Isolation and Functional Characterization of the Human Gene Encoding the Myeloid Zinc Finger Protein MZF-1†

Pei Hui,‡ Xin Guo, and Peter G. Bradford*

Department of Pharmacology and Toxicology, School of Medicine and Biomedical Sciences, State University of New York at Buffalo, Buffalo, New York 14214-3000

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ABSTRACT: The expression of the human myeloid zinc finger gene (MZF-1) by human bone marrow cells is necessary for granulopoiesis. We have analyzed the structure and function of the human MZF-1 gene by diagnostic polymerase chain reaction, genomic cloning, and promoter analysis. Comparison of human promyelocytic HL-60 cell cDNA with isolated MZF-1 genomic clones indicated that the human MZF-1 gene is without introns and spans approximately 3 kb. Restriction enzyme mapping and Southern analysis indicated further that the human MZF-1 gene is a single-copy gene. Primer extension studies identified the major transcription start site as a thymidine residue located 1102 bp upstream of the ATG translation start codon. A putative TATA box sequence (TAAAAA) was found at −66 bp and a CCAAT box at −130 bp relative to the transcription initiation site. In HL-60 cells, MZF-1 mRNA levels are increased by granulopoietic inducers including retinoic acid and GM-CSF. DNA upstream of the transcription start site contains tandem-repeated consensus retinoic acid response elements at −666 through −696 bp and paired putative GM-CSF-responsive sequences centered at −50 and −100 bp. CAT reporter gene constructs containing these DNA regions promoted transcription and conferred transcriptional responsiveness to retinoic acid and GM-CSF when transfected into HL-60 cells. Additional putative regulatory binding sites included conserved MZF-1 zinc finger binding sequences, the importance of which was suggested by the enhanced expression of the endogenous MZF-1 gene following vector-driven expression of MZF-1 constructs in K562 myeloblastic leukemia cells. These findings provide a clearer basis for understanding the role of MZF-1 gene expression in myeloid cell growth and differentiation.

The proliferation and differentiation of hematopoietic cells requires cell surface receptor stimulation by cytokines, the activation of signal transduction processes, and the expression of specific batteries of genes (Quesenbery, 1990; Metcalf, 1992). It is a goal of hematologic research to identify those genes that regulate cell growth and differentiation cascades. The myeloid zinc finger gene (MZF-1)[1] is a candidate gene for the hierarchical control of myelopoietic differentiation.

MZF-1 is a myeloid-specific transcription factor whose expression is essential for granulopoiesis. MZF-1 was discovered as a novel C_2H_2 zinc finger gene product in a cDNA library prepared from leukapheresed cells of a chronic myelogenous leukemia patient (Hromas et al., 1991). The MZF-1 cDNA encodes a protein of 485 amino acids containing a serine- and threonine-rich amino terminal domain followed by 13 consensus Krüppel-type zinc finger motifs that are contiguous except for a 24-residue, proline-rich insertion between fingers 4 and 5. The two zinc finger domains, composed of four and nine zinc finger sequences, are capable of high-affinity DNA binding and recognize similar consensus sequences containing a common G-rich core (Morris et al., 1994).

Experimental observations support the idea that MZF-1 expression is essential for myelopoiesis. Thus, MZF-1 mRNA is largely restricted to myeloid cells and is expressed in a stage-specific manner (Bavisotto et al., 1991). Expression is low in myeloblasts but increases dramatically in myelocytes and metamyelocytes and then ultimately declines to undetectable levels upon complete granulocytic differentiation. MZF-1 mRNA is also expressed in HL-60 promyelocytic leukemia cells, and this level of expression is increased several-fold following treatment with granulocytic inducers including retinoic acid and GM-CSF (Hromas et al., 1991; Hui & Bradford, 1993). On the other hand, treatment of HL-60 cells with inducers of macrophage differentiation, including tetradecanoylphorbol acetate (TPA), has no effect on MZF-1 mRNA levels. Furthermore, MZF-1 has been shown to have direct effects in regulating the induction of granulocytic differentiation of hematopoietic progenitor cells. In vitro granulocyte colony formation by bone marrow cells from normal human donors treated with granulocyte colony-stimulating factor is dramatically inhibited by antisense but not sense MZF-1 oligonucleotides (Bavisotto et al., 1991). Erythroid differentiation of the same cells by in vitro treatment with erythropoietin is unaffected by antisense MZF-1 oligonucleotides.

In order to elucidate the molecular processes controlling MZF-1 expression, we have isolated and characterized the

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* To whom correspondence should be addressed. Telephone: 716-829-2110. Telefax: 716-829-2801.

‡ Current address: Department of Pathology, Yale University School of Medicine, New Haven, CT 06510-8023.

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[1] Abbreviations: CAT, chloramphenicol acetyltransferase; [α-^{32}P]-dCTP, [α-^{32}P]deoxycytosine 5′-triphosphate; dNTP, deoxynucleotide triphosphate; GAS, interferon-γ activated site; GM-CSF, granulocyte−macrophage colony-stimulating factor; IL-3, interleukin-3; MoMLV, Moloney murine leukemia virus; MZF-1, myeloid zinc finger gene-1; PCR, polymerase chain reaction; PFU, plaque-forming units; SSC, standard saline citrate; TPA, tetradecanoylphorbol acetate.

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human gene encoding MZF-1. The MZF-1 genomic structure was analyzed by diagnostic polymerase chain reaction, by the isolation and sequencing of genomic DNA clones containing the MZF-1 gene, and by the identification of the major transcription start site and functional promoter regions. In addition, an autoregulation of MZF-1 expression was indicated through ectopic expression studies. The results help to explain observations concerning the patterns of MZF-1 expression in myelopoietic cells and provide a framework in which to understand the transcriptional control of myeloid differentiation.

EXPERIMENTAL PROCEDURES

Materials. HL-60 and K562 cells were obtained from American Type Culture Collection and cultured in RPMI 1640 medium with 10% fetal bovine serum as previously described (Bradford et al., 1992). Human placental genomic DNA λ_{FIXII} library was from Stratagene Inc. (La Jolla, CA). Restriction and modifying enzymes, Taq polymerase, and nucleoside triphosphates were purchased from Promega Corp. (Madison, WI). T7 DNA polymerase (Sequenase version 2.0) was purchased from U.S. Biochemicals (Cleveland, OH). [^{32}P]dCTP and [^{35}S]dATP were from DuPont NEN (Boston, MA). The random-primed oligolabeling kit was from Pharmacia LKB (Piscataway, NJ). All other chemicals and materials were of molecular biological reagent grade.

Polymerase Chain Reaction. The polymerase chain reaction (PCR) was performed essentially according to published procedures (Saiki, 1989). Genomic DNA templates were prepared from nonionic detergent lysates of cultured human leukemia HL-60 cells. Log phase HL-60 cells (6×10^6) were washed with phosphate-buffered saline (pH 7.4) and then lysed by incubation for 60 min at 55 °C in 1 mL of nonionic detergent buffer (50 mM KCl, 10 mM Tris-HCl at pH 8.3, 2.5 mM $MgCl_2$, 0.1% gelatin, 0.5% NP-40, and 0.5% Tween 20) containing 60 mg% proteinase K. The lysates were heated at 100 °C for 10 min, and aliquots of 15 μL were used as templates for PCR analyses. λ_{FIXII} clonal DNA templates were prepared by phenol−chloroform extraction of plaque-purified phage preparations. First strand cDNA templates were prepared by reverse transcription of poly A$^+$ RNA derived from HL-60 or K562 cells by guanidinium thiocyanate extraction, CsCl ultracentrifugation, and oligo-dT cellulose chromatography as previously described (Bradford et al., 1992). PCR amplifications were performed for 30 cycles in 50 μL reaction volumes containing template DNA in buffer (50 mM KCl, 10 mM Tris-HCl at pH 8.4, 0.7−2.25 mM $MgCl_2$, 100 μg/mL of gelatin, 0.25 μM primer, 0.2 mM dNTPs) with 1 unit of Taq polymerase. PCR products were sequenced by bidirectional dideoxy methods (Sanger et al., 1977) using either the PCR product itself or after subcloning into the pCR1000 vector (Invitrogen). PCR primers were synthesized by Bio-Synthesis Inc. (Lewisville, TX) and included p37s, AGTCAGGGTGTGGAAATGGCAGAT; p1069s, ACGGTGGGCATTCTGTCATGAATG; p1134s, GCGCAGATCTCCCTTGTGTATGCAGGGTTC; p1416a, GCGAATTCCCACGGCCGCCCCTAACCACCCC; p1873s, ACAAGTCCTTTGGCTGCGTCGAGT; p1897a, ACTCGACGCAGCCAAAGGACTTGT; and p2660s, ATTGGACACCTACAGTAGCCAAGC.

Isolation and Analysis of the Human MZF-1 Genomic Clone. The human placental DNA λ_{FIXII} library was plated and screened by plaque hybridization (Sambrook et al., 1989) using a column-purified 1.4 kb human MZF-1 cDNA probe labeled with [α-^{32}P]dCTP by the random primer method. The 1.4 kb cDNA was prepared by reverse transcription of HL-60 cell poly A$^+$ RNA followed by primer-specific PCR amplification (p37s sense primer, p1416a antisense primer). The gel purified amplification product was sequenced and shown to correspond to bp 37−1416 of the published human MZF-1 cDNA (Hromas et al., 1991). This region of the MZF-1 cDNA encodes ca. 1 kb of 5′ untranslated mRNA sequence plus 324 bp of DNA encoding the non−zinc finger N-terminus of MZF-1. For genomic library screening, the λ_{FIXII} phage were used to infect SRB/P2 host bacteria (Stratagene). The infected bacteria were plated at 5×10^4 PFU onto each of ten 150 mm plates and the resultant plaques transferred to Colony/Plaque Screen nylon hybridization membranes (DuPont NEN). The membranes were prehybridized and hybridized according to manufacturer's recommendations using 5×10^5 cpm/mL of [^{32}P]labeled MZF-1 cDNA probe (1×10^9 dpm/μg). Putative MZF-1 genomic clones were subjected to secondary and tertiary screening, amplified in liquid cultures, and then used for phage DNA preparation. Purified phage DNA was initially analyzed by PCR amplification, restriction enzyme digestion, and Southern blot analysis using cDNA probes. DNA from the λ clone (λ_{FIXII}9-2-1) was digested with *Sac*I and *Hind*III, producing a 1.8 kb DNA fragment that was subcloned into the pGEM-11Zf(+) vector (Promega), generating pGEM-1.8MZF. Likewise, the 3.2 kb *Hind*III fragment was subcloned into pBluescript II KS[+] (Stratagene) to generate pKS[+]-3.2MZF (Figure 1). Both inserts were sequenced bidirectionally by dideoxy chain termination methods. DNA sequences were analyzed via INTERNET using the FINDPATTERNS program of the Genetics Computer Group of the University of Wisconsin.

Genomic DNA Preparation and Southern Hybridization. Genomic DNA from exponentially growing HL-60 or K562 cells (5×10^7) was isolated by nonionic detergent lysis, nuclear isolation, proteinase K digestion, and fractionation through and elution from Qiagen (Chatsworth, CA) genomic DNA isolation columns according to the manufacturer's procedure. For restriction enzyme digestions, genomic DNA (20 μg) was incubated overnight at 37 °C with 10−20 units of enzyme. Digested DNA (5 μg) was electrophoresed through 0.65% agarose gels and transferred by capillary blot to Gene Screen Plus (Dupont NEN) nylon membranes. For λ phage DNA analysis, isolated DNA was digested with restriction enzymes, electrophoresed through 0.65% agarose gels, and transferred to Gene Screen Plus as above. Membranes were hybridized at 65 °C according to manufacturer's procedures in 6× SSC, 1% SDS, 10% dextran sulfate buffer containing $0.5-1.0 \times 10^6$ cpm/mL of [^{32}P]MZF-1 PCR-derived DNA probe corresponding to bp 1134−1416 of the MZF-1 cDNA, and washed under high-stringency conditions before autoradiographic exposure.

Primer Extension Analysis. The transcription start site of the human MZF-1 gene was analyzed by primer extension (Sambrook et al., 1989). Primers included antisense 18mer oligonucleotides corresponding to positions +82→+64 and −185→−201 as numbered from the published cDNA and continuing upstream into the MZF-1 genomic clone. Primers were end-labeled using [γ-^{32}P]ATP (3000 Ci/mmol) and T4 polynucleotide kinase for 1 h at 37 °C. The labeled

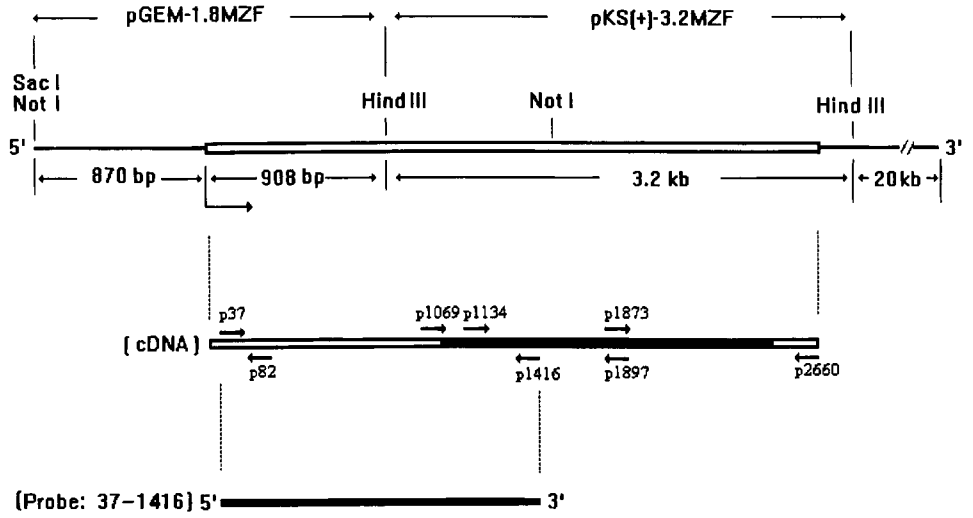

FIGURE 1: Schematic representation of the λ_{FIXII}9-2-1 MZF-1 human genomic clone and human HL-60 cell cDNA. Locations are shown for select genomic restriction enzyme sites, transcription start site, open reading frame, as well as subclones. Primer sequences are indicated as sense (above the cDNA) or antisense (below the cDNA) and are reported in Experimental Procedures. Numbering is based on the cDNA sequence determined by Hromas et al. (1991).

oligonucleotides were isolated free of residual [^{32}P]ATP by fractionation through Nuctrap Push Columns (Stratagene) and then diluted (10^5 cpm/reaction) in hybridization buffer (40 mM PIPES at pH 6.4, 1 mM EDTA, 0.4 M NaCl, 80% formamide) with total cellular RNA (100−150 μg) obtained from 3-day retinoic acid (1 μM)−treated HL-60 cells. The hybridization mixture was heated at 85 °C for 10 min and then incubated at 30 °C. After 12 h, the primer-RNA hybrids were precipitated with cold ethanol and dissolved in 20 μL of reverse transcription buffer containing 300 units of MoMLV reverse transcriptase (US Biochemicals). The reverse transcription reactions were carried out at 37 °C for 2 h and stopped by addition of EDTA to 5 mM and then incubated for 30 min at room temperature with RNase T1 and RNase A (5 units). The reaction was extracted with phenol−chloroform and DNA precipitated with ethanol before analysis on 6% denaturing polyacrylamide sequencing gels. Dideoxy sequencing reactions that used the same oligonucleotide primers and the same region of the DNA template were electrophoresed alongside the primer extension reactions.

CAT Reporter Constructs and Cell Transfection. The eukaryotic expression vector pCAT-basic (Promega), which lacks promoter and enhancer sequences, was used as the parent vector for the analysis of putative MZF-1 promoter and transcriptional regulatory sequences. Upstream MZF-1 genomic DNA (−837 through +48 bp) was directionally inserted into the multiple cloning site of the pCAT-basic vector upstream from the chloramphenicol acetyltransferase (CAT) gene to generate the pCAT/MZF reporter plasmid. Briefly, the pGEM-1.8MZF vector containing MZF-1 genomic DNA (−837 through +908) was digested with *Sac*I and *Pst*I (internal *Pst*I site at +48) and ligated to the *Pst*I/*Hind*III-digested pCAT-basic vector. This DNA was then blunt ended, ligated, and then recovered after $CaPO_4$ transfection of competent JM109 *E. coli*. All plasmids were purified by Qiagen plasmid kits and sequenced to confirm the constructions.

The ability of the pCAT/MZF constructs to promote and enhance transcription was determined by measuring CAT activity in lysates of transiently transfected HL-60 cells. The pCAT-control vector, containing SV40 promoter and enhancer sequences, was used as a positive control. Transfection efficiencies were monitored by β-galactosidase activity expressed from the cotransfection of the plasmid pActin/lacZ, a vector containing the lacZ gene driven by 300 bp of the human β-actin promoter. The pActin/lacZ plasmid was kindly provided by Dr. Te-Chung Lee (SUNY at Buffalo). For each experiment, 10×10^6 of log phase HL-60 cells were centrifuged (150*g*, 10 min), resuspended in 1 mL of RPMI serum-free media, and transferred to Bio-Rad Gene Pulser cuvettes (0.4 cm electrode gap). After 20 μg pCAT/MZF plasmid plus 2 μg pActin/lacZ plasmid were added and after the mixture was incubated on ice for 10 min, the mixture was subjected to electroporation using the Bio-Rad gene pulser set at 500 μF, 300 V, and time constants between 9.9 and 10.8 ms. Following electroporation, the cells were diluted into 37 °C RPMI 1640 medium containing 10% fetal bovine serum and cultured for 48 h in the presence or absence of 10^{-6} M retinoic acid or 50 U/mL (350 pM) of human recombinant GM-CSF (CalBiochem). The cells were then washed twice with phosphate-bufferred saline and lysed in 150 μL of 0.25M Tris-HCl, at pH 8.0, by repeated freeze−thaw cycles. The lysate was microcentrifuged for 10 min and portions of the resultant supernatant were used to measure β-galactosidase and CAT activities. β-Galactosidase activity in 30 μL of each supernatant was determined spectrophotometrically using freshly prepared substrate solution containing *o*-nitrophenyl-β-D-galactopyranoside as described (Rosenthal, 1987). After normalization for β-galactosidase activities, CAT activities in the lysate supernatants (usually ca. 100 μL) were determined by incubation at 37 °C overnight with 3 μL of [^{14}C]chloramphenicol (50−60 mCi/mmol; NEN DuPont), 5 μL of 5 mg/mL of acetyl coenzyme A, and 0.25 M Tris-HCl, at pH 8.0 in a volume of 150 μL. The mixtures were phase-extracted and subjected to thin layer chromatography (Seed & Sheen, 1988). Transfections were performed in duplicate and each experiment was repeated at least three times.

MZF-1 Expression Vector Construction and Cell Transfection. The mammalian expression vector pCEP-4 (Invitrogen Inc.) was used to construct an MZF-1 expression

vector. pCEP-4 was digested with *Xho*I, filled in at the 3′ end, and then dephosphorylated. DNA encoding the entire MZF-1 protein coding region (nucleotides 1069−2660) was amplified from HL-60 cell cDNA, subcloned into the PCR1000 TA cloning vector (Invitrogen Inc.), sequenced, and then excised with *Hind*III and *Nco*I (*Nco*I site at MZF-1 cDNA position 2574, 26 bp downstream of the TAG termination codon). This excised fragment, which does not include the p2660 priming site, was filled in with the large fragment of DNA polymerase (Klenow) and then blunt end−ligated to the prepared pCEP-4 vector to generate the recombinant vector pCEP4/MZF. Following bacterial transformation, the pCEP4/MZF vector was isolated and sequenced from both strands to confirm the orientation and fidelity of the insert. For stable transfection, the pCEP4/MZF DNA (15 μg) was added to 1×10^7 K562 human leukemia cells and the mixture subjected to electroporation using the Bio-Rad gene pulser set at 950 μF and 260 V. K562 cells were used as host cells because they have minimal or undetectable endogenous MZF-1 expression. After electroporation, the cells were resuspended in RPMI medium containing 10% fetal bovine serum, cultured for 2 days, and then stably transfected cells selected by culturing for 1 month in hygromycin B (0.2 mg/mL). Cells were expanded and analyzed for the expression of MZF-1 mRNA by Northern blot and RT-PCR (Bradford et al., 1992).

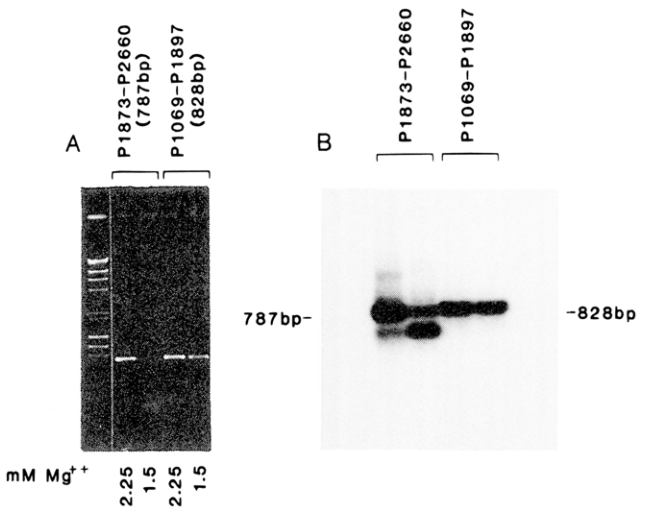

FIGURE 2: PCR analysis of the coding region of the human MZF-1 gene. (A) Human genomic DNA template was used with primer pairs encompassing the entire coding region of the MZF-1 cDNA (p1873−p2660, p1069−p1897) and with two concentrations of Mg^{2+} (1.5 mM and 2.25 mM). Products were identified by ethidium bromide staining after electrophoresis through agarose gels. λ-*Hind*III and ϕX174 *Hae*III DNA size markers are included (left lane). Major products migrate as 0.8 kb fragments. (B) Southern blot of the gel shown in (A) probed with an MZF-1 specific cDNA probe. Markers are product sizes predicted from cDNA sequence.

RESULTS

Analysis of MZF-1 Genomic DNA by PCR. The human MZF-1 cDNA is 2679 nucleotides and contains an open reading frame (nucleotides 1091−2545) predicted to encode a 54 463 MW protein with 13 consensus Krüppel-type zinc finger domains (Hromas et al., 1991). Based upon the cDNA sequence, oligonucleotide primers were designed for PCR amplification and initial analysis of the human MZF-1 gene. The primer designations and their corresponding locations on the MZF-1 cDNA are shown in Figure 1. Human genomic DNA was amplified using primer pairs (p1069s/p1897a and p1873s/p2660a) that encompass the entire predicted protein coding region of the MZF-1 cDNA. The result of one of these amplification experiments and the companion Southern blot are shown in Figure 2A. The major products of genomic DNA amplification were approximately 0.8 kb and corresponded roughly to the sizes predicted from the cDNA: 787 bp for the primer pair p1069s/p1897a and 828 bp for the primer pair p1873s/p2660a. Southern blot analysis shows that the amplification products hybridize specifically with an MZF-1 probe derived from the 1.6 kb MZF-1 cDNA (bp 1069−2660) (Figure 2B). The results are consistent with the lack of any large introns in the coding region of the MZF-1 gene.

A similar scheme was employed to analyze the region of the human MZF-1 gene upstream of the open reading frame. The primer pair (p37s/p1897a) encompassing cDNA bp 37−1897 was used for PCR amplification of human genomic DNA over a range of $MgCl_2$ concentrations. If there are no introns in this region of the MZF-1 gene, then a 1.86 kb product would be expected from amplification of genomic DNA. As shown in Figure 3A, the major amplification product is ca. 1.9 kb, although in this experiment there were several other, apparently nonspecific, products of smaller size. Southern blot analysis using an MZF-1 specific probe confirmed the 1.9 kb band to be the only authentic amplification product (Figure 3B).

Direct DNA sequencing of the PCR products shown in Figures 2 and 3 was performed using both sense and antisense single-stranded DNA sequencing templates generated from the PCR products by asymmetric amplification. The results (not shown) confirmed that the nucleotide sequences of the genomic DNA-derived PCR products matched the published MZF-1 cDNA over bp 37−2660 with no evidence of intervening sequences. A noted exception was the GGCC sequence starting at nucleotide 1253 which contrasts with the CGCG sequence as reported previously (Hromas et al., 1991). The change, which was confirmed by double-stranded sequencing of several PCR products and which was also found in the human MZF-1 genomic clone (see below), results in the amino acid changes of RV to GL at codons 55 and 56 in the non−zinc finger N-terminus. The fact that the PCR products amplified from genomic DNA were of sizes and sequences as predicted from the MZF-1 cDNA sequence suggests that the human MZF-1 gene lacks introns in both the 5′-untranslated and protein coding regions.

Isolation and Analysis of the Human MZF-1 Gene. A human placental genomic library was screened with the PCR-derived probe encoding bp 37−1416 of the human MZF-1 cDNA. The clone λ_{FIXII}9-2-1 containing a 25 kb insert was isolated. Digestion of the λ_{FIXII}9-2-1 DNA with the restriction enzyme *Not*I or *Hind*III resulted in the DNA fragment patterns shown in Figure 4A. Southern blot analysis with the MZF-1 cDNA probe (bp 37−1416) gave the hybridization pattern shown in Figure 4B. These results are consistent with the indicated MZF-1 map (Figure 1) and the *Not*I and *Hind*III restriction enzyme sites identified in the cDNA at bp 1510 and 890, respectively. A second *Hind*III site exists in the human DNA 3.2 kb downstream of the first site and companion *Not*I sites are found in the λ_{FIXII} polylinker regions. Thus, the *Hind*III digestion produced hybridizing

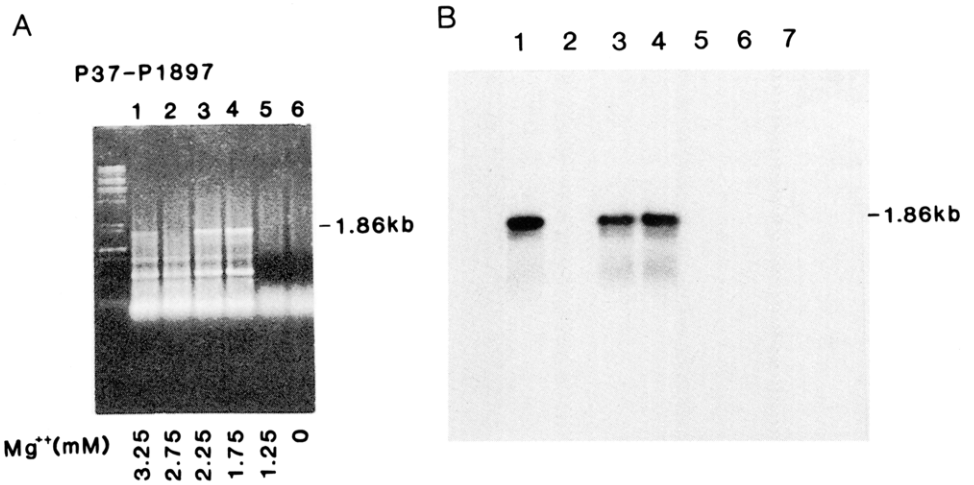

FIGURE 3: PCR analysis of the 5′ region of the MZF-1 gene. (A) Human genomic DNA was amplified with the p37-p1897 primer pair at varying Mg^{2+} concentrations. Products were electrophoresed and visualized as in Figure 2. λ-*Hind*III and φX174 *Hae*III DNA size markers are included (left lane). (B) Southern blot of gel shown in (A) probed with an MZF-1 specific cDNA probe. The major 1.86 kb specific amplification product is indicated.

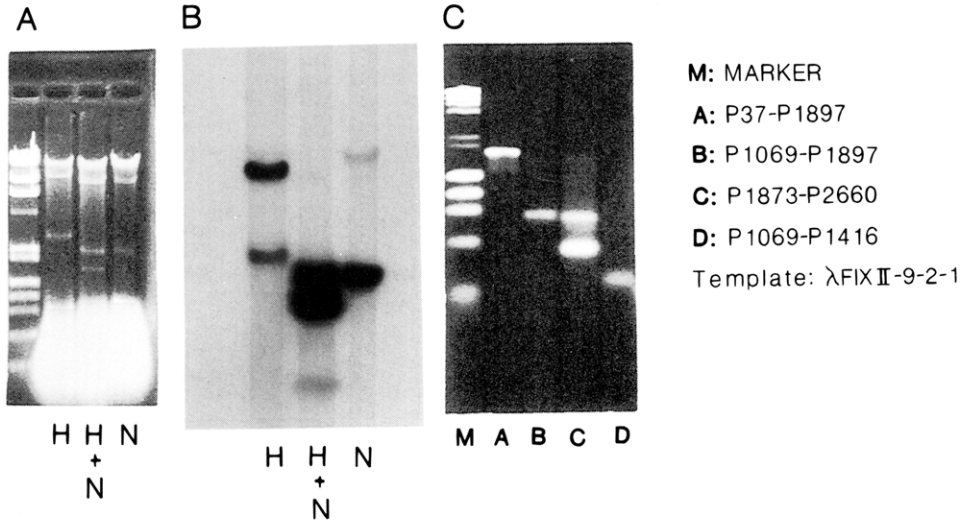

FIGURE 4: Restriction enzyme digestion, Southern analysis, and PCR amplification of MZF-1 genomic clone λ_{FIXII}9-2-1. (A) *Hind*III [H], *Not*I [N], and *Hind*III plus *Not*I [H + N] digestion pattern of λ_{FIXII}9-2-1 DNA. (B) Southern blot of gel in (A) probed with cDNA corresponding to bp 37-1416. (C) PCR amplification of λ_{FIXII}9-2-1 DNA using the indicated primers. λ-*Hind*III and φX174 *Hae*III DNA size markers are in left lane of each gel.

bands of 3.2 kb and 11 kb, the latter including the entire λ left arm plus 1.8 kb of human DNA. *Not*I digestion produced a single 2.4 kb hybridizing band. The combined digestion produced hybridizing species of 1.8 kb and 0.6 kb, plus the incompletely digested 2.4 kb *Not*I fragment.

PCR amplification of the isolated λ_{FIXII}9-2-1 DNA using the cDNA-defined primer pairs generated products of sizes predicted from the cDNA analysis (Figure 4C). Thus, amplification of the λ_{FIXII}9-2-1 DNA with primer pairs p37s/p1897a, p1069s/p1897a, p1873s/p2660a, and p1069s/p1416a yielded major products of 1.9 kb, 0.8 kb, 0.8 kb, and 0.35 kb, respectively, as predicted from the cDNA sequence. Direct sequencing of the entire genomic DNA fragments (pGEM-1.8MZF and pKS[+]-3.2MZF, Figure 1) containing the transcribed region of MZF-1 confirmed the intronless nature of the gene as deduced from PCR analysis. These results are all consistent with the contention that there are no intervening DNA sequences in the human MZF-1 gene.

Southern Hybridization Analysis of the Human MZF-1 Gene. Southern blot analysis of genomic DNA isolated from the K562 human myeloblastic cell line and digested with *Eco*RI or *Hind*III was performed with a probe corresponding to nucleotides 1130−1416 of the MZF-1 cDNA. This cDNA segment encodes the non−zinc finger amino terminal region of MZF-1. As shown in Figure 5, a single genomic fragment from each digestion specifically hybridized with the MZF-1 probe: a 12 kb fragment from *Eco*RI-digested DNA and a 3.2 kb fragment from *Hind*III-digested DNA. Identical results were obtained using high molecular weight genomic DNA from human promyelocytic HL-60 cells (not shown). A single 3.2 kb *Hind*III fragment was also observed after λ_{FIXII}9-2-1 DNA digestion (Figure 4). This hybridization pattern is consistent with the lack of *Eco*RI sites and the identified *Hind*III sites in the MZF-1 cDNA and genomic clones. These results indicate that MZF-1 is a single copy gene in the human genome, corresponding to the clone isolated from the placental library.

Identification of the Transcriptional Start Site. The major transcriptional start site in the human MZF-1 gene was identified by primer extension (Figure 6). Primer extension was carried out with two different synthetic antisense oligonucleotides (16mers), one mapping to position +82 bp

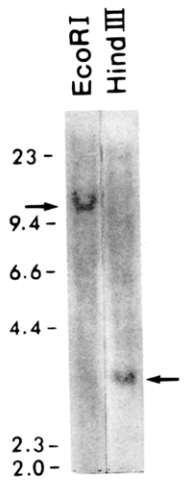

FIGURE 5: Southern analysis of the human MZF-1 gene. Genomic DNA from K562 cells was digested with *EcoR*I or *Hind*III, transferred to Gene Screen Plus nylon membranes, and probed with an [α-^{32}P]dCTP random-primed-labeled MZF-1 cDNA fragment (bp 1134−1416). Arrows indicate a single 12 kb hybridizing fragment from *EcoR*I-digested DNA and a single 3.2 kb hybridizing fragment from *Hind*III-digested DNA.

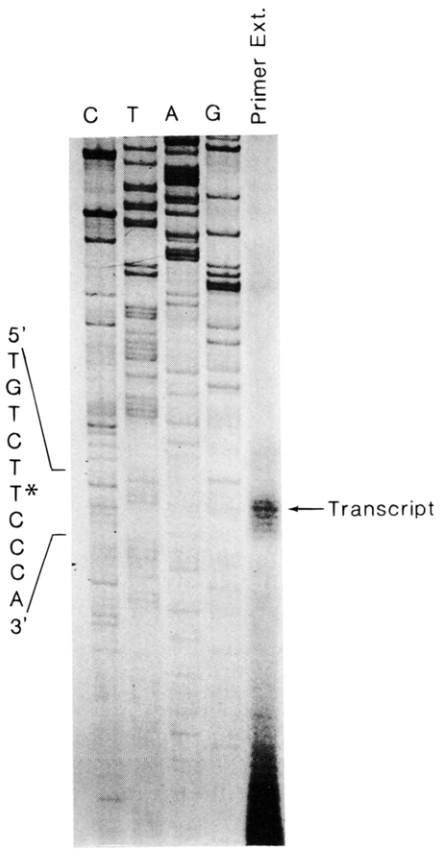

FIGURE 6: Transcription initiation site of the human MZF-1 gene. Primer extension was performed using poly A$^+$ RNA from 3-day retinoic acid−treated HL-60 cells and the p82 MZF-1 primer. The DNA sequence was determined using the same primer and the pGEM-1.8MZF template. Asterisk indicates the thymidine identified as the primary transcription start site.

and the other to position −105 bp relative to the cDNA start. The former primer was used for identification of potential start sites just upstream of the first base of the published cDNA, whereas the latter primer was used to analyze potential start sites in the vicinity of an identified "TATAAT" sequence in the genomic clone. Positive reaction product was observed only with the +82 primer. The primer extended products were electrophoresed alongside DNA sequence ladders derived from using the same oligonucleotide as primer and the pGEM-1.8MZF genomic clone plasmid. In two separate preparations, primer extension carried out with the +82 primer indicated a thymidine (T) nucleotide as the major transcription initiation site (shown by an arrow in Figure 6). Although most eukaryotic transcription start sites occur at nucleotide A, it is not unusual to start at nucleotide T (Breathnach & Chambon, 1981). The identified MZF-1 transcription start site is located 19 bp upstream of the first base of the previously identified cDNA (Hromas et al., 1991).

Sequence of the MZF-1 Promoter Region. In order to identify putative regulatory sequence elements, the 1.8 kb *Sac*I/*Hind*III fragment of the genomic clone (see Figure 1) containing ca. 840 bp of DNA upstream of the MZF-1 transcription start site was subcloned into pGEM-11Zf(+) vector and sequenced from both strands (Figure 7). Computer-aided inspection of the sequence identified a putative TATA box (TAAAAA) located 66 bp upstream from the transcription start site and a CAAT box sequence (CCATT) at −125 bp. In addition to these sites, there are other potential nuclear protein binding sites including consensus sequences for retinoic acid and retinoid X receptors (AGGTCA or TGACCT) (Umesono et al., 1988; Lee et al., 1993), interferon-γ activated Stat91 (TT[C/A]CNNNAA) (Khan et al., 1993; Shuai et al., 1993; Pelligrini & Schindler, 1993; Darnell et al., 1994), AP-2 (GCCTGGCC) (Imagawa et al., 1987), AP-3 (TTACCACA), heat shock transcription factor (NGAAN) (Harrison et al., 1994), c-myc/Max (CACGTG) (Davis, 1993), PU.1 (GAGGAA) (Klemsz et al., 1990), NF-1 (ATTGGG) (Gronostajski et al., 1985), and metal−response element binding proteins (TGCACNC) (Karin et al., 1987).

At positions −825, −696, −689, −675, and −666 bp are tandem repeats of the core hormone response element half-site (AGGTCA or related sequences) recognized by the retinoic acid and retinoid X receptors (Umesono et al., 1988; Vasios et al., 1989; de The et al., 1990; Lee et al., 1993). The −675 and −666 half sites are antisense and in the head-to-tail orientation with a 3 bp spacer that has been found to be the preferential enhancer element orientation for transcriptional activation by retinoic acid (Umesono et al., 1991; Näär et al., 1991). Retinoic acid treatment (1 μM) of HL-60 cells increases the level of the 3 kb MZF-1 mRNA over fourfold (Hromas et al., 1991; Hui and Bradford, unpublished). This effect of retinoic acid is observable within 20 h and is consistent with an increased rate of MZF-1 gene transcription in HL-60 cells (Hui and Bradford, unpublished). Retinoic acid response elements have been observed in several other genes shown to be regulated by retinoic acid in HL-60 cells including CD11b (Pahl et al., 1992; Hickstein et al., 1992), CD11c (López-Cabrera et al., 1993), and CD18 (Rosmarin et al., 1992; Agura et al., 1992).

Functional Promoter Analysis. It is important to demonstrate that the DNA upstream of the identified MZF-1 transcription start site can function to promote gene expression, particularly since this region lacks a typical TATA box. We generated pCAT/MZF constructs using the entire cloned upstream MZF-1 DNA to maximize the ability to detect promoter activity and transcriptional enhancement by potential hormone responsive elements. The pCAT/MZF construct was prepared by ligating the upstream MZF-1 DNA (−830 through +48 bp, relative to the identified transcription

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                    RAR                                   AP-2
-840  XXXTCATATG  AGGTCAGGAG  ATCGAGACCA  GCCTGGCCAA  CATGGTGAAA  CCATGTCTCT

-780  ACTAAAAATA  CAAAAAATTG  GCTAGGTGCG  GTGGCTCACG  CCTGCAATCC  CAGCACTTTG
                          RAR      RAR                RAR         RAR
-720  GGAGGCTGAA  GTGGGCAGAT  CACCTGAGGT  CGGGAGTTCG  AGACCAGCCT  GACCAACATG

-660  GAGAAACCCT  GTCTCTACTA  AAAATACAAA  ATTAGCCGGG  TGTGGTGGTG  CATGCCTGTA

-600  ATCCCAGCTA  CTCGGGAGAC  TGAGGCAGAA  GAACCGCTTG  AACCCAGGAG  ATGGAGGTTT
                                    MRE
-540  GCAGTGAGCA  GAGAACACGC  CATTGCACTC  CAACCTGGGC  AACAAGAGCA  AAACTATGTC
                                                        MRE
-480  TCAAAAAAAA  AAAAATACAA  ACAATTAGCC  AGGCATGGTG  GTGCACGCCT  ATAATCCCAG

-420  CTACTCAGGA  AGATGAAGCA  GCAGAATCGC  TTGAACCCGG  GAGGCAGAGG  TTGAGGTGAG
                       MRE          AP-2
-360  CCGAGATCAC  ACCACTGCAC  TCCAGCCTGG  GCGACACAGC  AAGACTCAGT  CTCAAAAAAA
                                                                  MZF-1
-300  AAAAAAAAAA  AAAAAAAACA  AAAAAAAAAA  CACACACATG  GATGCTTATT  CTCCACCCTG

-240  TTCCACATCT  GGCATTTTTT  GTGCATCTTC  CAACTTAAGT  ACACAGCAGC  AAGTGACTCT
                                MZF-1  myc/MAX      myc/MAX
-180  ACATCTTTGG  GTAGCTACAC  AGAACCCCAC  TGTGGCTCAC  TGTGGACACA  CCATTGAAAA
                          IFN-γ          PU
-120  CCTTGTATGT  AGGTCACTTA  GTACAAGGAG  GAACACAACT  GTTGAAGAAG  TACCTAAAAA
                IFN-γ                                                AP-3
-60   TTGAATTACT  TGAACAAGTG  TGGGACATTT  GCTGTTTTGA  TGGATTTTAC  CACACTGTCT
                          ▸          *
+1    TCCCATTTAT  GCTTACCAGC  AATACATAGG  AACACTTGGG  TCCCTGCAGT  CAGGGTGTGG

+61   AAATGGCAGA  TGAGTTCAGC  CCTAAGGTGC  ATTTTTCTTA  CTAGGAGGAG  ATGGAGTGTA

+121  TTTTATGGGA  TATAAGCATT  AGCTACATTT  CCTGTCCTGT  TCACATCCTT  TGCCCATGTG
```

FIGURE 7: Nucleotide sequence of the 5′ region of the human MZF-1 gene. The identified transcription start site (+1) is indicated by arrow. Potential regulatory sites (underlined) are indicated for the transcription factors retinoic acid/retinoid X receptor (RAR), AP-2, AP-3, PU.1, myc/MAX, interferon-stimulated STAT1 factor (IFN-γ), and metal response element (MRE) binding protein. The predicted MZF-1 autoregulatory sites (MZF-1) are also indicated. The previously identified cDNA start site (C) is indicated by an asterisk.

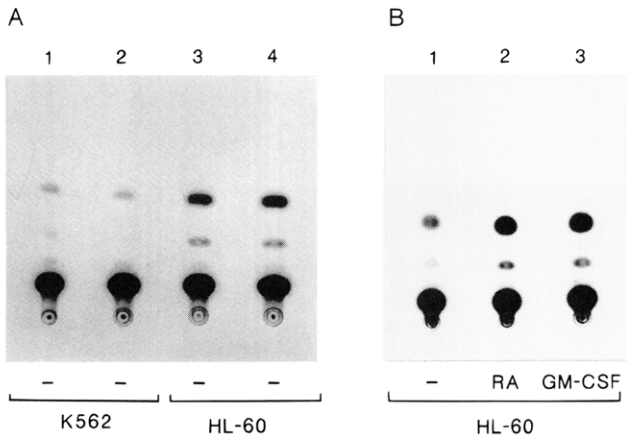

FIGURE 8: Functional activity of the MZF-1 promoter. Transient expression of the CAT gene was used to assess promoter and enhancer activities in K562 and HL-60 cells. Twenty micrograms of the pCAT/MZF reporter construct containing the 0.85 kb fragment of the 5′ upstream region of the MZF-1 gene and 2 μg of the β-actin/lacZ vector were electroporated with 1×10^7 cells. After culturing for 72 h in RPMI 1640 media containing 10% serum, the cells were lysed, and after normalization for β-galactosidase activities, CAT activities in the supernatants were analyzed by thin layer chromatography. (A) CAT activity in pCAT/MZF-transfected K562 cells (lanes 1 and 2) and HL-60 cells (lanes 3 and 4). (B) CAT activity in pCAT/MZF-transfected HL-60 treated for 72 h with vehicle (lane 1), 10^{-6} M retinoic acid (lane 2), or 350 pM GM-CSF (lane 3).

start site) in front of the promoterless bacterial CAT gene. Figure 8A shows CAT expression after transfection of the pCAT/MZF reporter into K562 myeloblastic cells versus expression in similarly transfected HL-60 cells. When normalized to expressed β-galactosidase activity, basal expression of pCAT/MZF in HL-60 cells averaged 16.5-fold ($n = 4$) greater than expression in K562 cells, indicating that the MZF-1 promoter region is more active in HL-60 cells. Since HL-60 cells are MZF-1-positive and K562 cells are MZF-1 negative, these results suggest that the cell specific expression of MZF-1 is dependent on the MZF-1 promoter. Furthermore, when the pCAT/MZF transfected HL-60 cells were treated with either retinoic acid (1 μM) or GM-CSF (350 pM), there was enhanced CAT activity compared to no hormone treatment (Figure 8B). Compared to no treatment, enhancement of CAT activities following retinoic acid or GM-CSF treatment averaged 3.1-fold and 4.4-fold, respectively ($n = 3$). No enhancement of CAT expression was observed for transfected K562 cells when treated with either GM-CSF or RA (not shown). These results clearly show that the upstream region of the MZF-1 gene functions to promote transcription in HL-60 cells and that this DNA can confer transcriptional enhancement in response to HL-60 cell stimulation by RA of GM-CSF.

Expression of pCEP4/MZF1 in K562 Cells. In order to investigate a role for MZF-1 as a potential regulator of myeloid differentiation, an MZF-1 expression vector pCEP4/MZF1 was constructed. This construct contains the complete protein coding region of the MZF-1 gene but lacks the ca. 1 kb untranslated leader sequence. Stably transfected K562 myeloblastic cells containing the electroporated pCEP4/MZF1 construct were selected by long-term culture in hygromycin B. In preliminary experiments K562 cells were shown to express very little endogenous MZF-1 mRNA, despite having some myeloid character. Following the pCEP4/MZF1-driven expression of MZF-1 in K562 cells, there was little demonstrable effect on cell differentiation

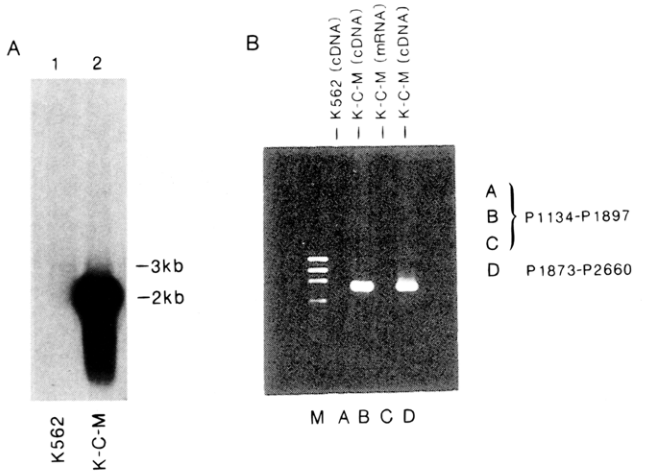

FIGURE 9: Expression of pCEP4/MZF1 in K562 cells. (A) Northern blot of poly A^+ RNA from parental K562 cells and pCEP4/MZF1-transfected K562 cells (K-C-M). The vector-driven transcript is 2 kb and endogenous MZF-1 mRNA is 3 kb. (B) PCR amplification from K562 and K-C-M cDNA and mRNA. Primers pairs are indicated at right. The p2660 priming site is found only in the endogenous 3 kb message.

even under a variety of cytokine treatment regimes (not shown); however, an enhanced expression of the endogenous MZF-1 gene was observed (Figure 9). Northern analysis and PCR amplification of reverse-transcribed mRNA indicates that under the experimental conditions there is no detectable mRNA expression in parental K562 cells (Figure 9A and 9B). However, as shown in Figure 9A, MZF-1 expression from the pCEP4/MZF1 vector in stably transfected K562 cells (K-M-C cells) resulted not only in the robust expression of the 2 kb MZF-1 vector-derived transcript but also in the expression of the endogenous 3 kb MZF-1 mRNA transcript. In the pCEP4/MZF1 transfected cells, MZF-1 mRNA was detectable after RT-PCR using primers not only within the vector construct (not shown) but also outside of the construct (p1873s/p2660a). The p2660a priming site exists only in the endogenous 3 kb MZF-1 mRNA and not in the 2 kb mRNA transcribed from the pCEP4/MZF1 vector construct. Amplification under these conditions can be directed only from the reverse-transcribed endogenous 3 kb MZF-1 mRNA. In control experiments, there was no amplification without prior reverse transcription of mRNA, indicating that there was no carryover of pCEP4/MZF1 vector DNA. These results suggest that the vector-driven expression of MZF-1 stimulated the expression of the endogenous MZF-1 gene.

DISCUSSION

The inhibition of in vitro G-CSF-stimulated granulocyte colony formation by human bone marrow cells following exposure to MZF-1 antisense oligonucleotides provides strong evidence that appropriate expression of the myeloid zinc finger gene, MZF-1, is essential for the growth and differentiation of myeloid progenitor cells (Bavisotto et al., 1991). Furthermore, the restricted expression of MZF-1 mRNA to myeloid cells and the rise and fall of MZF-1 mRNA levels during myelopoiesis and cytokine-driven differentiation suggest that this gene is tightly regulated. In this study we have used diagnostic PCR analyses and direct genomic cloning to examine the organization of the human MZF-1 gene. Primer extension, sequence analysis, and CAT reporter studies identified the major transcription start site, potential regulatory elements, and the functionality of the MZF-1 promoter. Our initial approach to analyze the human MZF-1 gene structure utilized an efficient PCR-based method starting simply with nonionic detergent cell lysates. The authenticity of all amplified products was established by Southern blot analysis, single-stranded DNA amplification, and sequencing. These results indicated that the human MZF-1 gene is a single-copy gene spanning approximately 3 kb and containing no introns. These observations were subsequently confirmed by direct genomic cloning and sequencing of the human MZF-1 gene.

Since zinc finger proteins have been found to regulate hierarchical gene transcription during cellular differentiation and development (Pevny et al., 1991; Nguyen et al., 1993), the lack of introns in the MZF-1 gene is of interest. Introns appear to be a ubiquitous feature of most mammalian genes, but the importance of this genetic organization is not fully realized (Hawkins, 1988). One hypothesis proposes that intronless genes have arisen from so-called retroposition, in which an active RNA species has been reverse-transcribed into DNA copies and dispersed back into the genome (Brosius, 1991). It is proposed that genomic diversity has been accomplished in part by retroposition of full-length functional cDNAs and, moreover, that such retroposition may be a preferred route towards evolutionary selection. The lack of introns as well as the lack of a classically located TATA box in the MZF-1 gene may be consistent with such a retroposition. Other examples of intronless genes in higher eukaryotes include the genes for members of potassium channel family, several G-protein linked receptors, α-interferons, and the heat shock proteins (Pellegrini & Schindler, 1993). It has been proposed that the lack of introns in the heat shock genes may contribute to their selective transcription and translation in the cell during times of stress including, for example, during cell differentiation (Schlesinger, 1990). In this regard, it is interesting that not only does the MZF-1 gene lack intron but the upstream region (−674 bp) contains an imperfect inverted triple repeat (CGGGAGTTCGAGACC) of the heat shock element (nGAAn box) recognized by the trimeric heat shock transcription factor (Harrison et al., 1994). These features may enable the efficient induction and maintained expression of MZF-1 during myelopoiesis and granulocyte differentiation.

The major transcription start site of the MZF-1 gene in HL-60 cells was identified as a thymidine residue. A TATA box sequence (TAAAAA) is found at −66 bp and a CAAT box-like sequence (CCATT) at −126 bp, relative to this identified start site. Despite this unusual TATA box location, the identified MZF-1 transcription start is located within the sequence (TTCCCAT) that closely resembles the "initiator" sequence associated with transcription initiation of many eukaryotic genes including several leukocyte-specific transcription units (Smale & Baltimore, 1989; López-Cabrera et al., 1993). It is interesting that the promoters for several other myeloid differentiation−associated genes, including the human N-formyl peptide receptor gene, the murine G-CSF receptor gene, and the human genes encoding the leukocyte integrin α_L (CD11a), α_X (p150,95), β_2 (CD18), and β_3 subunits, also lack classical TATA sequences (Shelley & Arnaout, 1991; Agura et al., 1992; Pahl et al., 1992; Hickstein et al., 1992; Rosmarin et al., 1992; Haviland et al., 1993).

Upstream of the identified transcription start site are multiple consensus regulatory elements that provide a basis for understanding the observed transcriptional control of MZF-1 by hormones and cytokines. Consensus DNA binding site sequences can be identified for the retinoic acid receptor, retinoid X receptor, and for the interferon-γ-activated protein STAT1. Retinoic acid treatment of human leukemic HL-60 cells promotes granulocytic differentiation and increases the level of the MZF-1 transcript (Hromas et al., 1991; Hui & Bradford, 1993). The presence of four tandem repeated retinoic acid receptor half sites (AGGTCA) from -666 bp through -696 bp including the strongest identified enhancer arrangement, a direct head-to-tail repeat arrangement of the half-site with a 3 bp spacer, suggests that this DNA region may promote MZF-1 transcription in response to retinoic acid. Likewise, the consensus interferon-γ activated site (GAS), TT(C/A)CNNNAA (Pellegrini & Schindler, 1993; Kahn et al., 1993; Darnell et al., 1994), is found at -47 through -55 bp and again at -95 through -102 bp upstream of the MZF-1 transcription start site. Interferon regulatory factor-1, which functions as a transcriptional regulator of interferon-inducible genes, exhibits growth inhibitory and antioncogenic activity and has been recently mapped to chromosome 5q31.1, a site of interstitial deletion frequently associated with leukemia and preleukemic myelodysplasia (Willman et al., 1993). Also, treatment of human peripheral blood monocytes and basophils with multiple cytokines including IL-3, IL-5, IL-10, and GM-CSF results in the activation of DNA binding proteins distinct from STAT1 or 2 that recognize the GAS-containing interferon-γ response element within the FcγRI gene (Larner et al., 1993). MZF-1 mRNA levels are increased by GM-CSF and to a lesser extent by IL-3 in HL-60 cells (Hromas et al., 1991; Hui & Bradford, 1993), suggesting that this hormonal regulation may be mediated by the GAS in the upstream region of the MZF-1 gene.

Other identified consensus sequences upstream of the MZF-1 transcription start site include binding sites for AP-2 and PU.1. AP-2 is a site-specific DNA binding protein and transcription factor, and AP-2 mRNA expression is increased by retinoic acid during differentiation of F9 human teratocarcinoma cells (Imagawa et al., 1987; Luscher et al., 1989). At the site -88 bp in the MZF-1 upstream sequence is the sequence GAGGAA, the PU.1 protein recognition sequence. PU.1 is a transcriptional activator expressed in macrophages and B cells, and has been associated with the expression of several hematopoietic colony stimulating factors and interleukins (Klemsz et al., 1990). Future studies are required to determine whether AP-2 or PU.1 is involved in MZF-1 gene expression.

A significant aspect of the experimental results is that the DNA upstream from the MZF-1 gene, despite lacking typical TATA box and CAAT promoter elements, can effectively promote transcription from the CAT reporter gene when transfected into HL-60 cells. Despite the reported practical difficulties of transfecting HL-60 cells, we were successful in demonstrating functional promoter activity of the cloned MZF-1 upstream DNA by using the pCAT-basic vector system combined with electroporation. Success in this regard required a high density of starting cell numbers for the electroporation and seemed to coincide with maintaining τ, the decay constant of the applied electric field, in the range of 8−12 ms. The MZF-1 promoter is active in HL-60 cells, a cell line that normally expresses MZF-1, but less active in K562 cells, another myeloid cell line but one that expresses little MZF-1. It is also significant that expression from the pCAT/MZF reporter was increased in HL-60 cells following treatment with either retinoic acid or GM-CSF. These observations are consistent with the increased expression of MZF-1 mRNA in HL-60 cells treated with retinoic acid or GM-CSF (Hromas et al., 1991; Hui & Bradford, 1993). Additional studies of the MZF-1 promoter will be required to determine those genetic elements conferring responsiveness to these hormones.

Of added significance is the observation that the ectopic expression of MZF-1 in K562 cells drives the expression of the endogenous MZF-1 gene. There is low basal MZF-1 expression in K562 cells, virtually undetectable under our conditions of Northern and PCR analyses. However, coincident with MZF-1 expression from the pCEP4/MZF vector, a 3 kb MZF-1 mRNA became detectable by Northern analysis. This 3 kb transcript was shown to be the endogenous MZF-1 gene product both by its size and by its ability to be amplified and sequenced using a PCR primer site (p2660) present only in the endogenous message. These results suggest the possibility for autoregulation, and support for this comes from a comparison of the consensus DNA binding sites for the MZF-1 zinc fingers (Morris et al., 1994) with the sequence of the cloned MZF-1 promoter determined in the present study. Consensus binding sites for each of the zinc finger domains of MZF-1 were determined by sequential gel shift enrichment (Morris et al., 1994). Near perfect matches (9 of 14 bp and 11 of 11 bp) of these consensus DNA binding sites are found in the MZF-1 gene promoter at -249 bp (TCCACCCTGTTCCA) and at -160 bp (AGAACCCCACT) (Figure 7). Without a determination of MZF-1 protein levels, it is impossible at this time to determine whether the observed autoregulation of MZF-1 transcription occurs at physiologically relevant levels of MZF-1. The myeloblastic K562 cells expressing the ectopic MZF-1 failed to undergo any discernible cell differentiation, as if cellular factors in addition to MZF-1 were required for differentiation. It is interesting that we failed to obtain stable cultures of MZF-1−transfected HL-60 cells; within several days of electroporation, these cells invariably underwent terminal differentiation, consistent with the upregulation of MZF-1 that occurs following HL-60 cell treatment with the granulocytic inducer retinoic acid.

In conclusion, these studies indicate that the human MZF-1 gene contains no introns and that the DNA upstream of the identified transcription start site contains elements with functional promoter activity. The promoter contains physiologically relevant regulatory sequences including consensus retinoic acid and GM-CSF response elements, consistent with the hormonal regulation of stage-specific MZF-1 expression in myeloid cells. The observation that MZF-1 may regulate its own transcription suggests that there are complex signal transduction mechanisms converging at this essential gene. More precise characterization of the critical regulatory elements in the MZF-1 gene including the identification of potential trans-acting factors should provide considerable insight into the molecular control of myelopoiesis.

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