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cDNA cloning of human myeloperoxidase: Decrease in myeloperoxidase mRNA upon induction of HL-60 cells

(myeloid differentiation/leukemic marker/λgt11 expression vector/promyelocyte)

SUSAN C. WEIL*†‡, GAYLE L. ROSNER*, MARTHA S. REID*, REX L. CHISHOLM§, NEAL M. FARBER¶, JOHN K. SPITZNAGEL||, AND MAURICE S. SWANSON**

Departments of *Pathology, †Medicine, and §Cell Biology, Northwestern University Medical Center, Chicago, IL 60611; ¶Biogen Research Corp., Cambridge, MA 02142; ||Departments of Microbiology and Immunology, Emory University, Atlanta, Georgia 30333; and **Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL 60201

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ABSTRACT Myeloperoxidase (MPO), the most abundant neutrophil protein, is a bacteriocidal component of the primary granules and a critical marker in distinguishing acute myelogenous leukemia from acute lymphoid leukemia. A cDNA clone for human MPO was isolated by immunologic screening of human hematopoietic λgt11 expression vector libraries with specific anti-MPO antibody. The identity of the cDNA clone was confirmed by finding that (*i*) epitope-selected antibody against this clone recognizes purified MPO and MPO in human promyelocytic (HL-60) cell lysates by immunoblot analysis, and that (*ii*) hybrid selection of HL-60 mRNA with this cDNA clone and translation *in vitro* results in the synthesis of an 80-kDa protein recognized by the anti-MPO antiserum. RNA blot analysis with this MPO cDNA clone detects hybridization to two polyadenylylated transcripts of ≈3.6 and ≈2.9 kilobases in HL-60 cells. No hybridization is detected to human placenta mRNA. Upon induction of HL-60 cells to differentiate by incubation for 4 days with dimethyl sulfoxide, a drastic decrease in the hybridization intensity of these two bands is seen. This is consistent with previous data suggesting a decrease in MPO synthesis upon such induction of these cells. The MPO cDNA should be useful for further molecular and genetic characterization of MPO and its expression and biosynthesis in normal and leukemic granulocytic differentiation.

Myeloperoxidase (MPO), a critical microbicidal protein of mature polymorphonuclear neutrophils, is a hemoprotein composing 3–5% of the protein weight of these cells (1, 2). It appears in the primary azurophilic granules during the differentiation of the neutrophil (3, 4) and has been used as a marker for these granules (5). Cytochemical staining of MPO activity is used clinically to distinguish acute myeloid leukemia from acute lymphoid leukemia (6). Furthermore, approximately 1 in every 2000 individuals has been shown to be deficient in MPO (7), an apparently benign but poorly understood hereditary disorder of polymorphonuclear neutrophils.

Although the function of MPO in the oxygen-dependent respiratory burst of polymorphonuclear neutrophils has been firmly established, relatively little is known about MPO biosynthesis, posttranslational modification, and genetic organization and expression. The exact structure of MPO remains a source of some controversy, and its amino acid sequence is not known.

The appearance of MPO in the primary granules marks the transition from the myeloblast to the promyelocyte in the course of normal neutrophilic differentiation (3). Promyelocytes, however, are not found in the peripheral blood of normal individuals and are therefore difficult to study biochemically. The HL-60 cell line, derived from a patient with acute promyelocytic leukemia, contains virtually 100% promyelocytes with azurophilic granules containing MPO (8). When HL-60 cells are cultured in the presence of compounds such as dimethyl sulfoxide (Me_2SO) (8) or retinoic acid (9), these cells mature into metamyelocytes, neutrophilic bands, and even mature polymorphonuclear neutrophils having some normal functional characteristics (10). HL-60 cells are therefore a good model in which to study both normal and leukemic granulopoiesis *in vitro*.

Recent studies using metabolic protein labeling of HL-60 cells and immunoprecipitation of *in vitro* translation products of total cellular HL-60 mRNA (11–13) suggest that MPO expression peaks during the promyelocyte stage and declines thereafter. Due to lack of cDNA probes, no direct examination to date of MPO mRNA levels at various stages of granulocytic differentiation has been possible. We have undertaken the isolation of cDNA clone(s) for MPO to obtain information about its amino acid sequence and to examine the organization and expression of the MPO gene(s) in both normal and leukemic hematopoietic cells. We report here the isolation of a cDNA clone for human MPO and describe its use to directly examine MPO mRNA during the induction of HL-60 cells.

MATERIALS AND METHODS

MPO Purification and Antibody Production. MPO was purified from a 0.2 M acetate (pH 4.0) extract of granules from leukocytes of a patient with chronic myelogenous leukemia as described (14). Rabbit antiserum to MPO was prepared by subcutaneous injection of 100 μg of purified MPO in Freund's complete adjuvant, followed by a booster injection (14). Antiserum was used at a 1:250 dilution.

Cells. The human promyelocytic cell line HL-60 was grown in RPMI 1640 medium supplemented with 1% L-glutamine/1% penicillin/1% streptomycin/15% heat-inactivated fetal calf serum at 37°C in humidified 5% CO_2/95% air. HL-60 cells were differentiated to granulocytes (8) by induction with 1.2% Me_2SO. On day 4, the cells were examined for viability and morphology by the ability to exclude trypan blue and Giemsa staining, respectively.

"Normal" human bone marrow was obtained from nonleukemic individuals after informed consent was obtained. Marrow mononuclear cells were isolated by Ficoll/Hypaque centrifugation (15).

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Abbreviations: Me_2SO, dimethyl sulfoxide; IPTG, isopropyl β-D-thiogalactoside; MPO, myeloperoxidase.

‡To whom reprint requests should be addressed at: Northwestern Memorial Hospital Wesley Pavilion, Room 323, 250 East Superior Street, Chicago, IL 60611.

NaDodSO$_4$/PAGE and Immunoblotting. All samples were heated in sample buffer prior to electrophoresis in gels containing 10% or 12.5% polyacrylamide using a discontinuous system described by Dreyfuss *et al.* (16) and electrophoretically transferred to nitrocellulose, essentially according to Burnette (17). Unreacted sites were blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS; 0.8% NaCl/0.02% KCl/0.02% KH_2PO_4/0.11% Na_2HPO_4). Filters were incubated overnight at 4°C in primary antibody. Unbound antibody was removed by four consecutive washes of PBS, PBS containing 0.05% Nonidet P-40 twice, and PBS again. To detect specifically bound antibody, the filters were incubated with ^{125}I-labeled goat anti-rabbit IgG or ^{125}I-labeled goat anti-mouse IgG (2–5 × 10^5 cpm/ml) for 2–4 hr at room temperature, washed as described above, and exposed to x-ray film for autoradiography (12–18 hr).

Immunologic Screening of λgt11 cDNA Libraries. RNA was isolated from both normal human bone marrow and HL-60 cells by the method of Chirgwin (18) and polyadenylylated mRNA was selected by chromatography on oligo(dT)-cellulose (19). Double-stranded cDNA was synthesized from HL-60 and bone marrow poly(A)$^+$ RNA using the S1 nuclease (20) and the Gubler and Hoffman (21) methods of second-strand synthesis, respectively. After synthetic *Eco*RI linker addition, the cDNA was ligated into the λgt11 expression vector (22). Independent clones (500,000) were initially screened by plating 10 plates of λgt11 recombinant phages on *Escherichia coli* strain Y1090 at 5 × 10^4 plaque-forming units per 150-mm agar plate. Plates were incubated for 4 hr at 42°C. Expression of β-galactosidase fusion proteins was induced by transferring the plaques to nitrocellulose filters previously saturated with 10 mM isopropyl β-D-thiogalactoside (IPTG) and air-dried. After transfer of the induced proteins to the filters by incubation for 5 hr at 37°C, filters were incubated in 5% nonfat dry milk in PBS to block unreacted sites. The anti-MPO antiserum was incubated on IPTG-induced filters containing only wild-type λgt11 in Y1090 to adsorb cross-reacting (anti-*E. coli*) antibodies prior to screening the libraries. The filters were washed as described above. The detection system (Bethesda Research Laboratories) utilized biotinylated goat anti-rabbit IgG diluted 1:500 in 2.5% bovine serum albumin in PBS. Filters were incubated 60 min followed by washes as described above. Filters were then incubated for 30 min in streptavidin conjugated to horseradish peroxidase diluted 1:100 in PBS, followed by washes. Finally, filters were incubated ≈15 min in 0.05% 4-chloro-1-naphthol in PBS containing 10% methanol and 0.02% hydrogen peroxide until a light purple color developed. Positive plaques were rescreened several times until purified to homogeneity.

Lysogenization and Preparation of Fusion Proteins. To confirm and characterize the immunoreactivity of the fusion protein, lysogens were prepared in *E. coli* strain Y1089 with aid of λh80MΔ selector phage (23). Lysogens were grown to OD_{600} = 0.6 at 30°C, upon which the temperature was shifted to 42°C for 20 min to induce the lytic cycle (22). IPTG (10 mM) was added, and the cultures were grown an additional 2 hr at 37°C. Bacteria were collected by centrifugation, resuspended, and boiled in 1/25th their original volume in reducing sample buffer. Samples were cleared by centrifugation before applying to 10% NaDodSO$_4$/polyacrylamide gels. Other samples of these lysogens were grown without induction by IPTG.

Epitope Selection. Bacteriophages (10,000 plaque-forming units) were used to infect Y1090 at 42°C for 4 hr on 85-mm agar plates. IPTG (10 mM) was used to induce expression of fusion proteins. Filters were blocked in 5% nonfat dry milk in PBS, incubated with rabbit antiserum to MPO, and washed as described above. Affinity-purified antibodies immobilized on each filter were eluted with three 2-ml 2-min washes in 5 mM glycine·HCl, pH 2.3/150 mM NaCl/0.5% Triton X-100/100 μg of bovine serum albumin per ml according to Weinberger (24). The combined washes were immediately neutralized with Tris·HCl (pH 7.4) to a final concentration of 50 mM. These antibodies were used to probe purified MPO and HL-60 cell lysates on immunoblots as described above.

Hybrid Selection and *In Vitro* Translation. The MPO insert was subcloned into the plasmid pGEM-1 (Promega Biotech), to yield pHMP7. Twenty micrograms each of pGEM-1 (with and without the MPO cDNA insert) was linearized with *Pst* I, denatured by both boiling and treatment with 1 M NaOH, and applied to nitrocellulose following neutralization in 0.25 M Tris·HCl, pH 8.0/1.5 M NaCl/0.15 M sodium citrate, then 1.0 M HCl/0.15 M sodium citrate. HL-60 poly(A)$^+$ RNA (10 μg) was used for each hybridization, which was performed at 50°C for 3 hr in 65% (vol/vol) formamide/20 mM pipes, pH 6.4/0.4 M NaCl/0.2% NaDodSO$_4$/140 μg of yeast tRNA per ml. After extensive washing at 65°C in 10 mM tetraethylammonium chloride, pH 7.6/0.15 M NaCl/1 mM EDTA/0.5% NaDodSO$_4$, specifically bound RNAs were eluted for 15 min at 20°C with 10 mM methylmercuric hydroxide containing 10 μg of tRNA per ml. Dithiothreitol was added to a final concentration of 200 mM. One volume of potassium acetate (pH 5.0) was added and the RNA was precipitated in 2 vol of ethanol. The resuspended RNA was translated *in vitro* in a nuclease-treated rabbit reticulocyte lysate system for 90 min at 37°C in 50-μl reaction volumes. Translated products were immunoprecipitated with 5 μl of anti-MPO antiserum and analyzed by NaDodSO$_4$/PAGE and fluorography (25).

RNA Blot Hybridization. Ten micrograms each of poly(A)$^+$ RNA, Me$_2$SO-induced HL-60 poly(A)$^+$ RNA, and human placenta poly(A)$^+$ RNA were resolved by electrophoresis in a 1.4% agarose gel in the presence of 2.2 M formaldehyde essentially as described (20) and transferred to nitrocellulose (26). The blot was probed with ^{32}P-labeled DNA produced by nick-translation of pGEM-1 containing the MPO cDNA insert. RNA blot hybridizations were carried out for 36 hr at 50°C in 50% formamide/5× SSC (1× SSC = 0.15 M NaCl/0.15 sodium citrate, pH 7.0)/50 mM NaPO$_4$, pH 6.5/5× Denhardt's solution (1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/10 μg of salmon sperm DNA per ml, followed by four consecutive 30-min washes at 50°C in 1× SSC containing 0.1% NaDodSO$_4$. The blots were exposed to x-ray film for autoradiography (36 hr).

RESULTS

A polyclonal antiserum against MPO purified from human neutrophils was used to screen two λgt11 expression vector libraries. One library contained cDNA prepared from uninduced HL-60 cells, and the second library contained cDNA prepared from normal human bone marrow mononuclear cells. Initial plaque screening of ≈500,000 recombinants of each library identified 29 putative MPO clones. Upon several rounds of purification and rescreening, 11 pure clones were obtained in which every plaque was reactive with anti-MPO antiserum. The cDNA inserts were isolated by digestion with *Eco*RI and their sizes were determined by gel electrophoresis. Inserts varying in size from 60 to 270 base pairs were obtained and the characterization of the largest of these clones, λHMP7 from the bone marrow library, is described in detail.

To confirm that the reactivity of this phage clone with the antiserum was due to an antigenic determinant for MPO encoded by the cDNA insert, lysogens were prepared, induced, and immunoblotted to test whether the lacZ–cDNA fusion protein bound specific MPO antibodies. Protein lysates from the lysogens induced by temperature shift and IPTG were prepared from λHMP7, as well as from λgt11

lacking an insert. These lysates were screened separately with anti-MPO antiserum and an anti-β-galactosidase monoclonal antibody by immunoblotting following NaDodSO$_4$/PAGE. Fig. 1 shows that a β-galactosidase-containing hybrid protein from λHMP7 of ≈125 kDa reacted with anti-MPO antiserum only upon induction. Lack of reactivity with both induced and uninduced λgt11 was also demonstrated. Thus, the cDNA insert encodes an MPO antigenic determinant. The same samples were probed with anti-β-galactosidase antibody (data not shown) to demonstrate that a β-galactosidase epitope as well as an MPO epitope was contained in this fusion protein. Reactivity with the anti-β-galactosidase antibody was seen in both the IPTG-induced λHMP7 and λgt11 lysates. No reactivity was seen in uninduced lysates of either λHMP7 or λgt11. In addition, β-galactosidase itself (from a battery of molecular size markers) was shown to react. Induction of clone λHMP7, therefore, results in the production of a β-galactosidase–MPO fusion protein of ≈125 kDa.

Two additional types of experiments were performed to further confirm the identity of clone λHMP7 as a true MPO clone. Epitope selection as described above demonstrated that specific antibodies from the anti-MPO antiserum affinity-purified on and then eluted from filters adsorbed with induced fusion protein of immunopositive clone λHMP7, continued to recognize MPO by immunoblot analysis. Both the "parent" antibody as well as the epitope-selected antibody clearly recognized the 55- and 39-kDa subunits in a sample of purified MPO (Fig. 2). Furthermore, the same epitope-selected antibody recognized, in HL-60 lysate, an 80-kDa protein, which is the MPO precursor, as well as bands corresponding to processed MPO (see *Discussion*). The somewhat lower molecular mass of the MPO bands seen in HL-60 cells is a variable finding and most probably results from proteolysis. Antibody epitope selected against wild-type λgt11, however, failed to recognize any of these MPO-specific proteins. The fusion protein therefore contains a moiety of MPO that can be used for successful immunoselection of specific anti-MPO antibodies from antiserum.

The cDNA insert of clone λHMP7 was used for the hybrid selection of poly(A)$^+$ mRNA from uninduced HL-60 cells. For this as well as subsequent experiments, the 270-base-pair cDNA insert from the initial clone λHMP7 was subcloned into the twin bacteriophage promoter plasmid pGEM-1. The mRNA hybrid-selected by pHMP7 specifically translated *in vitro* an 80-kDa protein that was immunoprecipitated with the anti-MPO antibody (Fig. 3). This is the expected size for the MPO precursor protein synthesized by HL-60 cells (11, 12, 27). No bands were seen when pGEM-1 alone was used in a similar experiment. Thus, additional confirmation for the identity of clone λHMP7 as a true MPO clone was provided.

To investigate the species of mRNA encoding MPO, poly(A)$^+$ RNA from uninduced and Me$_2$SO-induced HL-60

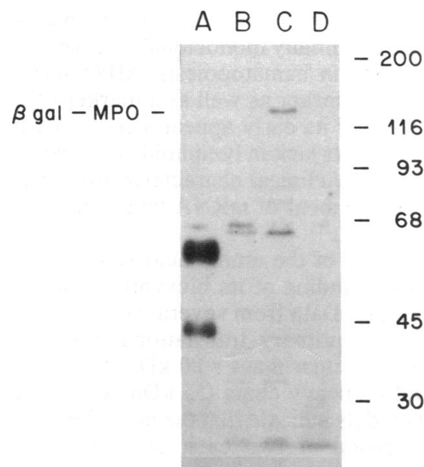

FIG. 1. Immunoblot of purified MPO (500 ng) (lane A), lysates of uninduced (lane B) and IPTG-induced (lane C) λHMP7 lysogen, and IPTG-induced λgt11 lysogen (lane D). The primary antibody was anti-MPO antiserum diluted 1:250. Positions and sizes of markers (in kDa) run in parallel are shown at right.

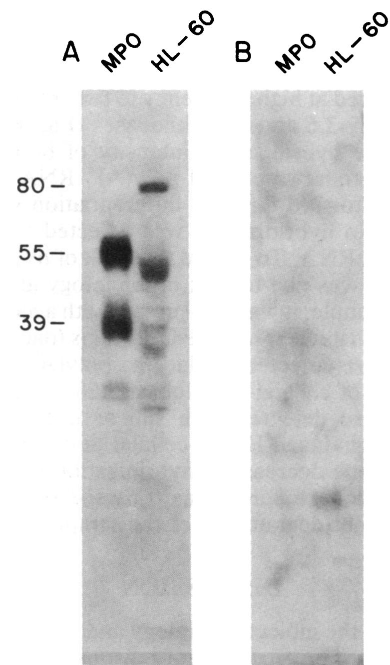

FIG. 2. Epitope selection. Immunoblot of purified MPO (500 ng) and HL-60 cell lysates (10^6 cells per lane) probed with anti-MPO antiserum affinity-purified by induced proteins of clone λHMP7 (*A*) and λgt11 (*B*). Positions and sizes of immunopositive bands (in kDa) are shown at left.

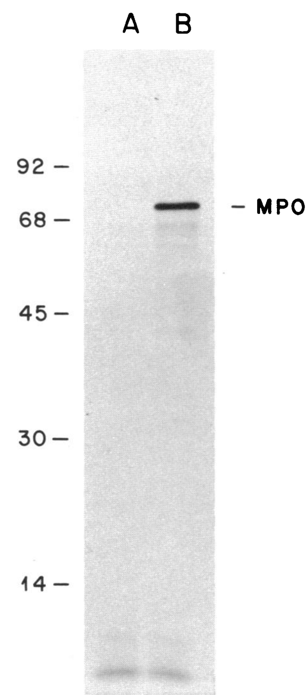

FIG. 3. Hybrid selection, *in vitro* translation, and immunoprecipitation of 20 μg of HL-60 poly(A)$^+$ RNA using pGEM-1 (lane A) and pHMP7 (lane B). Positions and sizes of markers (in kDa) run in parallel are shown at left.

cells was fractionated by electrophoresis in a denaturing formaldehyde/agarose gel, blotted onto nitrocellulose paper, and hybridized with nick-translated pHMP7. The radioactive cDNA hybridized at high stringency to two major bands from HL-60 cells at ≈3.6 and ≈2.9 kilobases (Fig. 4). A striking decrease in the hybridization intensity of both bands was seen when an equal amount of poly(A)$^+$ RNA from HL-60 cells, induced toward terminal differentiation with Me_2SO, was probed. No hybridization was detected to human placenta poly(A)$^+$ RNA. To ensure that lack of hybridization to placenta RNA was due to lack of homology and not degradation of the sample, it was also probed with a nick-translated clone for human nuclear hnRNP C proteins (data not shown). Two bands were detected in placenta poly(A)$^+$ RNA of the expected sizes of 1.9 and 1.4 kilobases (28). These same two bands were also detected with similar intensities in both induced and uninduced HL-60 cellular poly(A)$^+$ RNA. This indicated that the decrease in hybridization intensity of the 3.6- and 2.9-kilobase bands was truly the result of Me_2SO induction and not degradation of the sample.

DISCUSSION

Understanding the molecular biology and molecular genetics of MPO is important for several reasons. First, MPO biosynthesis has been intimately linked to myeloid differentiation. Data regarding regulation of MPO biosynthesis should provide insight into the events associated with both normal and leukemic granulopoiesis. Second, analysis of MPO at the protein level has been complicated by ambiguous reports regarding its structure and biosynthesis.

In myeloid cells, MPO activity has been cytochemically localized at both the light and electron microscope levels to the primary granules (3, 4). MPO is unique to and the most abundant component of myeloid cell primary lysosomes and therefore has been regarded as "the marker" of granulocytic lineage. MPOs first appearance morphologically marks the transition of the primitive myeloblast to the promyelocyte. MPO activity persists in the granules of terminally differentiated neutrophils, yet we (29) and others (30) have shown that these cells are essentially translationally inactive, with little or no detectable poly(A)$^+$ RNA. The mechanism of this shutdown may be best elucidated with probes for specific proteins such as MPO to examine their "window" of genetic expression.

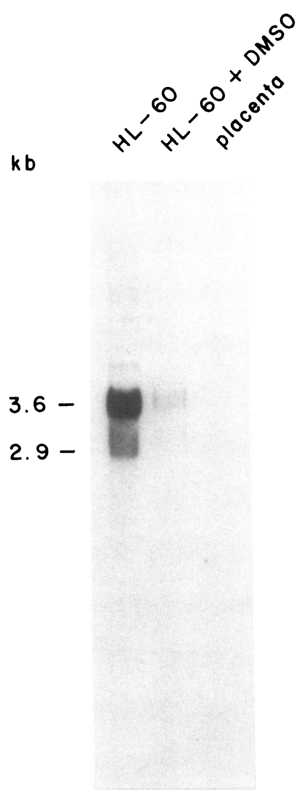

FIG. 4. RNA blot analysis. Blot of electrophoretically fractionated human poly(A)$^+$ RNAs from HL-60 cells, HL-60 cells induced with 1.2% Me_2SO (DMSO), and placenta, probed with nick-translated pHMP7. Sizes of hybridizing bands are shown at left. kb, Kilobases.

Studies of MPO biosynthesis, gene regulation, and expression require active granulopoiesis. The HL-60 cell line is useful because it produces azurophilic granules with high MPO content (8). Furthermore, when HL-60 cells are induced to differentiate into more mature granulocytes, MPO activity decreases (13). This was confirmed by decreased reactivity by immunoblot analysis (S.C.W. and R. Villanueva, unpublished results).

To examine whether the decrease of MPO during differentiation of HL-60 cells was due to degradation or reduced biosynthesis, several groups have conducted studies involving pulse-labeling and immunoprecipitation of translation products of total cellular HL-60 mRNA during various stages of chemical induction (11–13, 27). These studies confirmed that the ability of HL-60 promyelocytes to synthesize MPO was lost upon induction of differentiation, even though overall protein synthesis remained constant. These studies suggested that the decrease in MPO was due to the absence of translatable mRNA for MPO. To directly investigate MPO mRNA levels during granulocyte differentiation, a complementary DNA clone of human MPO is necessary.

Using a λgt11 expression vector library, we have isolated an MPO cDNA clone and directly measured the levels of MPO mRNA in induced and uninduced HL-60 cells. From RNA blot data, it appears that HL-60 MPO is translated from mRNA species of 3.6 and 2.9 kilobases, both of which decrease significantly upon induction with Me_2SO. Although the acute promyelocytic cell line HL-60 may be considered a model equivalent to clones of normal promyelocytes corresponding to this stage of differentiation, they nonetheless exhibit a transformed phenotype. It will be important, therefore, to demonstrate that normal bone marrow cells also show a similar pattern of MPO mRNA(s).

Studies of genetic regulation (i.e., control mechanisms for MPO protein biosynthesis) as a function of normal myeloid maturation will provide insight into the events associated wih the abnormal differentiation in myeloid neoplasia. As human leukemias are essentially monoclonal diseases resulting from specific blockage in hematopoiesis, MPO serves as an excellent leukemic marker as well as a marker of cell differentiation. In view of its early appearance during granulocytic development and its lack in lymphoid cells, MPO is extremely important in the clinical characterization of human acute leukemias. Assessment of mRNA levels may turn out to be far more sensitive.

The availability of the amino acid sequence of MPO will facilitate understanding of its biosynthesis and posttranslational processing. Data from several laboratories (11–13, 27) suggest that the primary translational protein is a direct precursor of molecular mass ≈80 kDa, which appears to be processed into a heavy chain (55 kDa) and a light chain (15 kDa). Most models indicate that the intact holoenzyme (≈150 kDa) is composed of two each of such heavy and light subunits. The source of a 39-kDa subunit seen in $NaDodSO_4$/PAGE remains somewhat a mystery, although nonreducing conditions or proteolysis of the 55-kDa subunit have been hypothesized (31). It is interesting to note that HL-60 mRNA has two mRNA species for MPO. This may provide insight into the origin of the 39-kDa subunit. It has been

suggested that some MPO-deficient patients synthesize the 80-kDa precursor but are unable to process it appropriately for ensuing transport into the primary granules. Obviously, this is a complex protein that will be best understood with information beyond the biochemical level. A full-length cDNA clone will also be important for isolating a genomic MPO clone and to study in detail the genetic organization and expression of this important granulocytic protein in both normal and abnormal hematopoiesis.

Note Added in Proof. In collaboration with Michelle M. LeBeau (University of Chicago) and Richard S. Lemons (St. Jude Children's Research Hospital), we have localized the myeloperoxidase gene to human chromosome 17 at bands q11–21 by using the analyses of *in situ* chromosomal localization and somatic cell hybrids.

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