

Individual Metal Responsive Elements of the Human Metallothionein-IIA Gene Independently Mediate Responses to Various Heavy Metal Signals

Kaoru SUZUKI and Shinji KOIZUMI*

Division of Hazard Assessment, National Institute of Industrial Health, 6–21–1, Nagao, Tama-ku, Kawasaki 214-8585, Japan

Received June 30, 1999 and accepted September 20, 1999

Abstract: Metallothioneins (MTs) are small metal-binding proteins that have a role in the defense against heavy metals. Mammalian MT genes are transcriptionally activated by metals such as Cd and Zn through multiple copies of the metal responsive element (MRE) present in the 5'-flanking region. To examine whether each MRE in a single promoter has a distinct role, we characterized seven MREs located upstream of the human MT-IIA gene. By transient transfection experiments using MRE-driven reporter gene constructs, individual MREs were assayed for the activity to mediate transcription in response to several heavy metal species. Four MREs including MREs a, b, e and g independently mediated reporter gene expression in response to Zn, Cd and Hg, while other MREs were not responsive to any of these metals. These results suggest that the multiplicity of MRE contributes to enhancing its activity, rather than providing functional diversity.

Key words: Metallothionein, Heavy metal, Transcription, Regulatory DNA element

Mammalian metallothioneins (MTs) are low molecular weight, cysteine-rich proteins that bind several heavy metals such as Cd and Zn, and are inducible by exposure to those metals¹. Based on these facts, MTs have been believed to play a role in heavy metal detoxification. This notion was supported by the findings that heavy metal resistance closely correlates with the ability to synthesize MTs among different mouse strains^{2, 3}, and with the amplification of MT genes among Cd-resistant cultured cell lines^{4, 5}. Recently, it was reported that MT-deficient mice show significantly enhanced sensitivity to Cd, consistent with the expected protective role of MTs^{6, 7}. The induction of MTs is therefore an undoubtedly important aspect in heavy metal toxicology.

The MT genes are transcriptionally activated by several heavy metal species, and a regulatory DNA element, the metal-responsive element (MRE), is essential for this process^{8, 9}. Multiple imperfect copies of MRE sharing the

highly conserved core sequence TGCRNC¹⁰ are found in the 5'-flanking region of MT genes. The multiplicity of MRE has been assumed to be effective in complimenting weak interaction between a specific transcription factor and a single short MRE sequence⁸, rather than providing their functional diversity. In fact, certain isolated MREs are able to mediate reporter gene expression in response to multiple heavy metal species^{10, 11}. On the other hand, it has been observed that MT isoform genes show differential responses to heavy metals^{12, 13}. For example, a certain isoform responds preferentially to Cd, but another responds to other metals as well. These findings raised a possibility that individual MREs could have unique metal responses. In this context, we examined whether the MREs of the hMT-IIA gene, that encodes a human MT isoform, have their own responses to various heavy metal species.

In the upstream region of the hMT-IIA gene, there are at least seven MRE-like sequences as shown in Fig. 1a¹⁴. To detect their possible functional differences, we examined

*To whom correspondence should be addressed.

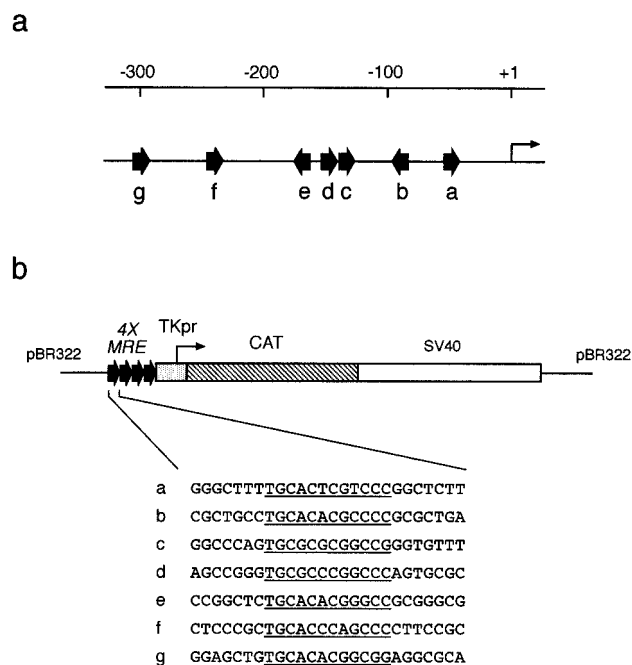


Fig. 1. Assay for estimating the activity of MREs.

a, MREs in the upstream region of the hMT-IIA gene. Nucleotides are numbered relative to the transcription start site (+1). The positions and orientations of MREs (a to g in the order of proximity to the transcription start site) are indicated by black arrows. b, Schematic representation of plasmid constructs used for the transient transfection assay. The basic reporter plasmid, derived from pBR322, carries the HSV-TK promoter fused to the CAT reporter gene. Four direct repeats of a double-stranded oligonucleotide containing one of the hMT-IIA MREs were inserted just upstream of the TK promoter. TKpr, TK gene promoter; SV40, the sequence derived from simian virus 40. Nucleotide sequences of the MRE oligonucleotides are indicated below the diagram (MRE sequences are underlined). Only the upper strand sequence without terminal linker sequences are indicated (see Ref. 14 for details).

metal response spectra of the individual MREs by a transient transfection assay. The basic reporter plasmid pTKprCAT carries the chloramphenicol acetyltransferase (CAT) reporter gene driven by the herpes simplex virus (HSV)-thymidine kinase (TK) gene promoter¹¹. Four direct repeats of an oligonucleotide containing each MRE sequence were inserted upstream of the TK gene promoter as described¹¹ (Fig. 1b). The resultant plasmids were designated p(MRE_x)TKprCAT (x=a to g). These constructs were transfected into HeLa cells (derived from human cervical carcinoma) by the standard calcium phosphate co-precipitation method and were cultured for 28 h. After incubation with heavy metals for 20 h further, cells were harvested and CAT levels in cell extracts were determined by an enzyme-linked immunosorbent assay¹⁴.

Table 1. Effects of heavy metals on reference gene expression

Metal	Concentration	Luciferase activity ^a (U/ μ g protein)	Induction ratio ^b
–		6.05 \pm 1.25	
CdSO ₄	2 μ M	8.75 \pm 0.55	1.45
CdSO ₄	20 μ M	9.40 \pm 1.70	1.55
ZnSO ₄	200 μ M	15.75 \pm 0.75	2.60

^aHeLa cells were transfected with pCAT (7.5 μ g) and pRSVL (2.5 μ g) in duplicates. After incubation for 28 h, cells were incubated with metals for 20 h further and levels of luciferase activity in cell extracts were determined. The pCAT plasmid without regulatory sequences was included to maintain the total plasmid amount equivalent to that in the standard assay. ^bLuciferase activity relative to that of control without metals.

In this kind of assay, we usually normalize CAT values relative to luciferase activity resulting from the co-transfected reference plasmid pRSVL, to minimize errors due to plate-to-plate differences in transformation efficiency¹⁴. ZnSO₄ at 100 μ M, which was used in our previous works, does not affect luciferase activity. For example, as an average from three independent experiments, the induction ratio of luciferase expression by 100 μ M Zn was 0.98 \pm 0.08 (standard error). However, we observed that heavy metals affected the reference gene expression in other conditions (Table 1). The level of luciferase activity was elevated approximately 1.5-fold by 2 to 20 μ M Cd, and 2.6-fold by 200 μ M Zn. These metals did not influence the enzyme activity when directly added to reactions (data not shown), suggesting that they probably modulate transcription from pRSVL. Consequently, normalization using pRSVL under such conditions appeared to be inappropriate. In our experience, however, reporter gene assays provide similar results with or without normalization, under a condition that does not affect the reference gene expression. When cells transfected with p(MRE_a)₄TKprCAT and pRSVL were induced with 100 μ M Zn, CAT induction ratios with or without normalization were 4.55 \pm 0.48 and 4.36 \pm 0.26 (as averages from three independent experiments), respectively, indicating that plate-to-plate differences in transformation efficiency are small in our system. To eliminate ambiguities resulting from unexpected alterations in the reference gene expression, we adopted the assay without normalization in estimating the effects of heavy metals on MRE-mediated transcription.

The reporter gene constructs each containing one of the hMT-IIA MREs (Fig. 1b) were analyzed for responses to several heavy metals. Transfected cells were incubated with 10 μ M CdSO₄, 100 μ M ZnSO₄, 10 μ M HgCl₂ or 100 μ M

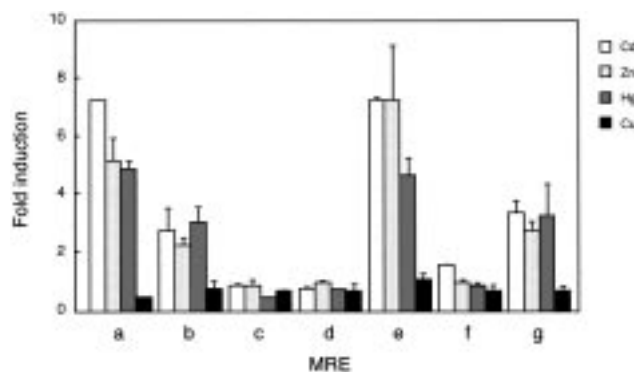


Fig. 2. Metal responses of the seven hMT-IIA MREs.

HeLa cells were transfected with 10 μ g each of p(MRE_x)₄TKprCAT plasmids (x = a, b, c, d, e, f or g), and incubated for 28 h. Cells were incubated with 10 μ M CdSO₄, 100 μ M ZnSO₄, 10 μ M HgCl₂ or 100 μ M CuSO₄ for 20 h further, and CAT levels in cell lysates were determined by enzyme-linked immunosorbent assay. The results from six independent experiments, each done in duplicates, were compiled. Fold induction relative to the CAT value of uninduced control is indicated.

CuSO₄ for 20 h, and CAT levels in cell lysates were determined. We previously reported that only four of these MREs, namely MREs a, b, e and g are able to respond to Zn¹⁴). Also to Cd and Hg, only these four MREs were responsive (Fig. 2). Induction ratios for MREa and MREe were higher than those for MREb and MREg, implying the former two MREs may play the major role in the overall promoter activity of the hMT-IIA gene. Other MREs (MREs c, d and f) were not responsive to any of these metals. None of the MREs was responsive to Cu, at least at the concentration used.

Our data demonstrated that the responses to Zn, Cd and Hg are restricted to the four MREs, indicating that only particular MREs are involved in the metal signal transduction. Furthermore, almost no specificity in the spectrum of metal response was observed among the four active MREs, indicating each MRE can independently respond to multiple heavy metal signals. In our previous study using model promoters with various numbers of MREa, it has been shown that multiple copies of MREa act synergistically¹⁴). Together with the present results, it appears likely that the multiplicity of MRE contributes to enhancing the activity, rather than providing functional diversity.

We previously reported that the four MREs mentioned above strongly bind the transcription factor human MTF-1, consistent with their ability to mediate Zn response¹⁴). This finding suggests that MTF-1 is involved in Zn signal transduction. From the data shown here, human MTF-1

could also be involved in the transduction of Cd and Hg signals. However, in protein-DNA binding reactions *in vitro*, human MTF-1 is activated only by Zn, not by other heavy metals including Cd and Hg¹⁴). Additional unknown mechanisms might therefore be involved in heavy metal-activated transcription *in vivo*.

In conclusion, the four active MREs of the hMT-IIA gene can independently mediate responses to multiple heavy metal signals, strongly suggesting that the multiplicity of MRE does not reflect their functional diversity, but provides a means to enhance their activity.

References

- 1) Kägi JHR, Nordberg M (1979) *Metallothionein*. Birkhäuser, Basel.
- 2) Tsunoo H, Nakajima H, Hata A, Kimura M (1979) Genetic influence on induction of metallothionein and mortality from cadmium intoxication. *Toxicol Lett* **4**, 253–6.
- 3) Piletz JE, Andersen RD, Berry W, Herschman HR (1983) Synthesis and degradation of hepatic metallothionein in mice differing in susceptibility to cadmium mortality. *Biochem Genetics* **21**, 561–78.
- 4) Rugstad HE, Norseth T (1975) Cadmium resistance and content of cadmium-binding protein in cultured human cells. *Nature* **257**, 136–7.
- 5) Beach LR, Palmiter RD (1981) Amplification of the metallothionein-I gene in cadmium-resistant mouse cells. *Proc Natl Acad Sci USA* **78**, 2110–4.
- 6) Michalska AE, Choo KHA (1993) Targeting and germline transmission of a null mutation at the metallothionein I and II loci in mouse. *Proc Natl Acad Sci USA* **90**, 8088–92.
- 7) Masters BA, Kelly EJ, Quaipe CJ, Brinster RL, Palmiter RD (1994) Targeted disruption of metallothionein I and II genes increases sensitivity to cadmium. *Proc Natl Acad Sci USA* **91**, 584–8.
- 8) Stuart GW, Searle PF, Palmiter RD (1985) Identification of multiple metal regulatory elements in mouse metallothionein-I promoter by assaying synthetic sequences. *Nature* **317**, 828–31.
- 9) Koizumi S, Otsuka F (1995) Transcriptional regulation of the metallothionein gene: metal-responsive element and zinc regulatory factor. In Sarkar B ed. *Genetic response to metals*. 397–410, Marcel Dekker, Inc., New York, NY.
- 10) Culotta VC, Hamer DH (1989) Fine mapping of a

- mouse metallothionein gene metal response element. *Mol Cell Biol* **9**, 1376–80.
- 11) Koizumi S, Yamada H, Suzuki K, Otsuka F (1992) Zinc-specific activation of a HeLa cell nuclear protein which interacts with a metal responsive element of the human metallothionein-II_A gene. *Eur J Biochem* **210**, 555–60.
 - 12) Richards RI, Heguy A, Karin M (1984) Structural and functional analysis of the human metallothionein-I_A gene: differential induction by metal ions and glucocorticoids. *Cell* **37**, 263–72.
 - 13) Varshney U, Jahroudi N, Foster R, Gedamu L (1986) Structure, organization, and regulation of human metallothionein I_F gene: differential and cell-type-specific expression in response to heavy metals and glucocorticoids. *Mol Cell Biol* **6**, 26–37.
 - 14) Koizumi S, Suzuki K, Ogra Y, Yamada H, Otsuka F (1999) Transcriptional activity and regulatory protein binding of metal-responsive elements of the human metallothionein-IIA gene. *Eur J Biochem* **259**, 635–42.