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Overexpression of the Zinc Finger Protein MZF1 Inhibits Hematopoietic Development from Embryonic Stem Cells: Correlation with Negative Regulation of *CD34* and c-*myb* Promoter Activity

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Zinc finger genes encode proteins that act as transcription factors. The myeloid zinc finger 1 (*MZF1*) gene encodes a zinc finger protein with two DNA-binding domains that recognize two distinct consensus sequences, is preferentially expressed in hematopoietic cells, and may be involved in the transcriptional regulation of hematopoiesis-specific genes. Reverse transcription-PCR analysis of human peripheral blood CD34$^+$ cells cultured under lineage-restricted conditions demonstrated *MZF1* expression during both myeloid and erythroid differentiation. Sequence analysis of the 5′-flanking region of the *CD34* and c-*myb* genes, which are a marker of and a transcriptional factor required for hematopoietic proliferation and differentiation, respectively, revealed closely spaced *MZF1* consensus binding sites found by electrophoretic mobility shift assays to interact with recombinant MZF1 protein. Transient or constitutive *MZF1* expression in different cell types resulted in specific inhibition of chloramphenicol acetyltransferase activity driven by the *CD34* or c-*myb* 5′-flanking region. To determine whether transcriptional modulation by MZF1 activity plays a role in hematopoietic differentiation, constructs containing the *MZF1* cDNA under the control of different promoters were transfected into murine embryonic stem cells which, under defined in vitro culture conditions, generate colonies of multiple hematopoietic lineages. Constitutive *MZF1* expression interfered with the ability of embryonic stem cells to undergo hematopoietic commitment and erythromyeloid colony formation and prevented the induced expression of *CD34* and c-*myb* mRNAs during differentiation of these cells. These data indicate that *MZF1* plays a critical role in hematopoiesis by modulating the expression of genes involved in this process.

The process of blood cell formation rests on the ability of a limited number of hematopoietic stem cells to undergo self-renewal or commitment into lineage-restricted progenitor cells. The mechanism(s) underlying these processes remains largely unknown, but the differentiation of committed progenitors is controlled by lineage-specific transcription factors that regulate, in a stage-specific manner, the appearance of markers that define the properties of morphologically recognizable blood cells.

The family of zinc finger genes represents a class of DNA-binding proteins, many of which regulate transcription events during developmental processes. Zinc finger genes encode proteins with a highly conserved domain of 28 to 30 amino acids that tetrahedrally binds a zinc molecule through two cysteines and two histidines; this motif, the zinc finger domain, interacts with specific nucleotides along the DNA major groove (7). Growing evidence indicates that zinc finger proteins regulate hematopoiesis, perhaps affecting directly the expression of hematopoietic cell-specific genes. For example, a functional *GATA-1* gene is essential for erythroid development in chimeric mice and in vitro (45, 64), and GATA-2-deficient mice have a marked defect in hematopoietic development (59). Constitutive expression of the *evi-1* zinc finger gene blocks the ability of 32D myeloid precursor cells to express myeloperoxidase and to terminally differentiate into granulocytes in response to granulocyte colony-stimulating factor (G-CSF) (37) and inhibits the capacity of erythroid marrow progenitors to respond to erythropoietin (EPO) by interfering with the GATA-1-regulated transcription of target genes (30). The zinc finger early growth response 1 gene (*Egr-1*) encodes a protein, expressed upon monocyte-macrophage differentiation, essential for differentiation of myeloblasts along the macrophage lineage (41). Other members of the zinc finger gene family, such as *AML1* (36), *ALL-1* (47), *ZnF23* (13), *ZnF32* (13), *HTRX1* (44), *PLZF* (15), and *bcl-5* (35), map to chromosome regions frequently involved in deletions and translocations in patients with hematopoietic malignancies.

The myeloid zinc finger *MZF1* gene was isolated from a cDNA library prepared from peripheral blood leukocytes of a patient with chronic myeloid leukemia (27) with an oligodeoxynucleotide encoding a 7-amino-acid linker (H-C links) present between two different zinc finger motifs as a probe. It encodes a 485-amino-acid protein that contains 13 zinc fingers, forming two different DNA-binding domains (zinc fingers 1 to 4 and 5 to 13) that interact with two different consensus sequences (38). This structure resembles that of the *Drosophila* Kruppel genes, characterized by multiple zinc fingers containing the conserved amino acid motif $CX_2CX_3FX_5LX_2HX_3H$ separated by highly conserved H-C links of the form TGEKPYX (17, 50). *MZF1* is expressed in cell lines representing early stages of myeloid differentiation, such as KG-1, HEL, and K562. In addition, *MZF1* mRNA levels were found to increase during granulocytic differentiation of HL60 cells induced by treatment with retinoic acid, dimethyl sulfoxide, or granulocyte-macrophage colony-stimulating factor (GM-CSF) (27). These observations suggest that *MZF1* might be involved in the early stages of hematopoiesis as well as in the induction of terminal differentiation. In support of a role of

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MZF1 during granulocytic differentiation, Bavisotto et al. (3) have shown that treatment of bone marrow cells with *MZF1* antisense oligodeoxynucleotides inhibits G-CSF-induced granulocytic colony formation. Genes preferentially expressed at early stages of hematopoietic differentiation, like *CD34* and c-*myb*, are attractive targets of *MZF1* regulation. The *CD34* gene encodes a surface glycoprotein that defines a subset of hematopoietic stem and progenitor cells able to reconstitute hematopoiesis in irradiated primates and marrow-ablated humans (6, 51). *CD34* may be involved in the interaction between hematopoietic and bone marrow stromal cells, perhaps serving as a ligand for stromal lectins (2, 51). The regulation of *CD34* expression in hematopoietic cells involves both transcriptional and posttranscriptional mechanisms (24, 34, 49). *CD34* is positively regulated by myb via binding to *myb* binding sites in the 5′-flanking region of the *CD34* gene (34).

The proto-oncogene c-*myb* is expressed in immature hematopoietic cells (14, 21) and plays a major role in controlling their in vivo and in vitro differentiation program. Constitutive expression of a full-length c-*myb* cDNA blocks murine erythroleukemia (MEL) differentiation induced by dimethyl sulfoxide (18, 33) or by erythropoietin (58). c-*myb* antisense oligodeoxynucleotides inhibit hematopoiesis and proliferation of myeloid leukemia cells (12, 23, 61), and mice homozygous for the inactivated c-*myb* gene show impaired hematopoiesis (39). As a transcriptional regulator (65), c-myb is able to transactivate hematopoiesis-specific genes (34, 40, 52).

We report here that the MZF1 protein negatively regulates *CD34* and c-*myb* promoter activity in hematopoietic and nonhematopoietic cells upon binding to the *MZF1* binding sites present in the 5′-flanking region of both genes. *MZF1* overexpression in undifferentiated ES cells inhibits the expression of *CD34* and c-*myb* mRNAs and markedly reduces the ability of these cells to undergo hematopoietic commitment and to form in vitro erythromyeloid colonies upon removal of leukemia inhibitory factor (LIF). These findings suggest that *MZF1* acts as a silencer of hematopoiesis-specific gene expression and might be necessary for a normal differentiation program involving a balance between positive and negative regulatory signals.

MATERIALS AND METHODS

Cell cultures and primary cells. TK^-ts13 Syrian hamster fibroblasts (kind gift of R. Baserga) are thymidine kinase-deficient cells derived from ts13 cells (57) and were maintained at 34°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 2 mM glutamine (GIBCO). The KG-1 myeloblastic leukemia cell line (American Type Culture Collection, Rockville, Md.) was grown at 37°C in RPMI 1640 medium supplemented with 10% FCS and 2 mM L-glutamine (GIBCO). ES-D3 cells have been described before (19). Undifferentiated ES cells were maintained in gelatinized tissue culture dishes in DMEM supplemented with 15% heat-inactivated FCS, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, and 1,500 U of recombinant LIF (Genetics Institute, Inc., Cambridge, Mass.). Human hematopoietic progenitor cells were purified from the peripheral blood of normal donors by a four-step procedure (55) and grown at 10^4 cells per ml in fetal bovine serum-free Iscove's modified Dulbecco's medium (IMDM) supplemented with bovine serum albumin (10 mg/ml), human transferrin (1 mg/ml), human low-density lipoprotein (40 μg/ml), insulin (10 μg/ml), sodium pyruvate (0.1 mM), L-glutamine (2 mM), ferrous sulfate (40 nM), nucleosides (each at 10 μg/ml), and recombinant human growth factors (for the granulocyte-macrophage differentiation culture, high dose of 100 U of interleukin-3 [IL-3] per ml and GM-CSF [10 ng/ml]; for the erythroid differentiation culture, low dose of IL-3 [0.01 U/ml] and GM-CSF [0.001 ng/ml] and standard amounts of EPO [3 U/ml]). Cultures were incubated in a humidified 5% CO_2 atmosphere at 37°C and periodically counted.

Plasmid and vector construction. Plasmid Zn1.8 (27), containing the full-length *MZF1* cDNA (nucleotides 993 to 2713 of the published sequence) cloned in the *Eco*RI site of pBlueScript SK^+ (Stratagene), was a kind gift of R. Hromas (Indiana University Medical Center, Indianapolis, Ind.). The *MZF1* cDNA was subcloned into the following vectors.

(i) pCMVMZF1. Plasmid Zn1.8 was digested with *Xba*I, Klenow treated, and digested again with *Xho*I. The released full-length *MZF1* cDNA was subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen) previously digested with *Eco*RV and *Xho*I.

(ii) pGEXMZF1. Because of a stop codon (TGA) in the *MZF1* cDNA before the ATG initiation codon, subcloning of full-length *MZF1* into the prokaryotic expression vector pGEX-2TK (Pharmacia) was done in two steps. A PCR fragment was amplified from Zn1.8 with a 22-mer (MZF1a: nucleotides 1090 to 1111 of the human *MZF1* cDNA published sequences) carrying the A→C mutation at position 1090 as the 5′ primer and a 19-mer (MZF1b: nucleotides 1312 to 1330) containing a *Bam*HI site as the downstream primer. The PCR mixture contained 100 pg of Zn1.8 plasmid, previously denatured at 100°C for 5 min, 300 ng of the 5′ and 3′ primers, 200 mM each deoxynucleoside triphosphate (dNTP), 1× *Taq* polymerase buffer supplemented with Mg^{2+} (Boehringer Mannheim), and 0.5 U of *Taq* polymerase (Boehringer Mannheim). PCR amplification was performed under standard conditions: 35 cycles of 30 s of denaturation at 94°C, 45 s of primer annealing at 54°C, and 45 s of elongation at 72°C. A 240-bp PCR product was phosphorylated, digested with *Bam*HI restriction endonuclease (Boehringer Mannheim), and purified. Plasmid Zn1.8 was also digested with *Bam*HI and *Eco*RI, and the 1.4-kb fragment was purified on an agarose gel. These two fragments were then subcloned into vector pGEX-2TK previously digested with *Bam*HI, blunted with Klenow fragment for 30 min at room temperature, and then digested with *Eco*RI restriction endonuclease.

(iii) pPolMZF1. The fragment containing *MZF1* cDNA was purified from plasmid pCMVMZF1 after partial digestion (20 min at 37°C) with *Eco*RI and *Xba*I and then subcloned into a eukaryotic expression vector containing the RNA polymerase II promoter (pPolIIshortneobpA; kind gift of W. Stratford May, Johns Hopkins University, Baltimore, Md.) previously digested with *Eco*RI and *Xba*I to remove the *neo* gene.

(iv) pXTMZF1neo. Plasmid Zn1.8 was digested with *Eco*RI restriction endonuclease and blunt ended. The full-length *MZF1* cDNA was then subcloned into the *Xho*I site, previously treated with Klenow fragment for 30 min at room temperature in the presence of an excess of dNTPs, of the eukaryotic expression vector pXT1neo (Stratagene, La Jolla, Calif.).

(v) pCMVHAMZF1. Hemagglutinin (HA)-tagged *MZF1* was prepared as follows. (i) A 159-bp PCR fragment encoding a triple HA epitope was amplified from plasmid pSKDR-nm23HA3 (62) with a 22-mer containing an *Hin*dIII restriction site at the 5′ end as the upstream primer and a 20-mer containing a blunted *Sal*I restriction site as the downstream primer. (ii) After phosphorylation and *Hin*dIII restriction endonuclease digestion, this HA fragment was subcloned into the *Hin*dIII-*Eco*RI-digested pcDNA3 vector (Invitrogen) after ligation to a 241-bp *Bam*HI-digested PCR fragment corresponding to nucleotides 1090 to 1330 of the *MZF1* cDNA and the contiguous 3′ 1.4-kb fragment obtained by digestion of Zn1.8 plasmid with *Bam*HI and *Eco*RI restriction endonucleases.

(vi) CD34CAT (pBLCD34CAT). A fragment of the *CD34* promoter region (nucleotides −666 to +234) (10, 24) was prepared by PCR amplification of human placenta genomic DNA and by cloning the amplified segment into the PCRII vector (Invitrogen Corp., San Diego, Calif.). This segment was subcloned in pBlueScript SK^+ digested with *Hin*dIII and *Bam*HI restriction enzymes and then subcloned into the *Hin*dIII and *Bam*HI sites of the chloramphenicol acetyltransferase (CAT) vector pBLCAT3 (30).

(vii) Myb-CAT (B1CAT). A plasmid containing nucleotides −687 to +204 of the c-*myb* gene linked to the CAT gene was constructed by ligating a blunt-ended 890-bp *Bam*HI-*Nco*I fragment from plasmid pE-3 (42) into a blunt-ended *Sal*I site in the polylinker of the pUCCAT vector (Promega, Madison, Wis.) in the sense orientation to the CAT gene.

(viii) ΔMybCAT. A plasmid containing nucleotides −47 to +204 of the c-*myb* gene linked to the CAT cDNA was constructed by ligating a blunt-ended fragment generated by PCR from plasmid P1CAT (43) into the vector pCATbasic (Promega) predigested with *Hin*dIII restriction enzyme and then blunt ended.

Constructs were automatically sequenced with the *Taq* Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems).

Recombinant protein purification, nuclear protein extracts, EMSA, and UV cross-linking. *Escherichia coli* DH5α cells were transformed with plasmid pGEX-2TK or plasmid pGEXMZF1, and colonies were screened for the production of the MZF1–glutathione-*S*-transferase (GST) fusion protein by induction of a logarithmically growing culture with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 2 h. Bacteria expressing the MZF1-GST fusion protein were washed and resuspended in phosphate-buffered saline (PBS) with 10 mM $ZnSO_4$ in the presence of protease inhibitors (1 mM phenylmethylsulfonyl fluoride [PMSF], 2 μg of aprotinin per ml, 2 μg of leupeptin per ml, and 1 μg of pepstatin A per ml) and sonicated. The supernatant (20 μl) was analyzed by sodium dodecyl sulfate–8% polyacrylamide gel electrophoresis (SDS–8% PAGE) and stained with Coomassie blue to determine the presence of the recombinant protein. Five microliters (10 μg) of the supernatant was used for electrophoretic mobility shift assays (EMSAs). KG-1-, ESneo-, and ES-MZF1-transfected cells were collected, washed twice in PBS, and then resuspended in 1.5 volumes of a buffer containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9), 1 mM EDTA, 60 mM KCl, and 1 mM dithiothreitol (DTT) in the presence of protease inhibitors. Deionized Nonidet P-40 was added to the cell suspension at a final concentration of 0.25% (vol/vol), and cells were disrupted with a Dounce homogenizer (B-type pestle). The nuclear pellet was resuspended in 1.5 volumes of 20 mM HEPES (pH 7.9)–0.4 M NaCl–25% glycerol–1 mM EDTA in the presence of protease inhibitors, and

nuclear proteins were obtained by three rounds of freezing in dry ice-ethanol and thawing at 37°C; lysates were clarified, and the amount of protein was quantitated by the Bradford assay (Bio-Rad). Fifteen micrograms of nuclear extracts was used for EMSAs. Lysates (bacterial and nuclear extracts) in binding buffer (25 mM HEPES-KOH [pH 7.5], 50 mM KCl, 10 μM $ZnSO_4$, 10% glycerol, 0.1% Nonidet P-40, 1 mM DTT) were incubated with 0.12 μg of poly(dI-dC) per μl for 10 min on ice. γ-^{32}P-end-labeled double-stranded oligonucleotide probes (5×10^4 cpm) containing either an *MZF1* binding site on the *CD34* promoter region from nucleotides −583 to −559 (CD34MZ1: 5′-ccagggCTGGAGAGGGGAT A aactgg-3′) or from nucleotides −524 to −499 (CD34MZ2: 5′-gatgat GGTGAT GGGGAActaaatgg) (10, 24) or an *MZF1* binding site on the c-*myb* promoter region (42) corresponding to nucleotides −71 to −45 (MybMZ1: agatttgGCGG GAGGGGGAGtgtcca) or to nucleotides −185 to −154 (MybMZ2: agggcac CGCGGGGGAGCCccgaatgg) were added to the binding reaction mixes and incubated for 15 min at room temperature. When indicated, EMSAs were performed in the presence of a 100-fold molar excess of 25-bp double-stranded DNA fragments containing the *MZF1* DNA consensus sequences (38) for fingers 1 to 4 (AGTGGGGAnGT) or 5 to 13 (cgGGNGAGGGGGAA) or a heterologous 21-bp DNA fragment (5′-gccaatttacctgtctaaccg-3′) used as a nonspecific competitor (38). Binding reaction mixes were electrophoresed in native 5% PAGE gels at low ionic stringency (0.25× Tris-borate-EDTA). Gels were dried and exposed to X-ray films for autoradiography. Bacterial lysates containing GST protein alone were used as a control in the EMSA. To cross-link proteins to DNA, ice-cold binding mixtures, prepared as described above, were exposed to UV irradiation (2.5×10^5 μJ) with a Stratalinker (Stratagene). UV cross-linked complexes were fractionated by SDS–8.5% PAGE. Gels were dried and exposed to X-ray films for autoradiography.

Western (immunoblotting) analysis. Equal numbers (2.5×10^6) of cells (ES CMVneo and ESCMVHAMZF1) were harvested, washed twice with ice-cold PBS, and lysed in 75 μl of HEPES buffer (10 mM HEPES [pH 7.5], 150 mM NaCl, 10% [vol/vol] glycerol, 1 mM EDTA, 1 mM DTT) containing 0.5% (vol/vol) Nonidet P-40 in the presence of protease inhibitors at the indicated concentrations (1 mM PMSF, 10 μg of leupeptin per ml, 25 μg of aprotinin per ml, 100 μg of pepstatin per ml, 1 mM benzamidine). After 20 min on ice, total lysates were frozen in dry ice, thawed at 37°C, and clarified. The lysates in 1× Laemmli buffer were fractionated by SDS–8.5% PAGE and transferred onto a nitrocellulose membrane (Schleicher & Schuell). Membrane blocking was performed at room temperature for 2 h in PBS containing 5% dry milk and 0.1% Nonidet P-40. The incubation with the anti-HA antibody (clone 12CA5; Boehringer Mannheim), used at a concentration of 10 μg/ml in PBS–1% dry milk, was carried out at room temperature for 2 h. After being washed four times for 10 min each with PBS–0.1% Tween 20, the membrane was incubated for 2 h with sheep anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Amersham) and washed again as described above. Bound proteins were detected by using chemiluminescent substrates according to the manufacturer's instructions (Amersham).

CAT assays. TK^-ts13 hamster fibroblasts were plated at a concentration of 10^6 cells per plate, grown for 16 to 18 h, and transfected by calcium phosphate precipitation (28) with 3 μg of the reporter plasmids (CD34CAT or Myb-CAT and the deletion constructs S-CD34CAT or ΔMybCAT) with or without 15 μg of effector plasmid (pPolMZF1) containing the human *MZF1* cDNA driven by the RNA polymerase II promoter. The rescue from *MZF1*-induced inhibition of CAT activity was assessed by adding to the cotransfection mixture the specific competitors Zn1-4 and Zn5-13, described above, each in a 100-fold molar excess. The vector pPol2neoshortbpA was used as a control. KG-1 cells were split to 10^5/ml and transiently transfected 24 h later as described before (26). Briefly, the day after plating, cells were washed twice with RPMI 1640 containing neither FCS nor L-glutamine, resuspended in the same medium (1.4×10^7 cells per 500 μl), and electroporated (Gene Pulser [BioRad]; 960 μF, 300 V) with 5 μg of reporter plasmid (CD34CAT or Myb-CAT) and 20 μg of effector plasmid (pC MVMZF1). After electroporation, cells were incubated for 5 min on ice and then transferred to 10 ml of RPMI 1640 containing 10% FCS plus 2 mM L-glutamine. The vector pcDNA3 (Invitrogen) was used as a control. Derivative ES cells (ESPolneo and ESpolMZF1) and the parental cell line ES-D3 were transiently transfected by electroporation (see below) with 15 μg of the reporter construct CD34CAT or Myb-CAT. Two micrograms of plasmid containing the bacterial β-galactosidase gene driven by the simian virus 40 early promoter was used as an internal control for transfection efficiency. Cells were harvested 48 h after transfection. Proteins were extracted in hypotonic buffer by freeze-thawing and normalized for transfection efficiency by the β-galactosidase assay, as suggested by the manufacturer (Promega). Cellular lysates were incubated with [^{14}C]chloramphenicol and acetyl coenzyme A (90 min) at 37°C. CAT levels were assayed by thin-layer chromatography followed by autoradiography, and the percentage of acetylated [^{14}C]chloramphenicol was determined by scintillation counting.

RNA isolation, cDNA synthesis, and PCR analysis. Total RNA was extracted as described before (16) with some modifications (30 μg of tRNA was added as a carrier to lysed cells). The first-strand cDNA reaction was performed with 10% of the extracted RNA (10 to 30 ng), 200 U of Moloney murine leukemia virus (MMLV) reverse transcriptase (RT) (GIBCO-BRL), 200 μM each dNTP, 15 U of RNasin (Promega), and 5×10^{-3} U of random hexamers (Pharmacia) in a 20-μl reaction mix containing 10 mM DTT, 50 mM Tris (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$. The RT reaction was carried out at 37°C for 90 min. Aliquots of the same first-strand preparation were used to analyze the expression of each gene. As an internal control, all cDNA samples were adjusted to yield relatively equal amplifications of either β-actin or $β_2$-microglobulin. Control PCRs were performed without template, and duplicates of each reaction were performed without RT. PCR amplifications (48) were performed with 20% of the first-strand reaction product previously denatured at 100°C for 5 min, 300 ng of the 5′ and 3′ primers (19- and 22-mer), 200 mM each dNTP, 1× *Taq* polymerase buffer supplemented with Mg^{2+} (Boehringer Mannheim), and 0.5 U of *Taq* polymerase (Boehringer Mannheim), in a reaction volume of 50 μl.

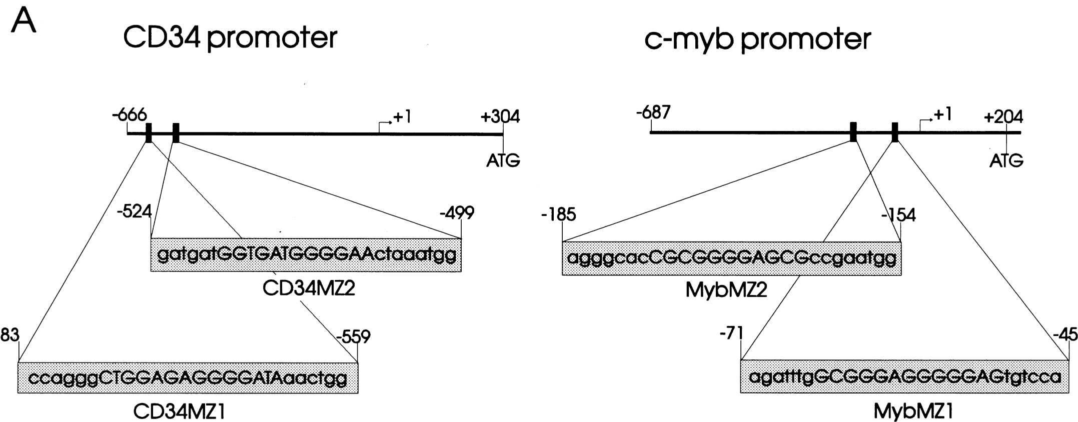
PCR amplification was performed under standard conditions at two different cycle numbers (25 and 50) by removing one-half of the reaction volume at the appropriate times during the amplification. PCRs were carried out in a Perkin Elmer-Cetus thermocycler for 25 to 50 cycles (30 s at 94°C, 45 s at 54 to 60°C [depending on the melting temperature of the primers], and 45 s at 72°C). The specificity of the PCR products was established by comparing the sizes of the amplified products with the expected cDNA bands and by hybridization with internal oligodeoxynucleotides unrelated to the PCR primers and specific for the respective amplification products. Amplified DNA was subjected to electrophoresis on 2.5% Seakem agarose gel (FMC Bioproducts), transferred with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) onto a Hybond-N nylon membrane (Amersham), fixed with a UV Stratalinker (Stratagene), and detected by Southern hybridization with [γ-^{32}P]ATP-end-labeled oligoprobes specific for the amplified sequences.

***MZF1*, *CD34*, and c-*myb* mRNA expression during in vitro erythroid and myeloid differentiation of peripheral blood $CD34^+$ cells.** RT-PCR was performed, as previously described, on RNA extracted from undifferentiated $CD34^+$ cells (day 0) and cells induced to differentiate for the indicated times in the presence of IL-3 and GM-CSF (low dose)/Epo (high dose) (erythroid differentiation) or high doses of IL-3 and GM-CSF (myeloid differentiation) (see under "Cell cultures and primary cells," above). The human recombinant factors used were purchased from Stem Cell Technologies Inc. (Vancouver, Canada) or kindly provided by the Genetics Institute (Cambridge, Mass.). Synthetic oligodeoxynucleotides specific for the human sequences of *MZF1* (27), *CD34* (24) and c-*myb* (32) cDNA were used as primers for PCR amplification. These were MZF1a (nucleotides 1090 to 1111) and MZF1b (nucleotides 1312 to 1330), hCD34a (nucleotides 973 to 992) and hCD34b (nucleotides 1177 to 1198), and myb7 (nucleotides 2558 to 2279) and myb8 (nucleotides 2466 to 2487). The oligoprobes were MZF1d (nucleotides 1204 to 1242), hCD34p (nucleotides 1109 to 1134), and myb9 (nucleotides 2351 to 2400). $β_2$-Microglobulin mRNA levels were evaluated to assess the amount of RNA in each sample. Primers for $β_2$-microglobulin amplification were nucleotides 280 to 301 (upstream primer) and nucleotides 510 to 531 (downstream primer) of the $β_2$-microglobulin cDNA sequence (56); the 50-mer oligodeoxynucleotide used as the probe for $β_2$-microglobulin corresponds to nucleotides 351 to 400 of the same cDNA sequence.

Transfection of ES cells, in vitro differentiation assay for hematopoietic progenitors within EB, and expression of hematopoietic markers in ES-derived colonies. (i) Transfection. ES-D3 cells (3×10^6 cells) were resuspended in 400 μl of DMEM, electroporated with a Gene Pulser (340 V, 250 μF) either with 20 μg of pPolMZF1 plus 2 μg of pPolIIneo or with 20 μg of pXTMZF1neo or pCMVHAMZF1, and, 24 h later, transferred to selective medium containing 1 mg of G418 per ml. After a 2-week selection, *MZF1* integration and expression were analyzed in several clones and in the mixed populations by PCR and RT-PCR, respectively, under the conditions described above. The upstream primers used to assess *MZF1* cDNA integration were derived from the promoter sequences in the expression constructs pPolIIMZF1 and pXTMZF1neo. Controls were ES cells transfected with the vector pPolIIneo, pXT1neo, or pCDNA3.

(ii) Cell cultures and detection of endogenous CD34 and c-myb in differentiating ES cells. Differentiation of parental ES cells and the indicated ES cell lines (ESPOLNEO, ESPOLMZF1, ESXTNEO, ESXTMZF1, ESCMVNEO, and ES CMVHAMZF1) derived from transfection to embryoid bodies (EB) was carried out in suspension culture. Briefly, undifferentiated cells (5×10^4/ml), after trypsinization, were plated in 100-mm bacterial petri dishes (Fischer) in 10 ml of DMEM supplemented with 15% heat-inactivated FCS and 2 mM glutamine in the absence of LIF. Fresh medium (3 ml) was added every 3 days to the EB cultures. Cultures were examined for the presence of EB with a light microscope. At day 0 and after 4, 7, 10, and 14 days in differentiation culture, EB were harvested and total RNA was extracted as described before (16). *CD34* and c-*myb* mRNA expression was analyzed by RT-PCR. The primers specific for the murine c-*myb* (4) and the murine *CD34* (9) sequences were used as described before (29, 34). β-Actin mRNA levels were measured to assess the amount of RNA in each sample; the upstream primer corresponds to nucleotides 224 to 244, the downstream primer corresponds to nucleotides 411 to 433, and the probe used to detect the amplification product corresponds to nucleotides 258 to 296 of the β-actin cDNA (59). RT-PCR to detect human *MZF1* was performed as an internal control.

(iii) Semisolid cultures and expression of hematopoietic markers in in vitro-differentiated ES colonies. At various times during differentiation, assays to develop hematopoietic colonies from differentiated ES cells in methylcellulose were carried out as described before (29). In particular, EB were disrupted by trypsinization, and 10^4 cells, plated in 35-mm-diameter bacterial grade dishes, were cultured in 0.9% methylcellulose (Methocult H4100; Stem Cell Technologies) in IMDM with 15% FCS, 10^{-4} M β-mercaptoethanol, 1% bovine serum



B

MZF1 DNA Consensus Sequences

rZN 1-4: 5′ A G T G G G G A n g t 3′

(%): 40 55 45 100 90 95 100 90 50 40

rZN 5-13: 5′ c g G G n G A G G G G G A A 3′

(%): 44 44 44 50 63 81 75 94 88 81 44 69 63

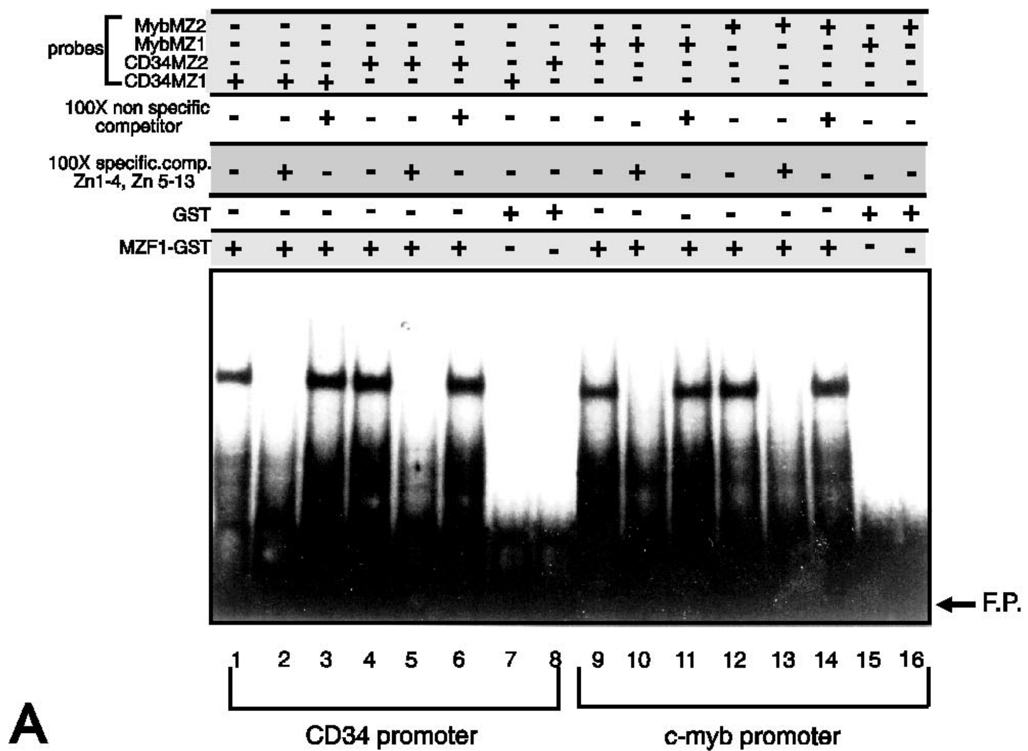
albumin, and 2 mM L-glutamine in the presence of the following growth factors: EPO (3 U/ml), Kit ligand (KL) (250 ng/ml), IL-3 (2% of the conditioned medium from WEH1-3B cells), IL-1α (1,000 U/ml), and GM-CSF (20 ng/ml). Colonies of >125 μm were scored 15 days later. Individual colonies were randomly aspirated (18 single colonies per dish), and RNA was extracted and used for single-colony RT-PCR as described before (54). Endogenous β-actin mRNA levels were also measured to ensure that similar amounts of RNA were used for mRNA expression analysis. Exogenous *MZF1* mRNA was measured as a positive control. RT-PCR analysis was performed to study the expression of (i) the murine embryonic β-globin (βH1) (8), using synthetic primers as described before (29), (ii) the murine myeloperoxidase (63), using primers corresponding to nucleotides 824 to 847 (5′ primer) and 1476 to 1500 (3′ primer) of the cDNA, and (iii) the murine c-*fms*, using specific primers as described before (46).

RESULTS

MZF1 interacts with the 5′-flanking region of the human *CD34* and c-*myb* genes. Two distinct DNA consensus sequences with a common G-rich core recognized by fingers 1 to 4 and 5 to 13 of the MZF1 protein (38) are present in the 5′-flanking region of the *CD34* and c-*myb* genes (Fig. 1). An EMSA with a bacterially produced MZF1 fusion protein and γ-^{32}P-end-labeled synthetic DNA fragments corresponding to the putative binding sites for zinc fingers 1 to 4 and 5 to 13 was performed to determine whether MZF1 protein interacts with these putative binding sites. The double-stranded DNA fragments containing the putative binding sites for fingers 1 to 4 correspond to nucleotides −524 to −499 (CD34MZ2) of the *CD34* 5′-flanking region (10, 24) and to nucleotides −185 to −154 (MybMZ2) of the c-*myb* promoter (42); the binding sites for fingers 5 to 13 correspond to nucleotides −583 to −559 (CD34MZ1) and −71 to −45 (MybMZ1) of the *CD34* and the c-*myb* promoters, respectively.

One retarded complex was detected after incubation of the probes with bacterial lysates containing the full-length human MZF1-GST fusion protein (Fig. 2A, lanes 1, 4, 9, and 12) but not with those containing the GST protein only (Fig. 2A, lanes 7, 8, 15, and 16). Binding specificity of MZF1 to these fragments was demonstrated by using a 100-fold molar excess of the DNA consensus sequence shown to interact with the recombinant zinc finger 1 to 4 (rZn1-4) or the rZn5-13 domain (38) as a specific competitor (Fig. 2A, lanes 2, 5, 10, and 13). The shifted complex was not affected by addition of a 100-fold molar excess of a heterologous double-stranded oligodeoxynucleotide to the binding reaction mixture (Fig. 2A, lanes 3, 6, 11, and 14). An additional putative MZF1 binding site for zinc fingers 1 to 4 (Zn1-4), present in the *CD34* 5′-flanking region (from nucleotide −146 to nucleotide −130), was also able to interact with recombinant MZF1 protein (not shown).

Experiments with nuclear extracts from *MZF1*-expressing KG-1 cells gave superimposable results (Fig. 2B). A retarded complex was detected after incubation of the KG-1 nuclear extract with oligomers containing MZF1 binding sites present in either the *CD34* or the c-*myb* 5′-flanking region (Fig. 2B, lanes 10, 12, 14, and 16). This complex comigrated with that resulting from the interaction of KG-1 nuclear extracts with the specific *MZF1* DNA consensus sequence Zn1-4 or Zn5-13 (lanes 1 and 5) (38) and was inhibited by adding to the binding reaction mix a 100-fold molar excess of oligomers including the Zn1-4 or Zn5-13 binding sites (lanes 11, 13, 15, and 17). The comigration of the KG-1 gel shift complex and the GST-MZF1 shift (Fig. 2B, lane 4) is probably due to substantial protein cleavage noted in the bacterial lysate, so that the full-length



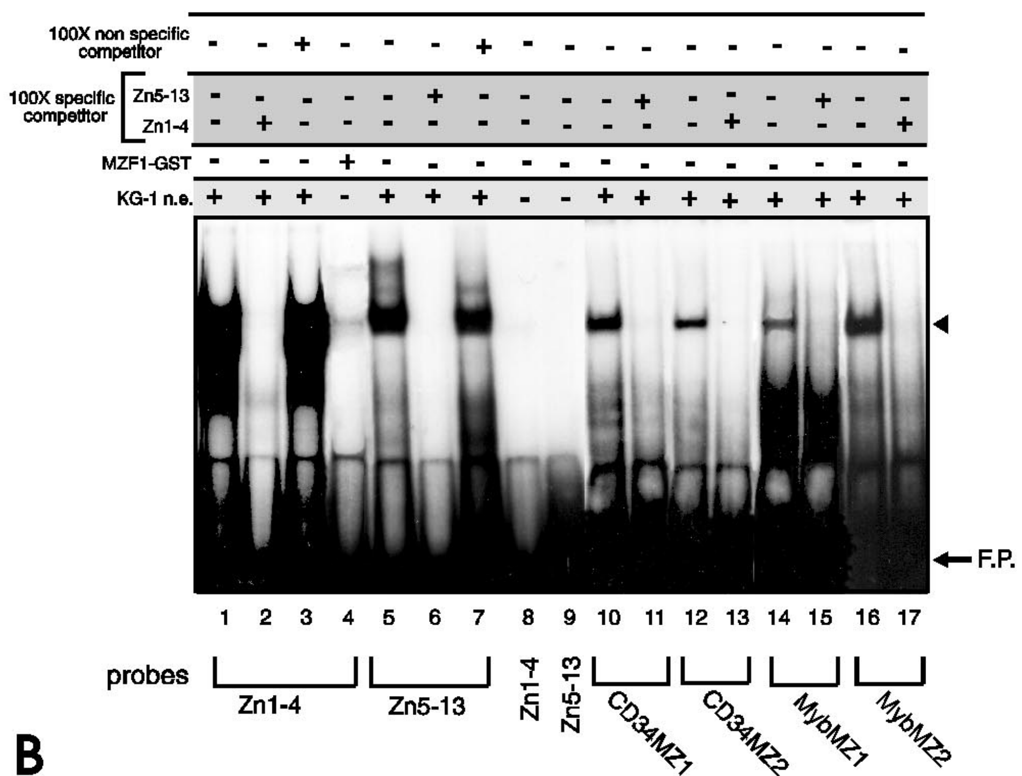


FIG. 2. MZF1 protein binding to *CD34* and c-*myb* 5′-flanking regions. (A) EMSAs were performed with bacterially synthesized MZF1 protein and γ-^{32}P-end-labeled double-stranded oligonucleotide probes as described in Materials and Methods. The specific probes and the presence in the reaction mixture of the specific or nonspecific competitor are indicated above each lane. (B) EMSAs were performed with KG-1 nuclear extracts (n.e.) (15 μg) and γ-^{32}P-end-labeled double-stranded oligonucleotide probes corresponding to the canonical binding sites of fingers 1 to 4 or 5 to 13 of MZF1 (38) or the putative binding sites in the *CD34* or c-*myb* promoter as indicated in Materials and Methods. The presence in the reaction mixture of specific or nonspecific competitor is indicated above each lane. F.P., free probe.

fusion protein is only present in a small amount. However, after long exposure of the gel (24 h versus 2 h), a slowly migrating complex, likely reflecting the presence of the full-length fusion protein, was noted (not shown).

MZF1 represses *CD34* and c-*myb* promoter activity. To determine whether MZF1 modulates the promoter activity of the *CD34* or the c-*myb* 5′-flanking region, two constructs in which a segment of the human *CD34* 5′-flanking region (nucleotides −666 to +234) and a segment of the human c-*myb* 5′-flanking region (nucleotides −687 to +204) drive the bacterial CAT gene were generated.

TK$^-$ts13 Syrian hamster fibroblasts (MZF1 nonexpressers) and KG-1 human myeloblastic leukemia cells (MZF1 expressers) were transiently transfected with the reporter plasmid CD34CAT or Myb-CAT (Fig. 3A and B, lanes 1 and 6) or cotransfected, at a 5:1 effector-to-reporter ratio, with the effector plasmid ppolMZF1 or pCMVMZF1 and assayed for CAT activity 48 h later. Compared with transfections with the insertless vectors (Fig. 3A and B, lanes 5 and 8), MZF1 induced approximately a 30- to 40-fold decrease in CAT activity driven by the *CD34* promoter in TK$^-$ts13 cells (Fig. 3A, lane 2) and a ~4-fold decrease in KG-1 cells (Fig. 3A, lane 7). CAT levels driven by the c-*myb* promoter were decreased by 15- to 20-fold in TK$^-$ts13 cells (Fig. 3A, lane 2) and by ~6-fold in KG-1 cells (Fig. 3B, lane 7). In TK$^-$ts13 cells transfected with the CD34CAT or the Myb-CAT construct, CAT assays were also performed in the presence of a 100-fold molar excess of double-stranded oligomers (see Materials and Methods) containing the consensus sequences for fingers 1 to 4 and 5 to 13 of MZF1 (38). *CD34* and c-*myb* promoter activities returned to control levels only in the presence of specific competitors (Fig. 3A and B, lane 3). MZF1-induced suppression of *CD34* and c-*myb* CAT activity was unaffected by a 100-fold molar excess of a heterologous double-stranded oligodeoxynucleotide used as a competitor (Fig. 3A and B, lane 4), further suggesting that the transrepression of the *CD34* and c-*myb* promoters depends directly on MZF1 interaction with its binding sites.

Identical results were obtained when the CD34CAT or the Myb-CAT construct was transfected into embryonic stem (ES) cells constitutively expressing MZF1 (Fig. 3C). Compared with parental ES cells and ES cells stably transfected with the empty ppolIIneo vector (Fig. 3C, lanes 1, 3, 4, and 6), approximately three- and sixfold decreases in CAT activity were observed in MZF1-transfected ES cells after transient transfection with the CD34CAT and Myb-CAT reporter constructs, respectively (Fig. 3C, lanes 2 and 5). Additional proof for the specificity of the MZF1-induced repression of *CD34* and c-*myb* CAT activity would be gained by using deletion mutants lacking MZF1 binding sites. As expected, the CAT activity of a chimeric construct driven either by a segment of the *CD34* promoter region (from nucleotide −67 to nucleotide +234), S-CD34 CAT (34), or by a segment of the c-*myb* promoter region (from nucleotide −47 to nucleotide +204), ΔMybCAT, which lacks MZF1 binding sites, was not suppressed by MZF1 in cotransfection experiments in TK$^-$ts13 cells (Fig. 4A and B, lane 5).

MZF1 is expressed during erythromyeloid differentiation in vitro. To determine the pattern of MZF1 expression during hematopoietic differentiation, human peripheral blood *CD34*$^+$ cells obtained from normal donors were induced, in the presence of human-specific growth factors, to differentiate toward the erythroid or the myeloid pathway (55).

At day 0 and after 3, 5, 7, 9, 12, 14, and 16 days in culture, cells were collected and assessed by RT-PCR for the expression of *MZF1*, *CD34*, and c-*myb* mRNAs. As expected, *MZF1* mRNA was expressed during granulocytic-macrophage differentiation (Fig. 5). It was also readily detectable during erythroid differentiation of *CD34*$^+$ cells (Fig. 5). *MZF1* mRNA levels remained essentially constant during the first 12 days of myeloid differentiation but were no longer detectable at later times; *MZF1* mRNA levels fluctuated during erythroid differentiation but remained at detectable levels until day 12 and decreased thereafter. By contrast, *CD34* mRNA levels declined sharply at early times during both erythroid and myeloid differentiation, whereas c-*myb* mRNA levels decreased with a more delayed kinetics (Fig. 5).

Derivation of ES cell lines expressing MZF1. The RNA polymerase II promoter, the thymidine kinase (TK) promoter from herpes simplex virus under the control of MMLV long terminal repeats (54), and the cytomegalovirus promoter function in ES cells without interfering with cell growth. Three eukaryotic expression constructs (ppolMZF1, pXTMZF1neo, and pCMVHAMZF1) were generated and introduced by electroporation into undifferentiated ES cells (ES-D3 cell line). Transfected cells were selected in the presence of G418. Genomic DNA and total RNA were extracted from a mixed population of ES cells transfected with ppolMZF1, pXTMZ F1neo, or pCMVHAMZF1 or with the *neo* expression vectors only. PCR and RT-PCR analysis to determine the integration and expression levels of *MZF1* in the transfected cells (Fig. 6A, B, and C) revealed higher *MZF1* mRNA levels in ESXTMZF1 than in the other two transfectants (Fig. 6B and C, top left). HL-60 cells and undifferentiated ES cells or ES cells transfected with *neo*-bearing vectors were used as positive and negative controls, respectively, for *MZF1* RNA expression. In the presence of LIF, *MZF1*-transfected ES cells grew at an even faster rate than insertless transfected ES cells (Fig. 7) and were morphologically indistinguishable (not shown). Upon LIF removal, ES differentiation and the development of newly formed EB were monitored by inverted light microscopy; the number and shape of the EB developed from the *MZF1*-transfected ES cells were not significantly different from those of control ES lines except for an increased frequency of aggregated EB in MZF1-transfected ES cells (not shown).

Exogenous MZF1 protein levels were evaluated in ES cells transfected with an expression construct containing the *MZF1* cDNA linked in frame to an HA epitope by using the anti-HA monoclonal antibody 12CA5. A band with an apparent molecular mass of ~55 kDa corresponding to HA-MZF1 protein was detected in ES cells transfected with pCMVHAMZF1 (Fig. 6C, top right). The ability of the exogenous MZF1 to bind DNA was assessed by EMSA with ^{32}P-labeled Zn1-4 and nuclear extract from the derivative ES cell lines transfected with ppolMZF1, pXTMZF1, and pCMVHAMZF1 constructs. Nuclear extracts from ES polneo and KG-1 cells were used as negative and positive controls, respectively. As shown in Fig. 6C (bottom left), comigrating retarded complexes were detected after incubation of the probe Zn1-4 either with the nuclear extracts obtained from the ES cell lines expressing the exogenous *MZF1* or with KG-1 nuclear extracts, but not after incubation of the probe (Zn1-4) with the ES CMVneo nuclear extracts (Fig. 6C, bottom left). To determine if MZF1 is present in the shifted complexes, UV cross-linking experiments were performed. The KG-1- and ESCMVHAMZF1-shifted DNA-protein complex contained a protein with an apparent molecular mass of ~55 kDa (Fig. 6C, bottom right), which is consistent with MZF1's molecular weight. Together, these data indicate that *MZF1*-transfected ES cells express a functional MZF1.

MZF1 overexpression inhibits hematopoietic differentiation of ES cells. After 4, 7, 10, and 14 days in liquid culture in the absence of LIF, EB from differentiated *MZF1*-transfected (polMZF1, XTMZF1, and CMVHAMZF1) and control ES

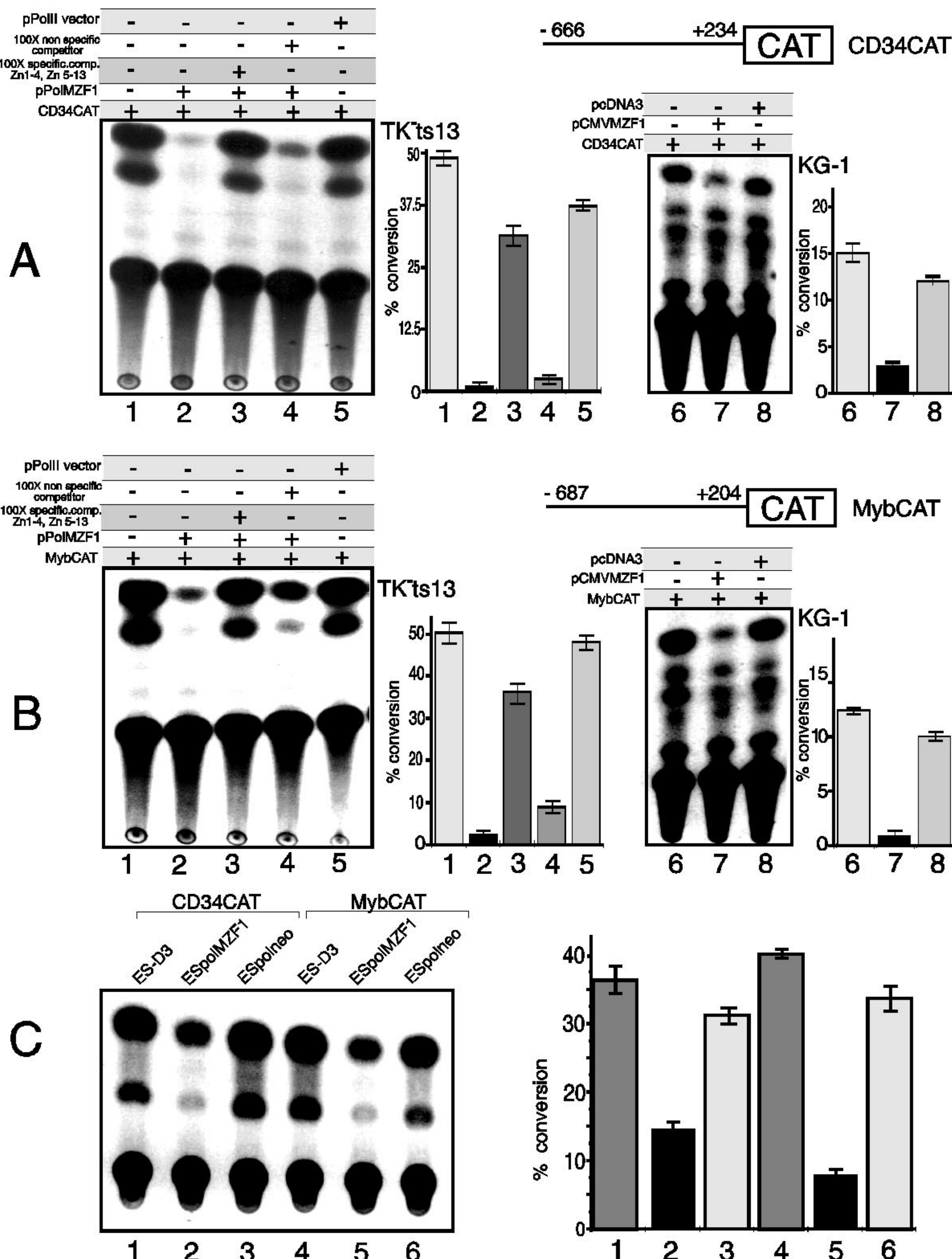


FIG. 3. Transrepression of *CD34* and c-*myb* promoters by MZF1. MZF1 transrepression of *CD34* (A) and c-*myb* (B) 5′-flanking region promoter activity in TK⁻ts13 hamster fibroblasts (lanes 1 to 5) and in KG-1 human myeloblastic leukemia cells (lanes 6 to 8). (A and B) Lanes 1 to 5, autoradiograms of CAT activity and CAT levels in lysates of TK⁻ts13 cells transfected with CD34CAT or Myb-CAT only (lane 1); CD34CAT or Myb-CAT plus pPolMZF1 (lane 2); CD34CAT or Myb-CAT plus pPolMZF1 in the presence of specific competitors (Zn1-4 and Zn5-13) in 100-fold molar excess (lane 3); CD3CAT or Myb-CAT plus pPolMZF1 in the presence of nonspecific competitor, 100-fold molar excess (lane 4); or CD34CAT or Myb-CAT plus pPolII vector (lane 5). Lanes 6 to 8, autoradiograms of CAT activity and CAT levels in lysates of KG-1 cells transfected with CD34CAT or Myb-CAT only (lane 6); CD34CAT or Myb-CAT plus pCMVMZF1 (lane 7); or CD34CAT or Myb-CAT plus pcDNA3 vector (lane 8). (C) Inhibition of *CD34* and c-*myb* promoter activity in MZF1-overexpressing ES cells. MZF1-CAT levels after transient transfection with CD34CAT (lanes 1 to 3) and Myb-CAT (lanes 4 to 6) were evaluated in parental ES cells (lanes 1 and 4), in ES cells transfected with insertless vector (lanes 3 and 6), and in ES cells overexpressing MZF1 (lanes 2 and 5). Autoradiograms are representative of three independent experiments with similar results.

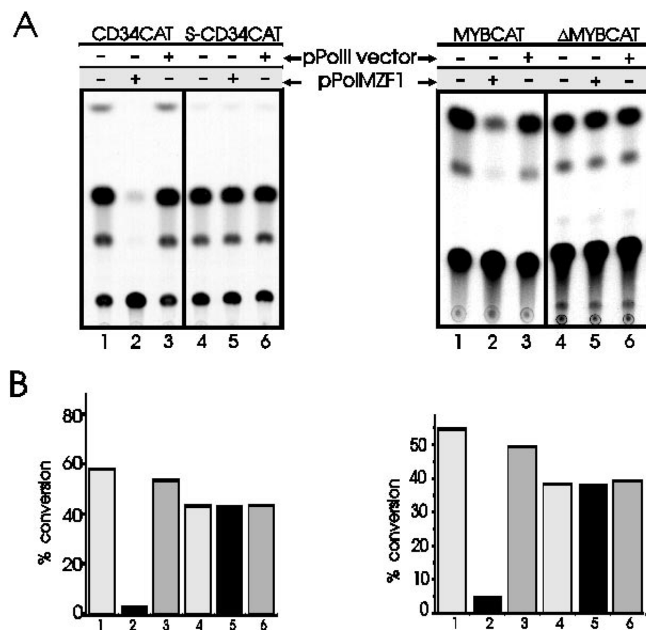


FIG. 4. Requirement for MZF1 binding sites for MZF1 transrepression of the *CD34* and c-*myb* promoters. (A) TK^-ts13 cells were transfected with a CD34CAT construct (from nucleotide −666 to nucleotide +234 of the *CD34* promoter) containing three MZF1 binding sites (lanes 1 to 3) or a CD34CAT construct (from nucleotide +67 to nucleotide +234 of the *CD34* promoter) lacking MZF1 binding sites (lanes 4 to 6) alone (lanes 1 and 4, respectively) or in the presence of pPOLMZF1 (lanes 2 and 5) or the insertless vector (lanes 3 and 6). (B) TK^-ts13 cells were transfected with a Myb-CAT construct (from nucleotide −687 to nucleotide +204) containing two MZF1 binding sites (lanes 1 to 3) or with the ΔMybCAT construct (from nucleotide −47 to nucleotide −204) lacking MZF1 binding sites (lanes 4 to 6) alone (lanes 1 and 4, respectively) or in the presence of pPOLMZF1 (lanes 2 and 5) or the insertless vector (lanes 3 and 6). CAT assays were performed as described in Materials and Methods. The autoradiograms and the histograms are representative of three independent experiments with similar results.

cells were disaggregated and replated in methylcellulose, as described in Materials and Methods. After 15 days, there was a marked reduction (80 to 90%) in the total number of hematopoietic cell colonies derived from *MZF1*-transfected ES cells compared with controls (Fig. 8). The inhibition of colony formation appeared to correlate with the levels of MZF1, since it was more pronounced in the ESXTMZF1 transfectants. Inverted light microscopy analysis revealed that 40 to 50% of the colonies derived from control ES cells were erythroid, based on the identification of hemoglobinized cells, whereas the large majority of colonies derived from the *MZF1*-transfected ES cells appeared to be hemoglobinized.

To define more precisely the colony phenotype, the expression of certain lineage-specific markers was analyzed. Day 4 EB were disaggregated, and the cells were plated in methylcellulose. After 15 days in semisolid culture, 18 colonies derived from differentiated ESpolMZF1 and ESpolneo cells were randomly picked up, and mRNA expression of erythroid and myeloid markers was evaluated by RT-PCR. The erythroid differentiation marker embryonic β-globin was detected in 40% of the colonies derived from the *neo* control cell lines, compared with 70% of the colonies derived from the *MZF1*-transfected cells (Fig. 9). Expression of the macrophage colony-stimulating factor (M-CSF) receptor (c-*fms*) (47) gene and of the myeloperoxidase (63) gene, markers of the macrophage and granulocyte lineages, respectively, was detected in 17 and 33%, respectively, of the colonies derived from the control cell lines, compared with only 11 and 5%, respectively, of the colonies derived from ES cells constitutively expressing exogenous MZF1; 11% of the colonies tested were negative for any of the mRNAs analyzed (Fig. 9). These data indicate that MZF1 overexpression in ES cells blocks both myeloid and erythroid differentiation, although the effect on myeloid differentiation appears to be slightly more pronounced.

MZF1 modulation of early hematopoietic gene expression: correlation between *MZF1*, *CD34*, and c-*myb* mRNA levels during EB differentiation. *CD34* and c-*myb* are among the earliest markers expressed in progenitor marrow cells, although both genes are expressed in other tissues (9, 11). RT-PCR analysis of total RNA extracted from ESpol1XTMZF1 and ESpolXTneo cells at time zero and after 4, 7, 10, and 14 days in differentiation culture revealed that almost no endogenous CD34 was expressed at day 0 in undifferentiated ES cells, transfected or not with *MZF1*, whereas *CD34* mRNA expression was detectable from day 7 to 14 in EB derived from the ES control *neo* cell lines. c-*myb* mRNA was detectable in undifferentiated ES control *neo* cell lines, and the mRNA levels increased during EB development (Fig. 10). In contrast, EB overexpressing exogenous *MZF1* showed either no or only low-level expression of endogenous *CD34* and c-*myb* mRNA (Fig. 10). In three separate experiments with ES cells transfected with ppolMZF1 or pXTMZF1neo, the low levels of *CD34* and c-*myb* mRNA expression directly correlated with the levels of exogenous *MZF1* mRNA, which were higher in ESX TMZF1 than in ESpolMZF1 cells.

DISCUSSION

Several transcription factors of the zinc finger family have a defined role in regulating hematopoiesis (41, 45, 60). Other members of the family have a less clear function (15, 30, 36,

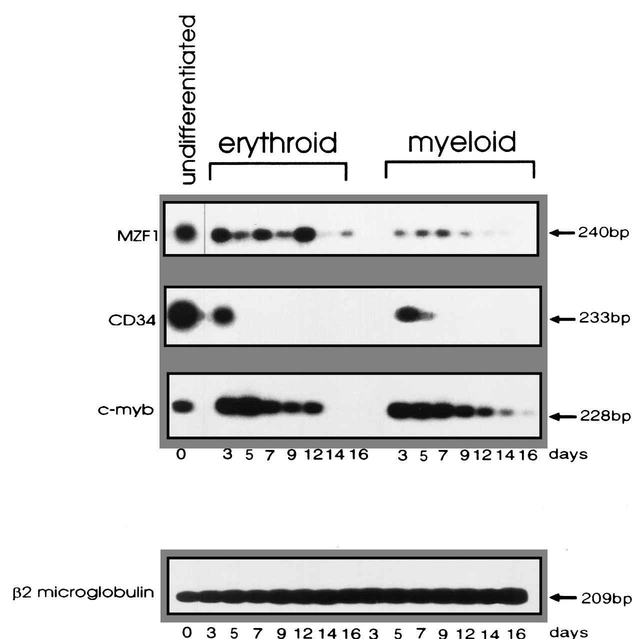


FIG. 5. *MZF1*, *CD34*, and c-*myb* mRNA levels during in vitro erythroid and myeloid differentiation of human $CD34^+$ progenitor cells. RT-PCR was performed on RNA from undifferentiated cells (day 0) and cells treated for the indicated days with IL-3 and EPO (left) or with IL-3 and GM-CSF (right). The specificity of PCR products was confirmed by Southern blot analysis, where the controls (RT-PCR without RNA and PCR without RT) were negative. Each lane is representative of three independent experiments with similar results.

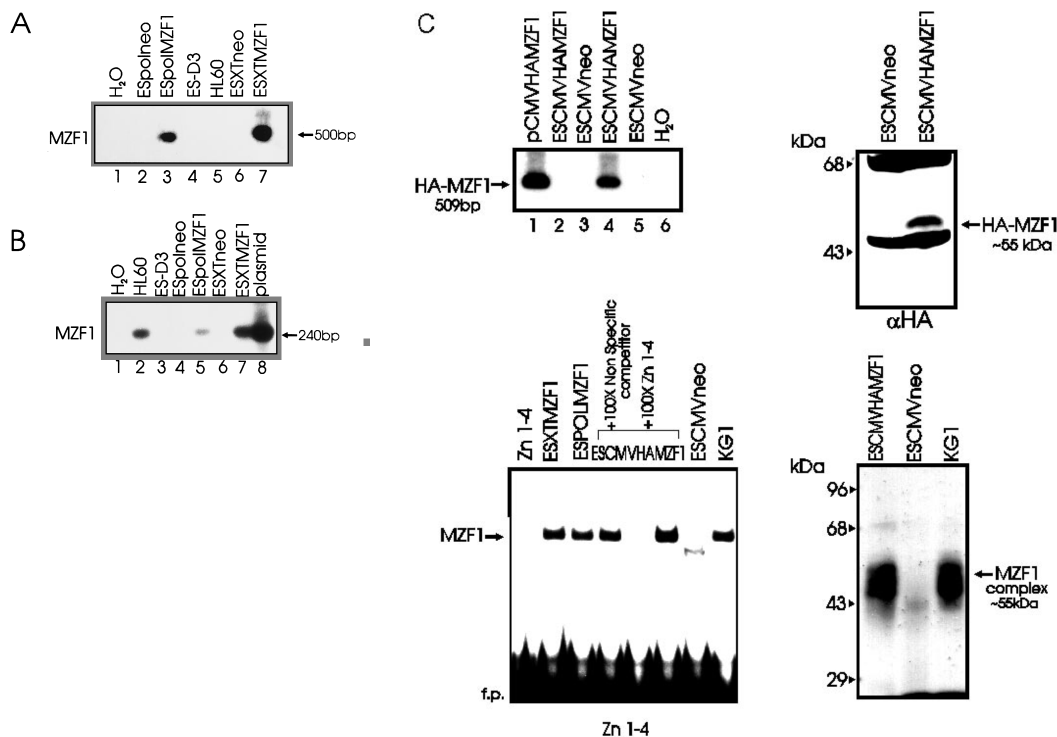


FIG. 6. Integration and expression of exogenous *MZF1* in derivative ES cell lines. (A) Integration of *MZF1* cDNA was evaluated by PCR performed on genomic DNA extracted from ES cells stably transfected with constructs (pPolMZF1 and pXTMZF1neo) encoding MZF1 (lanes 3 and 7, respectively). As negative controls, PCR was done on DNA extracted from ES cells transfected with a plasmid conferring neomycin resistance (lanes 2 and 6), on the parental ES cell line (lane 4), and on DNA extracted from HL60 cells expressing MZF1 (lane 5). (B) *MZF1* mRNA expression in derivative ES lines. RT-PCR was performed on RNA extracted from ESpolMZF1 and from ESXTMZF1neo cells (lanes 5 and 7, respectively) with primers mapping to the *MZF1* region not containing the DNA-binding domains. RT-PCR analysis was also performed on ES-D3, ESpolneo, and ESXTneo mRNAs (lanes 3, 4, and 6) as negative controls. The HL60 cell line was used as a positive control. Plasmid pPolMZF1 was amplified in lane 8. The specificity of the PCR products was confirmed by Southern blot analysis, where the controls (RT-PCR without RNA and PCR with non-reverse-transcribed RNAs) were negative. Each lane is representative of three independent experiments with similar results. (C) HA-tagged *MZF1* mRNA was detected (top left) by RT-PCR (lane 4) with a 22-mer oligomer of the HA sequence (5′-ATCAAGCTTATCGATACCGTGG-3′) as the 5′ primer and a 21-mer of the *MZF1* cDNA (5′-GCCACATACATCGCAACGGCC) as the 3′ primer. PCR was also performed on non-reverse-transcribed RNA (lanes 2 and 3). ESCMVNEO (lane 5) and plasmid pCMVHAMZF1 (lane 1) were used as negative and positive controls, respectively. HA-MZF1 protein levels (top right) were evaluated by Western blotting with the anti-HA antibody (see Materials and Methods); the EMSA (bottom left) was performed with nuclear extracts from *MZF1*-transfected ES cells. KG-1 and ESCMVNEO nuclear extracts were used as positive and negative controls, respectively. ^{32}P-end-labeled Zn1-4 (lane 1) was used as the probe. UV cross-linking (bottom right) of the EMSA complex detected with the nuclear extracts derived from KG-1 and ESCMVHAMZF1 cells. Sizes are shown in kilodaltons.

37), and among these, *MZF1* has been postulated to play a role in the regulation of granulocyte differentiation (3). The goal of the present study was to identify relevant targets of MZF1 that may be important for hematopoietic development and to assess whether MZF1-induced changes in gene expression correlate with hematopoietic commitment and differentiation of ES cells.

MZF1 acts as a transcriptional inhibitor of hematopoiesis-specific genes. Putative MZF1 binding sites are present in the promoters of several genes with hematopoiesis-specific functions, such as *GATA-1*, c-*kit*, *CD34*, GM-CSF, and c-*myb*. We focused on the regulation of the *CD34* and c-*myb* promoters by MZF1 because CD34 is a marker of the earliest hematopoietic progenitor cells, probably important for cell-cell and cell-matrix interactions (2), and c-*myb* encodes a nuclear protein required for hematopoietic proliferation and differentiation (11, 18, 23, 40). Bacterially synthesized MZF1 protein interacted specifically with putative DNA-binding sites present in the *CD34* and c-*myb* 5′-flanking sequences. Such binding correlated with inhibition of CAT activity in both $CD34^+$ MYB^+ cells (KG-1) and $CD34^-$ MYB^- cells (TK^-ts13). The inhibitory effect was clearly greater in TK^-ts13 than in KG-1 cells, probably reflecting the influence of endogenous *MZF1* expression on *CD34* and c-*myb* promoter activity in KG-1 cells. In this regard, He et al. (25) showed that a construct containing the *CD34* promoter region from nucleotides −1100 to −500, which includes functional MZF1 binding sites, was negatively regulated in KG-1 cells in comparison to a construct lacking that promoter region. The finding of a zinc finger protein that negatively regulates transcription is not unprecedented; the zinc finger protein encoded by the Wilms tumor suppressor *WT1* gene appears to function as a transcriptional repressor of the insulin-like growth factor II gene (20); similarly, IL-2 gene expression is transcriptionally repressed by a zinc finger gene named Nil-2-a (67). The recently cloned BZP (22) gene also appears to function as a transcriptional repressor, but the natural target(s) of the encoded protein is unknown.

MZF1 is a marker of erythromyeloid committed cells. A temporal correlation in the expression of *CD34*, c-*myb*, and *MZF1* mRNA was observed in human peripheral blood $CD34^+$ cells stimulated toward either the erythroid or the myeloid differentiation (55). Expression of the *MZF1* was not restricted to the myeloid lineage, as previously suggested (3), but was detectable during the early stages of in vitro erythromyelopoiesis and persisted as hematopoietic cells reached late stages of differentiation. This pattern of expression is consis-

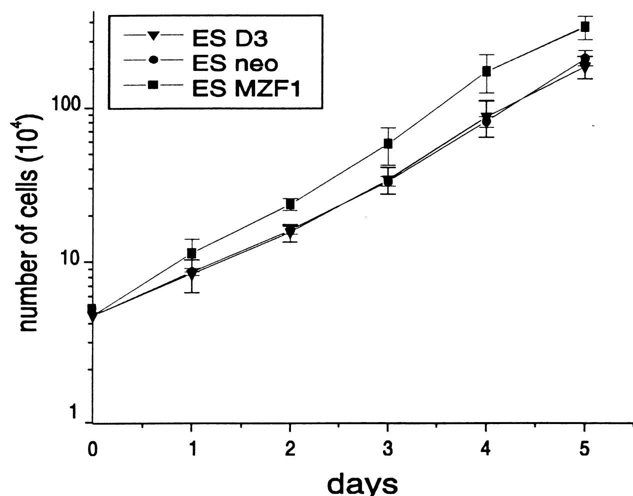


FIG. 7. Proliferation of *MZF1*-transfected ES cells in the presence of LIF. Cells were seeded at a density of 5×10^4 per ml and counted (in duplicate) at the indicated days by the trypan blue exclusion test. Error bars indicate ± standard deviation (SD) for three different experiments with similar results.

tent not only with a role for this protein during terminal myeloid differentiation (3, 27) but also with a function during early stages of hematopoietic differentiation. MZF1 function during early stages of hematopoiesis may derive from its ability to repress the promoter activity of hematopoiesis-specific genes upon binding to regulatory elements in the 5′-flanking region, as shown here for *CD34* and c-*myb*.

Constitutive MZF1 expression interferes with hematopoietic commitment and differentiation of ES cells. The cell line ES-D3 (19) retains its totipotency in vitro for many generations and, when reintroduced into the mouse blastocyst, contributes to all murine cell lineages. ES-D3 cells form complex EB with endoderm, basal lamina, mesoderm, and ectoderm after 3 to 8 days of differentiation in suspension culture. Under certain in vitro conditions, these cells are able to undergo commitment to different hematopoietic lineages (67), providing a unique model in which cellular and molecular events during the various stages of hematopoiesis can be analyzed.

Different clones or mixed populations of ES cells stably expressing a functional MZF1 protein were studied. ES cells constitutively expressing MZF1 and maintained in culture in the presence of LIF grew even faster than parental and insertless vector-transfected ES cells (Fig. 7), indicating that MZF1 overexpression does not affect proliferation of undifferentiated ES cells. When *MZF1*-transfected ES cells were induced to differentiate in vitro, erythromyeloid colony formation in methylcellulose decreased dramatically, and the extent of the decrease appeared to correlate with MZF1 expression levels; 1 to 3% of the cells obtained from the ESneo control cultures and plated in semisolid medium formed hematopoietic colonies, whereas only 0.1 to 0.4% of the cells derived from *MZF1*-transfected cell lines generated hematopoietic colonies.

In light of the ability of MZF1 to repress the promoter activity of hematopoiesis-specific genes and its pattern of expression in early stages of hematopoiesis, it seems likely that this protein begins to exert its role at a stage preceding that of erythroid or myeloid commitment or in early hematopoietic progenitors and that its overexpression prevents stem cell commitment and/or progenitor cell expansion and differentiation. However, a few progenitor cells were able to bypass the MZF1-dependent block and did generate morphologically mature colonies. Analysis of the expression of lineage-specific markers (i.e., embryonic β-globin, myeloperoxidase, and c-*fms*) in individual colonies derived from control *neo* ES cells or from MZF1-expressing ES cells suggested that erythroid progenitors are more likely than myeloid progenitor cells to escape the MZF1-induced hematopoietic block. This may reflect a preferential sensitivity of myeloid progenitors to the transcriptional repression function of MZF1 as a result of differences in (i) number of lineage-specific target genes carrying MZF1 binding

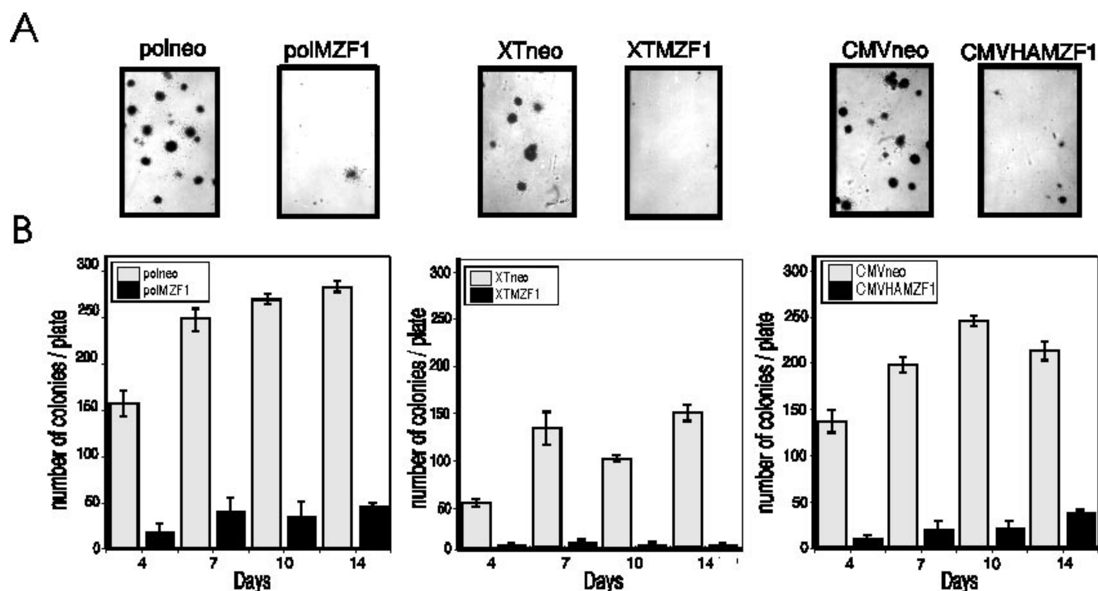


FIG. 8. Inhibition of hematopoietic cell colony development from ES cells. (A) Hematopoietic cell colonies were grown in methylcellulose from 10^4 *neo*-transfected (ESpolneo, ESXTneo, and ESCMVneo) and *MZF1*-transfected (ESpolMZF1, ESXTMZF1, and ESCMVHAMZF1) ES cells plated at different times after LIF removal and scored after 15 days in semisolid culture. (B) Histograms indicate the number of hematopoietic colonies in control and *MZF1*-expressing ES cells. Days of culture after LIF removal are indicated on the bottom. Error bars indicate ± SD for four independent experiments.

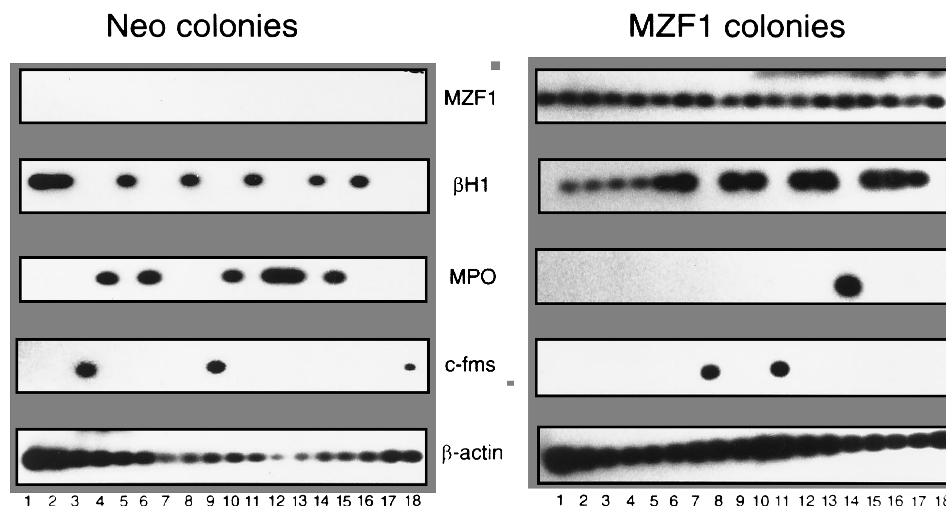


FIG. 9. Lineage specificity of ESneo- and ESMZF1-derived colonies. After 15 days in methylcellulose, the percentage of lineage-specific colonies was determined by scoring visibly hemoglobinized colonies or by RT-PCR detection of embryonic β-globin (βH1), M-CSF receptor (c-*fms*), or myeloperoxidase (MPO) mRNA in individual colonies. RT-PCR phenotyping was repeated twice, scoring each of 18 colonies derived from *neo*- or *MZF1*-transfected ES cells. *MZF1* mRNA expression was used as an internal control.

sites or (ii) binding affinity of MZF1 to regulatory sequences of myeloid versus erythroid MZF1-regulated genes. The ability of MZF1, when overexpressed, to inhibit erythromyeloid commitment and/or differentiation is only in apparent contrast to the "antisense" studies of Bavisotto et al. (3), suggesting an MZF1 requirement for granulocytic colony formation. It can be postulated that, depending on the stage of differentiation, MZF1 regulates hematopoiesis either negatively (this study) or positively (3).

MZF1 inhibits *CD34* and c-*myb* mRNA expression during EB differentiation. The transcriptional repressor activity of MZF1 on the *CD34* and c-*myb* promoters and the inhibition of hematopoietic colony formation induced by MZF1 in ES cells correlated with the lack of induced expression of *CD34* and c-*myb* mRNAs usually observed during EB development. Thus, the inhibition of hematopoietic development by MZF1 might reflect the inhibitory function of MZF1 on the transcription of genes (i.e., c-*myb*) that positively regulate the early stages of hematopoiesis. Myb-deficient mice appear to develop normally until the stage of hepatic hematopoiesis, when they die of anemia (39). Whether or not in vitro hematopoietic differentiation of ES cells strictly reflects yolk sac hematopoiesis (but the expression of adult β-globin and of other markers of definitive hematopoiesis [29] argues against this simple interpretation), c-*myb* expression appears to be required for in vitro erythromyeloid colony formation in ES cells (our unpublished data). However, c-*myb* is unlikely to be the only MZF1 target required for hematopoietic commitment and differentiation of ES cells. Putative MZF1 binding sites are also present in the promoter regions of other hematopoiesis-specific genes, such as c-*kit* and *GATA-1*, and MZF1 might well prove to bind to and negatively regulate the activity of these promoters; if so, the repression function of MZF1 will affect two genes whose lack of function is known to generate a distinctive hematopoiesis-deficient phenotype.

In conclusion, our studies of MZF1 overexpression might be revealing of the physiological function of the *MZF1* gene during hematopoietic commitment and/or early stages of hematopoietic differentiation, which depend not only on the activity of positive regulators but also on inhibitory signals, serving as a balance in the stimulatory pathway. Targeting of the same genes for positive and negative regulation would provide an efficient and economical mechanism to maintain the coordination of commitment, progenitor cell expansion, and terminal differentiation in the hematopoietic system.

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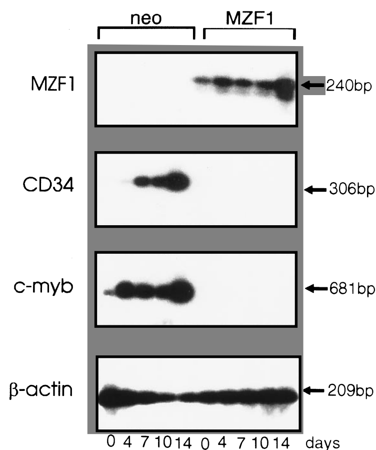

FIG. 10. *MZF1*, *CD34*, and c-*myb* mRNA levels during in vitro differentiation of *neo*- and *MZF1*-transfected ES cells. mRNA levels were determined by RT-PCR in *neo*-transfected (ESpolneo) (lanes 1 to 5) and in *MZF1*-transfected (ESpolMZF1) ES cells (lanes 6 to 10) on the indicated days (bottom) of in vitro differentiation following LIF removal. PCR was performed on non-reverse-transcribed RNAs to confirm the specificity of the PCR products. Each lane is representative of three independent experiments.

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