

Induction of Metallothionein I by Arsenic via Metal-activated Transcription Factor 1

CRITICAL ROLE OF C-TERMINAL CYSTEINE RESIDUES IN ARSENIC SENSING

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Metal-activated transcription factor 1 (MTF1) mediates the induction of metallothioneins I and II by zinc and stress signals. The mechanism of MTF1 activation has not been well understood. We analyzed the interaction between arsenic (As³⁺) and MTF1 for *Mt1* induction. As³⁺ potently induces *Mt1* mRNA expression in mouse hepalc1c7 cells. Induction is dependent upon functional MTF1 as induction is lost in *Mtf1* knockout cells but is restored upon reconstitution with *Mtf1*; moreover, As³⁺ induces the binding of MTF1 to the metal response elements of endogenous *Mt1*. Induction is not affected by modulating zinc concentrations but is markedly enhanced by cycloheximide. Phenylarsine oxide (PAO), which covalently binds to vicinal protein cysteine thiol groups, induces *Mt1* with a magnitude of higher potency than that of As³⁺. PAO affinity beads effectively pulls down the carboxyl half of MTF1 (MTF1_{321–675}) by binding to a cluster of five cysteine residues near the terminus. Preincubation with As³⁺, Cd²⁺, Co²⁺, Ni²⁺, Ag⁺, Hg²⁺, and Bi³⁺ blocks pulldown of MTF1_{321–675} by PAO beads *in vitro* and *in vivo*, indicating that binding of the metal inducers to the same C-terminal cysteine cluster as PAO occurs. Deletion of the C-terminal cysteine cluster or mutation of the cysteine residues abolishes or markedly reduces the transcription activation activity of MTF1 and the ability of MTF1 to restore *Mt1* induction in *Mtf1* knockout cells. The findings demonstrate a critical role of the C-terminal cysteine cluster of MTF1 in arsenic sensing and gene transcription via arsenic-cysteine thiol interaction.

Humans are constantly exposed to a wide variety of metals from dietary, environmental, and occupational sources. Trace elements, such as copper, iron, and zinc, are required for certain biological functions under physiological concentrations. However, most metals are toxic and cause a wide range of pathological conditions, including cancer, toxicity, and chronic diseases in humans (1, 2). Induction of metallothioneins (MT I and II),²

metal transporters, and antioxidative proteins represent a major means of metal detoxification in the body to maintain trace elements within a physiological range or to protect the body from the damage by metal overload. MTs are small, cysteine-rich, metal-binding proteins expressed in all eukaryotes and some prokaryotes. MTs I and II are highly inducible by heavy metals (such as Zn²⁺, Cd²⁺, Co²⁺, Ni²⁺, Ag⁺, Hg²⁺, and Bi³⁺), alkylating agents (e.g. iodoacetate), antioxidants, glucocorticoids, and inflammatory signals (such as lipopolysaccharide) (3–5). MTs regulate metal homeostasis by sequestering metals in protein-bound forms, providing a zinc reserve, and serving as a scavenger to quench ROS and other free radicals. On the other hand, metal transporters export metals out of the cells, and antioxidant proteins/enzymes antagonize metal-induced oxidative stress.

Transcriptional regulation of MTs, metal transporters, and certain antioxidant proteins is coordinated by metal-activated transcription factor 1 (MTF1), a member of the zinc finger family (6–10). MTF1 appears to be evolutionally conserved from *Drosophila*, to pufferfish *Fugu rubripes*, mouse, and human both structurally and functionally, reflecting a critical role of MTF1 in maintaining metal homeostasis across species (6, 9–13). MTF1 contains six pairs of Cys₂His₂ type of zinc fingers at the amino half as the signature DNA-binding domain (DBD) and three separable regions (acidic, proline-rich, and serine/threonine-rich) in the carboxyl half as the transcription activation domain (14).

In *Drosophila*, MTF1 (dMTF1) plays dual roles in copper homeostasis: at limiting copper concentrations, it induces the Ctr1B copper importer gene to increase cellular copper uptake; whereas at high copper concentrations, it induces MTs to chelate excess copper. Induction of both genes requires the binding of dMTF1 to the metal-responsive elements (MREs) located in the enhancers of the genes (15, 16). In mammalian species, MTF1 may have adapted into more complex biological functions. Disruption of the *Mtf1* gene in mice impairs liver development at gestation day 14 resulting in liver decay, generalized edema, and embryonic death (17), revealing a critical role of

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² The abbreviations used are: MT, metallothionein; ARE, antioxidant-responsive element; β -gal, β -galactosidase; ChIP, chromatin immunoprecipitation; CHX, cycloheximide; DBD, DNA-binding domain; HO1, heme

oxygenase 1; MRE, metal-responsive element; MTF1, metal-activated transcription factor 1; NAC, N-acetylcysteine; NQO1, NAD(P)H:quinone oxidoreductase; Nrf2, Nuclear factor erythroid 2-related factor 2; PAO, phenylarsine oxide; tBHQ, tert-butylhydroquinone; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagles medium; WT, wild type; KO, knockout; aa, amino acid(s); PBS, phosphate-buffered saline; HA, hemagglutinin; TA, transcription activation.

MTF1 in the regulation of liver-specific, developmental genes. This function of MTF1 may not involve *Mt1* and *Mt2*, because embryonic lethality is not observed in a double deletion of *Mt1* and *Mt2* (18, 19). Mice with conditional knockout of *Mt1* mature to adulthood but are highly sensitive to the toxicity of metals, such as Cd^{2+} (20). Adult *Mt1* null mice also exhibit relative leucopenia, in particular lymphocyte reduction (20), suggesting a role of MTF1 in lymphocyte development. The target genes of MTF1 responsible to its function in liver and lymphocyte development remain elusive. In addition, linkage analysis and haplotype mapping identified *Mt1* as a lymphoma susceptibility gene in mice, because a mutation in MTF1 (S424P) predisposes mice to ionizing radiation-induced thymic lymphomas, presumably by up-regulating radiation-resistant genes to promote tumor growth (21). In mammalian cells, coordinated regulation of MTs and zinc transporter 1 by MTF1 plays a major role in protection against the toxicity of zinc and other metals (22).

Induction of *Mt1* in the liver serves as a prototype of MTF1 action in response to metals. Quiescent MTF1 resides in the cytoplasm; MTF1 is activated by metals and is translocated into the nucleus, where it binds to multiple copies of MREs in the enhancer of *Mt1* to mediate gene transcription. A critical question on MTF1 action is how it senses metals. In one model, Zn^{2+} plays a central role: Zn^{2+} activates MTF1 by binding to a few or all of the zinc fingers of the protein leading to nuclear translocation and MRE-binding; other metals and stress signals, such as Cd^{2+} and ROS, mobilize Zn^{2+} from protein-bound Zn^{2+} pools to increase the concentration of intracellular free Zn^{2+} , which is then available to MTF1 zinc fingers for activation of the protein (23–26). This postulate is supported by the observations that Zn^{2+} binds to MTF1 zinc fingers and stabilizes the MTF1-MRE complex both *in vitro* and in chromatin (27, 28). On the other hand, Cd^{2+} does not appear to bind to the fingers in a way to preserve the canonical $\beta\beta\alpha$ -structure of the zinc fingers for optimal binding to DNA (29). Antioxidants, such as tBHQ, indeed increase intracellular free Zn^{2+} concentration (30); however, Cd^{2+} does not appear to affect the Zn^{2+} pool at 10 μM at which it significantly induces *Mt1* (31). Activation of MTF1 may also involve the removal of one or more inhibitory proteins, because inhibition of protein synthesis or exposing cells to heat shock synergizes with metal inducers to give rise to “superinduction” of MTs (7, 31, 32). However, the nature of the inhibitory protein(s) remains unclear. Other inducers, such as glucocorticoids and alkylating agents, may also interact with MTF1-associated proteins to activate MTF1, because these inducers are unlikely to interact with the zinc fingers directly. Phosphorylation of MTF1 was observed and may contribute to nuclear translocation of activated MTF1 (33–35).

Arsenic (As^{3+}) is a human carcinogen and a pleiotropic toxic metalloid that causes cancer (particularly of the skin, lung, bladder, and liver), neuropathy, cardiovascular lesions, ovarian dysfunction, aberrant embryonic development, and postnatal growth retardation in humans (36–42). As^{3+} is ubiquitous in the environment and has become a major public health concern worldwide, because millions of people are at risk of drinking water contaminated with arsenic (43). On the other hand, As^{3+}

has been effectively used as therapeutics, such as in the treatment of acute promyelocytic leukemia (44). The molecular targets of As^{3+} for many of its biological effects remain unidentified. As^{3+} appears to induce *Mt1* in rodents, but the mechanism of induction has not been addressed. Given the importance of MTF1 and MTs in metal detoxification, we examined the role and mechanism of action of MTF1 in the induction of *Mt1* by As^{3+} . The findings reveal that MTF1 mediates the induction of *Mt1* by As^{3+} and As^{3+} binds directly to the C-terminal cysteine thiol groups of MTF1 to induce the genes. The findings demonstrate a critical role of the C-terminal cysteine cluster of MTF1 in arsenic sensing and gene transcription by MTF1 *in vivo*.

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse hepa1c1c7 cells were provided by Dr. J. P. Whitlock, Jr. (Stanford University, Stanford, CA). The cells were cultured in α -minimal essential medium with 10% fetal bovine serum (FBS) and 5% CO_2 . Mouse Nrf2 wild-type (Nrf2 WT) and Nrf2 knockout (Nrf2 KO) embryonic fibroblast cells were derived from wild-type and Nrf2 null mice, respectively. The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS as described previously (45). MTF1 WT and MTF1 KO (dko7) fibroblast cells were kind gifts from Dr. W. Schaffner (Universitat Zurich, Zurich, Switzerland). The cells were cultured in DMEM supplemented with 10% FBS. The MRE- β Geo reporter cell line, BHK3038, was kindly provided by Dr. R. D. Palmiter (University of Washington, Seattle, WA). The cells were cultured in DMEM with 10% FBS medium. HEK293T cells were purchased from ATCC (Manassas, VA) and were cultured in DMEM with 10% FBS.

Chemical Reagents—Arsenic chloride (AsCl_3), bismuth ammonium citrate ($\text{C}_{12}\text{H}_{22}\text{BiN}_3\text{O}_{14}$), cadmium chloride (CdCl_2), cobaltous chloride (Cl_2Co), mercury chloride (HgCl_2), nickel chloride (NiCl_2), phenylarsine oxide (PAO), potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$), silver nitrate (AgNO_3), *tert*-butylhydroquinone (tBHQ), and zinc chloride (ZnCl_2) were purchased from Sigma-Aldrich. Affi-Gel 10 gel was from Bio-Rad (Hercules, CA). 4-Amino-phenylarsine oxide (*p*-aminophenyl arsenoxide, dihydrate) was purchased from Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada).

Plasmid Construction, Point Mutations, and Cell Transfection—The full-length coding region of mouse MTF1 cDNA (amino acid residues 1–675, aa_{1–675}) was amplified by reverse transcription and PCR from total RNA of hepa1c1c7 cells and was verified by sequencing. MTF1 was subcloned into the pcDNA3.1HA.his vector (Invitrogen) to generate pMTF1_{1–675}. A dominant-negative MTF1 (MTF1_{1–320}) was generated by cloning the DNA binding domain (aa_{1–320}) of MTF1 into pcDNA3.1HA.his. pMTF1_{321–675} was constructed by cloning the carboxyl half (aa_{321–675}) of MTF1 into the pcDNA3.1HA.his. pMTF1_{321–631} encodes the carboxyl half of MTF1 without the C-terminal 44 amino acid residues that contain the C-terminal five cysteine cluster. pMTF1_{1–631} was generated by cloning the aa_{1–631} fragment of MTF1 into pcDNA3.1HA.his. The pGMTF1 fusion constructs were generated by cloning the Gal4_{1–147} and a corresponding MTF1 fragment into pcDNA3.1HA.His to ob-

tain pGMTF1₁₋₆₇₅, pGMTF1₁₋₃₂₀, pGMTF1₃₂₁₋₆₇₅, and pGMTF1₃₂₁₋₆₃₁. pGVP16 was generated by cloning the VP16 transcription activation domain (aa₄₁₁₋₄₅₆) into pGal4₁₋₁₄₇. The pGMTF1₁₋₃₂₀VP16 was constructed by inserting MTF1₁₋₃₂₀ into the pGVP16. MTF1 C-terminal cysteine point mutation constructs were obtained by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The cysteine mutants include pGMTF1₃₂₁₋₆₇₅C632FC634F, pGMTF1₃₂₁₋₆₇₅C636FC638F, pGMTF1₃₂₁₋₆₇₅C653F, pGMTF1₃₂₁₋₆₇₅C636FC638FC653F, pGMTF1₃₂₁₋₆₇₅5CysMut, pMTF1₁₋₆₇₅5CysMut, and pMTF1₃₂₁₋₆₇₅5CysMut; 5CysMut = C632FC634FC636FC638FC653F. All plasmid DNA constructs and point mutations were confirmed by sequencing. The sequences of the primers used for plasmid construction and mutations are available upon request. The plasmids were transfected into cultured cells using the Lipofectamine plus reagents from Invitrogen.

RNA Preparation and Northern Blotting—Total RNA was isolated from cells using the Qiagen total RNA isolation kit. Total RNA of 3 μ g each was fractionated in a 1.2% formaldehyde agarose gel, transferred to a super-charged nylon membrane, and blotted with the digoxigenin-labeled riboprobe prepared with the digoxigenin-labeling reagents (Roche Applied Science, Indianapolis, IN). Plasmid constructs for riboprobes of mouse *Mt1*, *Ho1*, β -*Gal*, *actin*, and *Gapdh* were verified by sequencing. Northern signals were visualized by chemiluminescence using a digoxigenin RNA detection kit with CDP Star as a substrate (Roche Applied Science). *actin* and *Gapdh* were probed as loading control.

Measurement of β -Galactosidase Activity— β -Galactosidase (β -gal, encoded by β -*Geo*) was measured by using the β -galactosidase assay kit (Promega, Madison, WI). In a typical assay, BHK3038 cells were cultured in a 12-well plate and were treated with tBHQ (30 μ M) or As³⁺ (5 μ M) for 5 h. The cells were lysed in 100 μ l of 1 \times reporter lysis buffer (Promega) by shaking for 10 min at room temperature, followed by vortex for 15 s. Cell debris was removed by spinning in a microcentrifuge at the maximal speed for 2 min. Twenty-five μ l of supernatant was transferred to a 96-well plate containing 25 μ l of the 1 \times lysis buffer, and 50 μ l of the 2 \times assay buffer was then added. The 96-well plate was covered with a plastic wrapper and was cultured at 37 °C for 30 min to 3 h (until yellow color is visible). A stop solution (1 M sodium carbonate) of 150 μ l per well was added for 5 min. Absorbance at 420 nm (A_{420}) was measured on a 96-well plate reader. Each sample was repeated three to six times.

Luciferase Assay—Hepal1c7 cells were transiently transfected with appropriate plasmid DNA constructs with the *Renilla* internal control plasmid (Promega) by using Lipofectamine plus reagent. Cells were lysed with 1 \times passive lysis buffer (Promega) after 36–48 h of transfection. The plate was shaken for 10 min, and the lysate was collected and mixed by vortex for 15 s. Cell debris was removed by centrifugation for 2 min at 4 °C. Twenty microliters of cell lysate was mixed with 100 μ l of the luciferase assay reagent in a luminometer tube and the dual-luciferase activity was measured using the TD20/20 luminometer (Sunnyvale, CA); luciferase activity was normalized to both the protein concentration and *Renilla* luciferase activity of each sample.

Immunoblotting—Cells were lysed on ice with a radioimmune precipitation assay buffer containing protease and phosphatase inhibitors for 30 min. The cell lysate was sonicated briefly and was centrifuged at 14,000 \times g for 20 min to remove cell debris. Cell lysate (10–20 μ g each) was fractionated in 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes (Bio-Rad), and blocked with 5% nonfat milk in PBST (phosphate-buffered saline (PBS) plus 0.05% Tween 20). The membrane was blotted with primary antibody at 4 °C for overnight with shaking, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence detection reagents from Amersham Biosciences. Actin was blotted as loading control.

In Vitro Transcription and Translation—The TNT quick-coupled transcription/translation system (Promega) was used for *in vitro* transcription and translation of proteins. The reaction was incorporated with or without biotin tRNA for detection with streptavidin-horseradish peroxidase. Reaction products (1–5 μ l) were separated on 10% SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and immunoblotted with an antibody or with streptavidin-horseradish peroxidase, followed by detection with chemiluminescence.

PAO Beads Conjugation—10 mg of 4-aminophenylarsene oxide was dissolved into 3.05 ml of methanol and was mixed with 1.22 ml of Affi-Gel (50% slurry, Bio-Rad) for 2 h at room temperature. 100 μ l of aminoethanol was then added to block the remaining active groups of Affi-Gel. The mixture was washed three times with methanol and three times with PBS, followed by resuspension with 0.6 ml of PBS. An Affi-Gel control was prepared with 1.22 ml of Affi-Gel (50% slurry) mixed with 3.05 ml of methanol and 1 ml of aminoethanol with shaking at room temperature for 2 h.

Measurement of Protein Free Thiol Groups—Protein free thiol groups were measured as described previously with modifications (46, 47). Purified MT I protein (Sigma, 10 μ g) in 100 μ l was mixed with arsenic or PAO for 30 min. The protein was precipitated by adding 10 μ g of bovine serum albumin and 10% ice-cold trichloroacetic acid containing 1 mM dithiothreitol. The suspension was centrifuged at 14,000 \times g for 5 min at 4 °C. The precipitate was suspended in 400 μ l of Ellman's reagent (DTNB buffer, composed of, 0.5 M potassium phosphate, pH 7.4, containing 0.2 mM 5, 5'-dithiobis(2-nitrobenzoic acid) and 5 mM EDTA). The mixture was incubated at 4 °C for 30 min. Insoluble materials were removed by centrifugation for 5 min at 14,000 \times g at 4 °C, and the absorbance was measured at 412 nm.

ChIP Assay—Hepal1c7 cells were grown in a 10-cm dish to reach 90% confluence. The cells were treated with As³⁺ for 5 h. ChIP assay was performed as described previously with modifications (48). Briefly, DNA-proteins were cross-linked by incubation of cells with 1% formaldehyde at 37 °C for 10 min. Excess formaldehyde was quenched with 0.125 M glycine. Cells were collected in 1 ml of a lysis buffer (5 mM Pipes, pH 8.0, 85 mM KCl, and 0.5% IGEPAL CA-630) with proteinase inhibitors. Nuclei were pelleted by centrifugation. The nuclei were washed and resuspended in nuclei lysis buffer. DNA was sonicated to an average size of 200–1000 bp. Sheared chromatin was diluted in an immunoprecipitation dilution buffer (0.01% SDS, 1.1% Tri-

Activation of MTF1 by Arsenic

ton X-100, 1.2 mM EDTA, and 16.7 mM Tris/HCl, pH 8.0, and 167 mM NaCl) precleared with protein G and salmon sperm DNA; immunoprecipitation was performed with rabbit anti-mouse MTF1 antibodies or a normal rabbit IgG (as a control). The immunocomplex was reverse cross-linked by incubation with 5 M NaCl at 65 °C overnight. The DNA samples were purified and amplified by real-time PCR following standard procedures. Threshold cycles (C_T values) were determined using iCycler IQ software (Bio-Rad). Real-time PCR results were normalized using C_T values with 1% of input as an internal control. Relative DNA amounts were calculated from C_T values for each sample by interpolating into the standard curve obtained using a series dilution of standard DNA samples run under the same conditions. Primer set for amplifying the MRE of *Mt1* was as follows: forward, CGGAGGCCATTGTATTGTCT and reverse, GCTGGGTGGTCCGATACTA.

Data Quantification and Statistical Analysis—Quantification of RNA or protein bands were performed with Image-Quant software (Molecular Dynamics, San Jose, CA). Statistical

analysis was performed with one-way analysis of variance followed by *t* test using Prism (GraphPad Software, San Diego, CA).

RESULTS

Arsenic Induces *Mt1* via MTF1—To investigate the mechanism of *Mt1* induction by As^{3+} , we first characterized induction in hepa1c1c7 cells, a mouse hepatoma cell line highly responsive to toxic metals. Induction of *Mt1* mRNA by As^{3+} (10 μ M, 5 h) was compared with those by several known inducers of *Mt1*, including the phenolic antioxidant tBHQ (30 μ M), and carcinogenic metals Cr^{6+} (10 μ M) and Cd^{2+} (10 μ M). *Mt1* mRNA was nearly undetectable by Northern in the cells in the absence of the inducers (Fig. 1A, lane 1). tBHQ, Cr^{6+} , and Cd^{2+} induced *Mt1* as expected (lanes 2, 4, and 5). Notably, As^{3+} induced *Mt1* expression to a higher level than other inducers at an equal (for Cr^{6+} and Cd^{2+}) or lower concentration (for tBHQ) (Fig. 1A, compare lane 3 with lanes 2, 4, and 5). Induction by As^{3+} is concentration-dependent; induction was clearly observed at 2 μ M and reached >10-fold higher at 10 μ M (Fig. 1B).

The enhancer of the mouse *Mt1* gene contains several copies of MREs and an antioxidant responsive element (ARE) that overlaps with an upstream stimulatory factor-binding site (8, 49). Because As^{3+} is known to induce a number of cytoprotective genes, such as *Nqo1*, via ARE (50), we first examined if induction of *Mt1* by arsenic requires Nrf2, the principal transcription factor for ARE-dependent gene induction (Fig. 2A). tBHQ was used as a positive control, because it is known to induce *Nqo1* through ARE and *Mt1* through MRE, respectively (30, 45). As expected, tBHQ induced *Nqo1* in Nrf2 WT but not Nrf2 KO cells (Fig. 2A, middle panel, compare lanes 2 and 7); on the other hand, tBHQ induced *Mt1* in both Nrf2 WT and KO cells (upper panel, lanes 2 and 7). As^{3+} induced both *Mt1* and *Nqo1* in Nrf2 WT cells (Fig. 2A, upper and middle panels, compare lane 1 with lanes 3–5). Whereas induction of *Nqo1* was lost in the Nrf2 KO cells (Fig. 2A, middle panel, lanes 6 and 8–10), induction of *Mt1* was unaffected in Nrf2 KO cells (upper panel, compare lane 6 with lanes 8–10), indicating that Nrf2 is required for induction of *Nqo1* but not *Mt1*. Together, the data revealed that induction of *Mt1* by As^{3+} is unlikely to be mediated through ARE and Nrf2.

The role of MRE and MTF1 in *Mt1* induction by As^{3+} was examined. As^{3+} induced *Mt1* in MTF1 WT fibroblasts but not

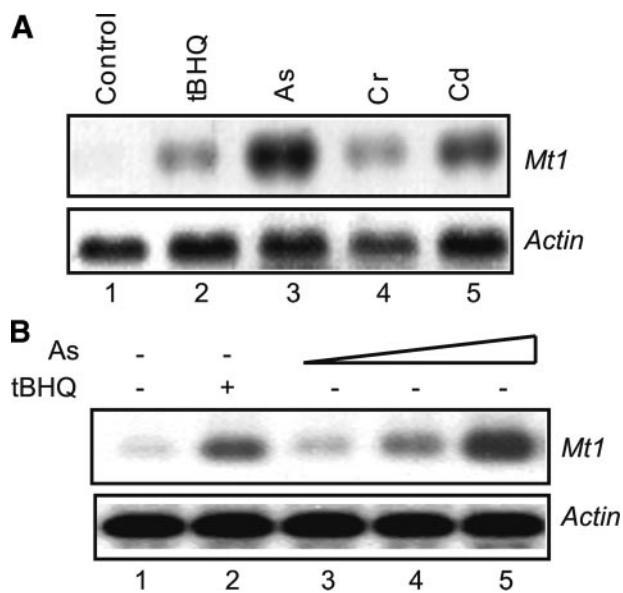


FIGURE 1. Induction of *Mt1* gene expression by arsenic. A, comparison of induction by metals and phenolic antioxidant. Hepa1c1c7 cells were treated with tBHQ (30 μ M), As^{3+} (10 μ M), Cr^{6+} (10 μ M), or Cd^{2+} (10 μ M) for 5 h. Total RNA was analyzed by Northern blotting. *Actin* was used as a loading control. B, concentration dependence. Cells were treated with tBHQ (30 μ M) or As^{3+} at 1, 5, and 10 μ M for 5 h. Total RNA was blotted with *Mt1* and *actin* riboprobes.

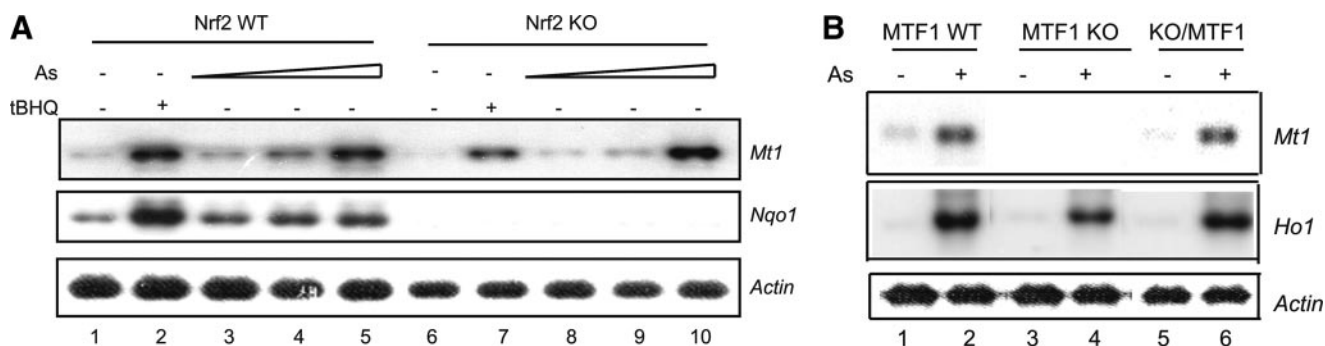


FIGURE 2. Induction of *Mt1* by arsenic requires MTF1. A, Nrf2 WT and KO cells were treated with tBHQ (30 μ M), or As^{3+} (1, 5 and 10 μ M) for 5 h. Total RNA was blotted with *Mt1*, *Nqo1*, and *actin* riboprobes. B, MTF1 WT, MTF1 KO, and MTF1 KO cells stably transfected with pMTF1_{1–675} were treated with As^{3+} (10 μ M) for 5 h. Total RNA was blotted for *Mt1*, *Ho1*, and *actin* mRNA expression.

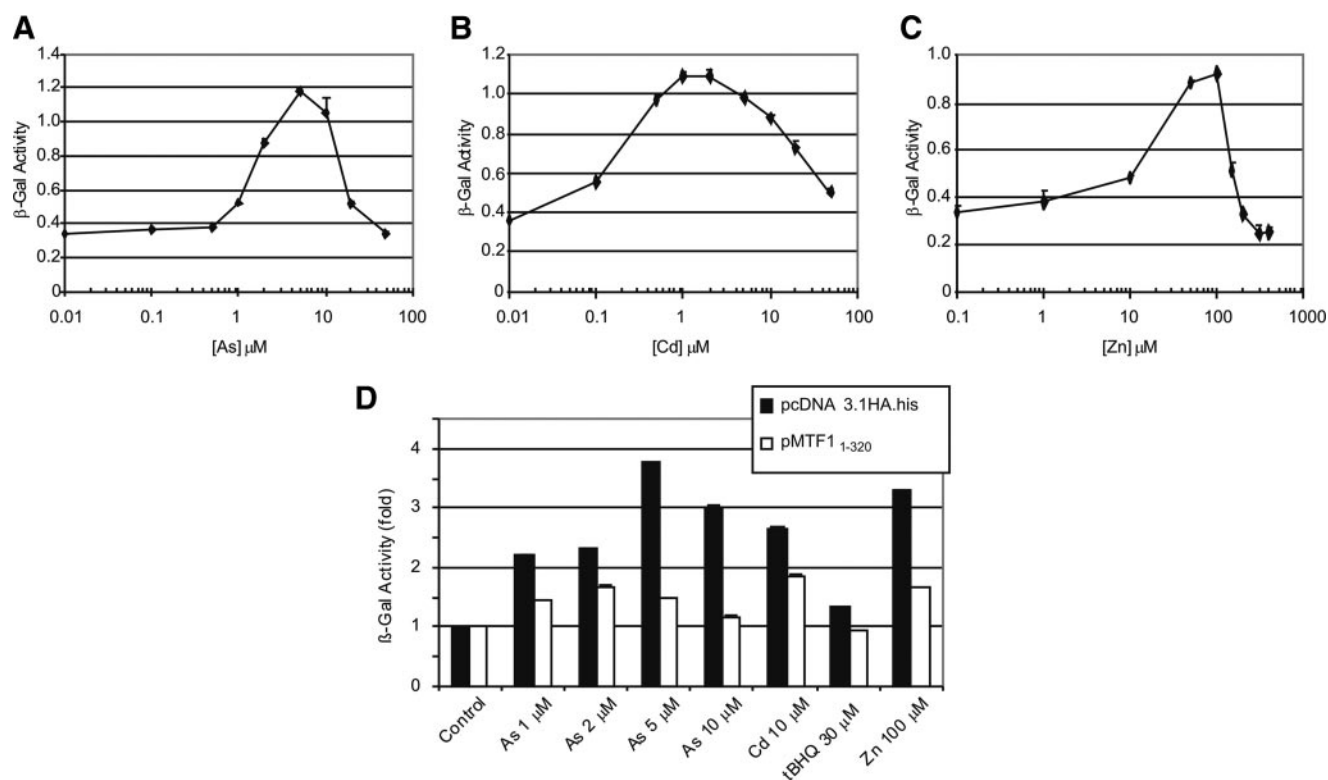


FIGURE 3. **Induction of MRE- β -gal reporter by arsenic.** BHK3038 were treated with metals for 5 h. Total cell lysate was measured for β -gal activity as described under "Experimental Procedures." A–C, concentration curves of β -gal induction by As^{3+} , Cd^{2+} , or Zn^{2+} , respectively. D, BHK3038 cells were transfected with pcDNA3.1HA.his (vector control) or pMTF1_{1–320} (dominant negative MTF1) for 48 h and were treated with As^{3+} (1, 2, 5, and 10 μM), Cd^{2+} (10 μM), tBHQ (30 μM), or Zn^{2+} (100 μM) for 5 h. β -gal activity was measured.

MTF1 KO cells (Fig. 2B, upper panel, lanes 1–4). Moreover, reconstitution of MTF1 expression in MTF1 KO cells (KO/MTF1) restored the induction (Fig. 2B, upper panel, lanes 5 and 6). *Ho1*, which does not depend on MRE for induction, was induced by As^{3+} in all three cell types independently of MTF1 (Fig. 2B, middle panel). The results indicate that induction of *Mt1* by As^{3+} requires MTF1.

As^{3+} stimulated β -gal reporter expression under the control of the MREs of *Mt1* in BHK3038 cells that express β -gal through the MTF1/MRE pathway (Fig. 3A). Induction was observed in a concentration range of 0.5–5 μM , above which the β -gal activity decreased concentration-dependently due to increased toxicity of As^{3+} at higher concentrations. Both the concentration range and the EC_{50} (~ 1.5 μM) of β -gal induction by As^{3+} were lower than those for the induction of the *Mt1* gene, possibly due to a higher sensitivity of reporter induction. Induction of the reporter by As^{3+} is similar to those by Cd^{2+} and Zn^{2+} in the overall shape of concentration-response curves (Fig. 3, B and C). Induction potency is in the order of $\text{Cd}^{2+} > \text{As}^{3+} > \text{Zn}^{2+}$ with estimated EC_{50} values of 0.25, 1.5, and 22 μM , respectively. To further ascertain the role of MTF1/MRE in *Mt1* induction, a dominant-negative form of MTF1 (MTF1_{1–320}) containing the DBD but lacking of the transcription activation domain was expressed in the BHK3038 cells. BHK3038 transfected with the empty vector served as a negative control. The β -gal reporter was induced by treatments with As^{3+} (from 1 to 10 μM), Cd^{2+} (10 μM), tBHQ (30 μM), or Zn^{2+} (100 μM) as expected. Induction was significantly inhibited in all cells expressing the dominant negative MTF1 compared with con-

trol (Fig. 3D), further supporting that MTF1 mediates *Mt1* induction by As^{3+} .

To examine if MTF1 directly mediates the induction of endogenous *Mt1* by As^{3+} , chromatin immunoprecipitation (ChIP) was performed. A polyclonal antibody preparation recognized the endogenous MTF1, *in vitro* expressed MTF1, and transiently expressed MTF1, all having an estimated apparent molecular mass of ~ 110 kDa (Fig. 4A) (31). MTF1 binds to the endogenous *Mt1* MREs constitutively at a low but detectable level; binding was markedly increased upon treatment with As^{3+} (Fig. 4B). Taken together, the results established that MTF1 mediates the induction of *Mt1* by As^{3+} through binding to its MREs. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay indicated that MTF1 WT cells were resistant to the toxicity of arsenic at concentrations of up to 50 μM , whereas the MTF1 KO cells had reduced survival in an arsenic concentration-dependent manner (data not shown). Moreover, KO cells reconstituted with MTF1 were resistant to arsenic toxicity similarly to the WT cells. Thus, MTF1 is required for defense against arsenic toxicity, which is, at least in part, attributable to MTF1-mediated induction of the *Mt* genes.

Role of Intracellular Free Zinc, ROS, and Labile Repressor—Some MTF1 inducers, such as tBHQ, activate MTF1 by mobilizing intracellular zinc from protein-bound forms to free zinc (30); increased intracellular free zinc can then bind and activate MTF1. Thus, modulating the concentration of intracellular free zinc may perturb induction of *Mt1* by tBHQ through MTF1. To examine if As^{3+} induces *Mt1* via zinc, hepa1c1c7 cells were cultured in a zinc-depleted medium to reduce the intracellular

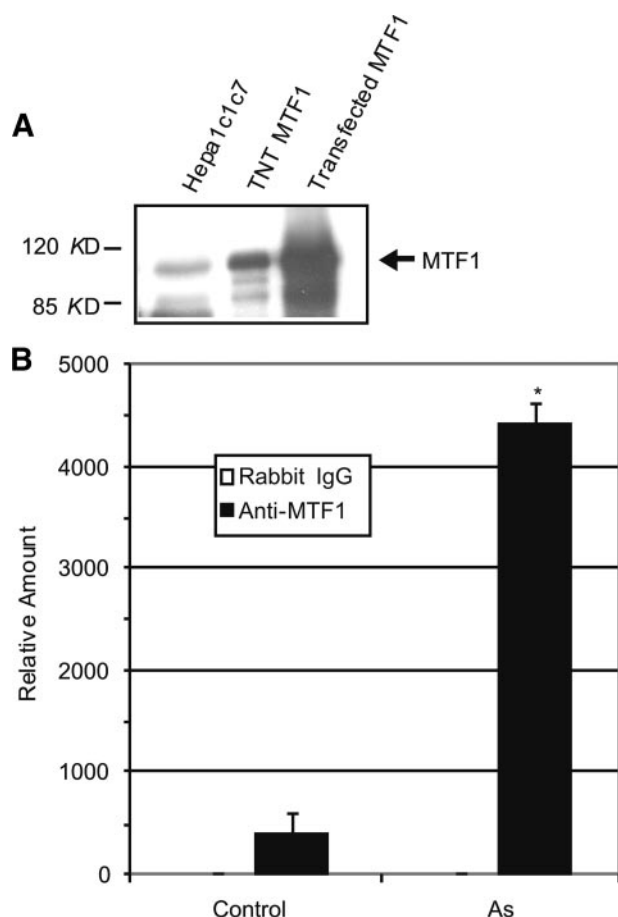


FIGURE 4. Recruiting MTF1 to endogenous MRE by As^{3+} . *A*, immunoblotting of MTF1. Cell lysate from hepa1c1c7, *in vitro* transcription/translation-produced MTF1 (TNT MTF1), and cell lysate from HEK293T cells transfected with pMTF1₁₋₆₇₅ were blotted with anti-MTF1 antibodies. *B*, ChIP assay. Hepa1c1c7 cells were treated with tBHQ (30 μM) or As^{3+} (10 μM) for 5 h. ChIP was performed and precipitated DNA was amplified by real time PCR with MRE primers. Quantitative data represent means and standard deviations from three experiments. Normal rabbit IgG was used as a negative control.

zinc concentration (30); induction of *Mt1* in the cells was compared with that of normal culture (Fig. 5). As^{3+} induced *Mt1* in MTF1 WT, but not KO cells, and stable expression of MTF-1 in KO restored the induction when the cells were cultured in a normal medium as expected (Fig. 5A, upper panels). A similar induction pattern was observed in the cells that were cultured in a zinc-depleted medium for a prolonged period of time (Fig. 5A, lower panels), indicating that depletion of extracellular zinc, which reduces the intracellular free zinc pool, does not appear to interfere with the induction of *Mt1* by As^{3+} . In Fig. 5B, induction of MRE- β -gal reporter was measured in BHK3038 cells that were cultured in a normal or zinc-depleted medium. Depletion of zinc in the medium did not decrease but increased reporter induction by either As^{3+} or Zn^{2+} , indicating that reduction of intracellular free zinc did not reduce induction but may have sensitized cells to MRE-inducers to give rise to increased induction.

EDTA chelates bivalent cations. Co-treatment of BHK3038 cells with EDTA and Cd^{2+} , As^{3+} , or Zn^{2+} , respectively, blocked the induction of β -gal by the metal inducers. However, treatment of BHK3038 cells with EDTA for 2 h followed by washing away of EDTA and replacing with a fresh medium did

not apparently affect induction of β -gal expression by either As^{3+} or Cd^{2+} (Fig. 5C), suggesting a direct effect of As^{3+} and Cd^{2+} on the induction. Together, the data imply that arsenic directly induces *Mt1* independently of Zn^{2+} concentrations in the culture medium or within the cells under the experimental conditions.

As^{3+} promotes the production of ROS in many cell types. A potential role of As^{3+} -induced oxidative stress in MTF1 activation was examined using the antioxidant, *N*-acetylcysteine (NAC), which inhibits ROS production. NAC itself did not affect the MRE- β -gal expression in a concentration range from 10 μM to 10 mM in BHK3038 cells (Fig. 6). Co-treatment with As^{3+} (5 μM) and NAC from 5 μM to 1 mM only slightly reduced reporter induction at high concentrations in which NAC was 100 to 200 times in excess of As^{3+} in comparison with treatment of As^{3+} alone. Only at a concentration of 2000 times excess (*i.e.* 10 mM) did NAC block the induction by As^{3+} . The apparently low efficiency of NAC in inhibiting the MTF1/MRE-dependent transcription of *Mt1* negates a direct role of ROS in MTF1 activation by As^{3+} .

Inhibition of protein synthesis induces *Mt1* and superinduces the gene in the presence of an activator of MTF1, such as Cd^{2+} and tBHQ, implicating a labile repressor in MTF1-mediated gene transcription (7, 31). The labile factor may suppress MTF1 as an MTF1-binding protein to sequester MTF1 in a dormant form (7) or as a nuclear factor that promotes the degradation of activated MTF1 in the nucleus (31). We examined if induction of *Mt1* by As^{3+} is also regulated by this labile repressor. In Fig. 7A, cycloheximide (CHX), a potent inhibitor of protein synthesis, induced *Mt1* in hepa1c1c7 cells or MRE-controlled β -gal in BHK3038 cells. As^{3+} induced both genes to a higher level than CHX. Co-treatment with arsenic and CHX increased the induction of the genes to much higher levels (*i.e.* superinduction). Furthermore, both the induction by CHX and superinduction by CHX plus As^{3+} required MTF1, because both activities were lost in the MTF1 KO cells (Fig. 7B). Thus, induction of *Mt1* by arsenic is likely regulated by the same labile repressor that suppresses MTF1 function constitutively and in the induction by Cd^{2+} and tBHQ.

As^{3+} and Other Metal Inducers Bind to MTF1 C-terminal Cysteine Residues *In Vitro* and *In Vivo*—The observation that induction of *Mt1* by As^{3+} does not depend on Zn^{2+} and ROS suggests a direct interaction of As^{3+} with MTF1 or an MTF1-associated protein. Because As^{3+} is reactive with protein thiol groups, we examined the possibility of As-thiol interaction in the activation of MTF1. Mouse MTF1 contains two clusters of cysteine residues (Fig. 8A). The N-terminal cluster includes the six pairs of cysteine residues within the six zinc finger structures plus two additional cysteine residues N-terminal to the zinc fingers. The C-terminal cluster contains five cysteine residues near the C-terminal end of MTF1. To investigate the potential role of the thiol clusters in MTF1 activation by As^{3+} , PAO, which forms stable covalent binding with two vicinal cysteine thiols of a protein, was used. We first showed that PAO potently induced *Mt1* in the concentration range of 0.01 to 1 μM (Fig. 8B, lanes 2–7); induction was reduced at 5 μM , which is attributable to increased toxicity of PAO at concentrations of >1 μM (data not shown). The potency of PAO for *Mt1* induc-

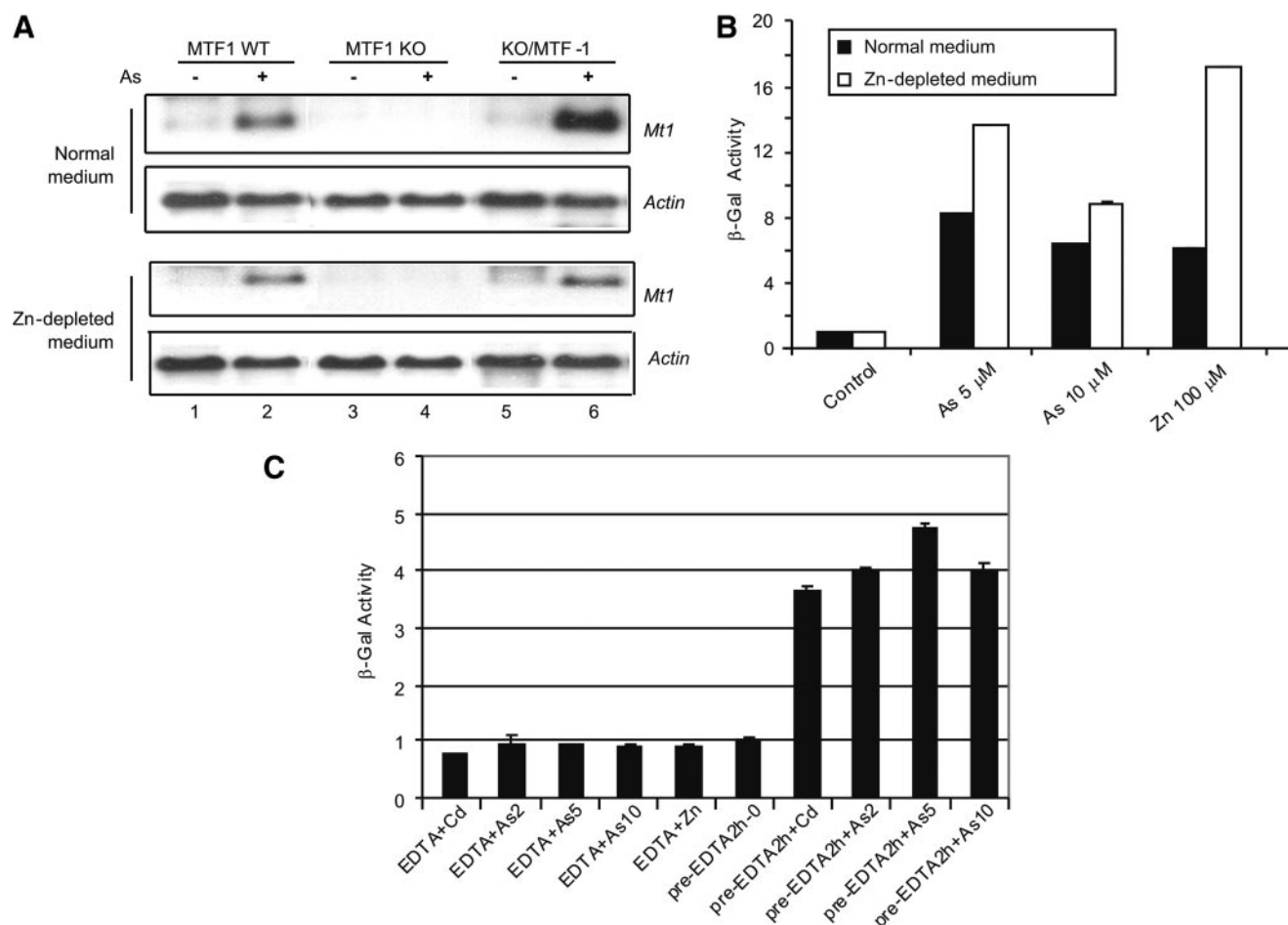


FIGURE 5. Induction of *Mt1* by arsenic does not depend on zinc. *A*, effect of zinc depletion. MTF1 WT, MTF1 KO, and MTF1 KO cells stably transfected with pMTF1₁₋₆₇₅ were cultured in a normal medium (DMEM plus 10% FBS) or a zinc-depleted medium (DMEM plus 10% of Chelex 100-treated FBS) for 48 h. The cells were treated with arsenic (10 μM) for 5 h. Total RNA was blotted for *Mt1* and *actin*. *B*, MRE-βGal induction. BHK3038 cells were cultured in a normal medium or a zinc-depleted medium for 48 h and were treated with arsenic (5 and 10 μM) or Zn²⁺ (100 μM) for 5 h. Cell lysate was assayed for β-gal activities. *C*, effect of EDTA. BHK3038 cells were treated with EDTA plus Cd²⁺ (10 μM), As³⁺ (2, 5, or 10 μM), or Zn²⁺ (100 μM) for 5 h, or the cells were pretreated with EDTA for 2 h, followed by washing with fresh medium and treating with Cd²⁺ 10 μM or As³⁺ at 2, 5, and 10 μM for 5 h. β-gal induction was measured.

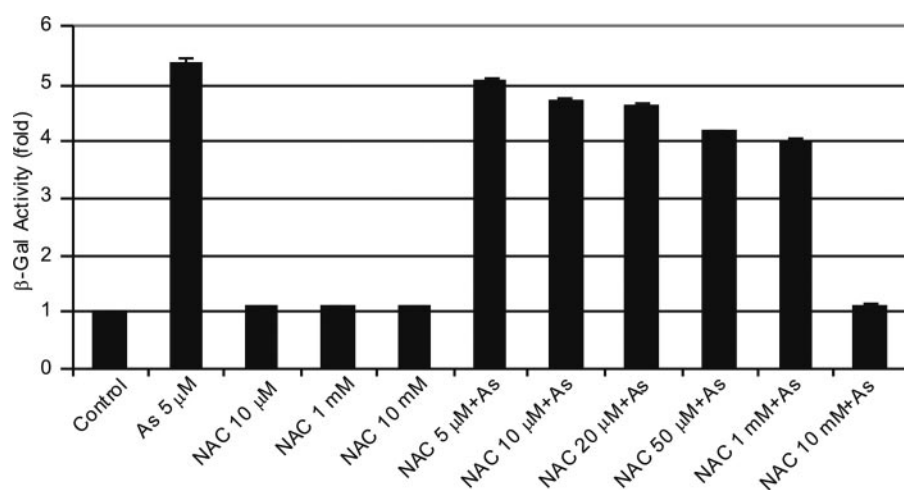


FIGURE 6. Effect of NAC on MRE reporter induction. BHK3038 cells were treated with NAC at 10 μM, 1 mM, and 10 mM alone or at 5 μM, 10 μM, 20 μM, 50 μM, 1 mM, and 10 mM plus arsenic (5 μM) for 5 h. β-gal was measured.

tion was estimated to be ~10 times higher than that of As³⁺ (EC₅₀ of 0.5 μM versus 5 μM). Furthermore, both As³⁺ and PAO were shown to bind the free thiol groups of proteins, as revealed

by reduction in the contents of free thiol groups of purified MTF1 protein after incubation with PAO or As³⁺ (Fig. 8C).

Binding of the N- or C-terminal cysteine clusters of MTF1 to As²⁺ was directly tested using PAO affinity matrix pulldown. MTF1 and deletion fragments were produced by *in vitro* transcription and translation (Fig. 9, *A* and *B*, upper panels). Both the full-length MTF1 (MTF1₁₋₆₇₅) and the DBD (MTF1₁₋₃₂₀) that comprises most of the six zinc fingers were precipitated by PAO pulldown as expected (Fig. 9A, lower panel). The C-terminal half (MTF1₃₂₁₋₆₇₅) and its deletion mutant that lacks the C-terminal cysteine cluster (MTF1₃₂₁₋₆₃₁) were examined in Fig. 9B. The PAO affinity matrix pulled down the C-terminal half of MTF1 but not the C-terminal deletion

Activation of MTF1 by Arsenic

mutant (*lower panel*), indicating that PAO binds to the C-terminal cysteine cluster. To further test the binding of PAO to the cysteine residues, the C-terminal cysteine residues were mutated to phenylalanine (MTF1_{321–675}5CysMut). PAO beads

efficiently pulled down MTF1_{321–675} but failed to precipitate MTF1_{321–675}5CysMut, even though both proteins were expressed at similar levels (Fig. 9C). The results demonstrate a direct binding of PAO with the C-terminal thiol cluster.

Binding of As³⁺ and other metal inducers with the C-terminal cysteine cluster was examined in Fig. 10. MTF1_{321–675} expressed *in vitro* was incubated with As³⁺ (10 μ M, 2 h) prior to PAO pulldown. Fig. 10A shows that preincubation with As³⁺ effectively abolished binding of PAO with the protein. Similarly, treatment of cells expressing MTF1_{321–675} with As³⁺ (10 μ M, 2 h) blocked pulldown of the peptide by PAO beads (Fig. 10B). Furthermore, most other metal inducers, Cd²⁺ (10 μ M), Co²⁺ (150 μ M), Ni²⁺ (150 μ M), Ag⁺ (10 μ M), Hg²⁺ (10 μ M), or Bi³⁺ (50 μ M), but not Zn²⁺ (150 μ M), blocked or significantly inhibited PAO pulldown of MTF1_{321–675} expressed in cells, which were treated with the inducers for 2 h prior to PAO pulldown (Fig. 10C). Taken together, these results demonstrated that As³⁺ and most other metal inducers bind directly to the C-terminal cysteine residues of MTF1 both *in vitro* and *in vivo*. The failure of Zn²⁺ to inhibit PAO pulldown could be due to the much lower affinity of Zn²⁺ to cysteine thiols than that of PAO.

MTF1 C-terminal Cysteine Residues Are Required for Transcription Activation by MTF1 and for Induction of Endogenous MTF1—We asked whether binding of As³⁺ to the C-terminal cysteine cluster plays one or more functional roles in MTF1 induction. Because MTF1 C-terminal half comprises mostly

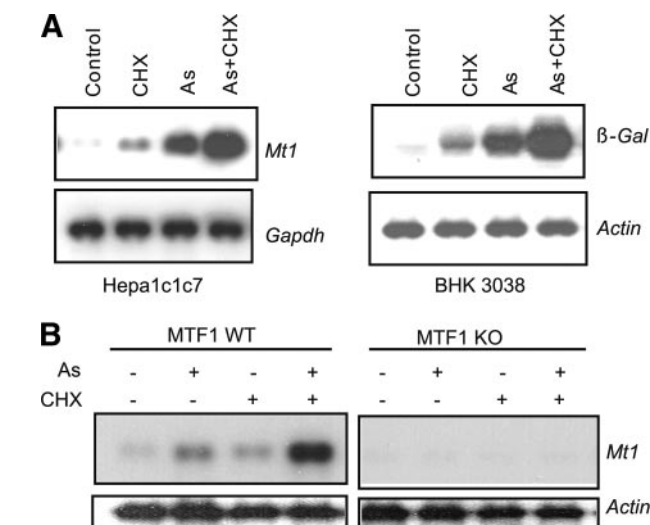


FIGURE 7. Superinduction of Mtf1 by As³⁺ and CHX. A, Hepa1c1c7 or BHK3038 cells were treated with CHX (10 μ g/ml), arsenic (10 μ M), or both for 5 h. Total RNA was blotted for mRNA expression of Mtf1 or β -Gal. Gapdh and actin were measured as loading controls. B, MTF1 WT or KO cells were treated with arsenic (10 μ M), CHX (10 μ g/ml), or both for 5 h. Expression of Mtf1 and actin mRNAs was measured by Northern blotting.

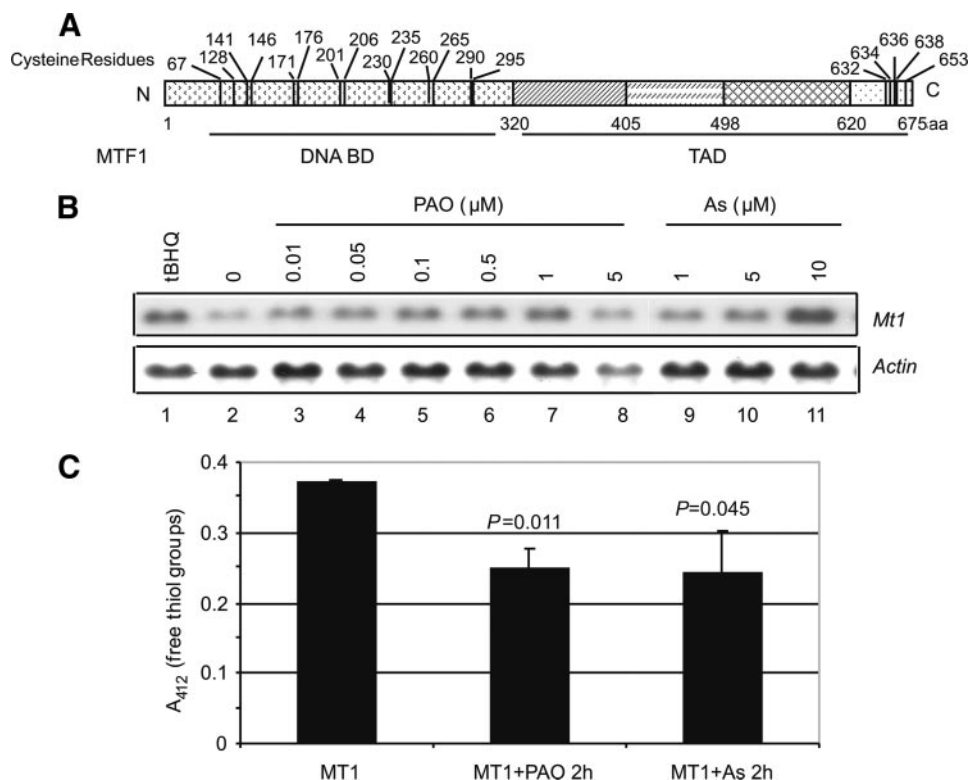


FIGURE 8. Characterization of PAO. A, schematic presentation of MTF1 and cysteine residues. B, induction of Mtf1 by PAO and arsenic. Hepa1c1c7 cells were treated with tBHQ at 30 μ M, PAO at 0.01, 0.05, 0.1, 0.5, 1, or 5 μ M, or arsenic at 1, 5, or 10 μ M for 5 h. Total RNA was blotted for Mtf1 and actin mRNA expression. C, direct binding of arsenic and PAO to the thiol groups of MTF1. Purified MTF1 protein was incubated with arsenic or PAO for 30 min; the mixture was precipitated with trichloroacetic acid, and free thiol groups were measured by using Ellman reagent with absorbance at 412 nm.

the TA domain, we examined the impact of the cysteine residues on the TA activity of MTF1. Gal4-MTF1 chimeric proteins, in which the Gal4-DNA binding domain (Gal4) and MTF1 or its deletion fragments were fused together (Fig. 11A), were expressed in cells to examine the TA activities of the fragments. Gal4 DBD (pGal4_{1–147}), which lacks a TA domain, was used as a negative control and showed little activity (Fig. 11B, lane 1), whereas Gal4-Vp16 (pGVP, lane 2), a positive control, activated reporter expression markedly. Full-length MTF1 (pGMTF1) exhibited a very low TA activity (lane 3) and MTF1 DBD (pGMTF1_{1–320}) showed no TA activity (lane 4). On the other hand, the C-terminal half (pGMTF1_{321–675}) had a high TA activity comparable to that of Vp16 (compare lane 5 with 2) and thus, is a strong TA domain. However, deletion of the C-terminal cysteine cluster (pGMTF1_{321–631}, lane 6) largely reduced the TA activity (to ~23% of pGMTF1_{321–675}), revealing a critical role of the C-terminal cysteine residues in MTF1 transcription activation.

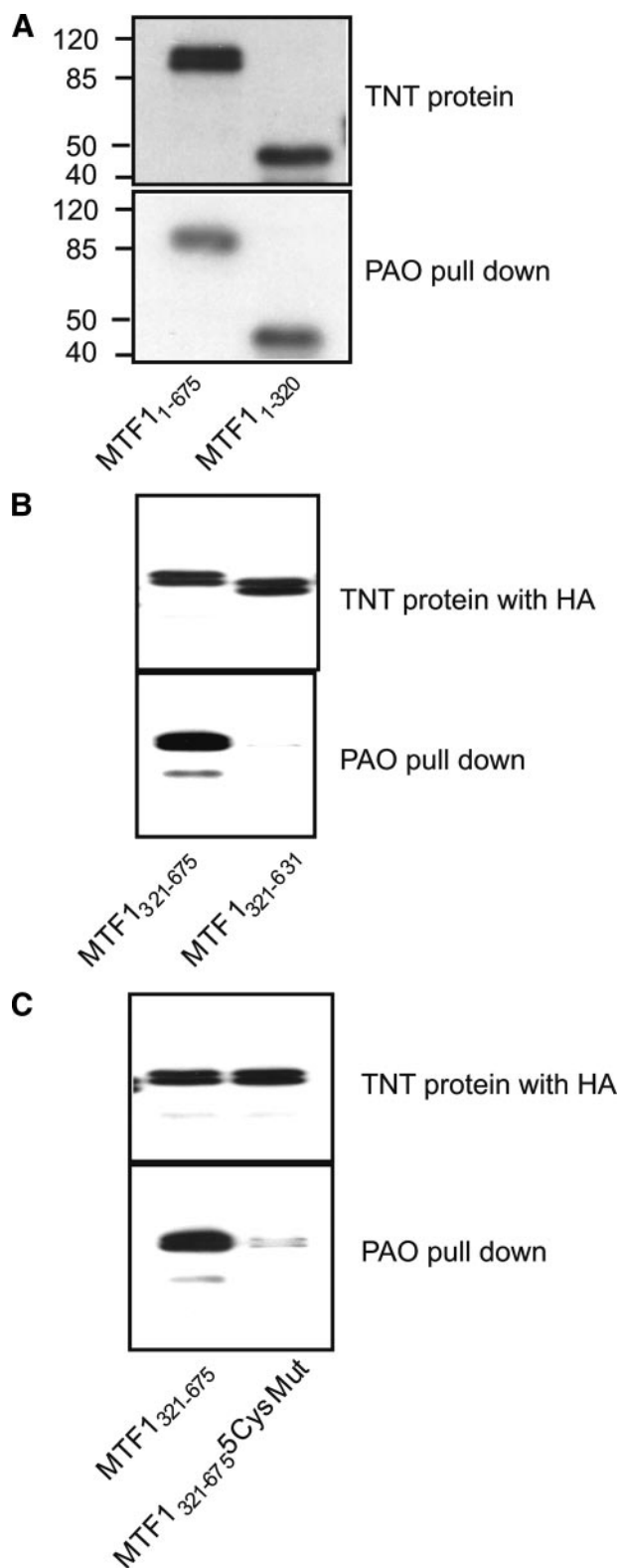


FIGURE 9. PAO directly binds to the C-terminal cysteine cluster of MTF1. A, biotin-labeled MTF1₁₋₆₇₅ and MTF1₁₋₃₂₀ was produced *in vitro* in TNT reticulocyte lysate and detected with streptavidin (upper panel). The proteins were pulled down with PAO matrix and detected with streptavidin (lower panel). B, MTF1₃₂₁₋₆₇₅ and MTF1₃₂₁₋₆₃₁ with a HA tag were produced *in vitro* and detected with an anti-HA antibody (upper panel). The proteins were pulled down with PAO matrix and detected with anti-HA (lower panel). C, HA-MTF1₃₂₁₋₆₇₅ and HA-MTF1₃₂₁₋₆₇₅ with all five cysteine residues mutated to phenylalanine were produced *in vitro*. The proteins were pulled down with PAO and detected with anti-HA. "5CysMut" = C632FC634FC636FC638FC653F.

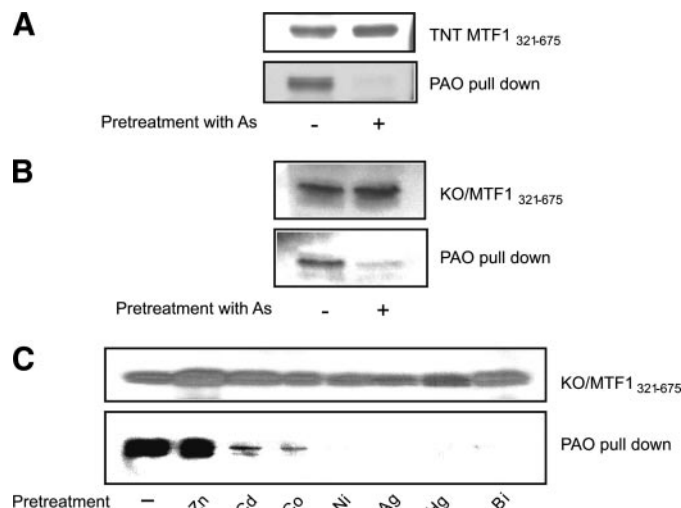


FIGURE 10. Metal inducers of Mtf1 bind to MTF1 C-terminal cysteine residues. A, binding with As³⁺ *in vitro*. TNT HA-MTF1₃₂₁₋₆₇₅ was incubated with As³⁺ (10 μ M) for 2 h and then pulled down with PAO and blotted with anti-HA. B, binding with As³⁺ *in vivo*. MTF1 KO cells were transfected with pMTF1₃₂₁₋₆₇₅ HA.His and treated with As³⁺ at 10 μ M for 2 h. MTF1₃₂₁₋₆₇₅ was pulled down with PAO and blotted with anti-HA. C, binding with other metal inducers *in vivo*. MTF KO cells transfected with pMTF1₃₂₁₋₆₇₅ HA.His were treated with Zn²⁺ (150 μ M), Cd²⁺ (10 μ M), Co²⁺ (150 μ M), Ni²⁺ (150 μ M), Ag⁺ (10 μ M), Hg²⁺ (10 μ M), or Bi³⁺ (50 μ M) for 2 h. MTF1₃₂₁₋₆₇₅ was pulled down with PAO and blotted with anti-HA.

The remarkably low activity of the full-length MTF1 compared with the C-terminal half suggest an inhibitory activity of the N-terminal fragment of MTF1. To test this notion, MTF1 DBD was inserted between Gal4 DBD and VP16. Remarkably, MTF1 DBD totally blocked the TA activity of VP16, demonstrating a potent inhibitory function of MTF1 DBD on a heterologous TA activity (Fig. 11B, compare lane 7 with 2). All Gal4 fusion proteins were expressed at comparable levels (Fig. 11C), excluding variable protein expression and stability, as contributing factors to the large differences in the TA activities observed from the fusion proteins. The results revealed that the C-terminal half of MTF1 contains a strong TA function that requires the C-terminal cysteine cluster, whereas the MTF1 DBD is a strong inhibitor of MTF1 or heterologous VP16 TA activities in addition to its DNA binding function.

Point mutation was performed to further dissect the role of the C-terminal cysteine residues in MTF1 transcription activation (Fig. 12). Replacing cysteine residues 632 and 634 with phenylalanine (pGMTF1₃₂₁₋₆₇₅C632FC634F) significantly reduced the TA activity compared with the wild-type (pGMTF1₃₂₁₋₆₇₅), but the reduction was only ~52%, much less dramatic than that of pGMTF1₃₂₁₋₆₃₁ (Fig. 12A, compare lane 4 with lanes 2 and 3). Double mutations at C636 and C638 (pGMTF1₃₂₁₋₆₇₅C636FC638F) resulted in a 33% reduction in the TA activity (compare lane 5 with 2). On the other hand, a single mutation at C653 (pGMTF1₃₂₁₋₆₇₅C653F) resulted in >80% inhibition. A triple mutation (pGMTF1₃₂₁₋₆₇₅C636FC638FC653F) or five cysteine mutation (pGMTF1₃₂₁₋₆₇₅5CysMut) caused further reduction in TA activity to a similar level to that of pGMTF1₃₂₁₋₆₃₁. The point mutant proteins were expressed at comparable levels (Fig. 12B). The findings revealed that most of the cysteine residues at the C terminus of MTF1 play a role in its TA function and may do so through a synergistic action

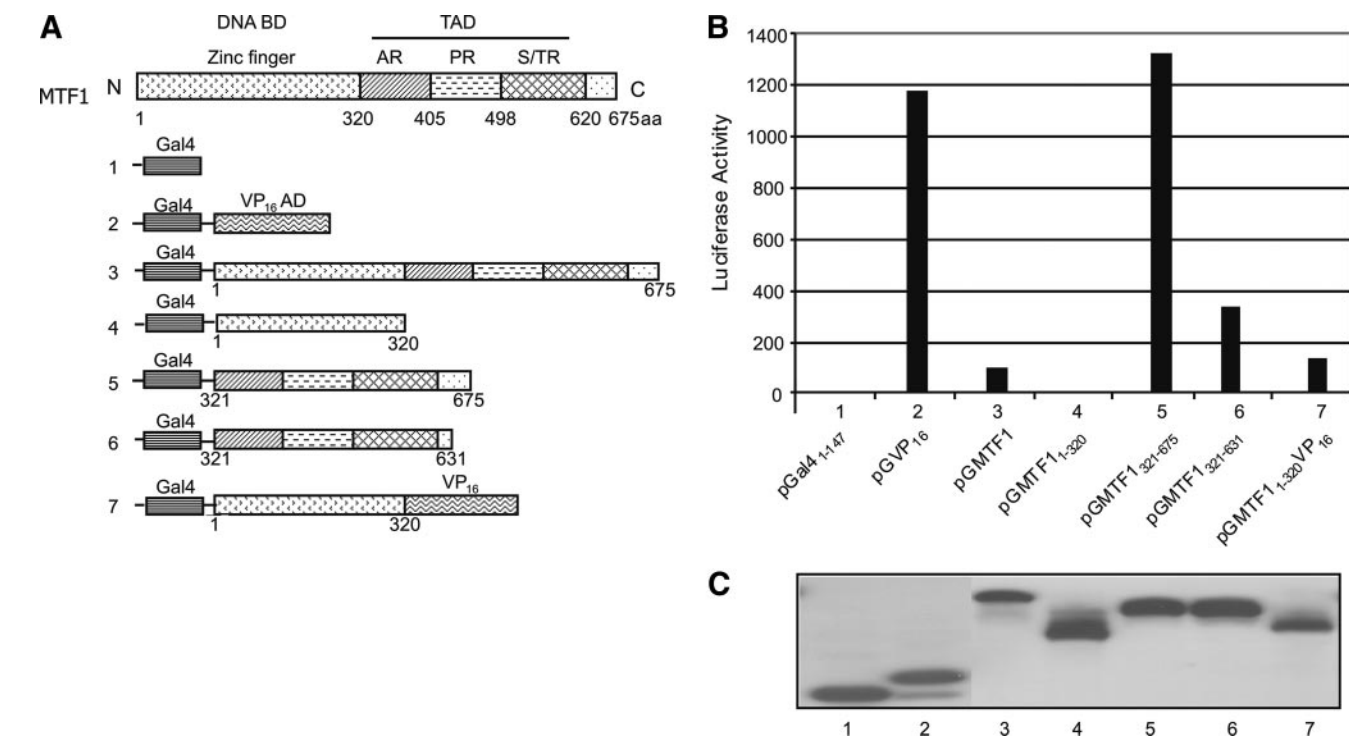


FIGURE 11. **MTF1 C-terminal cysteine cluster is required for transcription activation.** A, schematic presentation of MTF1 domains fused to the Gal4 DNA-binding domain (Gal4). B, Hepa1c17 cells were co-transfected with pG5IL2Luc and each construct shown above. 48 h later, the cells were lysed and measured for luciferase expression. C, protein expression of chimeric Gal4-MTF1 and Gal4-MTF1 deletion mutants.

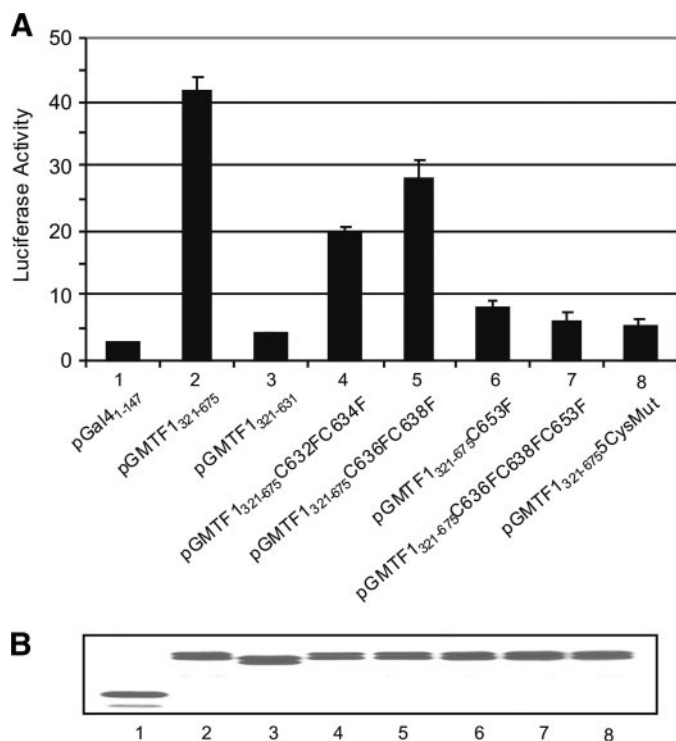


FIGURE 12. **Point mutations of MTF1 C-terminal cysteine residues.** A, point mutations of MTF1 C-terminal cysteine residues (cysteine to phenylalanine), C632FC634F, C636FC638F, C653F, C636FC638FC653F, and 5CysMut (C632FC634FC636FC638FC653F), were generated with pGMTF1₃₂₁₋₆₇₅ as the template, respectively. Co-transfection of pG5IL2Luc with one of the point mutation plasmids was performed, and luciferase activities were measured 48 h after transfection. B, protein expression of chimeric Gal4-MTF1 and Gal4-MTF1 cysteine mutants.

among the residues; however, of the five cysteines, Cys-653 is likely the most critical.

Lastly, the role of the C-terminal cysteine cluster in the induction of endogenous *Mt1* was examined by comparing the abilities of MTF1 and the deletion mutant (MTF1₁₋₆₃₁) or five cysteine mutant (MTF1₁₋₆₇₅5CysMut) to reconstitute *Mt1* induction in MTF1 KO cells (Fig. 13). MTF1₁₋₆₇₅ and MTF1₁₋₆₃₁ were stably expressed in MTF1 KO cells, respectively, as detected by immunoblotting with an anti-HA tag antibody (Fig. 13A). The MTF1 KO cells stably expressing MTF1 (KO/MTF1₁₋₆₇₅) showed a detectable basal expression and a strong induction of *Mt1* by As³⁺ compared with the KO cells (Fig. 13B, compare lanes 3 and 4 with lanes 1 and 2). Remarkably, two MTF1 KO clones expressing MTF1₁₋₆₃₁ (KO/MTF1₁₋₆₃₁-C1 and KO/MTF1₁₋₆₃₁-C2) did not show either the basal expression or induction of *Mt1* in the presence of As³⁺ (Fig. 13B, compare lanes 5–8 with lanes 1 and 2). In a separate experiment, MTF1₁₋₆₇₅ and MTF1₁₋₆₇₅5CysMut were expressed in KO cells (Fig. 13C). Cells expressing MTF1₁₋₆₇₅ are fully functional for induction of *Mt1*. On the contrary, one mutant clone (MTF1₁₋₆₇₅5CysMut-C4) showed only slight induction that is similar to that in KO cells (compare lanes 6 and 5 with lanes 1 and 2), and the other clone (MTF1₁₋₆₇₅5CysMut-C5) did not show detectable expression or induction of the gene (lanes 7 and 8). Together, the findings strongly support a critical role of the C-terminal cysteine clusters of MTF1 in the induction of *Mt1* by As³⁺ via As³⁺-MTF1 C-terminal cysteine thiol interaction.

DISCUSSION

MTF1 Mediates the Induction of Mt1 by As³⁺—As³⁺ elicits pleiotropic effects in animals and humans causing a wide range

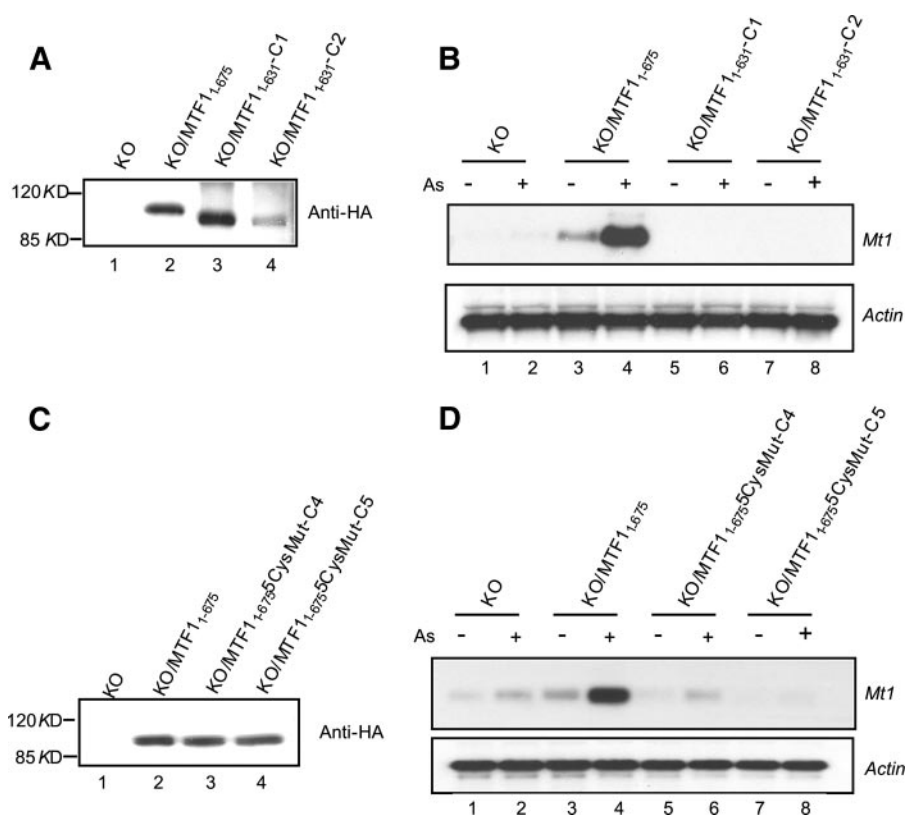


FIGURE 13. **MTF1 C-terminal cysteine residues are required for induction of endogenous *Mt1*.** A, MTF1 KO cells were stably transfected with pMTF1₁₋₆₇₅ or pMTF1₁₋₆₃₁. Cell lysate was blotted with anti-HA to show expression of MTF1 or the deletion mutant. B, cell clones stably expressing MTF1₁₋₆₇₅ or MTF1₁₋₆₃₁ (C1 and C2) were treated with arsenic (5 μ M) for 5 h. Total RNA was blotted for *Mt1* or *actin*. C, MTF1 KO cells were stably transfected with pMTF1₁₋₆₇₅ or pMTF1₁₋₆₇₅5CysMut in which all five C-terminal cysteine residues were mutated to phenylalanine. Cell lysate was blotted with anti-HA to show expressed MTF1 or the cysteine mutant. D, cell clones stably expressing MTF1₁₋₆₇₅ or MTF1₁₋₆₇₅5CysMut (C4 and C5) were treated with arsenic (5 μ M) for 5 h. Total RNA was blotted for *Mt1* or *actin*.

of lesions in multiple organs; moreover, arsenic is highly potent in many of the responses, suggesting multiple specific molecular targets and signaling pathways in arsenic responses. As³⁺ potently induces *Mt1* with EC₅₀ values of 5 μ M for induction of *Mt1* and 1.5 μ M for induction of MRE reporter, which are similar to those by Cd²⁺ and a magnitude higher than those by Zn²⁺. The pathway mediating *Mt1* induction by As³⁺ has not been analyzed.

The enhancer of *Mt1* contains a putative ARE that overlaps with a binding site of the upstream stimulatory factor (8). We have previously shown that As³⁺ activates Nrf2, a cap 'n' collar basic region leucine zipper transcription factor, to mediate induction of ARE-dependent phase II detoxification enzymes, such as NAD(P)H:quinone oxidoreductase 1 and glutathione *S*-transferase 1A, as well as several antioxidant enzymes/proteins, such as heme oxygenase 1, thioredoxin, and thioredoxin reductase 1 (50). Induction of the genes via Nrf2 by As³⁺ and other toxic metals is critical for protection against oxidative stress induced by the metals (51, 52). However, Nrf2 does not appear to be required for induction of *Mt1* by As³⁺, because induction remains unaltered in the absence of functional Nrf2, even though an ARE is present in the enhancer of mouse *Mt1*.

In this study, we provided several lines of convincing evidence demonstrating that MTF1 mediates the induction of *Mt1* by As³⁺. First, As³⁺ induces *Mt1* in MTF1 WT but not KO

cells, whereas reconstitution of the KO cells with full-length MTF1 restores the induction. Second, As³⁺ induces the MRE-driven β -gal expression similarly to Cd²⁺ and Zn²⁺. Third, As³⁺ induces the binding of MTF1 to the MREs of the endogenous chromatin *Mt1* in cells. Fourth, induction by As³⁺ is susceptible to inhibition by a labile repressor; inhibition of protein synthesis by cycloheximide in the presence of As³⁺ results in the superinduction of *Mt1*, which is characteristic of *Mt1* induction by a number of inducers through the MTF1/MRE pathway (31). Lastly, loss of MTF1 increases the sensitivity of cells to arsenic-induced toxicity, supporting MTF1 as a key mediator of the adaptive response for survival in the presence of As³⁺. We conclude that MTF1 is the critical mediator of *Mt1* induction by As³⁺.

MTF1 Is an Arsenic Sensor through the C-terminal Cysteine Residues—A central issue that remains elusive in understanding the regulation of metal homeostasis is how the body senses changes in the concentrations of metals. In light of the importance of MTF1 and MTs in metal response across

species, the MTF1/MRE pathway provides an opportunity for unraveling the molecular events of metal sensing. Much of the attention in the research of MTF1 activation by metals in the past has been focused on the zinc fingers of MTF1 in which six pairs of cysteine residues can bind a range of metals (9, 24, 26). Binding with Zn²⁺ stabilizes MTF1-MRE interaction. However, binding of the fingers with other metals, such as Cd²⁺, does not appear to induce conformations favored for DNA binding (9). It was proposed that Zn²⁺ plays a central role in MTF1 activation by interacting with the zinc fingers; other inducers, including metals and antioxidants, may mobilize Zn²⁺ in cells to increase free Zn²⁺, which binds to the zinc fingers and activates MTF1. One alternative hypothesis of MTF1 activation involves dissociation of MTF1 from one or more inhibitory proteins, because inhibition of protein synthesis or exposing cells to heat shock significantly increases the induction of *Mt1* (e.g. superinduction) (7, 31, 32). A third postulate of MTF1 activation suggests that a four-cysteine residue stretch at the C terminus of human MTF1 plays a role in metal-induced transcription of an MRE reporter gene, because the cysteine residues are conserved among the human, mouse, pufferfish, and *Drosophila* proteins and deletion of the cysteine cluster diminished the ability of MTF1 to support the induction of the reporter (53).

Our analysis of the mechanism of *Mt1* induction by As^{3+} does not support a central role of Zn^{2+} in arsenic sensing by MTF1, because modulating zinc concentrations did not affect the induction of *Mt1* or MRE β -gal reporter expression by As^{3+} . In fact, depletion of intracellular free Zn^{2+} did not decrease but consistently enhanced the induction of the MRE reporter. This result is consistent with our previous report that treatment with tBHQ but not Cd^{2+} elevates cytoplasmic free Zn^{2+} concentrations (30, 31). Therefore, it is plausible to postulate that some inducers, such as tBHQ and ROS, activate MTF1 via intracellular free Zn^{2+} , whereas metal inducers, such as As^{3+} , Cd^{2+} , Co^{2+} , Ni^{2+} , Ag^+ , Hg^{2+} , and Bi^{3+} , do not rely on Zn^{2+} for *Mt1* induction. In the latter scenario, Zn^{2+} may still be necessary for maintaining the zinc fingers in a conformation favorable for DNA binding; however, additional critical signaling events by As^{3+} and other inducers lead to the activation of MTF1 as discussed below.

Our study demonstrates a critical role of binding of metal inducers to the five-cysteine cluster at the C terminus of MTF1 for *Mt1* induction. First, PAO affinity beads efficiently bind and pull down the C-terminal half of MTF1 (MTF1_{321–675}) that comprises most of the transcription activation domain and contains the terminal five-cysteine cluster. Deletion or mutation of the cysteine residues abolishes the binding, thus providing the first direct evidence for the binding of an arsenic inducer to the C-terminal cysteine cluster of mouse MTF1. Second, a number of metal inducers, including As^{3+} , Cd^{2+} , Co^{2+} , Ni^{2+} , Ag^+ , Hg^{2+} , and Bi^{3+} , bind to the same cysteine cluster as PAO does both *in vitro* and *in vivo*, because the metal inducers effectively block PAO pulldown of MTF1_{321–675}. Third, deletion of the cysteine cluster abolishes the TA activity of the MTF1 TA domain; mutation of Cys-632, Cys-634, Cys-636, and Cys-638 of the cysteine cluster significantly reduces the TA activity, whereas mutation of Cys-653 largely diminishes and mutation of all five cysteine residues completely blocks the TA activity. Therefore, at a molecular level, the C-terminal cysteine cluster is critical for MTF1 transcription activation. In this regard, a synergistic action among the cysteine residues is likely, and Cys-653 appears to be essential. Finally, we showed that the C-terminal cysteine cluster is required for induction of endogenous *Mt1* in intact cells, because deletion of the cysteine cluster or mutation of the five cysteine residues totally repressed the ability of MTF1 to restore *Mt1* induction in MTF1 KO cells. Taken together, these findings strongly support the notion that interaction of As^{3+} and other metal inducers with the C-terminal cysteine cluster is critical for MTF1 activation and *Mt1* induction by the metal inducers. This metal-sensing function of the C-terminal cysteine cluster appears to be independent of the zinc fingers located in the amino half of MTF1.

A New Function of MTF1 Zinc Fingers: Direct Regulation of Transcription Activation—Examination of the transcription activation activity of MTF1 domains reveals a novel regulatory role of the zinc fingers of MTF1. Specifically, the DBD represses the transcription activation activity of MTF1 to give rise to a very low TA activity of the full-length MTF1, whereas deletion of the DBD results in the expression of a strong MTF1 TA activity that is comparable to that of the TA domain of VP16. Moreover, MTF1 zinc fingers totally block the heterologous

VP16 TA activity, indicating that the inhibitory activity of the zinc fingers is not dependent on the sequence context of MTF1 TA domain. Therefore, MTF1 zinc fingers appear to play multiple regulatory roles in MTF1 activation. In addition to mediating DNA binding and nuclear translocation, which is mediated through the nuclear import and export signaling sequences flanking the zinc fingers, our study demonstrates a new function of the zinc fingers, *i.e.* direct inhibition of the transcription activation activity of the C-terminal TA domain. We envision that repressing the TA function of MTF1 by the zinc fingers is necessary to keep MTF1 in a quiescent state in the absence of an inducer. The zinc fingers may directly interact with the TA domains to mediate suppression. In this model, a specific interface between the zinc fingers and the TA domain is required for effective interaction. Alternatively, the zinc fingers may interact with a repressor protein that in turn interacts with and inhibits the TA domains. Thirdly, the DNA-binding domain may take a conformation that hinders the interaction between the TA domain and transcription co-activators in the absence of an inducer. Because the zinc fingers also inhibit the TA activity of heterologous VP16 TA domain, the second and third hypotheses are more likely than the first postulate.

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