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# C-Terminal Deletion Mutant of MRE-Binding Transcription Factor-1 Inhibits MRE-Driven Gene Expression

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**Abstract** Heavy metal-induced transcriptional activation of the genes coding for metallothionein (MT) is mediated by a *cis*-acting DNA element, the metal-responsive element (MRE). MRE-binding transcription factor-1 (MTF-1) is a highly conserved heavy metal-induced transcriptional activator. MTF-1 also activates transcription in response to oxidative stress and regulates the expression of several cytoprotective factor genes, including MT, γ-glutamylcysteine synthetase, and Cu/Zn-superoxide dismutase. It is thus thought that MTF-1 plays a role in cellular stress response. The physiological role of MTF-1 remains unclear because of the lack of MTF-1-specific activators and/or inhibitors. To obtain an MTF-1-specific inhibitor, we constructed an MTFΔC (amino acids 1–317), a C-terminal deletion mutant of MTF-1. MTFΔC could bind MRE and competed with MTF-1 for MTF–MRE complex formation. Transient expression of MTFΔC in HepG2 cells reduced MRE-driven gene expression, demonstrating that MTFΔC is dominant to MTF-1. HepG2 cells stably expressing MTFΔC showed increased susceptibility to the cytotoxic effects of *tert*-butyl hydroperoxide (*t*BH). Furthermore, we constructed Ad5MTFΔC, a recombinant adenovirus that expresses MTFΔC. Infection with the virus induced MTFΔC expression and increased susceptibility to the cytotoxic effects of *t*BH. These results indicate that MTF-1 participates in controlling the cellular redox state. J. Cell. Biochem. 93: 609–618, 2004. 

**Key words:** MRE-binding transcription factor-1; dominant-negative mutant; oxidative stress

Metal-responsive element (MRE)-binding transcription factor-1 (MTF-1) is a highly conserved zinc finger transcription factor that regulates the transcription of target genes in response to heavy metals [Radtke et al., 1993; Heuchel et al., 1994; Otsuka et al., 1994]. The best-characterized target genes of MTF-1 are those encoding metallothioneins (MTs), a conserved family of cystein-rich heavy metal-binding proteins [Hamer, 1986; Vasak and Hasler, 2000]. MT-I and II display a wide tissue distribution and have been demonstrated to participate in the detoxification of transition metals such as cadmium and the homeostasis of zinc [Moffatt and Denizeau, 1997]. MT-I and II gene transcription is induced dramatically by heavy metals, especially cadmium and zinc. Heavy metal-induced transcriptional activation of the genes coding for MT is mediated by a *cis*-acting DNA element, the MRE [Stuart et al., 1985; Mueller et al., 1988; Ghoshal et al., 2002].

Several lines of evidence support a role for MTF-1 in oxidative stress response. To investigate the physiological role of MTF-1, Gunes et al., [1998] generated null mutant mice by targeted gene disruption and found that primary mouse embryo fibroblasts derived from MTF-1 null mutant embryos had increased sensitivity to hydrogen peroxide. Dalton et al. [1996, 1997a] have reported that oxidative stress activates MTF-1. Moreover, compounds

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that induce reactive oxygen species production also induce MT transcription, these compounds include paraquat, which accelerates the redox cycle [Sato, 1991], diethylmaleate, which depletes glutathione [Bauman et al., 1991], and inflammatory substances such as lipopolysaccharide [De et al., 1990] and cytokines [Karin et al., 1985; Sato et al., 1994, 1996]. Because MT has the potential to act as a free-radical scavenger, MTF-1 may help to control the cellular redox state. Taken together, these findings suggest that MTF-1 acts as cytoprotective factor against oxidative stress. Because of the lack of MTF-1-specific activators and/or inhibitors, the MTF-1 null mutant cell line is presently the only tool available to investigate the cytoprotective role of MTF-1.

The finding that MTF-1 requires an elevated concentration of zinc for strong binding to DNA suggests that MTF-1 DNA binding activity is controlled by allosteric regulation via metal [Bittel et al., 1998; Chen et al., 1999]. The MTF-1 DNA-binding domain contains six zinc fingers of the $C_2H_2$ type. The C-terminal portion of MTF-1 downstream of the zinc fingers contains three different transactivation domains (21). Though an N-terminal fragment of MTF-1 containing the zinc fingers (MTFΔC) could bind to MREs, MTFΔC could not induce MRE-driven gene expression [Radtke et al., 1995]. In resting cells, most MTF-1 localizes to the cytoplasm. Under several different stress conditions, however, it is translocated to the nucleus [Smirnova et al., 2000; Vanacore et al., 2000; Saydam et al., 2001]. The putative MTF-1 nuclear localization sequence $^{133}$KRKEVKR$^{139}$ is located in the N-terminal portion of MTF-1, upstream of the zinc fingers. The nuclear localization and zinc finger sequences might be important for MTF-1 function. It has been reported that phosphorylation is involved in the activation of MTF-1 in response to metals [LaRochelle et al., 2001; Saydam et al., 2002]. The unique phosphorylation site in human MTF-1, Tyr$^{140}$, is contiguous to the MTF-1 nuclear localization sequence $^{133}$KRKEVKR$^{139}$. Phosphorylation of this residue might contribute to the regulation of MTF-1 nuclear transport.

We set out to generate a dominant-negative mutant of MTF-1 that could be used as a specific inhibitor of the wild-type protein. We constructed vectors that express MTFΔC (amino acids 1–317), corresponding to the nuclear localization sequence and DNA-binding domain of MTF-1. The effects of the mutant on MTF-1 activity were examined. Furthermore, we tested cells expressing MTFΔC for susceptibility to the cytotoxic effects of the oxidative-stress-inducing agent, *tert*-butyl hydroperoxide (*t*BH).

## MATERIALS AND METHODS

### Plasmid Construction

To create the $MRE_d$-driven firefly luciferase reporter plasmid pGVP2 $MRE_{d4}$, we subcloned upstream of the minimal SV40 promoter in pGVP2 (Toyo Ink, Tokyo, Japan) four copies of a double-stranded oligonucleotide that includes $MRE_d$ sequences between −160 and −128 of the mouse MT-I promoter. To create the MTF-1 expression plasmid pRc/CMV-MTF, the full-length human MTF-1 cDNA (amino acids 1–753) was subcloned downstream of the cytomegalovirous (CMV) promoter in pRc/CMV. Similarly, to create the MTFΔC and MTFΔNΔC expression plasmids pRc/CMV-MTFΔC and pRc/CMV-MTFΔNΔC, DNAs encoding the MTF-1 N-terminal portion containing the zinc fingers (amino acids 1–317) and the zinc finger portion alone (amino acids 128–317), respectively, were subcloned downstream of the CMV promoter. All plasmids were prepared according to standard methods and purified using a high purity plasmid midiprep system (Marligen Biosciences, Inc., MD)

### Adenovirus Construction

The recombinant, replication-deficient adenovirus Ad5MTFΔC expresses MTFΔC from the CMV promoter-enhancer. It was constructed using the AdEasy$^{TM}$ adenoviral vector system according to the manufacturer's protocol (Stratagene, CA). Briefly, Ad5MTFΔC was constructed by a double-recombination event between cotransformed adenoviral backbone plasmid vector pAdEasy-1 and a shuttle vector carrying MTFΔC DNA. The adenovirus Ad5GFP, which expresses the enhanced green fluorescent protein gene, was used for experimental controls. All viruses were grown in HEK293 cells. Virus stock supernatant was prepared from cell suspension by four freeze–thaw cycles and then stored at −80°C.

### Cell Culture

Human HepG2 cells were cultured in Dulbecco's Modified Eagle's (DME) medium supplemented with 10% fetal bovine serum (FBS) at

37°C in 5% $CO_2$/95% air. For MTFΔC stable expression, HepG2 cells were transfected with pRc/CMV-MTFΔC by the calcium phosphate precipitation method [O'Mahoney and Adams, 1994]. After G418 selection and cloning, the existence of the transgene was analyzed by PCR. The following primers were used to amplify the 317-bp MTFΔC fragment from genomic DNA. The upstream primer was 5′-ACGAAGGAGAAGCCATTTGA-3′ and the downstream primer was 5′-TTTCTCACAGCCATTACTGGG-3′. Cells were infected with adenovirus at an MOI of 15 in DME medium supplemented with 0.1% bovine serum albumin (BSA) and kept at 37°C for 2 h. Cell viability was assessed by MTT assay [Mosmann, 1983].

### Luciferase Reporter Assays

Cell lysates were prepared and luciferase activities were measured on a luminometer (EG & G Berthold, Bad Wildbad, Germany). As an internal control, a *renilla* luciferase plasmid-RL-SV40 (Promega, Madison, WI), was used. All of the transfection experiments were performed in at least triplicate using two or three different preparations of DNA. The luciferase activities of the cells were normalized using the parental pGVP2 reporter plasmid, which contains only the minimal SV40 promoter. MTF-1 activity was defined as the ratio of luciferase activity in the stimulated cells to that in the unstimulated cells.

### In Vitro Transcription/Translation

Recombinant wild-type and deletion mutant proteins were synthesized in vitro with the TnT T7 quick coupled transcription/translation system (Promega), according to the manufacturer's instructions, using 1.0 μg of plasmid per reaction. To measure the amount of recombinant proteins synthesized, $^{14}$C-leucine was included in the reactions. Incorporated $^{14}$C-leucine was measured using a liquid scintillation counter.

### Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays

Nuclear extracts were prepared by the method of Dalton et al. [1996] in the presence of 1 mM DTT, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 0.3 μg/ml antipain, 1 mM NaF, 1 mM $Na_4P_2O_7$, and 0.1 mM $Na_3VO_4$. Binding reactions in the electrophoretic mobility shift assays (EMSA) took place in binding buffer containing 12 mM HEPES (pH 7.9), 5 mM $MgCl_2$, 60 mM KCl, 0.5 mM DTT, 2 μg of poly(dI-dC), 2.5 pmol of mutant MRE oligonucleotide (mut$MRE_s$; 5′-GATCCAGGGAGCTAATTACTCCGCCCGAAAAGTA-3′), and 5–50 fmol of $^{32}$P-labeled MRE oligonucleotide ($MRE_s$; 5′-GATCCAGGGAGCTCTGCACACGGCCCGAAAAGTA-3′), with 0.2–0.5 μl of a TnT lysate in a total volume of 20 μl. After incubation on ice for 30 min, protein–DNA complexes were separated electrophoretically at 4°C on 6% or 10% non-denaturing polyacrylamide gels at 15 V/cm. The gels were dried and the labeled complexes were detected by radioluminography.

### Statistical Analysis

The data were analyzed by ANOVA and Fisher's PLSD test. Differences between groups were considered significant at $P < 0.05$.

## RESULTS

### MTF-1 Activity and Sensitivity to *t*BH-Induced Cytotoxicity After Low-Dose *t*BH Pretreatment

To examine the effect of *t*BH on MTF-1 activity, HepG2 cells were transfected with the $MRE_{d4}$-luciferase reporter construct pGVP2-$MRE_{d4}$. Sixteen hours after transfection, the cells were treated with *t*BH at 200 μM final concentration. After 8 h of exposure to *t*BH, reporter gene expression was increased 2.5-fold relative to untreated cells (Fig. 1A). We also examined *t*BH-induced cytotoxicity in cells activated for MTF-1 by a 12-h pretreatment with a low dose of *t*BH (200 μM). After 20 h of exposure to indicated dose of *t*BH, the viability of control cells and MTF-1-activated cells was 9.2% and 53.4%, respectively (Fig. 1B).

### DNA-Binding Activity of MTFΔC and MTFΔNΔC

We examined whether the MTFΔC and MTFΔNΔC mutant proteins could bind to $MRE_s$ (Fig. 2A). MTF-1, MTFΔC, and MTFΔNΔC were synthesized in vitro in a coupled transcription-translation reaction mixture using the TnT lysate system. No MTF protein was detected in the TnT lysate that had no expression vector added. Lysates expressing pRc/CMV-MTFΔC produced an electrophoretic mobility shift (band shift) of the labeled $MRE_s$ DNA fragment, whereas lysates expressing pRc/CMV-MTFΔNΔC did not. Band shift formation was competed by the addition of unlabeled $MRE_s$. The zinc requirements of MTF-1 and MTFΔC

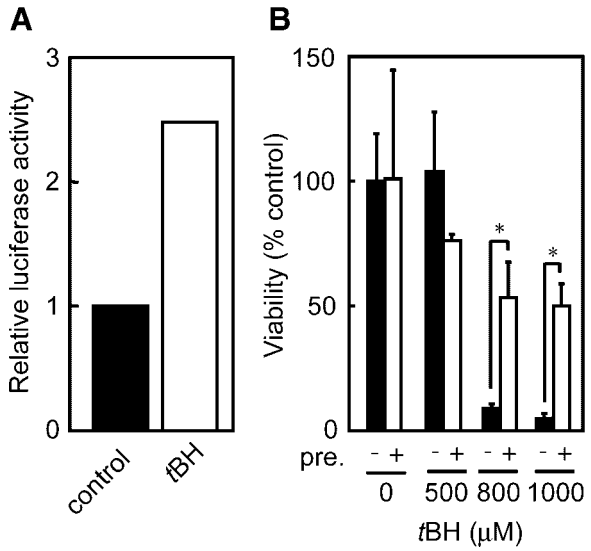


**Fig. 1.** Effect of *t*BH on MRE-driven gene expression and cytotoxicity. **A**: Fold induction of luciferase activities in HepG2 cells transiently transfected with pGVP2MRE$_{d4}$. Sixteen hours after transfection, cells were untreated (black bar) or treated (white bar) with *t*BH at 200 μM final concentration and luciferase activities were measured 8 h later. **B**: Cell viability 20 h after treatment with *t*BH at 800 μM final concentration. Cells were pretreated (white bars) or not pretreated (black bars) for 12 h with a low dose (200 μM final concentration) of *t*BH. Data represent means ± SD. *Significantly different from control group ($P < 0.01$).

for DNA-binding activity were also examined (Fig. 2B). Addition of zinc to 50 μM final concentration led to maximum DNA-binding activity for both MTF-1 and MTFΔC.

We also compared the relative DNA-binding activity of MTF-1 with that of MTFΔC. We used 200 fmol of each MTF-1 and MTFΔC in these experiments. For both MTF-1 and MTFΔC, 2.0 fmol of MRE$_s$ were necessary for detection of the complex (data not shown). At 25 fmol of MRE$_s$ addition, almost all MTF-1 and MTFΔC were bound to MRE$_s$ (Fig. 2C). To test the effect of the presence of MTFΔC on the formation of MTF-1–MRE complexes, increasing concentrations of MTFΔC were added to the MTF-1–MRE reaction mixtures. At equimolar MTFΔC and MTF-1 concentrations, the formation of MTF-1–MRE complexes was partly inhibited (data not shown). Inhibition of MTF-1–MRE complex formation increased in a dose-dependent manner with increasing concentration of MTFΔC, with complete inhibition by 12.5-fold molar excess of MTFΔC over MTF-1 (Fig. 2D).

To determine the inhibitory activity of MTFΔC against endogenous MTF-1, HepG2 cells were infected with Ad5MTFΔC. Twenty-four hours after infection, nuclear extracts were prepared and MTF DNA-binding activity was assessed by EMSA. In Ad5MTFΔC-infected HepG2 cells, MTFΔC–MRE complexes were detected, but MTF-1–MRE complexes were not (Fig. 2E).

### Inhibitory Effect of MTFΔC on MRE-Driven Gene Expression

To clarify the effect of MTFΔC on MRE-driven gene expression, HepG2 cells were cotransfected with the MRE$_{d4}$-luciferase reporter plasmid and an increasing concentration of the MTFΔC expression plasmid pRc/CMV-MTFΔC (Fig. 3). The amount of DNA transfected per sample was normalized by the addition of pRc/CMV, the parental expression plasmid. MRE$_{d4}$-luciferase reporter expression was decreased in a dose-dependent manner with increasing concentrations of pRc/CMV-MTFΔC. At the concentration of 273 ng pRc/CMV-MTFΔC per cell culture well, reporter expression was reduced to 20.0% of control cells not transfected with pRc/CMV-MTFΔC (Fig. 3).

### Establishment of MTFΔC Expressing Stable Cell Lines

To further investigate the effect of MTFΔC, we generated HepG2 cell lines that stably express MTFΔC. HepG2 cells were transfected with pRc/CMV-MTFΔC and cultured with G418 to select for stable integrants. Genotype analysis of surviving cell clones showed that all had the neomycin-resistance and MTFΔC genes (data not shown).

We used four HepG2 stable transfectant clones to examine the effects of MTFΔC on the expression of the MRE-driven luciferase reporter gene. The stable clones showed 110%, 45%, 51%, and 45% MRE reporter expression relative to wild-type HepG2 cells (Fig. 4, left panel). Treatment of the clones with 200 μM *t*BH for 8 h led to 54%, 25%, 49%, and 49% MRE reporter expression relative to *t*BH treated wild-type HepG2 cells (Fig. 4, right panel).

### Sensitivity of MTFΔC-Expressing Stable Cell Lines and Ad5MTFΔC-Infected Cells to *t*BH-Induced Cytotoxicity

To further examine the role of MTF-1 in protecting against *t*BH-induced cytotoxicity, we tested the sensitivity to *t*BH of the HepG2 cell lines stably expressing MTFΔC and

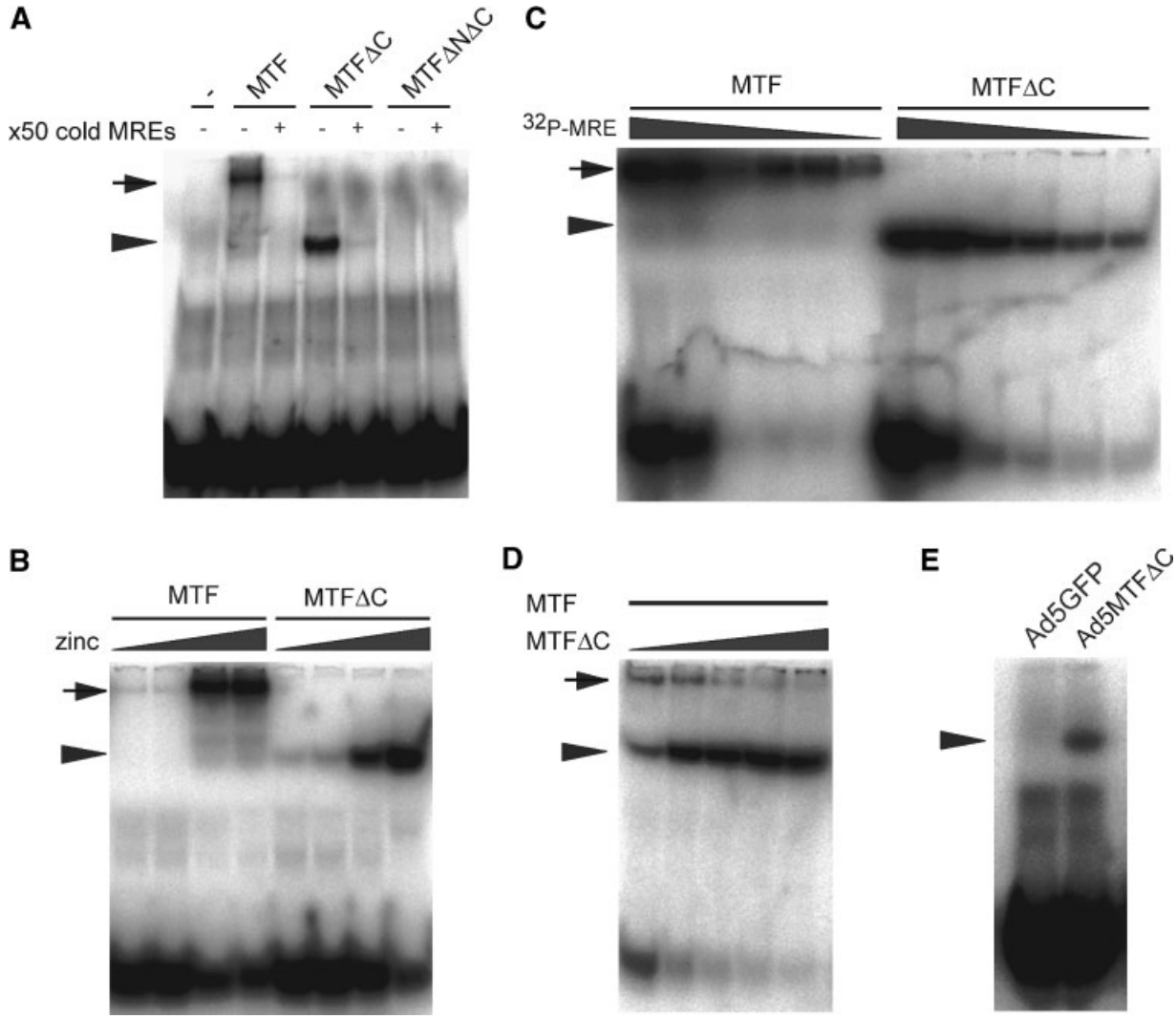


**Fig. 2.** DNA-binding activity of MTF-1, MTFΔC, and MTFΔNΔC. **A**: MTF-1, MTFΔC, and MTFΔNΔC proteins were synthesized in a coupled transcription/translation reaction. EMSA reactions using 1.0 pmol of MTF-1, MTFΔC, or MTFΔNΔC were carried out in buffer containing 50 μM zinc. **B**: EMSA reactions were carried in buffer containing 0, 10, 50, and 200 μM zinc. **C**: Comparison of the DNA-binding activities of MTF-1 and MTFΔC. EMSA reactions were carried out with 0.2 pmol of the MTF proteins and decreasing amounts of $^{32}$P-MRE (50, 25, 13, 8.3, 6.3, and 5.0 fmol). **D**: Competitive effect of MTFΔC on MTF–MRE complex formation. EMSA reactions using 0.20 pmol of MTF and increasing amounts of MTFΔC (0.20, 0.63, 1.3, 2.5, and 5.0 pmol). **E**: MRE binding proteins in nuclear extracts from Ad5GFP- and Ad5MTFΔC-infected HepG2 cells. The proteins were detected by EMSA reactions using 10 μg protein derived from nuclear extracts. In all panels, the arrows and arrowheads indicate the MTF-1–MRE and MTFΔC–MRE complexes, respectively.

Ad5MTFΔC-infected cells. The first experiment compared the wild-type to the four stable cell lines. After 20 h of exposure to 500 μM *t*BH, the viability of wild-type cells was 104.3% relative to untreated wild-type cells (Fig. 5). The viability of the MTFΔC expressing stable clones exposed to *t*BH was 49.0%, 12.1%, 35.4%, and 62.1% relative to untreated stable clones, respectively. In the second experiment, wild-type cells were infected with either Ad5MTFΔC or the control adenovirus Ad5GFP 12 h prior to *t*BH treatment. Ad5MTFΔC-infected cells had an increased sensitivity to the cytotoxic effects of *t*BH relative to Ad5GFP-infected control cells (Fig. 6).

## DISCUSSION

We demonstrated that MTFΔC, a C-terminal deletion mutant of MTF-1 containing the zinc fingers, binds to the MRE and competes with MTF-1 for MRE complex formation. Expression

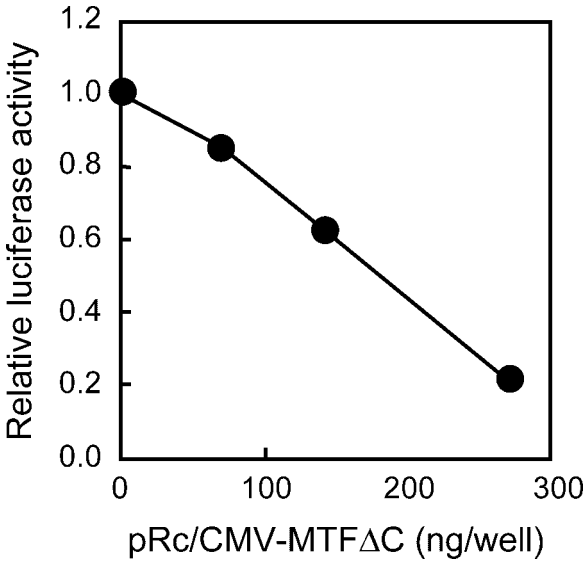


**Fig. 3.** Effect of MTFΔC on MRE-driven gene expression. pRc/CMV-MTFΔC and pGVP2MRE$_{d4}$ were transiently cotransfected into HepG2 cells and luciferase activities were measured 20 h later.

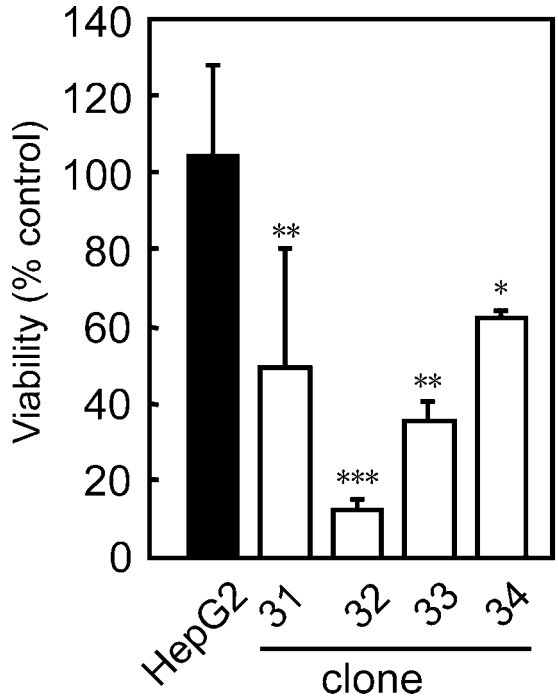


**Fig. 5.** Effect of MTFΔC on *t*BH-induced cytotoxicity. Cell viability was assayed by the MTT method 20 h after treatment with *t*BH at 500 μM final concentration. Data represent means ± SD. Significantly different from HepG2 cells (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

of MTFΔC in cells causes a decrease in MRE-driven gene expression. We also demonstrated that expression of MTFΔC in otherwise wild-type cells causes sensitization to the cytotoxic effects of *t*BH, presumably by inhibiting MTF-1 activity.

MTF-1 has been shown to be essential for basal and heavy metal-induced MT gene expression [Heuchel et al., 1994]. The role of MT in metal detoxification has been unambiguously demonstrated, because mice that lack MT-I and II by targeted gene disruption are particularly sensitive to cadmium [Michalska and Choo, 1993; Masters et al., 1994]. MTF-1 also activates transcription in response to oxidative stress [Dalton et al., 1996, 1997a] and regulates the expression of cytoprotective factor genes, including MT [Radtke et al., 1993; Otsuka et al., 1994], γ-glutamylcysteine synthetase (γ-GCS) [Gunes et al., 1998] and Cu/Zn-superoxide dismutase (Cu/Zn-SOD) [Yoo et al., 1999]. MRE-driven reporter gene expression in *t*BH-treated cells was 2.5-fold greater than that in untreated cells (Fig. 1). After 20 h of exposure to *t*BH, the viability of cells activated for MTF-1 by pretreatment with a low dose of *t*BH was greater than that of non-pretreated cells. It has been reported that the level of the heavy-chain subunit of γ-GCS, which is the key enzyme in the biosynthesis of GSH, is reduced in 13.5-day-old MTF-1 null mutant embryos relative to the wild-type [Gunes et al., 1998]. Moreover, primary mouse embryo fibroblasts derived from the MTF-1 null mutant showed increased sensitivity to hydrogen peroxide. These results

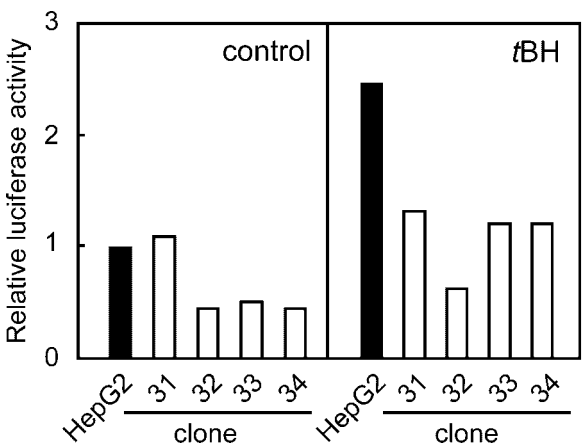


**Fig. 4.** Effect of MTFΔC on basal and *t*BH-stimulated MRE-driven gene expression. pGVP2MRE$_{d4}$ was transiently transfected into the indicated cells. Sixteen hours after transfection, cells were treated with *t*BH at 200 μM final concentration and luciferase activities were measured 8 h later. Control cells received no *t*BH treatment.

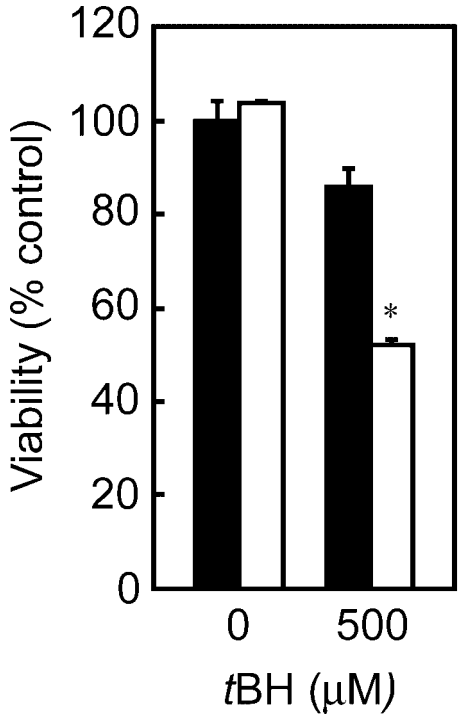


**Fig. 6.** Effect of Ad5MTFΔC infection on *t*BH-induced cytotoxicity. HepG2 cells were infected with Ad5GFP (black bars) or Ad5MTFΔC (white bars) at an MOI of 15 in DME medium supplemented with 0.1% BSA and kept at 37°C for 2 h. Cell viability was assayed by the MTT method 20 h after treatment with *t*BH at 500 μM final concentration. Data represent means ± SD. *Significantly different from Ad5GFP-infected HepG2 cells ($P < 0.01$).

indicate that MTF-1 might act as a mediator of cytoprotective signaling. However, since embryos lacking MTF-1 die in utero at approximately day 14 of gestation, it is difficult to clarify the function of MTF-1 in the cellular stress response.

To investigate the function of MTF-1 in cells, we set out to generate an MTF-1-specific inhibitor. MTF-1 contains six zinc fingers of the $C_2H_2$ type that function in DNA-binding. The C-terminal portion of MTF-1 downstream of the zinc fingers contains three different domains: an acidic domain, a proline-rich domain, and a serine/threonine-rich domain. It has been reported that these domains contribute to the transactivational activity of MTF-1; the expression of MTFΔC or MTFΔNΔC, which lack the three domains, did not promote MRE-driven gene expression in an $MTF^{-/-}$ cell line, even though the deletion derivatives could bind to the MRE [Radtke et al., 1995]. We considered the possibility that expression of MTFΔC and/or MTFΔNΔC could cause dominant inhibition of endogenous MTF-1-mediated gene expression.

We first characterized MTFΔC and MTFΔNΔC mutant proteins by assaying their DNA-binding activities. MTFΔC–MRE complexes were observed under our experimental conditions, whereas MTFΔNΔC–MRE complexes were not (Fig. 2A). Radtke et al. reported that, at the point of MTFΔNΔC–MRE complex formation, the zinc finger domain of mouse MTF-1 binds to MRE when expressed in vivo in transfected cells [Radtke et al., 1995]. However, Dalton et al. [1997b] reported that the zinc finger domain of mouse MTF-1, synthesized in TnT lysate, did not bind to MRE to form this complex. In this regard Dalton et al. data were similar to ours. They hypothesized that this lack of binding could reflect a limitation of the TnT lysate in supporting proper folding of the zinc finger domain. We agree with their hypothesis, and we consider that the Kd of the zinc finger domain alone was extremely high compared with that of full-length MTF-1. The N-terminal region might participate in stabilization of the complex. The zinc requirement of MTFΔC for DNA-binding activity was similar to that of MTF-1 (Fig. 2B); addition of zinc to 10–50 μM final concentration produced maximum DNA-binding activity for both MTF-1 and MTFΔC. Our results are consistent with those of Dalton et al. [1997b], who reported that the estimated Kd for zinc in activation of MTF-1 DNA-binding activity is 5–15 μM. MTF-1 and MTFΔC require the same relative concentration of $MRE_s$ for complex formation (data not shown), and the DNA-binding activity of MTFΔC is similar to that of MTF-1 (Fig. 2C). Scatchard plot analysis of MTF-1 and MTFΔC binding to MRE showed that the Kd of MTF-1 is 0.65 and that of MTFΔC is 1.1 nM. An excess (12.5-fold) of MTFΔC relative to MTF-1 could compete for the formation of MTF-1–MRE complex formation (Fig. 2D). Furthermore, binding of MTFΔC to the MRE was also observed using nuclear extracts prepared from Ad5MTFΔC-infected HepG2 cells (Fig. 2E). Adenovirus-mediated gene transfer provides the highest possible protein expression levels in a wide variety cells. Moreover, in adenoviral expression systems, successful transgene expression does not depend on active cell division; therefore, proteins can be expressed in dividing and non-dividing cells. If MTFΔC can inhibit both MTF-1 DNA-binding and MRE-driven gene expression,

Ad5MTFΔC would be a powerful tool for investigating MTF-1 function.

We next characterized the effect of MTFΔC on MRE-driven gene expression using HepG2 cells cotransfected with an $MRE_{d4}$-luciferase reporter construct and an increasing concentration of pRc/CMV-MTFΔC. Expression of the MRE-driven reporter construct was decreased in a dose-dependent manner with increasing concentrations of pRc/CMV-MTFΔC (Fig. 3). These results demonstrate that transient expression of MTFΔC inhibits MRE-driven gene expression. Expression of MTFΔC in stable HepG2 transfectants also inhibited MRE-driven gene expression under both untreated and *t*BH-treated conditions (Fig. 4).

The mechanism for *t*BH-induced MTF-1 activation remains unknown. Phosphorylation is involved in the activation of MTF-1 in response to metal ions [LaRochelle et al., 2001; Saydam et al., 2002]. Phosphorylation might also be involved in the activation of MTF-1 in response to *t*BH. The unique tyrosine kinase phosphorylation site in human MTF-1, $Tyr^{140}$, is contiguous with the MTF-1 nuclear localization sequence $^{133}$KRKEVKR$^{139}$. LaRochelle et al. proposed that phosphorylation of this residue might contribute to the regulation of MTF-1 nuclear transport. We note that the putative tyrosine phosphorylation site remains in the MTFΔC mutant. The putative kinase involved in MTF-1 phosphorylation might phosphorylate MTFΔC and increase its competitive inhibitory activity against MTF-1. Indeed, in MTFΔC stable cell clones 31 and 32, the ratio of inhibition of MRE reporter expression in *t*BH-treated cells was higher than that of inhibition in untreated cells; *t*BH treatment might activate the putative kinase. Experiment that investigates whether the putative phosphorylation site is important for nuclear localization of MTF-1 and MTFΔC will be of great interest.Our results suggest that in both transient and stable expression systems, MTFΔC can act as an inhibitor of MTF-1 activity in both untreated and *t*BH-treated cells.

Finally, using the stable MTFΔC-expressing cells lines and cells infected with Ad5MTFΔC, we tested whether MTFΔC could modulate the sensitivity of cells to oxidative stress. We have shown here that cells expressing MTFΔC are more sensitive to *t*BH treatment than wild-type (Figs. 5 and 6). Because mouse embryos that lack MTF-1 die in utero, it is impossible to investigate the function of MTF-1 after birth of the animal. Adenovirus-mediated gene transfer provides the highest possible protein expression levels in a wide variety of cultured cells and tissues. Ad5MTFΔC is expected to be a powerful tool to investigate MTF-1 activity.

In conclusion, we have constructed and characterized MTFΔC, a dominant inhibitor of MTF-1. MTFΔC is effective both in vitro, competing for MTF-1–MRE complex formation, and in living cells, decreasing expression of an MRE-driven reporter. Recently, RNA interference (RNAi) has emerged as a powerful tool for the silencing of gene expression [Zhang and Hua, 2004]. RNAi downregulates protein synthesis, whereas a dominant-negative mutant inhibits the function of wild-type protein. Because these actions are essentially different, we think that both dominant-negative mutants and RNAi will be valuable in the future. It is desirable that an RNAi technology for MTF-1 knockdown was to be reported. We have also shown that cells expressing MTFΔC, and presumably as a result having decreased MTF-1 activity, are more sensitive to the cytotoxic effects of *t*BH treatment. Our results suggest that MTF-1 mediates survival signaling in response to *t*BH. MTF-1 is activated under various stress conditions, such as heavy metal exposure and oxidative stress. Some cytoprotective factors, such as MT [Radtke et al., 1993; Otsuka et al., 1994], γ-GCS [Gunes et al., 1998], and Cu/Zn-SOD [Yoo et al., 1999], are the MTF-1 target genes. Additional MTF-1 candidate target genes that play a role in toxicity/cell-stress response have also been reported and include those encoding α-fetoprotein, the liver-enriched transcription factor C/EBPα, and tear lipocalin/von Ebner's gland protein [Lichtlen et al., 2001]. The finding that mouse embryos lacking MTF-1 die in utero [Gunes et al., 1998] is similar to the findings for mice lacking two other regulators of cellular stress response, namely c-Jun [Hilberg et al., 1993] and NF-κB [Beg et al., 1995]. Specific inhibitors of these regulators have been constructed, such as a deletion variant of c-Jun that acts as dominant-negative mutant [Brown et al., 1993, 1994] and a mutated form of IκBα that acts as a suppressor of NF-κB activity [Iimuro et al., 1998]. These inhibitors have been of great value in clarifying the roles of c-Jun and NF-κB. Similarly, the MTF-1-specific inhibitor MTFΔC should also be of great value in clarifying the role of MTF-1.

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