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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

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ABSTRACT

Synthesis of metallothionein-I (MT-I) and heme oxygenase mRNAs is rapidly and transiently induced by H_2O_2 in mouse hepatoma cells (Hepa) and this effect is blocked by catalase. Menadione, which generates free radicals, also induces these mRNAs. Deletion mutagenesis revealed that a region between −42 and −153 in the mouse MT-I promoter was essential for induction of a CAT reporter gene. A multimer of a 16 bp sequence (−101 to −86) that includes an antioxidant response element and overlapping adenovirus major late transcription factor binding site elevated basal expression and allowed induction by H_2O_2 when inserted upstream of a minimal promoter. However, deletion of this region (−100 to −89) from the intact MT-I promoter (−153) did not completely eliminate response. Multiple copies of a metal response element also permitted response to H_2O_2. These results suggest that induction of MT-I gene transcription by H_2O_2 is mediated by at least two different elements within the proximal MT-I gene promoter and suggest a previously undescribed function of the MRE. Induction of MT gene transcription by ROS and the subsequent scavenging of ROS by the MT peptide is reminiscent of the metal regulatory loop and is consistent with the hypothesized protective functions of MT.

INTRODUCTION

Generation of reactive oxygen species (ROS) occurs during numerous normal and pathological processes, including regression of the corpus luteum after ovulation, metabolism of compounds such as paraquat or menadione, ischemia reperfusion, and gram negative sepsis (1). In gram negative sepsis ROS are generated both in response to lipopolysaccharide (LPS) and in response to cytokines induced by LPS (2−4). ROS are cytotoxic, thus considerable effort has focused on elucidating the cellular mechanisms of protection from free radicals.

Metallothioneins (MT) are small cysteine-rich heavy metal binding proteins (5−7), whose function(s) are not clearly defined (8). Over-expression of MT can protect against heavy metal toxicity, whereas under-expression in cell lines or in mice with null mutations of the MT-I and MT-II genes leads to heightened sensitivity to cadmium (Cd) (5,9), suggesting a protective role for MT. In the absence of Cd, MT is isolated predominantly as Zn_7-MT, although copper (Cu) is also detected (7), and MT gene transcription is rapidly induced by these metals (10), suggesting a role for MT in essential metal homeostasis. MT is an efficient scavenger of hydroxyl radicals (11,12), and it has recently been shown that yeast and mammalian MTs can functionally substitute for superoxide dismutase in protecting yeast from oxidative stress (13). Furthermore, agents that induce oxidative stress can rapidly induce MT-I and MT-II mRNAs in the rodent liver (14−17). Thus, it has also been suggested that MT also plays a protective role from oxidative stress.

LPS and cytokines (18−22), phorbol esters (23), other non-metallic compounds (e.g. chloroform, turpentine, carbon tetrachloride), and xenobiotics (e.g. diethyl maleate, paraquat, menadione) (14−17,24) can induce MT, and each induces the formation of free radicals. The molecular mechanisms of induction of mouse MT by these oxidative stress-inducing agents, however, are unknown (5,15,16). This report documents that treatment of a mouse hepatoma cell line (Hepa cells) with H_2O_2 results in rapid transcriptional activation of the MT-I gene. This response is dose-dependent, rapid, transient and additive with metals. Mapping of the *cis*-acting MT-I promoter sequences required for this transcriptional response suggests roles for a composite major late transcription factor/antioxidant response element (MLTF/ARE) (25−27) as well as for metal response elements (MRE) (10).

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MATERIALS AND METHODS

Cell culture

The mouse hepatoma cell line, Hepa, was provided by Dr Ann Smith (University of Missouri at Kansas City) and has been described previously (28). Hepa cells were maintained in DMEM–high glucose supplemented with 2% fetal bovine serum. In all experiments, cells were grown to confluence in 100 mm dishes containing 5 or 10 ml of medium. The medium was changed, and 18 h later the cells were treated by direct addition of the indicated compound(s) into the culture medium. For preparation of RNA, cells were treated, scraped from the dish, the cell pellet collected by centrifugation at $500 \times g$ for 5 min, quickly frozen on dry ice, and stored at −70°C until needed. H_2O_2 and menadione (Sigma Chemical Co., St Louis, MO) were prepared fresh in water or DMSO, as 250–1000× concentrated stocks, respectively, and used immediately. Where indicated, catalase was added to H_2O_2 as follows: catalase [10 mg/ml in phosphate-buffered saline (PBS); Sigma Chemical Co.] was added in 10 μl aliquots to a solution of 1 M H_2O_2 until gas evolution was severely reduced. In parallel experiments, solutions of H_2O_2 were treated with the same final volume of a heat-denatured solution of catalase. $ZnCl_2$ was dissolved in acidified water at a 1000× concentration.

Isolation of total RNA

RNA was prepared by an initial guanidine thiocyanate/phenol/chloroform extraction as described by Chomczynski *et al.* (29) followed by sodium dodecyl sulfate/phenol/chloroform extractions as described by Andrews *et al.* (30).

Northern blot hybridization

RNA was denatured for 5 min at 65°C in a solution containing MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7), 50% formamide and 2.2 M formaldehyde. Denatured RNA (2 μg in 5 μl) was size separated by electrophoresis in a 1% agarose–formaldehyde gel as described (30), transferred to Nytran membranes (Schleicher and Schuell, Keene, NJ) and cross-linked by UV irradiation (Stratalinker, Spectronics Corp., Westbury, NY). Northern blots were prehybridized, hybridized and washed as described (19,30), and hybrids were detected by autoradiography at −70°C with intensifying screens and quantitated by radioimage analysis using the radioanalytic image system (Ambis Systems Inc., San Diego, CA). In all experiments, duplicate gels were stained with acridine orange to verify integrity and equal loading of RNA.

Hybridization probes

Mouse cDNA clones for MT-I (31), HSP70 (R.Morimoto, Northwestern University, Evanston, IL) and the rat cDNA clone for heme oxygenase (HO) (A.Smith, University of Missouri–Kansas City, Kansas City, MO) were inserted into pSP6 or pGEM vectors (Promega Biotec, Madison, WI) and used as templates for the synthesis of ^{32}P-labeled cRNA probes as described by Melton *et al.* (32). Probes had specific activities of $\sim 2 \times 10^9$ d.p.m./μg.

Preparation of nuclei and the nuclear run-on transcription assay

Hepa cells were scraped into ice cold PBS, collected by low speed centrifugation and washed two times with PBS. The cell pellet was resuspended at 5×10^7 cells/ml in ice-cold lysis buffer (10 mM Tris–HCl, pH 7.4, 3 mM $CaCl_2$, 2 mM $MgCl_2$). An equal volume of Nonidet P-40 (NP-40) lysis buffer (lysis buffer containing 1% NP-40) was added and the suspension was homogenized by 20 strokes in a Teflon–glass homogenizer. Nuclei were recovered by centrifugation at $500 \times g$ for 5 min at 4°C. Nuclei were resuspended in nuclei storage buffer (50 mM Tris–HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA) at a DNA concentration of between 0.7 and 1.2 mg/ml, frozen on dry ice, and stored at −70°C.

Nuclear run-on transcription was performed under the reaction conditions of Greenberg *et al.* (33). Each reaction (200 μl) contained 100 μCi of [^{32}P]UTP (800 Ci/mM; Dupont NEN, Boston, MA) and 70–120 μg DNA. Run-on reactions were carried out for 12 min at room temperature. Labeled transcripts ($1-2 \times 10^7$ c.p.m.) were recovered as described (33).

Run-on transcripts were hybridized to filter-bound DNAs [mouse MT-I gene, (34), chicken actin cDNA (D.Cleveland, Johns Hopkins University, Baltimore, MD) and pGEM 7 (Promega Biotec)]. Plasmids (3 μg) containing these DNAs were linearized by restriction enzyme digestion, denatured and bound to membranes as described (33). Nytran membranes (Schleicher and Schuell) were used in place of nitrocellulose and DNA was bound to the membrane by UV cross-linking. Prehybridization was performed as described for Northern blotting (30). Hybridization was also performed as above (30) with two exceptions. First, dextran sulfate was deleted, and second, hybridization was conducted for 48 h in 5 ml scintillation vials (10^7 c.p.m. RNA/1 ml/vial) at 68°C in a hybridization oven. After hybridization, vials were opened and immediately immersed in a large volume of 1× SSC. Nylon strips were then washed together as described (30).

Fusion gene constructs

All fusion gene constructs were in a modified pCATbasic (Promega Biotec) named pCATm, in which the Bluescript KS$^+$ multiple cloning region (MCR) replaced the majority of the pCATbasic MCR. This was accomplished by restricting pCATbasic with *Hin*dIII and blunt ending using S1 nuclease followed by restriction digestion with *Sal*I. Into this plasmid was cloned a Bluescript KS$^+$ MCR which was generated by PCR using Bluescript KS$^+$, T3 and T7 promoter primers (Promega Biotec) and Vent exo$^-$ (New England Biolabs) and cleaved by *Sal*I. The MCR of pCATm, therefore, contained from the T7 promoter to the *Sal*I site of Bluescript KS$^+$ and maintained the *Sal*I and *Xba*I sites of authentic pCAT basic. All MT-I–CAT fusions were cloned into pCATm between the *Sac*II and *Hin*dIII sites. MT-I promoter constructs were generated by PCR using oligos with 5′ MT-I promoter boundaries corresponding to the positions indicated by the construct name. These oligonucleotides included restriction sites at the 5′ end for ease in cloning. The construct named −153(−100/−89) was generated by ligation of two separate PCR products from the −153 CAT template; one from −153 to −100 and the other from −86 to +62. An antisense primer (CCGATATCACGCATAGTCACGGGCCC-CGCGTCC) complementary to the MT-I promoter from −86 to −111 introduced a G→C mutation at −100, thus creating an *Apa*I restriction site at that position. The sense primer beginning at −89 was synthesized with an *Apa*I site at the 5′ end. These two fragments were cloned as a *Sac*II–*Apa*I–*Hin*dIII fragment in pCATm. MRE-d′$_5$ and MLTF/ARE$_4$ were generated as described (35,36) (see Fig. 8). The MRE-d′ sequence (−150 to −134) (37) was cloned as 5 tandem copies

each in the forward orientation and the MLTF/ARE sequence (−101 to −86) was cloned as 4 tandem copies each in the forward orientation in front of a minimal (−42 bp) MT-I promoter in pCATm.

Transient transfection assays

Hepa cells at confluence were split 1/10 into 100 mm dishes in 10 ml of medium and allowed to attach for 18 h. The medium was changed, and after 3 h the cells were transfected using calcium phosphate (38). In brief, 1 ml of a freshly prepared calcium phosphate−DNA precipitate (containing 10 μg of DNA) was slowly added with gentle mixing to each culture dish and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 6 h. Cells were then washed twice with Dulbecco's PBS and refed with 10 ml of complete medium. After 24 h cells were treated by direct addition of the indicated compound into the culture medium. Cells were treated for 9 h, scraped from the dish, and the cell pellet was quickly frozen in a dry ice−ethanol bath and stored at −80°C until needed. All plasmids used for transfection were purified from CsCl gradients. The carrier plasmid used in transfections was pGEM7 (Promega Biotec), and SV-βGal (Promega Biotec) was co-transfected as an internal control for transfection efficiency.

Measurements of CAT and β-galactosidase activities in cell extracts

A cell lysate was prepared by repeatedly (4×) freezing the cell suspension for 4 min in a dry ice−ethanol bath and thawing for 4 min at 37°C. The lysate was centrifuged for 10 min at 11,000×g and the supernatant collected. CAT assays were conducted as described (39). Briefly, the cell lysate was heated for 10 min at 65°C and centrifuged at 11,000×g for 10 min. Chloramphenicol (0.5 M in absolute ethanol) was diluted to 10 mM in H_2O. CAT assays were conducted in 250 μl containing 20 μM [^{3}H]acetyl-CoA (1 μCi) (3 Ci/mmol; Amersham, Arlington Heights, IL) and 1 mM chloramphenicol. This was achieved by making a 5× substrate mix that was added to 200 μl of heat-treated cell extract. The reaction was then overlaid with 4.5 ml of Econofluor II (Dupont NEN) liquid scintillation fluid in a mini-scintillation vial. The amount of labeled chloramphenicol accumulating in the scintillation fluid was determined by liquid scintillation counting. CAT activity was normalized to β-galactosidase (β-Gal) activity in the same sample. Measurement of β-galactosidase was performed as described (40,41) in microtiter plates. Each well contained 70 μl of β-galactosidase buffer (90 mM phosphate buffer, pH 7.5, 9 mM KCl, 4.5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 2 mg/ml chloramphenol red α-D-galactopyranoside) and 30 μl of cell lysate plus lysis buffer. Incubation was conducted at room temperature. Under these transfection conditions, 10 μl of cell extract was sufficient to produce β-galactosidase activity 10-fold above background in 30 min.

RESULTS

Induction of MT-I mRNA by H_2O_2 and menadione

H_2O_2 rapidly induced MT-I mRNA in a dose-dependent manner in Hepa cells. Moderate induction occurred at 0.5 mM H_2O_2 and near maximal induction at 2.5 mM (Fig. 1). The magnitude of induction of MT-I mRNA was dependent not only on the final concentration of H_2O_2, but also on the volume of culture medium in which the cells were treated. Inductions of 4- to 20-fold were noted under conditions that had no discernible effects on cell viability as assessed by trypan blue exclusion 24 h post-treatment (data not shown). Induction of HO mRNA, a gene shown to respond to a variety of oxidative stresses, including H_2O_2 treatment of cultured cells (42−46), displayed a dose−response curve similar to that of MT-I mRNA (Fig. 1). In contrast, HO mRNA was not induced by Zn (Fig. 1). H_2O_2 or Zn treatment did not effect HSP70 mRNA levels (Fig. 1), suggesting the absence of a generalized stress response under these experimental conditions.

The MT-I gene can be effectively induced by several types of heavy metal ions (Zn, Cu, Cd), thus it was important to examine the possibility that induction of MT-I mRNA by H_2O_2 reflected contaminating heavy metals. An H_2O_2 stock solution

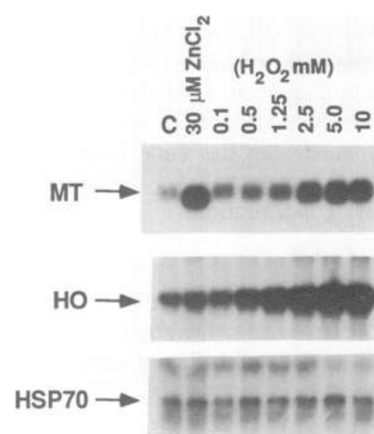


Figure 1. Dose−response effects of H_2O_2 treatment on MT-I, HO and HSP70 mRNA levels. Hepa cells were incubated for 5 h in 5 ml of medium containing the indicated concentrations of H_2O_2 or $ZnCl_2$. Total RNA was extracted, size-separated by denaturing agarose gel electrophoresis, and transferred to a nylon membrane. The membrane was hybridized successively with each of the ^{32}P-labeled antisense cRNA probes listed. Hybrids were detected by autoradiography, and the probe was stripped from the membrane before re-hybridization using another probe. MT, MT-I mRNA (0.4 kb); HO, heme oxygenase mRNA (1.6 kb); HSP70, heat shock protein 70 mRNA (2.4 kb).

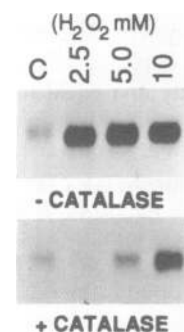


Figure 2. Effects of catalase on induction of MT-I mRNA by H_2O_2. H_2O_2 was titrated with catalase until the evolution of gas was severely reduced (+ catalase) or an equal amount of a solution of heat denatured catalase was added (− catalase). These solutions were then added in the indicated initial concentrations to Hepa cell culture medium, and the incubation was continued for 5 h in 5 ml of medium. Total RNA was extracted and analyzed by Northern blot hybridization using an MT-I cRNA probe. Hybrids were detected by autoradiography. MT, MT-I mRNA (0.4 kb).

was prepared, and one half was treated with catalase before addition to the culture medium in the indicated initial concentrations. The other half of the stock solution was similarly treated with heat-denatured catalase before use. Catalase pretreatment inhibited H_2O_2 induction of MT-I mRNA, and this effect was prevented by prior denaturation of the enzyme (Fig. 2).

To further examine potential roles of ROS in regulation of MT-I gene expression, Hepa cells were treated with menadione (Fig. 3). Menadione undergoes redox cycling leading to production of H_2O_2 and O_2^-. Menadione rapidly induced MT-I mRNA (Fig. 3) and HO mRNA (data not shown) in Hepa cells. Quantitation of the amount of radioactive probe specifically hybridized indicates that menadione, H_2O_2 and Zn are each effective inducers (Fig. 3).

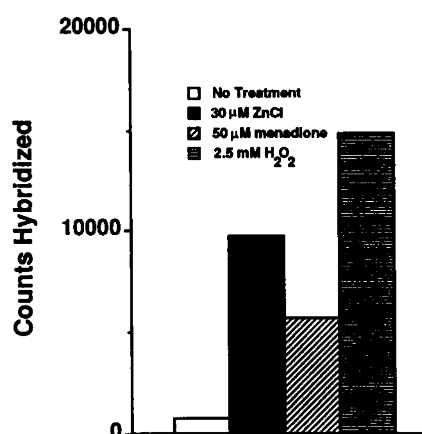


Figure 3. Induction of MT-I mRNA by menadione. Hepa cells were incubated for 5 h in 10 ml of medium containing H_2O_2, $ZnCl_2$, menadione at the indicated concentrations. The vehicle (DMSO) in which menadione was dissolved alone had no effect on MT mRNA levels (data not shown). Total RNA was extracted and analyzed by Northern blot hybridization using an MT-I cRNA probe. Hybrids were quantitated by radioimage analysis using a radioanalytic image system (Ambis Systems Inc).

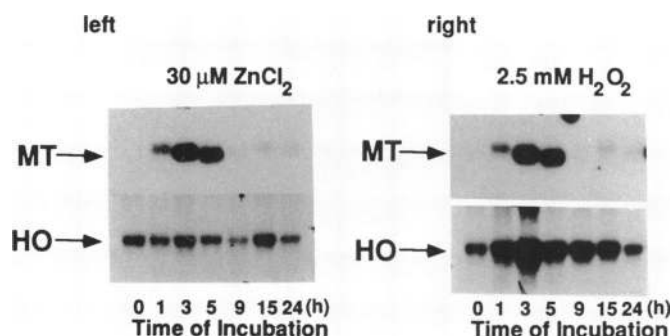


Figure 4. Time course for the effects of treatment with H_2O_2 or $ZnCl_2$ on MT-I and HO mRNA levels. Hepa cells were incubated in 10 ml of medium containing the indicated concentrations of H_2O_2 (right panel) or $ZnCl_2$ (left panel). Cells were collected at various time points. Total RNA was extracted, size-separated by denaturing agarose gel electrophoresis, and transferred to a nylon membrane. The membrane was hybridized successively with the ^{32}P-labeled HO and MT-I cRNA probes. Hybrids were detected by autoradiography, and the probe was stripped from the membrane before re-hybridization using the other probe. MT, MT-I mRNA (0.4 kb); HO, heme oxygenase mRNA (1.6 kb).

Analysis of regulation of MT-I mRNA levels by H_2O_2 and Zn

Incubation of Hepa cells with either 2.5 mM H_2O_2 or 30 μM Zn caused a rapid, transient accumulation of MT-I mRNA (Fig. 4). Increased MT-I mRNA levels were detected at 1 h, peaked by 3 h and returned to basal level by 9 h post-treatment (Fig. 4). HO mRNA levels also increased rapidly after H_2O_2 treatment and peaked at 3 h after treatment, but HO mRNA levels remained elevated for at least 15 h post-treatment (Fig. 4). As expected, Zn had no effect on HO mRNA levels. Neither Zn nor H_2O_2 altered steady-state levels of HSP70 mRNA at any time point assayed (data not shown). Transient induction of MT-I mRNA by H_2O_2 could occur if genes in treated cells became refractory to continued H_2O_2 treatment. To examine this possibility, cells were treated with 2.5 mM H_2O_2 and 3 h later additional H_2O_2 was added to the medium (either 0.5 or 2.5 mM) and the incubation was continued for 5 h (Fig. 5). Cells treated by the re-addition of 0.5 or 2.5 mM H_2O_2 maintained maximal levels of MT-I mRNA after 8 h of incubation. In comparison, MT-I mRNA levels had declined dramatically by 8 h after a single H_2O_2 treatment (Fig. 5). Thus, the MT-I gene does not become refractory to subsequent induction by H_2O_2. Although 2.5 mM H_2O_2 elicited maximal induction of MT-I mRNA on initial exposure of these cells, re-treatment with 0.5 mM H_2O_2 was sufficient to maintain maximal induction (Fig. 5).

To examine interactions between treatments with metal ions and H_2O_2 in the induction of MT-I mRNA, Hepa cells were treated with H_2O_2 along with different concentrations of Zn (Fig. 6). Treatment with 10 μM Zn did not induce MT-I mRNA under these experimental conditions, and treatment with both 10 μM Zn and 2.5 mM H_2O_2 induced MT-I mRNA to an extent indistinguishable from that of H_2O_2 alone (Fig. 6B). Treatment with 30 μM Zn induced MT-I mRNA levels similar to those observed with 2.5 mM H_2O_2, and in combination these compounds induced MT-I mRNA in an additive fashion (Fig.

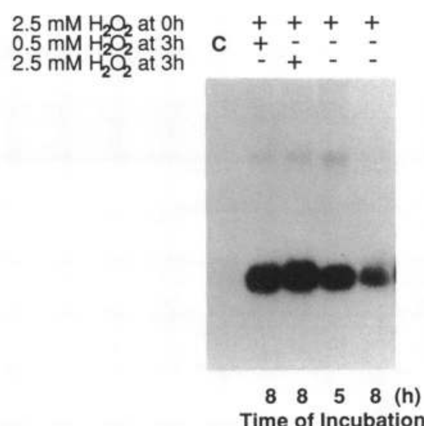


Figure 5. Effects of re-treatment with H_2O_2 on MT-I mRNA levels. Hepa cells were incubated for the indicated times in medium (10 ml) containing H_2O_2 (2.5 mM). Where indicated, fresh H_2O_2 (0.5 or 2.5 mM) was re-added after 3 h, and the incubation was continued for 5 h (8 h total incubation time). Total RNA was prepared and analyzed by Northern blot hybridization using an MT-I cRNA probe. Hybrids were detected by autoradiography. Similar results were obtained using 5 ml of culture medium. MT, MT-I mRNA (0.4 kb)

6B). Under these experimental conditions 100 μM Zn was a more effective inducer of MT-I mRNA than was 2.5 mM H_2O_2.

Induction of MT-I gene transcription by H_2O_2

To directly determine if H_2O_2 influences MT-I gene transcription, nuclear run-on assays were performed using nuclei obtained from Hepa cells treated with H_2O_2 or $ZnCl_2$ (Fig. 7). One hour after treatment with 2.5 mM H_2O_2 the relative rate of MT-I gene transcription had increased dramatically. This increase was transient, and by 3 h post-treatment the relative rate of MT-I gene transcription had returned to pretreatment levels. Treatment of Hepa cells with 30 μM Zn resulted in a similar increase in the relative rate of MT-I gene transcription at 1 h. This suggests that regulation of the MT-I gene by H_2O_2 is primarily a transcriptional response, as has been previously shown for metal ions (10,47).

Delineation of H_2O_2-responsive elements in the MT-I promoter

Transient transfection assays were employed to delineate sequences within the MT-I promoter that confer H_2O_2 response (Fig. 8). In these experiments transfected Hepa cells were treated in 10 ml of medium containing 1 mM H_2O_2 which resulted in a 3- to 5-fold peak increase in MT-I mRNA levels without significant cell toxicity. Transfected cells were found to be more sensitive to H_2O_2 toxicity. A fusion gene consisting of 742 bp of MT-I promoter (−742 CAT), numbered relative to the transcription initiation site, driving expression of the CAT gene responded both to $ZnCl_2$ and H_2O_2. Deletion of the 108 bp region located between −151 and −42 bp in the promoter abolished response to both of these inducers. This deletion also reduced basal activity, but the amount of reporter plasmid in all the transfections was adjusted (1−9 μg) to yield basal CAT activities 2- to 10-fold above background. −153 CAT responded both to $ZnCl_2$ and H_2O_2 with a similar magnitude of induction as did −742 CAT. Thus, sequences necessary for response to H_2O_2 are located within the proximal 151 bp of the MT-I promoter. Potential H_2O_2-responsive elements in this region of the MT-I promoter were identified initially by sequence similarity with the ARE consensus sequence (GTGACnnnGC) as described by Rushmore *et al.* (27). The ARE mediates H_2O_2 responsiveness of the rat glutathione S-transferase ya (GST ya) subunit and the human quinone reductase genes (48). A perfect match with the ARE consensus sequence was located in the mouse MT-I promoter (−98 to −89 bp) (Fig. 9). Interestingly, consensus AREs are also located in the proximal promoters of several other MT genes (Fig. 9). In the mouse MT-I promoter the ARE consensus sequence overlaps a previously identified MLTF binding site (−101 to −94) (49). The MLTF binding site enhances basal level expression, but does not affect metal induction of the MT-I gene (49). Deletion of the promoter region between −100 and −89 bp diminished H_2O_2 induction of −153 CAT by 2.3-fold ($P < 0.001$), but did not completely eliminate responsiveness of the fusion gene (Fig. 8). In agreement with previous studies (35,49) deletion of the MLTF/ARE reduced basal activity (6.6-fold), but had no effect on $ZnCl_2$ induction of −153 CAT. Four tandem copies of the GST ya ARE have been shown to increase basal activity from a minimal promoter and to direct induction by ROS (50). A similar approach was

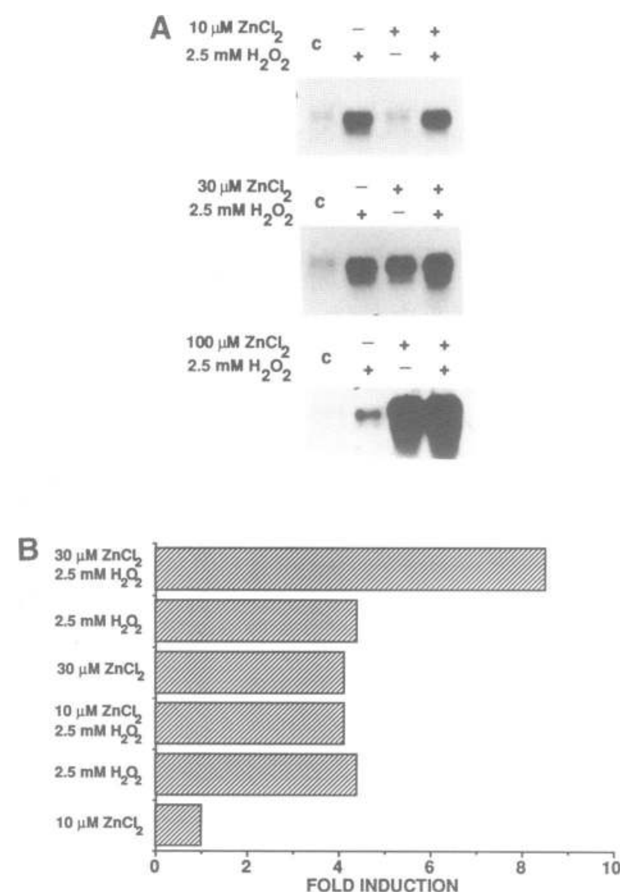


Figure 6. Effects of $ZnCl_2$ and/or H_2O_2 on MT-I mRNA levels. Hepa cells were incubated for 5 h in 5 ml of medium containing the indicated concentrations of H_2O_2 (2.5 mM) and/or $ZnCl_2$. Northern blot hybridization was performed on total RNA using an MT-I cRNA probe. (**A**) Hybrids were detected by autoradiography. (**B**) Hybrids were quantitated by radioanalytic image analysis (Ambis Systems Inc.)

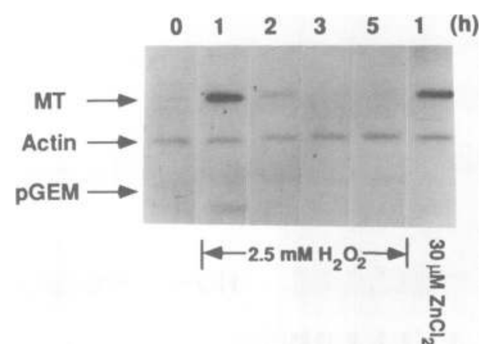


Figure 7. Nuclear run-on assay of the relative rate of MT-I gene transcription in Hepa cells treated with H_2O_2 or $ZnCl_2$. Hepa cells were treated with 5 ml of culture medium containing H_2O_2 (2.5 mM) or $ZnCl_2$ (30 μM). Nuclei were isolated from cells that had been treated for the indicated times and RNA synthesis was continued *in vitro* for 12 min in the presence of [^{32}P]UTP. Labeled RNAs were extracted and equal amounts (10^7 c.p.m.) from each time point reaction were hybridized in parallel to nylon strips containing equal amounts (3 μg) of immobilized mouse MT-I gene (MT), B-actin cDNA (actin), and plasmid vector (pGEM) DNAs. Hybrids for each time point assayed were detected by autoradiography in parallel.

taken to analyze the MLTF/ARE in the mouse MT-I promoter. Four tandem copies of the MLTF/ARE were inserted in the forward orientation immediately upstream of the TATA box in −42 CAT. The promoter in −42 CAT had low, but detectable, basal activity and did not respond to metals or H_2O_2 (data not shown). In contrast, MLTF/ARE$_4$ CAT had a 20-fold higher basal activity, and was responsive to H_2O_2, but unresponsive to $ZnCl_2$. These data establish that the MLTF/ARE plays a role in the response of the MT-I gene to H_2O_2. However, the involvement of other promoter elements in this response was also suggested. Metal ions are the best studied and most potent inducers of MT-I gene transcription. It has previously been demonstrated that the proximal 153 bp of the MT-I promoter contains 4 functional MREs (MRE-a−d) located between −140 and −42 bp which cooperate to confer metal responsiveness (37). In fact, all mammalian MT promoters contain multiple MREs within the region proximal to the transcription start site (5). The best studied of these is MRE-d from the mouse MT-I promoter

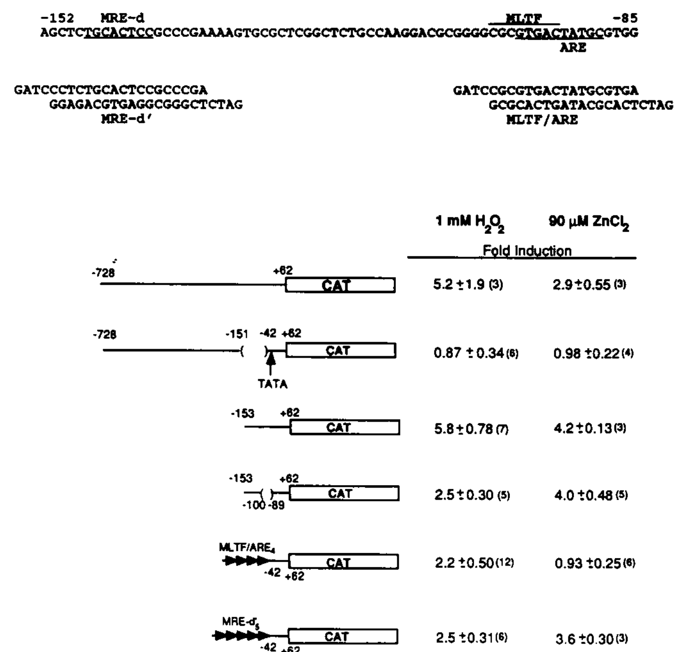


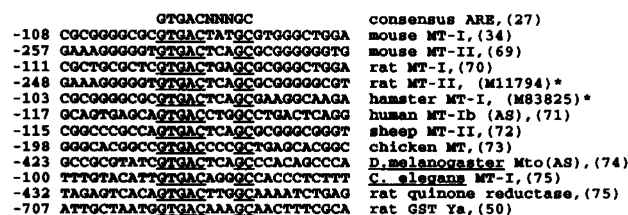
Figure 8. Mapping of mMT-I promoter elements involved in transcriptional induction by H_2O_2 in transient transfection assays. Hepa cells were transfected using calcium phosphate precipitates of the indicated mMT-I promoter−CAT fusion genes. Numbering of the mMT-I promoter refers to base pairs (bp) relative to the transcription start point (+1) in the fusion gene. The nucleotide sequence of the MT-I promoter region from −152 to −85 is shown and MRE-d and the MLTF/ARE sequences are underlined. The double-stranded MRE-d′ and MLTF/ARE oligos shown were inserted in front of a minimal (−42 bp) MT-I promoter in pCATm. DNA samples were each mixed with 1 μg of SV−βGAL and the plasmid pGEM7 such that the final DNA amount transfected was 10 μg/dish. Transfected cells were washed and allowed to recover for 24 h before addition of the indicated concentrations of Zn or H_2O_2 in 10 ml of medium. Cells were harvested after 9 h and CAT and βGAL activities were assayed in cell lysates. The experiments were repeated at least three times, and bracketed numbers represent the number of independent experiments with each vector and treatment. Data are normalized to βGAL activity as an internal control for transfection efficiency and are expressed as fold induction ± SD relative to control cells (vehicle-treated) transfected in parallel with the same plasmids. Under these transfection conditions basal CAT activities of minimal MT-I promoters was 2- to 10-fold above background.

(35,51). A fusion gene consisting of 5 tandem copies of MRE-d inserted in the forward orientation immediately upstream of the TATA box in −42 CAT was responsive to $ZnCl_2$, as has been previously reported (36). This fusion gene exhibited basal activity that was only 2-fold greater than that of −42 CAT. Interestingly, MRE-d′$_5$ CAT was induced by H_2O_2 (Fig. 8). These data suggest a role for MREs as well as MLTF/AREs in the H_2O_2 responsiveness of the MT-I promoter.

DISCUSSION

Induction of MT gene transcription by metal ions is an absolutely conserved response that is of fundamental importance in the metal regulatory roles of these proteins (5−7,52). However, MT genes are also induced by a wide variety of agents (cytokines, phorbol esters, organic solvents, xenobiotics) (16,17,19,20,22,24,53,54), but the functional role(s) of MT induced by this diverse group of agents is poorly understood. A commonality among many of these compounds is their induction of oxidative stress. Since MT has been shown to be an efficient scavenger of ROS (11,12), and mammalian MTs can functionally substitute for superoxide dismutase in protecting yeast from oxidative stress (13), a plausible role for MT in protection from ROS can be suggested. Thus, induction of MT gene transcription by ROS as shown herein, and the subsequent scavenging of ROS by the MT peptide is reminiscent of the metal regulatory loop and is consistent with the hypothesized protective functions of MT.

Induction of the mouse MT-I gene by H_2O_2 or menadione treatment of Hepa cells suggests a role for ROS in regulation of MT gene expression. H_2O_2 undergoes intracellular Fenton reactions (55) leading to the generation of hydroxyl radicals, whereas menadione undergoes redox cycling leading to ROS production (4). Furthermore, induction of HO is apparently dependent on the generation of hydroxyl radicals (46). Under the experimental conditions employed in this study, the concentration of H_2O_2 needed to evoke near maximal induction of MT-I mRNA was similar to that reported to induce maximal transcription of transfected CAT fusion genes driven by the peroxide-responsive GST ya and quinone reductase promoters (27,48). The concentration of H_2O_2 necessary for acute induction of specific gene expression depends on the culture conditions and/or the cell line examined. In human foreskin fibroblasts, HO is maximally induced by 0.1 mM H_2O_2 (43) and in the rat acinar cell line AR4-2J, MT is maximally induced by 0.5 mM H_2O_2 (data not shown). Other cell lines such as Hepa



	Sequence	Source
	GTGACNNNGC	consensus ARE, (27)
-108	CGCGGGGCGCGTGACTATGCGTGGGCTGGA	mouse MT-I, (34)
-257	GAAAGGGGGTGTGACTCAGCGCGGGGGGTG	mouse MT-II, (69)
-111	CGCTGCGCTCGTGACTGAGCGCGGGCTGGA	rat MT-I, (70)
-248	GAAAGGGGGTGTGACTCAGCGCGGGGGCGT	rat MT-II, (M11794)*
-103	CGCGGGGCGCGTGACTCAGCGAAGGCAAGA	hamster MT-I, (M83825)*
-117	GCAGTGAGCAGTGACCTGGCCTGACTCAGG	human MT-Ib (AS), (71)
-115	CGGCCCGCCAGTGACTCAGCGCGGGCGGGT	sheep MT-II, (72)
-198	GGGCACGGCCGTGACCCCGCTGAGCACGGC	chicken MT, (73)
-423	GCCGCGTATCGTGACTCAGCCCACAGCCCA	D.melanogaster Mto(AS), (74)
-100	TTTGTACATTGTGACAGGGCCACCCTCTTT	C. elegans MT-I, (75)
-432	TAGAGTCACAGTGACTTGGCAAAATCTGAG	rat quinone reductase, (75)
-707	ATTGCTAATGGTGACAAAGCAACTTTCGCA	rat GST Ya, (50)

Figure 9. Conservation of AREs in 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 start transcription site of the adjacent nucleotide shown in the figure. *These sequences are unpublished. Shown to the right is the accession number for the sequence.

1A and BHK cells are unresponsive, whereas NIH 3T3 cells show a modest response to H_2O_2. The precise reasons for the large variability between different cell lines in H_2O_2 activation of gene expression is not clear. It was noted during the course of these studies that increasing the volume of culture medium or decreasing the serum content during H_2O_2 treatment each increased the magnitude of induction of MT mRNA in Hepa cells. In addition, although near maximal induction of MT-I mRNA required 2.5 mM H_2O_2 in 5 ml of medium, this level of induction could be maintained by retreatment with as little as 0.5 mM H_2O_2. These observations are consistent with the idea that initial treatments with H_2O_2 must overcome antioxidants in the culture system (both in the medium and in the cells) before changes in gene expression occur. Thus, after an initial exposure, a lower concentration of H_2O_2 is sufficient to maintain response. Hence, induction of genes by H_2O_2 may depend on metabolism of H_2O_2 and the efficiency of ROS generation and scavenging reactions.

Interestingly, the coordinate induction of several prokaryotic ROS-responsive genes requires a common transcription factor, termed OxyR (56). OxyR apparently becomes oxidized and activated in response to an ROS-induced shift in the redox environment within the cell. Evidence suggesting the existence of a similar regulatory mechanism in eukaryotic cells is provided by the observations that diamide, an agent that changes the cells redox potential by depleting glutathione stores, induces HO in human fibroblasts (44,57,58) and that diethyl maleate, an agent with a similar action, induces MT in rat and mouse tissues *in vivo* (15,17). Although proposing a similar mechanism of regulation of MT and HO genes in response to ROS is speculative, the coordinate induction of MT and HO by H_2O_2 *in vitro* suggests this possibility.

Transcriptional induction of the mouse MT-I gene during oxidative stress is documented for the first time in these studies and the involvement of multiple *cis*-acting promoter elements is suggested. The mechanisms of this induction are poorly understood, but promoter regions involved in this response include a composite element consisting of overlapping MLTF binding site and ARE and/or MREs. Thus, ROS-induced transcription from the MT-I promoter differs from that induced by metals alone, and cannot simply arise as the result of H_2O_2-mediated redistribution of Zn or Cu. Although this study does not distinguish between MLTF binding site and ARE contributions to the ROS response, evidence from other investigators suggests the paramount importance of the ARE (27,48). The ARE was identified in the promoters of the GST ya subunit and the quinone reductase genes from several species by the ability to regulate transcription in response to redox cycling xenobiotics and H_2O_2 (27). The ARE can be bound by a factor(s) that is suggested to be activated by ROS. However, the mechanisms of this activation are poorly understood and constitutive ARE binding activity is detected in cell extracts (27,59). The ARE core sequence (GTGACnnnGC) bears homology with an AP1 binding site and can bind Fos and Jun *in vitro* if it contains the sequence TGACTCA (59). However, the ARE has been shown to bind with higher affinity to a novel but uncharacterized factor (48). Functionally, an AP1 binding site can respond to xenobiotics if a 3′ GC dinucleotide is present (59). In contrast, the MLTF binding site was originally identified in the adenovirus major late promoter as an element that recruited a factor present in HeLa cell extracts to increase basal transcription from this promoter (49,60). To date, no inducible activity has been associated with this element. The MLTF binding site in the MT-I promoter was first identified as an element that increased basal activity without affecting metal induction (35,49). However, in retrospect the deletion constructs used to evaluate activity of the MLTF binding site in the MT-I promoter were, in fact, MLTF/ARE deletions. The ARE had not been described at that time.

The finding that among the 29 MT promoter gene sequences in GenBank, 10 contain a single consensus ARE sequence (Fig. 9) is also consistent with the suggestion that this element is of functional importance. Two of these MT promoters (mouse and hamster MT-I) also contained a consensus MLTF binding site (CpuCGTGAC) (49,60) that overlapped the ARE sequence. Thus, as previously noted (49), MLTF does not appear to be a general regulatory element in MT promoters. In contrast, ARE sequences are common in MT-I promoters, and MLTF binding sites, when present, are a part of a composite element with the ARE. Further studies are required to delineate the relative contributions of the MLTF binding site and the ARE in ROS response and basal promoter activity.

An unexpected and novel finding is that part of the effect of H_2O_2 on MT-I promoter activity is mediated by factors interacting with MREs. A zinc-finger transcription factor, MTF-I, that binds MREs has recently been cloned (61). Inactivation of this factor by anti-sense RNA (36) or by targeted deletion of this gene in embryonic stem cells (62) prevents induction of MRE activity by metals. MTF-I is postulated to be controlled by a metal-sensitive inhibitor (36). ROS might liberate MTF-I from this inhibitor either directly by oxidizing thiols and releasing Zn (63,64) or by oxidation of the inhibitor.

The latter suggestion is analogous to the oxidative inactivation of I-κB which leads to activation of the transcription factor NF-κB (65,66). Alternatively, other transcription factors could interact with MREs in response to ROS. The activity of several different transcription factors can be modulated in response to redox potential in the cell (58,67,68).

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