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Embryonic lethality and liver degeneration in mice lacking the metal-responsive transcriptional activator MTF-1

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We have shown previously that the heavy metal-responsive transcriptional activator MTF-1 regulates the basal and heavy metal-induced expression of metallothioneins. To investigate the physiological function of MTF-1, we generated null mutant mice by targeted gene disruption. Embryos lacking MTF-1 die *in utero* at approximately day 14 of gestation. They show impaired development of hepatocytes and, at later stages, liver decay and generalized edema. MTF-1$^{-/-}$ embryos fail to transcribe metallothionein I and II genes, and also show diminished transcripts of the gene which encodes the heavy-chain subunit of the γ-glutamylcysteine synthetase, a key enzyme for glutathione biosynthesis. Metallothionein and glutathione are involved in heavy metal homeostasis and detoxification processes, such as scavenging reactive oxygen intermediates. Accordingly, primary mouse embryo fibroblasts lacking MTF-1 show increased susceptibility to the cytotoxic effects of cadmium or hydrogen peroxide. Thus, MTF-1 may help to control metal homeostasis and probably cellular redox state, especially during liver development. We also note that the MTF-1 null mutant phenotype bears some similarity to those of two other regulators of cellular stress response, namely c-Jun and NF-κB (p65/RelA).

Keywords: glutathione biosynthesis/knockout mice/liver degeneration/metallothionein gene expression/transcription factor MTF-1

Introduction

The metal-regulatory transcription factor 1 (MTF-1) is a highly conserved transcriptional activator (Radtke *et al*., 1993, 1995; Heuchel *et al*., 1994). We previously cloned mouse and human MTF-1, which share a 93% identity in amino acid sequence (Radtke *et al*., 1993; Brugnera *et al*., 1994). MTF-1 contains six zinc fingers of the C_2H_2-type as a DNA-binding domain and at least three distinct domains responsible for transcriptional activation. It binds to a number of metal responsive elements (MREs) present in the promoter regions of metallothionein genes I and II (MT-I and MT-II) and regulates their expression. The MREs present in metallothionein gene promoters are short DNA sequence motifs of 9–12 conserved base pairs, whereby a central core of 7 base pairs is strongly conserved (Stuart *et al*., 1985; Searle *et al*., 1987; Radtke *et al*., 1993; Ç.Günes, unpublished results). Binding of MTF-1 to MRE sequences is dependent on zinc cations, as has been shown by *in vivo* and *in vitro* experiments (Mueller *et al*., 1988; Westin and Schaffner, 1988; Searle, 1990; Radtke *et al*., 1993). However, MTF-1 activates the expression of metallothionein genes not only by zinc but also by other heavy metal cations such as cadmium or copper (Heuchel *et al*., 1994).

There are four types of metallothioneins in mammals, namely MT-I, MT-II, MT-III and MT-IV (Uchida *et al*., 1991; Palmiter *et al*., 1992; reviewed in Heuchel *et al*., 1995). The expression of MT-I and MT-II is stress inducible and ubiquitous, while MT-III and MT-IV are tissue-specific and only weakly stress-inducible. The expression of MT-III is restricted to neuronal tissue after embryonic day 17, whereas MT-IV expression is restricted to certain squamous epithelia and no transcripts can be detected before day 7 post partum (Palmiter *et al*., 1992; Quaife *et al*., 1994; Palmiter and Findley, 1995). The expression of MT-I/MT-II genes is enhanced by a great number of stimuli, most notably by adverse conditions such as an excess of heavy metal cations, reactive oxygen intermediates (ROI), UV- and X-irradiation (Searle *et al*., 1984; Kagi, 1987, 1991; Heuchel *et al*., 1995). Consequently, these proteins have been implicated in heavy metal detoxification, metal homeostasis and radical scavenging (Thornally and Vasak, 1985; Angel *et al*., 1986; Thomas *et al*., 1986; Kagi, 1991). Since constitutive MT-I and MT-II expression is very high in fetal liver (Kern *et al*., 1981; Ouellette, 1982; Quaife *et al*., 1986), it has been suggested that these MTs might play a pivotal role in fetal liver development. However, two groups have shown independently that mouse strains with combined deletion of the MT-I and MT-II genes, as a result of targeted gene disruption, show no altered phenotype under normal conditions. However, such null mutant mice were more sensitive to cadmium exposure, an observation which supports the proposed role of metallothioneins in metal detoxification (Michalska and Choo, 1993; Masters *et al*., 1994).

We have shown previously that MTF-1 is essential for basal and heavy metal-induced transcriptional activation of MT-I and MT-II genes in cultured cells (Heuchel *et al*., 1994). Here, using the power of knockout technology, we show that MTF-1 is essential for embryonic liver function in mice. At the molecular level, expression of the known MTF-1 target genes encoding MT-I and MT-II is undetectable. Furthermore, the mRNA for γ-glutamylcysteine synthetase (γ-GCS), an essential enzyme in glutathione (GSH) biosynthesis, is reduced in MTF-1$^{-/-}$ embryos.

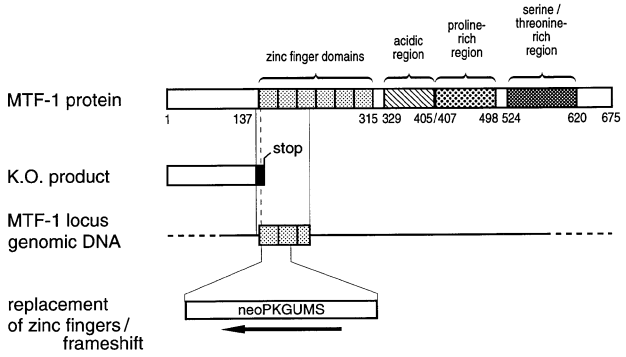


Fig. 1. Targeted disruption of the mouse MTF-1 locus (see also Heuchel *et al*., 1994). A schematic drawing of MTF-1 protein is shown in relation to the genomic locus with the 238 bp exon (which encodes the first two and part of the third zinc finger domains; dotted boxes) and part of the flanking introns. Part of the zinc finger exon was replaced by an inversely oriented neoPGK-UMS cassette, as described by Heuchel *et al*. (1994). Extensive analysis of MTF-1$^{-/-}$ embryos demonstrated that the targeted locus expresses only a short transcript encoding an N-terminal protein fragment of MTF-1 which ends in an out-of-frame stop codon (K.O. product).

Table I. Lethality of MTF-1 homozygous mutants

Stage	Total tested	Live MTF-1$^{-/-}$	[a]Dead MTF-1$^{-/-}$
E10.5-E12.5	41	10 (24%)	0
E13.5	54	13 (24%)	1 (2%)
E14.5	24	1 (4%)	3 (13%)
E15.5-E16.5	25	0	0
postnatal	41	0	–

No homozygous mutants were found among the born pups to date. Most of the homozygous mutants die after E13.5 and before E14.5. No living MTF-1$^{-/-}$ embryos older than E14.5 were observed. Before E13.5, living MTF-1$^{-/-}$ embryos were present in the expected Mendelian frequency.
E, embryonic day; [a]dead embryo or empty decidua.

Since both metallothionein and GSH share similar functions in that they are involved in cellular metal homeostasis and in detoxification processes, we propose that MTF-1 is involved in regulating cellular stress response.

Results

Targeted disruption of MTF-1 locus

To investigate the function of MTF-1 *in vivo*, we generated mice lacking functional MTF-1 by targeted disruption of the MTF-1 genomic locus (Figure 1). 129/SV ES cell clones containing one disrupted MTF-1 allele were injected into blastocysts to generate mice heterozygous for the mutated MTF-1 allele (see Materials and methods). These mice (MTF-1$^{+/-}$) with a mixed 129/SV×C57BL/6 genetic background appeared normal and were intercrossed. Among 41 born pups, none was homozygous for the MTF-1 mutation, implying that MTF-1$^{-/-}$ embryos die *in utero* (Table I). Genotype analyses of embryos from MTF-1$^{+/-}$ intercrosses revealed no viable homozygous mutant embryo older than E14.5 days (0 of 25). Before day E13.5, homozygous mutant embryos were obtained at about the expected Mendelian frequency (10 of 41), indicating that implantation and early post-implantation development were not impaired.

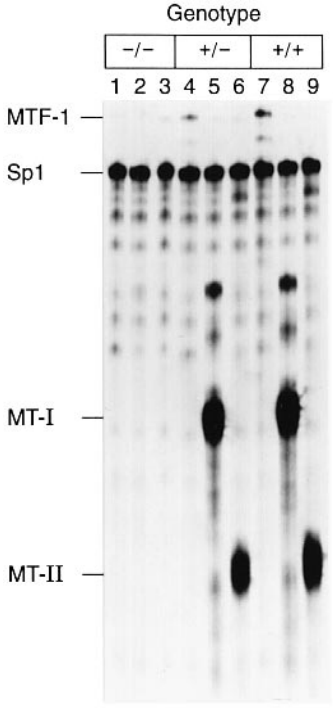


Fig. 2. Basal MT-I and MT-II expression is abrogated in MTF-1 knockout embryos. Total RNA from 13.5-day-old embryos was prepared and quantified by RNase protection using an antisense probe for MTF-1 (lanes 1, 4 and 7), the metallothioneins MT-I (lanes 2, 5 and 8), MT-II (lanes 3, 6 and 9) and the control, Sp1 (lanes 1–9). In the homozygous mutant, no expression of wild-type MTF-1 transcript was seen and transcripts of MTF-1 target genes MT-I and MT-II were missing altogether.

At day E13.5, only one of 14 MTF-1 homozygous mutant embryos was dead. Conversely, at day E14.5, only one homozygous mutant embryo was alive (Table I).

RT–PCR analysis of RNA from 13.5-day-old mutant embryos revealed an MTF-1 transcript with an internal deletion giving rise to a stop codon 39 bases after the point of deletion (Figure 1). As expected, total RNA from 13.5-day-old embryos did not show an MTF-1 signal with a probe covering the deleted segment (Figure 2, top lane). We also analyzed expression of the MT-I and MT-II genes in 13.5-day-old MTF-1$^{-/-}$ embryos by RNase protection assay and found no detectable MT-I and MT-II expression whereas expression of the unrelated transcription factor Sp1 was not affected (Figure 2). This result agrees with the previous analysis of MTF-1$^{-/-}$ ES cells (Heuchel *et al*., 1994). Hence, we conclude that homozygous mutant embryos do not produce any functional MTF-1 protein.

Phenotype of mice lacking MTF-1

Viable MTF-1$^{-/-}$ embryos at day E13.5 were macroscopically indistinguishable from their heterozygous and wild-type littermates. They were uniformly pink in color, well-vascularized and showed no internal bleeding. A histological analysis of whole embryos did not reveal any differences (data not shown) except pronounced liver damage (Figure 3). The liver structure of MTF-1$^{-/-}$ embryos at this stage was disrupted to a variable degree with enlarged, congested sinusoids and dissociation of the epithelial compartment (Figure 3D). Furthermore, although

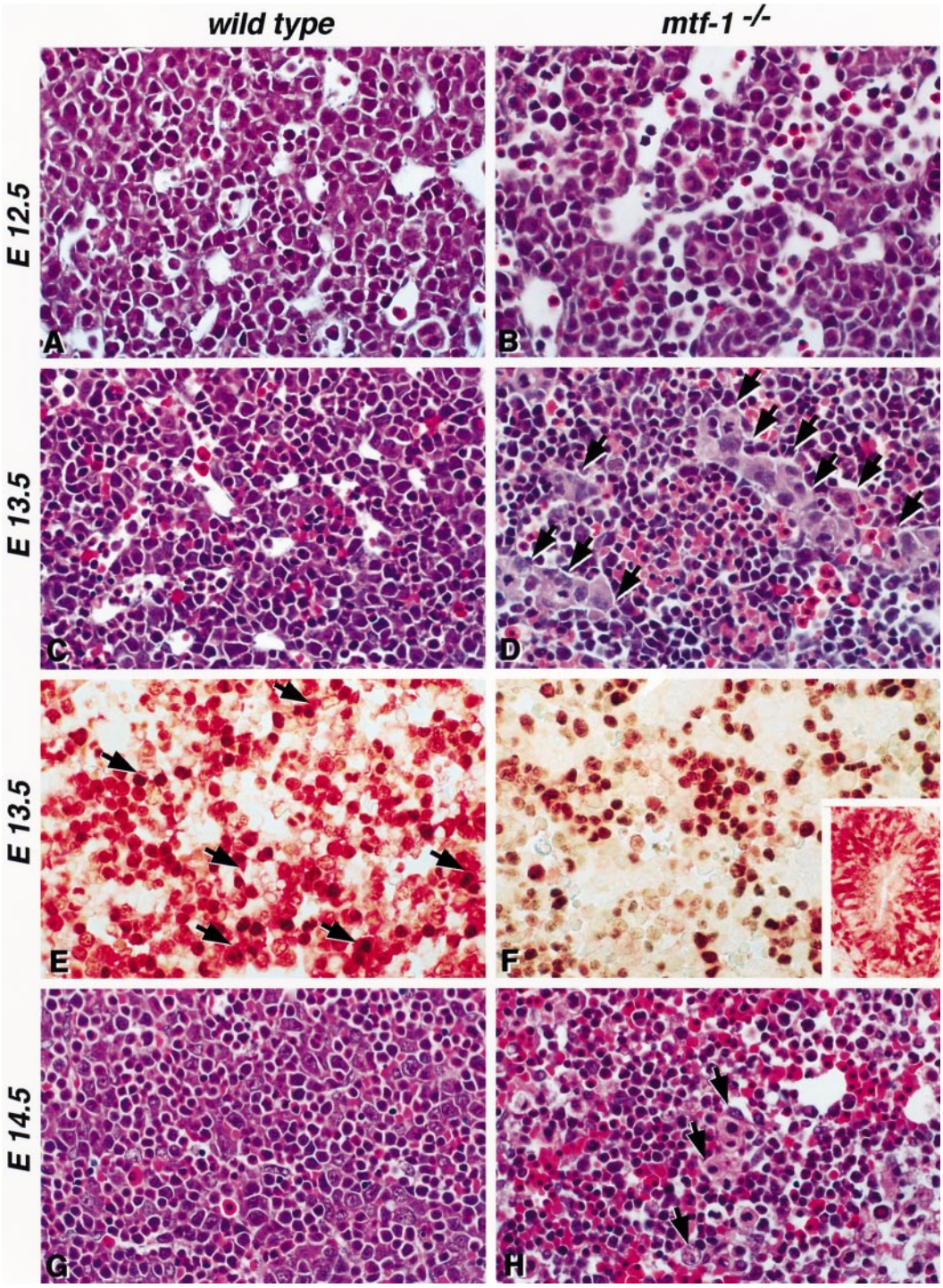


Fig. 3. Histological analysis of wild-type and MTF-1 knockout embryos showing disrupted liver phenotype in MTF-1$^{-/-}$ embryos. Hematoxylin and eosin-stained sagittal liver sections of wild-type embryos at days E12.5 (**A**), E13.5 (**C**) and E14.5 (**G**), as well as of MTF-1 knockout embryos at days E12.5 (**B**), E13.5 (**D**) and E14.5 (**H**), are shown. MTF-1$^{-/-}$ embryonic liver at day E12.5 (B) demonstrated no significant differences when compared with the wild-type littermate at the same stage (A) whereas MTF-1$^{-/-}$ embryonic liver at day E13.5 (D) showed enlarged, congested sinusoids and dissociation of the epithelial compartment, and scattered islands of hepatocytes are visible (arrows). Moreover, MTF-1$^{-/-}$ embryonic liver at day E14.5 (**H**) showed an almost complete disruption of the epithelial net. The remaining hepatocytes (arrows) are swollen and show picnotic nuclei. Double immunohistochemistry for BrdU (brown nuclear stain) and cytokeratin (red cytoplasmic stain) of day E13.5 wild-type (**E**) and MTF-1$^{-/-}$ (**F**) livers showed normal proliferation but a significant, selective loss of cytokeratin expression in the liver of MTF-1 mutant embryos at this stage. The inset shows cytokeratin expression in the gut of the same MTF-1 mutant mouse.

individual epithelial cells of MTF-1$^{-/-}$ liver at day E13.5 appeared morphologically normal in conventional histology, immunohistochemical analysis showed that these cells had a reduced cytokeratin expression (Figure 3F) in comparison with wild-type littermates (Figure 3E). This deficiency was liver-specific since cytokeratin expression

in the gut from the same embryo was not affected (Figure 3F, inset). At day E14.5, the only null mutant embryo still alive showed severe liver necrosis (Figure 3H), diffuse bleeding and generalized edema as well as an almost complete lack of cytokeratin expression in the liver (data not shown). By contrast, α-actin expression in the vessel walls was still indistinguishable from heterozygous or wild-type littermates at day E14.5 (data not shown). An obvious explanation for the absence of cytokeratin might have been a dependence of liver cytokeratin genes on MTF-1; however, we have not found any binding sites for MTF-1 in the known cytokeratin promoters, and it remains to be seen whether the absence of cytokeratin is a cause or a consequence of liver pathology.

At day E13.5, we could not detect any significant increase in the frequency of apoptotic cells in the liver of MTF-$1^{-/-}$ embryos, neither morphologically nor with the TUNEL *in situ* staining for DNA fragmentation (data not shown). At day E14.5, there was extensive DNA fragmentation, most likely resulting from necrosis rather than apoptosis. We also found that the cell proliferation rate in the MTF-1 homozygous mutant was not affected since bromodeoxyuridine incorporation at day E13.5 (Figure 3E and F) revealed no decrease when compared with wild-type or heterozygous littermates. Since the liver takes over as a blood-forming organ at this stage, we considered the possibility of a defect in the hematopoetic system in null mutant embryos. However, living 13.5-day-old homozygous mutant embryos showed not only no macroscopic color change indicative of anemia, but also no change in the number or appearance of erythrocytes or hematopoietic precursor cells observable in histological sections (data not shown). At day E13.5, the contribution of liver-derived enucleated erythrocytes is small, and thus a deficiency would not be easily detectable. However, the only living null mutant embryo at day E14.5 also showed no reduced representation of enucleated erythrocytes, again arguing against a defect in hematopoiesis (data not shown).

Live homozygous MTF-$1^{-/-}$ embryos at days E13.5 and E14.5 showed no reduced liver size which distinguishes them from the small liver phenotype seen in the knockout of HLX, a developmental control gene for liver formation (Hentsch *et al*., 1996). Also, the placenta of MTF-$1^{-/-}$ embryos did not reveal any abnormalities (data not shown), in contrast with the combined liver and placental defects seen in hepatocyte growth factor/scatter factor (HGF/SF) knockouts (Schmidt *et al*., 1995; Uehera *et al*., 1995). Hence, our findings render unlikely a loss of HLX or HGF/SF function as a downstream effect of MTF-1 deletion.

MTF-1 regulates the expression of the γ-GCS gene

As mentioned before, MTF-1 is essential for basal and heavy metal-induced expression of MT-I and MT-II genes. Since mice lacking the MT-I and MT-II genes are viable (Michalska and Choo, 1993; Masters *et al*., 1994) and other metallothionein isoforms cannot account for redundancy in MT-I and MT-II knockout mice because of their temporal and spatial expression (Palmiter *et al*., 1992; Quaife *et al*., 1994), we considered it most likely that deregulated expression of at least one additional target gene, perhaps in combination with loss of MT-I and MT-II expression, is responsible for the liver damage phenotype of MTF-$1^{-/-}$ mice.

Recently, MREs have been described in the promoter region of the heavy-chain subunit of γ-GCS (γ-GCS_{hc}), the key enzyme in the biosynthesis of GSH. We found additional putative MREs in the promoter of γ-GCS_{hc} and tested them for MTF-1 binding. By electrophoretic mobility shift assays (EMSA), we observed a strong, MTF-1-specific complex with one of these MREs (located at position –114) and a specific albeit weak interaction with the MRE located at position +296 (Figure 4A and B). No binding to other putative MREs could be observed in EMSA assays (data not shown).

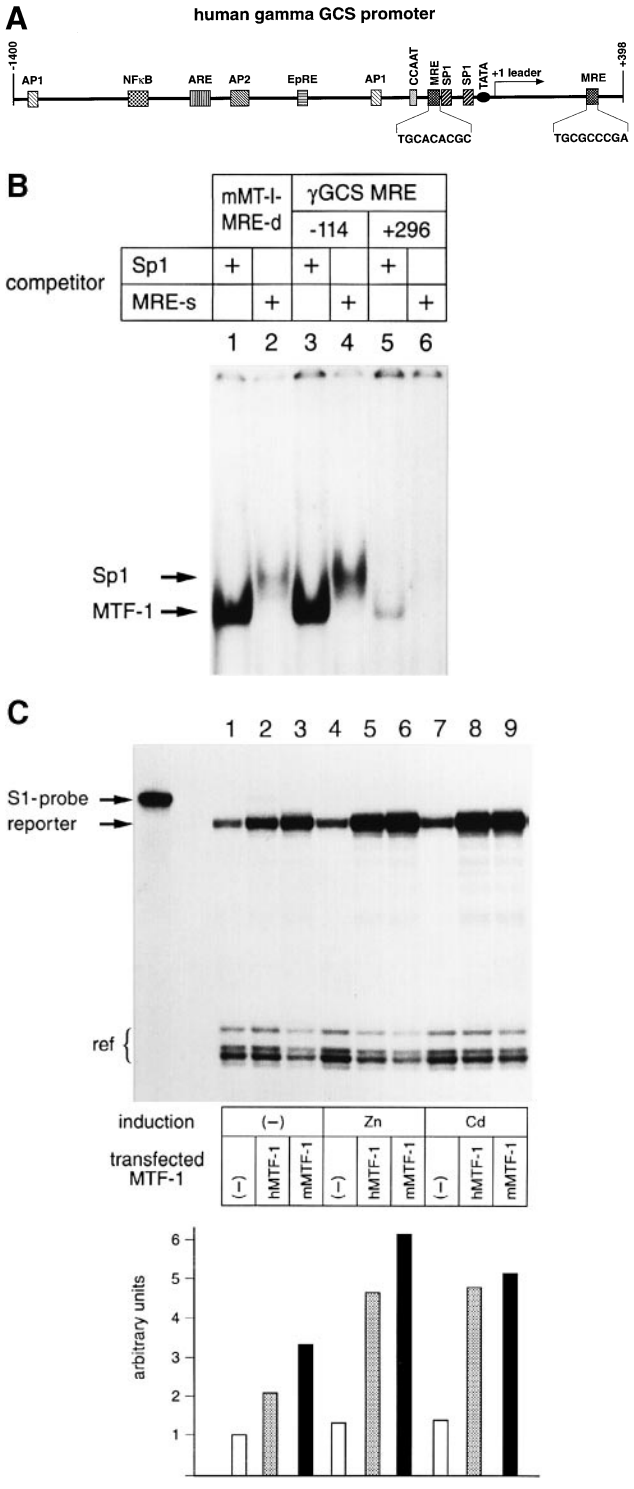
We then inserted a 1.8 kb fragment of the γ-GCS_{hc} promoter upstream of a β-globin reporter gene (Materials and methods) and tested the expression of the reporter gene in transient transfection assays in the human hepatoma cell line HepG2 (Figure 4C), which expresses MTF-1 endogenously. In addition to a basal level of expression (Figure 4C, lane 1), co-transfections with mouse or human MTF-1 expression vectors yielded an increased transcription of the reporter gene (Figure 4C, lanes 2 and 3). Moreover, induction with Zn or Cd further elevated reporter gene expression (Figure 4C, lanes 4–9). These results are in agreement with the recent observation that endogenous γ-GCS_{hc} expression can be induced by zinc or cadmium treatment (Ishikawa *et al*., 1996).

More importantly, by RT–PCR and Northern blot analysis we found reduced γ-GCS_{hc} gene expression in 13.5-day-old MTF-1 knockout embryos compared with wild-type embryos (Figure 4D and E). The expression of the 36B4 reference gene, which codes for the acidic ribosomal protein P0, was not altered in wild-type, heterozygous or knockout embryos whereas the expression of γ-GCS_{hc} was reduced in knockout embryos. In contrast, expression of the gene encoding the light chain subunit (regulatory subunit) of γ-GCS (γ-GCS_{lc}) or the gene for GSH synthetase (GSH-Syn), the second enzyme involved in GSH biosynthesis, was not altered (data not shown).

Primary mouse embryo fibroblasts from MTF-1 null mutant embryos are more sensitive to cadmium and hydrogen peroxide

To obtain more insights into the physiological role of MTF-1, we cultured primary mouse embryo fibroblasts (MEFs) from 12.5-day-old mouse embryos that were either wild-type, heterozygous or null mutant for the MTF-1 locus (Materials and methods). Since we observe a downregulation of genes responsible for metallothionein and GSH biosynthesis in the MTF-1 null mutant embryos, we decided to test the response of MTF-1-deficient cells against cadmium or H_2O_2-induced cytotoxicity. For this purpose, we treated primary MEFs with cadmium or H_2O_2 and determined the survival of MEFs by the neutral red assay (Figure 5A–D; see Materials and methods for details). As shown in Figure 5, MTF-1-deficient cells (MTF-$1^{-/-}$) are more susceptible to cadmium or H_2O_2 treatment when compared with wild-type cells (MTF-$1^{+/+}$). After 8 h of exposure to 40 μM cadmium, viability of the MTF-1-deficient cells was ~65% compared with that of wild-type cells (Figure 5A). This effect was even more pronounced when the cells were treated with 60 μM cadmium; the viability of MTF-1 deficient cells was

~40% compared with that of wild-type cells (Figure 5B). Similarly, deficient cells were more susceptible to exposure to H_2O_2; after an 8 h induction with 20 μM and 40 μM H_2O_2, ~75 and 60% of the knockout cells, respectively, were viable when compared with wild-type cells (Figure 5C and D). Primary MEFs heterozygous for the MTF-1 locus (MTF-$1^{+/-}$) were also more susceptible to the cytotoxic effects of cadmium and H_2O_2 when compared with wild-type cells. However, these MTF-$1^{+/-}$ cells showed more variation than wild-type or knockout cells from experiment to experiment (see Discussion).



Similar experiments were performed with permanent cell lines established from primary embryo fibroblasts. All MTF-1 knockout cell lines were more susceptible to cadmium and H_2O_2 than their wild-type counterparts (data not shown).

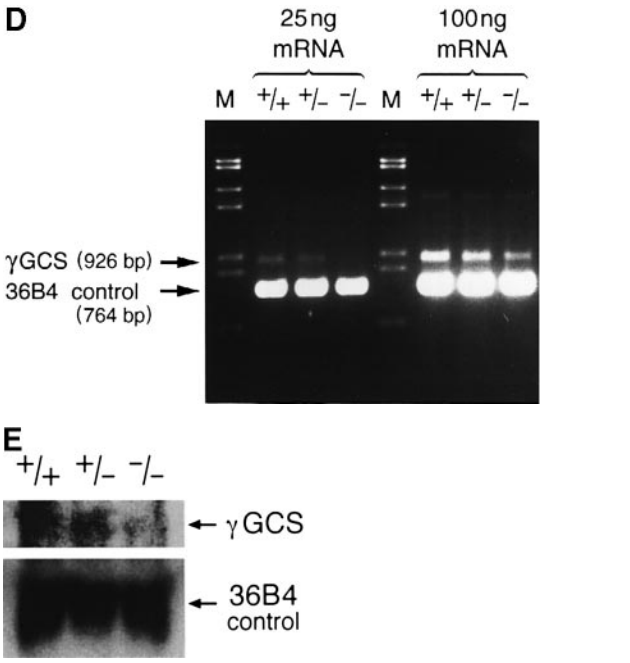


Fig. 4. γ-GCS_{hc} as a target gene of MTF-1. (**A**) The γ-GCS_{hc} gene promoter contains two MREs. A 1.8 kb fragment of human γ-GCS_{hc} gene promoter is shown schematically. The putative binding sites for transcription factors are shown as boxes; the TATA-box is shown as an oval. The transcription start point (tsp) is indicated as +1. Sequences of MREs are shown in the 5′ to 3′ direction. (**B**) MTF-1 binds to MREs present in γ-GCS_{hc} gene promoter. EMSA with MREs from γ-GCS_{hc} gene promoter using nuclear extracts from HepG2 cells, which were transfected with human MTF-1 expression vectors (see also Radtke *et al*., 1993). The respective positions within γ-GCS_{hc} promoter of the MREs used are indicated. MREd, the strongest MTF-1 binding site from the MT-I promoter, was used as a positive control. The MREd and γ-GCS_{hc} MRE/–114 sequences share an overlapping Sp1 binding site at their 3′ end which results in an additional Sp1–DNA complex in EMSA. Protein–DNA complexes specific for MTF-1 or the unrelated zinc finger factor Sp1 are indicated on the left. Approximately 250- to 500-fold excess of unlabeled competitor oligonucleotides, with binding sites for MTF-1 or for Sp1, were used to show specific binding of the respective proteins. (**C**) MTF-1 induces expression of a reporter gene from the human γ-GCS_{hc} promoter in transient transfection assays. S1 nuclease mapping of β-globin reporter gene from HepG2 cells. OVEC-ref was used as an internal control for transfection efficiency (Materials and methods). (**D**) The expression of γ-GCS_{hc} gene is downregulated in MTF-$1^{-/-}$ embryos. RT–PCR analysis of γ-GCS_{hc} gene expression in 13.5-day-old embryos. The One-Tube-RT–PCR analysis was performed with two different mRNA concentrations (25 or 100 ng, respectively). As an internal control, expression of the 36B4 gene, encoding the acidic ribosomal protein P0, was used. Both primer pairs for γ-GCS_{hc} and 36B4 were added to the same reaction (Materials and methods). (**E**) Northern blot analysis showing downregulation of the γ-GCS_{hc} gene in MTF-$1^{-/-}$ embryos. A concentration of 2 μg mRNA from total embryos (day E13.5) was separated on a 1% formaldehyde agarose gel and first probed with human γ-GCS_{hc} cDNA. The film was developed after 5 days exposure at –70°C. The same filter was stripped, rehybridized with mouse 36B4 control cDNA probe and developed after 36 h exposure at –70°C. The coarse and fine grains of γ-GCS_{hc} and 36B4 signals, respectively, are due to the different exposure times. The autoradiograms were recorded by a laser scanner. Positions of γ-GCS_{hc} and 36B4 gene transcripts are indicated by arrows.

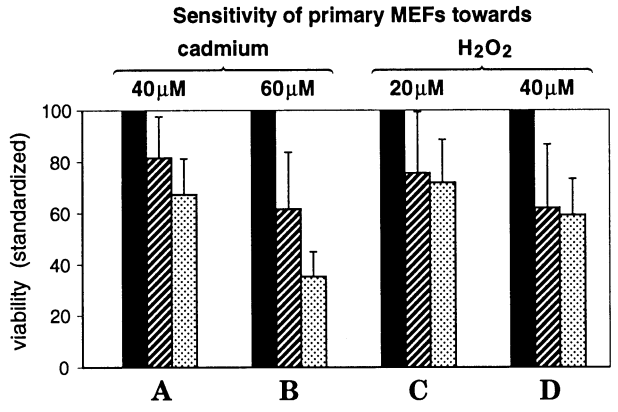


Fig. 5. MTF-1 deficient primary mouse embryo fibroblasts are more susceptible to cadmium or H_2O_2-induced cytotoxicity. Primary embryo cells were treated for 8 h with either 40 or 60 μM cadmium (**A** and **B**) or 20 or 40 μM H_2O_2 (**C** and **D**), respectively. Black bars represent wild-type (MTF-$1^{+/+}$), striped bars represent heterozygous (MTF-$1^{+/-}$) and dotted bars represent null mutant (MTF-$1^{-/-}$) cells. Each figure shows the average values of at least three independent experiments which were carried out in duplicate. Viability tests with control plates without cadmium or H_2O_2 treatment yielded the same numbers of cells for all three genotypes. Values obtained from experiments with MTF-$1^{+/+}$ cells were set at 100% and compared with those obtained with either MTF-$1^{+/-}$ or MTF-$1^{-/-}$ cells within each experiment.

Discussion

The metal-regulatory transcription factor MTF-1 is expressed in embryonic stem cells, throughout mouse gestation and in adult mice where it is ubiquitously expressed (Ç.Günes, unpublished results), and thus is likely to influence gene expression in many cell types. Nevertheless, the observed phenotype of liver degeneration in MTF-1 null mutant embryos implies that MTF-1 is particularly important for proper liver development/function at the embryonic stage.

As expected, no expression of the known target genes, MT-I and MT-II, could be detected in MTF-1 null mutant embryos. Metallothioneins are induced via MRE promoter sequences not only by heavy metal load but also by ROI and thus may help to protect the cell against oxidative stress (Thornally and Vasak, 1985; Girotti, 1986; Thomas *et al*., 1986; Kagi, 1991; Dalton *et al*., 1994, 1997). Null mutant mice for both of the metal-inducible metallothioneins, MT-I and MT-II, are viable but show greater sensitivity to cadmium toxicity (Michalska and Choo, 1993; Masters *et al*., 1994). Also, cultured hepatocytes from such mice are highly sensitive to both Cd-induced cytotoxicity (Zheng *et al*., 1996) or *tert*-butylhydroperoxide-induced oxidative stress (Lazo *et al*., 1995).

In addition to these known MTF-1 target genes, we observed a reduced transcript level of another gene, namely the gene coding for γ-GCS_{hc}. γ-GCS is the first and the rate-limiting enzyme in the biosynthesis of the GSH *de novo* pathway. γ-GCS is an essential enzyme (Maellaro *et al*., 1990; Meister, 1995). Similarly, malfunction of other enzymes involved in GSH metabolism in mice and human results in severe phenotypes (Liebermann *et al*., 1996; Shi *et al*., 1996). In spite of its ubiquitous presence, GSH seems of particular importance in the liver, perhaps due to high levels of mitochondrial respiration and lipid peroxidation within this organ. In fact, GSH homeostasis is mainly regulated by the liver.

GSH is essential to maintain the cellular redox-balance and is a scavenger of ROIs, which are mainly generated by respiration (Meister, 1995) or by exogenous cellular stress, such as that caused by heavy metal load (Bagchi *et al*., 1996). In addition, GSH has been shown to be the first line of defense against cadmium toxicity (Singhal *et al*., 1987; Shimizu and Morita, 1990; Chan and Cherian, 1992) and its biosynthesis can be induced by heavy metals (Iszard *et al*., 1995; Liu *et al*., 1995). A functional link between metallothionein and GSH in regulating the distribution of cellular zinc was proposed recently (Maret, 1995). This heavy metal-complexing ability of ubiquitous GSH could explain the viable phenotype of the MT-I and MT-II null mutant mice (Michalska and Choo, 1993; Masters *et al*., 1994). Conversely, it has been shown that Zn-MT, at least in part, can substitute for GSH as a cellular defense against ROI (Srivastava *et al*., 1993). Thus, GSH and metallothionein apparently have overlapping functions.

Using primary mouse embryo fibroblasts, we have shown that MTF-1-deficient cells are more susceptible to cadmium or H_2O_2 treatment when compared with their wild-type counterparts. Even though mice heterozygous for the MTF-1 locus develop normally and do not show any abnormalities under laboratory conditions, heterozygous (MTF-$1^{+/-}$) cells in culture were also more susceptible to cadmium or H_2O_2 treatment when compared with wild-type cells. MTF-1 is ubiquitously expressed, but its known target genes MT-I, MT-II and γ-GCS, although also expressed ubiquitously, play a particularly important role in the liver. It remains to be seen whether the knockout phenotype can be explained by the existence of a threshold for the accumulation of harmful agents during liver development, or whether MTF-1 has yet another role(s) in liver morphogenesis. Experiments with conditional MTF-1 knockout mice should help to resolve these questions.

The null mutant phenotype may be a compound effect of the aberrant expression of a number of genes. In fact, besides MT-I, MT-II and γ-GCS, we also found other candidate target genes involved in GSH synthesis and/or heavy metal homeostasis. Preliminary experiments suggest that MTF-1 may regulate the expression of the zinc transporter, ZnT-1 which is a ubiquitously expressed zinc export pump. As already described by Palmiter and Findley (1995), the promoter of ZnT-1 contains MTF-1 binding sites (MREs). We find that MTF-1 binds to these MREs and that the expression of the endogenous ZnT-1 transcript can be induced by zinc treatment in primary rat hepatocytes (Ç.Günes, unpublished results). In addition, we have identified MREs in the promoter of the γ-glutamyl transpeptidase gene which is involved in GSH metabolism and thus is another candidate target gene for MTF-1. Taken together, we consider it probable that MTF-1 plays a general role in cellular stress response.

Cellular stress response also relies on at least two further ubiquitous transcription factors, AP-1 (Jun/Fos) and NF-κB, which are induced by a number of common stimuli, notably stress signals, and are involved in antioxidant redox regulation (Herrlich *et al*., 1994; Pahl and Baeuerle, 1994; Angel, 1995; Wilhelm *et al*., 1995; Sen and Packer, 1996; Karin, 1997). It has already been shown

that the expression of some genes involved in GSH metabolism is regulated by AP-1 or NF-κB (Friling *et al*., 1992; Rahman *et al*., 1996; Sekhar *et al*., 1997). Interestingly, the promoter of the γ-GCS_{hc} gene contains two AP-1 binding sites and one NF-κB binding site (Mulchay and Gipp, 1995; Yao *et al*., 1995; Tomonari *et al*., 1997).

We note that c-Jun homozygous mutant embryos showed an embryonic liver phenotype similar, though not identical, to the one of MTF-1 null mutants (Hilberg *et al*., 1993), raising the possibility that one of these genes is epistatic over the other. However, preliminary experiments have not shown impaired c-Jun expression in MTF-1 knockouts (data not shown). Conversely, MTF-1 expression was not changed in fibroblasts derived from c-Jun knockout mice (A.Kolbus and P.Angel, personal communication). We also note that null mutants of the p65/relA subunit of NF-κB exhibit a similar failure in fetal liver development but at a slightly later stage and concomitant with a strong increase of apoptosis (Beg *et al*., 1995; Bladt *et al*., 1995). Again, no altered expression of p65/relA was found in MTF-1 null mutants (data not shown).

The similarity of the phenotypes of MTF-1, c-Jun and p65/relA null mutant mice, as well as the similar activation stimuli suggests that these transcription factors may have overlapping but nonredundant functions during embryogenesis, for example by synergistic regulation of common target genes involved in stress response.

Materials and methods

Targeted disruption of the MTF-1 gene

The construction of the targeting vector used in the targeted disruption of the MTF-1 locus in cultures of embryonic stem cells of mouse strain 129/5v, ES cell line ES-GS, was published in Heuchel *et al.* (1994). ES cells from one clone heterozygous for the mutated MTF-1 allele (26E7) were injected into blastocysts of C57BL/6 mice. Fourteen of the 31 male mice born were highly chimeric. Ten of those 14 were intercrossed with wild-type C57BL/6 females and showed germline transmission. Genotype identification was done by PCR with genomic DNA prepared from tail biopsies of 3- to 6-week-old born pups or of genomic DNA prepared from the yolk sac of embryos. The PCR was perfomed as described elsewhere (Heuchel *et al*., 1994).

Histological analysis

Embryos were isolated and fixed in 4% formaldehyde solution overnight. Their yolk sacs were used for genomic DNA preparation and PCR genotyping. After fixation, embryos were embedded in paraffin and processed for conventional histology. Sections were stained with hematoxylin-eosin or used for further treatments.

Two hours before isolation of the embryos, mice were injected intraperitoneally with 300 μl of bromodeoxyuridine (50 mg/ml)-containing solution. Bromodeoxyuridine integration was detected by incubation with peroxidase-conjugated Fab fragments. Sites of binding were visualized using the actin–biotin diaminobenzidine method. Double-staining of these sections was done using a monoclonal antibody (Lu 5, BMA) which detects all types of cytokeratins (concentration of 1:200), followed by an incubation with an AP-conjugated goat anti-mouse antisera.

RNA preparations, cloning of promoter constructs, transfections and transcript mappings

Total RNA from embryos was isolated using the guanidium thiocyanate/acidic phenol method (Chomczinski and Sacchi, 1987). mRNA from embryos was prepared directly by using the Qiagen direct mRNA midi/maxi kit. Preparation of cytoplasmic RNA from tissue culture was according to Radtke *et al*. (1993). RNase protection assay from MTF-1, Sp1 and MT-I and MT-II genes was performed as described by Heuchel *et al*. (1994). Northern blot analysis was performed according to standard procedures (Sambrook *et al*., 1989). To detect the γ-GCS_{hc} mRNA, 50 ng of the 2.9 kb human γ-GCS_{hc} cDNA was labeled by random hexanucleotide primers. Expression of 36B4 mRNA was used for RNA integrity. An 800 bp fragment of 36B4 cDNA was labeled by random hexanucleotide primers.

Transfections, cloning of promoter–OVEC constructs and S1 nuclease mapping of transcripts

Transfections and S1 nuclease mapping of transcripts were performed as described previously (Radtke *et al*., 1993). The following primers were used to amplify the 1.8 kb fragment of the γ-GCS_{hc} from a human genomic DNA library. The upstream primer (position –1442) was 5′-GGTCGAGCTCGAGCACTATTTAGTGTGGAGC-3′ and the downstream primer (position +398) was 5′-TGGCGACGTCTGTTCCTCCGGGCTGACGGCGGTCG-3′. The resulting 1.8 kb fragment was subsequently cloned upstream of the β-globin reporter gene (Westin *et al*., 1987). The human γ-GCS_{hc} promoter-reporter construct (10 μg) was transfected either with or without 2 μg of an expression plasmid carrying mouse MTF-1 or human MTF-1 cDNA. Cells were treated for 4 h with either 100 μM zinc sulfate or with 40 μM cadmium sulfate before harvest (as described previously by Radtke *et al*., 1993). The intensity of the bands was quantified by phosphoimager.

Electrophoretic mobility shift assays (EMSA)

EMSA was performed as described by Radtke *et al.* (1993). Binding reactions were performed by incubating 2–5 fmol end-labeled 31 bp long MRE-containing oligonucleotides with nuclear extracts obtained as described by Schreiber *et al.* (1989). Identification of the MTF-1 binding was performed by using wild-type control extracts in the presence or absence of an MRE-containing oligonucleotide. As an internal control, Sp1 binding to the oligonucleotides was performed.

RT–PCR analysis

Titan One-Tube-RT–PCR was performed according to the supplier's information (Boehringer Mannheim). Briefly, 25 or 100 ng mRNA was incubated with 25 pmol each of both primer pairs in the same tube for 1 min at 94°C and 2 min at 65°C for denaturation and was stored on ice immediately until the RT–PCR mix was added. The RT–PCR was performed as follows. A pre-cycle of 35 min at 58°C and 2 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 63°C and 1 min at 68°C, and a final cycle of 5 min at 68°C. As a control, the expression of the acidic ribosomal protein P0, which is encoded by the gene 36B4, was detected in the same RT–PCR. Aliquots from the RT–PCR were fractionated by agarose gel electrophoresis and bands were visualized by ethidium bromide (EtBr)-staining and the intensity of the bands was quantified by Fluor-Imager. The following primers were used to amplify respective fragments of either γ-GCS_{hc} or of 36B4 genes from 13.5-day-old embryos by RT–PCR. The first primer for the reverse transcriptase reaction and the following PCR for γ-GCS_{hc} was: 5′-CTATCATCTACAGATTCAGAAATCACTCCCCAGCG-3′ while the second primer for this gene in PCR was: 5′-GCGGGCATGGGGCTGCTGTCCCA-3′. The first primer for the reverse transcriptase reaction and the following PCR for 36B4 was: 5′-GCAAATGCAGATGGATCAGCCAGGAAGGCCTTGACC-3′ while the second primer for this gene in PCR was: 5′-GTGGGAGCAGACAACGTGGGCTCC-3′.

Primary mouse embryo fibroblasts

Primary embryo fibroblasts were isolated from 12.5-day-old mouse embryos by previously published methods. MTF-1 genotypes of cultured cells were determined by PCR and RT–PCR. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS).

Neutral red cell viability assay

Cytotoxicity of cadmium and H_2O_2 was assessed by determining the uptake of the neutral red dye essentially as described by Sigma, Biosciences™ according to Borenfreund and Puerner (1985). In brief, equal numbers of the indicated MEF cells were grown on 6-well dishes. After 12–16 h, media were replaced by fresh media containing the indicated amounts of cadmium or H_2O_2 for 8 h before adding the neutral red dye. Then 1/50 of a 0.33% neutral red solution was added to the plates and incubation was continued for 2 h in a standard 37°C incubator. Medium containing neutral red was removed and plates were carefully rinsed with 1/100 volume of fixation solution (0.1% $CaCl_2$ in 0.5% formaldehyde). A 1:1 volume of the neutral red solubilization solution (1% acetic acid in 50% ethanol) was then added and plates were placed on a shaker for ~2 h at room temperature. The viability of cells was

determined spectrophotometrically by measuring the absorbance of the neutral red dye extracted from the cells after solubilization at a wavelength of 540 nm.

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