

Negative Regulatory Role of Sp1 in Metal Responsive Element-mediated Transcriptional Activation*

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Transcription of mammalian metallothionein (MT) genes is activated by heavy metals via multiple copies of a cis-acting DNA element, the metal-responsive element (MRE). Our previous studies have shown that certain MREs of the human MT-IIA gene (MREb, MREc, MREd, and MREf) are less active than the others (MREa, MREe, and MREg). Gel shift analysis of HeLa cell nuclear proteins revealed that whereas the active MREs strongly bind the transcription factor MTF-1 essential for metal regulation, the less active MREs bind another distinct protein, MREb-BF. This protein recognizes the GC-rich region of MREb rather than the MRE core required for MTF-1 binding. All the MREs recognized by MREb-BF contain the CGCCC and/or CACCC motif, suggesting that the MREb-BF-MRE complex contains Sp1 or related proteins. Supershift analysis using antibodies against Sp1 family proteins as well as gel shift analysis using the recombinant Sp1 demonstrated that Sp1 represents the majority of MREb-BF activity. An MREb mutant with reduced affinity to Sp1 mediated zinc-inducible transcription much more actively than the wild-type MREb. Furthermore, when placed in the native promoter, this mutant MREb raised the overall promoter activity. These results strongly suggest that Sp1 acts as a negative regulator of transcription mediated by specific MREs.

Heavy metal-induced transcriptional activation of the genes coding for metallothioneins (MTs)¹ is mediated by multiple copies of a cis-acting DNA element, the metal-responsive element (MRE; Refs. 1–3). It has been shown that the mouse MRE-binding transcription factor-1 (MTF-1) is essential for MRE-mediated transcription from gene knockout (4) and antisense RNA expression studies (5). Nevertheless, it remains possible that metal regulation of MT genes involves additional transcriptional regulators. A number of MRE-binding proteins have been reported (reviewed in Ref. 6), and cDNAs encoding MRE-binding proteins distinct from MTF-1 have been cloned

(7, 8). However, no protein except MTF-1 has been demonstrated to be functionally relevant to metal regulation.

MREs were first identified by deletion analysis in duplicated sites upstream of the mouse MT-I (*mMT-I*) and human MT-IIA (*hMT-IIA*) genes (1, 2, 9). After that, a search for MRE-related sequences revealed that there are multiple MRE homologs in the upstream region of all the mammalian MT genes reported so far (3, 10). These facts imply that such a redundancy is probably essential for the high metal inducibility of MT gene promoters. However, MREs are not always functionally equivalent. In transfection assays using reporter genes driven by synthetic MRE-containing promoters, striking differences in zinc-induced transcriptional activity were observed among MREs of the *mMT-I* (3) and *hMT-IIA* (11) genes, despite the observation that all these sequences share the highly conserved MRE core (whose functional importance has been demonstrated; Refs. 12, 13) and the flanking semi-conserved GC-rich sequence. In addition, we have observed that the transcriptional activity of MRE does not always reflect its binding affinity to MTF-1; certain MREs of the *hMT-IIA* gene are able to bind the purified human MTF-1 *in vitro*, although they show no or very low transcriptional activity *in vivo* (11). We had assumed that additional transcriptional regulators might be responsible for such a discrepancy and explored HeLa cell crude nuclear extracts for another MRE binding activity. Here we show that a nuclear protein distinct from MTF-1, which was finally proven to be the transcription factor Sp1 (SV40 protein 1), binds to the less active MREs and probably acts as a negative regulator of MRE-mediated transcription.

EXPERIMENTAL PROCEDURES

Cell Culture and Preparation of Extracts—HeLa S3 cells (CCL2.2) were cultured in Eagle's minimum essential medium supplemented with 10% calf serum and non-essential amino acids at 37 °C under a 5% CO₂ atmosphere. In experiments, medium was supplemented with streptomycin (100 µg/ml) and penicillin (100 units/ml). Crude HeLa cell nuclear extracts (NEs) were prepared as described previously (14), except for the following modifications. For all buffer solutions, Hepes-Na, pH 8.0, and NaCl were replaced with Hepes-K, pH 7.9, and KCl, respectively (KCl was adjusted to 0.1 M in Buffer 3), and dithiothreitol was added to 1 mM.

Proteins—The human MTF-1 was purified ~2,000-fold from HeLa cell NEs by the biotin-streptavidin affinity purification procedure as previously described (15). Affinity-purified recombinant Sp1 was purchased from Promega Japan, Tokyo.

Synthetic Oligonucleotides—Preparation of double-stranded synthetic oligonucleotides containing the *hMT-IIA* MREs were described previously (11). Mutant MREb oligonucleotides (Figs. 4a and 8a, sequences) were prepared in the same manner. All double-stranded oligonucleotides carry *Bam*HI and *Bgl*II sites (at the 5'- and 3'-ends, respectively), which are convenient for multimerization and subcloning (11). An oligonucleotide containing two copies of the Sp1 binding site was previously described (16).

Electrophoretic Mobility Shift Assay (EMSA)—DNA binding activity of proteins was determined by EMSA. The MRE oligonucleotides de-

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¹ The abbreviations used are: MT, metallothionein; MRE, metal-responsive element; MTF-1, MRE-binding transcription factor-1; mMT-I, mouse MT-I; hMT-IIA, human MT-IIA; NE, nuclear extract; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase; HSV-TK, herpes simplex virus thymidine kinase.

scribed above were end-labeled with T4 polynucleotide kinase and [γ - 32 P]ATP and used as probes. The standard reaction mixture (12.5 μ l) contained 10 mM Hepes-K, pH 7.9, 2 mM MgCl₂, 50 mM KCl, 16 mM NaCl, 10 mM dithiothreitol, 10% glycerol, 0.2 mg/ml bovine serum albumin, 80 μ g/ml poly(dI-dC), 15 fmols [32 P]MRE probe. Proteins (NE or purified proteins; amounts are indicated in the figure legends) were incubated in the reaction mixture at 25 °C for 20 min and were electrophoresed in a 5% polyacrylamide gel in a buffer containing 22 mM Tris and 22 mM boric acid for 1.5 h. Protein/DNA complexes were detected by autoradiography.

Supershift Assay—The affinity-purified rabbit polyclonal antibody against human Sp1, Sp2, Sp3, and Sp4 were obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Each of these antibodies does not cross-react with other members of the Sp1 family proteins. The antibodies were added to EMSA reaction mixtures (2 μ g immunoglobulin G/reaction) prior to or after binding reaction and incubated at 25 °C for 20 to 30 min. Reactions were then analyzed by the standard EMSA procedure as described above.

Plasmid Construction—Plasmids with MRE-containing model promoters were derived from pTKprCAT, which carries the bacterial chloramphenicol acetyltransferase (CAT) gene driven by the herpes simplex virus thymidine kinase (HSV-TK) gene promoter (17). Construction of pTKprCAT variants with MREa or MREb tetramers placed upstream of the TK promoter was previously described (11). The plasmid containing the tetramer of an MREb mutant was prepared in the same manner.

A CAT reporter plasmid driven by the native *hMT-IIA* promoter was constructed as follows. The *Hind*III/*Bam*HI fragment of pSV2CAT containing the CAT gene (18) was subcloned between the *Hind*III and *Bam*HI sites of pUC19 to generate a plasmid pUC-CAT. The plasmid pMT-IIA, in which the 3-kilobase *Hind*III fragment containing the entire *hMT-IIA* gene (19) had been subcloned, was linearized by *Nco*I digestion and blunt-ended by Mung bean nuclease and Klenow polymerase. *Hind*III linkers were then ligated to the blunt ends. A fragment containing the *hMT-IIA* promoter (−767 to +71) was excised with *Hind*III and cloned into the *Hind*III site of pUC-CAT. The upstream *Hind*III site was eliminated by end-filling and religation to facilitate later mutagenesis manipulations. This plasmid was designated pUC-MTCAT.

Site-directed Mutagenesis—Under the background of pUC-MTCAT, the MREb sequence was converted to the mutant MREb m3 sequence (see Fig. 8a) using the Mutan-Express Km kit (Takara Shuzo, Osaka, Japan). The *Pst*I/*Hind*III fragment containing bases −624 to +71 of the *hMT-IIA* gene was excised and subcloned into a mutagenesis vector pKF-19k, and was subjected to *in vitro* mutagenesis according to the manufacturer's instructions. The mutated MRE-containing fragment was cloned back to the original pUC-MTCAT, and was inspected by dideoxy sequencing.

CAT Assay—Plasmid constructs to be tested (10 μ g each/10-cm plate or 4 μ g each/6-cm plate) and a reference plasmid pRSVL (2.5 μ g/10-cm plate or 1 μ g/6-cm plate) were transfected into HeLa cells by the standard calcium phosphate transfection procedure. After 28 h, cells were further incubated with or without 100 μ M ZnSO₄ for 20 h. Cell extracts were prepared, and CAT levels were determined by an enzyme-linked immunosorbent assay (ELISA). CAT values were normalized relative to the luciferase activity expressed by the reference pRSVL. Details of this procedure were previously described (11).

RESULTS

A Nuclear Protein Distinct from MTF-1 Binds to Less Active MREs—In the upstream region of the *hMT-IIA* gene, there are seven sites that contain sequences perfectly matching the MRE core consensus (10) as indicated in Fig. 1a. Three of these MREs (MREa, MREe, and MREg) efficiently mediate metal response of a reporter gene in transient transfection experiments and strongly bind the purified human MTF-1 *in vitro* (11), consistent with the idea that MTF-1 plays the major role in metal-induced transcription (4, 5, 11). However, the properties of certain MREs are quite different from these active MREs (see Fig. 1a). In particular, MREb has extremely low activity when compared with the three active MREs, although it is responsive to heavy metals and is able to bind MTF-1 (11). On the other hand, MREd and MREf do not respond to metals, although they appear to bind MTF-1 weakly (11). To unambiguously determine the affinity of MTF-1 with these less active MREs, we carried out competitive EMSA using the affinity-

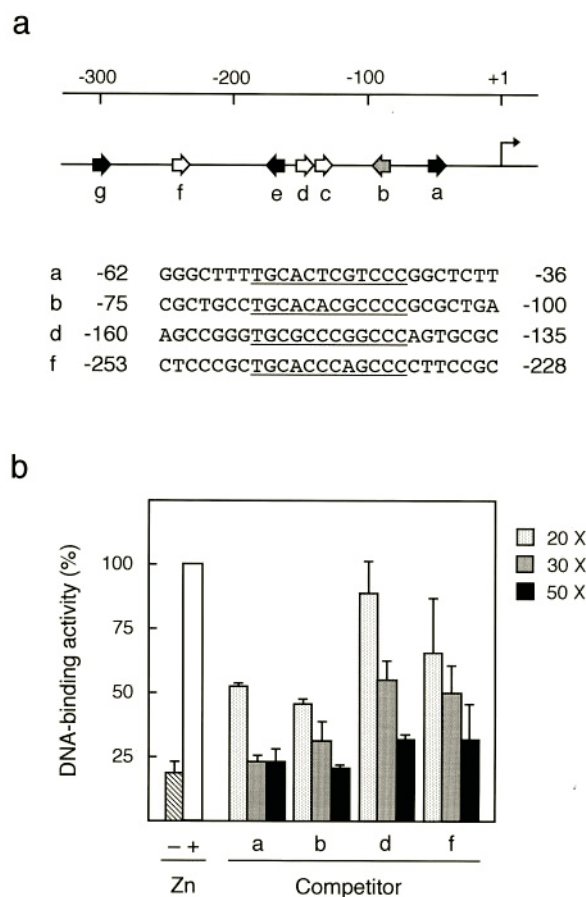


FIG. 1. Binding of human MTF-1 to MREs of the *hMT-IIA* gene. **a**, MREs located upstream of the *hMT-IIA* gene. The location and orientation of MREs are indicated by arrows: black arrow, zinc responsive MRE with high activity; gray arrow, zinc-responsive MRE with low activity; white arrow, MRE not responsive to zinc (11). MRE and flanking sequences contained in the oligonucleotides used as the probe and/or competitors in **b** are shown below the map. The MRE sequences are underlined. **b**, interactions of human MTF-1 with the less active MREs. Binding of the purified human MTF-1 (1 μ l; Ref. 15) to MREb, MREd and MREf was estimated by competitive EMSA using a [32 P]MREa oligonucleotide probe. Competitor oligonucleotides containing the MREa, MREb, MREd, or MREf sequence (labeled **a**, **b**, **d**, and **f**, respectively) were added to binding reactions containing 100 μ M ZnSO₄ as indicated (in a 20-, 30- or 50-fold molar excess). The MTF-1 complex bands on the autoradiogram were quantified by densitometry, and each value was normalized relative to the control included in every assay (with zinc and without competitor; taken as 100). Data compiled from six independent experiments are shown. Controls without competitor are indicated at the left; −, without metal; +, with 100 μ M ZnSO₄. Each column represents the average of 2–4 values from independent experiments with S.E.

purified human MTF-1 protein (15), a 32 P-labeled MREa probe, and varied amounts of unlabeled competitors each containing one of the MRE sequences. As shown in Fig. 1b, MREb competed with the MREa probe as efficiently as the unlabeled MREa competitor, confirming that MTF-1 has a high binding affinity to MREb, comparable with that of MREa. MREd and MREf also competed with the MREa probe, although less efficiently than MREb. These results demonstrated that MREb, MREd, and MREf are able to interact with the purified human MTF-1 *in vitro*, despite low or no activity for mediating metal-dependent transcription *in vivo*. MREc, another MRE not responsive to zinc, showed no affinity to human MTF-1 in similar competition experiments (data not shown).

Based on these observations, we assumed that additional regulator proteins could possibly interact with the less active MREs *in vivo*, resulting in their unusual properties. To detect

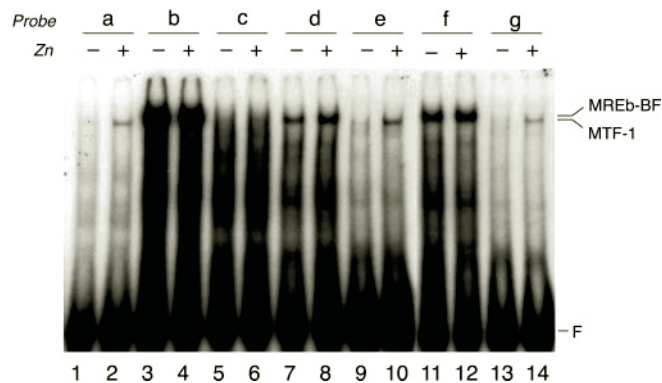


FIG. 2. **Detection of MRE-binding proteins in HeLa cell nuclear extract.** HeLa cell NE (2 μ g) was analyzed by EMSA using 32 P-labeled oligonucleotide probes each containing one of the seven MRE sequences (MREa to MREg; labeled a to g, respectively) in the absence (–) or presence (+) of 100 μ M ZnSO₄. F indicates the free probe.

additional MRE binding activity, we analyzed HeLa cell crude nuclear extract by EMSA using 32 P-labeled probes, each containing one of the seven *hMT-1IA* MREs (Fig. 2). MREe and MREg formed zinc-inducible complexes co-migrating with the MTF-1-MREa complex (lanes 10 and 14; compare with uninduced controls in lanes 9 and 13; also compare with MTF-1-MREa complex in lane 2), as expected from our previous competition experiments (11). However, a strong band migrating slightly slower than the MTF-1 complex was detected with the MREb probe (lanes 3 and 4). Formation of this complex was not affected by zinc. Complexes with identical mobility were also detected for MREd (lanes 7 and 8) and MREf (lanes 11 and 12), although the levels of complex formation were lower than that observed for MREb. The protein that binds to MREb was designated MREb-BF and was analyzed further. Neither MTF-1 nor MREb-BF bound to MREc (lanes 5 and 6).

Properties of MREb-BF—To determine the sequence recognition specificity of MREb-BF, competition experiments were done using the [32 P]MREb probe and unlabeled oligonucleotides containing the seven MREs (Fig. 3). In addition to MREb (lanes 4 and 5), MREd (lanes 8 and 9), and MREf (lanes 12 and 13) competed out the MREb-BF·[32 P]MREb complex, suggesting that an identical factor binds to these three MREs. Binding affinity to MREb-BF was higher in the order of MREb, MREf, and MREd, consistent with the levels of complex formation observed in Fig. 2. By contrast, MREa (lanes 2 and 3), MREc (lanes 6 and 7), MREe (lanes 10 and 11) and MREg (lanes 14 and 15) did not compete with the MREb probe, indicating that these are not the target sites of MREb-BF. We then tested two additional competitors containing mutated MREb (Fig. 4a), to locate the bases essential for MREb-BF binding within the MREb sequence. The mutant m1 has clustered base substitutions within the MRE core sequence that is important for MRE function and MTF-1 binding (11, 13). The other mutant m2 has mutations in the adjacent GC-rich region. As shown in Fig. 4b, m1 competed with the MREb probe as efficiently as the wild-type MREb (lanes 4 and 5; compare with lanes 2 and 3), but m2 showed no competition (lanes 6 and 7). These results indicate that the GC-rich region of MREb, not the MRE core, is recognized by MREb-BF. We noted that the GC-rich region of MREb is quite similar to the consensus recognition site of the transcription factor Sp1 (Ref. 20; see Fig. 5). Therefore we also tested a competitor containing two copies of the Sp1 binding site (GC box). In fact, this oligonucleotide strongly competed with the MREb probe (lanes 8 and 9).

In addition to MREb, MREd and MREf also contained subregions that resemble the Sp1 consensus sequence, although they are less similar than that in MREb (Fig. 5). Moreover,

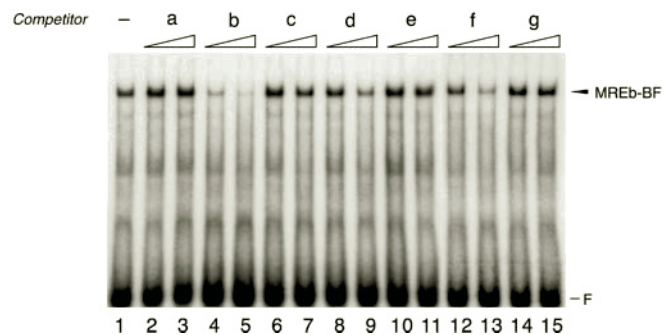


FIG. 3. **Interaction of MREb-BF with *hMT-1IA* MREs.** The affinity of MREb-BF to each of the seven *hMT-1IA* MREs was estimated by competitive EMSA using HeLa cell NE (2 μ g), [32 P]MREb probe and unlabeled competitors each containing one of the MRE sequences (MREa to MREg, labeled a to g, respectively; in a 30- or 100-fold molar excess). F indicates the free probe.

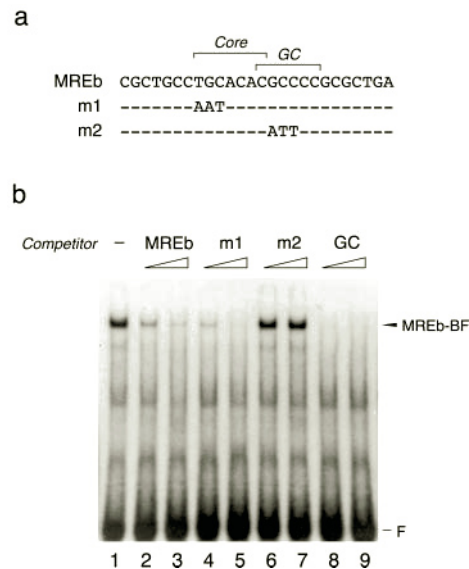


FIG. 4. **Nucleotides required for MREb-BF binding.** a, sequences of oligonucleotides containing MREb mutants. Clustered base substitutions introduced into MREb mutants m1 and m2 are indicated below the wild-type MREb sequence. b, competitive EMSA. Binding affinity of MREb-BF to the MREb mutants and GC box was examined by competitive EMSA as in Fig. 3, using HeLa cell NE (2 μ g), [32 P]MREb probe, and the competitors (in a 30- or 100-fold molar excess). The DNA sequence of the oligonucleotide containing two copies of the GC box was previously described (16). F indicates the free probe.



FIG. 5. **Sequence similarity of MREb, MREd, and MREf to the Sp1 recognition sites.** The consensus Sp1 binding sequence (20) is indicated at the top. MREs are underlined. Bases matching the Sp1 consensus are indicated by dots. Asterisks indicate the CACCC motif.

MREd and MREf contain the CACCC motif that is known as another recognition site for the Sp1 family proteins (21, 22); these nucleotides are marked by asterisks in Fig. 5. To confirm the binding of Sp1 to these MREs, we carried out a supershift analysis using an Sp1-specific antibody (Fig. 6a). The human MTF-1-MREa complex was not affected by this antibody (lane 2; compare with lane 1). By contrast, the MREb-BF complexes with MREb, MREd, and MREf probes were all supershifted

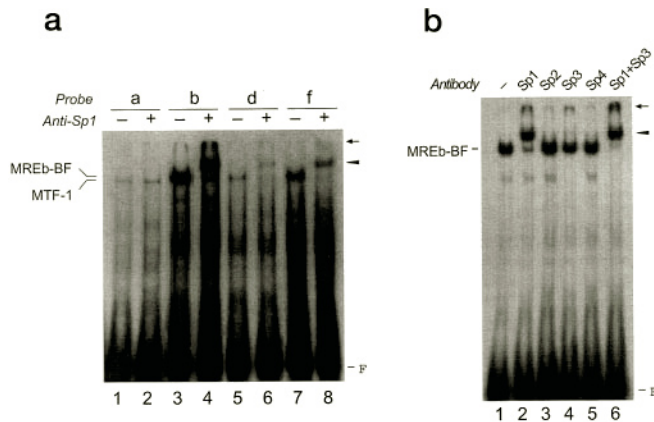


FIG. 6. *a*, supershift analysis of interactions between Sp1 and MREs. EMSA reaction mixtures containing HeLa cell NE (2 μ g) were incubated with (+) or without (–) an anti-Sp1 antibody for 25 $^{\circ}$ C, 30 min followed by a binding reaction with the addition of 32 P-labeled oligonucleotide probes. MREa, MREb, MREd, and MREf probes were added as indicated (labeled *a*, *b*, *d*, and *f*, respectively). The arrowhead indicates the supershifted band. The arrow indicates the slot position. *F* indicates the free probe. *b*, interaction of Sp1 family proteins with MREb. After the standard EMSA binding reaction with [32 P]MREb probe and HeLa cell NE (2 μ g), reaction mixtures were further incubated without antibody (control) or with antibody against Sp1, Sp2, Sp3, or Sp4, or their combination at 25 $^{\circ}$ C for 20 min before electrophoresis. The arrowhead shows the supershifted band. The arrow shows the slot position; note that supershifted signals can be seen also at this position. *F* indicates the free probe.

(lanes 4, 6, and 8; compare with lanes 3, 5, and 7, respectively). These results strongly suggest that Sp1 is responsible for the MRE-BF DNA binding activity. Because Sp1 family proteins are known to have recognition sequence specificity similar to that of Sp1 (21, 22), we further tested reactivity of the MREb-BF complex with antibodies against other Sp1 family proteins including Sp2, Sp3, and Sp4 (Fig. 6*b*). Antibodies against Sp2 and Sp4 did not supershift the MREb-BF complex (lanes 3 and 5, respectively). Anti-Sp1 antibody supershifted the majority of the MREb-BF complex (lane 2), but a part of the complex always remained unshifted. Even when the antibody was added in a large excess, this population was not shifted (data not shown). We observed that anti-Sp3 antibody supershifted a small part of the MREb-BF complex (lane 4). In addition, when both anti-Sp1 and anti-Sp3 antibodies were simultaneously added to the binding reaction, almost all of the MREb-BF complex was supershifted. These results indicate that the MREb-BF complex contains Sp1 as a major component and Sp3 as a minor component.

We further examined direct interactions of the purified MTF-1 and Sp1 proteins with the MREa and MREb sequences. As shown in Fig. 7, the purified human MTF-1 bound both MREa and MREb only in the presence of zinc (lane 4 in the upper and lower panels, respectively; compare with lane 3). The level of complex formation was similar for both complexes, consistent with the results of competition experiments shown in Fig. 1*b*. On the other hand, the affinity-purified recombinant Sp1 protein binds to MREb, either in the absence or presence of zinc (lanes 5 and 6 in the lower panel, respectively). The mobility of this complex was identical with that of MREb-BF (Fig. 2). By contrast, Sp1 formed no complex with MREa (lanes 5 and 6 in the upper panel). These results demonstrate that Sp1 specifically recognizes the MREb sequence in the same manner as MREb-BF does (Fig. 3).

Sp1 Probably Acts as a Negative Regulator of MRE-mediated Transcription—Competition between Sp1 and MTF-1 for binding to certain MREs could possibly be an important mechanism involved in the metal-regulated transcription of MT genes. To

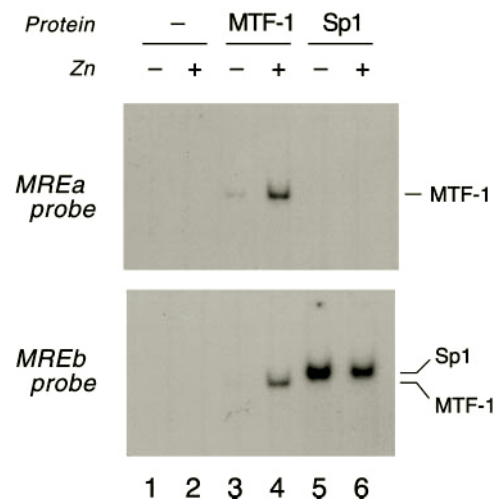


FIG. 7. **Binding of purified MRE-binding proteins to MREa and MREb.** Interactions of the purified human MTF-1 (1 μ g/reaction) and Sp1 (0.125 footprint units/reaction) with 32 P-labeled MREa and MREb oligonucleotides were examined by EMSA in the absence (–) or presence (+) of 100 μ M ZnSO₄. Only the regions of the autoradiogram containing the MTF-1 and Sp1 complexes are shown; no other complex was detected by this assay.

approach this possibility, we prepared and screened several MREb mutants to obtain one that is normal in MTF-1 binding but defective in Sp1 binding. Consequently, we were able to obtain a mutant satisfying those criteria, m3, that carries a single C→G base change within the GC-rich region (Fig. 8*a*). When assayed by competitive EMSA (Fig. 8*b*), m3 failed to compete with the [32 P]MREb probe (lanes 4 and 5), under a condition where the wild-type MREb competitor almost completely competed out the labeled MREb-Sp1 complex (Fig. 8*b*, lane 3; compare with control without competitors in lane 1). This result indicates that m3 almost completely lacks affinity for Sp1. By contrast, when assayed for MTF-1-binding with the [32 P]MREa probe (Fig. 8*c*), m3 competed with the probe (lanes 4 and 5) as efficiently as MREa and as wild-type MREb (lanes 2 and 3, respectively), indicating that the affinity of m3 for MTF-1 remains intact. These data demonstrate that the MREb mutant m3 has a defect in Sp1 binding but not in MTF-1 binding.

The mutant m3 was then assayed for the ability to mediate zinc-inducible transcription (Fig. 9). Four direct repeats of MREa, wild-type MREb, and m3 oligonucleotides were inserted upstream of the HSV-TK promoter in the pTKprCAT plasmid, respectively. These constructs were introduced into HeLa cells, and reporter CAT gene expression was monitored after incubation with or without 100 μ M ZnSO₄ for 20 h. The wild-type MREb responded to zinc, but the level of CAT expression was extremely low when compared with MREa both in the absence and presence of zinc. However, the single base change in the mutant m3 dramatically increased both induced and uninduced reporter gene expression, the level of which is almost comparable with that of MREa. These results strongly suggest that the defect in Sp1 binding confers high transcriptional activity on MREb.

We further examined the effect of m3 mutation on the overall *hMT-IIA* promoter activity (Fig. 10). Plasmids that carry the wild-type or mutated *hMT-IIA* gene upstream sequence (–767 to +71) linked to the CAT reporter gene were constructed, and tested for their transcriptional activity as in Fig. 9. In the mutant plasmid, MREb was replaced with m3 by site-directed mutagenesis. The wild-type promoter showed a typical zinc-inducible nature of the MT gene promoter (left columns), which represents the combined effect of multiple MREs. The tran-

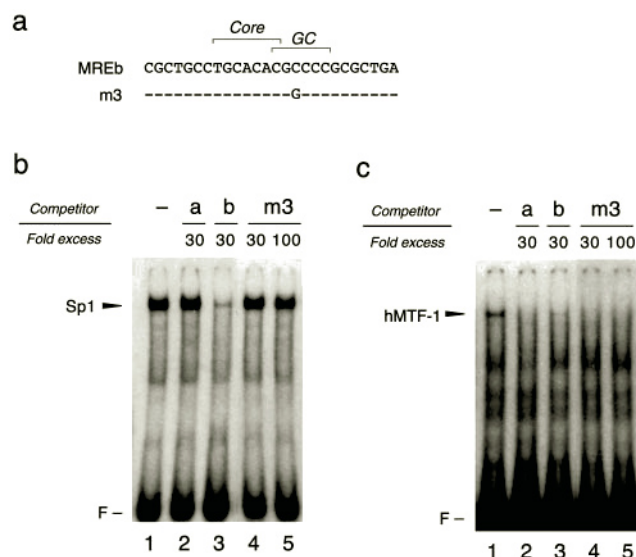


FIG. 8. Binding of Sp1 and MTF-1 to an MREb mutant with a single base change in the GC-rich region. *a*, nucleotide sequence of the MREb mutant m3. The mutated base in the MREb variant is indicated below the sequence of the wild-type MREb. *b*, binding affinity of Sp1 to m3. Protein-DNA interactions were assayed by competitive EMSA as described in the legend to Fig. 3. Reactions contained HeLa cell NE (2 μ g) and 32 P-MREb probe. Competitors (MREa, MREb, and m3; labeled *a*, *b*, and *m3*, respectively) were added to the reactions as indicated. *F* indicates the free probe. *c*, binding affinity of human MTF-1 to m3. Protein-DNA interactions were assayed as in *b* except that reactions contained [32 P]MREa probe and 100 μ M ZnSO₄.

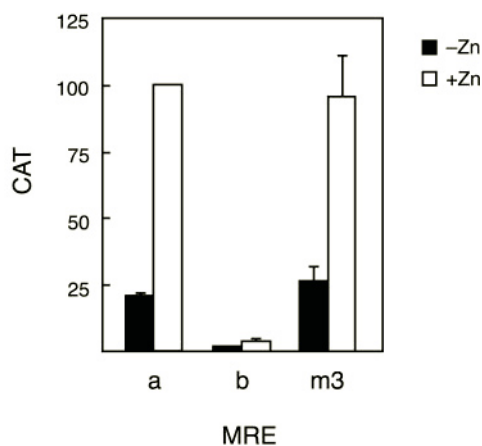


FIG. 9. Transcriptional activity of the MREb mutant m3. Four direct repeats of MREa, MREb, and m3, respectively, were inserted upstream of the HSV-TK gene promoter in the pTKprCAT reporter plasmid. These constructs were introduced into HeLa cells (in 10-cm dishes) by calcium phosphate transfection in duplicates. After incubation with (open columns) or without (filled columns) 100 μ M ZnSO₄ for 20 h, CAT levels in cell extracts were determined by ELISA. Values were normalized relative to the CAT level in cells transformed with the MREa construct and induced by zinc (taken as 100). The average values with S.E. from four independent experiments are shown.

scriptional activity of the mutant promoter was significantly higher than the wild type both under uninduced (3.1-fold) and zinc-induced (2.2-fold) conditions (right columns). These results demonstrate that Sp1 binding to MREb clearly has a negative effect under the native promoter background.

DISCUSSION

Binding of Sp1 to MREs—Our data demonstrate that Sp1 represents the majority of the MREb-BF DNA binding activity. Sp1 was originally identified as a transcriptional activator that recognizes the sequence containing the core CGCC motif (GC

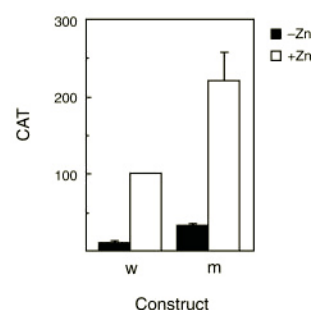


FIG. 10. Effect of MREb mutation on the overall transcriptional activity of the *hMT-IIA* promoter. Plasmids carrying the wild-type or mutant *hMT-IIA* promoter (-767 to +71) linked to the CAT gene were constructed. The mutant promoter carried MREb mutant m3 (see Fig. 8) instead of the wild-type MREb. These plasmids were tested for transcriptional activity as in Fig. 9, except that cells plated in 6-cm dishes were used. Values were normalized relative to the CAT level in cells transformed with the wild-type construct and induced by zinc (taken as 100). The average values with S.E. from three independent experiments are shown. Constructs: *w*, wild type; *m*, mutant.

box) located in the early promoter of SV40 as well as many other viral and cellular promoters (23–26). However, the target site of Sp1 is not restricted to the GC box; Sp1 can bind to the CACCC motif (21, 22, 27), which is known as a regulatory element in a variety of promoter and enhancer sequences (28–31) as well as other sequences (32–34). MREb contains a sequence highly homologous to the GC box consensus sequence (Fig. 5), and mutations within this site abolish Sp1 binding (Figs. 4 and 8), indicating that Sp1 recognizes the GC box region of MREb. The other two Sp1-binding MREs, MREd and MREf, also have GC box-like sequences, but they are less similar to the GC box consensus sequence (Fig. 5). However, these two MREs also contain another Sp1 recognition site, the CACCC motif. It remains to be determined which motif in these MREs is important for recognition by Sp1.

Negative Transcriptional Regulation by Sp1—The MREb mutant defective in Sp1 binding exhibited a much higher transcriptional activity than wild-type MREb in a simple model promoter (Fig. 9). Furthermore, when placed under the native *hMT-IIA* promoter background, this mutant MREb raised the overall promoter activity (Fig. 10). These findings strongly suggest that Sp1 could act as a negative regulator by competing with the positive regulator MTF-1 for binding to particular MREs. Although the Sp1 recognition site was originally regarded as a positive regulatory element (23, 26, 35), reports suggesting their negative regulatory roles have recently been published. Several DNA elements identified as those responsible for negative gene regulation contain GC-rich motifs, and the binding of Sp1 or closely related proteins to them is likely to be involved in down-regulation (34, 36–40). Transcriptional repression mediated by the Sp1 binding sites appears to occur through several different mechanisms. Proteins that bind to the sequences overlapping certain Sp1 sites appear to competitively inhibit Sp1 binding thereby abolishing transactivation by Sp1 (37, 41, 42). Alternatively, Sp1 transactivation could be inhibited by proteins that directly interact with Sp1 (43–45). In either case, Sp1 acts as a positive regulator, and other proteins appear to play roles in negative regulation. In contrast to this, DNA-binding of Sp1 itself could exert a negative effect (34). In MREb-mediated transcription, Sp1 appears to act in such a manner, namely its binding to MREb directly leads to transcriptional repression. In this particular case, however, the negative effect is likely to result from the competition between Sp1 and MTF-1. This is the first example that suggests the negative regulatory role of Sp1 through direct competition with another transcriptional activator.

In the *hMT-IIA* gene promoter, an Sp1 site located between MREa and MREb is known to be essential for promoter activity, both *in vivo* and *in vitro* (35, 46). These observations together with our present results suggest that Sp1 could act either positively or negatively, depending on its binding sites within the same promoter. There have been reports on the dual function of Sp1 that are similar to this (34, 39). It is curious to consider why DNA binding of a single transcription factor could result in apparently opposite effects. Positional effects exerted by surrounding DNA sequences might be important. Alternatively, the DNA sequence itself could directly affect the function of DNA-binding proteins through allosteric effects (47). It has been reported that the phosphorylated form of Sp1 is transcriptionally active (48). This finding raised the possibility that the non-phosphorylated, inactive form of Sp1 could act negatively on MRE-mediated transcription. This point remains to be studied.

It has been suggested that the level of Sp1 expression could directly affect the tissue-specific expression of particular genes (49, 50). It is possible that in tissues with high levels of Sp1 expression or in cells with increased Sp1 levels induced by certain extracellular stimuli, Sp1 displaces MTF-1 at certain MREs such as MREb, and consequently represses MT gene expression. Conversely, at low Sp1 levels MTF-1 might bind to these MREs, thereby increasing the level of MRE-mediated transcription. Functional modification of pre-existing Sp1 could also yield similar results. However, it remains to be demonstrated whether differential Sp1 levels among tissues or during induction could directly modulate MT gene transcription. Because multiple copies of MRE in model promoters act synergistically in transcriptional activation (11), the binding of MTF-1 to particular MREs could greatly affect the overall promoter activity and could be a regulatory switch. The data shown in Fig. 10 are consistent with this idea.

Several proteins related to Sp1 have been cloned (21, 22). Sp3 and Sp4 bind to both the CACCC motif and GC box with similar affinity and specificity (21, 22). Sp4 was shown to be a transcriptional activator (51), whereas Sp3 appears to be a transcriptional repressor that binds competitively to common target sites with Sp1 or Sp4 (41, 42, 51). It was also suggested that the relative levels of Sp1 and Sp3 could determine specific gene expression (49, 50). From our supershift analysis using specific antibodies against Sp1 family proteins, the majority of protein components contained in the MREb-BF complex is Sp1, and Sp3 was detected as only a minor component. These findings suggest a major role for Sp1 in negative regulation, although Sp3 may also have a contribution.

Although a lot of MRE-binding proteins have been reported since MRE was recognized as an element mediating heavy metal-activated transcription (6–8), no proteins with the exception of MTF-1 have been demonstrated to be involved in MRE-mediated gene regulation (4, 5). Our present results strongly suggest that Sp1 acts as the second regulator of MRE-mediated transcription by recognizing only particular MREs and repressing MTF-1 transactivation through competition for binding to the overlapping recognition sites.

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