BCR-ABL, ABL-BCR, BCR, and ABL Genes Are All Expressed in Individual Granulocyte-Macrophage Colony-Forming Unit Colonies Derived From Blood of Patients With Chronic Myeloid Leukemia

By Joana Diamond, John M. Goldman, and Junia V. Melo

It has been suggested that the BCR-ABL gene of chronic myeloid leukemia (CML) is not uniformly expressed in Philadelphia (Ph)-positive cells, and that BCR-ABL gene expression precludes transcription of the normal BCR or ABL genes. Therefore, we have analyzed granulocyte-macrophage colony-forming unit (CFU-GM) colonies derived from peripheral blood of 11 CML patients by cytogenetic and by reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of BCR-ABL, ABL-BCR, BCR, and ABL. All CFU-GM colonies with analyzable metaphases were found to contain a Ph chromosome. In 2 patients, the initial PCR screening failed to detect BCR-ABL transcripts in 2 of 11 and 1 of 7 Ph-positive colonies. However, when amplification for BCR-ABL was repeated in quintuplicate, all but 1 colony from a single patient showed one or more positive results. Amplifications of the four genes in each colony showed that BCR-ABL, ABL-BCR, and the normal BCR and ABL were simultaneously expressed in the majority of CFU-GM colonies. Duplicate PCR tests for BCR and for ABL in colonies initially scored as negative also uncovered previously undetected positive amplifications. We conclude that BCR-ABL expression does not suppress transcription from the normal BCR and ABL genes, and that Ph-positive, BCR-ABL-negative colonies derived from peripheral blood CFU-GM are rare or nonexistent.

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Thermocycling parameters were 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 50 seconds, and extension at 72°C for 1 minute, followed by a final 10-minute extension at 72°C. Each PCR experiment included a control reaction without template cDNA, a blank control for RNA extraction, and a blank control for cDNA synthesis. Rigorous precautions were taken to eliminate the possibility of false-positive results, as described elsewhere. The sequences of the synthetic oligonucleotide primers used in this investigation are shown on Table 1. The size of the PCR fragments obtained from amplifications of the various transcripts varied between 159 and 424 bp. Five microliters from each second-step PCR product were electrophoresed through ethidium bromide-stained 2% agarose gels, visualized, and photographed under UV light. Quantification of BCR-ABL transcripts. Competitive PCRs to estimate the number of BCR-ABL transcripts in CFU-GM colonies were performed as described elsewhere.

RESULTS

The number of colonies studied per patient was 7 to 32 (mean, 16). Additional colonies were harvested in assays from 4 patients for cell counts and preliminary cDNA titration experiments. The day-14 CFU-GM colonies contained on average 500 to 1,000 cells, as assessed by cell counts of 7 colonies from each of two independent methylcellulose assays.

Cytogenetics. Cytogenetic analysis of CFU-GM colonies was not uniformly successful (Table 2). Although a few metaphases could be found in preparations from the majority of colonies, most of them were of poor technical quality and, therefore, not reliable for identifying or excluding a Ph chromosome. Attempts to arrest a larger fraction of cells in metaphase by longer incubations of up to 24 hours with Colcemid resulted in a general worsening of chromosome morphology and were discontinued. Thus, cytogenetic results were recorded only for those colonies in which at least two well-spread metaphases could be analyzed. Except for patient no. 3 (Ph-negative, BCR-ABL-positive), all CFU-GM colonies with analyzable metaphases contained a Ph chromosome.

Assessment of gene expression by RT-PCR. Our main objective was to investigate whether all PB myeloid progenitors from CML patients at diagnosis carried a functional BCR-ABL gene and whether transcription from the normal ABL and BCR genes and from the reciprocal ABL-BCR fusion gene was somehow dependent on or suppressed by BCR-ABL expression. This screen required RT-PCR amplifications of six different transcripts (ie, BCR-ABL, normal BCR, normal ABL Ib and Ia, and ABL-BCR Ib and Ia) in six separate amplification reactions. We designed an RT-PCR screening in which 5 μL cDNA could be used for amplification of each transcript (Fig 1) and the remaining

Table 1. Synthetic Oligonucleotide Primers Used in This Study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'→3')</th>
<th>Gene Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1'</td>
<td>GAGGGTGCAAGTGGGAGGGAAC</td>
<td>BCR (b1)</td>
</tr>
<tr>
<td>B2A'</td>
<td>TCCAGAGCTCTCCCTGACAT</td>
<td>BCR (b2)</td>
</tr>
<tr>
<td>B2B'</td>
<td>ACACCATCCGGATCCATCAAA</td>
<td>BCR (b2)</td>
</tr>
<tr>
<td>BE16</td>
<td>CATGATATCCAGGCTGGGA</td>
<td>BCR (b5)</td>
</tr>
<tr>
<td>B4e</td>
<td>ACGTGAAATCCATGTCCTAGT</td>
<td>BCR (b4)</td>
</tr>
<tr>
<td>Nib'</td>
<td>CAGAAATCTGGGAAGGTCCTTTATA</td>
<td>ABL (Ib)</td>
</tr>
<tr>
<td>GLY'</td>
<td>GGATGCCGCGAGCACCTGTGAAG</td>
<td>ABL (Ib)</td>
</tr>
<tr>
<td>AAR'</td>
<td>TACGGGATCTATGCCAGATGCTGGAA</td>
<td>ABL (Ia)</td>
</tr>
<tr>
<td>L1A'</td>
<td>CGTGTTGGCTCAATCAGAAGA</td>
<td>ABL (Ia)</td>
</tr>
<tr>
<td>Jc'</td>
<td>GGAAGTGTCTCTCCAGACTTGTG</td>
<td>ABL (III)</td>
</tr>
<tr>
<td>C3A'</td>
<td>TGTGACTGGGTAGTGAGTGTGG</td>
<td>ABL (III)</td>
</tr>
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Table 2. Ph-Positivity Versus BCR-ABL Expression in Individual CFU-GM Colonies

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Ph-Positive</th>
<th>Informatory Colonies</th>
<th>Colonies Assayed</th>
<th>RT-PCR for BCR-ABL (No. of Positive Colonies)</th>
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<tr>
<td>1</td>
<td>3</td>
<td>3</td>
<td>7</td>
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<tr>
<td>2</td>
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<tr>
<td>3</td>
<td>0*</td>
<td>5</td>
<td>14</td>
<td>11/14</td>
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<tr>
<td>4</td>
<td>NA</td>
<td>0</td>
<td>11</td>
<td>7/11</td>
</tr>
<tr>
<td>5</td>
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<td>0</td>
<td>13</td>
<td>8/13</td>
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<tr>
<td>6</td>
<td>10</td>
<td>10</td>
<td>16</td>
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<td>7</td>
<td>NA</td>
<td>0</td>
<td>16</td>
<td>15/16</td>
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<td>11</td>
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<td>7</td>
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<td>10/14</td>
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<td>1</td>
<td>15</td>
<td>15/15</td>
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<tr>
<td>11</td>
<td>5</td>
<td>5</td>
<td>15</td>
<td>16/16</td>
</tr>
</tbody>
</table>

Abbreviations: NA, not available; ND, not done (all colonies BCR-ABL positive on first test).
* Ph-negative (BCR-ABL-positive) patient.

Fig 1. (A) BCR-ABL, (B) BCR, (C) ABL Ib, (D) ABL Ia, and (E) ABL-BCR Ib PCR products in 10 representative colonies from patient no. 6, as detected on ethidium bromide-stained agarose gels. Lanes 1 to 10, CFU-GM colonies; lane 11, control plucked methylcellulose with no cells; lane 12, blank for RNA extraction; lane 13, blank for cDNA synthesis; lanes 14 and 15, blanks for first- and second-step PCR, M, DNA molecular weight marker (pEMBL digested with Taq I).
phenomenon could be responsible for the apparent lack of colonies. It was impossible to ascertain the Ph status of the colony without detectable BCR-ABL transcription in patient no. 9, because no analyzable metaphases were found in the corresponding cytogenetic preparation.

To validate the results of these quintuplicate tests, BCR-ABL amplifications were also repeated 5 times in all the negative controls from 9 of the 11 patients. In no instance were PCR products detected in any of the negative controls, suggesting it is unlikely that the positive results obtained from test specimens were caused by trace contaminants. Similarly, repeat BCR-ABL amplifications in colonies from the normal individuals did not yield any detectable product.

Because the replicate PCRs for BCR-ABL uncovered previously undetected positive amplifications, we postulated that the same limitation could be applicable to the apparent lack of expression in some colonies of the other genes studied. Therefore, we repeated in quintuplicate the PCR amplifications for BCR and ABL on colonies from 1 patient (no. 8) for which cDNA was still available. BCR transcripts were now detected in 3 of 4 and ABL transcripts in 3 of 6 colonies initially scored as negative. Control quintuplicate ABL(1b) amplifications in 6 colonies from the KYO-1 cell line were all negative.

The overall results suggested that the apparently random pattern of expression of BCR-ABL and related genes by CFU-GM progenitors could be caused by poor reproducibility of amplifications from very low numbers of template transcripts. Assessment of the number of BCR-ABL transcripts in CML of 5 colonies from 1 patient by a competitive PCR assay showed an estimated number of less than 10 to 40 transcripts per amplification, with no significant difference between colonies initially scored as positive on the first test and those showing a positive signal only after quintuplicate analysis (Fig 3). For 2 of these colonies, triplicate competitive PCR tests showed that the number of transcripts detected in each amplification was not always concordant, but varied within the above range. These experiments confirmed a “sampling phenomenon” was probably the major contributor to the variability of results observed in amplifications from small numbers of transcripts.

DISCUSSION

The possible existence in CML of a Ph-positive cell population with a “silent” or nonfunctional BCR-ABL gene has important biologic and therapeutic implications, namely in the interpretation of data generated by PCR analysis of hematopoietic colonies. We have addressed this question by investigating the expression of BCR-ABL and the related ABL, BCR, and ABL-BCR genes in CFU-GM colonies derived from the PB progenitors of CML patients at diagnosis. To establish whether all circulating progenitors carry a BCR-ABL gene and are, therefore, part of the leukemic clone, one half of each colony was assessed by cytogenetics...
for the presence of the Ph-chromosome. However, this assessment was hampered by the lack of good-quality analyzable metaphases in many colonies. We think this limitation, rather than being caused by trivial technical reasons, is inherent to systems of cytogenetic evaluation of very small cell samples. Only 16% to 69% of the colonies were evaluable for cytogenetic analysis, and, although all of them were found to be Ph-positive, we could not, by this method, exclude the possibility that occasional colonies were derived from Ph-negative progenitors, as has been reported previously.

However, the high sensitivity of the RT-PCR assays used here was instrumental in showing the presence of an actively transcribed BCR-ABL gene in cells from the great majority of colonies. In only one colony from a single patient, were BCR-ABL transcripts repeatedly undetectable. Because no analyzable metaphases were available for this colony, we could not conclude whether this represented lack of the BCR-ABL gene (ie, Ph-negative, normal progenitor), lack of BCR-ABL transcription (ie, Ph-positive without BCR-ABL expression), or a level of transcription below the threshold of the RT-PCR detection system. Overall, the data suggest that Ph-positive, BCR-ABL-negative progenitors are probably extremely rare in the PB of CML patients.

Our data conflict with those of Keating et al., who reported that 23% of Ph-positive colonies derived from the BM of CML patients did not transcribe BCR-ABL. These discordant findings could be explained if the BM of CML patients contained two progenitor cell populations, but, by some unknown mechanism, only the progenitors from the Ph-positive, BCR-ABL-negative cells were not released into the PB. This hypothesis does derive some support from the work by Bedi et al., who reported the existence in CML BM of a primitive progenitor population exhibiting BCR-ABL gene rearrangement without BCR-ABL mRNA or P210 protein expression. However, our RT-PCR strategy differs technically from that of Keating et al. in two important respects. First, in their study, each colony was tested only once for BCR-ABL expression, and, as we have shown here, detection of small numbers of BCR-ABL transcripts may require repeated amplification tests. The extensive negative-control experiments of replicate RT-PCR reactions without cellular template or with normal BCR-ABL-negative cells confirmed that the positive detection of transcripts in CML colony cells was not the product of a contamination artifact. Second, the positive RT-PCR controls used in the study of Keating et al. were not cDNA-specific, as we have shown elsewhere, and lack of BCR-ABL amplification in these circumstances might have been the result of poor-quality cDNA template rather than actual absence of transcripts in these colonies.

Finally, our data show that the BCR-ABL and ABL-BCR hybrid genes and the normal ABL and BCR alleles are simultaneously expressed in the majority of CML CFU-GM colonies. These findings suggest that there is no suppression of transcription of one gene by the other(s), in contrast to reports that expression of BCR and BCR-ABL might be mutually exclusive in Ph-positive colonies. We conclude...
from our analysis that there is no evidence of allelic exclusion of the normal or the translocated BCR and ABL genes in CML progenitors, as previously postulated.15

REFERENCES


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