CHROMOSOME ABERRATIONS AND ONCOGENES IN HUMAN CANCER

CHROMOSOOM AFWIJKINGEN EN ONCOGENEN IN HUMANE KANKER

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ABBREVIATIONS

ALL	acute lymphocytic leukemia
AML	acute myeloblastic leukemia
AMMol	acute myelomonocytic leukemia
AMol	acute monocytic leukemia
A-MulV	Abelson murine leukemia virus
ANLL	acute nonlymphocytic leukemia
APL	acute promyelocytic leukemia
bcr	breakpoint cluster region
BL	Burkitt´s lymphoma
cDNA	complementary deoxyribonucleic acid
CML	chronic myelocytic leukemia
CLL	chronic lymphocytic leukemia
c-onc	cellular oncogene
CSF-1 (R)	colony stimulating factor-1 (receptor)
del	deletion
DM	double minute
DNA	deoxyribonucleic acid
dup	duplication
EGF (R)	Epidermal growth factor (receptor)
Ew.Sa	Ewing sarcoma
FAB	French-American-British classification
F.lym	follicular lymphoma
HSR	homogeneously staining region
i	iso
IgH	immunoglobulin heavy chain gene
IgL	immunoglobulin light chain gene
inv	inversion
ISCN	international system for cytogenetic nomenclature
kb	kilobase
kD	kilo Dalton
LTR	long terminal repeat
MDS	myelodysplastic syndrome

mpc	mouse plasmacytoma
mRNA	messenger ribonucleic acid
NB	neuroblastoma
N-terminus	amino terminus
Ov.Ca.	ovarian carcinoma
Ph^1	Philadelphia chromosome
PV	polycythemia vera
RA	refractory anemia
RB	retinoblastoma
Ren.Ca	renal cell carcinoma
RNA	ribonucleic acid
t	translocation
TCR	T-cell receptor
SCLC	small cell lung carcinoma
v-onc	viral oncogene
WT	Wilms tumor

1. INTRODUCTION

1.1 Chromosomal aberrations in neoplasia

The hypothesis that chromosomal changes play a fundamental role in the process of neoplastic transformation was postulated almost a century ago by Von Hansemann (1890) and Boveri (1914). The discovery in 1960, of a specific chromosomal marker, the Philadelphia (Ph^1) chromosome (Nowell and Hungerford, 1960) in chronic myelocytic leukemia (CML) supported this hypothesis. However, for a long time this Ph^1 chromosome was regarded as an unique example of a consistent karyotypic abnormality since other malignant disorders, especially solid tumors, showed a great variability in karyotype (Sandberg, 1980). The introduction and constant refinement of chromosome banding techniques (Hagemeijer et al., 1979; Yunis, 1981; Testa, 1984) made it possible to identify and define tumor specific chromosomal aberrations. Combination of these sophisticated cytogenetic techniques with cytological, cytochemical and immunological studies revealed that the malignant cells of most human tumors have a clonal karyotypic defect (Yunis et al., 1982; Yunis, 1983; Berger and Flandrin, 1984). Three major types of karyotypic changes can be distinguished that occur in tumor cells, either alone or in combination. 1) Numerical changes such as monosomy or trisomy, 2) structural changes such as translocations, deletions and inversions and 3) manifestations of gene amplification such as homogeneously staining regions (HSR's) or double minutes (DM's). In less than 10% of the cytogenetic studies the material was derived from solid tumors, the largest tumor group in humans. The majority of the cytogenetic data came from cytogenetic studies of hematopoietic disorders and lymphomas (70% and 20% resp.; Mitelman, 1986). This is in part due to the relatively simple collection of leukemic cells from bone marrow, blood or lymph- nodes, the well-defined conditions for culturing these cells and the adapted chromosome banding techniques. In Table 1 a list is shown of consistent chromosomal aberrations (primarily translocations), found in several types of leukemia and lymphoma . The list also contains a few

TABLE 1. CONSISTENT CHROMOSOMAL ABERRATIONS IN HUMAN CANCER

Leukemia		Chromosomal Aberration
CML[a]		[a]t(9;22) (q34;q11)
ANLL[b]	M1 (AML)	t(9;22) (q34;q11)
	M2 (AML)	t(8;21) (q22;q22)
	M3 (APL)	t(15;17)(q22;q21)
	M4 (AMMoL)	inv(16) (p13-q22)
		t(16;16)(p13;q22)
		del(16) (q22)
	M1, M2, M4	t(6;9) (p23;q34)
	M5 (M4)	t(9;*11) (p22;q23)
	M1- M6	del(5) (q13q31)
		del(7) (q31q36)
		-7; +8; 12p-
ALL	L1,L2	t(9;22) (q34;q11)
	L2	t(4;11) (q21;q23)
	L3	t(8;14) (q24;q32)
	pre B-cell	t(1;19) (q23;p13)
	T-cell	t(11;*14)(p13;q11)
	L1-L3	6q-
	L1, cALL	t or del 12p12
CLL	B-cell	t(11;*14)(q13;q32)
		dup(12) (q13-q22)
	T-cell	inv(14) (q11-q32)
		t(14;14)(q11;q32)
PV		del(20) (q11)
MDS		-7; +8; 5q-; 12p-

Lymphoma/Solid Tumor	Chromosomal Aberration
B cell lym. {	t(8;14) (q24;q32)
Burkitt's {	t(8;22) (q24;q11)
	t(2;8) (p12;q24)
follicular	t(14;18)(q32;q21)
diffuse small/large cell	t(11;14)(q13;q32)
T-cell lymphoma	t(11;14)(p11/p13;q11)
(Fam.) Renal Carcinoma	del(3)(p14)
	t(3;8)(p14;q24)
	t(3;11)(p14;p15)
Ovarian Carcinoma	del(3) (p21p13)
	del(6) (q15;q21)
	t(6;14)(q21;q24)
Salivary Gland Carcinoma	t(3;8) (p21;q12)
	del(12q); t(12q)
Testicular Carcinoma; Seminoma	i(12p)
Small lung carcinoma	del(3) (p14p23)
Rhabdomyosarcoma	del(3)(p14p21);t(3p)
(Alveolar)	t(2;13)(q37;q14)
Ewing Sarcoma	t(11;22)(q24;q12)
Neuroblastoma	del(1) (p32p36)
Retinoblastoma	del(13)(q14)
Wilms tumor	del(11)(p13)
Meningioma	-22; (del22q11)
Glioma	del(22)(q11qter)
Melanoma	del (6)(q15q23)
	t (1q11)

a: Abbreviations see page 4; b: FAB classification (Bennett et al., 1976); * Other chromosomes than * reported as acceptor. Compiled from: Berger et al., 1985; Bigner et al., 1984; Gibas et al., 1986; LeBeau, 1986; Rey et al., 1985; Trent, 1984; Yunis, 1983.

known recurring chromosomal abnormalities (primarily deletions) which can be observed in human solid tumors despite the technical difficulties (Yunis 1983; Trent, 1984). From the collected cytogenetic data (from over more than 5500 tumors; Mitelman, 1985) it is apparent that breakpoints of structural karyotypic aberrations are clustered to specific chromosomal regions (Mitelman, 1984; Mitelman, 1986). Some structural aberrations are exclusively found in one specific tumor subtype e.g. the t(15;17)(q22;q21)(for nomenclature see ISCN, 1985) is found solely in acute non-lymphocytic leukemia (ANLL-M3) (FAB classification for acute leukemias, Bennett et al., 1976)(Larson et al., 1984). However most of the chromosomal breakpoint regions are shared either by neoplastic disorders originating from different cell types or by related disorders. For example the chromosome 22q11 region is involved in structural aberrations observed in a variety of unrelated tumors such as meningiomas, gliomas, Di-George syndrome (del(22)(q11-qter)), Burkitt lymphoma (BL), acute lymphoblastic leukemia (ALL-L3) (t(8;22)(q24;q11)), ALL-L1,-L2, ANLL-M1, CML (t(9;22)(q34;q11)(Berger et al., 1985; Cannizzaro and Emanuel, 1985; Emanuel et al., 1986). Whereas the 14q11 region is frequently involved in structural aberrations (translocations and inversions) in T-cell leukemia/lymphoma (Hecht et al., 1984; Clare et al., 1986; Hecht et al., 1986) and similarly the 14q32 region in B-cell leukemia/lymphoma (Croce and Nowell, 1985; Cleary and Sklar, 1985a). At these latter two regions cell type specific genes have been localized: the T-cell receptor α-chain gene at 14q11 (Collins et al., 1985; Croce et al., 1985a) and the immunoglobulin heavy chain (IgH) genes at 14q32 (Croce et al., 1979; Kirsch et al., 1982). Previously a similar concordance has been notice between the localization of cell lineage specific, transcriptionally active genes and one of the translocation breakpoints in B-cell lymphomas, both in human and mouse (Klein, 1981). The author suggested that in analogy with the oncogene activation model in virally induced tumors (Hayward et al., 1981), the accidental transposition of specific, oncogene like genes to transcriptionally active regions could

alter the expression of these specific genes. Support for this hypothesis came from observations in BL cell lines with a characteristic t(8;14): the c-myc gene, normally located at chromosome 8 (q24)(Neel et al., 1982; Dalla-Favera et al., 1982a, 1982b) was translocated into the IgH gene locus (Taub et al., 1982; Dalla-Favera et al., 1983). This translocation of c-myc resulted in an aberrant and sometimes enhanced expression of the c-myc oncogene (Shen-Ong et al., 1982; Erikson et al., 1983a). An other example emphasized the fact that oncogenes might be involved in tumor specific translocations. The c-abl oncogene was translocated from its normal position on chromosome 9 (Heisterkamp et al., 1982) to the 22q- chromosome in the CML specific t(9;22)(q34;q11)(De Klein et al., 1982: Appendix Paper I).

1.2 Oncogenes

Several independent lines of cancer research have led to the discovery of cellular genes with a potential transforming activity. The majority of these cellular oncogenes were identified because they represent the cellular homologues (c-onc) of the transforming genes of acute RNA tumor viruses. In contrast to the slow transforming RNA tumor viruses these acute RNA tumor viruses contain an extra host cell derived sequence, v-onc, which is responsible for the acute form of transformation. Usually the v-oncogenes are truncated and/or mutated processed forms of normal cellular genes (Bishop, 1983; Bishop and Varmus, 1984). Slow transforming RNA tumor viruses cause malignant transformation after a protracted latent period. They can activate a cellular gene by proviral insertion next to it, placing this gene under the control of the strong promoter or enhancer sequences present in the viral long terminal repeat (LTR)(Varmus, 1982). Besides previously identified c-oncogenes like c-myc (Hayward et al., 1981; Payne et al., 1982; Corcoran et al., 1984), c-mos (Rechavi et al., 1982; Cohen et al., 1983; Gattoni-Celli et al., 1983); c-myb (Shen-Ong et al., 1984; Rosson and Reddy, 1986) and c-erbB (Fung et al., 1983; Raines

et al., 1985), other cellular genes can serve as targets for activation by proviral or transposon like integration, like int-1 (Nusse and Varmus, 1982; Nusse et al., 1984); pim-1 (Cuypers et al., 1984; Selten et al., 1985) or Mvli-1 (Tsichlis et al., 1983). Alternatively, rearrangements and/or mutations of cellular genes can be caused by chromosomal aberrations. They may affect known c-oncogenes as c-myc and c-abl in the BL and CML specific translocations respectively, or lead to the identification of new putative c-oncogenes as bcl-1 and bcl-2 (Nowell et al., 1984; Cleary and Sklar, 1985b) or pvt-1 (Webb et al., 1984; Cory et al., 1985). Like c-myc, these latter genes are joined to the immunoglobulin genes in the respective translocations. Similarly, the investigation of amplified DNA sequences in tumor cells showed the involvement of previously identified oncogenes: c-myc (Alitalo et al., 1983a) and K-ras (Schwab et al., 1983a) or led to the discovery of new, related oncogenes: N-myc (Schwab et al., 1983b) or L-myc (Nau et al., 1985).

Another approach, DNA-mediated gene transfer, led to the characterization of transforming genes present in tumor cells, competent to transform appropriate recipient cultured cells, e.g. the mouse fibroblast cell line NIH-3T3 (Cooper et al., 1980). One fifth of the DNA´s extracted from various tumors and tumor cell lines were able to induce focus formation in cultures of this immortalized, rodent cell line. The isolated transforming genes are in most cases mutated members of the ras gene family: H-ras, K-ras or N-ras (Cooper, 1982; Der et al., 1982; Shimizu et al., 1983; Hall et al., 1983; Cooper and Lane, 1984). Despite obvious limitation, probably due to the recipient cell line used, occasionally new and often truncated transforming genes have been isolated like met (Cooper et al., 1984; Dean et al., 1985, Park et al., 1986), trk (Martin-Zanca et al., 1986), B- and T-lym (Lane et al. 1982) or neu (Schlechter et al., 1984, 1985; Bargmann et al., 1986a, 1986b).

The normal cellular counter parts (proto-oncogenes) of the transforming and activated oncogenes are a heterogeneous group of genes which have been highly conserved throughout

TABLE 2 PROTO-ONCOGENES

Proto-oncogenes	V	I	C	A	T	Chromosomal Location
abl	+		+	+		9 (q34)
akt-1	+					14 (q32)
bcl-1			+			11 (q13)
bcl-2			+			18 (q21)
bcr			+			22 (q11)
B-lym					+	1 (p32)
dbl					+	
erbA	+					17 (q11-q12)
erbB-1	+	+				7 (p12-p14)
erbB-2 (neu; mac117)	+			+	+	17 (q12-q22)
ets-1	+					11 (q23)
ets-2	+					21 (q22)
fgr (src-2)	+					1 (p36)
fms	+					5 (q33)
fos	+					14 (q21-q31)
fps (fes)	+					15 (q26)
int-1		+				12 (pter-q11)
int-2		+				11 (q13)
int-3; 41		+				
kit	+					4
mcf-2					+	X (q27)
mel					+	19
met					+	7 (q21-q31)
mlvi 1-3		+				
mos	+	+			+	8 (q22/q11)
myb	+	+		+		6 (q22-q23)
c-myc	+	+	+	+		8 (q24)
L-myc				+		1 (p32)
N-myc				+		2 (p23-p24)
p53			+			17 (p13)
pim-1			+			6 (p21)
pvt-1 (mis-1; Rmo-int-1)			+	+		
raf-1 (mil; mht)	+	+			+	3 (p25)
raf-2 (ψ)	+					4
A-raf-1 (c-pks)	+					X (p11.4)
A-raf-2	+					7 (p14-q21)
H-ras-1	+	+			+	11 (p15)
H-ras-2 (ψ)	+					X
K-ras-1 (ψ)	+					6 (p23-q12)
K-ras-2	+	+		+	+	12 (p12.1)
N-ras				+	+	1 (p22/p11-p12)
rel	+					2 (p11-p12)
ret					+	
ros (mcf-3)	+				+	6 (q16-q22)
sis	+					22 (q13.1)
ski	+					1 (q22-qter)
src-1	+					20 (q13.3)
tck (lsk^t)		+				
tcl-1			+			14 (q32.3)
tcl-2			+			11 (p13)
tkns-1			+			
T-lym					+	
trk (onc D)					+	1 (q32)
tx 1-4					+	
yes-1	+					6
yes-2	+					18 (q21)

Mode of discovery: V = homologue of v-onc; I = target of proviral or transposon like element insertion; C = involved in chromosomal translocations; A = as amplified DNA; T = as transforming gene in DNA mediated gene transfer.

evolution (Shilo and Weinberg, 1981; Bishop, 1983; Land et al., 1983a). The tissue and stage specific expression (Müller et al., 1982, 1983, 1984a; Gonda and Metcalf, 1984; Duprey and Boettiger, 1985; Sariban et al., 1985; Thompson et al., 1986 and Zimmerman et al., 1986) and the rapid induction upon mitogenic signals (Kelly et al., 1983; Kruyer et al., 1984; Müller et al., 1984b) of some of these proto-oncogenes suggests that they play a role in regulation of normal cell differentiation and proliferation. Proto-oncogene products located in the nucleus (e.g. c-myc, c-myb and c-fos) may modulate the transcriptional activity of the cell, whereas cytoplasmic products (e.g. ras, c-abl, c-sis-, c-yes) could be related to the signal pathway in response to growth or differentiation signals. The identification of gene products of oncogenes as growth factors (Waterfield et al., 1983; Robbins et al., 1983) or growth factor receptors (Downward et al., 1984, Sherr et al., 1985; Yamamoto et al., 1986; Bargmann et al., 1986) provided strong support for such a role. Quantitative (enhanced or constitutive expression) and qualitative (mutations, truncations) alteration of these gene products will interfere with the normal cellular functions and could ultimately lead to transformation (see reviews by Varmus, 1984; Bishop, 1985; Weinberg, 1985).

The chromosomal location of many proto-oncogenes has been determined, using somatic cell hybrids and in situ hybridization (Table 2). The striking concordance between the position of proto-oncogenes and consistent chromosomal aberrations (Figure 1)(Rowley, 1983; Yunis, 1983; Pearson and Rowley, 1985) has led to numerous molecular and cytogenetic studies to verify this apparent association. Ample evidence has been provided for the involvement of the c-myc and c-abl oncogenes in BL and CML specific chromosomal translocations respectively. But also in other structural abnormalities, oncogenes which map closely to the respective chromosomal breakpoints, may prove to be directly involved. Alternatively, cloned sequences or genes located in the vicinity of chromosomal breakpoints may serve as tools for a further cytogenetic and molecular characterization of the abnormality. In particular when they are expressed in a cell

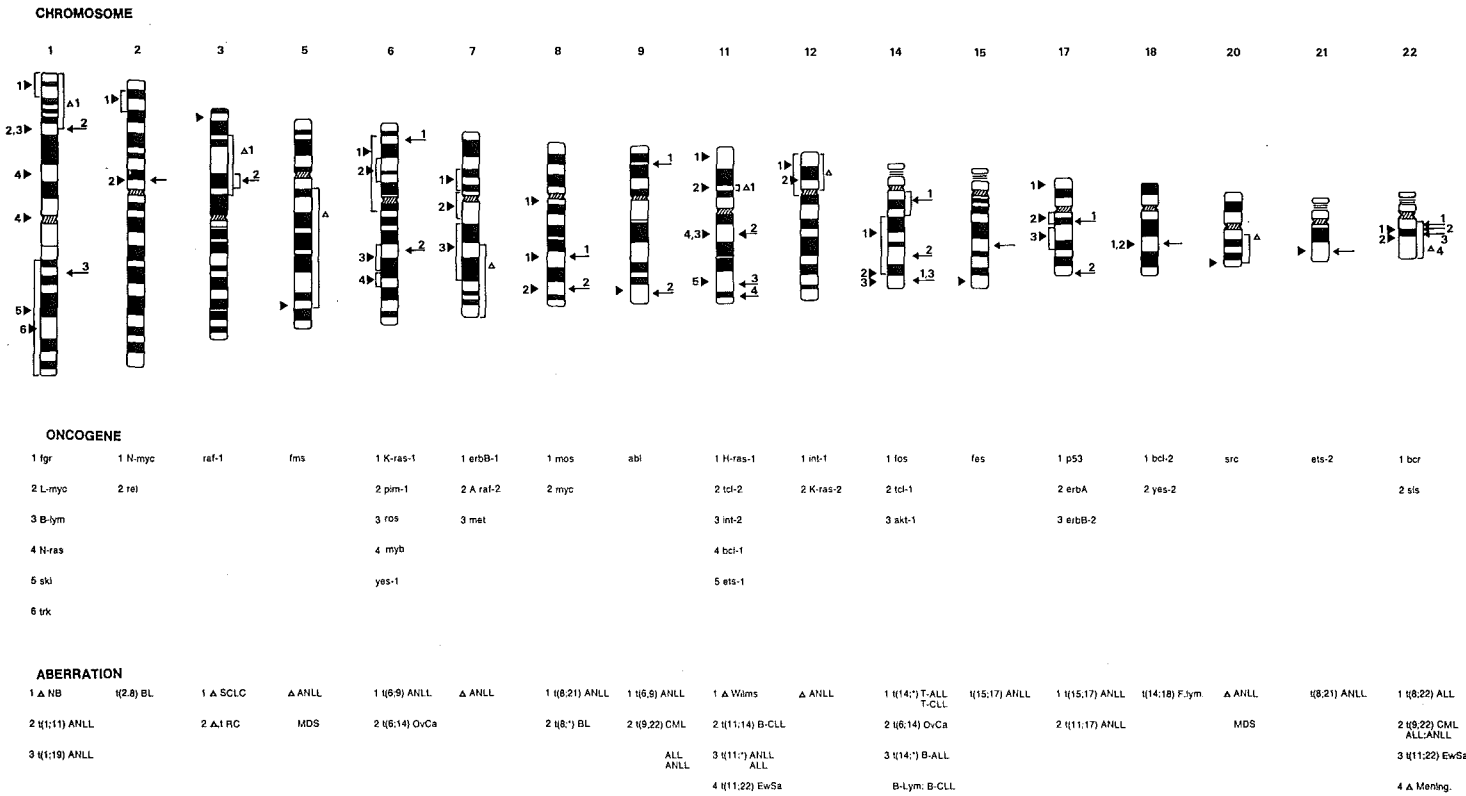

Figure 1.
Diagram of human chromosomes showing the concordance between the position of proto-oncogenes and the localization of breakpoints of consistent chromosomal aberrations. The position of proto-oncogenes is indicated by solid arrow heads on the left of each chromosome. The chromosomal breakpoints of translocations (⟵) or deletions (Δ) are indicated on the right of each chromosome. Abbreviations are listed on page 4.

type or/and differentiation stage-dependent manner. Eventually they can lead to the identification and isolation of new oncogenes. Other types of karyotypic changes such as deletions or amplifications could create a gene dosage effect of a specific oncogene, either direct, as demonstrated by the amplification of N-myc in neuroblastomas (Schwab et al., 1983b) or indirect by the deletion of an inhibitory sequence or tumor suppressor genes (anti-oncogene hypothesis), (Comings, 1973; Klein and Klein, 1985a). The following review will focus primarily on the involvement of oncogenes in chromosomal translocations consistently found in several hematopoietic disorders and in particular on the role of the c-abl oncogene in CML, since this has been the main subject of the experimental work preceding the completion of this thesis.

2. STRUCTURAL ABERRATIONS: TRANSLOCATIONS AND INVERSIONS

Among the acquired karyotypic changes in tumor cells, the most characteristic are the translocations and inversions. These structural aberrations are predominantly found in leukemia and lymphoma. Several morphological subtypes of these neoplasia are associated in varying degrees of specificity with a typical consistent translocation. Variant or complex forms of these consistent translocations can help to define the critical genetic rearrangement, such as the translocation products which are consistently found in the specific type of leukemia or lymphoma (Rowley, 1982, 1984). Apparently, these aberrations do not involve gain or loss of genetic material and point directly to the site of action: the genes or DNA sequences located at the critical chromosomal breakpoints. A number of oncogenes have been located near or at the chromosomal breakpoints, and cloned sequences of these genes were used as probe to analyse and characterize the consistent chromosomal abnormality in the respective neoplasia.

2.1 The Ph^1 translocation in chronic myelocytic leukemia

Chronic myelocytic leukemia (CML) is a pluripotent stem cell disease characterized by the presence of the Philadelphia (Ph^1) chromosome in the leukemic cells of 96% of all CML patients. Cytogenetic analysis revealed that this Ph^1 (or 22q-) chromosome is the result of either a standard translocation, t(9;22)(Rowley, 1973) in 90% of the cases, or of a variant translocation (3-8%) involving other chromosomes as well (Heim et al., 1985). A minority (3-7%) of the CML cases is without a Ph^1 chromosome (Ph^1 negative CML)(Rowley and Testa, 1982). The presence of a Ph^1 chromosome is regarded as a prognostic factor in CML: Ph^1-negative CML patients, with normal karyotypes or other karyotypic changes have a worse prognosis than the Ph^1-positive CML patients (Whang-Peng and Knutsen, 1982).

Cytogenetic studies and isoenzyme analysis have demonstrated that CML is a clonal disorder of pluripotent stem cells (Fialkow et al., 1977; Geurts van Kessel et al., 1982). The Ph^1 positive leukemic cells have a growth advantage over normal bone marrow cells and usually all nucleated cells in the bone marrow are Ph^1 positive at the time of diagnosis. The Ph^1 negative, normal cells persist but their growth is apparently suppressed by the leukemic cells (Dubé et al., 1984a, 1984b; Frassoni et al., 1986). Several reports (Lisker et al., 1980; Fialkow et al., 1981) suggest that the acquisition of the Ph^1 chromososome is not the initial abnormality in CML. This stage may be preceded by a clonal outgrowth of an abnormal hematopoietic stem cell without a marked proliferation advantage. This, in most cases clinically inapparent stage, is followed by the induction of the Ph^1 chromosome, which leads to the increased expansion of committed myeloid progenitors, characteristic of the chronic phase of CML (see reviews by Koeffler and Golde, 1981; Champlin and Golde, 1985). After this chronic phase, which lasts in general 3-4 years, most CML patients ultimately evolve to a lymphoid or myeloid acute phase (blast crisis). This latter stage is usuallly accompanied by additional non-random chromosomal aberrations like trisomy 8 or

19, isochromosome 17q, or a second Ph^1 chromosome (Sandberg, 1980; O´Malley and Garson, 1985; Sadamori et al., 1985a).

2.1.1 Cytogenetic and molecular characterization of the Ph^1 translocation

The Ph^1 chromosome usually results from a reciprocal translocation between chromosome 9 and 22, t(9;22)(q34;q11). Using somatic cell hybrids and *in situ* hybridization c-oncogenes have been assigned to both chromosomes: the *c-abl* oncogene to chromosome 9 (q34)(Heisterkamp et al., 1982; Jhanwar et al., 1984) and the *c-sis* oncogene to chromosome 22 (q13)(Swan et al., 1982; Dalla-Favera et al., 1982c; Jhanwar et al., 1984; Bartram et al., 1984). Molecular cloning of the breakpoint regions of chromosome 22 resulted in the identification of a breakpoint cluster region (*bcr*) (Groffen et al., 1984: Appendix Paper III; see section 2.1.3).

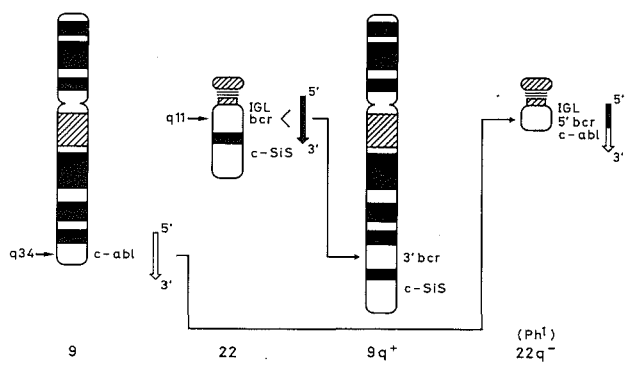

Figure 2.
Schematic representation of the standard Ph^1 translocation t(9;22)(q34;q11). The localization and orientation of the marker genes are indicated.

As a result of the Ph^1 translocation (Figure 2; Table 3), the c-abl oncogene is translocated from chromosome 9q34 to the proximal (5´) bcr sequences on chromosome 22q11 (De Klein et al., 1982: Appendix Paper I). The bcr is disrupted and the distal (3´) bcr sequences are translocated with the c-sis oncogene to the 9q+ derivative chromosome (Groffen et al., 1983a). Another genomic marker of the chromosome 22q11 region, the λ immunoglobulin light chain gene (IgL)(Erikson et al., 1981; McBride et al., 1982) remains on chromosome 22q- (Goyns et al., 1984). Similar studies of cytogenetic variant Ph^1 translocations (Table 3) revealed in all cases breaks on chromosome 22 within the bcr, and the c-abl oncogene was consistently translocated to the remaining bcr sequences on the Ph^1 chromosome. The c-sis oncogene was found to be located on the derivative chromosomes carrying the distal part of chromosome 22 (Table 3), indicating that the translocation of c-abl to a specific region on chromosome 22 was the critical event in these Ph^1 positive CML patients.

Recently this view has been strongly buttressed by the demonstration of a juxtaposition of c-abl and bcr sequences in the leukemic cells of some Ph^1 negative CML patients (Table 3; Bartram et al., 1985b; Morris et al., 1986). On the basis of molecular characteristics (bcr/c-abl rearrangement) these CML patients are identical to Ph^1 positive CML patients and possess also clinical and hematological features indistinguishable from Ph^1 positive CML patients. This in contrast to other Ph^1-negative CML patients who lack the bcr/c-abl rearrangement and differ clinically from classic CML. It has been suggested that these latter Ph^1-negative CML cases actually represent myelodysplasias (MDS) and reactive conditions other than CML with a clearly poorer clinical prognosis (Pugh et al., 1985). Therefore the detection of bcr/c-abl juxtaposition in CML may be of prognostic value.

These results, summarized in Table 3, indicate that both c-abl and bcr sequences may play a pivotal role in the pathogenesis of CML. The molecular characteristics of these two loci, the position of the breakpoints and the consequences of the

bcr/c-abl juxtaposition will be discussed in the following sections.

TABLE 3. Ph^1- CHROMOSOME IN CML

Karyotype	Chromosomal Localization of c-abl	c-sis	bcr	Reference
A. Standard Ph^1 translocation				
t(9;22)	9,22q-	22; 9q+	+	1,2,3,4,5,6
B. Variant Ph^1 translocation				
-simple t(*;22)	9;22q-	22, *	+	7,8,9
-complex t(*;9;22)	9;22q-	22, *	+	7,8,9
-masqued t(6;22)	9,22q+	n.d.	+	2,10
t(1;3;5;9;22)	9,22q+	n.d.	+	2,10
C. Ph^1-negative				
-with translocations				
t(9;12)	9,12q-	22	+	11,12
t(9;13;15)	n.d.	n.d.	+	13
-without translocations				
1)	9,22	22	+	14,15
2)°	9	n.d.	+	16
3)	9	22	-	3,6,8,14,15

°only one case; n.d. = not done; * stands for other chromosome as 9 or 22

References:

1. De Klein et al., 1982
2. De Klein and Hagemeijer, 1984
3. Bartram et al., 1984
4. Groffen et al., 1983a
5. Goyns et al., 1984
6. Groffen et al., 1984
7. Hagemeijer et al., 1984
8. Bartram et al., 1983
9. Bartram et al., 1985a
10. Hagemeijer et al., 1985
11. Bartram et al., 1985b
12. Hagemeijer et al., 1986
13. Hagemeijer et al., pers.comm.
14. Morris et al., 1986
15. Bartram and Carbonell, 1986
16. Bartram, 1985

2.1.2 The human c-abl oncogene

The proto-oncogene c-abl is the normal cellular homologue of the transforming gene, v-abl, of Abelson murine Leukemia virus (A-MuLV). This retrovirus is a recombinant between Moloney MuLV and mouse cellular c-abl sequences (Goff et al., 1980). It induces lymphoid tumors in vivo and transforms fibroblasts and hematopoietic cells in vitro (Rosenberg and Baltimore, 1980; Waneck and Rosenberg, 1981; Prywes et al., 1983; Waneck et al., 1986). Its transforming potential is tightly associated with the expression of a virus encoded protein $p160^{gag-abl}$ exhibiting tyrosine specific kinase activity (Sefton et al., 1981; Srinivasan et al., 1982; Reddy et al., 1983).

The cellular c-abl gene is strongly conserved during evolution and c-abl specific sequences have been cloned from nematoda (Goddard et al., 1986), Drosophila (Shilo and Weinberg, 1981; Hoffman-Falk et al., 1983), mouse (Goff et al., 1980; Goff and Baltimore, 1982; Wang et al., 1984; Ben-Neriah et al., 1986a), cat (Schalken et al., 1985) and human DNA (Heisterkamp et al., 1983a; 1983b; Shtivelman et al., 1985; Grosveld et al., 1986; Appendix Paper V). In human, v-abl homologous sequences are distributed discontinuously over a region of 32 kb and are dispersed over at least 9 exons (Figure 3: exons 2-10). Cloning of human c-abl cDNAs (Shtivelman et al., 1985; Grosveld et al., 1986: Appendix Paper V) allowed the identification of two additional exons 1 and A. Exon A is homologous to one of the four alternative first exons in mouse c-abl (Ben-Neriah et al., 1986a) and one other human first exon (exon B) is present 175 kb 5′ of exon A. This exon can also be linked to the c-abl body exons 1-10 by alternative splicing (Figure 3)(A. Bernards pers. comm.).

Southern blotting and the subsequent cloning and characterization of chimeric breakpoint fragments have demonstrated the presence of chromosome 9 breakpoints within or in the vicinity of the c-abl gene. Some of these breakpoints occur within the large intron of 17 kb, separating the first exon A and the c-abl body exon 1 (Heisterkamp et al., 1983b; Leibowitz

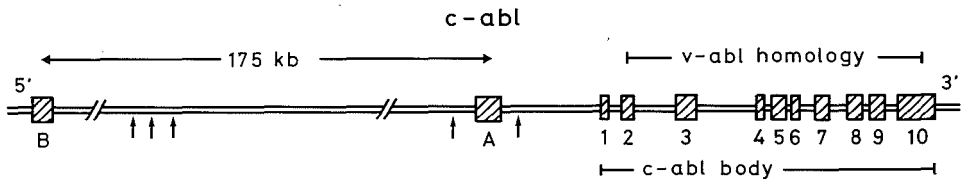

Figure 3.
The human c-abl gene. The two alternative first exons B and A and the c-abl body exons 1-10 are indicated as hatched boxes. Distance between exon A and B and the position of the v-abl homologous part are shown above the map. The cloned and analysed breakpoints are indicated by arrow below the map. ═══//═══ represents a gap of unknown distance.

et al., 1985a), whereas in other CML patients and the CML-derived cell line K562 (Lozzio and Lozzio, 1975), the breakpoints are located at variable distances, up to more than 80 kb upstream of exon A (Grosveld et al., 1986: Appendix Paper V; unpublished results). The localization of the K562 breakpoint is of particular interest because several reports (Heisterkamp, 1983b; Collins and Groudine, 1983; Selden et al., 1983) have shown that c-abl and the λ IgL constant region (Cλ), but not the c-sis oncogene are amplified at least four fold in this cell line. The amplification of chromosome 9 sequences starts at the breakpoint on chromosome 9 and extends in the direction of the telomere of the chromosome, including all known c-abl sequences. However, exon B the human homologue of one of the alternative 5′ exons of mouse c-abl (Ben Neriah et al., 1986) is not amplified in K562 DNA (A. Bernards, pers.comm.). This suggests that this 5′ c-abl exon B is located upstream of the K562 breakpoint on chromosome 9 and implies that even breakpoints, which map at a minimum of 80 kb 5′ of exon A, are still located within the c-abl gene.

2.1.3 The human bcr gene

In contrast to the breakpoints on chromosome 9 which are scattered over a region of more than 100 kb, the chromosome 22 breakpoints are all located within a small region of 5.0 kb termed the breakpoint cluster region or bcr (Groffen et al., 1984: Appendix Paper III). The isolation of bcr cDNAs established that bcr is part of a larger protein encoding region: the 'bcr' gene with an as yet unidentified cellular function (Heisterkamp et al., 1985: Appendix Paper IV). The bcr gene is orientated with its 5' end pointing towards the centromere of chromosome 22 and 2/3 of the coding regions, dispersed over a minimum of 18 exons encompassing 67 kb of genomic DNA, have been identified (Groffen et al., 1986). As shown in figure 4, three relatively small exons (designated 1-3), varying in size

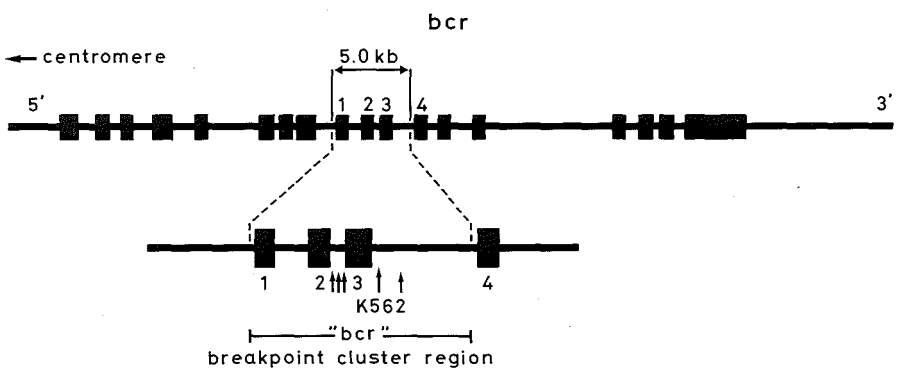

Figure 4.
The human bcr gene. The 18 identified exons are indicated as solid boxes. The three exons (1-3) within the 5.0 kb bcr and an adjacent exon are numbered. The position of the breakpoints are shown in the bottom part of the figure.

from 75 to 105 bases, are present within the bcr and all chromosome 22 breakpoints are clustered in the non-coding regions between exon 2 and 3 or exon 3 and 4 of the bcr (Heisterkamp et al., 1985: Appendix Paper IV; Groffen et al., 1986; De Klein et al., 1986a: Appendix Paper VI). Although all chromosome 22 breakpoints are located within the bcr, these observations indicate that a small variation in the number of bcr exons remaining on the Ph^1 chromosome occurs, some CML patients retain exon 3 and all sequences 5′ of it on Ph^1, whereas in other CML patients this exon 3 is translocated, together with the 3′ part of the bcr gene to the 9q+ derivative chromosome. In this respect the cell line K562 contains a genuine chromosome 22 breakpoint, located 3′ of exon 3 (Figure 4). However, in contrast with the leukemic cells of CML patients, K562 contains amplified remnants of the Ph^1 chromosome (Grosveld et al., 1986: Appendix Paper V) present on an acrocentric marker chromosome (Selden et al., 1983). The amplified region may be relatively large since it encompasses Cλ , 5′ bcr and at least 150 kb of c-abl sequences.

2.1.4 Analysis of CML breakpoints by DNA sequencing.

In the Ph^1 translocation, an illegitimate recombination takes place between sequences on chromosome 9 and 22. Sequence analysis and hybridization have indicated that the bcr gene has no homology to previously identified proteins sequences, including the c-abl oncogene (Heisterkamp et al., 1985: Appendix Paper IV). Since the DNA sequence of the translocation junction regions could provide additional information about the possible mechanism of chromosomal translocation we determined the DNA sequence of several breakpoint regions (Heisterkamp et al., 1985: Apppendix Paper IV ; Groffen et al., 1986; De Klein et al., 1986a: Appendix Paper VI). The breakpoint sequence of one CML patient showed a perfect conservative break without loss or gain of chromosome 9 or 22 sequences (Heisterkamp et al., 1985: Appendix Paper IV). However, in other CML patients deletions of both chromosome 9 and 22 sequences were observed.

Usually the chromosome 22 deletions are small (100-500 bp) whereas the chromosome 9 deletions could encompass more than 70 kb (De Klein et al., 1986a: Appendix Paper VI). Comparison of the DNA sequence of several breakpoints suggests that homologous recombination is unlikely, since there is no apparent homology between the chromosome 9 and 22 breakpoint sequences. Nor is there any evidence for crossing over within a homologous oligonucleotide. Similar results have been obtained from sequence analysis of the t(8;14) translocation in Burkitt lymphoma (Battey et al., 1983; Moulding et al., 1985). However, in CML there is some evidence that Alu-repetitive sequences are involved, since most of the breakpoint occur within Alu-repeats (Schmid and Jelinek, 1982) or Alu-repeat like sequences (Heisterkamp et al., 1985: Appendix Paper IV; Rogers et al., 1985; Groffen et al., 1986; De Klein et al., 1986a: Appendix Paper VI).

As illegitimate recombination within Alu-sequences has been reported in four independent cases of thalassemia (Vanin et al., 1983) and in one case of hypercholesterolemia (Lehrman et al., 1985), it is well conceivable that Alu-repetitive sequences are hot spots of recombination and play a role in the juxtaposition of 5′ bcr and 3′ c-abl sequences.

2.1.5 Consequences of the Ph^1 translocation

As a result of the Ph^1 translocation the bcr and c-abl genes are located on the Ph^1 chromosome; at the 5′ (centromeric) side the 5′ part of the bcr gene and at the 3′ (telomeric) side, the translocated c-abl sequences, in the same transcriptional orientation.

Several investigators have addressed the question whether the Ph^1 translocation influences the expression of these two genes. The c-abl mRNA transcripts of 6.0 and 7.0 kb are present in normal cells and tissues of both hematopoietic and non-hematopoietic origin (Westin et al., 1982; Wang and Baltimore, 1983; Gale and Canaani, 1984). In addition to these normal transcripts, a novel c-abl homologous mRNA of 8.5 kb is present

in the leukemic cells of CML patients and CML-derived cell lines (Canaani et al., 1984; Collins et al., 1984; Blick et al., 1984; Leibowitz et al., 1985b; Stam et al., 1985). Similar results were obtained when the expression of the bcr gene was studied. In addition to the normal bcr mRNAs of 7.5, 7.0 and 4.5 kb a 8.5 kb bcr specific mRNA transcript was present in the leukemic cells of Ph^1 positive CML patients and CML derived cell lines (Stam et al., 1985; Grosveld et al., 1986: Appendix Paper V). This 8.5 kb bcr mRNA migrated at the same position as the aberrant 8.5 kb c-abl mRNA in agarose gels and contained only the 5´ coding regions of the bcr. This aberrant 8.5 kb mRNA seems to be specific for Ph^1 positive cells, as it is not detected in either normal cells, tissues or other types of leukemia (Eva et al., 1982; Gale and Canaani, 1984; Romero et al., 1986; Grosveld et al., 1986: Appendix Paper V). However, the expression is not restricted to the myeloid cell lineage. Similar or often even enhanced expression of this 8.5 kb mRNA has been reported in both the myeloid and lymphoid acute phase of CML (Romero et al., 1986) in erythroleukemia or lymphoid CML-derived cell lines (Collins et al., 1984; Konopka et al., 1986) and in somatic cell hybrids between rodent fibroblasts and Ph^1 positive leukemic cells (Kozbor et al., 1986). In these latter somatic cell hybrids the 8.5 kb mRNA was only found in cell hybrids which retained the Ph^1 chromosome. These results strongly suggested that the 8.5 kb mRNA is a chimeric molecule that contains the 5´ part of the bcr gene in addition to the c-abl sequences. Direct proof of this hypothesis was achieved by the cloning of chimeric bcr/c-abl cDNAs (Shtivelman et al., 1985; Grosveld et al., 1986: Appendix Paper V). These cDNAs contained part of the 5´ coding regions of the bcr gene fused to the c-abl coding regions in a splice dependent manner following the GT-AG rules (Breathnach and Chambon, 1981). Transcription is likely to be initiated from the bcr promotor and probably stops at the 3´ end of the c-abl gene (Figure 5). By splicing of the precursor RNA the 8.5 kb mRNA is produced, in which the versatility of the splicing system accomodates for the large variation in intron size that links bcr and c-abl in

different patients. Depending on the chromosome 22 breakpoints, either bcr exon 2 or exon 3 will be spliced to the first available splice acceptor site of a c-abl exon: i.e. the second exon 1, since the first c-abl exon A or exon B probably lack such a 3´ splice acceptor site. Although DNA sequence data indicate that also other splices are compatible (e.g. between bcr exon 1 and c-abl exon 1 or 2) until now these have not been encountered in CML. In cases were the bcr breakpoint is located 3´ of bcr exon 3 both mRNAs are made (Shtivelman, pers. comm.). This indicates that the 8.5 kb chimeric mRNA can differ

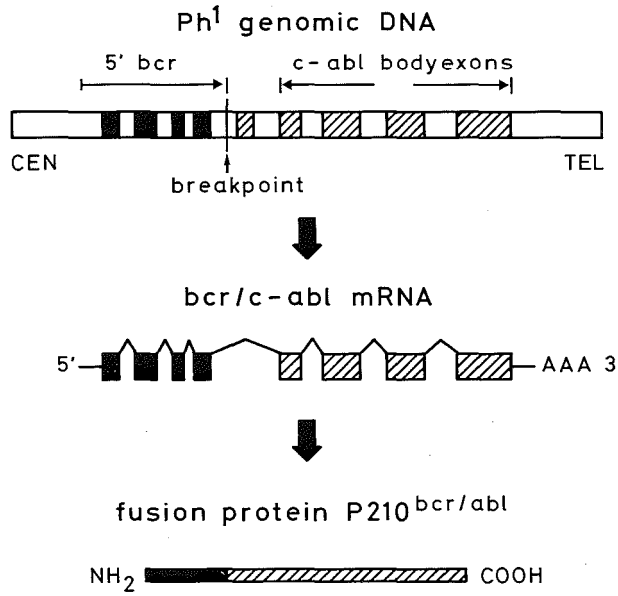

Figure 5.
Schematic representation of the molecular consequence of the Ph^1 translocation. The topline shows the physically joined 5' bcr and 3' c-abl regions on the Ph^1 chromosome. The exons are indicated by solid (bcr) or hatched (c-abl) boxes. This configuration allows the transcription of chimeric mRNA (consisting of 5' bcr exons fused to the 3' c-abl body exons (middle line). This mRNA is translated into a chimeric fusion protein with a bcr aminoterminus and a c-abl carboxy terminus (bottom line).

in the presence or absence of one bcr exon (bcr exon 3; 75 nucleotide in size). However, both mRNAs have one long open reading frame compatible with both the predicted bcr (Heisterkamp et al., 1985: Appendix Paper IV) and known c-abl (Wang et al., 1984) reading frames. Translation of this 8.5 kb mRNA into protein seems certain, because both c-abl and bcr antisera precipitate the same aberrant p210 kD bcr/c-abl protein in several CML-derived cell lines and Ph^1 positive CML patients (Konopka et al., 1984; 1985; Kloetzer et al., 1985, Naldini et al., 1986; Ben-Neriah et al., 1986b). This 210 kD bcr/abl protein is much larger than the normal 145kD c-abl protein and is like the viral protein $p160^{gag-abl}$ a fusion protein in which the N-terminal aminoacids of c-abl have been replaced by the 5´ bcr or gag residues respectively. In the viral protein the N-terminal gag sequences, or other N-terminal sequences which can replace the gag sequences, are required for protein stabilization and hence transformation of lymphoid cells (Prywes et al., 1985a; Matthey-Prevot and Baltimore, 1985). The bcr moiety of the $p210^{bcr-abl}$ fusion protein may serve a similar function, but whether the deletion of the c-abl N-terminus and/or its replacement by protein stabilizing sequences is the critical event for the conversion of the c-abl proto-oncogene into an oncogene is at the moment still unclear. However, the tyrosine kinase domain of v-abl is indispensable for transformation (Prywes et al., 1983, 1985b; Wang and Baltimore, 1985) and since this tyrosine kinase domain is also retained in the $p210^{bcr-abl}$ protein, it seems very likely that the tyrosine kinase activity of this protein plays an essential role in the altered growth pattern of the myeloid cells in CML. Initially it was suggested that the N-terminal substitutions could unmask the in vitro tyrosine kinase activity of the c-abl protein (Davis et al., 1985), but recent reports indicate that all c-abl proteins have similar in vitro kinase activities although the viral $p160^{gag-abl}$ and the $p210^{bcr-abl}$ differ from their normal cellular counterparts in the way they utilize themselves as substrate (Konopka and Witte, 1985a, 1985b). Besides this difference in substrate specificity in vitro, which probably

also exists *in vivo*, the $p160^{gag-abl}$ and the $p210^{bcr-abl}$ are phosphorylated on tyrosine *in vivo*. The normal mouse and human *c-abl* protein lack such a phosphorylation although they contain a tyrosine phosphorylation acceptor site with identical amino-acid sequence as the *v-abl* protein (Groffen et al., 1983b; Wang et al., 1984). The functional significance of *in vivo* phosphorylation is unclear but it often augments the catalytic activity of tyrosine kinases such as the EGF-receptor (Hunter and Cooper, 1985). Although *c-abl* lacks several characteristics of a receptor protein (e.g. membrane spanning sequence; extracellulair domain, glycosylation) it is well conceivable that *c-abl* is part of a growth factor/receptor complex and plays a role in the signal transduction (Konopka and Witte, 1985b). Hence the tyrosine kinase activity of the normal *c-abl* protein may be under strict control of such a growth factor/receptor complex. N-terminal substitution of the *c-abl* protein with *gag* or *bcr* sequences could change the substrate specificity of the tyrosine kinase, or could enable the protein to escape such a regulation and would result in the delivery of a continuous proliferation signal to the cell even in the absence of the growth factor. Consistent with this model is the observation that normal hematopoietic cells become growth factor independent in a non-autocrine manner, after infection with A-MulV (Cook et al., 1985; Pierce et al., 1985; Metcalf, 1986). The presence of the $p210^{bcr-abl}$ fusion protein may have similar stimulatory effects on the growth pattern of hematopoietic cells, or alternatively may help the neoplastic stem cell to ignore or override a negative regulatory signal produced by the normal adherent bone marrow cells (Eaves et al., 1986).

2.1.6 Other oncogenes involved in CML

There are several reports which indicate that other genes than the *bcr* or *c-abl* oncogene are involved in CML. By DNA mediated transfection of NIH3T3 cells it was shown, that some CML patients and CML derived cell lines contain an activated N or *K-ras* gene (Eva et al., 1983, 1985; Hirai et al.,

1985; H. Jansen, pers.comm.). Amplification and rearrangement of c-myc has been found in the acute phase of a CML patient (McCarthy et al., 1984). This may play a role in the progression of the disease. A similar role has been suggested for the expression of c-sis during accelerated/blast phase but not in the chronic phase of CML (Romero et al., 1986; Gale and Canaani 1984, 1985). Several other, as yet unidentified genes, are expressed specifically in CML (Mars et al., 1985; Birnie et al., 1983; Calabretta et al., 1986) and may also play a role in the progression of the disease. Therefore, analysis of the effect of $p210^{bcr-abl}$ on normal hematopoietic cell proliferation by using gene transfer techniques or alternative inhibition of the translation of this protein with antisense bcr/c-abl mRNA will help to define the precise role of this protein and hence the Ph^1 translocation in the pathogenesis of CML.

2.1.7 The Ph^1 chromosome in ALL and AML

The Ph^1 chromosome has been reported in other non-CML hematopoietic disorders (Sandberg, 1980), including different subtypes of acute leukemia in which no preceeding chronic phase has been observed (Beard et al., 1976; Oshimura and Sandberg, 1977; Bloomfield et al., 1977; 1978). The clinical distinction between blast crisis of CML and de novo Ph^1 positive ALL or AML is not always clear (Catovsky, 1979; Beard et al., 1976). Usually these latter cases have less than 100% Ph^1 positive cells in the bone marrow during the acute phase, and clinical remission is accompanied by the elimination of the Ph^1 chromosome from the bone marrow cells (Sandberg et al., 1980). The incidence of Ph^1 positive AML is low (< 1%) and restricted to the M1 subtype (Abe and Sandberg, 1979; Bloomfield et al., 1977; Yunis et al., 1984). A similar incidence (2-6%) has been reported for childhood ALL (L1-L2)(Chessells et al., 1979; Priest et al., 1980). However, in adult ALL the Ph^1 chromosome is the most frequent chromosomal abnormality with an incidence of 17-25% (Sandberg, 1980; LeBeau and Rowley, 1984b).

As in CML, the Ph^1 chromosome in ALL or AML is usually

the result of a t(9;22), although the incidence of variant translocations in ALL is higher than in CML or AML (Whang-Peng and Knutsen, 1982; De Klein et al., 1986b: Appendix Paper VII). Nevertheless, translocation of the c-abl oncogene to the Ph^1 chromosome was observed in all patients studied with Ph^1 positive ALL (Erikson et al., 1986a; De Klein et al., 1986b: Appendix Paper VII). However, molecular studies using bcr probes showed a hetereogeneous picture. In our own study (De Klein et al., 1986b: Appendix Paper VII), 6 Ph^1 positive ALL patients showed bcr rearrangements as observed in CML, and 3 other patients did show recombinations involving 5´ bcr sequences but the corresponding 3´ bcr sequences were not detectable. A group of 5 ALL patients did not show any bcr rearrangement at all. The presence of the 8.5 kb bcr/c-abl mRNA was demonstrated only in the leukemic cells of those ALL patients with bcr rearrangements. In patients without bcr rearrangements, only the normal bcr and c-abl mRNA species were present. Similar results were obtained by others (Rodenhuis et al., 1985; Erikson et al., 1986a; Saglio et al., 1986). Using somatic cell hybrids it was shown that the 22q11 breakpoint of one Ph^1 positive ALL patient was located proximal to the bcr region (Erikson et al., 1986a), probably distal of the λIgL locus. In another case in situ hybridization studies suggested a breakpoint within the λ IgL region (Cannizzaro et al., 1985).

In the limited number of Ph^1 positive AML patients studied, a similar heterogeneity in bcr rearrangements was observed (Saglio et al., 1986; Erikson et al., 1986a; Bartram pers.comm.). These results indicate that, if c-abl is involved in the Ph^1 positive ALL/AML patients without bcr rearrangement the genetic mechanism of activation must be different from that reported in CML, whereas in Ph^1 positive ALL and AML patients with bcr rearrangements probably the same or a similar $p210^{bcr-abl}$ protein is present. The molecular aspects observed in these latter ALL/AML patients are indistinguishable from CML, reinforcing the possibility that some of the Ph^1 positive ALL or AML patients may have a blast crisis, developed from subclinical CML (Beard et al., 1976; Catovsky, 1979).

2.2 Structural aberrations in acute non-lymphocytic leukemia

Concurrent with the constant refinement of cytogenetic techniques, clonal chromosomal abnormalities have been observed with increasing frequency (50-93%) in the bone marrow of patients with acute non-lymphocytic leukemia (ANLL)(Rowley and Potter, 1976; Yunis et al., 1981, 1984; Hagemeijer et al., 1981; Larson et al., 1983). The most frequent abnormalities associated with ANLL are a non-random loss or gain of chromosomes (Rowley et al., 1982; Rowley, 1984). Furthermore a number of unique structural aberrations have been described which are associated with specific morphological subtypes of ANLL (FAB, Bennett et al., 1976; Table 1). Since several oncogenes (Fig.1) map closely to these chromosomal breakpoints, a number of these consistent aberrations have been assessed for a possible involvement of these oncogenes.

2.2.1 t(8;21) in acute myeloblastic leukemia

The reciprocal translocation t(8;21)(q22;q22) is found in 18% of the karyotypic abnormal AML-M2 patients (acute myeloblastic leukemia with maturation)(Rowley, 1982; Rowley and Testa, 1982; LeBeau and Rowley, 1984b). Cytogenetic studies of variant forms of the translocation indicated that translocation of 21q material to the 8q- chromosome was the constant, and therefore probably critical event (Rowley 1982, 1984). At both breakpoints cellular oncogenes are located: the c-mos oncogene at chromosome 8q22 or 8q11 (Prakash et al., 1982; Neel et al., 1982; Caubet et al., 1985) and the c-ets-2 oncogene at chromosome 21q22 (Watson et al., 1985, 1986)(Figure 1).

Using in situ hybridization or somatic cell hybrids containing the 8q- or 21q+ recombinant chromosomes, it was established that both oncogenes were located at the critical 8q- chromosome, albeit without obvious DNA rearrangements (Drabkin et al., 1985; Sacchi et al., 1986). Translocation of c-ets-2 was concordant with an altered expression of this oncogene in the AML-M2 leukemic cells, as compared with its expression in

normal lymphocytes and control cell lines (Sacchi et al., 1986; Watson et al., 1985). The c-ets-2 gene maps in the chromosome 21 region that, when trisomic, confers the Down syndrome phenotype. This congenital aberration is associated with an increased risk of acute leukemia, in particular AML (Alimena et al., 1985; Evans and Steward, 1972). These data might indicate that c-ets-2 is involved in the pathogenesis of AML-M2, although its exact role remains to be established. Previous reports have demonstrated that c-mos can be activated by transposon like insertions in mouse myeloma cell lines (Rechavi et al., 1982; Cohen et al., 1983; Gattoni Celli et al., 1983). However, its recent reassignment to band 8q11 (Caubet et al., 1985) suggests that it could be located far more distant of the 8q22 breakpoint than was assumed initially. In this case its involvement in t(8;21) AML-M2 is not very likely.

2.2.2 t(15;17) in acute promyelocytic leukemia

The translocation t(15;17)(q22;q21) is exclusively found in acute promyelocytic leukemia (APL) (ANLL-M3) and several investigators have suggested that this reciprocal translocation (or variants) can be found in every patient with APL, if optimal cytogenetic techniques are used (Rowley et al., 1977; Kondo and Sasaki, 1982; Larson et al., 1984; De Braekelaar and Lin, 1986; Misawa et al., 1986; Mitelman et al., 1986). The constant recombinant chromosome is the 15q+ chromosome. This suggests that translocation of the distal part of chromosome 17q to chromosome 15q results in a critical gene rearrangement which leads to malignant transformation of the promyelocytic cell (Rowley, 1982, 1984; Misawa et al., 1986). A number of interesting genes have been assigned to the chromosomes involved (Figure 1). The c-fes (or c-fps) oncogene at 15(q26)(Heisterkamp et al., 1982; Dalla Favera et al., 1982a; Harper et al., 1983; Jhanwar et al., 1984) is located at a great distance from the 15 q22 breakpoint and its translocation to the non-critical 17q- chromosome seems fortuitous (Sheer et al., 1983). For the same reason it seems not very likely that the p53 or

the c-erbA-1 oncogenes are directly involved. These genes map proximal of the 17q21 breakpoint at 17p13 and 17q21, respectively (Isobe et al., 1986; Miller et al., 1986; McBride et al., 1986; Dayton et al., 1984; LeBeau et al., 1985a; Spurr et al., 1984). However, some genes may be involved that map closely distal to the 17q21 breakpoint such as the nerve growth factor receptor gene (Huebner et al., 1986) or another growth factor receptor gene c-erbB-2 (or neu)(Schechter et al., 1985; Coussens et al., 1985; Stern et al., 1986). Both map at 17q21 - 17q22. Using a mouse p53 cDNA probe, LeBeau et al. (1985a) have localized a p53 like sequence just distal to the 17q breakpoint and have proven its translocation to the critical 15q+ chromosome. These sequences which are not coding for the human p53 gene (Lamb and Crawford, 1986) could represent another as yet unidentified cellular gene which is possibly involved in the pathogenesis of promyelocytic leukemia.

2.2.3 inv(16) and t(16;16) in acute myelomonocytic leukemia

Recently, another cytogenetic-clinical association has been identified in acute myelomonocytic leukemia (AMMoL-M4) with abnormal eosinophils. Most of these AMMoL-M4 patients had an inv(16)(p13q22) or t(16;16)(p13;q22)(LeBeau et al., 1983; Arthur and Bloomfield, 1983; Testa et al., 1984). LeBeau et al. (1985b) have localized the metallothionin (MT) gene cluster to the 16q22 region and proved with the use of in situ hybridization, that this MT gene cluster is split by the t(16;16) or inv(16). This could indicate, that either activation of an as yet unknown oncogene by association with the strong MT gene control elements, or abnormal MT gene expression itself is involved in the leukemogenesis of this specific AMMol-M4 with abnormal eosinophils.

2.2.4 t(6;9) in acute non-lymphocytic leukemia

The t(6;9)(p23;q34) is a relatively rare translocation in ANLL (< 0.5%) and is observed in AML-M2; AMMOl-M4 and AML-M1

(Rowley, 1984; Vermaelen et al., 1983; Pearson et al., 1985; Carroll et al., 1985; Heim et al., 1986). This abnormality is interesting since, the chromosome 9 breakpoint is located in the same chromosomal band as in the t(9;22)(q34;q11) in CML and both CML and t(6;9) ANLL are associated with increased numbers of bone marrow basophils (Pearson et al., 1985). Recently, Westbrook et al. (1985) have demonstrated, that the chromosome 9(q34) breakpoint is located at a unknown distance 3´ of the c-abl gene. Moreover, no abnormal or higher levels of c-abl transcripts or proteins are present in the few t(6;9) ANLL samples tested (Westbrook et al., 1985; Von Lindern pers.comm.).

At chromosome 6(p21) the human homologue of pim-1 has been localized (Cuypers et al., 1986; Nagarajan et al., 1986). High levels of pim mRNA were observed in the cell line K562, which has an abnormal marker chromosome M2: t(6;6)(pter→p11::p21→ qter)(Chen, 1985; Nagarajan et al., 1986) and in one t(6;9) ANLL patient (Von Lindern, pers.comm.). As yet it is not clear, whether the elevated pim mRNA levels are a consequence of the translocations, or represent the normal transcription levels in myeloid cells. Sofar, no rearrangements have been observed within the 30 kb genomic DNA surrounding the known human pim sequences. We are currently investigating the involvement of the pim gene in the t(6;9) translocation and other malignancies associated with chromosome 6 aberrations (Miyamoto et al., 1984; Mecucci et al., 1985; Pedersen et al., 1986; Rey et al., 1985) using a human pim cDNA clone and proper control cells.

2.2.5 Translocations involving 11q23 in ANLL and ALL

Several investigators have noted the frequent involvement of chromosome band 11q23 in specific translocations associated with childhood ANLL (Berger et al., 1982; Yunis, 1983; Rowley, 1984; Hagemeijer et al., 1982, 1986a). Of the ANLL cases with a chromosome 11 translocation, 80% were classified as acute monoblastic leukemia (AMoL-M5) and 20% as AMMoL-M4 (FIWLC, 1984).

The t(9;11)(p21;q23) is strongly associated with AMMol-M5 (Hagemeijer et al., 1982), whereas other translocations such as t(11;17)(q23;q25), t(11;19)(q23;p13), t(10;11)(p15;q23) and t(6;11)(q27;q23) are found both in AMMoL-M4 and AMMoL-M5 (Yunis et al., 1984; Rowley, 1984; LeBeau, 1986). Moreover, all these translocations have also been associated with a poorly differentiated acute lymphoblastic leukemia (ALL-L1)(Kaneko et al., 1986; Hayashi et al., 1985). The t(4;11)(q21;q23) and t(1;11)(p32:q23) are usually found in poorly differentiated ALL-L1, often with some (inducible) myelo-monocytic morphology and markers and rarely associated with ANLL-M5 or M4 (Kaneko et al., 1986; Hagemeijer et al., 1986b; Mirro et al., 1986; Nagasaka et al., 1983; Crist et al., 1985; Meyers et al., 1986). These data suggest that the 11q23 translocation affects an early progenitor cell, capable of both lymphoid and myelo-monocytic differentiation. It has been suggested (Crist et al., 1985; Kaneko et al., 1986; Mirro et al., 1986) that due to the involvement of 11q23 in the translocation, the differentiation pattern is blocked in an early stage and a gene on the recipient chromosomes of the various translocations could influence the morphological and other clinical characteristics. In analogy with the 14q32 and 14q11 breakpoints in B or T-cell specific malignancies (sections 2.3) the 11q23 region could bear the locus of a cell type specific gene and the breakpoint regions on the recipient chromosomes loci for growth promoting genes (Rowley , 1984; Yunis, 1983, et al., 1984). However, as yet there is no evidence for a somatically rearranged gene nor for a cell or stage specific gene located at the chromosome 11q23 region. Interesting in this respect is that at several of the recipient chromosomes or chromosome regions involved in these 11q23 translocations, oncogenes or growth factor/receptor genes have been localized: At 1p32 the L-myc and B-lym oncogenes (McBride et al., 1985; Morton et al., 1984), at 9p21-p13 the interferon (IFN) α and β gene clusters (Trent et al., 1982), at 10p14-p15 the interleukin-2 receptor gene (Leonard et al., 1985) at 19p13 the insulin receptor gene (Yang-Feng et al., 1985), at chromosome 4 the c-raf-2 and c-kit oncogene (Bonner

et al., 1984; Besmer et al., 1986) and at chromosome 6 the c-yes-2 oncogene (Semba et al., 1985). Other genes in this segment of chromosome 11 are myeloid specific antigens (Geurts van Kessel et al., 1984; Rettig et al., 1985) located at 11q12-qter and four genes associated with cell-cell interactions located at 11q23: Thy-1 (van Rys et al, 1985; Seki et al., 1985), NCAM (N'guyen et al. 1986; Rutishauer and Goridis, 1985), and proximal of the t(4;11) breakpoint T_δ and T_ϵ genes of the T3-complex (Van den Elsen et al., 1986; Gold et al., 1986). All these genes may help to define and characterize the several 11q23 translocations in more detail. Another intriguing gene assigned to the 11q23 region is the c-ets-1 oncogene (Detaisne et al., 1984; Watson et al., 1985; 1986). The ets sequences were identified as a second cellular sequence transduced by the Avian acute leukemia virus E26 (Nunn et al., 1983; Leprince et al., 1983). The presence of v-ets in the virus is associated with a block in differentiation capacity of the transformed cells (Beug et al., 1984). In contrast to the organization in chicken, human c-ets sequences consist of two distinct domains located on different chromosomes. The 5' v-ets cellular homologue, c-ets-1 on chromosome 11q23 and the 3' v-ets cellular homologue, c-ets-2 on chromosome 21q22 (Watson et al., 1985; 1986). The c-ets-1 gene is located distal of the 11q23 breakpoint and is translocated to the recipient chromosome involved in the (4;11) or (9;11) translocations (Diaz et al., 1986; Sacchi et al., 1986). Rovigatti et al., (1986a; 1986b) have reported that in almost all leukemia patients with 11q23 abnormalities an alteration, often both amplification and rearrangement, of the c-ets-1 locus accompanied by an overall enhanced expression of c-ets-1 can be observed. Since other investigators (Sacchi et al., 1986; Diaz et al., 1986) report contradictary results, the involvement of c-ets-1 in these leukemias is not unambiguously proven.

2.3 Translocations associated with lymphocytic leukemia and lymphoma

As shown in Table 1, various characteristic structural aberrations have been described in patients with lymphocytic leukemia and lymphoma. The most frequently observed translocation in acute lymphoblastic leukemia (ALL), the t(9;22) translocation, is cytogenetically identical to the Ph^1 chromosome and prompted molecular studies to explore the involvement of the c-abl and bcr genes (see section 2.1.6). The t(8;14) translocation, observed in some (B-cell) ALL-L3 patients and the variant t(2;8) and t(8;22) are strongly associated with Burkitt's lymphoma. In these specific translocations the direct involvement of the c-myc oncogene and the three immunoglobulin loci was proven. In other B cell specific leukemias and lymphomas consistent translocations involving chromosome band 14q32 suggested a similar involvement of the IgH locus. An analogous situation is gradually emerging in T-cell specific malignancies: The T-cell receptor α-chain gene locus is localized at chromosome 14q11 ,and this 14q11 region is frequently involved characteristic chromosomal aberrations of T-cell specific leukemias and lymphomas.

2.3.1 The t(8;14) and variant (2;8) or (8;22) translocations in Burkitt's lymphoma

Burkitt's lymphoma (BL) is a B-cell malignancy, which is characterized by three specific chromosomal translocations (Zech et al., 1976; Bernheim et al., 1981). In 80% of the BLs a t(8;14)(q24;q32) is found in which the human c-myc oncogene is translocated from its normal position at chromosome 8q24 (Neel et al., 1982; Dalla-Favera et al., 1982a, 1982b) to the Immunoglobulin heavy chain (IgH) gene locus at chromosome 14q32 in a head to head orientation (Figure 6)(Kirsch et al., 1982; Taub et al., 1982; Dalla-Favera et al., 1983; Adams et al., 1983; Marcu et al. ,1983). In the variant t(2;8)(p12;q24) and t(8;22)(q24;q11) translocations, the c-myc gene remains on

chromosome 8 and part of the immunoglobulin light chain (IgL) genes (located at chromosome 2p12 and 22q11, respectively, Erikson et al., 1981; McBride et al., 1982) are translocated to the 3´ site of the c-myc gene in a head to tail orientation (De la Chapelle et al., 1983; Erikson et al., 1983; Malcolm et al., 1985)(Figure 6). A similar recombination between c-myc and the IgH or IgL genes has been observed in mouse plasmacytomas (mpc) bearing a t(12;15) or variant (6;15) translocation (Klein, 1981; 1983; Erikson et al., 1985a).

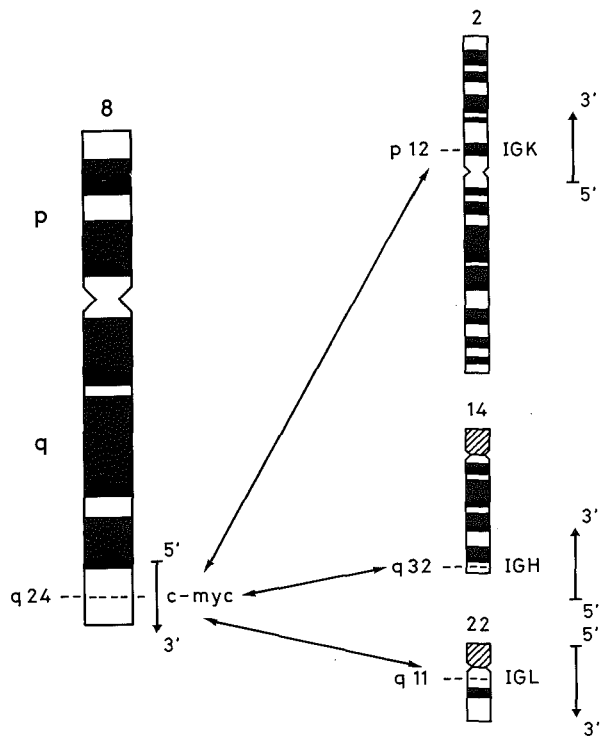

Figure 6.
Schematic representation of the chromosomes involved in Burkitt lymphoma specific translocations: t(2;8)(p12;q24), t(8;14)(q24;q32) and t(8;22)(q24;q11). The position of the breakpoints and the transcriptional orientation of the involved loci are indicated.

The c-myc oncogene, the cellular homologue of the transforming gene v-myc of Avian myelocytomatosis virus MC29, contains 3 exons (Figure 7). Only the exons 2 and 3, which are homologous to the v-myc gene, code for the nuclear c-myc protein (Alitalo et al., 1983b; Winquist et al., 1983; Eisenman et al., 1985). Transcription of the c-myc gene is initiated at two promotors (P1 and P2), located in the first untranslated exon and results in 2 mRNAs of 2.4 and 2.2 kb respectively (Taub et al., 1984; Zimmerman et al. 1986). The expression of c-myc is constant throughout the cell cycle in normal dividing cells and decreases rapidly during differentiation (Hann et al., 1985; Dony et al., 1985; Thompson et al., 1985). Conversely, the induction of cell proliferation by mitogens results in a drastic increase of c-myc mRNA levels (Kelly et al., 1983; Lacy et al., 1986). The c-myc mRNA and protein have an extremely short half life (Dani et al., 1984; Rabbitts et al., 1985a, 1985b) and regulation of c-myc expression takes place both at the transcriptional level (Bently and Groudine, 1986) and at a post-transcriptional level by regulating the stability of the mRNAs (Blanchard et al., 1985). In this latter post-transcriptional control, the 5´ untranslated exon seems to play an important role and loss of this exon results in a more stable

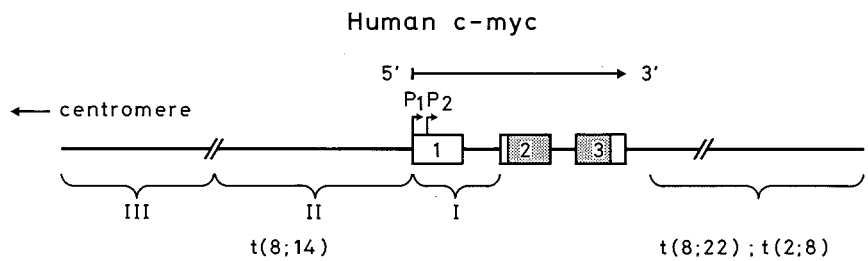

Figure 7.
The human c-myc gene. The three exons are indicated as boxes (hatched areas indicate protein coding regions). Transcriptional orientation (5'→ 3') and the position of the alternative initiation sites for transcription (P1 and P2) are indicated. Below the map the position of the variant translocation t(8;22) and t(2,8) breakpoints as well as the position of the class I, II and III t(8;14) breakpoints are shown.

c-myc mRNA (Piechaczyk et al., 1985; Eick et al. 1985; Rabbitts et al., 1985a).

The t(8;14) BLs all have chromosome 8 breakpoints 5′ of the two coding exons and can be divided in three classes: those with a breakpoint within the c-myc gene (class I; 50% of all BLs); those with a breakpoint immediately 5′ of the gene (class II); and those with a breakpoint at an unknown distance 5′ of the gene (class III). The variant t(2;8) or t(8;22) BL breakpoints are located 3′ of the c-myc gene (Cory, 1986; Figure 7). Although three breakpoints have been mapped in the vicinity of the gene (Hollis et al., 1984; Denny et al., 1985; Sun et al., 1986), the majority of these breakpoints are located more than 8 kb 3′ of c-myc. Recent cytogenetic data (Manolov et al., 1986) suggest that some of the t(8;22) chromosome 8q24 breakpoints are located at a different 8q24 subband than the t(8;14) or t(2;8) chromosome 8 breakpoints. In the mouse plasmacytoma variant t(6;15), these breakpoints 3′ of c-myc cluster in a segment of DNA (pvt-1 locus), located at least 86 kb 3′ of the c-myc gene (Webb et al., 1984; Cory et al., 1985; Banerjee et al., 1985). Proviral insertions found in this pvt-1 locus (in T-cell lymphomas) (Graham et al., 1985; Villeneuve et al., 1986) result in comparable c-myc mRNA levels as in plasmacytomas. This strongly suggests that a putative oncogene, involved in the regulation of c-myc expression is affected in both types of DNA rearrangements (Cory et al., 1985; Cough, 1985; Cory, 1986). At the moment it is unclear whether the variant human BL translocations cluster in a similar region 3′ of the c-myc gene.

The breakpoints in the Ig loci showed a similar disperse pattern. In the IgL loci breakpoints were observed 5′ of the Cλ or Ck genes or within the V_L gene regions. In the IgH locus the switch (S) region is frequently involved, although other breakpoints have been located more 5′ close to the enhancer (E_H) region or within the V_H genes. The Ig genes undergo several steps of somatic recombination during B-cell differentiation (Tonegawa, 1983; Hood et al., 1985). There is no evidence that a similar joining mechanism is involved in the generation

of the c-myc/Ig recombinations, nor is there any evidence for homologous recombination or crossing over within a homologous oligonucleotide. Usually these Ig/c-myc recombinations separate the coding regions of the Ig genes and hence prevent the expression of a functional Ig molecule from the translocated chromosome (Erikson et al., 1982, 1983b; Croce et al., 1983). However, in one case expression of a Igμ molecule was demonstrated from the translocated 14q+ chromosome (Versnel et al., 1986). Previously Lenoir et al. (1982) suggested that in the variant translocations, the expressed type of IgL chain is the same as the IgL locus involved in the variant translocation. However also λ IgL expressing t(2;8) BL and κIgL expressing t(8;22) BL are reported (Magrath et al., 1983; Denny et al., 1985; Hollis et al., 1984; A. Hagemeijer pers.comm.). Recently a striking correlation was observed between the breakpoints in the c-myc locus and the different stages of B-cell differentiation (Pellici et al., 1986). Class III breakpoints were only found in the endemic (African-type) BLs, whereas class I and II breakpoints were only found in the more mature sporadic (American-type)BLs. Furthermore, breakpoints within the S_H region are frequently found in either class I or II tumors, whereas class III tumors have only breakpoints 5′ of the E_H or in the V_H regions (Cory, 1986). This could indicate a correlation between certain sites of recombination and the stage of B cell differentiation. Consistent with this possibility are the differences in c-myc breakpoints in 4 (B-cell) ALL-L3 patients (all class I) and 2 (pre-B-cell) ALL patients (class (Peschle et al., 1984; Pegoraro et al., 1984; Blick et al., 1986; Care et al., 1986). However, despite the enormous variation in myc/Ig recombination, all the translocations result in a constitutive expression of the c-myc gene involved in the translocation. The other, normal c-myc gene is usually not expressed (Stanton et al., 1983; Nishikura et al., 1983; Croce et al., 1983; Erikson et al., 1983a; 1983b). Since the c-myc genes involved were frequently altered, either by truncation (class I) or by mutations and/or deletions in the conserved 5′ region or in the first exon (class II and III) of the gene, it

was assumed that these structural changes play a role in the deregulation of c-myc (Battey et al., 1983; Taub et al., 1984; Rabbits et al., 1984; Denny et al., 1985; Pellici et al., 1986). Several mechanisms are possible, both at the transcriptional or posttranscriptional level. For example, in cases where the c-myc gene is truncated, transcription is initiated at cryptic promoters in the first intron. These truncated mRNAs have an increased stability as compared to the normal c-myc transcripts (Piechaczyk et al., 1985; Eick et al., 1985; Rabbitts et al., 1985a). Mutations in the first exon could have a similar effect or alternatively could prevent the binding of repressor molecules which regulate the transcription and/or elongation of the c-myc mRNAs (Leder et al., 1983; Taub et al., 1984; Rabbitts et al., 1984; Pellici et al., 1986; Bentley and Groundine, 1986). In this respect an intriguing model has been proposed in which the constitutive expression of activated c-myc genes would have a negative effect, either direct or indirect on the expression of the normal c-myc gene (Rabbitts et al., 1984; Rapp et al., 1985; Adams et al., 1985). However, several other investigators suggest that the lack of normal c-myc expression is caused by normal regulation following the (B-cell) differentiation of the tumors (Feo et al., 1985; Croce et al., 1985b; Cole, 1985). Another negative transcriptional control element with properties opposite to an enhancer sequence (dehancer) has been found in the well conserved region 5′ of the c-myc gene (Remmers et al., 1986). Class II BL breakpoints (Fig.7) would result in the deletion or truncation of this dehancer element.

Apparently, there are several mechanisms which can lead to deregulation of c-myc transcription (Klein and Klein, 1985a; Rabbitts, 1985). However, from somatic cell hybrid experiments it is clear that the deregulation of the translocated BL c-myc gene requires a B-cell background (Nishikura et al., 1983; 1984) and that different genetic Ig elements play a role in the cis-activation of the c-myc gene (Croce et al., 1984; Nishikura et al., 1985). Besides the normal IgH chain gene enhancer, located 5′ of the Sμ-region, which can activate c-myc

transcription in both early and mature B-cell stages (Corcoran et al., 1985; Fahrlander et al., 1985; Feo et al., 1986) other sequences 3´ of the S_H (Sμ, Sγ or Sα) regions have been postulated. These can activate c-myc transcription in BL over a long distance but only in a more mature stage of B cell differentiation (Croce et al., 1985b; Croce and Nowell, 1985; Feo et al., 1986). Using transgenic mice bearing different c-myc genes, Adams et al. (1985) demonstrated, that enhancement and transcriptional activation of the c-myc gene is necessary to induce tumors in vivo, while neither truncation of the gene nor alteration of its normal chromosomal position are sufficient for activation. In their experiments, a c-myc gene driven by the IgH enhancer induced lymphoid tumors in 90% of the transgenic mice. Other enhancers, as those from SV40 or mouse mammary tumor virus (MMTV)-LTR are less effective (14-40% tumor induction, (Stewart et al., 1984; Adams et al., 1985; Leder et al., 1986). In these latter experiments using a MMTV- LTR driven c-myc gene a variety of tumor types arose, illustrating the broad transforming potential of the c-myc gene (Leder et al., 1986). However, high expression of the c-myc is not sufficient for tumor induction and the observation that most of the tumors were of clonal origin strongly suggested, that at least a second event was required to generate a fully transformed cell (Stewart et al., 1984; Adams et al., 1985; Cole, 1985; Leder et al., 1986). A similar situation exists in vitro, where an activated c-myc gene alone is not sufficient to cause transformation, although it can abolish the need for certain growth factors and can block differentiation (Rapp et al., 1985; Kelly, 1985; Coppola and Cole, 1986). Several oncogenes (e.g. ras; E1A) have been identified, that can complement the c-myc gene to transform primary embryonic fibroblasts in culture (Land et al., 1983a; 1983b; Ruley, 1983; Cooper and Lane, 1984). Consistent with this second hit model is the isolation of B-lym from BL cell lines, using a NIH-3T3 transformation assay (Diamond et al., 1983; Neiman, 1985) and the detection of additional DNA rearrangements in other oncogenes in mouse plasmacytoma cell lines (Cohen et al., 1983; Mushinski et al.,

1983; Perlmutter et al., 1984; Klein and Klein, 1985a, 1985b).

2.3.2 Translocations involving 14q32 in B cell leukemias and lymphomas

Translocations involving band 14q32 are the most common non-random abnormalities found in a variety of B-cell lymphomas and leukemias (Table 1; Sandberg, 1980; Rowley and Testa, 1983; Yunis, 1983). Besides the already mentioned t(8;14) found in BL and ALL (section 2.3.1), other non-random translocations involving the IgH gene bearing 14q32 have been reported in different subtypes of lymphoma and chronic lymphocytic leukemia (CLL). Among these are the t(11;14)(q13;q32), found in some patients with CLL and diffuse small or large cell lymphoma and the t(14;18)(q32;q21), found in 90% of the patients with follicular lymphoma (Yunis et al., 1982; 1984; Yunis, 1983; Nowell et al., 1986). Using IgH chain gene probes, cloning of the chromosomal breakpoints resulted in the identification of two B-cell lymphoma/leukemia breakpoint cluster regions: the 0.9 kb bcl-1 located at chromosome 11q13 and the 2.1 kb bcl-2 located at chromosome 18q21, respectively (Erikson et al., 1984; Pegoraro et al., 1984; Tsujimoto et al., 1984a, 1984b; Cleary and Sklar, 1985a). DNA sequence analysis of the breakpoint clones and normal chromosome 14 or 18 counterparts revealed that both types of translocations were the result of an aberrant V-D-J joining event (Tsujimoto et al., 1985b, 1985c; Cleary and Sklar, 1985b; Bakhshi et al., 1985). Since most 14q32 breakpoints were located just 5′ of the Ig enhancer, it was supposed that translocation to the proximity of the Ig enhancer could activate the expression of putative oncogenes located at the bcl-1 or bcl-2 breakpoint regions. Indeed a bcl-2 gene located at chromosome 18q21 has been identified. Translocation of this gene to the Ig Enhancer at chromosome 14q32 resulted in higher levels of transcription of a bcl-2 specific 6.0 kb mRNA (Tsujimoto et al., 1985a; Cleary and Sklar, 1985b). Recent results indicate that the t(14;18) breakpoints are located in the 3′ untranslated region of this gene. The translocation results in

the transcription of mRNAs of aberrant sizes but does not affect the bcl-2 protein coding sequences (Tsujimoto and Croce, 1986). No such putative oncogene has been identified at the bcl-1 region until now.

Fell et al. (1986) have recently analysed the t(2;14)(p13;q32) translocation in two children with B-CLL (Sonnier et al., 1983). Both the chromosome 14q32 breaks occurred just 5´ of the Cγ2 region of the IgH gene on the productive allele. Whether the chromosome 2p13 breakpoints cluster in a limited DNA region is unclear. However, the paradigm of the t(8;14) in BL and the t(14;18) in B-cell lymphomas justifies the assumption that the t(2;14) may have resulted in the somatic mutation or activation of an as yet unidentified gene on chromosome 2p13.

In 30% of the childhood pre B-ALLs a non-random translocation between chromosome 1 and 19 has been identified (t(1;19)(q23;p13.3), (Williams et al., 1984). The position of the chromosome 19 breakpoint coincides with the location of the insulin receptor gene (Yang-Feng et al., 1985). The insulin receptor is related to the tyrosine kinase family of oncogenes (Ullrich et al., 1985) and it will be interesting to see whether this gene is involved in the pre B-ALL specific (1;19) translocation.

2.3.3 Translocations and inversions involving 14q11 in T-cell leukemias and lymphomas

T-lymphocytes have a special set of genes which are involved in the recognition of antigen. The most common T-cell antigen receptor (TCR) is a heterodimer comprised of an α(TCRα) and β(TCRβ) gene chain. Recently a second TCR molecule has been identified and one of the chains of this heterodimer is encoded by the TCRγ chain gene (Brenner et al., 1986; Bank et al., 1986). Like the Ig genes in B-lymphocytes, these TCR genes are specifically rearranged and expressed during T-cell development (Hood et al., 1985; Goverman et al., 1986; Minden and Mak, 1986). The TCRα chain gene has been assigned to chromosome

14q11-q13 (Croce et al., 1985a; Rabbitts et al., 1985c; Collins et al., 1985). Since this 14q11-q13 region is frequently involved in chromosomal abnormalities in T-cell leukemia and lymphoma, this strongly suggested, that by analogy of the involvement of the Ig genes in B-cell tumors, the TCRα locus was involved in T-cell leukemias and lymphomas (Hecht et al., 1984, 1985, Ueshima et al., 1984; Sadamori et a;., 1985b; Clare et al., 1986; Dube et al., 1986).

The most common aberration in T-cell malignancies is an inversion of chromosome 14, inv(14)(q11q32), or the closely related t(14;14)(q11;q32)(Zech et al., 1984; Hecht et al., 1984; Sadamori et al., 1985b). Croce et al. (1985a) suggested that a putative oncogene tcl-1 (T-cell lymphoma/leukemia) was involved in these cases. However, molecular analysis revealed that the chromosome 14 inversion in a T-cell lymphoma cell line was caused by a site-specific recombination between IgH and TCRα loci (Baer et al., 1985; Denny et al., 1986). This resulted in the transcription of a chimeric Ig-TCR gene consisting of a Ig V_H gene segment and TCR Jα and Cα gene segments (Denny et al., 1986). A similar inv(14) or t(14;14) has also been observed in 'normal' lymphocytes and leukemic cells of Ataxia telangiectasia patients (Aurias et al., 1980, 1986). As yet it is not clear whether this chimeric Ig-TCR gene contributes to the malignant transformation of T-cells. However, it is possible that the formation of an aberrant Ig-TCR cell surface receptor molecule results in the delivery of an inappropriate mitogenic stimulus.

In T-ALL a specific t(11;14)(p13;q11) has been reported (Williams et al., 1984) and in this type of translocation it was shown that the chromosome 14q11 breakpoint occurred between the TCR Vα and Cα gene segments (Lewis et al., 1985; Erikson et al., 1985b). The chromosome 11p13 breakpoint coincides with the locus that is implied in Wilms tumor (Riccardi et al., 1980; Van Heyningen et al., 1985). This suggested that either this Wilms tumor gene or a yet unknown gene, tcl-2, located in this region is involved in T-cell malignancies (Erikson et al., 1985b). In three cases of T-cell leukemia (2 cell lines and an

ALL patient with a t(8;14)(q24;q11)), it was demonstrated, that part of the TCRα chain locus was translocated to the 3´ region of the c-myc gene (Mathieu-Mahul et al., 1985; Shima et al., 1986; Erikson et al., 1986b). In the two cell lines the chromosome 8 breakpoint was located in the 3´ flanking region of the c-myc gene, whereas in the T-ALL patients the breakpoint was located more than 38 kb downstream of the c-myc gene. Nevertheless, using somatic cell hybrids it was shown, that in this T-ALL patient the translocation of the TCRα locus to the 3´ c-myc region resulted in the deregulation of the transcription of this c-myc allele (Erikson et al., 1986b). This latter observation closely resembles the deregulation of the c-myc gene in the t(8;14)(q24;q32) in BL and B-ALL, described in section 2.3.2.

2.4. Chromosome translocations in solid tumors

Cytogenetic studies of solid tumors have also led to the identification of structural abnormalities, although hampered by technical difficulties. With a few exceptions, most of these abnormalities are not specific for one tumor type (Trent, 1984; Berger et al., 1985). Most of the structural abnormalities are deletions, but also recurring chromosomal translocations have been described: t(2;13)(q37;q14) in alveolar rhabdomyosarcoma (Turc-Carel et al., 1986), a t(3;8)(p21;q12) in salivary gland carcinoma (Mark et al., 1983), translocations involving 3p14 (t(3;8)(p14;q24) or t(3;11)(p14;p15)) in hereditary renal cell carcinoma (Cohen et al., 1979; Pathak et al., 1982; Yoshida et al., 1986), a t(6;14)(q21;q24) in ovarian carcinoma (Wake et al., 1980), and the t(11;22)(q24;q12) in Ewing sarcoma (Aurias et al., 1983; Turc-Carel et al., 1983). Molecular analysis of somatic cell hybrids revealed that in a renal cell carcinoma t(3;8) the c-myc oncogene was translocated to the 3p14 region (Drabkin et al., 1985). However, they could not detect any rearrangements in the 21 kb region surrounding the c-myc gene. Similar studies, using somatic cell hybrids between a Ewing sarcoma cell line and rodent cells, demon-

strated the chromosome 22 breakpoint to be proximal to the c-sis locus but distal to the bcr locus. Neither rearrangements nor an altered transcription of the c-sis gene were seen (Geurts van Kessel et al., 1985; Bechet et al., 1984). Recent experiments, using the chromosome 11q23 c-ets-1 probe indicated that the chromosome 11 breakpoint is distal to the c-ets-1 gene (Geurts van Kessel, pers.comm.). Since in all these experiments the same Ewing sarcoma cell line was used, further studies will be necessary to corroborate whether this is a common pattern among Ewing sarcomas.

3. DELETIONS AND AMPLIFICATIONS

A number of solid tumors and hematopoietic disorders are characterized by recurring deletions of part of a particular chromosome (Table 1). In contrast to the rather specific chromosomal breakpoints in recurring translocations, most of the breakpoints in the deletions are not consistent. Thus variable sizes of deleted fragments are observed among different patients. However, often a common deleted region can be determined which suggests, that the loss of genes located in that specific region is related to the particular tumor or disorder.

In a number of myeloid disorders such a common region coincides with the position of a proto-oncogene. This could suggest, that loss or decreased expression of this oncogene is implied in the disturbed growth pattern. Another mechanism of tumorigenesis has recently been discovered in embryonal tumors as retinoblastoma and Wilms tumor. Experimental data strongly suggest, that concomittant loss or inactivation of both alleles of a specific regulatory or tumor suppressor gene is necessary. Preliminary data indicate, that the loss of this gene function can lead to enhanced levels of specific oncogenes. A more direct mechanism which could contribute to enhanced levels of oncogene expression is gene amplification. Cytogenetically these gene amplifications are manifested as homogeneously Staining Regions (HSR) or double minutes (DM).

3.1 Deletions associated with MD, RA and ANLL

Abnormalities of the short arm of chromosome 12, predominantly deletions of 12p(p11-p13qter), are frequently observed in patients with ANLL or myelodysplastic syndromes (MDS)(Berger et al., 1986; Weh and Hossfeld, 1986). It has been suggested that this 12p- abnormality is a secondary event, although in some patients it is the only karyotypic abnormality (Berger et al., 1986). Two oncogenes, K-ras and int-1 have been localized in this 12p region(Jhanwar et al., 1983; Van 't Veer et al., 1984). However as yet no studies exploring the fate of these genes in the 12p- abnormality have been reported.

Loss of a whole chromosome 5 or loss of part of the long arm of chromosome 5(q13-q33) has been observed in patients with ANLL and refractory anemia (RA) (Wisniewski and Hirschhorn, 1983). At region 5q34, the c-fms oncogene is located (Groffen et al., 1983c). Recent data indicate that c-fms is related, if not identical to the mononuclear phagocyte growth factor receptor of CSF-1 (Sherr et al., 1985; Sacca et al., 1986). Although this oncogene was deleted in 5 cases of RA (Nienhuis et al., 1985; LeBeau et al., 1986), it was conserved in an ANLL patient with a 5q- deletion. A related gene, the human granulocyte macrophage colony stimulating factor (GM-CSF) located at 5q21-q32 (Huebner et al., 1985) was consistently deleted in all patients studied with a 5q- chromosome (LeBeau et al., 1986). This suggests that deletion of one or both of these genes may be important in the pathogenesis of RA or ANLL although the involvement of other genes located at the critical 5q- region cannot be excluded.

Another common abnormality in myeloid disorders is a deletion of chromosome 20q. LeBeau et al. (1985c) demonstrated, that these deletions were interstitial and that the c-src oncogene located at 20q13 was consistently conserved. Although no rearrangements were detected in the c-src gene, this oncogene may be located close to the deletion breakpoint and its expression may be altered.

3.2 Deletions and recessive mutations in cancer

Retinoblastoma (Rb) is an embryonal tumor which either occurs spontaneously (sporadic Rb), or to which predisposition can be inherited in an autosomal dominant manner. The inherited Rb is often bilateral and has in general an earlier onset than the unilateral sporadic form. Cytogenetic studies and isoenzyme segregation analysis revealed that both forms of Rb are associated with a common deletion at chromosome 13q14 (Vogel, 1979; Sparkes et al., 1983; Sparkes, 1984). Knudson (1971) suggested that Rb is caused by two mutational events. In the inherited form, one of these mutations is transmitted via the germline and a second mutation occurs in a somatic cell. In sporadic Rb both mutations occur in the same somatic cell. Comings (1973) extended this hypothesis, by suggesting that in these types of tumors, which have both hereditary and sporadic appearance, the two mutations involved the two allelic copies of a regulatory or tumor suppressor gene (anti-oncogene). Loss of this suppressor/regulatory function would result in the expression or activation of a transforming gene. Support for this hypothesis came from studies using polymorphic enzyme markers and restriction fragment length polymorphisms (RFLP)(Benedict et al., 1983; Cavenee et al., 1983, 1985; Dryja et al., 1984). These studies clearly demonstrated the development of hemi or homozygosity of a mutated Rb allele in both hereditary and sporadic Rb. Since loss of both wild type Rb alleles is associated with tumor formation, this strongly suggests that the nature of the Rb mutation is recessive at the cellular level. (Gilbert, 1983; Murphree and Benedict, 1984).

Wilms tumor (WT), like retinoblastoma, is a childhood tumor which occurs usually sporadic, but to which predisposition can be inherited in an autosomal dominant trait. Cytogenetically WT is associated with a deletion of chromosome 11p13. Patients with a constitutional or congenital deletion in this 11p13 region often have aniridia, which suggests that both loci are closely linked on chromosome 11p13 (Slater, 1986; Kaneko et al., 1981; Orkin, 1984; Van Heyningen et al., 1985). Molecular

analysis with RFLPs indicate that the specific loss of heterozygosity for the 11p region was a common event in WT (Koufos et al., 1984; Orkin et al., 1984; Reeve et al., 1984; Fearon et al., 1984; Solomon, 1984).

Patients, who have inherited the predisposition to either Rb or WT, have often an increased risk for the development of other specific tumors. These associated tumors can arise either simultaneously, or as a second primary tumor. For example, children with Beckwith-Wiedeman syndrome (BWS) have a predisposition to develop WT, rhabdomyosarcoma and hepatoblastoma. Koufos et al. (1985) demonstrated that these three types of embryonal tumors share a common pathogenic mechanism: The specific development of chromosome 11p homozygosity. In the BWS this predisposition is inherited as a single autosomal dominant mutation. This suggested that in each of these three embryonal tumors loss or inactivation of the same chromosomal region was involved. Whether they result from mutations in the same gene, or from an overlapping deficiency is as yet unknown. In addition, a specific loss of chromosome 11p heterozygosity has been observed in 42% of the patients with bladder cancer (Fearon et al., 1985). Hereditary Rb patients have an increased risk for the development of osteosarcomas and Hansen et al. (1985) have provided evidence for the specific loss of heterozygosity for the chromosome 13q14 region.

A third example of clustered tumors, which probably share a common pathogenic mechanism, has recently been reported by Seizinger et al. (1986). They demonstrated a specific loss of chromosome 22 heterozygosity in acoustic neuroma. This neural tumor can arise spontaneously or in a heritable manner and this latter form is often bilateral and frequently associated with meningiomas. Since meningiomas are cytogenetically characterized by a loss or deletion of chromosome 22 (Table 1), this strongly suggests that both neural tumors share a common mechanism of tumorigenesis. A number of other human tumors such as familial renal carcinoma, neuroblastoma and small cell lung carcinomas (Table 1) fit also the Rb and WT model of tumorigenesis (Knudson 1971; Comings 1973). This could indicate that

the human genome contains a number of genes or loci at which mutations can lead to a predisposition for the development of a clusters of specific associated tumors. Loss of a tumor suppressor or regulatory gene by conversion to hemi or homozygosity may therefore be a fundamental mechanism of tumorigenesis, especially in hereditary tumors (Murphree and Benedict, 1984). The exact molecular nature of the mutated genes and/or sequences in these tumors as well as their target genes are as yet unresolved. However, in two embryonal tumors, Rb and WT, the oncogene N-myc has been implied as a possible target gene. In WT the levels of N-myc expression are significantly elevated as compared to normal adult or fetal kidney tissue (F. Alt. pers.comm.). Amplification of N-myc, accompanied by increased levels of expression have been reported in some Rb tumors and cell lines (Kohl et al., 1984; Lee et al., 1984; Squire et al., 1985). In other Rb tumors, the expression of N-myc is similar to the expression observed in normal (8-12 week of gestation) fetal retina tissue (Squire et al., 1986). Based on this observation Squire et al. (1986) concluded, that the expression of N-myc in Rb is due to the embryonic origin of the tumor and not directly associated with the mutation in the Rb locus. However, this observation does not necessarily rule out a possible involvement of N-myc in Rb, since like the c-myc expression levels in BL (see section 2.3.1) constitutively expression of N-myc may be sufficient in the embryonic retina cells. It is even conceivable that this constitutive expression of N-myc prevents the differentiation of these cells, since recent reports (Coppola and Cole, 1986; Dmitrovsky et al., 1986) showed, that the constitutive expression of a transfected c-myc gene resulted in the inhibition of differentiation of the recipient cell lines.

3.3 Amplification of oncogenes

Chromosomal aberrations, as double minutes (DM) and homogeneously staining regions (HSR), are present in a variety of human tumors and tumor derived cell lines (Barker, 1982). These karyotypic abnormalities are cytogenetical markers for gene amplification (Hamlin, 1984; Schimke, 1984). It has been shown that in the tumor cells this amplified genetic material includes DNA sequences, that are identical or related to known cellular oncogenes. This amplification usually results in proportionally enhanced mRNA levels of the oncogene (reviews; Alitalo, 1984; Alitalo and Schwab, 1986). The reported examples of oncogene amplification in mammalian tumors can be divided in two types of appearance: A sporadic or tumor-specific type. Most cases of oncogene amplification in a variety of tumor derived cell lines, such as c-abl amplification in K562 cells (Collins and Groudine, 1983) belong to the first type since these are regarded as rare events in the respective tumor. Tumor specific oncogene amplification has been reported in four human malignancies. The c-erbB oncogene is amplified in several glioblastomas (Libermann et al., 1985), the c-myc oncogene is amplified in 32% of the breast carcinomas (Escot et al., 1986), the N-myc oncogene in neuroblastomas (Schwab et al., 1983b; Kohl et al., 1983; Schwab, 1985) and the c-myc, N-myc or L-myc oncogenes in small cell lung carcinomas (SCLC) (Nau et al., 1985, 1986; Wong et al., 1986). Wong et al. (1986) studied the amplification of N-myc and c-myc oncogenes in human SCLC tumors. Their results indicated that the amplifications are not associated with the development of metastatic lesions. In contrast to previous studies (Little et al., 1983; Gazdar et al., 1985) their results showed furthermore that the amplifications were not associated with the variant subclass of SCLC tumors in vivo. In neuroblastomas N-myc amplification is highly correlated with a morphologically more advanced disease stage and rapid tumor progression (Brodeur et al., 1984; Seeger et al., 1985). How these amplifications could contribute to the more agressive tumor type was recently suggested by the demon-

stration of an inverse correlation between N-myc or c-myc oncogene expression and the expression of class I major histocompatibility antigens (R. Versteeg; R. Bernards, pers.comm.). As mentioned before in section 3.2, neuroblastomas and SCLC resemble in many respects the WT or Rb type of tumors. In addition to the enhanced or constitutive expression of myc oncogenes and the heriditary background of some neuroblastomas, both SCLC and neuroblastomas are associated with a recurring chromosomal deletion of chromosome 3 (p14-p23) or chromosome 1 (p36-p32), respectively (Whang-Peng et al., 1982; Brodeur et al., 1981). Furthermore all these tumors arise in organs/or tissues which showed a high expression of myc oncogenes in the embryonic counterparts (Zimmerman et al., 1986). However, whether the enhanced or constitutive expression of these myc genes contributes to tumorigenesis or reflect the embryonic nature of the tumor cells remains as yet unknown.

4. CONCLUDING REMARKS

The combined application of cytogenetic and molecular genetic techniques has elucidated the involvement of cellular oncogenes in tumor specific chromosomal abnormalities. Although these studies further underline the fundamental role of chromosomal abnormalities in tumor-development, as yet virtually nothing is known of the generation of these aberrations. DNA sequence analysis of BL and CML specific chromosomal breakpoint regions revealed no clue to a possible translocation mechanism. However, a report by Fialkow et al. (1981) indicates that in CML, the acquisition of the Ph^1 chromosome is preceded by an initial phase of marked genetic instability. A similar phase of genetic instability of Ig or TCR loci may occur during the process of somatic rearrangements of these genes. During these phases, presumably various translocations occur and those with a selective growth advantage will eventually result in a clinically apparent leukemia.

It has been suggested that fragile sites may act as predis-

posing factors for certain specific chromosomal rearrangements (Yunis and Soreng, 1984; LeBeau and Rowley, 1984). The chromosomal location of a number of these fragile sites coincides with specific chromosomal breakpoint regions. Furthermore, leukemic patients were identified as carriers of a fragile site at the observed chromosomal breakpoint (Yunis, 1983; LeBeau, 1986). Although several genes, among which some oncogenes, have been mapped to an identical chromosomal region as a fragile site, at present the exact nature and function of the genes located at these sites remains an enigma.

Molecular techniques as Southern blotting and chromosomal walking have demonstrated in a few tumor specific aberrations the localization of (putative) oncogenes in the direct vicinity of the chromosomal breakpoint region. However, in other tumor specific aberrations the exact nature of the association between cytogenetic changes and alterations at the DNA or gene level remains obscure. The application of new techniques as Pulsed Field Gradient (PFG) gel electrophoresis (Schwartz and Cantor, 1984; Carle and Olson, 1984), which allows the separation of large (50-2000 kb) DNA fragments could help to corroborate a possible involvement of oncogenes in these cases. Furthermore, the use of PFG gels could lead to the detection of deletions which are not visible at the cytogenetic level. An example concerning deletions of part of chromosome 1p32, which resulted in the activation of the trk oncogene in a human colon carcinoma has recently been reported (Martin-Zanca et al., 1986).

SUMMARY

Extensive cytogenetic studies revealed that a considerable number of human tumors, especially leukemias and lymphomas is associated with consistent, specific chromosomal aberrations. These observations suggested that at the specific breakpoint regions of these chromosomal abnormalities, genes are located, which could play a role in the malignant transformation of a normal cell into a tumor cell. Attractive candidates for such genes are the cellular oncogenes. Some of these oncogenes are located at the same region as the specific chromosomal breakpoints. Recent data indicate that the protein products of these cellular proto-oncogenes are involved in the regulation of normal growth and differentiation. Disturbance of these functions (e.g. by chromosomal translocations) could result in an uncontrolled growth pattern.

The Philadelphia (Ph^1) translocation, present in about 96% of the patients with chronic myelocytic leukemia (CML), is one of the most typical and best documented examples of a consistent chromosomal aberration. Usually this translocation involves chromosome 9 and 22:t(9;22)(q34;q11) and results in two abnormal chromosomes designated 9q+ and 22q- (or Ph^1 chromosome). The human c-abl proto-oncogene has been localized at the long (q) arm of chromosome 9. By analysis of somatic cell hybrids, we have shown that this oncogene is translocated to the 22q- chromosome. This proved unequivocally the reciprocal nature of the Ph^1 translocation (Paper I). Study of variants forms of the Ph^1 translocation demonstrated that the c-abl oncogene was consistently translocated to the 22q- chromosome even in cases where there was no visible involvement of chromosome 9. The location of the c-abl oncogene adjacent to the translocation breakpoint in CML was shown by the isolation of a DNA fragment from the 9q+ chromosome of a CML patient: this fragment contained sequences of both chromosome 9 and 22. The breakpoint had occurred 14.5 Kb immediately 5′ of the v-abl homologous sequences and resulted in a 9q+ chromosome in which the tip of

chromosome 9, including the v-abl homologous sequences were replaced by sequences of chromosome 22 (Paper II). The isolated chromosome 22 sequences of this chimeric DNA fragment enabled us to clone the breakpoint region of chromosome 22 of this CML patient. A breakpoint cluster region (bcr) was identified on chromosome 22 and the DNAs of all Ph^1 positive CML patients examined to date (over 30) have breakpoints in this 5.0 Kb chromosome 22 region. As a consequence of the Ph^1 translocation part of bcr remains on the Ph^1 chromosome and part is translocated to the 9q+ chromosome (Paper III). Positive hybridization of bcr probes to cDNA or mRNA sequences suggested that this region contained protein encoding sequences. Part of this bcr gene has been characterized. However, as yet the bcr protein has an unknown cellular function. The 5.0 kb bcr, in which all the Ph^1 positive CML breaks occur, is an internal part of this gene and contains three small coding regions. The chromosomal breakpoints are located in the non-coding region between these exons (Paper IV). In contrast to the limited region in which all the 22q- breakpoints are clustered, the breakpoints on chromosome 9 are scattered over a very large area which may vary from 5 kb up to more than 100 kb upstream of the v-abl homologous exons. However, in the leukemic cells of all Ph^1 positive CML patients a new, larger c-abl mRNA of 8.5 kb can be detected. This RNA is also present in the CML derived cell line K-562. Although this cell line has lost the Ph^1 chromosome as such, it retained a Ph^1 like bcr/c-abl construct. Hybridization of c-abl and different bcr probes revealed that c-abl and 5′ bcr probes hybridized to the same mRNA species. This could imply that K562 cells contain a chimeric bcr/c-abl mRNA. Direct proof of this supposition was achieved by the cloning of a cDNA that contained the chimeric part of this mRNA molecule. This chimeric cDNA has one long open reading frame, that is compatible with both the predicted bcr and c-abl reading frames. Since this chimeric mRNA is also present in other Ph^1 positive CML patients, probably the versatility of the splicing system accomodates for the large variation in intron size linking the bcr and c-abl genes (Paper V).

DNA sequence analysis of several chromosomal breakpoints revealed that homologous recombination between chromosome 9 and 22 is unlikely. However, most of the breakpoints are located in Alu-repetitive sequences. This could suggest that these ALu-repeats are hot spots of recombination (Papers IV, VI).

The Ph^1 translocation in CML and ALL patients is cytogenetically identical: in both cases the translocation is reciprocal and the c-abl oncogene is translocated to the 22q- chromosome. At the molecular level some of the Ph^1 positive ALL patients differ from Ph^1 positive CML patients. In 30% of the ALL patients the chromosome 22 breakpoint was located outside the bcr. In 70% of the patients, however, we could demonstrate a 22q- breakpoint in the bcr region and the presence of a chimeric bcr/c-abl mRNA (Paper VII).

In CML, this 8.5 Kb chimeric mRNA is translated into a p210 KD bcr/c-abl fusion protein. This fusion protein resembles in many aspects the Abelson murine leukemia virus v-abl protein. However, as yet it remains uncertain whether this p210 fusion protein contains indeed a transforming activity.

SAMENVATTING

Uitgebreide cytogenetische studies hebben aangetoond dat een aanzienlijk aantal humane tumoren, vooral leukemieën en lymfomen geassocieerd is met consistente, specifieke chromosomale afwijkingen. Verondersteld werd dat op de specifieke breukpuntregio's van deze chromosomale afwijkingen genen liggen, welke betrokken kunnen zijn bij de transformatie van een normale cel tot kankercel. Aantrekkelijke kandidaten voor dergelijke genen zijn de cellulaire oncogenen. Enkele van deze genen zijn reeds gelokaliseerd in gebieden waar ook specifieke chromosomale breukpunten zijn gevonden. Uit recent onderzoek is gebleken dat de genproducten van deze cellulaire oncogenen betrokken zijn bij de regulatie van de normale celvermeerdering en celdifferentiatie. Verstoring van deze functies (b.v. door chromosoom translokaties) zou kunnen leiden tot een ongecontroleerde celproliferatie.

De Phildelphia (Ph^1) translokatie, aanwezig in ongeveer 60% van de patienten met chronische myeloide leukemia (CML), is een van de meest karakteristieke en best gedokumenteerde voorbeelden van een tumor specifieke chromosomale afwijking. Gewoonlijk zijn bij deze translokatie de chromosomen 9 en 22 betrokken (t(9;22)(q34;q11)) en ontstaan hierbij twee abnormale chromosomen, n.l. een 9q+ en een 22q- (Ph^1) chromosoom. Het humane c-abl oncogen is gelokaliseerd op de lange (q) arm van chromosoom 9. Met behulp van somatische celhybriden, toonden wij aan dat dit oncogen verhuisde naar het 22q- chromosoom. Dit leverde het onweerlegbare bewijs op voor het wederkerige karakter van de Ph^1 translokatie (Appendix Publikatie I). De studie van variante vormen van de Ph^1 translokatie toonde aan dat het c-abl oncogen ook daar verhuisde naar het 22q- chromosoom, zelfs in gevallen waar chromosoom 9 niet zichtbaar betrokken leek in de translokatie. De ligging van het c-abl oncogen op chromosoom 9 dichtbij het translokatie breukpunt in CML werd aangetoond door middel van de isolatie van een DNA fragment van het 9q+ chromosoom van een CML patient. Dit fragment bevatte sequenties

van zowel chromosoom 9 als 22. De chromosomale breuk in de leukemische cellen van deze patient vond plaats 14,5 Kb "stroomopwaarts" van de v-abl homologe sequenties. Dit resulteerde in een 9q+ chromosoom, waarvan de top van chromosoom 9, inclusief de v-abl homologe sequenties was vervangen door chromosoom 22 sequenties (Appendix Publikatie II). De geisoleerde chromosoom 22 sequenties van dit chimaere DNA fragment maakte het mogelijk om een breukpunt gebied van chromosoom 22 te kloneren. Dit leidde tot de identificatie van een gebied op chromosoom 22 waarin de breukpunten van de verschillende CML patienten geklusterd voorkomen (bcr). De DNA's van alle tot nu toe onderzochte Ph^1 positieve CML patienten bevatten een breukpunt in deze regio (Appendix Publikatie III). Ten gevolge van de Ph^1 translokatie blijft een deel van de bcr achter op het 22q- chromosoom en een deel verhuist naar het 9q+ chromosoom. Hybridisatie van bcr probes met cDNA en RNA sequenties toonde aan dat de bcr eiwit coderende sequenties bevatte. Een deel van dit bcr gen is gekarakteriseerd maar tot nu toe is de cellulaire functie van het bcr eiwit onbekend. Het 5.0 kb bcr fragment, waarin alle CML specifieke chromosoom 22q- breuken plaatsvinden, is een intern gedeelte van dit gen en bevat drie kleine coderende regio's. De breuken vinden plaats in het niet coderende gebied tussen de exonen (Appendix Publikatie IV).

In tegenstelling tot de situatie op chromosoom 22 zijn de breukpunten op chromosoom 9 verspreid over een groot gebied dat varieert van 5 kb tot meer dan 100 kb "stroomopwaarts" van de v-abl homologe exonen. Toch wordt in de leukemische cellen van alle Ph^1 positieve CML patienten een nieuw, groter dan normaal c-abl mRNA molecuul aangetroffen. Dit RNA is ook aanwezig in de CML cellijn K562. Hoewel de K562 cellen geen Ph^1 chromosoom bevatten, is in deze cellen ook een bcr/c-abl associaties opgetreden. Hybridizatie van K562 mRNA met c-abl en verschillende bcr probes toonde aan dat zowel een c-abl als een 5' bcr probe hybridiseerden met een groot, nieuw mRNA molecuul. Dit wijst erop dat de K562 cellen een chimaer bcr/c-abl mRNA bevatten. Een direct bewijs van deze veronderstelling werd geleverd

door de klonering van een cDNA dat het chimaere gedeelte van dit RNA bevatte. Dit cDNA heeft een lang open leesraam dat overeenstemt met de voorspelde bcr en c-abl leesramen. Aangezien dit chimere mRNA in alle Ph^1 positieve CML patienten wordt gevonden, is het waarschijnlijk dat door de grote flexibiliteit, het splicing systeem geen moeite heeft met de grote variatie in intron grootte tussen de bcr en c-abl genen bij de verschillende patienten (Appendix Publikatie V). Uit DNA sequentie analyse van verschillende chromosomale breukpunten bleek dat homologe recombinatie tussen de chromosomen 9 en 22 niet waarschijnlijk is. Aangezien de meeste breukpunten gesitueerd zijn in zogenaamde repetitieve Alu sequenties lijkt het aannemelijk dat deze sequenties fungeren als "hot spots" voor recombinatie (Appendix Publikaties IV, VI).

Het Ph^1 wordt ook in patienten met acute lymfatische leukemie (ALL) aangetroffen. Cytogenetisch zijn de Ph^1 translocaties in CML en ALL patienten identiek: in beide gevallen zijn de translocaties reciprook en verhuist het c-abl oncogen naar chromosoom 22q-. Maar op moleculair niveau bleken sommige van de translocaties bij deze Ph^1-positieve ALL patienten sterk te verschillen van die bij Ph^1-positieve CML patienten. In circa 30% van de Ph^1 positieve ALL patienten vindt de breuk in chromosoom 22 plaats buiten de bcr. In de overige 70% van de patienten konden we wel breuken in de bcr aantonen en bleek bovendien dat de leukemische cellen van deze patienten een chimaer bcr/c-abl) mRNA bevatte (Appendix Publikatie VII). In CML wordt dit chimaere mRNA molecuul vertaald in een p210 kD bcr/c-abl fusie eiwit. Dit fusie-eiwit lijkt in vele opzichten op het eiwit dat gecodeerd wordt door het v-abl gen van het Abelson muizen leukemie virus. Nog niet is aangetoond dat het p210 fusie-eiwit ook transformerende eigenschappen heeft.

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PAPER I

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

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

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

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

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

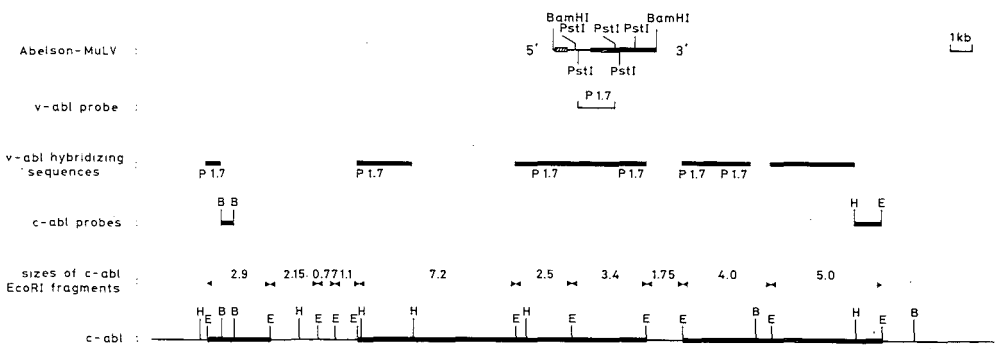

Fig. 1 Restriction enzyme map of the human c-*abl* region[19]. The upper line of the figure shows the *Bam*HI subclone of A-MuLV; the hatched box presents the long terminal repeat, the solid bar the acquired cellular sequences. Directly beneath the A-MuLV genome, a subgenomic *Pst* 1.7-kb fragment, used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

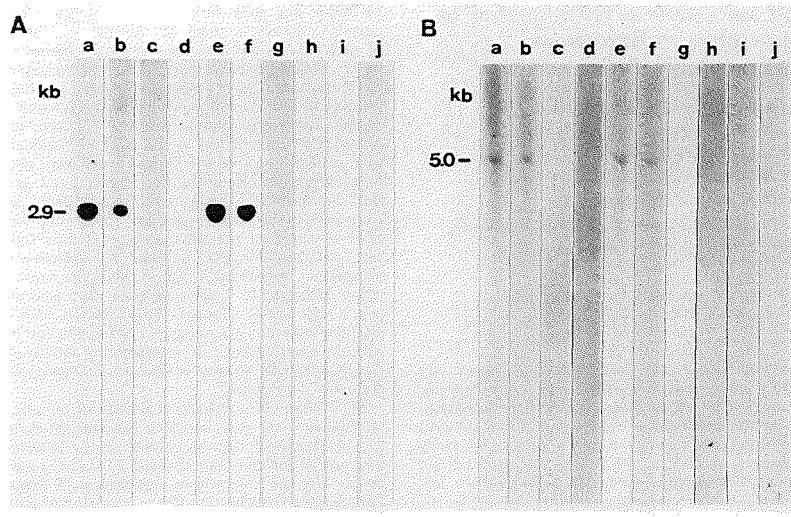

Fig. 2 Localization of human c-*abl* sequences on the Philadelphia chromosome, using hybrid cell lines and human c-*abl* probes. *A*, detection of the human 5′ end 2.9-kb *Eco*RI c-*abl* fragment in DNA from *a*, human placenta; *b*, 10CB-23B (chromosome 9); *c*, PgMe-25NU (chromosome 22q); *d*, 14CB-21A (chromosome $9q^+$); *e*, 1CN-17aNU (chromosome $22q^-$); *f*, WESP-2A (chromosome $22q^-$); *g*, mouse Pg19; *h*, Chinese hamster E36; *i*, mouse WEHI-3B; *j*, Chinese hamster a3. *B*, detection of the 3′-end 5.0-kb *Eco*RI c-*abl* fragment in DNAs as indicated in *A* (*a–j*). The derivations of all these cell lines and their complements of human chromosomes are summarized in Table 1.
Methods: All cell lines used in this experiment were grown in large batches (10^7–10^8 cells) and DNA was prepared as described by Jeffreys and Flavell[28]. *Eco*RI-restricted DNAs (10 μg per lane) from human placenta, hybrid cell lines, mouse and Chinese hamster fusion partners were electrophoresed on 0.7% agarose gels. *Hin*dIII and *Hin*dIII–*Eco*RI-digested λ DNAs were included as molecular weight markers (not shown). After blotting to nitrocellulose, the filters were hybridized to the 0.6-kb *Bam*HI c-*abl* (*A*) or 1.1-kb *Hin*dIII–*Eco*RI c-*abl* (*B*) restriction fragments described in Fig. 1. Hybridization and washing procedures (to 0.1 × SSC at 65 °C) were carried out according to the method of Bernards and Flavell[23].

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

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

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

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

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

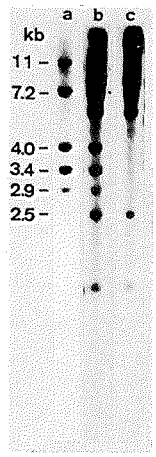

Fig. 3 Localization of human c-*abl* sequences on the Philadelphia chromosome, using a 22q$^-$ somatic cell hybrid and a v-*abl* probe. *Eco*RI-digested DNAs (10 μg) from human placenta (*a*), hybrid WESP-2A (*b*) and mouse WEHI-3B cells (*c*) were hybridized with the 1.7-kb *Pst* v-*abl* fragment (Fig.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

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

The above results show that both the 5′ and 3′ ends of the c-*abl* gene are translocated to chromosome 22q$^-$. Because all other c-*abl* *Eco*RI fragments, which hybridize to v-*abl* sequences, are flanked by the 2.9-kb and 5.0-kb *Eco*RI fragments, it seems highly probable that these fragments are also included in the translocation to the Philadelphia chromosome. To test this possibility directly, hybridization was performed using a 1.7-kb *Pst* v-*abl* probe (Fig. 1). Because of the problems with v-*abl* probes indicated above, only WESP-2A, the hybrid containing the most 22q$^-$ sequences (50% of the molar amount), was tested. As shown in Fig. 3, the viral probe detects human *Eco*RI c-*abl* fragments of 11, 7.2, 4.0, 3,4, 2.9 and 2.5 kb (weakly). Of these fragments, the 11-kb band has been shown to map outside the main human c-*abl* locus[19] and will not be considered here. The human 2.9-, 3.4-, and 4.0-kb c-*abl* fragments are readily detected in the WESP-2A DNA. In contrast, the 7.2-kb *Eco*RI fragment can only be seen in a short exposure of this filter (not shown), due to spill-over of radiation from strongly hybridizing mouse c-*abl* fragments in this area. The 2.5-kb *Eco*RI human c-*abl* fragment co-migrates with a mouse fragment of similar size and thus cannot be identified in this analysis.

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

In principle, the chromosomal translocation associated with CML could lead to elevated levels of c-*abl* expression which, by analogy to the c-Ha-*ras* gene in bladder carcinoma, would induce malignant transformation[24]. Elevated levels of c-*abl* expression could be the result of coupling of the gene to an enhancer sequence present on chromosome 22 or, alternatively, the gene could be linked to a strong promoter of another gene. To test these possibilities, we have initiated studies to clone the c-*abl* gene from the 22q$^-$ chromosome using WESP-2A DNA and a cosmid vector system. Finally, it is of interest that in some CML patients variant Ph^1 translocations are observed, in which the participation of chromosome 9 cannot be detected by classical cytogenetic analysis[3-5]. In another group of CML patients the Ph^1 translocation appears to be completely absent[25]. We are now investigating whether the c-*abl* gene is translocated to chromosome 22 in these cases also.

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PAPER II

CANCER SURVEYS 3: 515-529 (1984)

Cytogenetic and molecular analysis of the Ph[1] translocation in chronic myeloid leukaemia

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Keywords: Chronic myeloid leukaemia, Philadelphia chromosome, Ph[1] translocation, Ph[1] variants, cellular oncogenes, c-*sis*, c-*abl*, breakpoint cluster region.

Summary

The Ph[1] translocation is a consistent chromosomal abnormality associated with chronic myeloid leukaemia. Usually the Ph[1] results from a translocation (9;22)(q34;q11), but in a small percentage of cases variant forms are observed where other chromosomes are also involved. Analysis of the standard Ph[1] translocation using somatic-cell hybridization and recombinant DNA techniques showed that two cellular oncogenes c-*abl* and c-*sis* were translocated from chromosome 9 to 22q− and from chromosome 22 to the 9q+ derivative, respectively. Study of variant forms of the Ph[1] using the in-situ hybridization technique of single-copy DNA probes derived from these two cellular oncogenes revealed that c-*sis* was translocated from chromosome 22 to different chromosomes involved in the variant Ph[1] translocations,

concomitantly with the visible part of 22q. In contrast, c-*abl* was consistently translocated to the 22q− or Ph^1 chromosome even in cases where at first sight chromosome 9 was not involved.

Molecular analysis of breakpoint regions on the 9q+ and the 22q− derivatives was undertaken, trying to delineate further the molecular structure of the Ph^1 rearrangement and the possible mechanism for activation of the c-*abl* oncogene. A region of 5.8 kb (breakpoint cluster region) was cloned from chromosome 22 and all the breakpoints analysed so far from patients with chronic myeloid leukaemia were found to cluster within this region. At this point, it seems that the conjunction of the breakpoint cluster region, i.e. a specific region of chromosome 22, and the c-*abl* translocation constitute a rearrangement which could play a fundamental role in the pathogenesis of chronic myeloid leukaemia.

I Introduction

In 1960, Nowell and Hungerford described for the first time an abnormal small chromosome in the leukaemic cells of two patients with chronic myeloid leukaemia (CML). This abnormal chromosome was called Philadelphia (Ph^1) chromosome according to the location of the laboratory of discovery and was thought to be a deletion of one of the small acrocentrics.

In 1973, using banding techniques, Rowley showed that the Ph^1 did not result from a simple deletion of chromosome 22 but from a translocation: the deleted part of chromosome 22 is translocated to the long arm of chromosome 9. The rearrangement was described as t(9q+;22q−) or t(9;22)(q34;q11) using the International System for Cytogenetic Nomenclature (ISCN, 1978).

Cytogenetic analysis of chromosomal abnormalities in CML and other haematopoietic diseases revealed that: (1) The Ph^1 chromosome (22q—) is consistently found in more than 90% of the CML cases, where it appears as an acquired stem-cell abnormality observed in the majority of bone-marrow cells. (2) Cytogenetic variants of the Ph^1 translocation have been reported in about 3–8% of the Ph^1+ CML cases. These variants show a 22q− chromosome, with translocation of the distal part of 22 to a chromosome other than 9, although chromosome 9 also appears to be involved in complex variants. (3) A small percentage (3–7%) of CML cases are without a Ph^1 chromosome (Ph^1−negative); they tend to show much more rapid clinical deterioration and shorter survival than the Ph^1+ cases. (4) The Ph^1 chromosome is sometimes found in other haematopoietic disorders, particularly in the adult type of acute lymphoblastic leukaemia (ALL).

These cytogenetic findings in CML and their relevance for clinical diagnosis and prognosis have been discussed in a number of reviews (Lawler, 1977; Hagemeijer *et al.*, 1980; Rowley, 1980; Sandberg, 1980). In summary, the Ph^1 translocation still appears as the paradigm of the consistent chromosomal change associated with a specific type of malignancy, i.e. CML. The role played by the specific translocation in the pathogenesis of the disease remains to be established. The recent discovery of cellular oncogenes (Cooper, 1982)

and their localization near the breakpoints in several specific chromosomal rearrangements in various forms of cancer suggest a key role of these genes in the development of these neoplasms (Rowley, 1983). Of interest here are the c-*abl* and c-*sis* oncogenes which have been assigned to chromosomes 9 and 22, respectively (Heisterkamp *et al.*, 1982; Dalla-Favera *et al.*, 1982). The involvement of these cellular oncogenes in the standard and variants forms of the Ph^1 translocation have been studied by molecular techniques and by in-situ hybridization directly on metaphase chromosomes using cloned fragments of the genes as probes. We found that the c-*abl* oncogene was consistently involved in the Ph^1 rearrangement(s). Furthermore, molecular cloning of the breakpoint regions on chromosomes 9 and 22 was undertaken and was facilitated by the close proximity of the c-*abl* oncogene and the presumed breakpoints.

II The standard Ph^1 translocation

1 Cytogenetic studies

Using the methotrexate synchronization method (Hagemeijer *et al.*, 1979) a precise cytogenetic definition is obtained of the standard Ph^1 translocation, t(9;22), associated with CML (Fig. 1). The breakpoints are localized in bands 9q34 and 22q11 following the ISCN (1978) nomenclature. Translocation of the deleted part of chromosome 22 to the 9q+ derivative is clearly visible, but it is less obvious that a small segment of chromosome 9 is translocated to the 22q− derivative. Several studies using cytophotometric and cyto-densitometric measurements gave indications in favour of a reciprocal translocation but failed to produce unequivocal evidence (Mayall *et al.*, 1977; Watt and Page, 1978; Wayne and Sharp, 1982).

2 Somatic-cell genetic studies

Somatic-cell hybrids were obtained by fusion of rodent cells of Chinese hamster or mouse origin with blood or bone-marrow cells of patients with Ph^1+ CML. In these interspecies hybrid cell lines, random segregation of

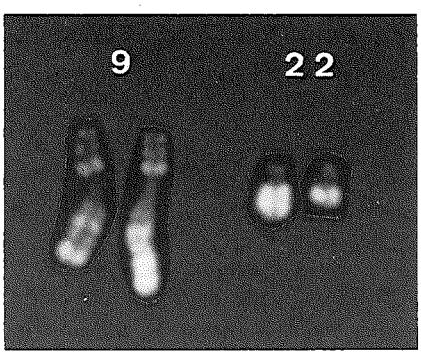

Fig. 1. Chromosomal pairs 9 and 22 from a bone-marrow metaphase of a CML patient illustrating the standard t(9;22)(q34;q11) (R-bands with acridine orange)

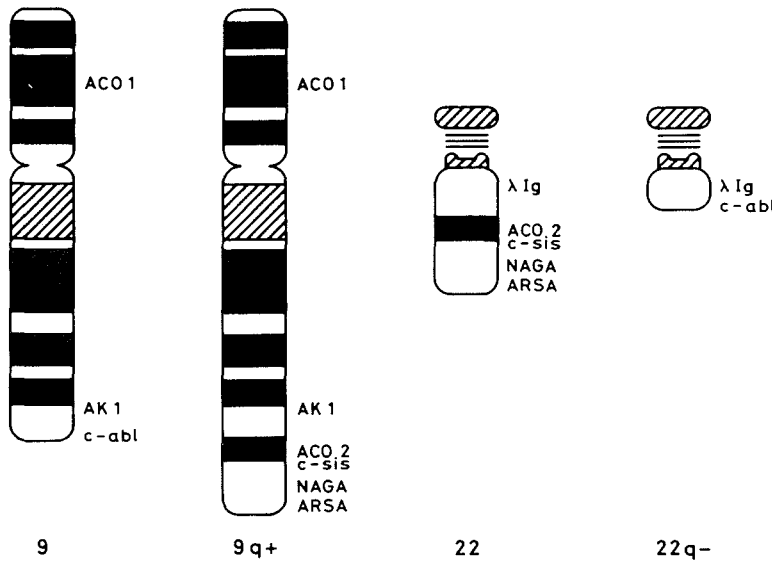

Fig. 2. Diagram showing the Ph[1] translocation (9;22)(q34;q11) and the location of the marker genes relative to the breakpoints

chromosomes of human origin takes place and it is possible to isolate a panel of hybrid clones containing, in addition to the rodent genome, only one of the human chromosomes involved in the Ph[1] translocation, i.e. either the 9q+ or the 22q− or their normal counterparts, 9 or 22. A panel of hybrid clones segregating these chromosomes have been isolated, originating from eight different CML patients carrying the standard t(9;22). These clones were assayed simultaneously for human chromosomal content and for the expression of genes known to be located on chromosome 22 or on the distal part of chromosome 9. A number of enzyme markers were assayed: adenylate kinase-1 (AK1) assigned to 9q34 (Mohandas *et al.*, 1978), the band involved in the Ph[1] translocation, and three markers of chromosome 22, i.e. mitochondrial aconitase (ACO2), arylsulphatase-A (ARSA) and *N*-acetyl-α-D-galactosaminidase (NAGA) (Geurts van Kessel *et al.*, 1980). These four enzyme markers segregated together, with the 9q+ derivative confirming the translocation of 22q to 9 but failing to demonstrate reciprocity (Fig. 2) (Geurts van Kessel *et al.*, 1981a).

3 Cellular oncogenes c-*abl* and c-*sis* as genetic markers

The localization of two human cellular oncogenes, c-*abl* and c-*sis* on chromosomes 9 and 22, respectively, stimulated an investigation of the fate of these oncogenes in the (9;22) translocation. The cellular homologue (c-*abl*) of the transforming gene (v-*abl*) of Abelson murine leukaemia virus is highly conserved in evolution. As for other cellular oncogenes, sequences homologous to c-*abl* have been found in several vertebrate genomes (Goff *et al.*, 1980) and in *Drosophila melanogaster* (Shilo and Weinberg, 1981;

Hoffman-Falk *et al.*, 1983). The human c-*abl* sequences homologous to the v-*abl* gene have been cloned (Heisterkamp *et al.*, 1983a). These c-*abl* sequences are distributed over a region of 40 kb of genomic DNA and contain several intervening sequences. The precise 5′ and 3′ boundaries of the human c-*abl* gene still remain to be determined. Experimental data obtained from the mouse c-*abl* gene indicate that the v-*abl* homologous sequences represent an internal part of the mouse c-*abl* gene (Wang, 1983; Wang *et al.*, 1984). The human c-*abl* sequences have been localized to the long arm of chromosome 9 (Heisterkamp *et al.*, 1982) at position 9q34 (Jhanwar *et al.*, 1984). Analysis of DNA from a panel of rodent–human somatic-cell hybrids already mentioned, containing either the 9q+ or the 22q− chromosome demonstrated the translocation of human c-*abl* from chromosome 9 to the Ph[1] chromosome (De Klein *et al.*, 1982).

c-sis represents the cellular homologue of the transforming gene (v-*sis*) of simian sarcoma virus (Josephs *et al.*, 1983). The human c-*sis* gene is localized on chromosome 22 (Dalla-Favera *et al.*, 1982) at position 22q13.1 (Jhanwar *et al.*, 1984). Hybridization of a c-*sis* probe to DNA of somatic-cell hybrids revealed that this gene is translocated to the 9q+ derivative chromosome in the Ph[1] translocation (Groffen *et al.*, 1983).

Another genomic marker of chromosome 22 is the λ light-chain immunoglobulin gene. The constant part of this gene (C λ) has been localized to the same band 22q11 as the Ph[1] breakpoint on chromosome 22 (McBride *et al.*, 1982). Hybridization of this probe (Cλ) to DNA of a selected panel of hybrid cells indicated that this gene remains on the Ph[1] chromosome (Goyns *et al.*, 1984).

In summary, although involving Ph[1] derivatives of different patients, all experiments showed the same segregation pattern of the markers tested which suggested that, in all patients studied, the breakpoints were consistent, at least relative to the markers tested. On chromosome 9 the breakpoint is distal to AK1 and proximal to c-*abl*, while on chromosome 22 the breakpoint is distal to C λ and proximal to ACO2 (Fig. 2). Furthermore, the translocation of the c-*abl* oncogene from chromsome 9 to 22q− constitutes the first unequivocal evidence that the standard Ph[1] translocation is a reciprocal exchange between chromosomes 9 and 22.

III Cytogenetic variants of the Ph[1] translocation in chronic myeloid leukaemia

1 Introduction

The demonstration that two cellular oncogenes were translocated in the standard Ph[1] translocation, t(9;22), suggested that one or both of these oncogenes could play a role in the pathogenesis of the disease. In order to study the specificity of the translocation of these oncogenes, we analysed their position in the cytogenetic variants of Ph[1], in CML.

Three forms of cytogenetic variants have been reported: firstly, 'simple' translocations between chromosome 22 and another chromosome, without

Table 1. Mapping of cellular oncogenes c-*abl* and c-*sis* to the derivative chromosomes observed in standard and variant forms of the Ph^1 translocation in CML

Number of cases	*Ph^1 translocation*	*Derivative chromosomes*	*Chromosomal localization of* c-abl	c-sis	*Techniques used*[a]
A. *Control with normal karyotype*					
3	None	None	9q34	22q13.1	S,H
B. *Standard Ph^1 translocation in CML*					
5	t(9;22) (q34;q11)	9q+, 22q−	9, 22q−	22, 9q+	S
2	t(9;22)	9q+, 22q−	9, 22q−		H
1	t(9;22)	9q+, 22q−		22, 9q+	H
C. *Complex translocation variants*[b]					
1	t(1;9;22) (p32;q34;q11)	9q+, 22q−, 1p−	9, 22q−	22, 1p−	S
1	t(9;11;22) (q34;q12;q11)	9q+, 22q−, 11q−	9, 22q−	22, 11q−	S,H
D. *Apparently 'simple' translocation variants*[c]					
1	t(4;9;22) (p16;q34;q11)	9q−, 22q−, 4p+	9, 22q−		H,B
1	t(9;12;22) (q34;p13;q11)	9q−, 22q−, 12p+	9, 22q−		H,B
1	t(7;9;22) (p22;q34;q11)[d]	9q−, 22q−, 7p+	9, 22q−		H,B
1	t(4;9;22) (p16;q34;q11)[e]	9q−, 22q−, 4p+			B
1	t(9;11;22) (q34;q23;q11)[f]	9q−, 22q−, 11q+			B
1	t(9;19;22) (q34;p13;q11)	9q−, 22q−, 19p+			B
E. *Masked Ph^1*					
1	t(6;22) (p21;q11)[g]	22q+, 6q−	9, 22q+		H,B
F. *Ph^1-negative CML*					
1	None	None	9	22	S
3	None[h]	None	9		H
1	None	None		22	H

[a]S, segregation analysis in somatic cell hybrids; H, in-situ hybridization technique using radioactive probes; B, high-resolution banding techniques

[b]Three additional cases were studied by banding only and were consistent with breakpoints in 9q34 and 22q11 resulting in clear 9q+ and 22q− derivatives: i.e. t(9;10;22) (q34;q24;q11), t(9;14;22) (q34;q32;q11) and t(9;16;22) (q34;p112;q11)

[c]Study of these cases with high-resolution banding showed involvement of one chromosome 9 resulting in a 9q− derivative undetectable when only standard cytogenetic techniques are used

[d]Referred by J. Fraisse, St. Etienne, France

[e]Referred by H. Van Den Berghe, Leuven, Belgium

[f]Referred by C. Turc-Carel, Dijon, France

[g]Referred by E. Godde-Salz, Kiel, West Germany

[h]Two of the cases were referred by M. F. Turchini, Clermont-Ferrand, France

apparent involvement of chromosome 9; secondly, complex translocations involving 9, 22 and at least one other chromosome (in these cases the third chromosome is recipient of the deleted part of 22q−, while 9 is recipient of the deleted part of the third chromosome); thirdly, a rare type of variant called 'masked Ph^1' where the Ph^1 is the recipient of a part of another chromosome (Tanzer *et al.*, 1977; Pasquali *et al.*, 1979; Sandberg, 1980; Oshimura *et al.*, 1982).

Examples of these variants were studied using high-resolution banding, segregation analysis of chromosomes and chromosomal markers in somatic-cell hybrids and in-situ hybridization of radioactive DNA probes. Chromosomal in-situ hybridization of radioactive probes allows the mapping of a gene to a chromosomal region, often restricted to one band. This technique is based on the property of single-stranded DNA to hybridize specifically to complementary sequences to form double-stranded structures. In these experiments we used single-copy DNA probes, tritiated by nick-translation: a 1.7-kb BamHI fragment of c-*sis* (Groffen *et al.*, 1983) and a 0.6-kb BamHI fragment and a 1.1-kb HindIII EcoRI fragment of c-*abl* (Heisterkamp *et al.*, 1983a). Results of these studies (Geurts van Kessel *et al.*, 1981b; De Klein *et al.*, 1982; Bartram *et al.*, 1983, 1984; Groffen *et al.*, 1983; Hagemeijer *et al.*, 1984; A. Hagemeijer *et al.*, unpublished observations) are summarized in Table 1.

2 c-*sis* in standard and variant Ph^1 translocations

By in-situ hybridization, c-*sis* was mapped on chromosome 22 at the junction between band q12 and q13 or at 22q13.1, far away from the breakpoint on 22q11 involved in the Ph^1 translocations. Consequently, in Ph^1+ CML c-*sis* was found to be located on the derivative chromosome carrying the visibly deleted part of 22q, i.e. the 9q+ in standard Ph^1 and another chromosome in the complex variants studied. Therefore, at first sight c-*sis* translocation in CML appeared as a trivial event associated with the translocation of the chromosomal part carrying the gene, and located at a relatively large distance from the critical breakpoint on 22q11.

3 c-*abl* in variant Ph^1 translocations

The c-*abl* oncogene maps consistently to the 22q− derivative or Ph^1 chromosome, in all cases of Ph^1+ CML, with either a standard or a variant translocation. In cases of complex translocations, all clearly involving chromosome 9, this finding was to be expected. In the so-called 'simple' translocation variants, the finding of the c-*abl* gene on the 22q− demonstrated the participation of the distal part of chromosome 9 in these variants as well (Fig. 3). Cytogenetic studies of these cases by high-resolution banding also showed a small deletion of one of the two chromosomes 9, confirming that all variants are complex translocations, involving chromosomes 22 and 9 and at least one other chromosome. In the so-called simple translocations, the breakpoint is in the telomeric region of the third chromosome and as a result subsequent translocation of this fragment to 9q34 is barely visible. In two cases of masked Ph^1 that we studied , c-*abl* maps on

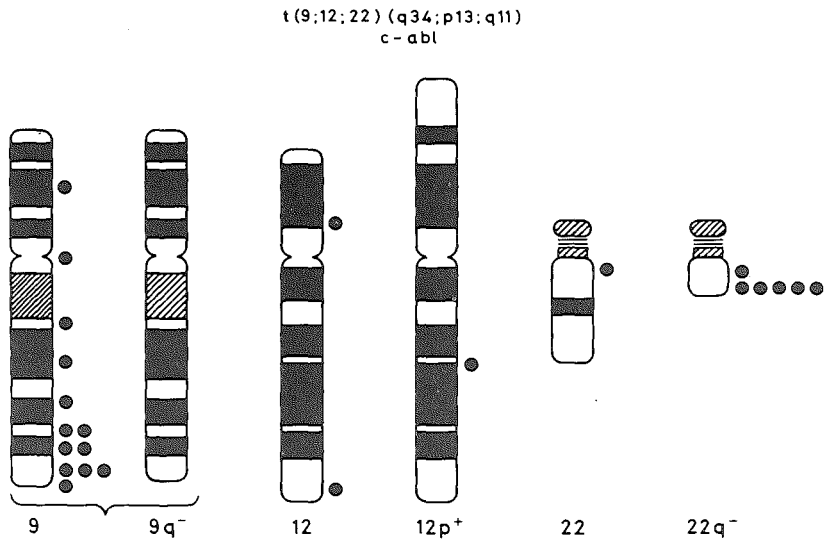

Fig. 3. Diagram illustrating the regional localization of labelling sites in a variant Ph^1 t(9;12;22), after in-situ hybridization of c-*abl* sequences. Only one chromosome 9 has been marked because distinction between 9 and 9q− is elusive in most metaphases. The accumulation of grains on the 22q− demonstrates the participation of chromosome 9 in this rearrangement

the 22q+ (masked Ph^1) at an interstitial site, adjacent to the 22q11 breakpoint; in one particular case, cytogenetic changes of chromosome 9 were not visible.

4 Conclusions

From these experiments we concluded that translocation of c-*abl* to chromosome 22 was a constant feature and therefore probably relevant to the genesis of Ph^1+ CML. In Ph^1− CML, translocation of c-*abl* or c-*sis* oncogenes was not observed. This is in agreement with the absence of chromosomal rearrangements in these patients and also in accordance with the view that CML without a Ph^1 chromosome is a different clinical entity with different origin, prognosis and survival.

IV Molecular analysis of the Ph^1 translocation

1 Cloning of the breakpoints

The localization of the breakpoint at the most telomeric band of chromosome 9 (band 9q34) and the consistent translocation to chromosome 22q− of a relatively small fragment of less than 5000 kb containing v-*abl* homologous sequences suggest that the breakpoint on chromosome 9 is located near or within the c-*abl* locus. We investigated the position of c-*abl* sequences relative to the breakpoint on chromosome 9 by cloning DNA fragments upstream to the known c-*abl* sequences from the leukaemic cells of a CML patient and by identification of chimeric fragments containing sequences from

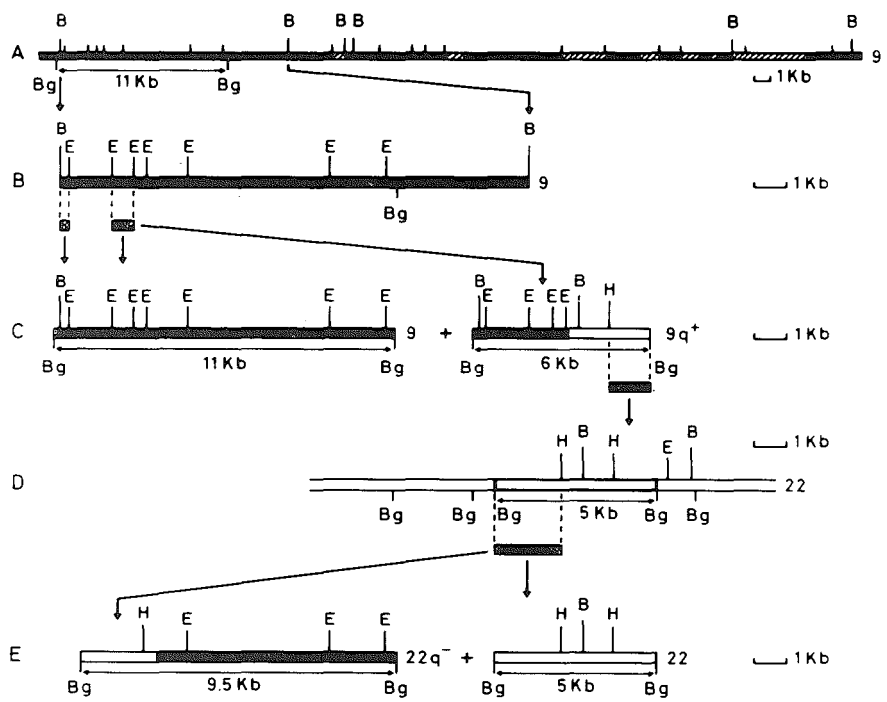

Fig. 4. Cloning strategy and analysis of the breakpoints in the Ph[1] translocation. The bars represent DNA fragments: solid bars indicate sequences from chromosome 9 and open bars sequences from chromosome 22. Restriction sites are indicated by: E, EcoRI; B, BamHI; Bg, BglII; H, HindIII. Probes isolated from the DNA fragments are indicated by hatched boxes beneath the bars at the appropriate sites. A: Restriction enzyme analysis of the c-*abl* region on chromosome 9. The hatched boxes indicate the v-*abl* homologous sequences. The EcoRI sites are indicated with small vertical lines. B: Enlargement of the 14.5-kb BamHI fragment of chromosome 9, situated in front of the known c-*abl* sequences, of which single copy DNA probes were isolated. C: Restriction-enzyme analysis of the two Bg1II fragments detected by the probes indicated in (B). The 11-kb fragment shows complete homology with sequences of chromosome 9, the 6-kb fragment shows homology with chromosome 9 and chromosome 22 sequences and therefore contained the breakpoint on 9 in this particular CML patient. A probe was made from the 22 part of the fragment. D: Restriction-enzyme analysis of a region of chromosome 22 containing the 5.0-kb BglII fragment that hybridizes to the probe indicated in C. From this fragment new probes were isolated, among others the BglII–HindIII probe represented in (D). E: Restriction-enzyme analysis of the two BglII fragments hybridizing to the probe indicated in (D). The 5.0-kb fragment is the chromosome 22 sequence illustrated in (D), while the 9.5-kb fragment shows only partial homology with chromosome 22 and partial homology with chromosome 9 sequences. The latter appears as the fragment carrying the breakpoint on chromosome 22 in this particular patient

chromosomes 9 and 22 (Heisterkamp *et al.*, 1983b). The strategy of cloning and analysis of the chimeric and normal DNA fragments is summarized in Fig. 4. From a region approximately 14.5 kb upstream (Fig. 4A) of the v-*abl* hybridizing sequences, two single-copy fragments were isolated. These probes (Fig. 4B) identify an 11-kb hybridizing BglII fragment in BglII-digested DNA of normal human cells and several CML patients. In the DNA of one CML patient an extra hybridizing BglII fragment of 6.0 kb was found. Analysis of

this fragment revealed that the 5′ part was colinear with the former cloned chromosome 9 sequences located at the 5′ side of the c-*abl* gene. The 3′ end of this fragment appeared to be derived from chromosome 22 sequences (Fig. 4C) and we concluded that this 6.0-kb BglII chimeric fragment represents the breakpoint at the 9q+ chromosome. Probes made from these chromosome 22 sequences only hybridized to a 5.0-kb BglII fragment in normal human DNA (Fig. 4D). With a probe made of the 5′ side of this 5.0-kb BglII fragment, we were able to detect an extra 9.5-kb BglII fragment in the DNA of this particular CML case. This fragment contained chromosome 22 sequences at the 5′ end, and chromosome 9 sequences at the 3′ end. Therefore it contained the breakpoint on chromosome 22q− (Fig. 4E). The presence of a breakpoint located 14.5 kb immediately upstream of the human v-*abl* homologous sequences seems to be unique for the DNA of this particular CML patient. Using the same probes from the c-*abl* locus, we were not able to localize other chromosome 9 breakpoints in the DNA of several other CML patients. Analysis of the chimeric fragments, however, suggests that the breakpoints on chromosomes 9 and 22 exhibit a perfect reciprocal exchange, at least at the level of detection by restriction-enzyme analysis.

2 Characterization of the breakpoint regions on chromosomes 22 and 9

To investigate and characterize further the location of the breakpoints on chromosome 22 in various Ph^1+ CML patients, a cosmid library of non-CML human DNA was screened and a region of 46 kb of human chromosome 22 DNA was cloned. The 5.0-kb BglII fragment cloned in the previous experiments (Fig. 4D) was situated in the centre of this region. Southern blot analyses of DNA from more than 20 CML patients, who were shown to be Ph^1-positive by cytogenetic techniques, was performed using probes specific for this 5.0-kb BglII fragment (Fig. 4C and 4D). In each case, in BglII-digested CML DNA, the expected 5.0-kb fragment originating from the normal chromosome 22 was found and in addition one or two fragments of different lengths. These fragments were shown by restriction-enzyme analysis to be the result of a break on chromosome 22 in the 5.0 kb region analysed by the specific probes for this region (Fig. 4C and 4D). The breakpoints on chromosome 22 are not situated at an identical site but they cluster in a specific region, the breakpoint cluster region (bcr), of up to 5.8 kb (Groffen *et al.*, 1984).

The bcr region was found to be normal in DNA isolated from other neoplastic tissues or cell lines tested, and in the DNA from Ph^1− CML patients or fibroblasts from Ph^1+ CML patients. These results indicate that the involvement of the bcr region is specific for the leukaemic cells in Ph^1+ CML patients. Up to now we have not been able to identify the function of the chromosome 22 region in which the breakpoints are situated. This region is a part of band 22q11 which also contains the λ light-chain constant region (C λ) (McBride *et al.*, 1982). However, no cross-homology was observed between our cosmid clones and C λ. At present we are investigating whether this region contains protein coding, or transcription-regulating sequences

which, in combination with the translocated c-*abl* sequences, might be responsible for the neoplastic transformation of the cell.

The probes from the bcr region provided the tools for cloning several 9q+ and 22q− breakpoint fragments of different CML patients. The pieces of chromosome 9 present in these fragments showed no cross-homology either with each other or with the cloned c-*abl* locus. Since they cover a region of about 100 kb of chromosome 9, we assume that location of the breakpoints in chromosome 9 may vary from less than 14.5 kb to more than 100 kb in front (5′) of the v-*abl* homologous sequences. We do not know whether these breakpoints actually occur within the human c-*abl* locus because the most 5′ exon of human c-*abl* has not been identified yet. However, the possibility must be considered that the human c-*abl* oncogene extends over a much larger region than that characterized by homology to the viral oncogene v-*abl* (Wang and Baltimore, 1983).

3 Transcription of c-*abl* in chronic myeloid leukaemia

Gale and Canaani (1984) investigated the expression of c-*abl* in cells of patients with a Ph^1+ CML, with Ph^1− CML, healthy controls, and patients with other types of leukaemia. In Ph^{1+} CML, enhanced c-*abl* expression was not observed. However, a new RNA transcript of 8 kb was found which sometimes replaced one of the normal 6 kb or 7 kb transcripts. We have studied c-*abl* expression in the K562 cell line which has been established from the leukaemic cells of a Ph^1+ CML patient in blast crisis. Although a typical Ph^1 chromosome is not visible in this cell line, the c-*abl* oncogene and C λ gene sequences are amplified and map on one of the newly formed marker chromosomes (Collins and Groudine, 1983; Selden *et al.*, 1983). In this cell line we found the new 8-kb RNA transcript replacing the 6-kb transcript.

Both breakpoint fragments from chromosomes 9 and 22 in this cell line have been cloned (Heisterkamp *et al.*, 1983b). The breakpoint on chromosome 9 is located about 100 kb in front of the known sequences of c-*abl*. As a consequence, the distance between the bcr and the v-*abl* hybridizing sequences must be over 100 kb. Nevertheless, this translocation resulted in a new c-*abl* RNA transcript. Experiments aimed at the cloning of the cDNA of this new c-*abl* RNA are in progress. This cDNA will enable us to investigate whether the altered gene product has transforming properties. Furthermore, it will help us to identify the origin of the new sequences in the enlarged c-*abl* transcripts. These new sequences could be either the result of alternative splicing of the c-*abl* RNA or derived from chromosome 22 sequences upstream of the breakpoint on chromosome 22.

4 Conclusions

The specificity of the bcr in chromosome 22 in Ph^1 positive CML suggests that rearrangements in this region may be involved in the pathogenesis of CML. Although the breakpoints on chromosome 9 are distributed over a large region 5′ to the v-*abl* hybridizing sequences, the translocation of the c-*abl* oncogene results in an altered RNA transcript of 8 kb. These observations

strongly suggest that human c-*abl* and bcr together may play a role in the generation of the neoplasm.

V Prospects

Molecular analysis of the CML-specific chromosomal translocation has revealed that both the bcr and the transposition of the c-*abl* oncogene in the vicinity of this region of chromosome 22 play an essential role in the pathogenesis of CML. These findings open new ways for investigations. Further molecular characterization of the bcr and of the cDNA coding for the new c-*abl* transcript may give information on the specific function of the bcr and on the role of the c-*abl* in the malignant transformation of the cells in CML. Using c-*abl* probes in in-situ hybridization experiments, we were able to demonstrate the translocation of this oncogene to the Ph^1 chromosome in all types of cytogenetic variants in CML. It is essential now to use bcr probes either in Southern blot analysis or by in-situ hybridization to study the eventual participation of chromosome 22 in cases which are atypical either clinically or cytogenetically. We have indications in some cases of ALL, carrying a Ph^1 chromosome, that the breakpoint on chromosome 22 is located outside the bcr. So far these are the only exceptions of Ph^1+ leukaemia showing breakpoints outside this region. These preliminary observations indicate that molecular analysis of this type may discriminate between true Ph^1+ ALL and CML presenting with a lymphoblastic blast crisis (Sandberg *et al.*, 1980). We have studied the DNA of CML patients in lymphoblastic and myeloblastic blast crisis with bcr probes. The hybridization pattern in Southern blot analysis was similar to that observed during the chronic phase in the same patients. Similar studies should be carried out on true Ph^1+ acute myeloblastic leukaemia (AML). Recently we had the opportunity to study the DNA from two patients with CML showing cytogenetically a translocation involving chromosome 9q34 and a chromosome other than 22: t(9;12) (referred by J. R. Teyssier, Reims) and t(9;13;15) (referred by M. R. Rivière, Brest). In both cases the cytogenetic picture of chromosome 22 appeared normal. Results obtained by Southern blot analysis with the bcr probes suggest that rearrangements did occur. These observations encourage further investigations into the molecular basis of Ph^1− CML. Clinically, these cases constitute a heterogeneous group. Many patients show rapid clinical deterioration and transformation in blast crisis, while others present with a rather typical CML chronic phase of 3–4 years. We have found that, in agreement with the cytogenetic absence of translocation, the position of the c-*abl* and c-*sis* oncogenes in this case is unaltered. It now becomes essential to analyse also the structural integrity of the bcr in these cases. In some patients a bcr–c-*abl* conjunction may have happened at chromosomal locations other than 22q−. The observation of normal c-*abl* RNA transcripts in the leukaemic cells of a Ph^1− CML patient (Canaani *et al.*, 1984; Gale and Canaani, 1984) suggests that, in at least some Ph^1− CML patients, c-*abl* is not rearranged either cytogenetically of molecularly. It is expected that further investigations along these lines would result in a better definition of CML, and

also of other Ph^1+ leukaemias where the classification on morphological grounds, as ALL, AML or blast crisis of CML has been regularly disputed.

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(The authors are responsible for the accuracy of the references.)

PAPER III

CELL 36: 93-99 (1984)

Philadelphia Chromosomal Breakpoints Are Clustered within a Limited Region, bcr, on Chromosome 22

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Summary

We have identified and molecularly cloned 46 kb of human DNA from chromosome 22 using a probe specific for the Philadelphia (Ph′) translocation breakpoint domain of one chronic myelocytic leukemia (CML) patient. The DNAs of 19 CML patients were examined for rearrangements on chromosome 22 with probes isolated from this cloned region. In 17 patients, chromosomal breakpoints were found within a limited region of up to 5.8 kb, for which we propose the term "breakpoint cluster region" (bcr). The two patients having no rearrangements within bcr lacked the Ph′ chromosome. The highly specific presence of a chromosomal breakpoint within bcr in Ph′-positive CML patients strongly suggests the involvement of bcr in this type of leukemia.

Introduction

Chronic myelocytic leukemia (CML) is characterized by the presence of the Philadelphia (Ph′) chromosome in the leukemic cells of 96% of all CML patients. The Ph′ chromosome is the result of a translocation between chromosome 22 and chromosome 9 (Rowley, 1973, 1982; Sandberg, 1980); its presence has important prognostic and diagnostic value. Previously we described the localization of the human c-*abl* oncogene (Heisterkamp et al., 1983a), to chromosome 9 (Heisterkamp et al., 1982) and demonstrated its translocation to the Philadelphia ($22q^-$) chromosome in CML (de Klein et al., 1982). This demonstrated unequivocally that the t(9;22) is reciprocal. As the breakpoint on chromosome 9 is at the most telomeric band on this chromosome, q34, human c-*abl* must be translocated on a relatively small fragment of less than 5000 kb to chromosome 22, suggesting a potential role for the c-*abl* oncogene in CML. This hypothesis was strengthened by the isolation of a chimeric DNA fragment from one CML patient containing sequences from chromosome 9 and 22 and located 14 kb immediately 5′ of human v-*abl* homologous sequences (Heisterkamp et al., 1983b). In the present study, we have used the chromosome 22-specific sequences of the chimeric DNA fragment to isolate a second chimeric chromosome 9/22 ($9q^+$) fragment from a different CML patient. The chromosome 9-specific sequences in this fragment must be localized at a minimal distance of 40 kb from the human v-*abl* homologous sequences. Using the same probe, we have isolated an extended region on chromosome 22 from non-CML human DNA. In contrast to the situation on chromosome 9, the breakpoints on chromosome 22 in the DNAs of these two CML patients had occurred in the same region, although not at an identical site. Subsequently, we investigated the genomic organization of this area in a number of other Ph′-positive CML patients: all exhibited abnormal restriction enzyme patterns, indicating that in Ph^+-positive CML a breakpoint occurs in a single well defined region of chromosome 22.

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Results

Isolation of a $9q^+$ Chimeric Fragment

Previously we have isolated a chimeric DNA fragment containing sequences originating from chromosomes 9 and 22 (Figure 1B) from a CML patient, 0319129. On chromosome 9, the breakpoint was located immediately to the 5′ of human v-*abl* homologous sequences (Figure 1A) and may even be within the human c-*abl* oncogene. However, the DNAs of two other CML patients did not contain rearrangements in this region; furthermore, we have molecularly cloned an additional 11 kb of DNA to the 5′ and have found no rearrangements in this area for these two DNAs (results not shown). Therefore, we decided to investigate whether we could localize the Ph′ translocation breakpoint to a specific site on chromosome 22. Using a 1.2 kb Hind III–Bgl II fragment (1.2 HBg, see Figure 1B) containing chromosome 22 sequences from the breakpoint region of CML patient 0319129 as a probe, we examined the DNA of the leukemic cells of a second patient (02120185). As shown in Figure 2A, this probe detects a normal 5.0 kb Bgl II fragment in control DNA (lane 1), in DNA of patient 0319129 (lane 2) and in DNA of patient 02120185 (lane 3). As expected, it also detects the breakpoint fragment of DNA 0319129 (Figure 1B and Figure 2, lane 2). In DNA of patient 02120185, an extra Bgl II fragment of 6.6 kb is visible (Figure 2, lane 3). Similarly, this probe hybridizes to a normal 3.3 kb Bam HI fragment in all three DNAs (Figure 2B), but detects an additional abnormal 11.3 kb Bam HI fragment in DNA 02120185 (lane 3). As we could detect additional restriction enzyme fragments with other restriction enzymes in DNA 02120185 (data not shown), we decided to examine whether these abnormal fragments were the result of the presence of a chromosomal breakpoint. Using the 1.2 kb HBg fragment as a probe, we molecularly cloned the 11.3 kb Bam HI fragment in charon 30. In this fragment only 1.2 kb of DNA, homologous to the probe, was defined as originating from chromosome 22; to determine all chromosome 22-specific sequences in the 11.3 kb Bam HI fragment, it was necessary to isolate the homologous region on chromosome 22 from non-CML DNA. For this

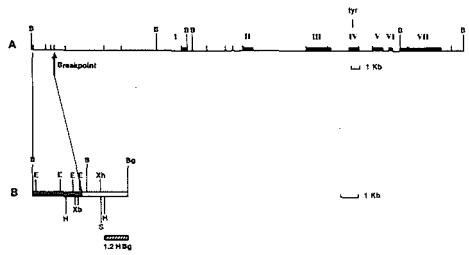

Figure 1. Position of the 9q+ Chromosomal Breakpoint in Spleen DNA of Patient 0319129 in Relation to Human v-*abl* Homologous Sequences

(A) The human v-*abl* homologous regions are designated I through VII and are indicated by solid bars; Eco RI sites are marked by small vertical lines. The vertical arrow points to the breakpoint in the DNA of CML patient 0319129. (B) The molecularly cloned region of DNA of patient 0319129 that contains a breakpoint. The solid bar indicates sequences from chromosome 9, while the open bar represents sequences from chromosome 22. The 1.2 kb HBg probe is indicated as a hatched box. Restriction enzymes include: Bam HI (B), Bgl II (Bg), Hind III (H), Sst I (S), Xba I (Xb), Xho I (Xh) and Eco RI (E).

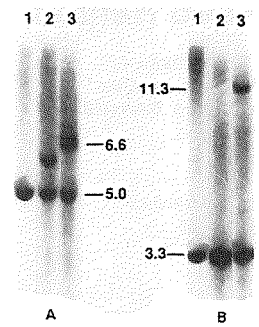

Figure 2. Restriction Enzyme Analysis of CML DNAs

Ten microgram of high molecular weight DNA was digested with Bgl II (A) or Bam HI (B), electrophoresed on 0.75% agarose gels, and blotted. DNAs analyzed were from human cell line GM3314 (lane 1), CML patient 0319129 (lane 2), and CML patient 02120185 (lane 3). Frozen spleen tissues, including those used as a source for the isolation of DNAs shown in Figure 5, were obtained through the Biological Carcinogenesis Branch, DCCP. Hybridization was with the 1.2 kb HBg probe (see Figure 1); filters were washed to 0.3 × SSC at 65°C. Molecular weights of fragments hybridizing to the probe are indicated in kilobases.

purpose, a previously described (Groffen et al., 1982) human lung carcinoma cosmid library was screened with the 1.2 kb HBg probe. Three cosmid clones were isolated, which contained overlapping portions of the same region.

Molecular Cloning of Ph′ Breakpoint Region of Normal Chromosome 22

As shown in Figure 3A, a region of approximately 46 kb was molecularly cloned; the 1.2 kb HBg probe hybridizes to a Bgl II fragment of 5.0 kb, located centrally in the cloned region. No homology is apparent between the restriction map of this region and that of human c-*sis* (Dalla-Favera et al., 1981), an oncogene situated on chromosome 22 but translocated to chromosome 9 in the Ph′ translocation (Groffen et al., 1983a). This confirms earlier reports that indicated that c-*sis* is not located in the immediate proximity of the Ph′ breakpoint (Bartram et al., 1983a). In variant Burkitt lymphoma, a t(8;22) has been described in which the immunoglobulin light chain was found to be involved (de la Chapelle et al., 1983). The light-chain constant region (Cλ) and the Ph′ chromosomal breakpoint have been localized to chromosome 22 band q11 (Rowley, 1973; McBride et al., 1982; Yunis, 1983); this suggests that c-*abl* could be translocated into Cλ in patients with CML. However, a probe isolated from the λ constant region showed no cross-homology with the above described chromosome 22 sequences. Additionally, no hybridization to a murine λ-variable region probe (Miller et al., 1981) was observed (results not shown).

To facilitate comparison of the 11.3 kb Bam HI fragment with homologous sequences on chromosome 22, the 5.0 kb Bgl II fragment was subcloned into pSV2-neo (Figure 3B). In concordance with our previous results (Heisterkamp et al., 1983b), in the 6.0 kb Bgl II breakpoint fragment from CML patient 0319129, restriction enzyme sites 3′ to the most 3′ Eco RI site originate from chromosome 22 (Figure 3C). In the 11.3 kb Bam HI fragment (Figure 3D) approximately 2.5 kb of DNA, including the 3′ Bam HI site and extending to the 3′ Xho I site, originates from chromosome 22.

The 11.3 kb Bam HI Fragment Also Contains a Breakpoint

To establish conclusively that the 11.3 kb Bam HI fragment represents a chimeric fragment of chromosomes 22 and 9, we isolated a 1.3 kb Eco RI fragment from the chromosome 22 nonhomologous region. Using this fragment as a probe, homologous sequences were detected in Bgl II-digested mouse DNA (Figure 4, lane 1) and Chinese hamster DNA (not shown). These bands, however, were clearly resolved from the Bgl II fragment visible in human DNA (lane 2). No human sequences homologous to the probe were detected in rodent–human somatic cell hybrids PgMe-25Nu, having human chromosome 22 as its only human component (lane 4) or in WESP-2A, (lane 3) containing a Ph′ chromosome but not chromosome 9 or 9q+ (de Klein et al., 1982). In the rodent–human somatic cell hybrids 10CB-23B (lane 5), containing human chromosome 9 and in 14CB-21A, containing a 9q+ chromosome (not shown), a Bgl II fragment of human origin homologous to the probe clearly was present. The only human DNA sequences these two hybrids have in common are those originating from chromosome 9. Therefore, the 11.3 kb Bam HI fragment possesses a breakpoint and represents a chimeric fragment containing chromosome 9- and 22-specific sequences isolated from a second CML patient.

Clustering of Ph′ Breakpoints on Chromosome 22 in CML Patients

Since in each of the above two CML DNAs the breakpoint in the t(9;22) on chromosome 22 was localized within a

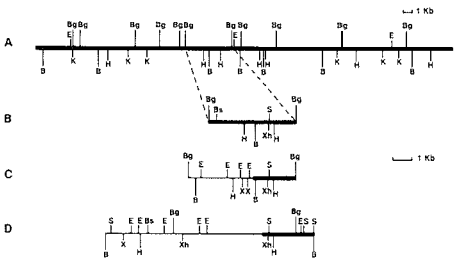

Figure 3. Comparative Restriction Enzyme Analysis of the Breakpoint Region on Chromosome 22 with Two 9q+ Breakpoint Regions

A restriction enzyme map of the cloned region in which chromosomal breaks occur on chromosome 22 is shown in (A); a subclone containing the 5.0 kb Bgl II fragment in (B) is compared with the 6.0 kb Bgl II and 11.3 kb Bam HI restriction enzyme fragments of the 9q+ chromosomes in (C) and (D); heavy lines indicate sequences from chromosome 22, whereas light lines indicate sequences originating from chromosome 9. B = Bam HI; Bg = Bgl II; Bs = BstE II; E = Eco RI; H = Hind III; K = Kpn I; S = Sst I; X = Xba I; Xh = Xho I.

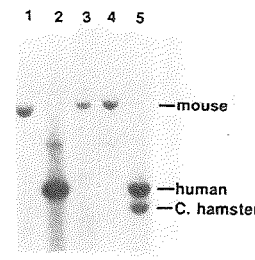

Figure 4. Origin of the 5′ Sequences of the 11.3 kb Bam HI Fragment

High molecular weight DNAs, including mouse (lane 1), human cell line A204 (lane 2), WESP-2A (chromosome 22q−, lane 3), PgMe-25Nu (chromosome 22, lane 4), and 10CB-23B (chromosome 9, lane 5) were digested with Bgl II, electrophoresed on an 0.75% agarose gel, blotted, and hybridized to a 1.3 kb Eco RI probe isolated from the 11.3 kb Bam HI fragment.

common region, we decided to investigate this area in other CML DNAs. As the 1.2 HBg probe had detected abnormal (9q+) Bgl II restriction fragments in DNAs 0319129 and 02120185, we subjected 17 additional independent CML DNAs to similar analysis; six of these were from spleen tissue, nine were from patient blood, and two were from bone marrow. As shown in Figure 5, lanes 1–13, CML DNAs from spleen, blood, and bone marrow all contained additional Bgl II fragments hybridizing to the 1.2 HBg probe; the DNAs of the patients shown in lanes 14–17 did not exhibit abnormal Bgl II fragments. Two of these (H81-258, lane 14 and C080, lane 15) showed deviant restriction enzyme patterns with other restriction enzymes (this will be discussed below).

To confirm that the 1.2 HBg probe detected chromosomal rearrangements and not merely DNA polymorphisms for the restriction enzyme Bgl II, all DNAs were subjected to digestion with at least one, but in most cases two or more different restriction enzymes. After hybridization with the 1.2 HBg probe, abnormal restriction enzyme fragments were detected in all Ph′-positive CML DNAs (also see below). Therefore, a polymorphism for Bgl II seems an unlikely explanation for the abnormal fragments shown in Figure 5; moreover, in the DNAs of most patients, abnormal fragments of different molecular weights are detected with the 1.2 HBg probe.

No extra Bgl II fragments were found in DNA isolated from cultured fibroblasts of patient H80-251 (lane 18) although an extra Bgl II fragment is clearly visible in DNA isolated from the leukemic cells of this patient (lane 7). Moreover, DNA isolated from the fibroblast cell line, AG 1732, established from a Ph′-positive CML patient, also lacked abnormalities (lane 19) in this region. Finally, in DNA isolated from leukemic cells of a Ph′-negative CML patient (lane 17) and of a two-year-old child with juvenile Ph′-negative CML, no visible rearrangements were found (lane 16), confirming our results of previous experiments (Bartram et al., 1983b) in which no translocations concerning *c-abl* to chromosome 22 or *c-sis* to chromosome 9 were found in Ph′-negative CML.

Sublocalization of Ph′ Breakpoints on Chromosome 22

As is apparent from the detailed restriction enzyme analysis of the breakpoint fragments of the DNAs of patients 0319129 and 02120185, the exact breakpoints are not localized at identical sites on chromosome 22. To sublocalize the breakpoints in the DNAs of the other CML patients more precisely, we arbitrarily divided the 5.0 kb Bgl II fragment into segments bordered by restriction enzyme sites for Bgl II, Bam HI, and Hind III (see Figure 6, bottom). Region 0 thus extends from the 5′ Bgl II site to the first 5′ Hind III site, region 1, a 0.6 kb Hind III–Bam HI fragment, is bordered by the same Hind III site at the 5′ and a Bam HI site 3′ to it. Region 2 is delineated by this Bam HI site at the 5′ and a Hind III site 3′ to it; region 3 is the 1.2 kb Hind III–Bgl II fragment (1.2 HBg) used as a probe in the experiments described above. Region 4 is outside the 5.0 kb Bgl II fragment and is bordered at the 5′ by the Bgl II site and at the 3′ by a Bam HI site.

As is evident from the restriction enzyme map of the 6.0 kb breakpoint fragment of CML DNA 0319129 (Figure 3C), the Bam HI site from chromosome 22 in region 1 is found on the 9q+ chromosome, whereas the Hind III site 5′ to it is missing; therefore, a break must have occurred between these two restriction enzyme sites in region 1. In accordance with this, only a (normal) 3.3 kb Bam HI fragment is detected with the 1.2 HBg probe, which originates from a region 3′ to the Bam HI site (Figure 2B, lane 2). In DNA 02120185, however, this Bam HI site is missing on the 9q+ chromosome (Figure 3D) and, therefore, the 1.2 HBg probe detects, in addition to the normal 3.3 kb Bam HI fragment, the 11.3 chimeric Bam HI fragment. As the 3′ Hind III site at region 2 is present in this 9q+ fragment, the breakpoint in this DNA is in region 2.

In DNA 0311068, as in DNA 0319129, only a normal 3.3 kb Bam HI fragment is detected with the 1.2 HBg probe,

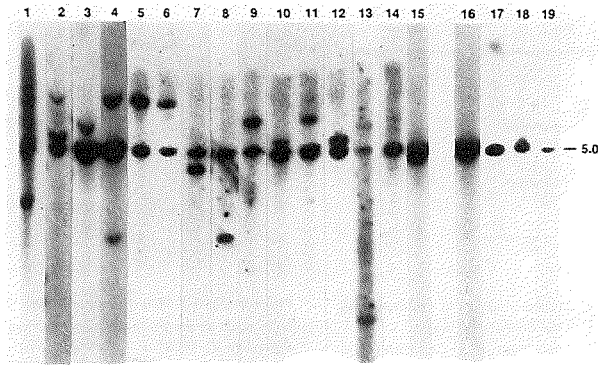

Figure 5. Analysis of DNAs from CML Patients

DNAs analyzed include isolates from CML patient 0311068 (lane 1), 7701C (lane 2), C999 (lane 3), C481 (lane 4), B79-100 (lane 5), B79-216 (lane 6), H80-251 (lane 7), CML 0 (lane 8), H81-164 (lane 9), H81-122 (lane 10), H81-118 (lane 11), H79-179 (lane 12), H77-94 (lane 13), H81-258 (lane 14), C080 (lane 15), C011 (lane 16), and H79-147 (lane 17). Also shown are DNAs isolated from fibroblasts of patient H80-251 (lane 18) and from the fibroblast cell line AG 1732 (lane 19).

DNAs in lanes 1–4 and 15–16 were isolated from the frozen spleens of CML patients; those from CML patients 0311068 and 7701C contained a very high percentage of leukemic cells; the percentage of leukemic cells in the other spleen tissues is not known. There are no data concerning the presence of a Ph′ chromosome in cells of these spleens. DNAs in lanes 7–14 and 17 were isolated from blood and those in lanes 5–6 from bone marrow. Cells from which DNA was isolated were examined for the presence of the Ph′ chromosomal marker; bone marrow and blood cells of all patients except patient H79-147 were Ph′-positive. The human fibroblast cell line AG 1732, obtained from the Human Genetic Mutant Cell Repository (Camden, New Jersey) was established from a CML patient carrying the Ph′ chromosome in her leukemic cells. DNA (10 μg) was digested with Bgl II, electrophoresed on an agarose gel, blotted, and hybridized to the 1.2 kb HBg probe.

indicating that no break has occurred within this fragment (Figure 6, lane 1). When the 0.6 kb Hind III–Bam HI fragment encompassing region 1 is used as a molecular probe, a normal 5.0 kb and two abnormal Bgl II fragments of 4.0 and 3.2 kb are visible. The 3.2 kb Bgl II fragment represents a $9q^+$ chimeric fragment containing the 3′ Bgl II site from the 5.0 kb Bgl II fragment on chromosome 22, as it is also detected with the 1.2 HBg probe (Figure 5, lane 1). The 4.0 kb Bgl II fragment is a $22q^-$ chimeric fragment with the 5′ Bgl II site originating from chromosome 22; it is not detected by the 1.2 HBg probe. The breakpoint in DNA 0311068 must be located in region 1.

The 1.2 HBg probe detects, in addition to a normal 3.3 kb Bam HI fragment, an abnormal 6.2 kb Bam HI fragment in CML DNA 7701C (Figure 6, lane 3). The Bam HI site bordering region 2 at the 5′ must, therefore, be absent from the $9q^+$ chromosome. The Hind III site at the 3′ of region 2 has been retained, as only one normal 4.5 kb Hind III fragment is visible after hybridization to 1.2 HBg (Figure 6, lane 4). This CML DNA must contain a breakpoint in region 2.

Patient C481 and H77-92 apparently have a breakpoint in region 3, encompassing the 1.2 HBg probe. For example, in DNA of patient C481 the 1.2 HBg probe hybridizes to three restriction enzyme fragments in every restriction enzyme digest: abnormal Bgl II fragments of 6.0 and 2.8 kb and a normal one of 5.0 kb (Figure 5, lane 4), abnormal Hind III fragments of 7.0 and 3.5 kb in addition to a normal 4.5 kb fragment (Figure 6, lane 7), abnormal Bam HI fragments of 7.5 and 5.2 kb and a normal 3.3 kb fragment (Figure 6, lane 8). Therefore, in this CML DNA, the 1.2 HBg probe detects both the $22q^-$ and $9q^+$ breakpoint fragments.

The situation in the DNA of patient C080 is less clear. As only one normal 5.0 kb Bgl II fragment is visible after hybridization to 1.2 HBg (Figure 5, lane 15), a chromosomal breakpoint most likely has occurred outside the Bgl II fragment. As the 1.2 HBg probe detects an abnormal 5.0 kb Hind III fragment in addition to the normal 4.5 kb Hind III fragment (Figure 6, lane 6), a chromosomal breakpoint may be situated immediately 3′ of the 5.0 kb Bgl II fragment. This is supported by the hybridization of the same probe to an abnormal 13 kb Bam HI fragment (and the normal 3.3 kb fragment, Figure 6, lane 5). The breakpoint has therefore been tentatively placed in region 4; in DNA of this patient, the 1.2 HBg probe would detect only $22q^-$ restriction enzyme fragments.

Using different restriction enzymes and probes from the 5.0 kb Bgl II fragment, we have analyzed the location of the breakpoint in all CML DNAs shown in Figure 5, lane 1–15. These results are summarized in Table 1.

Discussion

In the present studies we have identified and cloned a breakpoint cluster region (bcr) on chromosome 22, involved in the chromosomal translocation, t(9;22), of Ph′-positive CML. In total we have studied 19 CML patients; ten of these were shown to be Ph′-positive by cytogenetical analysis. All of the patients of this latter group pos-

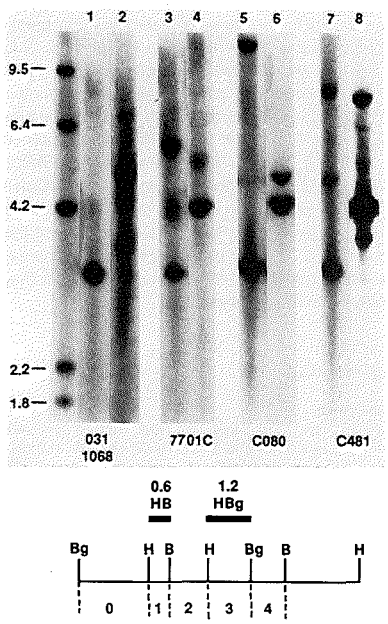

Figure 6. Analysis of Ph′ Translocation Breakpoints on Chromosome 22

Top: DNA of patients was digested with Bam HI (lanes 1, 3, 5, 7), Bgl II (lane 2), or Hind III (lanes 4, 6, 8). Hybridization was with the 1.2 kb HBg probe; lane 2 is hybridized with a 0.6 kb HB probe indicated in the bottom of the figure. Molecular weights of marker fragments in kilobases are indicated in the left of the figure. Bottom: bcr, the region within the cloned chromosome 22 sequences containing Ph′ translocation breakpoints identified in this study, is shown schematically. The numbers refer to the subregions mentioned in the text; the probes used are indicated above the figure.

sessed a chromosomal break within bcr. Of the remaining nine patients, one was cytogenetically characterized as Ph′-negative and a second patient has Ph′-negative juvenile CML; as expected, they did not exhibit a breakpoint in this region. Seven of seven nonkaryotyped patients were Ph′-positive because a chromosomal break could be identified within bcr. The involvement of bcr in the Ph′ translocation is highly specific for CML, as analogous rearrangements were not found in DNAs isolated from other neoplastic tissues or cell lines, including DNAs from four acute myeloid leukemia patients, one acute myelomonocytic leukemia patient, glioblastoma, melanoma, multiple myeloma, and teratocarcinoma cell lines (data not shown). Since abnormalities were not seen in a fibroblast cell line established from a Ph′-positive CML patient, in cultured fibroblasts of a Ph′-positive CML patient, or in leukemic cells of two Ph′-negative CML patients, we believe these rearrangements to be highly specific for the leukemic cells in Ph′-positive CML patients. In two patients these rearrangements were rigorously analyzed and shown to represent chromosomal breakpoints. Probes, isolated

Table 1. Breakpoint Location within bcr of Ph′-positive CML Patients

CML Patient	Breakpoint Location	CML Patient	Breakpoint Location
0311068	1	H81-122	2, 3
7701C	2	H81-118	2, 3
C999	1	H79-179	1
C481	3	H77-94	3
B79-100	2	H81-258	1, 2
B79-216	0, 1, 2	C080	4
H80-251	0, 1	0319129	1
CML-0	2	02120185	2
H81-164	2, 3		

The different breakpoint subregions (0–4) are as indicated in Figure 6, bottom. For some patients, the exact breakpoint subregion has not yet been determined; more than one subregion is indicated for these patients in the table.

from the bcr, in particular the 1.2 kb HBg probe, are highly specific tools for the identification of the Ph′ translocation in leukemic DNA and as such, may be of diagnostic value, in particular when no metaphase chromosome spread can be obtained.

We have molecularly cloned a region of chromosome 22 from non-CML DNA that contains the Ph′ breakpoints in CML DNA. The orientation of the chromosome 22-specific sequences in the two 9q$^+$ breakpoint fragments determines the orientation of the breakpoint cluster region on the chromosome: the most 5′ end will remain on chromosome 22 (Figure 7) and, depending on the exact position of a breakpoint, a smaller or larger region of bcr will be translocated to chromosome 9. Although the breakpoint on the Ph′ chromosome is in band q11 (Rowley, 1973; Yunis, 1983) and the λ light-chain constant region has been localized to the same band (McBride et al., 1982; our unpublished results) no cross-homology was observed between the chromosome 22 cosmid clones and Cλ. As of yet, these clones contain unidentified sequences. Experiments to determine if this region of chromosome 22 contains protein coding regions and/or enhancer sequences are in progress.

Previously we have reported the isolation of a Ph′ breakpoint fragment containing chromosome 9 sequences originating approximately 14 kb 5′ of human *v-abl* homologous sequences (Heisterkamp et al., 1983b). However, we were unable to detect chromosomal breakpoints in other CML DNAs up to 11 kb 5′ of this breakpoint. The 9q$^+$ breakpoint fragment from a second CML patient, isolated in the present study, contains 9 kb of DNA originating from chromosome 9. Preliminary results suggest that these sequences must be separated by, at minimum, 27 kb of DNA 5′ of the previously reported breakpoint. Therefore, the breakpoint on chromosome 9 appears to be variable in different Ph′-positive CML patients and may be found within a relatively large but limited region on chromosome 9; the region of chromosome 9

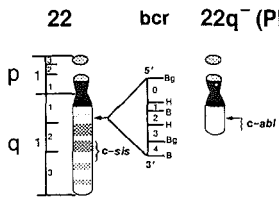

Figure 7. Diagrammatic Representation of the Ph' Translocation

The horizontal arrows indicate the chromosomal breakpoint in chromosome 22. Mapping of c-*sis* to the region of chromosome 22 (q12.3 to q13.1) translocated to chromosome 9 is as previously described (Groffen et al., 1983a; Bartram et al., 1983a). Localization of c-*abl* to the terminal portion of chromosome 9 (q34), which is translocated to chromosome 22 in the Ph' translocation, is as described in the text. The maximum size of bcr, from the 5' Bgl II to the 3' Bam HI site, is 5.8 kb.

containing human c-*abl* (q34-qter) that is translocated to chromosome 22 is too small to be visualized by cytogenetic analysis and does not exceed 5000 kb (Heisterkamp et al., 1983b; de Klein et al., 1982).

At present, we do not know whether the two breakpoints we have identified actually occur within human c-*abl* coding sequences: the most 5' exon of human c-*abl* has not yet been determined. However, the possibility must be considered that the human c-*abl* oncogene extends over a much larger region than that characterized by homology to the viral oncogene v-*abl*; whereas the v-*abl* oncogene is 3.5 kb in length (Goff et al., 1980). Homologous RNA species ranging in size from 5–7 kb (Ozanne et al., 1982; Westin et al., 1982), have been reported in humans. In contrast to the situation on chromosome 9, the breakpoints on chromosome 22 seem to be clustered in a very limited region. It is evident, however, even at the restriction enzyme level, that the breakpoints have not occurred in one specific site but rather are distributed over a region of up to 5.8 kb.

Analogous to the t(9;22) in Ph'-positive CML, a t(8;14) is found in many patients with Burkitt lymphoma: in the latter case, the human oncogene c-*myc*, located on chromosome 8, (Dalla-Favera et al., 1982; Taub et al., 1982) is often translocated into the immunoglobulin heavy-chain locus on chromosome 14 (Taub et al., 1982; Dalla-Favera et al., 1983; Adams et al., 1983; Erikson et al., 1983; Hamlyn and Rabbits, 1983). The breakpoints on chromosome 8 may be distributed over a relatively large region 5' of human c-*myc* (Dalla-Favera et al., 1983; Adams et al., 1983; Erikson et al., 1983), a situation analogous to that of human c-*abl* on chromosome 9. On chromosome 14, breakpoints in the variable (Erikson et al., 1982) and in the constant region of the heavy-chain locus have been reported in Burkitt lymphoma, indicating that neither the breakpoints on chromosome 8 nor those 14 can be found within a breakpoint cluster region as discussed in the present study. The bcr on chromosome 22 seems as of yet to be unique in human. However, the existence of bcrs on other human chromosomes is not unlikely taking into consideration the increasing number of reports of other highly specific translocations in neoplastic diseases (for a review, see Yunis, 1983).

The specificity of the presence of a chromosomal breakpoint on chromosome 22 within bcr in Ph'-positive CML indicates that this region may be involved in CML. Additionally, a human oncogene, c-*abl*, is consistently translocated to chromosome 22, even in patients with complex Ph' translocations (Bartram et al., 1983b) and is amplified in a CML cell line, K562, (Heisterkamp et al., 1983b). Although the breakpoints on chromosome 9 are distributed over a relatively large region of DNA 5' to human v-*abl* homologous sequences, the specific translocation of this oncogene in the t(9;22) must be of functional significance. Therefore, we believe that both human c-*abl* and bcr may be associated with Ph'-positive CML.

Experimental Procedures

Somatic Cell Hybrids

PgME-25 Nu is a human–mouse somatic cell hybrid obtained from fusion with mouse Pg19 cells; it contains as its only human component chromosome 22. Chinese hamster cell line E36 was used to obtain hybrids 10CB-23B and 14CB-21A. The hybrid 10CB-23B contains human chromosomes 5, 9, 11, and 19, whereas 14CB-21A has retained chromosomes 4, 7, 8, 14, 20, and 9q+ (Geurts van Kessel et al., 1981b; Geurts van Kessel et al., 1981C; de Klein et al., 1983). WESP 2A was obtained by fusion of mouse WEHI-3B cells with leukocytes of a Ph'-positive CML patient and contains human chromosomes 7, 8, and 14 in addition to the Ph' chromosome (de Klein et al., 1983).

Southern Blot Analysis

High molecular weight DNAs were isolated as described (Jeffreys and Flavell, 1977), digested with restriction enzymes, and electrophoresed on agarose gels. Blotting was according to Southern (1975) on nitrocellulose (Schleicher and Schuell, pH 7.9). Nick translation of probes and filter hybridizations were as described (Flavell et al., 1978; Bernards and Flavell, 1980). Specific activity of the probes were 2–5 × 10^8 cpm/μg. Filters were exposed to XAR-2 film (Kodak) at −70°C with Dupont Lightning Plus intensifying screens.

Isolation of Probes

DNA probes were prepared by digestion with appropriate restriction enzymes, followed by electrophoresis through low-melting-point agarose gels. Desired bands were cut from the gel and brought into solution by heating at 65°C for 30 min. Agarose was removed by two extractions with phenol equilibrated with 0.3 M NaOAc (pH 5.0), and one extraction with phenol/chloroform/isoamylalcohol (25:24:1). DNA was precipitated with ethanol and 0.2 M NaOAc (pH 4.8) in the presence of 20 μg/ml Dextran T-500 as carrier. Restriction enzymes and low-melting-point agarose were purchased from Bethesda Research Laboratories and were used according to the supplier's specifications.

Molecular Cloning

Subcloning of the 5.0 kb Bgl II fragment and cloning of the 11.3 kb Bam HI fragment was according to published procedures (Groffen et al., 1983b). A previously described (Groffen et al., 1982) human lung carcinoma cosmid library was screened with the 1.2 HBg probe according to the method of Grosveld et al. (1981). Three positive cosmid clones were isolated and mapped independently by digestion of individual restriction enzyme fragments isolated from low-melting-point agarose gels.

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PAPER IV

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

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

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

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

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

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

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

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

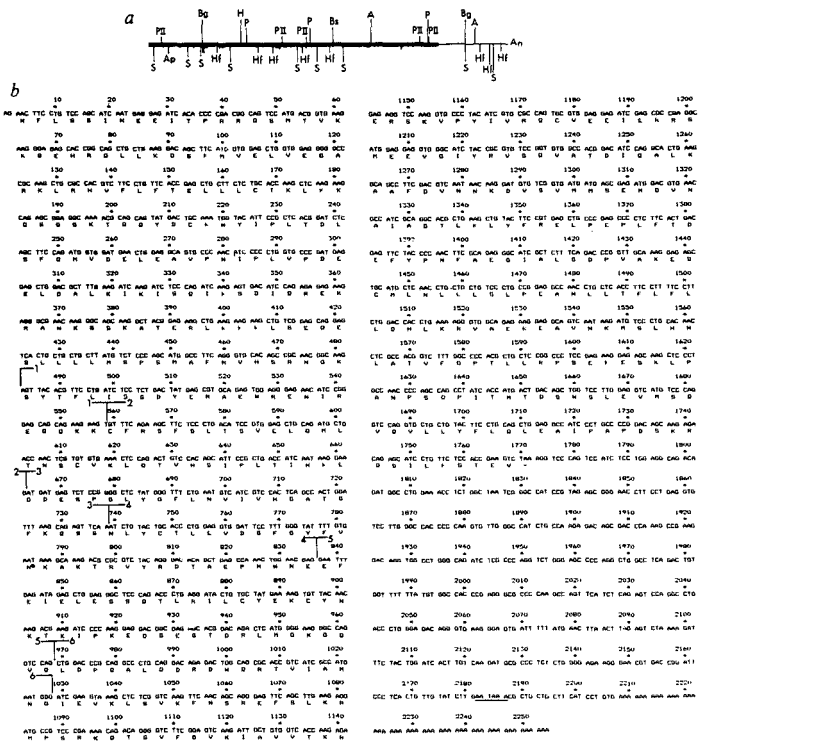

Fig. 1 *bcr* cDNA V1-3. *a*, Restriction enzyme map of V1-3. The black bar indicates the open reading frame sequences, the thin line non-translated sequences. The 3′ end of the cDNA containing the poly(A) tail is depicted as A_n. Restriction enzymes include: A, *Ava*I; Ap, *Apa*I; B, *Bgl*II; Bs, *Bst*EII;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

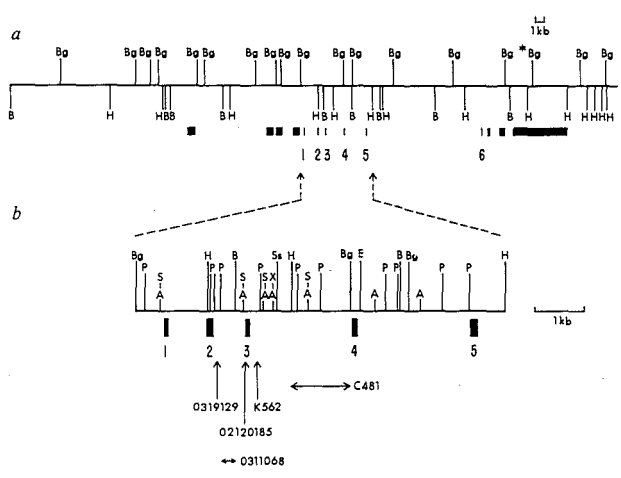

Fig. 2 Genomic organization of the *bcr* gene. *a*, Restriction enzyme map of chromosome 22 sequences encompassing *bcr*. Exons are indicated by black boxes below the restriction enzyme map. The position of the numbered exons has been determined by sequencing; all other exons were located by hybridization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

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

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

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

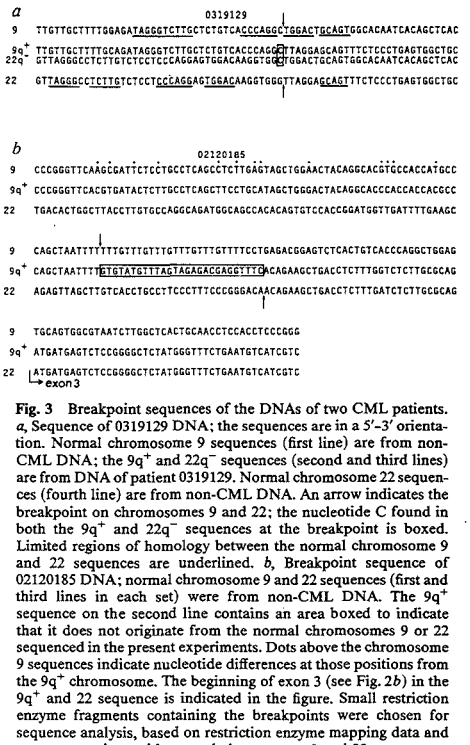

Fig. 3 Breakpoint sequences of the DNAs of two CML patients. *a*, Sequence of 0319129 DNA; the sequences are in a 5′–3′ orientation. Normal chromosome 9 sequences (first line) are from non-CML DNA; the $9q^+$ and $22q^-$ sequences (second and third lines) are from DNA of patient 0319129. Normal chromosome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

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

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PAPER V

MOL.CELL.BIOL. 6: 607-616 (1986)

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

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

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

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

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

MATERIALS AND METHODS

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

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

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

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

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

RESULTS

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

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

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

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

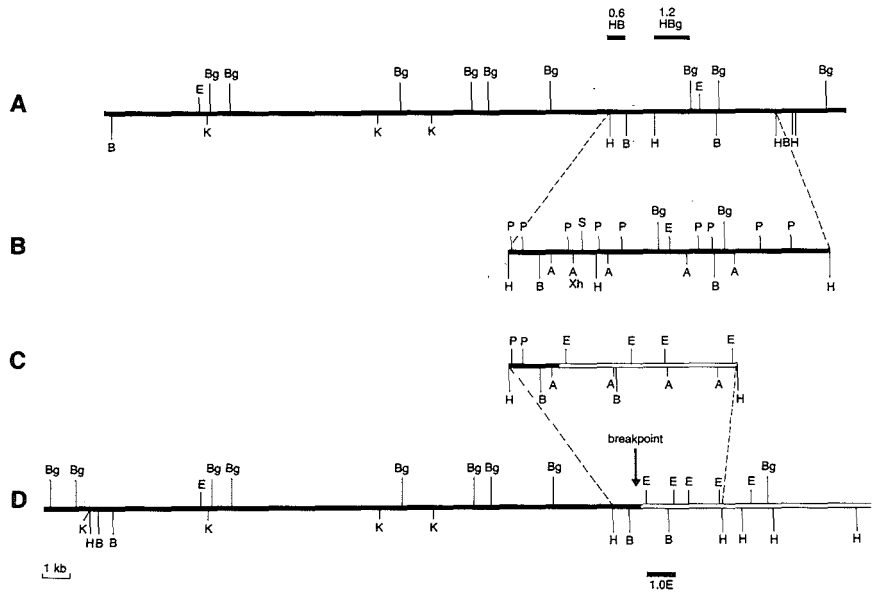


FIG. 1. Restriction enzyme map of the K562 breakpoint region on chromosome 22. A, Restriction enzyme map of human chromosome 22 sequences; the 5.8-kb *Bgl*II-*Bam*HI (*bcr*) region encompasses the 0.6-kb *Hin*dIII-*Bam*HI and 1.2-kb *Hin*dIII-*Bgl*II probes. D, Restriction enzyme map of the Ph^1 chromosome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

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

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

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

For the detection of c-*abl* mRNA we used a human c-*abl* riboprobe containing a 0.6-kb *Eco*RI-*Bam*HI fragment in pSP64 (28). This fragment contains the most 5′ human v-*abl* hybridizing exon as identified by DNA sequencing (see Fig. 5 and 6). This exon is homologous to the mouse c-*abl* exon containing the v-*abl*–*gag*-*abl* junction identified and sequenced by Wang et al. (37). To examine the effect of the amplification and translocation of the human c-*abl* oncogene on its expression, poly(A)$^+$ RNA was isolated from K562 and control Hela cells. In concordance with results obtained by others (6, 11), the c-*abl*-specific probe detects 6.0- and 7.0-kb mRNAs both in Hela cells and in K562. In addition, a novel c-*abl* homologous mRNA of 8.5 kb is detected in K562 (Fig. 3, left lane). An extra band of approximately 11 kb is also visible and may represent c-*abl* precursor RNA, which has not been studied in further detail. The abnormally sized mRNA seems to be characteristic of Ph^1-positive cells, as it is not detected in either normal control cells or cells of other types of leukemia (4, 11).

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

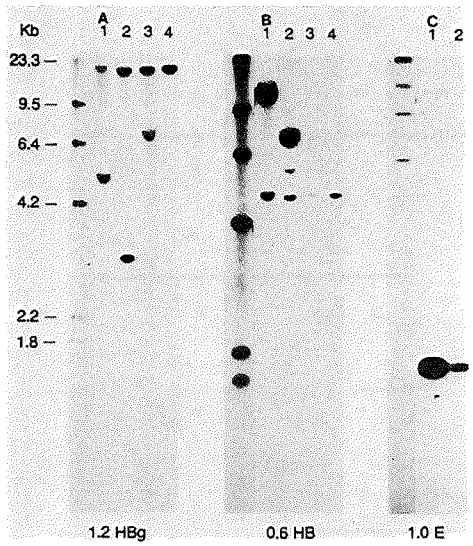


FIG. 2. Ph[1] chromosomal breakpoint in K562. A, *Eco*RI digest of 10 μg of DNA from CML patients 02120185 (lane 1), 0319129 (lane 2), and 0311068 (lane 3) and from the cell line K562 (lane 4). B, K562 DNA digested with *Sst*I (lane 1) and *Bgl*II (lane 2); DNAs of human cell lines AG1732 (lane 3) and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

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

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

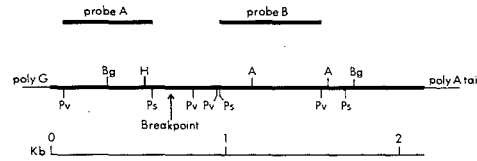


FIG. 3. Restriction enzyme map of the normal *bcr* cDNA, pV1-3. Three normal *bcr* cDNA clones were isolated from a fibroblast cDNA library (29) after screening with the 0.6-kb *Hin*dIII-*Bam*HI probe (Fig. 1A). Of the largest cDNA, pV1-3, only the 2.2-kb cDNA insert is shown flanked by poly(G) and poly(A)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

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

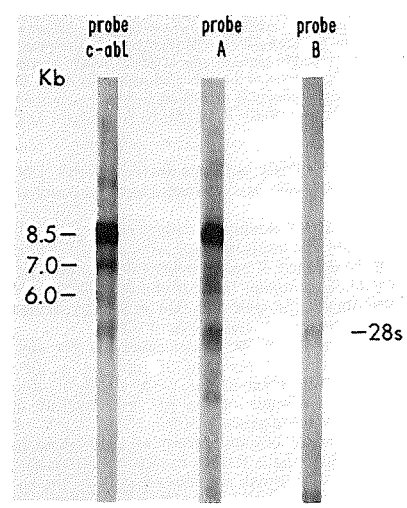


FIG. 4. Hybridization of K562 Northern blot with c-*abl* and *bcr* probes. Poly(A)$^+$ RNA (20 μg) of K562 was run on a 1% agarose gel and transferred to nitrocellulose. As probes we used a 0.6-kb *Eco*RI-*Bam*HI c-*abl* fragment (8), a 0.5-kb *Pvu*II-*Pst*I 5′ *bcr* fragment (probe A) and a 0.56-kb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

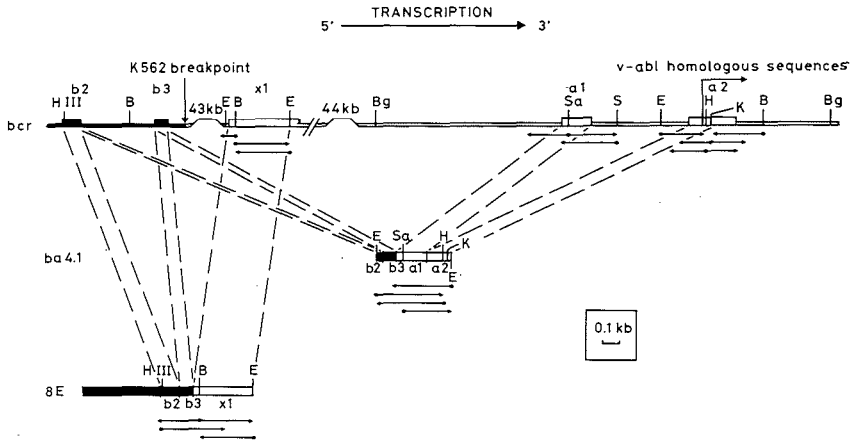
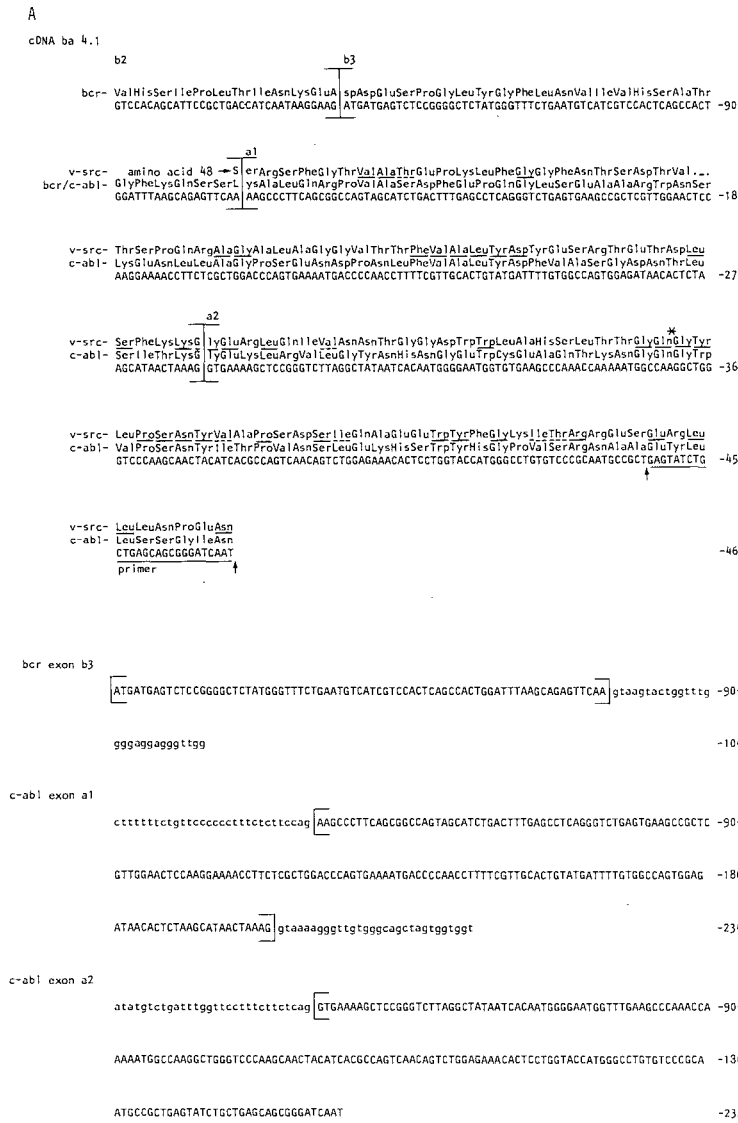


FIG. 5. Restriction enzyme maps of two chimeric cDNAs and the positions of the exon sequences in the K562 breakpoint region. The upper line represents the restriction map of the Ph^1 chromosome in K562. The chromosomal breakpoint is indicated by an arrow above the map, as is the region of c-*abl* that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

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

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

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



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

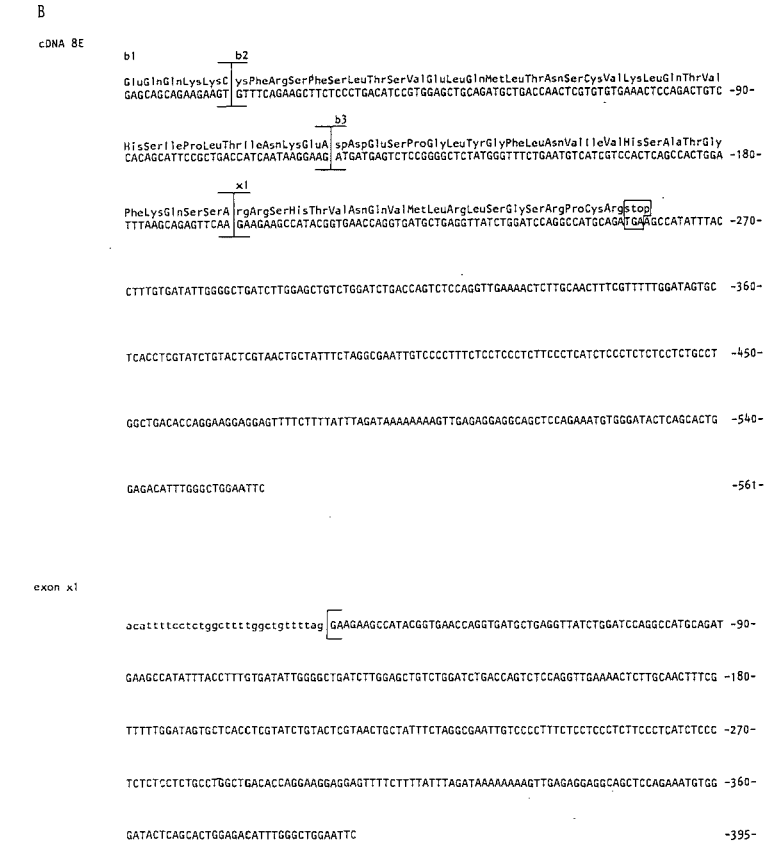


FIG. 6. DNA sequence analysis of cDNA ba 4.1 and cDNA 8E and the corresponding chromosomal exons. Sequence strategies and methods are as indicated in the legend to Fig. 4. A, (i) The nucleotide sequence of the 468-base-pair *Eco*RI fragment of cDNA ba 4.1; b2, b3, a1, and a2 indicate the *bcr* and c-*abl*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

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

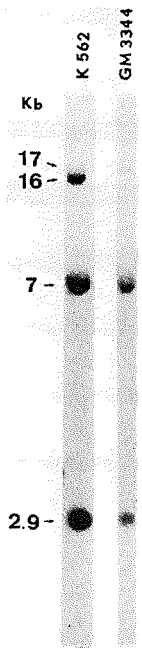


FIG. 7. Hybridization of cDNA ba 4.1 to chromosomal DNA of K562 and GM3344 cells. Southern blot of *Eco*RI digests of 10 μg of DNA from the cell line K562 and cell line GM3344. Both lanes were hybridized to the ^{32}P-labeled *Eco*RI insert of cDNA ba 4.1. Molecular masses are indicated at the side of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

DISCUSSION

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

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

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

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

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

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

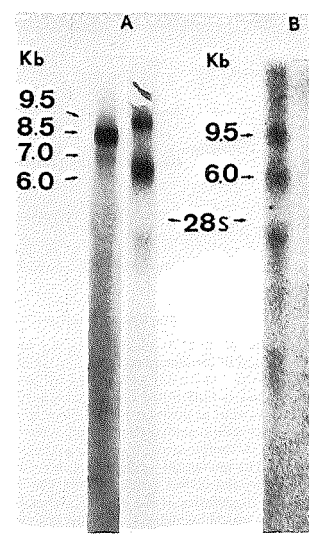


FIG. 8. Hybridization of exon x1 probe to Northern blots of K562 and HeLa poly(A)$^+$ RNA. Northern blots with K562 and HeLa poly(A)$^+$ RNA were prepared as described in the legend of Fig. 3. A, In the left lane, K562 RNA was hybridized with a 0.6-kb *Eco*RI-*Bam*HI c-*abl* probe (Fig. 5); in the right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

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

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

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

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

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

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PAPER VI

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MOLECULAR ANALYSIS OF BOTH TRANSLOCATION PRODUCTS OF A PHILADELPHIA-POSITIVE CML PATIENT.

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ABSTRACT

The breakpoint regions of both translocation products of the (9;22) Philadelphia translocation of CML patient 83-H84 and their normal chromosome 9 and 22 counterparts have been cloned and analysed. Southern blotting with bcr probes and DNA sequencing revealed that the breaks on chromosome 22 occurred 3´ of bcr exon b3 and that the 88 nucleotides between the breakpoints in the chromosome 22 bcr region were deleted. Besides this small deletion of chromosome 22 sequences a large deletion of chromosome 9 sequences (>70kb) was observed. The chromosome 9 sequences remaining on the 9q+ chromosome (9q+ breakpoint) are located at least 100 kb upstream of the v-abl homologous c-abl exons whereas the translocated chromosome 9 sequences (22q- breakpoint) could be mapped 30 kb upstream of these c-abl sequences. The breakpoints were situated in Alu-repetitive sequences either on chromosome 22 or on chromosome 9, strengthening the hypothesis that Alu-repetitive sequences can be hot spots for recombination.

INTRODUCTION

Chronic myelocytic leukemia (CML), a pluripotent stem cell disease is characterized by the presence of a Philadelphia (Ph^1) chromosome in the leukemic cells of more than 90% of all CML patients (1). This Ph^1 chromosome is the result of a reciprocal translocation between chromosomes 9 and 22 (2,3). Previous studies indicated that in all Ph^1 (+) CML patients a human oncogene c-abl, normally located on 9q34, was translocated to a specific, limited area on chromosome 22, the breakpoint cluster region (bcr)(4,5.) The 5.0 kb

bcr contains 4 small coding regions (6) and is an internal part of a large 'bcr' gene. Thusfar all chromosome 22 breakpoints (>30) map within two introns of this bcr. However, breakpoints on chromosome 9 are scattered over a very large area, which may vary from 14 kb (7) up to more than 100 kb (8) upstream of the v-abl homologous sequences of the c-abl gene. As a result of the translocation, the c-abl sequences are linked in a head-to-tail fashion to the 5' bcr sequences on the Ph^1 chromosome. Recent studies indicated, (8,9,10) by demonstration of the presence of a chimeric bcr/c-abl mRNA, that this region is transcriptionally active. The 5' bcr and c-abl coding sequences are linked by RNA splicing, apparently independent from the distance between the two genes on the Ph^1 chromosome. The detection of an abnormally sized c-abl protein (11) supports this hypothesis and is the presumable translation product of this chimeric mRNA found in the leukemic cells of Ph^1(+) CML patients and CML-derived cell lines. Virtually nothing is known about the mechanism of chromosomal translocation in CML. Sequence data from the breakpoints of two CML patients suggest that Alu-repetitive sequences may play a role (6,12). Here we report the cloning of the breakpoint regions of both translocation products of a CML patient with a t(9;22). Mapping and sequencing of the chromosomal breakpoint regions and their normal counterparts revealed that the translocation did not occur in a conservative manner: both chromosome 9 and 22 sequences were deleted. Furthermore Alu-repetitive sequences were located near or at the breakpoint in this CML patient.

MATERIALS AND METHODS

CML patient and cell lines

CML patient 83-H84 is an 18-year old male. In this study leukophoresis material from the chronic phase of CML (karyotype t(9;22)(q34;q11) prior to treatment was obtained. The cell line K562 (13) and Hela cells were used as controls.

Southern blotting and hybridization

High-molecular weight DNA's, isolated as described (14) were digested with restriction enzymes, electrophoresed on 0.7% Agarose gels and blotted according to Southern (15). Isolated DNA fragments (8) were labeled with ^{32}P as described by Feinberg and Vogelstein (16). Probes containing repetitive sequences were preincubated with sonicated human DNA to Cot=1 (10.pa mg/ml, 0.6M sodium phosphate buffer pH7, 3 hr at 65°C). Hybridization and washing conditions were as described in previous publications (3,5).

Genomic cloning

To isolate the breakpoint regions a genomic library of patient 83-H84 DNA was constructed in λ-EMBL-3 (17). Normal chromosome 9 counterpart of phage EMBL-3a (9q+) was isolated from another CML-EMBL-3 library. The normal chromosome 22 or chromosome 9 sequences were previously cloned from human cosmid or phage libraries (5,7,8).

DNA sequencing

Subcloned fragments of the breakpoint areas in pUC9 were sequenced according the methods of Maxam and Gilbert (18). In those cases where no suitable sites were available, a series of Bal 31 deleted subclones were generated and sequenced using end labeled sites of the pUC9 poly linker.

RESULTS

Identification and cloning of the chimeric DNA fragments

To identify the chimeric DNA fragments, BglII digested DNA of CML 83-H84 and control cell lines were hybridized to bcr probes. The 5' bcr probe (2.2 kb Bg-H, Fig. 1C) detects an abnormal fragment of 5.7 kb (Fig. 2D) besides the normal 5.0 kb BglII fragment. This 5.7 kb fragment is not present in Hela or K562 DNA and represents the 22q- breakpoint BglII fragment of CML 83-H84. In a similar manner we identified the 9q+ BglII fragment of 2.4 kb (Fig. 2A) using the 3' bcr probe (1.2 kb H-Bg, Fig. 1C). To analyse the aberrant fragments in more detail, a λ-EMBL-3 library was constructed of size-fractionated, partially MboI digested CML 83-H84 DNA. This

library ($1x10^6$ ph) was screened with both the 5′ and 3′ bcr probe. Positive phages, hybridizing only to either the 5′ or the 3′ bcr probe, were isolated and mapped by double digestion of individually isolated restriction enzyme fragments from low-melting point agarose gels (Fig. 1B, 1D). The phages hybridizing to the 5′ or 3′ bcr probe contained the expected abnormal BglII fragments of 5.7 and 2.4 kb resp. The 5′ end

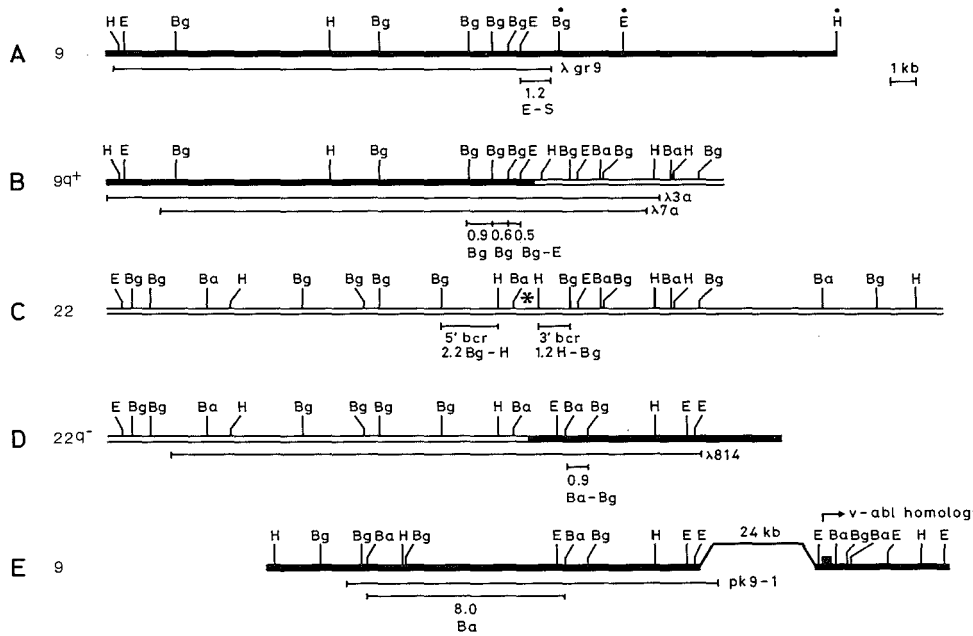

Fig. 1. Restriction enzyme map of the 9q+ (B) and 22q- (D) breakpoint regions and their normal chromosome 9 (A and E) and chromosome 22 (C) counterparts. Solid bars represent chromosome 9 sequences, whereas the open bars indicate sequences originating from chromosome 22 DNA fragments cloned in phage and probes used in the study are indicated below the relevant maps. A * marks the 0.7 kb Ba-H fragments in map C in which both chromosome 22 breakpoints are located. The restriction enzyme sites on the 3′ end of map A (indicated by a dot) are deduced from Southern blots. Ba = BamHI, Bg = BglII, E = EcoRI, H = HindIII, S = SalI.

of the 5.7 kb fragment was colinear with bcr sequences 5′ of the 0.7 BamHI - HindIII fragment (Fig.1C), whereas the 3′ end of the 5.7 kb fragment was identical to previously cloned chromosome 9 sequences (Fig. 1D,1E).These sequences, and as a consequence the 22q- breakpoint on chromosome 9, were located 30 kb at the 5′ side of the known c-abl exons (3,7). The 3′ end of the 2.4 kb chimeric fragment was colinear with the 3′ bcr sequences, confirming a breakpoint in the 0.7 kb BamHI-HindIII segment of the bcr. The chromosome 9 origin of the 5′ end of this 2.4 kb fragment was demonstrated using somatic cell hybrids containing either chromosome 9 or 22 (data not shown). We used two small BglII fragments of 0.9 and 0.6 kb as probes (Fig. 1B) to isolate recombinant phages that contained the normal chromosome 9 counterpart of this chimeric fragment. Although the CML 83-H84 EMBL-3 library contained several positive phages, restriction enzyme analysis revealed that these did not cover the 9q+ breakpoint. Therefore a positive phage (λgr.9) extending 0.6 kb 3′ of the 9q+ breakpoint was isolated from a CML-EMBL-3 library of another patient (gr)(Fig. 1A). To exclude the possibility of cloning artefacts we made chromosome 9 probes from the chimeric fragments and hybridized them to Southern blots of CML 83-H84 DNA and control cell lines. As shown in Fig. 2B the 0.5 kb, Bg-E probe (Fig. 1B) hybridizes to a normal 2.0 kb BglII fragment in all DNAs and only in the CML 83-H84 DNA the 2.4 kb 9q+ chimeric fragment is present. A 0.9 kb Ba-Bg probe (Fig. 1D) detects the 5.7 kb 22q- chimeric BglII fragment in the CML 83-H84 DNA (Fig. 2E). The normal 7.2 kb BglII fragment present in Hela and CML 83-H84 DNA is amplified at least 4 fold in K562 DNA.

Minimal distance between the two chromosome 9 breakpoints

In a recent report (8) we have demonstrated that in K562 the 22q- breakpoint is located at a distance of at least 100 kb upstream of the known c-abl sequences. This entire region, including the 5′ bcr (Fig. 2D, 8.0 kb BglII fragment), the more than 100 kb of chromosome 9 sequences upstream of the known c-abl sequences and c-abl sequences, is amplified at

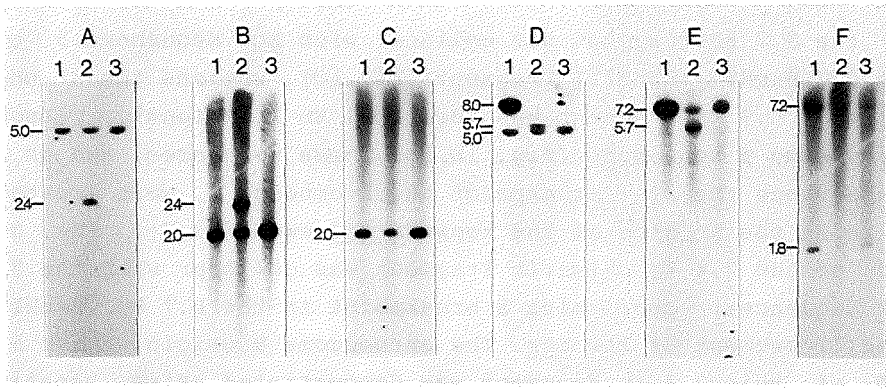

Fig. 2. Southern blots of BglII digested DNA of K562 (lane 1), CML 83-H84 (lane 2) and Hela (lane 3) hybridized with bcr probes (A,D); chromosome 9 probes (B,C,E,F). A: 1.2 kb HindIII - BglII (3´ bcr, 1C), B: 0.5 kb BglII - EcoRI (from λ 3a), C: 1.2 kb EcoRI - SalI (from λ gr.9), D: 2.2 kb BglII - HindIII (5´ bcr, 1C), E: 0.9 kb BamHI BglII (from λ 814) and F: 8 kb BamHI(from pK9-1). The used probes are indicated in figure 1 below the relevant restriction enzyme maps.

least four fold in K562. The amplification of the chromosome 9 sequences in K562 starts at the 22q- breakpoint and extends towards the telomere of chromosome 9. The 22q- breakpoint of CML 83-H84 maps 30 kb upstream the known c-abl sequences, and therefore the chromosome 9 sequences of the 22q- chimeric fragment of CML 83-H84 are also amplified in K562 (Fig. 2E, lane 1). The 2.0 kb normal BglII fragment containing the 9q+ breakpoint of CML 83-H84 is not amplified in K562 and therefore must map to the 5´ side of the K562 Ph^1-breakpoint (Fig. 2B, lane 1) and thus is located at a distance of more than 100 kb upstream of the known c-abl sequences. This indicates that the distance between the two chromosome 9 sequences is at least 70 kb.

We were not able to detect any additional hybridizing BglII fragments using either a 1.2 kb E-S probe (Fig. 1A) spanning the 9q+ breakpoint or a 8.0 kb Ba probe (Fig. 1E.

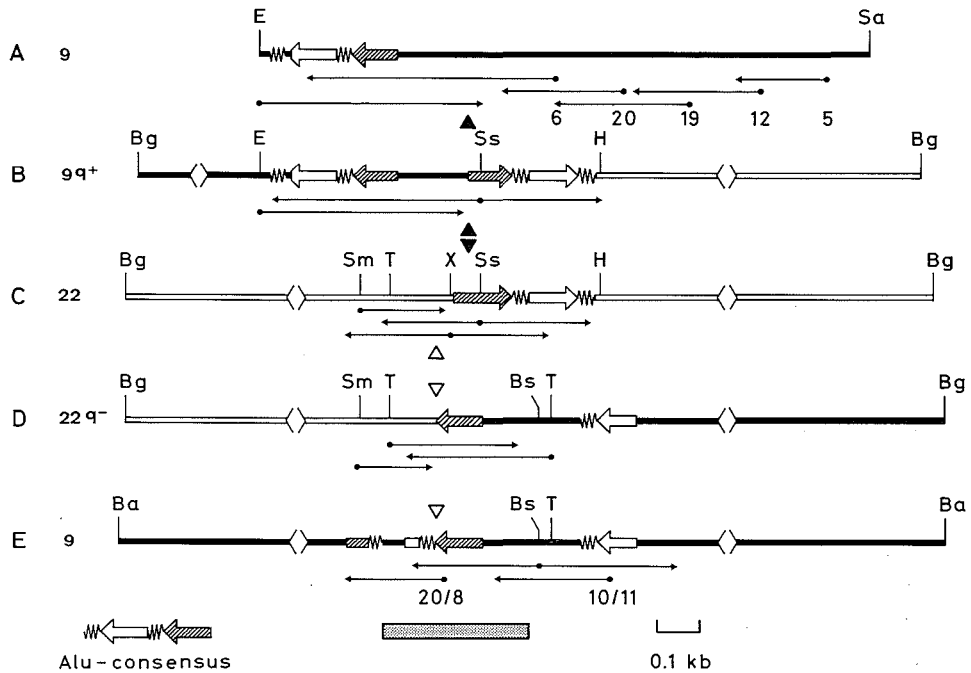

Fig. 3. Restriction enzyme maps of the subcloned breakpoint (B,D) and corresponding normal chromosome 9 (A,E) and 22 (C) fragments. Solid bars indicate chromosome 9 sequences and open bars indicate chromosome 22 sequences.The arrows below the relevant maps indicate the sequence strategies. All sequences were done by the method of Maxam and Gilbert (18) and dots indicate the position of the end label. Only the restriction enzyme maps and Alu-repetitive sequences around the breakpoints are shown (—≺ ≻— indicates known DNA ommitted from the figure). The hatched bar below the figure indicates the position of the 300 bases shown in figure 4. The position and orientation of Alu-repetitive sequences are indicated in the maps using the Alu-consenses symbol shown at the bottom. The 300 bp Alu-consensus (19) consist of two nearly homologous halves (arrows) each followed by an A-rich tract (zigzags)(12). The used subclone (see also figure 1): A: 1.2 kb EcoRI - SalI, B: 2.4 kb BglII, C:5.0 kb BglII, D: 5.7 kb BglII, E: 8.0 kb BamHI, restriction enzymes: Ba = BamHI, Bg = BglII, Bs = BstII, E = EcoRI, H = HindIII, Sa = SalI, Sm = SmaI, Ss = SstI, T = TaqI, X = XhoI.

spanning the 22q- breakpoint (Fig. 2C and 2F). The previously identified 9q+ and 22q- breakpoint BglII fragments of 2.4 kb and 5.7 kb, resp. hybridize only very faintly to these probes because these parts of the probes contained several repetitive sequences which where competed out with human cot-1 DNA during hybridization. However the remaining parts of the 1.2 kb E-S or 8.0 kb Ba probes, which were able to detect the normal hybridizing 2.0 and 7.2 kb BglII fragments did not detect any additional hybridizing fragments. Probably the chromosome 9 sequences between the two breakpoints are deleted although we cannot exclude the possibility that these sequences are present somewhere else in the genome.

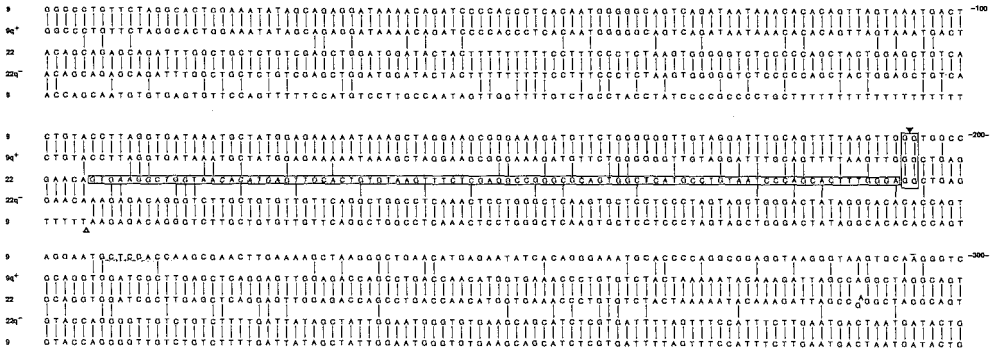

Fig. 4. DNA sequence analysis of the breakpoint subclones and their normal chromosome 9 and 22 counterparts. Sequence strategy and methods are indicated in figure 3. Only 300 nucleotides (indicated in figure 3) of the determined sequence is shown in this figure (other seqences are available on request from publisher). Sequence 9 represents the sequence of subclone A in figure 3, $9q^+$; the sequence of subclone B etc. Homology is indicated by vertical bars and the boxed sequences in the 22 sequence are the deleted chromosome 22 bcr sequences. ▲ : $9q^+$ breakpoint, △: 22q- breakpoint of CML 83-H84.

DNA sequences of breakpoints and normal counterparts

In order to determine the exact recombination site, subcloned breakpoint fragments (Fig. 3B, 3D) and corresponding normal chromosome 9 (Fig. 3A, 3E) and 22 (Fig. 3C) fragments were sequenced (18) as designated in Figure 3. The comparison of the breakpoint sequences with their normal counterparts is shown in Figure 4. At the breakpoints no nucleotides are inserted, but the boxed chromosome 22 sequences between the 22q- and 9q+ breakpoint (position 105, 193, resp.) are deleted. Comparison of the sequences with a human Alu repeat consensus sequence (19) revealed that both the breakpoints occurred in a Alu-repetitive sequence: the 9q+ breakpoint in a chromosome 22 Alu repeat and the 22q- breakpoint in a chromosome 9 Alu repeat.
The orientation and exact localization of the Alu repeats and other Alu related sequences in the vicinity of the breakpoints is shown in Figure 3.

DISCUSSION

Using a 5´ and 3´ bcr probe, we were able to identify the 22q- and 9q+ breakpoint fragments of a Ph^1-positive CML patient (CML 83-H84). The same bcr probes were used to screen a CML 83-H84 EMBL-3 phage library and positive phages containing the aberrant fragments were isolated and analysed. The chromosome 9 sequences present in the 22q- subclone were identical to previously cloned chromosome 9 sequences and map 30 kb upstream of c-abl exon a2 (8)(the first v-abl homologous exon of c-abl). These sequences were amplified in K562, a CML-derived cell line. The chromosome 9 sequences of the 9q+ breakpoint subclone were not amplified in K562. The amplification of chromosome 9 sequences in K562 starts with the chromosome 9 sequences located at the 22q- breakpoint of K562 and this whole area, including the 5´ part of the bcr gene and the c-abl oncogene is amplified. The 22q- breakpoint of this cell line maps at a distance of more than 100 kb 5´ of the c-abl exon a2 (8). Since the chromosome 9 sequences in the 9q+ breakpoint segment of CML 83-H84 are not amplified in

K562, these must be located at the 5′ side of the K562 breakpoint on chromosome 9, at a distance of >70 kb (>100-30) 5′ of the 22q- breakpoint of CML 83-H84. The chromosome 9 sequences inbetween these two breakpoints are probably deleted since no extra aberrant fragments were detectable when we used chimeric fragments as probes in a Southern blot of CML 83-H84 DNA and control lines DNA. However we cannot exclude the possibility that these sequences are present somewhere else in the genome. Sequencing confirmed that the breakpoints in the bcr of chromosome 22 were located 3′ of bcr exon b3 (6), but not at an identical site. The 88 nucleotides of bcr between the two chromosome 22 breakpoints are also deleted. Probably these deletions are the results of secondary recombination events, occurring at either the original Ph^1 or 9q+ chromosome (Fig. 5). Although we have no direct proof we favor the explanation that the second recombination event took place at the Ph^1 chromosome (Fig. 5, IIA) This would result in a shorter Ph^1 chromosome on which the 5′ bcr exon b3 is located 30 kb 5′ of c-abl exon a1, whereas on the original Ph^1 chromosome the distance between these two coding regions is more than 100 kb. This second recombination could either be necessary to remove chromosome 9 inhibitory or regulatory sequences in order to allow transcription of the chimeric bcr/c-abl mRNA, or it provides the resulting leukemic cell with growth advantages so that it has replaced the normal stem cells and original Ph^1 positive leukemic cells. In other CML′s, such as CML 0319129 (6) where the recombination took place at chromosome 9 sequences located 14 kb 5′ of c-abl exon a1, no such secondary recombinations are necessary. The breakpoint sequences of this patient (CML 0319129) showed a perfect conservative break without loss of chromosome 9 or 22 sequences. Comparison of the DNA sequences at the breakpoint regions of CML 83-H84, K562 (20) and two other CML patients (6) suggests that homologous recombination is unlikely since there is no apparent homology between chromosome 9 and 22 breakpoint sequences. Nor is there any evidence for crossover within an homologous oligonucleotide.

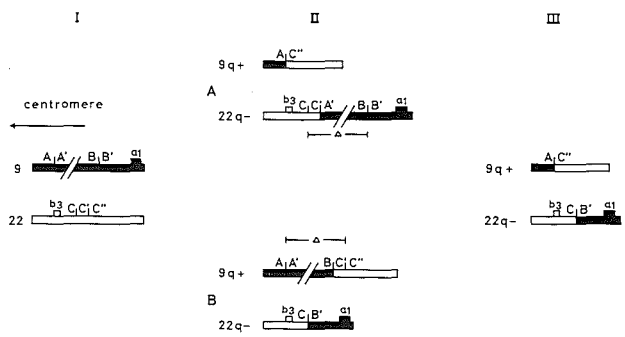

Fig. 5. Hypothetical model of the Ph^1 translocation and secondary recombination in CML patient 83-H854.
I. The normal chromosome 9 and (closed bars) chromosome 22 (open bars).
II. The resulting translocation products of the initial Ph^1 translocation and
III. The result of either a recombination of the 22q- chromosome (IIA) or the 9q+ chromosome (IIB) A,A´,B,B´are chromosome 9 sequences; C, C´,C´´ are chromosome 22 sequences, A/C´´ represents the 9q+ breakpoint whereas C/B´ represents the 22q- breakpoints shown in figure 1B and 1D resp. -//- indicates a gap of at least 70 kb. b3 = bcr exon b3 (6) a1 c-abl exon a1 (8). The distances are not in scale.

Similar results have been obtained from sequence analysis of the t(8;14) translocations in Burkitt lymphoma, in which no obvious homology was detectable between the recombined sequences on chromosome 8 or 14 (21,22). However, in CML there is some evidence that Alu-repetitive sequences are involved. In CML 83-H84 both the 22q- and the 9q+ breakpoint occur within an Alu-repetitive sequence. Similar homology to Alu-repetitive sequences are present at the breakpoints of two other CML patients (6,12) the cell line K562 (20) and the

22q- breakpoint of a Ph^1 positive Acute Lymphoblastic Leukemia (ALL) patient (23). Illegitimate recombination within Alu sequences has also been reported in non-CML related recombination events (24,25). Therefore it is well conceivable that Alu-repetitive sequences are hot spots for recombination and as such play a role in the juxtaposition of the 5´ bcr and c-abl sequences. The apparent transcription of a chimeric bcr/c-abl mRNA (9,26) could be facilitated by secondary recombination events, bringing the bcr exons in closer proximity to the c-abl coding regions. But the question remains open whether the translocation product of this mRNA, the 210 kD abnormal c-abl protein is the cause or merely a consequence of transformation in CML.

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PAPER VII

BLOOD: (IN PRESS)

bcr Rearrangement and Translocation of the c-*abl* Oncogene in Philadelphia Positive Acute Lymphoblastic Leukemia

By A. De Klein, A. Hagemeijer, C.R. Bartram, R. Houwen, L. Hoefsloot, F. Carbonell, L. Chan, M. Barnett, M. Greaves, E. Kleihauer, N. Heisterkamp, J. Groffen, and G. Grosveld

The Philadelphia (Ph[1]) chromosome, the cytogenetic hallmark of chronic myeloid leukemia (CML), has also been detected in a significant number of acute lymphoblastic leukemias (ALL). Using in situ hybridization, we demonstrate that in accordance with observations in CML the Ph[1] chromosome in ALL patients is the result of a reciprocal translocation of the c-*abl* oncogene to the Ph[1] chromosome. Southern blot analysis using *bcr* probes, however, suggests that Ph[1]-positive ALL includes heterogeneous leukemic subtypes: six ALL patients showed *bcr* rearrangements as observed in CML; in three other patients recombination involving 5′ *bcr* sequences could be demonstrated, but the corresponding translocated 3′ *bcr* sequences were not detectable. A third group of five patients did not show any *bcr* rearrangements at all. Northern blot analysis using RNA from three Ph[1]-positive ALL patients revealed that in the leukemic cells of two patients larger c-*abl* mRNA transcripts were present as in CML. In the RNA of one patient without a detectable *bcr* rearrangement, only the normal c-*abl* mRNA transcripts are present. The observed heterogeneity in *bcr* rearrangements of this group of Ph[1]-positive ALL patients is in contrast with the consistent results obtained in more than 50 Ph[1]-positive CML patients investigated in chronic and acute states.

THE PHILADELPHIA (Ph[1]) chromosome, an abnormal chromosome 22, is strongly associated with one type of human leukemia, chronic myeloid leukemia (CML).[1,2] It is found in the leukemic cells of more than 90% of all CML patients, both in the chronic and acute (blast crisis) phase of the disease.[3] The Ph[1] chromosome has also been reported in other malignant hematopoietic disorders,[4] including different subtypes of acute leukemias in which no preceding chronic phase has been observed.[5-8] The Ph[1] chromosome is found in the leukemic cells of 2% to 3% of the patients presenting with acute myeloid leukemia (AML) and a similar incidence (2% to 6%) is reported for childhood acute lymphoblastic leukemia (ALL).[9,10] In adult ALL the Ph[1] chromosome is the most frequent chromosomal abnormality with an incidence of 17% to 25%.[3,11] The clinical distinction between blast crisis of CML and de novo Ph[1]-positive acute leukemia is not always clear.[5,12,13] However, at the time of blast crisis the majority of the CML patients exhibit a karyotypic evolution characterized by additional nonrandom chromosomal aberrations such as trisomy 8 and 19, a second Ph[1] or isochromosome 17q[3]. Furthermore, the presence of Ph[1]-negative cells in the bone marrow during the acute phase and the elimination of Ph[1]-positive cells from the bone marrow during remission are typical features of cases presenting as Ph[1]-positive acute leukemias with no known prior CML.

In CML as well as in acute leukemias, the Ph[1] chromosome usually results from a translocation between chromosome 9 and 22t(9;22)(q34;q11). Previous studies using the leukemic cells of Ph[1]-positive CML patients indicated that in CML this translocation is reciprocal.[14] A consistent translocation of the human c-*abl* oncogene, normally located on chromosome 9q34, to the Ph[1] chromosome was observed in standard and variant translocations.[15] In all these Ph[1]-positive CML patients the c-*abl* oncogene was translocated to a specific, limited area on chromosome 22, the breakpoint cluster region (*bcr*).[16] Recently we have established that *bcr* is part of a gene[17] and as a consequence of the Ph[1]-translocation the "bcr" gene located in this area is disrupted and c-*abl* sequences are linked to the 5′ *bcr* sequences.[18] Transcription of this DNA segment results in a new 8.5-kb mRNA species, which contains 5′ *bcr* and 3′c-*abl* sequences.[19-23]

Since the Ph[1] chromosomes of CML and acute leukemias are cytogenetically indistinguishable, it was of interest to investigate whether the described molecular aspects of the Ph[1] chromosome in CML are also present in Ph[1]-positive acute leukemia. Here we report studies on the Ph[1] chromosome in ALL patients which demonstrate that similar molecular characteristics can be found in some but not all Ph[1]-positive ALL patients.

MATERIAL AND METHODS

Patients. We investigated the leukemic cells of 22 ALL patients referred to the cytogenetic units at Rotterdam (patients R1 through R7), Ulm (patients U1 through U6) and London (patients L1 through L9). The ALL diagnosis was based on clinical and hematologic data, bone marrow morphology, cytochemistry, and immunologic studies of the blast cells. Patient R1, a 23-year-old female, was studied during complete remission of Ph[1]-positive cALL. Cytogenetic studies revealed a normal karyotype (46,XX). Relevant clinical, hematologic, and cytogenetic data of all other patients are given in Tables 1, 2, and 3.

Southern blot analysis. DNA was prepared from bone marrow or peripheral blood samples as described.[24] DNA (10 μg) was digested with restriction enzymes, electrophoresed on 0.7% Agarose gels and blotted according to Southern.[25] Isolation of probes, hybridization, and washing conditions were as described in previous publications.[14,16] The *bcr* probes used are indicated in Fig 1.

In situ hybridization. Metaphases obtained from bone marrow or peripheral blood for cytogenetic analysis of the leukemic cells were also used for in situ hybridization. Technical procedures and

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Table 1. Ph¹-Negative ALL Patients

Patient No.	Age/Sex (Years)	WBC 10^9/L	% Blast BM	% Blast PB	Immunophenotype	Cytogenetics Cells Analyzed	Cytogenetics Karyotype	Rearranged *bcr**
L2	19 M	4.3	95	51	cALL	20	46, XY	—
L3	44 M	16.0	50	25	ALL†	20	46, XY	—
L4	67 F	60.6	80	78	T-ALL	20	46, XX	—
L5	36 M	70.4	99	89	cALL	20	46, XY	—
L6	25 M	15.1	99	57	cALL	50	46, XY	—
L7	17 M	21.7	95	69	cALL	8	46, XY	—

Abbreviations: BM, bone marrow; PB, peripheral blood; WBC, white blood cells.

*No *bcr* rearrangements.

†Unclassified ALL.

c-*abl* probes used were as described.[26]

In one case (U3), where the banding was poor, grain distribution on the chromosomes not involved in the translocation was calculated for the chromosomes in morphological groups (A, B, C, etc). In cases of mosaicism (R5, R6) only the hybridization data on the Ph¹-positive metaphases have been tabulated. The expected number of grains based on the relative DNA content of the given chromosome according to Mendelsohn[27] was corrected for sex and translocation of part of chromosomal material.

RNA analysis. Total RNA was isolated using the LiCl/urea[28] method (K562, Hela, human testes, and bone marrow of ALL patient U6) or using the guanidine thiocyanate[29] method (bone marrow of patient R6 and peripheral blood of patient R2). 20 μg of total RNA was electrophoresed on a 1% Agarose gel in the presence of formaldehyde.[29] After blotting, the nitrocellulose filters were hybridized to c-*abl* and *bcr* probes as described.[22] Recombinant plasmid and cosmid clones were handled under the containment conditions following the guidelines of the Dutch Committee on Recombinant DNA Research.

RESULTS

Bcr rearrangements in ALL patients. DNA from the leukemic cells of 21 different ALL patients was screened on Southern blots using the *bcr* probes I to IV indicated in Fig 1. In the DNA of six ALL patients having leukemic cells without a Ph¹ chromosome or other karyotypic markers (Table 1), no rearrangements of the *bcr* could be detected. *Bgl*II-digested DNA, hybridized to *bcr* probes I and III, showed a hybridization signal with the normal 5.0-kb *Bgl*II fragment on chromosome 22 (L4, shown in Fig 2A). Similarly, *Hind*III-digested DNA showed the normal 10.0-, 1.8- and 4.5-kb fragments after hybridization with probes I, II, and III, respectively (data not shown). Additional hybridizing bands were not observed.

One case (R1) of Ph¹-positive ALL was studied after achievement of complete remission. Cytogenetic studies of bone marrow cells revealed a normal karyotype (46,XX). In the DNA made at this Ph¹-negative stage, only the normal *bcr* fragments were detected (Fig 2B). Of the ALL patients showing a Ph¹ chromosome either in the acute or in the relapse phase, listed in Tables 2 and 3 the DNA of 14 patients was studied. Using different digests and probes we could detect *bcr* rearrangements in the DNA of nine of these ALL patients. In five patients no *bcr* rearrangements were observed. In the DNA of eight Ph¹-positive ALL patients *bcr* rearrangements were observed. In three of these DNAs (U3, U4, and R6) only the newly generated fragment present on chromosome 22q− could be detected (R6, Fig 2E) besides the normal 5.0-kb *Bgl*II fragment. While the rearranged 3′

Table 2. Ph¹-Positive ALL Patients

Patient No.	Age/Sex (Years)	Survival Week	WBC 10^9/L	% Blasts BM	% Blasts PB	Immunophenotype	Material	Cytogenetics Cells Analyzed	Cytogenetics Karyotype*	% Ph¹ Cells	Rearranged *bcr*
L8	19 F	44	22.7	90	90	cALL	BM	20	46,XX Ph¹	90	+
L9	52 F	28	121.0	84	86	cALL	BM	30	46,XX Ph¹	80	+
R2	3 M	67	88.2	87	89	cALL	PB	45	46,XY,t(9;22)(q34;q11) + add	91	−
R3	41 M	54	9.0	95	4.5	cALL	BM	80	46,XY,t(9;22)(q34;q11) + add	55	−
R4	19 M	46	3.6	99	45	cALL	BM‡	30	46,XY,t(14;22)(q32;q11)	83	−
R5	43 M	30	1.2	65	6	cALL	BM/PB	34	45,XY,t(9;22)(q34;q11) + add	70	−
U1	38 F	28	23.8	80	42	ALL§	PB	30	46,XX,t(14;22)(p22;q11)	30	+
U2	44 M	64	202.0	90	93	mixed AL‖	PB	31	46,XY,t(9;22)(q34;q11)	81	+
U3	39 M	48	129.0	50	15	mixed AL‖	BM	40	46,XY,t(5;9;22)(q13;q34;q11)	100	+
U4	31 M	12†	98.0	89	63	cALL	PB	30	46,XY,t(9;22)(q34;q11)	100	+
U5	21 M	12†	34.0	77	34	ALL§	BM	20	46,XY,t(9;22)(q34;q11)	100	ND

For abbreviations see Table 1; −, no *bcr* rearrangements; + with *bcr* rearrangements. ND, no data available.

*Ph¹ stands for 22q− without further information of karyotype changes.

The more complex karyotypes were as follows: R2: 46,XY,t(9;22), t(3;9)(q26;p21), t(4;9q+)(p14;q21), t(5;17)(p14;q11); R3: 46, XY, t(9;22)(12.5%)/47,XY, t(9;22), +22q−(11%)/49,XY,t(9;22), +18, +21, +22q−(31.5%), R5: 45,XY, t(1;9)(q25;p21), −8, 9p+q+ [8qter → q13::9p21 → q34::22q11 → qter], 22q−.

†Was known as Ph¹-positive CML at age 9, achieved clinical remission and normal blood count until age 19.

‡Frozen BM from diagnostic phase after Ficoll.

§Unclassified ALL.

‖Both cALL-antigen− and various myeloid-antigen-positive cells.

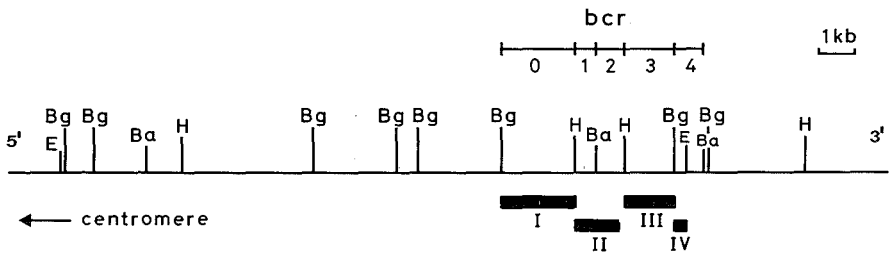

Fig 1. Restriction enzyme map of the human *bcr* locus on chromosome 22. *Bam*HI = Ba, *Bgl* II = Bg, *Eco*RI = E, *Hind* III = H. The 5.8-kb *bcr* indicated on top is artificially divided into five subregions (0-4). The probes used are indicated by solid boxes below the map. I, 2.0-kb Bg-H; II, 1.2-kb H-*Xho*II; III, 1.2-kb H-Bg; IV, 0.3-kb Bg-E.

bcr sequences (translocated to the 9q+ chromosome in CML) are not visible. Of patients R6 and U6 (Table 3) material was available from a relapse ALL phase. In each patient both *Bgl*II digested ALL- and relapse ALL-DNA showed identical aberrant *Bgl*II fragments (R6, Fig 2E).

Two of the ALL patients, L1 and R7 (Table 3) recurred with CML after 4 and 2 years of complete remission, respectively. Although the initial ALL phase of L1 was not karyotyped, the finding of a *bcr* rearrangement (Fig 2C) strongly suggests the presence of a Ph^1 chromosome. At the CML phase, a standard t(9;22) was found in the bone marrow cells, but unfortunately no DNA was available, and comparison of the molecular rearrangement of both phases was not possible. In patient R7, DNA from the ALL phase showed no *bcr* rearrangement; in contrast, DNA obtained in the CML phase did show a breakpoint in the *bcr* (Fig 2D). These results were confirmed by in situ hybridization of *bcr* probes showing segregation of the 5′ and 3′ *bcr* probes (data not shown).

Sublocalization of the bcr *breakpoints: Evidence for deletions in the* bcr. To localize the *bcr* breakpoints more precisely, we arbitrarily divided the 5.8-kb *bcr* into the five segments shown in Fig 1. In a manner analogous to the mapping of the Ph^1 breakpoints in CML patients (16) we sublocalized the 5′ and/or 3′ breakpoints of the 10 *bcr* (+) DNAs (nine ALL and the CML phase of patient R7). Usually the break in chromosome 22 splits the *bcr* in two segments: the 5′ *bcr* segment, oriented towards the centromere and remaining on the 22q− chromosome, and a 3′ *bcr* Ph^1 translocation. These segments are identified by Southern blot analysis using the 5′ and the 3′ *bcr* probe (probes I and III, respectively, Fig 1). The results are given in Table 4. In six patients the breakpoint regions determined with various probes and digests are not located in the same subregion of the *bcr*. In two of these patients (L1 and L9) both breakpoints could be localized, although in a different subregion. In patient L1 probe I and probe III (5′ *bcr* and 3′ *bcr*, respectively, Fig 1) detect two different new hybridizing bands in *Bgl*II-digested L1 DNA. This indicates that both breakpoints are situated within the *bcr*. In *Hind*III-digested L1-DNA, probe II and probe III detect different extra hybridizing bands. Since probe II detects only the 5′ *bcr* segment in *Bgl*II-digested L1-DNA, the 5′ breakpoint is located in the 1.8-kb *Hind*III fragment (region 1 + 2). The 3′ breakpoint is located in segment 3 and a minimum of 300 bp of *bcr* sequences is deleted. (The distance between the 3′ end of probe II and the 5′ end of probe III.) In four other patients (U3, U4, R6, and R7) only one of the breakpoints could be assigned. For instance, in patient R6 the 5′ breakpoint could be mapped 3′ of the *Bam*HI site in segment 2 of the *bcr*: abnormal *Bgl*II fragments with probe I and probe II, normal *Bam*HI fragments with both probes, and an aberrant *Hind*III fragment with probe II. Probes III or IV detect only

Table 3. Ph^1-Positive ALL Patients Studied in Acute and Relapse Phases

Patient No.	Age/Sex (Years)	Survival Week (w) Years (y)	WBC 10^9/L	% Blasts BM	% Blasts PB	Immunophenotype	Material	Cytogenetics Cells Analyzed	Cytogenetics Karyotype	% Ph^1 (+) Cells	Rearranged *bcr*
R6	61 F	56 w	209	75	60	cALL	BM D	38	46,XX,t(9;11;22)(q34;p15;q11)	60	+
			28.5	90	67	cALL	BM R*	57	idem/49,XXX,t(9;11;22),3p−,6q−,+7,+8p−,+21	51/32	+
U6	23 M	48 w	66.0	95	71	cALL	PB D	15	46,XY,t(9;22)(q34;q11)	53	+
			ND	ND	ND	cALL	PB R†	ND	ND	ND	+
L1	31 M	11 y	102	90	94	cALL‡	ND	ND	ND	ND	+
			17.5	2	0	CML	BM§	20	46,XY,Ph^1	100	ND
R7‖	50 F	156 w	33.7	61	71	cALL	BM	32	46,XX,t(9;14;22)(q33;q32;q11)	60	−
			89.8	3.2	4	CML	BM	21	46,XX,t(9;22)/46,XX,9q+,i(22q−)	70/30	+

Abbreviations: see Table 1. D, diagnostic; R, relapse; ND, no data available.

*Relapse ALL occurred after 11 months of clinical complete remission, but a Ph^1-positive bone marrow.

†Relapse ALL occurred after complete remission with Ph^1-negative bone marrow.

‡Common ALL immunophenotype but hematologic diagnosis of acute undifferentiated leukemia.

§CML occurred after 4 years of complete remission.

‖The karyotype of the ALL phase has been published.[39] During the 28 months of complete remission cytogenetic analysis of the bone marrow showed a normal karyotype with a residual standard t(9;22) in three out of 220 metaphases karyotyped. Leukemia relapsed as a Ph^1-positive AML that, after treatment, transformed in CML, chronic phase. Fatal myeloid blast crisis occurred 4 months later.

the normal *bcr* fragments in either a *Bgl*II, *Hind*III, *Bam*HI, or *Eco*RI digest. Thus apparently the 3′ part of the *bcr* is deleted and the 3′ breakpoint of patient R6 maps outside the *bcr*.

In situ hybridization. Translocation of the c-*abl* oncogene was studied by in situ hybridization of c-*abl* specific sequences to metaphase chromosomes from five Ph[1]-positive ALL patients (U3, U5, R2, R5, and R6). Distribution of silver grains was uniform and at random on all chromosomes except the specific signals ($P < 0.01$) on chromosomes 9(q34) and Ph[1] in all cases investigated (Table 5).

This indicates that c-*abl* sequences are translocated to the Ph[1] chromosome. In two of the patients (R2, R5) the *bcr* was not rearranged, which suggests that in these two cases recombination of c-*abl* with chromosome 22 sequences is different from that in CML.

c-abl *transcription in ALL.* For the detection of c-*abl* mRNA a human 0.6-kb *Eco*RI-*Bam*HI DNA fragment was used as a probe. This fragment contains the most 5′ human v-*abl* hybridizing exon.[30] In order to examine whether the translocation resulted in a modification of the mRNA transcript as found in CML, RNA was isolated from the leukemic cells of 3 Ph[1]-positive ALL patients. As a control, RNA isolated from human testes or Hela cells was used as well as the RNA of a Ph[1]-positive CML blast crisis cell line, K562. As shown in Fig 3, in all RNA species the c-*abl*–specific probe detects the normal 6.0- and 7.0-kb mRNA transcripts. In addition a novel c-*abl* mRNA of 8.5 kb is present in the RNA of patients U6 (lane 2) and R6 (lane 4) as well as in K562 RNA (lane 3). This 8.5-kb mRNA is not present in the RNA of Hela cells (lane 1) or normal human testes (lane 6) or the leukemic cells of patient R2 (lane 5) exhibiting no *bcr* rearrangements in Southern blots.

The Northern blot containing the RNA of K562, patient R6, and patient R2, and human testes RNA was also hybridized to a 5′ *bcr* cDNA probe.[22] In all RNA species the normal *bcr* transcripts of 7.0 kb and 4.5 kb were present. In the RNA of patient R6 and K562 cells, this 5′ *bcr*-specific probe detects an additional hybridizing band of 8.5 kb (not shown).

Table 4. Analysis of *bcr* Breakpoints

Patient No.	5′ Breakpoint in Segment	3′ Breakpoint in Segment
L8	1,2	1,2
L9	1,2	3*
U1	2	2
U2	1,2	1,2
U3	2	—*
U4	3	—*
R6†	2	—*
U6†	1	1
L1	1,2	3*
R7	—	2*

bcr segments are indicated in Fig 1.
*Deletion of *bcr* sequences has occurred.
†Identical breakpoints in initial and relapsed phase.

DISCUSSION

Recently we showed that in CML the Ph[1] chromosome is the result of a consistent juxtaposition of the c-*abl* oncogene and the chromosome 22 *bcr* sequences.[16,26] In the present studies, using in situ hybridization, we demonstrate a trans-

Table 5. Results of In Situ Hybridization Studies With c-*abl* Probes

Patient No.	Cells Analyzed	Total of Grains On Chromosome/Background	Chromosome*	Grains on Chromosomes Observed	Expected	χ^2
U3	26	189/68	5	5	6.0	0.2
			5q−	1	2.7	1.1
			9	13	4.5	16.1
			9q+	5	8.5	1.4
			22	3	1.6	1.2
			22q−	14	1.0	169.0
U5	43	243/75	9	27	5.8	77.5
			9q+	2	6.5	3.1
			22	0	2.1	2.1
			22q−	19	1.3	241.0
R2	30	113/54	9p−†	12	2.5	36.1
			9p+q+	4	3.1	0.3
			22	0	0.9	0.9
			22q−	5	0.6	32.3
R5	31	103/46	9p+‡	13	3.1	21.6
			9p+q+	3	3.7	0.1
			22	0	0.9	0.9
			22q−	9	0.5	144.5
R6	28	85/36	9	10	1.9	34.5
			9q+	2	2.1	0.0
			11	2	1.9	0.0
			11p+	2	1.9	0.0
			22	0	0.7	0.7
			22q−	7	0.4	108.9

*Karyotype of patients as given in Table 2 and 3. †9p− = der(9) t(3;9)(q27;p21); 9p+q+ = [4pter → p14::9p21 → q34::22q11 → qter]
‡9p+ = der(9) t(1;9)(q25;p21); 9p+q+ = [8qter → q13::9p21 → q34::22q11 → qter]

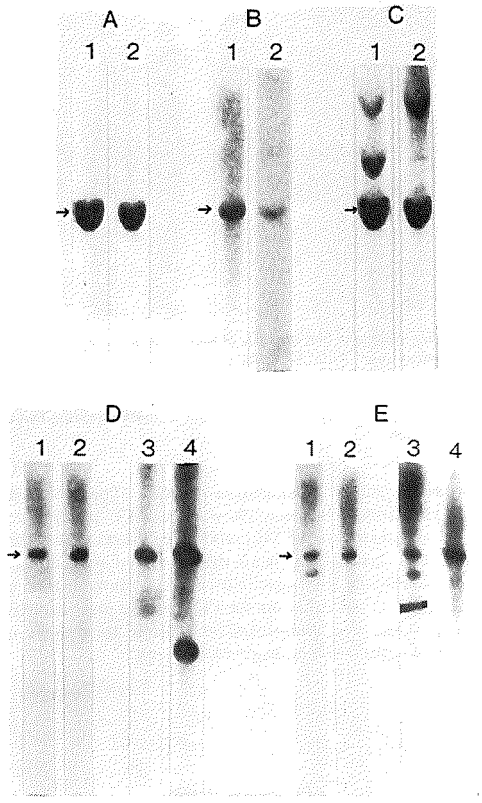

Fig 2. Southern blot analysis of *Bgl* II-digested DNA (10 μg) of the leukemic cells of five ALL patients. (A) ALL patient L4; (B) ALL patient R1; (C) ALL patient L1; (D) ALL (lane 1 and 2) and CML phase (lane 3 and 4) of patient R7; (E) ALL (lane 1 and 2) and relapse ALL (lane 3 and 4) of patient R6. Arrow indicates the 5.0 kb normal *Bgl* II fragment. Lanes 1 and 3 are hybridized with probe I (5′ *bcr*) and lanes 2 and 4 are hybridized with probe III (3′ *bcr*) indicated in Fig 1. In (C), lane 1, the hybridization signal (top band) of a previous hybridization of the same filter with probe III (3′ *bcr*) is still visible.

location of the c-*abl* oncogene from chromosome 9 to chromosome 22 in five Ph[1]-positive ALL patients. This observation proves that the Ph[1] chromosome in ALL is similar to that in CML[14] and derives from a reciprocal translocation between chromosome 9 and 22. The incidence of variant translocations (5/15) is higher than in CML, where only 5% to 8% of the patients show translocations different from t(9;22). However, in these variant translocations we also could demonstrate a translocation of the c-*abl* oncogene from 9q34 to the q11 band of chromosome 22.

In CML, a breakpoint is consistently found in the 5.8-kb *bcr* of the Ph[1] chromosome.[16] In contrast, Southern blot analysis of *bcr* of the leukemic cells from 14 Ph[1]-positive ALL patients only showed *bcr* rearrangements in nine patients. Sublocalization of the *bcr* breakpoints revealed that larger deletions involving *bcr* sequences frequently occurred in Ph[1]-positive ALL patients as compared to Ph[1]-positive CML patients. In the rare cases of CML where *bcr* deletions could be observed (D. Bootsma and A. de Klein, unpublished results), these measured 100 to 500 bp and did not result in a complete deletion of the 3′ *bcr* as in the ALL patients U3, U4, and R6. Although we did not observe *bcr* deletions during transition from chronic to acute phase of CML,[31] the 3′ *bcr* sequences are also absent in the cell line K562 established from pleural fluid of a CML patient in blast crisis.[22] However, cytogenetic analysis revealed a concordant absence of the 9q+ chromosome in K562,[32,33] whereas in Ph[1]-positive ALL patients the derivative chromosome containing the deleted part of chromosome 22 was present. Studies of other Ph[1]-positive hematologic disorders different from CML will be necessary to corroborate whether these large deletions are typical features of Ph[1]-positive ALL.

Absence of *bcr* rearrangements were found in 5 cases of Ph[1]-positive ALL. Recently, similar observations have been reported in two children with Ph[1]-positive ALL.[34] However, in two of our cases studied by in situ hybridization it was clear that one copy of the c-*abl* had moved to chromosome 22 despite the lack of *bcr* rearrangement. Since the complete *bcr* gene has not yet been identified, the possibility remains that in these cases c-*abl* is translocated to the more 5′ unidentified sequences of the *bcr* gene. The demonstration of a breakpoint within the immunoglobulin light chain gene[35] (by in situ hybridization) in one Ph[1]-positive ALL patient suggests that sequences which map more proximally on chromosome 22 than *bcr* can be involved in the Ph[1]-translocation in ALL. These observations suggest that a proportion of Ph[1]-positive ALL differ from CML by the absence of *bcr* rearrangements. The previously reported ALL cases were children, and the one childhood Ph[1]-positive ALL case that we studied (R2) was also without detectable *bcr* rearrangement; but since four other cases with no *bcr* rearrangements were adults, this does not seem restricted to childhood Ph[1]-positive ALL.

Ph[1]-positive ALL patients have a worse prognosis as compared to the Ph[1]-negative ALL patients.[36,37] In CML, Ph[1]-negative patients with no translocation of c-*abl*[38] and no *bcr* rearrangements[16] seemed to belong to a distinct subclass of leukemia with a poorer prognosis.[4] Although the number of patients we studied is small, our data indicate no difference in survival time for the Ph[1]-positive ALL patients with or without *bcr* rearrangements. A much larger series of ALL cases should be studied to ascertain any clinical relevance for diagnosis and prognosis of the distinction between Ph[1]-positive ALL with or without *bcr* rearrangement.

Two Ph[1]-positive ALL patients (U6 and R6) were studied at diagnosis and at relapse, with documented disappearance of the Ph[1] chromosome from the bone marrow during complete remission in one case (U6). Identical *bcr* rearrangements were observed in the initial and relapse ALL phase of each patient. This suggests that even if complete remission is achieved, the Ph[1]-positive progenitor cell still can remain in the hematopoietic system.

As reviewed by others,[12,13] a rarely observed feature of Ph[1]-positive ALL patients is that after achievement of complete remission, some recur with CML instead of ALL. Two such cases are included in this study: patients L1 and R7. Although we were unable, despite all efforts using frozen cells, to obtain a karyotype of patient L1, the finding of a *bcr*

rearrangement strongly suggests that this was a Ph[1]-positive ALL. The 4-year lapse between the ALL and CML phase makes it possible that CML is a secondary leukemia, but unfortunately we were not able to compare the *bcr* regions in these two phases, since no CML-DNA was available. Patient R7 showed a variant Ph[1] translocation in the initial ALL phase, and this variant Ph[1] was interpreted as a two-step rearrangement: first a standard t(9:22), followed by a t(9q+;14).[39] During the ALL phase this patient was without *bcr* rearrangements. When CML recurred a *bcr* breakpoint (with deletions) was found. This suggests either a secondary rearrangement of the original 9q+ or Ph[1] chromosome or the occurrence of a de novo Ph[1] translocation in another stem cell.

RNA isolated from Ph[1]-positive CML or Ph[1]-positive cell lines all contained the abnormally sized c-*abl* mRNA of approximately 8.5 kb.[19-22] In two of the three Ph[1]-positive ALL patients analyzed a similar 8.5-kb transcript was present. Both these ALL patients contained *bcr* rearrangements, in contrast to the third patient, and in patient R6 the same mRNA hybridizes to a 5′ *bcr* probe. This suggests that, as in CML,[21-23] in ALL the juxtaposition of c-*abl* or *bcr* sequences can result in the transcription of a chimeric mRNA, which contains 5′ *bcr* and 3′ c-*abl* sequences. Translation of this chimeric mRNA would results in a *bcr/abl* fusion protein. Evidence for the presence of such a protein in Ph[1]-positive CML cells and different CML cell lines has been reported.[40,41] An abnormally sized 210-kd c-*abl* protein is present in these cells which, in contrast to the normal 150-kd c-*abl*, has in vitro tyrosine kinase activity similar to the v-*abl* gene product.[42]

In the Ph[1]-positive ALL cases studied, translocation of the c-*abl* oncogene to the 22q− chromosome has been demonstrated by in situ hybridization. However, regarding the observed heterogeneity in respect to the *bcr* rearrangements of Ph[1]-positive ALL patients, the question remains open whether in all the ALL patients a *bcr/c-abl* chimeric mRNA is present. In those cases where neither *bcr* rearrangement nor an altered transcription of c-*abl* or *bcr* could be demonstrated, other genes or sequences could contribute to the generation and/or maintenance of ALL. The molecular aspects observed in some Ph[1]-positive ALL patients are indistinguishable from CML, reinforcing the possibility that in some of these ALL patients there may be lymphoid blast crises developing from subclinical CML.[5,12] Investigation of more patients and follow-up for a longer time may therefore finally reveal the clinical and/or biologic importance of molecular differences among these patients.

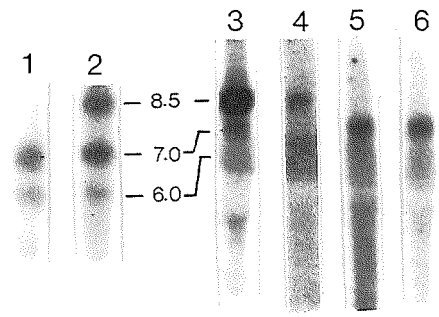

Fig 3. Northern blot analysis of RNA from three ALL patients and control cells. Total RNA (20 μg) was blotted and hybridized with a human c-*abl* probe. Lane 1, Hela cells; lane 2, ALL patient U6; lane 3, K562 cells; lane 4, ALL patient R6; lane 5, ALL patient R2; lane 6, human testes. The sizes (kb) of the hybridizing bands are indicated.

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