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Fine Mapping of a Mouse Metallothionein Gene Metal Response Element

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Metal-regulated transcription of metallothionein (MT) genes in higher eucaryotes involves multiple copies of a highly conserved 17-base-pair metal-regulatory element (MRE). We have assayed by transient transfection the ability of mouse MT-I element d (MREd) to confer metal responsivity to constructs containing the mouse MT-I TATA box and the bacterial chloramphenicol acetyltransferase indicator gene. A single copy of MREd works bidirectionally to afford a three- to fourfold induction, and dual copies act cooperatively to yield a 10- to 20-fold response. Element d responds to the same spectrum of heavy metals as doses the complete MT gene promoter. The sequences involved in induction by metals were delineated by analyzing point mutations in MREd. While nucleotides of the highly conserved core sequence TGCPuCXC are critical, substitutions in the less conserved regions affect the induction response only marginally. These sequences include residues of a potential Sp1-binding site, suggesting that if Sp1 binds to MREd, it has little if any role in induction by metals.

The regulated transcription of eucaryotic structural genes is accomplished through interactions between both general and gene-specific transcription factors and their target DNA sequences. The mammalian metallothioneins (MTs) provide a good example of the complexity of interactions that can occur at a single promoter. At least seven different *trans*-acting factors are thought to control the transcription of various MT genes: Sp1 and MLTF, which are involved in maintaining appropriate basal transcription levels (5, 14); AP1, which is involved in induction pathways utilizing protein kinase C (2, 14); AP2, which mediates induction involving cyclic AMP (9); AP4, whose function is unknown (16); glucocorticoid receptor, which binds to the human MT-IIA gene promoter but apparently not to several other MT genes (12, 13, 18, 19); and a putative metal regulatory factor(s) which induces transcription in response to heavy-metal ions (1, 17, 21). The first six proteins are all general factors in that they are known to interact with several chromosomal as well as viral promoter sequences (10). In contrast, the metal-regulatory factor appears to be gene specific in that its target DNA sequence, the metal-regulatory element (MRE), has so far been found only in MT genes. Because the induction of MT gene transcription by metals is the only response that plays a known physiological role, namely, to protect cells against metal toxicity (8), it is particularly important to elucidate the precise DNA sequences and *trans*-acting factors that mediate this form of induction.

The *cis*-acting MREs involved in transcriptional induction by heavy metals are organized as imperfect repeats upstream of the MT gene transcriptional start site. These 17-base-pair elements are highly conserved from fly to man and consist of a core heptanucleotide, TGCPuCXC, partially overlapping a less conserved GC-rich region (4, 13, 20, 23). In the case of the mouse MT-I gene, five MREs are present (MREa through MREe) between residues −40 and −175 upstream of the initiation site. Of these elements, MREd has been shown to confer the strongest induction response to a heterologous promoter in vivo; by these criteria, element e is nonfunctional (24). Interestingly, a potential binding site for mammalian transcription factor Sp1 has been noted in MREd, with the GC-rich region containing sequences of the Sp1 core hexanucleotide GGGCGG (11, 22, 24). Homologies to the Sp1 core site have also been noted in the GC-rich region of several other eucaryotic metal response elements (24), and recent studies have demonstrated weak binding of human Sp1 to mouse MT-I MREd in vitro (17). Whether or not this factor actually participates in metal-induced gene expression in vivo, however, is unclear.

In the experiments presented here, the role of individual MRE sequences in metal regulation was examined by assaying a series of constructs containing mouse MT-I MREd and point mutation derivatives thereof in a transient transfection system. We found that regulatory activity is primarily dictated by residues of the highly conserved MRE heptanucleotide core. In contrast, the GC-rich region containing the presumptive Sp1 core binding site appears to have a relatively minor role, if any, in the transcriptional induction by heavy metals.

Transcriptional induction by mouse MREd. All recombinant plasmids containing mouse MT-I MREd were derived from 5′Δ33, a construct containing the mouse MT-I TATA box and transcription initiation region (residues −33 to +18) fused to the bacterial chloramphenicol acetyltransferase (CAT) gene (Fig. 1A). MRE monomer plasmids were constructed by inserting synthetic oligonucleotides of MREd into 5′Δ33 in either orientation, preserving the natural spacing between the TATA box and the most proximal MRE of mouse MT-I (Fig. 1A). When these $MRED_1CAT$ constructs were transiently expressed in mouse L cells, a low level of induction of CAT activity by 2.5 μM $CdCl_2$ was observed (Fig. 1B, lanes 3 through 6). This 1.5- to 2-fold stimulation is in fact an underrepresentation of induction, since a reproducible decrease in CAT activity is obtained with noninducible promoters such as that of 5′Δ33 (Fig. 1B, lanes 1 and 2) or RSVCAT (7) (data not shown), presumably reflecting a metal toxicity effect. The corrected induction ratio for the $MRED_1CAT$ constructs is therefore three- to fourfold. When a second copy of MREd was inserted to yield either an inverted or a direct MRE repeat, a high cooperative level of induction was observed, ranging from 10- to 20-fold (Fig. 1B, lanes 7 and 8; see Fig. 3). This is comparable to the induction

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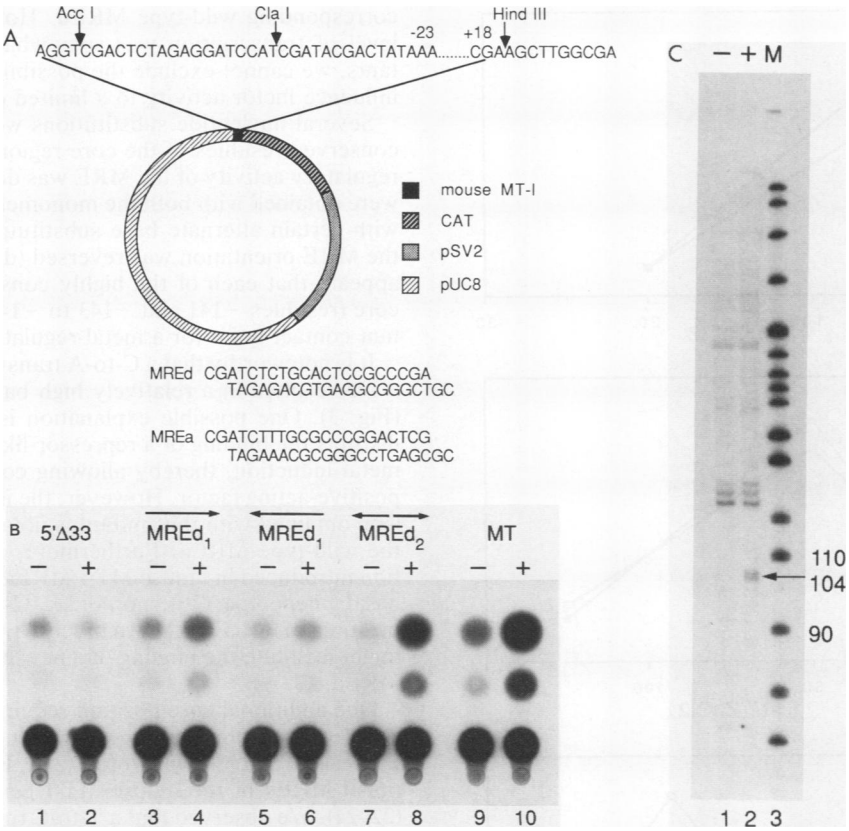


FIG. 1. (A) Construction of MT-CAT fusion plasmids. An oligonucleotide containing residues −33 to +18 of the mouse MT-I gene was cloned into the *Bam*HI and *Hin*dIII sites of p8CAT (6) to generate 5′Δ33. The sequence shown represents these MT residues flanked upstream by polylinker sequences and downstream by a *Hin*dIII site adjoining the CAT gene. MRE-monomer constructs were made by inserting oligonucleotides of MREd or MREa into the *Cla* site of 5′Δ33 and were screened for orientation by dideoxy sequencing of the *Bam*HI-*Eco*RI promoter-containing fragment mobilized into M13mp18. MRE dimers were constructed by inserting a second oligonucleotide into the *Acc*I site of the M13 monomer derivative and then mobilizing the *Pst*I-*Hin*dIII promoter-containing fragment into p8CAT. The p8MTCAT plasmid (construction not shown) was generated by inserting a 2-kilobase *Eco*RI-*Bgl*II fragment of the mouse MT-I promoter containing sequences −1900 to +68 into the *Eco*RI-*Bam*HI polylinker sites of p8CAT. (B) CAT activity of MT promoter-CAT fusion constructs. Mouse L cells were transiently cotransfected by the calcium phosphate precipitation procedure (7) with 20 μg of the indicated construct and 2 μg of pRSV/L, a plasmid containing a Rous sarcoma virus-firefly luciferase fusion gene (25). Cells were cultured for 15 h in the presence (+) or absence (−) of 2.5 μM $CdCl_2$ and harvested, and extracts were prepared as described elsewhere (7). Luciferase values were obtained by the method of Subramani (25), and after extract standardization, assays for CAT activity were conducted (7). Twenty times more extract was used for the CAT assays shown in lanes 1 through 8 than for those shown in lanes 9 through 10. Arrows indicate orientation of MREd oligonucleotide insertion with respect to the TATA box. (C) Primer extension analysis of RNA transcribed from $MRED_2CAT$. RNA from $MRED_2CAT$-transfected cells cultured in the presence (+) or absence (−) of 2.5 μM $CdCl_2$ was prepared by the guanidine thiocyanate procedure and fractionated on an oligo(dT) column (15). Approximately 2.5 μg of poly(A)$^+$ RNA was hybridized to a single-stranded ^{32}P-end-labeled CAT probe (RSV2CAT residues 4944 to 4921) (7) and extended with Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) as previously described (3). The expected 104-nucleotide product is a fusion of 86 CAT and 18 mouse MT-I residues. The origin of the higher-molecular-weight bands is not known, but it is apparent from panel B that they did not contribute to CAT activity. Lane M, ^{32}P-end-labeled pBR322 digested with *Msp*I.

ratio seen with the p8MTCAT construct containing the full-length 2-kilobase promoter of the mouse MT-I gene (Fig. 1B, lanes 9 and 10; note that these samples contained 20-fold less extract than samples in lanes 1 through 8). However, consistent with previous deletion analyses (4), the basal expression of the MREd constructs is only about 2% that of the intact promoter. Presumably the constitutive level of MT gene expression involves DNA sequences and factors distinct from those participating in induction by heavy metals.

The initiation site for the RNA transcribed from the $MRED_2CAT$ fusion gene was mapped by primer extension. A Cd-inducible RNA of the expected size was detected, and the levels of this RNA paralleled the levels of CAT activity (Fig. 1C). The origin of several additional primer extension bands is unknown, but clearly these noninducible RNAs do not contribute to CAT activity.

Induction of the complete MT gene promoter can be accomplished by a variety of heavy metals, including Cd, Zn, and Cu (8). To determine whether the isolated MREd is similarly responsive, cells transfected with p8MTCAT and $MRED_2CAT$ were treated in parallel with various doses of $CdCl_2$, $ZnCl_2$, and $CuSO_4$. The two constructs displayed virtually indistinguishable patterns of induction (Fig. 2). This result indicates that the multiplicity of MREs in the complete MT gene promoter does not function merely to allow induction by diverse metals. More probably, all the elements are responsive to the different metals and together act to facilitate a strong induction response.

Residues essential for induction by heavy metals. Since the isolated MREd of mouse MT-I is sensitive to the same spectrum of metals as the complete MT gene promoter, and since regulatory activity is observed with monomers as well

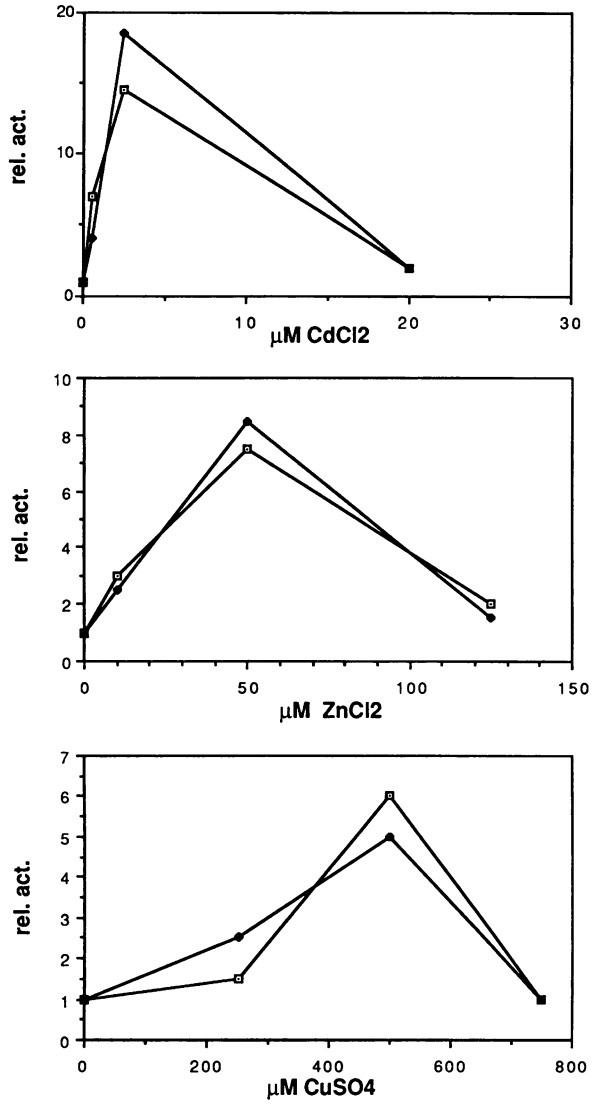


FIG. 2. Response of $MRED_2CAT$ versus p8MTCAT to various metals. Cells transfected with $MRED_2CAT$ (■) or p8MTCAT containing the complete mouse MT-I promoter (⊡) were treated with various doses of heavy metal as indicated. Relative activity (rel. act.) was calculated as CAT activity with metal/CAT activity without metal. Note that both the ordinates and abscissas are drawn to different scales for the different metals.

as multimers of this element, MREd was used as a model for delineating the MRE nucleotides essential for induction. For these studies, a series of synthetic oligonucleotides containing point mutations in MREd were inserted as monomers or dimers into 5′Δ33 and tested for regulatory activity in the transient transfection assay. The MREd oligonucleotide contains MT-I residues −134 to −150 (Fig. 3). Mutations were introduced in the highly conserved core region (nucleotides −141 to −147) as well as in the less conserved positions flanking this region. The results of these studies are summarized in Fig. 3 and are discussed below in the order of point mutations as they appear 5′ to 3′ on the MRE.

Two mutations were introduced into the semiconserved nucleotides upstream of the MRE core (at positions −148 and −149). In both cases, the mutations still allowed for an induction response comparable to that obtained with the corresponding wild-type MREd. However, since the basal level of transcription was somewhat altered in these mutants, we cannot exclude the possibility that these positions influence factor activity to a limited degree.

Several nucleotide substitutions were made in the highly conserved residues of the core region, and in each case the regulatory activity of the MRE was destroyed. These results were obtained with both the monomer and dimer constructs, with certain alternate base substitutions (Fig. 3) and when the MRE orientation was reversed (data not shown). It thus appears that each of the highly conserved positions of the core (residues −141 and −143 to −147) serves as an important contact point for a metal-regulatory factor(s).

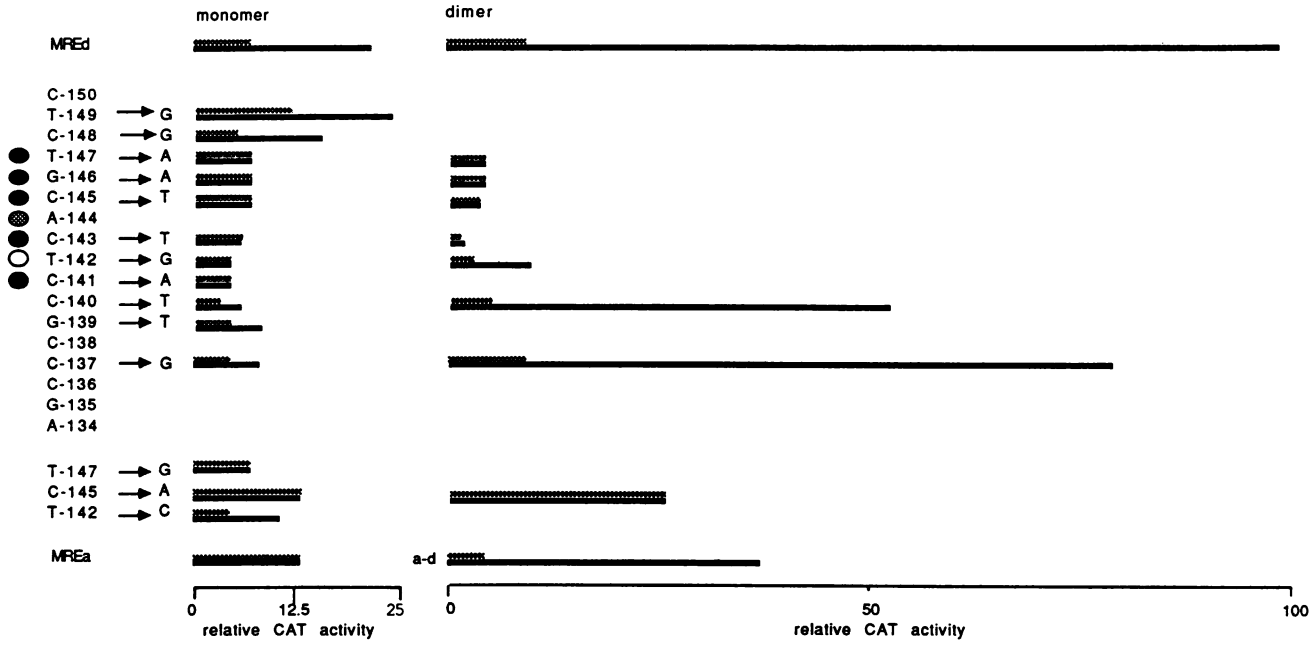
It is noteworthy that a C-to-A transversion at core position −145 resulted in a relatively high basal level of expression (Fig. 3). One possible explanation is that this substitution disrupts the binding of a repressor-like molecule involved in metal induction, thereby allowing constitutive binding of a positive-acting factor. However, the induced level of expression obtained with this mutant is notably lower than that for the wild-type MREd. Furthermore, a corresponding mutation introduced in mouse MT-I MREa did not produce a high basal phenotype (20), nor did a C-to-T transition at this position in MREd. Alternatively, the −145A substitution might facilitate the binding of a new positive-acting factor to MREd.

One additional core position requires discussion. Nucleotide −142 is the only core residue that is not strictly conserved across eucaryotes (24). However, in the functional MREs of the mouse MT-I gene it is always C or T (22–24). We observed that a T-to-C transition at this position was essentially neutral, while a T-to-G transversion dramatically reduced induction (Fig. 3).

Three point mutations were introduced in the semiconserved GC-rich region downstream of the MREd core. Such substitutions still allowed for a small level of induction in the monomer element and a large cooperative stimulation when assayed as dimer elements (Fig. 3). These results demonstrate that the individual nucleotides of the GC-rich area, unlike those of the core, are not essential for the activity of a putative metal-regulatory factor(s). A similar conclusion was drawn from an analysis of MREa, although in this weaker element position G-83 seems to be more important than the corresponding C-140 in MREd (20).

The role of the GC-rich region in metal induction was further examined by assaying constructs containing mouse MT-I MREa, which shares only three of seven GC-rich region residues with MREd (22–24). Although this element did not induce transcription as a monomer, it did interact with MREd in a cooperative manner to yield high levels of induction (Fig. 3). This result, together with the point mutation analyses described above, argues that the GC-rich region is not critical for induction by metals. However, since induction ratios obtained with point mutants in this region were less than wild-type levels (Fig. 3), and since the different MREs of mouse MT-I varied in their capacity to induce (Fig. 3) (24), the GC-rich region may act to modulate either the binding of the metal regulatory factor or its ultimate effect on transcription.

In summary, we found that the 17-base-pair MT gene MRE actually consists of two parts (Fig. 3, bottom). One is a highly conserved heptanucleotide core that plays a crucial role in the induction response; a single point mutation here completely abolishes MRE regulatory activity. Adjacent to this region is a semiconserved GC-rich region that in many eucaryotic MREs exhibits significant homology to the core



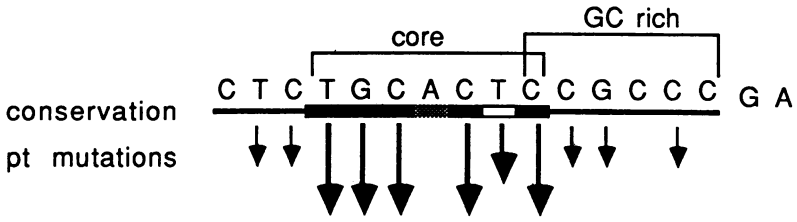


FIG. 3. Point mutational analysis of MREd. (Top) Residues of MREd are listed vertically, and each is marked with a number indicating its position in the natural mouse MT-I gene promoter. Symbols: ●, residues of the highly conserved MRE core; ⊛, purine; ○, pyrimidine conserved only in the functional MREs (a through d) of the mouse MT-I gene; ▩, CAT activity from control cells; ■, CAT activity from metal-treated cells; a-d, dimer construct containing MREa in the distal position and MREd in the proximal position; →, individual nucleotide substitutions introduced in MREd. The corresponding templates contained the MRE either as a monomer in the right orientation with respect to the TATA box or as a direct repeat dimer. The CAT activity measurement shown for each MRE-containing construct represents an average of two to five independent transfections standardized with luciferase. The average deviation was 15%. Values obtained from cells treated for 15 h with 2.5 μM $CdCl_2$ were corrected for the metal toxicity effect noted with noninducible constructs. Relative CAT activity is CAT measurements as a percentage of the induced activity of $MREd_2CAT$. (Bottom) Summary of point mutational analysis. The nucleotide sequence of MREd is shown, indicating residues of the highly conserved core region as well as the GC-rich region. Degree-of-conservation symbols: ——, 50 to 80% conserved in eucaryotes; ■, greater than 90% conserved; ▩, purine; ▭, pyrimidine conserved only in the functional MREs (a through d) of the mouse mMT-I gene; ↓, positions where nucleotide substitutions were made; arrow length and intensity represent the approximate relative degree of inhibition of metal induction observed with the corresponding mutant template.

binding site for mammalian transcription factor Sp1; this homology is extended to a consensus of 9 of 10 base pairs in the case of mouse MT-I MREd (11, 22, 24). However, sequences of the MRE GC-rich region appear to play a relatively minor role in induction by metals, suggesting that a Sp1-like protein is not the critical factor for induction. This is further supported by the observation that a partially purified mouse Sp1 preparation does not exhibit strong binding to this sequence (J. Imbert and D. Hamer, unpublished results). We suggest that the response to metals instead involves the binding of a positive-acting metal-regulatory factor to the MRE heptanucleotide core and that the flanking sequences can either influence this binding or affect interactions with other factors involved in MT gene transcription.

Although several attempts to detect factors that interact with the MREs have been reported (1, 17, 21, 22), none of these factors has been isolated or characterized in detail. Recent studies show that yeast MT is induced by copper through an MT-like specific DNA-binding protein that binds both to metals and to the MT gene control region (6a). The *cis*-acting mutants described here may facilitate the search for similar *trans*-acting factors in mammalian cells.

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