Regulation of Myeloblastin Messenger RNA Expression in Myeloid Leukemia Cells Treated With All-\textit{Trans} Retinoic Acid

By Catherine Labbaye, Jin Zhang, Jean-Laurent Casanova, Michel Lanotte, Jianye Teng, Wilson H. Miller Jr., and Yvon E. Cayre

Retinoic acid is known to induce differentiation of human myeloid leukemia cells in vitro. Recently, all-	extit{trans} retinoic acid has been used to induce remissions in patients with acute promyelocytic leukemia, probably through differentiation of the leukemia cells. Myeloblastin (mbn) is a protease that has been identified in the human leukemia cell line HL-60. Downregulation of this protease can inhibit proliferation and induce differentiation of HL-60-derived leukemia cells. Here we have investigated the regulation of mbn messenger RNA (mRNA) expression in two human leukemia cell lines, HL-60 and NB4, treated with all-	extit{trans} retinoic acid. Under this treatment, downregulation of mbn mRNA was observed in both cell lines, but was considerably delayed in NB4 cells that carry the t(15;17) translocation characteristic of acute promyelocytic leukemia. We have found that multiple mechanisms were involved in the control of mbn mRNA expression. These mechanisms were different in HL-60 and NB4 cells. Our results show that in HL-60 cells, all-	extit{trans} retinoic acid rapidly decreased transcription of mbn. In contrast, in the t(15;17)-positive NB4 cells treated with all-	extit{trans} retinoic acid, upregulation of mbn mRNA expression was followed by a late downregulation, both achieved via posttranscriptional mechanisms.

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mmol/L l-glutamine. All-trans RA (Sigma, St Louis, MO) was dissolved in 95% (vol/vol) ethanol and used at final concentration of 10⁻⁵ mol/L. Cycloheximide was dissolved in phosphate-buffered saline (PBS) and used at a final concentration of 10 μg/mL. Actinomycin D was dissolved in absolute ethanol and used at a final concentration of 10 μg/mL. During treatment of cells by all-trans RA, cycloheximide, or actinomycin D, compounds and cell concentrations were adjusted if necessary to remain unchanged. For morphologic assessment of differentiation, cells were washed three times in PBS, cytocentrifuged (1,250 rpm for 5 minutes), and stained with Wright/Giemsa.

Cell cycle analysis. The DNA content of individual cells was measured by flow cytometry as described.¹⁴

RNA isolation and Northern blot analysis. Total RNA was prepared as described.¹⁵ For Northern blots, RNA preparations were denatured and processed as described.¹⁶ All RNA blots contained 10 μg of total RNA per lane. The filters were hybridized to 2 × 10⁶ cpm/mL of the full-length mbn cDNA probe after labeling by random primer DNA-labeling system (Bethesda Research Laboratory, Gaithersburg, MD). Ethidium bromide–stained 28S and 18S ribosomal RNA (rRNA) were used as assessment of RNA quantities on filter after transfer.

Nuclear run-on assays. Nuclear run-on assay was conducted as described.¹⁷ Nuclei were isolated from HL-60 and NB4 cells before and after treatment with all-trans RA, stored at −70°C in suspension buffer containing 40% glycerol, and assayed as described.¹⁹,²⁰ After thawing and pelleting at 1,000g at 4°C for 10 minutes, approximately 10¹⁰ nuclei were resuspended and incubated at 37°C for 30 minutes in 0.25 mL of run-on assay buffer [5 mmol/L dithiothreitol, 5 mmol/L MgCl₂, 90 mmol/L KCl, 50% glycerol, 20 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.6), 1 mmol/L each of GTP and CTP, 3 mmol/L ATP, 1 μg/μL RNASin (Promega, Madison, WI), and 250 μCi [³²P]UTP (800 Ci/mmol; New England Nuclear, Boston, MA)]. The nuclei were then centrifuged as above, and the pellet was washed three times in ice-cold lysis buffer (10 mmol/L NaCl, 10 mmol/L Tris [pH 7.4], 3 mmol/L MgCl₂, and 0.5% Nonidet P-40) followed by centrifugation. The RNA was then purified as described¹⁵ and resuspended in hybridization buffer at 1 × 10⁶ cpm/mL, and equal numbers of 10% trichloroacetic acid-precipitable counts were added to each hybridization. For the preparation of cDNA probes to detect run-on labeled transcripts, supercoiled plasmid DNA-containing cDNA sequences were applied to a Nytran filter in a slot blot apparatus (Schleicher and Schuell, Keene, NH), as described.¹⁷ Enough plasmid DNA to provide 4.0 μg of each insert cDNA sequence was applied to each slot. Slots were then washed three times with 600 μL of 6× SSC. The filters were baked and then prehybridized, hybridized, and washed as above for Northern blot analysis, except that after the high-stringency wash they were incubated at 37°C for 30 minutes in 10 μg/mL RNase A in 2× SSC before drying and exposure to X-AR5 film (Eastman Kodak, Rochester, NY). The 28S rRNA cDNA²¹ was a gift from H. Hanafusa’s laboratory (Rockefeller University, New York, NY). 28S rRNA was unchanged during RA-induced differentiation of HL-60 cells.²¹ Human CD18 and myeloperoxidase (MPO) cDNAs were obtained from ATCC.

RESULTS

Different kinetics of mbn mRNA expression in HL-60 and NB4 cells treated with all-trans RA. In both HL-60 and NB4 cells, mbn was eventually downregulated, but at different times. In HL-60 cells, mbn mRNA was drastically downregulated as early as 2 hours after treatment with all-trans RA (Fig 1A). Early downregulation of mbn mRNA expression was not observed in NB4 cells treated under the same conditions with all-trans RA. Instead, upregulation was observed 24 hours after treatment. This upregulation was followed by downregulation starting 4 days after treatment (Fig 1B). At this time HL-60 cells were partially differentiated, whereas no differentiation was noted in NB4 cells (data not shown). Similarly to mbn and in contrast to previous observation with HL-60 cells,²² in NB4 cells c-myel mRNA was upregulated after 24 hours to 3 days of exposure to all-trans RA, as verified by hybridizing the NB4 Northern blot with a c-myel cDNA probe. This increase was preceded by an initial down-
regulation of this mRNA (data not shown). In order to correlate the different kinetics of mbn mRNA expression to the rates of differentiation and growth of HL-60 and NB4 cells treated with all-trans RA, cells were stained with Wright/Giemsa and submitted to cell cycle analysis. By 3 days of treatment, differentiation was detected in HL-60 cells but not in NB4 cells, whereas by 6 days the percent of neutrophils was almost identical in both cell lines (Table 1). In both HL-60 and NB4 cells, the percentage of cells in the S phase of the cell cycle was decreased by more than 30% after 3 days of treatment with all-trans RA. This decrease was more pronounced by 4 days in HL-60 (55.9%) than in NB4 cells (44.34%) (Table 2). In both HL-60 and NB4 cells, no precise time point correlation could be established between differentiation, withdrawal from the cell cycle, and the kinetics of mbn mRNA downregulation. However, our results showed that HL-60 cells, where mbn mRNA decreased early after treatment by all-trans RA, were more sensitive than NB4 cells to the differentiation effects of this inducer.

Early transcriptional downregulation of mbn mRNA expression in HL-60 cells. Nuclear run-on transcription assays were performed to examine whether all-trans RA mediated transcriptional regulation of mbn mRNA expression. In HL-60 cells, mbn mRNA expression was transcriptionally downregulated by 66.6% and 98.3% after 2 hours and 24 hours, respectively, of all-trans RA treatment as measured by scanning densitometry (Fig 2A). This was consistent with the results obtained on the Northern blot as described above (Fig 1A). In contrast, no significant transcriptional regulation was detected in NB4 cells treated with all-trans RA for 2, 16, and 24 hours (Fig 2B and Table 3). Therefore, upregulation of mbn mRNA observed on the Northern blot at 24 hours (Fig 1B) was likely to be the result of increased stabilization of mbn mRNA. No significant transcriptional regulation of mbn expression was observed in NB4 cells treated for 2, 3, and 4 days with all-trans RA, whereas the level of the 28S rRNA remained unchanged (Fig 2C and Table 3). As previously observed with HL-60 cells, 2, 3, 4 in NB4 cells CD18 mRNA was transcriptionally upregulated early after treatment with all-trans RA, whereas MPO was transcriptionally downregulated after 2 and 3 days (Fig 2C and Table 3). Therefore, downregulation of mbn mRNA observed in NB4 cells treated for 4 days with this inducer (Fig 1B) was not correlated with transcriptional mechanisms.

Protein factor(s) are involved in mbn mRNA expression. We have tested the effect of the protein synthesis inhibitor cycloheximide (CHX) on the expression of mbn mRNA in both HL-60 and NB4 cells. Treatment of HL-60 cells with CHX resulted in superinduction of mbn mRNA expression with a peak at 4 hours after treatment (Fig 3A). This result suggests that, in HL-60 cells, a protein factor(s) maintaining a low basal mbn mRNA level was inhibited by CHX. After this superinduction, mbn mRNA level decreased progressively and no mRNA was detected after 16 hours (Fig 3A). Similar results were observed when HL-60 cells were treated with both all-trans RA and CHX. These results suggest that transcriptional downregulation of mbn expression was factor dependent, although downregulation of mbn mRNA expression was still observed at 2 hours after treatment (Fig 3B). In contrast, treatment of NB4 cells with CHX did not result in superinduction of mbn mRNA expression. Instead, a progressive decrease was observed when CHX was used either alone (Fig 3C) or in association with all-trans RA (Fig 3D). These decreases in mbn mRNA levels were not the results of CHX toxicity on NB4 cells, because their viability after 8 and 24 hours of treatment by CHX was 96.5% and 94.5%, respectively, as assessed by the trypan blue exclusion test. Therefore, these results suggest that the increased mbn mRNA levels observed in NB4 cells treated with all-trans RA were dependent on protein synthesis. Inhibition of a protein factor(s) by CHX resulted in decreased levels of mbn mRNA.

Superinduction of mbn mRNA expression by actinomycin D. Because the effect of protein synthesis inhibitors such as CHX can occur at both transcriptional and posttranscriptional levels, cells were treated with actinomycin D (ActD), an inhibitor of transcription, in the presence or absence of CHX. Superinduction of mbn mRNA expression was observed in both NB4 and HL-60 cells treated for 45 and 90 minutes with ActD alone (Fig 4A and B, respectively). In NB4 cells, this superinduction was associated with an increased level of mbn mRNA between 45 and 90 minutes (Fig 4A). mbn mRNA was also increased when NB4 cells were treated simultaneously with either CHX and ActD (Fig 4A) or with all-trans RA and ActD (Fig 4C). As a control of transcriptional inhibition by ActD and as reported, 25 in HL-60 cells c-fos mRNA was downregulated by ActD and superinduced by CHX (data not shown). Although superinduction of mbn mRNA by ActD was surprising, one interpretation

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**Table 1. Differential Counts of HL-60 and NB4 Cells After Incubation With All-trans RA**

<table>
<thead>
<tr>
<th>Day</th>
<th>NB4</th>
<th>HL-60</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>98</td>
</tr>
<tr>
<td>3</td>
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<td>6</td>
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<td>34.5</td>
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<td>Total</td>
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**Table 2. Cell Cycle Analysis**

<table>
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<th>Day 0</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB4</td>
<td>44.2%</td>
<td>26.8%</td>
<td>24.6%</td>
</tr>
<tr>
<td>HL-60</td>
<td>35.2%</td>
<td>24.2%</td>
<td>15.5%</td>
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</tbody>
</table>

Percentages of cells in the S phase of the cell cycle are indicated before and after treatment of NB4 and HL-60 cells by all-trans RA for 3 and 4 days.
Fig 2. Regulation of mbn transcription by all-trans RA. Transcriptional run-on assays of nuclei isolated from HL-60 (A) and NB4 cells (B and C) before and after exposure to all-trans RA for 2, 16, and 24 hours (A and B), and 2, 3, and 4 days (C). cDNA samples loaded on the blots are mbn, pUC9 vector without insert DNA, CD18, MPO, and 28s rRNA. Transcription of the 28s rRNA remained unchanged after exposure of HL-60 and NB4 to all-trans RA. For (E) and (C), densitometry units are indicated in Table 3.

of our results could be that in NB4 cells, ActD inhibited a negative transcriptional control on the expression of a factor(s) that stabilizes mbn mRNA. The same hypothesis applies to the effect of all-trans RA, because it increased the level of mbn mRNA in NB4 cells through a posttranscriptional mechanism (Fig 1B). The hypothesis that a factor(s) that stabilizes mbn mRNA is present in NB4 cells is in agreement with the fact that treatment of NB4 cells by CHX results in a progressive decrease of mbn mRNA levels (Fig 3D). In contrast to NB4, in HL-60 cells the fact that both CHX and ActD resulted in superinduction of mbn mRNA (Figs 3A and 4B) suggests that treatment by these agents provoked downregulation of a negative transcription factor(s). This hypothesis was reinforced by the fact that transcriptional downregulation of mbn expression was not observed in HL-60 cells treated with both all-trans RA and CHX for 2, 4, and 8 hours (data not shown).

DISCUSSION

We have shown that although downregulation of mbn mRNA expression was eventually observed in both HL-60 and the t(15;17)-positive NB4 cells treated with all-trans RA, it was not obtained at the same time and not through the same mechanisms.

Our results indicate that multiple mechanisms regulate mbn mRNA expression in the two leukemia cell lines after treatment by all-trans RA. We have found that although in HL-60 cells mbn mRNA expression was controlled transcriptionally, it was mainly the result of posttranscriptional control in NB4 cells. In both HL-60 and NB4 cells, these mechanisms involve protein factors that might be regulated

Table 3. Quantification of Transcriptional Activities for mbn, 28S rRNA, CD18, and MPO in NB4 Cells Before and After Treatment by All-trans RA

<table>
<thead>
<tr>
<th>cDNAs</th>
<th>NB4</th>
<th>2 h</th>
<th>2 d</th>
<th>3 d</th>
<th>4 d</th>
<th>+ RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>mbn*</td>
<td>542</td>
<td>701</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28S*</td>
<td>1,020</td>
<td>1,150</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mbn†</td>
<td>351.7</td>
<td>—</td>
<td>375.8</td>
<td>351.7</td>
<td>419</td>
<td></td>
</tr>
<tr>
<td>CD18†</td>
<td>295.3</td>
<td>—</td>
<td>838.2</td>
<td>469.7</td>
<td>689.9</td>
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</tr>
<tr>
<td>MPO†</td>
<td>2,398</td>
<td>—</td>
<td>751.4</td>
<td>1,861</td>
<td>2,535</td>
<td></td>
</tr>
<tr>
<td>28S†</td>
<td>1,921</td>
<td>—</td>
<td>1,939</td>
<td>1,102</td>
<td>1,925</td>
<td></td>
</tr>
</tbody>
</table>

Values are densitometry units as measured by scanning densitometry.
* NB4 cells before and after exposure to all-trans RA for 2 h (Fig 2B).
† See Fig 2C.
Fig 3. Effect of a protein synthesis inhibitor on mbn mRNA expression. Autoradiograms of mbn mRNA in HL-60 (A and B) and NB4 cells (C and D). Total RNAs were prepared from cells either treated with CHX (A and C) or treated with both CHX and all-trans RA (B and D) for the indicated times. 28S and 18S rRNA are indicated as size markers. Bottom panels show ethidium bromide-stained 28S rRNA on the gels as assessment of RNA quantities in each lane.

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differently by all-trans RA at discrete stages of myeloid differentiation. The fact that mbn mRNA expression was not regulated through the same mechanisms in HL-60 and NB4 cells may reflect the possibility that these cells represent two discrete stages of myeloid differentiation where different sets of protein factors are present or activated. Alternatively, the differences observed between the two cell lines may be due to the existence of the t(15;17) in NB4 cells. This translocation may result in alteration of transcriptional control of either the mbn gene or protein factors that mediate the effects of RA on this gene.

Studies have suggested that the presence of t(15;17) in leukemia cells confers therapeutic sensitivity to all-trans RA resulting in in vivo differentiation of these cells. If, as suggested, PML-RAR acts to block or interfere with myeloid differentiation by altering transcription of genes that are critical to differentiation, posttranscriptional mechanisms as detected in NB4 cells may represent an
alternative for differentiation of cells carrying t(15;17). Therefore, the apparent paradox that t(15;17) (resulting in the expression of an altered RAR) seems to confer all-trans RA sensitivity to APL cells could be explained by the possibility that posttranscriptional mechanisms may regulate genes that are critical to growth and differentiation of these cells.

Further experiments to test for this possibility will require the identification of mbn transcriptional elements as well as factors and sequences that are involved in the stability of the mbn mRNA. Future experiments will also determine whether the mbn gene regulatory domains contain a sequence under the control of the PML part of t(15;17).

ACKNOWLEDGMENT

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REFERENCES

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