EMATOPOIESIS is a complex process during which pluripotent stem cells in the bone marrow proliferate, differentiate, and mature into individual peripheral blood cell types. At the molecular level, the process of hematopoiesis is best described as a tightly regulated signal transduction cascade. Environmental signals are received at the cell surface by growth factors binding to receptors. This initial signal is transduced from the cell surface, via secondary messengers, to the nucleus, where specific transcription factors are either activated or deactivated to alter the pattern of gene expression. Transcription factors have a pivotal role in translating the cell surface signal into a specific pathway of differentiation by specifying which genes will be actively used by a cell at a particular time in development.1,2

Transcriptional regulators can be classified into families based on the expression of distinct structural/functional domains characterized by conserved amino acid sequences.3,4 Zinc finger genes5 represent one family of proteins that form such a domain when the conserved amino acids cysteine and histidine bind a central zinc ion. Zinc finger genes can be further classified by their finger domain structures. The C2H2 zinc finger family, exemplified by Kruppel, a Drosophila gap gene,6 contains the highly conserved peptide motif CX2CX3FX5LX2HX3H. Zinc fingers usually occur in repeats, and individual fingers are separated by a conserved region called the H-C link. The Kruppel family members usually contain the H-C link motif of TEAKPXYY. Many members of the C2H2 zinc finger gene family regulate differentiation processes, and genetic mutations in some zinc finger genes have been associated with specific human diseases.7

The myeloid zinc finger gene 1, MZF-1, is a putative transcription factor of the C2H2 zinc finger gene family. MZF-1 was cloned from the peripheral leukocytes isolated from a patient with chronic myelogenous leukemia8 and exhibits several characteristics suggesting that MZF-1 is a necessary factor for granulocytic differentiation.9 MZF-1 is preferentially expressed in myeloid leukemia cell lines and myeloid progenitor cells from normal marrow.9,10 In addition, antisense, but not sense, oligonucleotides markedly inhibit in vitro granulocyte but not erythrocyte colony formation.10

To define the role of MZF-1 in regulating myeloid differentiation, we have initiated a comprehensive molecular, cel-
MZF-1 regulates CD34

There is an acidic region in the 5' portion of the protein.

Materials and Methods

Cell lines. All cell lines were obtained from the American Type Culture Collection (Rockville, MD). The cell lines NIH 3T3 (mouse embryonic fibroblast), 293 (human adenovirus-transformed kidney cells), and Hela (cervical carcinoma cells) were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% heat-inactivated fetal calf serum and antibiotics. The cell lines K562 (human erythroleukemia), Jurkat (human acute T-cell leukemia), and KG-1 (human acute myelogenous leukemia) were grown in RPMI 1640 plus 10% heat-inactivated fetal calf serum and antibiotics. Hela, NIH 3T3, 293, and Jurkat cell lines do not express MZF-1, and the K562 and KG-1 cells express MZF-1. 1 In addition, KG-1 cells are CD34+, whereas the other cell lines do not express CD34. Thus, these cell lines represent the possible variations in phenotype needed to test MZF-1 regulation of the CD34 promoter in this study, ie nonhematopoietic and hematopoietic, MZF-1 expressing and nonexpressing, and CD34+ and CD34-.

GAL4 assays. Chimeric proteins were produced that fuse the yeast GAL4 DNA binding domain to the full-length MZF-1 protein (Fig 1). Full-length MZF-1 was used because the zinc fingers domains may have transcriptional regulatory activity that would be masked if they were deleted. The coding region of MZF-1 is polynucleotide chain reaction (PCR)-amplified using a sense oligonucleotide that inserts an EcoRI restriction enzyme site (5'-TATAGAATTCACGTTTTAG-3') and an antisense oligonucleotide that inserts an XbaI restriction site (5'-TCATGTAGCTATAGCTTCTCTAGG-3'). The PCR product was digested with the restriction enzymes EcoRI and XbaI and subcloned into the GAL4 expression plasmid pSG424 to produce a GAL4-MZF fusion protein containing the entire MZF-1 coding region 3' of the GAL4 zinc finger region (Fig 2). The fusion construct was confirmed using deoxy DNA sequencing. The GAL4-MZF-1 fusion plasmid was cotransfected with a CAT reporter plasmid, pGL4-5tk-CAT, containing 5 GAL4 binding sites preceding the minimal thymidine kinase promoter regulating expression of the chloramphenicol acetyltransferase gene. 2 The GAL4 binding sites are distinct from the MZF-1 binding sites. This reporter construct had a modest basal activation rate in the cell lines used for these studies and was therefore used for both activation and repression studies. This allowed a more direct comparison of the activation seen in hematopoietic lines compared with the repression seen in nonhematopoietic lines. CAT activity was measured from whole cell lysates 36 hours after transfection.

CD34 promoter assays. A 1.2-kb BamHI-Sac I DNA fragment of the CD34 promoter (-1.1 to +78 from the transcriptional start site according to He et al 4) was subcloned into pBluescript KS+. The reporter fragment was then excised and subcloned onto the BamHI-Sac I sites of pGL2Basic (Promega, Madison, WI) to create the CD34 luciferase reporter construct 5 (here termed CD34-Luc) used in this study. This fragment contains three MZF-1 DNA binding sites. These three sites, termed A, B, and C, are at -584, -516, and -148, respectively (see Fig 4). 6 As shown below, the core guanines in these three MZF-1 binding sites were mutated to thymines using the PCR-based overlap extension method. 7 The CD34 promoter construct with the three MZF-1 sites mutated (termed CD34-ABC) was used to evaluate the binding site-dependence of MZF-1 regulation of CD34. Site A: wild-type, 5'-CTGGAGAGGGGATAACTG-3'; mutant, 5'-CTGGATATTATTATTATT-3'. Site B: wild-type, 5'-ATGTTGATGGGAACTAATTGGGA-3'; mutant, 5'-ATGGTTGATTTTACTAAATTTTTA-3'. Site C: wild-type, 5'-ATGGGTTTTTTAATTTTTTTTA-3'; mutant, 5'-AGTGGTTTTTTAATTTTTTTTA-3'. Sites A and B have DNA sequences that contain the full-length MZF-1 binding sites composed of the sequences recognized by ZN 1-4 and ZN 5-13 (Fig 4). Site C has only a single ZN1-4 binding site. Based on our binding site selection studies, MZF-1 will not bind to the mutated sites. 8

The entire open reading frame of MZF-1 was cloned into the EcoRI site of the expression vector CB6, 9 where the cDNA is driven off the CMV IE promoter. 10 This vector, termed CB6-MZF, was used in the cotransfection studies with the CD34 promoter.

Fig 1. Schematic of the MZF-1 protein and its peptide domains. The MZF-1 protein has 13 zinc finger domains (ZN 1-4 and ZN 5-13) separated into two sections by a glycine-proline rich region. There is an acidic region in the 5' portion of the protein.

Fig 2. Schematic of the GAL4-MZF fusion expression vector. Amino acids 1-147 from the GAL4 zinc finger gene, containing the DNA binding segment, were fused in frame to the coding region of MZF-1. Because some transcriptional regulatory domains are influenced by DNA binding domains, the DNA binding domain of MZF-1 was left intact. GAL4 DNA binding sites are distinct from MZF-1 binding sites. This cDNA was driven off the SV40 early promoter (SV40p), with the SV40 termination and polyadenylation signals (SV40 pA) placed at the 3' end. (B) Schematic of the GAL4-5tk-CAT reporter construct. Five GAL4 DNA binding sites (GAL4/S) were placed 5' to a truncated thymidine kinase promoter (TK) driving the chloramphenicol acetyltransferase cDNA (CAT).
and cotransfected with a CAT reporter construct with five GAL4 bind-
ferred by GAL4-MZF as compared with GAL4 alone was markedly
decreased; thus, the data are expressed as fold repression. Because
activity as compared with GAL4 expression vector alone, without
GAL4'ZF activated reporter CAT activity in the hematopoietic lines
properties of MZF-1. MZF-1 was fused to the GAL4 DNA binding domain
MZF-l, is proportional to the transcriptional activity conferred by
activity from conferred by GAL4 alone was normalized to a value of
1.0 for both nonhematopoietic and hematopoietic cell lines. Each
transfection was performed at least three separate times to obtain the
mean and standard errors.

RESULTS
Assessment of MZF-1 transcriptional regulation with GAL4 assays. MZF-1 belongs to the Kruppel family of

Gel mobility shift assays. The ability of recombinant MZF-1 protein to bind to the three sites in the CD34 promoter was assessed by the gel mobility shift assay. Recombinant MZF-1 protein was synthesized using the histidine-containing pDS56 expression vector and recombinant protein isolated by binding to a nickel-agarose column, as previously described. The 1.2-kb BamHI–Sac I CD34 promoter fragment was digested to completion with the restriction enzyme Mae I and end-labeled with the large fragment of DNA polymerase and α32P-dATP. Mae I digestion of the 1.2-kb DNA
fragment produced DNA fragments of 75 to 600 nucleotides. The
600-bp fragment contains both the A and B MZF-1 binding sites and
the 97-bp fragment contains site C. Consensus DNA binding sites
for MZF-1 were previously isolated, and duplex oligomers from these sites were used to compete binding of recombinant MZF-1 to a 234-bp CD34 promoter fragment containing sites A and B (−653 to −419, according to He et al14). This fragment was obtained by PCR using the primers 5′-GGTGGAGGTGGAGT-3′ and 5′-
TTCCCTGCAAGACC-3′, with the CD34-Luc as template.
Transfection analysis. The nonhematopoietic cell lines were
transfected using Lipofectin according to the manufacturer’s instruc-
tions (BRL, Bethesda, MD). Log phase cells were seeded at 103
per 100-mm culture dish and incubated overnight. Six hours before
transfection, the media was changed. Forty-eight hours after
transfection, cells were harvested by scraping and washed once in
phosphate-buffered saline (PBS), and whole cell lysates were prepared
by 3 cycles of freeze-thawing in 0.25 mol/L Tris-HCl (pH 8.0) with
protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/
ml of pepstatin A, and 1 μg/ml of leupeptin). Lysates were assessed
for CAT activity, as previously described.

The KG-1, K562, and Jurkat cells were transfected using a Bio-
Rad (Hercules, CA) electroporator at 0.24 kV and 960 μF. Briefly,
log phase cells were placed in fresh media overnight, and then 105
cells in 0.4 ml were placed in a cuvette with the appropriate plasmids.
Cuvettes were allowed to sit at room temperature for 5 minutes before
and after electroporation. Cells were then placed in 5 ml of media for
12 hours before lysis preparation. Lysate preparation were identical to that for nonhematopoietic cells described above.
Luciferase activity was assessed as described.

The quantity of expression vector or reporter construct varied
as required in the different cell lines to maintain CAT or luciferase
values within their respective linear ranges. When needed, empty
vector was added to maintain a constant amount of DNA in each
transfection. All transfections were normalized by transfecting RSV-
βgal, a β-galactosidase expression vector. Cell lysates were
assayed for β-galactosidase activity, as described, and individual
transfections were normalized for transfection efficiency. Each trans-
fection was performed at least three separate times to obtain the
mean and standard errors.

Fig 4. Schematic of the transcription factor binding sites in the
CD34 promoter. The MZF-1 full sites contain sequences nearly
identical to the selected consensus DNA binding sites for both ZN 1-
and ZN 5–13. The MZF-1 half site contains a site identical to the
consensus DNA binding site for ZN 1–4. The Materials and Methods
contains a full description of the sequences and exact location of
the MZF-1 binding sites.
zinc finger genes by amino acid homology. The members of this family that have been biochemically characterized are transcriptional regulators. The transcriptional regulatory properties of MZF-1 had not yet been proven. The transcriptional regulatory activity of proteins can be assessed by fusing the putative regulatory protein domain to a heterologous DNA binding domain, such as GAL4. To assess whether MZF-1 was a transcriptional activator or repressor GALA-MZF was cotransfected with pGAL4-5hk-CAT into two nonhematopoietic cell lines (3T3 and 293) and two hematopoietic cell lines (Jurkat and K562). In this assay, the transcriptional regulatory region is provided by MZF-1 and the DNA binding domain recognizing the reporter is provided by the GAL4 zinc fingers. There is no homology between the GAL4 zinc finger DNA binding sites and the MZF-I binding sites.

In the two nonhematopoietic cell lines, GALA-MZF-I repressed CAT activity (Fig 3). In 3T3 cells, GALA-MZF repressed CAT activity an average of 17-fold from GAL4 alone. In 293 cells, with the same transduction procedure, GALA-MZF repressed CAT activity by an average of 3.3-fold (Fig 3).

Surprisingly, GAL4-MZF behaved differently in hematopoietic cells. In contrast to the nonhematopoietic cells, it was found to activate CAT expression in both cell lines (Fig 3). In K562 cells, GAL4-MZF increased CAT activity by an average of 12.3-fold. In Jurkat cells, GAL4-MZF increased CAT activity an average of 31.0-fold.

Therefore, based on these assays, MZF-I had bifunctional transcriptional regulatory activity. In one intracellular environment it functions as a transcriptional repressor and in another as an activator.

MZF-1 regulation of the CD34 promoter. Once the GALA studies established MZF-l as a transcriptional regulator, myeloid promoters were searched for MZF-1 binding sites. Whereas single MZF-1 DNA binding sites were found in the lactoferrin and myeloperoxidase promoter, three MZF-1 binding sites (called A, B, and C; Fig 4) were found within the CD34 promoter. The CD34 promoter was chosen for further analysis based on the presence of these multiple MZF-I binding sites, making it a strong candidate for regulation by MZF-1.

To evaluate recombinant MZF-1 protein binding to these sites, mobility shift assays were performed (Fig 5). MZF-1 bound to fragments of the CD34 promoter produced by Mse I restriction digestion containing each of these sites. The 600-bp Mse I fragment contained two MZF-1 binding sites, termed A and B, that contained DNA consensus binding sites for both zinc finger domains 1 through 4 and 5 through 13 (Fig 1A). Recombinant MZF-1 protein fragments containing either zinc fingers 1 through 4 or 5 through 13 could bind to this CD34 fragment. A smaller Mse I fragment of 97 bp contained a binding site that was the exact consensus for MZF-1 zinc fingers 1 through 4. This DNA fragment also bound recombinant MZF-1 protein.

In addition, MZF-1 binding to a 234-bp PCR fragment of the CD34 promoter containing sites A and B could be specifically competed with the cold duplex oligomers containing the consensus MZF-I DNA binding sites previously (Fig 5). Thus, MZF-1 protein could bind specifically to the CD34 promoter at sequences homologous to MZF-1 consensus binding sites.

The previously described pGL-basic luciferase reporter under control of the 1.2-kb CD34 promoter fragment containing all three MZF-1 binding sites and the transcriptional start site was used to assess MZF-1 regulation of the CD34 promoter (Fig 6). Cotransfection of the expression vector CB6-MZF with this CD34-luciferase reporter plasmid (here termed CD34-Luc) into Hela cells repressed luciferase activity by an average of 5.2-fold over vector alone, at the highest CB6-MZF level. In 3T3 cells, CB6-MZF repressed
CD34-mediated luciferase activity by an average of 4.2-fold. This was consistent with the GAL4 studies. MZF-1 functioned as a repressor when transcription was initiated by the CD34 promoter in nonhematopoietic cells.

However, MZF-1 activated transcription from the CD34 promoter in hematopoietic cells (Fig 6). In Jurkat cells at the highest transfected amounts of CB6-MZF, CD34 luciferase activity was increased by an average of 7.5-fold. In K562 cells at similar cotransfection parameters, luciferase activity was increased by an average of 18.1-fold. In KG-1 cells, the average increase of luciferase activity produced by CB6-MZF was 6.1-fold. These data were also consistent with the previously described GAL4 assays.

Mutation of the three MZF-1 binding sites in CD34-Luc abrogated potential MZF-1 interactions; CB6-MZF no longer regulated expression of CD34-mediated transcription (Fig 6). In Jurkat and K562 cells, the activation of CD34-ABC by CB6-MZF was reduced to that of CD34-Luc baseline.

DISCUSSION

In the first portion of this study, the transcriptional regulatory properties of MZF-1 were investigated using cotransfec-
MZF-1 regulates CD34

Fig 6. (Cont'd). CD34-Luc/ABC indicates the CD34 reporter construct with the three potential MZF-1 binding sites mutated. When the three MZF-1 binding sites are mutated, MZF-1 loses its ability to regulate the CD34 promoter. All transfections were normalized to β-galactosidase. The luciferase activity of CD34-Luc cotransfected with empty vector DNA was given a value of 1.0, and all values from the MZF-1 expression vector cotransfections were compared with that. Each data point represents the average ± the standard error of at least three separate cotransfections.

The finding that a zinc finger gene can function as a transcriptional repressor in one type of intracellular environment and as an activator in another is interesting, but not entirely unique. There is preliminary evidence that at least three other Krupple class zinc finger genes may also have bifunctional

tion of GAL4-MZF fusion proteins and GAL4 binding site reporter plasmids. We found that the transcriptional regulation by MZF-1 was dependent on the intracellular environment. If MZF-1 was expressed in nonhematopoietic cells, then it functioned as a transcriptional repressor. If it was expressed in hematopoietic cells, then it functioned as an activator. This surprising finding was confirmed in several different types of hematopoietic and nonhematopoietic cell lines. Thus, it is unlikely to be due to variation within a single given cell line. In addition, in preliminary experiments, MZF-1 also activated a reporter construct via its own consensus DNA binding sites in the hematopoietic cell lines, indicating that this regulatory activity was not binding site-specific.
transcriptional regulatory properties. There is evidence that Kruppel itself can activate or repress transcription depending on its intracellular concentration.23 YY1 has been shown to repress transcription from the adenovirus P5 promoter. However, when YY1 can interact with adenovirus E1A, it mediates activation through this same promoter sequence.24 In addition, the Wilm’s tumor suppressor zinc finger gene WT1 can also mediate repression or activation.18,25,26 This latter case is more similar to MZF-1, as WT1 transcriptional regulatory properties appear to be dependent on which cell type transcription is assessed. Thus, this class of zinc finger genes has far more complex regulatory characteristics than previously thought.

The second portion of this investigation studied the binding to and transcriptional regulation of the CD34 promoter by MZF-1. Recombinant MZF-1 could bind to sites within the CD34 promoter by mobility shift assay. Cotransfection assays showed that, in nonhematopoietic cells, MZF-1 repressed the CD34 promoter, whereas, in hematopoietic cells, MZF-1 activated the CD34 promoter. This was entirely consistent with the GAL4 and heterologous promoter studies, indicating that even with a physiologically relevant promoter such as CD34 MZF-1 still had bifunctional transcriptional regulatory properties. The hematopoietic-specific activation of the CD34 promoter occurred in cells not expressing MZF-1 or CD34 (Jurkat), cells expressing MZF-1 but not CD34 (K562), and cells expressing both MZF-1 and CD34 (KG1). Thus, the activation of the CD34 promoter appeared dependent on whether the cell was of hematopoietic origin.

It is likely that the regulation of the CD34 promoter was dependent on the three MZF-1 binding sites, because mutation of these three sites returned CD34 expression to baseline levels in hematopoietic cells. The relative contribution of each binding site, and whether the full or half-MZF-1 binding sites have different regulatory roles, remains to be established. Still, it appears that MZF-1 mediates transcription through binding to sequences homologous to previously defined consensus MZF-1 binding sites.

In earlier reports we described the cloning and characterization of the CD34 promoter.14,15,27 The CD34 promoter had activity in many cell types, including cells not of hematopoietic origin. Although there is a 3′ region that may enhance hematopoietic specific expression of CD34,27 there are probably other sequence elements not yet isolated that control the complete tissue specificity of the gene. Other regions could produce silencing of the gene when levels of CD34 decline. It should be noted that, although MZF-1 can regulate the CD34 promoter in vitro as shown here, proving it has a biologically relevant role in regulating CD34 expression in marrow progenitors requires more definitive analysis, such as a knock-out of the MZF-1 gene.

It is also quite possible that MZF-1 is just a part of the complex activation of the CD34 promoter; there may easily be additional activating factors in the appropriate cells. Lending evidence to this view is another study that found that c-myb and c-ets-2 also activated the CD34 promoter, although in nonhematopoietic cells.28 C-ets-2 is expressed ubiquitously, whereas c-myb’s expression pattern resembles MZF-1. There are sequences within the CD34 promoter that are homologous to ets and myb binding sites. How c-ets-2 and c-myb might interact with MZF-1 is not known. However, what is clear is that the regulation of CD34 gene expression is multifactorial.

It is possible that MZF-1 is a general myeloid transcription factor. Searches of other myeloid promoters found several consensus MZF-1 DNA binding sites. There was one MZF-1 binding site in the human myeloperoxidase promoter at −278, according to Morishita et al.29 Recombinant MZF-1 protein will bind to this site in a mobility shift assay. There was one binding site within the human lactoferrin promoter that perfectly matched the consensus for ZNF-1 at −82, according to Johnston et al.30 Recombinant MZF-1 protein will also bind to this sequence. How MZF-1 would function in the complex regulation of these promoters is not known. Both lactoferrin and myeloperoxidase are expressed at different stages of myeloid development as compared with CD34. However, MZF-1 is expressed during the stages that all three of these genes are expressed.9,10 It is possible that MZF-1 may function as a necessary but not sufficient activator of many myeloid genes. Stably transfecting an expression vector containing MZF-1 into nonmyeloid cell lines and assessing myeloid gene transcription may answer some of these questions.

Two additional features of MZF-1 add other dimensions to the data presented here. First, we have found that overexpression of MZF-1 aggressively transforms 3T3 cells.31 These transformed 3T3 cells traverse the cell cycle more rapidly, form foci, form colonies in soft agar, and form tumors in nude mice when compared with control 3T3 cells. Second, we have localized the 5′ end of MZF-1 to within 12 kb of the telomere of human chromosome 19q (data submitted and available upon request). There is evidence that, in primitive hematopoietic progenitor cells, the telomeres shorten with age.32 Thus, it is possible that the degradation of aging telomeres may disrupt MZF-1, playing a role in the increased incidence of hematopoietic dysfunction seen in the elderly. This interesting possibility also remains to be investigated.

In summary, MZF-1 has bifunctional transcriptional regulatory properties depending on the cellular environment. In addition, it appears to regulate the CD34 promoter through specific binding sites. Based on this, it is possible that MZF-1 may have a role in governing the primitive hematopoietic phenotype.

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The myeloid zinc finger gene, MZF-1, regulates the CD34 promoter in vitro

JF Morris, FJ 3rd Rauscher, B Davis, M Klemsz, D Xu, D Tenen and R Hromas