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Sequence-specific transcriptional repression by an MBD2-interacting zinc finger protein MIZF

Masayuki Sekimata* and Yoshimi Homma

Department of Biomolecular Sciences, Institute of Biomedical Sciences, Fukushima Medical University School of Medicine, Fukushima 960-1295, Japan

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ABSTRACT

MBD2 is a member of the methyl-CpG-binding protein family that plays an important role in methylated DNA silencing. We have recently identified a novel zinc finger protein, MIZF, as an MBD2-binding partner. To understand the physiological function of MIZF in MBD2-mediated gene silencing, we investigated the DNA-binding properties of MIZF and its potential target genes. Using a cyclic amplification and selection of targets technique, the consensus sequence CGGACGTT, which contains a conserved CGGAC core, was determined as sufficient for MIZF binding. Deletion of individual zinc fingers revealed that five of the seven zinc fingers are required for DNA binding. Reporter assays demonstrated that MIZF represses transcription from the promoter including this DNA sequence. A database search indicated that a variety of human genes, including Rb, contain this sequence in their promoter region. MIZF actually bound to its recognition sequence within the Rb promoter and repressed Rb transcription. These results suggest that MIZF, through its DNA-binding activity, acts as a sequence-specific transcriptional repressor likely involved in MBD2-mediated epigenetic gene silencing.

INTRODUCTION

DNA methylation at CpG dinucleotides is associated with gene silencing and the formation of transcriptionally inactive chromatin enriched in deacetylated histones (1–3). Methyl-CpG-binding domain (MBD) family proteins, such as MeCP2 and MBD2, serve as a molecular link between DNA methylation and deacetylated histones by recruiting a co-repressor complex, Sin3, containing histone deacetylase (HDAC) (4–7). MBD2 has also been shown to recruit another multifunctional complex, Mi-2/NuRD, which possesses both HDAC and chromatin remodeling activities (4). This combination of MBD2 and Mi-2/NuRD is equivalent to the originally known MeCP1 repression complex (4,8). Alteration of the local chromatin structure via recruitment of these HDAC complexes may be one of the general mechanisms by which MBD proteins mediate transcriptional repression. On the other hand, accumulating evidence indicates that additional factors may be involved in methylation-dependent transcriptional repression (9–11). Recently, Kaiso, which associates with the p120 catenin, was revealed to be one component of the MeCP1 complex and a novel DNA-binding transcriptional repressor that recognizes both the specific consensus sequence and methyl-CpG dinucleotides (12). Therefore, the participation of repressor molecules, together with the HDAC pathway, may be a mechanism for MBD-mediated gene silencing. This idea is consistent with conventional findings that a specific HDAC inhibitor trichostatin A partially relieves the repressive effect of MeCP2 and MBD2 (1,2,8,13–15).

Previously, we have reported that MIZF, which we identified as a novel MBD2-binding partner, is a unique C2H2-type zinc finger protein involved in MBD2-mediated transcriptional repression (16). The C2H2 zinc finger motif was initially found in transcription factors, such as TFIIIA and Krüppel, and at present a number of transcription factors are known to utilize C2H2 zinc fingers as DNA-binding domains (17). Thus, it is possible that MIZF, like Kaiso, is a transcriptional repressor that binds to a specific DNA sequence. To address this issue, characterization of the DNA-binding properties of MIZF and identification of its potential target genes are required. In the present study, we determined a MIZF-specific DNA-binding sequence using a method for cyclic amplification and selection of targets (CASTing) (18) and showed that MIZF, through its DNA-binding activity, acts as a sequence-specific transcriptional repressor likely involved in MBD2-mediated gene silencing.

MATERIALS AND METHODS

Plasmids

The expression plasmid for glutathione *S*-transferase (GST) fusion proteins, pGST-MIZF, has been described (16). For the expression of His-tagged MIZF proteins, a full-length or truncated MIZF cDNA was subcloned into pET-15b (Novagen, Madison, WI) to obtain pET-MIZF or pET-MIZF-201–517. The FLAG-tagged full-length and the N-terminal MIZF constructs, pFLAG-MIZF and pFLAG-MIZF-1–201, were generated using pcDNA3 (Invitrogen, Carlsbad, CA). The myc-tagged MIZF constructs containing deletions of individual zinc fingers were generated by PCR. The human DNA polymerase β (pol β) promoter-luciferase

*To whom correspondence should be addressed. Tel: +81 24 547 1660; Fax: +81 24 548 3041; Email: matam@fmu.ac.jp

reporter construct, pGL3-polβ, was generated by inserting the SacI–HindIII fragment containing the pol β promoter from pGEM-polβ (16) into the corresponding sites of pGL3-enhancer (Promega, Madison, WI). Plasmid pGL3-M5polβ was constructed by inserting five copies of the MIZF-binding sequence upstream of the pol β promoter in pGL3-polβ. The Rb promoter-luciferase reporter construct, pGL3-Rb, was obtained by PCR amplification of the human Rb promoter between –507 and –33 relative to the translation start site as described elsewhere (19). The mutated Rb promoter-luciferase reporter constructs, pGL3-Rbm1 and pGL3-Rbm2, were obtained by site-directed mutagenesis of the putative MIZF-binding sites in pGL3-Rb using the primers 5′-GAGCCTCGCGTACGTGACGCCGC-3′ and 5′-GGAGGGCGCGTACGGTTTTTCTC-3′ (base changes underlined), respectively.

Binding site selection

CASTing was performed as described previously with some modifications (18). Single-stranded oligonucleotides containing a 23 bp random core sequence flanked on each side by 16 bp (5′-GCACTAGCGGATCCGT-N_{23}-CGAAGCTTGGTCACGC-3′) were synthesized. A double-stranded oligonucleotide library was generated by primer extension with the reverse primer (5′-GCGTGACCAAGCTTCG-3′). For the first round of capture, the library was mixed with an excess of GST-MIZF bound to glutathione–Sepharose beads in 100 µl of binding buffer containing 25 mM HEPES (pH 7.9), 50 mM KCl, 4 mM $MgCl_2$, 10% glycerol, 25 µM $ZnCl_2$, 250 µg/ml bovine serum albumin (BSA), 1 mM dithiothreitol (DTT), 250 µg/ml poly(dI·dC) and protease inhibitors. The mixture was incubated at room temperature for 30 min with continual rotation. After washing three times with binding buffer, the bound oligonucleotides were eluted by incubation of the beads with 148 µl of TE buffer and 2 µl of 10% SDS at 95°C for 10 min, precipitated and then amplified by PCR. The same procedure was repeated for five additional rounds, at which time the final PCR products were digested with BamHI and HindIII, subcloned into the corresponding sites of pBluescript (Stratagene, La Jolla, CA) and sequenced.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described (20) from HEK293 cells. Bacterial expression and purification of GST, GST-MIZF and His-MIZF proteins were carried out as previously described (16). A wild-type (Wt) probe was obtained by annealing two complementary oligonucleotides, 5′-CGCGGGAGCGGACGTTATTACCT-3′ and 5′-AGGTAATAACGTCCGCTCCCGCG-3′. A non-identical (Ni) probe was obtained by annealing two complementary oligonucleotides, 5′-ATGTTATGGTCGCGCATTTGCCG-3′ and 5′-CGGCAAATGCGCGACCATAACAT-3′. The other oligonucleotides used for EMSA analysis as labeled probes and competitors are listed in Figure 5. The probes were end-labeled using T4 polynucleotide kinase and [γ-^{32}P]dATP. Binding reactions were carried out for 10 min at room temperature using 500 ng of purified recombinant proteins in a final volume of 10 µl containing 10 mM HEPES (pH7.9), 1 mM DTT, 5 mM $MgCl_2$, 0.5 mM $ZnCl_2$, 60 mM KCl, 0.05% Nonidet P-40, 200 ng poly(dI·dC), 10% glycerol and 50 µg/ml BSA. Unlabeled double-stranded oligonucleotides were added as competitors at 20- or 200-fold molar excess. The DNA–protein complexes were resolved on 6% polyacrylamide gels in 0.5× Tris–glycine buffer (12.5 mM Tris and 100 mM glycine). In some reactions, an anti-MIZF antibody or normal rabbit serum was added prior to incubation with the probe.

Transfection, western blotting and luciferase assay

HEK293 cells were transiently transfected with various expression vectors using an Effectene reagent (Qiagen, Hilden, Germany). Expression levels of the expected proteins were confirmed by western blot analysis (16). Luciferase reporter assays were carried out in 24-well plates as described previously (16). The luciferase activity of each sample was determined and normalized using a dual luciferase reporter assay system (Promega).

Antibody production

An anti-MIZF antibody was raised in a rabbit using a recombinant His-MIZF-201–517 protein. The specificity of the antibody was checked for its ability to immunoprecipitate MIZF expressed in HEK293 cells and to detect MIZF by western blot analysis.

Chromatin immunoprecipitation (ChIP) assays

Cells (5×10^5) were fixed with 1% formaldehyde for 10 min. Crude cell lysates were sonicated to generate 200–1000 bp DNA fragments. After centrifugation, the supernatants were diluted 10-fold with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.1, and 167 mM NaCl) and incubated with an anti-MIZF antibody at 4°C overnight. Immunocomplexes were collected with protein A or G plus agarose beads and eluted by adding 200 µl of 1% SDS in 0.1 M $NaHCO_3$ and cross-links were reversed by heating at 65°C. Following proteinase K digestion, phenol/chloroform extraction and ethanol precipitation, the samples were subjected to 35 cycles of PCR amplification using the Rb promoter-specific primers 5′-GAGCCTCGCGGACGTGACGCCGC-3′ and 5′-TGGAGGAGCGCCGGGGAGGACG-3′.

RESULTS

Selection of a consensus MIZF binding sequence

The zinc finger domain of MIZF conforms to a classic C2H2 zinc finger domain with sequence-specific DNA-binding potential. To examine the binding sequence for MIZF, we employed CASTing analysis (18). Purified GST-MIZF was incubated with a random pool of double-stranded 55mer oligonucleotides, each of which carried 16 base fixed end sequences flanking 23 central bases of random sequence. Bound oligonucleotides were purified and subjected to PCR amplification using primers corresponding to the 16 fixed bases at both ends of the 55mer oligonucleotides. After six rounds of purification, 20 different oligonucleotides were cloned and sequenced. A consensus sequence CGGACGTT was derived from the sequence alignment of individual clones (Fig. 1A), and the CGGAC sequence therein was considered to be the core element.

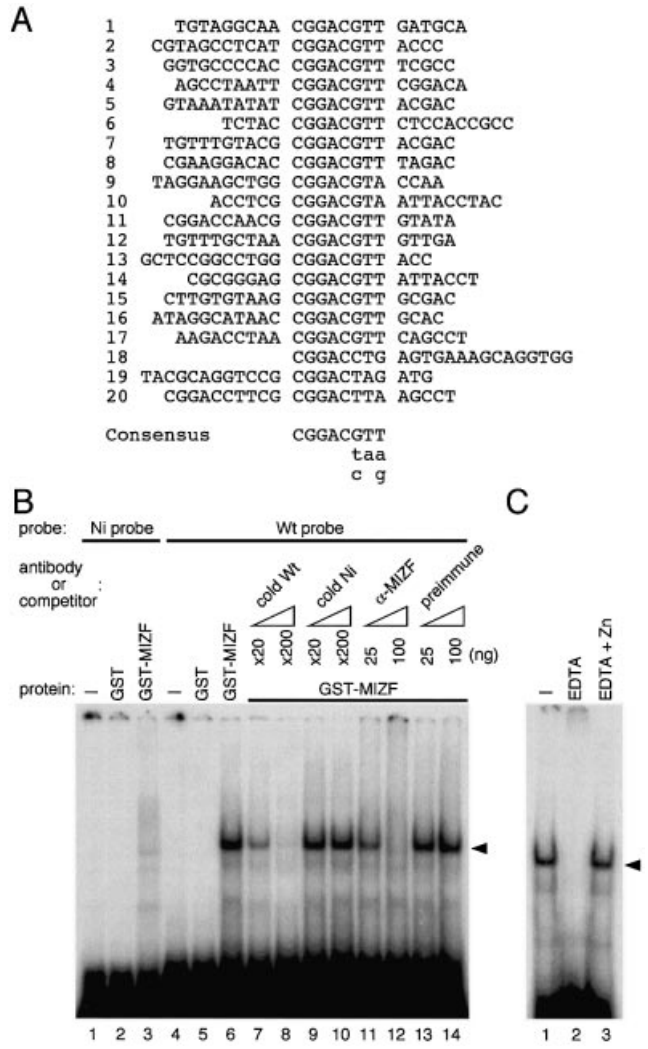

Figure 1. Binding of MIZF to a specific DNA sequence. (**A**) Alignment of individual DNA sequences recovered in the CASTing analysis. The deduced consensus MIZF-binding sequence, where the upper case letters indicate the preferred nucleotides, is shown below the individual aligned sequences. (**B**) EMSA analysis of the MIZF-binding sequence. EMSA was performed with a labeled Wt probe and either no added protein (lane 4), GST alone (lane 5) or GST-MIZF (lanes 6–14). A labeled Ni probe was incubated with either no added protein (lane 1), GST alone (lane 2) or GST-MIZF (lane 3). A 20- or 200-fold molar excess of unlabeled Wt probe (lanes 7 and 8) or Ni probe (lanes 9 and 10) was added to the binding reactions. An anti-MIZF antibody (lanes 11 and 12) or preimmune serum (lanes 13 and 14) was added to the binding reactions as indicated. (**C**) The effect of zinc ions on the binding specificity of MIZF. GST-MIZF proteins were incubated with a labeled Wt probe in the absence or presence of 10 mM EDTA (lanes 1 and 2) or in the presence of 10 mM EDTA and 50 mM $ZnCl_2$ (lane 3). The arrowhead indicates the MIZF complex.

To examine the binding specificity of MIZF for this consensus sequence, we performed EMSA analysis using an oligonucleotide corresponding to the insert sequence from clone 14 (wild-type probe, Wt) or an oligonucleotide identical in length but different in sequence (non-identical probe, Ni). As shown in Figure 1B, a single DNA–protein complex was formed by incubation of GST-MIZF with the labeled Wt probe (lane 6), but not with the labeled Ni probe (lane 3). In contrast, no complex formation was detected with the GST protein alone (lanes 2 and 5). Increasing amounts of the unlabeled Wt probe resulted in a gradual diminution of complex formation, whereas the unlabeled Ni probe did not interfere with complex formation (lanes 7–10). Moreover, the complex was disrupted by an antiserum against MIZF but not by preimmune serum (lanes 11–14). The binding specificity of MIZF was also confirmed by use of EDTA to abolish binding (Fig. 1C, lane 2). Complex formation was restored upon the addition of excess zinc ions (Fig. 1C, lane 3). Similar results were also obtained using recombinant His-MIZF (data not shown). These results indicate that MIZF interacts with this consensus sequence and that correct folding of the zinc finger of MIZF is necessary for this interaction.

Analysis of the zinc finger motifs required for DNA binding

To define which of seven zinc finger motifs within the MIZF proteins are required for DNA binding, we generated MIZF deletion constructs that lack the individual zinc finger motifs (Fig. 2A). Nuclear extracts from HEK293 cells transfected with these constructs were used for EMSA analysis. Mutant proteins within the nuclear extracts were as stable as the wild-type protein, as determined by western blot analysis (Fig. 2B). The conformational change may, at least in part, account for the discrepancy between the apparent and theoretical molecular weights of two deletion mutants (ΔZF1-myc and ΔZF3-myc). The result in Figure 2C revealed that zinc fingers 1–4 and 7 are required whereas zinc fingers 5 and 6 are dispensable for DNA binding. On the other hand, enhanced binding over the wild-type was observed in the ΔZF6-myc mutant. This may suggest a destabilizing effect of zinc finger 6.

Sequence-specific transcriptional repression by MIZF

To assess whether the MIZF-binding site affects gene transcription, we performed reporter gene assays using pGL3-M5polβ, which contains a luciferase gene driven by a DNA pol β promoter proximal to five copies of the MIZF-binding site. We transiently introduced pGL3-M5polβ and a construct expressing a full-length (pFLAG-MIZF) or N-terminal MIZF (pFLAG-MIZF-1–201) into HEK293 cells (Fig. 3A and B). Co-transfection of pFLAG-MIZF decreased the promoter activity of the reporter plasmid in a dose-dependent manner (Fig. 3C). In contrast, pFALG-MIZF-1–201 failed to repress transcription. This repression was specific, because co-transfection of a control reporter construct lacking the MIZF-binding sites with pFLAG-MIZF had no effect on luciferase activity. The C-terminus of MIZF contains the putative transcriptional repression domain that can interact with MBD2 (16). These results suggest that repression might occur in a sequence-specific manner through a co-repressive interaction mediated by the C-terminal half of MIZF.

Repression of the Rb promoter by MIZF

Identification of the downstream target genes for MIZF may provide a clue to its biological function in gene regulation. Therefore, we next searched for potential target genes that might be regulated by MIZF and found that a variety of genes, including human Rb, possess the recognition sequence in their 5′-upstream regulatory region (Table 1). Since the human and mouse Rb genes contain two MIZF-binding sites within the

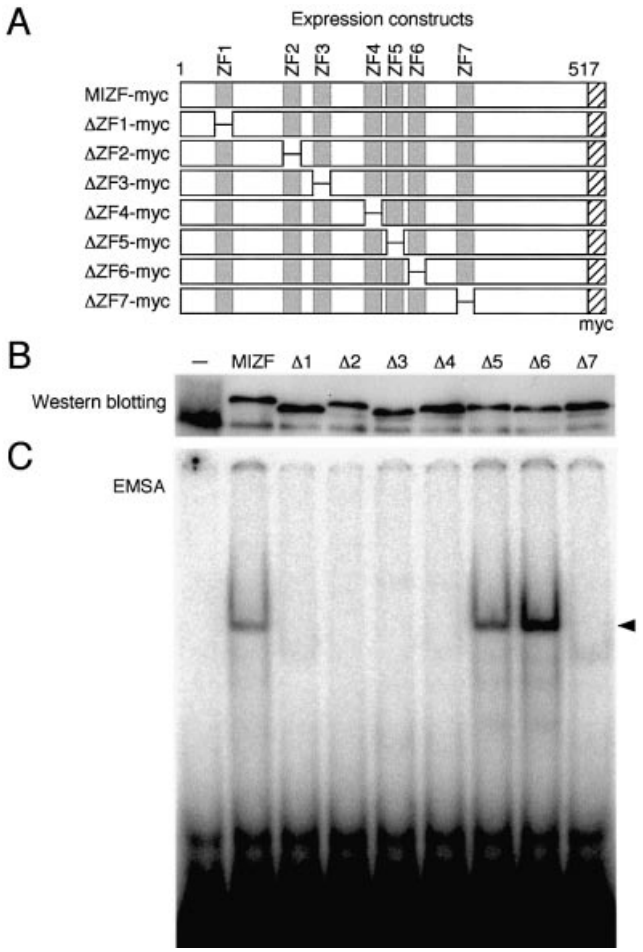

Figure 2. Mapping of the zinc finger motifs required for interaction with the MIZF-binding sequence. (**A**) Schematic representation of the MIZF expression plasmids used for EMSA analysis. The shaded boxes represent zinc finger motifs. (**B**) Protein expression analysis of the myc-tagged MIZF constructs shown in (A). The various MIZF deletion proteins within nuclear extracts from HEK293 cells were detected by western blotting with an anti-myc antibody. Note that all of the deletion proteins are expressed at similar levels to the wild-type protein. (**C**) EMSA analysis with various MIZF constructs lacking the individual zinc fingers. EMSA was performed with a labeled Wt probe and 2 mg of nuclear extracts from HEK293 cells expressing the various MIZF deletion proteins. The arrowhead indicates the MIZF complex.

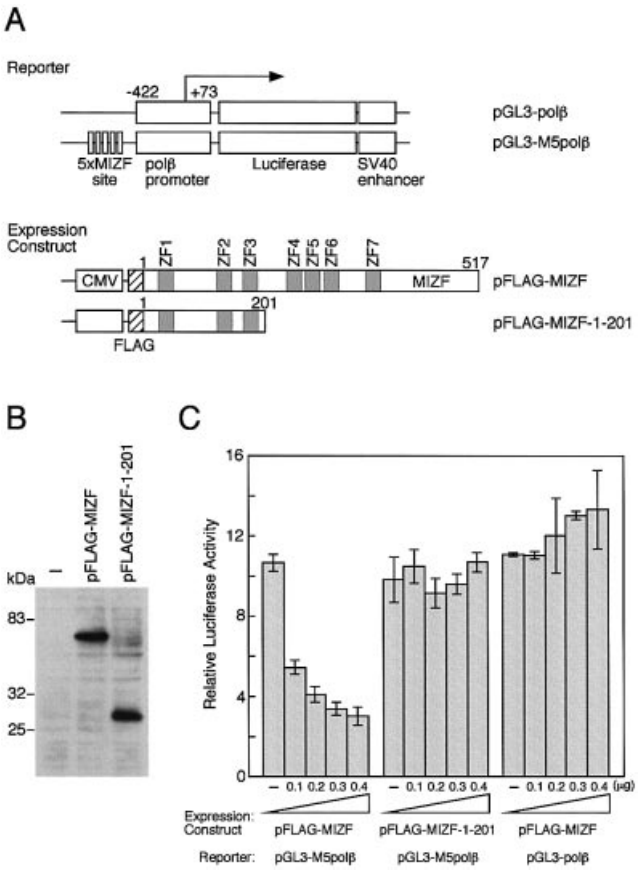

Figure 3. Transcriptional repression of a MIZF-binding site-containing luciferase reporter by MIZF. (**A**) Schematic representation of the plasmids used in transient transfection assays. The shaded boxes represent zinc finger motifs. (**B**) Protein expression analysis of the FLAG-tagged MIZF constructs shown in (A). Expressed molecules were detected by western blotting with an anti-FLAG antibody. (**C**) Transient transfection assays of the pol β-luciferase constructs in HEK293 cells. The relative luciferase activities of the reporter constructs transfected alone or together with increasing amounts of the FLAG-tagged MIZF constructs are shown. The data shown are the means ± standard deviation derived from three independent experiments.

promoter (Fig. 4A), we investigated the effect of these sites on Rb promoter activity. To address this issue, three human Rb promoter constructs were generated: a wild-type construct, pGL3-Rb, and two mutant constructs, pGL3-Rbm1 and pGL3-Rbm2 (Fig. 4A). Co-transfection of pGL3-Rb with a MIZF expression vector into HEK293 cells showed that the transcriptional activity of the Rb promoter was repressed by MIZF in a dose-dependent manner (Fig. 4B). A reporter with a mutated MIZF-binding site (pGL3-Rbm1), which is located between –217 and –212, was not subject to MIZF repression. However, pGL3-Rbm2, in which the mutation of the MIZF site is located between –140 and –135, exhibited a similar level of repression to pGL3-Rb. These results indicate that MIZF can potentially repress Rb transcription through binding to the MIZF-binding site at position –217 to –212. This site is located close to the region including the binding sites for RBF-1/E4TF1, ATF and E2F, which have been shown to be responsible for basal transcription of the human and mouse Rb promoters (21–24).

Binding of MIZF to its recognition site in the Rb promoter

To directly confirm whether MIZF binds to the Rb promoter, we performed EMSA with an Rb probe containing the MIZF-binding site located at position –217 to –212 in the human Rb promoter (Fig. 5A). Incubation of His-MIZF with the labeled Rb probe led to the formation of a single bound complex (Fig. 5B, lane 2). The binding specificity of the complex was demonstrated by competition with cold Rb and Rb-MIZF probes but not with the non-functional MIZF mutant, Rb-MIZFmut (lanes 3–5). We next examined the significance of this binding at the cellular level. When HEK293 cell nuclear extracts were subjected to EMSA in the presence of the labeled Rb probe, several retarded bands were detectable (lane 6). The binding was specific, because the intensity of these bands was reduced in the presence of a competing unlabeled Rb probe (lane 11). To investigate the effect of MIZF binding

Table 1. Sequences of potential MIZF binding sites

gene (human)	sequence
p14/ARF	-304 CGTCCG -299
	-705 AGTCCG -700
	-745 CGGACC -740
	-1322 CGTCCG -1317
p16/INK4a	-729 AGTCCG -724
	-858 CGTCCG -853
	-920 AGTCCG -915
Rb	-140 CGTCCG -135
	-217 CGGACG -212
p57Kip2	-779 CGGACC -774
	-821 GTCCGGACC -812
E-cadherin	-232 GGTCCG -227
GSTP1	-134 AGTCCG -129
Cyclin D1	-389 CGGACT -384
	-466 CGGACG -461
MLH1	-684 CGTCCG -679
MSH6	-812 CGGACT -807

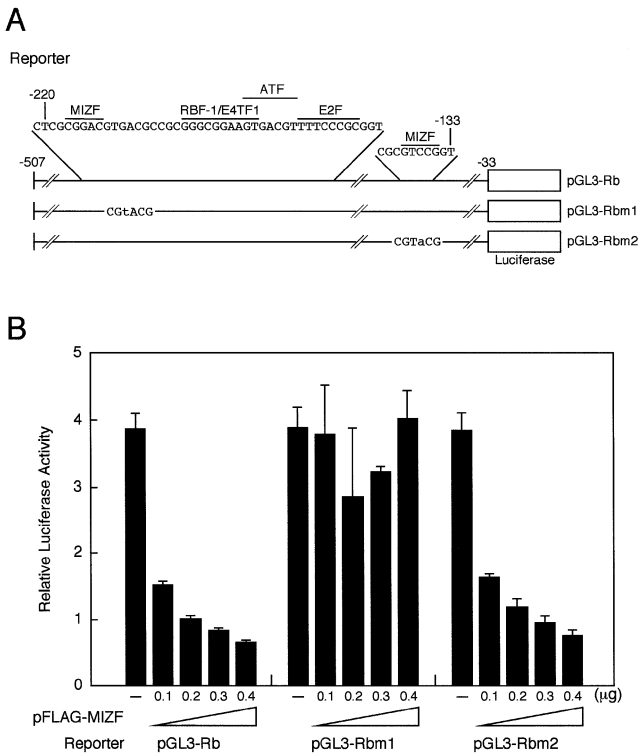

Figure 4. Suppression of the Rb promoter by MIZF. (**A**) Schematic representation of the wild-type (pGL3-Rb) and mutant (pGL3-Rbm1 and pGL3-Rbm2) Rb promoter-luciferase constructs used in transient transfection assays. The recognition sequences for transcription factors RBP-1/E4TF1, ATF and E2F are indicated. The MIZF-binding sites are indicated and the mutated nucleotide in the MIZF-binding site is in lower case. The sequence is numbered relative to the start site of translation. (**B**) Transient transfection assays of the Rb promoter-luciferase constructs in HEK293 cells. The reporter constructs were transfected with increasing amounts of pFLAG-MIZF. Firefly luciferase activity was measured and normalized to sea pansy luciferase activity using a dual luciferase reporter system. The data shown are the means ± standard deviation derived from three independent experiments.

on complex formation, we performed a factor-binding competition assay. Increasing amounts of His-MIZF diminished the most slowly migrating band in a dose-dependent manner (lanes 7–9). Since the binding was selectively inhibited by competition with the Rb-ATF probe (lane 10), the most slowly migrating band was thought to contain a member of the ATF family, which is consistent with a previous observation (25). These results indicate that MIZF and ATF bind to this region of the Rb promoter in a mutually exclusive manner.

ChIP was used to further investigate the binding of MIZF to the endogenous Rb promoter *in vivo*. Formaldehyde cross-linked chromatin fragments from HEK293 transfected with pFLAG-MIZF were immunoprecipitated with an antiserum against MIZF or a preimmune serum. DNA from the resulting immunoprecipitates was subjected to PCR amplification of a 193 bp fragment corresponding to the Rb promoter. Chromatin fragments containing the Rb promoter were specifically precipitated with an antiserum against MIZF but not with a preimmune serum (Fig. 5C), showing that MIZF binds to its cognate site in the Rb promoter *in vivo*.

DISCUSSION

We demonstrated that MIZF is a DNA-binding protein that recognizes the consensus sequence CGGACGTT containing a conserved CGGAC core via its C2H2 zinc finger (Fig. 1) and functions as a sequence-specific transcriptional repressor (Fig. 3). MIZF was originally identified as an MBD2-binding partner involved in MBD2-mediated transcriptional repression (16). MBD family proteins, such as MeCP2 and MBD2, serve as a molecular link between DNA methylation and deacetylated histones by recruiting a co-repressor complex, Sin3, containing HDAC (4–7). MBD2 also recruits another multifunctional complex, Mi-2/NuRD, which possesses both HDAC and chromatin remodeling activities (4). Thus, alteration of the local chromatin structure by recruiting HDAC complexes may be one of the general mechanisms underlying MBD-mediated transcriptional repression. On the other hand, the present findings indicate another pathway for MBD-mediated repression; the MBD2 complex contains repressors, including MIZF, that suppresses transcriptional activity by interacting directly with the recognition sequence present in the promoter region of specific genes. It has recently been reported that Kaiso, which belongs to the BTB/POZ family of C2H2 zinc finger proteins, is a constituent of the MBD complex and recognizes a specific consensus sequence (12,26). This protein repressed the transcription of specific target genes via its C2H2 zinc fingers (12). Therefore, in addition to HDAC and the chromatin remodeling mechanism, repressor molecules associated with the MBD complex may be responsible for MBD-mediated transcriptional repression. In this context, novel proteins with C2H2 zinc fingers are quite interesting, since C2H2 zinc fingers are known to function as the DNA-binding domains of many transcription factors (17).

Given that each zinc finger is expected to bind three or four bases (27,28), the 8 bp size of the MIZF-binding sequence

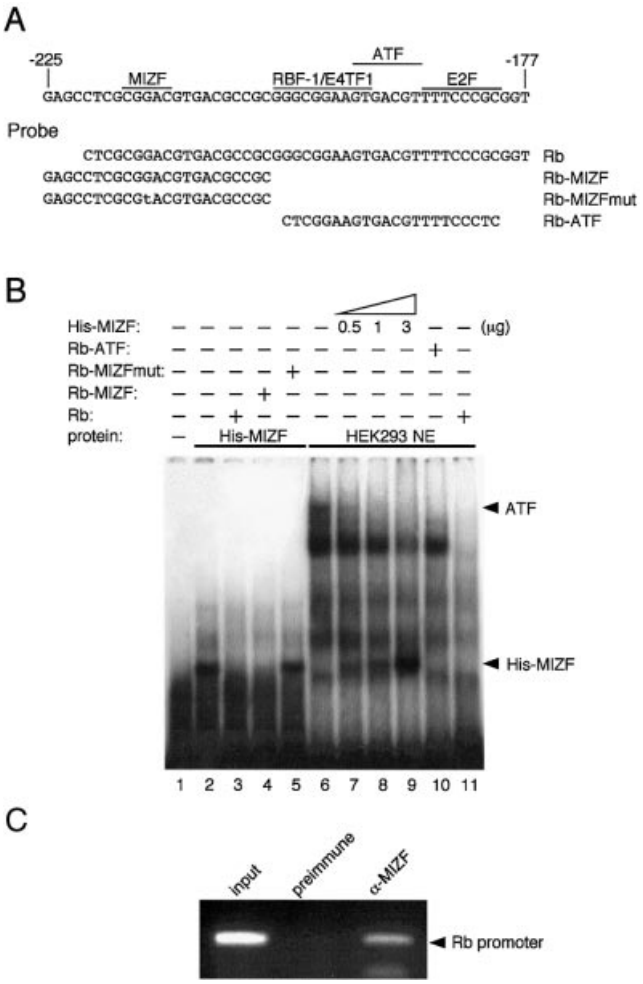

Figure 5. Binding of MIZF to the Rb promoter. (**A**) Sequences of oligonucleotide probes used in EMSA analysis. The mutated nucleotide in the MIZF-binding site is in lower case. (**B**) *In vitro* binding of MIZF to the Rb promoter in EMSA. EMSA was performed with a labeled Rb probe and either no added protein (lane 1), His-MIZF (lanes 2–5) or nuclear extracts prepared from HEK293 cells (lanes 6–11). A 200-fold molar excess of an unlabeled Rb probe (lanes 3 and 11), Rb-MIZF probe (lane 4), Rb-MIZFmut probe (lane 5) or Rb-ATF probe (lane 10) was added to the binding reactions. The indicated amounts of His-MIZF were added to constant amounts (2 μg) of nuclear extracts (lanes 7–9). The arrowheads labeled ATF and His-MIZF indicate a complex binding to the ATF and MIZF sites, respectively. (**C**) *In vivo* binding of MIZF to the Rb promoter in ChIP analysis. Formaldehyde cross-linked chromatin from HEK293 cells transfected with pFLAG-MIZF was immunoprecipitated with a preimmune serum or an anti-MIZF antibody. Precipitated chromatin samples were subjected to PCR amplification using Rb promoter-specific promoters. As a positive control (input), 10 ng of whole lysate DNA was amplified in parallel.

implies that only two or three of the seven MIZF zinc fingers are needed for sequence-specific DNA binding. Contrary to this prediction, however, our results demonstrated that zinc fingers 1–4 and 7 are necessary for DNA binding (Fig. 2). This suggests that all of the five zinc fingers are not directly involved in DNA binding. Therefore, it is possible that some of these zinc fingers function as DNA non-binding zinc finger motifs. These motifs are reminiscent of similar zinc finger motifs found in the Evi-1 protein. This protein, which is involved in regulating myeloid differentiation, is composed of seven N-terminal and three C-terminal zinc fingers. The first three zinc fingers do not directly bind DNA, but they influence the DNA binding specificity of the N-terminal domain (29). In this regard, some of the five zinc fingers within the MIZF proteins may contribute to the correct conformation of the protein by interacting with the DNA-binding zinc fingers.

It is noteworthy that the sequence recognized by MIZF contains one or two CpG dinucleotides and, therefore, is a candidate for regulation by CpG methylation. It is well known that methylation-sensitive transcription factors such as E2F are inhibited by the methylation of CpG dinucleotides, preventing their binding to DNA and resulting in the suppression of gene expression (30). In contrast, non-sensitive transcription factors such as Sp1 can bind to methylated CpG DNA and activate transcription (31). Preliminary results indicated that MIZF could bind to CGGACG and its methylated form with almost the same affinity. Thus, it is possible that MIZF can regulate a wide variety of genes containing both non-methylated and methylated CpG sequences.

The Mi-2/NuRD complex can be targeted to methylated DNA by interacting with MBD2, resulting in formation of the MeCP1 complex. Likewise, the Mi-2/NuRD complex also interacts with a variety of different DNA-binding proteins, such as Hunchback, Ikaros and p53 (32). Although it is unclear whether MIZF is a component of the Mi-2/NuRD complex, it may interact with the complex under certain cellular conditions. We previously observed that MIZF to some extent co-localizes with MBD2 in nuclei, but the main localization of the two molecules is quite different (16), indicating that MIZF does not always associate with MBD2. Rather, the diffuse localization of MIZF in the nucleus is similar to that of MBD3, which was identified as a core component of the Mi-2/NuRD complex (16,33). Moreover, MIZF could associate with MBD3 in a manner similar to the binding with MBD2 (data not shown). Thus, the association of MIZF with MBD3 is also likely to be involved in recruitment of the Mi-2/NuRD complex to the specific promoters. Further study of MIZF-interacting proteins will be necessary to clarify the mechanism of MIZF-dependent transcriptional regulation.

Identification of the downstream target genes for MIZF may provide insights into the biological function of MIZF. A search for MIZF-binding sites in the human genome database revealed that the recognition sequence was present in the putative transcriptional control regions of genes involved in cell cycle regulation, cell adhesion, DNA repair and transcription (Table 1). Among them, we focused on the Rb gene because it carries two perfect copies of the MIZF-binding site in the promoter region and is a gene whose transcription is negatively regulated by DNA methylation (34–37). Using EMSA and ChIP analyses, we demonstrated that MIZF binds specifically to the Rb promoter (Fig. 5) and represses transcription from the Rb promoter in a consensus sequence-dependent manner (Fig. 4). These results indicate that Rb is a gene target for MIZF. Although the mechanism underlying MIZF-mediated Rb repression is obscure, one of the most likely explanations is that the repression is based on a competitive mechanism. EMSA analysis revealed that recombinant MIZF could inhibit the binding of ATF to the Rb promoter (Fig. 5B). ATF is known to be a positive regulator of Rb transcription (38). Interestingly, MIZF and ATF had no

overlapping binding sites, however, MIZF specifically interferes with the binding of ATF without impeding the binding of other transcription factors, such as RBF-1/E4TF1 and E2F (Fig. 5B). These results suggest that local conformational changes resulting from protein–protein interactions between MIZF and MBD2 may be the basis for this inhibitory effect of MIZF.

To understand the molecular link between MIZF and MBD2, it would be interesting to analyze cancer cells in which aberrant DNA methylation is observed in the promoter region of the genes listed in Table 1. The transcriptionally silent p14/ARF, p16/INK4a and π-class glutathione *S*-transferase (GSTP1) are selectively associated with MBD2 in the promoter region, which is independent of MeCP2 (9–11). In addition, several studies using GSTP1 promoter-reporter constructs predicted the existence of a transcriptional repressor associated with the region (39–41). Since this region contains the MIZF-binding site between 134 and 129 upstream from the transcription start site, it is possible that synergetic action between MIZF and MBD2 produces an epigenetic silencing state in the methylated promoters.

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