Functional Heterogeneity in the Zinc Fingers of Metalloregulatory Protein Metal Response Element-binding Transcription Factor-1*

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Metal response element-binding transcription factor-1 (MTF-1) is a unique, zinc-inducible transcription factor that binds to metal response elements in the metallothionein promoter and activates transcription in response to metals and oxidative stress. MTF-1 contains six zinc fingers of the Cys2-His2 type. It was previously shown that MTF-1 is reversibly activated to bind DNA in response to changes in zinc status, unlike other zinc finger transcription factors, which do not appear to be reversibly activated by zinc in the cellular environment. Here we show that zinc fingers 2–4 constitute the core DNA-binding domain, whereas fingers 5 and 6 appear to be unnecessary for DNA binding in vitro. Deletion of finger 1 resulted in a protein that bound DNA constitutively in vitro. Furthermore, transfer of MTF-1 finger 1 to a position immediately preceding the three zinc fingers of Sp1 resulted in a chimera protein that required exogenous zinc to activate DNA binding in vitro, unlike native Sp1, which binds DNA constitutively. Transient transfection experiments demonstrated that intact MTF-1 activated a reporter 2.5–4-fold above basal levels after metal treatment in mouse MTF-1 knockout cells, Drosophila SL2 cells, and yeast. However, the metal response was lost in all three systems when finger 1 was deleted, but was unaffected by deletion of fingers 5 and 6. These data suggest that finger 1 of MTF-1 constitutes a unique metal-sensing domain that, in cooperation with the transactivation domains, produces a zinc-sensing metalloregulatory transcription factor.

Metallothioneins (MTs)1 constitute a conserved family of cysteine-rich heavy-metal-binding proteins (1). In the mouse, MT-I and MT-II display a wide tissue distribution and have been demonstrated to participate in zinc homeostasis (2), detoxification of cadmium (3, 4), and protection against oxidative stress (5). MT gene transcription is induced dramatically by heavy metals (especially zinc and cadmium) (6). Metal response elements (MREs) are essential for metal induction, and there are multiple copies of the MREs in the proximal promoters of MT genes. MREs were initially shown to mediate the transcriptional response of MT genes to metals (7) and more recently, to participate in the transcriptional response to oxidative stress (8, 9).

MRE-binding transcription factor-1 (MTF-1) was cloned (10, 11) and found to be a six-zinc finger transcription factor in the Cys2-His2 family. Targeted disruption of both MTF-1 alleles in mouse embryonic stem cells demonstrated its essential role for basal as well as heavy metal-induced MT gene expression (12). Unlike other zinc finger transcription factors, which appear to be constitutively active to bind DNA under normal physiological conditions, MTF-1 is reversibly activated to bind DNA in response to changes in zinc availability (9, 10, 13–16). This reversible activation involves direct interaction between zinc and the zinc finger domain of MTF-1. Activation of MTF-1 in response to zinc is reminiscent of copper activation of Ace1 in yeast. Ace1 binds to DNA in response to excess copper and activates transcription of the yeast MT (CRS5 and CUP) and superoxide dismutase (SOD1) genes (17, 18). Copper activation of transcription in yeast involves reversible binding of copper to the DNA-binding domain of Ace1.

Based on the observation that a single zinc finger interacts with 3 or 4 bases in DNA (19), it seems likely that the zinc fingers of MTF-1 are not all required for binding to the 12-base pair consensus MRE sequence (7). Chen et al. (20) reported that there is structural heterogeneity in the zinc-binding sites in the isolated MTF-1 zinc finger domain, and they detected at least two classes of zinc-binding sites. Their observations indicated that there are three or four high affinity zinc-binding fingers and that the remainder of the fingers have a much lower affinity for zinc. However, in their experimental system, no change in the DNA binding of the purified MTF-1 zinc finger domain was detectable when three zinc molecules were bound compared with six.

Multiple zinc fingers are often arranged in close proximity to each other, suggesting the potential for cooperative binding, which might, in turn, produce a metalloregulatory function inherent in zinc finger domains. Krizek et al. (21) examined the metal-binding characteristics of a peptide with two consensus zinc finger domains and found no evidence for cooperativity. They concluded that cooperative metal binding is not an inherent property of tandem zinc finger domains, but that specially evolved zinc fingers might still act as mechanisms sensing changes in free zinc concentration. Several lines of evidence suggest that MTF-1 may function as a metalloregulatory protein serving as an intracellular sensor of free zinc. First, treatment of mammalian cells with zinc in vivo has been reported to cause a rapid, dramatic increase in the DNA-binding activity of MTF-1 measured in vitro (14, 16). Second, zinc causes nuclear translocation of MTF-1 (22) and the concomitant occupancy of MREs in the MT promoter in vivo (9). Third, MTF-1 is more sensitive to metal chelators than are other zinc finger transcription factors (10, 14). Fourth, the DNA-binding activity of
native and recombinant MTF-1 can be reversibly modulated by zinc (12, 14, 23); and fifth, mouse MTF-1 can function as a zinc sensor in yeast.² The studies presented herein examined the roles that individual zinc fingers play in zinc-sensing, DNA-binding, and gene activation. The data indicate that three of the zinc fingers constitute the core DNA-binding domain, that two fingers have an unknown function, and that a single finger controls zinc-activated DNA binding.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatment**—Mouse dko7 (MTF-1⁻/⁻) cells were maintained in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The mouse dko7 cell line is a simian virus 40 large T-antigen-immortalized fibroblast line derived from embryonic stem cells lacking MTF-1 (MTF-1 double knockout) and was a generous gift of Dr. Walter Schaffner (University of Zurich, Zurich, Switzerland) (24). *Drosophila* SL2 cells were maintained in Schneider's *Drosophila* medium supplemented with 10% fetal bovine serum. The culture of *S. cerevisiae* was as described elsewhere.³ *S. cerevisiae* strain ZHY6 (25) was obtained from Dr. David Eide (University of Missouri at Columbia). ZnSO₄ and CdCl₂ were dissolved in acidified H₂O as 1000-fold concentrated stock solutions.

**Preparation of Whole Cell Extracts**—Whole cell extracts were prepared as described previously (26).

**Nuclear and Cytosolic Extracts**—Nuclear and cytosolic extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagent (Pierce) according to the manufacturer's suggestions.

**Immunoblotting**—Cell extracts (50–100 µg of protein) were separated by 10 or 12% SDS-polyacrylamide gel electrophoresis (27) under reduced conditions and transferred to nitrocellulose membranes. The preparation and development of the chemiluminescent signal were as described previously (22). Relative band intensities were quantitated using Biomax 1D image analysis software (Kodak Scientific Imaging Systems). Equal protein loading and transfer were verified visually by staining membranes with Ponceau solution.

**In Vitro Transcription/Translation**—The cDNA clones encoding mouse MTF-1 and human Sp1 were previously described (14, 15). Recombinant and chimeric proteins were synthesized in vitro using a TnT coupled reticulocyte lysate system (Promega, Madison, WI) containing 1 µg of the plasmid and Sp6 RNA polymerase according to the manufacturer's instructions (15).

**Electrophoretic Mobility Shift Assays (EMSA)**—EMSA, using 1 µl of a TnT lysate and MRE and/or Sp1 oligonucleotide, was carried out as described in detail previously (14, 15).

**Plasmid Preparation**—DNA sequences coding for chimeric proteins were constructed by polymerase chain reaction amplification of individual domains and ligating domains together using endogenous restriction sites or sites introduced by altering nucleotides at redundant codon positions. The resulting chimeric proteins (Fig. 1A) retained the exact amino acid sequences of the original domains.

MTF-1 deletion constructs were prepared by amplifying MTF-1 in two components containing the 5’ and 3’ termini of the coding sequence. Fingers were deleted from the first cysteine of the deleted finger to the amino acid preceding the first cysteine of the next finger. The two amplified fragments were ligated using an endogenous KpnI site for deletion of finger 1, fingers 1 and 2, and fingers 1–3 and a BspHI site for deletion of finger 6, fingers 5 and 6, and fingers 4–6. This allowed the amino acid sequence to be maintained exactly as that of full-length MTF-1 with the exception of the indicated deletion. All constructs were verified by sequencing at the Biotechnology Support Facility of the University of Kansas Medical Center.

F1Sp1 (Fig. 1B) was constructed by polymerase chain reaction amplification of the Sp1 5’ terminus with the addition of a KpnI site preceding the zinc finger coding sequence. The KpnI site was added by modifying the redundant nucleotides without altering the resulting amino acid sequence. The 3’ terminus of Sp1, including the zinc finger region, was amplified with the addition of an EcoRI site to the 5’-end. MTF-1 finger 1 was amplified by polymerase chain reaction and included an endogenous KpnI site on the 5’-side and an added EcoRI site on the 3’-side. To introduce the EcoRI site, a phenylalanine codon was added between MTF-1 finger 1 and the zinc fingers of Sp1. This resulted in seven amino acids between MTF-1 finger 1 and the first zinc finger of Sp1. The first three amino acids following MTF-1 finger 1 were from a TnT lysate and MRE and/or Sp1 oligonucleotide, was carried out as described (28). The yeast reporter gene vector pYEp363:MRE5-βGal was engineered to express β-galactosidase under the control of five tandem copies of mouse MRE5, as described (8), and for LEU2 auxotrophy.

**Transfection**—LipofectAMINE (Life Technologies, Inc.)-mediated transfections were performed according to the manufacturer's suggestions. Briefly, dko7 (MTF-1⁻/⁻) cells were transfected and assayed for reporter gene expression as described (22, 29).

SL2 cells were transfected using the calcium phosphate transfection system kit (Life Technologies, Inc.) according to the manufacturer's suggestions. Briefly, cells were plated at a density of 1 x 10⁶ cells/ml in 6-well plates. The following morning, the DNA/calcium phosphate suspension was prepared as recommended in a volume equivalent to 200 µl/well. Each 200-µl aliquot contained 5 ng of pcAC-MTF-1, 5 µg of

⁴ G. K. Andrews, D. Bittel, I. Smirnova, R. Ravindra, and D. Winge, submitted for publication.

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**Fig. 1. Diagram of chimeric proteins.** A, the zinc fingers of MTF-1 and Sp1 were switched without gain or loss of amino acids (AA) in the backbone of each. The domains that were exchanged encompass the first cysteine of finger 1 to the last histidine of the final finger. The Sp1 peptide backbone with the MTF-1 finger cassette is termed SMS, whereas the MTF-1 peptide backbone with the Sp1 finger cassette is termed MTF-1. B, the F1Sp1 fusion construct contains the Sp1 amino acid sequence to lysine 632, followed by 29 residues encompassing finger 1 of MTF-1, including the three amino acids immediately preceding the first cysteine and the three amino acids following the last histidine. C, the Sp1 zinc fingers were inserted following finger 1 of MTF-1, replacing MTF-1 zinc fingers 2–6. The amino acid sequence at the fusion of finger 1 and Sp1 finger 1 was the same as in F1Sp1. Sp1 finger 3 ended with the last histidine of the finger and was followed by aspartate, which is the first amino acid following the final histidine of finger 6 of MTF-1.
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RESULTS

Comparison of the Concentrations of Exogenous Zinc Required to Activate DNA Binding by MTF-1 and Sp1—MTF-1 in whole cell extracts from control cells and recombinant MTF-1 synthesized in vitro require micromolar concentrations of exogenous zinc to activate DNA binding (14, 15, 23, 31). To make a more direct comparison between the zinc concentrations required to activate DNA binding by MTF-1 and Sp1, both constructs were simultaneously transcribed and translated in the same TnT reaction, and their individual DNA-binding activities were detected by EMSA. Most of the DNA-binding activity of Sp1 (60%) was activated in the TnT lysate and did not require the addition of exogenous zinc to the EMSA reaction (Fig. 2, lanes 2, 4, and 5). In contrast, without exogenous zinc, there was little detectable (<5%) MTF-1 DNA-binding activity (Fig. 2, lanes 4 and 5). Furthermore, if zinc was added to the reaction, but the reaction was kept on ice, very little activation of MTF-1 occurred, whereas there was a significant Sp1 complex formed (Fig. 2, lane 8). Thus, MTF-1 requires exogenous zinc and an increase in temperature for activation of DNA binding to occur (Fig. 2, lanes 1 and 3).

In Vivo and in Vitro Analysis of Chimeric Proteins—Previous studies support the concept that zinc induction of DNA binding is contained within the zinc fingers of MTF-1 (14, 15, 20). To further examine the roles of the zinc fingers in zinc activation, the zinc fingers of Sp1 and MTF-1 were exchanged to produce chimeric proteins (Fig. 1A). The Sp1 peptide backbone with the MTF-1 zinc fingers is termed SMS, whereas the MTF-1 peptide backbone with the Sp1 zinc fingers is called MSM. The chimeric DNA constructs were prepared for transcription and translation in TnT lysates and for expression in dko7 (MTF-1−/−) cells. When expressed in a TnT lysate, there was a low, but detectable level of MRE binding by SMS (Fig. 1A) without exogenous zinc being added to the EMSA (Fig. 3, lane 3). However, there was a dramatic increase in binding after zinc was added (Fig. 3, lane 1).
of MTF-1 zinc finger deletion constructs. A. EMSAs reactions containing 1 μl of the indicated TnT lysate were brought to 30 μM zinc, and the reactions were carried out as indicated under “Experimental Procedures.” The arrow indicates the specific complex. B, MTF-1 was detected with the anti-MTF-1 polyclonal antibody as described under “Experimental Procedures.” 1 μl of the TnT lysate used for EMSA in A was loaded onto each lane of a 10% SDS-polyacrylamide gel.

![Image](image-url)

FIG. 4. Analysis of the DNA binding of MTF-1 zinc finger deletion constructs. A, EMSA reactions containing 1 μl of the indicated TnT lysate were brought to 30 μM zinc, and the reactions were carried out as indicated under “Experimental Procedures.” The arrow indicates the specific complex. B, MTF-1 was detected with the anti-MTF-1 polyclonal antibody as described under “Experimental Procedures.” 1 μl of the TnT lysate used for EMSA in A was loaded onto each lane of a 10% SDS-polyacrylamide gel.

As expected, this chimeric protein did not bind to the Sp1-binding site (Fig. 3, lanes 5 and 6). In contrast, MSM (Fig. 1) bound to the Sp1-binding site without the addition of exogenous zinc (Fig. 3, lane 9), and only a small increase in DNA binding occurred after exogenous zinc was added (lane 10). The Sp1 oligonucleotide contains two Sp1-binding sites, which likely accounts for the secondary EMSA band detected after the addition of exogenous zinc (Fig. 3, lane 10).

The intramolecular and intermolecular interactions that combine to induce transcription in a nucleus cannot be assessed merely by EMSA. Therefore, to test the in vivo function of the chimeric protein, the SMS construct was transfected into dko7 (MTF-1<sup>−/−</sup>) cells along with a luciferase reporter construct driven by five copies of MREd. Transfection with SMS caused an increase in basal luciferase activity with increasing DNA concentration. However, there was no significant increase in luciferase activity in response to added zinc or cadmium (Fig. 3B). In contrast, cells transfected with MTF-1 displayed an increased basal luciferase activity compared with cells transfected only with reporter. The luciferase activity in extracts from cells transfected with MTF-1 was increased up to 2.5-fold in response to zinc or cadmium (Fig. 3B).

In Vitro Analysis of Zinc Finger Deletions—To examine the contribution of the individual MTF-1 zinc fingers to DNA binding and zinc response, finger deletion constructs were made. Surprisingly, deletion of finger 1 (D1) led to the constitutive DNA-binding activity of MTF-1 (Fig. 4A, lanes 2 and 3). In the absence of finger 1, most of the DNA binding (~60%) was present without the addition of exogenous zinc.

Deletion of fingers 1 and 2 (D1,2) produced a peptide that bound MREs poorly (Fig. 4A, lanes 5 and 6). Deletion of fingers 1–3 (D1,2,3) resulted in a peptide with no detectable DNA-binding activity (Fig. 4A, lanes 7 and 8). Similarly, deletion of fingers 4–6 (D4,5,6) resulted in the drastic reduction of DNA binding, although a small zinc-inducible MTF-1-MRE complex was detectable by EMSA (Fig. 4A, lanes 9 and 10). The loss of DNA binding were not the result of poor translation of the deletion constructs, as each TnT lysate reaction produced similar amounts of immunoreactive peptide (Fig. 4B).

Deletion of finger 6 (D6) or of both fingers 5 and 6 (D5,6) had little detectable effect on DNA-binding activity in an EMSA reaction (Fig. 4A, lanes 11–14). The resulting peptide required concentrations of exogenous zinc to activate DNA binding that were similar to concentrations required to activate the native protein. The intensities of the bands detected by immunoblotting of lysates containing native MTF-1, D5,6, or D6 were similar (Fig. 4B), as were the intensities of the respective MTF-1-MRE complex in EMSA reactions.

Expression of MTF-1, D1, and D5,6 in Mouse dko7 (MTF-1<sup>−/−</sup>) Cells—The effects of finger deletions on the in vivo function of MTF-1 were examined by transient transfection of mouse cells as well as insect cells and yeast (see below). D1 and D5,6 in a CMV expression vector were transfected into dko7 (MTF-1<sup>−/−</sup>) cells. A putative nuclear localization sequence just before finger 1 could have been affected by the deletion of finger 1, disturbing correct nuclear trafficking. Therefore, proteins were isolated from nuclear and cytoplasmic fractions of dko7 cells after transfection with MTF-1 or D1 to verify that the proteins could be transported to the nucleus. Both MTF-1 and D1 were detected in the nuclear fraction before and after zinc treatment (Fig. 5A). However, before zinc treatment, the majority of MTF-1 was found in the cytoplasmic fraction, as previously reported (22). In contrast, the majority of D1 was present in the nucleus in control cells as well as in zinc-treated cells (Fig. 5A). The nuclear fractions from these transfected cells were also analyzed by EMSA (Fig. 5B). Extracts from non-transfected dko7 cells contain no detectable MTF-1 (14). In contrast, nuclear extracts from dko7 cells transfected with CMV-MTF-1 or CMV-D1 contained a protein that could bind MREs (Fig. 5B).

MTF-1, D1, and D5,6 were transfected into dko7 cells using conditions that were optimal for detecting changes in expression of the MREd5 reporter in response to zinc treatment (22). In cells transfected with MTF-1 or D5,6, expression from the reporter increased ~2-fold when the medium was adjusted to 100 μM zinc or 10 μM cadmium (Fig. 5C). In contrast, in cells transfected with D1, there was a 3-fold increase in basal level reporter activity compared with cells transfected with reporter alone, but no change in reporter activity after zinc or cadmium treatment of the cells (Fig. 5C).

Expression of MTF-1, D1, and D5,6 in Drosophila SL2 Cells—We examined the behavior of MTF-1 in Drosophila SL2 cells to determine if MTF-1 could function as a zinc-activated transcription factor in a heterologous system and to further
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examine the effects of finger deletions. SL2 cells transfected with pAC-MTF-1 produced a protein recognized by the mouse anti-MTF-1 antibody that was of the expected size (Fig. 6A). Drosophila expression vectors encoding MTF-1, D5,6, or D1 were cotransfected with the MREd5 reporter into Drosophila SL2 cells. This resulted in an increase in reporter activity compared with that found in cells transfected with the reporter alone. Zinc treatment (1 mM) of SL2 cells transfected with MTF-1 or D5,6 led to a 3.5- or 2.5-fold increase in luciferase activity (Fig. 6B), in agreement with the results from dko7 cells expressing D1 (Fig. 7A). In contrast, there was no change in the level of β-galactosidase mRNA after the addition of zinc in cells expressing MTF-1 or D5,6 (4- and 3-fold, respectively) (Fig. 7B). The change in mRNA was observed 1 h after the addition of zinc, indicating a rapid transcriptional response (Fig. 7B). In contrast, there was no change in the level of β-galactosidase mRNA after the addition of zinc in cells expressing D1.

Six independently selected colonies expressing either D5,6 or D1 were examined for their response to zinc. The β-galactosidase activity in cells expressing D5,6 increased significantly (up to 3-fold) by 8 h after the addition of zinc to the medium (Fig. 7C), similar to cells expressing MTF-1. In contrast, the β-galactosidase activity in cells expressing D1 was unchanged or even slightly reduced after the addition of zinc to the medium (Fig. 7C).

MTF-1 Finger 1 Transferred in Front of the Sp1 Zinc Fingers Confers Zinc-inducible DNA Binding in Vitro—The experiments described above indicated that finger 1 was responsible for the zinc-activated DNA binding of MTF-1. Whether this function was transferable to a constitutive DNA-binding zinc finger domain, such as that in Sp1, was examined. The coding sequence for finger 1 was transferred from MTF-1 to a position immediately preceding finger 1 of Sp1 (F1Sp1) (Fig. 1B). The chimeric protein was expressed in the TnT reaction and ana-
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FIG. 7. Function of MTF-1, D1, or D5,6 in yeast. A, proteins extracted from three independent colonies of yeast strain ZHy6 transformed with D1 or D5,6 were separated by 10% SDS-polyacrylamide gel electrophoresis. Specific bands were detected by anti-MTF-1 antibody. Recombinant MTF-1 synthesized in a TnT lysate was used as a positive control in the first lane. The ZHy6 lane contained protein from the non-transfected parental yeast strain. The arrow indicates the position of the 98-kDa molecular mass marker. B, shown is a Northern blot probed with an antisense β-galactosidase riboprobe (as described under Experimental Procedures.) Using 3 μg of total RNA extracted from yeast strains cotransfected with MTF-1, D1, or D5,6 plus an MREδ5-β-galactosidase reporter. Cells were grown overnight in medium containing 2 μM zinc and brought to 60 μM zinc as indicated for 1 h. Arrows indicate the presence of two β-galactosidase (β-gal) transcripts. C, independent colonies transfected with D1 or D5,6 were grown overnight in 2 μM zinc and then brought to 60 μM zinc for 8 h before harvest. β-Galactosidase is expressed as units/min/μg of protein. Note that the axes are different. The basal β-galactosidase activities in cells expressing D1 and D5,6 were ~3 and 10 times that of the background β-galactosidase activity in cells transfected with reporter only. Each bar represents the mean ± S.E. of three experiments.

lyzed by EMSA (Fig. 8A). In contrast to the constitutive DNA binding of Sp1, F1Sp1 had little detectable DNA-binding activity when zinc was not added to the EMSA reaction (Fig. 8A). However, the addition of 1 μM zinc and raising the temperature to 37 °C caused a dramatic increase in F1Sp1 DNA-binding activity. MTF-1 behaved similarly to F1Sp1 under these experimental conditions (Fig. 8A). Immunoblotting using an anti-Sp1 antibody demonstrated that similar amounts of protein were made in the Sp1 and F1Sp1 reactions (Fig. 8B). A protein extract from mouse Hepa cells was included as a reference (Fig. 8B, Hepa).

To examine the in vivo function of F1Sp1, a Drosophila expression construct encoding F1Sp1 was transfected into Drosophila SL2 cells, which are devoid of Sp1-like DNA-binding activity. Although a 5–10-fold increase in the basal activity of the Sp1 reporter was detected when either F1Sp1 or Sp1 was transfected into these cells, zinc (1 mM) had no detectable effect on Sp1 reporter expression (data not shown). Neither pVT-F1Sp1 nor pVT-SMS were zinc-responsive in SL2 cells, even though both demonstrated zinc-activated DNA binding in vitro. Furthermore, it was noted that the F1Sp1 zinc finger cassette in the context of the MTF-1 protein backbone (F1SMS) (Fig. 1C) was also not zinc-responsive in SL2 cells (data not shown).

DISCUSSION

In mammals, zinc homeostasis appears to be maintained, in part, by zinc-dependent activation of the transcription factor MTF-1, which, in turn, modulates gene expression. The MT genes are activated by MTF-1, and MTs chelate zinc and provide a labile pool of zinc during periods of zinc deficiency (2, 32). MTF-1 also regulates the expression of other homeostatic genes such as ZnT1 (zinc transporter 1), which functions to remove excess zinc from the cell.

The mechanisms by which MTF-1 senses zinc and subsequently activates gene expression are not well understood. The zinc finger domain of MTF-1 appears to be essential for zinc activation of DNA binding, and this likely reflects heterogeneity in the zinc-binding properties of the zinc fingers. Chen et al. (20) have described at least two classes of zinc-binding sites in MTF-1. Three or four high affinity binding sites were detected, as were two or three low affinity zinc-binding sites. The evidence presented here suggests that three fingers (fingers 2–4) are essential for DNA binding and that these fingers display constitutive DNA-binding activity in the absence of zinc. This suggests that these fingers represent the high affinity zinc-binding sites in MTF-1 observed by Chen et al. (20). This, in turn, is consistent with the fact that MTF-1 interacts specifically with a 12-base pair MRE-binding site, which would be expected to require three zinc fingers.

Remarkably, zinc finger 1 of MTF-1 was found to mediate zinc activation of the DNA-binding activity of adjacent zinc fingers of MTF-1 or Sp1 and to be essential for zinc activation of gene expression by MTF-1. This suggests that finger 1 represents a low affinity zinc-binding site that, in the absence of zinc, can actually mask the constitutive DNA-binding activity of adjacent zinc fingers. The mechanisms by which finger 1 senses zinc and masks DNA binding are unclear. The length of

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finger 1 and the placement of the cysteine and histidine residues are the same as those of each of the other zinc fingers in MTF-1. However, finger 1 is significantly different in primary amino acid sequence relative to the other fingers in MTF-1. In addition, four of the seven residues in the linker between fingers 1 and 2 are unique compared with the residues in those positions in the other linking sequences (the linker region between zinc fingers has been shown to modulate the folding of the adjacent finger (33)). Presumably, these differences in sequence influence the affinity of finger 1 for zinc, resulting in a finger with unique zinc-binding characteristics. Alternatively, these residues may influence the interaction of finger 1 with the other fingers, thus affecting the zinc response.

The majority of MTF-1 in control cells is found in the cytoplasm, but it is re-localized to the nucleus after zinc treatment (22). Although deletion of finger 1 could have potentially inhibited accumulation of MTF-1 in the nucleus, thus attenuating the zinc response, transient transfection experiments demonstrated that D1 was recovered primarily from the nuclear fraction of transfected cells and was competent to bind to DNA. Therefore, deletion of finger 1 does not interfere with nuclear localization.

In the course of these experiments, it was noted that fingers 5 and 6 of MTF-1 are dispensable for DNA binding in vitro and for zinc activation of gene expression in vivo. These findings contradict a recent report (34) that suggested that fingers 5 and 6 are low affinity zinc-binding sites that are essential for the DNA-binding affinity and specificity of MTF-1. The reason for this discrepancy is unclear. However, in those studies, the zinc finger domain, in isolation from surrounding amino acids, was analyzed for in vitro activity. Thus, it is conceivable that the functions (in vitro and in vivo) of these zinc fingers are not accurately reflected when the isolated finger domain is studied. Nevertheless, the functions of fingers 5 and 6 remain unclear. The high degree of conservation of the amino acid sequence between fingers 5 and 6 of mouse and human (10, 35) and fish (Fugu rubripes) (36) suggests that these fingers have an important function. Thus, it seems likely that transfection assays and in vitro DNA binding assays do not measure all functions of MTF-1. In addition to protein-DNA interactions, zinc fingers have been shown to participate in protein-protein interactions (37). Perhaps, fingers 5 and 6 are important for intermolecular interactions between MTF-1 and coactivators.

Cadmium is a potent inducer of MT, and MTF-1 is required for metal activation of MT gene transcription (38). However, cadmium does not cause measurable changes in the DNA binding of MTF-1 in vitro (15, 16) and does so only at high concentration in vivo (22). Mutational analysis demonstrated that loss of zinc activation in vitro and in vivo was accompanied by a loss of cadmium induction in vivo. This finding is consistent with the concept that cadmium might displace zinc in vivo, making it available for activation of MTF-1 (39). Cadmium activation of MT gene expression may require only a small change in the amount of activated MTF-1 in the nucleus in combination with other components, e.g. protein modification or a coactivator. These questions remain to be resolved.

Intramolecular interactions are clearly important for MTF-1 function (24). Substitution of the Sp1 zinc fingers or transactivation domains for their counterparts in MTF-1 resulted in a chimeric protein that was not zinc-responsive in vitro. Therefore, both the zinc fingers and the transactivation domains of MTF-1 are necessary for efficient zinc response in vivo. Activation of DNA binding by MTF-1 is not sufficient for zinc activation of MT transcription. MTF-1 is a complex protein with multiple domains that may have an equally complicated folded structure. The chimeric proteins used in these studies may not be able to achieve a structure capable of responding to zinc in vivo.

In summary, our studies suggest that within the context of an intact protein, finger 1 is necessary for the zinc activation of DNA binding by MTF-1 as measured in vitro and for an efficient response to zinc and cadmium in vivo. Furthermore, fingers 2–4 appear to be the core DNA-binding fingers. Our studies indicate that there is an intricate interplay between the zinc fingers and other domains within MTF-1 that is required for an efficient response to increased zinc concentrations in vivo. The function of fingers 5 and 6 is unknown. These two fingers apparently behave differently in their interaction with zinc compared with the other fingers, and our data indicate that they do not play a significant role in the zinc or cadmium response, either in vitro or in vivo, at least under these experimental conditions. Studies examining protein-protein interactions and modification of MTF-1 in response to inducers may help resolve these questions.

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