Superinduction of Metallothionein I by Inhibition of Protein Synthesis: Role of a Labile Repressor in MTF-1 Mediated Gene Transcription

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ABSTRACT: Induction of metallothioneins (MTs) through the metal-activated transcription factor-1 (MTF-1) provides a model response for analyzing transcriptional gene regulation by heavy metals. Here, we report inhibition of protein synthesis by cycloheximide (CHX) increases induction of *Mt1* by approximately five-fold, a phenomenon designated as "superinduction." Characterization of superinduction revealed it is time- and concentration-dependent of CHX, requires the presence of an MTF-1 activator, and occurs at a transcriptional level, suggesting a labile repressor in the control of *Mt1* induction. Genetic analyses using *Mtf1* null cells and a metal response element (MRE)-driven reporter construct showed that superinduction of *Mt1* is mediated through MTF-1 and MRE-dependent transcription. Analyses of intracellular zinc content by inductively coupled plasma emission spectroscopy and fluorescence imaging demonstrated that treatment with CHX alone or CHX plus an inducer does not increase the total zinc accumulation or the concentration of free zinc in cells under the conditions in which superinduction occurs. Moreover, superinduction was observed in cells cultured in a zinc-depleted medium, suggesting that superinduction does not involve elevation of intracellular zinc concentration. Northern blotting showed that Cd, CHX, or Cd + CHX does not affect the expression of the mRNA of MTF-1. Immunoblotting using antibodies specific for MTF-1 demonstrated that Cd induces a down-regulation of the MTF-1 protein, whereas cotreatment with Cd and CHX blocked the Cd-induced degradation of MTF-1. The findings reveal a new mechanistic aspect of the superinduction of *Mt1*, in which a labile repressor negatively controls agonist-induced turnover of the MTF-1 protein. © 2006 Wiley Periodicals, Inc. J Biochem Mol Toxicol 20:57–68, 2006; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jbt.20116

KEYWORDS: **Superinduction; Cycloheximide; Transcription Repression; MTF-1; MT-1**

INTRODUCTION

Heavy metals are ubiquitous in the environment and are frequently encountered from industrial and dietary sources. Heavy metals include trace elements that are essential for life, such as zinc and copper, and nonessential metals, such as cadmium, arsenic, and mercury. Overexposure to heavy metals can cause various adverse effects including toxicity, cancer, and organ-specific chronic diseases in humans and experimental animals [1,2]. Evidence reveals that cells cope with toxic metals by eliciting a number of adaptive responses, among which induction of metallothioneins (MTs) is essential in protection against the toxicities of heavy metals such as Cd, Cu, Hg, Ag, and Zn [3,4]. MTs are a group of small, cysteine-rich, metal-binding proteins. The cysteine residues coordinate multiple zinc and copper atoms under physiological conditions, but they can also bind toxic metals with high affinities. The genes encoding MTs are grouped into four subfamilies, *Mt1* through *Mt4*. Whereas *Mt3 and 4* are found in specific tissues and are minimally inducible, *Mt1 and Mt2* are expressed in most cell types and are highly inducible by heavy metals, antioxidants,

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glucocorticoids, alkylating agents, and inflammatory signals. Induction of MTs increases the sequestration of metals in protein-bound metal pools, thereby reducing the concentrations of free metals in cells leading to decreased metal toxicity. Because of their high contents of cysteine residues, MTs can also confer protection against oxidative stress, electrophilic anticancer agents, ionizing irradiation, and nitric oxide.

Genetic and biochemical evidence implicates metal-activated transcription factor 1 (MTF-1) as a key transcription factor in the induction of MT-I/II by heavy metals, antioxidants, and certain stress stimuli [5–7]. Consistent with its role in MT and metal transporter induction, MTF-1 is found to be essential in protection against heavy metal toxicity through evolution, as mice, insects, or cells lacking a functional MTF-1 are highly sensitive to the toxic effects of heavy metals such as cadmium, zinc, and copper [8–11]. In addition, MTF-1 is required for a normal hepatic development; disruption of the *Mtf1* gene results in embryonic death due to a failure in liver development [12]. Because this phenotype is not observed in a combined deletion of *Mt1* and *Mt2* [13,14], the finding suggests a role of MTF-1 in the transcription of liver-specific genes other than *Mt1 and Mt2* during embryonic development, though such target genes of MTF-1 for hepatic development have not been identified.

MTF-1 is a member of the zinc finger family of transcription factors. Structurally, it consists of several modular domains that are highly similar between the human and murine proteins. A DNA-binding domain composed of six zinc fingers of the C_2H_2-type is located between residues 137 and 315 of mouse MTF-1. Juxtaposed to the zinc fingers is the transcription activation domain consisting of an acidic, a proline-rich, and a serine/threonine-rich transactivation modules in the carboxyl half [6,15,16]. In addition, a nuclear localization and two nuclear export-signaling sequences have been suggested to co-localize with the DNA-binding and transactivation domains, respectively [17].

Analysis of the mechanism by which MTF-1 mediates the induction of MT-I/II reveals a xeno-chemical activated transcriptional response in the maintenance of metal homeostasis in cells. Unactivated MTF-1 exists in the cytoplasm. MTF-1 translocates into the nucleus upon activation by metals. Nuclear MTF-1 binds to specific DNA sequences termed as metal response elements (MREs), which are located in the enhancer regions of *Mt1* and *Mt2*. Interaction between MTF-1 and MRE activates the transcription of the genes. Several issues critical to the induction of *Mt1* by MTF-1 remain elusive. For instance, the mechanism of MTF-1 activation by heavy metals has not been resolved. Direct binding of zinc (or other metals) to regulatory sites on MTF-1 such as the zinc fingers, activation of signal transduction pathways leading to covalent modifications of MTF-1 such as phosphorylation of MTF-1, and release of MTF-1 from regulatory molecules have all been proposed but are not conclusive [5,6,18–20]. In addition, regulation of MTF-1 in the nucleus after activation remains poorly understood.

To elucidate the molecular events of *Mt1* transcription by MTF-1, we attempted to identify new protein factors that modulate MTF-1 function. We found that inhibition of protein synthesis by cycloheximide (CHX) increases induction of *Mt1* by Cd, Zn, and tBHQ by three- to five-fold, a phenomenon designated as "superinduction." Superinduction of *Mt1* is both concentration- and time-dependent of cycloheximide and requires new mRNA synthesis. Cell genetic analyses reveal that superinduction of *Mt1* is mediated through MTF-1 and MRE-dependent transcription. Inhibition of protein synthesis by CHX does not increase intracellular total or free zinc concentrations; moreover, superinduction is observed in cells cultured in a zinc-depleted medium, indicating that superinduction does not involve modulation of the intracellular free zinc concentration. Finally, analyses of MTF-1 by Northern and immunoblotting analyses demonstrated that Cd does not affect the expression of MTF-1 mRNA, but induces a down-regulation of MTF-1 protein, whereas CHX blocks the Cd-induced turnover of the protein. Together, these findings implicate a labile repressor that negatively regulates the stability of agonist-activated MTF-1.

MATERIALS AND METHODS

Materials

Restriction endonucleases and other molecular biology reagents were purchased from New England Biolabs (Beverly, MA), Roche Molecular Biochemicals (Indianapolis, IN), Invitrogen (Carlsbad, CA), and Promega (Madison, WI). DMSO, tBHQ, cadmium chloride (Cd), zinc chloride (Zn), dexamethasone (Dex), cycloheximide (CHX), and actinomycin D were purchased from Sigma (St. Louis, MO). Cell culture materials were from Invitrogen and HyClone (Logan, UT).

Cell Culture and Treatments

Mouse hepa1c1c7 cells were provided by Dr. J.P. Whitlock, Jr. (Stanford University, Stanford, CA). The cells were grown as monolayer in α-minimal essential medium (α-MEM), containing 10% fetal bovine serum

(FBS) and 5% CO_2. Cells were treated with chemicals as described in figure legends. In a typical induction experiment, mRNA was measured after 4 h of treatment as specified in figure legends. DMSO was used as a solvent control for tBHQ and water for Cd and Zn. Mouse *Mtf1*$^{+/+}$ and *Mtf1*$^{-/-}$ (dko7) cells were kindly provided by Dr. W. Schaffner (Universitat Zurich, Zurich, Switzerland) [15]. The cells were cultured as described elsewhere [15]. BHK cell clones 3038 (kindly provided by Dr. R.D. Palmiter, University of Washington, Seattle, WA), which were stably transfected with MRE-βGeo, were cultured in DMEM with 10% FBS as described before [6,21]. *Mtf1*$^{-/-}$ cells (dko7) were transfected with plasmids, allowing expression of full-length MTF-1 driven by the CMV promoter (CMV-MTF-1). Stable clones were isolated after selection for resistance to cadmium toxicity.

Analysis of mRNA

Northern blotting was performed as follows. Total RNA was isolated from cells using a Qiagen total RNA isolation kit (Valencia, CA). RNA samples (5 μg) were fractionated in a 1% agarose–formaldehyde gel and transferred to a Nytran membrane. The blot was probed with a DIG-labeled riboprobe prepared with the DIG-labeling kit (Roche Molecular Biochemicals) for mouse *Mt1*, according to the established procedures [22]. Signals were visualized by chemiluminescence using a DIG RNA detection kit with CDP Star as a substrate (Roche Molecular Biochemicals). Parallel blots of the same samples were probed with DIG-labeled mouse actin probe to ensure equal loading. Results shown were repeated two more times in separate experiments with consistent observations. Quantification of Northern results was performed using ImageQuant software (Molecular Dynamics, San Jose, CA). Quantification data represent means and standard deviations from three experiments.

β-Galactosidase Induction and Measurement

MRE-βGeo encodes β-galactosidase fused to neomycin phosphotransferase under the control of five MRE-d elements located upstream of the basal mouse *Mt1* promoter (−42 to +60) [6]. BHK cell line 3038, which is stably transfected with MRE-βGeo, was treated with agents as described for each figure. After a brief rinse in phosphate-buffered saline, the cells were fixed for ∼5 min in glutaraldehyde and formaldehyde, rinsed and then incubated with *o*-nitrophenyl-β-galactopyranoside for ∼1 h, and the absorbance at 405 nm was measured [8].

Generation of Polyclonal Antibodies Against MTF-1

Mouse MTF-1 carboxyl half was expressed in bacteria using the pET-28 expression system (Novagen, Madison, WI) according to procedures from Novagen. The expressed protein (MTF1-CT) was purified using the Ni-NTA His.Bind purification kit (Novagen) as instructed by the manufacturer. Rabbit polyclonal antibodies (serum) were raised, prepared, and characterized following standard protocols as described previously [23].

Zn Depletion and Measurement of Zinc Content

FBS (HyClone), which contains ∼55 μM Zn, was mixed with Chelex 100 (Sigma) to generate Zn-depleted FBS according to instructions from Sigma and then sterilized by filtration. Aliquots of α-MEM, FBS, Zn-depleted FBS, and α-MEM with 10% FBS were analyzed to determine Zn and Cd contents using inductively coupled plasma emission spectroscopy. To determine Zn content in cells, cells were cultured and treated as specified in figure legends, followed by washing with PBS for three times. Total cell lysate was collected, lyophilized, and analyzed to determine Zn content using inductively coupled plasma emission spectroscopy.

Fluorescence Imaging of Intracellular "Free" Zinc

Hepa1c1c7 cells were seeded in a Lab-Tek 4-chamber coverglass systems (Electron Microscopy Sciences, Fort Washington, PA) in α-MEM with 10% FBS at a density of 1.5×10^4/mL. Cells were grown at 37°C, 5% CO_2 for 24 h to reach 80% confluency. The culture was changed to α-MEM containing 10% FBS, or Zn-depleted media, which was treated with Chelex 100. FluoZin-3AM (Molecular Probes, Eugene, OR) and pluronic (Molecular Probes) were added to the culture medium at final concentrations of 1 μM or 0.2%, respectively. The culture was continued for 30 min, followed by treatments, as indicated in figure legends. The cells were kept at 37°C with 5% CO_2 in a temperature and CO_2-controlled chamber during treatments. Fluorescence response of the cells was examined using a laser scanning confocal microscope (LSM 510, Carl Zeiss, Thornwood, NY) fitted with an argon-ion laser (Ex: 488 nm; Em: 520 nm). Images were scanned at 512 × 512 pixels. Using only the green channel, the mean gray value of each field was extracted using Optimas 6.51 image analysis software (Media Cybernetics, Silver Spring, MD). Quantitative data represent means and standard

deviations from six separate fields for each treatment. One-way ANOVA analysis and Tukey's multiple comparison test were performed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

RESULTS

Inhibition of Protein Synthesis Superinduces *Mt1* Transcription in the Presence of MTF-1 Activator

To dissect the molecular events governing the signal transduction of MTF-1 in *Mt1* gene regulation, we first examined if *Mt1* transcription by MTF-1 requires new protein synthesis. We have previously shown that phenolic antioxidants such as tBHQ induce the mouse *Mt1* gene transcription through the MTF-1 and MRE-dependent pathway [7]. Figure 1 shows that *Mt1* is expressed at a low but detectable level in mouse hepatoma cells (lane 1). TBHQ at a concentration of 100 μM induces *Mt1* mRNA by six-fold (lane 2). Cycloheximide, which inhibits up to 95% of protein synthesis in the cells at a concentration of 10 μg/mL, induces *Mt1* similarly to tBHQ (lane 3). Cotreatment of the cells with tBHQ and CHX markedly increased *Mt1* mRNA expression (i.e., four- to five-fold higher than those with either tBHQ or CHX alone), a phenomenon designated as "superinduction." Therefore, inhibition of protein synthesis by CHX induces and superinduces *Mt1* mRNA expression. Puromycin, which inhibits protein synthesis with a similar potency to but through a different mechanism from cycloheximide, superinduces *Mt1* similarly to cycloheximide (data not shown), further supporting that inhibition of protein synthesis accounts for the superinduction. Two conclusions can be made from the observation. First, the basal and inducible transcription of *Mt1* does not require new protein synthesis (i.e., representing a primary transcriptional event). Second, both the basal and inducible transcriptions of *Mt1* are negatively regulated by a labile repressor that is susceptible to inhibition of protein synthesis.

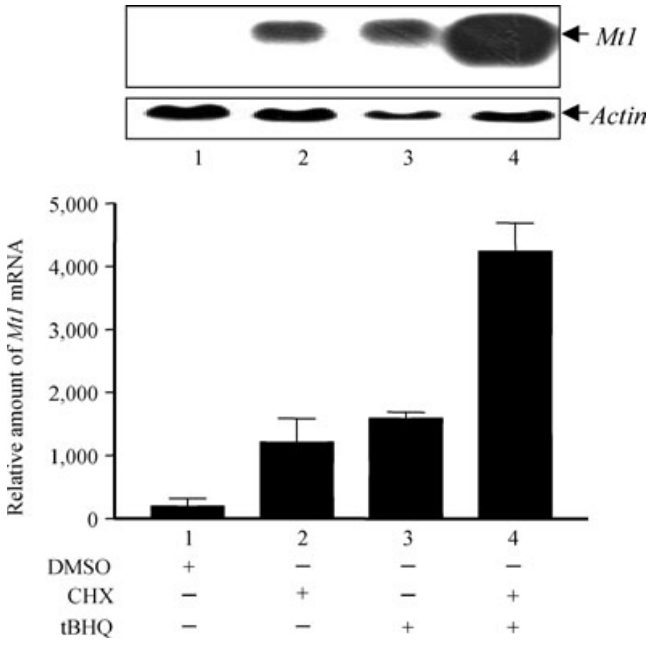


FIGURE 1. Superinduction of *Mt1* by CHX. Hepa1c1c7 cells were treated with CHX (10 μg/mL) in the absence or presence of tBHQ (100 μM) for 4 h. *Mt1* mRNA was analyzed by Northern blotting (upper panel). *Actin* mRNA was measured as a control for equal loading. Quantification of mRNA bands (lower panel) was performed using ImageQuant software (Molecular Dynamics, San Jose, CA). Quantification data represent means and standard deviation from three experiments.

Superinduction by CHX is Dose-Dependent

Superinduction was first observed at 100 ng/mL of CHX with 100 μM of tBHQ (lane 6) (Figure 2A). Superinduction reached a high level at 10 μg/mL of CHX (lane 8), but was reduced at concentrations larger than 10 μg/mL due to toxicity to the cells (data not shown). The results indicated that a nearly total inhibition of protein synthesis in cells was required for superinduction by CHX. A time-course study revealed that superinduction occurred as early as 15 min after cotreatment with tBHQ and CHX and was strongly induced at 1 h, reaching a plateau at the 2.5 h time points (Figure 2B and data not shown).

Mt1 can be induced by a range of inducers including divalent metals and glucocorticoids. Thus, we examined the specificity of superinduction for *Mt1* inducers. Cd or Zn induces *Mt1* through MTF-1 [5,6] (Figure 3A, compare lanes 3 and 5 with lane 1), whereas Mg does not (lane 7). Cotreatment with Cd or Zn with CHX dramatically increased the induction of *Mt1* mRNA (lanes 4 and 6). On the contrary, Mg + CHX did not superinduce *Mt1* (lane 8). Dexamethasone induces *Mt1* through the glucocorticoid receptor (GR) and glucocorticoid response element (GRE) [24]. Dex (100 μM, 3 h) was shown to induce *Mt1* (Figure 3B; compare lanes 7 and 1). However, Dex + CHX did not superinduce *Mt1* (compare lanes 8 and 2), whereas Cd or Zn + CHX (positive controls) superinduced the gene under the same conditions (lanes 4 and 6). Taken together, superinduction of *Mt1* by CHX and an inducer correlates with the capacity of the inducer to activate MTF-1, implying a labile repressor in MTF-1 mediated transcription.

We further exploited the labile nature of the repressor. In Figure 4, CHX was added at different time points

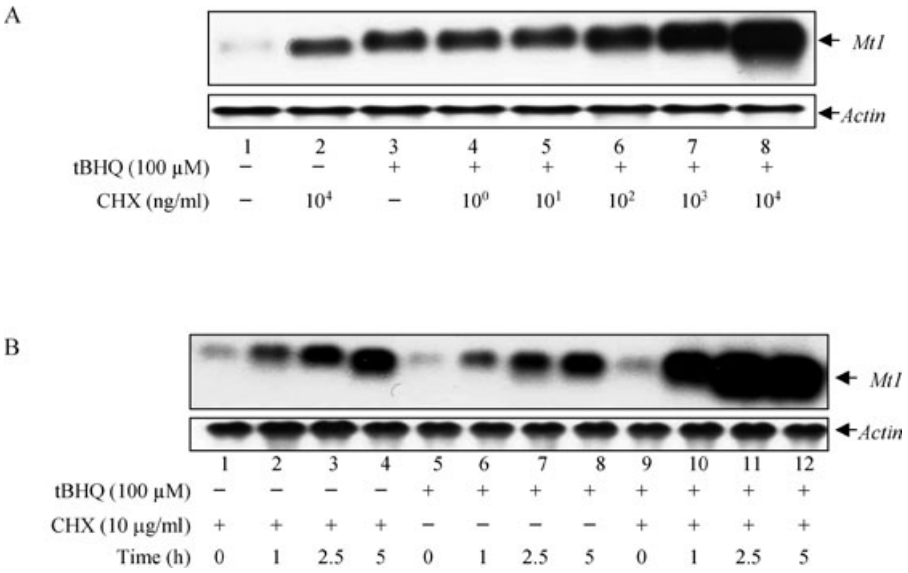


FIGURE 2. Concentration and time dependence of *Mt1* superinduction. (A) Cells were treated with tBHQ (100 μM) and increasing amount of CHX (ng/mL) for 4 h as indicated; (B) cells were treated with tBHQ (100 μM), CHX (10 μg/mL), or both for various time periods. Total RNA was analyzed for *Mt1* expression by Northern blotting. *Actin* was used to control loading variations.

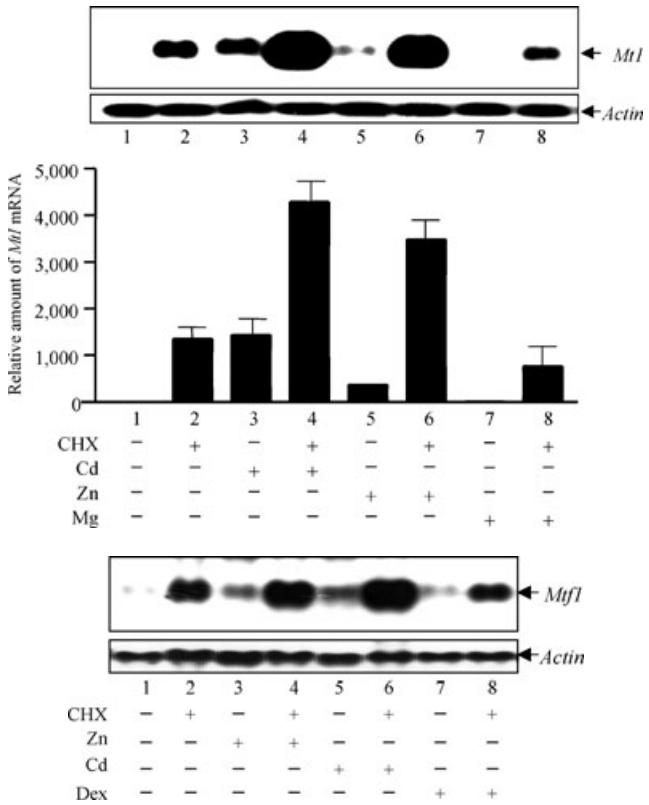


FIGURE 3. Specificity of *Mt1* superinduction of *Mt1* for inducers. Hepa1c1c7 cells were treated with CHX (10 μg/mL) in the absence or presence of Cd (10 μM), Zn (100 μM), or Mg (100 μM) for 4 h (A); or Cd (10 μM), Zn (100 μM), or Dex (100 μM) for 3 h (B). *Mt1* mRNA was analyzed by Northern blotting. *Actin* mRNA was measured as a control for equal loading.

during induction by Cd. Pretreatment with CHX for 2 h prior to Cd (lane 5), cotreatment with CHX during the first 2 h of Cd treatment (lane 6), cotreatment with CHX during the last 2 h of Cd treatment, or cotreatment with CHX and Cd for a total of 4 h (lane 4) all resulted in superinduction (compare lanes 5, 6, 7, and 4 with lanes 2 and 3). The magnitude of superinduction was highest when cells were cotreated for 4 h (lane 4), followed (in

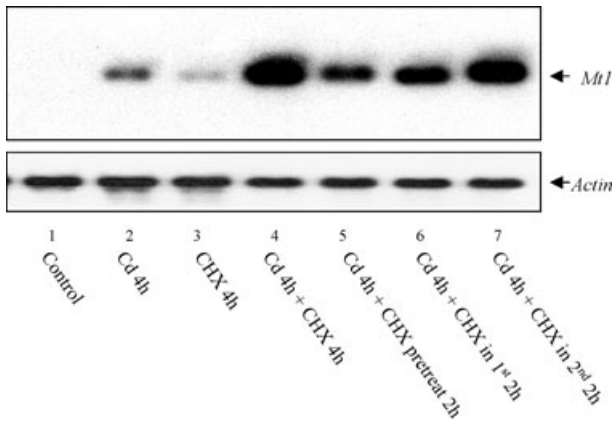


FIGURE 4. Effect of treatment time on superinduction. Cells were treated with Cd (10 μM, 4 h), CHX (10 μg/mL, 4 h), Cd + CHX (4 h), Cd (4 h) + CHX pretreatment (2 h), Cd (4 h) + CHX cotreatment during the 1st 2 h of Cd treatment, or Cd (4 h) + CHX during the 2nd 2 h of treatment. Total RNA was analyzed for *Mt1* and *Actin* expression by Northern blotting.

a decreasing order) by cotreatment in the last 2 h (lane 7), cotreatment in the first 2 h (lane 6), and pretreatment of 2 h (lane 7). Decreased superinduction at earlier than later time points of cotreatment (lanes 5 and 6) further supported the notion that the CHX-sensitive repressor is a short-lived protein and thus functionally labile.

Superinduction of *Mt1* mRNA expression can result from an increase in the rate of transcription or enhanced mRNA stability of *Mt1*. To distinguish the possibilities, *Mt1* mRNA stability was examined in the presence or absence of CHX. Cells were pretreated with tBHQ to induce *Mt1* mRNA. After extensive washing, the cells were treated with CHX, actinomycin D (inhibitor of mRNA synthesis), or both. *Mt1* mRNA was examined by Northern blotting. In Figure 5, *Mt1* mRNA is induced by tBHQ (lane 2, compare with lane 1). CHX did not affect the steady-state level of *Mt1* mRNA for 5 h, during which superinduction was normally observed (lanes 3–5). Inhibition of mRNA synthesis did not change the mRNA level (lanes 6–8), indicating the mRNA was stable within 5 h. The mRNA level was not changed by a cotreatment with CHX and actinomycin D (lanes 9 and 10), suggesting that superinduction by CHX in the presence of an inducer was not attributed to enhance stability of the *Mt1* mRNA, but was due to an increase in the transcription of the gene.

Superinduction of *Mt1* Requires MTF1 and MRE

CHX may superinduce *Mt1* mRNA expression through MTF1 and MRE; alternatively, superinduction can be mediated via an MTF1-independent transcriptional pathway(s). To distinguish the possibilities, we examined the MTF1 and MRE-dependence of *Mt1* superinduction by CHX using genetically altered cell lines. In Figure 6A, embryonic fibroblast cells derived from wild type or $Mtf1^{-/-}$ mouse stem cells with the same genetic background were used. As expected, CHX, tBHQ, and Cd induced *Mt1* mRNA expression in $Mtf1^{+/+}$ cells (lanes 2, 3, and 5); CHX superinduced *Mt1* in the presence of tBHQ or Cd (lanes 4 and 6). However, no induction or superinduction was observed by CHX, tBHQ, Cd, or in combinations in $Mtf1^{-/-}$ cells (lanes 7–12). In a separate experiment, $Mtf1^{-/-}$ cells were reconstituted with functional MTF-1 ($Mtf1^{-/-}$ + *Mtf1*). Induction by CHX or tBHQ and superinduction by CHX + tBHQ were restored in the reconstituted cells (Figure 6B, lanes 5–8) compared with $Mtf1^{-/-}$ cells (lanes 1–4). Thus, superinduction by CHX is dependent on the presence of functional MTF-1.

We tested if MRE is required and sufficient for superinduction of *Mt1*. Figure 7A showed that CHX, tBHQ, or Cd induced the expression of β-*Gal* mRNA, a reporter gene under the control of five copies of MRE (lanes 2, 3, and 5); CHX superinduced β-*Gal* mRNA in the presence of tBHQ or Cd (lanes 4 and 6). In Figure 7B, *β-Gal* activity was examined. Cells were treated with CHX for 2 h (with or without tBHQ or Cd) and then were washed with a fresh medium for three times, followed by 3 h of incubation in a fresh medium with or without tBHQ or Cd. CHX, tBHQ, or Cd was shown to induce *β-Gal* activity (lanes 2, 3, and 5); cotreatment with CHX further increased the induction of *β-Gal* activity by tBHQ or Cd (lanes 4 and 6). The results demonstrated that MRE is sufficient to drive superinduction of *Mt1* by CHX.

Zn Homeostasis and *Mt1* Superinduction

Mt1 transcription by MTF1 appears to be closely related to Zn homeostasis in cells for two reasons: (1) Zn is required for maintaining the zinc fingers of MTF1 in proper configurations for DNA binding; and (2) Zn activates the cytoplasmic MTF1 for transcription. Furthermore, intracellular Zn concentration can be altered by changing membrane permeability to zinc or by mobilizing Zn pools within the cells. A plausible hypothesis of superinduction would be that CHX modulates intracellular zinc homeostasis and thereby superinduces *Mt1*. In Figure 8, cells were treated with CHX, Zn, Cd, pyrithione, or in combinations. The total cellular Zn

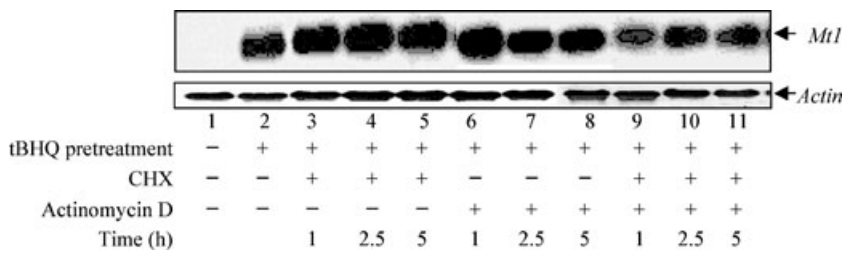


FIGURE 5. Effect of CHX on *Mt1* mRNA stability. Cells were treated with DMSO (lane 1) or tBHQ (100 μM, lanes 2–11) for 3 h. The cells were washed with fresh medium for three times and then were treated with CHX (10 μg/mL), actinomycin D (2 μg/mL), or both for indicated time periods. Total RNA was analyzed for *Mt1* and *Actin* by Northern blotting.

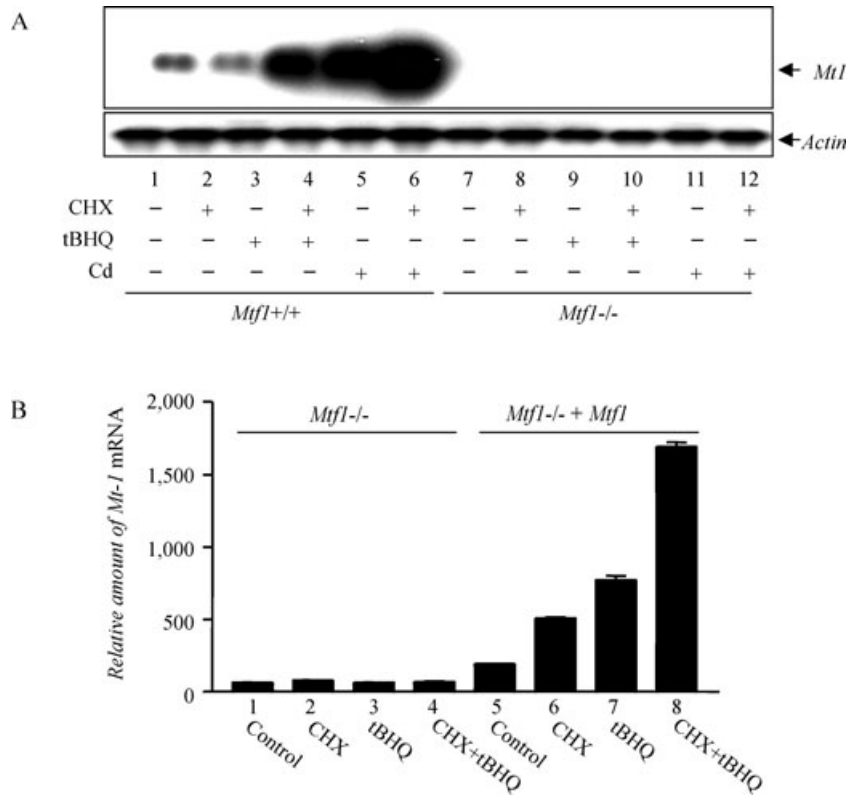


FIGURE 6. MTF-1 dependence of superinduction. (A) Embryonic fibroblast cells $Mtf1^{+/+}$ or $Mtf1^{-/-}$ were treated with CHX (10 μg/mL), tBHQ (100 μM), Cd (10 μM), or in combination as indicated for 4 h. (B) $Mtf1^{-/-}$ cells or $Mtf1^{-/-}$ cells reconstituted with full length MTF-1 ($Mtf1^{-/-} + Mtf1$) were treated with CHX, tBHQ, or both as indicated for 4 h. Total RNA was harvested and analyzed for *Mt1* and *Actin* expression by Northern blotting.

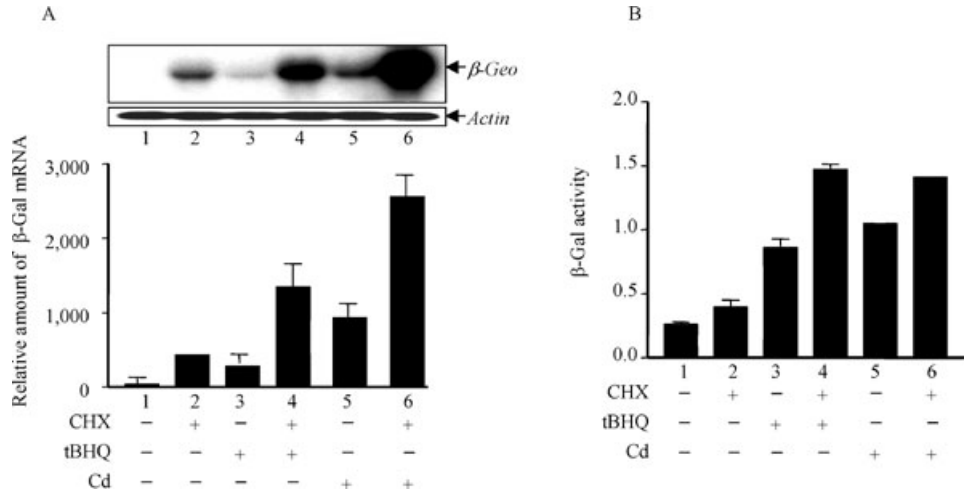


FIGURE 7. Superinduction of MRE-driven reporter gene expression. (A) BHK 3038 cells were treated with CHX (10 μg/mL), tBHQ (100 μM), Cd (10 μM), or in combinations for 4 h as indicated. Induction and superinduction of *β-Geo* mRNA were analyzed by Northern blotting. (B) BHK 3038 cells were treated as indicated for 2 h, followed by washing with fresh media for three times. The cells were then incubated in fresh media for additional 3 h to allow expression of β-galactosidase. Total cell lysate was prepared and β-galactosidase activities were measured as described under "Materials and Methods".

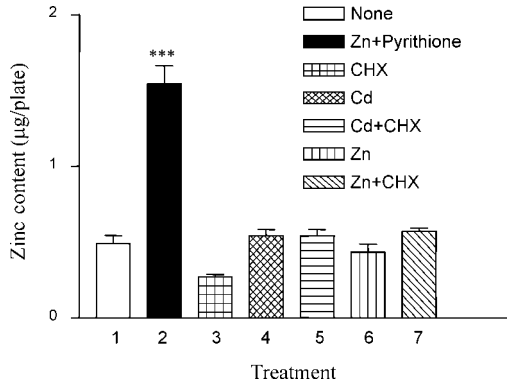


FIGURE 8. Effect of CHX on intracellular zinc content. Hepa1c1c7 cells were treated with Zn (100 μM) + pyrithione (10 μM), CHX (10 μg/mL), Cd (10 μM), Zn (100 μM), Cd + CHX, or Zn + CHX for 4 h. The cells were washed with PBS for three times. Total cell lysate was prepared, lyophilized, and analyzed for Zn content using inductively coupled plasma emission spectroscopy.

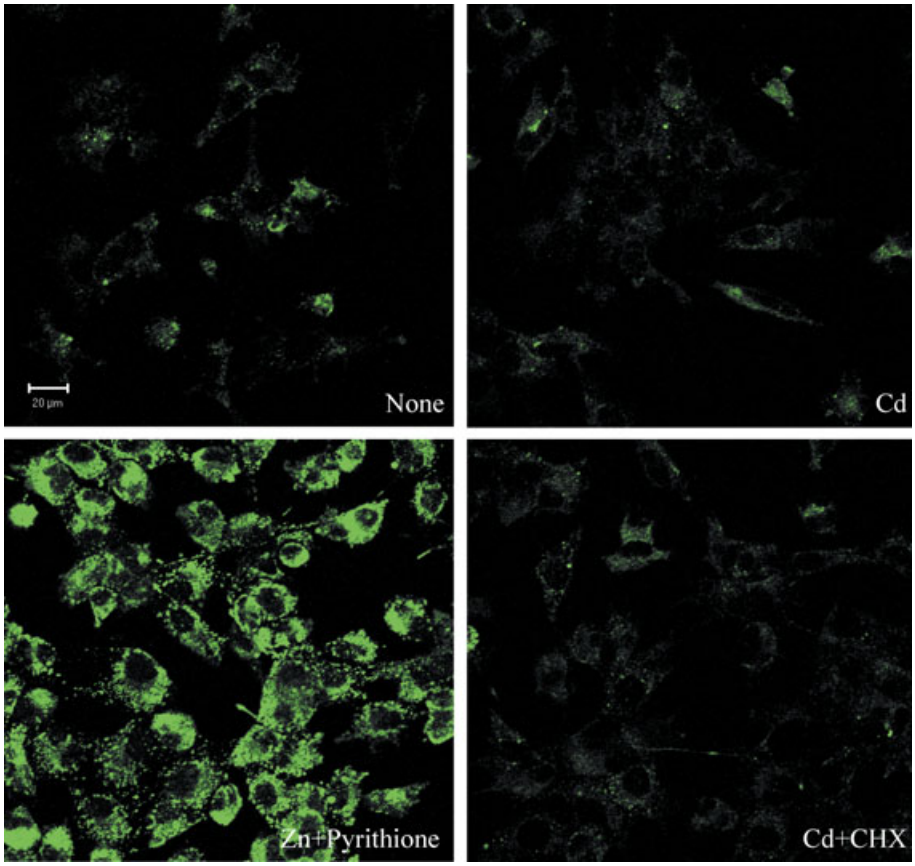
content was measured. As expected, pyrithione, which increases Zn permeability of the plasma member, increased the Zn content in cells by three-fold when cotreated with Zn (lane 2, $p < 0.001$). CHX alone did not increase but reduced the content (lane 3); Zn or Cd alone did not affect the content (lanes 4 and 6). CHX in the presence of Cd or Zn did not change Zn content in cells (lanes 5 and 7). Thus, CHX does not change the total zinc load in cells or the membrane permeability to zinc.

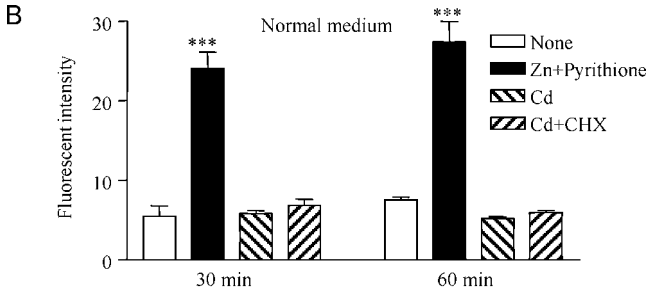
Inhibition of protein synthesis by CHX may reduce the capacity of Zn-binding by Zn-binding proteins in cells, resulting in mobilization of Zn from protein-bound Zn pool giving rise to increased intracellular free Zn concentration. FluoZin-3AM, a fluorescence probe with high affinity and selectivity for cytoplasmic Zn^{2+}, was used to measure the cytoplasmic free Zn concentration. In Figure 9A, cells were cultured in complete media in the presence of FluoZin-3AM. Fluorescence images of the cells were taken at 30 or 60 min after exposure to treatments. As a positive control, pyrithione is shown to dramatically increase the fluorescence intensity of the cells (lower left) in comparison with nontreated control (upper left), indicating increased intracellular free Zn^{2+} concentration by pyrithione. Treatment with Cd or Cd + CHX did not change the fluorescence intensity (upper and lower right panels). Quantification of fluorescent intensity at 30 and 60 min after treatments was shown in Figure 9B. To exclude the possible effect of Zn^{2+} in the normal media (~4.3 μM) on intracellular free zinc, cells were cultured in a Zn-depleted medium ([Zn] <0.7 μM) for 3 days before treatment. As shown in Figure 9C, pyrithione significantly increased the intracellular free Zn concentration at the 30 and 60 min time points similarly to pyrithione treatment with a normal medium (Figure 9B). Treatment with Cd or Cd + CHX did not affect the fluorescence intensity. Taken together, these results demonstrated that CHX does not affect the total and free Zn content in cells as well as Zn permeability of plasma membrane under the experimental conditions.

Next, we examined if modulating intracellular zinc homeostasis affects superinduction of *Mt1*. Treatment with EDTA reduces the free Zn concentrations in both the media and cells by chelating divalent cations by EDTA. In Figure 10A, CHX, tBHQ, and Cd (alone or in combinations) failed to induce or superinduce *Mt1* in cells cultured in the presence of EDTA (1 mM) (lanes 7–12), compared with those in normal media (lanes 1–6). The data indicate that induction and superinduction of *Mt1* by the inducers require a divalent cation, which may be Zn or Cd in the case of Cd treatment. In Figure 10B, intracellular Zn was depleted by prolonged culture of the cells in a Zn-free medium. As expected, CHX, tBHQ, and Cd induce *Mt1* mRNA in cells cultured in normal media (lanes 8, 9, and 11); CHX superinduces *Mt1* in the presence of tBHQ or Cd (lanes 10 and 12). In cells cultured in Zn-depleted media, CHX and tBHQ failed to induce *Mt1* (lanes 2 and 3), whereas cotreatment with CHX and tBHQ induces *Mt1* (lane 4). The result indicates that CHX and tBHQ require a certain level of intracellular Zn to significantly induce *Mt1*; however, CHX + tBHQ can induce the gene with a substantially lower level of intracellular Zn. Cd alone is shown to induce *Mt1* in cells cultured in Zn-depleted media (lane 5) and CHX superinduces when cotreated with Cd (lane 6). Furthermore, both the induction by Cd and superinduction by CHX + Cd are higher in Zn-depleted media than in normal media (compare lane 5 with 11 and lane 6 with 12). Taken together, these results suggest that superinduction by CHX does not depend on elevation of intracellular zinc concentration.

CHX Inhibits the Turnover of Activated MTF-1

The finding that CHX targets the MTF-1/MRE pathway prompted us to examine the effect of CHX on MTF-1. In Figure 11A, the mRNA expression of MTF-1 was analyzed by Northern blotting. CHX, Cd, or CHX + Cd did not affect the expression of MTF-1 mRNA (lanes 2, 3, and 4). To examine the protein expression of MTF-1, polyclonal antibodies against the carboxyl terminal half of the mouse MTF-1 were prepared. Immunoblotting analysis revealed that treatment with Cd (10 μM, 5 h) induced a large decrease in the level of the MTF-1 protein (Figure 11B, compare lanes 2 and 1). CHX alone did not change the MTF-1 protein level significantly. However, cotreatment with CHX and Cd inhibited the Cd-induced





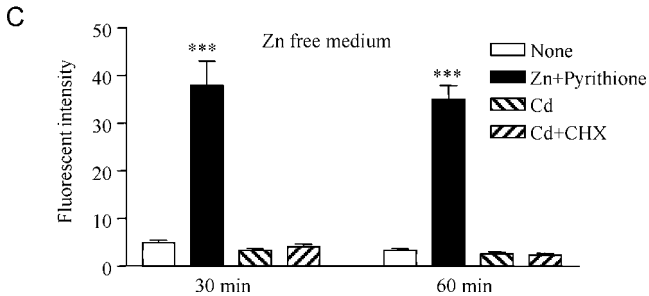


FIGURE 9. Effect of CHX on intracellular free zinc concentration. (A) Hepa1c1c7 cells were grown in a Lab-Tek 4-chamber coverglass system. The cells were treated with FluoZin-3AM (1 μM) and pluronic (0.2%) for 30 min, followed by treatments with DMSO, Zn + pyrithione, Cd, or Cd + CHX. The cells were cultured at 37°C with 5% CO_2. Fluorescence image of FluoZin-3AM was taken at 30 min after treatment. (B) Cells were cultured in normal α-MEM media with 10% FBS and were treated as described for (A). Quantification of fluorescence intensities of the cells was performed using Optimas 6.51 image analysis software. Data represent means and standard deviations from six separate fields for each treatment. (C) Cells were cultured in Zn-depleted α-MEM medium with FBS. Treatments and quantification of fluorescence intensity were performed as described for (B). *** $P < 0.001$.

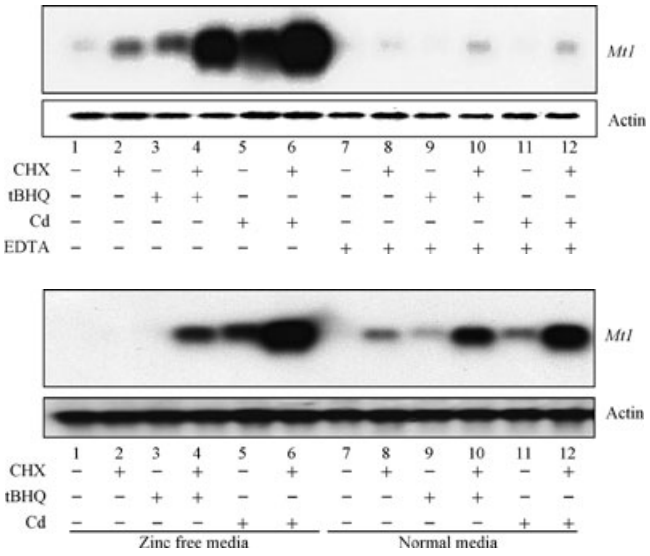


FIGURE 10. Effect of Zn depletion on superinduction. (A) Hepa1c1c7 cells were treated with CHX (10 μg/mL), tBHQ (100 μM), or Cd (10 μM) in the absence or presence of EDTA (1 mM) for 4 h. (B) Cells were cultured in Zn-depleted media or normal α-MEM media and were treated with CHX, tBHQ, Cd, or in combination for 4 h. Total RNA was analyzed for expression of *Mt1* and *Actin*.

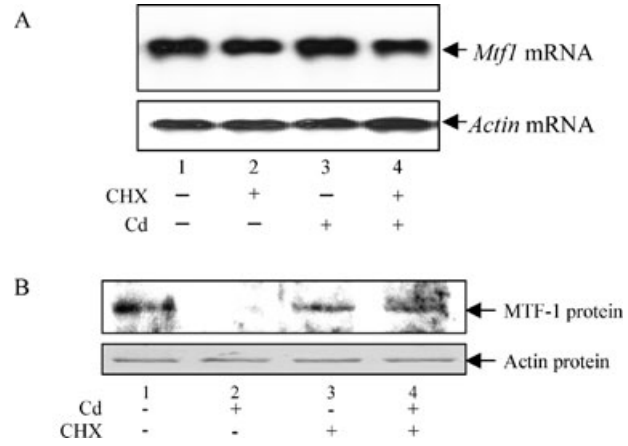


FIGURE 11. Effect of CHX on MTF-1. (A) Northern blotting: cells were treated with CHX (100 μM) and Cd (10 μM) for 4 h. Total RNA (5 μg each) was analyzed for mRNA expression of *Mtf1* and *Actin*. (B) Immunoblotting: cells were treated with CHX and Cd as described for (A). Total cell lysate (5 μg each sample) was analyzed by immunoblotting with antibodies against mouse MTF-1 or actin.

down-regulation of the MTF-1 protein. The findings suggest that a CHX-sensitive factor represses the agonist-induced degradation of the MTF-1 protein.

DISCUSSION

Heavy metals consist of a large group of essential and toxic elements. Heavy metals elicit a variety of transcriptional responses, either toxic or protective, in cells. The molecular mechanism(s) by which metals regulate gene transcription is not well understood at present. In this regard, induction of *Mt1* and *Mt2* through the MTF-1/MRE-dependent pathway by a range of heavy metals can serve as a model system for analyzing the mechanisms of gene regulation by metals. Induction of *Mt* through MTF-1 involves a series of signaling processes including activation of the latent form of MTF-1 in the cytoplasm, translocation of MTF-1 into the nucleus, binding of MTF-1 to MRE, and transcription of *Mt* genes [6,16,25,26]. Knowledge on the molecular events underlying these signaling steps of MTF-1 is currently lacking. In particular, protein factors that regulate MTF-1 have not been identified. In this study, we report that inhibition of protein synthesis by cycloheximide increases the induction of *Mt1* by heavy metals, a phenomenon known as "superinduction." The findings suggest that MTF-1 mediated induction of *Mt-1* does not require synthesis of new proteins, but involves a labile factor(s) that negatively regulates MTF-1 function.

A number of observations support the conclusion that inhibition of the synthesis of a labile transcriptional repressor of MTF-1 accounts for "superinduction." First, superinduction is both time- and concentration-dependent of CHX; maximal superinduction is observed at a concentration at which larger than 95% of total protein synthesis in cells is inhibited. Second, inhibition of protein synthesis by different classes of inhibitors results in superinduction, suggesting that inhibition of protein synthesis is a common component of superinduction by cycloheximide or puromycin. Third, superinduction is transcriptional. Fourth, superinduction occurs in the presence of a wide range of *Mt1* inducers such as Cd, Zn, or tBHQ, which activates MTF-1, but not Dex, which induces *Mt1* through the GR/GRE-dependent pathway; thus, activation of MTF-1 is required for superinduction.

The molecular step at which the labile repressor regulates MTF-1 is unclear. It has been proposed that zinc activates MTF-1 via binding to the protein, possibly at the zinc fingers, whereas other inducers, such as cadmium and tBHQ, mobilize intracellular zinc from protein-bound zinc pools by replacing zinc or modifying zinc-binding proteins, resulting in an increase in free zinc concentration and consequently activation of MTF-1 [7,27]. This mechanism provides a plausible explanation for superinduction: inhibition of protein synthesis by CHX reduces the total amount of zinc-binding proteins, thereby increasing intracellular free zinc for activation of MTF-1 and superinduction of *Mt1*. We tested this notion by measuring the total as well as free zinc concentrations in cells under the condition of superinduction. The results reveal that treatment with cycloheximide in the absence or presence

of an *Mt1* inducer does not change the total or free zinc concentrations in cells. Furthermore, cells retain the capability of superinduction after prolonged culture in a zinc-depleted medium ($[Zn^{2+}] < 0.7$ μM). Together, these findings preclude modulation of intracellular zinc concentration as a mechanism of superinduction.

It has been reported that incubation of cells at an elevated temperature (41 or 42°C) enhances induction of *Mt1* by metals [28]. Enhanced induction of *Mt-1* by heat shock does not appear to be a consequence of activation of HSF1, a principle transcription factor mediating heat shock responses, as transfection of HSF1 increases the transcription of a reporter gene driven by the *Hsp70* promoter, but does not affect the promoter activity of *Mt1*. Instead, heat shock was found to increase intracellular accumulation of zinc by 2.5- and 2.3-fold in the presence of zinc or cadmium, respectively [28]. How heat shock increases intracellular zinc content is not clear. Nonetheless, the observation does provide a clue to the mechanism by which heat shock affects *Mt-1* induction through MTF-1. Because superinduction of *Mt1* by CHX is not accompanied by an elevation of intracellular zinc content, superinduction of *Mt1* by inhibition of protein synthesis and by heat shock are mediated through distinct mechanisms.

We have previously reported superinduction of *Cyp1a1* (cytochrome P450 1a1) and TiPARP (2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-inducible poly (ADP-ribose) polymerase) by CHX in the presence of TCDD, an aryl hydrocarbon receptor (AhR) agonist [22,29]. Superinduction of the genes requires activation of cytoplasmic AhR, nuclear translocation of the receptor, and binding of AhR to dioxin-response elements in the enhancer regions of the genes, similarly to the superinduction of *Mt1* by MTF-1. Superinduction of AhR target genes involves inhibition of the agonist-induced turnover of the AhR protein [22,30]. The findings suggest that a labile repressor, designated as ADPF (AhR degradation promoting factor), negatively regulates AhR transcription activity by promoting the turnover of activated AhR. Inhibition of the synthesis of ADPF blocks the turnover of AhR, giving rise to superinduction of the genes. In this study, we found that activation of MTF-1 by Cd does not affect the expression of MTF-1 mRNA, but dramatically downregulates the protein level of MTF-1; whereas cotreatment with CHX and Cd increases the stability of activated MTF-1. A simple explanation of the result is that Cd activates MTF-1; at the same time, Cd induces an accelerated turnover of activated MTF-1, which serves as a mechanism by which the MRF-1 mediated transcription of *Mt1* is controlled at certain levels to maintain the homeostasis of the metal inducers. Degradation of activated MTF-1 is promoted by a labile factor; inhibition of protein synthesis by CHX or puromycin blocks the action of the labile factor, leading to increased concentration of activated MTF-1 and consequently, superinduction of *Mt1* transcription. Whether ADPF is involved in the control of MTF-1 degradation remains unclear at present. Our observations provide new directions for elucidating the molecular pathway of *Mt1* induction by MTF-1 in future. Given the broad range of metal and stress inducers of *Mt1* and the apparent protective role of MTs in metal toxicity and certain pathological processes, understanding the mechanism of action of MTF-1 in *Mt-1* induction would generate new mechanistic insights into the biological responses to environmental/occupational metals and stress signals.

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