Entire ABL Gene Is Joined With 5'-BCR in Some Patients With Philadelphia-Positive Leukemia

By Christine M. Morris, Nora Heisterkamp, John Groffen, and Peter H. Fitzgerald

In four patients, the chromosome 9 breakpoint of the t(9;22)(q34;q11) occurred at different sites within an 8.25-kilobase (kb) region situated 5' of ABL exon 1B. Chromosome in situ hybridization and field inversion gel electrophoresis (FIGE) studies showed that ABL exons 1A and 1B were present on the Ph chromosome. Yet this large fusion gene produced an mRNA conventional for chronic myelogenous leukemia (CML). Splicing from BCR exon 3 to ABL exon 2 crossed more than 200 kb and deleted exons 1A and 1B. This breakpoint site may occur in about 10% of all CML patients. Three of our patients have pronounced thrombocytosis, and two had been diagnosed as having Ph-positive essential thrombocytemia. The platelet count of the other patient was not available.

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MATERIALS AND METHODS

Cytogenetics and In Situ Hybridization

Leukemic cells from peripheral blood (PB) or bone marrow (BM) samples, taken for clinical evaluation when the patients were first examined or during the later course of their disease, were cultured, synchronized, and prepared for cytogenetic analysis as previously described.7 Metaphase cells were hybridized in situ with the 3'H-labeled ABL exon 1B probe, as described previously.5

Probes

The origin and identity of the BCR probes (M-Bcr-3', M-Bcr-5') and ABL probes (T-39-2-2, ABL exon 1B, T-39-1-2, ABL exon III) used in this study have been described in detail elsewhere.7 Their precise genomic locations are shown in Figs 1 and 2.

Patient DNA Analysis

High-molecular-weight (high-mol-wt) DNA was isolated from PB or BM cells, digested with restriction enzymes according to the manufacturer's guidelines, electrophoresed on 0.8% agarose, and transferred to nylon membrane (Gene Screen Plus, New England Nuclear, Boston, MA) according to the method of Southern.9 Probes were isolated from plasmid in low-melting-point agarose (Bethesda Research Laboratories, Gaithersburg, MD), oligolabeled with 32P-dCTP (Amersham International, Amersham, England), and hybridized to the filters overnight at 65°C. Filters were exposed to Kodak X-ray film for 1-3 days at ~80°C with intensifying screens. Methods for cloning and restriction mapping of patient 1 have been described previously.10,12

Field Inversion Gel Electrophoresis (FIGE)

DNA of very high mol wt, prepared from frozen cells, was digested in agarose plugs.11 The plugs were placed in a 1% agarose gel alongside mol-weight markers of S. cerevisiae chromosomes. FIGE was performed as described12,14 except that forward pulse times were increased from 9 to 116 s during a 48-hour period. The DNA was transferred to Gene Screen Plus membrane and hybridized with labeled probes. The filters were washed and sequentially rehybridized according to the manufacturer's recommendations.

Oligonucleotides

Primers CML-A and CML-B, and the oligonucleotide CML-C corresponded exactly to those described previously.15

Polymerase Chain Reaction (PCR) Analysis

RNA (2 μg), extracted by a guanidine isothiocyanate method,16 was precipitated with ethanol, washed once with 70% ethanol, and...
solved in 10 µL ddH₂O. Two-microliter aliquots (100 ng) were heated for 5 minutes at 65°C and then cooled on ice. The reverse transcriptase reaction was performed in a final volume of 20 µL in 1 x TaqI buffer (20 mmol/L Tris-HCl, pH 8.4, 50 mmol/L KCl, 2.5 mmol/L MgCl₂, 100 µg/mL nuclease-free bovine serum albumin, BSA), 1 mmol/L dNTPs, 4 mmol/L dithiothreitol (DTT), 20 U RNAsin (Promega, Madison, WI), 200 U M-MLV (BRL), and 50 pmol CML-B primer. Taq polymerase amplification was performed in a 100-µL final volume in 1 x Taq I buffer with 2 U Taq I (Stratagene, La Jolla, CA), 0.5 µL gene 32 protein (Pharmacia, Uppsala, Sweden) and 50 pmol each of CML-A and CML-B. One half of each sample was electrophoresed on a 1.5% agarose gel, blotted to nylon (Schleicher and Schuell, Dassel, Germany) and cross-linked under short-wave ultraviolet light. Blots were prehybridized for 2 hours at 65°C in 3 x SSC, 10 x Denhardt's solution, 200 µg/mL salmon sperm DNA, 10% dextran sulfate, 7% sodium dodecyl sulfate (SDS) and hybridized to the γ-32P-dATP end-labeled CML-C probe overnight at 53°C. Filters were washed at 53°C in 3 x SSC, 10 x Denhardt's, and 0.5% SDS.

RESULTS
Identification of Breakpoints 5' to the ABL Gene on Chromosome 9

Patient 1. Detailed restriction enzyme analysis of the breakpoint fragment cloned from leukemic DNA of patient 1 was described previously as patient 02120185 of Groffen et al. A 1.3-kb EcoRI fragment (probe T-39-2-2), isolated from chromosome 9 sequences of this breakpoint fragment (Heisterkamp and J. Groffen, unpublished observations, 1983), was used to isolate normal chromosome 9 sequences from two human cosmid libraries. These overlapping clones spanned more than 50 kb. A 19-kb region, mapped in detail (Fig 2A), shows that the T-39-2-2 probe is located 10.0 kb 5' of ABL exon 1B, a site also demonstrated independently. The chromosome 9 breakpoint of patient 1 was 4.2 kb 5' of ABL exon 1B (Fig 2A). The chromosome 22 breakpoint was between exons 3 and 4 of M-BCR, and the breakpoint junction had been sequenced.

Patients 2 through 4. Two groups of patients were screened for chromosome 9 breakpoints 5' of ABL. The first was a previously reported group of six patients who had clinical features of essential thrombocythemia. Five were Ph-positive, and all six showed rearrangement of M-BCR. DNA from blood cells was digested with BamHI, Bgl II, HindIII, and EcoRI restriction enzymes, and Southern blots were hybridized with the ABL exon 1B probe (Table 1, Fig 3). Patients 2 and 3 showed rearranged fragments in HindIII digests, but only germline fragments with the remaining three enzymes, which indicated that the breakpoints were 5' of ABL exon 1B (Fig 2A). A third patient of this group (patient 5) showed rearrangements in EcoRI and HindIII digests, but germline bands only with Bgl II and HindIII. Sequential hybridization with T-39-2-2, M-BCR-5' and M-BCR-3' probes indicated a chromosome 9 breakpoint approximately 1 kb 3' of ABL exon 1B (patient 5, Fig 2A, data not shown). The remaining three patients showed no rearrangement in the vicinity of ABL exon 1B.

Leukemic DNA of a second group of 28 patients, diagnosed as CML and showing rearrangement of M-BCR, was digested with the same four restriction enzymes as above, and blots were hybridized with the ABL exon 1B probe. One patient showed rearrangements in all four digests, which indicated a chromosome 9 breakpoint close to and 5' of exon 1B (patient 4, Fig 2A, Table 1). One other patient showed rearrangements in BamHI and EcoRI digests but not in Bgl II or HindIII digests of leukemic DNA. Sequential hybridization with the 3' and 5' M-BCR probes and analysis of the structure of the junction fragments showed a breakpoint site approximately 3-4 kb 3' of ABL exon 1B (patient 6, Fig 2A, data not shown). There was no rearrangement in the vicinity of ABL exon 1B in the remaining 25 patients.

Mapping the BCR-ABL Junction Fragments

Probes from each side of the breakpoints in chromosome 9 were used to determine the structure of the breakpoint regions on the 9q+ (ABL/BCR junction) and Ph (BCR/ABL junction) derivative chromosomes of patients 2 through 4 (Table 1, Fig 2).

Patient 2. The M-BCR-3' probe identified two rearranged fragments in BamHI, Bgl II, and HindIII digests of leukemic DNA on Southern blots. One rearranged fragment was much fainter than the other and suggested that the breakpoint in M-BCR had occurred close to one end of the HindIII-Bgl II region characterizing the probe (Table 1, Fig 2D). A breakpoint at the 5' end of that region was confirmed when the M-BCR-5' probe identified a rearranged Bgl II fragment of the same size as the faint 8.0-kb rearranged Bgl II fragment identified by M-BCR-3' (Table 1, Fig 2D). The T-39-2-2 probe identifies the 5' side of the breakpoint on chromosome 9, and we anticipated that it would be juxtaposed with M-BCR-3' sequences and the rest of the 22 long arm (Fig 2C). This was confirmed when both T-39-2-2 and M-BCR-3' probes hybridized independently to rearranged 6.9-kb BamHI and 7.2-kb HindIII fragments in leukemic DNA (Table 1, Figs 2C and 3). Consistent with this interpretation, M-BCR-3', but not T-39-2-2, detected a rearrangement in Bgl II and EcoRI digests (Table 1, Fig 3).

A restriction map of the ABL/BCR breakpoint junction (9q+) was drawn from these results (Fig 2C). Likewise, a restriction map of the BCR/ABL breakpoint junction on the Ph chromosome was drawn from the results of sequential hybridization of ABL exon 1B and M-BCR-5' probes to the same filters (Table 1, Fig 2D).

Patient 3. The breakpoint junctions were more complex in patient 3. The M-BCR-3' probe identified a single rearranged fragment in all four restriction digests: BamHI, Bgl II, HindIII, and EcoRI (Table 1, Fig 3), but the absence of a second rearranged band made it unlikely that a breakpoint
Fig 2. Restriction enzyme maps of the germline region 5' of the ABL gene (A) and M-BCR (B) showing the respective breakpoints of patients (Pt) 1 through 6 and probes used in this study. ABL regions are indicated by an open line; BCR regions are indicated by a solid line. M-BCR exons 1 through 5 are marked by black boxes beneath (6) and A81 exon 1B by a hatched box in map (A). Restriction enzyme maps of the 9/22 breakpoint junction and 22/9 breakpoint junction are shown for patient 2 (C and D), patient 3 (E and F) and patient 4 (G and H), respectively. Restriction sites are BsmHl (B), BstEII (Bs), Bg II (Bg), EcoRI (E), HindIII (HI. K#n (K). Mot I (N). SstI (Ss), XbaI (Xb), and Xho I (Xh).
ENTIRE ABL GENE IS JOINED WITH 5'-BCR

Table 1. Sizes of Abnormal Restriction Fragments (in Kilobases) Detected on Southern Blots of Leukemic DNA From Patients 2 Through 4

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<thead>
<tr>
<th>Patient No. (Probe)</th>
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<th>HindIII</th>
<th>EcoRI</th>
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had occurred in the HindIII-Bgl II region of the probe as these results suggested. The T-39-2-2 and M-Bcr-3' probes identified rearranged fragments that migrated identically in BamHI and HindIII digests (Table 1, Fig 3), and we concluded that these were chimeric 9/22 fragments. From these results, we believe that a breakpoint had occurred within the 1.3-kb BamHI-HindIII region 5' of the M-Bcr-3' probe, accompanied by deletion of a small segment extending within the adjacent HindIII-Bgl II region (Fig 2B,E,F).

Patient 4. The M-Bcr-3' probe showed rearranged fragments in Bgl II, BamHI, and EcoRI digests, but not in the HindIII digest (Table 1). These results placed the breakpoint within the BamHI-HindIII region 5' of the M-Bcr-3' probe (Fig 2b). The 2.4-kb EcoRI fragment detected with M-Bcr-3' (Table 1) indicated that the breakpoint on chromosome 9 was very close to the 5' end of the EcoRI fragment containing ABL exon 1B, and therefore within a kilobase 5' of that exon (Fig 2A). Maps of the breakpoint junctions on the 9q+ and Ph chromosomes are shown in Fig 2G and H.

BCR-5' Juxtaposes the Entire ABL Gene

Chromosome in situ hybridization and FIGE techniques were used to confirm that a breakpoint had occurred 5' of ABL and that the entire gene including exon 1B was translocated next to 5' M-BCR on the Ph chromosome.

Chromosome in situ hybridization. The ABL exon 1B probe was hybridized to Ph-positive, t(9;22) metaphase cells of patient 3. One hundred thirty-six bands on 70 metaphases were scored. Specific labeling was detected on the normal chromosome 9, at band q34 (10 grains; 7.4% of total grains scored), and also on the Ph chromosome (12 grains; 8.8% of total grains scored). There was no specific labeling on the 9q+ derivative, the normal chromosome 22, or other chromosomes. Clearly, the first exon of the ABL gene was present on the Ph chromosome of this patient.

FIGE analysis. Probes from ABL and BCR were used to study the long-range restriction fragment structure of the 5' ABL-BCR fusion gene. Restriction mapping studies of cloned fragments extending 5' of ABL have previously shown a Not I site 18 kb 5' of the Not I site within exon 1B (Fig 2A) (N. Heisterkamp and J. Groffen, unpublished observations, 1989). We showed that the germline fragment recognized by the T-39-2-2 probe on a Southern blot of Not I-digested DNA is 18-kb (Fig 4A; data not shown) and not 40 kb as previously reported.18 The sizes of the smaller rearranged bands in Sfi I digests of DNA from patients 2 and 3 were estimated by comparison with this 18-kb fragment (Figs 4 and 5).

Patient 2. Both Not I and Sfi I digests of leukemic DNA showed germline fragments only with the T-39-1-2 and ABL exon III probes (Fig 5). ABL exon 1B identified a single z175-kb germline fragment in the Not I digest (Fig 5). These results showed that the region 3' of ABL exon 1B on the Ph chromosome was entire and in the germline configuration (Fig 4A and B).

A rearranged Sfi I fragment of about 20-kb was identified by each of the four probes M-Bcr-5', M-Bcr-3', T-39-2-2, and ABL exon 1B (Fig 5). We assumed from our previous studies of this patient (Fig 2C and D) that M-Bcr-5', a small part of M-Bcr-3', and ABL exon 1B had hybridized to a z20-kb Sfi I fragment that contained the BCR/ABL junction on the Ph chromosome (Fig 4B), whereas the larger part of M-Bcr-3' and T-39-2-2 identified a different z20-kb Sfi I fragment derived from the ABL/BCR junction on the 9q+ chromosome (Fig 4C). This was in keeping with our prediction of an abnormal Sfi I junction fragment of about

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**Fig 3. Hybridization of the M-Bcr-5', M-Bcr-3', T-39-2-2, and ABL exon 1B probes to Southern blots of HindIII (H) and EcoRI (E) digested DNA of patients 2 and 3. Arrows indicate rearranged fragments.**

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Fig 4. Long-range restriction maps surrounding the normal ABL and BCR genes and the rearrangements of these genes in patient 2. Chromosome 9 regions are indicated by an open line; BCR is indicated by a solid line. Rare cutting enzyme sites are Not I (N) and Sfi I (S). Distances are in kilobases. (A) The normal ABL gene and adjacent parts of chromosome 9. Exons are shown as black boxes. Fragment sizes are after refs 18 and 20 except for the 18-kb Not I fragment (this study). The breakpoint sites of patients 1 through 4 are indicated at top left. (B) The BCR/ABL junction of the Ph chromosome in patient 2. (C) The ABL/BCR junction of the 9q+ chromosome in patient 2. (D) Long-range map of BCR based on this study.

25 kb on the Ph chromosome based on germline Sfi I sites located 4.5 kb 5' of the breakpoint in M-BCR and 21 kb 3' of the breakpoint on chromosome 9 (Fig 4). The abnormal ±20-kb Sfi I band detected with T-39-2-2 was smaller than the ±55-kb fragment we expected from previous reports of an Sfi I site ±30 kb 5' of T-39-2-2.19 (Fig 4A and C). The position of this Sfi I site requires confirmation. Alternatively, a deletion or other rearrangement had occurred in this region on the 9q+ derivative of patient 2.

The M-Bcr-5' and M-Bcr-3' probes both hybridized to a rearranged ±300-kb fragment in Not I digests (Fig 5). Because 5'-BCR joins with chromosome 9 eight kb 5' of the Not I site at ABL exon 1B (Fig 2A and D), most of this 300-kb fragment must derive from sequences 5' to M-BCR on chromosome 22. From this result, we estimate that the germline Not I site 5' of BCR is about 290 kb 5' of M-BCR (Fig 4D). Both T-39-2-2 and M-Bcr-3' probes hybridized to an abnormal ±240-kb Not I fragment, which contained the breakpoint junction on the 9q+ derivative (Figs 4C and 5). Because the germline Not I fragment containing T-39-2-2 is 18 kb, we place the chromosome 9 breakpoint 10 kb downstream of the 5' Not I site (Fig 4A and C), and conclude that ±230 kb of the rearranged T-39-2-2 Not I fragment in patient 2 must derive from the 3' part of the Not I fragment containing the BCR gene (Fig 4A and C).

If no secondary rearrangements occurred during the translocation, we estimate from our data that M-BCR lies almost centrally within a NotI fragment of approximately 520 kb (Fig 4D). We were unable to confirm this germline size in the present study. Both control and patient germline BCR fragments migrated within a very high-mol-wt range (Fig 5) in which our yeast markers were unreliable. We believe it is unlikely that these germline fragments correspond with the 1,600-kb Not I fragment described in some patients by Rubin et al.20 Our control cells were from the same source as used for a previous study when, under slightly different running conditions, the BCR germline band migrated at ≥450 kb.

Patient 3. M-Bcr-5' and ABL exon1B probes detected the same ±16-kb rearranged Sfi I band (Fig 5), supporting their contiguity on the Ph chromosome (Fig 2F and compare Fig 4B). The 3' ABL probes T-39-1-2 and ABL exon 3

Fig 5. FIGE analysis of high-mol-wt DNA from leukemic cells of patients 2 and 3 (lanes 2 and 3 of each column) and from blood cells of a control donor (lane C) digested with Not I and Sfi I restriction enzymes. Probes indicated above the column were hybridized sequentially to the same membrane and show germline (open arrows) and rearranged bands (solid arrows). Size markers (in kilobases) are of S cerevisiae chromosomes.
showed germline SfI fragments only, indicating that the segment containing the first ABL intron and the main body of ABL exons was intact (Figs 4A and 5). M-BCR-3' and T-39-2-2 probes hybridized to the same abnormal ±25-kb SfI fragment (Fig 5) containing the 9q+1 junction (compare Fig 4C). Probes hybridized to Not I digests of this patient’s DNA yielded complex and uninterpretable results.

The Fusion Gene Produces a Conventional BCR-ABL mRNA

BCR-ABL sequences were amplified by PCR from RNA of patients 2 and 3, a Ph-positive CML patient who lacked rearrangement in the vicinity of ABL exon 1B, and the CML-derived cell line K562, using the CML-specific amplification primers CML-A (from M-BCR exon 2) and CML-B (from ABL exon 2).13 CML-C, an oligonucleotide that spans the b3 and a2 junctions, hybridized to a 200-base pair (bp) fragment on blotted PCR products from all four samples. The PCR sample from a reaction control was negative. We concluded that patients 2 and 3 were producing M-BCR exon 3/ABL exon 2 mRNA junctions typical of CML.

DISCUSSION

The 5' part of M-BCR usually involved in the BCR-ABL gene association in CML, is joined in our four patients with the entire ABL gene plus some kilobases of chromosome 9 upstream of that gene. The chromosome 9 breakpoints did not occur at a single site but were spread over a 8.25-kb region 5' of exon 1B. This is the first demonstration of a group of patients with breakpoints upstream of the entire ABL gene, although the breakpoint site in our patient 1 was surmised previously.14 We also showed, using in situ hybridization and FIGE studies, that both ABL exon 1A and exon 1B existed on the Ph chromosome of patients 2 and 3. There was no secondary rearrangement that might delete these 5' parts of ABL. Therefore, BCR-ABL fusion gene was formed by the basic (9;22) in these patients. Surprisingly, this large fusion gene produced an mRNA that is standard for CML.

The ABL gene is unusual in two respects. First, it has two alternative first exons 1A and 1B which characterize different mRNAs, each of which encodes a different peptide. Two distinct transcriptional promoters and their coding regions begin with the two alternative first exons 1A and 1B.22 Second, these alternative exons are separated by 175 kb, and splicing normally occurs over a very long distance. When transcription initiates at exon 1B it is assumed that both exons 1A and exon 1B are spliced out to produce the conventional chimeric RNA transcript that is standard in CML. The splice from M-BCR exon 3 to ABL exon 2 covers more than 200 kb and includes the overridden transcriptional promoters of exons 1A and 1B.

Breakpoints upstream of the ABL gene may not be uncommon in CML. Three patients among the 34 Christchurch patients showed such a breakpoint, ie, 9%. A separate study showed that most ABL breakpoints occurred between exons 1B and 1A, but the breakpoints of four patients in 37 could not be located and were suggested to be upstream of the ABL gene.4 Our results show that this is probable. The combination of these two studies suggest that about 10% of CML patients have breakpoints upstream of the ABL gene.

Is the breakpoint site upstream of ABL associated with any hematologic features of leukemia? We first identified the breakpoint in two patients diagnosed as having Ph-positive essential thrombocythemia (ET), which can be considered a clinical variant of CML. The remaining four ET patients showed conventional breakpoints in ABL, however, although one was very close to exon 1B on the 3’ side (patient 5, present study). Our patient 4 was diagnosed as having CML but had a platelet count of 1,521 × 10^9/L, which is very high for CML. This strengthens the possibility that a breakpoint 5' of ABL is associated with thrombocytosis; however, we have another CML patient with a similar platelet count whose ABL breakpoint was 3' of exon 1B. Unfortunately, information on the platelet count of patient 1 is not available. Further studies are needed to establish whether any association exists between a breakpoint 5' of ABL and thrombocytosis. Special hematologic features would not be expected from this breakpoint 5' of ABL exon 1B because splicing produces the conventional chimeric mRNA. Problems such as recognition of correct splice sites over a distance approximating 200 kb may influence the kinetics of mRNA production, however, and thus the amount of protein product, which in turn may influence differential maturation of the neoplastic hematopoietic stem cells.

ACKNOWLEDGMENT

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