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Review

Cellular zinc sensors: MTF-1 regulation of gene expression

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Abstract

Zinc metabolism in higher eukaryotes is complex, being controlled by uptake, efflux, and storage in individual cells, as well as in peripheral tissues and organs. Recently there have been advances in the understanding of the genes involved in these processes and their regulation. Metal-response element-binding transcription factor-1 (MTF-1) functions as a cellular zinc sensor which coordinates the expression of genes involved in zinc homeostasis, as well as protection against metal toxicity and oxidative stresses. In mice, these are known to include the metallothionein (MT), the zinc-transporter-1 (ZnT1) and the γ-glutamylcysteine synthetase heavy chain (γGCS_{hc}) genes. The cysteine-rich MTs function as an intracellular metal-chelators that bind zinc with high affinity, whereas the transmembrane protein ZnT1 exports zinc from the cell. γ-Glutamylcysteine synthetase controls the rate limiting step in glutathione (GSH) biosynthesis. GSH, which is present in mM concentrations in cells, effectively chelates large amounts of zinc *in vitro*. Both MT and GSH also function as antioxidants. The current model suggests that the zinc-finger domain of MTF-1 directly (and reversibly) binds to zinc. This metalloregulatory protein then adopts a DNA-binding conformation and translocates to the nucleus, where it binds to metal-response elements in these gene promoters leading to increased transcription. The six zinc-finger domain of this factor is highly conserved from insects to mammals, and biochemical studies confirm that the zinc-fingers are heterogeneous in function and in zinc-binding. Furthermore, the mouse MTF-1 gene is essential for development of the embryo, thus underscoring the importance of this transcription factor.

Abbreviations: γGCS_{hc}, γ-glutamylcysteine synthetase heavy chain; GSH – glutathione; MRE – metal-response element; MTF-1 – metal-response element-binding transcription factor-1; MT – metallothionein; TnT lysate – coupled transcription-translation lysate; USF – upstream stimulatory factor; ZIP – zinc-iron related transport protein; ZnT1 – zinc-transporter-1

Introduction

Regulation of gene expression by transition metals has been demonstrated in organisms ranging from bacteria to mammals (O'Halloran 1993). Metals regulate genes involved in protection against metal toxicity, as well as those involved in the homeostasis of essential metals, which themselves can be toxic. Transcription factors (activators and repressors), which directly interact with metal ions, and subsequently signal changes in gene expression are known as metalloregulatory proteins (reviewed in O'Halloran 1993; DeMoor & Koropatnick 2000). These cellular metal sensors can regulate gene transcription, and/or mRNA stability and translation. Transition metals are ubiquitous in our diet and environment and have a great impact on gene expression and, therefore, organismal function. Of particular focus here is the essential metal zinc.

The transcription factor MTF-1 coordinates the expression of genes which are important in the homeostasis of zinc and in protection against metal-toxicity and oxidative stress. In mice, these are known to include the MT-I/II, ZnT1 and γGCS_{hc} genes although it seems likely that other genes are also regulated by MTF-1. This article provides a brief overview of the functions of these proteins and then describes our current understanding of the mechanisms by which MTF-1 senses zinc and regulates their expression.

Overview of higher eukaryotic MTs

In prokaryotes, lower eukaryotes, plants and throughout the animal kingdom, one of the most intensely-studied examples of metal-regulation of gene transcription is that of the MT genes (Andrews 1990; Thiele 1992; Klaassen *et al.* 1999; DeMoor & Koropatnick 2000; Miles *et al.* 2000). In mammals, birds and fishes, the MT genes are remarkably responsive to zinc. Induction of these after exposure to zinc has been documented in many cell-types in culture and in intact animals. Furthermore, these genes are dramatically depressed in specific tissues (e.g., intestine, pancreas) taken from animals exposed to dietary zinc deficiency.

The metal-inducible MT genes from higher eukaryotes encode proteins of 60 to 68 amino acids in length which contain 20 cysteine residues and no aromatic amino acids (Kägi & Schäffer 1988). The placement of cysteine residues is absolutely conserved, and the majority of other changes in amino acid sequence are conservative. The cysteine-rich metal-binding clusters are distributed in α and β domains of the protein (Nordberg & Nordberg 2000). MTs adopt their specific, biologically unique tertiary structure only upon metal binding. These proteins can generally bind seven zinc or cadmium ions or up to 12 copper ions. Although MT is isolated as a $zinc_7$-complex from most mammalian tissues, it is a cadmium/zinc-complex under toxicological conditions (Kägi & Schäffer 1988; Kägi 1991). MT is also isolated as a copper/zinc-complex from animals with inherited disorders of copper metabolism, such as the Menkes and Wilson diseases in humans (Vulpe & Packman 1995) or the LEC rat (Sugawara *et al.* 1991). Furthermore, in *Drosophila melanogaster*, MT is naturally found complexed with copper (Maroni *et al.* 1995).

MTs are the most-abundant intracellular zinc-binding proteins in higher eukaryotes (Kägi & Schäffer 1988), and a significant percentage (5 to 20%) of the total cellular zinc is found complexed with MT under normal physiological conditions. Multiple isoforms of the protein are often present and the complexity of the MT gene family vary among organisms (1 in bacteria, 4 in mice, 16 in humans) and levels of expression of individual genes varies among tissues in higher eukaryotes (Miles *et al.* 2000). None of the MT genes that have been genetically inactivated in any species is an essential gene for that organism (Michalska & Choo 1993; Masters *et al.* 1994; Jensen *et al.* 1996). However, neither the effects of loss-of-function mutation in the mouse MT-IV gene, nor those of a complete loss of all four mouse MT genes have been examined. Functions for MTs in protection against metal toxicity, zinc-deficiency and oxidative stress have been demonstrated. For example, mouse MT-I and -II can provide a biologically important reservoir of zinc under zinc-limiting conditions (Dalton *et al.* 1996a; Andrews & Geiser 1999), and protect the animal against cadmium toxicity and oxidative stress (Lazo *et al.* 1995; Palmiter 1998; Lazo *et al.* 1998; Klaassen *et al.* 1999). In contrast, MT predominantly functions to protect against copper toxicity in yeast and the fruit fly (Karin *et al.* 1984; Mehra & Winge 1991; Jensen *et al.* 1996; Zhang *et al.* 2000). MT can sequester reactive oxygen and hydroxyl radicals, and provide for zinc, copper, or cadmium exchange with other proteins (Roesijadi 2000). Although primarily a metal-binding cytoplasmic protein, MT can translocate to the nucleus and may protect DNA from oxidative damage and participate in zinc exchange with zinc-dependent transcription factors (Cherian & Apostolova 2000; Roesijadi 2000).

Overview of a mouse zinc-transporter (ZnT) family

Recently, four mammalian genes involved in efflux or vesicular transport of zinc have been identified (McMahon & Cousins 1998a). Genes involved in the uptake of zinc (ZIP genes) are discussed elsewhere in this journal issue. Mouse ZnT1 through 4 are peptides of 359 to 503 amino acids, with six membrane-spanning domains, a histidine-rich intracellular loop, and a long intracellular carboxyl-terminal tail (Palmiter & Findley 1995; Palmiter *et al.* 1996a,b). The functional domains of the ZnT proteins are not well defined, but similar histidine-rich regions in other metal-transporters suggest a role of this domain in zinc chelation and transport. Transport function of these proteins is not energy depen-

dent, and it is thought that they function as multimers (Palmiter & Findley 1995). ZnT1 is homologous to zinc and cobalt resistance genes of yeast (Palmiter & Findley 1995). It functions to efflux zinc from cells, is localized to the plasma membrane, and is apparently expressed in most cell- and tissue-types (Palmiter & Findley 1995; Palmiter *et al.* 1996a). Exceptionally high level expression of the ZnT1 gene occurs in the visceral endoderm of the early mouse embryo and in the placenta (Langmade *et al.* 2000). These cells surround the developing mouse embryo and play a key role in nutrient transport and protection. Cultured cells which actively express ZnT1 are more resistant to zinc-toxicity (Palmiter & Findley 1995; Langmade, Ravindra & Andrews, unpublished observation). Mouse ZnT2 causes the vesicular accumulation of zinc in endosomal vesicles (Palmiter *et al.* 1996a), and is most similar in structure to ZnT3 which is responsible for the accumulation of zinc in synaptic vesicles in the brain (Wenzel *et al.* 1997; Cole *et al.* 1999). Targeted deletion of ZnT3 is not lethal (Cole *et al.* 1999). ZnT4 was discovered to be the Lethal Milk locus in the mouse (Huang & Gitschier 1997). This zinc-efflux protein is highly expressed in the mammary gland. Aberrant expression of ZnT4 causes severe zinc-deficiency to develop in the pups of mutant mothers. ZnT4 may also be involved in more general zinc homeostasis in the adult (Huang & Gitschier 1997).

Except for the finding of cell-specific expression patterns of ZnT genes, little else is known about their regulation. Among the ZnT genes, zinc-induction of ZnT1 has been documented in cultured neurons, and fibroblasts (Palmiter & Findley 1995; Tsuda *et al.* 1997; Langmade *et al.* 2000), and in the rat intestine after oral gavage with zinc (McMahon & Cousins 1998b; Davis *et al.* 1998). Furthermore, ZnT1 expression in enterocytes and the visceral endoderm of the embryo is responsive to changes in dietary zinc levels (McMahon & Cousins 1998b; Langmade *et al.* 2000). Furthermore, ZnT1 is an essential gene and homozygous knockout of the ZnT1 gene is lethal to the developing embryo (R.D. Palmiter, personal communications). Thus, ZnT1 appears to play a key role in zinc homeostasis.

Overview of γGCS

The enzyme γGCS is a key regulatory enzyme in the synthesis of GSH (Anderson 1998; Griffith & Mulcahy 1999; Wild & Mulcahy 2000; Soltaninassab *et al.* 2000). GSH is an important intracellular tripeptide (γ-glutamylcysteinylglycine) with multiple functions ranging from antioxidant defense to cell proliferation (Lu 1999). GSH is present in higher eukaryotic cells in millimolar concentrations (Griffith 1999), and it interacts with hydroxyl radicals, peroxinitrite, and hydroperoxides, as well as reactive electrophiles (Griffith & Mulcahy 1999). GSH also chelates metal ions with relatively high affinity. It binds zinc with an equilibrium constant of 2×10^{-8} M (Chaberek & Martell 1959; Ballatori 1994), and might compete for or facilitate metal interactions with proteins. When oxidized, GSH facilitates release of zinc from MT (Maret 1994), and when reduced it facilitates transfer of copper to MT (Da Costa Ferreira *et al.* 1993). Thus, GSH plays a role in zinc metabolism.

The synthesis of GSH is tightly regulated at the key step which is the ATP-dependent synthesis of γ-glutamylcysteine by the enzyme γGCS (Griffith & Mulcahy 1999). It is a heterodimeric zinc metalloprotein that belongs to a unique class of enzymes that gain activity due to the formation of a reversible disulfide bond (Soltaninassab *et al.* 2000). In the rat, a $\approx$28 kDa light chain and 73 kDa heavy chain form the holoenzyme. Details of the catalytic mechanism and structure of this enzyme have been recently reviewed elsewhere (Griffith & Mulcahy 1999). The enzyme's two subunits are encoded by separate genes which display both differential and coordinate regulation (Wild & Mulcahy 2000; Soltaninassab *et al.* 2000). In the mouse, the γGCS_{hc} (heavy chain) gene is essential for development of the embryo past d8.5 of gestation, but not for cell growth in culture (Shi *et al.* 2000). Expression of these genes is up-regulated in response to oxidants and metals (Griffith 1999; Wild & Mulcahy 2000; Soltaninassab *et al.* 2000). Recent studies suggest that the transcription factor Nrf2 in combination with other bZIP proteins mediates gene induction in response to oxidants, but AP-1 and NF-κB may also be involved (Wild & Mulcahy 2000). The mouse γGCS_{hc} gene is a target for MTF-1 (Günes *et al.* 1998), as is discussed below.

MTF-1: A zinc-dependent, positive transcriptional regulator in higher eukaryotes

Metal response elements mediate zinc-induction

All of the zinc-activated MT genes have promoter elements termed metal response elements (MRE), which

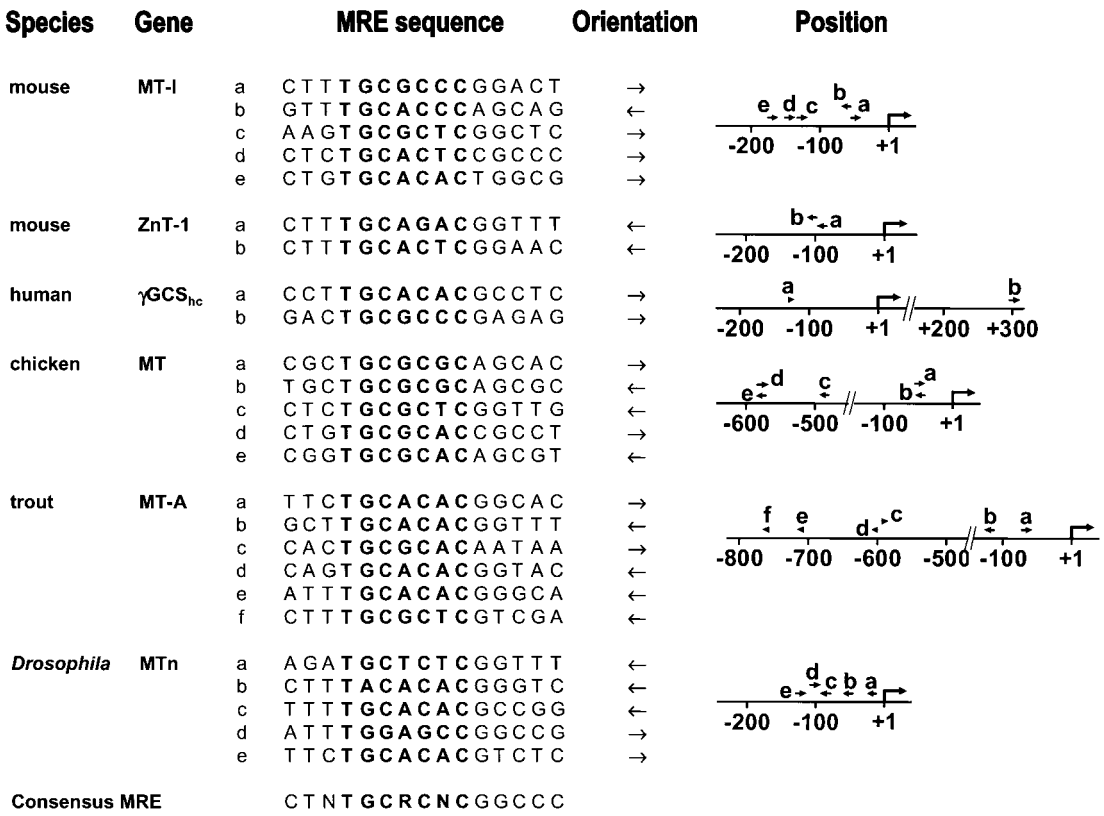

Fig. 1. Metal response elements are found in the proximal promoters of MTF-1 regulated genes. The proximal promoter region (+1 designates the transcription start site) of MT genes from higher eukaryotes (insects to mammals) contains multiple copies of metal response elements (MRE_{a-f}) which represent binding sites for the metalloregulatory protein MTF-1. The mouse ZnT1 and γGCS_{hc} genes contain two MREs each. Arrows indicate the sense or antisense orientation of each MRE, and their positions in each promoter. The 12 bp MRE sequence is well conserved and the core bases are essential for MTF-1- binding.

are present in multiple copies in the proximal promoters. Two MREs are present in the mouse ZnT1 gene promoter (Palmiter & Findley 1995) and two MREs are present in the mouse γGCS_{hc} gene (Günes *et al.* 1998). Multiple MREs function synergistically to confer response to zinc, cadmium and oxidative stress in transfected mammalian cells (Stuart *et al.* 1984, 1985; Koizumi *et al.* 1999), and it is thought that two or more MREs are required for a promoter to exhibit significant metal responsiveness. Neither the spacing nor orientation of MREs in these promoters appears to be critical for function. Metal responsiveness is dependent on the MRE core consensus sequence **TGCRCNC** (Stuart *et al.* 1984, 1985; Cizewski Culotta & Hamer 1989) which is found within an extended consensus sequence of 12 bp (Figure 1).

MTF-1 is an essential protein that binds to MREs in a zinc-dependent manner

The MRE is a binding site for the transcription factor MTF-1. MTF-1 was first cloned from the mouse by screening an expression library for MRE-binding activity (Radtke *et al.* 1993). The native protein was also purified from human cells by binding-site affinity chromatography, and the human MTF-1 gene was cloned (Brugnera *et al.* 1994; Otsuka *et al.* 1994; Koizumi *et al.* 1999). MTF-1 genes were subsequently cloned in *Drosophila* (Zhang *et al.* 2000), pufferfish (Auf der Maur *et al.* 1999), and the chicken (Jiang & Andrews, unpublished data) (Figure 2), which revealed that MTF-1 is a conserved protein in higher eukaryotes.

Binding of MTF-1 to the MRE is dependent on zinc and is easily disrupted when zinc is depleted (Andrews 2000). Furthermore, MTF-1 binds tightly to functional MREs, but not to nonfunctional MRE-like

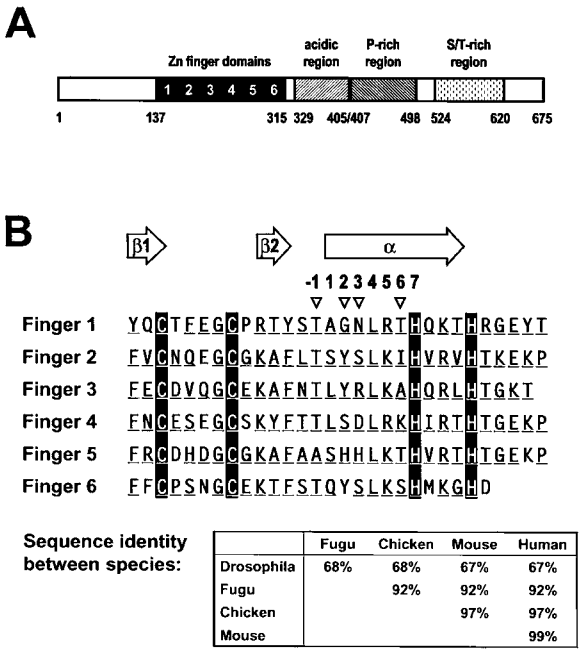

Fig. 2. The transcription factor MTF-1 contains several functional domains, and the zinc-finger domain is highly conserved among higher eukaryotes. **A**: Diagrammatic representation of the functional domains of mouse MTF-1. An amino terminal domain precedes the six zinc-finger domain, which is followed by three transactivation domains located in the carboxyl-terminal region of the protein. These domains are acidic, proline-rich and serine-threonine-rich, respectively (Radtke *et al.* 1995). Except for the zinc-fingers, other regions of the protein are not highly conserved. **B**: The highly conserved six zinc-finger domain of MTF-1 is shown here as a consensus sequence which was derived by comparisons of the amino acid sequence of the fingers from the five indicated species of mammals, birds, fish and insects. The cysteine and histidine residues (boxed) coordinate a zinc atom in similar C_2H_2-type zinc-fingers. The underlined amino acids are present at that position in 4 out of the 5 species examined, and the amino acid sequence identity in this domain is over 90% between fish and man. The *Drosophila* sequence is more divergent (67% identity). The reversible binding of zinc to specific zinc-fingers of MTF-1 apparently modulates DNA-binding activity of this transcription factor.

sequences (Koizumi *et al.* 1999). High affinity binding is dependent on the MRE core bases (Figure 1). MTF-1 binds with high affinity to the MREs from the mouse ZnT1 and γGCS_{hc} promoters (Günes *et al.* 1998; Langmade *et al.* 2000).

Homozygous knockout of the mouse MTF-1 gene in cultured cells eliminates heavy metal-induced MT and ZnT1 gene expression, as well as basal expression of these genes (Heuchel *et al.* 1994; Langmade *et al.* 2000). Homozygous knockout of this gene in mice abolishes expression of the MT-I gene, and significantly attenuates the expression of the ZnT1 and γGCS_{hc} genes in the embryonic liver and visceral endoderm of the yolk sac (Günes *et al.* 1998; Langmade *et al.* 2000; Andrews *et al.* 2001). Furthermore, the mouse MTF-1 gene is essential for fetal development (Günes *et al.* 1998). Embryos homozygous for MTF-1 null mutations die on d14 of gestation, which is after development of the yolk sac and initial formation of the liver. However, the fetal liver fails to develop properly (or degenerates) in these mice, while development of the nervous system and visceral yolk sac is not impaired (Günes *et al.* 1998; Lichtlen *et al.* 1999; Andrews *et al.* 2001).

MTF-1 structure

MTF-1 is a zinc-finger transcription factor in the Cys_2His_2 family (Figure 2A). The six zinc-finger domain has been highly conserved during evolution (Figure 2), while significant divergence has occurred in the remainder of the protein (Auf der Maur *et al.* 1999). Although, the precise mechanisms by which MTF-1 activates MT gene expression in response to metals remain unknown, this observation is consistent with the concept that the zinc-finger domain of MTF-1 is critical for both its metalloregulatory and DNA-binding functions in response to zinc (Müller *et al.* 1995; Dalton *et al.* 1997). The transactivation domains of MTF-1 are less well understood than the zinc-finger domain, but intra-molecular interactions are important for optimal MTF-1 function (Radtke *et al.* 1995; Müller *et al.* 1995). The carboxyl-termini of human and mouse MTF-1 contain three transactivation domains which are acidic, proline-rich and serine-threonine-rich, respectively (Radtke *et al.* 1995; Müller *et al.* 1995) (Figure 2A). The transactivation domain from the VP16 transcription factor can replace the function of these MTF-1 transactivation domains to produce a metal-responsive factor in transfected cells (Palmiter 1994; Radtke *et al.* 1995), but the transactivation domain of the zinc-finger protein Sp1 cannot (Bittel *et al.* 2000).

MTF-1 binding activity, nuclear translocation and occupancy of MREs in vivo is responsive to zinc

Treatment of cells with zinc *in vivo* causes a rapid, dramatic increase in DNA-binding activity of MTF-1 measured *in vitro* (Dalton *et al.* 1997; Koizumi *et al.* 1999), and this is accompanied by the nuclear translocation of MTF-1 (Smirnova *et al.* 2000; Otsuka *et al.* 2000). Western blot analysis revealed that zinc-treatment causes the rapid accumulation of immunoreactive MTF-I in the nuclear fraction (Figure 3A). In

the untreated cells, 80% of the MTF-1 was detected in the cytosolic fraction and it was not active in DNA-binding assays (Smirnova *et al.* 2000). Within 30 min of treatment of cells with zinc, essentially all of this protein was found in the nucleus and electrophoretic mobility shift assays using the MRE binding site detected a parallel increase in DNA-binding activity (Smirnova *et al.* 2000) (Figure 3B).

The activation and translocation of MTF-1 in response to zinc (or oxidative stress) is accompanied by the occupancy of MREs in the mouse MT-I promoter *in vivo* (Palmiter 1987; Dalton *et al.* 1996b). *In vivo* genomic footprinting using ligation-mediated PCR provided evidence for increased protein-DNA interactions with bases in the MRE core sequences and surrounding bases in cells treated with zinc for 1 h (Figure 4). Shown here are footprints for MRE-c and MRE-d of the mouse MT-I promoter. In these same samples there was no apparent change in protein-DNA interactions with Sp1 or USF1 binding sites in this promoter (Dalton *et al.* 1996b). These transcription factors are constitutively active to bind DNA. The activation and nuclear translocation of MTF-1 in response to zinc parallels increases in the relative rate of transcription of the MT-I gene.

Studies of the *in vivo* occupancy of the MREs in the mouse ZnT1 and γGCS_{hc} genes in zinc-treated cells have not been reported. However, the γGCS_{hc} proximal promoter drives MTF-1-dependent and zinc-dependent expression of a reporter gene in a transient transfection assay (Günes *et al.* 1998). In contrast, the ZnT1 promoter has been reported to be unresponsive to zinc under similar assay conditions (Palmiter & Findley 1995). However, MTF-1 binds *in vitro* to the MREs in the ZnT1 promoter, and the expression and metal regulation of the endogenous gene is MTF-1-dependent and is lost in cultured cells from MTF-1 knockout mice. In addition, zinc elicits a rapid transcriptional response of the ZnT1 gene which parallels that of the MT-I gene in cultured cells (Langmade *et al.* 2000). Thus, it is assumed here that MTF-1 directly modulates expression of the ZnT1 gene by interacting with MREs in that promoter.

MTF-1 binding activity is reversibly induced in vitro by zinc

MTF-1 in the cytosol of untreated cells is not active to bind DNA, but it can be fully activated *in vitro* by low micromolar (5 to 15 μM) concentrations of exogenous zinc at temperatures above 4 °C (Dalton *et al.* 1997; Bittel *et al.* 1998; Smirnova *et al.* 2000; Otsuka *et al.* 2000). Similarly, human and mouse MTF-1 synthesized *in vitro* in a coupled transcription-translation system (TnT lysate) is not active to bind DNA, but can be activated by exogenous zinc (1 to 5 μM) at elevated temperature (Bittel *et al.* 1998) (Figure 5A). This activation does not occur at 37 °C in the absence of exogenous zinc, nor does it occur at 4 °C in the presence of exogenous zinc. The three zinc-finger (Cys_2His_2) protein Sp1 synthesized under these conditions is largely constitutively active to bind DNA without the addition of exogenous zinc. Once activated by zinc to bind DNA, MTF-1 is more sensitive to metal chelators than is Sp1 (Radtke *et al.* 1993; Dalton *et al.* 1997). Furthermore, the DNA-binding activity of native and recombinant MTF-1 can be reversibly modulated by zinc (Okajima *et al.* 1993; Heuchel *et al.* 1994; Otsuka *et al.* 1994; Dalton *et al.* 1997). Diluting active MTF-1 into buffer in which the zinc concentration is below 0.6 μM, leads to a time – and temperature – dependent loss of binding, which can be completely restored by readjusting the zinc concentration in the reaction to 30 μM (Figure 5B).

In contrast to these effects of zinc, other transition metals (e.g., cadmium) which are potent inducers of MT, ZnT1 and γGCS_{hc} gene expression, do not activate the DNA-binding activity of mammalian MTF-1 *in vitro* and exert only modest effects *in vivo* on this activity (Bittel *et al.* 1998; Koizumi *et al.* 1999; Smirnova *et al.* 2000). These metal ions may cause the redistribution of zinc which in turn activates some MTF-1 to bind DNA (Palmiter 1994), but other mechanisms may also be involved. It should also be emphasized that increased DNA-binding activity alone seems unlikely to completely explain the metalloregulatory functions of MTF-1. Mouse MTF-1 can activate MRE-driven reporter gene expression in yeast only in response to zinc, and not to cadmium or oxidative stress (Andrews *et al.* unpublished results). Mouse MTF-1 functions as a zinc-sensor to activate MRE-driven gene expression in mammalian, yeast and *Drosophila* cells (Bittel *et al.* 2000; Andrews *et al.* unpublished results), and *Drosophila* MTF-1 can activate gene expression in response to zinc in transfected mammalian cells (Zhang *et al.* 2000). Thus, this metalloregulatory function of MTF-1 has been highly conserved during evolution.

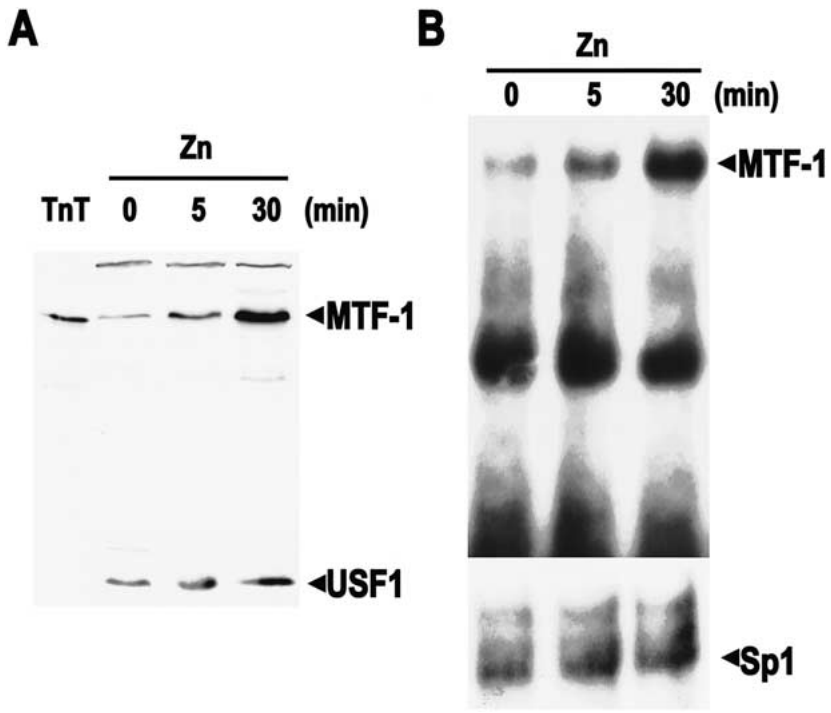

Fig. 3. Western blot and mobility shift assay detection of MTF-1 in nuclear extracts from Hepa cells at different times after zinc-treatment. Hepa cells were treated with 100 μM $ZnSO_4$ for 5 or 30 min and then separated into cytosolic and nuclear fractions (shown), as described (Smirnova *et al.* 2000). **A**: Nuclear extracts were analyzed by Western blotting for MTF-1. *Upper panel*: Recombinant mouse MTF-1 synthesized *in vitro* in a TnT lysate was used as a positive control for MTF-1. *Lower panel*: the extracts were Western blotted for USF1. **B**: *Upper panel*: nuclear extracts were analyzed for DNA-binding activity using a labeled MRE oligonucleotide. *Lower panel*: the same extracts were assayed using an Sp1 specific oligonucleotide. Arrows point to specific MTF-1 and Sp1 complexes with their respective oligonucleotides. Reproduced with permission from (Smirnova *et al.* 2000).

MTF-1 activation by zinc reflects functional heterogeneity of its zinc-fingers

Zinc-activation of the DNA-binding activity of MTF-1 involves reversible interactions with zinc (Radtke *et al.* 1993), and these interactions occur with the zinc-finger domain (Dalton *et al.* 1997). This was first demonstrated by deletion mutagenesis of the protein (Dalton *et al.* 1997), and subsequently by finger swapping experiments (Bittel *et al.* 2000), finger mutation experiments (Koizumi *et al.* 2000), and analyses of the purified recombinant MTF-1 finger domain (Chen *et al.* 1998, 1999). Replacing the zinc-finger domain of Sp1 with that of MTF-1 results in a chimeric protein that requires exogenous zinc for activation of MRE-binding activity, similar to native MTF1 (Bittel et al. 2000) (Figure 6). In contrast, the three zinc-fingers of Sp1, in the context of the MTF-1 peptide backbone, are constitutively active to bind DNA and do not require exogenous zinc.

The precise mechanisms by which zinc reversibly interacts with the zinc-fingers of MTF-1 and facilitates a DNA-binding conformation remain to be determined. Although, contradictory evidence has been reported with regard to the functions of the individual zinc-fingers of MTF-1, it is clear in each of these studies that the six zinc-fingers exhibit functional heterogeneity (Chen *et al.* 1999; Koizumi *et al.* 2000; Bittel *et al.* 2000). Some are involved in DNA-binding whereas others appear to be important for sensing zinc.

Analyses of the purified recombinant zinc-finger domain of human MTF-1 suggests that three or four zinc-fingers play a structural role in folding, DNA-binding and DNA-bending (Chen *et al.* 1998). The reduced peptide which bound enough zinc to fold three or four fingers was able to bind an MRE with high affinity (K_{app} 3.8×10^8 M^{-1}) and specificity. This finding is consistent with the fact that a single zinc-finger of this type can interact tightly with three or four bases in DNA (Rebar *et al.* 1996). Thus, effective MRE-binding could be accomplished with three or

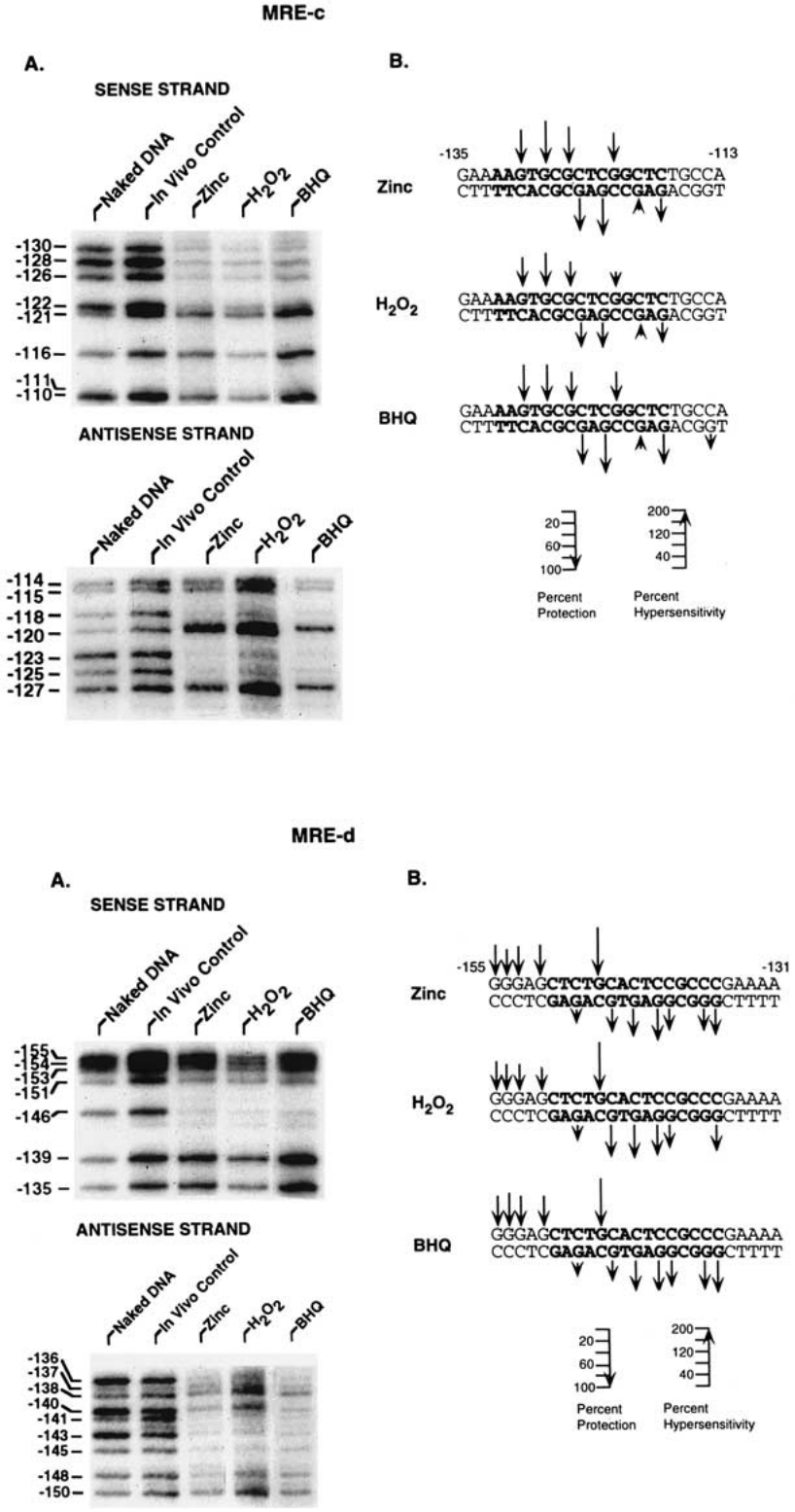

Fig. 4. In vivo genomic footprints over MRE-c and MRE-d in cells treated with zinc or oxidative stress-inducing agents. Mouse Hepa cells were treated with 2.5 mM H_2O_2 for 0.5 h, or 400 μM tBHQ (tert-butyl hydroquinone) or 100 μM $ZnCl_2$ for 1 h, and *in vivo* genomic footprints in the MT-I promoter were determined using ligation-mediated PCR of DNA from cells treated with dimethylsulfate. **A**: Shown are the results of the regions of the sequencing gel corresponding to MRE-c (Upper panel) and MRE-d (Lower panel). **B**: Band intensities were quantitated by phosphoimage analysis of dried gels and protection of $\geq$20% and hypersensitivity of $\geq$40% of individual guanine residues in treated cells compared with those in control cells were calculated for both the sense and antisense strand. Reproduced with permission from (Dalton *et al.* 1996b).

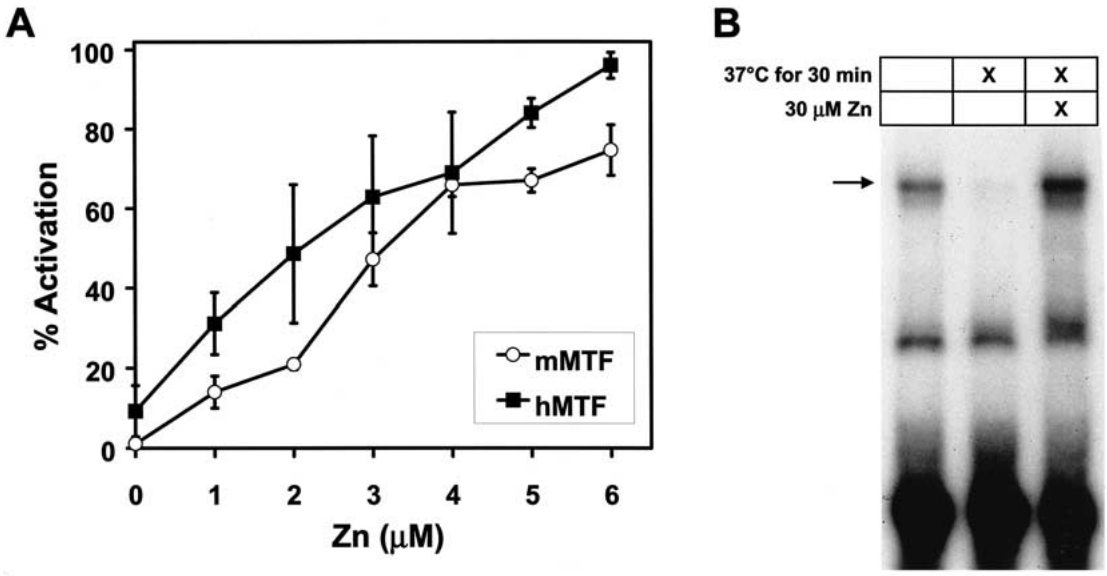

Fig. 5. Zinc reversibly activates MTF-1 DNA-binding activity. 'Recombinant' mouse and human MTF-1 were synthesized *in vitro* in a TnT lysate, as described (Dalton *et al.* 1997), and its DNA-binding activity was detected by a mobility shift assay using labeled MRE oligonucleotide. **A**: The effects of zinc concentration on the DNA-binding activity of mouse MTF-1 (mMTF) or human MTF-1 (hMTF) were examined (Bittel *et al.* 1998). Binding reactions were assembled with the indicated concentrations of exogenous zinc and incubated at 37 °C for 15 min before labeled MRE-s oligonucleotide was added and the reactions were subjected to electrophoresis. The amount of MTF-1/MRE complex was quantitated by phosphorimage analysis. Reactions containing 30 μM Zn served as the 100% activation standards and values shown represent the average ± SEM of three determinations. **B**: Mobility shift assay was performed using recombinant mouse MTF-1 that was activated (37 °C, 15 min) after addition of zinc (60 μM) directly to the TNT lysate. Binding reactions, minus labeled MRE-s, were then assembled in which the activated recombinant MTF-1 and the exogenous zinc were diluted over 100-fold (<0.6 μM). Binding reactions were then incubated at 4 °C (Lane 1) or at 37 °C for 1 h, as indicated. After incubation at 37 °C for 1 h, one binding reaction was placed at 4 °C (Lane 2), whereas the other binding reaction was readjusted to 30 μM zinc (Lane 3) and incubated further at 37 °C for 15 min. Labeled MRE-s was then added and all binding reactions were analyzed by electrophoresis. The gel was dried and labeled MRE-s detected by autoradiography. The arrow indicates the specific MTF-1/MRE-s complex. Reproduced with permission from (Dalton *et al.* 1997; Bittel *et al.* 1998).

four zinc-fingers. These studies also demonstrated that MTF-1 binding alters the DNA structure of the MRE. (Chen *et al.* 1998). The additional, and lower affinity binding of two or three zinc atoms did not cause a further conformational change in the finger domain. This finding is unexpected because zinc-finger domains adopt their conformation upon zinc-binding. This heterogeneity of zinc-binding is, however, consistent with a zinc-sensing function within the zinc-finger domain. Subsequent analysis of deletion mutants of the purified MTF-1 zinc-finger domain suggested that fingers 5 and 6 correspond to the weak zinc-binding fingers which stabilize the constitutive tight DNA-binding activity of fingers 1 to 4 (Chen *et al.* 1999).

However, the studies reviewed earlier reveal that native MTF-1 exists in a latent DNA-binding form, not a constitutively active DNA-binding form. The temperature requirement for zinc-activation of MTF-1 suggests that zinc-binding induces a conformation change in the native protein (Dalton *et al.* 1997, 2000). Although much evidence supports this concept, a genetic study using transfected cells suggested that the function of MTF-1 may be inhibited in the cell by a zinc-sensitive inhibitor (Palmiter 1994). Inactivation of the putative inhibitor could lead to heightened expression of the MT-I gene. No such inhibitor has been identified, and mouse MTF-1 functions in insect cells and yeast cells (Bittel *et al.* 2000), which suggests that a specific zinc-sensitive inhibitor is not required for MTF-1 to sense zinc. It is conceivable that the peptide domains of MTF-1 which surround the zinc-fingers, also influence their folding and DNA-binding, as was recently suggested (Koizumi *et al.* 2000).

No one has reported the successful purification of full-length recombinant MTF-1, and this problem has impeded detailed physical analyses of the native and mutant proteins. However, the effects of finger deletions in the mouse MTF-1 zinc-finger domain in the context of the native peptide backbone have been examined using mobility shift assays with

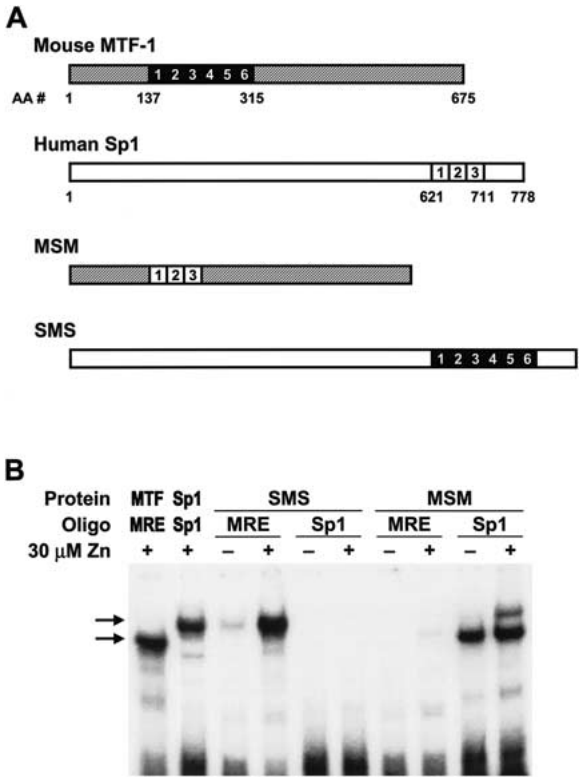

Fig. 6. The zinc-finger domain of MTF-1 mediates reversible activation by zinc: Zinc-finger swapping between MTF-1 and Sp1. **A**: Diagram of the chimeric proteins created by swapping the zinc-finger domains of MTF-1 and Sp1. The zinc-fingers of MTF-1 and Sp1 were switched without gain or loss of amino acids in the backbone of each. The domains which were exchanged encompass the first cysteine of finger 1 to the last histidine of the final finger. The Sp1 peptide backbone with the MTF-1 finger cassette is termed SMS, whereas the MTF-1 peptide backbone with the Sp1 finger cassette is called MSM. **B**: Full-length MTF-1 and Sp1 and chimeric SMS and MSM constructs were used to program TnT lysates, and zinc-activated DNA-binding (Dalton *et al.* 1997) was assessed by mobility shift assay using the labeled MRE or Sp1 oligonucleotides, as indicated. Arrows indicate the specific protein-DNA complexes. Reproduced with permission from Bittel *et al.* (2000).

proteins synthesized in a TnT lysate system (Figure 7A). This approach precludes measurements of binding constants, and can only provide qualitative information on relative DNA-binding activity. However, functional activity of the expressed proteins was also monitored using transient transfection assays in yeast (Figure 7B), MTF-1 knockout cells or *Drosophila* cells (Bittel *et al.* 2000).

Consistent with studies of the isolated finger domain, these studies also demonstrated functional heterogeneity of the zinc-fingers of MTF-1 and mapped core DNA-binding activity to fingers 2, 3 and 4 (Bittel *et al.* 2000). However, zinc-dependent activation of both DNA-binding and of reporter gene expression was mapped to zinc-finger 1 (Bittel *et al.* 2000). Deletion of finger 1 resulted in a protein which bound DNA constitutively, and zinc-response, but not basal expression, was lost in all three transfection systems (Figure 7). In contrast, these functions were unaffected by deletion of fingers 5 and 6 (Figure 7B). This observation was confirmed in a recent study of human MTF-1 which demonstrated that mutation of the second cysteine residue (cys to tyr) in finger 5 or 6, which would preclude zinc-binding and folding of the finger, had little affect on DNA-binding or transcriptional activation in transfected cells (Koizumi *et al.* 2000). These results are inconsistent with a major role of fingers 5 or 6 in the DNA-binding or gene activation processes of MTF-1, yet these fingers are highly conserved during evolution. Thus, it seems very unlikely that these zinc-fingers have no function.

Surprisingly, finger 1 of MTF-1 may constitute a unique zinc-sensing domain which is important for its metalloregulatory function (Bittel *et al.* 2000). Consistent with this concept, transfer of MTF-1 finger 1 to a position immediately preceding the three zinc-fingers of Sp1 resulted in a chimeric protein which requires exogenous zinc to activate DNA-binding *in vitro*, unlike native Sp1 which binds DNA constitutively (Figure 8). This suggests that in the absence of sufficient zinc, zinc-finger 1 of MTF-1 adopts a conformation which impedes the DNA-binding activity of adjacent zinc-fingers. Zinc binding apparently relieves that inhibition, which suggests that a conformational change in this finger occurs upon binding zinc. Consistent with these concepts, mutation of the second cysteine residue in finger 1 created a protein which did not bind DNA or activate transcription in response to zinc (Koizumi *et al.* 2000). Thus, zinc-finger 1 of MTF-1 is apparently the zinc-sensitive inhibitor of MTF-1 activity.

Future perspectives

Most studies of MTF-1 structure and function suggest that this protein serves to sense zinc levels in the cell by a direct and reversible interaction of zinc with a subset of zinc-fingers; the precise mechanism of this process remains to be determined. Clearly, structural studies of purified recombinant MTF-1 are needed. Unfortunately, purification of sufficient, full-length recombinant MTF-1 from bacteria has proven difficult.

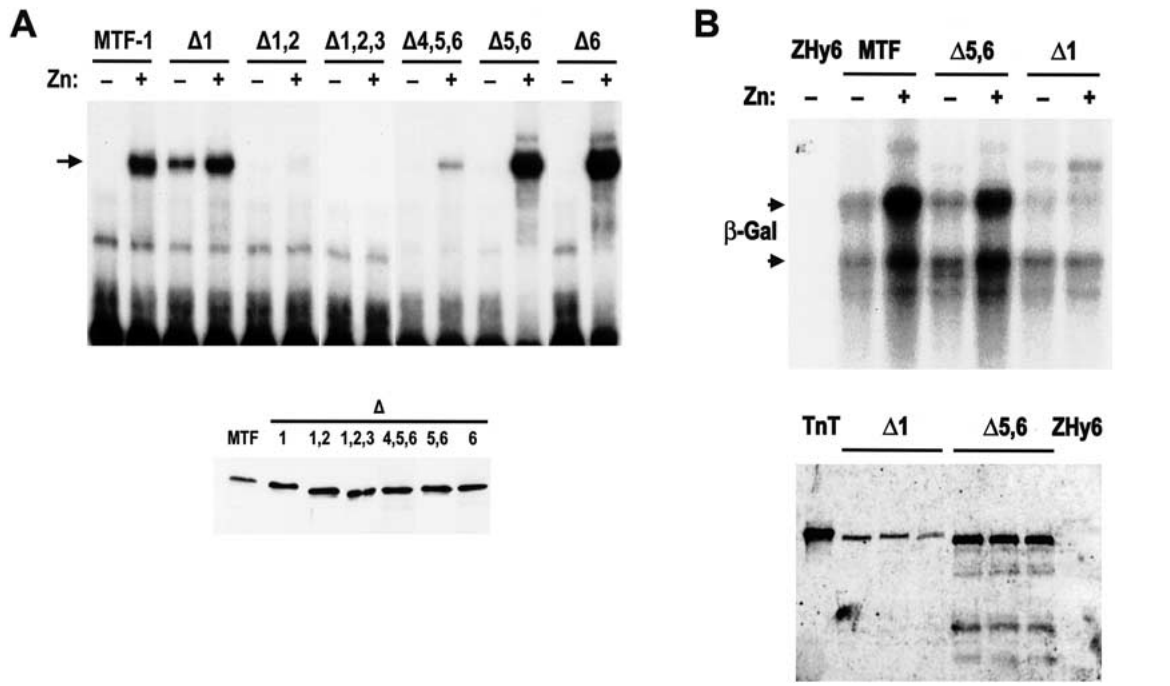

Fig. 7. Analysis of the DNA-binding of mouse MTF-1 zinc-finger deletion constructs, and their function in yeast. **A**: Upper panel: fingers were deleted (△) from the first cysteine of the deleted finger to the amino acid preceding the first cysteine of the next finger, as indicated (Bittel *et al.* 2000). Proteins were synthesized *in vitro* in a TnT lysate and analyzed by mobility shift assay for binding to labeled MRE-s. DNA-binding activity was assessed before (−) and after (+) the addition of 30 μM zinc, and incubation at 37 °C. The arrow indicates the specific protein-DNA complex. Lower panel: the synthesis of MTF-1 and its finger deletion mutants was confirmed by Western blotting (Smirnova *et al.* 2000). **B**: Function of MTF-1, △1 or △5,6 finger deletion mutants in yeast. The *Saccharomyces cerevisiae* strain ZHy6 has a mutation in the *ZAP1* gene which results in severely attenuated zinc transporter gene expression, and increased dependence of these cells on zinc levels in the culture medium (Zhao & Eide 1997; Davis *et al.* 1996). Top panel: Northern blot probed for β-galactosidase expression in ZHy6 cells cotransfected with an MTF-1, △1 or △5,6 expression vector plus an MRE-d5-β-galactosidase reporter. Cells were grown overnight in medium containing 2 μM zinc and brought to 60 μM zinc as indicated for 1 h. Arrows indicate the presence of two β-galactosidase transcripts. Bottom panel: proteins extracted from three independent colonies of yeast strain ZHy6 transformed with the △1 or △5,6 expression vector were analyzed by Western blotting. Recombinant mouse MTF-1 synthesized in a TnT lysate was used as a positive control in lane 1. The lane labeled ZHy6 contained protein from the non-transfected parental yeast strain. Reproduced with permission from Bittel *et al.* (2000).

One of the complications of this experimental system is the fact that zinc is a ubiquitous and essential metal ion. Defining assay conditions *in vitro* which control zinc availability is relatively simple, but defining such conditions *in vivo* in transfection assays is difficult. Cells adapt to zinc availability to maintain homeostasis. Thus, transfection studies may not accurately reveal some details of MTF-1 function (e.g., fingers 5 and 6 function?). Furthermore, the redox environment in the cell may affect zinc-activation of MTF-1 (Bittel *et al.* 1998; Koizumi *et al.* 2000). GSH alters the sensitivity of MTF-1 to zinc-activation *in vitro* (Bittel *et al.* 1998; Koizumi *et al.* 2000). This effect probably involves competition for zinc binding, although the redox state of the cysteine residues in the zinc-finger of MTF-1 also influences zinc binding (Chen *et al.* 1998).

It also remains to be determined how cadmium, and other metal ions, affect MTF-1 activity leading to the activation of gene expression. Cadmium treatment, using concentrations that are maximally effective to induce MT and ZnT1 gene expression in mammalian cells (Langmade *et al.* 2000), causes only a modest increase in MTF-1 binding activity *in vivo* and this metal has no effect *in vitro* (Koizumi *et al.* 1992; Bittel *et al.* 1998; Koizumi *et al.* 1999; Smirnova *et al.* 2000). It is conceivable that these metals may utilize specific co-activators of MTF-1 and/or activate signal transduction cascades that impinge on MTF-1 to affect MT gene transcription.

The transactivation domains of MTF-1 contain several potential sites for phosphorylation by known kinases, but functional phosphorylation, or other modifications (acetylation, methylation) of MTF-1 has not

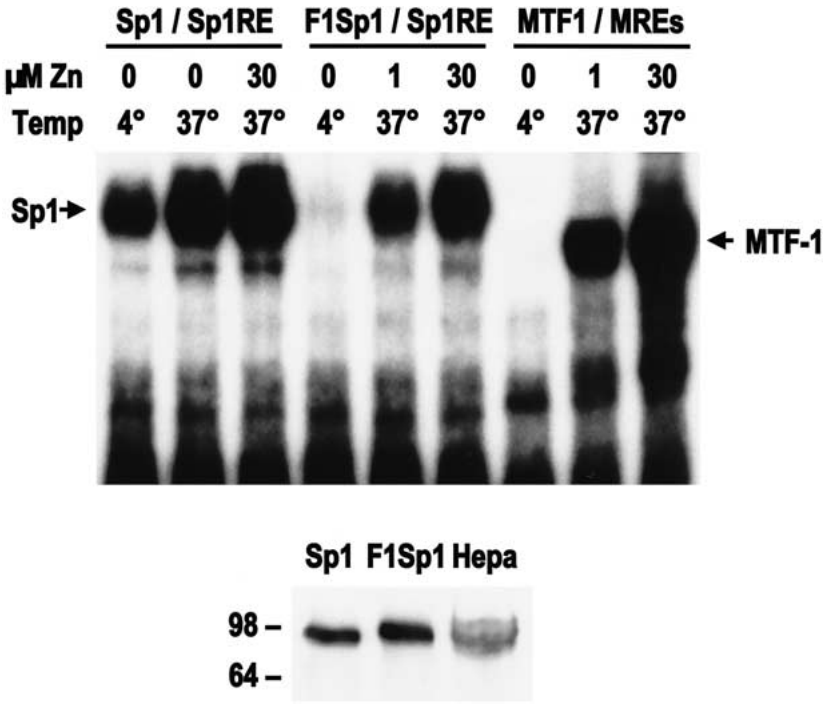

Fig. 8. Comparison of zinc-activation of DNA-binding of MTF-1, Sp1 and a chimeric F1Sp1 which contains the first zinc-finger from mouse MTF-1. The F1Sp1 fusion construct contains the Sp1 amino acid sequence to lysine 632 which is followed by 29 residues encompassing finger 1 of MTF-1 including the 3 amino acids immediately preceding the first cysteine and the 3 amino acids following the last histidine. This is followed by the three zinc-fingers of Sp1 and the remainder of the Sp1 peptide backbone (Bittel *et al.* 2000). Upper panel: TnT lysates were programmed with MTF-1, Sp1 or chimeric F1Sp1. Mobility shift assays containing an aliquot from the indicated TnT lysates were adjusted to the indicated concentrations of zinc and incubated at 4 °C or 37 °C for 15 min before electrophoresis. Binding to the Sp1 oligonucleotide (Sp1RE) or MREs was monitored. Arrows indicate the specific protein-DNA complexes. Lower panel: Western blot detection of Sp1 or F1Sp1 in these TnT lysates or in a whole cell extract from Hepa cells using an Sp1 antibody. The arrow indicates the position of the 98 kDa molecular weight marker. Reproduced with permission from Bittel *et al.* (2000).

been demonstrated. A recent study reported that MTF-1 from cadmium treated cells displays altered mobility during native gel electrophoresis, consistent with a possible posttranslational modification or conformation change in the protein (Otsuka *et al.* 2000). The nature and function (if any) of that potential modification are unknown. Inhibition of histone deacetylase activity renders cultured cells hypersensitive to metal-induction of MT gene expression (Andrews & Adamson 1987), which suggests that the threshold for sensitivity to metals may have different set points in different cells based on nucleosome structures. That MTF-1 molecules may interact is suggested by the findings that two or more MREs cooperate to confer metal-responsiveness (Stuart *et al.* 1984; Koizumi *et al.* 1999), and a single palindromic MRE directs metal-regulation in avian MT promoters (Shartzer *et al.* 1993). However, dimerization of MTF-1 has not been demonstrated.

MTF-1 may also cooperate with other transcription factors to regulate gene expression. The bHLH protein upstream stimulatory factor-1 (USF1) may play a role in the cadmium activation of MT-I gene expression in cultured cells (Li *et al.* 1998), and this protein apparently cooperates with MTF-1 to regulate the high level expression of MT genes in the visceral endoderm cells of the early mouse embryo (Andrews *et al.* 2001). Whether or not these proteins directly interact with each other is under investigation. Finally, methylation status of the mouse MT-I gene represses its activity and inducibility by metal ions (Lieberman *et al.* 1983; Lu *et al.* 1999), although methylation of the MRE does not appear to inhibit the *in vitro* binding of MTF-1 (Radtke *et al.* 1996).

In summary, current models of the mechanisms by which metals regulate MT gene expression suggest that the reversible interaction of zinc with the metalloregulatory protein MTF-1 modulates its DNA-binding structure. Reversible occupancy of perhaps

a single zinc-binding site in a zinc-finger may stabilize the DNA-binding form of the protein, leading to nuclear translocation, binding to MRE and increased transcription of MT, ZnT1 and γGCS_{hc} genes. These genes protect the cell from metal deficiency, metal toxicity and oxidative stresses. Further validation of these models for MTF-1 function awaits the crystal structures of active DNA-bound MTF-1 and inactive MTF-1.

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