**ABL1** Promoter Methylation Can Exist Independently of BCR-ABL Transcription in Chronic Myeloid Leukemia Hematopoietic Progenitors

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**ABSTRACT**

Formation of the hybrid BCR-ABL gene is responsible for >95% of chronic myeloid leukemia (CML). The alternative, downstream ABL promoter (Pa), which is usually retained in this chimeric oncogene, was reported to be methylated in many CML patients, but there has been controversy as to whether this methylation is a frequent change in bone marrow (BM) in early chronic phase (CP) or only past this stage. Also, the relevance of Pa promoter methylation to BCR-ABL expression in CML is unclear. We examined methylation of the ABL Pa promoter in uncultured BM samples and in colonies derived from their hematopoietic precursor cells by bisulfite and PCR-based assays (combined bisulfite restriction analysis and methylation-specific PCR). BM from seven CP CML patients at diagnosis had about 20–60% of the copies of the ABL Pa promoter methylated. No Pa methylation was detected in normal BMs or colonies derived from them. In contrast, most colonies from CP CML patients had Pa methylation. Surprisingly, 18–49% of the CML-derived colonies with this methylation reproducibly had no detectable BCR-ABL RNA by nested reverse transcription-PCR. Furthermore, the percentage of BCR-ABL RNA-positive colonies was almost same among the colonies not displaying Pa methylation as among the colonies in which this methylation was found. We conclude that ABL Pa methylation is often an early marker of CML in hematopoietic precursors and in total mononuclear BM cells but that it is not associated with an increased frequency of BCR-ABL RNA-positive cells. This methylation might be emblematic of cancer-associated hypermethylation elsewhere in the genome with the consequent silencing of tumor suppressor genes seen in many malignancies.

**INTRODUCTION**

The t(9;22; q34;q11) translocation that produces the activated oncogene-containing Ph chromosome is responsible for >95% of the cases of CML (1–3). This oncogene contains the 5′ end of the BCR gene fused to the second exon, exon 1a, of the ABL gene. It encodes a fusion protein, BCR-ABL, which, like ABL, is a tyrosine kinase. However, the fusion protein has altered kinase activity, which is the source of its oncogenicity by affecting signal transduction pathways and gene expression (4, 5). The normal ABL gene is constitutively expressed with transcription initiating either from the Pa promoter or the upstream Pb promoter (6). These transcripts specify functional proteins, albeit with different NH2-termini. The ABL Pa promoter is usually retained within the chimeric gene on the Ph chromosome (7). Ben-Yehuda et al. (8) have proposed that transcription from Pa could interfere with expression of the BCR-ABL product from the upstream BCR promoter and that Pa methylation might abrogate this down-regulation and thereby facilitate blastic transformation. Methylation of CpGs, especially in promoter regions, can repress gene expression in vivo (9), including in cancers. Abnormal promoter hypermethylation is frequently one of the steps of oncogenesis (10–12). Methylation of the ABL Pa promoter has been found in CML BM and PB samples but not in analogous samples from normal individuals or in patients with acute lymphocytic leukemia, acute myelogenous leukemia, lymphoma, or myelodysplastic syndrome (8, 13, 14). There is controversy about the frequency of this methylation in CML. Ben-Yehuda et al. (8) reported that only 26% of 148 CP CML patients displayed ABL Pa methylation as assessed by the ability of this DNA region to be amplified by PCR after digestion with CpG methylation-sensitive restriction endonucleases. In contrast, Issa et al. and Nguyen et al. (13, 14) found that 78% (73/93) and 77% (10/13) of CP patients were positive for such methylation by Southern blot analysis or a bisulfite and PCR-based assay (methylation-sensitive single nucleotide primer extension). Ben-Yehuda et al. (8, 15) observed large increases in Pa methylation correlated with the stage of disease in analyses of uncultured BM and PB samples and in derivative hematopoietic colonies. Recently, Issa et al. reported only modest increases (13) and Nguyen et al. reported no increase (14) in Pa methylation with the stage of the disease in studies of blood and BM. For evaluation of the hypotheses that this methylation contributes to blastic transformation and may be a predictor of the response to IFN-α therapy (8, 15), it is important to assess the frequency of ABL Pa methylation and its relationship to BCR-ABL RNA levels in hematopoietic colonies as well as in uncultured BM and PB samples. These colonies arise from hematopoietic progenitor cells, which are the repository for maintenance and progression of CML.

In the present study, we examined hematopoietic colonies both for their ABL Pa methylation status and the presence of BCR-ABL transcripts. During the course of this study we established conditions for RT-PCR and methylation analysis from individual colonies that give reproducible results circumventing problems described previously with stochastic effects on PCR of hematopoietic colonies (16). We also quantitated ABL Pa methylation in BM samples from CP CML patients and correlated our findings with the relative concentration of BCR-ABL transcripts in these samples and with clinical cytogenetic observations. We confirmed the high incidence of Pa methylation in CP BM samples and the lack of such methylation in normal BM (13, 14). However, unexpectedly, many BM-derived hematopoietic colonies from CML patients displayed ABL Pa promoter methylation but not BCR-ABL transcripts.

**MATERIALS AND METHODS**

**Clinical Samples, Colony Formation, and DNA Isolation.** BM samples were from 10 early CP CML patients (7 female and 3 male; ages 42–68). Patient CML10 had been treated with IFN-α and low-dose AraC for 6 months. The only PB sample was a stem cell pheresis product from a patient (CML9) after 8 months of IFN-α/AraC therapy and then treatment with idarubicin/AraC and granulocyte colony-stimulating growth factor (protocol approved by the Investigational Review Board of Tulane Medical Center). The other patient samples were obtained at diagnosis. The CML patients and four healthy BM...
donors (two female and two male, ages 9, 31, 42, and 43) gave informed consent for study of their tissues. The MNC fraction was immediately plated for colony formation with a 14-day incubation in methylcellulose medium containing GM-CSF, stem cell factor, erythropoietin, and interleukin 3 (MethoCult GF H4434, Stem Cell Technologies, Vancouver, British Columbia, Canada) or frozen for viability and later plated and incubated for 14 or 21 days. Only well-separated colonies with the typical features of either CFU-GM or BFU-E colonies were harvested. As a control for PCR, methylcellulose without cells was aspirated and processed analogously. Genomic DNA was isolated from the MNC by standard methods (DNeasy kit; Qiagen, Valencia, CA).

**In Vitro Methylation of Normal DNA.** Normal lymphocyte DNA (12 μg) was incubated with 120 units of M.SssI Cpg methylase (New England Biolabs, Beverly, MA) and 320 μM AdoMet at 37°C in a 1:2:1 reaction volume. After 3 h, an equal amount of AdoMet was added, and incubation continued for 2 h. The extent of in vitro methylation was determined by methylation in parallel of a plasmid containing a single SmaI site, digestion with SmaI, electrophoresis, and analysis of the ratio of signal in the intact plasmid band to the total signal. This ratio was 0.95, and so we estimated the extent of in vitro methylation of the ABL Pa promoter in normal lymphocyte DNA, which is unmethylated at tested sites in vivo (14), to be ~95%.

**Bisulfite Modification of DNA.** BM or PB DNA (at least 2 μg in 25 μl of TE buffer) was treated with bisulfite (17) at 55°C for 4 h and 2 μg of salmon sperm DNA was used as the carrier for ethanol-purification. The DNA was dissolved in 50 μl of 10 mM Tris (pH 8)-1 mM EDTA and stored at −20°C for up to 1 week before PCR. For hematopoietic colonies, cells from half of a colony were incubated for 60 min at 37°C in 25 μl of water containing 2 μg of salmon sperm DNA, 1 mM SDS, and 280 μg/ml proteinase K (18); treated in a boiling water bath for 15 min under mineral oil; and treated with bisulfite as described above.

**COBRA Analysis of ABL Pa Methylation.** PCR was performed on 10 μl of bisulfite-treated BM or PB DNA in 50 μl of reaction mixture containing 1.5 mM MgCl₂, 0.2 mM of each deoxynucleotide triphosphate, 2.5 units of Taq polymerase (HotStarTaq, Qiagen), and 150 ng each of COBRA1 and COBRA2 primers for the noncoding strand of the Pa promoter (Fig. 1; Table 1). DNA was denatured at 94°C for 15 min; amplified for 35 cycles of 94°C for 45 s; 48°C for 45 s; 72°C for 45 s; and incubated at 72°C for 10 min. An aliquot from colonies that gave no band with the methylation-specific primer pair were always assayed with the unmethylation-specific primer pairs (MSP1unmeth and MSP2 followed by MSP1unmeth and MSP3; 58°C, Tₐₘₐₐₚ; colonies that gave no band with either set of primers were discarded from the analysis. For one of the untreated patients, PCR was done with both unmethylation-specific and methylation-specific primer pairs on all of the colonies.

**RNA isolation and RT-PCR for BCR-ABL RNA.** RNA was extracted (20) from MNC (3–5 × 10⁵) and reverse transcribed with M-MLV reverse transcriptase (300 units; Life Technologies, Inc., Gaithersburg, MD) and random hexamer primers in a 40-μl reaction. PCR was done as described above but with primers NB+ and ABL3– (Table 1; Ref. 21); 4 μl of the reverse-transcriptase reaction product; and 32 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s. Samples that were negative for the BCR-ABL transcript were amplified with primers A2N (for exon 2 sequences) and A4– (for exon 4 sequences; Table 1, 58°C, Tₐₘₐₐₚ) for the ubiquitously expressed ABL transcript. For quantitation of BCR-ABL transcripts, competitive RT-PCR to determine equivalence points was done with ABL as an internal standard, as described previously (22). For the MNC sample that gave no BCR-ABL product after the first round of PCR (CML9), nested PCR was done with a 200-fold dilution of the first-round product and primers B2A and CA3– (Table 1; 30 cycles; 60°C, Tₐₘₐₐₚ); competitors were added only during the first round. For BCR-ABL RT-PCR on colony samples, RNA was isolated after lysing the cells in 0.5 ml of denaturing buffer (20; 4 mM guanidinium thiocyanate, 5 mM EDTA, 25 mM sodium citrate, 0.5% N-lauroyl sarcosine) containing 4 μg of Escherichia coli RNA carrier. cDNA was prepared and nested PCR was done as above, in 25-μl reactions using 50 pM of the reverse-transcriptase reaction product and, in the second round of PCR, 2 μl of the first-round product. For BCR-ABL RNA-negative colonies, two rounds of PCR (30 cycles each) were performed with the ABL primers. The colonies that did not give the expected product with ABL primers (~10% of colonies) were excluded from the analysis.

**RESULTS**

**ABL Pa Promoter Methylation Analyses.** To standardize conditions for quantitation of methylation of the ABL Pa promoter (Fig. 1)
in BM and PB samples, we needed analogously methylated and unmethylated human DNA samples with known amounts of methylation in this promoter region. First, we prepared normal lymphocyte DNA exhaustively methylated at CpG sites with M.SssI, which was deduced to be ~95% methylated at its CpGs, as described in “Materials and Methods.” The unmethylated standard was the untreated normal lymphocyte DNA, which we showed to be unmethylated at the site monitored by COBRA (Figs. 2 and 3). COBRA and MSP assays of DNA methylation involve bisulfite modification and PCR and rely on the resistance of 5-methylcytosine residues and the sensitivity of unmethylated C residues to bisulfite modification (23, 24). On treatment with bisulfite and then alkali, an unmethylated C residue is converted to U, on which PCR becomes T.

COBRA is a quantitative assay of C methylation in which restriction sites can be created because of C→T conversions only at unmethylated C residues (25). The bisulfite and alkali treatments and PCR are followed by digestion with the appropriate restriction endonuclease. In the ABL Pa promoter there is a 5′-CCGAGGCCGA-3′ sequence amenable to methylation analysis by COBRA with TagI (Fig. 2). With various mixtures of the M.SssI-methylated and untreated lymphocyte DNA preparations, we showed that this COBRA assay was quantitative (Fig. 3). There was no appreciable bias for amplification of the unmethylated or the methylated ABL Pa sequence, a complication that is sometimes found when other DNA sequences (Table 2). By cytogenetic analysis of unstimulated BM, the similar percentages of methylation deduced from MSP and COBRA were generally in agreement about the approximate percentage of copies of the Pa promoter that were methylated (Table 2).

The similar percentages of methylation deduced from MSP and COBRA suggest that methylation is often widespread along the ABL Pa promoter region as concluded from genomic sequencing of CML patient samples in a previous study of accelerated and blast phase patients (15). The COBRA assay monitored methylation of sequences in the Pa promoter at position −133 to −142 (all positions given relative to the downstream Pa transcription start site; Ref. 6). At least one of the two CpGs at the assayed sequence should be methylated for the sequence to score as methylated in the COBRA assay (Fig. 2). For MSP, most or all of the seven CpGs at the genomic sequence from position −16 to +14 (MSPI primer region, Fig. 1) should be methylated if the bisulfite-treated sample is to hybridize specifically to the MSPI-meth primer and not to the MSPI- unmeth primer and thereby score as methylated. Despite the requirement for more mC residues at the assayed sequence in the MSP than in the COBRA assay, similar results were obtained from these two analyses (Table 2) suggesting that when the promoter region is methylated, it is usually extensively methylated.

The frequency of methylation of the Pa promoter in these CML samples was compared with the steady-state level of BCR-ABL transcripts determined by competitive RT-PCR, as illustrated in Fig. 6. The constitutively expressed transcripts initiated from either the Pa or Pb promoter from the unstimulated BM. Table 1) can be designed that semiquantitative MSP was confirmed with different ratios of M.SssI-methylated and untreated normal lymphocyte DNA (Fig. 4).

Quantitation of Methylation of the ABL Pa Promoter Region versus BCR-ABL Gene Expression in CP CML BM Samples. Using both COBRA and MSP, methylation of the ABL Pa promoter in BM samples obtained from four normal BM donors, seven untreated early CP CML patients, and one IFN-α/AraC-treated patient was quantitated. A stem cell pheresis PB sample of an IFN-α/AraC- and idarubicin/AraC-treated patient was also analyzed (Table 2). The normal BM samples showed no evidence of methylation of this promoter by either assay (Fig. 5). In contrast, all BM samples from the CML patients had appreciable methylation of this promoter at the assay sites (Fig. 5). The results from semiquantitative MSP and COBRA were generally in agreement about the approximate percentage of copies of the Pa promoter that were methylated (Table 2).

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those patients had $\geq45\%$ Ph$^+$ cells, which is consistent with their high level of BCR-ABL transcripts. As expected, only the PB sample from CML9 obtained after treatment with idarubicin/AraC and the BM sample from patient CML10 obtained after treatment with IFN-α/AraC showed low levels of BCR-ABL transcripts as well as the absence of detected Ph-positive cells on routine cytogenetic analysis (Table 2). The former had no detectable methylation, and the latter had only $\sim5\%$ of the copies of the Pa promoter methylated.

Comparison of Methylation of the ABL Pa Promoter and Expression of BCR-ABL Transcripts in CFU-GM and BFU-E Colonies from CML Samples. Because concerns have been raised about the reliability of PCR assays on hematopoietic precursor-derived colonies (16), we first checked the reproducibility of such assays. For each analyzed BM-derived CFU-GM colony ($\sim800$–1,500 cells/colony) or BFU-E colony ($\sim8,000$–10,000 cells/colony), we assayed half of the cells by MSP and half by BCR-ABL RT-PCR by amplifying $1/10$ of the cDNA or the bisulfite-treated genomic DNA. Triplicate MSP amplifications of DNA from three Pa methylator-positive CML colonies and three Pa methylation-negative colonies were uniformly positive or negative, and the positive replicate samples gave PCR product bands of similar intensities. Also, the same reproducibility among replicate aliquots from a given colony was observed in triplicate or quintuplicate amplifications of cDNA for BCR-ABL using 12 CML colonies. Furthermore, we tested the reproducibility of the RT-PCR not only by doing replicate amplifications on cDNA from a colony, but also by isolating RNA in quintuplicate from half of the cells of a colony and preparing replicate cDNA samples for RT-PCR. For this analysis we used five CFU-GM and five BFU-E colonies from BM of an untreated CML patient. All of these colonies were found to be positive for the BCR-ABL transcript when amplifying one-fourth of the cDNA product from each of these quintuplicate RNA preparations. The reproducibility of these findings was established by showing that whenever an RNA preparation was verified to be intact by amplification of the control ABL transcript, which was the case for $3–5$ of the replicate aliquots from each of the colonies, the RT-PCR for BCR-ABL was also positive. In the other replicate RNA preparations where the BCR-ABL primers gave no PCR product, the ABL primers also gave no product, probably indicating a problem with RNA preparation on such a small scale, smaller than we routinely used. Such ABL RNA-negative samples were never incorrectly scored as BCR-ABL-negative, because in Tables 3 and 4 we included colonies that were negative for BCR-ABL RNA only if they were positive for ABL RNA. Only $1$ exceptional aliquot of the $50$ tested was negative for BCR-ABL RNA and positive for ABL RNA, whereas the replicate aliquots from the same colony were positive for both RNA species. Therefore, although we were examining BCR-ABL expression and ABL Pa methylation in rather small numbers of cells, we did not have previously reported findings was established by showing that whenever an RNA preparation was verified to be intact by amplification of the control ABL transcript, which was the case for $3–5$ of the replicate aliquots from each of the colonies, the RT-PCR for BCR-ABL was also positive. In

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**Table 2** Comparison of cytogenetics, BCR-ABL RT-PCR, and methylation analyses (COBRA and MSP) on CML samples

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Cytogenetics: (No. Ph$^+$ cells/examined cells)$^a$</th>
<th>BCR-ABL/ ABL RNA$^b$</th>
<th>COBRA (%) meth. of ABL Pa$^c$</th>
<th>MSP (%) meth. of ABL Pa$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CML1 BM</td>
<td>9/20</td>
<td>0.3</td>
<td>41 $\pm$ 1</td>
<td>31</td>
</tr>
<tr>
<td>CML2 BM</td>
<td>20/20</td>
<td>0.15</td>
<td>20 $\pm$ 6</td>
<td>15</td>
</tr>
<tr>
<td>CML3 BM</td>
<td>20/20</td>
<td>0.2</td>
<td>29 $\pm$ 3</td>
<td>30 $\pm$ 3</td>
</tr>
<tr>
<td>CML4 BM</td>
<td>20/20</td>
<td>0.3</td>
<td>62 $\pm$ 3</td>
<td>60</td>
</tr>
<tr>
<td>CML5 BM</td>
<td>182/222</td>
<td>0.6</td>
<td>57 $\pm$ 1</td>
<td>34</td>
</tr>
<tr>
<td>CML6 BM</td>
<td>20/20</td>
<td>1.0</td>
<td>57 $\pm$ 4</td>
<td>45</td>
</tr>
<tr>
<td>CML7 BM</td>
<td>20/20</td>
<td>0.8</td>
<td>39 $\pm$ 0</td>
<td>40 $\pm$ 3</td>
</tr>
<tr>
<td>CML9 BM IFN-α/AraC,</td>
<td>0/20</td>
<td>0.0003</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CML10 BM IFN-α/AraC,</td>
<td>0/10; I-FISH, 0/25</td>
<td>0.03</td>
<td>4</td>
<td>7 $\pm$ 1</td>
</tr>
<tr>
<td></td>
<td>4 normal BMs</td>
<td>–</td>
<td>–</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$ BM from 7 CP CML patients at diagnosis, 1 treated CP CML patient, and 4 normal BM donors and 1 PB sample from a treated CP CML patient was examined.

$^b$ Cytogenetic analysis was by metaphase karyotyping unless indicated as I-FISH and was from clinical data at the time of BM sampling.

$^c$ The ratio of BCR-ABL:ABL transcripts was determined. All of the samples showed either the b2a2 type or the b3a2 BCR-ABL translocation by RT-PCR except for CML6, which displayed both the b2a2 and the b3a2 type of PCR product.

$^d$ Where PCR was done in duplicate, the mean and the SD are shown.
promoter methylated (Figs. 3 and 4) and methylation of the Pa promoter was seen in only a small number of MNC fraction of BM are clonogenic hematopoietic progenitors. In should be noted that only a very small percentage of the cells in the BM samples (Tables 2 and 3). However, in comparing these results it positive for Pa methylation were even higher than the percentage of their relative levels within a colony.

promoter were amplified with the unmethylation-specific primer pair, aliquots of DNA from CML colonies with methylated copies of the Pa promoter were present. There- they always gave the same size product indicating that both unmethy- lated and methylated copies of the Pa promoter were present. Therefore, unmethylated copies of the Pa promoter were present along with the methylated copies; the colony assays did not allow quantitation of their relative levels within a colony.

problems with stochastic effects on RT-PCR from colony samples (16) nor on MSP.

MSP and RT-PCR were done on about 20–40 colonies each from three BM samples from untreated CML patients who were strongly cytogenetically Ph-positive, a BM sample of a patient in complete cytogenetic remission after treatment with IFN-α-AraC (CML9 BM), and a stem cell pheresis PB sample obtained from this patient after additional treatment with idarubicin/AraC (CML9 PB). For the three untreated patients, 65–100% of the CFU-GM colonies and 36 or 34 cycles, respectively. No bands were visible for normal BM samples amplified with the methylation-specific primer pair. C, MSP analysis of Pa methylation in the same CML samples as in A. Std. is the standard mixture of in vitro-methylated and unmethylated lymphocyte DNA with ~50% of the copies of the ABL Pa promoter methylated (Figs. 3 and 4) and M is the marker DNA.

A surprising result from the comparison of the ABL Pa methylation and the BCR-ABL transcript status of CML BM-derived colonies was that about 15–40% of the total examined colonies from three BM samples obtained at diagnosis contained Pa methylation but no detectable BCR-ABL RNA even after two rounds of PCR on the cDNA (Table 3, column D). Moreover, the percentage of BCR-ABL RNA-positive colonies was no higher among the colonies in which the Pa promoter was methylated than in the colonies in which it was not (Table 4). The RT-PCR and MSP results from three colonies that were BCR-ABL RNA-negative and Pa methylation-positive and two colonies that were BCR-ABL RNA-positive and Pa methylation-negative were among those verified by quintuplicate RT-PCR and triplicate MSP assays, as described above. Furthermore, the percentage of BCR-ABL RNA-negative colonies was consistently higher for BFU-E than for CFU-GM colonies for untreated patient samples despite the larger size of the BFU-E colonies and the use of about 7–10-fold more cells for RT-PCR on these colonies than on the CFU-GM. This additionally validates the significance of our finding of BCR-ABL RNA-negative and Pa methylation-positive colonies from CML samples.

**DISCUSSION**

ABL Pa is the second downstream promoter of the ABL gene, and it is the promoter that is generally retained within the chimeric BCR-ABL gene of the Ph chromosome in CML hematopoietic cells. Methylation of this promoter has been proposed to be important in disease progression by repressing expression of the normal ABL transcript from the translocated gene thereby allowing more synthesis of the leukemic BCR-ABL transcript (26). If so, there should be only a low percentage of methylation of this promoter in early CP CML patients. With two assays for DNA methylation, COBRA and MSP, we have shown that a high percentage of early CP CML patients have Pa methylation in BM samples. Similar results were obtained by Issa et al. and Nguyen et al. (13, 14) with different methylation assays. Furthermore, in this study we demonstrated that most of the hematopoietic progenitor-derived colonies from early CP CML patients had ABL Pa methylation. This contrasts with the results of Asimakopoulos et al. (15), who found that only one CP patient of six displayed such methylation using the same MSP assay for analysis.

In this study, we quantitated both the level of ABL Pa methylation and of BCR-ABL transcripts in uncultured BM samples from CML

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**Fig. 5.** Analysis of ABL Pa methylation in normal BM samples and representative CML BM samples by COBRA and MSP. A, COBRA analysis of Pa methylation in CML BM samples (2, 10, 7, and 3) and normal BM samples (1–4). The 162- and 113-bp bands for CML2 and CML10 were clearly seen in the original gel. B, MSP analysis of Pa methylation in normal BM samples using methylation-specific or methylation-unspecific primer pairs and 36 or 34 cycles, respectively. No bands were visible for normal BM samples amplified with the methylation-specific primer pair. C, MSP analysis of Pa methylation in the same CML samples as in A. Std. is the standard mixture of in vitro-methylated and unmethylated lymphocyte DNA with ~50% of the copies of the ABL Pa promoter methylated (Figs. 3 and 4) and M is the marker DNA.

**Fig. 6.** Quantitation of BCR-ABL transcripts by competitive RT-PCR: a representative analysis. A, RT-PCR with BCR-ABL primers on CML1 BM (Table 2). B, RT-PCR on the same sample with ABL primers as an internal control. The number of molecules of competitor plasmid added before PCR is indicated above the Lanes for A. For this sample, the equivalence point, the concentration of competitor DNA at which the competitor and sample-derived PCR product bands are of equal intensity (22), was estimated to be ~3 × 10¹⁰ molecules for BCR-ABL transcripts and 1 × 10¹⁰ for the internal control, ABL transcripts. Therefore, the BCR-ABL/ABL ratio for this sample was 0.3 (Table 3). The negative control that was included in this set of assays was electrophoresed on another gel.

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With careful controls, we demonstrated that we should have negligible numbers of false negative results. Whether cells in these Pa-methylated, BCR-ABL RNA-negative colonies had a transcriptionally silent Ph chromosome or have CML-associated Pa methylation in the absence of the Ph chromosome remains to be determined by three-way cytogenetic analysis, Pa methylation assays, and BCR-ABL RT-PCR on hematopoietic progenitor-derived colonies having Pa methylation but no BCR-ABL transcripts. It has been reported that there are no BCR-ABL RNA-negative hematopoietic progenitor-derived colonies from CML samples that contain the Ph chromosome (29, 30), although evidence to the contrary has also been presented (31).

Importantly, we found that among the CML samples plated for colony formation, hematopoietic precursor-derived colonies that displayed ABL Pa methylation were no more likely to express BCR-ABL RNA than those without such methylation (Table 4). Nonetheless, ABL Pa methylation was never seen in this or other studies in uncultured non-CML samples (Fig. 5; Refs. 8, 13, 14) nor in cultured non-CML BM samples (Table 3). Therefore, methylation of this promoter is clearly associated with CML, although it does not appear to be linked to BCR-ABL expression.

Increases (and decreases) in DNA methylation in cancer cells (and in embryos) occur in many genomic sequences, only some of which affect the cellular phenotype (9, 11). Cancer-linked and transcription-repressing de novo promoter methylation of tumor suppressor genes is very often found in diverse types of malignancies (10–12). Although some tumor suppressor gene promoters were examined for hypermethylation in CP CML and found to be normal (14, 15, 32, 33), hypermethylation in the 5’ region of the calcitonin1 gene has been observed to track with CML progression (34, 35), and hypermethylation of BCR sequences has been reported in ~40% of CP CML patients (36). Because our results indicate that ABL Pa methylation does not favor BCR-ABL expression and our results and others (13, 14) demonstrate that it shows little or no correlation with leukemic progression, this methylation might just be a marker for coordinate methylation-regulated silencing of tumor suppressor genes in CP CML. Such DNA methylation might play a role in the generation of CML or in determining the variability in the clinical course of the disease.

ACKNOWLEDGMENTS

We thank Sherry Price for help with BM and PB sample preparation, Jean-Pierre Issa for sharing his unpublished sequences for COBRA primers.
with us, and Nicholas Cross and John Goldman for detailed information about competitive RT-PCR conditions and for plasmids.

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