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Cloning and characterization of a tilapia (*Oreochromis aureus*) metallothionein gene promoter in Hepa-T1 cells following the administration of various heavy metal ions ☆

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Abstract

Metallothioneins (MTs) are highly conserved intracellular metal-binding proteins that contribute to the homeostasis of essential metals and the detoxification of non-essential heavy metals. MT gene expression is induced by various heavy metal ions, and Zn^{2+} is able to bind and activate a transcription factor associated with the MT gene that is known as the metal responsive element (MRE) binding transcription factor-1 (MTF-1). Heavy metals other than Zn^{2+}, such as Cd^{2+} and Cu^{2+}, fail to activate the binding of MTF-1 to MREs despite their ability to induce the transcription of the MT gene. To study how different metal ions regulate MT gene expression, a tilapia (ti)-MT gene promoter was cloned and its responses to activation by various metal ions measured using a Hepa T1 cell culture model. The tiMT gene promoter contains six functional MREs within 2118 bp 5′ of the translational start site. A transient gene expression study showed the tiMT gene promoter fragment to be responsive to Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, and Zn^{2+}. Deletions from the 5′ end and the site-directed mutagenesis of individual MREs in the tiMT gene promoter confirmed that both proximal and distal clusters of MREs were required for the maximal metal induction of the tiMT gene. The distal cluster of MREs greatly enhanced the induction of tiMT gene expression by several of the heavy metal ions, and especially the non-Zn^{2+} ions. Individual MREs showed a different responsiveness to metal ions, with MREe being the most potent, MREb being responsive to Zn^{2+} but not to other metal ions, and MREa being mainly for the basal expression of the tiMT gene. Electrophoretic mobility shift assay (EMSA) identified a transcription factor that was able to bind most of the MREs, with the exception of MREd, but the binding was only activated by the *in vivo* administration of Zn^{2+}, not the administration of Cd^{2+} or Cu^{2+}. In conclusion, the results of this study on a Hepa T1 cell model suggest that the mechanism of MT gene activation by non-Zn^{2+} metal ions is different from that of activation by Zn^{2+}, and that different MREs may be involved in the activation of the tiMT gene by different metal ions without enhancing the binding of MTF-1 to MREs.
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Keywords: LC50 values of metal ions; Gene transcription; Gel-shift assay; Metal induction; Transient gene expression

1. Introduction

Metallothionein (MT) is an intracellular metal-binding protein of Cu^{2+} and Zn^{2+} (Hamer, 1986; Kägi and Kojima, 1987; Bremner, 1987) originally discovered as a cadmium-binding protein (Margoshes and Vallee, 1957; Kägi and Vallee, 1961). Metal ions, including Cr^{3+}, Cr^{6+}, Fe^{2+}, Pb^{2+}, Mn^{2+}, Hg^{2+}, and Ni^{2+} increase MT tissue levels *in vivo* and *in vitro* (Waalkes and Klaassen, 1985; Andrews, 1990), and MT is important in reducing the toxicity of these non-essential metals when they enter cells (Goering and Klaassen, 1984). Knockout experiments have confirmed the protective role of MT against Cd^{2+}, with MT-null mice being found to be more vulnerable to cadmium-induced lethality, aberrant gene activations, liver injury, bone mass loss,

Abbreviations: AP1, activator protein 1; bp, base-pair; cDNA, complementary deoxyribonucleic acid; CMV, cytomegalovirus; EC_{50}, median effective concentration; EDTA, ethylene diamine tetraacetic acid; EMSA, electrophorectic mobility shift assay; GRE, glucocorticoid regulatory element; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; MRE, metal responsive element; mt, metallothionein; MTF-1, MRE-binding transcription factor 1; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RACE, rapid amplification of cdna ends; RNA, ribonucleic acid; RT, reverse transcription; Sp1, specific protein 1; ti, tilapia; TSP, target specific primer; U, unit; UV, ultra-violet.

☆ GenBank accession number for the nucleotide sequence of tilapia (*Oreochromis aureus*) MT gene reported in this paper: **EU005543**.

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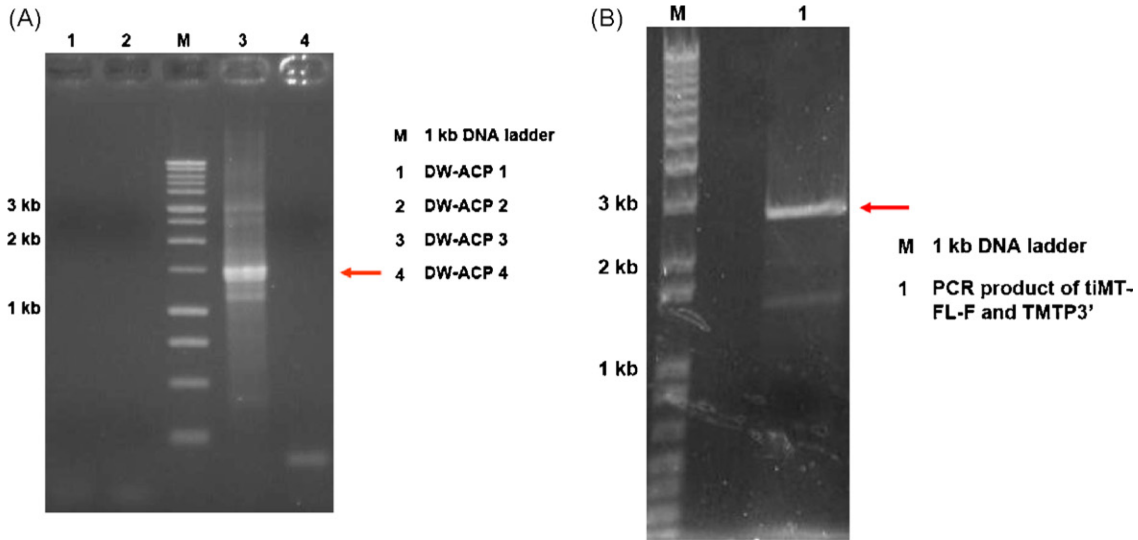
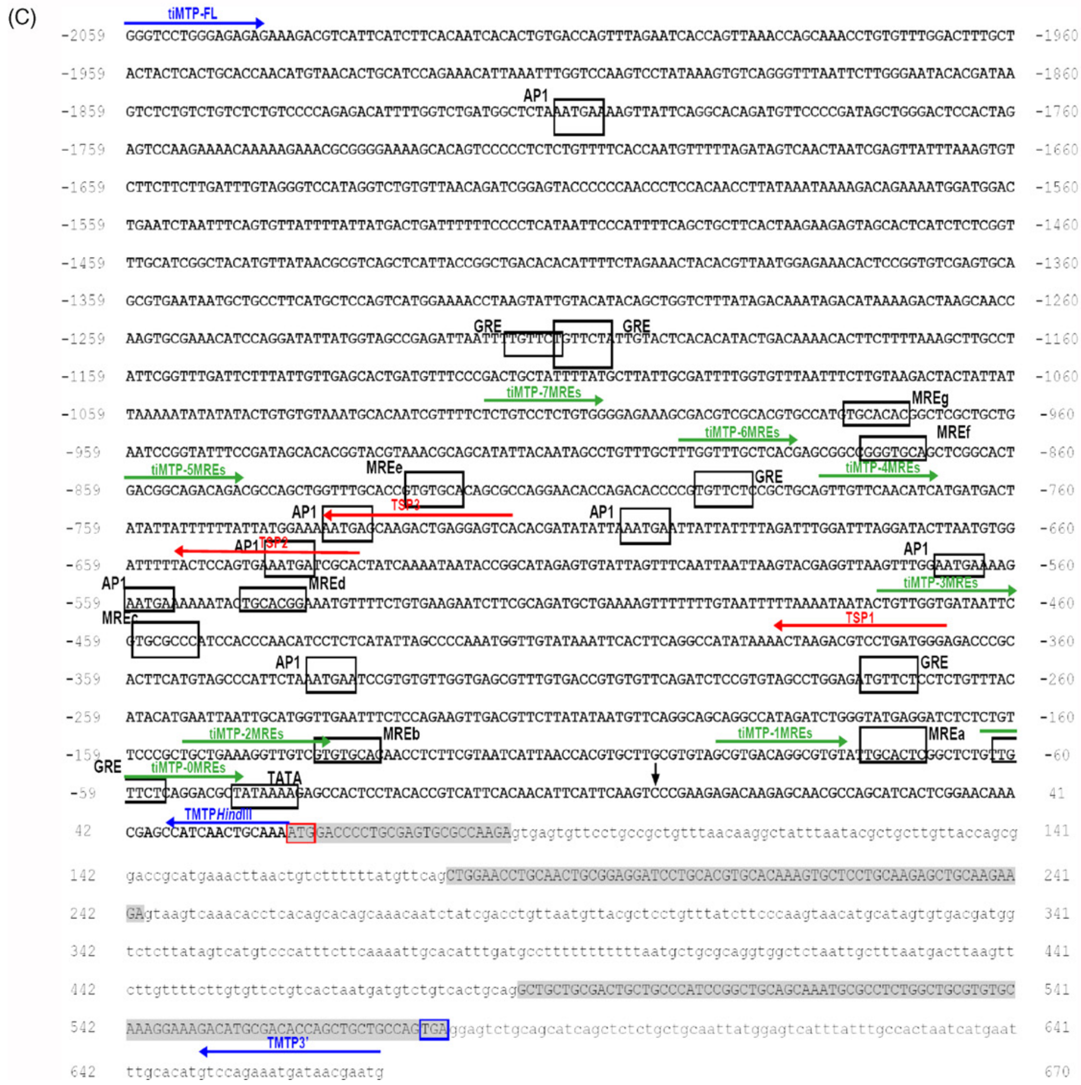


(A)
1
2
M
3
4
3 kb
2 kb
1 kb
M 1 kb DNA ladder
1 DW-ACP 1
2 DW-ACP 2
3 DW-ACP 3
4 DW-ACP 4
(B)
M
1
3 kb
2 kb
1 kb
M 1 kb DNA ladder
1 PCR product of tiMT-FL-F and TMTP3'



(C)
tiMTP-FL
AP1
GRE
GRE
tiMTP-7MREs
MREg
tiMTP-6MREs
MREf
tiMTP-5MREs
MREe
GRE
tiMTP-4MREs
AP1
TSP3
AP1
AP1
TSP2
AP1
AP1
MREd
tiMTP-3MREs
MREc
TSP1
AP1
GRE
tiMTP-2MREs
MREb
tiMTP-1MREs
MREa
GRE
tiMTP-0MREs
TATA
TMTP*HindIII*
TMTP3'

and bone injury than control mice (Liu et al., 1995; Zheng et al., 1996; Habeebu et al., 2000). Many toxic metals are able to displace Zn^{2+} from MT because their relative affinity for MT is higher than that of Zn^{2+} ($Zn^{2+} < Cd^{2+} < Cu^{2+} < Hg^{2+}$) (Day et al., 1984; Funk et al., 1987). The displaced Zn^{2+} may stimulate MT synthesis to reduce the toxicity of the displacing metal ion, such as Cd^{2+} (Waalkes and Goering, 1990).

In vertebrates, MT gene promoters contain a TATA box with multiple copies of *cis*-acting regulatory elements called metal responsive elements (MREs), which control MT gene transcription at basal and induced levels following the administration of metal ions (Hamer, 1986). An MRE is a conserved motif that acts as a binding target for a transcription activating protein factor called *M*RE-binding *t*ranscription *f*actor 1, or MTF-1 (Stuart et al., 1985; Brugnera et al., 1994). MTF-1 regulates MT gene expression in a conserved motif that is present in the MT gene promoters of many organisms, from invertebrates to vertebrates. MREs consist of a highly conserved 7-bp core and consensus sequence (bold) surrounded by the semi-conserved flanking sequence CTN**TGCRCNC**GGCCC (Westin and Schaffner, 1988). Multiple copies of MREs are always present in the MT-gene promoters found in animals, and differences in the basal activity of MREs seem to be determined by both the sequence of the MRE and its position in the promoter region (Samson and Gedamu, 1998). Some MREs may be more potent than others. For instance, among the five putative MREs in the mouse MT-I promoter, MREd is the most potent (Stuart et al., 1985), whereas in the human MT-IIA promoter, MREa is the most active *cis*-acting element (Koizumi et al., 1999).

In teleosts, MT gene promoters from rainbow trout (Zafarullah et al., 1988; Olsson et al., 1995), sockeye salmon (Chan and Devlin, 1993), pike (Kille et al., 1993), stone loach (Kille et al., 1993), icefish (Scudiero et al., 2001), zebrafish MT-II (Yan and Chan, 2002, 2004; Chen et al., 2004), common carp MT-I (Chan et al., 2004), crucian carp MT-I (He et al., 2007) and MT-II (Ren et al., 2006), and tilapia (Cheung et al., 2005) have been reported to contain multiple MREs to maximize gene activity. The MREs in these teleost MT promoters are divided into two clusters, with 2 to 3 MREs being located within 300 bp of the TATA box and additional MREs in the distal region further upstream forming another cluster in the promoters of fish MT genes.

Tilapia is a useful model fish species for eco-toxicological studies, and its hepatic MT level may be a biomarker of exposure to metal ions in the waters of Hong Kong, mainland China, and Southeast Asian countries (Chan, 1994; Cheung et al., 2004, 2005; Lam et al., 1998; Wong et al., 2000). In a series of studies, a single form of MT was purified in an ion-exchange DEAE Sephadex A25 column and its protein expression levels at larval stages and induction by Cd^{2+} were studied (Wu et al., 1999, 2000, 2007). Genomic PCR was performed to isolate tiMT gene sequences from *O mosambicus* and *Oreochromis aureus* with their gene promoters in the dessert topminnow *Poecilliopsis lucida* hepatocellular carcinoma cell-line PLHC-1 (Cheung et al., 2005). Four putative MREs were identified in the tilapia MT gene promoter, but transient gene expression assay revealed that although the promoter was responsive to Zn^{2+} and other metal ions, such as Ni^{2+} and Cu^{2+}, it was a poor inducer despite strong inductions of mRNA levels following *in vivo* treatments of the non-zinc metal ions tested (Cheung et al., 2004, 2005). In this paper, we report the cloning and characterization of the further upstream tiMT gene promoter in the homologous cell-line Hepa T1.

The aim of this project is to study the molecular mechanism of the response of MT genes to various heavy metal ions and to determine which of the genes are primary inducers of MT in tilapia using the Hepa T1 cell-line as a model. We report the cloning of an extended tiMT promoter from *O. aureus*, the aim of which was to dissect the functions of MREs in the tiMT gene promoter following the administration of different heavy metal ions and study the binding of *trans*-acting factors from Hepa-T1 cells using electrophoretic mobility shift assay (EMSA) on putative MREs in the tiMT gene promoter.

2. Materials and methods

2.1. Tilapia stock and gene cloning

The tilapia (*O. aureus*) was obtained from a broodstock from the Xijiang Aquaculture Institute, Guangdong, China. Muscle tissue (3 g) from the tilapia was quickly ground into fine powder with liquid nitrogen in 30 ml of frozen grinding buffer (100 mM of NaCl, 10 mM of Tris pH 8, 25 mM of EDTA pH 8, 0.5% sodium dodecyl sulfate) and then digested with Proteinase K (5 mg) overnight at 65 °C. The mixture was then extracted

Fig. 1. Amplification of the tilapia MT gene fragments by using polymerase chain reaction. (A) Gel electrophoresis of the DNA walking products from the 3rd round of nested PCR. The arrow indicates the PCR product (∼1.5 kb) that was subsequently subjected to TA cloning. (B) Primers were designed from the 5′ end of the 5′ flanking regions obtained from (A) and a primer of the 3′ end of the cDNA to amplify the whole MT gene. The arrow indicates the full-length tilapia MT gene, which is around 2.7 kb in size. (**C**) Nucleotide sequence of the tilapia (*Oreochromis aureus*) MT gene, as shown in (B). The seven putative MREs identified with a TATA box in the tilapia MT promoter are boxed. The putative AP1 site, GRE, and Sp1-binding site are also boxed. The transcriptional start site is marked as +1, and the three exons are shown in uppercase letters and shadowed. The primers used to amplify the whole gene fragment are indicated (tiMTP-FL and TMTp3′). The other primers used for the nested PCR, 5′RACE for start site determination, and the creation of the deletion mutants for the transient gene expression assay is also shown. The nucleotide sequences of primers used are: TSP1 (5′-CCCATCAGGACGTCTTAGT-3′); TSP2 (5′-TGCGATCATTTCACTGGAGTA-3′); TSP3 (5′-GACTCCTCAGTCTTGCTCATT-3′); tiMTP-FL-F (5′-GAT*GAGCTC*GGGTCCTGGGAGAGA-3′); TMTP3′ (5′-CATTCGTTATCATTTCTGGAC-3 ′); tiMTP-7MREs (5′-GAT*GAGCTC*TCTGTCCTCTGTG-3′); tiMTP-6MREs (5′-GAC*GAGCTC*TTGGTTTGCTCAC-3′); tiMTP-5MREs (5′-GAT*GAGCTC*GACGGCAGACAGA-3′); tiMTP-4MREs (5′-GC*GAGCTC*GGTTGTTCAACATC-3′); tiMTP-3MREs (5′-GAT*GAGCTC*CTGTTGGTGATAATTCG-3′); tiMTP-2MREs (5′-GAT*GAGCTC*TGCTGAAAGGTTGTCGT-3′); tiMTP-1MREs (5′-GAT*GAGCTC*GCGTGACAGGCGTGT-3′); tiMTP-0MREs (5′-GAT*GAGCTC*GTTGTTCTCAGGACGCT-3′); and TMTP*Hind*III (5′-GC*AAGCTT*TTTGCAGTTGA-TGG-3′).

by repeated phenol–chloroform extractions until finally a high molecular weight DNA was precipitated in ethanol (Scott et al., 1985). The genomic DNA obtained was dissolved in Tris–EDTA buffer (pH 8.0), and its purity and quantity were determined by measuring the absorbance at 260 and 280 nm using a UV spectrophotometer (Hitachi U2800).

Three target specific primers (TSPs, Fig. 1C) were designed from the known tiMT promoter sequence (Cheung et al., 2005) using a DNA Walking SpeedUp™ Premix Kit (Seegene, Korea). Nested PCR was performed by using the DNA Walking Annealing Control Primers (DW-ACP) provided in the Kit and the three TSPs. Each of the DW-ACPs contained a specific ACP primer-binding site at its 3′-end (5′-AGGTC, 5′-TGGTC, 5′-GGGTC, 5′-CGGTC). The amplification contained 100 ng of *O. aureus* genomic DNA, 4 μl of 2.5 μM DW-ACP (one of DW-ACP 1, 2, 3, and 4), 1 μl of 10 μM TSP 1, 25 μl of 2X SeeAmp™ ACP™ Master Mix II, and sufficient distilled water to make up a 50 μl reaction. In the second PCR, four PCR reactions were set up, each of which contained 3 μl of the purified PCR product, 1 μl of the 10 μM DW-ACPN provided in the kit, 1 μl of 10 μM TSP 2, 10 μl of 2X SeeAmp™ ACP™ Master Mix II, and 5 μl of distilled water to make up a 20 μl reaction. In the third PCR, four PCR reactions were prepared, each of which contained 1 μl of the second PCR products, 1 μl of the 10 μM universal primer provided in the kit, 1 μl of 10 μM TSP 3, 10 μl of 2X SeeAmp™ ACP™ Master Mix II, and 7 μl of distilled water to make up a 20 μl reaction. All of the PCRs were performed on a TaKaRa PCR Thermal Cycler Dice. The extracted PCR products were cloned into the pGEM®-T Easy Vector System (Promega) and sent to a commercial DNA sequencing service (Tech Dragon Limited, Hong Kong) for nucleotide sequence determination. After the upstream tiMT promoter sequence was cloned and sequenced, two primers were designed to obtain the whole tiMT gene using the Expand High Fidelity PCR System (Roche Applied Science).

The genomic PCR contained 200 ng of *O. aureus* genomic DNA, 5 μl of 10X Expand High Fidelity buffer with 15 mM of $MgCl_2$, 1.5 μl of 10 μM tiMTP-FL-F primer, 1.5 μl of 10 μM TMTP3′ primer, 1 μl of 10 mM of dNTP mix, 0.75 μl of Expand High Fidelity Enzyme Mix, and sufficient distilled water to make up a 50 μl reaction. The gel-extracted PCR product was cloned into the pGEM®-T Easy Vector System (Promega) and sent to a commercial sequencing service provided by Tech Dragon Limited for nucleotide sequence determination of the seven clones on both strands.

The GeneRacer™ Kit (Invitrogen) was used according to the manufacturer's instruction manual to identify the transcriptional start site. The total RNA was prepared by using Tri-Reagent (Roche) and 3 μg of purified RNA that was treated with 10 U of calf intestinal phosphatase (CIP) in the presence of 1× CIP buffer and 40 U of RNaseOut™ at 50 °C for 1 h. The dephosphorylated RNA was purified by phenol:chloroform extraction and ethanol precipitation, and was resuspended in 7 μl of DEPC water and treated with 0.5 U of tobacco acid pyrophosphatase (TAP) in 1× TAP buffer at 37 °C for 1 h. After phenol–chloroform extraction and ethanol precipitation, the RNA (7 μl) was denatured with GeneRacer™ RNA Oligo (0.25 μg) at 65 °C for 5 min and then incubated with 5 U of T4 RNA ligase in 1× ligase buffer at 37 °C for 1 h. The reverse transcription of the mRNA to cDNA was performed using SuperScript™ III RT (Invitrogen). The RNA was first denatured with random primer (Invitrogen) at 65 °C for 5 min and then incubated with 200 U of reverse transcriptase at 50 °C for 60 min. The Expand High Fidelity PCR System (Roche Applied Science) was used to amplify the 5′ cDNA ends with the MT gene-specific primer (TMTD) (5′-TCACTGGCAGCAGCTGGTGT-3′) as the reverse primer and the GeneRacer™ 5′ Primer (Invitrogen) as the forward primer. The PCR products obtained were subjected to nucleotide sequence determination after TA cloning with the pGEM®-T Easy Vector System (Promega) with the gene-specific primer (TMTD) using a commercial sequencing service provided by Tech Dragon Limited, Hong Kong.

2.2. Cell-culture and Alamer Blue assay

The Hepa-T1 cell-line, which for this study was obtained from the Institute of Physical and Chemical Research (RIKEN) in Japan, comprises epithelial-like hepatocytes from the tilapia *Oreochromis niloticus*. The cells were cultured in a combination of media: 50% (v/v) Leibovitz's L-15 medium with 2 mM of L-glutamine (Gibco), 35% (v/v) Dulbecco's modified Eagle medium with 4.5 g/L of glucose and 4 mM of L-glutamine (Gibco), and 15% (v/v) Ham's F12 with 1 mM of L-glutamine (Gibco). As none of the media contained sodium bicarbonate, they were supplemented with 0.15 g/L of sodium bicarbonate and 15 mM of HEPES. The pH was adjusted to 7.2, and the media was then filter-sterilized through a 0.22-μ membrane (Millipore). Heat-inactivated fetal bovine serum (Gibco) and 5% (v/v) and 1% (v/v) penicillin–streptomycin (Gibco) were freshly added before use. The cell-line was sub-cultured after trypsin digestion and maintained as a stationary monolayer in a humidified incubator at 28 °C.

Cadmium chloride monohydrate, copper(II) chloride dehydrate, mercury(II) chloride, nickel(II) chloride hexahydrate, lead(II) nitrate, and zinc chloride of reagent grade were purchased from Sigma–Aldrich. Metal stock solutions in concentrations of 100 mM of heavy metal ions were prepared in double distilled water followed by 0.22-μM filtration in a syringe. The Hepa-T1 cells (1×10^3) were seeded to individual wells of a 96-well plate. Solutions of the heavy metal ions (Cd^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+}) of various concentrations were obtained by the serial dilution of the stock solution (100 mM) with fresh medium. The solutions were applied to the Hepa-T1 cells the next day. After 24 h of treatment, the media were aspirated and the cells incubated with 10% alamarBlue™ (BioSource) at 28 °C for about 2 h. Fluorescence readings at an excitation wavelength of 530 nm and an emission wavelength of 590 nm were obtained by using a Tecan GENios™ fluorescence plate reader. The cytotoxicity of the various heavy metal ions was plotted with EC_{50} values that were determined by using GraphPad Prism 4 on a personal computer.

2.3. Transient gene expression assay

Eight forward primers (tiMTP-7MREs to TMTP D6*Sac*I, Fig. 1C) and one reverse primer (TMTP*Hind*III, Fig. 1C) were designed from the 5′-flanking region of the tiMT gene for the generation of the eight different PCR fragments using the Expand High Fidelity PCR System (Roche Applied Science). The restriction enzyme sites *Sac*I and *Hind*III at the ends were adopted for the subsequent cloning into the pGL3-Basic vector (Promega) with a firefly luciferase reporter gene digested with *Sac*I and *Hind*III. The clones were further confirmed by nucleotide sequencing.

The transfection conditions were pre-optimized by mixing 200 ng of pGL3-tiMT construct with the seven putative MREs in the 5′-flanking region of the tiMT gene (pGL3-tiMTP-7MREs), 2 ng of the pRL-CMV normalization construct (Promega), and a serum-free medium, which consisted of 1 μl of Lipofectamine reagent (Invitrogen) and 1 μl of PLUS Reagent (Invitrogen) as per the manufacturer's recommendations. The mixture was added into 1.5×10^5 Hepa-T1 cells in a 24-well plate for transfection at about 80% confluence for 3 h. A promoterless negative control plasmid pGL3-Basic (Promega) (200 ng) was also transfected with pRL-CMV (200 ng) in the same way. After 3 h of incubation at 28 °C, the transfection reagents were removed from the cells and replaced with fresh culture medium with 5% serum. The cells were then allowed to recover at 28 °C in an incubator for 24 h before the addition of the metal ion solutions.

Different doses (0%, 10%, 25%, 50%, 75%, and 100% of the 24 h-LC_{50}) of one of the heavy metal ions (Cd^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, or Zn^{2+}) were added for 12, 24, or 48 h, and the luciferase activity was measured using a Lumat LB 9501 luminometer with one fourth of the volume of the reagents, as recommended in the technical manual of the Dual-Luciferase® Reporter Assay System (Promega). The activity of the different constructs was shown as the relative luciferase activity, presented as the average of the mean ± standard deviation (S.D.) of three replicates, and analyzed using GraphPad Prism 4 on a personal computer.

Eight deletion mutant constructs (200 ng each) from which the seven MREs in the 5′-flanking region of the tiMT gene were successively deleted (pGL3-tiMTP-7MREs to pGL3-tiMTP-0MRE) and the pRL-CMV normalization construct (Promega) (200 ng) were transfected into Hepa-T1 cells seeded into the individual wells of a 24-well plate, as has been described. The optimal doses of heavy metal ions to induce tiMT promoter activity were chosen (75% of the 24 h-LC_{50}) to treat the Hepa-T1 cells transfected with the deletion mutant constructs. The luciferase activity after 12 or 24 h of metal ion treatment was measured as has been described. The results are presented as the average of the mean ± standard deviation (S.D.) of three replicates, and were analyzed using GraphPad Prism 4 on a personal computer.

2.4. Site-directed mutagenesis of the tiMT promoter

Two strategies based on PCR were adopted to generate the mutants. In each of the strategies, mutations (TGCRCNC → **ATT**RCNC) were introduced to one of the seven putative MREs in the 5′-flanking region of the tiMT gene. Constructs that had undergone site-directed mutagenesis at the individual MRE sites (MREa, MREd, MREe, MREf, and MREg) in the tiMT promoter were prepared using a method modified from Allemandou et al. (2003). In brief, the pGL3-tiMTP-7MREs plasmid (5855 bp) was used as the template in the PCR to generate DNA fragments (one with mutations in the desired MRE to another with a phosphate group at the 5′ end), each of which represented half of the original template. The two DNA fragments were digested with *Apa* I and ligated by T4 DNA ligase at the *Apa* I restriction sites and the 5′ end with a phosphate group. Successful mutations were confirmed by DNA sequencing. The amplification reaction (100 μl) contained 100 ng of the native plasmid (pGL3-tiMTP-7MREs), 20 pmol of each primer, 10 nM of each dNTP (Roche), and 3 U of *Pfu* polymerase (Promega) in 1× *Pfu* buffer.

The second strategy was a two-step mega primer-based PCR mutagenesis method modified from Tyagi et al. (2004). Two rounds of PCR amplification were performed. The first round of PCR reaction of 5 cycles was performed with the mutagenic primer carrying mutations in the desired MRE (MREb and MREc) and the reverse primer of the tiMT promoter to synthesize the mutagenic mega primer. The first round was carried out in a total volume of 50 μl that contained 3 U of *Pfu* polymerase, 1× *Pfu* buffer, 100 ng of the native plasmid (pGL3-tiMTP-7MREs), 0.2 mM of dNTP, 1 pmol of the internal mutagenic forward primer (mut-b or mut-c), and 0.05 pmol of the reverse primer TMTP*Hind*III (Fig. 1C). The lower concentration of the reverse primer TMTP*Hind*III in this round of PCR was used to reduce the chance of the reverse primer pairing up with the forward primer in the second round of PCR and generating a tiMT promoter fragment without any of the desired MRE mutations. The clones were confirmed by nucleotide sequence determination.

A pGL3-tiMT plasmid with site-directed mutations in one of the MREs (200 ng) and pRL-CMV (200 ng) were transfected into the Hepa-T1 cells in a 24-well plate as has been described. Two hundred nanograms of pGL3-tiMT with the tiMT promoter region embracing the seven putative MREs was also transfected for comparison. Metal ion doses that were 75% of the 24 h-LC_{50} of the heavy metal ions (Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, Zn^{2+}) were used to treat the transfected Hepa-T1 cells. After 12 or 24 h of treatment, the luciferase activity was measured as has been detailed and the results are presented as the average of the mean ± standard deviation (S.D.) of three replicates, and were analyzed using GraphPad Prism 4 on a personal computer.

2.5. Electrophoretic mobility shift assay (EMSA)

To prepare the cell extracts, Hepa-T1 cells were grown to a 90% confluence and the serum concentration in the growth medium reduced to 1% the night before metal ion treatment. The cells were treated with or without 75% 24-h EC_{50} of cadmium, copper, and zinc (74.0 μM for $CdCl_2$, 727.7 μM for $CuCl_2$, and 565.5 μM for $ZnCl_2$) for 24 h. The control and treated cells were harvested for whole-cell protein extraction as previously

Table 1
Double-stranded oligonucleotide pairs for the binding experiments in the electrophoretic mobility shift assay, EMSA

Oligos	Sequences of complementary strands
MREa	5′-GATCCCGTGTAT**TGCACTC**GGCTCTGTTGTTA-3′ 3′-GGCACATA**ACGTGAG**CCGAGACAACAATCTAG-5′
MREb	5′-GATCCGGTTGTC**GTGTGCA**CAACCTCTTCGTA-3′ 3′-GCCAACAG**CACACGT**GTTGGAGAAGCATCTAG-5′
MREc	5′-GATCCTAATTCG**TGCGCCC**ATCCACCCAACAA-3′ 3′-GATTAAGC**ACGCGGG**TAGGTGGGTTGTTCTAG-5′
MREd	5′-GATCCAAAATAC**TGCACGG**AAATGTTTTCTGA-3′ 3′-GTTTTATG**ACGTGCC**TTTACAAAAGACTCTAG-5′
MREe	5′-GATCCTTGCACC**GTGTGCA**CAGCGCCAGGAAA-3′ 3′-GAACGTGG**CACACGT**GTCGCGGTCCTTTCTAG-5′
MREf	5′-GATCCAGCGGCC**GGGTGCA**GCTCGGCACTGAA-3′ 3′-GTCGCCGG**CCCACGT**CGAGCCGTGACTTCTAG-5′
MREg	5′-GATCCTGCCATG**TGCACAC**GGCTCGCTGCTGA-3′ 3′-GACGGTAC**ACGTGTG**CCGAGCGACGACTCTAG-5′

The MRE sequences are in bold and shaded.

described by Dalton et al. (1997). After treatment, the cells were placed on ice and the cultured medium removed by washing once with ice-cold phosphate-buffered saline. The cells were scraped off the dish in 5 ml of cold phosphate-buffered saline and were collected by centrifugation at 1500 × *g* for 5 min at 4 °C. The cell pellet was frozen in liquid N_2 and resuspended in three volumes of extraction buffer (20 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 400 mM KCl, 0.5 mM of dithiothreitol, 0.2 mM of phenylmethylsulfonyl fluoride, 25% glycerol). The cell lysate was centrifuged at 89,000 × *g* for 5 min, and the supernatant was collected and stored in aliquots at −80 °C. The protein concentrations of the extracts were determined by using the Coomassie Blue staining (Bio-Rad) method.

Complementary oligonucleotide pairs of 32 bp in length were designed to incorporate the seven putative tiMT MREs (MREa to MREg) and the flanking sequences with the addition of four overhanging bases, as shown in Table 1. The complementary oligonucleotides (300 pmol) were air-dried, resuspended in 54 μl of autoclaved distilled water and 6 μl of 10× annealing buffer (200 mM Tris–HCl, pH 7.5, 100 mM $MgCl_2$, 500 mM NaCl), and heated at 80 °C for 10 min. The samples were then cooled slowly at room temperature for the annealing of the complementary strands. A labeling reaction was assembled with 2 μl of the annealed oligonucleotides, 5 μl of [γ-^{32}P]dATP, 10 μl of autoclaved distilled water, 2 μl of kinase 10× reaction buffer, and 10 u of T4 polynucleotide kinase (Promega), and incubated at 37 °C for 1 h. The radiolabeled probes were purified with a Microspin G-25 column (GE Healthcare) and stored at −20 °C.

The DNA–protein-binding reactions of the seven putative MREs and Hepa-T1 cell extracts were performed as previously described (Dalton et al., 1997), with some modifications. Ten micrograms of the cell extract was incubated with buffer that contained 12 mM of HEPES (pH 7.9), 60 mM of KCl, 0.5 mM of dithiothreitol, 12% glycerol, 5 mM of $MgCl_2$, 0.2 μg of dI-dC/μg protein, and 2–4 fmol of end-labeled double-stranded oligonucleotide (5000 cpm/fmol) in a total volume of 20 μl for 20 min on ice. The binding reactions without the addition of cell extract (free probe only) and with the addition of 200-fold excess unlabeled double-stranded oligonucleotides as competitors of the labeled probes were prepared as controls. The protein–DNA complexes were separated at 4 °C by PAGE using a 5% polyacrylamide gel of acrylamide–bisacrylamide (30:0.8) running at 15 V/cm in Tris–glycine buffer (0.19 M of glycine, pH8.5, 25 mM of Tris, and 0.5 mM EDTA). After electrophoresis, the gel was dried and the radioactivity detected by using a Molecular Imager PharosFX Plus System (Bio-Rad).

3. Results

3.1. PCR-cloning of a tilapia MT (tiMT) gene

The 5′ upstream region of the tiMT gene was obtained once the nested PCR (Fig. 1A) and the whole tiMT gene had been obtained with a 2.7 kb fragment (Fig. 1B). The nucleotide sequence of the tiMT gene fragment (2729 bp) is shown in Fig. 1C. It had a 5′-flanking region of 2059 bp in length and contained seven putative metal responsive elements (MREs) with a consensus sequence of (TGCRCNC or TGCRCNG). The MRE sequences were named MREa, MREb, MREc, MREd, MREe, MREf, and MREg in order of their proximity to the TATA box, with MREa being the closest. Additional putative recognition sites of various transcription factors, including the activator protein-1 (AP1) binding sites (5′-AATGA-3′) and glucocorticoid response element (GRE) half sites (5′-TGTTCT-3′) were also identified *in silico*.

3.2. Determination of heavy metal ion toxicity by alamarBlue^{TM} assay

The median effective concentrations (EC_{50}) of the heavy metal ions on the Hepa-T1 cell-line was determined using alamarBlue^{TM} assay. The dose response curves with the 24 h-EC_{50} were plotted using GraphPad Prism 4 (Fig. 2). The cytotoxicity of the heavy metal ions in descending order was as follows: Cd^{2+} (98.71 μM) > Hg^{2+} (118.3 μM) > Zn^{2+} (754.0 μM) > Cu^{2+} (970.2 μM) > Ni^{2+} (2365 μM) > Pb^{2+} (3157 μM).

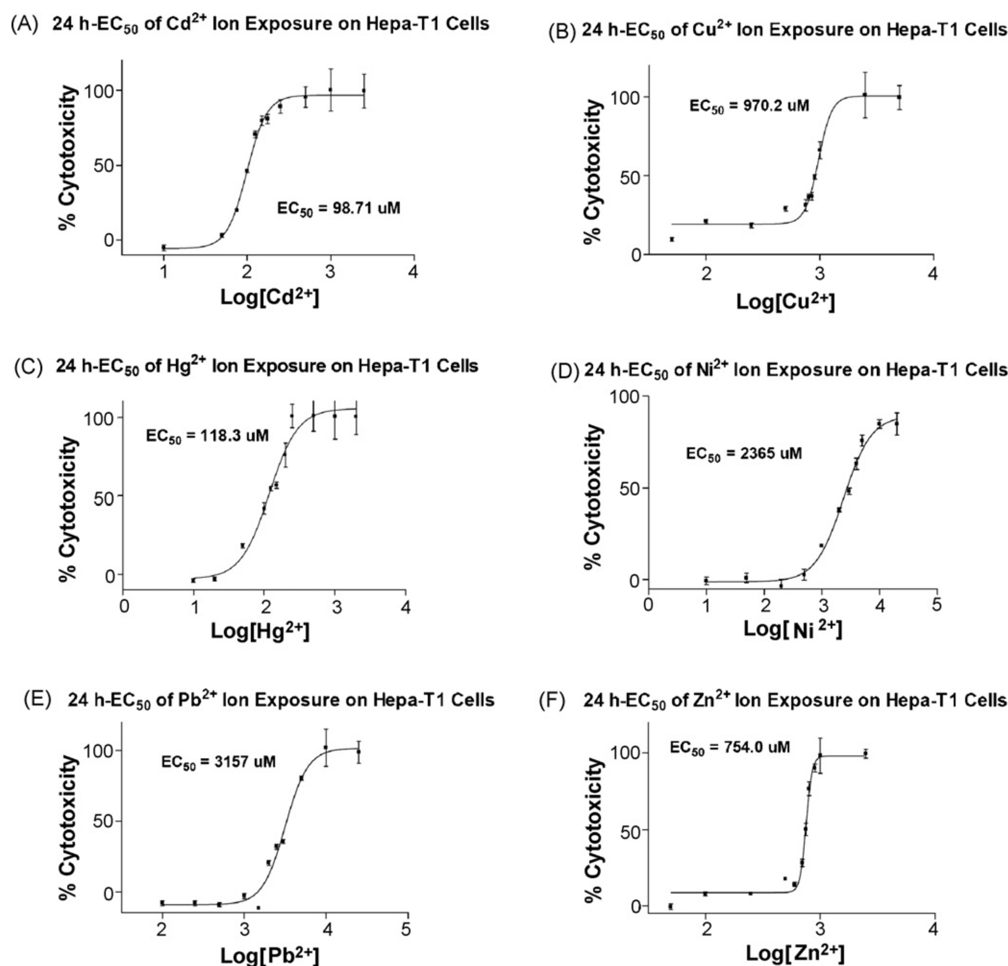


Fig. 2. Cytotoxicity of heavy metal ions on the Hepa-T1 cell-line as determined by using alamerBlue™ assay. The cells were exposed to (A) Cd^{2+}, (B) Cu^{2+}, (C) Hg^{2+}, (D) Ni^{2+}, (E) Pb^{2+}, and (F) Zn^{2+} for 24 h. The median effective concentrations (EC_{50}) were determined by alamarBlue™ assay and are shown in the graphs.

3.3. Study of tiMT promoter activity by heavy metal ion exposure

The tiMT gene promoter carrying seven putative MREs (1078 bp) with the 5′UTR was ligated into a pGL3-Basic (Promega) reporter vector upstream to the firefly luciferase (*Luc*) gene and designated as pGL3-tiMT-7MREs. The tiMT gene promoter activity was measured after the administration of Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, and Zn^{2+} for 12 (Fig. 3A), 24 (Fig. 3B), and 48 (Fig. 3C) h. A promoterless pGL3-Basic (Promega) vector was also used as a negative blank control. The relative luciferase activity is presented with the fold induction written on the histograms.

Only the tiMT promoter activity that was induced by the 12-h exposure to Ni^{2+} is presented, because Ni^{2+} did not activate the tiMT gene promoter, whereas the other metal ions induced the tiMT promoter at 12 and 24 h with clear dose responses. Cd^{2+} and Zn^{2+} provoked a rapid induction, with the highest tiMT promoter activity occurring 12 h after exposure (7.64-fold and 12.84-fold, respectively), whereas Cu^{2+}, Hg^{2+}, and Pb^{2+} caused a higher activation of promoter activity after 24 h of exposure (2.83-fold, 5.98-fold, and 8.68-fold, respectively). The tiMT promoter induction was found to be lowest after 48 h of exposure (data not shown), probably due to the toxic effects of the metal ions.

To study each putative MRE in the tiMT promoter, eight deletion mutants of the promoter were constructed by using PCR, and successive MREs were removed from the 5′ end to produce different promoter fragments that were cloned into the pGL3-basic reporter vector at the *Sac*I and *Hind*III restriction sites. The pGL3-tiMTP-7MREs contained the tiMT promoter fragment (1078 bp) with all of the seven putative MREs. The other deletion mutants contained promoter fragments with a number of MREs proximal to the tiMT gene translation start codon according to the designated name of mutants. In the mutant pGL3-tiMTP-0MRE, all of the MREs were deleted such that only the TATA box remained.

The results of the deletion mutant study after 24 h of metal ion treatment (Fig. 4) show that the deletion of MREg significantly reduced the tiMT promoter activity induced by Cd^{2+} ($P < 0.001$), Cu^{2+} ($P < 0.001$), Pb^{2+} ($P < 0.001$) and Zn^{2+} ($P < 0.001$). The further deletion of MREf in the promoter reduced the activity induced by Cu^{2+} ($P < 0.05$), Hg^{2+} ($P < 0.05$), and Pb^{2+} ($P < 0.05$). The further removal of MREe drastically reduced the induction of promoter activity for all five heavy metal ions ($P < 0.001$). No significant induction of the tiMT promoter was

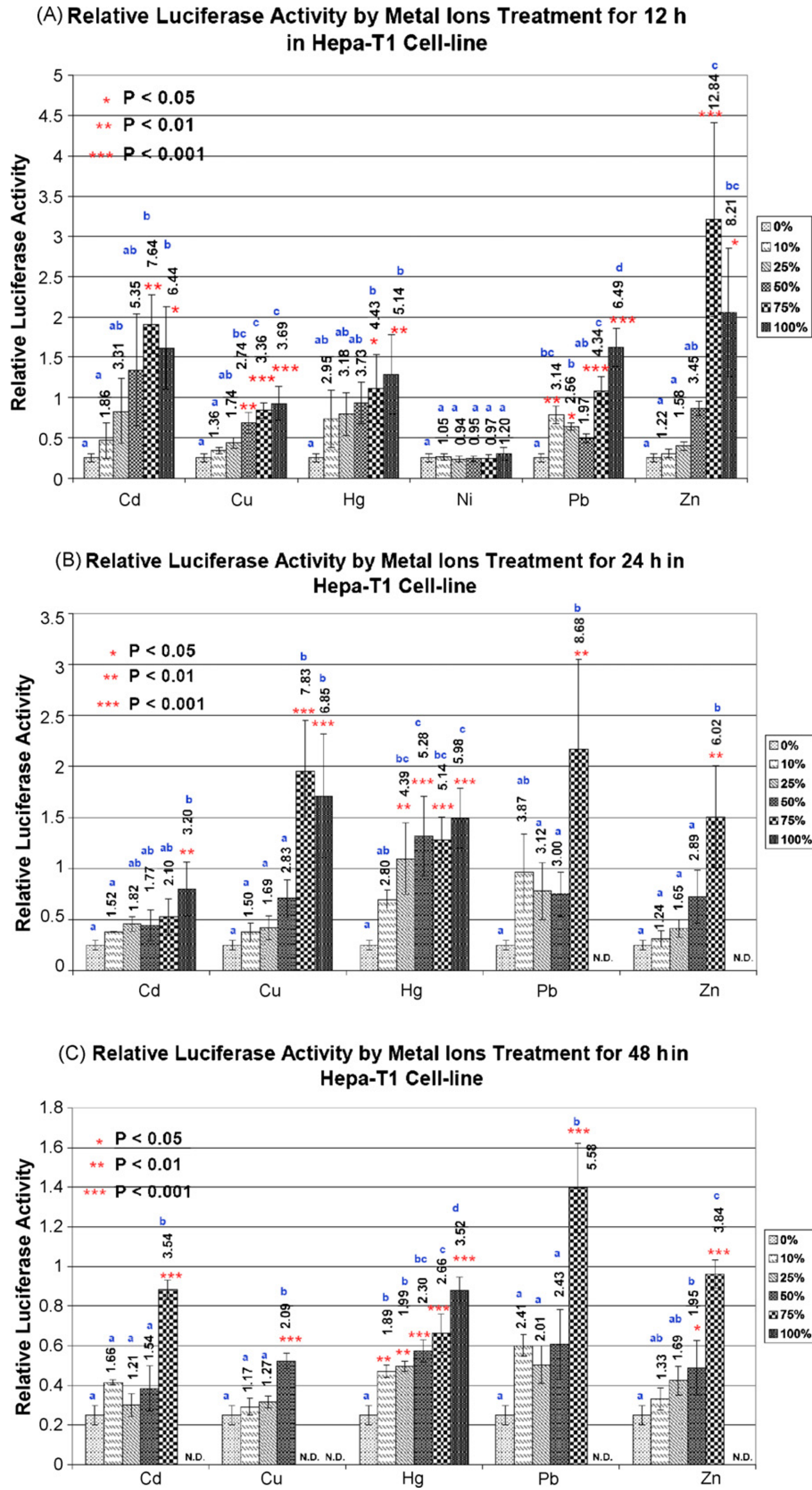


Fig. 3. Relative luciferase activity after the administration of metal ions for (A) 12, (B) 24, and (C) 48 h in the Hepa-T1 cell-line. Different doses (0%, 10%, 25%, 50%, 75%, and 100% 24 h-EC_{50}) of Cd^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+} were applied to Hepa-T1 cells for different durations. The fold inductions of the tiMT promoter activity are given above each metal-treated bar, which was calculated by dividing the normalized relative luciferase activity of the metal treated groups with the average of normalized relative luciferase activity of the untreated group. The data were analyzed by one-way ANOVA test.

found after the administration of Cd^{2+}, Cu^{2+}, Hg^{2+}, and Pb^{2+} (but not Zn^{2+}) with the further deletion of the MREs after MREd (inclusive) (Fig. 4). The deletion of MREb significantly reduced the tiMT promoter activity activated by Zn^{2+} treatment (Fig. 4E). The tiMT promoter activity was completely removed after deleting the six upstream MREs.

3.4. Site-directed mutagenesis of MREs in the tiMT promoter

The results of the site-directed mutagenesis of the tiMT promoter show that the mutations of each distal MRE (MREe, MREf, and MREg) reduced the induction of the promoter activ-

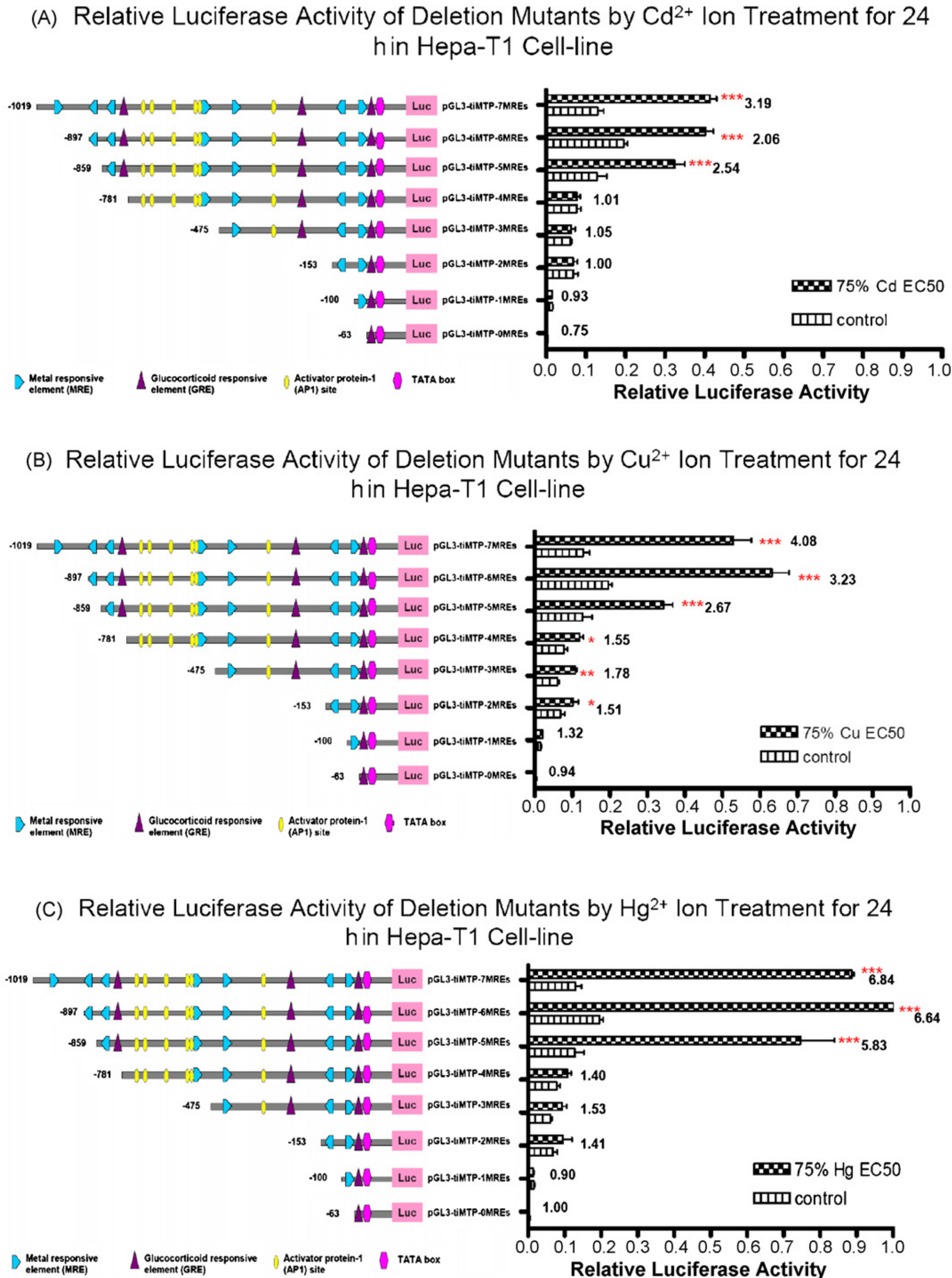
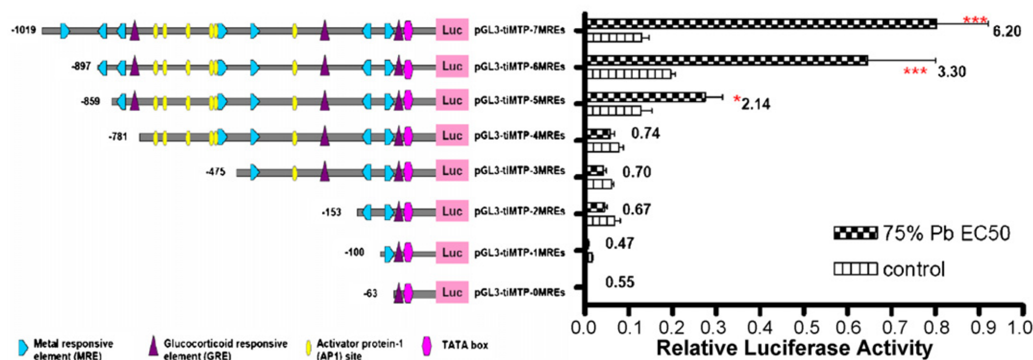


Fig. 4. Functional analysis of tiMT 5′ deletion mutants in Hepa-T1 cells subjected to 24-h heavy metal ion treatment. Hepa-T1 cells were transfected with different deletion mutants and the relative luciferase activity was determined after 75% 24 h-EC_{50} treatment with (A) Cd^{2+}, (B) Cu^{2+}, (C) Hg^{2+}, (D) Pb^{2+}, and (E) Zn^{2+} for 24 h. The fold inductions of the tiMT promoter activity are given above each metal-treated bar, and were calculated by dividing the normalized relative luciferase activity of the metal-treated groups with the average of normalized relative luciferase activity of the untreated group. The data were analyzed by one-way ANOVA test. The schematic diagrams of the eight deletion mutants of the tiMT gene promoter are shown on the left.

(D) Relative Luciferase Activity of Deletion Mutants by Pb^{2+} Ion Treatment for 24 h in Hepa-T1 Cell-line



(E) Relative Luciferase Activity of Deletion Mutants by Zn^{2+} Ion Treatment for 24 h in Hepa-T1 Cell-line

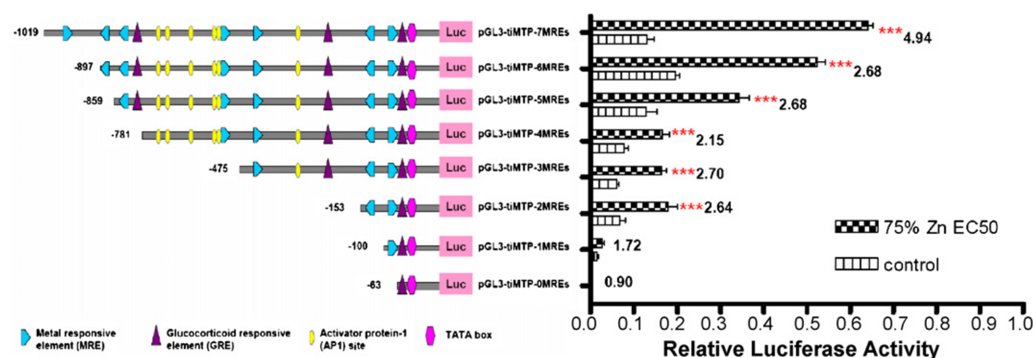


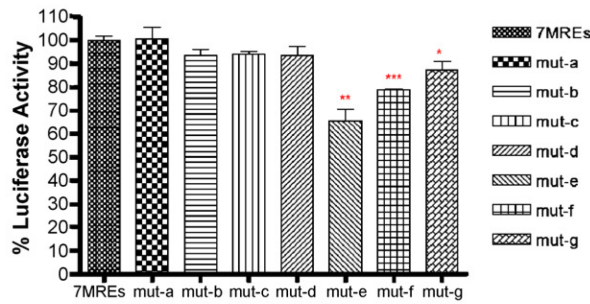
Fig. 4. (*Continued*).

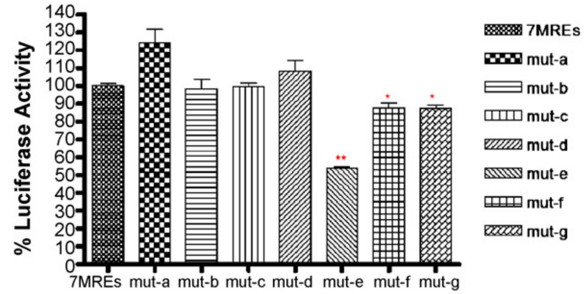
ity by Cd^{2+} treatment by 20–30% (Fig. 5A). The mutations of MREe, MREf, and MREg decreased the tiMT promoter activity following 24 h of Hg^{2+} treatment by 30%, 10%, and 20%, respectively (Fig. 5C). The mutations of the three distal MREs also decreased the promoter activity, by about 50% for the mutation of MREe and 10% for the mutation of MREg and MREf (Fig. 5D) after 24 h of exposure to Pb^{2+}. However, the mutation of MREg had no significant impact on the induction of tiMT promoter activity by Cu^{2+}, whereas both MREe and MREf reduced the promoter activity induction by around 30% (Fig. 5B).

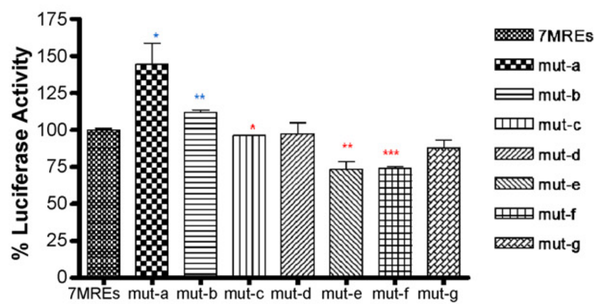
Compared to the mutations of the distal MREs, the mutations of the other four MREs were much less effective in reducing the tiMT promoter activity induced by non-Zn^{2+} metal ions. Only the mutation of MREc significantly decreased the tiMT promoter activity induced by 24 h of Cu^{2+} treatment and 12 h of Pb^{2+} treatment (data not shown). Distinct from other heavy metal ions, mutating MREb seemed to have a more significant effect in reducing tiMT promoter activity induced by Zn^{2+} (Fig. 5E). The promoter activity after 24 h of Zn^{2+} exposure was found to decrease by about 20% when MREb was mutated. Mutated MREe was found to significantly reduce the induction of tiMT promoter activity after Zn^{2+} treatment by around 40%, respectively. The promoter activity decreased by about 20% when MREg was mutated after Zn^{2+} treatment. The site-directed mutation of MREf seemed to have little effect in aborting promoter activity induction by Zn^{2+} treatment compared to that induced by other metal ions. The mutation of MREa was found to increase the Zn^{2+} induced tiMT promoter activity, mainly due to the fact that MREa contributes to a significant basal tiMT expression level.

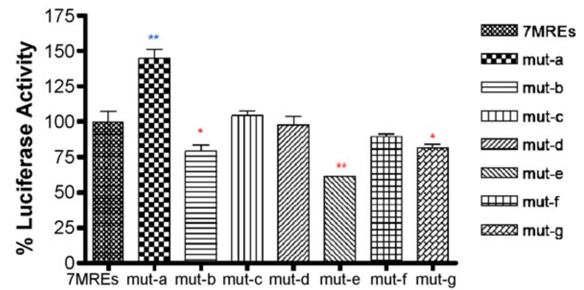
3.5. Electrophoretic mobility shift assay (EMSA)

EMSA was used to study the ability of the seven putative MREs in the tiMT promoter to bind with the cellular protein in Hepa-T1 cells. Fig. 6 shows that except for MREd (Fig. 6D), a basal level of MRE-binding was identified in the cell extract from untreated cells for all of the MREs. Hepa-T1 cells were treated with 75% 24-h EC_{50} of Cd^{2+}, Cu^{2+}, and Zn^{2+} for 24 h before extract preparation. The MRE-binding of these cell extracts was analyzed by EMSA. The incubation of the whole-cell extracts from the treated cells with radiolabeled MRE oligonucleotides yielded a binding complex that was the same size as the complexes in the extracts from the control cells, except for MREd. A comparison of the binding complexes of Zn^{2+}-treated Hepa-T1 cells and untreated cells (Table 2) revealed an increase in the MRE-binding for MREb (1.5-fold), MREe (1.7-fold), and MREg (2.0-fold) after the cells were treated with Zn^{2+}. How-









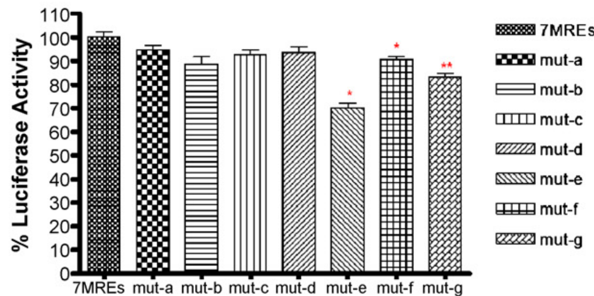


Fig. 5. Functional analysis of the tiMT promoter with specific MRE abortion by site-directed mutagenesis following exposure to heavy metal ions for 24 h. Hepa-T1 cells were transiently transfected with reporter constructs with mutations at one of the seven putative MREs in the tiMT promoter. The transfected cells were treated with 75% 24-h EC_{50} of (A) Cd^{2+}, (B) Cu^{2+}, (C) Hg^{2+}, (D) Pb^{2+}, and (E) Zn^{2+} for 24 h and the luciferase activity determined. The fold inductions of the relative luciferase activity were calculated by dividing the relative luciferase activity of the treated cells by the activity of the control cells. An experiment with the original construct without any mutation in any of the MREs was also conducted, and its calculated fold induction of tiMT promoter activity was assigned as 100%. The percentage luciferase activity of cells transfected with the different mutants was calculated with reference to the non-mutated reporter construct (pGL3-tiMTP-7MREs). Blue asterisks indicate a significant increase in promoter activity by specific mutations of MRE. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

ever, there were no observable changes in the binding intensity of MREa, MREc, and MREf after Zn treatment. The binding of the MRE oligonucleotides was not induced in extracts from Cd^{2+} and Cu^{2+} treated cells, in contrast to the results for the untreated controls.

MREa, MREc, and MREf binding of the transcription factor were not inducible by Zn^{2+} treatment, whereas MREb, MREe, and MREg were responsive to Zn^{2+} treatment. The DNA-binding activity of this transcription factor was inducible by Zn^{2+} but not by Cd^{2+} and Cu^{2+}, which actually reduced the MRE-binding activity of the transcription factor.

4. Discussion

DNA walking was performed to obtain a further upstream region of the tiMT gene with a total of six functional MREs with

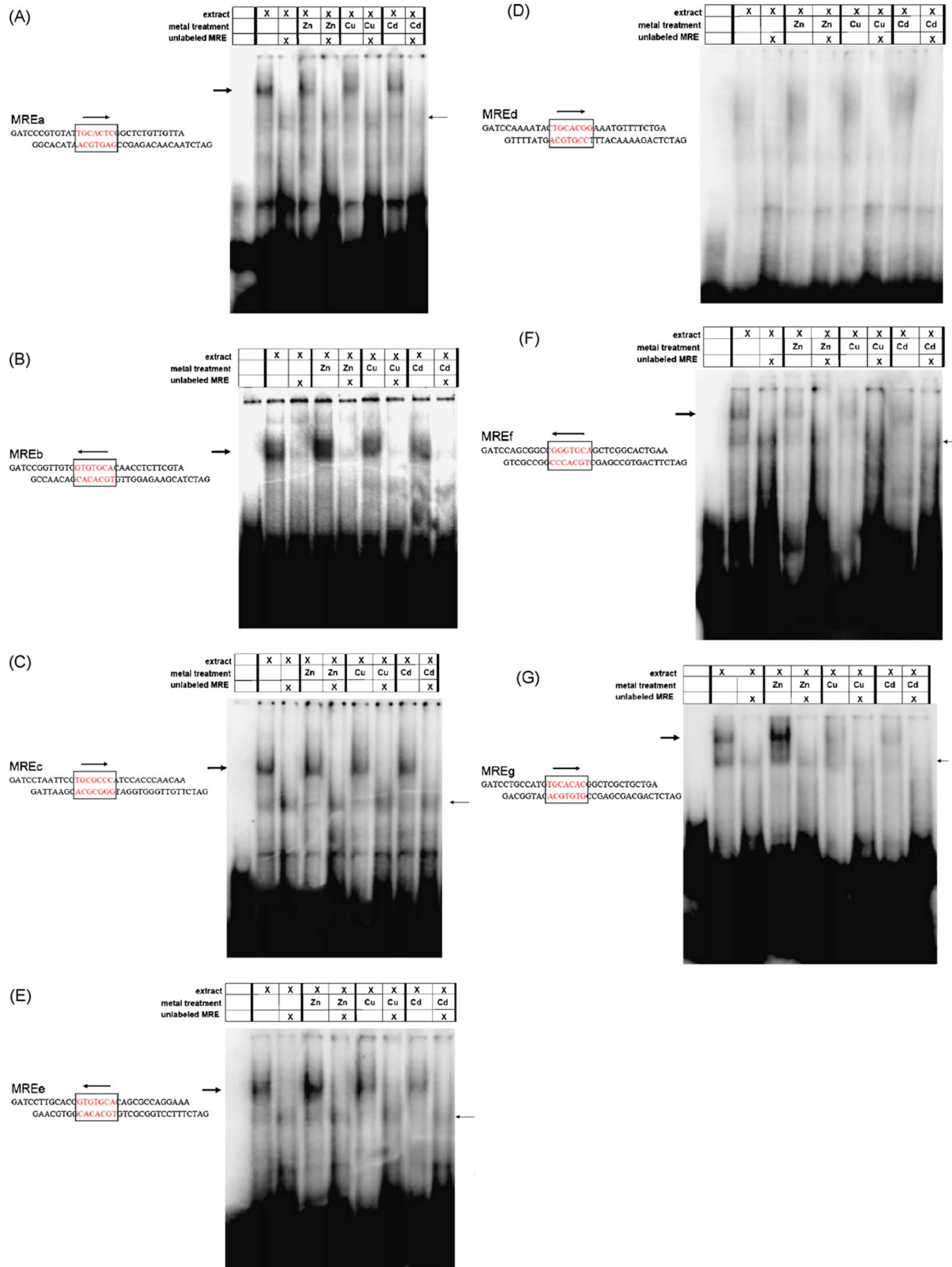


Fig. 6. Electrophoretic mobility shift assay (EMSA) of the seven putative MREs identified in the tiMT gene promoter. Whole-cell extracts from control, Zn^{2+}-treated (75% 24-h EC_{50}), Cu^{2+}-treated (75% 24-h EC_{50}), and Cd^{2+}-treated (75% 24-h EC_{50}) Hepa-T1 cells were incubated with radiolabeled (A) MREa, (B) MREb, (C) MREc, (D) MREd, (E) MREe, (F) MREf, and (G) MREg oligonucleotides on ice for 20 min. The first lane of each photo represents the free labeled MRE probes without any cell extract. The binding reactions of the whole-cell extract from Hepa-T1 cells subjected to different treatments (Zn^{2+}, Cd^{2+}, and Cu^{2+}) is annotated above each lane, except for the extract from the untreated cells. The arrows indicate the DNA–protein complexes with specific MRE-binding as confirmed by the addition of 200-fold excess unlabeled competitors (lane 2). The presence of a lower molecular weight binding complex is indicated by a small arrow on the right, but the binding is non-specific because it exists in the presence of cold-competitors.

Table 2
Quantification of the MRE-binding activity of the transcription factor from Hepa-T1 extracts

	Control	Zn^{2+}	Cu^{2+}	Cd^{2+}
MREa	1	0.91	0.77	0.75
MREb	1	1.52	0.88	0.86
MREc	1	1.07	0.95	0.95
MREd	–	–	–	–
MREe	1	1.69	0.88	0.65
MREf	1	0.98	0.61	0.80
MREg	1	2.03	0.66	0.58

Hepa-T1 cells were treated with Zn^{2+}, Cu^{2+}, and Cd^{2+} for 24 h before preparation of the cell extracts. Cell extracts from Hepa-T1 with no metal ion treatments were also prepared. Radiolabeled MRE oligonucleotides were added and incubated with the extracts at 4 °C. The band shifts were separated by PAGE and detected by autoradiography with a Molecular Imager Pharos FX Plus System (Bio-Rad), and the intensity of the MRE-binding complexes was quantified by using Multi-Analyst (Bio-Rad). The numbers in this table are the averaged value of three gels from each MRE.

5′TGCRCNC3′. In neither the EMSA or reporter gene assay did the MREd with 5′TGCACGG3′ show any binding signal with luciferase in the Hepa T1 cell transient gene expression studies. The MREs in the tiMT promoter can be divided into two clusters. MREa, MREb, and MREc occur in the proximal cluster that is located near to the TATA box, whereas MREe, MREf, and MREg occur in the distal region more upstream from the transcription start site. Similar to other teleost MT gene promoters, both the distal and proximal clusters of MREs are essential for the maximal metal induction of fish MT genes (Olsson and Kille, 1997; Samson et al., 2001; Scudiero et al., 2001; Yan and Chan, 2002). However, the distal MRE cluster provokes a high induction of gene activity after metal ion treatment, especially for non-Zn ions. The distal MREs may act as distal enhancers, which come into close proximity of the promoter by DNA ‘looping’ through physical interactions between distal and proximal bound MRE-binding proteins (Samson and Gedamu, 1998).

The transient transfection of the tiMT gene promoter reporter construct (pGL3-tiMTP-7MREs) in the Hepa-T1 cells revealed Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, and Zn^{2+} to be strong inducers of tiMT gene promoter activity, but that Ni^{2+} did not induce the TiMT gene promoter. Although it has been shown that the binding of MTFs can only be activated by Zn^{2+} and not by other metal ions (Seguin, 1991; Bittel et al., 1998), metal ions such as Cd^{2+} can activate the MT gene transcription indirectly by altering the distribution of Zn^{2+} in cells. These metal ions may displace Zn^{2+} from the weakly bound cellular zinc-storage protein (such as MT), which then activates MTF-1 binding to MREs (Palmiter, 1994). Non-zinc stressors may also be involved in some metal–responsive signal transduction cascades to activate MT gene expression by changing the level of MTF-1 phosphorylation (LaRochelle et al., 2001; Saydam et al., 2002).

An *in vivo* study in tilapia treated with intra-peritoneal injection of metal ions (Cheung et al., 2004) showed hepatic MT mRNA level to be induced by Cd^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+}. In rats, Zn^{2+}, Cd^{2+}, Hg^{2+}, Co^{2+}, and Ni^{2+} can induce MT both *in vivo* and *in vitro*, yet Pb^{2+}, Mn^{2+}, As^{3+}, and several organic compounds (e.g., ethanol) were not found to increase the MT content of hepatocyte cultures, although they did increase the hepatic MT content *in vivo* (Waalkes et al., 1984; Albores et al., 1992; Kreppel et al., 1993). These chemicals may induce MT as a general stress response, but they are not primary inducers of MT genes. The mechanism of this indirect induction is unknown, but may be due to changes in the homeostasis of Zn^{2+} (Waalkes and Klaassen, 1985) or an alteration in the post-transcriptional processing of MT mRNA or protein (Albores et al., 1992).

The results of this study suggest that Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, and Zn^{2+} are primary inducers of the tiMT gene, as they were able to induce MT promoter activity *in vitro* using cultured cells. The zebra fish zMT-II gene promoter was characterized by transient transfection in the zebrafish caudal fin cell-line SJD.1, and was found to be responsive to Cd^{2+}, Cu^{2+} and Zn^{2+} but inert to Hg^{2+}, Ni^{2+} and Pb^{2+} (Yan and Chan, 2002, 2004). However, the two MT promoters are similar in the sense that Cd^{2+} and Zn^{2+} are the most potent inducers of MT promoter activity, despite the fact that the response of the zMT promoter (with only four MREs) in HepG2 cells to Cd^{2+} and Zn^{2+} was much stronger than the tiMT promoter. The higher temperature of the HepG2 cell culture (37 °C) may be the reason for the higher transcriptional rate of the zMT gene promoter in this study.

The deletion analysis and site-directed mutagenesis study of the tiMT promoter indicated the significance of the three distal MREs, and especially MREe, in the induction of MT by non-Zn^{2+} heavy metal ions (Cd^{2+}, Cu^{2+}, Hg^{2+} and Pb^{2+}). Both the deletion of MREg and the further deletion of MREf significantly reduced the induction of tiMT promoter activity by Pb^{2+}. Both the site-directed mutagenesis of MREf and MREg caused a 10–30% reduction in tiMT promoter activity induction, which implies that both MREs are required for the maximum induction of tiMT by Pb^{2+}.

The deletion of MREg also significantly reduced the responsiveness of the tiMT promoter to exposure to Cd^{2+}, but the additional deletion of MREf seemed to have no effect, meaning that MREf may be inert to Cd^{2+} or that it cooperates with MREf in tiMT promoter activation. The site-directed mutagenesis of MREf and MREg confirmed the relative the importance of these two MREs for the metal activation of the tiMT gene.

The deletion analysis showed that the removal of MREg (6MREs vs. 7MREs) was able to reduce the induction of tiMT promoter activity after 24 h of Cu^{2+} exposure ($P < 0.001$), but not after 12 h of Cu^{2+} exposure (data not shown). The mutations of MREg similarly failed to show any effect on tiMT promoter inducibility, although a small and insignificant decrease in tiMT promoter activity was observed for the 24-h exposures. It is not therefore possible to draw any conclusions about the role of MREg during Cu^{2+} exposure. However, the further deletion and specific mutation of MREf attenuated the fold induction of the promoter activity caused by Cu^{2+} by around 30%, which indicates that MREf plays a significant role in tiMT induction by Cu^{2+}.

The deletion of MREg reduced the tiMT promoter activity induction after 12 h of Hg^{2+} exposure, but caused only a mild decrease in induction after 24 h of Hg^{2+} exposure. The site-directed mutagenesis of the MT promoter revealed the significance of MREg in the induction of MT by exposure to Hg^{2+},

indicating that this distal MRE should be active in the Hg^{2+} activation of tiMT. The additional deletion of MREf (5MREs vs. 6MREs) reduced the activation of the tiMT promoter after 24 h of exposure to Hg^{2+}, indicating that the site-directed mutation of MREf may reduce the response of the tiMT promoter to Hg^{2+}. It can therefore be concluded that MREf is also likely to participate in tiMT promoter activation by Hg^{2+}.

The results of the deletion analysis and site-directed mutagenesis of MREe were most consistent for the four non-Zn^{2+} metal ions at exposures of 12 and 24 h. A comparison of the promoter activity of the two deletion mutants (5MREs and 4MREs) showed tiMT promoter activity induced by the non-Zn^{2+} heavy metal ions to be rendered insignificant after the removal of MREe. This indicates either that MREe is the most active MRE through which Cd^{2+}, Cu^{2+}, Hg^{2+}, and Pb^{2+} bring about MT gene transcription, or that MREe is required in cooperation with the other MREs to cause the activation of MT gene expression by these metal ions. The site-directed mutation of MREe, which consistently reduced the tiMT promoter activity induction by the metal ions, supported this notion. Furthermore, the mutations of the proximal MREb and MREc caused a reduction in the activation of the tiMT promoter in some cases, which further indicates that these two proximal MREs may cooperate with the distal MREs to bring about the full induction of tiMT gene in MT induction by the non-Zn^{2+} metal ions, although they do not in themselves cause a major effect.

Distinct from the other metal ions, Zn^{2+} resulted in the significant induction of tiMT promoter activity after the deletion of the distal cluster of MREs. The results for the deletion mutants with 2MREs (MREa and MREb) and 1MRE (MREa only) indicate that only MREb is required to enable Zn^{2+} to activate MT gene transcription. The induction of the tiMT gene promoter by Zn^{2+} ceased after the removal of the six upstream MREs. This finding, together with the results of the deletion analysis study for the other metal ions, makes it possible to speculate that MREb is the most responsive to Zn^{2+}, but not to the other non-Zn^{2+} ions. The site-directed mutagenesis of MREb and MREg caused a reduction in tiMT promoter activity induction by around 20% each, whereas the mutation of MREe decreased the induction of promoter activity by 30–40%. These results agree with the results from the deletion analysis that show the three MREs (MREb, MREe, and MREg) to play an important part in tiMT gene activation by Zn^{2+}.

The observed metal specificity of the individual MREs was similar to a previous study that found that an additional MREc in the rainbow trout MT-B promoter caused a significantly higher induction in response to Cd^{2+} exposure than a promoter fragment that contained only MREa and MREb (Zafarullah et al., 1988). The distal cluster of the MREs in rainbow trout MT-B (Samson et al., 2001) and in this study also contributed to the Zn^{2+}-induced MT promoter activity co-operatively.

Although the results of the site-directed mutagenesis of MREa in the tiMT promoter consistently show an increased induction of tiMT promoter activity by Zn^{2+}, in fact the specific mutation of MREa greatly reduced the basal level of luciferase activity by over 50%, and a strong EMSA signal was observed in the MREa in the untreated cells. These results indicate the significance of MREa in maintaining the basal level of MT promoter activity without the additional administration of metal ions to Hepa T1 cells.

Similarly sized band shifts were observed in the incubation of the cell extract from Hepa-T1 cells, with all of the synthetic oligonucleotides flanking the MREs in the tiMT promoter, except for MREd. Thus, these six MREs (MREa, MREb, MREc, MREe, MREf, and MREg) are identified as functional in binding with a specific common transcription factor in tilapia liver cells. The binding of the transcription factor to MREb, MREe, and MREg intensified in the whole-cell extract from the Zn^{2+}-treated cells compared with the control (Table 2), which is consistent with the deletion and site-directed mutagenesis, and also with previous studies that found that both fish and mammalian cells respond to Zn^{2+} by increasing MTF-1 binding (Heuchel et al., 1994; Dalton et al., 1997, 2000). Although the binding of the protein factor with MREa, MREc, and MREf was not induced by the Zn^{2+} pretreatment, the binding may contribute to the basal expression of the MT gene.

Cd^{2+} and Cu^{2+} are notable for inducing MT expression, and were found in this study to activate the tiMT gene promoter, but the results of the EMSA indicate that there was no increase in band shift, and that the MRE-binding of the transcription factor from Hepa-T1 cells was not activated by Cd^{2+} and Cu^{2+} *in vivo*. Instead, the results (Table 2) imply that they may inhibit the MRE-binding of the transcription factor. Similar results have been found in the study of human MTF-1 in HeLa cells (Murata et al., 1999). This finding was observed for all six functional MREs in the tiMT promoter. However, the MREs, and especially MREe, MREg, and MREf, were found to be responsible for the induction of the MT gene by Cd^{2+} and Cu^{2+}, as shown in the tiMT promoter activity studies. It may thus be the case that the EMSA conditions that were used in this study were unable to reflect the changes in the binding of MTF-1 to MREs, and that perhaps chemical reactions other than binding are involved in the activation of gene expression.

The binding of MTF-1 to MRE requires the occupancy of zinc finger 1 with zinc (Bittel et al., 2000), and the occupancy of the zinc fingers by other transition metals inhibits MTF-1 binding to DNA *in vitro* (Bittel et al., 1998). Therefore, non-Zn^{2+} metal ions, such as Cd^{2+} and Cu^{2+} can activate MT gene transcription by indirect mechanisms that do not involve inducing MTF-1 to bind with MREs. It has been proposed that other metal ions induce MT gene expression indirectly by causing the redistribution of a small fraction of Zn^{2+} by displacing Zn^{2+} from the weakly bound cellular zinc-storage protein, which in turn activates MTF-1 to bind MRE (Palmiter, 1994; Zhang et al., 2003). This activation of the MT gene by non-Zn^{2+} metal ions through an intracellular Zn^{2+} pool cannot be excluded, because a subpopulation of MTF-1 bound to the MT promoter may be sufficient to carry out its metalloregulatory functions. However, the results of this study imply that this mechanism only accounts for a small part of MT induction at the sub-lethal dose of non-Zn^{2+} metal ions adopted in the study, and that other events that increase the trans-activation potential of MTF-1 are likely to be involved. It is speculated that, at low concentrations, Cd^{2+} can induce MT gene transcription through the acquisition of greater

trans-activity from that portion of the MTF-1 protein that is known to be able to bind with MRE under low Zn^{2+} conditions (Dalton et al., 2000).

Recent studies have suggested that the six zinc fingers of MTF-1 are different in terms of their functions in DNA binding and the formation of a stable chromatin complex on the MT promoter (Chen et al., 1999; Bittel et al., 2000; Apuy et al., 2001; Jiang et al., 2003). Overall, it has been suggested that zinc fingers 2–4 exhibit a high affinity zinc binding, and constitute the core DNA-binding domain. Among the other zinc fingers, zinc finger 1 is speculated to be metal sensing, and to suppress the DNA-binding activity of adjacent fingers in the absence of sufficient Zn^{2+} and enhance the DNA binding activity when Zn^{2+} is replete. Finger 1 achieves this function either by directly interacting with finger 4, or by stabilizing an intra-molecular interaction between finger 4 and adjacent fingers (Apuy et al., 2001). Finger 1 may also unmask the adjacent NLS in MTF-1 protein on exposure to exogenous metal ions, thus promoting the nuclear import of MTF-1 (Jiang et al., 2003; Chen et al., 2007). The functions of fingers 5 and 6 are poorly understood, but they are highly conserved during evolution and are likely to be of functional importance in MTF-1. A recent study demonstrated the importance of the linker peptides between the zinc fingers in the zinc sensing function of mouse MTF-1. In the study, the mutation of the linker peptide between zinc fingers 1 and 2 resulted in constitutive DNA binding, nuclear translocation, and the transcriptional activation of the mouse MT-I gene, which was independent of exogenous Zn^{2+} (Li et al., 2006). In addition, the mutation of the linker between fingers 3 and 4 caused MTF-1 to be less sensitive to Zn^{2+}-dependent activation (Li et al., 2006). However, these proposed mechanisms fail to explain the activation of MT gene transcription by non-Zn^{2+} metal ions. Our results indicate that the mechanism by which these metal ions activate MT gene expression through the displacement of the Zn^{2+} in the zinc-binding protein to increase the intracellular Zn^{2+} concentration may not be as effective as Zn^{2+} treatment alone in enhancing MTF-1 binding, as Cd^{2+} and Cu^{2+} activate the MT gene promoter activity to a level similar to Zn^{2+} despite their inability to activate the MRE-binding activity of MTF-1. It is probable that these non-Zn^{2+} metal ions regulate MT gene expression by other indirect mechanisms that remain to be investigated.

The phosphorylation of MTF-1 plays a significant role in its translocation to activate MT gene transcription in the nucleus (Bittel et al., 1998; Chen et al., 2007; LaRochelle et al., 2001). It has been shown that the level of MTF-1 phosphorylation is modified after exposure to Zn^{2+} or Cd^{2+} *in vivo* (LaRochelle et al., 2001; Saydam et al., 2002). Indeed, a protein motif analysis (PROSITE) (Hofmann et al., 1999) of MTF-1 from different species indicated the presence of several evolutionarily conserved, potential phosphorylation sites, including protein kinase C, casein kinase II, and tyrosine kinase. Similarly, an analysis of the tilapia MTF-1L amino acid sequence (Cheung and Chan, unpublished data from a cloned tilapia MTF cDNA) by PROSITE predicted eight putative protein kinase C sites, eight casein kinase II sites, and a tyrosine kinase site. It is therefore possible that the level of phosphorylation in tilapia MTF-1L also changes during heavy metal exposure. A model in which the transcriptional activation of MT via the MTF-1/MRE interaction is controlled by several signal transduction cascades that ultimately affect the level of MTF-1 phosphorylation has been proposed. Studies in this area have shown that the addition of signal transduction inhibitors for protein kinase C, c-Jun N-terminal kinase, phosphoinositide 3-kinase, and tyrosine kinase attenuate or abolish MT mRNA induced by heavy metal ions (LaRochelle et al., 2001; Saydam et al., 2002), whereas the activator of signal transduction cascades causes an increase in the steady-state level of MT mRNA (Garrett et al., 1992; Kelly et al., 1997; Laychock et al., 2000). Metal ions, oxidative stress, and radiation, all of which are known to induce MT transcription, have also been found to modulate the activity of intracellular signal transduction cascades (Beyersmann and Hechtenberg, 1997; Karin, 1998; Stohs et al., 2000). This further supports the idea of the model that the regulation of MT gene expression by metal ions, including Zn^{2+} and Cd^{2+}, involves the modulation of several kinases, some of which may overlap with the MTF-1 kinase transduction pathway, thus stimulating MTF-1 transcriptional activity.

However, Jiang et al. (2004) suggested that MTF-1 phosphorylation may not play such an important role in the regulation of MTF-1 activity, as the results from their study show that metal treatment did not cause a significant change in the phosphorylation level or modification pattern of MTF-1. Instead, they suggested that signal transduction cascades activated by metal ions may act on cofactor(s) that interact with MTF-1, rather than causing the phosphorylation of the MTF-1 directly. Although a coactivator model and the existence of a metal-sensing MTF-1 inhibitor have been proposed (Palmiter, 1994), no cofactors have as yet been identified.

Other putative regulatory elements in the tiMT promoter have also been predicted by computer programs. These elements include three glucocorticoid regulatory elements (GRE), six activator protein-1 (AP1) binding sites, and three specific protein-1 (Sp1) binding sites. Sp1 sites were found in common carp MT, zebrafish MT, and icefish MT-I genes, but not in rainbow trout, pike, or stone loach MT promoters (Chan et al., 2004; Olsson et al., 1995; Olsson and Kille, 1997; Scudiero et al., 2001; Yan and Chan, 2002). Sp1 is an ubiquitous zinc-finger transcription finger for housekeeping genes. It has been proposed that Sp1 may bind with the GC box that overlaps the MREs to act as a negative regulator of MT gene transcription in mice (Ogra et al., 2001). The AP1 site has been found in rainbow trout MT-A, pike MT, stone loach MT, icefish MT-II, common carp MT, and zebrafish MT. 5′ deletion studies of rainbow trout MT-A promoter and common carp MT promoter have revealed the role of the AP-1 site in MT gene regulation in inducing oxidative stress (Olsson et al., 1995; Chan et al., 2004), whereas the zebrafish MT promoter has been shown to be inert to H_2O_2 treatment (Yan and Chan, 2004). The functional roles of Sp1 and AP1 sites in the MT promoter thus remain to be elucidated.

In summary, in this study a 5′-flanking region (2118 bp) with six functional MREs in the tiMT gene promoter was identified. Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, and Zn^{2+} induced tiMT promoter activity significantly *in vitro*. These metal ions are primary inducers of

the tiMT gene, but only Zn^{2+} activated the binding of the protein factor to the MREs. Different MREs showed differential responsiveness toward the metal ions. Deletion analysis indicated that the induction of tiMT promoter activity by non-Zn^{2+} metal ions was terminated, whereas Zn^{2+} still caused a significant induction after the removal of the three distal MREs. Site-directed mutagenesis revealed that cooperation between MREs in the tiMT promoter is important in MT gene activation, as mutations of the distal or proximal MREs reduced induction by the metal ions. The most proximal MREa was responsible for the basal and induction of tiMT promoter activity. To summarize the two promoter characterization experiments, the distal cluster of MREs, and especially MREe, was essential for enabling metal ions to induce MT promoter activity, whereas the proximal MREs were most responsive to Zn^{2+}. The results of the EMSA show that a Zn^{2+}-responsive transcription factor from tilapia liver cells was able to bind with the MREs (except MREd) in the promoter. However, other indirect mechanisms that activate tilapia MTF-1 and MT by different metal ions remain to be elucidated. Although the activation of tiMT gene expression through the intracellular Zn^{2+} pool was unable to significantly induce transcription factor binding activity, cellular modifications such as the phosphorylation of the transcription factor may be more important in the induction of tiMT gene expression in tilapia liver by metal ions other than Zn^{2+}.

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