The ABL-BCR Fusion Gene Is Expressed in Chronic Myeloid Leukemia

By Junia V. Melo, D.E. Gordon, N.C.P. Cross, and J.M. Goldman

Although the BCR-ABL hybrid gene on chromosome 22q—plays a pivotal role in the pathogenesis of chronic myeloid leukemia (CML), little is known of the reciprocal chimeric gene, ABL-BCR, formed on chromosome 9q+. By reverse transcription/polymerase chain reaction (RT/PCR) amplification, we have detected ABL-BCR mRNA sequences in cells from 26 patients and of both ABL(1b)-BCR and ABL(1a)-BCR mRNA species in 6 patients. The ABL-BCR transcripts encoded one or, more rarely, both of the two potential junctions, designated ABL-b3 and ABL-b4, which differed in size by 75 bp. In 2 patients, the BCR exon b3 was not present in either the BCR-ABL or the corresponding ABL-BCR transcript, whereas in 5 patients exon b3 was present in both transcripts. Direct sequencing of PCR fragments representing the full-length coding sequence of ABL-BCR cDNAs type ib-b3, la-b3, lb-b4, and la-b4 showed an open reading frame predicted to encode fusion proteins of 370 to 414 amino acids. If an ABL-BCR gene product is produced in CML cells, it may be relevant as a mechanism for deregulating the GTPase activating protein (GAP) function of BCR.

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Patients and cell lines. Cells from a total of 44 CML patients were studied: 20 in chronic phase (CP) and 24 in blast crisis (BC). Among the latter, 15 were myeloid BC, 4 lymphoid, 1 mixed myeloid/lymphoid, and 4 of unknown phenotype. Thirty-eight patients showed characteristic Ph and 9q+ chromosomes; 1 had an atypical 22q—without a 9q+, and 5 were Ph-negative. All patients had clonal BCR rearrangement by Southern blot analysis.

Five BCR-ABL-positive cell lines were also investigated. These were well-characterized lines from patients with CML in blast crisis: K562, KCL-22, KYO-1, BV173, and LAMA-84. The HL60 promyelocytic cell line was used as a negative control for BCR-ABL expression in all tests.

Polymerase chain reaction (PCR) amplification. Amplifications of specific sequences on the ABL, BCR, BCR-ABL, and ABL-BCR genes were performed by reverse transcription (RT) of cDNA, followed by PCR (RT/PCR) by standard methods. Briefly, PB white blood cells (WBC) were obtained by dextran sedimentation or by red cell lysis of centrifuged buffy coat preparations. The patients’ WBC and freshly explanted cells from lines in culture were washed twice in phosphate buffered saline and processed for RNA extraction by the guanidinium thiocyanate/CsCl gradient method. The RNA was reverse transcribed into cDNA with Mo-MuLV reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) using random hexamers and, in some samples, oligo-dT primers. PCR amplification of cDNA was performed as described elsewhere. Precautions towards eliminating the possibility of false PCR results were based on the recommendations by Kwok and Higuchi. In brief: (1) cells, RNA, and cDNA preparations were always handled in a room separate from that specifically dedicated to the analysis of PCR products; (2) different sets of pipettes were dedicated to sample preparation and PCR product

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handling, and plugged tips (aerosol resistant) were used in all steps; (3) all reagents were prepared under sterile conditions in a laminar-flow cabinet, and stored as single-use aliquots; and (4) each PCR experiment included 6 to 8 test (CML) cDNA samples plus 2 known negative controls: BCR-ABL- and ABL-BCR-negative cDNA from HL60 cells, and a H2O-blank (ie, no cDNA). In no instance were ABL-BCR products detected in either negative control. Furthermore, test samples showing no ABL-BCR amplification alongside samples that did yield an ABL-BCR PCR product were always found in any given experiment, reinforcing the validity of the positive results. Five microliters from each PCR was electrophoresed through ethidium bromide stained 1% to 2% agarose mini-gels, visualized, and photographed under UV light. Samples that showed no ABL-BCR product were submitted to a second round of amplification with nested primers and 1 μL of the original PCR products as template.

Primers for PCR, Southern hybridization, and sequencing. The sequences of the synthetic oligonucleotide primers used in this investigation are shown on Table 1. Some of the primers were designed to contain natural or forced restriction enzyme sites at their 5' ends to facilitate future cloning of the PCR fragments into phage or plasmid vectors. The size of PCR fragments amplified with each primer pair is shown in Table 2.

Southern hybridization. Electrophoresed PCR products from some samples were transferred from agarose gels to nylon membranes by Southern blotting, and tested for hybridization to a synthetic oligonucleotide probe (primer B1)’s labeled with γ-32P-ATP. Hybridization and high-stringency washings were carried out at the appropriate discriminating temperatures for the oligonucleotide as described elsewhere.19

Sequencing. Direct sequencing of PCR products was performed by the linear amplification sequencing method20 and/or by the Taq cycle sequencing method [USB, Cleveland, OH]. PCR products were used directly as templates on an estimated basis of 100 fmol ss-cDNA per base per primer. Denaturing sequencing gels were prepared, electrophoresed, and autoradiographed by conventional techniques.

RESULTS

BCR-ABL gene. BCR-ABL amplification of cDNA with primers B2* and CA3* yielded fragments 385 and 465 bp long, representing the b2a2 and b3a2 type transcripts respectively (Fig 1A).

The types of BCR-ABL transcript expressed in the 44 patients and the five CML cell lines were as follows. In the CP group, 45% of patients expressed only b2a2 and 50% only b3a2 BCR-ABL transcripts. A relatively similar distribution for b2a2 and b3a2 patients was observed in the BC group, which includes the five cell lines (59% and 38%, respectively). One CP and one BC patient expressed both b2a2 and b3a2 transcripts.

Normal ABL and normal BCR genes. Expression of the 2 major alternative transcripts from the normal ABL allele was observed in all the 44 patients. This was shown by RT/PCR amplification of the sequence spanning exon Ia to exon III (PCR PAa* ↔ Jc*) and, likewise, the sequence from exon Ib to exon III (PCR PAb* ↔ Jc*). Of the five cell lines studied, only LAMA-84 and BV 173 did not express either ABL transcript, which agrees with the absence of a normal chromosome 9 in these lines.14,21

The normal BCR allele was also found to be expressed in all patients and cell lines, as assessed by PCR amplification of a 1,208-bp long fragment spanning the major breakpoint cluster region (M-bcr), and extending downstream of exon c7,22,23 This fragment includes the BCR-GAP coding region in the normal allele.2,23

ABL-BCR gene. The formation of an ABL-BCR fusion gene, the reciprocal product of the BCR-ABL translocation, should theoretically yield different transcripts, depending on the positions of the breakpoints in both ABL and BCR (Fig 2). If the breakpoint in ABL occurs upstream of exon Ib, no

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence* (G → 3)</th>
<th>Gene Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>As*</td>
<td>TGGAGACTTTCCATCTGGAA</td>
<td>ABL (la)</td>
</tr>
<tr>
<td>Ab*</td>
<td>CTTCTGAGAAAGGGTTACCTTTTA</td>
<td>ABL (lb)</td>
</tr>
<tr>
<td>Pa*</td>
<td>CCGTTGAGAATTTCTGCAGCTTCA</td>
<td>ABL (la)</td>
</tr>
<tr>
<td>Ca*</td>
<td>GGAGTGTTCATTCAGAATTTGC</td>
<td>ABL (lb)</td>
</tr>
<tr>
<td>B1*</td>
<td>GCAGCACTCAGGAGTGAGG</td>
<td>ABL (la)</td>
</tr>
<tr>
<td>B2*</td>
<td>TCTGAAGACCTTTCCTCCTGACAT</td>
<td>ABL (lb)</td>
</tr>
<tr>
<td>B3*</td>
<td>TCTAGAGCTCCCTCCACCTCAGCA</td>
<td>ABL (la)</td>
</tr>
<tr>
<td>B4*</td>
<td>TCTAGATCTTCACTCTCCCTCCCTGCTC</td>
<td>ABL (lb)</td>
</tr>
<tr>
<td>B7*</td>
<td>GCAGAAGAATTTCTGCAGCTTCA</td>
<td>ABL (la)</td>
</tr>
<tr>
<td>C4*</td>
<td>ATAGGAAATTTCTGCAGCTTCA</td>
<td>ABL (lb)</td>
</tr>
<tr>
<td>C5*</td>
<td>ATAGGAAATTTCTGCAGCTTCA</td>
<td>ABL (lb)</td>
</tr>
<tr>
<td>G+</td>
<td>GTGTTGACTGGCGTGAT</td>
<td>ABL (la)</td>
</tr>
<tr>
<td>PB*</td>
<td>TGGAGATCTCCCTGAAG</td>
<td>ABL (lb)</td>
</tr>
</tbody>
</table>

*Lower case indicates nucleotides that are not homologous to the cDNA sequence, but incorporate restriction enzyme recognition sites.
ABL-BCR transcript is formed. If the breakpoint is between exons Ib and Ia, only transcripts originating from the exon Ib promoter are possible (Ib-BCR); and if between exons Ia and II, two RNA species can be transcribed from the independent promoters in exons Ib and Ia (Ib-BCR and Ia-BCR).

The 49 samples were tested for expression of ABL-BCR transcripts by RT/PCR amplification using sense primers on ABL exons Ib (Ah') and Ia (PAa'), and anti-sense primers on BCR 3' end (G- or PB-). A total of 34 out of 49 patients (69%), including 2 Ph-negative patients and the patient without a 9q+ chromosome, showed ABL-BCR amplification of the Ib-BCR type and 6 of these 34 also expressed Ia-BCR. No case expressed Ia-BCR alone (Table 3). Likewise, abnormally large ABL-BCR fragments containing both exons Ib and Ia were never found. In 7 of the 34 samples the ABL-BCR products were only detected by nested PCR. In all the others, the level of ABL-BCR transcripts seemed comparable to that of ABL, BCR, and BCR-ABL amplified products, as estimated within the limitations of a standard, nonquantitative PCR assay. Among the 15 ABL-BCR negative samples, 4 (3 patients and the cell line K562) were Ph-negative, although BCR-rearranged and BCR-ABL positive.

Like BCR-ABL, the ABL-BCR transcripts are predicted to vary in length, depending on whether BCR exons b3 or b4 are joined to the 5'ABL. This was confirmed by Ib-BCR products 1,148 bp and/or 1,223 bp long when primers Ah' (in ABL exon Ib) and G' (in BCR), representing fragments 1,223 bp (lb-b3) and/or 1,148 bp (lb-b4) long. Lanes 1 to 10 are CML patient samples; lane 11 is the HL60 cell line (negative control), M, DNA molecular weight marker (pEMBL digested with Taq I).

Therefore, the ABL-BCR expressing cases fell into 3 categories (Table 4): 12 showing Ib-b4 junction, 19 with Ib-b3 junction, and 3 cases with double transcripts (Ib-b4 and Ib-b3). In the 6 patients who also showed Ia-BCR transcription, the junction was of Ia-b4 type in 3 and Ia-b3 in 3. The presence or absence of BCR exon b3 in each transcript was confirmed by Southern hybridization of the ABL-BCR fragments with a 26-mer oligonucleotide (primer B3') spanning a sequence specific for exon b3 (Fig 3) and, in some cases, by direct sequencing of the junction region in each PCR fragment.

Overall, the proportion of ABL-BCR-expressing patients who showed only b2a2 (56%), only b3a2 (38%), and both b2a2 and b3a2 (6%) BCR-ABL transcripts matched the pro-
Fig 2. Schematic representation of the ABL, BCR, BCR-ABL, and ABL-BCR genes. Arrows indicate the most frequent regions for breakpoints in the ABL and the BCR genes. The possible ABL-BCR transcripts arising from the different breakpoints in BCR are shown underneath the corresponding BCR-ABL transcripts type b2a2 and b3a2, respectively.

portion of patients in each BCR-ABL category in the whole series. The frequency of ABL-BCR expression within each BCR-ABL major group was 73% and 62% in the b2a2 and the b3a2 groups, respectively.

When the expression of the BCR-ABL and the ABL-BCR fusion genes was compared in each individual sample (Table 5), it was found that, in 27 patients (79%), the expression of BCR-ABL was exactly reciprocal to the expression in BCR-ABL. Thus, from the 19 b2a2-positive samples, 15 expressed an ABL-b3 type of junction, and 2 expressed both ABL-b3 and ABL-b4 transcripts. Similarly, 8 out of 13 patients with single b3a2 and 2 patients with both b2a2 and b3a2 transcripts from BCR-ABL showed ABL-BCR expression of the ABL-b4 type, as expected. However, in 7 samples the junction type in the ABL-BCR transcripts did not correlate with the reciprocal BCR-ABL products: these were 2 b2a2 patients who expressed only ABL-b4 transcripts, and 5 b3a2 patients in whom ABL-b3 type of transcripts were found either alone (4 samples) or together with ABL-b4 transcripts (1 sample). The junction region of BCR-ABL and ABL-BCR products from this group of patients was sequenced (primers PAu+ → B4) and results confirmed that, in 2 cases, BCR exon b3 was not expressed in either BCR-ABL or ABL-BCR, whereas, in 5 cases, this exon was present in both fusion-gene products. The same result was obtained by Southern hybridization of BCR-ABL and ABL-BCR products with the oligonucleotide probe B3- (Fig 3). In 5 of the 7 cases it was possible to repeat the BCR-ABL and ABL-BCR amplifications in duplicate blood samples obtained at different times, and/or in duplicate RNA/cDNA preparations from the original blood samples. The initial results were always reproducible. In 2 patients duplicate samples were not available for analysis.

Coding sequence of the ABL-BCR gene. PCR amplified ABL-BCR fragments from cDNA of 2 samples were sequenced in overlapping segments, using 4 pairs of sense and antisense primers (Table 2). The full-length PCR products represented ABL-BCR type Ib-b3 and 3a-b4 from 1 patient, and Ib-b4 and 1a-b4 from another. Each fragment started 60 bp (Ib) or 10 bp (Ia) upstream of ABL’s initiation ATG, and ended 86 bp downstream the stop codon for PI60RCR.

**Table 3. ABL-BCR Transcripts Found in CML Patients**

<table>
<thead>
<tr>
<th>Stage of Disease (no. of cases)</th>
<th>None</th>
<th>lb-b3</th>
<th>lb-b4 and la-b4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic phase (20)</td>
<td>5 (25%)</td>
<td>13 (65%)</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>Blast crisis (29)*</td>
<td>10 (34%)</td>
<td>15 (52%)†</td>
<td>4 (14%)</td>
</tr>
<tr>
<td>TOTAL (49)</td>
<td>15 (31%)</td>
<td>28 (57%)</td>
<td>6 (12%)</td>
</tr>
</tbody>
</table>

† Including the 5 CML cell lines.

**Table 4. Junction Type of the ABL(b)-BCR Transcripts in the Positive Cases**

<table>
<thead>
<tr>
<th>Stage of Disease (no. of cases)</th>
<th>Ib-b3</th>
<th>Ib-b4</th>
<th>Ib-b3 and Ib-b4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic phase (15)</td>
<td>7 (47%)</td>
<td>6 (40%)</td>
<td>2 (13%)</td>
</tr>
<tr>
<td>Blast crisis (19)*</td>
<td>12 (63%)†</td>
<td>6 (32%)</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>TOTAL (34)</td>
<td>19 (56%)</td>
<td>12 (35%)</td>
<td>3 (9%)</td>
</tr>
</tbody>
</table>

† Including the cell lines KCL-22, KYO-1, and BV173.
In the 4 products, the sequences matched exactly ABL exon lb or la which joined in phase BCR exons b3 or b4, maintaining the open reading frame (ORF) up to the normal BCR stop codon. The ORFs of the ABL-BCR hybrid cDNAs code for predicted fusion proteins of 414 amino-acids (AA), 395 AA (lb-b3), 389 AA (lb-b4), and 370 AA (la-b4).

DISCUSSION

In Ph-positive CML, the coding sequences of two genes, ABL and BCR, are disrupted as a result of the reciprocal exchange between chromosomes 9 and 22, and two hybrid genes are formed, BCR-ABL (on 22q−, Ph) and ABL-BCR (on 9q+). Expression of the BCR-ABL gene was shown in all cases of Ph-positive CML when the sensitive method of RT/PCR amplification was used. However, it was not known whether the reciprocal ABL-BCR hybrid gene is likewise functionally active in this disease. This possibility was raised previously in a single report when a 3.5 M oligonucleotide probe sequence is specific for BCR exon b3. Note that in samples 2 and 8 both BCR-ABL and ABL-BCR transcripts hybridize to this probe, indicating the presence of BCR exon b3 in the two hybrid genes. Conversely, neither BCR-ABL nor ABL-BCR transcripts from sample 9 hybridize to the probe, which confirms the absence of exon b3 in both genes.

Table 5. BCR-ABL Transcript Versus ABL(lb)-BCR Transcript

<table>
<thead>
<tr>
<th>BCR-ABL (no. of cases)</th>
<th>ABL(lb)-BCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>lb-b3</td>
</tr>
<tr>
<td>b2a2 (19)</td>
<td>15</td>
</tr>
<tr>
<td>b3a2 (13)</td>
<td>4*</td>
</tr>
<tr>
<td>b2a2 and b3a2 (2)</td>
<td>0</td>
</tr>
</tbody>
</table>

* Cases in which the ABL-BCR transcripts are not reciprocal to the BCR-ABL transcripts.

Fig 3. Southern-blot hybridization with oligonucleotide (primer) B3 of (A) BCR-ABL and (B) ABL-BCR PCR products of the same representative samples shown in Fig 1. The oligonucleotide probe sequence is specific for BCR exon b3.
The fifth (CP), additional i(Ph) chromosomes probably arose greater than within the second intron. The fact that a patients who express both b2a2 and b3a2 transcripts presumably result from translocation breakpoints in chromosome 9 upstream of ABL exon Ib, or from deletions in BCR sequences 3' to the chromosome 22 breakpoint. In neither case would an ABL-BCR hybrid message be present.

The higher proportion (82%) of patients expressing only ABL-BCR transcripts of the Ib-BCR type, compared with those (18%) with transcripts initiated from both exons Ib and Ia (Ib-BCR and Ia-BCR) is probably a reflection of the frequency of breakpoints between exons Ib and Ia, and Ia and II. Because the distance between exons Ib and Ia is greater than 200 kb, approximately 10 times that between exons Ia and II (19 kb), the probability of a break occurring within the first large ABL intron might be 10-fold greater than within the second intron. The fact that a single transcript carrying both exons Ib and Ia was never found shows that the normal mechanism of alternative splicing of these two exons is maintained in the ABL-BCR hybrid gene.

The overall similar distribution of ABL-BCR–positive patients among the b2a2 and b3a2 BCR-ABL categories suggests that there is no simple correlation between the breakpoint sites in ABL and in BCR. On the other hand, the structure of the BCR moiety of the BCR-ABL and ABL-BCR transcripts from 7 patients showed unexpected patterns. In 2 of these, neither ABL-BCR nor ABL-BCR transcripts include the BCR exon b3. This means that the breakpoint may be in exon b3 itself, or that this exon was included in deletions at the breakpoint site. Another possibility is that b3 was spliced out in the mature mRNA from whichever of the two chimeric primary transcripts that retained it. Precedents for alternative splicing of exon b3 in BCR-ABL are well established in those patients who express both b2a2 and b3a2 transcripts and, in the present study, by the 3 patients with both Ib-b3 and Ib-b4 ABL-BCR. More surprising are the 5 patients in this study in whom both BCR-ABL and ABL-BCR transcripts contain BCR exon b3. In 3 of these (3 BC), the karyotype showed single Ph and 9q+ chromosomes, in 1 (CP), the 22q— was longer than a typical Ph and there was no 9q+, and in the fifth (CP), additional i(Ph) chromosomes probably arose from duplication of the original Ph. Therefore in these cases, there is no evidence that the BCR-ABL and the ABL-BCR hybrid genes are not part of the same clone, which implies that BCR exon b3 alone was duplicated in the t(9;22). Whether this b3 duplication took place before or during the translocation is not known. The breakpoint regions of both BCR-ABL and ABL-BCR in these 5 cases are being cloned for further studies.

The nucleotide sequence of the 4 different ABL-BCR hybrid cDNAs found in our series shows that in each case the 5'ABL-3'BCR junction is in phase as expected. Therefore, ABL-BCR fusion proteins of about 390 AA could be translated, the exact size of each varying according to the exon contribution of ABL and BCR to the transcript. The fact that ABL-BCR amplifications were obtained from oligo-dT primed cDNAs indicates the presence of poly(A) on these transcripts and suggests that translatable RNA is produced. Investigations on the presence of such ABL-BCR proteins in cells from patients with CML are in progress.

The significance of ABL-BCR expression in CML is still unclear. It seems unlikely that the hybrid ABL-BCR gene has any primary oncogenic role, because it is not present in at least one third of the CML patients. Furthermore, the fact that ABL-BCR is expressed in CP as well as in BC of CML argues against a role in causing disease progression. On the other hand, ABL-BCR expression could well be associated with specific clinical and/or hematological features in subsets of CML patients, which may reflect prognosis and response to treatment. Data from our present series are still insufficient to address this question.

If a functional ABL-BCR fusion protein is indeed produced, as predicted from the cDNA coding sequences, it would contain a GAP-BCR domain in its C-terminus linked to an N-terminal ABL sequence. Such an arrangement could alter the racGAP function of BCR leading to either constitutive activation or inactivation of the rac activity. Moreover, the Ib-BCR-encoded fusion protein, like the ABL type II P145 would have a myristoylation site at its N-terminus and, therefore, unlike p160BCR, it could become associated with the cell membrane. Although mRNA expression from the normal BCR allele can be detected in CML cells, it is not clear whether the level of P160BCR production is the same as in normal leukocytes. Coincidence or normal ABL and BCR genes could result in competition for the same target protein, rac, and imbalance in the rate of rac activation. The biologic effects of a deregulated BCR are unknown. However, because the rac proteins display relative myeloid specificity and are involved in activation of the NADPH oxidase system of neutrophils, it is tempting to speculate that an ABL-BCR protein with altered GAP activity might have a role in granulocyte function.
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