

Activation of Gene Expression by Metal-Responsive Signal Transduction Pathways

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Metallothioneins are small, cysteine-rich, metal-binding proteins that play important roles in maintaining intracellular metal homeostasis and in transition metal detoxification. MTF-1 (metal transcription factor-1) plays a central role in regulating the metal-inducible, transcriptional activation of metallothionein. Here we report that the phosphorylation of MTF-1 plays a critical role in the activation of MTF-1/metal-responsive element-mediated transcription. Inhibitor studies indicate that signal transduction cascades, including those mediated by protein kinase C, tyrosine kinase, and casein kinase II, are essential for zinc- and cadmium-inducible transcription. In addition, calcium signaling is also involved in regulating transcription. In contrast, cAMP-dependent protein kinase may not be directly involved in the metal response. Contrary to what has been reported for other transcription factors, the inhibition of transcriptional activation does not impair the binding of MTF-1 to DNA, suggesting that phosphorylation is not regulating DNA binding. Elevated phosphorylation of MTF-1 is observed under conditions of protein kinase C inhibition, suggesting that dephosphorylation of this transcription factor mediates its activation. **Key words:** cadmium, casein kinase II, metallothionein, metals, MTF-1, phosphorylation, protein kinase C, signal transduction, transcription, zinc. *Environ Health Perspect* 110(suppl 5):813–817 (2002).

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Metallothioneins (MTs) are a family of evolutionarily conserved, low molecular-weight, cysteine-rich, metal-binding proteins (1,2). The precise physiological role of MT has not been fully elucidated. However, proposed roles include *a*) participation in maintaining the homeostasis of essential transition metals, *b*) detoxification of nonessential metals, and *c*) protection against intracellular oxidative stress (1–3).

Metallothionein expression is primarily controlled at the level of transcription. Transcription can be induced by a variety of physiological agents and environmental stressors, including transition metals (1,4–11). Transition metal-induced activation of MT transcription is mediated by regulatory elements, designated metal-responsive elements (MREs). MREs contain a 7-bp core sequence (TGCRNC) and are present in multiple copies in the promoter/enhancer regions of almost all metal-inducible MTs (12,13).

Metal-inducible MT transcription is regulated by the transcription factor MTF-1 (metal transcription factor-1) (14,15). MTF-1 is an evolutionarily conserved protein that specifically binds to MREs and has been characterized in several species (14,16–19). MTF-1 contains six zinc finger domains and several *trans*-activation domains: acidic-rich, proline-rich, and serine/threonine-rich, all of which are required for metal-inducible transcription (20).

Two models have been proposed to describe how the interaction among MREs, MTF-1, and zinc activates MT transcription (21,22). Although the current models can account for the regulation of MT transcription

by zinc, they do not adequately explain the control of MTF-1 activity by nonzinc stressors. There is a scarcity of information regarding the molecular mechanism of MT transcriptional activation by other transition metals and environmental stressors.

We propose a model in which the regulation of MT transcription, via the MTF-1/MRE interaction, is controlled by multiple signal transduction cascades that affect MTF-1 phosphorylation. This model is based on several observations. First, MTF-1 contains several evolutionarily conserved, potential phosphorylation sites: protein kinase C (PKC), casein kinase II, and tyrosine kinase. Second, exposure of cells to activators of signal transduction cascades causes an increase in the steady-state level of MT mRNAs (4,6,11,23). Similarly, the addition of signal transduction inhibitors attenuates or abolishes metal-inducible MT mRNA expression (24). Finally, many of the effectors that induce MT transcription (metals as well as other environmental stressors) modulate the activity of intracellular signal transduction cascades (25–28).

In this report, we show that MTF-1 is phosphorylated on serine and tyrosine residues. In addition, several signal transduction cascades have been identified that modulate cadmium- and zinc-inducible MTF-1/MRE-mediated activation of MT transcription.

Materials and Methods

Cell Culture and Transfection

COS-7 cells were maintained in complete Dulbecco's modified Eagle's medium

(DMEM) containing 10% fetal bovine serum (FBS), 5% nonessential amino acids, 5% L-glutamine, and penicillin/streptomycin. HEK293 and HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum.

For transient transfection studies, HEK293 and HeLa cells were grown in DMEM supplemented with 5% FBS before transfection. These cells were then transfected using the calcium phosphate transfection method (29,30). Transient transfection of COS-7 cells was accomplished by lipofection, using Lipofectin per manufacturer instructions (Life Technologies, Carlsbad, CA, USA).

Expression Plasmids and Fusion Genes

Plasmids encoding either a vesicular stomatitis virus (VSV) G protein-tagged human MTF-1 (hMTF-1) fusion protein, designated phMTF-1-VSV, or a *myc*-his₆-tagged mouse MTF-1 (mMTF-1) fusion protein, designated pmMTF-1-*myc*-his, were engineered. The expressed fusion proteins contain either a VSV or a *myc* epitope that can be used *a*) to identify the expressed protein by Western immunoblot analysis and *b*) for purification by immunoprecipitation. Expression of both the hMTF-1-VSV and mMTF-1-*myc*-his mRNAs is under the control of the cytomegalovirus promoter, which allows for high-level expression in a variety of cell types.

A series of reporter plasmids were used to assess the levels of MT transcription. MT transcription in HEK293 and HeLa cells was determined from the amount of luciferase produced using mMT-1-Luc or 4xMREd-Luc reporter constructs (29). The level of MT transcription in COS-7 cells was determined by measuring the level of chloramphenicol acetyltransferase (CAT) produced using –42CAT, –153CAT, and MRE-d'5CAT reporter genes (31).

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In Vivo ³³P and ³²P Labeling

HEK293 cells were transfected with pMTF-1-VSV. After transfection the medium was replaced with phosphate-free DMEM containing ³³P-orthophosphate. Either zinc (100 μM ZnCl₂) or cadmium (60 μM CdCl₂) was added to the ³³P-containing medium. Incubation proceeded for 3 hr, after which nuclear and cytoplasmic extracts were prepared (14). The hMTF-1-VSV fusion protein was isolated by immunoprecipitation, and the amount of ³³P incorporated into hMTF-1-VSV was determined after SDS-PAGE by PhosphorImager analysis.

COS-7 cells were transfected with pmMTF-1-*myc*-his, after which they were washed with phosphate-free DMEM and then incubated in phosphate-free DMEM containing ³²P-orthophosphate. Metals (50 μM CdCl₂ or 100 μM ZnCl₂) were added, and the cells were incubated an additional 4 hr. After this incubation, the mMTF-1-*myc*-his fusion protein was purified by immunoprecipitation.

Amino acid residues that are phosphorylated in MTF-1, and the effects of metal exposure on their level of phosphorylation were determined by Western immunoblot analysis. COS-7 cells were transfected with pmMTF-1-*myc*-his and exposed to metals. The fusion protein was purified by immunoprecipitation and resolved by SDS-PAGE.

Phosphorylated amino acid residues were identified after Western immunoblot analysis using anti-phosphothreonine, anti-phosphoserine, or anti-phosphotyrosine antibodies.

Signal Transduction Cascade Inhibition

The effects of exposing HEK293, HeLa, and COS-7 cells to kinase inhibitors on the levels of MRE-mediated MT transcription were determined using luciferase- and CAT-based reporter genes.

To measure luciferase activity, HEK293 and HeLa cells were transiently transfected with mMT-I-Luc or 4xMREd-Luc reporter plasmids. After incubating under serum-free conditions, cells were treated with kinase inhibitors (Table 1). Inhibitor treatments were initiated 30 min before the addition of cadmium (60 μM CdCl₂) or zinc (100 μM ZnCl₂). Cells were exposed to metals for 4 hr in both the presence and absence of the inhibitors. Cell lysates were prepared and luciferase activity measured according to manufacturer instructions.

CAT concentrations were measured using a CAT-enzyme linked immunosorbent assay. COS-7 cells grown in complete DMEM were transfected with -42CAT, -153CAT, or MRE-d'5CAT reporter genes. Chemical inhibitors were added to the transfected cells

in complete DMEM. After a 0.5- to 1-hr incubation, either no metal, 50 μM CdCl₂, or 100 μM ZnCl₂, was added, and cells were incubated for an additional 3 hr. Extracts were prepared, and CAT concentrations and β-galactosidase activities were determined.

Electrophoretic Mobility Shift Assays

HEK293 cells were incubated with 100 μM ZnCl₂ or 60 μM CdCl₂ for 3 hr in the presence or absence of kinase inhibitors. Binding reactions were performed by incubating 2–5 fmol of a ³²P-end-labeled, double-stranded oligonucleotide that contains the core MRE consensus sequence with nuclear extracts as previously described (14).

Results**Phosphorylation of MTF-1**

Radiolabeled proteins corresponding to the MTF-1 fusion proteins were isolated from transfected HEK293 and COS-7 cells. *In vivo* labeling of both the hMTF-1 and mMTF-1 fusion proteins showed that MTF-1 is constitutively phosphorylated in the absence of added metal. The phosphorylated form of MTF-1 is located primarily in the cytoplasm of nonexposed cells (Figure 1). After zinc or cadmium exposure, the level of MTF-1 phosphorylation increased. This increase was primarily observed as a higher level of phosphorylation in the nuclear form of MTF-1. However, significant levels of phosphorylated MTF-1 were still observed in the cytoplasmic fraction (Figure 1).

To identify which types of amino acid residues are phosphorylated *in vivo*, immunopurified mMTF-1-*myc*-his was treated with anti-phospho antibodies. Both anti-phosphoserine and anti-phosphotyrosine antibodies cross-reacted with mMTF-1-*myc*-his (Figure 1); however, the binding of anti-phosphothreonine antibodies was not observed (data not shown). These results indicate that MTF-1 is phosphorylated at multiple sites: serine and tyrosine residues. Similar to that observed in the *in vivo* radiolabeling experiments, the protein is constitutively phosphorylated, and the level of phosphorylation increases in response to metal exposure (Figure 1).

Effects of Signal Transduction Cascade Activators and Inhibitors on MT Transcription

The effects of the PKC inhibitors, staurosporine, H-7, and BIM-I on zinc- and cadmium-inducible MT transcription were investigated. Exposure to these inhibitors reduced mMT-I-based and MRE-based promoter activity to levels below that observed in cells not exposed to metal (Figure 2, Table 1).

Treatment of HEK293 cells with the casein kinase II inhibitor DRB significantly

Table 1. Effect of signal transduction cascade activators and inhibitors on metallothionein reporter gene activity.

Chemical agent	Cell type	Metal	Concentration	Target pathway	% Change in activity
Inhibitors					
Staurosporine	HeLa	Zn	1 μM	PKC	-68
	HEK293				-91
H-7	HeLa	Zn	100 μM	PKC	-68
	HEK293				-100
BIM-I	HeLa	Zn	10 μM	PKC	-72
	HEK293				-93
DRB	HeLa	Cd	50 μM	Casein kinase II	-94
		Zn			-99
	HEK293	Cd			-86
		Zn			-90
Rp-cAMP	HeLa	Cd	100 μM	PKA	-22
		Zn			-53
	HEK293	Cd			-15
		Zn			-53
Herbimycin A	HeLa	Cd	5 μM	Tyrosine kinase	-25
		Zn			-62
	HEK293	Cd			-44
		Zn			-69
BAPTA-AM	HeLa	Cd	30 μM	Calcium-dependent kinases	-97
		Zn			-90
Activators					
(-)-Indolactam	HeLa	NM	25 μM	PKC	-23
TPA	COS-7	NM	1 μM		-10
Dibutylryl-cAMP	HeLa	NM	1 μM	PKA	-23
	COS-7	NM			-5
A-23187	COS-7	NM	5 μM	Calcium-dependent kinases	+300
Spermidine	COS-7	NM	2 mM	Casein kinase II	+36

Abbreviations: NM, no metal added; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; BIM-I, bisindolylmaleimide; KN-62, 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-1-tyrosyl]-4-phenylpiperazine; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzene; HA1004, N-(2-guanidinoethyl)-5-isoquinolinesulfonamide; BAPTA-AM, bis-(o-aminophenoxy)-N,N,N',N'-tetraacetic acid; TPA, 12-O-tetradecanoyl phorbol-13-acetate; A-23187, calcium ionophore ionomycin.

reduced the level of cadmium- and zinc-inducible reporter gene expression (Table 1). Treatment of COS-7 cells with heparin (2 $\mu\text{g}/\text{mL}$) significantly inhibited cadmium-inducible reporter gene expression. This concentration of heparin, however, did not affect zinc-inducible expression (Figure 2). Consistent with observation, treatment of cells with spermidine, a casein kinase II activator, slightly activated reporter gene expression in COS-7 cells in the absence of metal (Table 1).

Inhibition of tyrosine kinase activity with herbimycin A significantly reduced the level of cadmium- and zinc-inducible reporter gene activity (Table 1). These results suggest that phosphorylation of the tyrosine residue

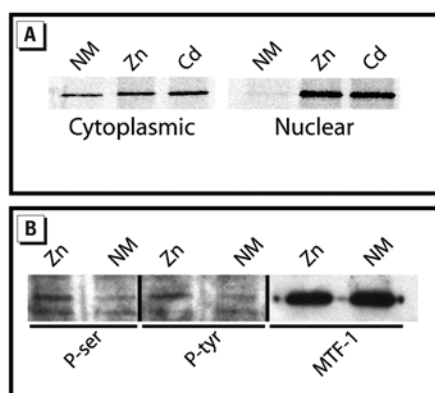


Figure 1. Phosphorylation of MTF-1 *in vivo*. (A) HEK293 cells were transfected with 5 μg of pMTF-1-VSV for 24 hr and labeled with ^{32}P -orthophosphate for 3 hr in either the absence or presence of 100 μM zinc or 60 μM cadmium. Nuclear and cytoplasmic extracts were then prepared and hMTF-1 fusion proteins purified by immunoprecipitation. (B) COS-7 cells were transfected with 16 μg of pmMTF-1-*myc*-his and exposed to 100 μM zinc (Zn) for 4 hr or no metal (NM). The fusion protein was purified by immunoprecipitation and resolved by SDS-PAGE. Identification of MTF-1-*myc*-his (MTF-1) and phosphorylated serine (P-ser) or tyrosine (P-tyr) residues was accomplished by Western immunoblot analysis using anti-mMTF-1, anti-phosphoserine, and anti-phosphotyrosine antibodies, respectively.

in MTF-1 is involved in the activation of MRE/MTF-1-regulated MT transcription.

Calcium is required for the activation of many kinases; therefore, the effects of intracellular calcium chelators and calcium ionophores on MTF-1/MRE-mediated transcription were investigated. Exposure to the intracellular calcium chelator BAPTA-AM reduced the level of cadmium- and zinc-inducible promoter activity (Table 1). Consistent with these results, exposure of COS-7 cells to the calcium ionophore A-23187 increased the level of promoter activity in the absence of added metals (Table 1). These results suggest that calcium-mediated signal transduction pathways are involved in the activation of MTF-1.

Inhibition of cAMP-dependent protein kinase (PKA) with Rp-cAMP significantly reduced the levels of zinc-inducible reporter gene expression in HeLa and HEK293 cells (Table 1). However, this change in expression can be attributed to an inhibition of basal activity, suggesting that PKA may not directly mediate metal-inducible transcription. Treatment of COS-7 cells with the PKA inhibitor HA1004 reduced cadmium-inducible reporter gene expression by a small yet significant degree. Zinc-inducible transcription was not affected (Figure 2). Exposure to a PKA activator, dibutyryl-cAMP, did not significantly affect the MT gene expression (Table 1). The PKA inhibitor and activator data suggest that signaling pathways regulated

by PKA may not be directly involved in the regulation of MTF-1 activity.

Electrophoretic Mobility Shift Assay

Exposure to PKC, tyrosine kinase, or casein kinase II inhibitors decreases the level of MTF-1/MRE-mediated gene expression. In contrast, treatment of HEK293 cells with kinase inhibitors did not significantly affect the cadmium- or zinc-inducible binding of MTF-1 to the MRE (Figure 3). In addition, the level of MTF-1-MRE complex formed increased in the presence of inhibitors. These results suggest that the regulation of MRE-mediated transcription by these kinases, or their related signal transduction pathways is not mediated by inhibiting the DNA binding activity of MTF-1.

Effect of PKC Inhibition on MTF-1 Phosphorylation

The inhibition of PKC activity prevents both cadmium- and zinc-inducible MT transcription. When PKC activity was inhibited in zinc-treated COS-7 cells, the amount of ^{32}P incorporated into mMTF-1-*myc*-his increased, compared with zinc-treated cells not exposed to H-7 (Figure 4). Similarly, the amount of phosphorylation on the serine residues significantly increased for both zinc- and cadmium-treated cells exposed to H-7 (results not shown). It should be noted that treatment with H-7 did not affect the steady-state level of MTF-1.

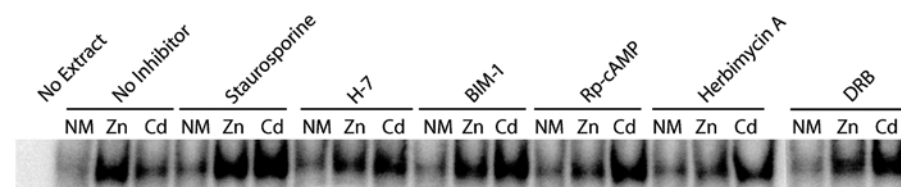


Figure 3. Effect of kinase inhibitors on the DNA binding activity of MTF-1. HEK293 cells were incubated with the indicated kinase inhibitors for 30 min before exposure to 100 μM zinc or 60 μM cadmium, or no metal (NM). After a 3-hr incubation, nuclear extracts were prepared and analyzed using a ^{32}P -labeled MRE-containing oligonucleotide. Nuclear extracts were also prepared from cells that were exposed to cadmium or zinc but not inhibitors.

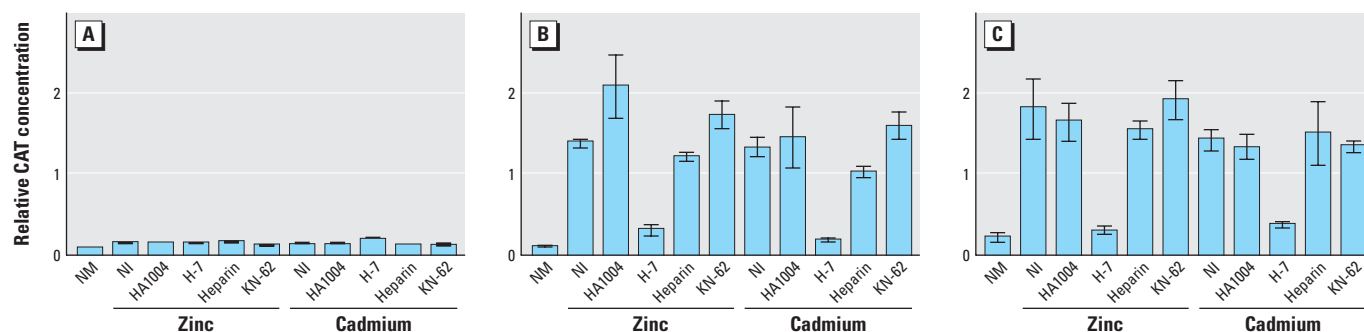


Figure 2. Effect of kinase inhibitors on metal-inducible transcription in COS-7 cells. Cells were transfected with (A) -42CAT, (B) -153CAT, or (C) MRE-d'5CAT and pSV- βGal (s), for 3 hr. After a 24-hr recovery period, cells were treated with kinase inhibitors and metals. Reporter gene activity in the presence of metal, but in the absence of inhibitors (NI), and in cells not exposed to metals or inhibitors (NM) was also determined. The data presented are the mean values from three independent experiments with the standard deviations.

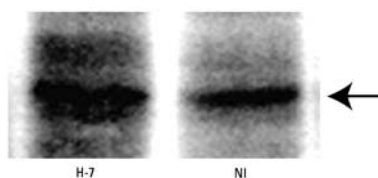


Figure 4. Effect of PKC inhibition on the level of MTF-1 phosphorylation. COS-7 cells were transfected with 16 μ g of pmMTF-1-*myc*-his for 24 hr. Cells were exposed to 100 μ M zinc and 32 P-orthophosphate for 4 hr in the presence (H-7) or absence (NI) of the PKC inhibitor H-7. The fusion protein was purified by immunoprecipitation, immunocomplexes were then resolved by SDS-PAGE and transferred onto a nitrocellulose membrane, and the amount of 32 P-labeled MTF-1 was determined by PhosphorImage analysis (Molecular Dynamics, Sunnyvale, CA, USA). The arrowhead indicates the location of the MTF-1 fusion protein.

Discussion

It is well documented that metal-activated MT transcription is mediated primarily through the MRE-binding transcription factor MTF-1. Previous studies indicate that metal-inducible transcription requires several processes: nuclear translocation, DNA binding, and transcriptional activation of MTF-1. Here we report that activation of MT transcription via the MRE is dependent on interactions with several signal transduction pathways and a change in the level of MTF-1 phosphorylation.

The initial examination of MTF-1 activity demonstrated that transcription depends on the binding of this factor to the MRE (14). DNA binding, however, is not synonymous with transcriptional activation. This is supported by the observations that metal-inducible, MTF-1/MRE-mediated transcription is significantly blocked with PKC and casein kinase II inhibitors (Figure 2, Table 1), but these agents do not inhibit MTF-1 binding to DNA (Figure 4).

Recent reports identified a second component in the activation of mammalian MT transcription: the metal- and stress-inducible translocation of MTF-1 from the cytoplasm to the nucleus (29,32). Our studies demonstrate that after metal exposure, the phosphorylated form of MTF-1 accumulates in the nucleus (Figure 1). It is reasonable to hypothesize that modifications of specific residues could control nuclear translocation and transcriptional activation. The unique tyrosine kinase phosphorylation site in human MTF-1, tyr¹⁴⁰, is contiguous with the MTF-1 nuclear localization sequence 133KRKEVKR¹³⁹ (29). Phosphorylation of this residue may contribute to the regulation of MTF-1 nuclear transport. It should be noted that the location of the unique, putative tyrosine phosphorylation site that is contiguous with nuclear localization sequence is conserved in vertebrate MTF-1.

The observations that *a*) MTF-1 is phosphorylated, *b*) its level of phosphorylation increases in response to metal exposure, and *c*) several kinase inhibitors block or attenuate metal-inducible MT transcription support a model in which transcriptional activation via the MTF-1/MRE interaction is controlled by several signal transduction pathways that ultimately affect the level of MTF-1 phosphorylation. The activation of the signal transduction pathways that involve PKC, casein kinase II, tyrosine kinase, and calcium by metals and other environmental stressors has been well documented (25,33–35). A codicil to this hypothesis is that the interaction among several signaling pathways may “fine-tune” the basal and inducible activity of MTF-1.

In the presence of the PKC inhibitor H-7, the level of MTF-1 phosphorylation increases (Figure 4); however, H-7, staurosporine, and BIM-1 are potent inhibitors of metal-inducible MT transcription (Figure 2, Table 1). Although contradictory, these results are consistent with a model in which metal-activated transcription is controlled by the dephosphorylation of MTF-1. A similar mechanism is responsible for activating the binding of c-Jun to the AP-1 promoter elements. (36). In the dephosphorylation model, only transcriptional activation of MTF-1 is controlled by the dephosphorylation of specific phospho residues. The total level of MTF-1 phosphorylation may be greater after exposure to metals or stress, as shown in Figure 1; however, specific residues are dephosphorylated to activate transcription. The dephosphorylation of MTF-1 is performed by a yet unidentified “MTF-1 phosphatase,” whose activity may be controlled by upstream regulatory proteins.

Exposure to metals, organic chemicals, physical stress, intracellular damage, and physiologic agents causes an increase the steady-state level of MT mRNA (5,37–40). In addition, several of these nonmetal stressors activate MT transcription via MREs (10,22,41,42). The observation that multiple signal transduction pathways ultimately converge at MTF-1 to activate MT transcription provides a mechanistic link between exposure to structurally unrelated stressors and the activation of MT transcription. Potentially, any stressor that can activate an MTF-1–regulating signal transduction pathway could increase MT transcription. Furthermore, agents that activate parallel signal transduction pathways that converge with pathways regulated by PKC, casein kinase II, and tyrosine kinase will affect MT transcription.

In the future a detailed analysis of the specific residues in MTF-1 that are modified after exposure to metals or other stressors, and various kinase inhibitors, will help to define how each signaling pathway affects the level of

MTF-1 phosphorylation. The effects of individual and combinatorial mutations on the ability of MTF-1 to regulate MT transcription will help to determine the precise mechanism by which metals and other stressors activate transcription via MTF-1/MRE interactions.

REFERENCES AND NOTES

- Hamer DH. Metallothionein. *Annu Rev Biochem* 55:913–951 (1986).
- Kagi JH, Schaffer A. Biochemistry of metallothionein. *Biochemistry* 27:8509–8515 (1988).
- Thornalley PJ, Vasak M. Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. *Biochim Biophys Acta* 827:36–44 (1985).
- Kelly EJ, Sandgren EP, Brinster RL, Palmiter RD. A pair of adjacent glucocorticoid response elements regulate expression of two mouse metallothionein genes. *Proc Natl Acad Sci USA* 94:10045–10050 (1997).
- Durnam DM, Palmiter RD. Induction of metallothionein-1 mRNA in cultured cells by heavy metals and iodoacetate: evidence for gratuitous inducers. *Mol Cell Biol* 4:484–491 (1984).
- Garrett SH, Xiong X, Arizono K, Brady FO. Phorbol ester induction of rat hepatic metallothionein *in vivo* and *in vitro*. *Int J Biochem* 24:1669–1676 (1992).
- Rimoldi D, Flessate DM, Samid D. Changes in gene expression by 193- and 248-nm excimer laser radiation in cultured human fibroblasts. *Radiat Res* 131:325–331 (1992).
- Sato M, Bremner I. Oxygen free radicals and metallothionein. *Free Radic Biol Med* 14:325–337 (1993).
- Tamai KT, Liu X, Silar P, Sosinowski T, Thiele DJ. Heat shock transcription factor activates yeast metallothionein gene expression in response to heat and glucose starvation via distinct signalling pathways. *Mol Cell Biol* 14:8155–8165 (1994).
- Murphy BJ, Andrews GK, Bittel D, Discher DJ, McCue J, Green CJ, Yanovsky M, Giaccia A, Sutherland RM, Laderoute KR, et al. Activation of metallothionein gene expression by hypoxia involves metal response elements and metal transcription factor-1. *Cancer Res* 59:1315–1322 (1999).
- Nebes VL, DeFranco D, Morris SM Jr. Cyclic AMP induces metallothionein gene expression in rat hepatocytes but not in rat kidney. *Biochem J* 255:741–743 (1988).
- Searle PF, Stuart GW, Palmiter RD. Building a metal-responsive promoter with synthetic regulatory elements. *Mol Cell Biol* 5:1480–1489 (1985).
- Culotta VC, Hamer DH. Fine mapping of a mouse metallothionein gene metal response element. *Mol Cell Biol* 9:1376–1380 (1989).
- Radtke F, Heuchel R, Georgiev O, Hergersberg M, Gariglio M, Dembic Z, Schaffner W. Cloned transcription factor MTF-1 activates the mouse metallothionein I promoter. *EMBO J* 12:1355–1362 (1993).
- Heuchel R, Radtke F, Georgiev O, Stark G, Aguet M, Schaffner W. The transcription factor MTF-1 is essential for basal and heavy metal-induced metallothionein gene expression. *EMBO J* 13:2870–2875 (1994).
- Brugnera E, Georgiev O, Radtke F, Heuchel R, Baker E, Sutherland GR, Schaffner W. Cloning, chromosomal mapping and characterization of the human metal-regulatory transcription factor MTF-1. *Nucleic Acids Res* 22:3167–3173 (1994).
- Dalton TP, Solis WA, Nebert DW, Carvan MJ III. Characterization of the MTF-1 transcription factor from zebrafish and trout cells. *Comp Biochem Physiol B Biochem Mol Biol* 126:325–335 (2000).
- Auf der Maur A, Belser T, Elgar G, Georgiev O, Schaffner W. Characterization of the transcription factor MTF-1 from the Japanese pufferfish (*Fugu rubripes*) reveals evolutionary conservation of heavy metal stress response. *Biol Chem* 380:175–185 (1999).
- Zhang B, Egli D, Georgiev O, Schaffner W. The *Drosophila* homolog of mammalian zinc finger factor MTF-1 activates transcription in response to heavy metals. *Mol Cell Biol* 21:4505–4514 (2001).
- Radtke F, Georgiev O, Muller HP, Brugnera E, Schaffner W. Functional domains of the heavy metal-responsive

- transcription regulator MTF-1. *Nucleic Acids Res* 23:2277–2286 (1995).
21. Andrews GK. Regulation of metallothionein gene expression by oxidative stress and metal ions. *Biochem Pharmacol* 59:95–104 (2000).
 22. Palmiter RD. Regulation of metallothionein genes by heavy metals appears to be mediated by a zinc-sensitive inhibitor that interacts with a constitutively active transcription factor, MTF-1. *Proc Natl Acad Sci USA* 91:1219–1223 (1994).
 23. Laychock SG, Duzen J, Simpkins CO. Metallothionein induction in islets of Langerhans and insulinoma cells. *Mol Cell Endocrinol* 165:179–187 (2000).
 24. Yu CW, Chen JH, Lin LY. Metal-induced metallothionein gene expression can be inactivated by protein kinase C inhibitor. *FEBS Lett* 420:69–73 (1997).
 25. Beyersmann D, Hechtenberg S. Cadmium, gene regulation, and cellular signaling in mammalian cells. *Toxicol Appl Pharmacol* 144:247–261 (1997).
 26. Stohs SJ, Bagchi D, Hassoun E, Bagchi M. Oxidative mechanisms in the toxicity of chromium and cadmium ions. *J Environ Pathol Toxicol Oncol* 19:201–213 (2000).
 27. Whisler RL, Goyette MA, Grants IS, Newhouse YG. Sublethal levels of oxidant stress stimulate multiple serine/threonine kinases and suppress protein phosphatases in Jurkat T cells. *Arch Biochem Biophys* 319:23–35 (1995).
 28. Karin M. Mitogen-activated protein kinase cascades as regulators of stress responses. *Ann NY Acad Sci* 851:139–146 (1998).
 29. Saydam N, Georgiev O, Nakano MY, Greber UF, Schaffner W. Nucleo-cytoplasmic trafficking of metal-responsive transcription factor MTF-1 is regulated by diverse stress signals. *J Biol Chem* 276:25487–25495 (2001).
 30. Ausubel FM. *Current Protocols in Molecular Biology on CD-ROM*. New York: John Wiley & Sons, 1998.
 31. Dalton T, Palmiter RD, Andrews GK. Transcriptional induction of the mouse metallothionein-I gene in hydrogen peroxide-treated Hepa cells involves a composite major late transcription factor/antioxidant response element and metal response promoter elements. *Nucleic Acids Res* 22:5016–5023 (1994).
 32. Smirnova IV, Bittel DC, Ravindra R, Jiang H, Andrews GK. Zinc and cadmium can promote rapid nuclear translocation of metal response element-binding transcription factor-1. *J Biol Chem* 275:9377–9384 (2000).
 33. Pfundt R, van Vlijmen-Willems I, Bergers M, Wings M, Cloin W, Schalkwijk J. *In situ* demonstration of phosphorylated c-jun and p38 MAP kinase in epidermal keratinocytes following ultraviolet B irradiation of human skin. *J Pathol* 193:248–255 (2001).
 34. Sayed M, Kim SO, Salh BS, Issinger OG, Pelech SL. Stress-induced activation of protein kinase CK2 by direct interaction with p38 mitogen-activated protein kinase. *J Biol Chem* 275:16569–16573 (2000).
 35. Masuya Y, Hioki K, Tokunaga R, Taketani S. Involvement of the tyrosine phosphorylation pathway in induction of human heme oxygenase-1 by hemin, sodium arsenite, and cadmium chloride. *J Biochem* 124:628–633 (1998).
 36. Boyle WJ, Smeal T, Defize LHK, Angel P, Woodgett JR, Karin M, Hunter T. Activation of protein kinase C decreases phosphorylation of c-Jun at sites that negatively regulate its DNA binding activity. *Cell* 64:537–584 (1991).
 37. Arizono K, Peterson KL, Brady FO. Inhibitors of Ca²⁺ channels, calmodulin and protein kinases prevent A23187 and other inductions of metallothionein mRNA in EC3 rat hepatoma cells. *Life Sci* 53:1031–1037 (1993).
 38. Hermes E, Abraham M, Nemcsok J. Tissue-specific expression of two metallothionein genes in common carp during cadmium exposure and temperature shock. *Comp Biochem Physiol C Toxicol Pharmacol* 128:457–465 (2001).
 39. Karin M, Andersen RD, Slater E, Smith K, Herschman HR. Metallothionein mRNA induction in HeLa cells in response to zinc or dexamethasone is a primary induction response. *Nature* 286:295–297 (1980).
 40. Imbra RJ, Karin M. Metallothionein gene expression is regulated by serum factors and activators of protein kinase C. *Mol Cell Biol* 7:1358–1363 (1987).
 41. Samson SL, Gedamu L. Molecular analyses of metallothionein gene regulation. *Prog Nucleic Acid Res Mol Biol* 59:257–288 (1998).
 42. Dalton TP, Li Q, Bittel D, Liang L, Andrews GK. Oxidative stress activates metal-responsive transcription factor-1 binding activity. Occupancy *in vivo* of metal response elements in the metallothionein-I gene promoter. *J Biol Chem* 271:26233–26241 (1996).