Oxidative stress (tert-butylhydroquinone) rapidly induced metallothionein-I gene expression in mouse Hepa cells, and this effect was mediated predominantly through metal response promoter elements in transient transfection assays. In vivo genomic footprinting of the mouse metallothionein-I promoter after treatment of Hepa cells with hydrogen peroxide, tert-butylhydroquinone, or zinc suggested a rapid increase in occupancy of the metal response elements. More subtle changes also occurred in the constitutive genomic footprint at the composite major late transcription factor/antioxidant response element. This element may, in part, mediate induction by hydrogen peroxide. Electrophoretic mobility shift assays demonstrated a rapid (30 min) increase in the DNA binding activity of metal-responsive transcription factor-1 in Hepa cells treated with any of these inducers. In control cells, upstream stimulatory factor binding with the major late transcription factor site, and a nuclear protein complex distinct from AP-1, but specific for the antioxidant response element, were detected. The amounts of these complexes were not altered after these treatments. These studies indicate that metal-responsive transcription factor-1 plays a role in activating mouse metallothionein-I gene transcription in response to oxidative oxygen species.

Reactive oxygen species (ROS) are generated as a result of many normal and pathological processes (1), and their generation has been shown to promote a number of deleterious cellular and organismal pathologies including aging, cancer, and cell death (2). Cells synthesize a battery of proteins and other antioxidants whose function is to protect from ROS (1). Metallothioneins (MT) constitute a family of small cysteine-rich heavy metal-binding proteins. MT-I and -II display a broad tissue distribution and were initially characterized for their ability to sequester and protect cells against heavy metals (3). The functional roles for MT include zinc homeostasis (4) and protection from ROS (5–7).

A hallmark of MT-I and MT-II genes is their rapid transcriptional induction both in vivo and in vitro by agents against which MT protects. Essential to induction by heavy metals are cis-acting sequences termed metal response elements (MRE) (8–10). The metal-responsive transcription factor, termed MTF-1, that binds to MREs and activates MT gene transcription has been cloned from mouse and human (11, 12). The mechanisms by which metals regulate transcription through MTF-1 are poorly understood.

It is well documented that agents that redox cycle, such as paraxquat, agents that deplete glutathione, such as diethylmaleate, and inflammatory substances, such as lipopolysaccharide and cytokines, induce ROS production (1) and MT (13, 14) in rodents. We found that transcription of the mouse MT-I gene in mouse Hepa cells is rapidly induced by hydrogen peroxide (H2O2) (15). In that study transient transfection analysis using the mouse MT-I promoter and isolated transcription elements driving the CAT reporter gene implicated MREs and a composite major late transcription factor/antioxidant response element (MLTF/ARE) in the response of the MT-I gene to ROS. The MLTF binding site has been shown to effect basal transcription from the mouse MT-I promoter (16) and upstream stimulatory factor (USF), a member of the bHLH-bZip protein superfamily, can bind to this site (17, 18). The ARE is an element best characterized in the promoters of phase II drug metabolizing enzyme genes and directs response to electrophilic xenobiotics and H2O2 (19). The transcription factor(s) responsible for transactivation through the ARE is not clear. Some reports implicate AP-1 complexes and some a novel factor (19).

In the present study, we employ transient transfection analysis, in vivo footprint analysis, and electrophoretic mobility shift assays (EMSA) to elucidate the transcription factors involved in the transcriptional response of the mouse MT-I gene to ROS. Our results indicate that pro-oxidant conditions activate mouse MTF-1 binding activity and MT-I gene transcription during oxidative stress.

EXPERIMENTAL PROCEDURES

Cell Culture, Treatment, and Transfection—Hepa cells were cultured and treated by direct addition to the culture medium from...
concentrated stocks of H$_2$O$_2$ and ZnSO$_4$ as described (15). tert-Butyl-
hydroperoxide and β-naphthol (β-NF) were purchased from Sigma (St. Louis, MO) and 
Me$_2$SO as a 1000 times concentrated stock. Hepa cells were transfected 
using the calcium phosphate precipitation method and reporter 
gene activity (CAT) was normalized to β-gal activity by cotransfection of 
SV-β-gal as described (15).

Isolation of RNA and Northern Blot Hybridization—RNA was iso-
lated, size fractionated by formaldehyde agarose gel electrophoresis, 
transferred to nylon membranes and hybridized with 32P-labeled mouse 
MT-1 cDNA probe as described (15, 20). The probe had a specific activity of 
approximately 2 x 10$^6$ cpm/µg.

Fusion Gene Construction—The construction of fusion genes used in 
this study has been described previously (15).

In Vivo Genomic Footprinting—After treatment with the indicated inducers, 
Hepa cells were exposed to 0.25% dimethyl sulfoxide (Aldrich) 
for 24 h at 37 °C; genomic DNA was purified and subjected to piperidine 
(Aldrich) cleavage at positions of methylated guanines as described 
(21). The cleaved DNA (2 µg) was then amplified by ligation-mediated 
PCR (LM-PCR), using mouse MT-I promoter-specific primers that have 
been described previously (22, 23), except that the following primer (GATAGGCCGCGGTATATCGGGGAAAGCAC) was used as the third 
primer for LM-PCR. Reaction conditions were as described (22, 23) 
with the following exceptions. 1) The initial primer extension reaction con-
tained 2 units of Vent Exo DNA polymerase (New England Biolabs, 
Beverly, MA), and denaturation was conducted for 5 min at 95 °C, 
annealing was for 30 min at 60 °C and primer extension was for 10 min 
at 72 °C; 2) during PCR, denaturation, annealing, and primer extension 
temperatures were as above; 3) the labeling reaction contained 2.3 pmol 
of end-labeled oligonucleotide (4–8 x 10$^6$ cpm/µmol) and was denatured 
for 3.5 min at 95 °C, annealed for 2 min at 66 °C, and extended for 10 
min at 76 °C; and 4) at the end of the labeling cycle, one half of the 
sample was frozen at –20 °C for later use, and the remainder was 
fractionated on a 6% DNA sequencing gel. Based on band intensities in 
each lane of the initial sequencing gel, a second gel was run in which the 
radioactivity in each lane was “normalized.” Samples with low signal 
were subjected to another cycle of labeling before further analysis.

Quantitation of Footprints—Dried sequencing gels were exposed to a 
phosphor screen, which was scanned with a Phosphorimager SI, and 
the radioactivity in each lane was quantitated using the ImageQuaNT software (Molecular 
Dynamics, Sunnyvale, CA). To account for variations in the amount of 
radioactivity in bases within the footprinted region (base) was compared with that of bases flanking the 
footprinted region (standard), and the ratio (P$_{base}$/P$_{standard}$) was calcu-
lated for each base as described (24). Data were verified for consistency 
by examining the ratios obtained using several standard bases. Protec-
tion or hypersensitivity of individual guanines was determined by sub-
tracting the ratio that base in the uninduced sample in vivo control sample 
from that in the induced sample, and the difference was divided by the 
ratio of the in vivo control.

Preparation of Nuclear Extracts—Nuclear extracts were prepared with modifications of the method of Dignam et al. (25). After treatment, 
cells (1 x 10$^8$) were placed on ice, the medium was removed, and the 
cells were washed once with ice-cold phosphate-buffered saline. Cells. 
were scraped off the dish in 5 ml of cold phosphate-buffered saline. Cells 
were centrifuged at 1500 x g for 5 min at 4 °C. The cell pellet was 
resuspended in 5 ml of cell lysis buffer (10 mM HEPES (pH 7.9), 1.5 
mm MgCl$_2$, 10 mM KCl, 0.5 mM DTT, and 0.2 mM phenylmethylsulfonyl 
fluoride) and immediately centrifuged as above. Cells were resuspended 
in 2 ml of the original packed cell volume of cell lysis buffer, allowed to 
swell on ice for 10 min, and homogenized with 10 strokes of a Dounce 
homogenizer (B pestle). Nuclei were collected by centrifugation at 
3300 x g for 15 min at 4 °C, the supernatant was removed and the 
nuclei were suspended, using 6 strokes of a Teflon glass homogenizer, 
in 3 volumes (about 750 µl) of nuclear extraction buffer (20 mM HEPES 
(pH 7.9), 1.5 mM MgCl$_2$, 400 mM KCl, 0.5 mM DTT, 0.2 mM phenylmethyl-
sulfonyl fluoride, and 25% glycerol). The nuclear suspension was 
stirred for 30 min on ice, and then centrifuged at 89,000 x g for 30 min 
at 4 °C. The supernatant was collected, and concentrated in a Micro-
con3 microconcentrator (Amicon Inc., Beverly, MA) by centrifugation at 
12,000 x g for 3 h at 4 °C. The concentrated extract was diluted in half 
with diluent buffer (extraction buffer without KCl) and frozen in aliquots 
at –80 °C. Proteins were determined using a Bradford protein assay 
(Bio-Rad) with rabbit IgG as a standard. A nuclear extract from 
Tpa-treated NIH 3T3 cells, and antibodies against the c-jun family and USF 
recognizes both USF-1 and USF-2; Ref. 26) were purchased from 
Santa Cruz Biochemicals (Santa Cruz, CA).

EMSA—Nuclear proteins (5–10 µg) were incubated in binding reac-
tion buffer containing 12 mM HEPES (pH 7.9), 60 mM KCl, 0.5 mM DTT,
12% glycerol, 5 mM MgCl$_2$, 0.2 µg of di-dC/dµ of protein, 2–4 fmol of 
ed-labeled double-stranded oligonucleotide (5000 cpm/µmol) (27), and 
where indicated 60 µM ZnSO$_4$, in a total volume of 20 µl for, unless 
otherwise stated, 20 min at room temperature. In binding site com-
petition experiments, binding reactions also contained a 250-fold molar 
excess of the indicated unlabeled oligonucleotide. In antibody competi-
tion experiments, the indicated antisemur (1 µg) was added to the 
complete binding reaction and the mixture was incubated for 20 min 
at room temperature. The oligonucleotide sequences were as follows 
(bold bases denote the functional core, and unlabeled bases denote 
mutations).

| MRE-d(8): | GATCCAGGAGGTCGACATCAGGGAAAGAATA |
| MRE-s(11): | GATCCAGGAGGTCGACATCAGGGAAAGAATA |
| mutMRE-d(28, 29): | GATCCAGGAGGTCGACATCAGGGAAAGAATA |
| Spi(30): | GATCCCGGGGCGGGCACTGATACGAGCAGCCCCAATCAG |
| MTFLT/ARE (15): | GATCCCGGGGCGGGCACTGATACGAGCAGCCCCAATCAG |
| mutMTFL/ARE (16): | GATCCCGGGGCGGGCACTGATACGAGCAGCCCCAATCAG |
| MTFLT/mutARE (31): | GATCCCGGGGCGGGCACTGATACGAGCAGCCCCAATCAG |
| TRE (32): | GATCCCGGGGCGGGCACTGATACGAGCAGCCCCAATCAG |

Protein-DNA complexes were separated electrophoretically at 4 °C in 
a 4% polyacrylamide gel (acylamide:bisacylamide/80:1) at 15 Vcm. 
The gel was polymerized in, and the running buffer consisted of 0.19 M 
glycine (pH 8.3), 25 mM Tris, 0.5 mM EDTA, and where indicated 60 µM 
ZnSO$_4$. After electrophoresis, the gel was dried and labeled complexes 
were detected by autoradiography.

Proteolytic EMSA—The effects of digestion with thrombin or trypsin 
on MRE-s binding activity were examined as follows. Nuclear proteins 
(30 µg) from H$_2$O$_2$- and ZnSO$_4$-treated Hepa cells (1 h after treatment) 
and recombinant MTP-1 (0.25 µl of a 50-µl reaction described below) 
were added to the complete binding reaction containing labeled MRE-s 
and incubated at 4 °C for 20 min to allow DNA binding. In some binding 
reactions, a solution of 2.5% trypsin (Life Technologies, Inc.) diluted 
1:100 in Hanks’ balanced salt solution was added (1 µl/20 µl binding 
reaction), and they were incubated for 15 min at 37 °C. Soybean trypsin 
inhibitor (Life Technologies, Inc.; 100 µg in 1 µl) was then added, and 
the samples were analyzed by gel electrophoresis. In other binding 
reactions, thrombin (Boehringer Mannheim) was added (8 x 10$^{-3}$ 
units/2 µl) and the mixtures were incubated at room temperature for 15 
min. These samples were immediately chilled on ice and analyzed by gel 
electrophoresis. Specificity of the binding was confirmed by competition 
with excess unlabeled MRE-s, and protein-DNA complexes were sepa-
rated electrophoretically, as described above.

In Vitro Transcription/Translation of MTP-1—A cDNA clone for 
mouse MTP-1 was generated using reverse transcriptase-PCR from 
mouse liver RNA with the following primers.

| MTP-1(S)+91: | 5’-GGATCGTGAAGGATCATGCCCAGACAGGACACACAG-3’ |
| MTP-1(AS)+2125: | 5’-GGTCTAGAGAAAGATCTGGAAGGGGCGGCGGAGC-3’ |

Sequences 9 and 10

Primers contained XbaI or ClaI sites (bold bases), plus two terminal 
stabilizing bases, for ease of cloning and they directed amplification of 
the complete coding sequence of mouse MTP-1. Reverse transcription-
PCR was conducted as described (33) using mouse liver total RNA. The 
MTP-1 cDNA was cloned between the XbaI-ClaI sites of pGEM7 (Prom-
ega Biotech, Madison, WI) and sequenced using Sequenase (Strat-
agen). MTP-1 was synthesized in vitro using a TnT coupled reticulo-
cyte lysate system (Promega Biotech), containing 1 µg of the MTP-1 
plasmid, according to the manufacturer’s suggestions.
Oxidative Stress Activates MTF-1

RESULTS

Activation of Transcription from the MT-I Promoter by tBHQ Involves MREs—We previously reported that transcriptional induction of the mouse MT-I gene by H$_2$O$_2$ requires the promoter region between –153 and –42, relative to the transcription start point (15). This region contains five MREs and a composite MLTF/USF/ARE. We also reported that four or five tandem copies of these elements independently conferred response to H$_2$O$_2$ on a reporter gene. In contrast, only MREs conferred response to zinc. To further examine the regulation of MT-I gene expression by oxidative stress, the effects of the planar aromatic compound β-NF and the phenolic antioxidant tBHQ on MT-I gene expression were determined. β-NF is a bifunctional inducer that activates both phase I (e.g. CYP1a1 P-450) and phase II (e.g. glutathione S-transferase Ya) detoxification systems, whereas tBHQ is monofunctional and induces phase II genes (34–36).

Incubation of Hepa cells in medium containing β-NF (50 or 100 μM) did not induce MT-I mRNA, which suggested that this compound may not be metabolized by these Hepa cells. Thus, effects of β-NF were not further examined. In contrast, tBHQ caused a concentration-dependent induction of MT-I mRNA (Fig. 1), and 400 μM tBHQ caused an approximately 40-fold increase in MT-I mRNA by 8 h. Treatment of cells with tBHQ also dramatically induced heme oxygenase-1 mRNA, but did not result in significant induction of the MLTF/ARE construct; RSV-CAT, driving CAT. Treatment of Hepa cells with 200 μM tBHQ resulted in 4.4-fold increase in CAT activity from the MRE-d5-CAT construct; RSV-CAT, driving CAT. Treatment with tBHQ did not increase CAT activity from an RSV-CAT vector or from the minimal MT promoter construct (–42). These results demonstrate the mouse MT-I gene is transcriptionally regulated by tBHQ, consistent with a role for ROS in regulating MT gene expression, and suggest that this induction is mediated by MREs.

In Vivo Genomic Footprinting of the MT-I Promoter Reveals That Occupancy of the MREs Is Induced in Response to H$_2$O$_2$, tBHQ, and Zinc—In vivo genomic footprint analysis was accomplished by LM-PCR of bases –200 to –30 in the MT-I promoter (Figs. 3 and 4). Cells, before or after treatment with an inducer, were incubated with dimethyl sulfate, and genomic DNA was isolated, cleaved with piperidine, and MT-I promoter fragments were specifically amplified. Guanine residues involved in protein-DNA interactions were visualized as either less intense (protected) or more intense (hypersensitive) compared with invariant G residues in the promoter, and by comparison with DNA from untreated control cells. Naked genomic DNA was methylated and similarly amplified to provide a G-ladder and to reveal constitutive interactions in the MT-I promoter in control cells. Footprint experiments with each inducer were conducted several times, and the results were reproducible.

In control cells, a strong constitutive footprint was evident on the guanine-rich sense strand (Fig. 3A) over an Sp1 binding site in the proximal promoter region. Oxidative stress activates MTF-1 26235

Fig. 1. Concentration-dependent induction of MT-I mRNA in Hepa cells by tBHQ but not β-NF. Hepa cells were incubated for 6 h in medium containing β-NF or tBHQ in the indicated concentrations (0–400 μM). As a positive control, Hepa cells were also treated for 3 h with 2.5 mM H$_2$O$_2$. Total RNA was analyzed by Northern blotting using a $^{32}$P-labeled MT-I antisense RNA probe. Hybridization signals were visualized by autoradiography and quantitated by phosphoimage analysis.

Fig. 2. Delineation of MT-I promoter elements involved in the transcriptional response to tBHQ. Hepa cells were cotransfected with calcium phosphate precipitates of the indicated CAT fusion genes plus SV-β-gal as an internal control. Promoter constructs were as follows: –153 to –62 relative to the transcription start point in the MT-I gene; deletion of the MLTF/ARE region (–100 to –89) in –153; MRE-d5, five tandem copies of MRE-d ligated in front of a minimal MT-I promoter that extends to –42; MLTF/ARE4, four tandem copies of the MLTF/ARE in the –2 construct; RSV-CAT, driving CAT. Treatment of Hepa cells with 200 μM tBHQ resulted in 4.4-fold increase in CAT activity from the MRE-d5-CAT construct; RSV-CAT, driving CAT. Treatment with tBHQ did not increase CAT activity from an RSV-CAT vector or from the minimal MT promoter construct (–42). These results demonstrate the mouse MT-I gene is transcriptionally regulated by tBHQ, consistent with a role for ROS in regulating MT gene expression, and suggest that this induction is mediated by MREs.

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site (−187 to −179); in addition, there was a constitutive footprint over the MLTF/ARE (Figs. 3, A and B, and 4c). Similar results have been reported previously (41). In contrast, little constitutive footprinting of MRE core sequences, with the exception of a weak footprint over MRE-d, was detected (Figs. 3, A and B, and 4b). An apparently stronger constitutive footprint over MRE-d was reported previously (41), which may reflect the cell type or culture conditions in those experiments. Please note that the histograms do not indicate constitutive footprints, as the data were derived by comparisons of footprints from induced cells with those from uninduced cells (in vivo control).

After treatment with H$_2$O$_2$, tBHQ, or zinc, footprints were rapidly induced over five of the MREs (Fig. 3, A and B). Footprinted regions at the time of maximal occupancy are shown enlarged and quantitated for MRE-c and MRE-d (Fig. 4, panels a and b), because these two MREs were more strongly footprinted. In contrast, the footprint over MRE-a was weak. It is noteworthy that G residues within each MRE core sequence (TGRCnC) that are functionally essential (28) were protected from methylation. Remarkably, with all three inducers the footprint patterns were essentially identical over the MREs, but the temporal appearance of these footprints differed. Footprints induced by H$_2$O$_2$ appeared within 30 min of treatment, but were absent by 5 h. This is consistent with the rapid, transient induction of MT-I gene transcription by H$_2$O$_2$ (15). In contrast, the MRE footprints induced by 200 μM tBHQ were detected by 1 h, but were maintained for at least 5 h. These footprints were notably less intense than those induced by H$_2$O$_2$ or zinc. However, 400 μM tBHQ, which maximally induced MT mRNA (Fig. 1), induced strong and prolonged footprints over these MREs. It is certainly conceivable that MRE occupancy and subsequent transcriptional activation of the MT gene are both modulated.

In addition to footprints induced over MREs, all of these treatments also induced footprints over the MLTF/ARE region (Fig. 3, A and B; Fig. 4, panel c). 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 MLTF binding site and immediately downstream of the ARE. The footprint

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**Fig. 3.** In vivo genomic footprinting of the mouse MT-I promoter in H$_2$O$_2$-, tBHQ-, and zinc-treated Hepa cells. Hepa cells were incubated in medium containing the indicated concentrations of zinc, H$_2$O$_2$, or tBHQ. After the indicated treatment period, Hepa cells were incubated in medium containing 0.1% dimethyl sulfate to methylate guanine residues and genomic DNA was then purified. To generate a G-ladder, purified MT-I promoter DNA was methylated in vitro. Methylated DNA was cleaved with piperidine, and the MT-I promoter region from −200 to −30 of the sense strand (A) and antisense strand (B) were specifically amplified using LM-PCR. LM-PCR products were separated on a 6% sequencing gel and detected by autoradiography. Locations of known regulatory elements in the MT-I promoter are indicated. Sp1, Sp1 binding site; MRE, metal regulatory elements; MLTF/ARE, overlapping major late transcription factor binding site and antioxidant response element. Constitutive footprints can be detected in the 0 h control.
over the MLTF/ARE induced by H$_2$O$_2$ was transient, while those induced by tBHQ and zinc were prolonged. It is somewhat surprising that zinc induced a footprint over the MLTF/ARE; however, a similar result was reported previously (41). This region does not contain an MRE core sequence, and an oligonucleotide with this sequence does not interact with MTF-1 (see below). These results revealed little evidence of protein interactions with guanine residues within the ARE functional core (GTGACnnnGC) (31, 42).

H$_2$O$_2$, tBHQ, and Zinc Each Rapidly Induce MRE Binding Activity in Hepa Cells—EMSA was used to detect MRE binding activity in nuclear extracts from Hepa cells. MRE-d is the best studied MRE in the mouse MT-I promoter (8, 29, 43), but it is bound by two transcription factors, Sp1 and MTF-1 (43). To reduce the complexity of analyzing MTF-1 binding to the MRE, we employed a consensus MRE oligonucleotide (MRE-s) that is a strong MTF-1 binding site, but does not bind Sp1 (11).

EMSA analysis using nuclear extracts obtained from Hepa cells treated for 45 min with H$_2$O$_2$ revealed an 8-fold increase in MRE-s complex formed (Fig. 5A). Formation of this complex was effectively inhibited by a 250-fold molar excess of unlabeled MRE-s or MRE-d oligonucleotides, but not by a similar molar excess of a mutant MRE in which the bases that constitute the MRE functional core were mutated (Fig. 5, A and B). Furthermore, Sp1 binding site and MLTF/ARE oligonucleotides did not compete for MRE-s complex formation. Thus, formation of this complex displayed similar DNA sequence specificity to recombinant MTF-1 (11, 43) and was rapidly increased in amount during oxidative stress. This complex has the same electrophoretic mobility shift as that formed between MRE-s and mouse MTF-1 synthesized in vitro in a coupled transcription/translation system (Fig. 6).

Antibodies that supershift MTF-1 are not available; therefore, to provide further evidence that the MRE-s binding activity in nuclear extracts from Hepa cells induced with H$_2$O$_2$ is MTF-1, a proteolytic EMSA was performed in which the proteinase-resistant MRE-s complexes from Hepa cells were compared with those from recombinant MTF-1 (Fig. 6, A and B). Trypsin digestion of preformed MRE-s complexes resulted in the rapid appearance of a predominant smaller complex that displayed the same mobility shift using Hepa cell nuclear extracts and recombinant MTF-1 (Fig. 6A). Thrombin digestion of preformed MRE-s complexes resulted in the appearance of a cluster of three predominant smaller complexes that displayed the same mobility shifts using Hepa cell nuclear extracts and recombinant MTF-1 (Fig. 6B). Identical results were obtained using nuclear extracts from Hepa cells treated with zinc. A relatively weak signal in the proteinase digested nuclear extract lanes (Fig. 6, A and B, lane 4) was noted in both experiments, but we found that mixing those extracts with recombinant MTF-1 before EMSA also resulted in a weakened signal (data not shown). Proteinase digestion of these nuclear extracts and of recombinant MTF-1 in the absence of MRE-s led to the rapid and complete loss of subsequent MRE-s binding activity (data not shown). These results strongly suggest that the major MRE-s complex in Hepa cell nuclear extracts during oxidative stress contains MTF-1 (Fig. 6).

The MT-I promoter are shown in Fig. 3. These samples represent the treatment time and dosage for maximal occupancy of the MREs with these inducers. For these selected DNA samples, the regions of the LM-PCR sequencing gel corresponding to MRE-c (panel a), MRE-d (panel b), and MLTF/ARE (panel c) are shown enlarged (A). Band intensities were quantitated by phosphoimage analysis of dried gels and protection of $\pm$20% and hypersensitivity of $\pm$40% of individual guanine residues in treated cells compared with those in control cells were calculated as detailed under “Experimental Procedures” and are shown for both the sense and antisense strands (B).
Saturation analysis of MRE-s binding activity in nuclear extracts from control and H2O2-treated Hepa cells suggested that differences in MTF-1 activity between these extracts reflected increased binding activity, without an apparent change in the affinity of MTF-1 binding (data not shown). Although MTF-1 binding activity was increased in nuclear extracts from H2O2-treated cells, the binding activities of Sp1 (Fig. 7) and USF (Figs. 8 and 9) were not.

During preparation of Hepa cell nuclear extracts (25), EDTA was omitted from all buffers to avoid potentially removing zinc from MTF-1. During the EMSA shown in Fig. 5, zinc (60 μM) was included in the binding reaction and electrophoresis buffer, as described previously (11). However, exogenous zinc

![Figure 5](image-url)  
**FIG. 5.** Detection of MTF-1-like binding activity in nuclear extracts from H2O2-treated Hepa cells using EMSA. Hepa cells were incubated in medium containing H2O2 (2.5 mM) for 45 min, and nuclear proteins were extracted. Nuclear extracts (5 μg protein) from control and H2O2-treated cells were incubated with a 32P-labeled MRE-s oligonucleotide, which represents a high affinity binding site for MTF-1. The binding reaction and electrophoresis buffer contained 60 μM zinc. The arrow indicates the specific MTF-1 complex. A, where indicated, the binding reaction contained a 250-fold molar excess of unlabeled MRE-s, MRE-d, or HSV Sp1 oligonucleotide. B, where indicated, the binding reaction contained a 250-fold molar excess of unlabeled MRE-s, MRE-d, a mutant MRE in which the functional core was mutated (mutMRE), HSV Sp1, or MLTF/ARE oligonucleotide.

![Figure 6](image-url)  
**FIG. 6.** Proteolytic EMSA comparison of MRE-s complexes formed in Hepa cell nuclear extracts with those formed with recombinant mouse MTF-1 synthesized in a coupled *in vitro* transcription/translation system. EMSA was performed using labeled MRE-s and a nuclear extract from H2O2-treated Hepa cells (see Fig. 5) or an aliquot (0.25 μl of a 50-μl reaction) from a coupled transcription/translation reaction containing *in vitro* synthesized mouse MTF-1 (INTL MTF1). The *in vitro* translated MTF-1 was activated to bind DNA by the addition of 30 μM zinc to the binding reaction, followed by incubation at 37 °C for 15 min before EMSA. In the absence of exogenous zinc, as well as in the “coupled” reaction lacking MTF-1 cDNA template, no specific MRE-s binding activity (left side arrow) was detected (data not shown). Specificity of binding was confirmed by competition with excess unlabeled MRE-s as described above (data not shown). A, the effects of trypsin on MRE-s-protein complexes with recombinant MTF-1 (*lanes 1 and 3*) and in nuclear extracts from H2O2-treated Hepa cells (*lanes 2 and 4*) were examined. Nuclear proteins (30 μg) and recombinant MTF-1 in the complete binding reaction were incubated with trypsin (described under “Experimental Procedures”) for 15 min at 37 °C. Soybean trypsin inhibitor was then added, and the samples were analyzed by gel electrophoresis. The right side arrow indicates the trypsin-resistant specific MRE-s protein complex.

B, thrombin was added to binding reactions as in A, which were incubated at room temperature for 15 min immediately before gel electrophoresis. *Lanes 1 and 3*, recombinant MTF-1; *lanes 2 and 4*, Hepa nuclear extract. The right side arrows indicate thrombin-resistant specific MRE-s protein complexes.
was not required for MRE-s complex formation in these nuclear extracts (Fig. 7), and it was omitted in subsequent EMSA. Under these experimental conditions, nuclear extracts from H₂O₂-treated (30 min) cells had increased MTF-1 binding activity, as did those from cells treated with zinc. Furthermore, tBHQ-treated (200 μM) cells also displayed increased MTF-1 binding activity. Although the amount of complex formed was much less than that from extracts of untreated or H₂O₂-treated cells, it was noted that 400 μM tBHQ induced much more MTF-1 binding activity (data not shown). The electrophoretic mobility shift of the MRE-s complex from zinc-, tBHQ-, and H₂O₂-treated cells was identical. In Fig. 7, MTF-1 and Sp1 binding activities in the same extracts were compared to confirm the specificity of activation of MTF-1 by zinc and oxidative stress, and as an internal control for extract loading and quality. The HSV Sp1 binding site was used in this analysis. This oligonucleotide has two Sp1 binding sites, and thus gives rise to a complex shift pattern as reported previously (43).

**FIG. 7.** Detection of MTF-1 and Sp1 DNA binding activities in nuclear extracts from Hepa cells treated with H₂O₂, zinc, or tBHQ using EMSA. Hepa cells were incubated in medium containing H₂O₂ (2.5 mM), zinc (60 μM), or tBHQ (200 μM) for 30 min. Nuclear proteins (10 μg) were extracted and EMSA was performed using ³²P-labeled MRE-s and Sp1 oligonucleotides, as indicated. EMSA was performed as described in the legend to Fig. 5, except that exogenous zinc was omitted from all buffers. These results were reproduced in three separate experiments.

**FIG. 8.** Detection of MTF/ARE binding activities in nuclear extracts from control and H₂O₂-treated Hepa cells. Hepa cells were incubated for 30 min in medium containing H₂O₂ (2.5 mM). Nuclei from control and treated cells were then isolated and nuclear extracts prepared. EMSA was performed using a ³²P-labeled MTF/ARE oligonucleotide. Where indicated, the binding reaction also contained a 250-fold molar excess of the following unlabeled competitor oligonucleotides: MTF/ARE, MTF/mutARE in which the terminal GC nucleotides in the ARE consensus sequence were mutated, and mutMTF/ARE in which the first three bases in the MTF binding site were mutated. MLTF Ab denotes an reaction in which antibody against USF-1 and -2 was added to the binding reaction before EMSA as detailed under "Experimental Procedures."

**FIG. 9.** Examination of AP-1 binding activity in nuclear extracts from TPA-treated NIH 3T3 cells and from Hepa cells treated with H₂O₂ or tBHQ. NIH 3T3 cells incubated in medium containing phorbol ester (10 ng of TPA/ml) for 1 h before nuclear extract preparation. Nuclear extracts were prepared from Hepa cells incubated in medium containing H₂O₂ (2.5 mM) or tBHQ (200 μM) for 30 min. A, EMSA was performed using a ³²P-labeled MTF/ARE oligonucleotide. B, EMSA was performed using a ³²P-labeled TRE oligonucleotide. AP-1 Ab denotes an antibody competition experiment in which antibody against the c-jun family was added to the binding reaction before EMSA, as described under "Experimental Procedures." Arrows point to specific protein-DNA complexes.
binding activities were sequence-specific, and complex formation was inhibited by a 250-fold molar excess of unlabeled MLTF/ARE oligonucleotide (Fig. 8). Mutation of the two functionally important (31, 42) terminal bases (GC) in the ARE consensus sequence (GTGACnnnGC) in the competitor oligonucleotide (MLTF/mutARE) completely inhibited formation of the faster migrating complex, but had less of an inhibitory effect on formation of the slower migrating complex. In contrast, mutation of the functionally important (16) first three bases of the MLTF binding site (CCCGTGAC) in the competitor oligonucleotide completely inhibited formation of the slower migrating complex, but had no effect on the faster migrating complex. These results suggested that two factors bind to the MLTF/ARE. The faster migrating complex contains USF, whereas the slower migrating complex likely contains ARE-specific binding activity. That the faster migrating complex contained USF was confirmed by antibody competition analysis (Fig. 8). In these experiments, the USF antiserum did not alter the ARE-specific complex or Sp1 binding activity (data not shown). The identity of the factors that bind to the ARE is investigated below.

AP-1 Is Not a Major ARE Binding Activity and Is Not Rapidly Induced in H$_2$O$_2$- or tBHQ-treated Hepa Cells—AP-1 has been shown to bind to AREs (19), but several studies suggest that ARE function is not mediated by nor dependent on AP-1 (31, 35, 36). To examine AP-1 activity in Hepa cell extracts, an antibody against the c-jun family, that blocks binding of c-jun-containing AP-1 complexes, was used in EMSA. Nuclear proteins from TPA-treated NIH 3T3 cells served as a positive control for AP-1 activity, and using the MLTF/ARE oligonucleotide, formation of one major complex was detected in this extract. The AP-1 antibody inhibited formation of this complex by greater than 90% (Fig. 9A). Thus, Jun-containing AP-1 complexes can also bind to the mouse MT-I MLTF/ARE. However, this AP-1 antibody did not significantly prevent the formation of either of the two MLTF/ARE-specific complexes in nuclear extracts isolated from control, H$_2$O$_2$-, or tBHQ-treated Hepa cells. In addition, the AP-1 antibody did not affect Sp1 binding activity in these extracts (data not shown).

To further examine AP-1 activity in Hepa cells during oxidative stress, nuclear extracts from H$_2$O$_2$- or tBHQ-treated cells were used in EMSA with a consensus TRE oligonucleotide (Fig. 9B). As a positive control, a single specific complex was readily detected in nuclear proteins from TPA-treated NIH 3T3 and the AP-1 antibody inhibited greater than 80% of this binding. In contrast, only a low amount of a TRE-specific complex was detected in nuclear extracts from H$_2$O$_2$- and tBHQ-treated Hepa cells, and formation of this complex was reduced 30–50% by the AP-1 antibody. The amount of this TRE complex was not increased after H$_2$O$_2$- or tBHQ treatment, and cross-competition experiments demonstrated that excess unlabeled TRE oligonucleotide did not efficiently inhibit formation of the MLTF/ARE slow migrating complex. Thus, AP-1 activity is not rapidly induced during oxidative stress in Hepa cells, and AP-1 does not represent a major component of the MLTF/ARE binding activity in these cells.

DISCUSSION

MT protects cells against oxidative stress. In yeast, mammalian MT can functionally replace superoxide dismutase (5), and mouse cells with targeted disruption of the MT-I and -II genes are more susceptible to organic peroxides than are cells with a single or two wild-type alleles (6). Furthermore, overexpression of MT protects mammalian cells against oxidative stress and can dramatically reduce the level of intracellular oxygen radicals (7). In vitro, MT is an efficient scavenger of hydroxyl radicals (44). Consistent with this hypothesized protective function, mouse MT gene transcription is rapidly and dramatically induced by oxidative stress (H$_2$O$_2$, menadione, tBHQ) in Hepa cells (15), as well as by tBHQ in AR42J, NIH3T3, and L929 cells. Recently, H$_2$O$_2$ has been reported to induce MT in human retinal pigment epithelial cells (45). Thus, induction of MT genes by oxidative stress occurs in many cell types.

Herein, we further explored the mechanisms of regulation of MT-I gene transcription during oxidative stress. A number of convergent experimental approaches yielded results consistent with the conclusion that induction of mouse MT-I gene transcription by tBHQ, as well as by H$_2$O$_2$ (15), is mediated, in part, by MREs in the proximal promoter region. Interestingly, the heme oxygenase-I (46) and several acute-phase genes are also induced by metals (47), which may suggest that MREs are important promoter elements in several protective genes. In vivo genomic footprint analysis and EMSA suggest that MT-I gene MRE activity is modulated during oxidative stress by the binding of the transcription factor MTF-1. Manipulation of MTF-1 expression by targeted deletion of both genes in ES cells (48) or by expression of antisense MTF-1 (49) eliminates metal responsiveness of the MT-I gene. Thus, this transcription factor plays a key role in regulating MT gene expression in response to metal ions and oxidative stress.

Mouse MTF-1 contains six zinc fingers and three different putative transactivating domains (50). Mechanisms of activation of MTF-1 are not well understood. It has been postulated that this transcription factor is constitutively active in the absence of a metal-sensitive inhibitor (11, 49), although such an inhibitor has not been isolated. Another model for MTF-1 activation, proposed previously (11), suggests that MTF-1 has a lower affinity for zinc than other transcription factors and that it is reversibly activated in response to free zinc levels in the cell. Our finding that mouse MTF-1 DNA binding activity is rapidly activated (10-fold in 30 min) in Hepa cells treated with zinc or oxidative stress-inducing agents, and the distinct similarities in the genomic footprints of the MT-I promoter induced by zinc and oxidative stress are consistent with a central role for free zinc in the activation of this transcription factor during oxidative stress. Oxidative stress may cause the release of bound intracellular zinc by oxidation of thiols and lipids. Oxidized glutathione, which is increased during oxidative stress, has been shown to mobilize zinc from metallothionein (51).

Data presented herein do not allow one to distinguish between these proposed models for mechanisms of activation of MTF-1 by zinc. Furthermore, other mechanisms of activation of MTF-1 are plausible. If MTF-1 binding is controlled by an inhibitory molecule, a transcription factor that might serve as a paradigm for the control of MTF-1 is NF-kB. This transcription factor is regulated by a family of inhibitory proteins, IxB, that dissociate in response to a number of different signals including oxidative stress (52). It is also possible that MTF-1 is activated in a manner similar to that of the bacterial transcription factor OxyR (53). OxyR apparently becomes oxidized and activated in response to an ROS-induced shift in the redox environment within the cell. Once MTF-1 is activated to bind to DNA, its transactivating potential may also be regulated. Oxidative stresses can enhance tyrosine phosphorylation of the epidermal growth factor receptor (54), phosphorylation of IxB (55, 56), and Jun kinase (mitogen-activated protein kinase) activity (57). Mouse MTF-1 contains several potential sites of phosphorylation (50), but no studies of the phosphorylation state of the protein have been reported.

Evidence from other investigators suggests the paramount
importance of the ARE in response to oxidative stress (19), but deletion of the MLTF/ARE in the MT-I promoter reduced but did not eliminate response to H$_2$O$_2$ (15), and had little effect on response to tBHQ. We obtained similar results in studies of the chicken MT promoter. Furthermore, the MLTF/ARE element alone (four copies) can direct response of a minimal promoter to H$_2$O$_2$ (15), but not to tBHQ in transient transfection assays. Transcription of the mouse glutathione S-transferase Ya gene during oxidative stress is controlled by two adjacent electrophile response elements that are identical in core sequence to the ARE and similar to AP-1 binding sites. These elements cooperate with the transcription factor Ets to mediate response to oxidative stress (58). Thus, interactions between MT-I promoter elements may modulate activity of the MLTF/ARE.

Although the mouse MT-I gene MLTF/ARE was found to interact with AP-1, this was a minor binding activity in Hepa cell nuclear extracts, and it was not increased under these experimental conditions. However, the demonstration that AP-1 can interact with the MLTF/ARE suggests that it could potentially be involved in regulating mouse MT-I gene expression. AP-1 is thought to regulate mouse glutathione S-transferase Ya (58, 59) and human quinone reductase (19) gene expression via induction bound to the ARE/TRE. In addition to the well-documented regulation of AP-1 at the transcriptional level, AP-1 binding is redox regulated by Ref-1 (60). Furthermore, its ability to stimulate gene transcription during oxidative stress is regulated by phosphorylation (57). Thus, the transactivating potential of preexisting AP-1-TRE complexes can be modulated. In this light, it is also important to note that electrophilic quinones, in particular tBHQ, have recently been shown to preferentially induce Fra-1, which forms an inactive heterodimer with Jun, and thus antagonizes AP-1 activity (36).

Induction of Fra-1 by tBHQ in Hepa cells might explain the inability of this construct to transactivate through the MT-I MLTF/ARE. H$_2$O$_2$, on the other hand, is a poor inducer of Fra-1 (61).

In contrast to AP-1 interactions with the ARE, the constitutive binding of as yet uncharacterized proteins to the ARE has been demonstrated (19, 31). Consistent with those studies, EMSA demonstrated the binding of USF and a predominant ARE-specific non-AP-1 factor to the mouse MT-I gene MLTF/ARE. A functional ARE from the rat glutathione S-transferase Ya gene does not bind AP-1, but if it is mutated to an ARE-TRE, it can bind AP-1 yet retain inducibility by oxidative stress (31). Thus, protein interactions with the ARE are complex in several genes, and the functional significance of the ARE may depend upon its context within a promoter, flanking nucleotides, inducer, and/or the cell-type being analyzed. Due to the complex regulation of factors that interact with AREs, understanding the nature of the transcriptional control through the MLTF/ARE will require further experimentation using alternative approaches.

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Oxidative Stress Activates Metal-responsive Transcription Factor-1 Binding Activity: OCCUPANCY IN VIVO OF METAL RESPONSE ELEMENTS IN THE METALLOTHIONEIN-I GENE PROMOTER

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