Ph-Negative Chronic Myeloid Leukemia: Molecular Analysis of ABL Insertion Into M-BCR on Chromosome 22

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

Leukemic cells from a patient with Ph-negative chronic myeloid leukemia (CML) had a normal karyotype. M-BCR was rearranged and chromosome in situ hybridization showed an ABL insertion between 5' and 3' M-BCR on an apparently normal chromosome 22. The association of 5' BCR and 3' ABL at the 5' junction of the chromosome 9 insert was typical of that found for the BCR-ABL fusion gene in other patients with the standard t(9;22) and CML. With an M-bcr-3' probe, we cloned and characterized a 3' junction fragment. Field inversion gel electrophoresis and chromosome in situ hybridization studies using a probe isolated from genomic DNA 5' of the junction showed that 3' M-BCR was joined to a region of chromosome 9q34 rich in repetitive sequences and lying some distance 3' of ABL. The chromosome 9 insert was at least 329 kilobases long and included 3' ABL and a larger portion of chromosome 9q34. Our results allowed us to exclude transposon- or retroviral-mediated insertion of ABL into chromosome 22. Instead, we favored a two-translocation model in which a second translocation reconstituted a standard t(9;22)(q34; q11) but left the chromosome 9 insert, including 3' ABL, in chromosome 22.

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MATERIALS AND METHODS

Previous Studies of Ph-Negative CML Patient

Patient M.B., a 45-year-old white man, was diagnosed with CML in 1981 and died in myeloid blast crisis in March 1986. At diagnosis, he did not have the Ph chromosome which characterizes this leukemia and was karyotypically normal. Southern blot studies showed a rearrangement in M-BCR, and chromosome in situ hybridization analysis indicated insertion of ABL sequences into an otherwise normal chromosome 22.10-13 These findings confirmed the importance of the BCR-ABL fusion gene but posed a question about the mechanism of movement of ABL from its usual place on chromosome 9 to chromosome 22, apparently without the aid of cytogenetic translocation.

Our earlier in situ hybridization and genetic studies of one of these patients had shown the insertion of a small part of chromosome 9, at least that part carrying the 3' ABL sequences having homology with v-abl, between the 5' and 3' sequences of the BCR gene on chromosome 22. The involved chromosome 22 appeared to be whole and morphologically normal. We report the results of our investigations of the size of this chromosome 9 insert within BCR, the nature of the breakpoint junctions, and the mechanism of ABL transposition.

Probes

The following molecular probes were used: M-bcr-u, a "universal" M-BCR probe; M-bcr-3', a 1.2-kilobase (kb) HindIII-BglII fragment from 3'M-BCR (probe 1, Oncogene Science, Manhasset, NY); M-bcr-5', a 1.45-kb BglII- HindIII fragment from 5'M-BCR;11 a 0.4-kb BamHI probe isolated from the 5' end of a cloned 3'M-BCR breakpoint fragment from patient M.B. and considered to derive from the 3' end of the chromosome 9 sequence inserted into chromosome 22 (Fig 1B); ABL exon 1B, a 1.2-kb KpnI-EcoRI fragment containing exon 1B of the ABL gene (Groffen et al, unpublished observations); T-39-1-2, a 1.0-kb EcoRI fragment containing chromosome 9 sequences immediately 3' to the translocation breakpoint in the CML cell line K562; T-39-2-2, a 1.4-kb EcoRI fragment representing the chromosome 9 translocation breakpoint from the DNA of a CML patient (not M.B.) (Groffen et al, unpublished observations); and ABL exon 111, a 0.65-kb HindIII-BamHI probe containing ABL exon III (c-abl probe 1, Oncogene

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ABL INSERTION INTO M-BCR

Fig 1. (A) Diagram of part of chromosome 22 and the BCR gene (not to scale). A segment of chromosome 9 including ABL lies between the 5' and 3' parts of a divided M-BCR. The 5' and 3' chimeric junctions and the sites of the M-BCR probes are indicated. (B) Restriction map of a cloned 9.5-kb genomic fragment (9.5A4) containing the 3' chimeric junction (9q34/3'M-BCR). The part that has homology with BCR (thin line) is aligned over a restriction map of the BCR gene which also shows the breakpoint site (double-headed arrow) and the locations of M-BCR probes used in this study. The location of the 0.4-kb BamHI probe is indicated above the segment from chromosome 9 (heavy line). Restriction sites are BamHI (B); BglII (Bg); SstI (Ss); XbaI (Xb); HindIII (H); XhoI (Xh); EcoRI (E).

Science). Genomic locations of these probes are indicated in Figs 1 and 2.

Southern Blot Analysis

High-molecular-weight (mol wt) DNA was isolated from peripheral blood or bone marrow cells, digested with restriction enzymes according to the manufacturer's guidelines, electrophoresed on 0.8% agarose gels, and transferred to nitrocellulose (Schleicher and Schuell, Dassel, West Germany) or nylon membrane (Gene Screen Science, New England Nuclear, Boston, MA) according to the method of Southern. Probes were isolated from plasmid in low-melting-point agarose (Bethesda Research Laboratories, Gaithersburg, MD), oligolabeled with "P-dCTP (Amersham International, Amersham, UK), and hybridized to the filters overnight at 65°C. Probes contaminated with repeat sequences were prehybridized with driver DNA. Final posthybridization washes were in 0.3 x SSC, 0.1% sodium dodecyl sulfate (SDS), 0.1% sodium pyrophosphate at 65°C. Filters were exposed to Kodak XAR-2 or XAR-5 film for 1 to 7 days at -80°C with intensifying screens.

Construction and Screening of a Genomic Library

DNA isolated from blast crisis leukemia cells of patient M.B. was digested with BglII and electrophoresed in a 0.8% low-melting-temperature agarose gel (BRL), and fragments in the size range of 7 to 20 kb were isolated and purified. The DNA was ligated to the arms of the bacteriophage vector EMBL 3 (Clontech Laboratories, Palo Alto, CA), and packaged in vitro with the Gigapack Gold system of Stratagene (La Jolla, CA). Phage libraries were screened using nitrocellulose filters as described for Southern hybridization. Inserts from selected colonies were subcloned into pUC19, was nick-translated with 3HdATP and "HdCTP (Amersham) to a specific activity of 1 to 2 x 106 cpm/µg and hybridized in situ to metaphase chromosomes as described. Sonicated total human DNA (2 mg/mL) was added to the hybridization mix to quench repetitive sequences in the probe.

Field Inversion Gel Electrophoresis (FIGE)

DNA of very high mol wt prepared from frozen cells of patient M.B. and a normal male control was digested in agarose plugs. The plugs were placed in a 1% agarose gel and electrophoresed alongside mol wt markers of Saccharomyces cerevisiae chromosomes. After FIGE, 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.

RESULTS

Our earlier studies of leukemic cells from this Ph-negative CML patient indicated that a part of chromosome 9 carrying ABL sequences was inserted between 5' and 3' parts of BCR on chromosome 22 (Fig 1A). The chromosome 9 fragment will have two breakpoints, a 5' breakpoint which we assume is adjacent to 5' sequences of M-BCR as has been demonstrated in other cases of CML, and a 3' breakpoint which will be associated with 3' sequences of M-BCR. We refer to these as the 5' and 3' junctions of the chromosome 9 insert. We describe the isolation and study of the 3' junction.

Identification of a 3'M-BCR Rearranged Fragment

DNA isolated from leukemic blood sampled during terminal blast crisis was Southern blotted, and the filter was hybridized with M-bcr-u. Abnormal bands were detected in BglII and BamHI digests, and the germline 5.0-kb BglII and 3.3-kb BamHI bands were absent (Fig 3), consistent with our
previously reported findings.\textsuperscript{10,14} M-bcr-u detects abnormal bands representing both the 5'M-BCR/3'ABL breakpoint junction on the Ph chromosome, and the 5'ABL/3'M-BCR junction on the 9q+ in leukemic DNA of CML patients carrying the standard t(9:22).\textsuperscript{23} Independent hybridization of the M-bcr-S' and M-bcr-3' probes showed that the 9.5-kb $\text{BamH}1$ and 3.8-kb $\text{BamH}1$ bands represented 3' M-RCR breakpoint fragments in our patient's DNA (Fig 3).

\textit{Isolation of a Chimeric 9:22 Junction}

A size-selected library of $\text{BglII}$ fragments in the range of 7 to 15 kb was prepared, and approximately $1.7 \times 10^6$ recombinant phages were screened with M-bcr-u, yielding 31 positive colonies. All 31 cohybridized with the M-bcr-3' probe and were therefore presumed to derive from the 3' junction fragment.

Eight positively hybridizing phage colonies were subcloned and restriction mapped. All genomic inserts showed homology with a small region of M-BCR-3', including the HindIII-BglII region encompassed by the M-bcr-3' probe (Fig 1B), but diverged from normal M-BCR in the 8-kb region 5' of a $\text{Xho}$ site (Fig 1B). These sequences represented the 5' part of the chimeric 3' junction fragment. We could not isolate a repeat-free probe from this region because of the large number of repeat sequences present; however, a 0.4-kb $\text{BamH}1$ fragment (Fig 1B) provided a discrete signal after prehybridization with excess sonicated total human DNA.\textsuperscript{20}

\textit{Fig 2.} Long-range restriction maps surrounding the normal ABL and BCR genes and the deletion/insertion junctions of the Ph-negative CML patient. Rare-cutting enzyme sites include $\text{SfiI}$ ($\text{Sfi}$), NotI (N), and SacII (S). Fragment sizes are in kilobases. (A) Map of normal ABL locus. Exons are shown as vertical boxes, vertical arrows indicate the approximate locations of the genomic probes T39-1-2 and T39-2-2. (B) Estimated structure of the abnormal chromosome 9 showing deletion of 3' ABL and rejoining of 5' ABL with more distal chromosome 9 sequences. (C) Long-range map of the BCR gene and restriction map of the 9S fragment containing M-BCR (inset). Restriction sites are $\text{BglII}$ (Bg), HindIII (H), $\text{BamH}1$ (B), and EcoRI (E). (D) Estimated structure of chromosome 9 sequences (thick line) inserted into chromosome 22 (thin line). Insets show the 5' and 3' breakpoint junctions and locations of the M-bcr-5' and M-bcr-3' probes. The derivation of chromosome 9 sequences (~329 kb), inserted within M-BCR is shown at top. This includes an unknown number (?) of NotI fragments between the fragment containing ABL and the 3' junction.

\textit{Fig 3.} Southern blot analysis of DNA from leukemic blood of patient with Ph-negative CML after hybridization with the M-bcr-u probe. Rearranged (R) and germline bands (G) are indicated by arrows for both the M-bcr-5' and M-bcr-3' components of the probe, hybridized independently. DNA was restricted with EcoRI (E), HindIII (H), $\text{BamH}1$(B) and $\text{BglII}$ (Bg) enzymes. Molecular weight markers (column 1) are a HindIII digest of DNA. Germline 5.0-kb $\text{BglII}$ and 3.3-kb $\text{BamH}1$ M-BCR fragments are absent, consistent with homozygosity of the BCR-ABL rearrangement.\textsuperscript{10,14} Sizes and derivations of the fragments can be determined from Fig 1B.
Table 1. Results of In Situ Hybridization of 0.4-kb BamH1 Probe to Metaphase Cells of Patient With t(9;22) CML and to PHA-Stimulated Lymphocyte Cells of Normal Male Donor

<table>
<thead>
<tr>
<th>Karyotype</th>
<th>No. of Metaphase Cells Analyzed</th>
<th>Total on Genome</th>
<th>On Chromosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal donor</td>
<td>46,XY</td>
<td>150</td>
<td>233</td>
</tr>
<tr>
<td>CML patient</td>
<td>46,XY.t(9;22)</td>
<td>150</td>
<td>257</td>
</tr>
</tbody>
</table>

Abbreviation: PHA, phytohemagglutinin.

*Number of grains at 9q34 in parentheses.

This probe cohybridized to the 19-kb EcoRI and 9.5-kb BglII fragments detected with M-bcr-3' in our patient's DNA and also detected the rearranged 6-kb HindIII fragment predicted by our restriction map (Figs 1B and 3, data not shown).

The 0.4-kb BamH1 probe was hybridized in situ to normal metaphase chromosomes. Significant labeling on chromosome 9 at band 9q34 (Table 1) allowed us to conclude that the 9.5-kb BglII fragment (Figs 1B and 3) represented the chimeric 9q34/3'M-BCR junction present in our Ph-negative CML patient's leukemic DNA.

**M-BCR-3’ Recombines With Sequences Outside and Distantly 3’ of the ABL Gene on 9q34**

FIGE and chromosome in situ hybridization techniques were used to determine more precisely the origin of the 0.4-kb BamH1 probe representing chromosome 9q34 sequences that had recombined with M-bcr-3' in our patient.

**FIGE analysis.** FIGE analysis allows examination of the entire length of the ABL and BCR genes using two enzymes, NotI and SfiI, and appropriate ABL or BCR probes. Long-range physical maps of the normal ABL and BCR genes20-28 show the restriction fragments identified by ABL and BCR probes used in this study (Fig 2A and B). We hybridized the 0.4-kb BamH1 probe to FIGE-separated NotI, SacII, SfiI, MluI, and BssHII-restricted DNA from a normal donor. The probe did not hybridize with any of the germline-size ABL fragments detected by T-39-2-2 or ABL exon III probes when they were hybridized serially to the same membranes (Fig 4, data not shown). The T-39-1-2 and ABL exon 1B probes did hybridize to similar-sized fragments in NotI and SacII digests but detected SfiI, MluI, BssHII, and NruI fragments of different size than the 0.4-kb BamH1 probe (Fig 4, data not shown). These results showed that the part of chromosome 9q34 that had recombined with M-bcr-3' in the junction fragment was not part of the ABL gene.

**Chromosome in situ hybridization analysis.** The 0.4-kb BamH1 probe specifically labeled the normal chromosome 9 at band q34 and the Ph chromosome in metaphase cells carrying a standard (9;22) translocation. The 9q + chromosome showed no significant labeling (Table 1). This result

![FIG 4](https://www.bloodjournal.org/content/102/15/1815/F4)

**Fig 4.** FIGE analysis of high-mol-wt DNA from leukemic cells of the Ph-negative patient and blood cells of a control donor (left- and right-hand lanes of each column, respectively). Probes indicated above columns were hybridized sequentially to the same membrane and show germline (open arrows) and rearranged bands (solid arrows). A small solid arrow marks the rearranged ABL SfiI fragment detected with T-39-1-2. Size markers (in kb) are of S cerevisiae chromosomes.
showed that the 0.4-kb BamHI fragment was contained within the region 3' ABL → 9qter, which is translocated to the Ph chromosome as a result of the t(9;22). Because the 0.4-kb BamHI probe was not part of the ABL gene (FIGE results above), we concluded that it was located telomeric of ABL on 9q34.

At Least 300 kb of DNA From Chromosome 9 Is Inserted Into BCR on Chromosome 22

Probes from the 5' and 3' sides of the M-BCR breakpoint have been used to identify long-range restriction fragments originating from the Ph and 9q+ chromosome, respectively, in two CML lines. We applied a similar approach to analysis of the 5' and 3' M-BCR breakpoint junctions in our patient. According to our model (Fig 1A), the M-bcr-5' probe will detect DNA fragments containing the 5' BCR/ABL fusion gene (5' junction), whereas the M-bcr-3' probe will identify the other end of the insertion which contains the 9q34/3'-BCR junction (3' junction) and the remainder of chromosome 22.

NotI-digested leukemic DNA showed a 340-kb rearranged fragment that hybridized with M-bcr-3' and the 0.4-kb BamHI probe (Fig 4A). Calculations based on a previously published long-range map of BCR indicate that most of this abnormal 340-kb NotI fragment derives from chromosome 22 sequences distal to 3' M-bcr; approximately 28 kb may derive from the 3' part of the 9q34 insertion (Fig 2C and D). Hybridization of the same filter with the ABL exon IB and T-39-2-2 probes showed germline NotI and SfiI fragment sizes only, indicating that the first ABL intron was intact, as was the 75-kb segment immediately upstream of ABL (Figs 2A and B). The T-39-1-2 probe showed germline NotI fragments and a rearranged 275-kb SfiI band (Fig 4). This result suggested that a breakpoint in ABL had occurred in the second intron between exon 1A and the first common exon II (Fig 2A and B).

M-bcr-5' detected a 75-kb rearranged SfiI fragment, which also hybridized to a 3' ABL probe (ABExon III) (Fig 4B). Because an SfiI site exists 3 kb 5' of the M-bcr breakpoint, 72 kb of the rearranged fragment must derive from the 3' ABL region (Fig 2C and D). This places the breakpoint in ABL close to exon IA, a location consistent with that which we found using the 5' ABL probes (Fig 2). A rearranged 450-kb NotI fragment was detected by the ABL exon III probe (Fig 4A). The M-bcr-5' probe hybridized to a single fragment of this size in both patient and control DNA. Because our patient's leukemic cells lack germline M-BCR alleles, however, we concluded that the 450-kb M-bcr-5' fragment in his DNA was actually rearranged and cohybridized with the ABL exon III probe. Previous reports have placed a germline NotI site 150 kb 5' of M-bcr on chromosome 22 (Fig 2C). If we assume that this 5' region is not rearranged in our patient, the remaining 300 kb of the rearranged 450-kb NotI fragment must derive from ABL and from sequences 3' of ABL to the next most 3' NotI site on chromosome 9 (Fig 2A, C, and D). When regions containing ABL sequences and the sequences 3' of ABL on 9q34 are combined, we estimate that a minimum of 329 kb of chromosome 9 DNA is inserted into BCR on chromosome 22 (Fig 2D).

DISCUSSION

We wished to determine the size of chromosome 9 insert and features of its 5' and 3' junctions with the BCR gene. We know that the breakpoint in M-BCR occurred within the BamHI-XhoI fragment, which includes the third exon of M-BCR, a breakpoint site that is conventional for the standard t(9;22). We assumed for our experimental model that these broken ends of M-BCR represented the two junctions of the chromosome 9 insert. FIGE studies showed that the 5' junction required a breakpoint in ABL between exon IA and exon 2, again a conventional breakpoint for the standard t(9;22). We conclude that the 5' junction of BCR and ABL was typical of those described for other patients with CML; this emphasizes the precise association of 5' BCR and ABL sequences, within a latitude allowed by messenger splicing, as we might expect for proper functioning of the fusion gene.

The 3' junction, on the contrary, is distinct from any in the standard t(9;22), where BCR-3' sequences on the 9q+ derivative chromosome (Fig 2). Using the M-bcr-3' probe as a starting point to investigate the chromosome 9 sequences adjacent to BCR-3', we isolated a 9.5-kb 3' junction fragment. An 0.4-kb BamHI probe isolated 5' of the breakpoint characterized an 8-kb region of chromosome 9q34 lying 3' of ABL and rich in unidentified repetitive sequences. The chromosome 9 insert into the BCR gene on chromosome 22 was at least 329 kb long and encompassed those 3' ABL sequences important for leukemia and also a larger portion of chromosome 9 distal to the ABL gene.

The BCR-ABL fusion gene arises in most patients in whom CML or any leukemia of the Ph group develops, as a consequence of cytogenetic translocation, most commonly the t(9;22)(q34;q11). The presence of the fusion gene in Ph-negative leukemia can be explained by cytogenetic mechanisms that are merely variants of the usual t(9;22). The apparently normal karyotype of our patient could have arisen by a standard t(9;22)(q34;q11) followed by a second translocation between the derivative 9 and 22 chromosomes that reversed the original translocation and reconstituted chromosome 22, leaving in it the chromosome 9 insert containing ABL (Fig 5A). In that event, the reconstituting breakpoint in the derivative chromosome 9 occurred at, or close to, the site of the earlier union of ABL-5' and M-BCR-3' (Fig 5A). The breakpoint was not 5' of that site because our FIGE studies showed no association of ABL-5' and M-bcr-3', and the breakpoint was not 3' of that site because we earlier in situ hybridization results showed M-Ber-3' to be present on the reconstituted chromosome 22 rather than the derivative 9 chromosome. The reconstituting breakpoint in the derivative chromosome 22 occurred 3' of ABL within 9q34 (Fig 5A).

Other researchers have suggested that Ph-negative CML showing BCR rearrangement arises from reconstitution of the standard t(9;22), and there is clear evidence for this in one case. Using genomic markers, we demonstrated...
ABL INSERTION INTO M-BCR

Fig 5. Diagram of two cytogenetic mechanisms leading to BCR-ABL gene fusion in Ph-negative CML. (A) A standard t(9;22)(q34;q11) produces derivative 9q and 22q chromosomes which undergo a second translocation, reconstituting the original 9 and 22 chromosomes but leaving a chromosome 9 insert including 3' ABL sequences within M-BCR. Small arrowheads and dotted lines show translocation breakpoints. (B) A two-break deletion of chromosome 9 and its insertion between the ends of a single breakpoint in M-BCR. Dotted lines indicate reunion of broken ends.

that the apparently normal karyotype in the leukemic cells of another patient with Ph-negative, BCR-rearranged CML arose from a standard t(9;22), which was followed by a complex three-way rearrangement between the derivative 9 and 22 chromosomes and chromosome 20.

The genomic rearrangement in the leukemic cells of our present patient fits the reconstitution model but, alternatively, it may have resulted from a single cytogenetic event involving a two-break deletion of chromosome 9q34, including ABL, and its insertion between the ends of a single breakpoint within M-BCR (Fig 5B). We cannot distinguish between this mechanism and one involving two translocations.

Although our studies do not indicate a precise mechanism by which ABL was inserted within the BCR gene in Ph-negative CML, they do allow us to exclude some possible mechanisms. The most intriguing of these concerned the movement of ABL by some transpozon mechanism, perhaps mediated by retroviruses. Such action is rare in humans, but is a usual mode of oncogene transmission in experimental animals. Our findings clearly exclude any such mechanism.

The chromosome 9 insert including ABL sequences was more than 300 kb long and of greater size than sequences that are moved by transposition mechanisms. FIGE studies of leukemic DNA showed dimensions consistent with genomic ABL; we showed previously that the leukemic ABL has retained intron sequences: These data exclude any involvement of an RNA intermediate.

The 0.4-kb BamHI probe from 9q34 will be useful in analyzing regions 3' of ABL in other leukemias. We showed that segments containing this probe site were not rearranged in conventionally restricted leukemic cell DNA of three other patients with Ph-negative CML who had normal karyotypes and rearranged BCR. The 3' junction appears to occur at different chromosome 9 sites in different patients. This finding might argue against the presence of “recombinant” sites on 9q34 that favored reconstitution of (9;22) translocations unless several of these sites, possibly associated with similar repetitive sequences, were distributed over a wide region of 9q34 telomeric of ABL. FIGE analysis of other Ph-negative patients using the 0.4-kb BamHI probe should clarify this question.

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Ph-negative chronic myeloid leukemia: molecular analysis of ABL insertion into M-BCR on chromosome 22

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