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Myeloid-specific transcriptional activation by murine myeloid zinc-finger protein 2

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ABSTRACT **Myeloid zinc finger protein 2 (MZF-2) is a zinc-finger transcription factor that is expressed in myeloid cells, particularly in the cells committed to the neutrophilic lineage. Here we examine the ability of murine MZF-2 (mMZF-2) to activate transcription. The mMZF-2 protein binds to a DNA element (MZF-binding site) through its zinc-finger domain. When the intact mMZF-2 was cotransfected with a reporter gene, it did not activate transcription. However, N-terminal deletion mutants greatly enhanced transcription specifically in myeloid cells. Furthermore, in an *in vivo* competition assay, the middle region of MZF-2 inhibited the mMZF-2-mediated transcription activation. These results suggest that mMZF-2 is a transcriptional factor that can specifically work in myeloid cells and can be divided into at least three functional domains. The N-terminal domain inhibits transactivation by masking the effect of the activation domain. The middle region recruits a coactivator, which is responsible for myeloid-specific transcriptional activation. The C-terminal zinc-finger domain functions as a DNA-binding domain.**

Hematopoiesis is controlled by the combined effects of extracellular signals that permit cellular proliferation and differentiation and of transcription factors that activate lineage-specific genes (1). Granulopoiesis, or development of neutrophils, is a well characterized model system of hematopoiesis. Granulocyte colony-stimulating factor (G-CSF) binds to its receptor to regulate proliferation and differentiation of the progenitor cells of neutrophilic granulocytes (2, 3). We and others have tried to reconstitute the system by introducing the cloned G-CSF receptor (G-CSFR) into various cell lines (4–6). When G-CSFR was exogenously expressed in myeloid precursor cells that normally do not express it, the transformants responded to G-CSF by proliferating and differentiating into neutrophils. However, when G-CSFR was introduced into pro-B cells, G-CSF stimulated the growth of the transformants, but it did not induce morphological differentiation (7). Furthermore, a T cell line expressing G-CSFR did not respond to G-CSF, not even by proliferating. These results indicate that cells must be committed to the neutrophilic lineage to respond G-CSF for neutrophilic differentiation.

Zinc-finger transcription factors play an important role in cellular commitment to specific lineages. Several have been identified in hematopoietic cells (1). For example, GATA-1, Egr-1, and Ikaros are specific zinc-finger transcription factors, which seem to determine the cell lineage of erythrocytes and megakaryocytes, macrophages, and lymphocytes, respectively (8–10). One candidate transcription factor for controlling the development of neutrophils that we and others have cloned is MZF (myeloid zinc-finger protein), which is expressed in myeloid cells, in particular in the cells committed to neutrophilic cell lineage (11–13). There are two forms of MZF, MZF-1 and MZF-2, which seem to be produced by alternative uses of two transcriptional initiation sites (11). Human MZF-1 (hMZF-1) is a protein of 485 amino acids that contains 13 Krüppel-type zinc fingers (12). Human and murine MZF-2 (hMZF-2 and mMZF-2) proteins differ in length, consisting of 775 and 814 amino acids, respectively. MZF-2 contains 13 zinc fingers in its C-terminal half, and a leucine-rich (LeR) domain (also called SCAN box) in its N-terminal region (11). Previously, Bavisotto *et al.* (13) reported that an antisense oligonucleotide of hMZF-1 inhibits G-CSF-induced neutrophilic colony formation in bone marrow cells, suggesting that MZF may play a role in the development of neutrophils.

In this report, we studied the transcriptional regulatory ability of mMZF-2 by cotransfecting various cell lines with a reporter gene carrying the MZF-binding site. Although the full-length mMZF-2 could not activate transcription, its N-terminal deletion mutants markedly enhanced transcription, suggesting the existence of an inhibitory domain in the N terminus and an activation domain in the middle region of the molecule. The mMZF-2-mediated enhancement of transcription was observed predominantly in myeloid cells, suggesting that MZF-2 is a transcription factor that can specifically work in myeloid cells. An *in vivo* competition assay suggested that a myeloid-specific cellular protein(s) binds to the activation domain of mMZF-2.

MATERIALS AND METHODS

Plasmid Construction. The reporter plasmid pBPA-TATA carrying the chloramphenicol acetyltransferase (CAT) gene downstream of mouse G-CSF promoter has been described previously (14). The CAT gene of pBPA-TATA was replaced by the luciferase gene of pUC18FOS-Luc (15) to generate pBPA-TATALuc. The following complementary oligonucleotides (F113B) carrying the binding sequence for hMZF-1 (16) were synthesized:

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GATCTAAAAGTGGGGAGAAAGGATCTGGCTGGTGAGGGGGATCG
    ATTTTCACCCCTCTTTCCTAGACCGACCACTCCCCCTTAGCCTAG
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The oligonucleotides were annealed, subcloned in pUC19BEX and tetramerized as described by Kamachi and Kondoh (17). The F113B tetramer was then inserted into the *Xho*I site of pBPA-TATALuc.

An expression vector, pEF-BOS-EX, which is a derivative of pEF-BOS (18), was constructed by inserting the following polylinker sequence into the *Eco*RI–*Xba*I portion in the stuffer region, and by disrupting the other *Eco*RI site at the poly(A) region.

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AATTCGGTACCGCGGCCGCCCGGGGATCCGCTAGXGTCGACT
     GCCATGGCACCGGCGGGCCCCTAGGCGATCGCAGCTGAGATC
EcoRI KpnI  NotI   SmaI BamHI NheI  SalI  XbaI
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Abbreviations: G-CSF, granulocyte colony-stimulating factor; G-CSFR, G-CSF receptor; LeR, leucine-rich region; MZF-2, myeloid zinc-finger protein 2; hMZF-2, human MZF-2; mMZF-2, murine MZF-2.

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The expression plasmids for the myc epitope-tagged mMZF-2 and its derivative were constructed as follows. Plasmids pmMZF2 and pKM3 carrying the full-length mMZF-2 cDNA and its truncated form, KM3, in pBluescript II have been described previously (11). They were digested with *Sal*I at the 5′ terminus, blunt-ended, and ligated with a fragment encoding the myc epitope (19), to generate pMYC-MZF2 and pMYC-KM3. These plasmids were digested with *Kpn*I and *Xba*I, and the fragment coding for the myc-tagged mMZF-2 or KM3 was inserted between the *Kpn*I and *Xba*I sites of pEF-BOS-EX to generate pEF/MYC-MZF2 and pEF/MYC-KM3. The blunt-end ligation of the myc epitope sequence to the *Sal*I site of pKM3 created a *Bam*HI site at the junction (Fig. 1). mMZF-2 cDNA carries a unique *Bam*HI site upstream of the zinc-finger domain (at nucleotide number 1507). The plasmid pEF/MYC-ZF1–13 coding for the zinc-finger domain was therefore prepared by deleting a 499-bp *Bam*HI fragment from pEF/MYC-KM3. To generate pEF/MYC-ΔLeR, pEF/MYC-MZF2 was digested either with *Stu*I at 663 and *Bam*HI at the 5′ end or partially with *Hin*dIII at 1022 and completely with *Eco*RI at the 3′ end. Oligonucleotides carrying the sequence of bases 663–680 with an overhanging *Hin*dIII site at the 3′-termini were synthesized. The *Bam*HI–*Stu*I fragment containing the myc and cDNA sequences (307–662), the oligonucleotides for bases 663–680, and the *Hin*dIII–*Eco*RI fragment containing the cDNA sequence of 1022–2869 were ligated between the *Bam*HI and *Eco*RI sites of pEF-BOS-EX. To construct pEF/MYC-ΔN64 and pEF/MYC-ΔN117, the fragments containing the cDNA sequences of 500–941 or 659–941 were amplified by PCR using an upstream primer containing a *Bam*HI site. The PCR products were digested with *Bam*HI in the upstream primer and *Hin*cII at nucleotide 904. The 603-bp *Bam*HI–*Hin*cII fragment carrying the 5′ portion (bases 307–903) of mMZF-2 cDNA in pEF/MYC-MZF2 was then replaced by each PCR product. To generate pEF/MYC-EX5, pMYC-KM3 was digested partially with *Pst*I at 1332 and completely with *Xba*I at the 3′ end. Complementary oligonucleotides carrying the sequence of nucleotides 1317–1331 with an overhanging *Bam*HI site and *Pst*I site were synthesized. The *Pst*I–*Xba*I fragment containing the cDNA sequence of 1332–2869 and the oligonucleotides for bases 1317–1331 were ligated to *Bam*HI–*Xba*I-digested pMYC-KM3.

To generate pEF/N1–124 and pEF/N1–238, pMYC-ΔLeR and pMYC-MZF2 were digested with *Hin*dIII and blunted, and a *Spe*I linker (CTAGACTAGTCTAG) carrying termination codons in three different frames was inserted. For pEF/N1–416 and pEF/N239–416, pMYC-MZF2 and pMYC-KM3 were digested with *Sph*I and *Eco*RI and blunted, and the *Spe*I linker was inserted as described above.

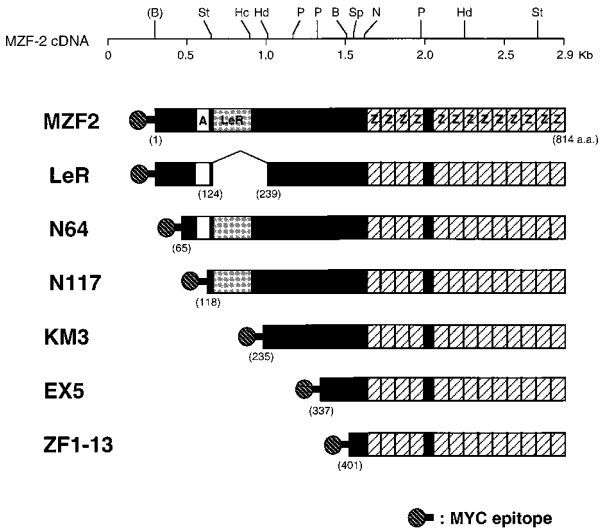

FIG. 1. The myc epitope-tagged mMZF-2 protein and its derivatives are shown schematically. The myc epitope is located at the N terminus. The LeR domain and zinc fingers are indicated by LeR and Z, respectively. At the top, the major restriction sites used for construction of the mMZF-2 mutants are indicated.

Transfection and Luciferase Assay. LGM-1, LG, LyD9 (20), and WR19L cells (ATCC TIB52) were grown in RPMI medium 1640 containing 10% fetal calf serum (GIBCO/BRL). NIH 3T3 (ATCC CRL 1658) and L929 cells (ATCC CCL 1.1) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Transfection of L-GM-1, LG, LyD9, and WR19L cells with plasmid DNA was carried out by electroporation (7). In brief, $4–6 \times 10^6$ cells were washed with serum-free RPMI medium 1640 containing 10 mM Hepes, resuspended in 0.25 ml of the same medium, and mixed with 5 μg of the reporter, 5 μg of the effector, and 1 μg of pSV-β-gal plasmid (Promega). After electroporation at 280 V with a capacitance of 960 μF by using a Gene Pulser (Bio-Rad), cells were placed for 10 min at room temperature, transferred into 5 ml of RPMI 1640 containing 10% fetal calf serum, and cultured for 24 or 48 h. Transfection of NIH 3T3 and L929 cells (5.0×10^5 cells) with 2.5 μg of the reporter, 2.5 μg of the effector, and 1 μg of pSV-β-gal were carried out by using the DEAE-dextran method as described (21) except that the glycerol shock step was omitted. For the *in vivo* competition assay, LGM-1 cells were transfected by electroporation with a total of 10 μg of DNA, which consists of 3.0 μg of the reporter, 1.0 μg of the effector, 1 μg of pSV-β-gal, an increasing amount of the competitor DNA, and a compensating amount of pEF-BOS-EX.

Preparation of the cell extracts and the luciferase assay were carried out as described (22). The β-galactosidase activity in the cell lysates was assayed by using methylumbelliferyl β-D-galactopyranoside (MUG) as described (23), and the luciferase activity of each sample was normalized to the β-galactosidase activity.

Immunoblotting and Gel Mobility Shift Assay. Expression plasmids for mMZF-2 or its derivatives were transfected into COS-7 cells by the DEAE-dextran method (21). The cells were cultured at 37°C for 48 h, and the nuclear extracts were prepared as described (24) except that all buffers contained a mixture of protease inhibitors (2.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml pepstatin, and 2.5 μg/ml of leupeptin). Immunoblotting was performed essentially as described previously (5). In brief, proteins were resolved by electrophoresis on a 4–20% or 10–20% gradient polyacrylamide gel (Dai-Ichi Pure Chemicals, Tokyo) in the presence of 0.1% SDS, and transferred onto GVHP membranes (Millipore). The membranes were incubated with 1000-fold diluted mouse anti-c-myc monoclonal antibody, 9E10 (Babco, Richmond, CA), then with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Babco). The myc-tagged proteins were visualized by the enhanced chemiluminescence system (Renaissance; DuPont/NEN).

The gel mobility shift assay was carried out as described (17). To prepare the probe for the gel shift assay, a 69-bp *Hin*dIII–*Bam*HI fragment, carrying a single copy of the F113B sequence described above, was excised from pUC19BEX and labeled with the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP. The labeled F113B probe ($1–2 \times 10^4$ cpm, about 1.0×10^6 cpm/pmol) was incubated with 25 μg of nuclear extract in binding buffer [25 mM Hepes–NaOH, pH 7.9/10% (vol/vol) glycerol/50 mM NaCl/100 μM $ZnSO_4$/0.1% Nonidet P-40/1 mM DTT/0.1 mg/ml poly(dI-dC)] for 20 min at room temperature. The mixtures were analyzed by electrophoresis on a native 5% polyacrylamide gel in 0.25× TBE as described (17).

RESULTS

DNA-Binding Activity of mMZF-2 and Its Deletion Mutants. mMZF-2 consists of several domains, including an acidic domain, an LeR domain, and a domain containing 13 zinc fingers (11). To examine the ability of mMZF-2 to activate transcription, expression plasmids for the intact mMZF-2 and its deletion mutants

were constructed. The ΔLeR mutant lacks the LeR domain, while ΔN64, ΔN117, KM3, EX5, and ZF1–13 are mutants carrying successive deletions from the N terminus of the protein (Fig. 1). To monitor the expression, all constructs were tagged with a myc epitope at the N terminus. Each construct was introduced into COS-7 and LG cells, and the nuclear extracts were analyzed by immunoblotting with anti-c-myc antibody. As shown in Fig. 2*A*, mMZF-2 and its derivatives, except for KM3, were expressed at a similar level in COS cells. The KM3 protein was expressed at level several times higher than the others. The molecular masses estimated by SDS/PAGE of mMZF-2 and ΔLeR, ΔN64, ΔN117, and KM3 were about 10 kDa larger than those calculated from their amino acid sequences. EX5 and ZF1–13 migrated in the gel to the positions predicted from the amino acid sequences. All constructs that showed abnormal mobility contained the amino acid sequence from positions 235–336 of mMZF-2. The effect on mobility is presumably due to the high proline content (15.7%) of this polypeptide and/or to posttranslational modification. The wild-type and mutant mMZF-2 proteins were also produced in LG cells in a similar fashion as in COS cells (data not shown), but the total amount of the protein synthesized in LG cells was less than that in COS cells.

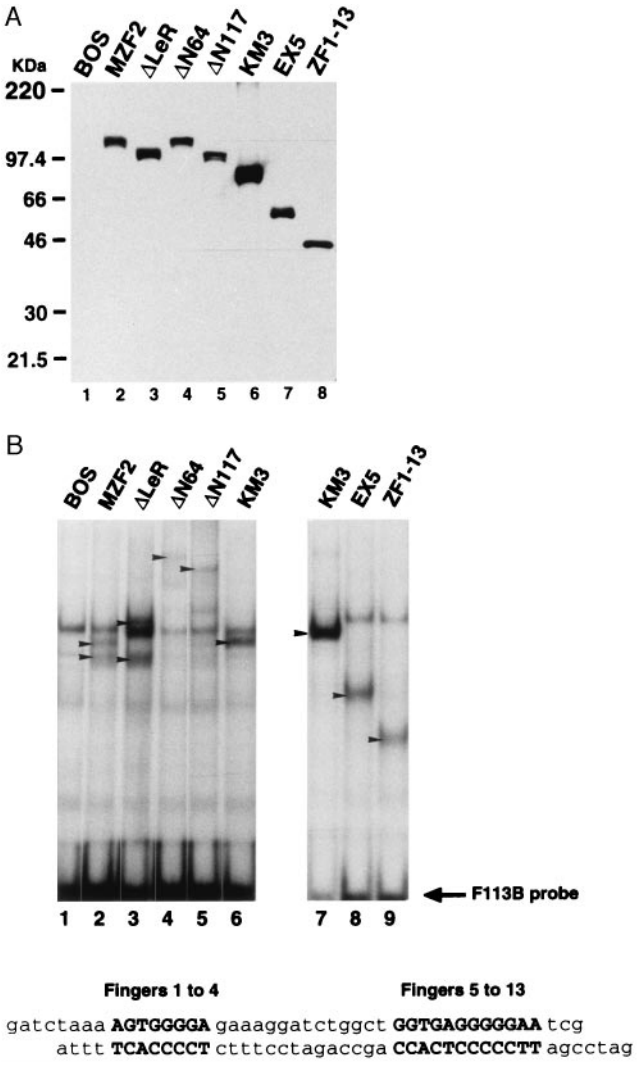

FIG. 2. Expression and DNA-binding activities of mMZF-2 and its derivatives. (*A*) Western Blotting of mMZF-2 and its derivatives. The expression plasmid without insert (lane 1), or with the insert for mMZF-2 (lane 2), or its derivatives (lanes 3–8) was introduced into COS-7 cells. Nuclear extracts (25 μg) were analyzed by Western blotting with anti-c-myc monoclonal antibody (9E10). The decorated bands were visualized with enhanced chemiluminescence. Migration positions of molecular mass standards are shown on the left. (*B*) Gel-shift assay. The nuclear extracts from COS cells transfected with the expression plasmid for mMZF-2 or its derivatives were analyzed by gel mobility-shift assay using a F113B probe carrying the MZF-1 consensus binding sequence. The positions of specific DNA–protein complex bands are indicated by arrowheads. At the bottom, the nucleotide sequence of the F113B fragment is shown; in it the consensus DNA-binding sequences for zinc fingers 1–4 and 5–13 are indicated by bold letters.

The zinc-finger transcription factors usually bind to the cognate DNA element through their zinc-finger domain. The consensus DNA-binding sequence for hMZF-1 was determined previously (16). Because the amino acid sequence of the zinc-finger domain of mMZF-2 is highly homologous to that of hMZF-1 (96% identity), it is likely that mMZF-2 binds to the same DNA element. To confirm this assumption, a DNA fragment (F113B) carrying the DNA-binding site for hMZF-1 was synthesized and used as a probe for a gel mobility-shift assay. As shown in Fig. 2*B*, nuclear extracts from the COS cells transfected with the mMZF-2 expression plasmid showed a retarded band, whose formation could be inhibited by competition with an excess of unlabeled F113B DNA (data not shown). When nuclear extracts from the COS cells transfected with the expression plasmids for MZF-2 derivatives were examined, they all showed specific retarded bands (Fig. 2*B*, lanes 2–9), indicating that the zinc-finger domain is responsible for binding of mMZF-2 to the DNA element. However, the intensities of the bands were relatively weak with mMZF-2 containing the LeR domain (intact mMZF-2, ΔN64, and ΔN117), which may indicate that the LeR domain has an inhibitory effect on the binding of mMZF-2 to the DNA element. Furthermore, the deletion of the N-terminal domain that has an inhibitory effect on the transcriptional activation (see below) caused retardation of the shifted band (Δ64). Because this region seems to fold to mask the activation domain in the middle of the molecule (discussed below, Fig. 6), this retardation is probably due to the conformational change of the molecule.

Myeloid-Specific Transcriptional Activation. To examine the ability of mMZF-2 to activate transcription, a reporter plasmid containing four copies of the MZF binding site upstream of the TATA box and the firefly luciferase gene was constructed (Fig. 3*A*). Because mMZF-2 mRNA is specifically expressed in myeloid cells (11), mouse myeloid leukemia LG cells were used as the host. When the intact mMZF-2 was cotransfected into LG cells together with the reporter gene, it did not activate transcription. However, some of its derivatives greatly activated transcription. As shown in Fig. 3*B*, the derivative lacking the LeR domain did not activate transcription. But the ΔN64, ΔN117, and KM3 constructs, in which the N-terminal 64, 117, and 234 amino acids were deleted, enhanced transcription 30-, 160-, and 100-fold, respectively. It should be noted that ΔN64 and ΔN117 constructs could only weakly bind to the MZF-2 binding site (Fig. 2*B*), yet they showed a very high transactivation (Fig. 3*B*). When the MZF-2 binding site was placed in the opposite orientation upstream of the TATA box, the mMZF-2 derivatives could still activate transcription (data not shown). However, the activation of transcription by ΔN64, ΔN117, and KM3 was not observed with the control reporter that lacked the MZF-2 binding site (Fig. 3*B*). When mMZF-2 was further deleted to amino acid positions 337 and 401 (EX5 and ZF1–13), the mutants did not show transactivation activity (Fig. 3*B*). The internal deletion of mMZF-2 (amino acids from 381 to 415) also abolished its ability to activate the transcription (data not shown). These results indicate that mMZF-2 has the ability to transactivate transcription through a domain of amino acids that includes residues 235–336, and that this activity seems to be masked by the N-terminal domain of 117 amino acids in intact mMZF-2. Furthermore, the LeR domain of mMZF-2 that can be found in various zinc-finger proteins plays no apparent role in regulating transcription, as measured by using this assay system.

To examine the tissue specificity of mMZF-2 in transactivating transcription, the expression plasmids for mMZF-2 and its de-

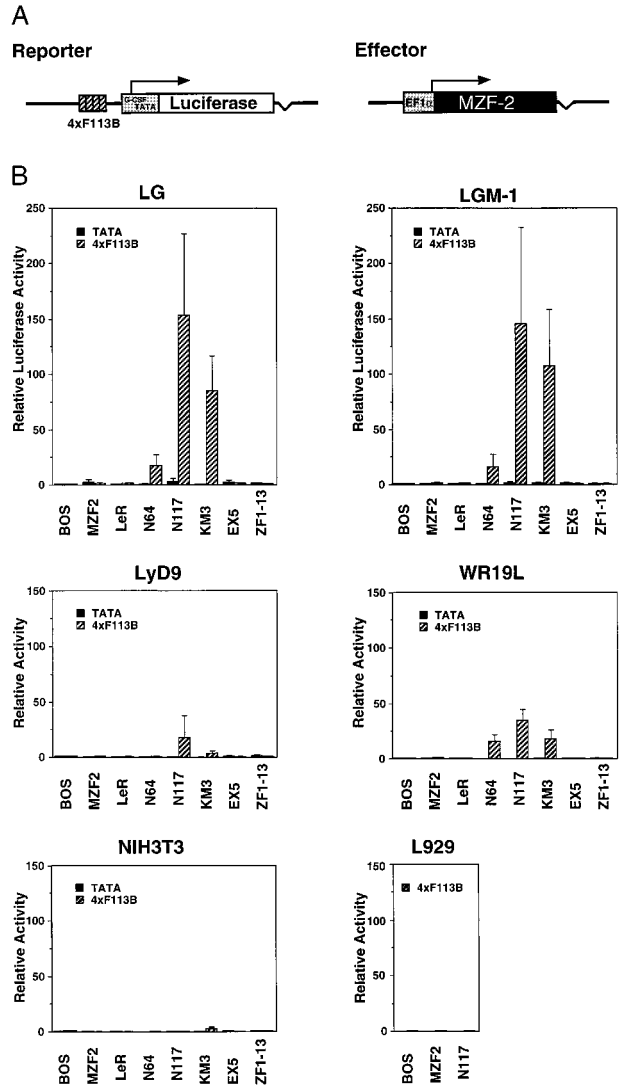

FIG. 3. Myeloid-specific transcriptional activation by mMZF-2. (*A*) Schematic drawing of the reporter and effector plasmids. The reporter plasmid (4xF113B) carries a tetramer of the MZF binding site (F113B) upstream of the TATA box of the murine G-CSF and luciferase genes. The reporter gene (TATA) without MZF-binding site was also used as a control. The effector plasmid harbors the coding sequence for the epitope-tagged mMZF-2 or its derivative under the human elongation factor (EF)-1α promoter. (*B*) Transcriptional activation by mMZF-2 and its derivatives. Mouse LG, LGM-1, LyD9, WR19L, NIH 3T3 and L929 cells were transfected with the indicated effector plasmids together with the reporter plasmids of either pBPA-4xF113B-Luc (4xF113B) or pBPA-TATALuc (TATA). As a control for transfection efficiency, a β-galactosidase plasmid (pSV-β-gal) was also included. The cells were harvested after 24 (LGM-1, LG, LyD9, WR19L) or 48 h (NIH 3T3, L929) for the luciferase assay. The luciferase activity of each sample was normalized to the β-galactosidase activity. Each bar represents the average of at least three independent transfection experiments. Luciferase activities detected in the presence of mMZF-2 or its derivatives are shown as values relative to the activity observed with the empty vector (pEF-BOS-EX, indicated as BOS). The absolute luciferase activity obtained with the reporter plasmid and the empty vector was low in any cell lines we have used.

rivatives were transfected into a myeloid cell line (LGM-1), a multipotent stem cell line (LyD-9), a T cell line (WR19L), and fibroblast cell lines (NIH 3T3 and L929) together with the reporter plasmid. As shown in Fig. 3*B*, the deletion mutants of mMZF-2 (ΔN64, ΔN117, and KM3) activated transcription in LGM-1 cells as potent as in LG cells. In contrast, their ability to activate transcription in LyD9 and WR19L cells was about 1/5 of that in LG cells, and almost no transactivation was observed in NIH 3T3 and L929 cells. These results indicate that mMZF-2 is a transcription factor that works preferentially in myeloid cells.

Involvement of a Cellular Factor(s) in the mMZF-2-Mediated Transactivation. The tissue-specific transcriptional activation by mMZF-2 suggested an involvement of a cellular factor(s) in this process. To examine this possibility, an *in vivo* competition assay was carried out (25–27). Four constructs: N1–124, N1–238, N1–415, and N235–415, which code for mMZF-2-derived polypeptides containing the amino acid sequences 1–124, 1–238, 1–416, and 239–416, respectively, were prepared (Fig. 4*A*). All constructs produced a similar amount of the protein in COS cells (data not shown). These mutant constructs were then introduced into LGM-1 cells, together with the reporter gene. The mutant proteins do not carry zinc fingers, thus alone they could have no effect on the transcription of the reporter gene (Fig. 4*B*). The transactivation ability of the intact mMZF-2 or ΔLeR was not potentiated by these constructs. In contrast, when these constructs were transfected into LGM-1 cells together with ΔN117 or KM3, the N235–415 construct that encodes the middle region of mMZF-2 (amino acids 235–415), inhibited the transactivation

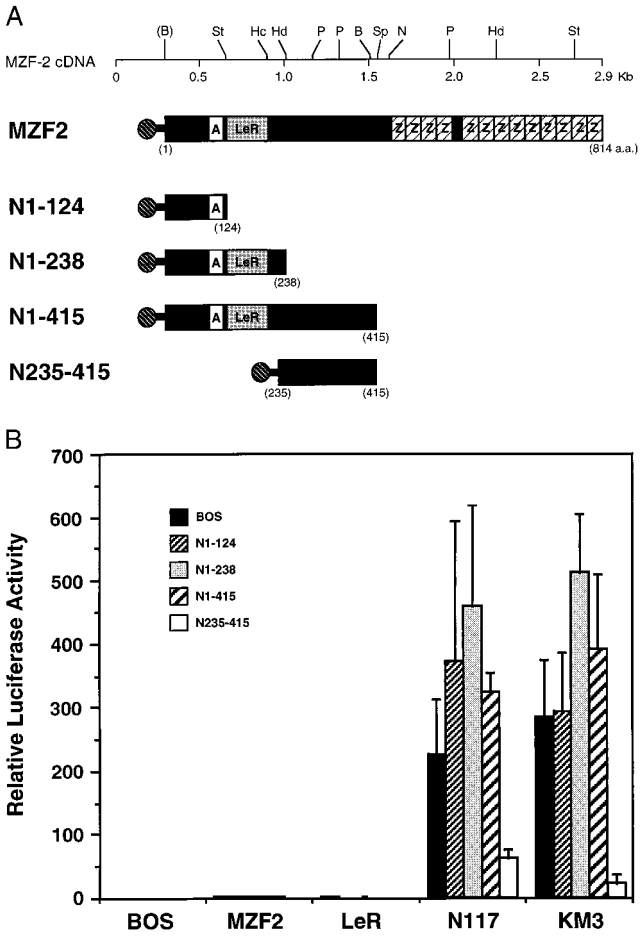

FIG. 4. *In vivo* competition assay for mMZF-2-mediated transactivation. (*A*) Schematic representation of the mMZF-2 derivatives used as competitors. (*B*) Inhibition of mMZF-2-mediated transactivation by competitors. LGM-1 cells were cotransfected with 4 μg of the reporter plasmid pBPA-4xF113B-Luc, 3 μg of the effector plasmids pEF-BOS (BOS, no insert), mMZF-2, ΔLeR, ΔN117 or KM3, and 3 μg of the competitor plasmids of pEF-BOS, N1–124, N1–238, N1–415, and N235–415. As a control, 1 μg of β-galactosidase plasmid (pSV-β-gal) was also included. At 24 h after transfection, the luciferase activities were determined as described above, and normalized with the value of β-galactosidase activity. The luciferase activity is expressed relative to that obtained with the empty effector plasmid (pEF-BOS). The average values from at least two independent experiments are shown with standard deviations (thin bars).

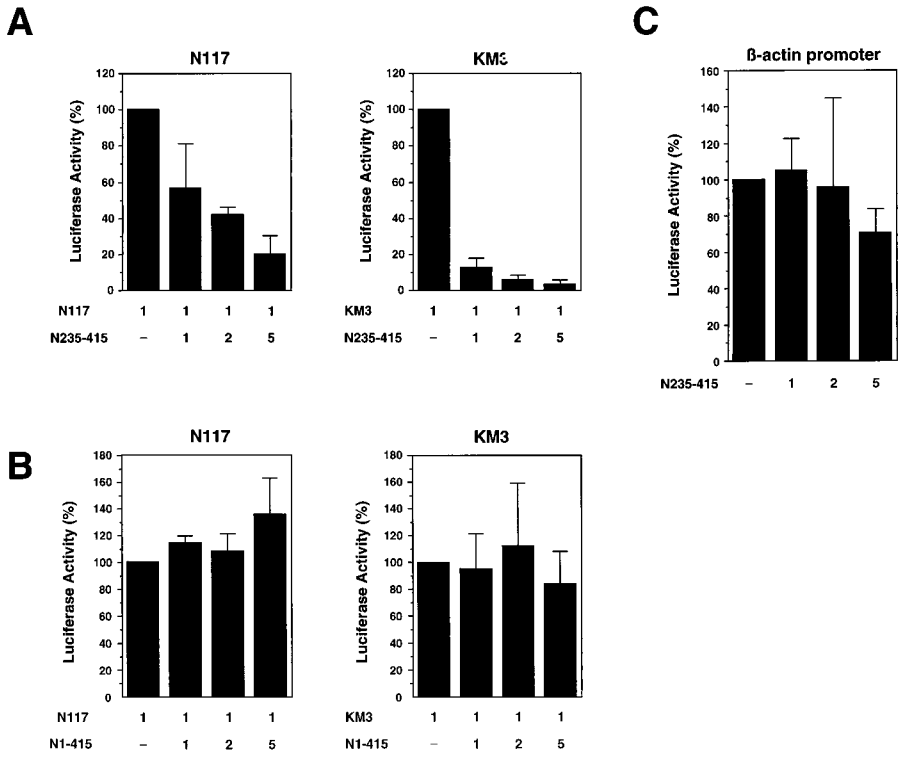

FIG. 5. Dose-dependent inhibition of transcription by competitors. (*A* and *B*) Effect of competitors on the ΔN117- and KM3-mediated transcriptional activation. LGM-1 cells were cotransfected with 3 μg of pBPA-4xF113B-Luc, 1 μg of ΔN117 or KM3, and increasing amounts of the competitor plasmids N235–415 (*A*) or N1–415 (*B*). The total amount of the competitor plasmid was kept at 5 μg by adding the empty expression plasmid. The transfection efficiency was monitored by including 1 μg of pSV-β-gal plasmid as described in the legend of Fig. 4. The luciferase activities are expressed as percentages of the values obtained in the absence of competitor plasmid. (*C*) Lack of effect of the competitor on transcription driven by β-actin promoter. LGM-1 cells were cotransfected with 0.5 μg of the reporter plasmid carrying the luciferase gene under the β-actin promoter, 1 μg of pSV-β-gal, and increasing amounts of N235–415. The luciferase activities are expressed as percentages of the value obtained without the addition of competitor. The assay was done at least three times, and the average values are shown with standard deviations (thin bars).

(Fig. 4*B*). The inhibitory action of N235–415 was dose-dependent, and it inhibited the KM3-mediated transactivation more efficiently than the ΔN117-mediated transactivation (Fig. 5*A*). N235–415 showed little effect on transcription driven by the β-actin promoter (Fig. 5*B*), suggesting that it is not a general inhibitor of transcription. In contrast to N235–415, the other constructs (N1–214, N1–238, N1–415) did not have a significant effect on the transactivation induced by ΔN117 or KM3 (Fig. 4*B*). In particular, the N1–415 construct, which shares the cDNA sequence encoding the amino acids from 235 to 415 with N235–415, did not inhibit the ΔN117 or KM3-mediated transactivation at any concentration (Fig. 5*C*). These results suggest that the middle region (amino acids 235–415) of mMZF-2 interacts with another cellular factor or factors to promote transcriptional activation. The N-terminal region, in contrast, may inhibit transcriptional activation by binding to the activation domain through an intramolecular mechanism and preventing the interaction of the activation domain with its cellular cofactor.

DISCUSSION

mMZF-2 is a zinc-finger transcription factor that is specifically expressed in myeloid cells (11). In this report, we showed that the mMZF-2 protein of 814 amino acids can be divided into three functional domains: one negative and one positive regulatory domain and the DNA-binding domain (Fig. 6). The negative regulatory region was assigned to the N-terminal portion of mMZF-2. Various transcription factors such as GAL4, c-jun, and myb contain such inhibitory regions. In the cases of GAL-4 and c-jun, an inhibitory protein(s) binds to this region and inhibits transcription (26–31). The negative regulatory domain of c-myb is phosphorylated, which inhibits the ability of c-myb to activate the transcription of the CD34 promoter (32). In the case of mMZF-2, its N-terminal region inhibited the mMZF-2-induced transactivation of transcription, but an *in vivo* competition assay with the N-terminal domain did not rescue the inhibition. These results suggest that the N-terminal domain-mediated inhibition of the transactivation is due to an intramolecular interaction with its own activation domain rather than to the recruitment of an inhibitory factor(s). It is possible that an external signal(s) post-translationally modifies the inhibitory or activation domains of mMZF-2, which prevents the interaction of the inhibitory domain with the activation domain.

When the N-terminal inhibitory domain was deleted from mMZF-2, the truncated mMZF-2 greatly activated transcription in a myeloid cell-specific manner. Previously, Morris *et al.* (33) reported that hMZF-1, another form of MZF, activates transcription in hemopoietic cells. However, its enhancement of transcription was at most severalfold. Here, a greater than 100-fold enhancement of transcription was observed with the mMZF-2 construct that coded for amino acid residues 235–415. When a further deleted form of mMZF-2 (EX5) that corresponds to MZF-1 was examined for its transactivation ability, it showed very little transcription activation, indicating that the domain consisting of the amino acid residues 235–415 is necessary for the full transactivation. As found with the activation domains of various transcription factors (34), the activation domain of mMZF-2 is rich in proline, serine, and threonine, and is well conserved (62.4% identity) between murine and human MZF-2s. Recently, cellular proteins binding to the transcriptional activation domain have been identified in several systems (34, 35). Some of them are general transcription factors, whereas others are coactivators that relay the activation signal to a general transcription factor. The ability of mMZF-2 to transactivate transcription was myeloid-cell-specific, indicating that the protein(s) binding to the activation domain of mMZF-2 may not be

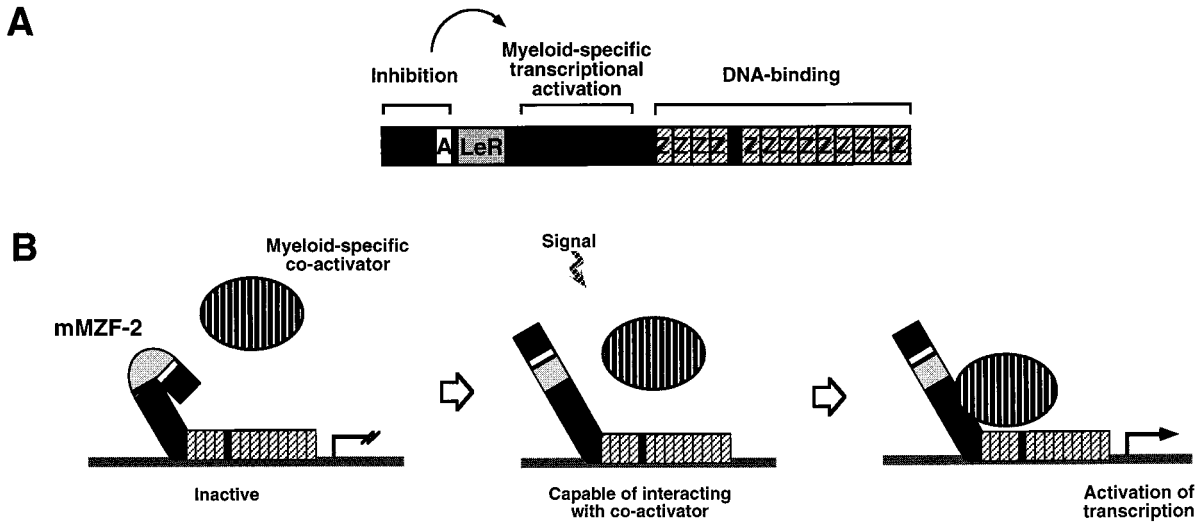

FIG. 6. A model for MZF-2-mediated transcriptional activation. (*A*) The structure of mMZF-2 is schematically shown. It carries an inhibitory region at the N terminus, an activation domain in the middle of the molecule, and a DNA-binding domain consisting of 13 zinc-finger motifs (Z). A and LeR indicate an acidic region and leucine-rich region, respectively. (*B*) Activation of mMZF-2. In the resting stage, the inhibitor domain of MZF-2 masks its activation domain. A signal, not yet identified, releases the activation domain, and a myeloid-specific coactivator(s) binds to the activation domain. MZF-2 then activates transcription.

a general transcription factor(s). In any case, the ability of the activation domain to inhibit transactivation in the *in vivo* competition assay suggests that the molecule interacting with the activation domain of mMZF-2 can be analyzed by biochemical methods.

The C-terminal half of mMZF-2 carries 13 zinc fingers, which can be divided into two clusters, zinc fingers 1–4 and 5–13. Using an *in vitro* selection assay from a pool of random oligonucleotides, Morris *et al.* (16) reported that the first cluster of zinc fingers binds to AGTGGGGA, whereas the second cluster binds to CGGGNGAGGGGGAA. The intact mMZF-2 protein and its derivatives containing the zinc-finger domains bound to the DNA element containing these sequences, suggesting this domain is responsible for MZF-2 binding its DNA element. Because MZF-2 functions specifically in myeloid cells, it is reasonable to assume that the target genes of MZF-2 are those that are specifically transcribed in myeloid cells. Although the consensus binding sequence of MZF can be found on the promoter of some myeloid-specific genes such as those encoding CD34, c-myb, lactoferrin, and myeloperoxidase (16), it is not well established yet whether these genes are actually regulated by MZF-2. In this study, we showed that overexpression of the activation domain of MZF-2 inhibits the MZF-2-mediated transactivation, suggesting that this dominant-negative form of MZF-2 can be used to investigate MZF-2-regulated genes. In fact, transformants overexpressing the activation domain could not express myeloperoxidase upon G-CSF stimulation, suggesting that myeloid-specific myeloperoxidase gene is directly or indirectly regulated by MZF-2 gene (K.M. and S.N., unpublished results). Furthermore, the well conserved LeR domain in the N-terminal half of mMZF-2 was dispensable for ability of MZF-2 to inhibit and/or activate transcription. However, the role of the LeR domain should be reanalyzed, using the promoter of an actual target gene.

In summary, MZF-2 is a transcription factor that bears negative and positive regulatory domains and works rather specifically in myeloid cells. For MZF-2 to be activated, the inhibition must be released, and a coactivation factor must bind to its activation domain. Such complex regulation of its activity may indicate that mMZF-2 functions transiently in response to some signals during myeloid development. To understand the role of mMZF-2 in myeloid cell development, it will be necessary to identify the signal(s) that inactivate the negative regulatory domain, the cofactor(s) that binds to the activation domain, and MZF-2's target genes.

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1. Orkin, S. H. (1995) *J. Biol. Chem.* **270,** 4955–4958.
2. Nicola, N. A. (1989) *Annu. Rev. Biochem.* **58,** 45–77.
3. Nagata, S. (1994) In *The Cytokine Handbook*, ed. Thomson, A. W. (Academic, London), 2nd Ed., pp. 371–385.
4. Fukunaga, R., Ishizaka-Ikeda, E. & Nagata, S. (1993) *Cell* **14,** 1079–1087.
5. Yoshikawa, A., Murakami, H. & Nagata, S. (1995) *EMBO J.* **14,** 5288–5296.
6. Dong, F., van Buitenen, C., Pouwels, K., Hoefsloot, L. H., Löwenberg, B. & Touw, I. P. (1993) *Mol. Cell. Biol.* **13,** 7774–7781.
7. Fukunaga, R., Ishizaka-Ikeda, E., Pan, C.-X., Seto, Y. & Nagata, S. (1991) *EMBO J.* **10,** 2855–2865.
8. Orkin, S. (1992) *Blood* **80,** 575–581.
9. Georgopoulos, K., Bigby, M., Wang, J. H., Molnar, A., Wu, P., Winandy, S. & Sharpe, A. (1994) *Cell* **79,** 143–156.
10. Nguyen, H. Q., Hoffman, L. B. & Liebermann, D. A. (1993) *Cell* **72,** 197–209.
11. Murai, K., Murakami, H. & Nagata, S. (1997) *Genes to Cells* **2,** 581–591.
12. Hromas, R., Collins, S. J., Hickstein, D., Raskind, W., Deaven, L. L., O'Hara, P., Hagen, F. S. & Kaushansky, K. (1991) *J. Biol. Chem.* **266,** 14183–14187.
13. Bavisotto, L., Kaushansky, K., Lin, N. & Hromas, R. (1991) *J. Exp. Med.* **174,** 1097–1101.
14. Nishizawa, M. & Nagata, S. (1990) *Mol. Cell. Biol.* **10,** 2002–2011.
15. Kato, G. J., Barrett, J., Villa, G. M. & Dang, C. V. (1990) *Mol. Cell. Biol.* **10,** 5914–5920.
16. Morris, J. F., Hromas, R. & Rauscher, F. 3. (1994) *Mol. Cell. Biol.* **14,** 1786–1795.
17. Kamachi, Y. & Kondoh, H. (1993) *Mol. Cell. Biol.* **13,** 5206–5215.
18. Mizushima, S. & Nagata, S. (1990) *Nucleic Acids Res.* **18,** 5322.
19. Evan, G. I., Lewis, G. K., Ramsay, G. & Bishop, J. M. (1985) *Mol. Cell. Biol.* **5,** 3610–3616.
20. Lee, K. H., Kinashi, T., Tohyama, K., Tashiro, K., Funato, N., Hama, K. & Honjo, T. (1991) *J. Exp. Med.* **173,** 1257–1266.
21. Fukunaga, R., Ishizaka, I. E., Seto, Y. & Nagata, S. (1990) *Cell* **61,** 341–350.
22. Shibuya, H., Kohu, K., Yamada, K., Barsoumian, E. L., Perlmutter, R. M. & Taniguchi, T. (1994) *Mol. Cell. Biol.* **14,** 5812–5819.
23. Young, D. C., Kingsley, S. D., Ryan, K. A. & Dutko, F. J. (1993) *Anal. Biochem.* **215,** 24–30.
24. Schreiber, E., Matthias, P., Muller, M. M. & Schaffner, W. (1989) *Nucleic Acids Res.* **17,** 6419.
25. Chen, X. & Bieker, J. J. (1996) *EMBO J.* **15,** 5888–5896.
26. Baichwal, V. R. & Tjian, R. (1990) *Cell* **63,** 815–825.
27. Baichwal, V. R., Park, A. & Tjian, R. (1992) *Genes Dev.* **6,** 1493–1502.
28. Johnston, S. A., Salmeron, J. J. & Dincher, S. S. (1987) *Cell* **50,** 143–146.
29. Lohr, D., Venkov, P. & Zlatanova, J. (1995) *FASEB J.* **9,** 777–787.
30. Lue, N. F., Chasman, D. I., Buchman, A. R. & Kornberg, R. D. (1987) *Mol. Cell. Biol.* **7,** 3446–3451.
31. Ma, J. & Ptashne, M. (1987) *Cell* **50,** 137–142.
32. Miglarese, M. R., Richardson, A. F., Aziz, N. & Bender, T. P. (1996) *J. Biol. Chem.* **271,** 22697–22705.
33. Morris, J. F., Rauscher, F. J., III, Davis, B., Klemsz, M., Xu, D., Tenen, D. & Hromas, R. (1995) *Blood* **86,** 3640–3647.
34. Burley, S. & Roeder, R. (1996) *Annu. Rev. Biochem.* **65,** 769–799.
35. Goodrich, J., Cutler, G. & Tjian, R. (1996) *Cell* **84,** 825–830.