the CML patient (T) and normal thymus DNA (N). DNA was digested with the restriction enzymes Bg1II (Bg) and BamH1 (B). The following probes were used: 1.2 kb HindIII–Bg1II BCR fragment (3'-BCR), 2.0 kb Bg1II–HindIII BCR fragment (5'-BCR), and a 0.9 kb SauI–KpnI fragment spanning the intron between *abl* exon a2 and a3. The sizes of the germline fragments are given in kilobases (kb). Additional restriction fragments are indicated by arrowheads. Lower panel: A simplified restriction map of the normal BCR region and the part of the *abl* gene surrounding *abl* exons a2 and a3 is depicted. (Bg) Bg1II; (B) BamH1; (K) KpnI; (S) SauI. Exons are represented by black boxes. The locations of the 3'-BCR, 5'-BCR, and ABL fragments used as probes in the Southern blot analysis are shown.

To check which of the explanations was correct, it was investigated if *bcr-abl* mRNA was expressed in the leukemic cells of this patient. PCR analysis was performed on cDNA preparations obtained from total RNA of the bone marrow cells of the patient. A scheme indicating the localization of the PCR primers and the expected size of the amplified fragments is shown in Figure 2. No fragment of the expected size, i.e. 298, 319, or 394 bp, corresponding to mRNA with an e1a2, b2a2, or b3a2 junction, was visible on gel, nor was such a fragment detected after hybridization with oligonucleotides which specifically recognize these *bcr-abl* fusion regions in the amplified fragment (data not shown). However, a fragment of 145 bp was amplified instead (Figure 4), using primers 1 and 2. The fragment failed to hybridize under stringent conditions to e1a2, b2a2, or b3a2 specific oligonucleotide probes. It also failed to hybridize to an oligonucleotide containing exon a2 sequences, but hybridized readily to an oligonucleotide containing exon b2 sequences. The most likely explanation for these hybridization data and the size of the amplified fragment (319 − 174 = 145 bp, i.e. b2a2 − a2 = b2a3) is that the leukemic cells of this CML patient express a *bcr-abl* mRNA with a b2a3 junction.

This was checked by determining the breakpoint in the *abl* gene by Southern blotting (Figure 3). The Southern blot containing the Bg1II and BamHI digested DNA was hybridized to a 0.9 kb SauI–KpnI genomic fragment spanning the *abl* intron between exon a2 and a3. Indeed, both in Bg1II and BamHI digested DNA an aberrant restriction fragment was detected, indicating a breakpoint in this region.

Final proof for the presence of a b2a3 junction in the *bcr-abl* mRNA was provided by hybridization of the PCR product to an oligonucleotide specific for the b2a3 fusion region. This oligonucleotide readily hybridized to the 145 bp PCR fragment of the CML patient, whereas it failed to hybridize to the PCR product of a Ph-negative BCR unrearranged CML patient (Figure 4), or to the 319 bp PCR product of a CML patient with a b2a2 junction (data not shown), which served as negative controls on the PCR reaction and the hybridization, respectively.

In conclusion, the leukemic cells of this CML patient

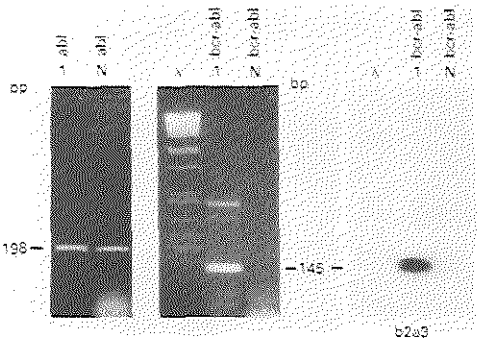

Figure 4. The two panels on the left show the UV picture of the ethidium bromide stained agarose gel containing the PCR products of the blood cells of the CML patient (1), and of a Ph-negative CML patient without *bcr-abl* rearrangement (N), serving as control. Bacteriophage λ DNA digested with the restriction enzyme PstI is used as molecular weight marker. Abl indicates a lane in which primers 1 and 4 (see Figure 2) are used to amplify a 198 bp *abl* fragment, which should always be present irrespective of the presence of the Ph translocation. Bcr-abl on top of a lane indicates that primers 1 and 2 are used to amplify a *bcr-abl* fragment. A 145 bp fragment was detected in the patient (1). As expected, no *bcr-abl* fragment was amplified in the negative control patient (N). The panel on the right shows the autoradiogram of the same agarose gel after blotting onto nylon membrane and hybridization to an oligonucleotide which specifically recognizes the junction between BCR exon b2 and *abl* exon a3 (b2a3). The 145 bp PCR product of the patient (1) readily hybridized to the b2a3 specific oligonucleotide, whereas no hybridization signal is detected in the negative control patient (N).

express *bcr-abl* mRNA, in which BCR exon b2 is fused to *abl* exon a3. In the BCR region the breakpoint is located between exon b2 and b3, in the *abl* gene the breakpoint occurs at an unusual location, i.e. in the intron between exon a2 and a3.

To the best of our knowledge the CML patient described in this paper and the two ALL patients described by Soekarman *et al.* (1) are the only three cases reported with a breakpoint in the *abl* gene 3′ of exon a2. Theoretically the predicted frequency of occurrence of *bcr-abl* mRNA lacking *abl* exon a2 is 0.3% of the *bcr-abl* rearranged patients (0.6 kb:200 kb = 0.003), assuming that the breakpoints in *abl* are randomly distributed (1). However, in the leukemia patients, in whom the breakpoint location in the *abl* gene has been determined, it was always found 5′ of *abl* exon a2 (5,7,18–20). In this respect a paper by Hooberman *et al.* (21) is very interesting. These authors reported that in one out of six Ph-positive and in one out of five Ph-negative ALL or blast crises CML patients breakpoints in the BCR gene were found, but no *bcr-abl* mRNA expression could be detected by PCR analysis. These patients possibly express the *bcr-abl* mRNA lacking *abl* exon a2, which was not detected in the PCR because of the use of a 3′-primer corresponding to *abl* exon a2 sequences. To our opinion it would be worth checking a selected group of patients for the expression of mRNA with a fusion between one of the *bcr* exons and *abl* exon a3. This especially concerns all Ph-positive ALL or AML patients and all CML patients, in whom a breakpoint in the BCR region is detected using Southern blotting, but none of the known *bcr-abl* mRNAs has been found using PCR analysis. The additional analysis, which is required in these cases is either PCR analysis using an *abl* primer containing *abl* exon a3 instead of a2 sequences, or hybridization of the Southern blot to the SauI–KpnI *abl* probe. It is expected that using this strategy more patients will be identified with this unusual breakpoint location in the *abl* gene.

Concerning the disease caused by this new *bcr-abl* rearrangement, the following data reported in this paper are important. Firstly, the expression of *bcr-abl* mRNA with a b2a3 junction is not restricted to acute leukemia or blast crisis of CML, but also occurs in the chronic phase of CML. Secondly, the CML patient reported in this paper and the two ALL patients reported by Soekarman *et al.* (1) were clinically indistinguishable from other Ph-positive CML or ALL patients. In the ALL patients the tyrosine kinase activity of the p210 proteins in the autophosphorylation assay did not differ from other Ph-positive ALL patients. In the CML patient this assay could not be performed because the percentage of blasts was too low for this assay. In the bcr-abl fusion protein of the patients, 17 out of 50 amino acids, forming the SH3 region, are missing. The SH3 domain is a negative regulator of the kinase domain of the abl protein (11–13).

Important for understanding the mechanism of activation of the *abl* oncogene is to compare these patient data to the data reported by Jackson *et al.* (22) and van Etten *et al.* (23), who investigated the effect of deletions in the mouse abl type IV protein. Jackson *et al.* (22) and van Etten *et al.* (23) reported that in mice, the deletion of 53 amino acids in the SH3 domain of the abl type IV protein, which is homologous to the human SH3 domain, results in full activation of the transforming potential, both with respect to fibroblast and B-lymphoid cell transformation *in vitro* and leukemogenic activity *in vivo*. The same is found in v-abl, in which the SH3 domain is absent. Activation of the kinase domain of the abl protein can be reached either by replacement of 5′-abl by bcr sequences or viral gag sequences or by deletion of the SH3 domain. As argued by Soekarman *et al.* (1) partial deletion of the SH3 domain in the three patients has no extra effect on kinase activity of the fusion proteins due to the fact that the inhibiting function of the SH3 domain is most probably already overruled by the presence of 5′-*bcr* sequences in the bcr-abl proteins.
possible clinical and prognostic implications of this finding. It is expected that molecular analysis in patients such as these will contribute to a better understanding of the mechanisms of regulation of the kinase activity of the abl protein, and thus of leukemogenesis.

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