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A Novel Cysteine Cluster in Human Metal-responsive Transcription Factor 1 Is Required for Heavy Metal-induced Transcriptional Activation *in Vivo**

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Metal-responsive transcription factor 1 (MTF-1) specifically binds to metal response elements (MREs) associated with a number of metal- and stress-responsive genes. Human MTF-1 contains a cysteine-rich cluster, -^{632}Cys-Gln-Cys-Gln-Cys-Ala-Cys^{638}-, conserved from pufferfish to humans far removed from the MRE-binding zinc finger domain and just C-terminal to a previously mapped serine/threonine-rich transcriptional activation domain. MTF-1 proteins containing two Cys→Ala substitutions (C632A/C634A) or a deletion in this region altogether (Δ(632–644)) are significantly impaired in their ability to induce Zn(II)- and Cd(II)-responsive transcription of a MRE-linked reporter gene in transiently transfected mouse dko7 (MTF-1−/−) cells in culture under moderate metal stress but retain the ability to drive basal levels of transcription in a MRE-dependent manner *in vivo* and *in vitro*. In addition, the mutated proteins respond to induction by Zn(II) or Cd(II) with nuclear translocation and MRE binding activities comparable with wild-type MTF-1. Attempts to rescue the Δ(632–644) deletion mutant phenotype by inserting similar Cys-rich sequences from *Drosophila* MTF-1 were unsuccessful, suggesting that the structure of this motif within intact human MTF-1, rather than the simple presence of multiple closely spaced Cys residues, is required for function. This cysteine cluster therefore functions at a step subsequent to nuclear translocation and MRE-binding DNA to naked promoter-containing DNA and appears to be specifically required for MTF-1 to activate transcription in the presence of inducing heavy metal ions.

Zinc and other transition metal ions play diverse roles in many biological processes, serving as structural components of proteins (1) or as essential cofactors in enzyme-catalyzed reactions (2, 3). However, all metal ions become cytotoxic at high intracellular concentrations. Metal-responsive control of gene expression allows organisms to regulate the available concentration of beneficial metal ions such as zinc, copper, and iron within an acceptable range while removing toxic heavy metals that play no biological function such as cadmium, lead, mercury, and arsenic.

Metal-responsive transcription factor 1 (MTF-1)[1] was identified as a protein in higher eukaryotes that specifically binds to metal response element (MRE) DNA sequences associated with the genes encoding metallothioneins (4–7), a family of conserved cysteine-rich, metal-chelating proteins (8). MRE sequences and MTF-1 are required for both basal and metal-inducible metallothionein gene transcription (9). MTF-1 binding to MRE sequences is also required for expression of ZnT-1, the major zinc efflux transporter in the plasma membrane (10, 11), as well as several other genes (12). MTF-1 is constitutively expressed in mouse and human cells, and targeted disruption of the gene encoding MTF-1 in transgenic mice leads to embryonic lethality (13). MTF-1 genes from mouse (7), human (14), the pufferfish *Fugu rubripes* (15), zebrafish (16), and *Drosophila melanogaster* (17) have been cloned. Candidate MTF-1 genes most closely related to *D. melanogaster* MTF-1 have also been identified in the genomes of *Drosophila pseudoobscura*[2] and the malaria-transmitting mosquito *Anopheles gambiae*.[3]

The primary distinguishing feature of the domain organization of MTF-1 proteins (Fig. 1*A*) is a highly conserved MRE-binding domain composed of six tandemly arranged Cys_2-His_2 zinc fingers that are known (18) or thought to fold into canonical $\beta\beta\alpha$-structures (19), a subset of which is anticipated to bind within the major groove of the MRE (20–22). The zinc finger domain is flanked by nuclear localization and nuclear export signals required for intracellular trafficking in and out of the nucleus (23–25). Multiple C-terminal transcriptional activation domains including acidic, proline-rich, and serine/threonine-rich regions are thought to mediate the interaction of MTF-1 with the transcriptional machinery under both basal and metal-induced conditions (26). Endogenous MTF-1 is extensively phosphorylated and becomes more so upon heavy metal stress. This post-translational modification clearly plays an important role in the signal transduction pathway of this stress response (27, 28).

Several studies are consistent with the hypothesis that one or more of the zinc fingers might play a metalloregulatory role by activating MRE-binding and/or transcriptional activation directly upon reversible binding of zinc. Current evidence suggests that zinc fingers F5 and F6 increase the stability of the

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[1] The abbreviations used are: MTF-1, metal-responsive transcription factor 1; MRE, metal response element; DME, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HEK, human embryonic kidney; DTT, dithiothreitol; F, finger; ZF, zinc finger; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

[2] D. Egli and W. Schaffner, unpublished observations.

[3] D. Giedroc and W. Schaffner, unpublished observations.

MRE complex formed by structural fingers F1-F4 (29–31) and that fingers F1, F5, and F6 are essential for MTF-1 to activate gene expression from an endogenous metallothionein promoter packaged in chromatin (32) but not from a promoter containing tandemly linked MREs present on extra-chromosomal DNA (33, 34).

In this study, we identify and characterize a cysteine-rich regulatory site outside of the zinc finger domain, C-terminal to the serine/threonine-rich transcriptional activation domain. This region of the protein is required for Zn(II) and Cd(II) sensing *in vivo* under moderate metal stress and appears to function directly in transcriptional activation.

EXPERIMENTAL PROCEDURES

Plasmids—The expression plasmid pChMTF-1 (26) in which transcription of the human MTF-1 cDNA is driven by the cytomegalovirus promoter was modified by fusing coding sequences for a c-Myc epitope tag (EQKLISEEDL) to the C terminus of the MTF-1 gene to generate c-Myc-pChMTF-1. Cysteine-to-alanine mutations, deletion of MTF-1 amino acids 632–644, and human/*Drosophila* MTF-1 chimeras were constructed with the QuikChange protocol (Stratagene) using appropriate primers and pChMTF-1 or c-Myc-pChMTF-1 as a template. All of the constructs were completely sequenced to verify that the desired mutations were present and that other alterations had not been inadvertently introduced. The luciferase reporter plasmid pLucMRE was constructed by inserting four tandem repeats of the MREa sequence (GGCTTTTGCACTCGTCCCGGCT) from the human metallothionein-IIA gene (35) into the HindIII and SalI sites of pLuc-MCS (Stratagene) upstream of a basal promoter that drives transcription of firefly luciferase. pSVβ-galactosidase (Promega) contains the *E. coli lacZ* gene, transcription of which is driven by the SV40 early promoter and enhancer. The reporter (4×MREd-OVEC) and β-globin reference (OVEC-REF) genes used in the *in vitro* transcription experiments have been described previously (36).

Cell Culture—Mouse dko7 cells (9), which express no MTF-1, were maintained in high glucose Dulbecco's modified Eagle's medium (DME, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 units/ml penicillin, and 100 mg/ml streptomycin (pen-strep, Hyclone) at 37 °C in a water-saturated atmosphere containing 5% CO_2. HEK293 cells were cultured as described previously (17).

Transient Transfection—Chelex-treated DME (used as described below) was prepared by passing 500 ml of FBS- and pen-strep-supplemented DME through a column (2.5 × 9 cm) containing Chelex 100 Resin (Bio-Rad) to remove metals. $CaCl_2$ (1.55 mM), $MgSO_4$ (0.67 mM), and $FeCl_3$ (3.9 μM) were then added to restore the original concentration. In transfection experiments for luciferase assays, 3×10^5 cells were plated in each well of 6-well tissue culture plates. After waiting for the cells to attach (1–2 h), each well was washed with 1 ml of DME (without FBS and pen-strep) and 1.5 ml of Chelex-treated DME was then added. After 18–24 h, the cells were washed with 1 ml of DME (without FBS and pen-strep) and 1 ml DNA solution in DME (without FBS and pen-strep) containing pLucMRE (200 ng/ml), pSVβ-galactosidase (200 ng/ml), and MTF-1 expression plasmid (concentration as indicated in the figure legends) and 4 μl/ml LipofectAMINE reagent (Invitrogen) was added. After 4 h, 1 ml of DME (without FBS and pen-strep) was added to each well. 18–20 h after DNA addition to the cells, medium was replaced with DME (without FBS, but with pen-strep) containing metals as indicated in the figure legends. 10 h after metal induction, cells were lysed by washing each well twice with 2 ml of phosphate-buffered saline and adding 250 μl of 0.1 M sodium phosphate buffer (pH 7.8) containing 0.5 mM DTT and 0.2% Triton X-100. The wells were briefly scraped, and the cell lysate was transferred to a Microfuge tube and centrifuged at 12000 rpm for 10 min at 4 °C. The supernatant was transferred to a clean Microfuge tube, quick frozen in liquid N_2, and stored at −80 °C until luciferase and β-galactosidase assays were performed.

Transfection experiments for immunoblotting were performed as above with the exception that 3×10^6 cells were plated on 150-mm tissue culture plates and volumes of media, DNA solution, and so forth were 10-fold larger than those for the experiments performed in 6-well dishes. Metal treatment was for 8 h. After metal treatment, the cells were washed twice with 10 ml of phosphate-buffered saline and harvested by scraping in 5 ml of ice-cold phosphate-buffered saline. Cells were recovered by centrifugation, and cytosolic and nuclear extract fractions were prepared using the NE-PER kit (Pierce) as recommended by the manufacturer, quick frozen in liquid N_2, and stored at −80 °C. Protein concentrations were determined by the method of Bradford (37) with bovine serum albumin as the standard.

Luciferase and β-Galactosidase Assays—Luciferase and β-galactosidase assays were performed in a Packard Lumicount luminometer in 96-well assay plates. Luciferase assays contained 100 ml of a solution containing 40 mM Tricine (pH 7.8), 0.5 mM ATP, 10 mM $MgSO_4$, 0.5 mM EDTA, 10 mM DTT, 0.5 mM coenzyme A, 0.15 mg/ml luciferin (Sigma), and 5–50 μl of cellular (or cytosolic) extract. β-Galactosidase assays of 5–50 μl of cellular (or cytosolic) extract were performed in the same instrument using the Galacto-Light Plus chemiluminescent reporter assay kit (Tropix) as recommended by the manufacturer.

Immunoblotting—Protein samples were fractionated by SDS-polyacrylamide gel electrophoresis and then electrophoretically transferred to PVDF membrane (Hybond-P, Amersham Biosciences). The membrane was blocked by incubation in solution TBS-T (25 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.1% Tween 20) containing 5% nonfat dry milk and then probed with a horseradish peroxidase-conjugated antibody directed against the c-Myc epitope (Invitrogen) diluted (1:5000) in TBS-T. After incubation for 1 h at 25 °C, the filter was washed in TBS-T at 25 °C, once for 15 min and then 3 times for 5 min each. Bound antibody was detected with the ECL Plus Western blotting detection system (Amersham Biosciences) using Kodak X-Omat AR Blue film (Eastman Kodak Company).

Electrophoretic Mobility Shift Assays—Electrophoretic mobility shift assays were performed according to Radtke *et al.* (7) with a 30-bp ^{32}P-labeled oligonucleotide probe containing a single copy of a consensus MRE-s sequence (7). The binding reaction (20 μl) was carried out in a buffer containing 12 mM HEPES, pH 7.9, 12% glycerol, 50 mM KCl, 5 mM NaCl, 5 mM $MgCl_2$, 0.6 mM DTT, 0.5 μg of poly(dI·dC), 20 μg of nuclear extract protein, and 20 fmol of probe. Reactions were fractionated by polyacrylamide gel electrophoresis (4% gel) in 0.0225 M Tris borate buffer (pH 8.0) containing 0.5 mM EDTA at room temperature. Labeled bands were detected with a PhosphorImager (Amersham Biosciences).

In Vitro Transcription—In vitro transcription assays were performed according to Mueller-Storm *et al.* (38) and Zhang *et al.* (39). Reactions (15 μl) contained 40 μg of nuclear extract protein from HEK293 cells, 40 ng of a MRE-linked reporter construct (4xMREd-OVEC), and 40 ng of a reference plasmid in which β-globin transcription was driven by the SV40 early promoter (OVEC-REF). Reactions also contained 10 mM HEPES, pH 7.9, 8.5% glycerol, 20 mM KCl, 7 mM NaCl, 5 mM $MgCl_2$, 0.15 mM DTT, 1.5 units of RNasin, 5 mM creatine phosphate, 0.5 mM each of ATP, GTP, CTP, and UTP, and Zn(II) as indicated in the legend to Fig. 6. Some reactions (see legend to Fig. 6) were complemented with 39 μg of nuclear extract protein prepared from 293 cells that had been transfected with an MTF-1 expression plasmid prepared according to Schreiber *et al.* (40). Reactions were performed at 30 °C for 60 min and fractionated by polyacrylamide gel electrophoresis in the presence of 8 M urea. Products were visualized with a PhosphorImager.

RESULTS

Mutations in the C-terminal Region of MTF-1—MTF-1 includes a region of 32 amino acids beginning in the C-terminal portion of the serine/threonine-rich domain (residues 613–644 in human MTF-1) (26, 41) that is strikingly conserved (Fig. 1*A*) (42). This region is composed largely of hydrophobic residues, but it also contains the sequence ^{629}EEACQCQCACRD^{641}, which includes a number of potential metal-liganding amino acids. The role of these amino acids in metal-responsive transcription has not been directly tested, but it is possible that they could function in zinc recruitment or participate in the response of MTF-1 to metals other than zinc.

To examine the role of cysteines in the C-terminal region of MTF-1, we constructed several mutations in the protein coding sequence. The mutations included deletion of 13 amino acids (Δ(632–644)) as well as cysteine-to-alanine mutations at positions 632 (C632A), 653 (C653A), or 696 (C696A) and a double substitution at positions 632 and 634 (C632A/C634A). In addition, two chimeric proteins were created in which sequences from the MTF-1 of *D. melanogaster* (17) were inserted into the deletion site of Δ(632–644). The insertions contained amino acids 547–559 (Δ(632–644)+dMTF-(547–559)) or 547–565 (Δ(632–644)+dMTF-(547–565)) of the *Drosophila* protein. Although the exact sequence of the C-terminal region of the fly

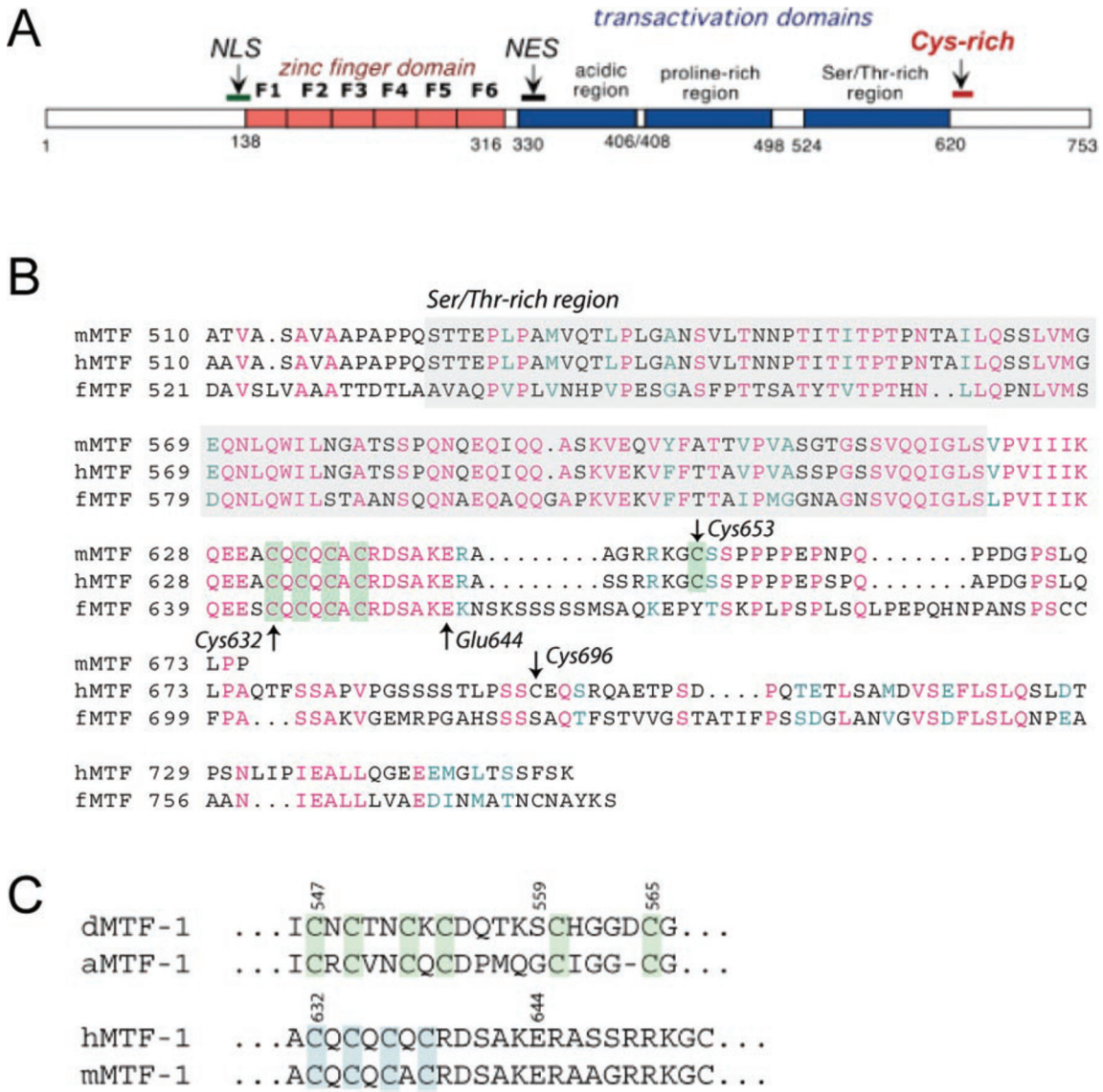

FIG. 1. **Structure of MTF-1.** *A*, domain organization of human MTF-1. Positions of the six zinc fingers, nuclear localization (*NLS*), and nuclear export (*NES*) signals as well as acidic, proline-rich, and serine/threonine-rich activation domains are indicated. *B*, partial sequence alignment of MTF-1 from human (*h*), mouse (*m*), and pufferfish (*f*). *C*, sequence comparison of cysteine-rich regions of MTF-1 from human (*h*), mouse (*m*), *D. melanogaster* (*d*), and mosquito (*a*).

protein is not closely related to the human, mouse, and pufferfish sequences, *Drosophila* MTF-1 as well as the putative MTF-1 from mosquito contains an analogous cysteine-rich peptide in the same general region within the protein (Fig. 1*C*) (42). As described below, these constructs allowed us to test whether this region could replace the endogenous cysteine-rich element in the human protein.

Metal-responsive Transcription Mediated by MTF-1 Derivatives—To assess the ability of our MTF-1 derivatives to affect transcription, we took advantage of a transient transfection assay with mouse dko7 cells, which express no endogenous MTF-1 (9). Plasmids directing expression of MTF-1 were based on pChMTF-1 (26) in which transcription of human MTF-1 cDNA is driven by the cytomegalovirus promoter. The MTF-1 expression plasmids were transformed along with reporter plasmid pLucMRE, which contains four tandem MRE sequences inserted just upstream of a basal promoter driving transcription of firefly luciferase. To control for transfection efficiency, each transfection also included the plasmid pSVβ-galactosidase in which transcription of *Escherichia coli lacZ* is driven by the SV40 early promoter.

When transient transfection assays were performed with the wild-type MTF-1 expression plasmid (pChMTF-1), we observed metal-induced expression of the luciferase reporter gene (Fig. 2). Treatment of transfected cells with 20 μM Zn(II) or 6 μM Cd(II) resulted in ~2-fold and 10-fold induction of luciferase activity, respectively. Normalized levels of luciferase expression were calculated from the ratio of activities of luciferase and β-galactosidase in extracts of transfected cells. This value was arbitrarily set to 1 for the ratio obtained with the wild-type construct at a concentration of 10 ng/ml in the absence of metal treatment. To allow for quantitative comparisons between experiments performed with different constructs or with different metal treatments, at least four independent "normalization" transfections using these conditions were included in each set of assays described below. The level of luciferase expression detected under these conditions is many-fold over the background (see below). Interestingly, we observed increased levels of metal responsiveness if the cells were cultured briefly (18–24 h) using medium that had been treated to remove contaminating heavy metals (see "Experimental Procedures"). Presumably, this depletes endogenous stores of zinc (43) and possibly

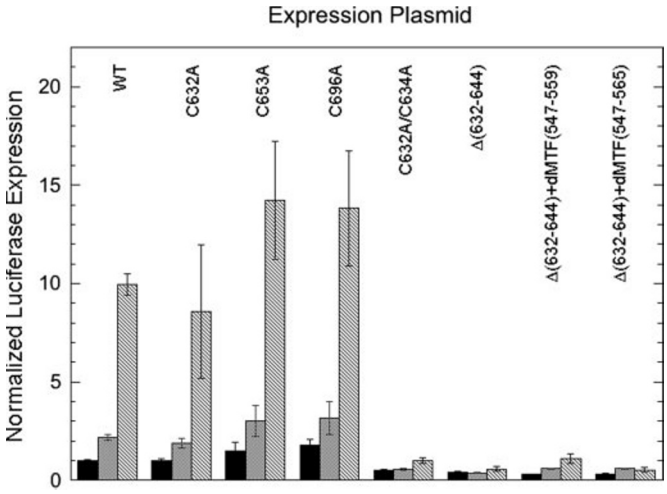

FIG. 2. **Metal-responsive transcription mediated by MTF-1 derivatives.** Transient transfections in mouse dko7 cells were performed with an expression plasmid containing the MTF-1 derivative indicated (10 ng/ml) and a MRE-linked luciferase reporter plasmid. When present, $ZnCl_2$ (20 μM) and $CdCl_2$ (6 μM) were added 10 h before harvest. Luciferase expression was normalized to the β-galactosidase activity obtained from a cotransfected pSVβ-galactosidase control plasmid. The ratio of luciferase activity to β-galactosidase activity obtained from wild-type MTF-1 in the absence of metal induction was arbitrarily assigned a value of 1. Each transfection was performed a minimum of three times. Transfections with wild-type (*WT*) MTF-1 and the C632A/C634A and Δ(632–644) variants were performed 20, 6, and 6 times, respectively. *Error bars* reflect the mean ± S.E. of the measurements.

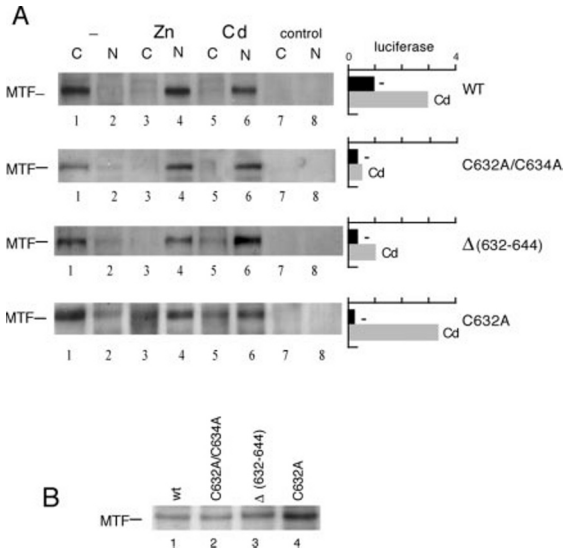

FIG. 3. **Expression of MTF-1 in transfected cells.** *A*, metal-induced nuclear localization of MTF-1. Transient transfections in mouse dko7 cells were performed with an expression plasmid containing the c-Myc-tagged hMTF-1 derivative indicated (200 ng/ml). Cytosolic (*C*) and nuclear (*N*) fractions were prepared from transfected cells treated with $ZnCl_2$ (20 μM), $CdCl_2$ (6 μM), or no metal as well as those from untransfected control cells. When present, metals were added 8–10 h before harvest. Samples (100 μg of cytosolic protein (*lanes 1*, *3*, *5*, and *7*) and 30 μg of nuclear protein (*lanes 2*, *4*, *6*, and *8*) were fractionated by SDS-polyacrylamide gel electrophoresis and subjected to immunoblot analysis with an antibody directed against the c-Myc epitope tag. The band corresponding to MTF-1 is indicated. Transfections also contained a MRE-linked luciferase reporter plasmid. Cytosolic fractions from untreated and Cd(II)-treated cells were assayed for luciferase activity, which was normalized to the β-galactosidase activity obtained from a cotransfected pSVβ-galactosidase control plasmid. Luciferase levels are shown in the *bar graph* adjacent to the corresponding immunoblot. *B*, expression levels of MTF-1 derivatives. Nuclear fractions (30 μg) of the zinc-treated and transfected cells (same samples as in *part A*, *lanes 4*) were fractionated by SDS-polyacrylamide gel electrophoresis and subjected to immunoblot analysis with an antibody directed against the c-Myc epitope tag. The band corresponding to MTF-1 is indicated.

other metals. All of the dko7 cell transfections were performed in this manner.

We performed several controls for the transient transfection assays. Metal induction was dependent on the presence of the MTF-1 cDNA in the expression plasmid as well as on the presence of MRE sequences in the reporter, and luciferase expression increased in a dose-dependent fashion as the amount of pChMTF-1 was increased (data not shown).

Although cysteine-to-alanine substitutions at residues 632, 653, and 696 had no effect on MTF-1 function, both basal and metal-induced luciferase expression were markedly decreased in transfections with plasmids containing cDNAs encoding the C632A/C634A and Δ(632–644) variants (Fig. 2). With the possible exception of the moderate induction (approximately 2-fold) by Cd(II) with the C632A/C634A variant, metal induction is largely lost. Transfections with constructs containing coding sequences for the two human *Drosophila* chimeric proteins also resulted in very low levels of luciferase expression, similar to that of the parent Δ(632–644) construct in which the *Drosophila* sequences were inserted. Interestingly, the Δ(632–644)+dMTF-(547–559) construct while mediating low absolute levels of expression did appear to mediate 2- to 3-fold induction by Zn(II) and Cd(II).

Expression and Metal-induced Nuclear Translocation of MTF-1 Derivatives—In the absence of inducing metals, MTF-1 is localized primarily in the cytosol of cells and metal treatment leads to rapid nuclear translocation (23–25), which is accompanied by the appearance of MTF-1 bound to the MREs associated with metal-responsive genes (32).

To facilitate immunological detection of our MTF-1 derivatives, we constructed expression plasmids for MTF-1 proteins that included a C-terminal 10 amino acid extension derived from c-Myc. This c-Myc tag provided an epitope for recognition of the fusion proteins by a highly specific anti-c-Myc monoclonal antibody directed against the 10 residue peptide. Luciferase expression levels mediated by MTF-1 and c-Myc-MTF-1 in transfections performed under the conditions used in the experiments in Fig. 2 were essentially identical (data not shown). To assess nuclear localization, transient transfections were performed with the c-Myc-MTF-1 expression plasmids and extracts of cytosolic and nuclear proteins were prepared, fractionated by SDS-polyacrylamide gel electrophoresis, and subjected to immunoblot analysis with the anti-c-Myc antibody (Fig. 3*A*). In the absence of metal treatment, proteins with the mobility expected for MTF-1 (≈100 kDa) (28, 44) were detected in the cytosolic extracts of cells transfected with MTF-1 expression plasmids containing coding sequences for the wild-type protein and the Δ(632–644), C632A, and C632A/C634A derivatives (*lanes 1*). These bands were not detected in cytosolic extracts of untransfected cells (*lanes 7*) nor in cells transfected with the empty expression vector (data not shown). The corresponding nuclear extracts (*lanes 2*) had low levels of MTF-1, consistent with previous reports (23–25) that in the absence of metal induction MTF-1 is predominantly in the cytosol. The amounts of protein loaded on the gels depicted in Fig. 3*A* (100 μg of cytosolic and 30 μg of nuclear) reflect the ratio of protein recovered in the cytosolic and nuclear extracts, and thus the intensities of the bands in the immunoblot reflect the relative amounts of protein in the two cellular compartments. In contrast to untreated cells when extracts were prepared from Zn(II)- or Cd(II)-treated transfected cells, all four MTF-1 derivatives were recovered largely in the nuclear fraction (*lanes 3–6*). Thus, the Δ(632–644), C632A, and C632A/C634A derivatives, similar to wild-type MTF-1, respond to metals by translocating to the nucleus.

The immunoblots in Fig. 3*A* do not directly address the question of the relative levels of expression of the various MTF-1 derivatives. To directly compare expression levels, nuclear extracts (30 μg of protein) derived from Zn(II)-treated transfected cells (the same samples used in Fig. 3*A*, *lanes 4*) were fractionated in side-by-side lanes by SDS-polyacrylamide gel electrophoresis and subjected to immunoblot analysis with the anti-c-Myc antibody (Fig. 3*B*). All four MTF-1 derivatives were expressed at comparable levels.

The transfections used to assess nuclear localization (Fig. 3) were performed under somewhat different conditions than those used in experiments whose primary focus was to test metal-responsive transcription (Fig. 2). The low concentration of MTF-1 expression plasmid used to test transcriptional regulation (10 ng/ml) while optimizing metal responsiveness did not provide sufficient MTF-1 expression for detection on immunoblots (data not shown). Therefore, we used a higher concentration of MTF-1 expression plasmid (200 ng/ml) in the nuclear localization experiments. At this concentration, basal levels of luciferase expression mediated by wild-type MTF-1 were ~3-fold higher than at the lower concentration, whereas the Zn(II)- and Cd(II)-induced levels were essentially independent of MTF-1 expression plasmid concentration in the 10–200-ng/ml range (data not shown). We included the pLucMRE luciferase reporter and pSVβ-galactosidase control plasmids in the transfections used to assess nuclear localization, which allowed us to directly assess the effects of MTF-1 on transcription in the same samples in which we compared expression levels and nuclear localization (Fig. 3*A*, *right*). Cytosolic fractions from untreated and Cd(II)-induced cells (samples from *lanes 1* and *5* of the immunoblots) were assayed for luciferase and β-galactosidase activity. These assays confirmed the transcriptional defects of the C632A/C634A and Δ(632–644) derivatives under conditions in which we know they are expressed and translocated to the nucleus in a manner indistinguishable from the wild-type protein. It is important to emphasize that the absolute values of the luciferase activities shown in Fig. 3 are not directly comparable with those in Fig. 2. The higher concentration of MTF-1 expression plasmid used in the transfections reported in Fig. 3 increases luciferase expression by a factor of approximately 3 relative to the conditions used in Fig. 2.

Dependence of Basal Transcription on the MRE—It was shown in some of the earliest characterizations of the MRE that even in the absence of metal induction, metallothionein transcription was markedly MRE- and MTF-1-dependent (9). In our transient transfection assays, we observed that luciferase expression in the absence of metal induction was consistently lower when expression plasmids for the C632A/C634A or Δ(632–644) derivatives were used in place of wild-type MTF-1 (Fig. 2) but that this decreased level was still substantially above background. We tested whether this effect was dependent on the presence of the MRE in the luciferase reporter plasmid (Fig. 4). These transfections were performed at a concentration of MTF-1 expression plasmid of 200 ng/ml to enhance basal expression. To normalize the absolute level of luciferase expression, parallel transfections were also done with wild-type MTF-1 at a concentration of 10 ng/ml (the same conditions used in the transfections in Fig. 2) and the luciferase expression levels shown in Fig. 4 were normalized to the level obtained under these conditions. The empty pC expression vector yielded only a low level of luciferase activity, and the presence of the MRE in the luciferase reporter did not enhance expression. In contrast, whereas the absolute luciferase expression levels were decreased with the C632A/C634A and Δ(632–644) MTF-1 variants relative to that obtained with wild-type MTF-1, luciferase expression with all three MTF-1 derivatives

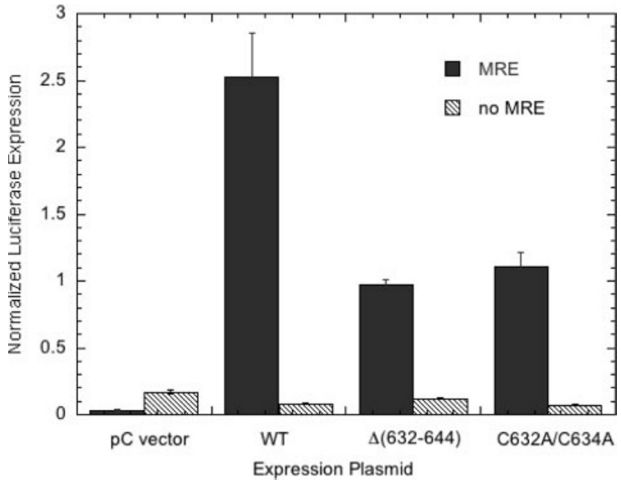

FIG. 4. **MRE dependence of basal transcription mediated by MTF-1.** Transient transfections in mouse dko7 cells were performed with an expression plasmid containing the MTF-1 derivative indicated (200 ng/ml) and luciferase reporter plasmids that differed only in the presence or absence of linked MRE sequences. Luciferase expression was normalized to the β-galactosidase activity obtained from a cotransfected pSVβ-galactosidase control plasmid. The ratio of luciferase activity to β-galactosidase activity obtained from wild-type (*WT*) MTF-1 at a concentration of 10 ng/ml (performed in parallel with the other transfections) was arbitrarily assigned a luciferase expression value of 1.0. All of the values shown here are normalized relative to this value. Each transfection was performed three times. *Error bars* reflect the mean ± S.E. of the measurements.

was markedly dependent on the presence of the MRE in the reporter plasmid. Thus, the C632A/C634A and Δ(632–644) variants were able to mediate basal levels of transcription within a factor of ≈2.5 of wild-type MTF-1, but were far more strongly attenuated in their transcriptional response to metal induction (Fig. 2).

MRE Recognition by MTF-1 Derivatives—The MRE-dependence of basal transcription mediated by the MTF-1 C632A/C634A and Δ(632–644) derivatives (Fig. 4) suggests that both of these variants can specifically recognize the MRE. To directly test this idea, we performed gel electrophoresis mobility shift assays. Nuclear extracts of dko7 cells transiently transfected with the c-Myc-MTF-1 expression plasmids served as a source of MTF-1, and a ^{32}P-labeled oligonucleotide containing a single copy of a consensus MRE designated MRE-s (7) was used as a probe. A shifted band corresponding to a protein-DNA complex was observed with nuclear extracts derived from cells transfected with the wild-type MTF-1 expression plasmid. Formation of the complex could be competed with an unlabeled oligonucleotide containing MREd but not with a control oligonucleotide lacking MRE sequences, and it could be supershifted with an antibody directed against the C-terminal c-Myc peptide tag (data not shown). Furthermore, the complex migrated with a mobility identical to a complex observed with nuclear extracts of HEK293 cells, which contain endogenous MTF-1 (*lane 1*). We conclude that this band corresponds to a specific complex between MTF-1 and the MREs. Essentially identical results were obtained with nuclear extracts from cells transfected with expression plasmids for the C632A, C632A/C634A, and Δ(632–644) derivatives and the human *Drosophila* chimeric proteins Δ(632–644)+dMTF-(547–559) and Δ(632–644)+dMTF-(547–565). Consistent with previous observations (9), the amount of MTF-1-MREs complex in all of the cases was increased when nuclear extracts from untreated cells were supplemented *in vitro* with Zn(II) (compare *lanes 3*, *6*, *9*, *12*, *15*, and *18* with *lanes 2*, *5*, *8*, *11*, *14*, and *17*, respectively) or when extracts were prepared from nuclei of cells treated with Zn(II) (*lanes 4*, *7*, *10*, *13*, *16*, and *19*), which would promote nuclear translocation of

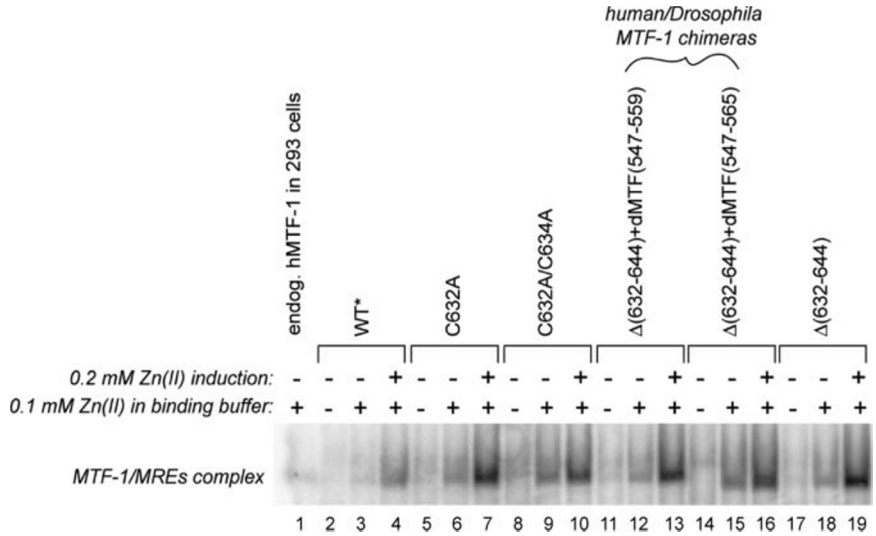

FIG. 5. **Specific MRE binding by MTF-1 derivatives.** Electrophoretic mobility shift assays were performed with nuclear extract and a ^{32}P-labeled oligonucleotide probe that contained a single MRE-s sequence. *Lane 1*, control nuclear extract from 293 cells, which contain endogenous MTF-1. *Lanes 2–19*, nuclear extracts from dko7 cells transiently transfected with the c-Myc-MTF-1 expression plasmid indicated. In *lanes 4*, *7*, *10*, *13*, *16*, and *19*, $ZnCl_2$ (0.2 mM) was added to the cells 8–10 h before harvest. In *lanes 1*, *3*, *4*, *6*, *7*, *9*, *10*, *12*, *13*, *15*, *16*, *18*, and 19, $ZnCl_2$ (0.1 mM) was added to the DNA binding assay. After electrophoresis, bands were visualized with a PhosphorImager.

MTF-1 (see Fig. 3). All of the MTF-1 derivatives therefore retain the ability to bind specifically to the MRE.

In Vitro Transcription Mediated by MTF-1 Derivatives—As a further test of the activity of the MTF-1 derivatives, we exploited an *in vitro* transcription assay. In this assay (39), RNA polymerase II and general transcription factors were supplied by a nuclear extract derived from HEK293 cells. MTF-1 function was assessed by the addition of complementing nuclear extracts derived from HEK293 cells transfected with various MTF-1 expression plasmids. Transcription templates were based on the oligonucleotide vector (OVEC) system previously described (36, 39). The reporter template consisted of a basal TATA box linked to four tandem MRE sequences, and a control template contained the β-globin gene and served as an internal normalization standard. mRNA transcripts were identified by S1 nuclease protection and gel electrophoresis.

A low level of transcription was observed with the basal, uncomplemented system (Fig. 6, *lanes 1* and *2*) or after complementation with nuclear extract from mock-transfected cells (*lanes 5* and *6*). However, when the basal system was complemented extracts of cells transfected with a plasmid-driving expression of wild-type MTF-1 (*lanes 3* and *4*) or c-Myc wild-type MTF-1 (*lanes 7* and *8*), transcription from the reporter template was increased as much as 7–8-fold relative to this basal level. This increase was dependent on the presence of the MRE in the reporter plasmid (39) and reflects a specific transcriptional activation by MTF-1. As MRE binding by MTF-1 in nuclear extracts is stimulated by the addition of exogenous zinc (9, 45) (see Fig. 5), we included 0.1 mM $ZnCl_2$ in our transcription reactions. In nearly all of the cases, this addition of zinc stimulates the yield of transcribed mRNA ≈1.5–3-fold. Interestingly, c-Myc wild-type MTF-1 (*lanes 7* and *8*) appears to give maximal mRNA expression in the absence of added zinc under these conditions. The reason for this is unknown but may reflect the higher levels of activated MTF-1 in these extracts relative to the others, giving rise to maximal transcription levels under both conditions.

Nuclear extracts derived from HEK293 cells transfected with MTF-1 expression plasmids driving synthesis of the Δ(632–644), C632A, and C632A/C634A derivatives as well as the human-*Drosophila* chimeric proteins Δ(632–644)+dMTF-(547–559) and Δ(632–644)+dMTF-(547–565) were all able to stimulate transcription when added to complement the basal *in vitro* system (Fig. 6, *lanes 9–16*). The level of stimulated transcription in each case was comparable with that obtained with the wild-type protein. Because a detailed analysis of the behavior of MTF-1 variants in this assay has not yet been performed, the functional domains of MTF-1 that are required to observe transcriptional activation with this system have not yet been defined. However, this *in vitro* induction probably relies strongly on the zinc finger MRE-binding domain (39), which itself gives ≈3-fold Cd(II) induction *in vivo* when fused to the strong transcriptional activator domain VP16 (41). Thus, although we cannot definitively say what activation functions are operating in the *in vitro* system, all of the variants with mutations in the C-terminal cysteine-rich region retain significant activation function required by this system.

DISCUSSION

MTF-1 as a Zinc Sensor—A number of experiments strongly suggest that MTF-1 functions as a sensor of the intracellular levels of free zinc. Treatment of cultured mammalian cells with zinc induces rapid nuclear localization of MTF-1 (23, 24), activates the specific MRE binding activity of MTF-1 (45), and results in association of MTF-1 with MRE sequences linked to metal-responsive genes packaged in chromatin (32). The zinc finger domain of MTF-1 (Fig. 1*A*) has been implicated in the mechanism by which MTF-1 senses changes in zinc concentration. Studies with a purified recombinant zinc finger domain derived from the human protein and expressed in bacteria suggest that zinc fingers F1-F4 bind zinc tightly and form the core of the MRE-binding domain, while fingers F5 and F6 bind zinc with much lower affinity and increase the stability of the MRE complex formed by fingers F1-F4 (29–31). More recent functional studies have implicated fingers F1, F5, and F6 as being essential for MTF-1 to activate gene expression with F5 and F6 only required when the target gene promoter is packaged in chromatin (32).

A Cysteine-rich Region of MTF-1 Important for Metal-induced Transcription—Our experiments have identified a region of MTF-1 outside of the zinc finger domain that is required for metal-induced transcription. This region, near the C terminus of the protein, is highly conserved among human, mouse, and pufferfish MTF-1 sequences (Fig. 1) (42) but is not present in the published zebrafish MTF-1 sequence (16). This might suggest a more specialized role for the cysteine-rich domain in higher eukaryotes. We have shown that proteins with alterations in this region are significantly impaired in their ability to induce metal-responsive transcription of a MRE-linked reporter gene (Fig. 2) but retain the ability to drive significant basal levels of transcription in a MRE-dependent manner *in vivo* (Fig. 3) and *in vitro* (Fig. 6). In addition, the mutated

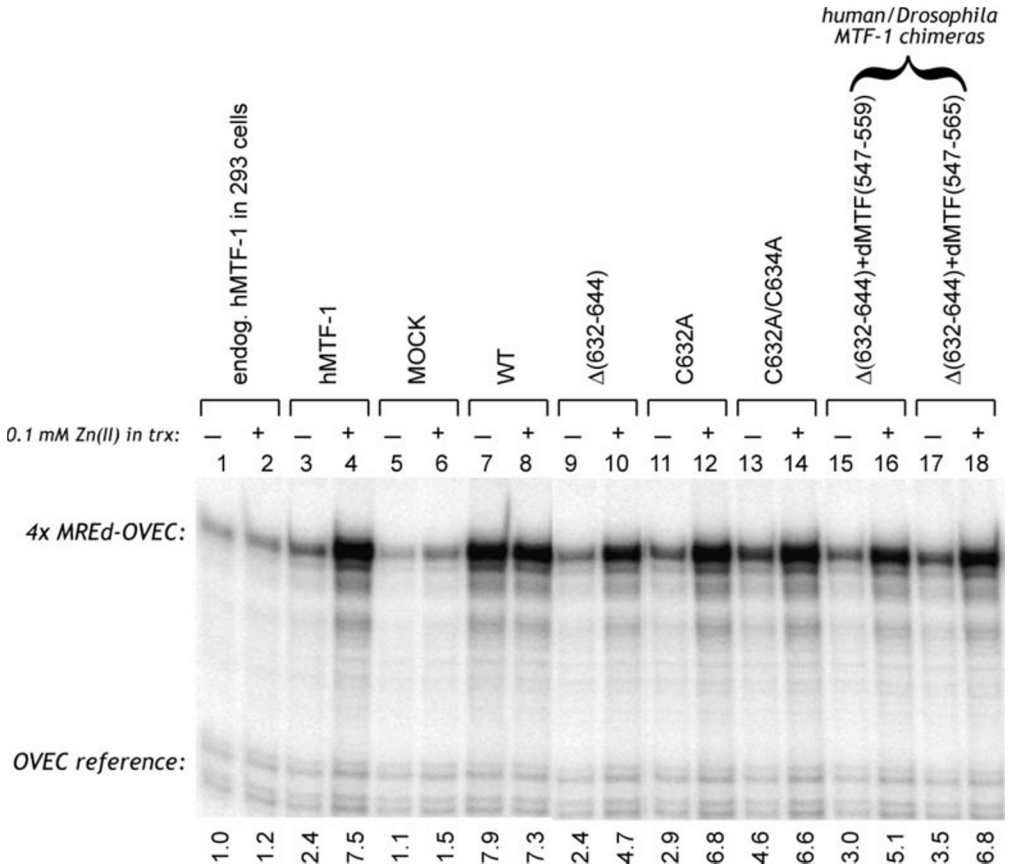

FIG. 6. **Analysis of MTF-1-driven transcription *in vitro*.** *In vitro* transcription reactions contained nuclear extract protein from 293 cells, a MRE-linked reporter template (4xMREd-OVEC) and a reference template (OVEC-REF). $ZnCl_2$ (0.1 mM) was added to reactions in *even-numbered lanes*. Reactions in *lanes 1* and *2* had no further additions. Reactions in *lanes 3–18* were supplemented with nuclear extracts derived from 293 cells transfected with the indicated MTF-1 expression plasmid. Transcripts were detected as S1 nuclease-protected fragments after gel electrophoresis and visualization with a PhosphorImager. *Numbers* below *each lane* reflect the normalized ratio of transcription from the MRE-linked template to that from the control template (*lane 1*).

proteins are expressed in transfected cells at levels comparable with wild-type MTF-1 (Fig. 3*B*) and respond to metal induction with nuclear translocation (Fig. 3*A*) and MRE binding activity (Fig. 5) comparable with the wild-type protein. Thus, we conclude that this cysteine-rich domain of the protein is specifically required for MTF-1 to activate transcription in the presence of metals *in vivo* while playing a correspondingly minor role in basal transcription (Fig. 4). The fact that this defect in transcriptional activation *in vivo* cannot be recapitulated by the simple *in vitro* system employed (Fig. 6) means that the molecular requirements for each process must differ.

Molecular Mechanisms by which the Cysteine-rich Domain May Function—Zinc and cadmium both promote nuclear translocation and activation of specific gene transcription by MTF-1 (23–25). However, cadmium cannot replace zinc in the MTF-1 zinc fingers (18) and is inhibitory toward MRE binding (45). The zinc finger domain itself does not function as the sole metal-sensing domain in metal-responsive transcription, because the region just C-terminal to this domain is responsible for the differential metal inducibility mediated by the human and mouse MTF-1 proteins *in vivo* (41). Furthermore, MTF-1 is extensively phosphorylated *in vivo* (27, 28) and a number of protein kinase inhibitors can disrupt MTF-1-induced transcription. These observations suggest that there are multiple potential sites for metal regulation and that more than one point in the signal transduction pathway may be subject to control by metal ions in a manner that might also depend on the precise induction protocol. In these studies, relatively low concentrations of metal inducers (20 μM Zn; 6 μM Cd) were used on cultured cells that had been acclimated to media stripped of Zn(II) prior to metal induction. These are conditions that are expected to significantly reduce the intracellular levels of total zinc in these cells (43).

One attractive model is that the cysteine-rich domain of MTF-1 could function in metal sensing by binding metal ions directly and altering intramolecular or intermolecular protein-protein interactions important for metal-inducible transcription activation. Consistent with this idea, bacterially expressed and purified full-length human MTF-1 contains approximately 7–8 mol eq Zn(II).[4] Because six Zn(II) ions bind to the six zinc fingers (29), the remaining 1–2 mol eq of Zn(II) must bind elsewhere. We have expressed and purified a domain of human MTF-1 consisting of amino acids 567–753 in bacteria. Preliminary metal binding studies reveal that this domain binds stoichiometric Zn(II), Co(II), and Cd(II) with a relatively low affinity of approximately 1×10^6 M^{-1}.[4] This finding suggests that Zn(II) and Cd(II) could activate MTF-1 by binding to the cysteine-rich domain directly and forming a metal complex. The complex would then alter the manner in which MTF-1 interacts with other regions of the protein, coactivators, or other components of the transcription apparatus. In particular, it will be important to determine how this domain influences metal-dependent activation from an MRE packaged in chromatin (32). Clearly, however, this Cys-rich domain is capable of exerting its influence on metal-induced transcription from naked DNA. On the other hand, this domain could function in zinc recruitment, exchange, or insertion into the zinc finger domain

[4] X. Chen and D. Giedroc, unpublished observation.

of MTF-1 in a manner analogous to that proposed for a pair of cysteines in domain III of hCCS, the Cu(I) chaperone of human Cu,Zn-superoxide dismutase 1 (46) or C-terminal cysteine pair of mercuric ion reductase (47, 48).

The idea that a transcription factor could contain a metal-responsive transcription activation function is not without precedent. *Saccharomyces cerevisiae* Mac1 is a transcriptional activator in copper-deficient cells, but its activity is inhibited in copper-replete cells. Copper regulates the activity of Mac1 through a C-terminal cysteine-rich motif by forming a polynuclear Cu(I) cluster that leads to an intramolecular interaction between its DNA-binding domain and the C-terminal copper-binding domain, which also possesses a transactivation activity (49). This interaction suppresses both DNA-binding and activation functions of Mac1. Interestingly, *Drosophila* MTF-1 (17) and a candidate MTF-1 gene from *A. gambiae*, which are not similar in sequence to the human, mouse, and pufferfish sequences in the C-terminal region, do contain a cysteine-rich sequence with six cysteines in this part of the protein (Fig. 1*C*) (42). Because six closely spaced cysteines are a hallmark of known polynuclear Cu(I)-metalloregulatory sites (50, 51), this domain, like the activation domains of Mac1 and Ace1, may be specific for Cu(I). Consistent with this finding, MTF-1/MRE-dependent metallothionein expression in *Drosophila* is strongly induced by Cu(I) and to a lesser extent by Zn(II) (17, 52). Furthermore, the major *Drosophila* metallothioneins are known to be copper-thioneins (53).

Another *S. cerevisiae* transcriptional activator, Zap1, appears to have zinc-regulated transcription activation function (54). The DNA-binding domain of Zap1 contains five C-terminal zinc fingers (ZF3-ZF7) that are necessary and sufficient for mediating the specific interaction with the zinc-responsive element (55) with primary roles played by ZF4 and ZF7 (56). Zap1 also contains two additional Cys_2-His_2 zinc fingers (ZF1/ZF2) embedded in the transcriptional activation domain 2 that play no role in zinc-responsive element binding (55). Recent findings show that zinc binding to these zinc fingers specifically inhibits the activity of activation domain 2 (57). Therefore, the ZF1/ZF2 finger pair in Zap1 may be functionally analogous to the Cys-rich domain of MTF-1 mapped here, with the exception that zinc occupancy represses rather than activates transcription.

In summary, our studies reveal that the cysteine-rich domain of MTF-1 plays a strong modulatory role in transcriptional activation *in vivo* at a step separable from metal-stimulated nuclear translocation and zinc-activated MRE binding. The precise molecular mechanism by which this domain exerts its function remains to be elucidated.

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