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Identification and functional analysis of multiple murine myeloperoxidase (MPO) promoters and comparison with the human MPO promoter region

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Myeloperoxidase (MPO) is an important component of the oxidative antibacterial defense system of granulocytes. Mammalian MPO gene expression has been most extensively studied in human and murine cells. Transcription of the human MPO gene appears to begin at a single initiation site and we have recently described the isolation and characterization of the corresponding human MPO promoter. On the other hand, MPO transcripts in murine myeloid cells show several distinct 5′-termini, suggesting the existence of multiple murine MPO promoters. However, significant levels of endogenous murine MPO promoter activity have not been demonstrated heretofore, although several murine MPO enhancers have been described. We now report the identification and preliminary functional characterization of four distinct murine MPO promoters. Sequence comparison of the human and murine MPO promoter regions reveals homologues of three out of four of these murine promoters within the human MPO gene. However, only one of these sites appears to be functionally active in human myeloid cells, possibly because of the interposition of Alu sequences between the putative promoter sites in the human gene.
Keywords: myeloperoxidase; murine; promoter

Introduction

The enzyme myeloperoxidase (MPO; donor: H_2O_2 oxidoreductase EC1.11.1.7) is an important constituent of the oxygen-dependent bacterial killing system of granulocytes and monocytes.[1] Since it is expressed only in cells of the myeloid lineage, MPO has been widely used to classify and diagnose acute leukemias.[2] A constituent of the primary granules of neutrophils and monocytes, MPO is synthesized only during the late myeloblastic and promyelocytic stages of myeloid maturation.[3,4] MPO enzyme activity has been seen in mature myeloid cells of all mammalian species examined, but the expression of this gene has been studied most extensively in murine and human cells. Both human and murine MPO appear to be encoded by a single gene[5,6] and the respective genes have been cloned and sequenced.[7,8] Regulation of MPO gene expression appears to occur primarily at the transcriptional level.[9–11] Human myeloid cells appear to contain several size classes of MPO RNA, but all cDNAs which have been examined to date appear to share the same initiation site,[12–14] suggesting the presence of only one functionally important human MPO promoter. On the other hand, analysis of murine MPO RNA has revealed several distinct 5′-termini, suggesting the possible existence of multiple murine MPO promoters.[15,16]

We recently reported the identification and characterization of the human MPO promoter,[17] and have subsequently described the mapping and functional analysis of a series of important nuclear protein binding sites within the human MPO promoter region.[18–20] Studies in other laboratories, however, have failed to detect significant endogenous promoter activity in the proximal 5′-flanking region of the murine MPO gene, although several DNA segments showing enhancer activity for minimal viral promoters have been described.[21–25] We now report the identification of four distinct sites with promoter activity in the proximal 5′-flanking region and adjacent coding region of the murine MPO gene. These promoters appear to be responsible for the different size classes of murine MPO RNA previously reported by Venturelli *et al*[15] and Friedman *et al.*[16] We also demonstrate that the human MPO gene contains homologues of three out of four of these murine promoters and the three human homologues show functional activity *in vitro*. We postulate that the insertion of an Alu sequence into the human MPO gene in the region of the promoter cluster interferes with the function of the more upstream human MPO promoters *in vivo*, leaving the most proximal promoter (P1) as the major site of initiation of MPO transcription in human myeloid cells.

Materials and methods

Propagation of cells

The murine myeloblastic cell line 32D c13 was graciously provided by Dr Joel S Greenberger (University of Pittsburgh) and was propagated in Iscove's modified Dulbecco's medium supplemented with 10% heat-inactivated fetal bovine serum and 5% WEHI-3B conditioned medium, as a source of IL-3. Granulocytic differentiation of these cells was induced by treatment with 5 ng/ml of recombinant murine granulocyte colony-stimulating factor (G-CSF) (R&D Systems, Minneapolis, MN, USA). The murine monocytic cell line WEHI 3 and the murine fibroblast cell line 3T3 were obtained from the American Type Culture Collection (Rockville, MD, USA). These cell lines were maintained in Iscove's modified Dulbecco's medium. The human cell lines HL-60 (myeloblastic), K-562 (erythroleukemic), and HeLa (squamous carcinoma) were obtained from the American Type Culture Collection. Except where otherwise noted, the cell lines were grown in humidified 5% CO_2 at 37°C in RPMI 1640 (Gibco BRL, Gaithersburg, MD, USA) supplemented with 1% l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum. Cells were harvested by centrifugation and washed in phosphate-buffered saline (pH 7.4) before processing.

Protein concentration in cell extracts was determined by measuring absorbance at 280 and 260 nm. Genomic DNA was extracted from urine or human cells as described by Sambrook *et al.*[26]

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Analysis of 5′-flanking DNA sequences of murine and human DNA

The published sequences of the 5′-flanking region of the human and murine MPO genes were obtained from GeneBank R86.0, December 1994 version, compiled by the US National Institutes of Health and distributed by Hitachi Software Engineering (San Bruno, CA, USA). Regions of homology between the murine and human MPO genes were compared using the software program MacDNASIS Pro version 3.4 Sequence Analysis Software (Hitachi Software Engineering).

Construction of luciferase reporter plasmids containing portions of the murine or human MPO genes

Selected segments of the 5′-flanking or adjacent coding regions of the murine or human MPO genes were amplified by PCR using GeneAmp kits (Perkin-Elmer Cetus, Norwalk, CT, USA) as previously described.[17,18] To prepare luciferase reporter plasmids containing portions of murine or human MPO promoter DNA, the amplified promoter segments were inserted into *Bgl*II and *Hin*dIII sites of pGL-2 Basic vector or pGL-3-Basic vector (Promega, Madison, WI, USA). Luciferase reporter plasmids containing different length segments of MPO promoter DNA were prepared by amplifying these various MPO DNA segments using primers containing *Bgl*II and *Hin*dIII handles, and inserting the products into the *Bgl*II and *Hin*dIII sites of plasmid pGL-2-Basic or pGL-3-Basic (Promega). Plasmid pSV-β-galactosidase Control, which was used as an internal control for transfection studies, was obtained from Promega and was grown in *Escherichia coli* HB101. β-galactosidase activity was measured by conversion of substrate ONPG (*o*-nitrophenyl-β-d-galactopyranoside) to a yellow product which was determined by measuring absorbance at 420 nm.[27]

Transient transfection by electroporation or using Lipofectamine reagent

Leukemic cell lines were transfected by electroporation as previously described,[17] using a Gene Pulser System (BioRad, Richmond, CA, USA) or a PZ200 Progenator II (Hoeffer, San Francisco, CA, USA). Electroporetic transfections were carried out in a volume of 400 μl OPTI-MEM I (Gibco BRL Life Technologies, Gaithersburg, MD, USA) without serum at 250 V, 960 μF, and Tau of 39 ms using 30–40 μg of plasmid DNA and 0.8 to 1.0×10^7 cells. HeLa cells were transfected using Lipofectamine reagent (Gibco BRL) using 5 μg plasmid DNA and 10^6 cells. To normalize for efficiency of transfection, cells were cotransfected with plasmid pSV-β-galactosidase control vector (Promega) (0.5 μg for HeLa cells, 1.0 μg for the electroporetic procedure).[28] For luciferase reporter plasmids aliquots of 3×10^6 cells were lysed in 200 μl lysis buffer (Promega) for 15 min at room temperature. Luciferase activity was assayed with a luciferase assay kit from Promega using 20 μl of cell extract and an ML3000 Microtiter Plate Luminometer (Dynatech Laboratories, Chantilly, VA, USA). Results were expressed as relative light units (RLU) luciferase activity per OD_{420} β-galactosidase.

Results

Our recent studies of the human MPO promoter[17–20] demonstrated the existence of seven discrete, functionally important nuclear protein binding sequences (which we termed DP1–DP7) in the proximal 5′-flanking region of the human MPO gene. Mutation of sites DP1, DP2, DP3, DP4 or DP5 reduced promoter activity, while mutation of sites DP6 or DP7 resulted in increased reporter expression. Because the endogenous murine MPO promoter had not been localized and characterized previously, we compared the DNA sequences of the murine and human MPO genes to look for sequences in the murine MPO gene which were homologous to the nuclear protein binding sequences in or adjacent to the minimal promoter of the human MPO gene. As shown in Figure 1, a 218-bp segment from the 3′-end of exon 0 of the murine MPO gene (terminology of Friedman *et al*[16]) contains sequences which are highly homologous to sites DP1 to DP4 of the human MPO promoter region. Sites DP1–DP4 of the human MPO gene lie in the 5′-flanking region between bp −157 and bp +10, and include the three major nuclear protein binding sites which comprise the minimal promoter, as well as an adjacent sequence that enhances promoter activity. The four corresponding sites in the murine MPO gene lie between bp 1547 and bp 1690 (numbering from the 5′ end of the gene; see GeneBank accession number X 15378) in the same relative orientation as in the human gene. The DP1 homologue in the murine gene is situated at the 3′-end of exon 0, directly upstream from a major initiation site for murine MPO transcription in G-CSF-treated 32D cl3 cells.[16–22]

To determine if this fragment of murine MPO DNA had promoter activity, we amplified a DNA segment consisting of bp 1513 to bp 1713 of the murine MPO gene, which includes the sequences homologous to sites DP1 to DP4 of the human MPO gene. We inserted this segment into plasmid pGL-2 Basic directly upstream from a luciferase reporter, and assayed it for promoter activity in transfected cells. As shown in Figure 2, this DNA segment exhibited substantial promoter activity, comparable to that which we previously reported for the minimal human MPO promoter (Refs 17 and 18; see also Figure 5 below). We named this murine promoter ‘P1’. As shown in Table 1 below, this promoter showed no specificity for MPO-expressing cells, consistent with it being a minimal promoter. A plasmid containing the murine DNA segment from bp 1513 to bp 1649 (lacking the murine homologue of DP1 and part of the DP2 homologue) showed essentially no promoter activity compared with the promoterless negative control plasmid (Figure 2). Hence, the segment of the murine MPO gene between bp 1650 and bp 1713 is required for minimal promoter activity, as predicted from its homology with the human MPO promoter. In the human promoter a CATAA sequence located from bp −26 to bp −22 (along with adjacent 3′ sequences up to bp +10) is essential for nuclear protein binding to site DP1 and for promoter activity. The homologous CATAA sequence in the murine P1 promoter is located from bp 1668 to bp 1672, about 25 bases upstream from the putative initiation site for transcription. A plasmid containing a longer segment of 5′-flanking murine MPO DNA (bp 1213 to bp 1713) showed similar promoter activity to that of the 200 bp P1 promoter, suggesting that the entire P1 minimal promoter was contained between bp 1513 and bp 1713. However, a still longer segment (bp 978 to 1713) contained increased activity, suggesting the presence of either enhancer elements or additional promoters upstream from promoter P1. A plasmid containing the DNA segment from bp 978 to 1649

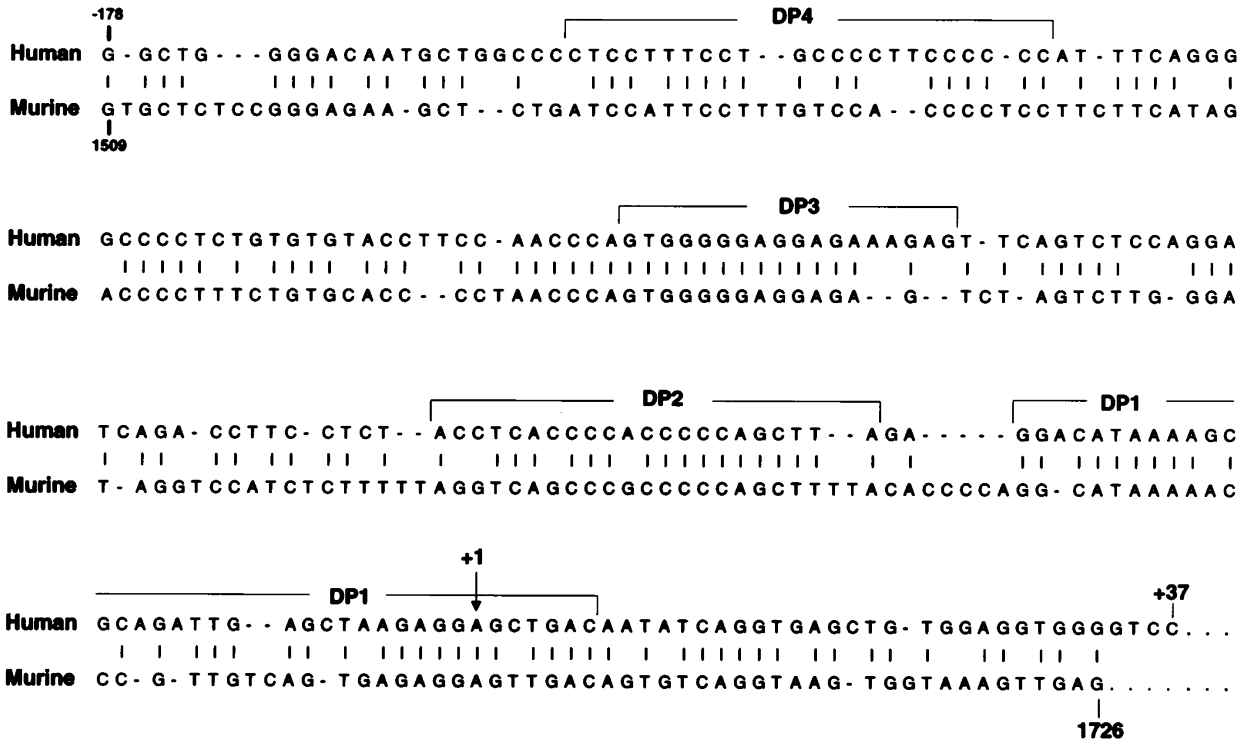

Figure 1 Sequence homologies between the principal human MPO promoter and the 3′-end of exon 0 of the murine MPO gene. DP1, DP2, DP3 and DP4 are functionally important nuclear protein binding sites in the human MPO promoter region. Base numbers for the murine MPO gene are from GeneBank R860, December 1994, compiled by the US National Institutes of Health. The base numbering system for the human MPO gene is that of Johnson *et al.*[7] Hyphens indicate bases missing from either the human or murine DNA sequence.

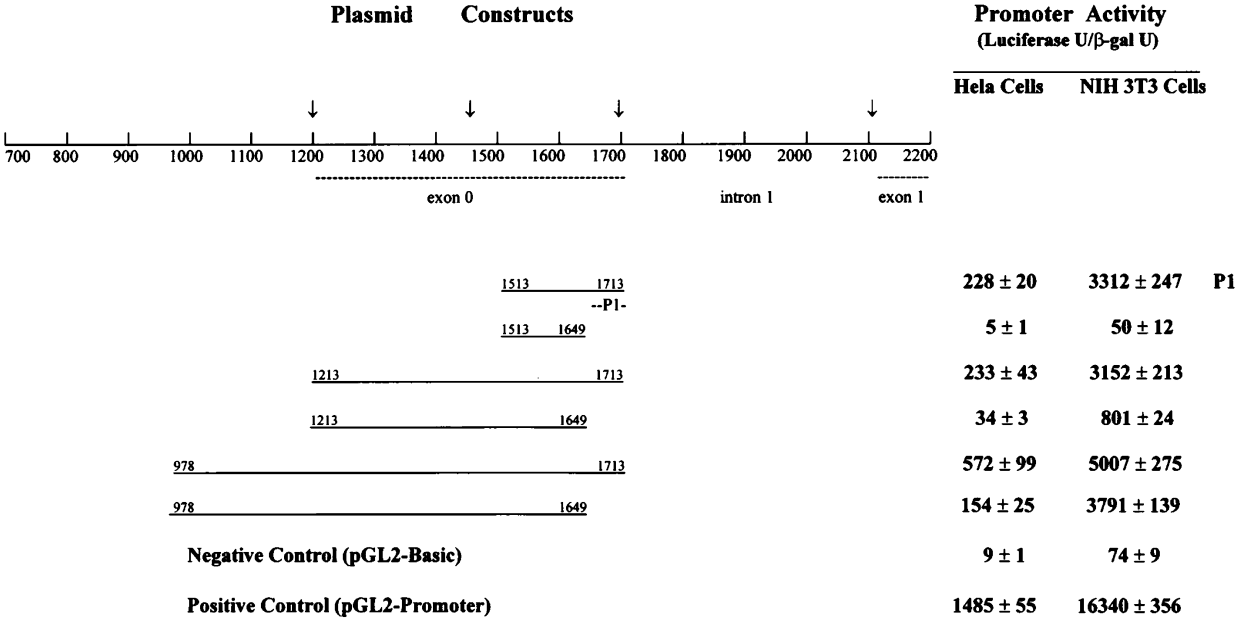

Figure 2 Demonstration of promoter activity in segments of exon 0 of the murine MPO gene which contain sequences homologous to the human MPO promoter. Plasmid constructs containing the indicated portions of the murine MPO gene in luciferase reporter plasmid pGL-2 were assayed in transiently transfected cells. The murine MPO promoter (P1) homologous to the principal human MPO promoter is located between bp 1650 and bp 1713 of the murine MPO gene. Arrows indicate the apparent transcription sites for MPO RNA species, as reported by Friedman *et al.*[16] Luciferase activities represent mean ± s.e.m. for five to seven experiments. The negative control plasmid (pGL-2-Basic) lacks a promoter; the positive control plasmid (pGL2-Promoter) contains an SV40 early promoter.

Table 1 Comparison of murine promoter activities in MPO-expressing and MPO-non-expressing murine cell lines

Plasmid	*Promoter activity (luciferase U/β-galactoside U)*[a]			
	NIH 3T3	*WEHI-3*	*32D cl3*	*32D cl3 (G-CSF)*[b]
pGL3-mP1[c]	1045 ± 158 (4)[d]	80 ± 7 (3)	149 ± 16 (5)	184 ± 21 (4)
pGL3-mP2	884 ± 49 (4)	46 ± 4 (3)	26 ± 7 (5)	30 ± 9 (4)
pGL3-mP3	4770 ± 392 (4)	264 ± 18(3)	1990 ± 272(5)	2762 ± 321(4)
pGL3-mP4	2418 ± 175 (4)	112 ± 9 (3)	880 ± 86 (5)	806 ± 251(4)
pGL3-Promoter	69146 ± 3813(4)	761 ± 50(3)	1162 ± 112(5)	1026 ± 34 (4)
pGL3-Basic	65 ± 24 (4)	6 ± 2 (3)	3 ± 1 (5)	2 ± 1 (4)

[a]All assays were performed 24 h after electroporation.
[b]Recombinant murine G-CSF (5 mg/ml) was added to 32D cl3 cells immediately after transfection.
[c]pGL3-mP1 contains murine MPO promoter P1, etc; pGL3-Promoter contains an early SV40 promoter; and pGL3-Basic lacks any promoter.
[d]Mean ± s.e.m. (*n*).

(thus lacking the minimal P1 promoter) had significant promoter activity, providing further support for the existence of additional upstream promoters.

As mentioned above, murine MPO mRNA has been shown to be comprised of several size classes.[15,16] Venturelli *et al*[15] studied the sequence of several murine MPO cDNA clones and showed them to have differing 5′-termini, suggesting the existence of multiple transcription initiation sites. Friedman *et al*[16] analyzed murine MPO RNA in 32D cl3 cells which had been treated with the growth factor G-CSF. By Northern blotting and primer extension experiments these authors provided evidence for four distinct initiation sites for murine MPO transcription. The two major initiation sites for these transcripts were at the 3′-end of exon 0 (corresponding with promoter P1, which we have just described) and in intron 1, adjacent to the 5′-end of exon 1. The initiation sites for the remaining 10–20% of the murine MPO transcripts were thought to be further upstream, at the 5′ end of exon 0 and in the middle of that exon.

We examined each of these additional segments of the murine MPO gene for sequences which might resemble those of known promoters. CATAA sites were found at bp 1036 to bp 1040 (about 170 bp upstream from the 5′ end of exon 0), and in the middle of exon 0 (bp 1417 to bp 1421), suggesting that these might be the sites of the promoters rsponsible for the longer transcripts observed *in vivo*. In addition, a TATAA site was found at bp 1810 to 1814, near the middle of intron 0. This site is about 300 bp upstream from the putative initiation site for the shortest MPO RNA transcripts. To determine if DNA segments containing these CATAA or TATAA sites had promoter activity, we prepared a series of plasmid constructs (in the luciferase reporter plasmid, pGL-2) containing portions of exon 0, segments of DNA directly upstream from exon 0, or portions of the first intron of the murine MPO gene. Figure 3 shows the promoter activity of each of these constructs in transiently transfected murine or human cells.

As indicated above, a plasmid containing a 564 bp DNA segment directly upstream from promoter P1 (bp 978 to bp 1649) showed substantial promoter activity, suggesting that it contained one or more additional promoters. Likewise, a plasmid containing a 328 bp segment from bp 1213 to bp 1541, lacking the extreme 5′-end of exon 0, but including the remainder of the 5′-end and the middle of exon 0, showed some luciferase reporter activity above background, suggesting the presence of a weak promoter within this segment. A plasmid containing the segment from bp 1213 to bp 1400 (but lacking the segment from bp 1400 to bp 1541) showed substantially lower activity. Hence, the segment from bp 1400 to bp 1541, which includes the CATAA site at bp 1417 to bp 1421, is the apparent location of a weak promoter, which we term ‘P2’. The promoter activity in this segment does not appear to be simply residual P1 activity (due to the 28 bp overlap with P1), since the segment from bp 1513 to bp 1649 shows no promoter activity.

A plasmid containing the murine MPO DNA segment from bp 978 to bp 1224 (including the CATAA site at bp 1036 to bp 1040) showed high luciferase activity, suggesting that it contains the promoter responsible for the longest murine MPO transcripts (which initiate at bp 1207, Ref. 15). We named the putative promoter contained within this segment ‘P3’. A plasmid containing additional murine sequences downstream from bp 1224 (bp 978 to bp 1400) showed lower promoter activity than P3, suggesting that the region between bp 1224 and bp 1400 did not contain a promoter. Likewise, a plasmid containing additional DNA sequences at the 5′-end of P3 (bp 793 to bp 1224) showed only about one half as much activity as P3, suggesting that it contained additional inhibitory sites but no additional promoters. Plasmids containing various portions of the DNA segment between bp 793 and bp 1103 exhibited no promoter activity. Hence, the 122 bp segment between bp 1103 and bp 1224 is required for, and apparently contains the minimal P3 promoter. This segment includes the proximal enhancer (AACCACA, bp 1102–1108) previously described by Friedman[16] and colleagues,[21,22] which may account for the relatively high activity of promoter P3 in transfected cells, compared with the reportedly small fraction of MPO RNA initiated at this site. This segment does not include a TATAA sequence but does contain a GATAA sequence at bp 1158–1162 which may perhaps be involved in promoter function. A CATAA sequence and an initiator-like sequence lie further upstream at bp 1036–1048 but, based upon their location, these seem unlikely to be constituents of the minimal P3 promoter.

The remaining putative initiation site for MPO transcription, which has been described in murine leukemic cell lines is at the 3′-end of the first intron, near bp 2100. To look for the promoter responsible for these transcripts, a plasmid containing a 431 bp murine MPO DNA segment from bp 1695 to bp 2125 was constructed and examined in transfection experiments. This segment showed significant, though low promoter activity. A plasmid containing a slightly shorter (398 bp) segment (from bp 1728 to bp 2125) showed greater activity and was named ‘promoter P4’. This segment contained a TATAA sequence at bp 1811 to bp 1815. However, a plasmid con-

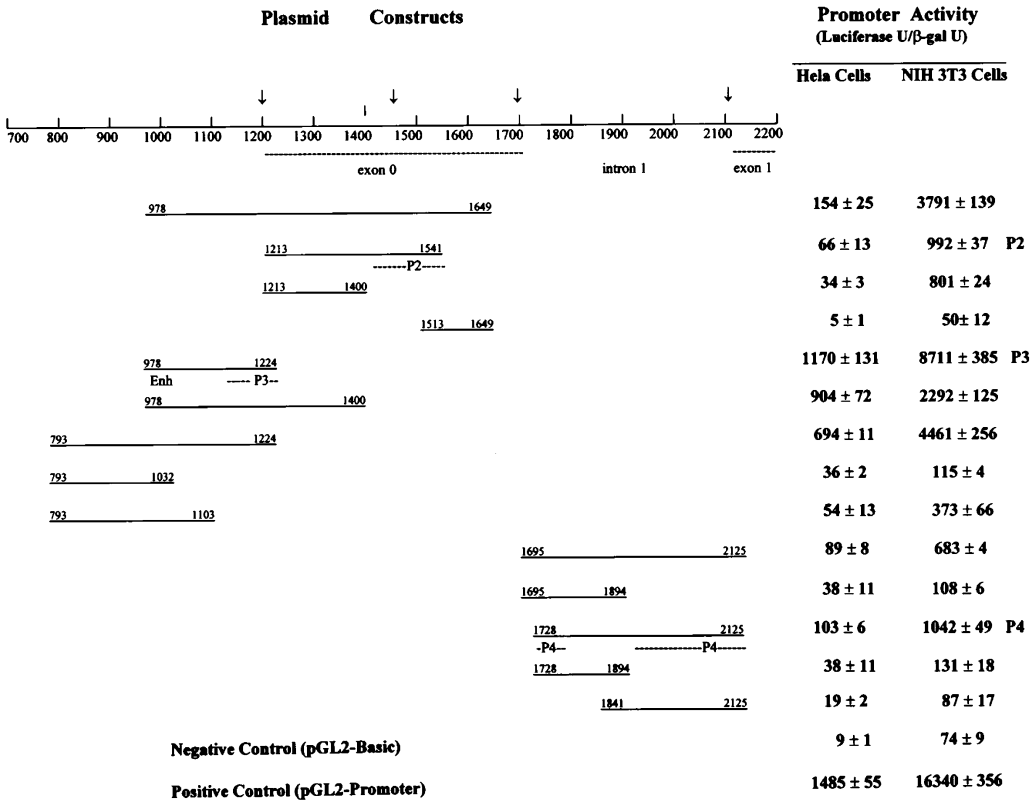

Figure 3 Localization of promoters P2, P3 and P4 in the 5′-flanking and adjacent coding region of the murine MPO gene. Plasmid constructs containing the indicated portions of the murine MPO gene in luciferase reporter plasmid pGL-2 were assayed for luciferase activity in transiently transfected cells. Arrows indicate the apparent transcription sites for MPO RNA species, as reported by Friedman *et al.*[16] Luciferase activities represent mean ± s.e.m. for five to seven experiments. The negative control plasmid (pGL2-Basic) lacks a promoter; the positive control plasmid (pGL2-Promoter) contains an SV40 early promoter.

taining only the upstream half of P4 (bp 1728 to bp 1894), including the TATAA sequence, showed much lower activity than did the entire P4 sequence. Likewise, a plasmid containing the 3′-end of P4 (bp 1841 to bp 2125) but lacking the TATAA sequence showed little activity. Hence, P4 appears to require both upstream sequences, probably including the TATAA sequence, and also downstream sequences (between bp 1895 and bp 2125) for full activity. A pyrimidine-rich sequence closely resembling a consensus initiator sequence[28] is located between bp 2080 and bp 2100, and could possibly be important for promoter function. However, further studies will be required to clarify this issue.

Plasmid constructs created using pGL-2 vectors (Promega) generally show low levels of reported activity when transfected into MPO-expressing leukemic cell lines, possibly because of the high levels of degradative enzymes present in the primary granules of these cells. Hence, to study the activity of the four murine MPO promoters in leukemic cells we used the recently available pGL-3 vector (Promega), which has been modified to improve luciferase expression. Table 1 compares the relative activities of murine MPO promoters P1, P2, P3 and P4 in three murine leukemic cell lines: NIH 3T3 (which does not express MPO *in vivo*), WEHI-3 (which expresses MPO at a very low level *in vivo*), and 32D c13 (which expresses low-to-moderate levels of MPO in the uninduced state and high levels of MPO following treatment of the cells with granulocyte colony-stimulating factor (G-CSF).[16] All four murine MPO promoters showed greatest activity in NIH-3T3 cells, lowest activity in WEHI-3 cells, and intermediate levels of activity in uninduced 32D cl3 cells. Using this vector promoter P3 was the most active of the four murine MPO promoters in all three cell lines tested, P4 the next most active, and P1 the third most active, while P2 showed the lowest activity. Following treatment of the cells for 24 h with G-CSF, promoter P3 showed about a 40% increase in activity (20–70% in individual experiments), while the activity of the other three murine MPO promoters as well as that of the SV40 promoter in the positive control plasmid remained essentially unaffected by G-CSF. Table 2 illustrates the results of another series of experiments, in which luciferase activity in uninduced and G-CSF-treated 32D cl3 cells was compared at 18, 24 and 48 h after transfection. Again, promoter P3 showed slightly greater activity in G-CSF-treated cells at all time-points, compared with untreated cells, whereas the activities of promoters P1, P2 and P4 were essentially unaffected by G-CSF treatment.

Extensive work by Morishita *et al*,[11] Hashinaka *et al*[14] and Chang *et al*[12] has demonstrated that most, if not all, MPO transcription in human myeloid cells employs a single initiation site, whose location corresponds with that of our previously described MPO promoter (human P1). However, a homology plot comparing the sequences of human and murine MPO DNA (Figure 4) shows extensive sequence similarity between the segments of murine MPO DNA containing promoters P2 and P3 (bp 1000 to bp 1550) and the region of the human MPO gene lying between bp −1100 and bp −500. (The murine P4 region shows no homology with the human MPO gene.) Furthermore, Rovera reported evidence for minor usage of more upstream human MPO initiation sites (cited by Friedman *et al*[16] and Nausfeef[29]). Hence, we prepared luciferase

Table 2 Activity of murine MPO promoters in 32D cl3 cells at various times after treatment with G-CSF

Plasmid	*Promoter activity (luciferase U/β-galactoside U)*					
	18 h[a]		*24 h*		*48 h*	
	No G-CSF	*G-CSF*	*No G-CSF*	*G-CSF*	*No G-CSF*	*G-CSF*
pGL3-mP1[b]	312 ± 6[c]	389 ± 12[c]	279 ± 27[c]	252 ± 20[c]	236 ± 33[c]	185 ± 27[c]
pGL3-mP2	27 ± 2	33 ± 2	36 ± 4	29 ± 1	20 ± 3	32 ± 3
pGL3-mP3	5164 ± 199	6193 ± 193	5460 ± 445	6024 ± 543	5531 ± 272	5985 ± 202
pGL3-mP4	1418 ± 35	1555 ± 48	730 ± 40	689 ± 60	274 ± 29	440 ± 26
pGL3-Promoter	2324 ± 65	2025 ± 39	1663 ± 105	1513 ± 167	ND	ND
pGL3-Basic	6 ± 3	6 ± 2	4 ± 1	5 ± 1	ND	ND

[a]Time after induction. Fresh medium with or without G-CSF was added to the cells immediately after electroporation.
[b]Plasmid pGL3-mP1 contains murine promoter P1, etc; Plasmid pGL3-Promoter contains an SV40 early promoter; and Plasmid pGL3-Basic lacks a promoter.
[c]Mean ± s.e.m. for three identical experiments.
ND, not done.

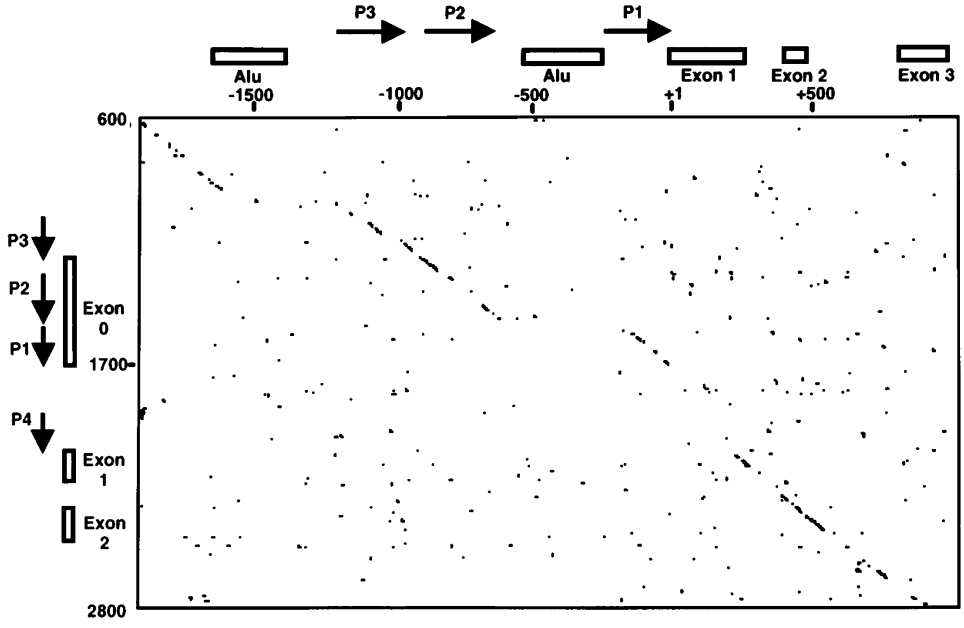

Figure 4 Homology plot showing areas of sequence homology between the human and murine MPO genes. Note that murine promoters P1, P2 and P3 have human homologues in the 5′-flanking region of the human MPO promoter, while murine promoter P4 does not. Each dot represents a match of 10 out of 12 sequential bases.

reporter plasmid constructs containing segments of 5′-flanking human MPO DNA which showed sequence homology to murine MPO promoters P2 and P3, and analyzed these plasmids for promoter activity in transient transfection studies. These results are shown in Figure 5.

Based upon sequence comparisons, the human homologue of murine MPO promoter P2 should lie between bp −700 and bp −500 of the human MPO gene. Although no TATAA or CATAA sequence is located in this part of the human gene, a plasmid containing the human MPO DNA segment from bp −1088 to bp −523 does, in fact, show low but detectable promoter activity in transient transfection experiments (Figure 5). On the other hand, a plasmid containing the DNA segments from bp −1088 to bp −924 shows almost no activity. Hence, a weak homologue of murine promoter P2 appears to lie in the expected position in the human MPO gene, between bp −924 and bp −523.

Likewise, based upon sequence homology, the human equivalent of murine P3 should lie between bp −1200 and bp −1000, although no CATAA or TATAA sequence is present within this region. In fact, a plasmid containing the human DNA segment between bp −1271 and bp −1013 shows appreciable promoter activity (Figure 5), though much less than that seen with the corresponding murine P3 promoter. Since the plasmid containing the murine DNA segment from bp −1088 to bp −924 has no promoter activity, the human P3 equivalent appears to lie in the 182 bp segment between bp −1271 and bp −1089.

Discussion

Our transfection results clearly demonstrate promoter activity in each of four distinct segments of the murine MPO gene.

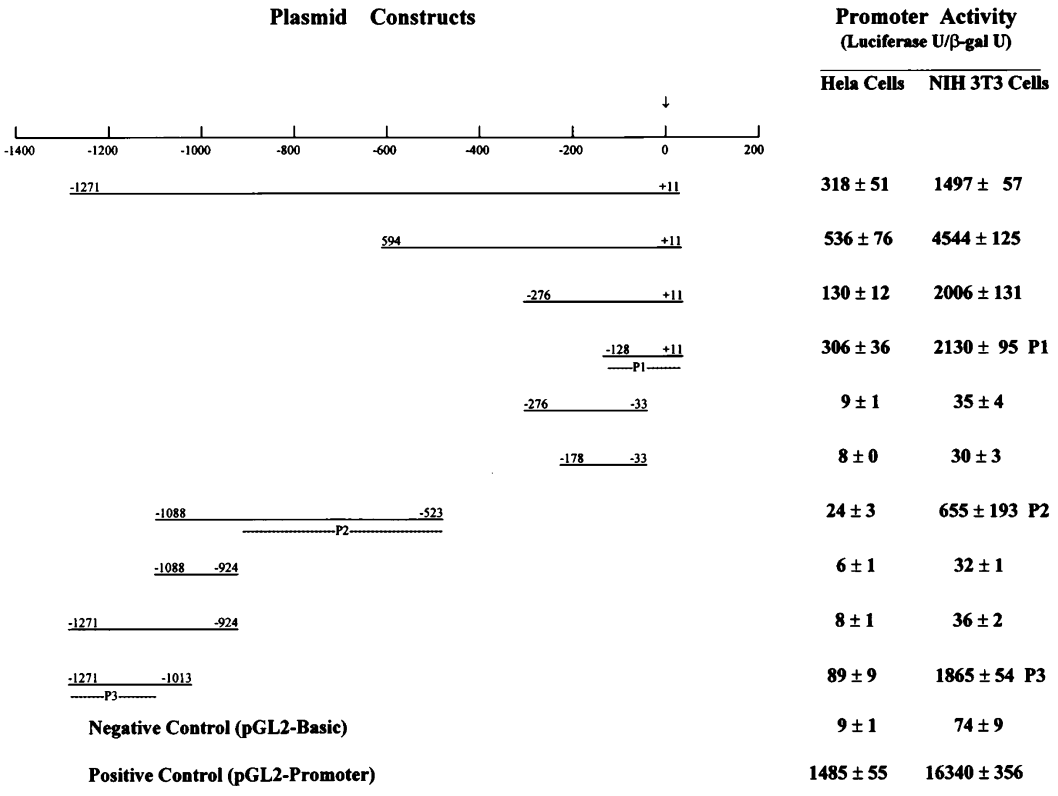

Figure 5 Identification of weak promoter activity in segments of the 5′-flanking region of the human MPO gene which are homologous to murine promoters P2 and P3. Luciferase reporter plasmids containing the indicated portions of the human MPO gene inserted into the pGL-2 Basic vector were assayed in transiently transfected cells. Luciferase activities represent mean ± s.e.m. for five to seven experiments. The negative control plasmid (pGL2-Basic) lacks a promoter; the positive control plasmid (pGL2-Promoter) contains an SV40 early promoter.

The locations of these four promoters correspond closely to the four MPO transcriptional initiation sites previously demonstrated by analysis of MPO RNA populations and sequencing of cDNA clones.[15,16] However, several discrepancies are evidence between the relative activities of the four promoters which we observed in transfection experiments, and the relative frequencies with which each of the corresponding initiation sites are reported to be used for MPO transcription *in vivo*. Evidence referred to above indicates that most MPO RNA species have 5′-termini which correspond in location to promoters P1 and P4. In contrast, our current transfection studies show P3 promoter to have the highest activity, both in MPO-expressing cells such as 32D cl3 and WEHI-3, and in MPO-non-expressing cells such as NIH-3T3 or HeLa. The reasons for the relatively strong activity of P3 relative to the activity of the other promoters are not yet clear. It is possible that the presence within the P3 promoter segment of the proximal enhancer previously described by Suzoh and Friedman[21] is responsible for the relative strength of the P3 activity in transfected cells. Perhaps *in vivo* this enhancer may stimulate the activity of all four promoters.

Promoters P1 and P4 also show substantial activity in transfected cells. Using the pGL3 vector, promoter P4 showed greater activity than P1 in all murine cell lines tested. However, for reasons not yet clear, using the pGL2 vector, P1 activity is greater than P4 activity in both HeLa and NIH 3T3 cells. Finally, promotor P2 shows the lowest activity of the four promoters in all cell lines tested, in agreement with previous evidence that the corresponding initiation site is responsible for only a small minority of the MPO transcripts synthesized *in vivo*.

While our work suggests that all four promoters are capable of initiating transcription of the MPO gene, none of the minimal promoters, in itself, shows specificity for cells which express MPO *in vivo*. Additional regulatory elements must be required to produce the high level of tissue-specific and maturation stage-specific transcription of the MPO gene observed *in vivo*. We have recently described a series of *cis*-elements in the promoter region of the human MPO gene which are capable of modulating the activity of the minimal human MPO P1 promoter, and similar elements undoubtedly exist in the murine MPO gene.[17,18] It will be necessary to characterize the interactions of these various *cis*-elements and their associated transacting protein factors, with each of the four murine MPO promoters, in order to understand the way in which MPO expression is regulated in normal and neoplastic myeloid cells.

It is not entirely clear why significant levels of endogenous murine MPO promoter activity have not been detected previously by other investigators who have studied this problem. It is true that promoter P1 is located downstream from the site examined in detail by Suzoh and Friedman[21] but promoter P3 lies within the segment of the proximal 5′-flanking region which those workers studied. One reason undoubtedly is the difficulty in obtaining high levels of luciferase reporter activity in transfected leukemic cells with most currently available plasmids. This difficulty appears due, at least in part, to the numerous proteases present in the primary granules of myeloid precursors. It appears that the luciferase reporter plasmid pGL-2 (Promega) which we and others have previously employed in transfection studies involving leukemic cells, is targeted to the lysosomal granules present in many of these

cell lines. This problem seems not to apply to cell lines of other lineages which have fewer lysosomal granules. Our studies demonstrate that the recently available pGL-3 vectors (Promega) partially overcome this problem. We were able to obtain substantially higher luciferase reporter activity in 32D cl3 cells using pGL-3 plasmids than we obtained using plasmids derived from the pGL-2 vectors. The availability of these newer vectors should facilitate studies of transcription in leukemic cell lines.

Of the four murine MPO promoters we have detected, only promoter P4 contains a true TATA element (bp 1811–1816). However, activity of this promoter appears to be dependent also upon sequences substantially downstream from the TATA element. A pyrimidine-rich sequence closely resembling a consensus initiator sequence is present near bp 2090, but further studies will be required to determine if this sequence has functional importance.

On the other hand, murine P1, P2 and P3 are all TATA-less promoters. Promoters P1 and P2 both contain CATAA sequences located a few bases upstream from their respective transcription initiation sites. We have previously shown that the CATAA sequence in the human homologue of promoter P1 appears to be critical for activity of that promoter,[18] suggesting that the same may be true for murine promoter P1 and perhaps also for murine promoter P2. The murine P1 segment also contains three sequences resembling a consensus initiator sequence, but all are located upstream from the CATAA site and the reported Cap site. Hence, it is unlikely that these sequences are functionally significant for this promoter. Promoter segment P3 contains both a CATAA sequence and a consensus initiator sequence immediately downstream from it, but both of these elements lie 150 bp upstream from the reported site of transcriptional initiation. Further work will be required to determine if these elements contribute to promoter function.

The *in vivo* evidence available to date indicates that all or almost all transcription of the human MPO gene employs a single initiation site. Promoter P4, which seems to be responsible for a significant fraction of MPO transcription in intact murine cells, lacks a human homologue. However, our data indicate that homologues of the other three murine MPO promoters are present in human MPO DNA, and that each of these homologues exhibits promoter activity in transfected cells. Why the homologues to P2 and P3 are apparently not used substantially for MPO transcription in human cells is not yet evident. One possible explanation is the existence of Alu sequences downstream from the P2 and P3 homologues in the human gene. It is possible that the runs of dA:dT base pairs in these Alu regions may abort any transcription which is initiated by the upstream promoters.

The majority of eukaryotic and prokaryotic genes appear to employ a single promoter for their transcription. However, recent studies have identified a number of genes whose transcripts show heterogeneity at their 5′-ends, and such genes commonly contain several functional promoters.[30–32] In these instances, the selective use of multiple promoters may provide a powerful mechanism for transcriptional regulation.[33–35] One example, reported by Timmusk *et al*,[33] is the rat brain-derived neurotrophic factor (BDNF) gene. These authors demonstrated that the tissue-specific and seizure-induced expression of BDNF mRNA is controlled by the alternative usage of four promoters within the BDNF gene, together with differential RNA splicing. Our current studies have demonstrated four distinct functional promoters in the murine MPO gene and three in its human counterpart. The fact that murine MPO RNA shows heterogeneity at its 5′-end suggests that this form of regulation may occur in the MPO gene as well. Indeed, our initial studies of changes in MPO promoter activity in 32D cl3 cells following treatment with G-CSF suggest that the increase in MPO transcription following induction of maturation with this agent may be due primarily to a stimulation of the activity of promoter P3. Furthermore, Chang *et al*[12] showed changes in the expression level of different size classes of human MPO RNA after induction of HL-60 cells with TPA. Although those authors attributed the changes they observed to differential splicing it is possible that the use of multiple promoters may be involved as well. Further studies will be needed to clarify the relative roles of these and other mechanisms in the modulation of MPO expression in developing myeloid cells and in myeloid neoplasms.

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