Transcriptional Regulation of Two Myeloid-Specific Genes, Myeloperoxidase and Lactoferrin, During Differentiation of the Murine Cell Line 32D C13

By Alan D. Friedman, Brent L. Krieder, Donatella Venturelli, and Giovanni Rovera

The transcriptional regulation of myeloperoxidase (MPO) and lactoferrin (LF) was examined during terminal myeloid differentiation of the murine cell line 32D C13. The rates of transcription initiation for MPO and LF, determined by an in vitro nuclear run-on assay, increased approximately ninefold. The accumulation of MPO mRNA in 32D C13 cells, determined by Northern blot analysis, correlated temporally with the observed increase in MPO transcription initiation. On the other hand, accumulation of LF mRNA lagged behind the observed increase in LF transcription initiation. In mouse L cells, the LF gene was transcribed more frequently than the MPO gene, though neither mRNA accumulated. Finally, murine MPO transcription is shown, by Northern blot and primer extension analysis, to initiate at multiple sites. These results indicate that whereas transcription induction may largely account for the accumulation of MPO mRNA during terminal myeloid differentiation, both transcriptional and posttranscriptional mechanisms operate to allow accumulation of LF mRNA. The 32D C13 cell system will be a useful model for elucidating these mechanisms.

© 1991 by The American Society of Hematology.
cDNA. The LF probe is a 563-bp PstI fragment derived from the 5' end of the murine LF cDNA (N. Shirsat and G.R., 1990, submitted). The tubulin probe is a 1.6-kb fragment of the cDNA of murine β-5-tubulin. The Bluescript probe (Stratagene, La Jolla, CA) is a plasmid vector.

The MPO exon 0 5'-end oligonucleotide probe was 5'-GTCACTTGTGAAGGCAGCAGCAGGACATCTGTCG-3' (with the upstream end located 1,053 bp 5' to the initiating ATG, 70 bp from the proximal end of exon 0, as mapped by primer extension); the MPO exon 0 middle probe was 5'-GGACAAGAATGGATCAAGTCCTCTTACC-3' (442 bp 5' to the ATG, representing the last 25 bp of exon 0); the MPO exon 1 probe was 5'-CTGGGATGGCATTGGAGGTCTGAAG-3' and the MPO exon 10 probe was 5'-GGCTGGAGGAGATGGGGTCAATGCCAC-3'. The actin probe was the pHF β-1 probe for β-actin. RNA isolation and blotting. Total 32D C13 or NIH-3T3 cell RNA was purified using the guanidine isothiocyanate/CsCl method. For Northern blotting, RNA samples were separated in a 1% formaldehyde/agarose gel and transferred to a nylon membrane (Genescreen; New England Nuclear, Boston, MA). For duplex DNA probes, filters were hybridized at 42°C for 16 to 24 hours in 5x SSPE (1 x SSPE = 150 mmol/L NaCl, 1 mmol/L Na₂ EDTA, 10 mmol/L NaH₂PO₄, pH 7.4), 5x Denhardt's, 0.1% sodium dodecyl sulfate (SDS), 50% formamide, and 250 µg/mL boilded salmon sperm DNA to probes labeled with ³²P by random priming. Filters were then washed to a stringency of 0.1 M NaCl, 0.15 M NaCl, 1% SDS, 0.5% SDS, 0.5% NaPP, and 100 µg/mL denatured salmon sperm DNA to oligonucleotides labeled with ³²P using polynucleotide kinase. Filters were then washed with 6x SSC, 0.05% NaPP at 45°C for 1 hour and exposed to film with an intensifying screen at -80°C overnight.

In vitro nuclear run-on assay. To isolate nuclei, 32D C13 cells were first pelleted, and L cells were first scraped and pelleted. Cell pellets were washed with cold RSB (10 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 7.4, 3 mmol/L MgCl₂) and then disrupted on ice in RSB containing 0.1% NP40 and 20 U RNase inhibitor/mL with three to five passes of a glass dounce homogenizer. Nuclei were pelleted and washed with RSB, and resuspended in nuclear storage buffer. Nuclei in storage buffer were stored at -80°C. Nuclear transcription and RNA isolation were performed as described. RNA transcripts (0.5 to 1 x 10⁶ cpm per 10⁶ nuclei) were hybridized to nitrocellulose strips in which appropriate DNA probes had been applied using a Mini-fold II slot blot apparatus (Schleicher and Schuell, Keene, NH). To prepare slot blots, DNA probes were first depurinated by treatment with 0.24 mol/L HCl for 6 minutes at room temperature, followed by neutralization, and application to the filter in 0.5 mL of 1.2 mol/L NH₄Ac. Filters were then air-dried and baked at 80°C for 2 hours. Filters were hybridized as described. In brief, hybridization was for 36 to 42 hours at 42°C in 4x SET (1 x SET = 0.15 mol/L NaCl, 0.05 mol/L Tris-HCl, pH 8.0, 5 mmol/L EDTA), 50% formamide, 1x Denhardt's, 100 µg boiled salmon sperm DNA per milliliter, and 100 µg yeast tRNA per milliliter. Filters were then washed to a stringency of 0.1x SET/0.1% SDS at 60°C for 60 minutes and exposed to x-ray film.

Primer extension. Primer extension of MPO mRNA was performed using the exon 0 oligonucleotide 5'-CTGAGGAGGAG- CAGGAAAT-3' (with the upstream end located 860 bp 5' to the initiating ATG) or the exon 1 sequence 5'-TGGAGGTCATGAG- CATGG-3' (with the downstream end located 62 bp 3' to the initiating ATG). Total RNA (25 µg) extracted from 32D C13 cells on day 4 of G-CSF treatment or from NIH-3T3 cells was mixed with 20 ng of 5' end-labeled oligonucleotide in 10 mmol/L Tris-HCl, pH 8.3, 250 µmol/L KCl. The mixture was heated to 85°C for 3 minutes and reannealed at 55°C for 45 minutes. Recombinant MoMLV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) and RNase inhibitor (Promega Biotech, Madison, WI) were added to a final concentration of 20 U/µL and 1 U/µL, respectively, to a solution containing the annealed RNA/primer, 50 mmol/L Tris-HCl, pH 8.3, 75 mmol/L KCl, 10 mmol/L dithiothreitol, 3 mmol/L MgCl₂, and 50 µg/mL nuclelease-free bovine serum albumin. The reaction mixture was incubated for 60 minutes at 37°C, and the DNA was phenol-extracted, precipitated with ethanol, and sized on a 6% polyacrylamide, 7 mol/L urea gel. Size markers were provided by a sequencing ladder obtained using the dyeoxy DNA chain termination method.

Results

Increased rate of transcription initiation of MPO and LF during 32D C13 differentiation. To correlate the morphologic state of 32D C13 cell differentiation and MPO or LF mRNA accumulation with MPO or LF initiation rate, 32D C13 cells growing exponentially in IL-3-containing media were washed and placed in G-CSF-containing media. Before transfer, and on days 3, 6, and 8, an aliquot of cells was Wright-stained and examined by light microscopy. A morphologic differential was determined for each culture. Similar to previous results, uninduced cells were blasts; day 3 cells were mainly promyelocytes; day 6 cells were mainly myelocytes; and day 8 cells were mainly metamyelocytes.

Aliquots of cells were taken from the same cultures used to generate the differentials described and total RNA was extracted. An equal cell number equivalent of each RNA sample was subjected to Northern blotting. The resulting filter was hybridized sequentially to probes for LF, MPO, and β-5-tubulin (Fig 1A). The amount of β-5-tubulin mRNA, consisting of two RNA species as previously described, remained constant during this 8-day period. Consistent with previous observations, MPO mRNA was already maximal by day 3, the promyelocytic stage. (Cross-hybridization was present with 18S rRNA using the MPO probe.) LF mRNA was only minimally detected on day 3, but was maximal by day 6, the myelocyte stage. (Cross-hybridization was present with both 18S and 28S rRNA using the LF probe.) The small amount of LF mRNA detected on day 3 may have derived from the small fraction of cells (2%) that had reached the myelocyte stage on this day. Similarly, it is unclear whether the small amount of MPO mRNA detected in uninduced cells derived from the entire population or a subset thereof.

Aliquots of cells, 10⁵ each, were taken from the same cultures used to generate the differentials and Northern blots described, and nuclei were isolated. Radiolabeled run-on RNAs were then generated from these nuclei in an in vitro transcription reaction. These RNAs were used to probe duplicate filters to which had been bound plasmids containing partial cDNAs for murine MPO, murine LF,
murine β-5-tubulin, α1 Bluescript DNA as a control for nonspecific RNA binding (Fig 1B). There was no detectable nonspecific binding, and the rate of initiation of the tubulin gene remained constant during the 8-day differentiation period. MPO and LF both had a low, but reproducibly detected, rate of transcription initiation in uninduced 32D C13 cells. By day 3 of G-CSF treatment, both the MPO and LF genes had increased their rate of transcription initiation approximately ninefold, relative to that of tubulin. This increased rate of transcription, which was reproduced in two independent experiments, was maintained on days 6 and 8 of induction.

RNA polymerase II specificity, immediate early G-CSF effect, and cell-type specificity of MPO and LF transcription. To verify that the transcription observed in 32D C13 cells grown in IL-3 is dependent on RNA polymerase II, the nuclear run-on assay was performed, using $2 \times 10^7$ nuclei from these cells, either in the absence or presence of $1 \mu g/mL$ α-amanitin (Fig 2, left panels). The vast majority of hybridization detected in the absence of α-amanitin was eliminated by its inclusion.

Given that the increased rates of both MPO and LF transcription initiation were already maximal by day 3, we examined whether any portion of these increases might be an immediate early response to G-CSF, independent of new protein synthesis. 32D C13 cells, a portion of the same culture used to determine the effect of α-amanitin, were induced with G-CSF for 4 hours in the presence of either cycloheximide, an inhibitor of protein synthesis, or the ethanol vehicle. The nuclei were then isolated and used for an in vitro nuclear run-on reaction (Fig 2, middle panels). The rate of tubulin initiation decreased several-fold during this 4-hour period; however, the rate of LF initiation, relative to tubulin, increased during this same interval. For cells growing in IL-3, the rate of LF initiation was threefold less than that of tubulin (Fig 2, IL-3/-AM panel), whereas after 4 hours of G-CSF treatment, this rate was approximately twofold greater than tubulin (Fig 2, G4HR/-CHX
Fig 2. Nuclear run-on assays. Nuclei were prepared from $4 \times 10^6$ 32D C13 cells (IL3) growing in IL-3, or from $2 \times 10^6$ mouse L cells (L Cells), and these nuclei were divided into two groups and used in an in vitro nuclear run-on reaction, in the absence (−AM) or presence (+AM) of α-amanitin. Radiolabeled RNAs generated by these reactions were used to probe filters similar to those described in Fig 1 (left and right panels). Two groups of cells from this same 32D C13 culture, $2 \times 10^6$ each, were pretreated for 30 minutes with either ethanol (−CHX) or cycloheximide (+CHX), and were then induced with G-CSF for 4 hours (G4HR) in the presence of these same reagents. Nuclei were then isolated from these cultures and used in an in vitro nuclear run-on reaction. The radiolabeled RNA was used to probe similar filters to those described (middle panels).

Multiple murine MPO transcription initiation sites. As a first step toward determining the mechanism(s) responsible for the observed induction of MPO and LF transcription during 32D C13 cell induction, we undertook to map the site(s) of transcription initiation for these genes. The cap site for the LF gene is located 24 bp downstream from a TATAA homology (Shirshat NV, Bittenbender S, Kreider BL, Rovera G: Structure of the murine lacto-transferrin gene is similar to the structure of other transferrin genes and shares putative regulatory region with the murine myeloperoxidase gene. Gene [in press]). For MPO, the observation of three RNA species on Northern blots (see for example Fig 1A), along with the cloning of cDNAs with diverse 5′ ends, suggested that multiple initiation sites are used during the transcription of this gene. To test this hypothesis, RNA was prepared from 32D C13 cells induced with G-CSF for 4 days. This RNA was subject to Northern blotting and probed with oligonucleotides specific to either the 3′ end, middle, or 5′ end of murine MPO exon 0, an untranslated exon represented fully only in the longest cDNA clones; the same RNA was also probed with exon 1 and exon 10 specific probes, exons containing protein coding sequences (Fig 3). The exon 0 5′-end probe did not detect any bands. The exon 0 middle probe detected only the central band and the minor, highest band. The exon 0 3′-end probe detected the lower two bands, with equal

<table>
<thead>
<tr>
<th>EXON-SPECIFIC PROBES</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
</tr>
<tr>
<td>0-3′</td>
</tr>
<tr>
<td>0-m</td>
</tr>
<tr>
<td>0-5′</td>
</tr>
</tbody>
</table>

Fig 3. Northern blot analysis using MPO-specific oligonucleotide probes. Total RNA prepared from 32D C13 cells induced with G-CSF for 4 days was subject to Northern blotting and probed with oligonucleotides specific to murine MPO exon 10 (10), exon 1 (1), exon 0 3′ end (0-3′), exon 0 middle (0-m), or exon 0 5′ end (0-5′).
intensity. The third, higher molecular weight, band is possibly lost in the background. The exon 1 and exon 10 probes detected the two lower bands, but unlike with the exon 0 3'-end probe, the lowest band is of far greater intensity than the central band, as was also seen in Fig 1A. (Cross-hybridization with 18S and 28S rRNA bands is also evident in several lanes.) As will be discussed, these data suggest the existence of at least three initiation sites, including a very minor far upstream site. The increased intensity of the lower, compared with the central and upper MPO mRNA bands, suggests that the downstream site (or region) is the most used site.

To better define these initiation sites, primer extension was performed, using oligonucleotides from MPO exons 0 and 1. The Exon 0 oligonucleotide, corresponding to a region near the 5' end of the longest isolated MPO cDNA clone, leads to the detection of a 280-bp band in 32D cells, but not in NIH-3T3 cells (Fig 4, left panel), indicating the position of the most 5' murine MPO cap site, 1,123 bp upstream from the initiating ATG. The exon 1 probe leads to the detection of 73 and 77 bp bands (Fig 4, right panels), indicating the positions of the most used cap sites, either 11 and 15 bp upstream from the initiating ATG, or within the 3' end of exon 0, as will be discussed.

DISCUSSION

We have used the murine 32D C13 cell line, a model of terminal myeloid differentiation, to investigate the expression of two myeloid-specific genes, MPO and LF. The rate of transcription initiation was shown to increase approximately ninefold for both of these genes after induction of 32D C13 cells with G-CSF. Interestingly, however, several differences in the regulation of these two genes became apparent.

Both MPO mRNA level and transcription initiation rate reached maximal levels by 3 days of G-CSF treatment. Brief, 4-hour treatment of 32D C13 cells with G-CSF had no affect on MPO transcription. Finally, MPO transcription was only minimally detected in mouse L cells.

In contrast to the MPO profile, LF mRNA had only accumulated to a fraction of its maximal level by day 3 of G-CSF treatment, although the rate of LF transcription initiation was already maximal. Furthermore, brief treatment of 32D C13 cells with G-CSF did increase LF transcription, relative to that of tubulin, even in the absence of new protein synthesis. Finally, significant LF transcription was detected in mouse L cells. Therefore, in addition to transcriptional induction, posttranscriptional mechanisms may also play a significant role in regulating LF mRNA accumulation in myeloid cells.

Thus, these data suggest, strongly for MPO and less conclusively for LF, that myeloid-specific transcription factors, and the unique DNA elements they interact with, participate in the specification of terminal myeloid differentiation. Interestingly, 32D C13 cells maintained in IG3 have a doubling time of 13 hours, whereas their doubling time increases to 48 hours on induction with G-CSF. Perhaps the same changes that result in this marked diminution in growth rate also allow full activity of regulatory proteins that specify terminal myeloid differentiation.

As a first step toward elucidating such proteins, we have mapped the transcription initiation sites for MPO. MPO transcription appears to initiate over a broad region devoid of a TATAA sequence, reminiscent of several housekeeping and tissue-restricted genes, including dihydrofolate reductase (DHFR), transforming growth factor-α, nerve growth factor receptor, and terminal deoxynucleotide transferase (TdT). Several TATAA-less promoters have been shown to contain an “initiator” element, thought to be
capable, like TATAA, of directing RNA polymerase to a particular start site.\textsuperscript{40,41} However, neither the MPO exon 0, exon 1, nor the intervening intron contain a strong homology to either the TdT or DHFR initiators. Perhaps an alternate initiator element is used.

The relative intensities of the three murine MPO bands detected on Northern blots suggests that the major site of transcription initiation might be located at the beginning of the first intron, just 11 or 15 bp upstream of the ATG that initiates translation. These sites are also 9 or 13 bp upstream from the exon 1 splice acceptor site used by transcripts initiated within exon 0. Of note, MPO cDNAs have been isolated that contain sequences from the first intron at their 5' end (G.R., unpublished observations, December 1989), indicating that initiation indeed occurs within this intron.

Alternatively, the major site of transcription initiation may be located within exon 0, just 9 or 13 bp upstream from its splice donor site. The exon 0 3'-end probe, used in Northern blotting (Fig 3), would then only contain 12 or 16 bases homologous to the shortest MPO transcript. This short homology might account for this probe hybridizing equally to the smallest and middle MPO transcripts, whereas the exon 1 and 10 probes displayed far stronger hybridization to the shortest transcript. Furthermore, transcripts initiating in the first intron would not hybridize with the exon 0 3'-end probe; thus, some initiation apparently also occurs near the 3' end of exon 0.

Interestingly, there is a CATATAA sequence, a weak TATAA homologue, located 40 bp upstream from the exon 0 splice donor site. Furthermore, the human MPO transcript has been shown to initiate 26 bp downstream from a CATATAA sequence,\textsuperscript{42} and the 160-bp region of the human promoter containing this sequence is 72\% homologous to the downstream end of exon 0. Determining which of these two initiation sites, the 3' end of exon 0 or of the first intron (Fig 5), is the most used will likely require functional promoter studies with mutant versions of the murine MPO promoter.

However, perhaps 10\% to 20\% of initiation appears to take place farther upstream within exon 0. The data presented are consistent with a single weak initiation site at the 5' end of exon 0 and a site, or short region, of intermediate strength located at least 192 bp upstream (the position of the exon 0 middle probe, Fig 3), but no more than about 436 bp upstream (the position of the exon 0 primer, Fig 4), from the 3' end of exon 0 (Fig 5). The exon 0 middle probe only detected these two sites on Northern blotting, and the exon 0 primer only detected the more upstream site on primer extension. The exon 0 5'-end oligonucleotide probe did not detect the longest MPO transcript, perhaps because this transcript is expressed at low levels. Interestingly, we have found evidence for minor usage of more upstream human MPO initiation sites in HL-60 cells, in a region homologous to murine exon 0 (data not shown, G.R., January 1990).

These results are being used to aid the design of MPO and LF promoter/reporter gene constructs, which in turn are being used to search for cis-DNA elements within these promoters that direct their myeloid-specific expression.

**ACKNOWLEDGMENT**

We thank D. Cleveland for the tubulin probe. We are grateful for the technical assistance of Susan Bittenbender.

**REFERENCES**


2. Grosschedl R, Baltimore D: Cell-type specificity of immunoglobulin gene expression is regulated by at least three DNA sequence elements. Cell 41:885, 1985


41. Means AL, Farnham PJ: Transcriptional initiation from the dihydrofolate reductase promoter is positioned by HIP-1 binding at the initiation site. Mol Cell Biol 10:653, 1990

Transcriptional regulation of two myeloid-specific genes, myeloperoxidase and lactoferrin, during differentiation of the murine cell line 32D C13

AD Friedman, BL Krieder, D Venturelli and G Rovera