— Review —

Molecular Mechanism of the Metallothionein Gene Expression Mediated by Metal-Responsive Transcription Factor 1

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(Received August 22, 2001)

Metal-responsive transcription factor 1 (MTF-1) is essential for activating transcription of the metallothionein genes in response to multiple species of heavy metals by binding to the metal-responsive element (MRE) located in the upstream region of the genes. In contrast, MTF-1 binding to the MRE *in vitro* is induced only by zinc ions, suggesting that MTF-1 acts as a zinc sensor protein. Although the mechanism allowing such a zinc-responsive factor to respond to various metal species *in vivo* remains ambiguous, recent information regarding the metal response of MTF-1 and the genes expressed downstream of MTF-1 provides important clues to unraveling the intrinsic mechanism and the biological significance of the MTF-1 functions *in vivo*.

Key words —— metallothionein, metal-responsive element, MTF-1, heavy metal, transcription

INTRODUCTION

Metallothioneins (MTs) are some of the most familiar proteins to researchers in the health science field for their protective role against the toxicities of heavy metals.[1,2] MTs have also been implicated in the defense against reactive oxygen species and in the homeostatic regulation of essential heavy metals such as zinc; thus MTs are now assumed to be multifunctional proteins with additional unidentified physiological roles.[3–6] Another hallmark of MTs is their induction by multiple heavy metal species at the transcriptional level. Therefore the MT genes serve as a valuable model for investigating the mechanism of cellular response to heavy metals. The clarification of heavy metal-dependent gene regulation will in turn contribute to our understanding of the physiological roles of MTs.

The molecular outline of heavy metal-dependent MT gene expression was established in the 1980s. During that period, researchers focused on identifying the enhancer that confers heavy metal inducibility on the MT gene promoter and the protein factors that bind to the enhancer in a heavy metal-dependent fashion. Functional dissection of the upstream region of the mouse MT-I (mMT-I) gene revealed multiple imperfect repeats, termed metal-responsive elements (MREs); similar sequences were found in the regulatory regions of other MT genes[7,8] (Fig. 1). Subsequently, various protein factors with different biochemical characteristics were identified by means of DNA binding assays using MRE sequences as probes (reviewed in Refs. 9 and 10). Most of these factors have not been investigated further, and only the zinc-dependent MRE binding factor designated metal-responsive transcription factor 1 (MTF-1) is now accepted as an essential factor for both basal and heavy metal-dependent expression of the MT genes. Although 8 years have passed since the cDNA for mouse MTF-1 (mMTF-1) was cloned,[11] the actual mechanism of how it responds to heavy metals has not yet been clarified. This article reviews recent information on MTF-1 to begin unraveling the mechanism and physiological significance of the heavy metal response mediated by MTF-1.

General Features of MTF-1

MTF-1 was first discovered as a zinc-dependent

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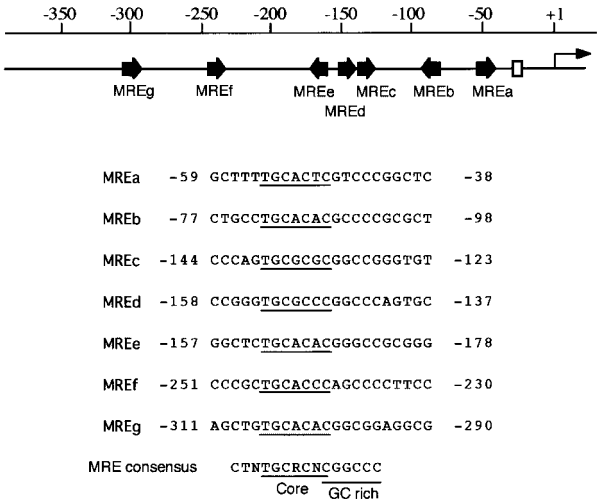

Fig. 1. The Arrangement of MREs in the Upstream Region of the hMT-IIA Gene

MREs carrying the consensus core sequence and the TATA box are indicated by filled arrows and an open box, respectively. The sequence of each MRE is shown below the figure, and the core sequence is underlined. The MRE consensus is also shown according to Refs. 7 and 8.

MRE binding factor,[12] and cDNA for MTF-1 was cloned from an expression library of mouse L cells using a synthetic MRE probe.[11] Human (hMTF-1)[13,14] and fish (fMTF-1)[15] homologues have since been isolated, and their amino acid sequences have been compared.[15]

The overall structure of MTF-1 is well conserved among species (Fig. 2). The remarkable feature of MTF-1 is six tandem repeats of the TFIIIA-type zinc finger structure in the N-terminal half of the protein. The amino acid residues in this domain are more than 90% identical among these species.[15] Also, three transcriptional activation domains (acidic, proline-rich, and serine/threonine-rich regions) are commonly found in the C-terminal half. The N-terminal adjacent to the zinc finger domain and the C-terminal adjacent to the transcriptional activation domains are unique and their functions have not been clarified. Unlike mMTF-1, both hMTF-1 and fMTF-1 have a 78- and 79-amino acid extension at the C-terminus, respectively. It is suggested that this extension was truncated in the mouse and rat during evolution.[13,15]

In yeast cells, the transcription factor ACE 1 regulates copper-dependent expression of the MT gene.[16] ACE 1 contains the MT-like cysteine cluster structure in its N-terminal half, and copper-binding to this structure converts ACE 1 into the conformationally active, DNA-binding form. MTF-1 is not structurally related to ACE 1, suggesting that the mechanism of MT induction is not evolutionarily conserved between yeast and higher eukaryotes despite the functional similarity of their MTs.

We have previously reported the zinc-dependent MRE binding factor termed zinc regulatory factor (ZRF) in HeLa cells,[17,18] which differs from mMTF-1 in biochemical characteristics. Protein purification[19] and cloning of its cDNA[14] revealed that ZRF is evidently a variant of hMTF-1 with a single amino acid exchange in the second zinc finger motif (Fig. 2), so we renamed ZRF hMTF-1b. Because this exchange suggests the existence of polymorphism

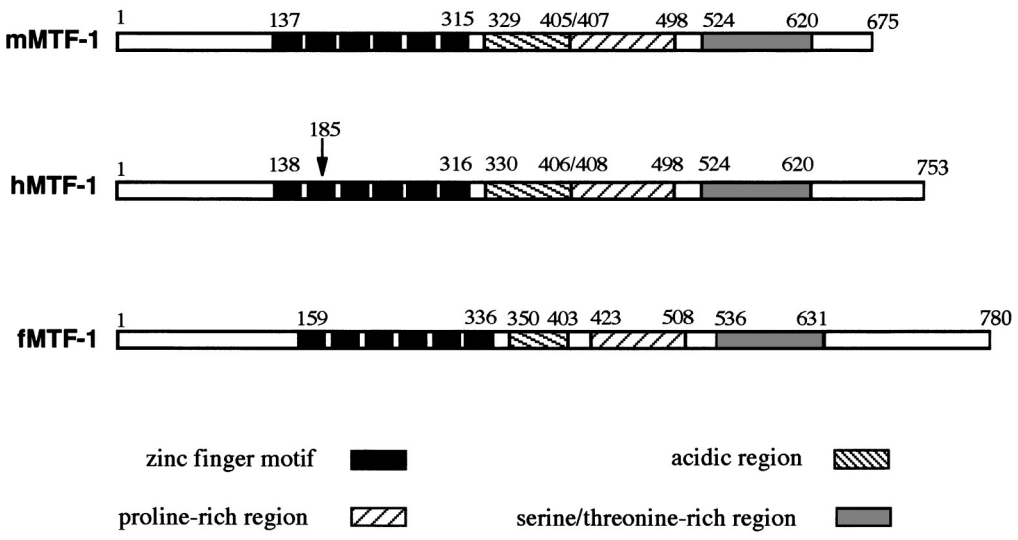

Fig. 2. Schematic Representation of hMTF-1, mMTF-1, and fMTF-1

The regions of the zinc finger, acidic, proline-rich, and therine/threonine-rich domains are indicated by boxes and amino acid numbers (Ref. 15). The position of the His → Tyr conversion appearing in hMTF-1b (Ref. 14) is indicated by an arrow in the diagram of hMTF-1.

of the hMTF-1 gene, we sequenced the cDNA prepared from blood samples obtained from Japanese donors, but could not find any sequence type other than that of hMTF-1b.[14] Among the other previously reported MRE-binding factors, MEP-1 may be a proteolytic fragment of mMTF-1.[20] However, the MRE binding factor MREBP[21] that we have purified from HeLa cells is not related to hMTF-1: the MRE binding activities of MREBP and hMTF-1b are separable during the purification.[14,18]

MTF-1 is constitutively expressed in various cell lines at both the mRNA and protein levels, which are not influenced by the treatment of cells with heavy metals.[11,14] This is supported by recent analysis of the upstream regions of the mMTF-1 and hMTF-1 genes; the MRE does not exist in the regulatory region of the MTF-1 gene and its mRNA is transcribed from a TATA-less promoter that is often found in housekeeping genes.[22]

MTF-1 is ubiquitously expressed in mouse heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testes.[22,23] It is noteworthy that significantly greater expression of MTF-1 was observed in testes compared with other tissues.

Characteristics of MTF-1 Binding to MRE

Through identification and purification of hMTF-1, we have exhaustively characterized its binding to MRE *in vitro* with mobility shift assays using MREa, the most potent MRE among those of the human MT-IIA (hMT-IIA) gene,[24] as a probe. hMTF-1 binds to MREa in a zinc-dependent manner under the relatively high reducing conditions generated by 10 mM dithiothreitol.[17] Other heavy metal ions, including cadmium, copper, and mercury, cannot facilitate the binding,[17,24] indicating that zinc is the only metal that can activate hMTF-1 to bind to MREa *in vitro*. The hMTF-1 binding to MREa is extremely specific to the core sequence (TGCRCNC), and a few bases just 5′ to the core are also important.[17,19,24,25]

In the upstream region of the hMT-IIA gene, seven MREs carry the consensus core sequence. Of the seven, hMTF-1 preferentially binds to MREa, MREb, MREe, and MREg.[24] Consistent with this, only these four MREs can mediate zinc responsiveness of a reporter gene in transient transfection experiments, indicating that the hMTF-1 binding to MRE is critical in the zinc-dependent activation of the MT genes. Despite the strong affinity to MTF-1, these MREs are different in their ability to activate transcription. In particular, MREb exhibits only very low activity. Koizumi's group explains the inconsistency between the hMTF-1 binding and transcriptional activation with MREb by participation of Sp1 as a negative regulator.[26] Adjacent to the core sequence of MREb is the binding site for the Sp1 family of proteins, and the actual binding of Sp1 to the sequence has been shown by supershift analysis. The functionally weak MREb can be converted to a strong MRE when a reporter gene carries MREb with a disrupted Sp1 target sequence. Moreover, a significant increase in the overall *hMT-IIA* promoter activity was also observed when the corresponding sequence in MREb was mutated. These results suggest that the MT gene regulation by heavy metals is not simply explained by only MTF-1, but involves complex protein interactions on the regulatory region of the genes. Andrews and colleagues have recently reported that mMTF-1 and upstream stimulatory factor 1 (USF1) possibly cooperate in the activation of the MT-I gene by maternal zinc in the endoderm cells of the visceral yolk sac during early development of the mouse embryo.[27]

Possible Mechanism of Heavy Metal-Dependent MT Induction by MTF-1

The inability of MTF-1 to respond to heavy metals other than zinc *in vitro* contradicted the idea that MTF-1 is essential in MT induction by various heavy metal species. In addition, MTF-1, when overexpressed in the culture cells, constitutively activates transcription *via* MRE.[11,23,28] In mouse embryonic stem cells lacking both MTF-1 alleles, however, MT induction by any of the heavy metals tested was depressed,[29] indicating that MTF-1 is a prerequisite for MT induction by multiple species of heavy metals. We recently conducted a chromatin immunoprecipitation assay using an antibody raised against hMTF-1 to ascertain the *in vivo* binding of endogenous MTF-1 in response to cadmium and copper as well as to zinc (F. Otsuka, unpublished observations). To account for the discrepancy in responses, Palmiter has proposed a hypothesis that zinc is the second messenger of various heavy metals, and constitutively active MTF-1 is associated with a zinc sensory inhibitor that liberates MTF-1 by receiving the zinc signal.[23]

Andrews' group has argued that the cadmium signal transduced to the MT gene involves oxidative stress,[30] based on their finding that a sequence responsive to hydrogen peroxide, located between –101 and –86 upstream of the mMT-I gene,[31] is also responsive to cadmium but not to zinc.[32] This se-

quence includes an overlap sequence consisting of a USF binding site and an antioxidant-responsive element; at least one specific USF, USF1, is reportedly involved in the induction.[32] Moreover, Dalton and colleagues have shown that MRE and mMTF-1 are also responsive to oxidative stress,[31,33,34] sugesting that the intracellular free zinc level increases by oxidative stress.[30] These results imply that the end of the transduction pathway of the cadmium signal is bipartite, whereas that of the zinc signal ends only at the MRE. These lines of investigation are presently being conducted primarily in the mouse system; it remains to be shown that the mechanism is universal.

Zinc Sensory Function of the Zinc Finger Domain

The DNA binding activity of MTF-1 is ascribed to the zinc finger domain,[28,35] although the binding capacity of the isolated domain is much weaker than that of the full-length MTF-1.[28] In addition to this classical role, a zinc-sensing function is now ascribed to the zinc finger domain of MTF-1, first suggested by its lower affinity to zinc compared with that of another zinc finger transcription factor, Sp1.[11,12]

To address this hypothesis, Chen and colleagues analyzed a bacterially produced zinc finger domain of hMTF-1 by various biophysical approaches.[36,37] They have shown that the four N-terminal zinc fingers have relatively high affinity to zinc compared to the two C-terminal fingers, and these two groups of zinc fingers contribute to MRE binding in a different fashion. That is, the four N-terminal fingers are required for high affinity and specific binding to the MRE core sequence, while the two C-terminal fingers bind to the GC-rich sequence adjacent to the core, and fulfill a metalloregulatory role by modulating the MRE binding of the N-terminal fingers. Contrary to this, Bittel and colleagues have recently reported that the zinc response of MTF-1 resides in the first zinc finger, shown by the deletion analysis of mMTF-1.[38] They further showed that Sp1 can be converted to a zinc-dependent DNA-binding protein by inserting the first finger motif of mMTF-1 just N-terminal to the zinc finger domain of Sp1.

The MTF-1 function is vulnerable to deletions, suggesting interplay between the domains in MTF-1.[35] Koizumi and colleagues have investigated the functional roles of each zinc finger using a series of mutants that carry an amino acid substitution at the second chelating cysteine residue in the individual finger structure.[28] They have shown that the transcriptional activity of hMTF-1 is reduced when any of the four N-terminal fingers are defective, while the defect in the two C-terminal fingers does not affect either transcriptional activity or DNA binding of hMTF-1, suggesting that the four N-terminal fingers are functionally distinct from the other two fingers. This result is consistent with that reported by Chen and colleagues, although the zinc regulatory role of the two C-terminal fingers has not been confirmed due to the high constitutive activity of overexpressed hMTF-1.

Although the zinc finger domain of MTF-1 appears to have a zinc sensory function, whether that function can be ascribed to specific fingers awaits further evidence. Sites other than the zinc finger domain, such as the N-terminal domain[28] and the segment overlapping with the acidic region,[39] reportedly influence the zinc-dependent functions of MTF-1. Hence, the zinc response of MTF-1 may be caused by collaborative interactions between the zinc finger domain and other portions of MTF-1.

Novel Responses of MTF-1 to Heavy Metals

Overexpression of MTF-1 constitutively activates transcription by MRE, which presents a significant obstacle to investigating the function of MTF-1 *in vivo*. The recent observations regarding new aspects of the metal response of MTF-1 may serve as an alternative approach to clarify the mechanism of the metal response of MTF-1.

We have found that the amounts of hMTF-1 in HeLa cell nuclear extracts increase after treatment with various heavy metals, although the amounts of hMTF-1 in whole-cell extracts remain unchanged.[14] This increase cannot be due to the nuclear translocation of hMTF-1 because endogenous hMTF-1 is distributed exclusively within the nucleus. In native gel electrophoresis, hMTF-1 recovered in the nuclear extracts migrates to a different position from that recovered in the cytosol fraction. These results suggest that alterations in hMTF-1, such as structural changes, occur in response to heavy metals, which probably facilitate the nuclear recovery of hMTF-1 during cell fractionation.

A similar increase in the amount of nuclear mMTF-1 has also been reported by Smirnova and colleagues,[40] which was observed in the nuclear extracts prepared from mouse Hepa cells treated with zinc and cadmium. However, they have shown that mMTF-1 expressed in MTF-1-null dko7 cells distributes within the cytoplasm and translocates to the nucleus after treatment with zinc. Their results apparently conflict with ours, but the underlying rea-

son is unclear.

Recently, Saydam and colleagues have reported that the nuclear translocation of hMTF-1 occurs if cells are cultured under serum-starved conditions.[41] Translocation is caused by various stress conditions, including heat shock, hydrogen peroxide, low pH, and cycloheximide, as well as by zinc and cadmium, suggesting that MTF-1 responds to these stimuli *in vivo*. A functional nuclear exporting signal was identified in the acidic region of hMTF-1, but a canonical nuclear localization signal found in the N-terminal direction adjacent to the zinc finger domain is not essential to nuclear transport. Based on our preliminary data, the third zinc finger is involved in the nuclear localization of hMTF-1, suggesting that the zinc finger domain itself may function as a nuclear localization signal. The nuclear translocation of hMTF-1 by itself cannot activate the transcription mediated by the promoter region of the mMT-I gene, so the physiological significance of this phenomenon remains to be clarified.

Genes under the Regulation of MTF-1

MTF-1 knockout mice die around the day 14 of gestation from hepatic decay,[42] while MT knockout mice develop normally even though they show increased sensitivity to cadmium toxicity.[43] These facts imply that MTF-1 is not the transcription factor solely for MT genes, but regulates developmentally important genes other than those for MTs. By comparing MTF-1 knockout embryos with their wild-type littermates, Lichtlen and colleagues investigated genes downregulated in the MTF-1-null embryo, using the SABRE selective amplification method, microarray screening, and database searches of the MRE sequences.[44] Among the many genes downregulated in the MTF-1-null embryo, three genes, α-fetoprotein, C/EBPα and tear lipocalin/von Ebner's gland protein were finally selected, and functional MREs were found in the upstream region of these genes. The authors have concluded that the downregulation of the α-fetoprotein and C/EBP α genes were the most plausible explanation for the MTF-1-null phenotype, based upon the involvement of each gene product in the defense system in the liver. However, previously reported downregulation of the γ-glutamylcysteine synthetase gene has not been confirmed in their investigation, and it might be a secondary phenomenon accompanied by liver damage.

MTF-1 has also been reported to regulate the gene for zinc transporter-1 (ZnT1) that functions to efflux zinc from cells.[45] In addition, transcriptional activation of placental growth factor by hypoxia has been recently reported to require MTF-1.[46] Although their relevance to the MTF-1-null phenotype is unclear, these results suggest that MTF-1 may be involved in zinc homeostasis and the cellular response to the oxidation-reduction status.

It is important to remember that the MT genes are members of the MTF-1-regulated genes, and the coexpression of MTs has been confirmed. In this context, the significance of the coexpression should be considered to understand the biological functions of MT induction. Through investigations of the gene set controlled by MTF-1 in various cell systems, the biological roles of both MTF-1 and MTs are expected to be clarified.

Conclusions

MTF-1 has been well established as essential for the MRE-mediated transcriptional activation of the MT genes, and its zinc sensory function has become accepted. Although the mechanism of its response to heavy metals *in vivo* is still unclear, recent findings of novel responses by MTF-1 to heavy metals may provide clarity. In addition, from a survey of genes regulated by MTF-1, various genes involved in the cellular defense system or in the regulation of zinc homeostasis have been identified, which may lead to the discovery of new networks of cellular defense systems, including crosstalk between stress pathways. Thus MTF-1 should be recognized as a factor necessary for a broad range of cellular functions rather than for only MT gene expression, and its roles warrant further investigation.

Acknowledgements Our investigations described in this article were done in collaboration with Dr. Shinji Koizumi and colleagues (National Institute of Industrial Health, Japan). I greatly appreciate Dr. Motoyasu Ohsawa (Teikyo University, Japan) for valuable discussions and kind support of our research.

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