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Genetics

Heterogeneity of chromosome 22 breakpoint in Philadelphia-positive (Ph^+) acute lymphocytic leukemia

(c-*abl*/breakpoint cluster region/oncogene translocation/genetics of human leukemia)

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ABSTRACT **In chronic myelogenous leukemias (CML) with the t(9;22)(q34;q11) chromosome translocation the breakpoints on chromosome 22 occur within a 5.8-kilobase segment of DNA referred to as "breakpoint cluster region" (bcr). The same cytogenetically indistinguishable translocation occurs in approximately 10% of patients with acute lymphocytic leukemias (ALL). In this study we have investigated the chromosome breakpoints in several cases of ALL carrying the t(9;22) translocation. In three of five cases of ALL we found that the bcr region was not involved in the chromosome rearrangement and that the 22q11 chromosome breakpoints were proximal (5′) to the bcr region at band 22q11. In addition, we observed normal size bcr and c-*abl* transcripts in an ALL cell line carrying the t(9;22) translocation. We conclude, therefore, that if c-*abl* is inappropriately expressed in ALL cells without bcr rearrangements, the genetic mechanism of activation must be different from that reported for CML.**

More than 90% of chronic myelogenous leukemias (CML) carry a small marker chromosome, the Philadelphia (Ph) chromosome (1), that is the result of a reciprocal translocation between chromosomes 9 and 22 with breakpoints at 9q24 and 22q11 (2). Recent studies have indicated that the human homologue (c-*abl*) of the Abelson leukemia virus oncogene is located at band 9q24 and is translocated to band 22q11 in CML (3). In CML the breakpoints on chromosome 22 seem to cluster within a short segment of DNA, approximately 5.8 kilobases (kb) in length, for which the name breakpoint cluster region (bcr) has been proposed (4). Analysis of CML cells for the expression of c-*abl* transcripts has indicated that an aberrant 8.0-kb c-*abl* RNA is present (5). In addition, immunoprecipitation of CML extracts with antisera specific for the c-*abl* gene product has demonstrated that CML cells express an aberrant p210 c-*abl* product that has protein kinase activity (6). Further investigation of the 8.0-kb aberrant c-*abl* transcripts has indicated that they contain sequences derived from chromosome 22 (the bcr gene) and from chromosome 9 (the c-*abl* oncogene) (7). Thus, the genetic defect in CML seems to be the result of the fusion of two genes, bcr and c-*abl*, leading to the expression of a hybrid mRNA and of a hybrid protein that acquires protein kinase activity (7). Interestingly, because of the bcr c-*abl* gene fusion, the c-*abl* gene product loses its amino terminus similarly to what occurs in the case of Abelson leukemia virus (6, 7).

Approximately 10% of human acute lymphocytic leukemias (ALL) carry a t(9;22) chromosome translocation (8). We investigated whether the 22q11 breakpoints in different cases of ALL with this rearrangement occur within bcr or whether in these cases a different gene rearrangement is involved.

MATERIALS AND METHODS

Cells. Leukemia cells were obtained from peripheral blood from patients 1–6 and were isolated by Ficoll-Paque separation (9). The leukemia cells from patient 4 after relapse were hybridized with BW5147 mouse T-cell leukemia cells deficient in hypoxanthine phosphoribosyltransferase in the presence of polyethylene glycol 1000 as described (9). Hybrids were selected in hypoxanthine/aminopterin/thymidine (HAT) medium containing 0.1 μM ouabain (9). Cell lines K562 and Bv173 are derived from CML patients (10, 11) and Daudi has been established from a Burkitt lymphoma (12). The ALL-1 cell line was established from bone marrow cells from patient 5 with 10% conditioned medium from the Mo T-cell line (M.V. and G.R., unpublished results).

Chromosome Analysis. Parental and hybrid cell chromosomes were studied by the trypsin/Giemsa banding method as described (12).

Gel Electrophoresis and Southern Transfer. High molecular weight DNA was digested with restriction endonucleases for 5 hr and 10-μg samples were fractionated on 0.8% agarose gels and blotted to nitrocellulose filters essentially as described by Southern (13).

Preparation of Labeled DNA Probes. Hybridization probes were prepared with calf thymus primers to specific activities of approximately 10^9 cpm/μg (9). The C_λ probe is a genomic clone that contains an 8.0-kb fragment that includes Ke^-Oz^- and Ke^-Oz^+ (14). The c-*sis* oncogene probe is a 1.7-kb *Bam*HI fragment that comprises the 3′ exon of the human c-*sis* gene (15). The C_ε probe is a 2.7-kb *Bam*HI fragment that has been subcloned from λCH38 (16, 17). This probe also hybridizes with the pseudo-ε gene that has been mapped to chromosome 9 (16). The c-bcr probe is a 430-base-pair (bp) *Eco*RI–*Pst* I fragment derived from the 5′ end of clone K38 cDNA (7). The bcr genomic probe was purchased from Oncogene Science (Mineola, New York) and consists of a 1.2-kb human bcr restriction fragment that has been used to detect rearrangement in >30 Ph^+ CML patients (4). The c-*abl* probe is a 700-bp fragment isolated from a K562 cDNA library using a 1.6-kb *Bgl* II–*Bgl* II v-*abl* probe (G.R., unpublished).

Hybridization. DNA on nitrocellulose sheets was hybridized to a [^{32}P]DNA probe in 0.6 M NaCl/60 mM sodium citrate, pH 7.0/50% formamide at 37°C for 36 hr. Final washes were 15 mM NaCl/1.5 mM sodium citrate/0.1% $NaDodSO_4$ at 65°C. After air-drying, the filters were exposed to Kodak XAR film for various times.

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Abbreviations: CML, chronic myelogenous leukemia(s); ALL, acute lymphocytic leukemia(s); bcr, breakpoint cluster region; kb, kilobase(s); bp, base pair(s); TdT, terminal nucleotidyltransferase.

***In Situ* Hybridization.** The bcr- and C_λ-containing plasmids were nick-translated with all four [^{3}H]dNTPs and hybridized to human metaphase chromosome preparations from peripheral blood cells of a normal male and from patients 4 and 5. The techniques used for *in situ* hybridization were essentially as described by Harper and Saunders (18). Human metaphases were hybridized in 50% formamide, 0.3 M NaCl/30 mM sodium citrate, 10% dextran sulfate, and 100 μg of sonicated salmon sperm DNA per ml at 37°C for 15 hr. Autoradiography was performed by using NTB-2 Kodak emulsion for 10–16 days before development. The slides were stained for 5 min in a mixture of six parts of borate buffer (pH 9.2) to one part of Wright's Giemsa stain solution (19). Because of the technical limitations on the quality of the banding, 22 and Ph were identified individually when possible, or they were included with unidentifiable G-group chromosomes. The long arms of the C-group chromosomes were treated collectively (Cq) and included the 9q+.

RNA Transfer Analysis. Poly(A)$^+$ cytoplasmic RNA was extracted by subjecting cells to a brief hypotonic treatment, lysing the cytoplasmic membrane in 0.5% Nonidet P-40 in the presence of Sarkosyl, and spinning the lysate through a cushion of 5.7 M CsCl, followed by oligo(dT) column chromatography as described (20). RNA was denatured at 65°C in 50% formamide/2.2 M formaldehyde, electrophoresed in 1% agarose, and transferred to a nitrocellulose membrane. Prehybridization and hybridization to nick-translated c-*abl* and bcr cDNAs were performed as described by Thomas (21).

Phenotype Analysis of Leukemia Cells. The expression of terminal nucleotidyltransferase (terminal transferase; TdT) was determined by indirect immunofluorescence microscopy. Leukemia cells were also examined by indirect immunofluorescence using an Ortho cytofluorograph cell sorter (22) after reaction with mouse monoclonal antibodies against surface markers of human hematopoietic cells. The OKT3, OKT11, OK11, and OKM1 antibodies were purchased from Ortho Diagnostics. The B1 and J5 monoclonal antibodies were purchased from Coulter.

RESULTS

Phenotypic Characterization of Ph$^+$ ALL. Table 1 summarizes the results of the analysis of five cases of Ph$^+$ ALL for the expression of surface markers of hematopoietic cells. These five cases included two children (patients 5 and 7) (Fig. 1), two young adults (patients 3 and 4), and a 70-year-old woman (patient 6) (Table 1). We also included two additional

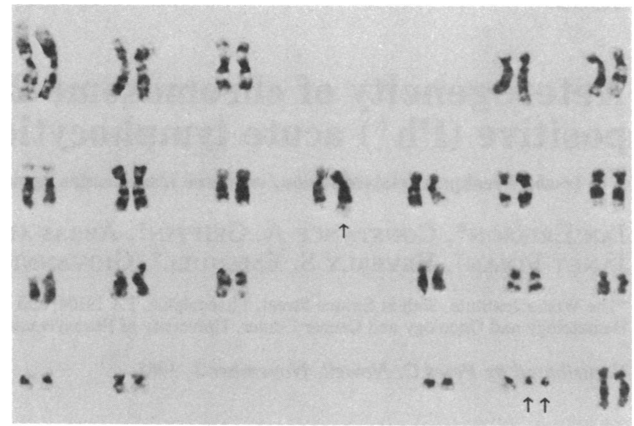

FIG. 1. Karyotype of the leukemia cells of patient 5. The leukemia cells carried a t(9;22)(q34;q11) translocation and two copies of the Ph chromosome.

patients, one with acute myelogenous leukemia and the other with acute undifferentiated leukemia; both had a t(9;22)(q34;q11) translocation (patients 1 and 2).

As shown in Table 1, analysis of the leukemia cells of all ALL patients for the expression of hematopoietic surface antigens and of TdT indicated that they were pre-B cells, since they reacted with the J5 and the Ia1 antibodies, which recognize the common ALL antigen and the DR specificities, respectively, and were positive for TdT.

Analysis of Leukemia Cells for bcr Rearrangements. The *Bam*HI and *Bgl* II restriction enzymes have been used to detect bcr rearrangements in CML (4, 7). We investigated our patients' leukemia DNAs with a number of restriction enzymes. As shown in Fig. 2*A*, by using a bcr probe we did not observe rearrangement of the bcr gene in *Bam*HI-cleaved DNA derived from patients 3 (lane 2), 4 (lane 3), 5 (lane 7), and 7 (lane 8). In all of these cases we observed only a germ-line 3.3-kb *Bam*HI band. In a Ph$^+$ CML cell line, Bv173 (11) (lane 4), and in a fresh case of CML (lane 1) we detected a germ-line band and a rearranged bcr band. With the *Bam*HI restriction enzyme, we also detected a germ-line band and a rearranged bcr band in patients 1 and 2 (lanes 5 and 6, respectively). Cleavage with *Bgl* II confirmed the rearrangement in control Bv173 cells and in patients 1 and 2, whereas we did not detect rearrangement in patients 3, 4, 5, and 7 or in Daudi Burkitt lymphoma cells (data not shown). Utilizing other enzymes, as shown in Fig. 2*B*, we detected bcr

Table 1. Characterization of acute leukemias carrying a t(9;22)(q34;q11) chromosome translocation

Patient	Age	Sex	Diagnosis	TdT	Surface membrane marker						bcr rearrangement
					OKT3	OKT11	Ia1	B1	J5	OKM1	
1	60	M	AML	Neg	0	1	25	ND	0	95	+
2	37	M	AUL	Pos	9	9	53	2	4	26	+
3	27	F	ALL	Pos	4	5	80	55	89	17	+
4	18	F	ALL	Pos	6	8	90	80	83	14	−
5	7	F	ALL	Pos	0	1	88	10	96	10	−
6	70	F	ALL	Pos	28	37	65	58	46	19	+
7	4	F	ALL	Pos	ND	0	98	ND	75	ND	−

M, male; F, female; AML, acute myelogenous leukemia; AUL, acute undifferentiated leukemia; Neg, negative; Pos, positive; ND, not done. Numbers indicate the percentage of cells reacting with each monoclonal antibody as determined by immunofluorescence flow cytometry. Leukemia cells were >90% of cells analyzed except patient 6. The T11 and T3 positivities in this patient were due to contaminating normal cells. Diagnosis was determined by French–American–British morphological classification. TdT activity of leukemia cells as determined by immunofluorescence microscopy. A result was considered positive if >10% of cells were reactive. OKT3, mature peripheral blood T cells and late thymocytes; OKT11, erythrocyte rosette receptor on T cells; Ia1, nonpolymorphic determinant of DR; B1, mature peripheral blood B cells and malignant B cells from disorders, including CML, Burkitt lymphoma, B-cell lymphomas, and non-T-cell acute lymphoblastic leukemia (Coulter); J5, common ALL antigen (CALLA) (Coulter); OKM1, monocytes, granulocytes, natural killer cells, and some myeloid precursor cells.

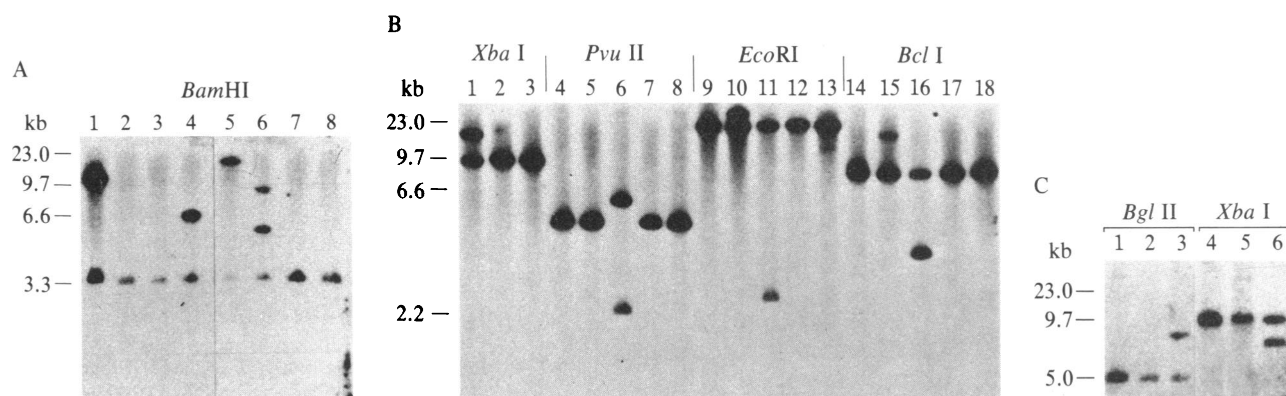

FIG. 2. Southern blotting analysis of patient and control DNAs hybridized with the bcr genomic probe: (A) *Bam*HI-digested DNA detects rearrangement in a CML Ph$^+$ patient (lane 1) and a CML cell line, Bv173 (lane 4), as well as in patients 1 and 2 (lanes 6 and 5) but not in patients 3, 4, 5, and 7 (lanes 2, 3, 7, and 8). (B) Use of additional enzymes on those patient DNAs that showed no detectable bcr rearrangement with *Bam*HI and *Bgl* II reveals rearrangements in patient 3 using *Eco*RI and *Bcl* I (lanes 10 and 15) but not *Pvu* II (lane 5). Bv173 control DNA shows rearrangement of bcr with *Xba* I, *Pvu* II, *Eco*RI, and *Bcl* I (lanes 1, 6, 11, and 16), whereas Daudi Burkitt DNA shows no rearrangement (lanes 4, 9, and 14). Patients 4 and 5 show no rearrangement with any of the enzymes used (lanes 2 and 3, lanes 7 and 8, lanes 12 and 13, lanes 17 and 18). (C) Patient 6 shows bcr rearrangement with *Bgl* II and *Xba* I (lanes 3 and 6) as compared with patients 3 and 4, who show no rearrangement with either enzyme (lanes 1 and 2 and lanes 4 and 5, respectively).

rearrangements in *Xba* I-cut DNA of Bv173 (lane 1) but not in patients 5 and 4 (lanes 2 and 3). We also detected bcr rearrangement in *Pvu* II-cut DNAs of Bv173 (lane 6) but not in DNAs of Daudi (lane 4), patient 3 (lane 5), patient 5 (lane 7), and patient 4 (lane 8). However, *Eco*RI- and *Bcl* I-cut DNAs of patient 3 (lanes 10 and 15, respectively) and of control Bv173 (lanes 11 and 16, respectively) cells showed bcr rearrangements. These last results indicate a rearrangement in proximity to the bcr region in patient 3 with ALL.

No rearrangements following *Eco*RI and *Bcl* I digestion were detected in DNA derived from Daudi Burkitt lymphoma (lanes 9 and 14, respectively), from patient 5 (lanes 12 and 17, respectively), and from patient 4 (lanes 13 and 18, respectively (Fig. 2*B*). In patient 6 (Fig. 2*C*) we also detected bcr rearrangement following digestion with *Bgl* II (lane 3) and *Xba* I (lane 6). The DNA of patient 5 is in lanes 1 and 4, whereas the DNA of patient 4 is in lanes 2 and 5 (Fig. 2*C*).

Taken together, these results indicate heterogeneity of breakpoints on chromosome 22 in patients with ALL. In three of the five ALL patients (nos. 4, 5, and 7), we could not find rearrangements of the bcr locus. In one patient (no. 3) we could detect rearrangement only after digestion with *Eco*RI and *Bcl* I, and in patient 6 we did find bcr rearrangement following digestion with *Bam*HI (not shown) and *Bgl* II, as has been shown consistently in CML (4).

***In Situ* Hybridization to Chromosomes of Ph$^+$ ALL.** We have hybridized metaphase chromosomes of patients 4 and 5, who had no detectable rearrangements of the bcr locus, with a human C_λ genomic DNA probe (14) and with a human bcr probe (4) according to standard procedures (19). As shown in Table 2, following hybridization with a C_λ probe we detected grains on the normal chromosome 22 and on the Ph chromosome and on G group chromosomes that could not be further identified. However, we did not observe a significant increase in grain count over distal Cq, including 9q+. These results indicate that the C_λ genes remain on the Ph chromosome of ALL cells, similarly to what occurs in CML (3, 4, 23). We cannot rule out, however, the possibility that a small portion of the C_λ locus translocates to chromosome 9 in these ALL cases, and similar findings were obtained in an earlier study of patient 7 by *in situ* hybridization (24).

As shown in Table 2, following hybridization with a bcr probe we detected grains on normal chromosome 22 and on the distal Cq, including 9q+, but not on the Ph chromosome in patients 4 and 5. These results indicate that bcr is translocated to the involved chromosome 9 in these two Ph$^+$ ALL cases, which had no bcr rearrangement.

Somatic Cell Hybridization Studies. The karyotypes of the ALL cells derived from patient 4 are shown in Fig. 3. At diagnosis, the leukemia cells had only a t(9;22)(q34;q11) chromosome translocation (Fig. 3*A*). Following therapy and remission, the patient relapsed. Additional chromosomal changes were observed in the leukemia cells after relapse: a t(14;20)(q11;q13) chromosome translocation and a second copy of the Ph chromosome (Fig. 3*B*). We hybridized these leukemia cells obtained at relapse with BW5147 mouse T-cell leukemia cells deficient in hypoxanthine phosphoribosyltransferase (12). The characterization of two resultant hybrids is shown in Table 3. Two hybrid clones containing the Ph chromosome of patient 4 and without the other relevant chromosomes retained the human C_λ and c-*abl* genes but lost the bcr gene (Table 3). This result is consistent with the *in situ* hybridization data, and it also indicates that the c-*abl* oncogene is translocated to the Ph chromosome in this case of

Table 2. *In situ* hybridization to chromosomes of Ph$^+$ ALL with C_λ and bcr probes

				No. of grains over chromosome			
Probe	Case	No. of metaphases	No. of grains	22	Ph	G	Distal Cq, including 9q+
C_λ	Patient 4	106	201	32 (16%)	12 (6%)	32 (16%)	29 (13%)
	Patient 5	101	180	19 (11%)	24 (13%)	20 (11%)	28 (15%)
	Normal male	50	129	16 (12%)	NA	NA	13 (10%)
bcr	Patient 4	101	168	10 (6%)	0 (0%)	9 (6%)	43 (26%)
	Patient 5	100	148	24 (16%)	0 (0%)	0 (0%)	34 (23%)
	Normal male	50	71	14 (20%)	NA	NA	7 (10%)

NA, not applicable.

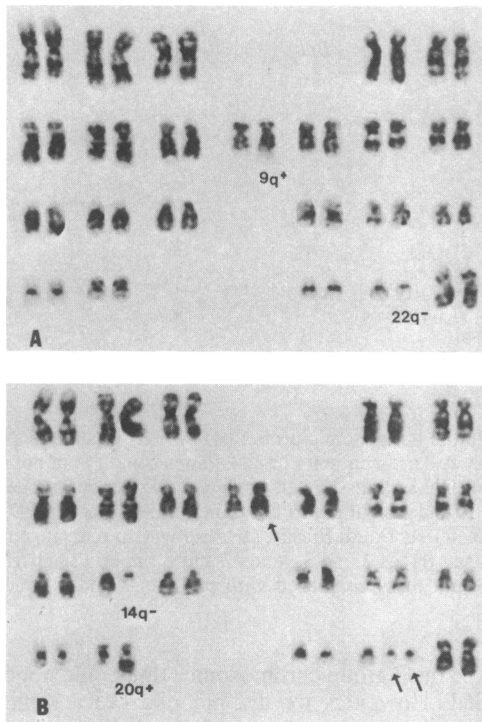

FIG. 3. Karyotypes of the leukemia cells of patient 4. (*A*) Representative trypsin/Giemsa-banded karyotype from unstimulated peripheral blood at time of diagnosis, showing Ph chromosome derived from typical t(9;22)(q24;q11) translocation. (*B*) Representative Giemsa-banded karyotype of evolved subclone in bone marrow following relapse, with second Ph chromosome (arrows) and t(14;20)(q11;q13) translocation. Twenty-nine of 30 metaphases examined had this karyotype.

ALL with the t(9;22) chromosome translocation, which had no bcr rearrangement (Fig. 4).

Thus, the results from various methods suggest that the chromosome breakpoint on chromosome 22 in those cases of ALL with no bcr rearrangement is 5′ (proximal) to the bcr and 3′ (distal) to the C_λ locus (Fig. 5).

We also examined the hybrids derived from patient 4 for the presence of the V_α and C_α regions of the locus for the α chain of the T-cell receptor to determine if this gene was involved in the t(14;20) translocation observed in the ALL cells in relapse (Fig. 3*B*). The results of the analysis indicate that the breakpoint on chromosome 14 occurred within the locus for the α chain and that the V_α genes remain on the 14q− chromosome, whereas the C_α gene translocates to the involved chromosome 20 (20q+) (data not shown). These

Table 3. Characterization of hybrids between BW5147 leukemia mouse and patient 4's leukemia cells

	Chromosome				DNA probe			
Cells	9	9q+	22	22q−*	c-*abl*†	Igλ	bcr	c-*sis*†
BW5147	—	—	—	—	—	—	—	—
Patient 4	+++	+++	+++	+++	+++	+++	+++	+++
Hybrid AA2-A2	−	−	−	+	+	+	−	−
Hybrid AA2	−	−	−	+	+	+	−	−

Percentage of cells containing the relevant human chromosome: ± = <10%; + = 10–30%; ++ = 30–50%; +++ = >50%.
*22q− and 14q− are indistinguishable cytogenetically, but the presence of Igλ and c-*abl* in hybrids that have lost the human c-*sis* gene indicates that they have retained the 22q− chromosome.
†Human.

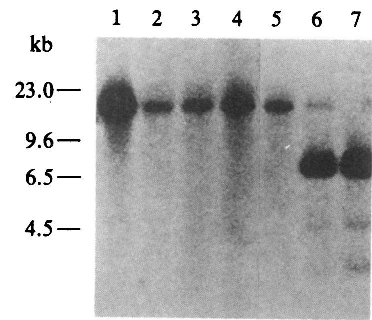

FIG. 4. Southern blotting analysis of *Hin*dIII-digested DNAs hybridized with the c-*abl* probe. Lane 1, K562 DNA; lane 2, patient 5 DNA; lane 3, patient 3 DNA; lane 4, Daudi Burkitt lymphoma DNA; lane 5, patient 4 DNA; lane 6, hybrid 514AA2-A2 DNA; lane 7, BW5147 DNA. The hybrid that contains the Ph chromosome and not the normal 9 or 9q+ chromosome is positive for the c-*abl* gene (lane 6).

results are consistent with the known orientation of the α locus of the T-cell receptor (9). Rearrangements of both immunoglobulin and T-cell receptor genes are not uncommon in B- and T-cell malignancies (25, 26), but the significance of these genes in the evolution of the disease in this patient, who had a predominantly B-cell phenotype, is not readily apparent.

Expression of the bcr and c-*abl* Genes in All Cells with No bcr Rearrangement. We have derived a cell line (ALL-1) from the leukemia cells of patient 5. The ALL-1 cell line shows the typical t(9;22)(q34;q11) chromosome translocation and contains two copies of the Ph chromosome (Fig. 1); it does not contain a rearranged bcr. RNA transfer blotting analysis of

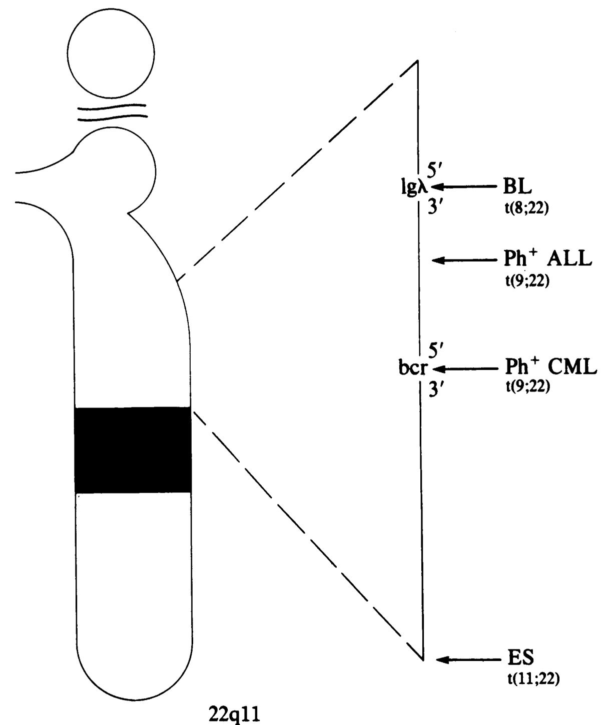

FIG. 5. Schematic representation of 22q11 breakpoints in different human malignancies. The 22q11 breakpoint in Burkitt lymphoma (BL) with the t(8;22) translocation involves directly the immunoglobulin λ (Igλ) locus. The breakpoint in Ph+ ALL of childhood with the t(9;22) chromosome translocation is proximal to the bcr and distal to the λ locus. The 22q11 breakpoint in Ph+ CML is within the bcr. The breakpoint in Ewing sarcoma (ES) is at 22q11-q12, distal to the breakpoints observed in the hematopoietic disorders.

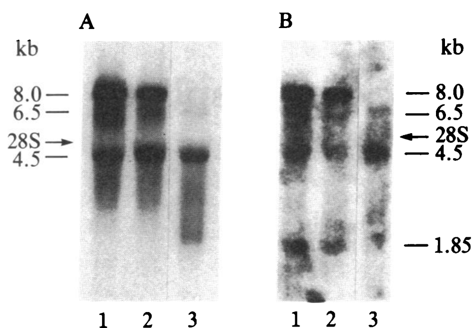

FIG. 6. RNA transfer blotting analysis of poly(A)$^+$ RNA derived from leukemia cells. The RNA from K562 (lane 1), Bv173 (lane 2), and ALL-1 cells (lane 3) was hybridized with the bcr cDNA probe (*A*) and the c-*abl* probe (*B*).

poly(A)$^+$ RNA derived from ALL-1 cells and from two other leukemia cell lines (K562 and Bv173) having bcr rearrangement is shown in Fig. 6. K562 cells carry amplified V_λ (27), C_λ (27-29), and c-*abl* (28, 29) sequences on the same marker chromosome, presumably derived from a Ph chromosome (29). Bv173 cells derived from a CML patient in blastic crisis (11). As shown in Fig. 6*A*, K562 and Bv173 cells show 8.0-kb bcr transcripts in addition to the normal 4.0- and 6.5-kb bcr transcripts. These results are consistent with previous reports (4, 7). The 8.0-kb band demonstrated by the bcr probe represents the hybrid transcript of the fused bcr and c-*abl* genes on the Ph chromosome (7). In ALL-1 cells, however, we detected the expression of the 4.0- and 6.5-kb band but not of the 8.0-kb hybrid transcript. We also did not detect altered c-*abl* transcripts (8.0 kb) in ALL-1 cells when the c-*abl* probe was used (Fig. 6*B*).

DISCUSSION

The results of this study indicate heterogeneity in the breakpoints on chromosome 22 of Ph$^+$ ALL. In a 70-year-old ALL patient, we detected rearrangement using the bcr probe and cleaving the DNA with *Bam*HI and *Bgl* II, as in cases of CML (4). We found bcr rearrangement in the ALL cells of a 27-year-old patient only after digestion with other restriction enzymes, indicating a different breakpoint within the bcr locus. We did not find, however, any bcr rearrangements in three other cases of ALL. Interestingly, two of these latter three cases were very young children (4 and 7 years old), whereas the third was an 18-year-old girl. Thus, it seems possible that ALL of childhood may have a molecular genetic basis different from that of adults.

Analysis of chromosomes of childhood ALL by the *in situ* hybridization technique and analysis of hybrids between mouse cells and ALL cells indicated that the breakpoint in childhood ALL is more proximal than the bcr on 22q11. Further information on the critical issue of whether the bcr locus is in fact involved in ALL of childhood was obtained by examining the expression of bcr and c-*abl* transcripts in a leukemia cell line from one of these children (ALL-1). Since we did not observe the characteristic CML 8.0-kb bcr and c-*abl* hybrid transcripts or any other abnormal bcr or c-*abl* transcripts in ALL-1 cells, we conclude that the bcr locus may not be involved in the t(9;22) translocation in many patients with Ph$^+$ ALL. We did find normal size c-*abl* transcripts in ALL-1 cells, and, thus, if c-*abl* is inappropriately expressed in ALL cells without bcr rearrangements, the genetic mechanism of activation must be different from that reported for CML. It will be important to determine whether the translocation of the c-*abl* oncogene 3′ (distal) to the C_λ locus may represent an "activating" mechanism or whether mutations within the c-*abl* locus itself may be involved in deregulation of c-*abl* gene expression.

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