Chromosomal Gene Structure of Human Myeloperoxidase and Regulation of Its Expression by Granulocyte-Colony-stimulating Factor

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Myeloperoxidase (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7) is a glycoprotein which is present in azurophilic granules (primary lysosomes) of polymorphonuclear neutrophils (1). In the presence of the hydrogen peroxide and chloride ion, myeloperoxidase catalyzes the formation of hypochlorous acid, which works as a potent microbicidal agent. Since azurophilic granules appear only in cells of myeloid lineage, cytochemical staining of myeloperoxidase activity is used clinically to distinguish myeloid leukemia WEHI-3B D+ and NFS-60 cells. When these cells were treated with human recombinant granulocyte colony-stimulating factor, the steady-state level of myeloperoxidase mRNA declined to zero within 36 h in WEHI-3B D+ cells, but not in NFS-60 cells.

By using the full-length cDNA as a probe, the chromosomal gene for human myeloperoxidase was isolated from a human gene library. Comparison of the nucleotide sequence of the chromosomal gene with that of the cDNA has revealed that the human myeloperoxidase gene is composed of 12 exons and 11 introns. S1 mapping analysis of human myeloperoxidase mRNA identified the single transcription initiation site at 180 base pairs upstream of the ATG initiation codon. In the 5′-flanking region of the human myeloperoxidase gene, there are several blocks of sequences which are homologous to the sequences found on the 5′-promoter region of the human c-myc proto-oncogene. The myeloperoxidase gene is expressed in mouse myeloid leukemia WEHI-3B D+ and NFS-60 cells. When these cells were treated with human recombinant granulocyte colony-stimulating factor, the steady-state level of myeloperoxidase mRNA declined to zero within 36 h in WEHI-3B D+ cells, but not in NFS-60 cells.

Myeloperoxidase has been purified from various sources, and the enzyme isolated from mature human neutrophils has a molecular mass of 120,000-160,000 daltons and is composed of two heavy chains (55,000-60,000 daltons) and two light chains (10,000-15,000 daltons) (3-8). Recently, the cDNA for human myeloperoxidase was isolated by us (9) and others (10), and the nucleotide sequence analysis of the cDNA has indicated that the heavy and light chains of human myeloperoxidase are coded for by a single mRNA. By electron microscopic analysis of human bone marrow cells, it has been suggested that myeloperoxidase is synthesized only in the early stages of myeloid cell differentiation, especially in promyelocytes, and stored in azurophilic granules (11). On the other hand, human promyelocytic leukemia HL-60 cells produce myeloperoxidase constitutively; and when cells are induced to differentiate into granulocytes or monocytes by chemical inducers such as dimethyl sulfoxide, retinoic acid, and phorbol ester, the synthesis of myeloperoxidase ceases (10, 12-15). It is also known that the expression of the c-myc proto-oncogene is suppressed during differentiation of HL-60 cells by chemical inducers (16-18).

In addition to chemical inducers, colony-stimulating factors regulate the proliferation and differentiation of the precursor cells of granulocytes and monocytes (19, 20). In order to understand the mechanism of the regulation by colony-stimulating factors, we have isolated the cDNAs for human and mouse granulocyte colony-stimulating factor (G-CSF) (21-23), which is a member of colony-stimulating factors. Human G-CSF was produced in large quantity by using recombinant DNA technology and purified to homogeneity (24). G-CSF can induce terminal differentiation of mouse myeloid leukemia WEHI-3B D+ cells (25, 26) while it supports proliferation of other myeloid leukemia NFS-60 cells without inducing differentiation (23, 27).

In this report, we show that G-CSF can suppress the expression of myeloperoxidase in WEHI-3B D+ cells, but not in NFS-60 cells. The chromosomal gene structure for human myeloperoxidase is also elucidated, and several blocks of sequences on the 5′-flanking region of the gene are found to be homologous to the sequences on the corresponding region of the human c-myc proto-oncogene.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases were purchased from Takara Shuzo Co. or Toyobo Co. and were used essentially as recommended by the supplier except for the use of 200 μg/ml gelatin instead of bovine serum albumin. Klonef fragment of Escherichia coli DNA polymerase I and T4 DNA ligase were products of Takara Shuzo Co., and T4 polynucleotide kinase was obtained from Toyobo Co. S1 nuclease was purchased from Pharmacia Biotechnology, Inc. [γ-32P]ATP was prepared from carrier-free H3PO4. (Du Pont-New England Nuclear) as described by Walsh and Johnson (28), and [α-32P]dCTP was purchased from Amersham Corp. Recombinant human G-CSF was produced by using recombinant human G-CSF cDNA (21, 22) and purified to homogeneity as described previously (24). The purified recombinant G-CSF has a specific activity of 9.1×106 units/mg on proliferation assay using murine NFS-60 cells (23). Plasmid pHISR1 carrying the human c-myc chromosomal gene (29) was obtained from

1 The abbreviations used are: G-CSF, granulocyte colony-stimulating factor; kb, kilobase; IL-3, interleukin 3.

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American Type Culture Collection (ATCC 41010). The 0.9-kb EcoRI-ClaI fragment bearing the 3' exon of the human c-myc gene was used as a probe. The cDNA for human elongation factor 1α was provided by Dr. F. Lee (DNAX Institute, Palo Alto, CA).

Cloning of the Chromosomal Gene for Human Myeloperoxidase—The plasmid pGM706 (9) was digested with EcoRI and SalI, and an EcoRI fragment of about 3.0 kb containing full-length human myeloperoxidase cDNA was isolated by electrophoresis on 1% agarose gel (low melting temperature, Bio-Rad). The cDNA was labeled with 32P by nick translation (32) or the random primer method (33). The mouse myeloid leukemia NFS-60 cells were maintained in RPMI 1640 medium (Nissui Seiyaku, Tokyo) containing 10% fetal calf serum (M. A. Bioproducts). Mouse myeloid leukemia NFS-60 cells were maintained in RPMI 1640 medium containing 10% fetal calf serum. The cDNA for human elongation factor 1α was isolated from a human cDNA library. The structure is essentially identical to that described by Brands et al. (30). The 2.2-kb BamHI fragment containing the full-length cDNA was used as a probe.

Cell Lines—Mouse myelomonocytic leukemia WEHI-3B D+ cells (25) were kindly supplied by Dr. Metcalf (Walter Eliza Hall Institute, Melbourne, Australia) and grown in Dulbecco's minimal essential medium (Nissui Seiyaku, Tokyo) supplemented with 3.5 g/liter glucose and 10% fetal calf serum (M. A. Bioproducts). Mouse myeloid leukemia NFS-60 cells were maintained in RPMI 1640 medium (Nissui Seiyaku, Tokyo) containing 10% fetal calf serum, 50 μM β-mercaptoethanol, and 4% of the COS cell supernatant transfected with mouse IL-3 expression plasmid (31). The mouse IL-3 expression plasmid was provided by Dr. F. Lee (DNAX Institute, Palo Alto, CA).

Human promyelocytic HL-60 cells were maintained in RPMI 1640 medium containing 10% fetal calf serum.

RESULTS

Isolation of the Human Myeloperoxidase Chromosomal Gene—By screening about 4 × 10^6 plaques of the human genomic library with the 32P-labeled EcoRI fragment of pGM706 (9) as a probe, 12 strongly hybridizing clones and several weakly hybridizing clones were obtained. Three clones (AMP04, AMP012, and AMP018) were picked up at random from these strongly hybridizing clones and plaque-purified. EcoRI restriction enzyme digestion of DNAs from these clones released inserts of 13, 12, and 12 kb, respectively, and no EcoRI site was found within the inserts. The EcoRI fragments were subcloned at the EcoRI site of pBR327, and the resultant plasmids were designated as pMP04, pMP012, and pMP018. These clones were characterized by restriction enzyme mapping and Southern hybridization analysis using either the 5' or 3' specific DNA probes.

Preparation of RNA and Northern Hybridization—Total cellular RNA was extracted from cells by the guanidine thiocyanate/cesium chloride method as described by Chirgwin et al. (39) and quantitated by measuring A260. Usually, about 50-60 μg of RNA was recovered from 2 × 10^6 cells of WEHI-3B D+ or NFS-60 cells. When RNA was analyzed on 1.5% agarose gel in Tris acetate/EDTA buffer (pH 7.8) containing 0.5 μg/ml ethidium bromide, clear bands of 28 S and 18 S RNA were recognized. For Northern hybridization analysis, 20 μg of RNA was denatured by heating at 60 °C for 5 min in 2.2 M formaldehyde, 50% deionized formamide and electrophoresed through 1% agarose gel containing 2.2 M formaldehyde (32). The fractionated RNA was transferred to a nitrocellulose filter (Schleicher & Schuell).

A. Naito, T. Uetsuki, and S. Nagata, unpublished data.

Fig. 1. Organization of the human myeloperoxidase chromosomal gene. Boxes and lines between them represent the 12 exons and 11 introns, respectively. The coding sequence is shown by the dark area. The top line indicates the size scale in kilobases; the initiation codon ATG is numbered as 1. The location of the major recognition sites for restriction enzymes are given above the gene. The sequencing strategy is shown under the gene. Arrows represent the direction and the length of the sequence determined by each independent experiment.
Fig. 3. Nucleotide sequence of the human myeloperoxidase gene. The coding sequence of exons is translated and numbered from the ATG initiation codon. On the 5'-flanking region, the TATA-like box, and the core element of an enhancer are underlined. The putative initiation site for transcription is marked by an asterisk. The nucleotide sequence of the chromosomal gene differs from the published sequence of cDNA (6) at two positions. An insertion of 9 nucleotides (GTACAAGGG) at nucleotide 122 and a deletion of a guanine at nucleotide 133 of cDNA (9) are the result of a typing error. bp, base pairs.
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Fig. 4. Northern hybridization analysis of RNA from WEHI-3B D+ cells. WEHI-3B D+ cells (2 x 10⁷/ml) were incubated in the presence of 20 ng/ml human G-CSF for various lengths of time. Cellular RNAs were prepared from cells either untreated (lane 1) or treated with G-CSF for 12 h (lane 2), 24 h (lane 3), 36 h (lane 4), 48 h (lane 5), 72 h (lane 6), or 96 h (lane 7). About 20 µg of RNA was electrophoresed on 1% agarose gel and analyzed by Northern hybridization using 32P-labeled DNA probes. The DNA probes used were human myeloperoxidase cDNA (a), the 3' exon of the human c-myc proto-oncogene (b), and cDNA for human elongation factor 1α (c). To the left, the sizes of 32P-labeled marker DNA are shown in kilobases. Ori, origin of electrophoresis.

Fig. 5. Dose response of the effect of G-CSF on expression of the myeloperoxidase gene in WEHI-3B D+ cells. WEHI-3B D+ cells (2 x 10⁷/ml) were incubated with various concentrations of human G-CSF and 20 µg of cellular RNA was analyzed by Northern hybridization using human myeloperoxidase cDNA as a probe. The concentrations of G-CSF added to the culture were 0 (lane 2), 0.5 ng/ml (lane 3), 1.0 ng/ml (lane 4), 2.0 ng/ml (lane 5), 4.0 ng/ml (lane 6), and 8.0 ng/ml (lane 7). As size markers, 32P-labeled DNA fragments (lane 1) were electrophoresed in parallel, and sizes of DNA fragments are shown in kilobases. Ori, origin of electrophoresis.

cellular RNA prepared from human promyelocytic leukemia HL-60 cells. After digestion with S1 nuclease, the size of the protected DNA fragment was analyzed by electrophoresis on 15% polyacrylamide gel containing 8.3 M urea. As shown in Fig. 3, a distinct band of 56 nucleotides could be observed by using two different concentrations of S1 nuclease. From the size of the protected fragment and taking into consideration the fact that the cap structure on the 5' end of mRNA hinders the digestion of DNA by S1 nuclease (40), we tentatively conclude that myeloperoxidase mRNA starts from adenine at nucleotide −180, which is marked with an asterisk in Fig. 2. Twenty-one nucleotides upstream of the putative cap site, there is the 5'-ATAAAA-3' sequence flanked on both sides with G + C-rich segments which may correspond to the Hogness box (41). Further upstream, we notice the 5'-CTTCC-3' sequence which matches the core element of an
enhancer found in the promoter of several viral and cellular genes (43).

*Effect of G-CSF on the Expression of the Myeloperoxidase Gene*—G-CSF is known to induce differentiation of mouse myeloid leukemia WEHI-3B D* cells into granulocytes and monocytes (25, 26). Since human myeloperoxidase cDNA can hybridize well with mouse myeloperoxidase mRNA (9) and human G-CSF can work on mouse cells (24, 44), we have examined the effect of human G-CSF on the expression of the myeloperoxidase gene in these myeloid leukemia cells.

WEHI-3B D* cells were incubated in the presence of 20 ng/ml human recombinant G-CSF (24). Aliquots of cells were withdrawn before G-CSF treatment and at various time points afterwards. Total cellular RNA was extracted from cells and analyzed by Northern hybridization using human myeloperoxidase cDNA as a probe. As shown in Fig. 4a, the myeloperoxidase gene is transcribed in WEHI-3B D* cells, giving rise to a single 2.4-kb RNA species. On the other hand, when the cells were treated with G-CSF, the steady-state level of myeloperoxidase mRNA declined to zero within 36 h (Fig. 4a). The expression of c-myc proto-oncogene was reported to be suppressed when WEHI-3B D* cells were treated with G-CSF (26). To correlate the expression of the myeloperoxidase gene with that of c-myc, Northern hybridization was carried out using c-myc DNA (29) as a probe. As shown in Fig. 4b, c-myc mRNA was detected for 48 h after exposure to G-CSF, and then its level decreased dramatically. This result agrees with the observation of Gonda and Metcalf (26), although their system contained actinomycin D in addition to G-CSF. In contrast to myeloperoxidase and c-myc, the mRNA level of elongation factor 1α, one of the housekeeping enzymes (30), increased approximately 5-fold by treatment with G-CSF for 36 h (Fig. 4c) and remained constant afterwards. This result indicates that the reduction of the myeloperoxidase and c-myc mRNAs is specific and not due to the generalized degradation of the RNA sample. To confirm the effect of G-CSF on the expression of the myeloperoxidase gene, WEHI-3B D* cells were incubated with various concentrations of G-CSF for 36 h, and the RNAs were analyzed as described above. As shown in Fig. 5, the effect of G-CSF was almost dose-dependent, and as little as 2 ng of G-CSF/ml could significantly reduce the steady-state level of myeloperoxidase mRNA.

Recently, Holmes et al. (45) have established several IL-3-dependent myeloid leukemia cell lines from mouse spleen cells transfected with Cas-Br-M leukemia virus. Most of these cell lines require IL-3 for growth and do not respond to G-CSF.

However, G-CSF can support the proliferation of one of these cell lines, NFS-60, although it does not induce terminal differentiation (27). Since the myeloperoxidase gene is expressed in NFS-60 cells in the presence of IL-3 (9), we have studied the effect of G-CSF on the expression of the myeloperoxidase gene. As shown in Fig. 6, the steady-state level of myeloperoxidase mRNA remained almost constant in NFS-60 cells during G-CSF treatment for at least 96 h.

**DISCUSSION**

In this report, the chromosomal gene for human myeloperoxidase was isolated from a human gene library and characterized by restriction enzyme mapping and nucleotide sequence analysis (Figs. 1 and 2). Previously, it was suggested that there are one or two genes for human myeloperoxidase per haploid genome (9) since Southern hybridization analysis of human genomic DNA shows six bands in *Bam*HI and three bands in *Hind*III-cleaved DNA using myeloperoxidase cDNA as a probe (see Fig. 6 in Ref. 9). The chromosomal DNA fragment isolated in this report contains three *Bam*HI sites and no *Hind*III site within the myeloperoxidase gene locus (Fig. 1). This result suggests that at least two genes must exist for human myeloperoxidase per haploid genome. In this regard, it is noteworthy that we have recently isolated the second cDNA clone for human myeloperoxidase, which has more than 80% homology with the cDNA reported previously (9, 10). G-CSF can induce differentiation of mouse myelomonocytic leukemia WEHI-3B D*, but not NFS-60 cells, into granulocytes and monocytes on soft agar (25, 27). However, when WEHI-3B D* cells are cultured in liquid, complete differentiation cannot be induced in a few days by G-CSF treatment (26, 46). Nevertheless, G-CSF could diminish the steady-state level of mRNAs for myeloperoxidase and the c-myc proto-oncogene in WEHI-3B D* cells after 2 days of exposure to G-CSF (Fig. 4), although the kinetics are slightly different between myeloperoxidase and c-myc. It seems that during differentiation of WEHI-3B D* cells into granulocytes and monocytes by G-CSF, a multistep event such as down-regulation of myeloperoxidase and c-myc genes precedes the morphological change of cells. Similar results were also reported in human promyelocytic leukemia HL-60 (17) or murine embryonal carcinoma F9 (47) cells, where the suppression of the c-myc gene by vitamin D₃ or dimethyl sulfoxide precedes morphological changes of cells. On the other hands, G-CSF could not regulate myeloperoxidase mRNA in NFS-60 cells (Fig. 6) which can proliferate in response to G-CSF (27). These results may suggest that the expression of the myeloperoxidase gene can be suppressed only in cells which are programmed for differentiation. It will be interesting to study the regulation of the myeloperoxidase gene by G-CSF in various human and mouse myeloid leukemia cells.

Coordinate suppression of myeloperoxidase and c-myc mRNAs was also reported when human promyelocytic leukemia HL-60 cells were induced to differentiate into granulocytes and monocytes by chemical inducers (10, 12-18). In this case, the down-regulation of the steady-state level of c-myc mRNA is controlled at transcriptional elongation (18), although the c-myc gene can be regulated at the transcriptional initiation (48) or post-transcriptionally (47) in other systems. From our results described here, we cannot conclude at which level myeloperoxidase and c-myc genes in WEHI-3B D* cells are regulated by G-CSF. However, as shown in Table I, the promoter regions of human myeloperoxidase and human...
c-myc genes (49) have several blocks of sequences which are homologous to each other. To clarify the significance of these sequences, it is necessary to study the transcription of myeloperoxidase and the c-myc gene both in vivo and in vitro using cloned cDNAs and chromosomal genes for human myeloperoxidase and the c-myc gene.

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