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Regulation of Metallothionein Gene Expression by Oxidative Stress and Metal Ions

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ABSTRACT. The metallothioneins (MT) are small, cysteine-rich heavy metal-binding proteins which participate in an array of protective stress responses. Although a single essential function of MT has not been demonstrated, MT of higher eukaryotes evolved as a mechanism to regulate zinc levels and distribution within cells and organisms. These proteins can also protect against some toxic metals and oxidative stress-inducing agents. In mice, among the four known *MT* genes, the *MT-I* and *-II* genes are most widely expressed. Transcription of these genes is rapidly and dramatically up-regulated in response to zinc and cadmium, as well as in response to agents which cause oxidative stress and/or inflammation. The six zinc-finger metal-responsive transcription factor MTF-1 plays a central role in transcriptional activation of the MT-I gene in response to metals and oxidative stress. Mutation of the *MTF-1* gene abolishes these responses, and MTF-1 is induced to bind to the metal response elements in proximal MT promoter in cells treated with zinc or during oxidative stress. The exact molecular mechanisms of action of MTF-1 are not fully understood. Our studies suggest that the DNA-binding activity of MTF-1 *in vivo* and *in vitro* is reversibly activated by zinc interactions with the zinc-finger domain. This reflects heterogeneity in the structure and function of the six zinc fingers. We hypothesize that MTF-1 functions as a sensor of free zinc pools in the cell. Changes in free zinc may occur in response to chemically diverse inducers. MTF-1 also exerts effects on *MT-I* gene transcription which are independent of a large increase in MTF-1 DNA-binding activity. For example, cadmium, which has little effect on the DNA-binding activity of MTF-1 *in vivo* or *in vitro*, is a more potent inducer of *MT* gene expression than is zinc. The basic helix-loop-helix-leucine zipper protein, USF (upstream stimulatory factor family), also plays a role in regulating transcription of the mouse *MT-I* gene in response to cadmium or H_2O_2. Expression of dominant negative USF-1 or deletion of its binding site from the proximal promoter attenuates induction of the mouse *MT-I* gene. USF apparently functions in this context by interacting with as yet unidentified proteins which bind to an antioxidant response element which overlaps the USF-binding site (USF/ARE). Interestingly, this composite element does not participate in the induction of *MT-I* gene transcription by zinc or redox-cycling quinones. Thus, regulation of the mouse *MT-I* gene by metals and oxidative stress involves multiple signaling pathways which depend on the species of metal ion and the nature of the oxidative stress. BIOCHEM PHARMACOL **59**;1:95–104, 2000. © 1999 Elsevier Science Inc.

KEY WORDS. metallothionein; zinc; cadmium; oxidative stress; USF; ARE

MT† are the most abundant intracellular, metal-binding proteins, and they are isolated from tissues as $zinc_7$–MT complexes [1, 2]. In the mouse, four *MT* genes have been cloned (*MT-I* to *-IV*) [3], but MT-I and MT-II are the most widely distributed MT isoforms [1]. The mouse *MT-I* and *MT-II* genes are actively expressed in many cell types in different organs and tissues, as well as in most cultured cells. In contrast, the *MT-III* and *MT-IV* genes show a very restricted cell type-specific pattern of expression [4]. As discussed below, expression of the mouse *MT-I* gene in response to zinc is regulated by the zinc-finger transcription factor MTF-1 [5]. Our studies suggest that MTF-1 is a metalloregulatory protein whose DNA-binding activity is reversibly activated in response to changes in free zinc concentrations in the cell [6, 7]. The preponderance of experimental evidence suggests that the mouse *MT-I* and *MT-II* genes evolved, in part, as a mechanism to control zinc homeostasis. Analyses of transgenic mice that overexpress MT-I [8] or that have loss-of-function mutations (gene knockout) in the *MT-I/II* genes [9] have provided evidence for this hypothesis. Transgenic mice that overexpress MT-I accumulate more zinc in maternal organs and are significantly more resistant to the teratogenic effects of dietary zinc deficiency during pregnancy than are control mice [10]. In contrast, mice with knockout of the *MT-I/II*

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† *Abbreviations:* MT, metallothioneins; MTF-1, metal-responsive transcription factor-1; MRE, metal response element; ARE, antioxidant response element; USF, upstream stimulating factor; tBHQ, *tert*-butylhydroquinone; EMSA, electrophoretic mobility shift assay; and TnT, coupled transcription and translation system.

genes accumulate less zinc in tissues and are much more sensitive to the effects of dietary zinc deficiency during pregnancy than are control mice [11]. Neonatal development of the kidney is also abnormal in knockout mice fed a zinc-deficient diet [12]. Recent studies of cells exposed to extreme zinc deprivation suggest that MT can be part of a zinc-scavenging mechanism for cell survival [13]. MT may also remove zinc from inhibitory sites on enzymes and thus protect against zinc toxicity [14]. Although genetic experiments do not support the notion of a generalized essential cellular function of MT [15], the ubiquitous need to maintain adequate and appropriate levels of the essential metal zinc in higher eukaryotes likely provides significant selective pressure for conservation of MT.

MT is a stress protein, and critical functions of mammalian MT have, therefore, been revealed only during exposure to environmental insults. Mammalian MT can also provide protection against cadmium toxicity and oxidative stress. Cells which contain more MT are protected against heavy metal toxicity and oxidant stress, whereas underexpression in cell lines or in mice with null mutations of the *MT-I* and *MT-II* genes leads to heightened sensitivity to cadmium toxicity and oxidative stress [1, 16–18]. *MT* gene transcription is also rapidly induced by cadmium and by oxidative stress [19, 20]. MT is an efficient scavenger of hydroxyl radicals [21, 22], and yeast and mammalian MTs can functionally substitute for superoxide dismutase in protecting yeast from oxidative stress [23]. Recent studies of zinc release and binding to MT demonstrated that the redox status of the cell, measured by the ratio of reduced to oxidized glutathione, and energy metabolism together affect binding and release of zinc from MT [24, 25]. Thus, MT appears to be a multifunctional stress protein in higher eukaryotes.

A hallmark of the mouse *MT-I* and *MT-II* genes is their transcriptional induction by zinc and cadmium [1]. Essential for this induction are DNA motifs, termed metal response elements (MRE), present in multiple copies in the proximal promoters of *MT* genes. MREs were shown to confer response to zinc and cadmium [26, 27] and to oxidative stress [20]. A protein responsible for transactivation through the MRE has been cloned from fish, mouse, and human, and is termed MTF-1 [28–30]. MTF-1 is a zinc-finger transcription factor in the Cys_2His_2 family. Targeted disruption of both MTF-1 alleles in embryonic stem cells demonstrated its essential role for basal as well as heavy metal-induced *MT* gene expression [5] and oxidative stress-induced *MT-I* gene expression [31] in the mouse.

We have also discovered an oxidative stress response and cadmium response element in the MT-I promoter that maps to the -101-bp region [20, 31]. This region contains an antioxidant response element (ARE; TGACnnnGC) [32]. The ARE (also called electrophile response element) mediates induction of glutathione *S*-transferase Ya subunit and the quinone reductase genes in response to redox-cycling xenobiotics and H_2O_2 [32, 33]. Many MT promoters contain a single perfectly matched consensus ARE sequence [20]. The ARE may be negatively regulated by the bZip proteins Fos and Fra-1, and positively regulated by Nrf2-small Maf heterodimers in response to electrophilic agents [34, 35]. In the mouse MT-I promoter (and the hamster MT-I promoter), the ARE overlaps a previously identified USF-binding site (CRCGTGRY) [36]. USF is a member of the basic-helix-loop-helix-Zip protein superfamily which includes Myc, Max, Mad, and TFE3 [37, 38]. There are three isoforms of USF (USF-1, USF-2a, and USF-2b) [39–41]. The 43 kDa USF-1 and the 44 kDa USF-2 polypeptides are encoded by separate genes [39, 40]. Alternate splicing gives rise to the USF-2a and USF-2b isoforms [41]. USF forms DNA-binding USF homo- and heterodimers [40–42], but can also interact with several different bZip transcription factors [43–46]. USF is ubiquitously expressed [39], and has been shown to positively or negatively influence the expression of a myriad of genes [41, 47–51].

Herein, I describe our studies of mechanisms by which MTF-1 regulates *MT* gene transcription in response to metals and oxidative stress [6, 7, 20, 31], and our studies which indicate that the composite USF/ARE promoter sequence participates in cadmium and H_2O_2 regulation of *MT-I* gene transcription [20, 52] and that USF plays a role in this regulation.

RESULTS AND DISCUSSION

Oxidative Stress-Induced Transcription of the Mouse MT-I Gene

MT mRNA in the rodent liver is induced, for example, after injection of diethyl maleate, paraquat, and menadione [53–57], each of which causes formation of free radicals. The molecular mechanisms of induction of mouse MT by these oxidative stress-inducing agents, however, were unknown. We first addressed this issue by treating mouse Hepa cells with H_2O_2, menadione, or tBHQ and examining the induction of MT-I mRNA. During the course of these studies, we obtained similar results when we examined the effects of these agents on MT-I mRNA in NIH 3T3 and L929 cells. H_2O_2 undergoes Fenton chemistry to produce hydroxyl radicals, whereas menadione and tBHQ are thought to redox cycle and produce superoxide anions. tBHQ, for example, induces phase II genes [58–60] and undergoes autoxidation to form a redox-active semiquinone anion radical [61, 62].

We found that H_2O_2, tBHQ, and menadione each rapidly induced MT-I mRNA in a dose-dependent manner in Hepa cells. Inductions of 4- to 20-fold within 6 hr were noted under conditions that had no discernible effects on cell viability as assessed by trypan blue exclusion 24 hr posttreatment. Induction of heme oxygenase-I mRNA, a gene shown to respond to a variety of oxidative stresses including H_2O_2 treatment of cultured cells [63–67], displayed a dose–response curve similar to that of MT-I mRNA. Induction of MT-I mRNA by H_2O_2 and suboptimal concentrations of zinc was rapid and transient. In-

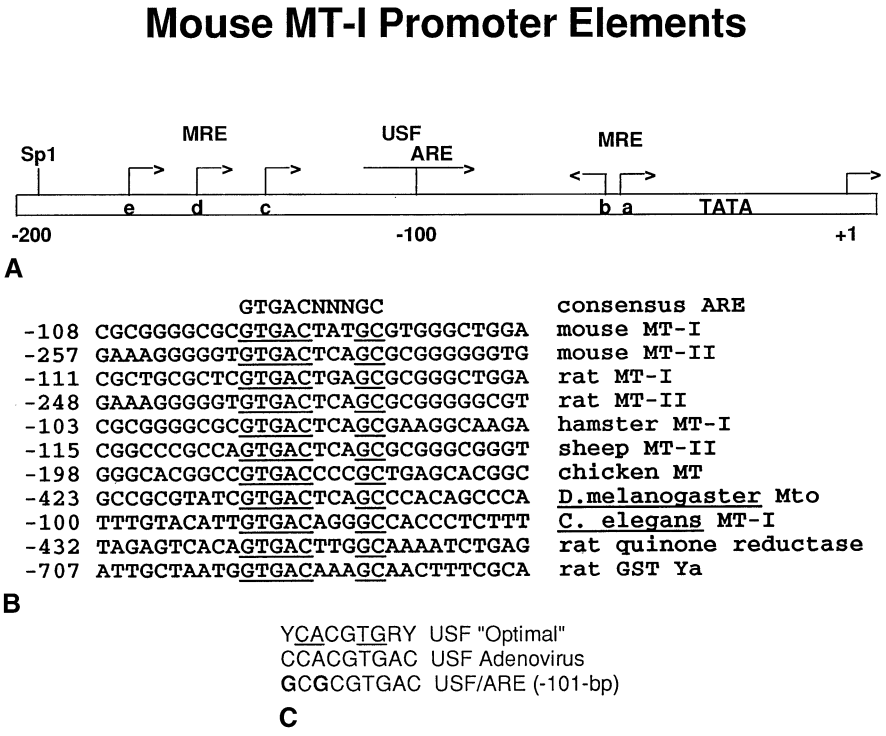

FIG. 1. (A) A schematic representation of the mouse MT-I proximal promoter (+1 to −200 bp relative to the transcription start point). The locations of known regulatory elements in the MT-I promoter are as indicated: MRE, metal regulatory elements (a through e); Sp1, Sp1-binding site; USF/ARE, composite upstream stimulating factor/antioxidant response element. (B) AREs are found in many MT proximal promoters. MT promoters submitted to GenBank were searched for consensus AREs in both the sense and antisense orientation. Numbers to the left represent the number of nucleotides upstream of the transcription start site of the adjacent nucleotide shown in the figure. (C) Alignment of the optimal USF-binding site, which contains an underlined E-box motif, the adenovirus promoter USF-binding site, and the USF site which overlaps the ARE in the MT-I promoter. Y refers to pyrimidine, R refers to purine, and the bases displayed in bold type in the USF/ARE do not match the optimal binding site. The USF-binding site in the MT-I promoter binds USF poorly relative to the consensus sequence E-box [52].

creased MT-I mRNA levels were detected at 1 hr, peaked by 3 hr, and returned to basal level by 9 hr posttreatment. In contrast, induction by tBHQ was sustained overnight. The oxidative stress induction of MT-I mRNA was additive and not synergistic with zinc.

These data suggested that oxidative stress activates transcription of the MT-I gene. This was directly demonstrated using nuclear run-on assays. One hour after treatment with H_2O_2, the relative rate of *MT-I* gene transcription had increased dramatically. This increase was transient, and by 3 hr posttreatment the relative rate of *MT-I* gene transcription had returned to pretreatment levels. Treatment of Hepa cells with 30 μM zinc resulted in a similar increase in the relative rate of *MT-I* gene transcription at 1 hr. Thus, regulation of the *MT-I* gene by H_2O_2 is primarily a transcriptional response, as has been previously shown for metal ions [19, 68].

Delineation of Oxidative Stress-responsive Elements in the MT-I Promoter: Roles of the MRE and USF/ARE

Transfection assays in Hepa cells were employed to delineate sequences within the MT-I promoter that confer responsiveness to oxidative stress. Deletion mutagenesis revealed that the 108-bp region located between -151- and -42-bp in the promoter was essential for response in transiently transfected cells. Among other potential *cis*-acting sequences, this region contains the 5 MREs in the MT-I promoter (consensus MRE: CTN**TGCRC**N**CG**-GCCC) (Fig. 1a), and an ARE consensus sequence (GTGACnnnGC) (-101-bp to -94-bp), as described by Rushmore *et al.* [69]. Interestingly, consensus AREs are also located in the proximal promoters of several other *MT* genes (Fig. 1b) [20]. In the mouse MT-I promoter, the ARE consensus sequence overlaps a previously identified USF-binding site (Fig. 1c) [36]. The USF/ARE was known to enhance basal level expression of the mouse *MT-I* gene [26, 36]. We determined the roles of the MREs and the USF/ARE in induction of the *MT-I* gene by metals and oxidative stress [20, 31, 52]. Metal ions are the best studied and most potent inducers of *MT-I* gene transcription and the MREs cooperate to confer metal responsiveness on this gene [27]. The best studied of these is MRE-d [26, 70]. A fusion gene consisting of 5 tandem copies of MRE-d inserted in the forward orientation immediately upstream of a minimal promoter was constructed to drive reporter gene expression (chloramphenicol acetyltransferase or Lucif-

erase) in transfected Hepa cells. Functions of the USF/ARE were examined by deletion of the USF/ARE from the proximal -153-bp of the MT-I promoter. Also, four tandem copies of the ARE in the glutathione *S*-transferase Ya promoter have been shown to increase basal activity from a minimal promoter and to direct induction by oxidative stress [71]. A similar approach was taken to analyze the USF/ARE in the mouse MT-I promoter. Four tandem copies of the USF/ARE were inserted in the forward orientation immediately upstream of a minimal promoter driving a reporter gene. In addition, the functions of both a single copy of this element and of this element containing mutations in the USF or the ARE site were tested.

The results of several studies from our lab [20, 31, 52], which used stably transfected cells as well as transiently transfected cells, demonstrated that the mouse MRE-d is responsive to zinc, cadmium, H_2O_2, and tBHQ. In subsequent experiments, we discovered that an MRE from the chicken *MT* gene promoter is responsive to metal ions and oxidative stress in transfected mouse cells and in transgenic mice [72]. In contrast, the USF/ARE directed responsiveness to cadmium and H_2O_2, but not to zinc or tBHQ. Responsiveness required an intact USF and intact ARE consensus sequences. Selective deletion of the USF/ARE (-100 to -89) from the proximal promoter of the *MT-I* gene reduced cadmium and H_2O_2 responsiveness in half, with no effect on zinc or tBHQ responsiveness. In subsequent experiments, we found that co-expression of a dominant negative USF-1 in transfected cells severely attenuated cadmium responsiveness of the USF/ARE and the intact MT-I promoter [52]. These results suggest that optimal induction of the mouse *MT-I* gene by cadmium and H_2O_2 requires at least two distinct elements in the proximal promoter of the mouse *MT-I* gene, whereas induction by zinc and tBHQ is dependent only on the MREs.

Metal Ion- and Oxidative Stress- Induced Protein–DNA Interactions In Vivo *with MREs and the USF/ARE*

To examine protein–DNA interactions in the proximal region of the MT-I promoter, we employed *in vivo* genomic footprint analysis by ligation-mediated polymerase chain reaction of bases -200 to -30 in the MT-I promoter [31]. Cells, before or after treatment with an inducer, were incubated with dimethyl sulphate, and genomic DNA was isolated, cleaved with piperidine, and MT-I promoter fragments were specifically amplified. The results of this assay revealed a strong constitutive footprint over an Sp1 binding site (-187 to -179), a constitutive footprint over the USF/ARE, and a weak footprint over MRE-d. Similar results were reported previously [73]. In contrast, the other MREs did not footprint in control Hepa cells. However, treatment with H_2O_2, tBHQ, or zinc rapidly induced protein interactions over all of the MREs. Guanine residues within each MRE core sequence (TGCRCnC) that are functionally essential [70] were protected from methylation, and with all three inducers the footprint patterns were essentially identical over the MREs. The footprints were detected within 30 min of treatment. Footprints induced by H_2O_2 were absent by 5 hr, consistent with the transient induction of *MT-I* gene transcription by H_2O_2 [20]. In contrast, the MRE footprints induced by tBHQ were maintained for at least 5 hr, as were those induced by high levels of zinc. We did not examine footprinting of this promoter after cadmium treatment.

In addition to footprints induced over MREs, all of these treatments also induced changes in the footprint over the USF/ARE region. Footprint patterns with the various inducers were qualitatively similar, but quantitatively distinct. Major changes in guanine methylation were noted in residues immediately upstream and within the USF-binding site and immediately downstream of the ARE. The footprint over the USF/ARE induced by H_2O_2 was transient, while those induced by tBHQ and zinc were prolonged. It is somewhat surprising that zinc induced a footprint over the USF/ARE; however, a similar result was previously reported [73]. These results revealed little evidence of protein interactions with guanine residues within the ARE functional core (GT**G**ACnnn**G**C) (Fig. 1b) [74, 75].

Rapid Induction by Oxidative Stress and Zinc, but Not Cadmium, of MTF-1 DNA-binding Activity In Vivo

The above results suggest that metal ions (particularly zinc) and oxidative stress can activate the binding of MTF-1 to the MREs in the MT-I promoter. This was documented using EMSA to detect MRE-binding activity in nuclear and whole cell extracts. In these assays, we employed a consensus MRE oligonucleotide (MRE-s) that is a strong MTF-1-binding site [29]. EMSA analysis using nuclear extracts or whole cell extracts obtained from Hepa cells treated for as little as 15 min with zinc, H_2O_2, or tBHQ revealed a 4- to 10-fold increase in MTF-1-binding activity in the nucleus, but no change in the constitutive DNA-binding activity of Sp1 [6, 31]. MTF-1-binding activity from Madin–Darby canine kidney (dog) and HeLa (human) cells incubated in medium containing excess zinc (120 μM) was also rapidly (30 min) increased. We proved that MTF-1 binding was measured specifically in these experiments using supershift assays with a polyclonal antiserum against recombinant mouse MTF-1, and by demonstrating the sequence specificity for the binding. In subsequent experiments, we found that the specific MRE-s–protein complex was absent when extracts from the mouse dko7 cell line, which lacks MTF-1, were analyzed [5, 76]. In that cell line, induction of the endogenous *MT-I* gene is refractory to zinc, cadmium, and oxidative stress [31]. These results confirmed that MTF-1 plays a central role in the activation of *MT-I* gene expression by these agents. In contrast to the above EMSA results, we detected little change in the binding activity of MTF-1 in cells treated with cadmium [7]. This suggests that cadmium activation of the *MT-I* gene involves a distinct mechanism relative to the other agents examined. These results also established that MTF-1 is not constitutively

active in untreated cells, but is instead rapidly activated to bind to DNA by zinc and oxidative stress.

Zinc and cadmium apparently activate MT-I promoter function by overlapping, yet distinct signal transduction pathways. Cadmium can cause oxidative stress and the depletion of glutathione [77], as well as affect the activity of signal transduction molecules [78–81] and evoke superoxide anion production by macrophages [82]. Our studies suggest that in Hepa cells, USF also plays an *in vivo* role in cadmium induction of the mouse *MT-I* gene. In addition to MTF-1, the USF/ARE could serve to specifically augment or prolong the responsiveness of the *MT-I* gene to cadmium relative to zinc and to the other mouse *MT* genes. USF could help maintain an open chromatin structure to facilitate interactions of the MT-I promoter with other factors. The cell specificity of this role of USF warrants investigation.

Increased ARE-binding Activity with Cadmium, but Not Zinc or Oxidative Stress

Protein interactions with the USF/ARE were examined. Two complexes were detected by EMSA using the mouse USF/ARE oligonucleotide and Hepa cell nuclear extracts. One of these complexes contained both USF-1 and USF-2, based on supershift assays using specific USF antisera and oligonucleotide competition assays [31, 52]. Formation of the ARE complex was sequence-specific, and mutation of two functionally important [74, 75] terminal bases (GC) in the ARE consensus sequence (GTGACnnnGC) blocked complex formation. Supershift assays suggested that activator protein-1 is not a component of this complex. The two complexes formed with the USF/ARE did not differ in amount or mobility among nuclear extracts from control and cells treated with zinc or oxidative stress-inducing agents. In contrast, increased ARE-binding activity was detected in cadmium-treated cells [52].

Our studies suggest that USF participates in activation of the *MT-I* gene in response to cadmium and H_2O_2 by interacting with ARE-binding factors through the USF/ARE composite element. USF can, in fact, interact with several different bZip transcription factors [43, 44], including the ARE-binding proteins Fra1 [45] and c-Maf [46]. USF activity can involve other proteins and adjacent promoter elements. A composite CCAAT-binding protein/USF-binding site mediates transforming growth factor-β1 induction of the human type 1 plasminogen activator inhibitor gene [83], and a three-protein complex containing USF occurs at the immunoglobulin μ heavy chain gene enhancer in B-cells [84]. The proteins which interact with the ARE in the MT-I promoter are unknown, but our data suggest that they play an important role in cadmium induction of this gene. A recent study also suggested a role for USF in cadmium induction of the rat heme oxygenase-I gene [85]. We noted that dominant negative USF significantly antagonized cadmium induction of the USF/ARE, as well as that of the intact MT-I promoter. Previous studies did not report a significant effect of deletion of the USF/ARE on metal induction of the MT-I promoter [26], but manipulation of MTF-1 expression by targeted deletion of both genes in embryonic stem cells [5] or by expression of antisense MTF-1 in baby hamster kidney cells [86] eliminated metal responsiveness of the *MT-I* gene. Thus, MTF-1 plays a key role in regulating *MT* gene expression in response to metal ions.

Rapid and Reversible In Vitro *Activation of MTF-1 DNA-binding Activity by Zinc, but Not Cadmium*

EMSA was used to monitor MTF-1 binding activity after *in vitro* activation of whole cell extracts from control Hepa, HeLa, and Madin–Darby canine kidney cells or of recombinant MTF-1 synthesized *in vitro* in a TnT system. In the absence of exogenous zinc, little MTF-1-binding activity was detected in extracts from control cells or in the TnT lysate reaction containing recombinant MTF-1. In contrast, the addition of 2 to 5 μM zinc caused a modest, but reproducible activation of MTF-1, and 30 μM zinc resulted in maximal activation in whole cell extracts. Half-maximal activation of mouse and human recombinant MTF-1 in the TnT lysate required 2 to 3 μM zinc, and was maximal with 15 μM zinc. Zinc activation of recombinant MTF-1 and of MTF-1 in the whole cell extracts was temperature- and time-dependent [6, 7]. In contrast to these results, the binding activity of the three zinc-finger protein Sp1 is maximal in control cells, and is not increased by treatment of the cells or extracts with zinc or oxidative stress. Furthermore, recombinant Sp1 synthesized in the TnT system is competent to bind to DNA without the addition of exogenous zinc. Cadmium, at concentrations from 1 nM to 760 μm, was ineffective in inducing MTF-1-binding activity in extracts from these cell lines. Likewise, micromolar concentrations of H_2O_2 failed to activate MTF-1-binding activity *in vitro* in control extracts. Similarly, no transition metals other than zinc were found to activate recombinant MTF-1 in the TnT lysate [7]. During the course of these studies, we noted that MTF-1-binding activity is more sensitive to metal chelators than is that of Sp1. In addition, we noted that "active" MTF-1 from zinc- or oxidative stress-induced cells (nuclear extracts or whole cell extracts) or the TnT lysate that was diluted in buffer such that the zinc concentration was < 0.5 μM lost DNA-binding activity when incubated at 37° [6]. Sp1 did not lose DNA-binding activity under these conditions. Remarkably, the DNA-binding activity of MTF-1 could be completely restored by readdition of 15 to 30 μM zinc to the sample.

The demonstration that MTF-1-binding activity, found in nuclear and whole cell extracts, is rapidly increased *in vivo* by inducers (zinc, H_2O_2, tBHQ) of *MT-I* gene transcription, and the finding, by us and others, that MREs in the MT-I promoter are not protein-bound *in vivo* before induction [73] establish that the ability of MTF-1 to stimulate *MT-I* gene transcription is controlled initially at

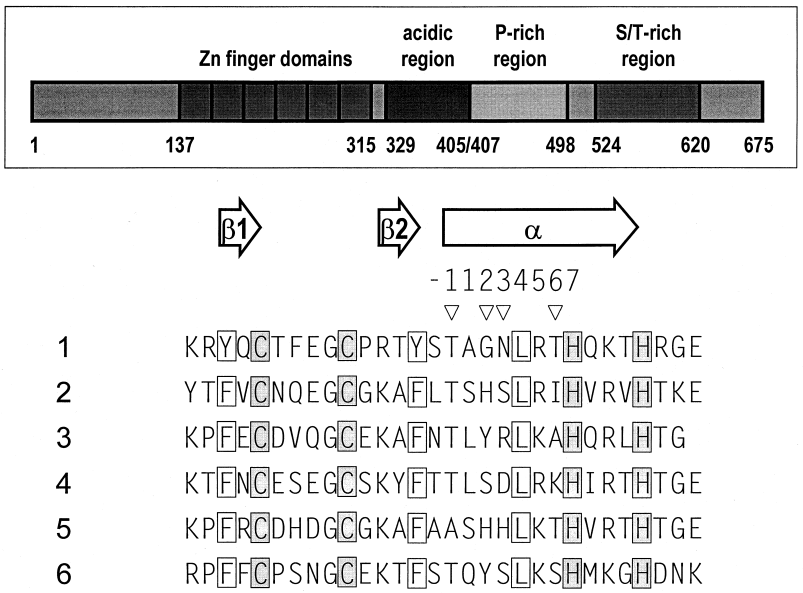

FIG. 2. Mouse MTF-1 structure and amino acid alignment of the six zinc fingers. The functional domains of mouse MTF-1 were delineated by Walter Schaffner's group in Zurich [29, 30]. The acidic, proline (P)-rich and serine/threonine (S/T)-rich regions are transactivation domains. The six zinc fingers of MTF-1 are aligned and the conserved cysteine and histidine residues are highlighted with shaded boxes, while the hydrophobic residues which stabilize the structure folded around a zinc atom are highlighted with open boxes. Two regions of β-sheet and one α-helical region are found in folded zinc fingers, and locations of residues in the α-helix which may play a role in DNA recognition are indicated (-1, 2, 3, and 6). Finger 1 differs from the other five zinc fingers in that tyrosine residues replace phenylalanine residues at these sites. This structure is conserved in mice [29], humans [30], fish [28], and birds (data not shown).

a step prior to specific DNA binding. Our data suggest that the DNA-binding activity of MTF-1 is specifically and reversibly activated by zinc. This supports the hypothesis that MTF-1 serves as a sensor for free zinc in the cell, and suggests that oxidative stress may activate *MT-I* gene expression indirectly by causing a redistribution of zinc within the cell. Based on these studies, we conclude that MTF-1 is not controlled by a zinc-sensitive inhibitor [86].

Regulation of DNA-binding Activity by Zinc Interactions with the Zinc-Finger Domain of MTF-1

To explore structure–function relationships in mouse MTF-1 (Fig. 2) in relation to zinc-dependent activation of DNA binding, polymerase chain reaction was used to prepare deletion mutants that lacked the entire region before or after the zinc-finger domain, EMSA using MRE-s revealed zinc-dependent activation of MRE-s-binding activity in a coupled reaction containing either the amino- or carboxyl-terminus-deleted MTF-1 peptides. Activation of these MTF-1 peptides by zinc was reversible. These MTF-1 peptides share the zinc-finger domain (Fig. 2), which suggests a role for the zinc-finger domain in the zinc-dependent activation of MTF-1 [6]. In recent studies we found, using finger domain swapping and deletion mutagenesis of the MTF-1 zinc fingers, that zinc activation of MTF-1 does involve the zinc-finger domain and that the zinc fingers of MTF-1 are heterogeneous in function. A recent study of a bacterially expressed human MTF-1 zinc-finger domain confirms the concept of structural and functional heterogeneity in the MTF-1 zinc fingers [87]. Further studies are underway in our laboratory to delineate the functions of the MTF-1 zinc fingers, and preliminary results suggest a central role for finger 1 in the reversible activation of the DNA-binding activity by zinc. Zinc binding could lead to an allosteric change in MTF-1, causing exposure of zinc fingers involved in DNA binding in a process reminiscent of the activation of steroid hormone receptors by ligand binding [88]. However, in contrast to the steroid receptors, the ligand-binding domain of MTF-1 may be located within the zinc-finger domain itself rather than in a separate carboxyl-terminal domain. Alternatively, the folding of the DNA-binding zinc finger(s) might be directly and reversibly regulated by zinc. This latter model is reminiscent of the activation of the yeast transcription factor ACE1. ACE1 regulates yeast MT genes in response to Cu [89], and its DNA-binding activity is reversibly activated by the cooperative binding of Cu(I) [90]. The Cu(I) and DNA-binding domains are located in the amino-terminal half of ACE1 [91]. Interestingly, this factor has an apparent K_d of 6 μM for Cu(I).

CONCLUDING REMARKS

The findings that *MT* gene expression is induced by oxidative stress and that MT can, in turn, exert antioxidant activities suggest that one of the functions of these proteins

is to protect the cell from free radicals. Whether this is an essential function remains to be determined. However, limiting free radical damage is a high priority for the cell, and efficiency at this task provides a positive selective advantage. Our studies suggest that activation of *MT* gene expression by oxidative stress is mediated, in part, by an increase in free zinc in the cell. This free zinc then serves as a second messenger to activate the DNA-binding activity of MTF-1. Unfortunately, we have no reliable method to directly determine free zinc levels in cells, and the source of zinc apparently liberated by oxidative stress is unknown. However, one candidate for such a zinc pool is MT itself. An increase in oxidized glutathione in the cell can facilitate release of zinc from MT, and MT binds from 5 to 20% of the total cellular zinc in Hepa cells. This hypothesis can now be tested using cells which lack functional *MT-I* and *-II* genes. Redistribution of zinc in the cell may serve to transiently inhibit metabolic enzyme activity, protect free thiols from oxidation, and stabilize membranes, as well as restore activity to zinc-finger proteins.

Our studies suggest that other signal transduction cascades may be involved in activation of *MT* gene expression by MTF-1 in cells treated with cadmium. The identity of these signals has yet to be determined. It is possible that the ability of MTF-1 to transactivate MT gene transcription may be enhanced in the absence of increased DNA binding in cells treated with cadmium. This could be accomplished by modifications such as acetylation or phosphorylation of MTF-1. Furthermore, it is apparent that multiple transcription factors participate in cadmium and H_2O_2 regulation of this gene. Thus, MTF-1 interactions with other transcription factors may bring it to the MT-I promoter, reminiscent of one mechanism by which MyoD activates muscle-specific gene expression. Candidate transcription factors for interactions with MTF-1 include Sp1, USF and ARE-binding proteins. Certainly, co-activators of transcription, such as those in the histone deacetylase family, may also interact with MTF-1. Further studies of MTF-1 are needed to address these questions.

Finally, an interesting finding in our studies is that the USF/ARE element mediates response to H_2O_2, but not to tBHQ. This suggests that specific signal transduction cascades may mediate response to different forms of oxidative stress. This would be reminiscent of the specific bacterial oxidative stress response pathways for superoxide anions versus hydroxyl radicals. Clearly this observation warrants further investigation.

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References

1. Andrews GK, Regulation of metallothionein gene expression. *Prog Food Nutr Sci* **14:** 193–258, 1990.
2. Kagi JHR, Overview of metallothionein. *Methods Enzymol* **205:** 613–626, 1991.
3. Palmiter RD, Sandgren EP, Koeller DM, Findley SD and Brinster RL, Metallothionein genes and their regulation in transgenic mice. In: Metallothionein III: *Biological Roles and Medical Implications* (Eds. Suzuki KT, Imura N and Kimura M), p. 399. Birkhauser Verlag, Basel, 1993.
4. Liang L, Fu K, Lee DK, Sobieski RJ, Dalton TP and Andrews GK, Activation of the complete metallothionein gene locus in the maternal deciduum. *Mol Reprod Dev* **43:** 25–37, 1996.
5. Heuchel R, Radtke F, Georgiev O, Stark G, Aguet M and Schaffner W, The transcription factor MTF-I is essential for basal and heavy metal-induced metallothionein gene expression. *EMBO J* **13:** 2870–2875, 1994.
6. Dalton TD, Bittel D and Andrews GK, Reversible activation of the mouse metal response element-binding transcription factor-1 DNA binding involves zinc interactions with the zinc-finger domain. *Mol Cell Biol* **17:** 2781–2789, 1997.
7. Bittel D, Dalton T, Samson S, Gedamu L and Andrews GK, The DNA-binding activity of metal response element-binding transcription factor-1 is activated *in vivo* and *in vitro* by zinc, but not by other transition metals. *J Biol Chem* **273:** 7127–7133, 1998.
8. Palmiter RD, Sandgren EP, Koeller DM and Brinster RL, Distal regulatory elements from the mouse metallothionein locus stimulate gene expression in transgenic mice. *Mol Cell Biol* **13:** 5266–5275, 1993.
9. Masters BA, Kelly EJ, Quaife CJ, Brinster RL and Palmiter RD, Targeted disruption of metallothionein I and II genes increases sensitivity to cadmium. *Proc Natl Acad Sci USA* **91:** 584–588, 1994.
10. Dalton TP, Fu K, Palmiter RD and Andrews GK, Transgenic mice that over-express metallothionein-I resist dietary zinc deficiency. *J Nutr* **126:** 825–833, 1996.
11. Andrews GK and Geiser J, Expression of the mouse metallothionein-I and -II genes provides a reproductive advantage during maternal dietary zinc deficiency. *J Nutr* **129:** 1643–1648, 1999.
12. Kelly EJ, Quaife CJ, Froelick GJ and Palmiter RD, Metallothionein I and II protect against zinc deficiency and zinc toxicity in mice. *J Nutr* **126:** 1782–1790, 1996.
13. Suhy DA, Simon KD, Linzer DIH and O'Halloran TV, Metallothionein is part of a zinc-scavenging mechanism for cell survival under conditions of extreme zinc deprivation. *J Biol Chem* **274:** 9183–9192, 1999.
14. Maret W, Jacob C, Vallee BL and Fischer EH, Inhibitory sites in enzymes: Zinc removal and reactivation by thionein. *Proc Natl Acad Sci USA* **96:** 1936–1940, 1999.
15. Palmiter RD, The elusive function of metallothioneins. *Proc Natl Acad Sci USA* **95:** 8428–8430, 1998.
16. Liu J, Liu YP, Hartley D, Klaassen CD, Shehin-Johnson SE, Lucas A and Cohen SD, Metallothionein-I/II knockout mice are sensitive to acetaminophen-induced hepatotoxicity. *J Pharmacol Exp Ther* **289:** 580–586, 1999.
17. Pitt BR, Schwarz M, Woo ES, Yee E, Wasserloos K, Tran S, Weng WL, Mannix RJ, Watkins SA, Tyurina YY, Tyurin VA, Kagan VE and Lazo JS, Overexpression of metallothionein decreases sensitivity of pulmonary endothelial cells to oxidant injury. *Am J Physiol* **273:** L856–L865, 1997.
18. Lazo JS, Kondo Y, Dellapiazza D, Michalska AE, Choo KHA and Pitt BR, Enhanced sensitivity to oxidative stress in cultured embryonic cells from transgenic mice deficient in metallothionein I and II genes. *J Biol Chem* **270:** 5506–5510, 1995.

19. Palmiter RD, Molecular biology of metallothionein gene expression. *EXS* **52:** 63–80, 1987.
20. Dalton TP, Palmiter RD and 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.
21. Abel J and de Ruiter N, Inhibition of hydroxyl-radical-generated DNA degradation by metallothionein. *Toxicol Lett* **47:** 191–196, 1989.
22. Thornalley PJ and 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.
23. Tamai KT, Gralla EB, Ellerby LM, Valentine JS and Thiele DJ, Yeast and mammalian metallothioneins functionally substitute for yeast copper-zinc superoxide dismutase. *Proc Natl Acad Sci U S A* **90:** 8013–8017, 1993.
24. Maret W, Oxidative metal release from metallothionein via zinc-thiol/disulfide interchange. *Proc Natl Acad Sci USA* **91:** 237–241, 1994.
25. Jacob C, Maret W and Vallee BL, Control of zinc transfer between thionein, metallothionein, and zinc proteins. *Proc Natl Acad Sci U S A* **95:** 3489–3494, 1998.
26. Stuart GW, Searle PF, Chen HY, Brinster RL and Palmiter RD, A 12-base-pair DNA motif that is repeated several times in metallothionein gene promoters confers metal regulation to a heterologous gene. *Proc Natl Acad Sci USA* **81:** 7318–7322, 1984.
27. Stuart GW, Searle PF and Palmiter RD, Identification of multiple metal regulatory elements in mouse metallothionein-I promoter by assaying synthetic sequences. *Nature* **317:** 828–831, 1985.
28. Maur AAD, Belser T, Elgar G, Georgiev O and 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.
29. Radtke F, Heuchel R, Georgiev O, Hergersberg M, Gariglio M, Dembic Z and Schaffner W, Cloned transcription factor MTF-1 activates the mouse metallothionein I promoter. *EMBO J* **12:** 1355–1362, 1993.
30. Brugnera E, Georgiev O, Radtke F, Heuchel R, Baker E, Sutherland GR and Schaffner W, Cloning, chromosomal mapping and characterization of the human metal-regulatory transcription factor MTF-1. *Nucleic Acids Res* **22:** 3167–3173, 1994.
31. Dalton TP, Li QW, Bittel D, Liang LC and 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.
32. Favreau LV and Pickett CB, Transcriptional regulation of the rat NAD(P)H:quinone reductase gene. Characterization of a DNA–protein interaction at the antioxidant responsive element and induction by 12-*O*-tetradecanoylphorbol 13-acetate. *J Biol Chem* **268:** 19875–19881, 1993.
33. Jaiswal AK, Antioxidant response element. *Biochem Pharmacol* **48:** 439–444, 1994.
34. Venugopal R and Jaiswal AK, Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase$_1$ gene. *Proc Natl Acad Sci U S A* **93:** 14960–14965, 1996.
35. Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M and Nabeshima Y, An Nrf2 small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem Biophys Res Commun* **236:** 313–322, 1997.
36. Carthew RW, Chodosh LA and Sharp PA, The major late transcription factor binds to and activates the mouse metallothionein I promoter. *Genes Dev* **1:** 973–980, 1987.
37. Beckmann H, Su LK and Kadesch T, TFE3: a helix-loop-helix protein that activates transcription through the immunoglobulin enhancer muE3 motif. *Genes Dev* **4:** 167–179, 1990.
38. Bendall AJ and Molloy PL, Base preferences for DNA binding by the bHLH-Zip protein USF: Effects of $MgCl_2$ on specificity and comparison with binding of Myc family members. *Nucleic Acids Res* **22:** 2801–2810, 1994.
39. Sirito M, Lin Q, Maity T and Sawadogo M, Ubiquitous expression of the 43- and 44-kDa forms of transcription factor USF in mammalian cells. *Nucleic Acids Res* **22:** 427–433, 1994.
40. Gregor PD, Sawadogo M and Roeder RG, The adenovirus major late transcription factor USF is a member of the helix-loop-helix group of regulatory proteins and binds to DNA as a dimer. *Genes Dev* **4:** 1730–1740, 1990.
41. Viollet B, Lefrancois-Martinez AM, Henrion A, Kahn A, Raymondjean M and Martinez A, Immunochemical characterization and transacting properties of upstream stimulatory factor isoforms. *J Biol Chem* **271:** 1405–1415, 1996.
42. Sirito M, Walker S, Lin Q, Kozlowski MT, Klein WH and Sawadogo M, Members of the USF family of helix-loop-helix proteins bind DNA as homo- as well as heterodimers. *Gene Expr* **2:** 231–240, 1992.
43. Blanar MA and Rutter WJ, Interaction cloning: Identification of a helix-loop-helix zipper protein that interacts with c-Fos. *Science* **256:** 1014–1018, 1992.
44. Meier JL, Luo X, Sawadogo M and Straus SE, The cellular transcription factor USF cooperates with varicella-zoster virus immediate-early protein 62 to symmetrically activate a bidirectional viral promoter. *Mol Cell Biol* **14:** 6896–6906, 1994.
45. Pognonec P, Boulukos KE, Aperlo C, Fujimoto M, Ariga H, Nomoto A and Kato H, Cross-family interaction between the bHLHZip USF and bZip Fra1 proteins results in down-regulation of AP1 activity. *Oncogene* **14:** 2091–2098, 1997.
46. Kurschner C and Morgan JI, USF2/FIP associates with the b-Zip transcription factor, c-Maf, via its bHLH domain and inhibits c-Maf DNA binding activity. *Biochem Biophys Res Commun* **231:** 333–339, 1997.
47. Reisman D and Rotter V, The helix-loop-helix containing transcription factor USF binds to and transactivates the promoter of the *p53* tumor suppressor gene. *Nucleic Acids Res* **21:** 345–350, 1993.
48. Gao E, Wang Y, Alcorn JL and Mendelson CR, The basic helix-loop-helix-zipper transcription factor USF1 regulates expression of the surfactant protein-A gene. *J Biol Chem* **272:** 23398–23406, 1997.
49. Kirschbaum BJ, Pognonec P and Roeder RG, Definition of the transcriptional activation domain of recombinant 43-kilodalton USF. *Mol Cell Biol* **12:** 5094–5101, 1992.
50. Carter RS, Ordentlich P and Kadesch T, Selective utilization of basic helix-loop-helix-leucine zipper proteins at the immunoglobulin heavy-chain enhancer. *Mol Cell Biol* **17:** 18–23, 1997.
51. Lun Y, Sawadogo M and Perry M, Autoactivation of *Xenopus* MyoD transcription and its inhibition by USF. *Cell Growth Differ* **8:** 275–282, 1997.
52. Li QW, Hu NM, Daggett MAF, Chu WA, Bittel D, Johnson JA and Andrews GK, Participation of upstream stimulatory factor (USF) in cadmium-induction of the mouse metallothionein-I gene. *Nucleic Acids Res* **26:** 5182–5189, 1998.
53. Bauman JW, Liu J, Liu YP and Klaassen CD, Increase in

metallothionein produced by chemicals that induce oxidative stress. *Toxicol Appl Pharmacol* **110:** 347–354, 1991.
54. Bauman JW, Liu YP, Andrews GK and Klaassen CD, Examination of potential mechanism(s) of metallothionein induction by diethyl maleate. *Toxicol Appl Pharmacol* **117:** 226–232, 1992.
55. Bauman JW, Madhu C, McKim JM Jr, Liu Y and Klaassen CD, Induction of hepatic metallothionein by paraquat. *Toxicol Appl Pharmacol* **117:** 233–241, 1992.
56. Bauman JW, McKim JM Jr, Liu J and Klaassen CD, Induction of metallothionein by diethyl maleate. *Toxicol Appl Pharmacol* **114:** 188–196, 1992.
57. Min K-S, Terano Y, Onosaka S and Tanaka K, Induction of hepatic metallothionein by nonmetallic compounds associated with acute-phase response in inflammation. *Toxicol Appl Pharmacol* **111:** 152–162, 1991.
58. Pinkus R, Weiner LM and Daniel V, Role of quinone-mediated generation of hydroxyl radicals in the induction of glutathione *S*-transferase gene expression. *Biochemistry* **34:** 81–88, 1995.
59. Prestera T and Talalay P, Electrophile and antioxidant regulation of enzymes that detoxify carcinogens. *Proc Natl Acad Sci USA* **92:** 8965–8969, 1995.
60. Yoshioka K, Deng T, Cavigelli M and Karin M, Antitumor promotion by phenolic antioxidants: Inhibition of AP-1 activity through induction of Fra expression. *Proc Natl Acad Sci USA* **92:** 4972–4976, 1995.
61. Schilderman PA, van Maanen JM, Smeets EJ, ten Hoor F and Kleinjans JC, Oxygen radical formation during prostaglandin H synthase-mediated biotransformation of butylated hydroxyanisole. *Carcinogenesis* **14:** 347–353, 1993.
62. Bergmann B, Dohrmann JK and Kahl R, Formation of the semiquinone anion radical from *tert*-butylquinone and from *tert*-butylhydroquinone in rat liver microsomes. *Toxicology* **74:** 127–133, 1992.
63. Keyse SM, Applegate LA, Tromvoukis Y and Tyrrell RM, Oxidant stress leads to transcriptional activation of the human heme oxygenase gene in cultured skin fibroblasts. *Mol Cell Biol* **10:** 4967–4969, 1990.
64. Keyse SM and Tyrrell RM, Both near ultraviolet radiation and the oxidizing agent hydrogen peroxide induce a 32-kDa stress protein in normal human skin fibroblasts. *J Biol Chem* **262:** 14821–14825, 1987.
65. Keyse SM and Tyrrell RM, Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. *Proc Natl Acad Sci USA* **86:** 99–103, 1989.
66. Applegate LA, Luscher P and Tyrrell RM, Induction of heme oxygenase: A general response to oxidant stress in cultured mammalian cells. *Cancer Res* **51:** 974–978, 1991.
67. Keyse SM and Tyrrell RM, Induction of the heme oxygenase gene in human skin fibroblasts by hydrogen peroxide and UVA (365 nm) radiation: Evidence for the involvement of the hydroxyl radical. *Carcinogenesis* **11:** 787–791, 1990.
68. Durnam DM and Palmiter RD, Transcriptional regulation of the mouse metallothionein-I gene by heavy metals. *J Biol Chem* **256:** 5712–5716, 1981.
69. Rushmore TH, Morton MR and Pickett CB, The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *J Biol Chem* **266:** 11632–11639, 1991.
70. Culotta VC and Hamer DH, Fine mapping of a mouse metallothionein gene metal response element. *Mol Cell Biol* **9:** 1376–1380, 1989.
71. Rushmore TH and Pickett CB, Transcriptional regulation of the rat glutathione *S*-transferase Ya subunit gene. Characterization of a xenobiotic-responsive element controlling inducible expression by phenolic antioxidants. *J Biol Chem* **265:** 14648–14653, 1990.
72. Dalton T, Paria BC, Fernando LP, Huet-Hudson YM, Dey SK and Andrews GK, Activation of the chicken metallothionein promoter by metals and oxidative stress in cultured cells and transgenic mice. *Comp Biochem Physiol B Biochem Mol Biol* **116:** 75–86, 1997.
73. Mueller PR, Salser SJ and Wold B, Constitutive and metal-inducible protein: DNA interactions at the mouse metallothionein I promoter examined by *in vivo* and *in vitro* footprinting. *Genes Dev* **2:** 412–427, 1988.
74. Favreau LV and Pickett CB, The rat quinone reductase antioxidant response element. Identification of the nucleotide sequence required for basal and inducible activity and detection of antioxidant response element-binding proteins in hepatoma and non-hepatoma cell lines. *J Biol Chem* **270:** 24468–24474, 1995.
75. Nguyen T, Rushmore TH and Pickett CB, Transcriptional regulation of a rat liver glutathione *S*-transferase Ya subunit gene. Analysis of the antioxidant response element and its activation by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate. *J Biol Chem* **269:** 13656–13662, 1994.
76. Radtke F, Georgiev O, Müller H-P, Brugnera E and Schaffner W, Functional domains of the heavy metal-responsive transcription regulator MTF-1. *Nucleic Acids Res* **23:** 2277–2286, 1995.
77. Stohs SJ and Bagchi D, Oxidative mechanisms in the toxicity of metal ions. *Free Radic Biol Med* **18:** 321–336, 1995.
78. Tang N and Enger MD, Cd^{2+}-induced c-*myc* mRNA accumulation in NRK-49F cells is blocked by the protein kinase inhibitor H7 but not by HA1004, indicating that protein kinase C is a mediator of the response. *Toxicology* **81:** 155–164, 1993.
79. Chao SH, Bu CH and Cheung WY, Stimulation of myosin light-chain kinase by Cd^{2+} and Pb^{2+}. *Arch Toxicol* **69:** 197–203, 1995.
80. Vig PJS and Nath R, *In vivo* effects of cadmium on calmodulin and calmodulin regulated enzymes in rat brain. *Biochem Int* **23:** 927–934, 1991.
81. Smith JB, Dwyer SD and Smith L, Cadmium evokes inositol polyphosphate formation and calcium mobilization. Evidence for a cell surface receptor that cadmium stimulates and zinc antagonizes. *J Biol Chem* **264:** 7115–7118, 1989.
82. Amoruso MA, Witz G and Goldstein BD, Enhancement of rat and human phagocyte superoxide anion radical production by cadmium *in vitro*. *Toxicol Lett* **10:** 133–138, 1982.
83. Riccio A, Pedone PV, Lund LR, Olesen T, Olsen HS and Andreasen PA, Transforming growth factor β1-responsive element: Closely associated binding sites for USF and CCAAT-binding transcription factor-nuclear factor I in the type 1 plasminogen activator inhibitor gene. *Mol Cell Biol* **12:** 1846–1855, 1992.
84. Rao E, Dang W, Tian G and Sen R, A three-protein-DNA complex on a B cell-specific domain of the immunoglobulin mu heavy chain gene enhancer. *J Biol Chem* **272:** 6722–6732, 1997.
85. Maeshima H, Sato M, Ishikawa K, Katagata Y and Yoshida T, Participation of altered upstream stimulatory factor in the induction of rat heme oxygenase-1 by cadmium. *Nucleic Acids Res* **24:** 2959–2965, 1996.
86. 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.
87. Chen XH, Agarwal A and Giedroc DP, Structural and functional heterogeneity among the zinc fingers of human MRE-binding transcription factor-1. *Biochemistry* **37:** 11152–11161, 1998.

88. Beato M, Transcriptional control by nuclear receptors. *FASEB J* **5:** 2044–2051, 1991.
89. Zhou P, Szczypka MS, Sosinowski T and Thiele DJ, Expression of a yeast metallothionein gene family is activated by a single metalloregulatory transcription factor. *Mol Cell Biol* **12:** 3766–3775, 1992.
90. Fürst P and Hamer D, Cooperative activation of a eukaryotic transcription factor: Interaction between Cu(I) and yeast ACE1 protein. *Proc Natl Acad Sci USA* **86:** 5267–5271, 1989.
91. Dameron CT, Winge DR, George GN, Sansone M, Hu S and Hamer DA, Copper–thiolate polynuclear cluster in the ACE1 transcription factor. *Proc Natl Acad Sci USA* **88:** 6127–6131, 1991.