

Heterogeneity of genomic fusion of *BCR* and *ABL* in Philadelphia chromosome-positive acute lymphoblastic leukemia

(chronic myelogenous leukemia/protooncogene/translocation)

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ABSTRACT Philadelphia chromosome-positive acute lymphoblastic leukemia occurs in two molecular forms, those with and those without rearrangement of the breakpoint cluster region on chromosome 22. The molecular abnormality in the former group is similar to that found in chronic myelogenous leukemia. To characterize the abnormality in the breakpoint cluster region-unrearranged form, we have mapped a 9;22 translocation from the Philadelphia chromosome-positive acute lymphoblastic leukemia cell line SUP-B13 by using pulsed-field gel electrophoresis and have cloned the DNA at the translocation junctions. We demonstrate a *BCR-ABL* fusion gene on the Philadelphia chromosome. The breakpoint on chromosome 9 is within *ABL* between exons Ia and II, and the breakpoint on chromosome 22 is ≈50 kilobases upstream of a breakpoint cluster region in an intron of the *BCR* gene. This upstream *BCR* breakpoint leads to inclusion of fewer *BCR* sequences in the fusion gene, compared with the *BCR-ABL* fusion gene of chronic myelogenous leukemia. Consequently, the associated mRNA and protein are smaller. The exons from *ABL* are the same. Analysis of leukemic cells from four other patients with breakpoint cluster region-unrearranged Philadelphia chromosome-positive acute lymphoblastic leukemia revealed a rearrangement on chromosome 22 close to the breakpoint in SUP-B13 in only one patient. These data indicate that breakpoints do not cluster tightly in this region but are scattered, possibly in a large intron. Given the large size of *BCR* and the heterogeneity in breakpoint location, detection of *BCR* rearrangement by standard Southern blot analysis is difficult. Pulsed-field gel electrophoresis should allow detection at the DNA level in every patient and thus will permit clinical correlation of the breakpoint location with prognosis.

A Philadelphia chromosome (Ph¹) resulting from a 9;22 translocation, t(9;22)(q34;q11), characterizes the malignant cells in virtually all patients with chronic myelogenous leukemia (CML) (1, 2), in 10% of those with acute lymphoblastic leukemia (ALL) (3), and in rare cases of acute myelogenous leukemia (4). The t(9;22) is the most common chromosomal abnormality in adults with ALL (3); it is found in one of five of these patients and in well over half of those with specific chromosomal translocations. In children with ALL, ≈6% are found to have the t(9;22). In CML and in a subgroup of Ph¹-positive ALL, the breakpoints of the translocation occur in a 200-kilobase (kb) area within the 5' introns of the *ABL* gene (5) and in the 5.8-kb breakpoint cluster region (bcr) of the *BCR* gene (6, 7) on chromosomes 9 and 22, respectively. This results in a 5' *BCR-ABL* 3' fusion gene on the Ph¹ (8, 9) that is expressed as an 8.5-kb

hybrid *BCR-ABL* mRNA. The translation product of the hybrid message is a 210-kDa tyrosine kinase (P210^{BCR-ABL}) (10). The P210 is thought to play a central role in the malignant process (11). In contrast, in many cases of Ph¹-positive ALL, the breakpoint on chromosome 22 is not in a bcr (12-14), and the 8.5-kb mRNA and the P210 are absent (15-17). Instead, the breakpoint is an unknown distance upstream from the bcr (12-14), and an abnormal *ABL* mRNA of 6.5-7.4 kb (15, 16) and a 185-kDa *ABL* tyrosine kinase (P185) are found (15-17). The mRNA and the protein have been identified as *BCR-ABL* hybrids (18-20), and analysis of DNA breakpoints in several patients are consistent with the hypothesis that the *BCR* and *ABL* genes are fused (18).

To identify the DNA sequences on chromosome 22 that are involved in the t(9;22) of ALL and that play a role in production of the P185, we studied the cell line SUP-B13, which was established from the malignant cells of a child with Ph¹-positive ALL. As has been found in CML, we observed that the t(9;22) in SUP-B13 interrupts the *ABL* gene on the 5' side of exon II. In contrast to CML, the breakpoint on chromosome 22 did not occur in the bcr; instead it occurred ≈50 kb upstream from the bcr but still within the *BCR* gene. Thus, a *BCR-ABL* fusion occurs at the genomic level, corresponding to the structure of the associated message and protein (18-20). A similar breakpoint was identified in bone marrow cells from another patient with Ph¹-positive ALL, demonstrating that the findings are not isolated to the cell line.

MATERIALS AND METHODS

Cells. The cell line SUP-B13 was established from the malignant cells of an 8-year-old child with Ph¹-positive ALL in relapse. The cell line contains a typical t(9;22)(q34;q11), lacks rearrangement of the 5.8-kb bcr (S.D.S., unpublished data), and expresses the P185 (S. S. Clark, S.D.S., and O. N. Witte, unpublished data). The complete karyotype of SUP-B13 at the time of our molecular analysis was 46,XY,-16, del(15)(q22q24),t(1;4)(p13;q31),t(4;14)(p11;q2?4),t(9;22)(q34;q11),+der(16)t(8;16)(q12;p13) (100%) (M. M. Le Beau and C.M.R., unpublished data).

Cells from the bone marrows of four adult patients were obtained at diagnosis (patients 1 and 2) or relapse (patients 3 and 4) of Ph¹-positive ALL. The patients' ages in years were 49 (patient 1), 52 (patient 2), 33 (patient 3), and 48 (patient 4). Patients 1 and 4 were female, and patients 2 and 3 were male. These cases were selected for this study, because no re-

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Abbreviations: Ph¹, Philadelphia chromosome; CML, chronic myelogenous leukemia; ALL, acute lymphoblastic leukemia; bcr, breakpoint cluster region(s).
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arrangements of the bcr were detected on Southern blot analysis.

Control human cells included normal placenta, the human T-cell leukemia cell line MOLT-16 (21), and the CML cell line BV173 (22).

Standard Southern and RNA Gel Blot Analysis. Standard methods of nucleic acid preparation, gel electrophoresis, transfer, and hybridization were performed as described for DNA (23) and RNA (24). Probes were labeled with ^{32}P by using the random primer method (25). Individual probes are described in the *Results* section and in the figure legends.

Southern Blot Analysis with Pulsed-Field Gel Electrophoresis. Preparation and restriction enzyme digestion of very high molecular weight DNA was performed as described (26). Orthogonal-field-alternation gel electrophoresis was performed by using the electrical configuration designed by Carle and Olson (27) and the apparatus designed by Schwartz and Cantor (28), as described (26). The orientation of the field was alternated every 35 seconds. Bacteriophage λ multimers and intact yeast chromosomes were used as molecular weight markers. DNA was transferred to Gene-ScreenPlus (New England Nuclear) following acid treatment of the gel (0.25 M HCl for 20 min) (26) and hybridized to ^{32}P -labeled DNA probes (25).

Construction and Screening of a Genomic Library. SUP-B13 DNA was digested to completion with *Bam*HI, size-selected on a sucrose gradient, and ligated to the arms of the bacteriophage λ vector EMBL3 (29, 30). The ligated DNA was packaged into bacteriophage with the Gigapack Plus system (Vector Cloning Systems) and screened by using Colony/PlaqueScreen hybridization filters (New England Nuclear) as described by the manufacturers.

RESULTS

Identification of Abnormal ABL Messages in SUP-B13. By using a human cDNA probe containing *v-abl* homologous exons from the 3' part of *ABL* (31), RNA gel blot analysis of polyadenylated RNA from SUP-B13 revealed the expected normal *ABL* mRNA sizes of 6.0 kb and 7.0 kb, corresponding to the sizes of the two normal alternative mRNA species, type Ia and type Ib, respectively (32). The normal type Ia and type Ib messages differ only at the 5'-most exon that is either Ia or Ib, respectively (32). There was no 8.5-kb message similar to that of CML. However, the 7.0-kb message was present in ≈ 10 -fold excess compared to a control lymphoid cell line MOLT-16 (data not shown). To distinguish this abundant mRNA species from the normal 7.0-kb *ABL* type Ib message that is generally expressed at a low level, we rehybridized the blot to a specific exon Ib probe pHab15' (5). MOLT-16 and SUP-B13 were shown to have relatively low, but approximately equal, amounts of type Ib message, demonstrating that the abundant message lacks *ABL* exon Ib. These results indicate that SUP-B13 expresses an abnormal *ABL* mRNA species similar in size to that reported in other cases of Ph¹-positive ALL (15, 16), but different from that of CML. The *ABL* exon Ib probe also recognized an abnormal 4.5-kb mRNA that is likely to be an aberrant transcript from the 9q+ chromosome, as we have observed in CML (24).

Identification of the 9:22 Translocation Breakpoint in SUP-B13 Within *ABL*. The location of the translocation breakpoint within the 240-kb *ABL* gene was determined by using pulsed-field gel electrophoresis. Two *Not*I and two *Sfi*I sites are present in the normal *ABL* gene (Fig. 1A) (5, 24, 26). In *Not*I and *Sfi*I digests of control DNA, appropriate probes identify 175-kb and 225-kb fragments, respectively (Fig. 1B); both fragments encompass the large region of *ABL* between exons Ib and Ia, whereas only the *Sfi*I fragment encompasses the region between exons Ia and II. These two

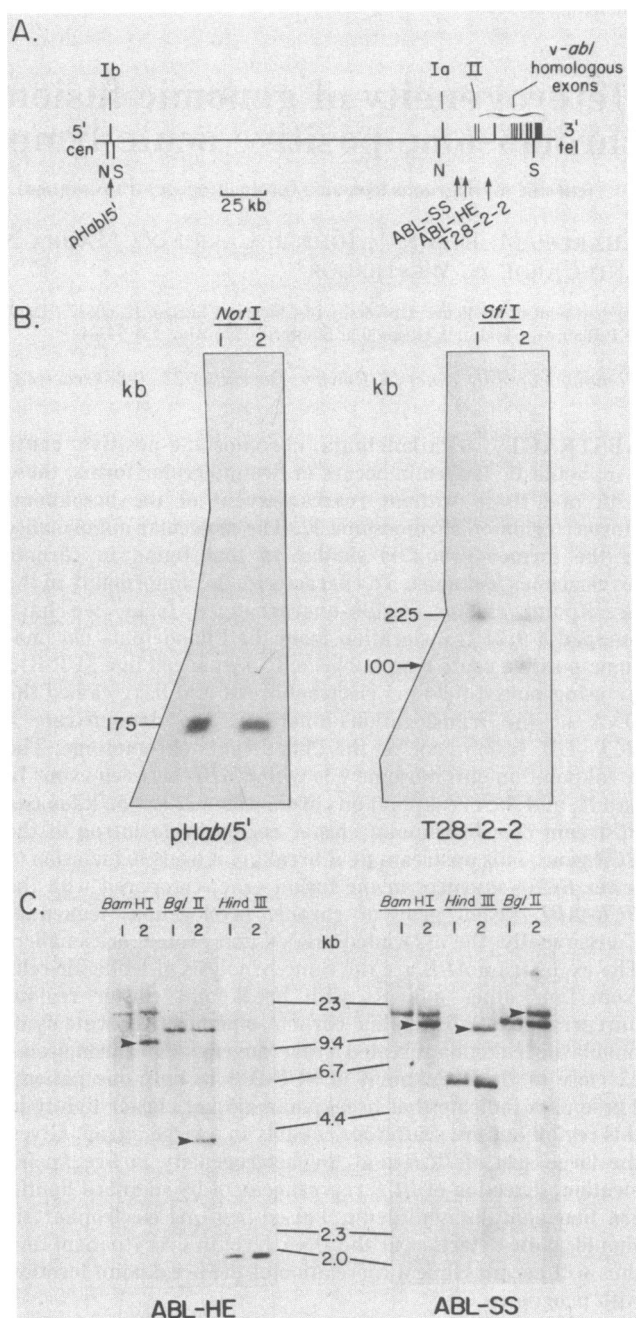


FIG. 1. Identification of a rearrangement of the *ABL* gene in SUP-B13. (A) Restriction map of *ABL* including *Not*I (N) and *Sfi*I (S) sites and the locations of known exons (5, 24, 26). The locations of the sequences recognized by the probes used in this study are indicated with arrows. (B) *Not*I- and *Sfi*I-digested DNA from a control cell line MOLT-16 (lanes 1) and from SUP-B13 (lanes 2) were hybridized to the human genomic *ABL* probes pHab15' (containing exon Ib) (5) and T28-2-2 (from an intron in the *v-abl* homologous region of *ABL*) (26), respectively. Each lane contains 10 μg of DNA prepared and digested in agarose blocks and fractionated with orthogonal-field-alternation gel electrophoresis in 1% agarose (26). Germ-line and rearranged bands are indicated by lines and arrows, respectively. (C) Southern blot analysis of genomic DNA from SUP-B13 with two probes from the intron of *ABL* between exons Ia and II (33). Each lane contains 10 μg of DNA from normal human placenta (lanes 1) or SUP-B13 (lanes 2) digested with *Bam*HI, *Bgl*II, or *Hind*III and fractionated by electrophoresis in a 0.8% agarose gel. The DNA was transferred and hybridized to the probes ABL-HE and ABL-SS. Arrowheads indicate the rearranged bands.

regions together represent the major sites of chromosomal translocation breakpoints in CML (5, 24). In SUP-B13, only

the germ-line 175-kb *Not* I fragment was present, whereas the 225-kb *Sfi* I fragment was accompanied by an abnormal 100-kb band (Fig. 1B). Therefore, we focused our attention on the region between exons Ia and II by using standard Southern blot analysis. Two probes, ABL-SS and ABL-HE derived from this intron (33), recognized different-sized rearranged bands in *Bam*HI and *Bgl* II digests (Fig. 1C). ABL-SS also demonstrated a rearrangement in a *Hind*III digest but not in an *Eco*RI digest (data not shown), whereas ABL-HE demonstrated a rearrangement in an *Eco*RI digest (data not shown) but not in a *Hind*III digest. These results indicate that the breakpoint occurred between the sequences seen by these probes, ≈ 10 kb on the 5' side of *ABL* exon II (refer to map in ref. 33). Another patient with Ph¹-positive ALL has been reported to have a breakpoint in this region (17).

Identification of the 9;22 Translocation Breakpoint in SUP-B13 Within *BCR*. Localization of the breakpoint in *ABL* enabled us to improve upon our earlier estimate of the distance between the *bcr* and the breakpoint on chromosome 22 (34). By using pulsed-field gel electrophoresis, a 350-kb rearranged band was identified in *Not* I digests of SUP-B13 with a 3' *bcr* probe (1.2-kb *Hind*III-*Bgl* II fragment) (6) (Fig. 2), in comparison with the germ-line polymorphic fragments of either >700 kb or 450 kb (34). The 350-kb band was found to cross-hybridize with the ABL-SS probe (data not shown); hence, this band contains the 9;22 junction of the 9q+ chromosome. Since there is a *Not* I site just upstream of the breakpoint in *ABL* (see Fig. 1A), virtually all of the 350-kb fragment is of chromosome 22 origin including sequences both upstream and downstream of the *bcr*. Three hundred kilobases are accounted for by the distance from the *bcr* to the next cleavable *Not* I site in the 3' direction (24), leaving ≈ 50 kb of unrearranged DNA on the 5' side of the *bcr*. This estimate of 50 kb from the *bcr* to the breakpoint is consistent with pulsed-field analysis with the enzyme *Sfi* I, which has excluded rearrangement up to ≈ 50 kb on the 5' side of the *bcr* (34).

The *Not* I digests were then rehybridized to a probe from the 5'-most exon of *BCR* (*BCR* exon 1) obtained from the 8.5-kb *BCR*-*ABL* message of CML (31). The germ-line

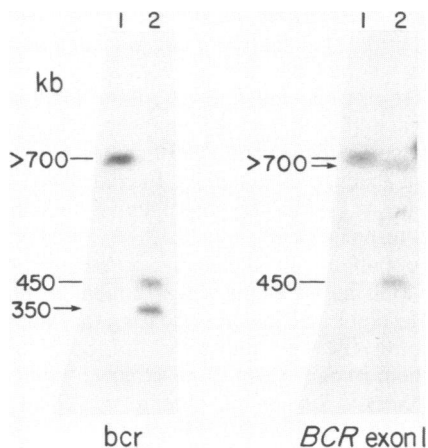


Fig. 2. Identification of a rearrangement of the *BCR* gene in SUP-B13. *Not* I-digested DNA from a control cell line MOLT-16 (lanes 1) and from SUP-B13 (lanes 2) were sequentially hybridized to the *BCR* probes *bcr* and *BCR* exon 1. Each lane contains 10 μ g of DNA prepared and digested in agarose blocks and fractionated with orthogonal-field-alternation gel electrophoresis in 1% agarose. Germ-line and rearranged bands are indicated by lines and arrows, respectively. Two germ-line fragment sizes are indicated that result from the presence of a restriction fragment length polymorphism (34). The two bands labeled together as >700 kb occur in an area of low resolution in the gel.

>700-kb and 450-kb bands in MOLT-16 and SUP-B13, respectively, were recognized by this probe, indicating that *BCR* exon 1 is normally located in the same *Not* I fragments as the *bcr* (Fig. 2). In addition, a rearranged band was observed that migrates somewhat faster than the large >700-kb germ-line band; this band cross-hybridized with the ABL-HE probe (data not shown), indicating that it contains the 9;22 junction of the 22q- chromosome. These results demonstrate that the breakpoint has occurred within the *BCR* gene between exon 1 and the *bcr*.

Isolation of the 9;22 Translocation Junctions of SUP-B13. To isolate the junctions of the t(9;22), we screened 500,000 recombinants of a genomic library made from a complete *Bam*HI digest of SUP-B13 DNA, which had been size-selected and ligated into the EMBL3 bacteriophage λ vector (29, 30). Restriction maps of the *Bam*HI inserts found in three of six clones that hybridized to the probes ABL-SS or ABL-HE are shown in Fig. 3. The map of λ abl-F16, which hybridized to both probes, corresponded to that of the normal 16-kb *ABL Bam*HI fragment (8, 33). λ abl-C10.5, which hybridized only to ABL-HE, contained a 10.5-kb *Bam*HI fragment that matched with the normal *ABL* only at the 3' end. λ abl-A11.5, which hybridized only to the ABL-SS probe, contained an 11.5-kb *Bam*HI fragment that matched with *ABL* only at the 5' end. Thus, we presumed that the latter two clones represented the rearranged bands found on Southern blot analysis (Fig. 1C) and that these contained the 9;22 junctions from the 22q- and 9q+ chromosomes, respectively.

We verified that λ abl-C10.5 and λ abl-A11.5 contained material from chromosome 22 by using a Chinese hamster ovary-human somatic cell hybrid EyeF3A6 that contains a chromosome 22 as its only human chromosome (35). The probes were the 6-kb *Bam*HI-*Sal* I fragment from the 5' end of λ abl-C10.5 and the 2.3-kb *Eco*RI-*Bam*HI fragment from the 3' end of λ abl-A11.5 (see Fig. 3). Because the former contained repetitive sequences, hybridization was performed following preassociation of the probe with a large excess of sonicated total human DNA (36). The probes hybridized to bands in *Bam*HI- and *Hind*III-digested EyeF3A6 DNA that were identical in size to those seen in normal human DNA (data not shown).

To determine whether exons were present within the cloned region of chromosome 22, the same fragments of λ abl-C10.5 and λ abl-A11.5 representing a total of 6 kb of DNA from chromosome 22 were hybridized to an RNA gel blot containing poly(A)-selected RNA from three human leukemia cell lines, SUP-B13, BV173, and MOLT-16; no homologous messages were detected (data not shown). Thus, the cloned regions represent part of an intron within the *BCR* gene.

Analysis of Patient Samples. To determine whether other cases of Ph¹-positive ALL had rearrangements near the SUP-B13 breakpoint on chromosome 22, we used the 2.3-kb *Eco*RI-*Bam*HI fragment at the extreme 3' end of the λ abl-A11.5 clone as a probe. In normal individuals, this probe recognizes a restriction fragment length polymorphism in *Bam*HI, *Bgl* II, *Eco*RI, and *Hind*III digests that is caused by a 1-kb deletion/insertion. The polymorphic fragment sizes are 5.7 kb and 4.7 kb in *Bam*HI digests and 14.5 kb and 13.5 kb in *Hind*III digests. The polymorphism is also identified in *Bgl* II (9.3 kb and 8.3 kb) and *Eco*RI (8 kb and 11 kb) digests. Preliminary data from 17 individuals indicates allele frequencies of 0.74 and 0.26 for the longer and shorter alleles, respectively, and a heterozygote frequency of 0.48 (C.M.R. and C.A.W., unpublished data).

By using the 2.3-kb *Eco*RI-*Bam*HI probe, rearrangement of one allele was detected in *Bam*HI- and *Hind*III-digested DNA from the bone marrow of a patient (patient 3) with the typical clinical and cytogenetic features of Ph¹-positive ALL

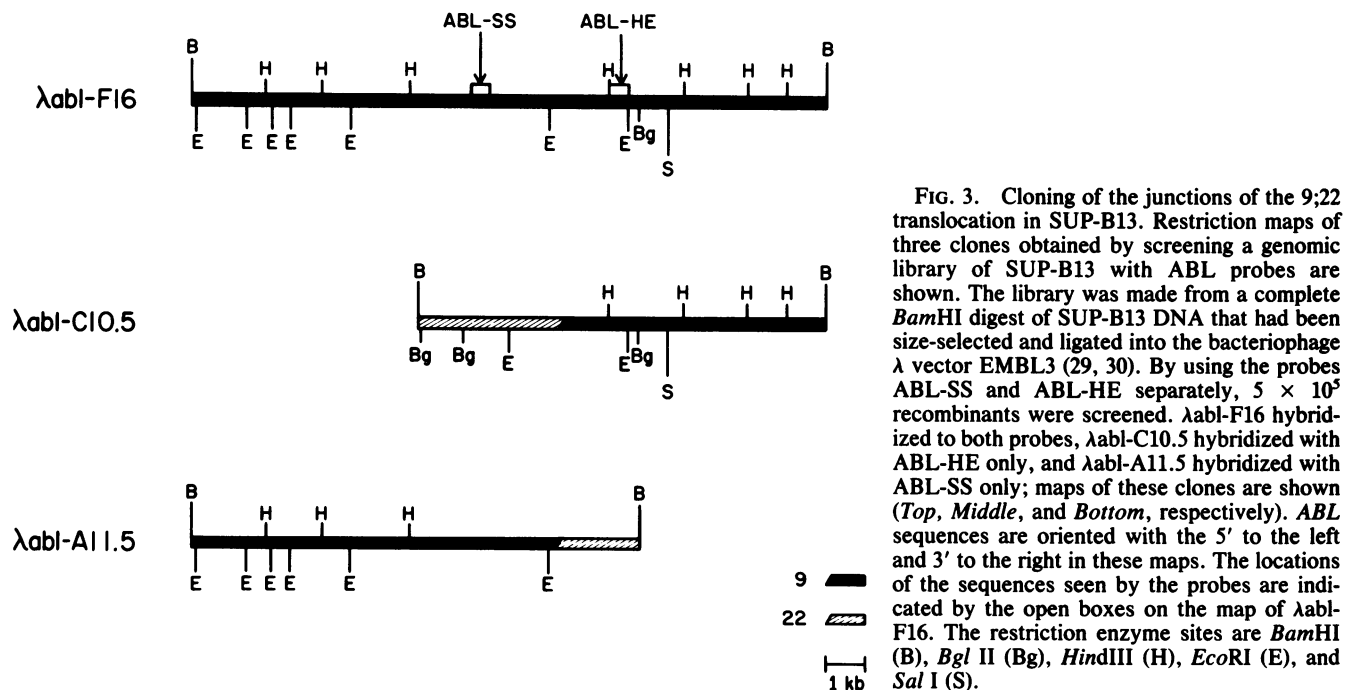


FIG. 3. Cloning of the junctions of the 9;22 translocation in SUP-B13. Restriction maps of three clones obtained by screening a genomic library of SUP-B13 with ABL probes are shown. The library was made from a complete *Bam*HI digest of SUP-B13 DNA that had been size-selected and ligated into the bacteriophage λ vector EMBL3 (29, 30). By using the probes ABL-SS and ABL-HE separately, 5×10^5 recombinants were screened. λ abl-F16 hybridized to both probes, λ abl-C10.5 hybridized with ABL-HE only, and λ abl-A11.5 hybridized with ABL-SS only; maps of these clones are shown (Top, Middle, and Bottom, respectively). ABL sequences are oriented with the 5' to the left and 3' to the right in these maps. The locations of the sequences seen by the probes are indicated by the open boxes on the map of λ abl-F16. The restriction enzyme sites are *Bam*HI (B), *Bgl* II (Bg), *Hind*III (H), *Eco*RI (E), and *Sal* I (S).

in whom no *bcr* rearrangement was present (Fig. 4), demonstrating that the SUP-B13 breakpoint is not unique in its location. No rearrangement was detected with this probe in *Bam*HI, *Bgl* II, *Hind*III, and *Eco*RI digests of DNA from three other patients with Ph¹-positive ALL in whom the *bcr* was unrearranged (patients 1, 2, and 4). The analysis surveys a 14-kb area surrounding the sequences recognized by the probe. Thus, not all breakpoints upstream of the *bcr* occur in the immediate vicinity of the SUP-B13 breakpoint.

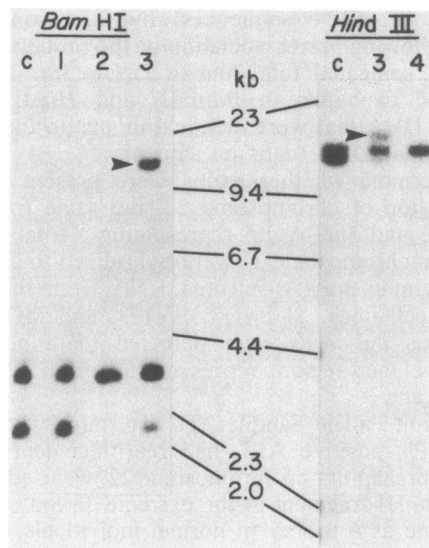


FIG. 4. Southern blot analysis of bone marrow cells from patients with Ph¹-positive ALL lacking the *bcr* rearrangement. Each lane contains 10 μ g of DNA from a control (lanes C) or patients 1–4 (lanes 1–4, respectively) digested with *Bam*HI or *Hind*III and fractionated by electrophoresis on an 0.8% agarose gel. The DNA was transferred to a nylon membrane and hybridized with the 2.3-kb *Eco*RI–*Bam*HI fragment from the extreme 3' end of the λ abl-A11.5 clone shown in Fig. 3. The control and patients 1 and 3 are heterozygous for a 1.0-kb deletion/insertion polymorphism that occurs within the *Bam*HI and *Hind*III restriction fragment seen by this probe; patients 2 and 4 are homozygous for the longer allele. In patient 3, the shorter allele is rearranged; however, faint germ-line bands are seen due to the presence of residual normal bone marrow cells. Arrowheads indicate the rearranged bands.

DISCUSSION

We have mapped and cloned the translocation junctions of the t(9;22) of the cell line SUP-B13, which represents a *bcr*-unrearranged case of Ph¹-positive ALL. We have demonstrated that the t(9;22) in SUP-B13 leads to fusion of *ABL* and *BCR* by breakage of DNA within these genes followed by reciprocal chromosomal exchange. This process appears to be similar to that which occurs in CML and, presumably, in the *bcr*-rearranged cases of Ph¹-positive ALL; however, the breaks in *BCR* occur on the 5' side of the *bcr* and lead to inclusion of 50 fewer kb of *BCR* DNA in the *BCR*–*ABL* fusion gene on the Ph¹. The *ABL* exons translocated to the Ph¹ are equivalent to those translocated in CML. The abnormal protein (P185) associated with the 5' rearrangement is smaller than the P210 presumably due to fewer amino acids encoded by *BCR* sequences. Since the substrates for these proteins have not yet been identified, it is not known whether the structural difference between the P185 and the P210 corresponds to a functional difference in the leukemic cell. It is possible that the difference is significant, however, because the P185 has not been observed in CML in the absence of the P210. Furthermore, Clark *et al.* (15) have shown that these proteins have different phosphorylation patterns *in vitro*.

In cells obtained from the bone marrow of a patient with Ph¹-positive ALL, we found a rearrangement in *BCR* close to the breakpoint of SUP-B13 indicating that the findings are not unique to the cell line. We did not find a nearby rearrangement in cells from three other patients that lacked *bcr* involvement, suggesting that a significant degree of heterogeneity exists. This may simply be due to the presence of a large intron, so that the breakpoints can vary without a change in the spliced transcript from the fusion gene. Supporting this possibility, we have shown here that the SUP-B13 breakpoint has occurred within an intron of at least 6 kb. An alternative possibility is that the breakpoints in these other patients have occurred in a different gene on chromosome 22. *BCR*-related genes have been identified on chromosome 22 and may play a role in this disease as well (37).

DNA analysis with pulsed-field gel electrophoresis appears to be a simple way to screen the *ABL* and *BCR* genes for rearrangement (24, 26, 34). Because both of these genes

are large and because translocations occur in variable locations within them, standard Southern blot analysis is ill-suited for this purpose. We show here that most of the length of the *ABL* gene and the entire *BCR* gene can be examined with only one enzyme and one probe each. The *ABL* gene can be studied best with the enzyme *Sfi* I and any of a number of *ABL* probes that recognize the 225-kb fragment produced by *Sfi* I. The *BCR* gene can be studied with *Not* I and any *BCR* probe, because the entire *BCR* gene is included in a single *Not* I fragment. Although careful study of the sensitivity and specificity of this technique is required, pulsed-field gel electrophoresis is likely to provide a rapid diagnostic test to identify the presence of a t(9;22) and to distinguish the more 5' breakpoints in *BCR* seen in some ALL patients from the 3' breakpoint in CML. This will permit prospective studies of a large series of Ph¹-positive ALL patients to correlate the location of the breakpoint with clinical features such as patient age and survival. These data will have important implications for optimal management of these patients who presently have a very poor prognosis (3).

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