Chromatin Structural Analysis of the 5' End and Contiguous Flanking Region of the Myeloperoxidase Gene

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Myeloperoxidase (MPO) synthesis is known to be associated with the promyelocyte stage of myeloid differentiation. In particular the downregulation of MPO gene transcription is associated with myeloid cell maturation. We examined the changes in the deoxyribonuclease I hypersensitive sites within the 5’ end of the MPO gene and its 5’ flanking region during dimethyl sulfoxide (DMSO)-induced differentiation of HL-60 cells to determine the changes in chromatin structure that accompany this process. The locations of hypersensitive sites surrounding the 5’ end of the gene in proliferating, uninduced cells were determined: three were observed in the 5' flanking region and one within the gene. Progressive changes in all sites accompanied the downregulation of MPO transcription after treatment with DMSO. No evidence of hypersensitivity was observed in the chromatin region examined after 8 days of DMSO exposure. The results provide an example of the changes that occur in the chromatin structure of a gene as it is inactivated during differentiation.

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The myeloperoxidase (MPO) enzyme has important functions in the granulocyte and is crucial in the diagnosis of acute nonlymphoid leukemia.2,3 Synthesis of the enzyme occurs in a narrow interval of myeloid differentiation, being limited primarily to the promyelocyte stage and diminishing rapidly as myeloid maturation progresses.3,4 Cells of the human promyelocytic leukemia line HL-60, which constitutively produce MPO, have been a major source of material for the studies that elucidated its properties.5 HL-60 cells differentiate into mature myeloid cells when cultured in the presence of dimethyl sulfoxide (DMSO) or retinoic acid.6 This process is accompanied by a reduction of the MPO message and a concomitant cessation of MPO synthesis within the first 24 hours after exposure to DMSO.7,8 Thus, DMSO-induced differentiation of HL-60 cells is a model system that allows the study of the mechanism of MPO gene downregulation during the later stages of myeloid differentiation. Several cDNAs for MPO have been isolated and the gene structure has been determined.7,9,10,11 The gene is contained in a DNA fragment about 10 kb long and consists of 12 exons and 11 introns. The complete nucleotide sequences of each of the exons and partial sequences of the introns are known. In addition, the sequence of approximately 500 nucleotides located immediately upstream of the first exon has been determined.

Preferential sensitivity to digestion by nucleases and a variety of other DNA-cleaving agents is a feature of chromatin that correlates with gene expression and is associated with transcribable genes.12 This sensitivity is generalized and extends over the entire active domain of the chromatin. Superimposed on this generalized sensitivity are localized regions, 50 to 400 bp long, that are about an order of magnitude more susceptible to nuclease digestion and are known as hypersensitive (HS) sites. HS sites are ubiquitous in eukaryotic chromatin, and many have been associated with transcriptional control elements such as promoters and enhancers.15 These have often been localized to the 5’ ends and the regions immediately upstream of actively transcribed genes. In native chromatin these sites are believed to represent discontinuities in the arrangement of nucleosomes that act as “windows,” allowing trans-acting regulatory factors access to cis-nucleotide sequences.14,15 Deoxyribonuclease I (DNaseI) is frequently used to assay HS sites because it is both highly selective for nucleosome-free regions and relatively low in nucleotide sequence specificity.12

Certain HS sites arise transiently in developing biologic systems at locations surrounding genes whose expression is linked to maturation, and the appearance of these sites correlates with the expression of the associated gene.13 The behavior of the c-myc gene is of special interest in the hematopoietic system because several HS sites are markedly altered by DMSO-mediated induction of the terminal differentiation of HL-60 cells.17,20 Thus, in addition to identifying regions likely to be associated with transcriptional control elements, the DNaseI hypersensitivity assay also offers the possibility of describing hematopoietic differentiation at the level of chromatin structure.

In the work reported here the chromatin structure of the 5’ end of the MPO gene and its 5’ flanking region in both uninduced and DMSO-induced HL-60 cells was investigated using DNaseI. The goals of these studies were both to identify segments of DNA likely to be associated with transcriptional control and to determine the behavior of the HS sites as differentiation progresses.

MATERIALS AND METHODS

Cells. HL-60 (ATCC No. CCL-240) and RPMI-8392 cells were grown in RPMI 1640 (Hazelton Biologics, Lexana, KS) supplemented with 5% fetal calf serum, 10% defined supplemented bovine calf serum (both from Hy Clone Laboratories, Logan, UT), and 2 mM glutamine in a humidified atmosphere of 95% air and 5% CO2 at 37°C. Cells were passaged twice weekly and maintained.

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at a concentration between $5 \times 10^6$ and $2 \times 10^6$/mL. Cell viability was determined by trypan blue exclusion. Peripheral blood was drawn from normal donors after informed consent as required by the local Institutional Review Board. Purified populations of granulocytes were separated from mononuclear cells using Mono-Poly Resolving Medium (Flow Laboratories, McLean, VA) according to the manufacturer’s instructions.

**Differentiation induction.** HL-60 cells at a concentration of 5 to $8 \times 10^6$/mL were grown in tissue culture medium containing 1.25% (vol/vol) DMSO (Sigma, St Louis, MO). The cell concentration was readjusted at 24-hour intervals to 5 to $8 \times 10^6$/mL by the addition of fresh medium containing 1.25% DMSO. Aliquots were harvested at 24-hour intervals for both assessment of viability, differentiation, and DNasel digestion experiments. In all cases cell viability was determined to be greater than 90%.

**Evaluation of cellular morphology.** Cell suspensions (0.25 mL) were centrifuged for 5 minutes at 400 rpm onto microscope slides in a Cytospin 2 centrifuge (Shandon Southern Products, Cheshire, England). The slides were air dried and stained with either Wright-Giemsa or an MPO-specific stain before evaluation by light microscopy.

**Immunofluorescence and flow cytometry.** Cells were stained with the fluorescein isothiocyanate (FITC)-conjugated CD11b monoclonal antibody Mo1 (Coulter, Hialeah, FL) according to procedures that have been described. Analysis was performed on a System 50H Cytocounter and the data recorded by a 2150 computer system (Ortho Diagnostic Systems, Westwood, MA).

**Preparation of molecular probe.** A pWE15 cosmid library of human placenta DNA in *Escherichia coli* (Stratagene, La Jolla, CA) was plated on Luria broth agar containing 30 μg/mL ampicillin. A 5'P-labeled MPO cDNA probe, pMPO62 (a gift of Dr G. Rovera, Wistar Institute, Philadelphia, PA), was used to screen the collection (ATCC No. 41010). The former is a 1.53-kb genomic SsaI-BamHI fragment (Fig 1B) was then subcloned with DNasel at $8 \times 10^6$/mL. Cell viability was determined by trypan blue exclusion. Peripheral blood was drawn from normal donors after informed consent as required by the local Institutional Review Board. Purified populations of

**RESULTS**

We adopted the indirect end-labeling method to map the DNasel HS sites of the 5' end and 5' flanking regions of the MPO gene in uninduced, proliferating HL-60 cells. (A) Nuclei digested with increasing concentrations of DNasel (arrow above figure). Lane 1, no DNasel; lanes 2 through 8 are the results of digestions with DNasel at 8, 11, 17, 23, 34, 46, and $68 \mu g/mL$, respectively. Four lanes corresponding to digestions at low enzyme concentrations that showed no effect are not shown. The HS sites are labeled with roman numerals I through IV, g represents the genomic BamHI fragment that hybridized to the probe, and the horizontal arrow (upper right) labels the minor band of about 10 kb (see text). (B) Locations of 5' HS sites relative to the MPO gene. Boxes and lines represent exons and introns, respectively. The coding sequence is denoted by dark boxes and the approximate centers of HS sites are indicated by vertical arrows labeled with roman numerals. The sizes of these fragments are shown above the horizontal arrows in the lower portion of the figure. B, BamHI; S, SsaI; g, genomic BamHI fragment.

![Fig 1. DNasel HS sites of the 5' end and 5' flanking regions of the MPO gene in uninduced, proliferating HL-60 cells. (A) Nuclei digested with increasing concentrations of DNasel (arrow above figure). Lane 1, no DNasel; lanes 2 through 8 are the results of digestions with DNasel at 8, 11, 17, 23, 34, 46, and $68 \mu g/mL$, respectively. Four lanes corresponding to digestions at low enzyme concentrations that showed no effect are not shown. The HS sites are labeled with roman numerals I through IV, g represents the genomic BamHI fragment that hybridized to the probe, and the horizontal arrow (upper right) labels the minor band of about 10 kb (see text). (B) Locations of 5' HS sites relative to the MPO gene. Boxes and lines represent exons and introns, respectively. The coding sequence is denoted by dark boxes and the approximate centers of HS sites are indicated by vertical arrows labeled with roman numerals. The sizes of these fragments are shown above the horizontal arrows in the lower portion of the figure. B, BamHI; S, SsaI; g, genomic BamHI fragment.](www.bloodjournal.org)
the fragments and the probe that detected them were generated by a cut at a common 3' BamHI site they were coterminal at that end. Therefore, their sizes defined HS sites relative to the BamHI site (Fig 1B). Thus, the positions of the HS sites relative to the start of the coding region in exon 1 could be determined from the available gene map.15

Four major bands corresponding to specific regions, or sites, of hypersensitivity were consistently observed following DNaseI digestion of nuclei from uninduced cells (Fig 1A, I through IV). The positions of these bands correspond to fragments of approximately 7.7, 3.8, 2.8, and 1.8 kb. Sites I, II, and III are located 4.5, 1.3, and 0.3 kb upstream of the start of the coding region, respectively. HS site IV was located in the neighborhood of the start of exon 3, and the band resulting from it was weak relative to the other bands in all experiments with uninduced cells. The lane corresponding to the mock digestion control indicates that there was a small amount of endogenous nuclease activity at the 5' end of the gene and its associated flanking region.

A minor band of about 10 kb was observed just below that for the genomic fragment (arrow at right, Fig 1A). The intensity of this band relative to that of the 14.5-kb band varied among different DNaseI digestion experiments. Furthermore, this fragment appeared to be more resistant to DNaseI digestion than the MPO genomic BamHI fragment. This band could have resulted either from endogenous nuclease activity or from an untranslated gene sequence with homology to the probe. For example, the eosinophil peroxidase gene is known to be homologous to the MPO gene within the region of the probe, and the available restriction map indicates that a fragment of about 9.8 kb would be produced by BamHI digestion and detected by the 2-kb MPO genomic probe.28

Our observations of the changes in DMSO-treated HL-60 cells were consistent with those that have been reported previously.7,8,29 Briefly, proliferation was markedly reduced by 48 hours and negligible at 96 hours. The morphology at the later time was consistent with granulocytic differentiation in the majority of cells.5,29 After 72 hours of growth in medium containing DMSO, greater than 90% of the cells were strongly positive for the expression of the CD11b cell surface molecule, detected by monoclonal antibody Mo1.29 Furthermore, after 24 hours of induction there was a dramatic reduction in MPO mRNA,27,29 which was barely detectable by Northern analysis (data not shown).

Progressive changes in the DNase I hypersensitivity of the 5' end of the gene and its associated flanking region accompanied the downregulation of MPO transcription following DMSO induction. Figure 2 shows the patterns of DNaseI HS sites for HL-60 cells cultured in medium containing 1.25% DMSO for 1, 3, and 8 days. At day 1 (Fig 2A) the fragment from site III, located in the near 5' flanking region, was barely detectable while that from site IV, which is located in the 5' end of the gene, produced a more intense band. In the far 5' flanking region the band corresponding to site I broadened and exhibited evidence of the presence of a doublet. At the same time, a region of increased generalized sensitivity between sites I and II also broadened, and a possible doublet appeared while site II remained relatively unchanged.

Growth in DMSO-containing medium for 2 additional days resulted in further devolution of the hypersensitivity pattern in the distant 5' flanking region. Thus, at 3 days (Fig 2B) two bands were apparent both at site I and in the region between sites I and II. In addition, a reduction in the intensity of the band resulting from site II was observed. The band corresponding to site III remained weak, and the one from site IV was essentially unchanged from that seen at day 1.

After 3 days the intensity of all of the bands associated with HS sites began to diminish, and samples from days 4
and 6 showed progressively weaker bands (data not shown). Finally, after 8 days of culture in the presence of DMSO, when the majority of cells exhibited granulocytic morphology and were not proliferating, the 14.5-kb genomic fragment demonstrated an increased resistance to DNase I (Fig 2C), consistent with the absence of generalized sensitivity, a characteristic of inactive genes. Furthermore, no evidence of hypersensitive sites was observed in the 5' end or the 5' flanking region of the MPO gene. These results after 8 days of DMSO-induced differentiation are similar to those obtained with populations of both mononuclear cells and granulocytes purified from peripheral blood and with cells of the RPMI-8392 lymphoblastoid line (Fig 3, A through C). None of these cell populations contained detectable MPO message.

The sensitivity of the method used to detect these MPO gene HS sites was evaluated by digesting DNA isolated from DNase I-treated nuclei of the control and induced HL-60 cells with either the BglII or XbaI restriction endonucleases. After electrophoresis and transfer the resulting filters were hybridized with the c-myc probes. The results of all of these parallel experiments were in agreement with those presented in previous reports and confirm the adequacy of the method used in this work (data not shown).17-20

**DISCUSSION**

The DNase I HS sites in the 5' end and 5' flanking region of the c-myc gene in both uninduced and induced HL-60 cells have been thoroughly characterized.17-20 In proliferating, uninduced cells the patterns of HS sites in the c-myc and MPO gene are quite similar: both have HS sites in the distant (≥ 1 kb) 5' flanking regions and near the start of the first exon. In addition, each has a site within its 5' end; for c-myc it is near the 3' end of intron 1,17 for MPO it is near the 3' end of intron 2.

In view of these similarities it was interesting to determine the behavior of the HS sites in the MPO gene when HL-60 cells are induced to terminal differentiation and compare it with that of the c-myc gene under the same culture conditions. In the distant 5' flanking region the c-myc HS sites behave differently from the comparable MPO sites in that one c-myc site persisted at 8 days.17 Site III of the MPO gene, in the 5' flanking region near the start of exon 1, developed almost complete resistance to DNase I at day 1 after the initiation of DMSO induction. In the comparable region of the c-myc gene, three HS sites surround the two promoters in exon 1.17,20 The bands resulting from DNase I digestions at two of these sites became faint at 48 hours and almost disappeared at 72 hours of continuous exposure to DMSO,20 while the band representing the third site changed minimally at 72 hours and was still detectable at 8 days.17 The intensity of the bands resulting from the sites within both genes is either unchanged or is increased at day 1 of induced differentiation, but at day 8 the band from the c-myc gene was still present and that of the MPO gene (site IV) was undetectable.17 Thus, although the c-myc gene retains HS sites within its 5' end and in the 5' flanking region when it reaches the inactive state, here we show the MPO gene does not.

The two genes also differ in the timing of the disappearance of HS sites near their 5' ends during differentiation compared with the time of disappearance of their transcripts. Transcription of c-myc mRNA stops about 6 hours after exposure to DMSO,20 while the corresponding time
for MPO mRNA is almost 24 hours. Thus, the downregulation of full-length c-myc transcription occurs soon after induction, the loss of HS sites near the 5' end of the gene much later; analogous changes in this region of the MPO gene are more closely correlated, in time, with the cessation of message production. It has been suggested that hypersensitivity in this region of the c-myc gene disappears closer in time to downregulation of transcription initiation than does full-length transcription. Little is now known regarding the kinetics of initiation of MPO transcription, so no comparisons with c-myc can be made.

The timing of the disappearance of HS site III of the MPO gene suggests that it plays a role in transcriptional regulation. It is also noteworthy that the octamer motif 5'-CTCCCTTC-3' is present both in the region of the MPO III and at the 3' end of intron 2, in the neighborhood of site IV. This motif shares seven of eight nucleotides with the enhancer “core” element, CACCTTTC, which is common to several other transcription enhancers. Because DNaseI HS sites are near DNA sequences that bind transcriptional regulatory factors, the regions surrounding these sites warrant further investigation.

New DNaseI HS sites appear in the 5' flanking regions of genes transcriptionally activated during differentiation of hematopoietic cells. However, the disappearance of HS sites from the 5' end and the 5' flanking region of the c-myc gene in HL-60 cells after terminal differentiation is one of the few examples of changes in chromatin structure that accompany the inactivation of a gene in hematopoietic cells. But the c-myc gene is putatively associated with capacity for proliferation, and the disappearance of these sites in HL-60 cells is correlated with growth arrest as well as with what may be a coincidental commitment to differentiation. In contrast, MPO synthesis occurs primarily during a well-defined stage of myeloid differentiation and therefore represents a differentiation-associated process. Thus, the changes we have observed in the HS sites of the MPO gene in HL-60 cells may be more characteristic of a differentiation-linked process than those that have been reported for the c-myc gene.

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