Putative Zinc-sensing Zinc Fingers of Metal-response Element-binding Transcription Factor-1 Stabilize a Metal-dependent Chromatin Complex on the Endogenous Metallothionein-I Promoter*

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The metalloregulatory functions of metal-response element-binding transcription factor-1 (MTF-1) have been mapped, in part, to its six highly conserved zinc fingers. Here we examined the ability of zinc finger deletion mutants of mouse MTF-1 to regulate the endogenous metallothionein-I (MT-I) gene in cells lacking endogenous MTF-1. MTF-1 knockout mouse embryo fibroblasts were transfected with expression vectors for FLAG-tagged MTF-1 (MTF-1flag) or finger deletion mutants of MTF-1flag and then assayed for metal induction of MT-I gene expression, nuclear translocation, and in vitro DNA-binding activity of MTF-1 and its stable association with the endogenous chromosomal MT-I promoter. In intact MTF-1flag restored metal responsiveness of the MT-I gene, underwent nuclear translocation, displayed increased DNA binding in response to zinc and less so to cadmium, and rapidly formed a stable complex with the MT-I promoter chromatin in response to both of these metals. In contrast, although deletion of finger 1, fingers 5 and 6, or finger 6 only had variable effects on the nuclear localization and in vitro DNA-binding activity of MTF-1, each of these finger-deletion mutants severely attenuated metal-induced MTF-1 binding to the MT-I promoter chromatin and activation of the endogenous MT-I gene. These results demonstrated that the metal-induced recruitment of MTF-1 to the MT-I promoter is a rate-limiting step in its metalloregulatory function and that an intact zinc finger domain is required for this recruitment. During the course of these studies, it was discovered that mouse MTF-1 is polymorphic. The impact of these polymorphisms on MTF-1 metalloregulatory functions is discussed.

Metal-response element (MRE)\(^1\)-binding transcription factor-1 (MTF-1) is an essential metalloregulatory transcription factor that coordinates the expression of genes involved in zinc homeostasis and protection against metal toxicity and oxidative stress. These include, but are not limited to, metallothionein (MT) (1), zinc transporter-1 (2), and \(\gamma\)-glutamylcysteine synthetase heavy chain genes (3). However, the mechanisms by which MTF-1 activates gene expression in response to metals are not well understood.

Treatment of mammalian cells with zinc in vivo promotes rapid nuclear translocation of MTF-1 (4, 5) and causes a dramatic increase in MTF-1 DNA-binding activity measured in vitro (6–8) concomitant with the occupancy of MREs in the MT-I promoter in vivo (6, 9, 10) and the activation of MT-I gene expression (11). Direct interactions between zinc and the zinc finger domain of MTF-1 regulate its DNA-binding activity (7). This suggests a model in which MTF-1 senses free zinc, adopts a reversible DNA-binding conformation, moves to the nucleus, and activates gene expression by associating with the promoter (12, 13). However, cadmium, a more potent inducer of MT-I gene expression than zinc, is less effective than zinc at driving MTF-1 to the nucleus (4, 5), has little effect on the DNA-binding activity of MTF-1 in vitro (8), yet requires MTF-1 to activate gene expression. Furthermore, studies of the metalloregulatory activity of mouse MTF-1 in yeast demonstrated that MTF-1 can function as a zinc sensor but not as a cadmium sensor in this system (14). These studies suggest that distinct co-activators or signal transduction cascades are required to mediate MTF-1 activation by zinc versus cadmium. In that regard it has been shown recently (15, 16) that MTF-1 is phosphorylated in vivo, and kinase inhibitor studies (15–17) suggest that multiple protein kinases may also play a role in MTF-1 activation. Thus, the mechanism of action of MTF-1 appears to involve several potential metal-specific regulatory steps that are not well defined.

MTF-1 is a zinc finger transcription factor in the Cys2-His2 family (18). Its zinc finger domain plays an essential role in its metalloregulatory functions and consists of six fingers that have been highly conserved during evolution (13). In contrast, significant divergence has occurred in the remainder of the protein (19). The zinc finger domain (7, 18, 20) binds specifically to DNA sequences termed metal-response elements (MREs) that are found in the promoter regions of MTF-1-regulated genes (21). The six zinc fingers display structural and functional heterogeneity (22–27). Studies of the purified recombinant zinc finger domain of human MTF-1 suggest that

MTF-1 KO, MTF-1 knockout mice.
about half of the fingers exhibit high affinity zinc binding and play a structural role in protein folding, DNA binding, and DNA bending, whereas the other half exhibit lower affinity zinc binding and appear to be important for zinc sensing and further stabilization of DNA binding (22–25).

Functional analyses of mouse MTF-1 finger-deletion mutants using transient transfection assays and in vitro DNA-binding assays suggest that the core DNA-binding activity resides in fingers 2–4, and the metal-dependent activation of DNA-binding activity may be influenced by finger 1 (26). In contrast, deletion of mouse MTF-1 fingers 5 and 6 (26) or mutation of the second cysteine residue (Cys to Tyr) in human MTF-1 finger 5 or 6 (27), which would preclude zinc binding and folding of the fingers, had little effect on zinc-induced MTF-1 DNA-binding activity measured in vitro or reporter gene activation in transiently transfected cells. Similarly, studies of the metalloregulatory activity of mouse MTF-1 in yeast did not reveal a function for fingers 5 and 6 (14, 26). However, these zinc fingers are highly conserved during evolution and must be of functional importance in MTF-1. Although the in vivo roles of the putative metal-sensing zinc fingers of MTF-1 remain unknown, it is clear that the zinc finger domain of MTF-1 plays a crucial role in its responses to both zinc and cadmium.

An intrinsic disadvantage of using reporter genes in transient transfection studies is that the reporter gene is not packaged into chromatin. Chromatin structure has been shown to play an important role in MT-I gene expression. DNase-I-hypersensitive sites in regions flanking the mouse MT-I and MT-II genes confer position-independent expression to reporter transgenes (28) and may represent locus control regions in the MT gene locus. DNA methylation and histone acetylation have been suggested in MT-I gene silencing and re-activation in mouse lymphosarcoma cells, respectively (10, 29). Furthermore, histone deacetylase inhibitors can potentiate heavy metal-induced MT-I gene expression (10, 30). Therefore, we hypothesized that zinc fingers 5 and 6 of MTF-1 may play a role in its metal-sensing functions on native chromatin templates.

Here we examined the roles of the putative metal-sensing zinc fingers of mouse MTF-1 in the metal-dependent regulation of expression of the endogenous, chromatin packaged MT-I gene. Evidence is presented that the metal-induced recruitment of MTF-1 to the MT-I promoter and formation of a stable MTF-1-chromatin complex is a rate-limiting step in zinc- as well as cadmium-induced activation of gene expression by MTF-1, and that MTF-1 zinc fingers 1, 5, and 6 play an essential role in this process.

**EXPERIMENTAL PROCEDURES**

**RT-PCR**—Total RNA was prepared from CD-1 mouse liver using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Briefly, cells were plated in 10-cm dishes so that they were 70–90% confluent at the time of transfection. The next morning, DNA-PLUS-LipofectAMINE reagent complexes were prepared as recommended in a volume of 1.5 ml containing expression vectors (0.1–8.1 μg) for mouse MTF-1 flag or its finger-deletion mutants. A green fluorescence protein (GFP) expression vector (9 μg) pEGFP-C2 (Clontech, Palo Alto, CA) was included in each transfection as an internal control for transfection efficiency. The complexes were added directly to cells in fresh serum-free medium. After 5 h of incubation, DMEM with 20% FBS was added to bring the final concentration to 10% serum and to split 1:2 into 10-cm dishes. Total RNA was prepared using TRIzol reagent (Invitrogen) from untreated cells or cells treated with 12 h with 100 μM ZnSO4.

Stable cell lines were generated by transfecting MTF-KO cells, as described above, with expression vectors coding for mouse MTF-1 or its finger-deletion mutants. All expression vectors carry the hygromycin resistance gene. The transfected cells were selected in 200 μg/ml hygromycin B (Invitrogen). Pools of stably transfected cells were collected after 7–10 days of selection. Single colonies of stably transfected cells were picked after 2 weeks of selection, and expression was confirmed by Western blotting using an anti-FLAG antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Two independent cell lines for each construct were analyzed.

**Northern Blotting**—Cells (80–90% confluency) were treated with ZnSO4 or CdCl2, as indicated, prior to harvest. Total RNA was isolated using TRIzol reagent (Invitrogen), separated by denaturing agarose/formaldehyde gel electrophoresis, transferred, and UV cross-linked to Nytran Supercharge nylon membranes (Schleicher & Schuell). Northern Blots were prehybridized, hybridized with an MT-I cRNA probe, and washed as described previously (31). Hybridization signals were detected by autoradiography at −70 °C with intensifying screens and quantitated using the PhastGnome Storage Phosphor System (Packard, Meriden, CT). A β-actin cRNA probe was used as a control to normalize for RNA amount, integrity, and transfer efficiency.

**Preparation of Nuclear, Cytoplasmic, and Whole Cell Extracts**—Nuclear and cytoplasmic extracts were prepared from cells (80–90% confluent), untreated or treated for 1 h with ZnSO4 (100 μM) or CdCl2 (10 or 20 μM), as described previously (6) with modifications. Briefly, the cells were collected in 1× phosphate-buffered saline and homogenized in cell lysis buffer (6). Nuclei were collected, and supernatant was saved as a cytoplasmic extract. The nuclei were resuspended in 1× electrophoresis buffer, were stirred on ice for 30 min and centrifuged, and the supernatant was collected as nuclear extract (6). Cytoplasmic extracts were adjusted to 140 mM KCl and cleared by ultracentrifugation. Whole cell extracts were prepared by snap-freezing and -thawing cell pellets in nuclear extraction buffer and cleared by ultracentrifugation. Protein concentration was determined using Bio-Rad Protein Assay reagent (Bio-Rad) with bovine serum albumin as the standard.

**Electrophoretic Mobility Shift Assay (EMSA)**—EMSA was performed using nuclear extracts with end-labeled double-stranded oligonucleotide called MRE-s, as described previously (7). Due to the different protein expression levels in each cell line, different amounts of nuclear protein from each cell line, as indicated in the figure legend, were used to obtain signals within the detection range.

**Western Blotting**—Western blotting was performed using an anti-MTF-1 antibody, or an anti-FLAG antibody (Santa Cruz Biotechnology), followed by a goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology), as described previously (4). Due to the different protein expression levels in each cell line, different

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*H. Jiang and G. K. Andrews, unpublished data.*
Metalloregulatory Mechanisms of MTF-1

Fig. 1. Diagram of the structure of MTF-1* and its finger-deletion mutants and delineation of polymorphisms in mouse MTF-1. MTF-1* contains a short amino-terminal region followed by six consensus Cys-His-zinc fingers and three transactivation domains (acidic domain, proline-rich domain, and serine/threonine-rich domain). The 8-amino acid FLAG peptide is located at the carboxyl terminus. MTF-1 finger-deletion mutants, D1 flag, D5&6 flag, and D6 flag, have finger 1, fingers 5 and 6, or only finger 6 deleted, respectively. Confirmed polymorphisms (ΔQ590, and P656T) and the amino-terminal polymorphism (I1IT) are as indicated. MTF-1* contains two additional amino acid substitutions (R164Q and L181F) in the zinc finger domain that are not present in MTF-1*. These were not confirmed polymorphisms.

amounts of protein from each cell line, as indicated in the figure legend, were used to obtain signals within the detection range.

Chromatin Immunoprecipitation (ChIP)—ChIP assays were carried out as described previously (32, 33), with modifications. Cells were treated with 1% formaldehyde, and cross-linking was stopped by the addition of glycine to a final concentration of 0.125 M. After homogenization of cells as described previously (6), the nuclei were lysed in sonication buffer (50 mM Tris·HCl, pH 8.1, 10 mM EDTA, 10% SDS, 1 mM protease inhibitor mixture (Roche Applied Science), and the chromatin was sonicated to an average length of 600–1000 bp and clarified by centrifugation at 12,000 × g for 10 min at 4 °C. The chromatin was diluted 10-fold in ChIP dilution buffer (33), pre-cleared with protein A-Sepharose (Sigma), and immunoprecipitated with anti-FLAG M2-Sepharose. The beads were washed, the immune complexes eluted, and the cross-links reversed as described previously (33). The DNA was phenol:chloroform-extracted, reconstituted in TE buffer (pH 8.0), and analyzed by PCR using primers that span –230 to +123 bp (relative to the transcription start site) of the mouse MT-I gene. [32P]dCTP-incorporated PCR products (29 cycles) were separated by electrophoresis in TBE gels, quantified using the Packard Cyclone Storage Phosphor System (Packard Instruments), and normalized to input products. Fold induction was calculated from triplicate PCRs, and for initial experiments, input and immunoprecipitated DNA samples were analyzed at various cycles (28–32 cycles) to ensure that the PCR products were obtained in the linear range of amplification.

RESULTS

MTF-1 Is Polymorphic in Mice—During the course of these studies, we discovered that mouse MTF-1 is polymorphic. A mouse MTF-1 cDNA (GenBank™ accession number AY260849) cloned by RT-PCR from the CD-1 outbred strain of mice showed several differences throughout the protein (Fig. 1) compared with the sequence published in GenBank™ (accession number X71327), resulting in several amino acid substitutions and an amino acid deletion. To determine whether these differences represent polymorphisms, these regions of MTF-1 mRNA were cloned by RT-PCR using total RNA extracted from six individual CD-1 mouse livers, and individual clones were then sequenced. The results confirmed that the differences in the serine/threonine-rich domain and the carboxyl-terminal region of our MTF-1 cDNA clone are present in many individual mice (GenBank™ accession numbers AY260851 and AY260852) and thus represent true polymorphisms. The difference in the amino-terminal region of our MTF-1 cDNA clone was not detected among these RT-PCR products. However, a different change resulting in a different amino acid substitution was found in this region among the clones (GenBank™ accession number AY260850), and a computer search of the GenBank™ EST data base revealed yet another polymorphism in this region (accession number XM124539). Thus, the amino terminus of MTF-1 is highly polymorphic, and the differences we discovered in this region is very likely to represent a true polymorphism. In contrast, we were unable to confirm that the two differences in the zinc finger domain of this MTF-1 cDNA clone represent true polymorphisms. Screening of cDNAs and genomic DNAs from 35 CD-1 mice did not reveal a sequence with those changes. Thus, it cannot be excluded that these differences represent RT-PCR-induced changes in the zinc finger coding region, although the probability of that is very low.

Deletion of the Putative Metal-sensing Fingers of MTF-1 Attenuates Its Metalloregulatory Functions on the Endogenous MT-I Gene in Transiently Transfected Cells—Changes found in the zinc finger domain of this MTF-1 cDNA (MTF-1*), Fig. 1) may not represent true polymorphisms. Therefore, an expression vector coding for a FLAG-tagged MTF-1 (MTF-1flag) containing the consensus MTF-1 zinc fingers in a backbone bearing the confirmed polymorphisms in the amino terminus, the serine/threonine-rich domain, and the carboxyl terminus was created and used in the remaining studies (Fig. 1).

In initial experiments, expression vectors for MTF-1flag and its finger-deletion mutants (D1flag, D5&6flag, and D6flag, diagrammed in Fig. 1) were transiently transfected into mouse embryo fibroblasts derived from MTF-1 knockout mice (MTF-KO), and the ability of the transiently expressed MTF-1flag and its finger-deletion mutants to restore the metal responsiveness of the endogenous MT-I gene was examined. A GFP expression vector was co-transfected to verify consistent transfection efficiency among the vectors (20–25%, data not shown). Total RNA was prepared from the transfected cells before or after treatment with zinc, and MT-I mRNA levels were determined by Northern blotting. Very low levels of MT-I mRNA were detected in MTF-KO cells transfected with the GFP expression vector alone, before and after zinc treatment (Fig. 2, the 0-DNA lanes). In contrast, basal expression of the endogenous MT-I gene was enhanced in cells transiently transfected with the MTF-1flag expression vector. Basal levels of MT-I mRNA in these cells increased as the amount of transfected expression vector increased (Fig. 2A). Expression of MTF-1flag also restored the metal responsiveness of the endogenous MT-I gene. Zinc treatment, as well as cadmium treatment (not shown, see Fig. 3), caused an increase in MT-I mRNA abundance (Fig. 2A), unless a very large amount of expression vector had been transfected. Thus, MTF-1flag can regulate basal and metal-induced MT-I gene expression in vivo as does native MTF-1 (1).

In contrast to intact MTF-1flag, metal responsiveness of the endogenous MT-I gene was not restored in cells transiently transfected with expression vectors coding for the MTF-1*flag finger-deletion mutants (D1flag, D5&6flag, and D6flag), regardless of the amount of expression vectors transfected (Fig. 2, B–D). Furthermore, basal levels of MT-I mRNA in cells transiently transfected with expression vectors for D1flag or D5&6flag were increased only modestly even after a large amount of expression vector had been transfected (Fig. 2, B–C). In cells transiently transfected with the D6flag expression vec-
Northern blot analyses of MT-I mRNA in pools of stably transfected cells. Increasing amounts of expression vectors for MTF-1flag (A), and its finger-deletion mutants, D1flag (B), D5&6flag (C), and D6flag (D), were transiently transfected into MTF-KO cells. Total RNA was extracted from untreated cells (−) or cells treated for 12 h with 100 μM ZnSO₄ (+) and analyzed for MT-I mRNA levels by Northern blotting. Transfection efficiency was monitored by co-transfection of a GFP expression vector and ranged from 20 to 25% of the cells in each sample (data not shown).

Finger-deletion Mutants Reflect a Diminished Metal-induced Responsiveness of the Endogenous MT-I Gene in Pools of Stably Transfected Cells. A, Northern blot analyses of MT-I mRNA in pools of stably transfected cells expressing MTF-1 flag and its finger-deletion mutants. MTF-KO cells were transfected with expression vectors for MTF-1 flag or its finger-deletion mutants (D1 flag, D6 flag, and D5&6 flag) and selected by hygromycin B, and pools of selected cells were collected and analyzed. Total RNA was extracted from untreated cells (−) or cells treated for 12 h with 100 μM ZnSO₄ (Zn) or 20 μM CdCl₂ (Cd). MT-I mRNA levels were determined by Northern blotting. B, Western blot detection of MTF-1 protein levels in these cell lines expressing D6 flag displayed clearly elevated basal MT-I mRNA levels but attenuated metal induction of the MT-I gene. MT-I mRNA levels increased less than 2-fold after zinc or cadmium treatment in these cells (Fig. 3A). Differences in MT-I gene expression and metal responsiveness in these cells did not reflect differences in protein expression levels. Western blotting suggested that MTF-1 protein levels were similar in all four pools of stably transfected cells (Fig. 3B).

To examine further the effects of these zinc finger deletions on the activity of MTF-1, individual stably transfected cell lines were cloned and analyzed. The metal ion dose response and the time course for induction of MT-I mRNA by zinc and cadmium were determined using Northern blotting (Fig. 4). Two independent cell lines expressing MTF-1 flag (lines 9 and 42) were selected that contain different amounts of this protein (Fig. 4A, lower panel). Both cell lines displayed increased MT-I mRNA levels after zinc and cadmium treatment, and the response of the MT-I gene was similar to that seen in wild type MEF (Fig. 4A, upper panel). However, basal levels of MT-I mRNA were higher in cells expressing higher amounts of MTF-1 flag (cell line 42). Dose-response experiments revealed that the induction of MT-I mRNA was clearly detectable in cells (line 9) treated with as little as 20 μM zinc or 10 μM cadmium (Fig. 4B, left panels). Induction of MT-I mRNA by zinc and cadmium was evident as soon as 4 h after treatment (Fig. 4B, right panel). The time course assay was repeated in the number 42 cell line, and similar results were obtained (data not shown).

Interestingly, basal levels of MT-I mRNA were undetectable in cells stably transfected with an expression vector for MTF-1 flag* (Fig. 4A), which bears two amino acid changes in the zinc finger domain (see Fig. 1). However, zinc greatly induced MT-I mRNA levels in these cells, whereas cadmium treatment only caused a modest induction of MT-I mRNA (Fig. 4A). These results indicate that MTF-1 flag* is zinc-responsive but exhibits a diminished ability to regulate basal and cadmium-induced expression of the MT-I gene.

In contrast to cells expressing MTF-1 flag, MT-I gene induction by zinc and cadmium was not restored in stably transfected cell lines expressing the MTF-1 flag finger-deletion mutants (Fig. 5). Two separate cell lines were analyzed for each expression construct, and similar results were obtained. Results from only one cell line for each construct are shown here. Northern blot analysis of total RNA prepared from cells expressing D1 flag, D5&6 flag, and D6 flag revealed detectable basal levels of MT-I mRNA in these three cell lines (Fig. 5, A–C). However, induction of MT-I mRNA by zinc and cadmium was severely attenuated in all three cell lines, even at an exposure to toxic concentrations of these metals and regardless of the length of exposure. The maximum induction of MT-I mRNA after zinc or cadmium treatment was less than 2-fold in cells expressing D5&6 flag and D6 flag (Fig. 5, B and C).

The Attenuated Metalloregulatory Functions of MTF-1 flag Finger-deletion Mutants Reflect a Diminished Metal-induced Association with the MTF-1 Promoter in Vivo, but Not the Loss of Nuclear Translocation or in Vitro DNA-binding Activity—These above results demonstrate that the metal-sensing zinc...
fingers of MTF-1 are critical for its functions on the endogenous MT-I gene. The mechanisms by which these finger deletions affect the activity of MTF-1 was examined using these individual stably transfected cell lines expressing MTF-1\textsubscript{flag} and its finger-deletion mutants. These cells were analyzed for metal-induced nuclear translocation of MTF-1\textsubscript{flag}; its MRE-binding activity was measured in \textit{vitro}, and its association with the MT-I promoter was measured in \textit{vivo}. Two separate cell lines were analyzed for each construct, but only representative results from one line are shown.

The impact of finger deletions on the subcellular localization of MTF-1\textsubscript{flag} in response to zinc and cadmium was examined by Western blotting of nuclear and cytoplasmic extracts (Fig. 6). An equal quantity of nuclear and cytoplasmic protein from each cell line was Western-blotted, although about 4-fold more protein is found in the cytoplasmic extract from a given number of cells. Consistent with our previous studies (4), the results revealed that the majority of MTF-1\textsubscript{flag} was detected in the cytoplasm of untreated cells (Fig. 6A). In contrast, zinc (100 \( \mu \text{M} \)) and cadmium (20 \( \mu \text{M} \)) treatment caused MTF-1\textsubscript{flag} to rapidly (1 h) translocate into the nucleus (Fig. 6A). Notably, 10 \( \mu \text{M} \) cadmium was less effective than 20 \( \mu \text{M} \) cadmium at causing MTF-1\textsubscript{flag} nuclear translocation (Fig. 6A). These results are consistent with our previously published findings (26) that deletion of fingers 5 and 6 does not impair metal-induced activation of \textit{in vitro} DNA binding of MTF-1. Similar to the increased nuclear localization of MTF-1, an increase in its DNA-binding activity alone does not ensure metal-dependent activation of the MT-I gene.

To address directly whether metal induction of \textit{MT-I} gene expression is associated with increased recruitment of MTF-1\textsubscript{flag} to the promoter as well as to determine whether zinc finger deletions impair this function, we employed a ChIP assay using these stably transfected cell lines expressing MTF-1\textsubscript{flag} and its finger-deletion mutants. Briefly, soluble, cross-linked chromatin prepared from control and metal-treated cells was immunoprecipitated using anti-FLAG M2-agarose affinity gel, and the amount of the \textit{MT-I} promoter in the immunoprecipitate was quantified by PCR using gene-specific primers. Controls
consisted of an anti-USF-1 antibody plus protein A-Sepharose or beads alone in the immunoprecipitation reaction. In addition, ChIP assays of chromatin from MTF-KO cells confirmed the specificity of the FLAG immunoprecipitation (data not shown). It is important to note that each of these stably transfected cell lines expressed significantly different amounts of MTF-1flag and its finger-deletion mutants. Thus, one cannot compare the relative amounts of the MT-I promoter in the immunoprecipitates from different cell lines in these experiments.

The ChIP assay demonstrated that zinc (100 μM) or cadmium (10 μM) rapidly (1 h) and significantly increased recruitment of MTF-1flag to the MT-I promoter chromatin (4-5- and 9-fold, respectively, Fig. 7B). Notably, cadmium concentrations of 10 and 20 μM had similar effects (Fig. 7B). These results were similar to those obtained from non-transfected wild type MEFs, where ChIP was carried out using an antibody against native MTF-1 (data not shown). In contrast, binding of the bZipHLH transcription factor USF-1 to the MT-I promoter chromatin was constitutive and not increased by treatment with zinc or cadmium, consistent with previous in vitro DNA-binding assays (34, 35). A ChIP assay of the MT-I promoter from stably transfected cells expressing MTF-1flag revealed that zinc (100 μM) and cadmium (20 μM) also increased the amount of MTF-1flag associated with the chromosomal MT-I promoter by 5.4- and 3.8-fold, respectively (Fig. 7F). This metal-dependent formation of a stable MTF-1-chromatin complex is consistent with the ability of these two forms of MTF-1 protein to mediate metal induction of the MT-I gene measured using Northern blotting.

In sharp contrast to the above results, ChIP assays showed that D1flag is constitutively associated with the MT-I promoter.

Fig. 5. Effects of zinc finger deletions on metal-dependent activation of the endogenous mouse MT-I gene by MTF-1flag. Left panels, dose response for induction of MT-I mRNA by zinc and cadmium in stably transfected cell lines expressing D1flag (A), D5&6flag (B), and D6flag (C). Total RNA was extracted from untreated cells and cells treated for 12 h with increasing concentrations of ZnSO4 or CdCl2, as indicated. MT-I mRNA levels were analyzed by Northern blotting. Right panels: transfected cells were treated for the indicated times with zinc (100 μM) or cadmium (20 μM). Total RNA was extracted from untreated cells (−) and cells treated with metals for the indicated times and analyzed for MT-I mRNA levels by Northern blotting.
chromatin at low levels, and this association is not significantly increased after zinc treatment (Fig. 7C). Similarly, only a modest increase in the constitutive binding of D5&6_R and D6_R to the MT-I promoter was noted after zinc treatment (less than 2-fold) (Fig. 7, D and E). Thus, the deletion of finger 1, fingers 5&6, or finger 6 alone greatly impaired the ability of MTF-1 to form a stable complex with the chromosomal MT-I promoter in response to zinc. Given this result, we did not extend the ChIP assay to include cadmium treatment of these cells. Taken together the results of the ChIP assays suggest that the formation of a metal-dependent, stable MTF-1-chromatin complex requires an intact zinc finger domain and the participation of each of the six zinc fingers of MTF-1. In contrast, its ability to activate basal expression of the MT-I gene is apparently not as dependent on all six fingers.

**DISCUSSION**

The results of these studies suggest that zinc and cadmium induce the formation of a complex between MTF-1 and the MT-I promoter. Formation of this complex is essential for metals to induce transcription of this gene and is dependent on an intact zinc finger domain. Mutations of the putative metal-sensing zinc fingers revealed that its ability to activate gene transcription can be independent of increased nuclear translocation and in vitro DNA-binding activity. This latter result is consistent with recent studies indicating that neither MTF-1 nuclear translocation nor its ability to bind DNA in vitro is sufficient to activate target gene transcription (5, 15, 16). It is well established that the chromatin structure of the MT-I gene and the MT locus is important in its regulation and expression (10, 28, 29), and our results indicate that the three putative metal-sensing zinc fingers in the zinc finger domain of MTF-1 serve their metalloregulatory functions in the context of the chromatin-packaged MT-I gene. Stabilizing the interactions between MTF-1 and the MT-I promoter in response to metals may involve the recruitment of chromatin remodeling factors, as well as increased affinity and specificity of DNA binding in response to zinc occupancy and/or post-translational modifications.

Studies of the human MTF-1 zinc finger domain suggest that fingers 5 and 6 are important in stabilizing MTF-1-DNA interactions (23-25). Thus, the ability of fingers 5 and 6 to stabilize MTF-1-chromatin complexes may reflect, in part, the fact that zinc increases their DNA-binding affinity thus allowing a stable association with the chromatin-packaged MT-I promoter. The packaging of this promoter with nuclear proteins could reduce the accessibility of the MREs, leading to the requirement for an increase in the binding affinity of MTF-1 to allow metal-induced binding to occur. This increase in affinity would not be detected in an EMSA that does not measure the actual binding affinity of MTF-1, only the relative amount of binding activity. An increase in binding affinity might also not be apparent if the function of these fingers was being addressed in transiently transfected cells bearing multiple copies of an MRE reporter gene vector that is not packaged with proteins in a similar manner to that of the endogenous MT-I promoter.

Our results regarding the MTF-1 finger 1 deletion mutant are consistent with previous data (26), which suggested that zinc finger 1 functions to suppress the DNA-binding activity of adjacent fingers in the absence of sufficient zinc but enhances their DNA binding activity when zinc is replete. A recent study of the recombinant human MTF-1 zinc finger domain lacking finger 1 (MTF-zf26) demonstrated that it has a lower affinity for binding to an MRE than does the wild type zinc finger domain. MREd is one of several MREs in the native mouse MT-I promoter. That study also suggests that finger 1 either directly interacts with finger 4 or stabilizes an intramolecular interaction between finger 4 and the adjacent fingers, leading to an overall stabilization of the MTF-1 zinc finger domain-MRE complexes (24). Our results suggest that the deletion of zinc finger 1 does not completely prevent MTF-1 from reactivating basal expression of the endogenous MT-I gene, but it prevents all metal responsiveness. Despite its lower affinity for MRE binding, this protein appears to be able to transiently associate with MREs in the MT-I promoter, but it cannot form a stable metal-induced complex. The constitutive nuclear localization of the finger 1 deletion mutant protein suggests that this finger may also mask the nuclear localization signal, located near its amino terminus in the wild type protein. Therefore, unmasking the nuclear localization signal upon exposure to exogenous metals may also be a part of the metal-sensing mechanism of zinc finger 1.

Zinc and cadmium appear to activate MT-I gene expression via distinct mechanisms, yet our studies demonstrate that the zinc-sensing fingers of MTF-1 are essential for its responsiveness to both of these metals. The large nuclear influx of MTF-1, activation of its DNA-binding activity, and formation of a stable MTF-1-chromatin complex on the MT-I promoter are consistent with previous models of its mechanism of action, at least as a zinc sensor (13). Taken in context with these previous studies, our results suggest that cadmium may cause redistribution of intracellular zinc (or zinc in the medium), leading to the occupancy of the metal-sensing zinc fingers in only a small portion of the total MTF-1 in the cell. Treatment of cells with higher concentrations of cadmium (50 μM) can exaggerate this effect and cause the nuclear translocation and DNA-binding activation of most of the MTF-1 in the cell (4). However, ChIP assays demonstrated that cadmium in low concentrations (10 μM) and zinc (100 μM) each rapidly increased the formation of similar amounts of the MTF-1-chromatin complexes in vivo. Because cadmium does not cause a large increase in the nuclear translocation of MTF-1 under these conditions, these results suggest that only a small increase in nuclear MTF-1 is sufficient to drive the formation of stable MTF-1-chromatin complexes in response to this metal. Zinc fingers 5 and 6 may serve an important role in this process.

It is possible that cadmium causes a unique post-translational modification(s) of MTF-1 that leads to an increase in the stability of its association with the MT-I promoter. This could occur through the recruitment of specific co-activators that interact with MTF-1 and/or by increasing the affinity of MTF-1 for binding to the MRE. Several signal transduction cascades...
have been suggested in the activation of MT-I gene expression in response to heavy metals (15–17). Identification of phosphorylation sites in MTF-1 and proteins that interact with MTF-1 will help to elucidate the differences between zinc and cadmium regarding their respective mechanisms of activating MT-I gene expression. Whether protein kinase inhibitors can block the formation of the MTF-1-chromatin complex or act subsequent to that step remains to be determined.

A puzzling finding in these studies was that activation of basal level expression of the MT-I gene was, in part, independent of an intact zinc finger domain. This was particularly notable with deletion of the metal-sensing zinc fingers 5 and 6 and was exaggerated in cells expressing very high levels of D5&6flag and D6flag. Furthermore, a measurable MTF-1-chromatin complex was detected in cells expressing each of the finger-deletion mutants. These results may suggest that the core DNA-binding zinc fingers 2–4 can bind to the MT-I promoter and activate its expression in the absence of exposure of MT-I promoter activity.
the cell to exogenous zinc or cadmium. Endogenous zinc levels could be sufficient to drive this process which appears to be enhanced by zinc finger 1. Perhaps differences in the metal-sensing properties of the zinc fingers of MTF-1 allow it to sense metal concentrations or exposure over a wide range. In normal zinc conditions, zinc finger 1 may augment the DNA binding of the core zinc fingers 2–4, whereas fingers 5 and 6 may be of paramount importance in the response to high concentrations of zinc.

During the course of this study, we discovered that mouse MTF-1 is highly polymorphic in regions outside of the zinc finger domain, and we also obtained evidence suggesting that even the zinc fingers exhibit polymorphism. A recent study (36) also identified what must be considered a polymorphism in the zinc finger domain of human MTF-1. No such polymorphisms had been reported previously for mouse MTF-1, and the extent of the polymorphisms we found was surprising. The fact that an MTF-1 containing polymorphisms outside of the zinc finger domain (MTF-1\textsubscript{pol}) induced MTF-1 gene expression in response to metals and behaved indistinguishably from endogenous MTF-1 suggests that these polymorphisms do not alter its metalloregulatory functions, at least under our experimental conditions. These regions of the protein have rapidly diverged during evolution, consistent with this finding. However, the zinc finger domain of MTF-1 is highly conserved during evolution, and we also obtained evidence suggesting that these polymorphisms outside of the zinc finger domain, and the protein factors that interact with MTF-1 may help resolve these questions.

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REFERENCES

1. Heuchel, R., Radtke, F., Georgiev, O., Stark, G., Auet, M., and Schaffner, W. (1994) EMBO J. 13, 2870–2875
2. Langmade, S. J., Ravindra, R., Daniels, P. J., and Andrews, G. K. (2000) J. Biol. Chem. 275, 34803–34809
3. Ganes, C., Heuchel, R., Georgiev, O., Müller, K. H., Lichtlen, P., Blüthmann, H., Marino, S., Aguzzi, A., and Schaffner, W. (1998) EMBO J. 17, 2846–2854
4. Smirnova, I. V., Bittel, D. C., Ravindra, R., Jiang, H., and Andrews, G. K. (2000) J. Biol. Chem. 275, 19577–19584
5. Saya, D., Georgiev, O., Nakano, M. Y., Greber, U. F., and Schaffner, W. (2001) J. Biol. Chem. 276, 25487–25495
6. Dalton, T. D., Li, Q., Bittel, D., Li, L. C., and Andrews, G. K. (1996) J. Biol. Chem. 271, 26233–26241
7. Dalton, T. D., Bittel, D., and Andrews, G. K. (1997) Mol. Cell. Biol. 17, 2761–2769
8. Bittel, D., Dalton, T., Samson, S., Gedamu, L., and Andrews, G. K. (1998) J. Biol. Chem. 273, 7127–7133
9. Mueller, P. R., Sailer, S. J., and Wold, B. (1988) Genes Dev. 2, 412–427
10. Ghoshal, K., Datta, J., Majumder, S., Bai, S. M., Dong, X. C., Parthun, M., and Jacob, S. T. (1997) Mol. Cell. Biol. 17, 8302–8319
11. Durnam, D. M., and Palmiter, R. D. (1981) J. Biol. Chem. 256, 11161–11165
12. Andrews, G. K. (2001) Bioessays 23, 1010–1017
13. Lichtlen, P., and Schaffner, W. (2001) Bioessays 23, 423–427
14. Daniels, P. J., Bittel, D., Smirnova, I. V., Winge, D., and Andrews, G. K. (2002) Nucleic Acids Res. 30, 3130–3140
15. LaRuschelle, O., Gagne, V., Charron, J., Seh, J. W., and Seguin, C. (2001) J. Biol. Chem. 276, 41878–41884
16. Saya, D., Adams, T. K., Steiner, F., Schaffner, W., and Freedman, J. H. (2002) J. Biol. Chem. 277, 20438–20445
17. Yu, C. W., Chen, J. H., and Lin, L. Y. (1997) FEBS Lett. 420, 69–73
18. Radtke, F., Heuchel, R., Georgiev, O., Hergersberg, M., Gariglio, D., Membic, Z., and Schaffner, W. (1993) EMBO J. 12, 1365–1365
19. Auf der Maur, A., Belser, T., Elgor, G., Georgiev, O., and Schaffner, W. (1999) Biol. Chem. 380, 175–185
20. Westin, G., and Schaffner, W. (1998) EMBO J. 7, 3763–3770
21. Stuart, G. W., Searle, P. F., Chen, H. Y., Brister, R. L., and Palmer, R. D. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 7318–7322
22. Chen, X. H., Agarwal, A., and Giedroc, D. P. (1998) Biochemistry 37, 11151–11161
23. Chen, X. H., Chu, M. H., and Giedroc, D. P. (1999) Biochemistry 38, 12915–12925
24. Apuy, J. L., Chen, X., Russell, D. H., Baldwin, T. O., and Giedroc, D. P. (2001) Biochemistry 40, 15164–15175
25. Giedroc, D. P., Chen, X., Agarwal, A., and Giedroc, D. P. (2001) J. Biol. Chem. 276, 41225–41232
26. Giedroc, D. P., Chen, X., Fennella, M. A., and LiWang, A. C. (2001) J. Biol. Chem. 275, 37194–37201
27. Kuzumaki, S., Suzuki, K., Ogra, Y., Gong, P., and Otuska, F. (2000) J. Cell. Physiol. 185, 464–472
28. Palminteri, R. D., Sandgren, E. P., Koeller, D. M., and Brister, R. L. (1993) Mol. Cell. Biol. 13, 5266–5275
29. Majumder, S., Ghoshal, K., Li, Z. L., Bo, Y., and Jacob, S. T. (1999) Oncogene 18, 6287–6295
30. Andrews, G. K., and Adamson, E. D. (1987) J. Biol. Chem. 262, 3879–3884
31. Langmade, S. J., Ravindra, R., Daniels, P. J., and Andrews, G. K. (2000) J. Biol. Chem. 275, 28539–28544
32. Boyd, K. E., Wells, J., Gutman, J., Bartley, S. M., and Farnham, P. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13887–13892
33. Chen, H., Lin, R. J., Gie, W., Wilpitz, D., and Evans, R. M. (1999) Cell 98, 675–686
34. Li, Q., Hu, N. M., Daggett, M. A. P., Chu, W. A., Bittell, D., Johnson, J. A., and Andrews, G. K. (1998) Nucleic Acids Res. 26, 5182–5189
35. Andrews, G. K., Lee, D. K., Ravindra, R., Lichtlen, P., Siritos, M., Sawadogo, M., and Schaffner, W. (2001) EMBO J. 20, 1114–1122
36. Habeebu, S. S., Liu, Y., Park, J. D., and Klaassen, C. D. (2001) Toxicol. Appl. Pharmacol. 177, 200–207
37. Liu, J., Corton, C. D., Li, Y., and Klaassen, C. D. (2001) Toxicol. Appl. Pharmacol. 176, 1–9
38. Hotau, D. N., Jr., Machado, A. F., Scott, W. J., Jr., and Collins, M. D. (1999) Teratology 60, 13–21
39. King, L. M., Andersen, M. B., Sikka, S. C., and George, W. J. (1998) Arch. Toxicol. 72, 650–655
Putative Zinc-sensing Zinc Fingers of Metal-response Element-binding Transcription Factor-1 Stabilize a Metal-dependent Chromatin Complex on the Endogenous Metallothionein-I Promoter

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