

## The Murine Myeloperoxidase Promoter Contains Several Functional Elements, One of Which Binds a Cell Type-Restricted Transcription Factor, Myeloid Nuclear Factor 1 (MyNF1)

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**The myeloperoxidase (MPO) gene is expressed specifically in myeloid cells. There is significant homology between the murine and human MPO genes in the 1.6-kb region located upstream of the murine MPO transcription initiation sites. 5', 3', and internal deletions of this DNA segment localized several *cis*-acting DNA elements in the murine MPO promoter which are functional in 32D cl3 cells, a murine myeloblast cell line which expresses MPO. These DNA elements did not function well in mouse L-cell fibroblasts. Additional mutagenesis of the most active promoter region allowed the delimitation of a functional 20-bp segment. Mutation of the enhancer core motif within this segment was functionally deleterious, and an oligonucleotide containing these base pairs increased the activity of a minimal promoter. This same oligonucleotide, but not a mutant variant, could bind a set of nuclear proteins, myeloid nuclear factors 1 $\alpha$  and 1 $\beta$  (MyNF1 $\alpha$  and -1 $\beta$ ), present in 32D cl3 cells but absent from L cells, murine erythroleukemia cells, and SP2 lymphoid cells.**

The differentiation potential of the pluripotent hematopoietic stem cell becomes stochastically restricted until unipotent progenitors, which subsequently differentiate into mature blood cells, arise (56). The ability of these progenitors to grow in culture in the presence of appropriate protein hormones has allowed the cellular and hormonal bases of hematopoiesis to be analyzed in detail (6, 27, 66), and several hematopoietic cell-specific protein kinases which may carry hormonal signals into the nucleus have been described (30, 41, 54).

Transcriptional events which allow the various progenitors and their differentiated progeny to be endowed with specific phenotypes have been best studied in the erythroid and lymphoid lineages. Analysis of the regulation of globin, immunoglobulin, and other erythroid and lymphoid cell-specific genes has led to the identification of lineage-specific *cis*-regulatory elements (2, 15, 18, 38, 55, 62) and transcription factors (9, 16, 20, 44, 50, 58, 64). A similar analysis of transcriptional regulation in other marrow lineages is necessary before a complete understanding of hematopoietic differentiation can be achieved.

Myeloid cells, which consist of granulocytes, monocytes, and their precursors, account for approximately half of the hematopoietic cells in murine or human marrow. Recently several groups have begun investigating the transcriptional regulation of myeloid cell-specific genes (17, 37, 42, 48, 51). Myeloperoxidase (MPO) is a microbicidal protein present in the primary granules of myeloid cells (35). This protein, and its cognate mRNA, is not found in other cellular lineages (22, 57), and transcriptional regulation is in part responsible for its lineage-specific expression (10, 43). MPO is an early marker of the granulocytic and monocytic myeloid lineages (40). In contrast, secondary granule proteins such as lactoferrin are later markers of granulocytic cells (10).

The murine and human MPO genes have been cloned and

sequenced (21, 60). They both lack a TATAA box, though transcription of the human gene is thought to initiate near a CATAA element adjacent to the first exon (33). The murine MPO gene initiates transcription from two major sites and a minor site, leading to the accumulation of mRNAs of three sizes (10). These sites are located within a 700-bp untranslated exon termed exon 0 (shown in the diagram at bottom of Fig. 1A). The sequence of exon 0 and the adjacent, upstream 900 bp of DNA is approximately 60% homologous to the region of the human MPO gene located upstream of its first exon (21, 42a, 60). In addition, when DNase I-hypersensitive sites were mapped in this region of the human MPO gene, three were found: one near the CATAA motif; one 0.7 kb upstream of this motif, in the center of this 1,600-bp homologous region; and one 3 kb further upstream (24, 29).

These findings suggest that exon 0 and/or DNA located just upstream, in the murine MPO gene, contains conserved transcriptional regulatory elements. Therefore, we determined the ability of this region to stimulate transcription from a minimal, heterologous promoter in a cellular environment wherein MPO is actively transcribed, induced 32D cl3 cells (59). These Friend virus-infected murine cells require interleukin-3 (IL-3) to proliferate as myeloblasts. Upon replacement of IL-3 with granulocyte colony-stimulating factor (G-CSF), these cells differentiate, over an 8- to 12-day period, to mature granulocytes. Within 24 h of G-CSF treatment, MPO mRNA levels increase (59). The MPO transcription initiation rate also increases, although uninduced 32D cl3 cells have a basal level of MPO transcription (10).

We have found that the murine MPO promoter region contains several functional elements which, together, are only minimally active in mouse L-cell fibroblasts. One of the strongest positive elements contains an enhancer core motif and binds a set of cell type-restricted transcriptional activators, myeloid nuclear factors 1 $\alpha$  and 1 $\beta$  (MyNF1 $\alpha$  and -1 $\beta$ ).

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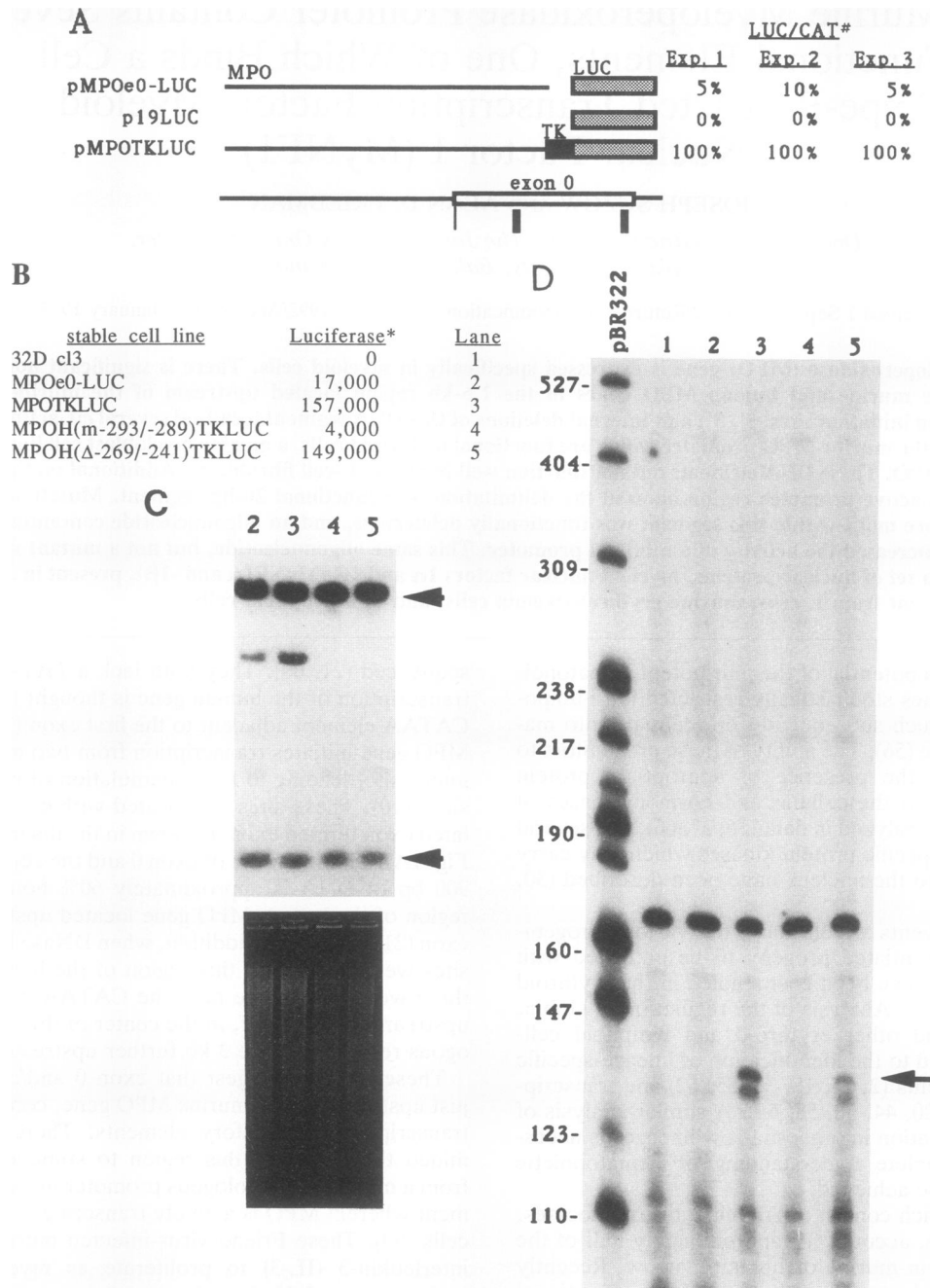


FIG. 1. Assay of MPO promoter function in 32D cl3 cells. (A) Aliquots (10  $\mu$ g) of the diagrammed plasmids, along with pMSVCAT, were transiently transfected into 32D cl3 cells growing in IL-3. After transfection, the cells were placed in G-CSF-containing medium for 2 days. Cell extracts were then assayed for luciferase and CAT activities. A diagram of a portion of the murine MPO gene is shown. Bars indicate the mapped sites of transcription initiation. The thinner bar indicates the weakest site. The absolute ratios of luciferase to CAT activities (LUC/CAT) are expressed relative to that of pMPOTKLUC. (B) Activities of various MPO-luciferase constructs in stably transfected 32D cl3 cells. Light units of luciferase per  $10^6$  cells are shown. Southern blot analysis (C) was carried out to show that cells in the four transfected pools contain similar average numbers of MPO-luciferase genes. Genomic DNAs were digested with *Eco*RI and probed with the luciferase cDNA. The expected 2.7- and 0.6-kb fragments are indicated by arrows (top panel). Equivalence and integrity of digested DNAs were verified by ethidium staining (bottom panel). (D) Primer extension analysis of luciferase RNAs in stably transfected 32D cl3 cells. The origin of the RNA used for each lane is shown in panel B. Sizes (in base pairs) of *Msp*I-digested pBR322 markers are shown; the arrow indicates the location of the transfection-specific extension product, originating from the TK segment, in lanes 3 and 5.

## MATERIALS AND METHODS

**Cells and transfection.** 32D cl3 cells (59) were maintained at 37°C in a 5% CO<sub>2</sub> environment in Iscove modified Dulbecco medium (IMDM) supplemented with 10% heat-inactivated (HI) fetal bovine serum (FBS) and 5% WEHI-3B supernatant as a source of IL-3 (63). For induction of granulocytic differentiation, cells were washed twice with phosphate-buffered saline (PBS) and placed in IMDM-10% HI FBS supplemented with 500 U of G-CSF (Amgen) per ml. Mouse L cells (25) were maintained in Dulbecco modified Eagle medium (DMEM)-10% FBS. Murine erythroleukemia (MEL) (12) and SP2 (49) cells were maintained in RPMI-10% HI FBS. For induction of erythroid differentiation, MEL cells were treated with 2% dimethyl sulfoxide.

Transient transfection of 32D cl3 cells was accomplished by using a method initially described for transfecting lymphoid cells (1), with some modifications. A total of  $13 \times 10^6$  cells per DNA, growing in IL-3, were washed twice with PBS-0.5 mM EDTA and once with TS (137 mM NaCl, 25 mM Tris [pH 7.5], 5 mM KCl, 0.37 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.68 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>). The cells were then resuspended in TS-0.25 mg of DEAE-dextran per ml, 1 ml per DNA. pMSVCAT, 0.25 to 0.5 µg per test sample, was then added to the cells as an internal control. The test luciferase plasmids were then added to 1-ml aliquots, and the cell-DNA mixtures were left at room temperature for 30 min; 10 ml of IMDM-10% HI FBS was then added to each sample, and incubation was continued for 30 min at 37°C. Cells were then spun, resuspended in 10 ml of IMDM-10% HI FBS-G-CSF, and cultured for 40 to 48 h. Transient transfection of L cells was accomplished by using DEAE-dextran and dimethyl sulfoxide shock as described previously (11).

Stable transfection of 32D cl3 cells was accomplished as described previously (26). In brief,  $5 \times 10^6$  cells were washed twice and then resuspended in 0.8 ml of PBS; 12 µg of linearized test plasmid and 2 µg of *EcoRI*-digested pSV<sub>2</sub>NEO (53) were then added. After 10 min on ice, cells were subject to a 800-V, 25-µF pulse, using a Bio-Rad Gene Pulser. After an additional 10 min on ice, cells were placed in 20 ml of medium. Two days later, 600 µg of G418 (active) per ml was added. Stably transfected cells were maintained in G418.

**Luciferase and CAT assays.** Luciferase (7a) and chloramphenicol acetyltransferase (CAT) (46) assays were carried out as described previously, with minor modifications. 32D cl3 cells were poured, and L cells were scraped, into a 15-ml conical tube and spun at  $210 \times g$  for 5 min at 4°C. Cells were then washed with 5 ml of 0.1 M Tris (pH 7.8), resuspended in 1 ml of this same solution, and microcentrifuged at  $1,300 \times g$  for 3 min at 4°C. Cell pellets were resuspended in 120 µl of 0.1 M Tris (pH 7.8)-1 mM dithiothreitol (DTT)-0.1 mg of bovine serum albumin (BSA) per ml and then lysed by three freeze-thaw cycles. Extracts were then microcentrifuged at  $16,000 \times g$  for 2 min at 4°C. A 76-µl aliquot of each extract was mixed with 24 µl of 46 mM MgCl<sub>2</sub>-25 mM ATP; 100 µl of luciferin (0.3 mg/ml) was then injected, and luciferase activity was assayed by using a Monolight 1500C luminometer (Analytical Luminescence), with no delay and a 10-s count time. The balance of each extract was then incubated at 55°C for 15 min and microcentrifuged; 20 µl of each extract was then added to 80 µl containing 18 µg of *n*-butyryl coenzyme A, 0.1 M Tris (pH 7.8), and 0.15 µCi of [<sup>14</sup>C]chloramphenicol (NEN). After incubation at 37°C for 1 h, the mixtures were extracted with 200 µl of xylene. The xylene phase was then back-extracted once with 85 µl of TE (10 mM

Tris [pH 8.0], 1 mM EDTA), added to scintillant, and counted.

**Primer extension.** Total cellular RNA was prepared by the acid phenol-guanidinium isothiocyanate procedure (5). A 29-bp oligonucleotide (5'-GGCGTCTTCCATTTTACCAACAGTACCGG-3') homologous to bases 36 to 64 of luciferase mRNA (7a) was synthesized. This oligonucleotide was phosphorylated with [ $\gamma$ -<sup>32</sup>P]ATP. Ten nanograms of oligonucleotide (20,000 cpm) was annealed with 30 µg of total cellular RNA at 68°C in 250 mM KCl-10 mM Tris (pH 8.3) for 2 h. Extension was then carried out for 30 min at 42°C in a solution containing 25 mM Tris (pH 8.3), 10 mM MgCl<sub>2</sub>, 6 mM DTT, 200 µM each of the four deoxynucleoside triphosphates, 100 µg of actinomycin D (Sigma) per ml, and 4,000 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories). Reaction products were resolved on a 7% acrylamide-urea gel. *MspI*-digested and radiolabeled pBR322 was run as a molecular weight standard. The gel was dried and subject to autoradiography at -70°C with Kodak XAR film.

**Plasmid constructions.** Plasmid pMPO-SR, containing a *SalI-EcoRI* genomic subclone of a portion of the murine MPO gene, which extends from the 5' upstream region to the second exon (bases 1 to 2370 as listed in EMBL accession number X15378), was kindly provided by G. Rovera. This plasmid was treated sequentially with *NcoI*, mung bean nuclease, and *SalI*, and the resulting 1,305-bp fragment was ligated into *SalI-SmaI*-digested p19LUC (59a) to yield pMPOe0-LUC. The same MPO genomic fragment was subcloned into ptk/ $\Delta$ 5'-32 (31), which encodes a herpesvirus thymidine kinase (TK) gene deleted from the 5' direction to just upstream of the TATAA homology, which had been sequentially treated with *BamHI*, Klenow enzyme, and *SalI*. A *SalI-BglII* subfragment (*BglII* cuts in the TK 5' untranslated region) of this plasmid was subcloned into *SalI-BamHI*-digested Bluescript (BS; Stratagene), and a *SalI-SstI* fragment of the resulting plasmid was then subcloned into similarly digested p19LUC to yield pMPOTKLUC. This plasmid is also termed pMPO(-1305)TKLUC, where the juncture between the MPO and the TK portion of this plasmid is numbered as position 0. This same numbering system was used in all subsequent plasmid names. A *BamHI-HindIII* subfragment of ptk/ $\Delta$ 5'-32 was subcloned into BS. The resulting plasmid was treated sequentially with *XbaI*, Klenow enzyme, and *PstI*, and the resulting small fragment was inserted into *PstI-EcoRV*-digested BS. A *SalI-SstI* subfragment of the resulting plasmid was ligated into similarly digested p19LUC to yield ptkLUC. A 12-bp *NheI* linker (U.S. Biochemical) was ligated just upstream of the TATAA homology in ptkLUC, after a *ClaI* linear of this plasmid was treated with Klenow enzyme, to yield pTKLUC.

Clustered point mutations were introduced into pMPO(-1305)TKLUC by oligonucleotide-mediated mutagenesis (52), replacing various 6-bp pair regions, having their 3' bases located at -1107, -984, -884, -695, -552, -414, -215, -297, -288, -276, -264, -241, -173, and -74, with *NheI* sites. Some of these plasmids were digested with *HindIII*, and the resulting larger fragment was religated to yield the pMPOH series of clustered point mutations (bases -1305 to -685 are thus deleted). A 5' deletion series was created by treating plasmids containing clustered point mutations with *SalI*, *NheI*, and Klenow enzyme, after which the resulting larger subfragments were ligated. Internal deletions were created by ligating various smaller *HindIII-NheI* inserts into various *HindIII-NheI* vectors derived from

the described set of clustered point mutations. Similarly, a 3' deletion series was created by ligating the same inserts into *HindIII-NheI*-digested pTKLUC.

A MyNF1 oligonucleotide, obtained by annealing 5'-CTAGACTGACCATTAACCACAACCAGTTG-3' and 5'-CTAGCAACTGGTTGTGGTTAATGGTCAGT-3', was ligated into pTKLUC, which had been digested with *NheI*, to yield the p(MyNF1)<sub>x</sub>TKLUC series of plasmids ( $x = 1$  [one site in forward orientation], 1r [reverse orientation], or 2). This same oligonucleotide as well as one carrying a mutant MyNF1 binding site, obtained by annealing 5'-CTAGACTGACCAGGTAGCACAACCAGTTG-3' and 5'-CTAGCAACTGGTTGTGCTACCTGGTCAGT-3', were ligated into pMPOH ( $\Delta-552/-241$ )TKLUC, which had been digested with *NheI*, to yield pMPOH( $\Delta-552/-241$ +MyNF1 or mMyNF1)TKLUC.

**Nuclear extracts and electrophoretic mobility shift assay (EMSA).** Nuclear extracts were prepared by the method of Dignam et al. (8) as modified by Lee et al. (28). In brief,  $3 \times 10^8$  to  $4 \times 10^8$  cells were washed with PBS and incubated in 5 ml of buffer A-0.5 mM DTT-0.4 mM phenylmethylsulfonyl fluoride (PMSF) for 10 min on ice. Cells were then spun and resuspended in 2.5 ml buffer A-DTT-PMSF-0.5 mM spermidine-2 mM benzamidine-1 mM sodium orthovanadate-10 mM NaF-10  $\mu$ g of leupeptin per ml-1  $\mu$ g of pepstatin A per ml-1  $\mu$ g of antipain per ml-1  $\mu$ g of chymostatin per ml-2  $\mu$ g of soybean trypsin inhibitor per ml-10  $\mu$ g of aprotinin per ml. Cells were then lysed by 10 passes of a Dounce homogenizer. Nuclei were pelleted at  $850 \times g$  for 5 min, resuspended in 1 ml of this same solution, and microcentrifuged at  $16,000 \times g$  for 5 min. Nuclei were then lysed by addition of 0.6 ml of buffer C containing DTT, PMSF, and the protease and phosphatase inhibitors listed above. After rocking for 30 min at 4°C, the extract was microcentrifuged and the supernatant was dialyzed against 50 ml of buffer D-DTT-PMSF-sodium orthovanadate-NaF-spermidine-benzamidine for 5 h. Extracts were quick-frozen in liquid nitrogen and stored at -70°C. Analysis by the Bradford assay (Bio-Rad) showed that the resulting extracts contained 4 to 5 mg of protein per ml.

EMSA was performed as follows. Six to 20  $\mu$ g of extract was preincubated 5 min in 20  $\mu$ l with 2  $\mu$ g of dI-dC-0.1 mg of BSA per ml-50 mM KCl-70 mM NaCl-10 mM Tris (pH 7.5)-1 mM DTT-0.5 mM EDTA-1 mM PMSF-10% glycerol. When desired, unlabeled competitor oligonucleotides were included in this preincubation; 1 ng of MyNF1 oligonucleotide, radiolabeled and blunted by Klenow fill-in, was then added, and incubation was continued on ice for 30 min. The mixtures were then resolved on a 5% acrylamide gel run at 20 mA at 4°C for 3 h in 0.33 $\times$  Tris-borate-EDTA. The resulting gel was dried and exposed to Kodak XAR film. The C/EBP oligonucleotide used as a competitor was obtained by self-annealing 5'-GTTGAGCTGCAGATTGCGCAATCTGCAGCTC-3'. The USF oligonucleotide was obtained by annealing 5'-TCGAGGTAGGCCACGTGACCGGGTA-3' with 5'-AGCTTACCCGGTCACGTGGCCTACC-3'.

## RESULTS

**Assay for MPO promoter region regulatory elements.** For transient transfection of 32D cl3 cells, we modified a protocol first developed for transfecting lymphoid cells (1) (see Materials and Methods). The luciferase reporter gene was placed immediately downstream of the most 5' ATG in exon 0, removing this ATG and creating pMPOe0-LUC. This construct gave a low level of luciferase activity in 32D cl3 cells (Fig. 1A).

Because of concern that pMPOe0-LUC might lack a functional initiation site in this assay, a TATAA homology and cap site from the herpes simplex virus TK gene were inserted. The resulting construct, pMPOTKLUC, gave a 10- to 20-fold-stronger signal (Fig. 1A). We chose to use this paradigm, the ability of MPO genomic segments to stimulate the activity of the heterologous TK promoter, to search for functional elements in the MPO promoter region. Although the quantitative availability of basal transcription factors (e.g., TFIID) varies between minimal promoters (39), a similar approach, the use of a heterologous promoter, has led to the identification of functional elements regulating other genes (13, 61). As TATAA-containing and TATAA-less promoters employ similar basal transcriptional proteins (32, 47), transcription factors which stimulate a TATAA-containing promoter would likely also stimulate a TATAA-less promoter. Our ability to obtain biochemical data which affirm functional data gained with use of this paradigm (see below) provides strong justification for its use.

Primer extension was used to verify that the higher activity of pMPOTKLUC resulted from utilization of the TK initiation site. pMPOe0-LUC and pMPOTKLUC were stably transfected into 32D cl3 cells, and pools of clones were analyzed for luciferase activity (Fig. 1B and C). Consistent with the results of transient transfection, pMPOTKLUC was more active than pMPOe0-LUC, although integration sites have not been controlled for. Extracts of stably transfected 32D cl3 cells contain 100-fold more luciferase activity per cell than do cells transiently transfected with the same constructs. These increased levels of luciferase expression make detection of luciferase mRNAs feasible. Total cellular RNAs from untransfected 32D cl3 cells and from these two pools of stably transfected cells were analyzed for luciferase mRNAs by primer extension (Fig. 1D). Several nonspecific bands are present in all lanes. However, whereas no additional bands are evident with use of RNA prepared from pMPOe0-LUC-transfected cells, an additional doublet is detected with use of RNA from pMPOTKLUC-transfected cells. The larger, more intense band in this doublet is 130 bases in length. This result indicates that the large majority of RNAs transcribed from the pMPOTKLUC template initiate within the TK segment, 36 bp downstream from the TATAA homology. We confirmed this conclusion by sequencing the appropriate region of pMPOTKLUC (not shown). In particular, no initiation is evident from the MPO promoter region. The additional data in Fig. 1D will be discussed after the appropriate mutant derivatives of pMPOTKLUC are described.

**The MPO promoter contains several tissue-restricted functional elements.** Plasmid pMPOTKLUC contains 1,305 bp of murine MPO promoter region sequences. To develop a nomenclature for derivatives of this plasmid, the 3' end of the MPO segment in pMPOTKLUC is designated 0 and the 5' end is designated -1305. In initial experiments, the activity of pMPOTKLUC was compared with that pMPOTKLUC<sub>H</sub>, in which base pairs between -1305 and a *HindIII* site located at -685 were deleted from pMPOTKLUC. In four experiments, deletion of this segment decreased activity 1.1-, 1.1-, 0.8-, and 1.0-fold. Therefore, we focused on DNA segments proximal to -685 in subsequent experiments.

A set of 5' and 3' deletion mutants of pMPOHTKLUC was created, and the activities of these constructs were determined in induced 32D cl3 cells and also in L cells (Fig. 2A). This set of constructs allows the activity of seven different DNA segments, located between -685 and 0, to be determined. Deletion of base pairs between -685 and -414 did

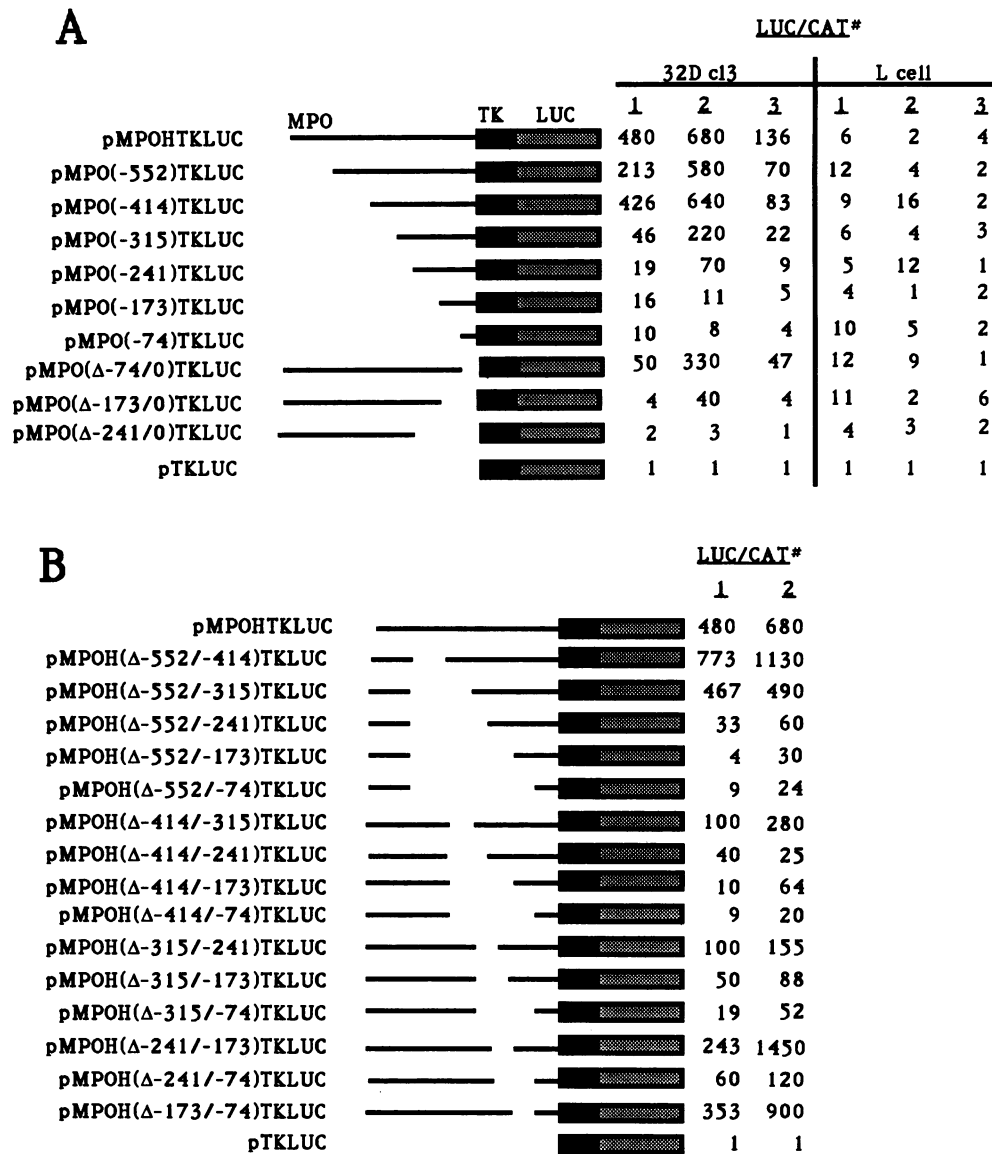


FIG. 2. Assay of 5', 3', and internal MPO promoter deletion constructs in 32D cl3 cells and mouse L cells. (A) Aliquots (10 μg) of the diagrammed plasmids, containing 5' or 3' MPO promoter deletions, were cotransfected with pMSVCAT into 32D cl3 cells, which were then cultured in the presence of G-CSF. Aliquots (5 μg) of these same plasmids were similarly cotransfected into mouse L cells. Cell extracts were assayed for luciferase and CAT activities 2 days later. The results of three experiments are shown. The absolute ratios of luciferase to CAT activities (LUC/CAT) are expressed relative to that of pTKLUC in each experiment for each cell type. The absolute luciferase and CAT activities for pMPOHTKLUC in experiment 1 were 1,620 light units and 45,800 cpm/μg of pMSVCAT in 32D Cl3 cells and 6,290 light units and 211,600 cpm/μg of pMSVCAT in L cells. (B) Activities of the diagrammed plasmids, containing internal MPO promoter deletions, were assessed identically in 32D cl3 cells.

not significantly reduce activity in induced 32D cl3 cells. Deletion of segments -414/-315 (base pairs between -414 and -315), -315/-241, -241/-173, -173/-74, and -74/0, from the 5' and/or 3' direction, reduced activity in these cells. Quantitative differences between experiments may reflect variability in the expression level of transcription factors in 32D cl3 cells.

The activities of these constructs were also determined in uninduced 32D cl3 cells (not shown). A pattern of effects very similar to that found in induced cells was evident. Therefore, the MPO promoter region does not contain a DNA element mediating the increased transcriptional rate

observed during induction (10), at least as far as this assay can discern. Perhaps such an element is present in further upstream or downstream DNA. Note that significant MPO transcription was evident in uninduced 32D cl3 cells (10).

These data from 32D cl3 cells may be contrasted with those obtained from L cells. The activity of plasmid pMPO HTKLUC, relative to pTKLUC, was, on average, approximately 100-fold less in L cells. The much lower level stimulations of pTKLUC noted in L cells did not show a consistent pattern, inhibitory or stimulatory, from deletion of any segment. The only exception is segment -74/0. In these and other experiments, pMPO(-74)TKLUC was more



TABLE 2. Clustered point mutations and positive functional assays with MyNF1 oligonucleotide in 32D cl3 cells

Plasmid	Luciferase/CAT activity <sup>a</sup>			
	Expt 1	Expt 2	Expt 3	Expt 4
pMPOHTKLUC	68	46	250	401
pMPOH(m-302/-297)TKLUC	19	29	52	98
pMPOH(m-293/-289)TKLUC	3	5	4	8
pMPOH(m-281/-276)TKLUC	174	216	123	582
pMPOHTKLUC(m-269/-264)	135	ND	133	200
pMPOHTKLUC(m-246/-241)TKLUC	30	45	ND	ND
p(MyNF1)1fTKLUC	7	6	2	2
p(MyNF1)1rTKLUC	3	4	2	3
p(MyNF1)2TKLUC	3	2	2	6
pMPOH( $\Delta$ -552/-241)TKLUC	4	17	6	8
pMPOH( $\Delta$ -552/-241+MyNF1)TKLUC	131	165	208	313
pMPOH( $\Delta$ -552/-241+mMyNF1)TKLUC	7	5	12	18

<sup>a</sup> Promoter activities of the listed plasmids are expressed relative to that of pTKLUC, which was assigned a value of 1. ND, not done.

Alternately, deletion of bp -269 to -241 may bring closer two interacting transcription factors.

Results of the remaining data may be summarized as follows. Deletion of bases between -281 and -269 had no significant effect. On the other hand, deletion of bases -315 to -297 had a consistently deleterious effect on activity, as did deletion of bases between -296 and -288.

A set of clustered point mutations, diagrammed in Fig. 3B, was then analyzed (Table 2). Strikingly, mutation of bases -293 to -289 resulted in 9- to 62-fold diminution in activity. These bases are at the center of an inverted enhancer core motif (Fig. 3A). Mutation of bases -302 to -297, which resulted in two- to fivefold-diminished activity, may either weaken the binding of a factor whose binding site centers on bases -293 to -289 or weaken the binding of a second factor. In L-cell fibroblasts, mutation of bases -293 to -289 in pMPOTKLUC resulted in a 0.5-, 0.8-, 1.3-, 1.5-, or 2.1-fold decrease in luciferase activity in five experiments.

**Primer extension assay confirms the presence of the MyNF1-binding site.** To confirm that mutagenesis of the MyNF1 site in pMPOTKLUC markedly reduces transcription initiation from the TK initiation site, pMPOH(m-293/-289)TKLUC was stably introduced into 32D cl3 cells. The average luciferase activity of the resulting pool of clones is shown in Fig. 1B. As was seen in the transient assays, mutation of the MyNF1 site markedly reduces luciferase activity in these stable transformants. Total RNA from these cells was analyzed for luciferase mRNAs by primer extension (Fig. 1D, lane 4). The extension products originating from the TK cap site (lane 3) were not detected. Also, no additional extension products were detected, indicating that mutation of the MyNF1 site does not activate a cryptic promoter in the MPO segment.

Plasmid pMPOH( $\Delta$ -269/-241)TKLUC, lacking the putative repressor-binding region, was also stably introduced into 32D cl3 cells. The luciferase activity of the resulting pool of clones was similar to that of the clones containing pMPOTKLUC (Fig. 1B). The average level of luciferase mRNA in these cells was less than in cells transformed with pMPOTKLUC (Fig. 1D, lane 5). Note that both the pMPOTKLUC and pMPOH( $\Delta$ -269/-241)TKLUC transfectants initiate luciferase mRNA transcription only within the TK segment.

**Positive functional assay of a MyNF1 site.** Mutational

dissection of segment -315/-241 suggests the presence of binding sites for at least one transcriptional activator and perhaps a transcriptional repressor. We next focused on the activator-binding site, mapped between bases -302 and -281 by the deletional analysis described above. Synthetic oligonucleotides were annealed to produce a double-stranded oligonucleotide containing bases -301 to -278. The E box located between bases -303 and -298 was not completely included in this oligonucleotide. The putative transcriptional activator binding this oligonucleotide was named MyNF1. When one or two copies of the MyNF1 oligonucleotide were linked directly upstream of the TATAA homology in pTKLUC, the oligonucleotide was found to stimulate transcription in the forward or reverse orientation (Table 2). In L-cell fibroblasts, one forward-oriented MyNF1 site increased the activity of TKLUC 0.5-, 0.7-, 0.9-, 1.0-, or 1.5-fold in five experiments.

The absolute activities of these constructs were fairly low in 32D cl3 cells. Therefore, we also assessed the activity in these cells of the MyNF1 oligonucleotide in the presence of more proximal murine MPO promoter region sequences. The MyNF1 oligonucleotide was inserted just upstream of base -241 in pMPO( $\Delta$ -552/-241)TKLUC in the forward orientation. The MyNF1 oligonucleotide consistently stimulated transcription dramatically in this context (Table 2). We similarly inserted a mutant MyNF1 oligonucleotide (mMyNF1), carrying clustered point mutations in bases -293 to -289. These mutations were markedly deleterious to promoter activity in this context, as they had been in the context of pMPOTKLUC. Thus, the results of these positive functional assays are consistent with the predictions of the mutational data and indicate that a transcriptional activator interacts with the MPO promoter between -301 and -281.

**A set of myeloid nuclear proteins binds the MyNF1 site.** To identify MyNF1 biochemically, nuclear extracts were prepared from 32D cl3 myeloblasts growing in IL-3, 32D cl3 cells which had been cultured in the presence of G-CSF for 4 days, and mouse L-cell fibroblasts. These extracts were incubated with radiolabeled 29-bp MyNF1 oligonucleotides, and bound proteins were resolved by polyacrylamide gel electrophoresis (Fig. 4A). A quantitatively similar MyNF1 band shift pattern was obtained with two independent sets of uninduced and induced 32D cl3 cell extracts. Extracts from 32D cl3 cells maintained in IL-3 contain two proteins, MyNF1 $\alpha$  and MyNF1 $\beta$ , which are not present in L-cell extracts. Induced 32D cl3 extracts contain these two proteins and a third protein, MyNF1 $\gamma$ . As will be shown, the increased intensity of the MyNF1 $\beta$  and MyNF1 $\gamma$  band shifts in induced 32D cl3 cells may have resulted from degradation of larger proteins. In addition to the MyNF1 shifts, both L-cell and 32D cl3 extracts produced one or two additional band shift species which migrated more slowly in the gel.

To assess whether clustered point mutation of bases -289 to -293, which affected MPO-TK promoter function, also affect MyNF1 DNA binding, a competition experiment was performed (Fig. 4B). Free probe and band shifts obtained with L-cell extracts are included for comparison. Nuclear extracts from 32D cl3 cells maintained in IL-3 or induced with G-CSF were incubated with radiolabeled wild-type MyNF1 oligonucleotide in the presence of no competitor or a 500-fold excess of wild-type or mutant competitor. The wild-type competitor completely inhibited MyNF1 interaction with the radiolabeled oligonucleotide, whereas significant binding was still apparent in the presence of excess mutant oligonucleotide.

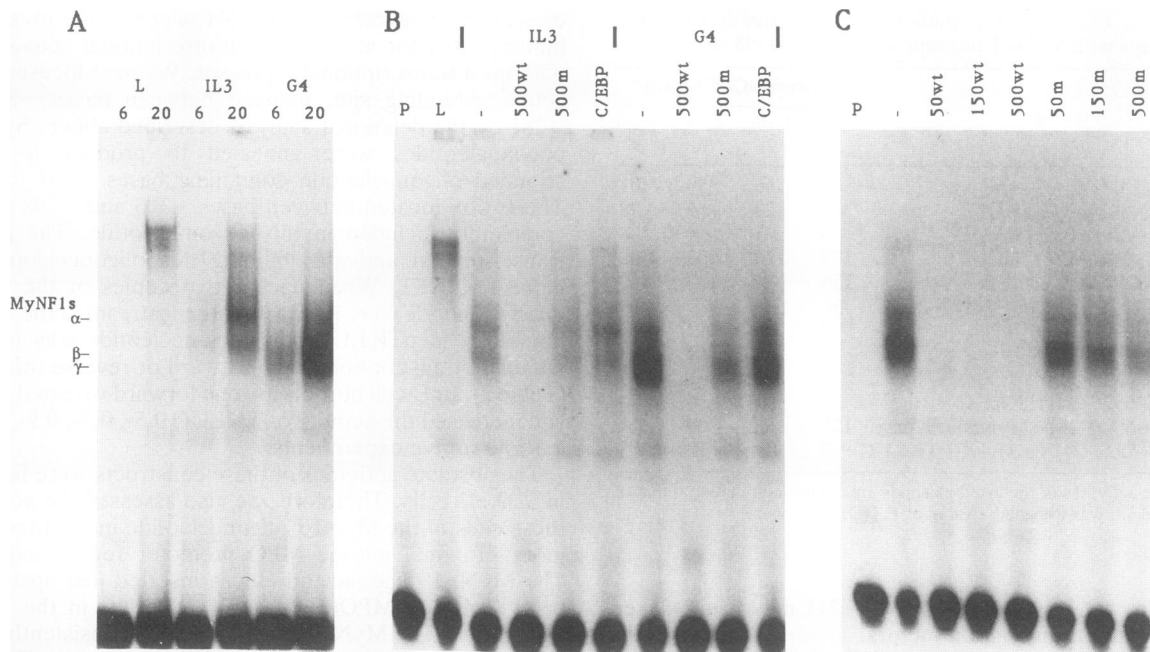


FIG. 4. EMSAs with MyNF1 oligonucleotide and nuclear extracts from 32D cl3 and L cells. (A) Six or 20  $\mu$ g of nuclear protein from L cells (L), from 32D cl3 cells growing in IL-3 (IL3), or from 32D cl3 cells induced for granulocytic differentiation by exposure to G-CSF for 4 days (G4) was incubated with 1 ng of radiolabeled MyNF1 oligonucleotide. The resulting mixtures were then resolved on a 5% polyacrylamide gel. The positions of MyNF1 $\alpha$ , - $\beta$ , and - $\gamma$  are indicated. (B) Aliquots (12  $\mu$ g) of nuclear extracts from L cells (L) and uninduced (IL-3) or induced (G4) 32D cl3 cells were incubated with 1 ng of the same oligonucleotide. The 32D cl3 extracts were coincubated with either no specific competitor (-), a 500-fold excess of wild-type MyNF1 oligonucleotide (500wt), a 500-fold excess of mutated MyNF1 oligonucleotide (500m), or a 500-fold excess of an oligonucleotide containing a C/EBP binding site consensus (C/EBP). The probe alone (P) is shown for comparison. (C) Twelve micrograms of nuclear extract from 4-day-induced 32D cl3 cells was incubated with radiolabeled MyNF1 oligonucleotide in the presence of either no competitor or a 50-, 150-, or 500-fold excess of wild-type or mutated MyNF1 oligonucleotide as the competitor.

The MyNF1 oligonucleotide contains an inverted enhancer core motif (65) (Fig. 3), and this motif is disrupted in the mutant oligonucleotide. C/EBP $\alpha$  was originally purified on the basis of its ability to bind either an enhancer core motif (23) or a CCAAT motif (14). Therefore, we attempted to inhibit MyNF1 interaction with the MyNF1 site by using an oligonucleotide containing an inverted GCAAT sequence. Such an oligonucleotide has been shown to interact with members of the C/EBP family (3). An excess of this oligonucleotide did not affect MyNF1 binding to the MyNF1 oligonucleotide (Fig. 4B). Therefore, MyNF1 is likely not a member of the C/EBP family.

To titrate the inhibitory effect of the mutant MyNF1 oligonucleotide, an additional competition experiment was performed (Fig. 4C). A 50-fold excess of wild-type competitor completely inhibited MyNF1 binding, whereas a similar excess of mutant oligonucleotide was only mildly inhibitory. Apparently the MyNF1s have a weak affinity for the mutant oligonucleotide compared, for example, with their lack of affinity for the C/EBP oligonucleotide.

**MyNF1 is cell type restricted.** To begin to determine whether MyNF1 is present in cells derived from other hematopoietic lineages, nuclear extracts were prepared from uninduced and induced MEL cells, which were derived from the erythroid lineage, and from murine SP2 cells, which were derived from B-lymphoid cells. These extracts, and those from mouse L and 32D cl3 cells, were used in an EMSA with the MyNF1 oligonucleotide (Fig. 5A). MyNF1 $\alpha$ , - $\beta$ , and - $\gamma$  were detected only in extracts from 32D cl3 cells. Several additional minor species were detected in all six extracts.

These same extracts were used in an EMSA with a USF oligonucleotide (Fig. 5B). As USF is ubiquitously expressed (4), this assay serves as a control for extract protein integrity. USF is intact in extracts from uninduced 32D cl3 cells and from the nonmyeloid cell lines. However, despite the presence of a large number of protease inhibitors, USF is largely degraded in the induced 32D cl3 cell extract. This finding is not surprising, as induced 32D cl3 cells contain a high concentration of proteases in their primary granules. On the other hand, MyNF1 $\alpha$ , as well as three lower-intensity bands, remain unchanged between uninduced and induced extracts, suggesting that the MyNF1s might all be more stable than USF in these extracts.

## DISCUSSION

We have investigated the regulation of transcription of a myeloid cell-specific gene, murine MPO. Our focus has been a 1.3-kb promoter region which shares sequence homology with the analogous region from the human MPO gene. Our deletion data identified several positive functional elements and a negative functional element within this region. Together, these elements stimulated TK promoter activity to a much greater extent in induced 32D cl3 myeloblasts than in L-cell fibroblasts, suggesting that they interact with tissue-restricted transcription factors. These elements are located within the upstream 400 bp of the 700-bp exon 0, where they surround two endogenous MPO initiation sites. As we could not detect RNAs originating from either of these cap sites, the effects of the various functional elements on initiation



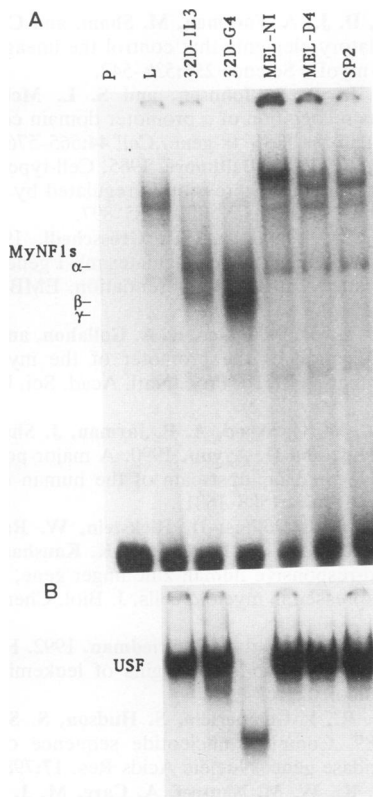


FIG. 5. MyNF1 and USF DNA-binding activities in several cell lines. Aliquots (12  $\mu$ g) of nuclear extract from L cells (L), uninduced and 4-day-induced 32D cl3 cells (32D-IL3 and 32D-G4), uninduced and 4-day-induced MEL cells (MEL-NI and MEL-D4), or SP2 cells (SP2) were incubated with a radiolabeled MyNF1 (A) or USF (B) oligonucleotide. The resulting mixtures were then resolved on a 5% polyacrylamide gel. Positions of the MyNF1s and USF are indicated. Unbound probe (P) is shown for comparison.

from these sites could not be assessed. Perhaps addition of upstream or downstream MPO genomic DNAs to our constructs would increase utilization of endogenous initiation sites in stably transformed 32D cl3 cells. Nevertheless, the strong, cell type-restricted activity of this region, its proximity to endogenous MPO initiation sites, and its strong homology to a region of the human MPO promoter region which contains a DNase I-hypersensitive site suggest that elements identified in this region function in the endogenous murine MPO gene.

We have focused on a 74-bp region,  $-315/-241$ , which was the strongest positive functional region identified in the initial deletions, which functioned more efficiently in 32D cl3 than in L cells, and which shares 77% homology with a region of the human MPO promoter. This region is located upstream of the highly utilized, endogenous MPO initiation site located at approximately  $-190$  and just downstream of the weakly utilized, endogenous MPO initiation site located at  $-322$ . Surprisingly, though the activity of our initial constructs had indicated that this region as a whole was stimulatory in induced 32D cl3 cells, finer dissection indicated the presence of a negative functional element located downstream of a strong positive functional element(s). This repressor-binding site had variable activity. Finer mutagenesis of this region, as well as biochemical analysis, will be necessary to determine whether a repressor of transcription

binds this region or whether alteration in spacing between other DNA elements accounts for the effects observed. Of note, deletion of bases  $-269$  to  $-241$  or  $-281$  to  $-241$  had a similar effect. An ubiquitous repressor, CCAAT displacement protein, has been shown to participate in the regulation of the myeloid-specific gp91-phox gene (51). Region  $-269/-241$  does not contain a CCAAT site, however.

Mutational analysis of region  $-315/-264$  indicates the presence of a binding site for a strong transcriptional activator, centered on bases  $-293$  to  $-289$ . Interestingly, these base pairs are at the center of an enhancer core motif (65), 5'-TGTGGTTA-3'. Positive functional and mobility shift assays, using wild-type and mutant oligonucleotides centered on these bases, confirmed that at least a portion of this motif interacts with a transcriptional activator termed MyNF1. The precise position of MyNF1 interaction, between bases  $-301$  to  $-282$ , remains to be identified. Other proteins may well bind just upstream of this site. For example, bases  $-303$  to  $-298$  contain an E box, mutation of these base pairs was functionally deleterious, and E-box-binding factors have been detected in induced 32D cl3 cells (26).

The enhancer core motif has been shown to bind several transcription factors, including AP-3 (34), TEF-2 (7), and C/EBP (23). As several members of the C/EBP family have recently been described in myeloid cells (45), we determined the ability of a consensus C/EBP oligonucleotide, containing two inverted GCAAT half-sites, to inhibit interaction of the MyNF1s with the MyNF1 oligonucleotide. Our results indicate that the MyNF1s are not likely C/EBPs. Identification of the MyNF1s will require their purification and/or cDNA cloning.

Interestingly, the MyNF1s were not detected in nuclear extracts from an erythroid, lymphoid, or fibroblast cell line. Precise determination of their cell type distribution will again require development of antibody and cDNA reagents. Extracts from uninduced 32D cl3 cells contain MyNF1 $\alpha$  and MyNF1 $\beta$ , whereas extracts from 4-day-induced 32D cl3 cells also contain MyNF1 $\gamma$ . The increased levels of MyNF1 $\beta$  and MyNF1 $\gamma$  in induced 32D cl3 cells may result from proteolysis. In vivo footprinting of the MyNF1-binding site may allow its occupancy, in the endogenous MPO gene, to be quantified without interference by primary granule proteases.

Further study of other functional elements identified in the murine MPO promoter, especially those located in segment  $-414/-315$  and between bases  $-241$  and 0, will likely provide additional insight into MPO gene regulation during myelopoiesis. Also, additional regions of the murine MPO gene will need to be examined for the presence of important functional elements, especially given the identification of a DNase I-hypersensitive site 4 kb upstream of the human MPO gene initiation site (24, 29). In addition, we have not located an induction-specific DNA element. In some experiments, pMPOHTKLUC was significantly more active in induced than in uninduced 32D cl3 cells (data not shown). Unfortunately, this finding was not reproducible over time, even when cells that were stored when MPO induction was evident were used at later times. We have previously commented on the variability of 32D cl3 cells in culture (45), suggesting that uninduced cells may be more differentiated under some conditions. Therefore, we could not determine which elements become more active when MPO transcription increases during myeloblast differentiation. This determination may require transfection of other myeloblast cell

lines which do not transcribe MPO under any culture conditions.

A myeloid-specific zinc finger protein, MZF-1, has been cloned. However, this protein is expressed in later-stage granulocytic cells (19) and thus likely does not regulate MPO gene activation. *c-myc* has been shown to induce early-myeloid-specific transcripts (36). Study of the MyNFs in normal hematopoietic cells will be necessary to determine their precise contribution to the regulation of MPO gene expression. Identification of transcription factors which regulate the early stages of myelopoiesis will facilitate investigations of how the differentiation potential of the pluripotent hematopoietic stem cell becomes restricted during hematopoiesis.

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