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Transcription of Metallothionein Isoform Promoters Is Differentially Regulated in Cadmium-Sensitive and -Resistant CHO Cells

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Abstract Transcription regulation of metallothionein (MT) isoform promoters was investigated in Chinese hamster ovary (CHO) K1 and MT gene amplified, cadmium-resistant (Cd^R) cells. The transfected promoter of Chinese hamster MTI and MTII genes can be activated in both cell lines by stimulation with Cd or Zn ions, although no MT mRNA can be detected in CHO K1 cells after challenge with metal ions. Neither MT promoter used in this study can be activated by induction with dexamethasone, regardless of whether a sequence homologous to glucocorticoid responsive element is present. During induction by metal ions, differential promoter activities of the MT genes occurs in both CHO K1 and Cd^R cells where MTII promoter has a stronger activity than that of MTI. As indicated by a time course study in both cell lines, the relative induction ratios of both MTI and MTII promoters are similar at each time interval. This result is consistent with a differential transcriptional factor–promoter interaction for the two MT promoters. By using the CHO K1 and Cd^R cells as a model system, the occurrence of autoregulation for yeast CUP1 (MT) gene was examined in mammalian cells. Both MT promoters consistently show a lower basal activity but a higher induction ratio in CHO K1 than Cd^R cells; a result different from that of yeast CUP1 gene. When MTF-1 mRNA was examined, no difference in relative quantity was observed in CHO K1 and in Cd^R cells treated with metal ions or with metal ions absent. J. Cell. Biochem. 68:174–185, 1998.

Key words: metallothionein; isoform; differential expression; autoregulation; Chinese hamster ovary cell; cadmium-resistant cell

Metallothionein (MT) is a metal-binding protein that was isolated from equine renal cortex [Margoshes and Vallee, 1957]. This protein was further demonstrated to be present in all the organisms examined. Although their structural features differ among animals, plants, and microorganisms [Kagi et al., 1984; Hamer, 1986], low molecular weight, cysteine-rich composition, and high metal-binding capacity are common characteristics of the proteins. The biological function of MT is not well defined. Nevertheless, it has been demonstrated to play a prominent role in metal detoxification [Michalska and Choo, 1993; Masters et al., 1994]. Other biologically significant roles, i.e., metal homeostasis, free radical scavenging, and anticancer drug resistance, have also been attributed to this protein [Karin, 1985; Kelley et al., 1988].

In mammals, MTs were initially divided into two families, i.e., MTI and MTII, according to the elution profile of the isoforms in anion exchange column chromatography. Subtypes of the MTs are also noted in certain species [Karin and Richards, 1984; Stennard et al., 1994]. MTI and MTII are induced by metals, hormones, stressors, and several drugs primarily in liver and kidney [Hamer, 1986]. Recently, a growth inhibitory factor (GIF) was found in the study of Alzheimer's disease. It occurs only in brain and has extensive homology with MTI and MTII [Uchida et al., 1991]. GIF is constitutively expressed in brain and can inhibit neuron growth [Kobayashi et al., 1993]. Subsequent studies demonstrated that the GIF gene is located at the 5' end and upstream of MTII gene of the same chromosome [Palmiter et al., 1992; Quaife et al., 1994]. This protein is therefore a member of MT family, designated MTIII. Another MT

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family, MTIV, is expressed in the squamous epithelium cells and may be associated with zinc (Zn) homeostasis in these cells [Quaife et al., 1994].

Regulation of MTI and MTII gene expression has been studied extensively. Molecular analyses indicate that several cis-regulatory elements are located in the 5'-flanking region of MT genes [Stuart et al., 1984, 1985]. Among them, metal-responsive elements (MREs) and a glucocorticoid-responsive element (GRE) are the best characterized [Stuart et al., 1984; Plisov et al., 1994]. Upon induction by metal ions, MT genes can be expressed only when repeated consensus sequences, i.e., MREs, occur in the promoter region [Stuart et al., 1995]. An MT transcriptional factor (MTF-1) interacts with Zn, leading first to interaction of the factor with MREs [Heuchel et al., 1994] and then to the transcription of MT genes. There also is evidence to suggest the existence of an inhibitor that regulates MTF-1 activity, although no such protein has been isolated [Palmiter, 1994]. It has been proposed that the inhibitor binds with MTF-1 before the cell is stimulated. Binding of the inhibitor with Zn releases MTF-1 as the cell is stimulated, and the transcriptional factor is then free to interact with MREs.

Studies on the comparison and transcriptional control of MT isogenes are limited. The occurrence and biological significance of MT isoforms in mammalian cells remain ambiguous. During stimulation by metal ions, MT isoforms are usually coordinately but differentially induced, although the level and time course of induction may vary for different metals [Sadhu and Gedamu, 1988]. Gene methylation [Jahroudi et al., 1990], variation in TATA box sequence [Shworak et al., 1993] and chromatin structure [Jahroudi et al., 1990] have been demonstrated to be involved in differential expression between MTIF and MTIG genes in human cells. Further study is required to determine whether these factors are involved in differential MT expression in all systems.

An interesting regulatory mechanism of the MT gene was reported in yeast cells where activity of transfected yeast MT (CUP1) promoter is related to the copy number of CUP1 gene in the cells [Hamer et al., 1985]. The transfected CUP1 promoter exhibits a greater level of activity as it presents in yeast cells without CUP1 gene, and the activity drops progressively with increasing copy number of the CUP1 gene in the cells. This phenomenon is known as autoregulation of the yeast MT gene. However, this mechanism has not been investigated in mammalian cells. In the work reported here, we use CHO K1 cells and a cadmium-resistant cell line (Cd^R) derived from the CHO K1 cells with MT genes amplified 60- to 100-fold [Morris and Huang, 1987] to study the activities of MT promoters. CHO K1 cells are very sensitive to Cd toxicity, the LD_{50} was estimated to be 2 mM after exposure to Cd for 4 h [Yang et al., 1996]. However, the Cd^R cells can grow in medium containing 100 μM Cd and are thus resistant to Cd toxicity [Morris and Huang, 1987]. The major goal of this work is to investigate (1) the differential expression of Chinese hamster MTI and MTII promoters; (2) transcriptional control of the isoform genes in cells with varied copies of MT gene; and (3) whether the autoregulation of yeast CUP1 gene can be observed in mammalian cells by using CHO K1 and Cd^R cells as a model system.

MATERIALS AND METHODS

Cell Culture and DNA Transfection

The CHO K1 and Cd^R cells were cultured as monolayers at 37°C in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS); 0.22% sodium bicarbonate, 100 U/ml penicillin, and 100 mg/ml streptomycin, in an atmosphere of 5% CO_2. MTII promoter was inserted in front of chloramphenicol acetyltransferase (CAT) gene in the pCAT-Basic vector (Promega, Madison, WI). Nested deletions of the MT promoter were created by using *Exo*III/Mung bean nuclease kit (Stratagene, La Jolla, CA). Plasmids for transfection were prepared by Qiagen purification kit (Qiagen, Germany). Before transfection, the cells were cultured in a density of 5×10^5 cells/60 mm petri dish for 18 h. Transfections were conducted by the cationic liposome method. The liposome was prepared followed the procedures described by Rose et al. [1991]. Five μg of promoter-CAT construct was cotransfected with 5 μg of pSV-β-galactosidase plasmid into the cells in each experiment as an internal control so that the transfection efficiency could be adjusted. Optimal transfection efficiencies were obtained for both CHO K1 and Cd^R cells when 30 μl of liposome was used. However, 5 and 30 μl of liposomes were used, respectively, for CHO K1 and Cd^R cells in the autoregulation study so that the transfection efficiencies, as demonstrated by β-galactosi-

dase activities, were similar for both cell lines. At 16 h after transfection, the medium was replaced by fresh medium, and the cells were harvested 24 h later with metal added to the medium at the required time point before cell harvest. The cells were removed with a rubber policeman and lysed by three successive cycles of freezing and thawing in liquid nitrogen and at 37°C. After centrifugation, the supernatant fraction was collected. Appropriate amounts of cell extract with the same level of β-galactosidase activity were heated at 60°C for 10 min and used to analyze the CAT activity by thin-layer chromatography (TLC) [Sambrook et al., 1989]. Alternatively, 70 µl of 0.25 M Tris–HCl (pH 7.8) was added to the heated cell extract (30 µl) to make the final volume of 100 µl. Then, 1 µl of ^{3}H-acetyl CoA (0.25 µCi/µl), 2 µl of 125 mM chloramphenicol, and 147 µl of H_2O was mixed with the cell extract, followed by the addition of 3 ml of scintillation fluid (Econofluor-2, Du Pont). Radioactivity in the sample was determined by scintillation counting (model 1600CA, Packard). At least five radioactivity determinations were made at different time intervals. The radioactivities were plotted against the reaction time, and the slope of each sample was estimated after linear regression. The slope is used to estimate the amount of CAT by using a known concentration of CAT as a standard. The level of enzyme expressed in the sample was expressed as nmole CAT/min/unit β-galactosidase activity.

Determination of MTF-1 mRNA Level

Two sets of primers were synthesized according to the reported mouse MTF-1 cDNA sequence [Radtke et al., 1993]. The first set was from nucleotide number 493–512 (5' CGGAAAGAAGTAAAGCGGTA 3') and 1615 to 1634 (5' GCATCAGCAGCAACAGTGGC 3'). The second set was from nucleotide number 543–562 (5' CTACAGCACAGCAGGCAACC 3') and 1588–1607 (5' GGACTTTCTGTTGTTGCTGG 3'). These DNA sequences are exactly the same between mouse and human MTF-1 [Radtke et al., 1993; Brugnera et al., 1994]. To determine the level of MTF-1 mRNA, a reverse transcriptase-polymerase chain reaction (RT-RCR) and a PCR were employed. For RT-PCR, the first set of primers was used. After 30 cycle-reaction, 1 µl of the product and the second set of primers were used for another PCR. A single DNA product can be obtained after 15-cycle reaction as analyzed by agarose gel electrophoresis. The quantity of the DNA fragment elevates linearly with the increase of reaction cycles and reached saturation after 30 cycles. PCR products at the 20-cycle reaction were compared in this study. For internal control, two primers were synthesized corresponding to the hamster tubulin cDNA sequences (GenBank). The primer sequences are 5' TGGAGCACTCTGATTGTGCC 3' and 5' TGGCTGTGGTGTTGCTCAGC 3', which will amplify a 0.6-kb DNA fragment in the RT-PCR. The PCR product was observed in the agarose gel after the 20-cycle reaction and increased up to 40 cycles. The 25-cycle reaction product for each treatment was compared.

Chemicals and Others

The methods for metal content determination, RNA extraction, Northern blot analysis and primer extension were described previously [Wang et al., 1994]. Chinese hamster MTII cDNA [Griffith et al., 1983] was used as a probe for Northern blot analysis. The primer synthesized for the determination of MTII transcription start site was from nucleotide number of +68 to +86 of CHO MTII gene. FBS and culture medium were purchased from GibcoBRL (Gaithersburg, MD). Restriction enzymes and Taq polymerase were obtained from Promega. Radioisotopes and labeling kit were from Amersham (Buckinghamshire, UK). Other chemicals were obtained from Sigma (St. Louis, MO).

RESULTS

Expression of two MT isoforms (MTI and MTII) can be detected in cadmium-resistant Chinese hamster ovary cells after induction by metal ions [Griffith et al., 1983]. The MTI and MTII genes have been cloned along with their promoter regions [Hung et al., 1991; Yu and Lin, 1995]. The MTI promoter was recently studied [Yu and Lin, 1995], and the description of the MTII promoter in the present report facilitates comparisons of the transcriptional regulation of both MT isogenes. Figure 1 shows the determination of the transcription start site of the MTII gene by primer extension. This site was the same as that predicted by sequence comparison [Hung et al., 1991]. For the 5'-flanking region of MTII gene, five putative MREs, an AP1 site and an Sp1 site were noted. This region was nestedly deleted and inserted in front of a reporter gene so that the significance of the cis-regulatory elements could be

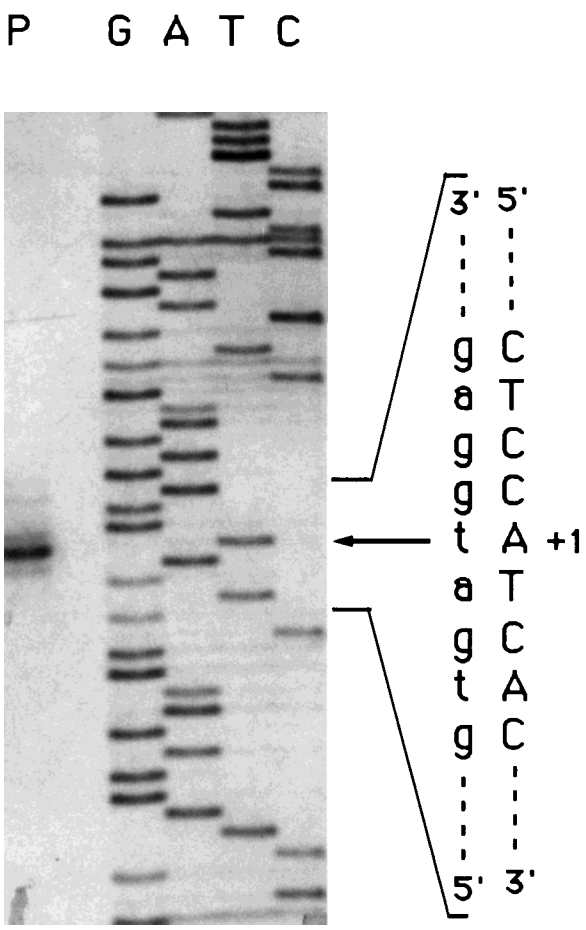

Fig. 1. Determination of transcription start site of Chinese hamster MTII gene. The position was determined by primer extension using an oligonucleotide complementary to the sequence of +68 to +86 of MTII gene as the primer. The sequence ladder shown on the left side was obtained using a DNA fragment containing the MTII flanking region as template and the same oligonucleotide for extension as primer. The ladder represents the complementary sequence of the transcribed DNA strand. P, result of primer extension.

studied (Fig. 2A). The constructs were then transfected into CHO K1 cells and followed by Cd treatment. Figure 2B shows that the promoter can be induced with two MREs present. However, all the identified cis-regulatory factors must present in order to obtain a maximum level of either basal or induced activities. Because of a high basal level expression of the constructs, the induction ratio after metal treatment is approximately 2- to 4-fold increase in chloramphenicol conversion percentage in this study. Similar results have been noted for MT promoters of other species. Examples can be found in human MTIF, MTIG [Foster and Gedamu, 1991], MTIH, MTIX [Stennard et al., 1994], mouse MTI [Stuart et al., 1985], rat MTI [Andersen et al., 1986] and yeast CUP1 [Hamer et al., 1984] promoters. All revealed a 2- to 5-fold induction ratio when examined in transient transfections.

MTI and MTII promoter activities were also examined by transfecting the promoter-reporter gene constructs into CHO K1 and Cd^R cells, and followed by induction by metal or hormone. As indicated in Figure 3, both MTI and MTII promoters could be activated after Cd or Zn administration in either CHO K1 or Cd^R cells. However, the basal and induced activities were lower for MTI promoter than those of MTII in both cell lines. Basal level expression was consistently higher in Cd^R than K1 cells for both promoters, while the relative inducibility (as calculated from percentage conversion) was higher in CHO K1 than Cd^R cells. Furthermore, dexamethasone did not significantly induce the promoter in either cells even though a putative GRE was found in the 5'-flanking region of MTI gene. To confirm the lack of inducibility by Cu and dexamethasone in both cell lines, Northern analysis was performed (Fig. 4). The results indicated that MT genes could respond only to stimulation by Cd and Zn in Cd^R cells. Data in this study agreed with the results of promoter analyses. However, CHO K1 cells did not accumulate MT mRNA, regardless of what inducers are used. The results demonstrated that the lack of MT mRNA expression in CHO K1 cells is not related to the absence of transcriptional machinery, since the transfected MT promoter functioned properly in those cells.

To further confirm the low relative inducibility of the MT promoter in Cd^R cells and the differential expression of the promoters in both cell lines, a time course study was conducted. MT promoters were respectively transfected into CHO K1 and Cd^R cells, and the cells were harvested at various time intervals after metal administrations. Figure 5 shows the results of promoter activities determined at each time interval. The promoters again revealed a higher basal level expression (0 h) in Cd^R than CHO K1 cells; in addition, at each time interval, MTII promoter showed greater activity than that of MTI. However, similar patterns were observed as the magnitudes of induction (ratio of CAT activity between induced and uninduced cells) for both promoters were estimated. The promoter activities elevated with a greater rate in CHO K1 cells as the duration of stimulation by metal ions increased; meanwhile, it

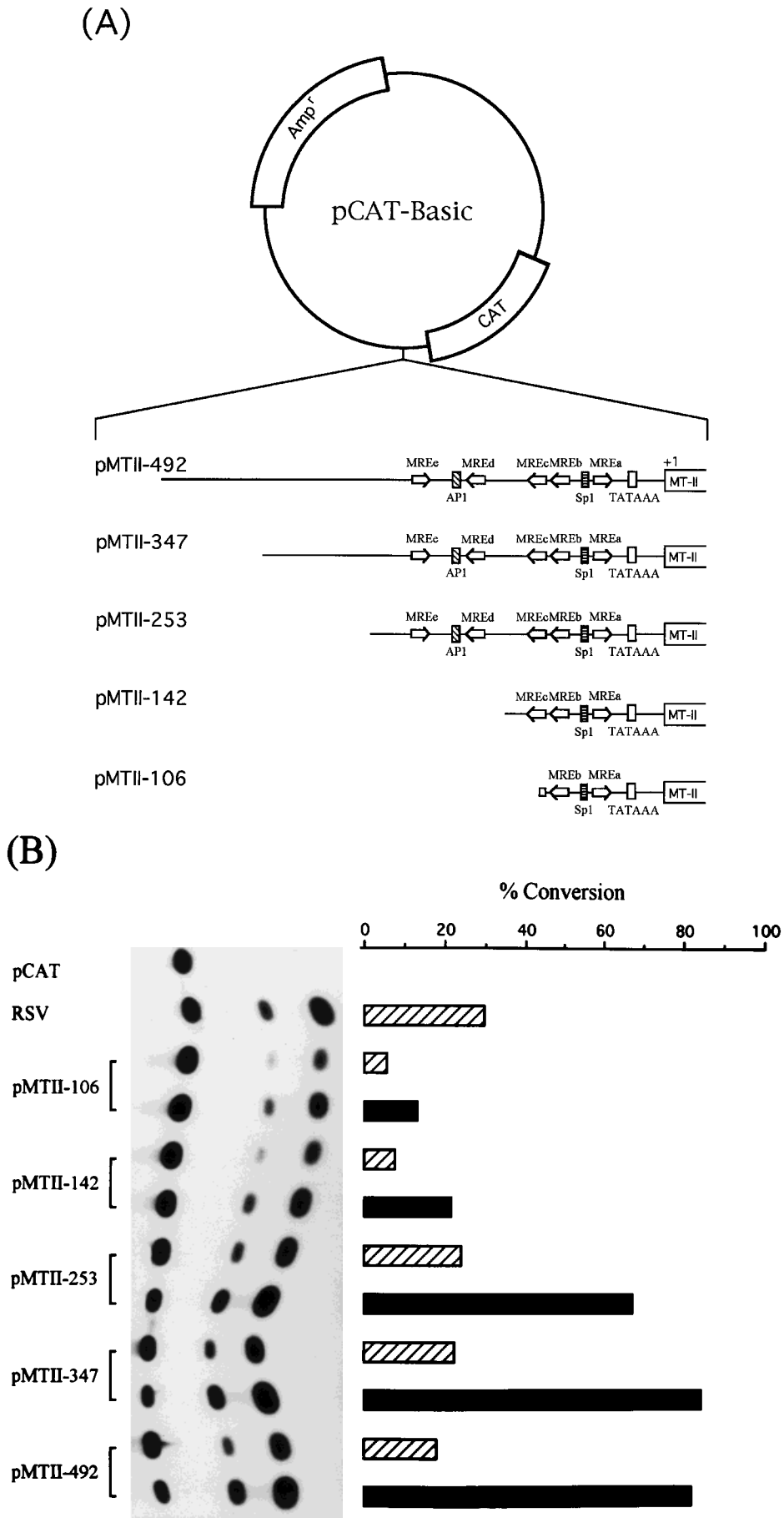
(A)
Amp r
pCAT-Basic
CAT
pMTII-492
MREe MREd MREcMREb MREa +1
AP1 Sp1 TATAAA
MT-II
pMTII-347
pMTII-253
pMTII-142
pMTII-106
(B)
% Conversion
0 20 40 60 80 100
pCAT
RSV
pMTII-106
pMTII-142
pMTII-253
pMTII-347
pMTII-492

Figure 2.

changed slowly in Cd^R cells. Results of this study provides further indication that the differential expression of MT isogenes is primarily associated with the characteristics of their promoters.

By using CHO K1 and Cd^R cells as a model, the phenomenon of yeast MT (CUP1) gene autoregulation can also be investigated in mammalian cells since the cells contain single and multiple MT genes, respectively. Experimental design is similar to that of autoregulation study in yeast [Hamer et al., 1985]. MT promoters were transfected into CHO K1 and Cd^R cells and their activities were measured after induction by various concentrations of Zn. The transfection efficiency has been adjusted to a similar level by altering the amount of liposome used since a higher transfection efficiency was noted for CHO K1 than Cd^R cells when the same level of liposome was employed. As shown in Figure 6, both MT promoter activities remain consistent and higher in Cd^R than CHO K1 cells when low doses of Zn are administered. At least 50 μM of Zn is required to initiate the MT promoter activities in CHO K1 cells, while 100 μM is necessary for Cd^R cells. Once the MT promoters are activated, their activities elevate at a higher rate in CHO K1 cells with an increasing metal concentrations; they become even higher than those in Cd^R cells. Results in this study are in contrast with those of yeast MT gene.

It has been shown that MT transcriptional factor (MTF-1) is essential for both basal and metal-induced MT gene expression [Heuchel et al., 1994]. To investigate whether the level of MTF-1 mRNA changes followed by the amplification of MT gene, the relative quantities of MTF-1 mRNA in CHO K1 and Cd^R cells were estimated. Two sets of primers (see Materials and Methods) were designed according to reported mouse MTF-1 sequences [Radtke et al., 1993] to conduct polymerase chain reaction (PCR). The same amount of RNA (equal quantity of 18S rRNA as analyzed by Northern blot) prepared from both cell lines and the first set of primers were used in the reverse transcriptase-PCR (RT-PCR). After 30-cycle reaction, 1 μl of the RT-PCR product was then served as a template and the nested primers (second set) were used for the second PCR. The reactions were performed for various cycles, and the results were analyzed in the agarose gel (Fig. 7A). A DNA fragment with an expected size (1.1 kb) was observed in the gel that increased progressively in density as the reaction elongated. Density of the specific DNA fragment was also increased progressively when elevated amounts of the RT-PCR product were used as templates. The DNA fragment was subsequently cloned and partially sequenced. The nucleotide sequence reveals a high homology with that of reported mouse MTF-1 [Radtke et al., 1993]. Attempts to analyze the MTF-1 mRNA expression by Northern hybridization were unsuccessful. We therefore used semi-quantitative PCR to compare the MTF-1 mRNA level in both CHO K1 and Cd^R cells. Figure 7B shows the results obtained from the 20-cycle reaction. Tubulin cDNA was also amplified and used as an internal control. The relative quantity of MTF-1 mRNA did not change, as demonstrated clearly by the density of the amplified DNA fragment, in CHO K1 and Cd^R cells, with or without the induction of metals.

Fig. 2. Plasmid construction and determination of promoter activity of the CHO MTII 5′-flanking region. **A:** The cloned MTII promoter region was inserted in front of CAT gene of pCAT-Basic vector (Promega) and inserted DNA was selected to various lengths using an *Exo* III/Mung bean deletion kit. The constructs were then used for transfection. The number of each DNA construct refers to the location of the nucleotide from the transcription start site. The MREa to MREe locates at nucleotide numbers -56 to -45, -84 to -95, -103 to -114, -158 to -168, and -218 to -203, respectively. Among them, MREa and MREe are in forward orientation, and the others are in reverse orientation, **B:** The various constructs were transfected into CHO K1 cells, and the CAT activities after transfection were determined by TLC. Conversion ratio was determined by dividing the radioactivity of acetylated chloramphenicol by the total radioactivity. Solid and slash bars, samples with and without Cd treatment, respectively.

DISCUSSION

We investigated the differential regulation and transcriptional regulation of MTI and MTII promoters in CHO K1 and Cd^R cells. Since Cd^R cells are derived from CHO K1 cells with the MT gene amplified 60- to 100-fold [Morris and Huang, 1987], results in this study also reveal the regulatory mechanisms of MT isoform expressions in cells of different MT copy numbers. In CHO K1 cells, MT genes are not expressed upon stimulation with metal ions, while Cd^R cells respond rapidly. However, as demonstrated by results in Figure 3, the regulatory factors required for MT transcription are clearly present in these cells. Methylation of the MT gene in CHO K1 cells may be one explanation

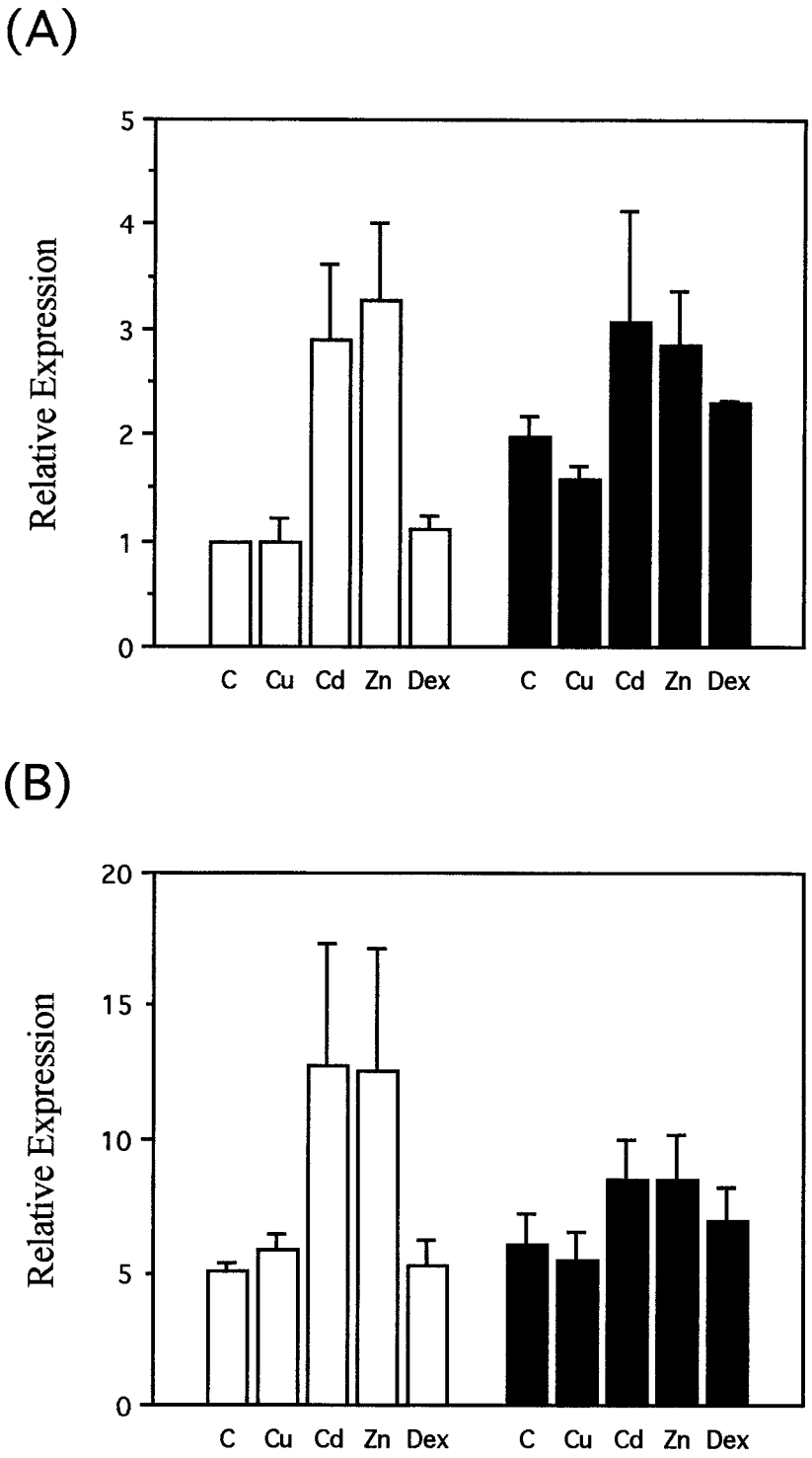

Fig. 3. Induction of MT isogene promoter activities by hormone and various metals. Chinese hamster MTI (pMTI-558) **(A)** or MTII (pMTII-492) **(B)** promoter was cotransfected with pSV-β-gal plasmid into CHO K1 (open bars) and Cd^R (solid bars) cells. At 10 h before harvest, the cells were treated with $CuSO_4$ (150 μM), $CdCl_2$ (5 μM), $ZnSO_4$ (100 μM) or dexamethasone (Dex, 10 μM). Cell extracts from each treatment were subjected to TLC analysis. Conversion ratio was determined by dividing the radioactivity of acetylated chloramphenicol by the total radioactivity. Relative expression was calculated by dividing the conversion ratio of treated sample by that of untreated sample in each experiment. Each value represents a mean ±SD of three (Cd- and Zn-treated) or two (Cu- and Dex-treated) samples. C, untreated sample.

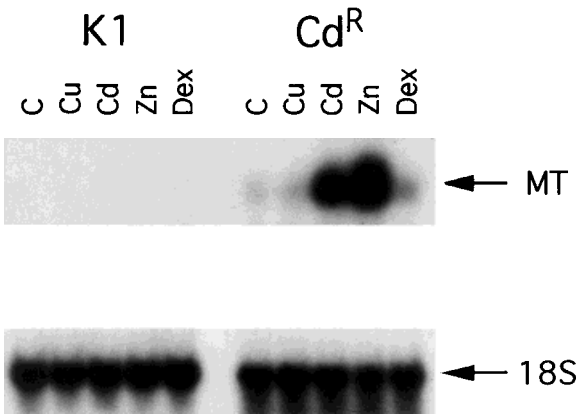

Fig. 4. Northern analysis of MT mRNA expressions in CHO K1 and Cd^R cells after various inductions. CHO K1 and Cd^R cells were treated with either $CuSO_4$ (150 μM), $CdCl_2$ (5 μM), $ZnSO_4$ (100 μM) or dexamethasone (Dex, 10 μM) for 10 h and MT mRNAs in the cells were analyzed. 18S rRNA was also probed with yeast 18S rRNA and used as an internal control.

for lack of responsiveness to metal ions. The methylation of MT genes may not occur after gene amplification (Compere and Palmiter, 1981), and thus MT mRNA can be expressed in Cd^R cells.

In CHO Cd^R cells, both MTI and MTII genes are activated in response to challenge with Cd or Zn, but not with Cu and dexamethasone (Dex). The amount of Cu administered may not be sufficiently high to stimulate the MT genes and may explain the lack of MT mRNA expression. Previous studies indicate that the dose of Cu used to stimulate significant MT synthesis is cell type dependent; it may range from 50 μM to 600 μM [Durnam and Palmiter, 1981; Leone et al., 1985]. On the other hand, the dose may not be the reason for the nonresponsiveness of MT promoter to Dex induction, since a series of Dex concentrations have been examined. The loss of regulation of MT genes by glucocorticoid after gene amplification has also been observed in a cadmium-resistant mouse cell line [Mayo and Palmiter, 1982]. It has been proposed that element(s) for glucocorticoid induction were not coordinately amplified with MT genes, thereby resulting in a lack of MT mRNA expression after Dex treatment. However, this is not a general phenomenon since a recent study indicated that inducibility by glucocorticoid can be retained in another mouse cell line in which the MT gene was amplified [Thibodeau et al., 1992]. It is predicted that the cis-acting element(s) required for the induction by Dex is also amplified in the later cell line so that those cells can respond to the stimulation of Dex and express MT mRNA [Thibodeau et al., 1992]. For transfection experiments, the size of MT promoter used might be insufficiently large to cover all of the elements required for induction by Dex, and thus no response could be observed [Mayo and Palmiter, 1982].

In this study, we investigated differential promoter activities of MT isoforms. MT isoforms are commonly found in mammals, though the physiological significance of the different isoforms remains unclear. MT isoforms are expressed coordinately in response to stimulation by various metals. However, differential expression of MT isoforms is frequently observed. For induction of MT by metals, interactions between Zn ions, MTF-1 and MREs are required [Heuchel et al., 1994]. Therefore, exhibition of differential MT expression in response to metals should also be related to interactions between those factors. In Cd^R cells, there is approximately 2.5-fold more MTII than MTI mRNA after induction with Zn [Morris and Huang, 1987]. This finding corresponds to the promoter activities of MTI and MTII reported (Figs. 3, 5). Since the CHO MT promoters were transfected into cells and transiently expressed, the differential expression is due neither to the effect of chromatin structure [Jahroudi et al., 1990] nor to the variation of TATA box sequence [Shworak et al., 1993] as was found with human MTIF and MTIG genes. When the sequences of CHO MTI and MTII promoters (nucleotide -1 to -300) are compared, approximately 42% in identity is found. Although both promoters contain the same number of putative MRE, AP1, and Sp1 recognition sequences, other element(s) in the MT promoter region may play roles in determining the promoter activity. There are two possible explanations for the differential activity of CHO MTI and MTII promoters. First, MTF-1 binds coordinately with MTI and MTII promoters upon induction by metal ions. However, a more effective induction complex is formed for MTII promoter, which results in a stronger promoter activity. A second explanation is that MTF-1 binds differentially with MT promoters, and this is the determinant factor for the differential promoter activity. Further experiments are required to test between the possibilities.

When MT promoter was transfected into CHO K1 and Cd^R cells, a higher induction ratio was observed in the former cell line upon stimula-

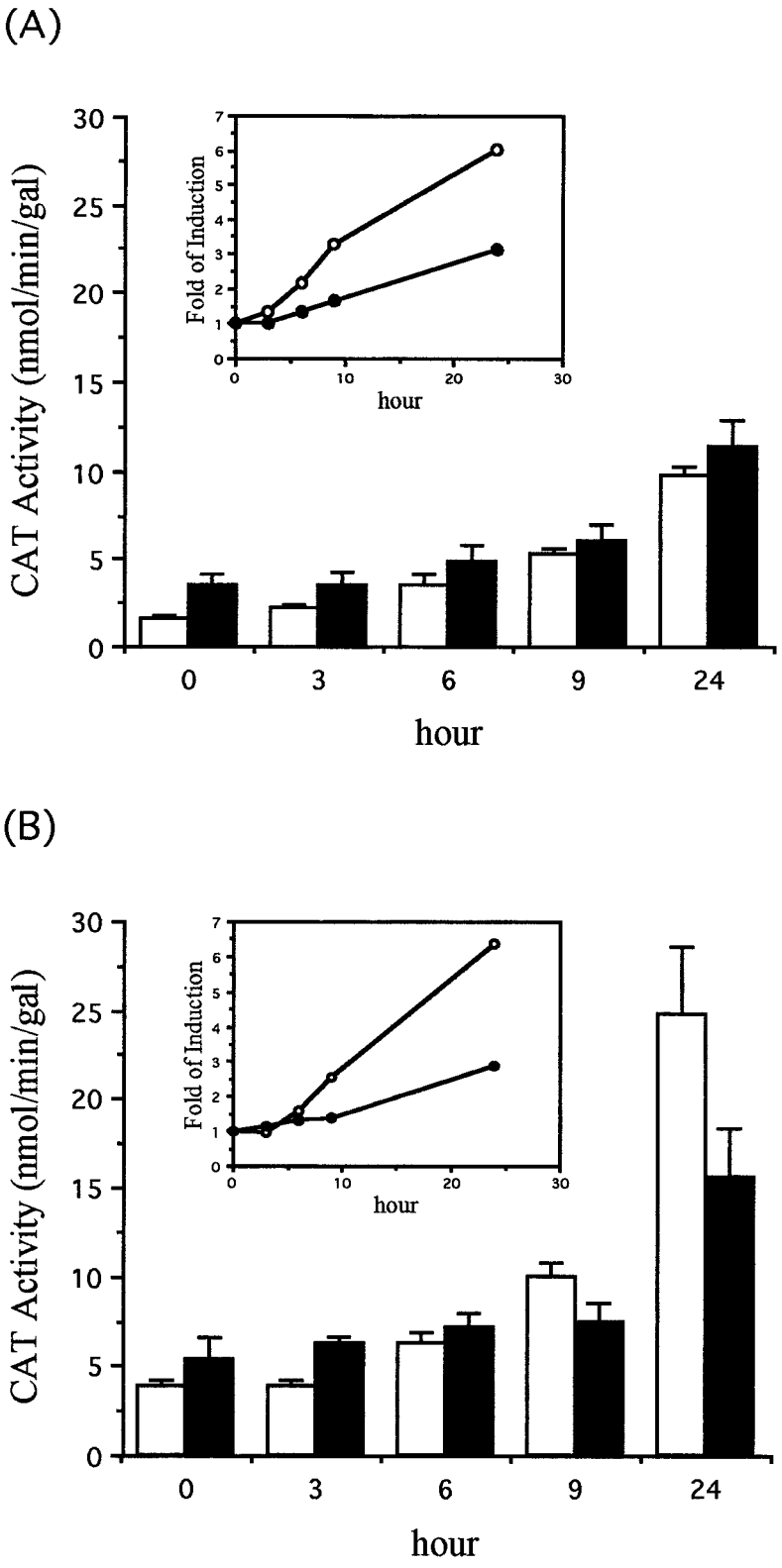

Fig. 5. Time course studies of MTI and MTII promoter activities in CHO K1 and Cd^R cells after metal induction. Chinese hamster MTI (pMTI-558 **(A)** or MTII (pMTII-253 **(B)** was transfected into CHO K1 and Cd^R cells. $ZnSO_4$ (100 μM) was added to the culture medium at various time intervals before harvest. The cells were harvested at the same time and cell extract with the same β-gal activity was analyzed for CAT activity. The activities were determined by scintillation counting (see Materials and Methods). Each value represents a mean ±SD of five samples. Open and solid symbols (bars and circles) indicate the results for CHO K1 and Cd^R cells, respectively. The fold of induction in CAT activity of each cell type was estimated by dividing the activity of pMTI-558 and pMTII-253 at various time intervals by that of 0 h.

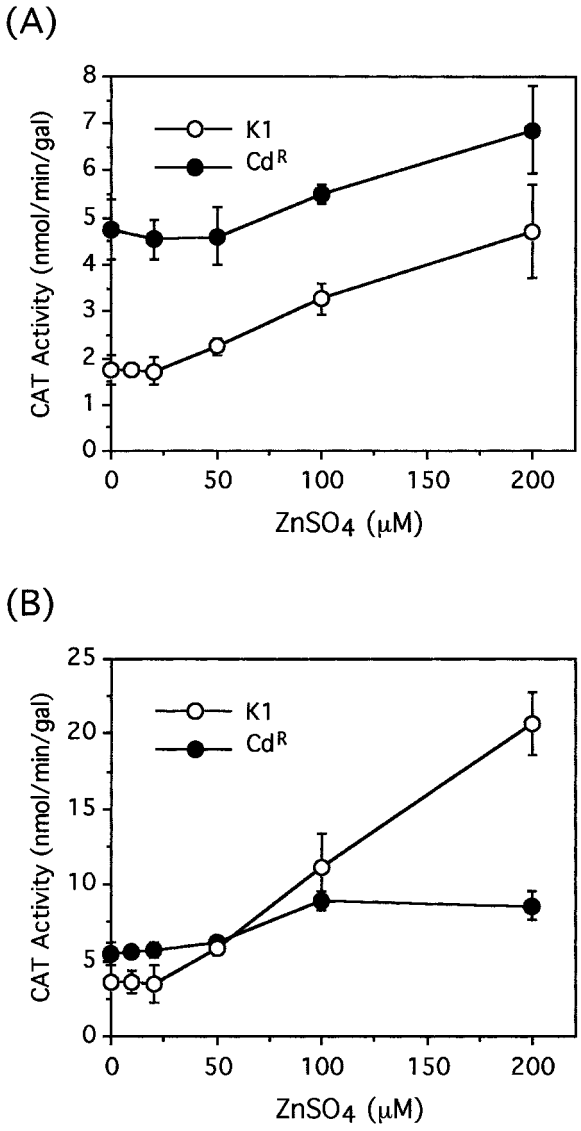

Fig. 6. Expression of MT promoter activities in CHO K1 and Cd^R cells after various amounts of metal treatment. Chinese hamster MTI (pMTI-558) **(A)** and MTII (pMTII-352) **(B)** promoters were transfected into CHO K1 and Cd^R cells. At 10 h before harvest, the cells were treated with various concentrations of $ZnSO_4$. Cell extract with the same β-gal activity was used to determined the CAT activity. The activities were analyzed by scintillation counting (see Materials and Methods). Each value represents a mean ±SD of five samples.

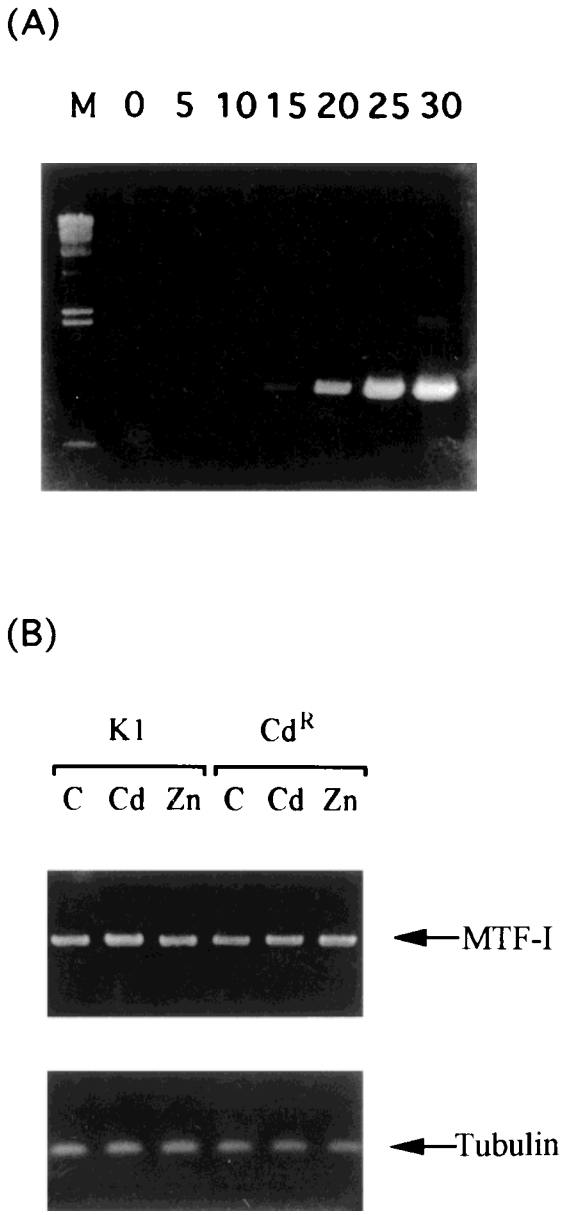

Fig. 7. Evaluation of relative MTF-1 mRNA level in CHO K1 and Cd^R cells by RT-PCR. CHO K1 and Cd^R cells were treated with 5 μM of $CdCl_2$ or 100 μM of $ZnSO_4$ for 10 h. Cytoplasmic RNA was extracted to perform RT-PCR (see Materials and Methods). The reaction product (1 μl) was used as template to conduct the second PCR. **A:** The second PCR was conducted for various cycles and the result was analyzed by agarose gel electrophoresis. **B:** The relative MTF-1 mRNA level after various treatments was compared using the 20-cycle products of the second PCR. The amount of tubulin mRNA was also analyzed by RT-PCR and used as an internal control. C, control.

tion with metal ions (Fig. 5). This result can be explained as a competition of factors required for MT transcription. For CHO K1 cells, the transfected MT promoter more easily accesses MTF-1 since only one copy of endogenous MT gene is present in the cells. Furthermore, MT is not synthesized in CHO K1 cells, which subsequently leads to less efficiency in competing Zn ions from MTF-1 or its inhibitor to terminate the transcription as proposed by Palmiter [1994]. Therefore, the transfected MT promoter can quickly respond to stimulation by metal ions and exert its activity effectively. However, a completely different situation may occur for

Cd^R cells. Although the MT gene is highly amplified in the Cd^R cells, MTF-1 gene expression is essentially the same in both CHO K1 and Cd^R cells (Fig. 7). Upon induction by metal ions, the transfected MT promoter in Cd^R cells should compete with 60 to 100 copies of endogenous MT promoters for MTF-1. In addition, MT is synthesized in Cd^R cells and is possibly able to remove Zn ions from MTF-1 or its inhibitor to inactivate the activity of MTF-1 [Palmiter, 1994]. The combined effects may explain the poor inducibilities of the MT promoters in Cd^R cells.

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