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Functional Integrity of Metallothionein Genes in Testicular Cell Lines

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Abstract The presence and inducibility of the major cadmium (Cd) chelating protein metallothionein (MT) in testicular cells has been controversial. In this study, the induction and production of MT in testicular cells were studied using mouse Leydig and Sertoli cell lines. Metal accumulation was studied by subjecting the cells to increasing levels of Cd. The presence of transcription factors for MT synthesis was analyzed by transfecting the cells with a reporter gene under the control of the MT promoter. The dose- and time-dependent induction of MT were conducted by Northern analyses. Expression of MT genes occurred in both Leydig and Sertoli cells. To avoid cross hybridization of the MT probe with mRNAs encoding testicular metal binding proteins and to investigate the integrity of MT mRNA, isoMT mRNA identification and primer extension experiments were performed. Those studies show that the induced mRNA indeed encodes MT. The biosynthesis of MT was confirmed by following ^{35}S-cysteine incorporation into the protein. Finally, cadmium tolerance of testicular cells is compared with that of fibroblast cells. By these studies, we conclude that the MT genes are functional and inducible in testicular cells.

Key words: Metallothionein, gene expression, Leydig cell, Sertoli cell

Among the tissues studied, testis is one of the most sensitive target sites for cadmium (Cd) toxicity. At doses at which no obvious effects can be observed in other tissues, testes progressively show severe hemorrhage and necrosis within 48 hr [Parizek and Zahor, 1956]. However, only a very small fraction of the metal accumulates in the testes compared with other tissues [Singh et al., 1974; Shaikh and Tewari, 1990]. Besides tissue damage, Cd has also been reported as a potent carcinogen in certain cells of rodent testes [Gunn et al., 1963; Waalkes et al., 1988, 1989].

For most cells, high levels of metallothioneins (MTs) are synthesized during Cd challenge [Karin, 1985; Hamer, 1986]. MTs are low molecular weight, metal binding proteins with high cysteine content. They can be induced by metals, hormones, interferon, interleukin, and lipopolysaccharides [Hamer, 1986; Cousin, 1985]. Although their main function is probably directly linked to heavy metal detoxification, they may also be involved in the homeostatic control of certain essential trace elements (e.g., zinc and copper). In addition, they possibly participate in embryonic development, free radical scavenging, cell differentiation, and proliferation [Karin, 1985]. Recently, there is more cumulative evidence showing that MTs may play a role in carcinogenesis and multiple drug resistance [Kelly et al., 1988; Waalkes and Ward, 1989; Muller et al., 1991]. It is also reported that one of the MT isoforms may be involved in preventing neuronal sprouting and development of neurofibrillary tangles [Uchida et al., 1991; Palmiter et al., 1992].

The presence or inducibility of MTs in testes by Cd is still controversial. High basal levels of either MT-like protein or mRNA have been observed in rodent testes [Onosaka and Cherian, 1982; Durnam and Palmiter, 1981], but the level did not increase after Cd induction [Shaikh and Tewari, 1990; Onosaka and Cherian, 1982; Abel et al., 1991]. Using immunohistochemical techniques, Danielson et al. [1982] identified the presence of MT in Leydig and Sertoli cells but

Abbreviations: CAT, chloramphenol acetyl transferase; EDTA, ethylenediamine tetraacetic acid; MT, metallothionein; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; SDS, sodium dodecyl sulfate.

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not in developing spermatogonial cells. However, other reports have indicated that MT is absent in Leydig cells but present in germ cells [Nishimura et al., 1990; De et al., 1991].

Although MT-like proteins have been identified in the testes [Chen et al., 1974; Webb, 1975; Brady and Webb, 1981] based on physical characteristics (e.g., molecular mass, charge, metal binding), successful attempts to purify MTs from testes have not been reported. Instead, Cd binding proteins, which are totally different from MT, were found in testes of several animal species [Waalkes et al., 1984a,b; Deagen and Whanger, 1985; Waalkes and Perantoni, 1986, 1988]. The inability to induce MT synthesis after metal challenge has thus been suggested as an explanation for the susceptibility of testes to Cd.

In order to clarify whether MT can be faithfully synthesized in testicular cells, we investigated the expression of MT in mouse Leydig and Sertoli cells at transcriptional and translational levels. We found that MT is inducible in two testicular cell lines.

MATERIALS AND METHODS

Cell Culture

Mouse Leydig (TM3), Sertoli (TM4), and embryo fibroblast (STO) cell lines were obtained from ATCC (Rockville, MD). TM3 and TM4 cells were cultured in Ham's F-12/Dulbecco's modified Eagle's medium (DMEM, 1:1) supplemented with 5% horse serum and 2.5% fetal bovine serum. STO cells were cultured in DMEM medium supplemented with 10% fetal bovine serum. All media contained penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were grown in 5% CO_2, 95% air at 37°C, and 80% confluent cell culture (in 100 mm petri dish) was used for the experiments. Stock solutions of $CdCl_2$, $ZnSO_4$, and $CuSO_4$ were prepared in deionized water, and dexamethasone was dissolved in ethanol, filter sterilized, and added to the medium at the indicated final concentrations.

Measurement of Cd Content

Cellular metal content was quantitated by the method of Gagne et al. [1990]. Briefly, cells were treated with various concentrations of Cd for 12 hr before washing twice with PBS (phosphate-buffered saline) and removed using a rubber policeman. The cells were resuspended in PBS to the final volume of 10 ml, and 0.5 ml of the suspension was removed for cell counting. The remaining cells were collected by centrifugation at 2,000 rpm for 5 min, and resuspended in 2 ml of 10 mM of Tris-acetate (pH 7.4), 50 mM of sucrose, 1 mM of β-mercaptoethanol, 0.002% of NaN_3, and 8 N HNO_3. After incubating at 60°C for 24 hr, the samples were diluted at least fourfold with deionized water and the metal contents determined with a graphite atomic absorption spectrophotometer (Varian).

Transcriptional Factor Assay

The 5′ upstream flanking sequence of Chinese hamster metallothionein-II gene containing the promoter region [Hung et al., 1991] was inserted in front of the chloramphenicol acetyl transferase (CAT) gene of pCAT®-Basic vector (Promega). The resulting plasmid (pMTCAT) was cotransfected with pSV-β-galactosidase control vector (Promega) into TM3 and TM4 cells by the calcium phosphate method as described by Sambrook et al. [1989]. Rous sarcoma virus long terminal repeat fused to CAT (RSVCAT) plasmid [Gorman et al., 1982] was also used as a positive control. Forty hours after transfection, 10 μM of Cd was added and the cells were further incubated for an additional 10 hr. The cells were harvested, lysed by three successive freezing–thawings in liquid nitrogen and at 37°C, and the supernatant collected after centrifugation. Galactosidase activities were measured using o-nitrophenyl-β-D-galactopyranside as substrate, and an appropriate amount of cytosols with the same enzyme activity was used to conduct the CAT assay [Sambrook et al., 1989].

Extraction of Cytoplasmic RNA and Northern Blot Analysis

Cells were washed twice with chilled PBS, and 1 ml of the same buffer was added to the petri dish. Cells were then removed using a rubber policeman and collected into 1.5 ml microcentrifuge tubes. Following centrifugation at 2,000 rpm for 5 min at 4°C, cell pellets were resuspended in 0.5 ml of NTE (100 mM NaCl, 10 mM Tris-Cl, pH 7.5, 1 mM EDTA). After adding 25 μl of Nonidet P-40 and standing on ice for 10 min, the samples were spun at 12,000 rpm for 5 min in an Eppendorf microcentrifuge. Supernatants were then transferred to new tubes and 50 μl of 10% SDS was added. The supernatants were successively extracted with 0.5 ml of water-saturated phenol and a phenol/chloroform/isoamyl alcohol (25:24:1) solution. RNA in the

supernatant was precipitated with 1 ml of ethanol at −70°C for 1 hr. The RNA was pelleted by centrifugation at 12,000 rpm for 10 min, and resuspended in diethylpyro-carbonate-treated water. An equal amount of RNA from each treatment was separated by electrophoresis on a 1.2% formaldehyde agarose gel and transferred onto a nitrocellulose membrane. Hybridization was conducted as described by Ausubel et al. [1989] using Chinese hamster metallothionein-II cDNA [Griffith et al., 1983] as probe. Human α-tubulin mRNA was used as an internal standard for normalization of the amount of RNA applied.

Isoform Detection and Primer Extension

Oligonucleotides (27 mer) for detecting MT isoforms were synthesized as described by De et al. [1991]. These sequences are complementary to the 3′ untranslated region of MT-I or MT-II [Glanville et al., 1981; Searle et al., 1984]. These oligonucleotides were labeled using γ-^{32}P-ATP and T4 polynucleotide kinase and served as probes for Northern analysis. Another oligonucleotide (5′-CAGGAGCAGTTGGGGTCCAT-3′), complementary to the coding region of the first seven amino acids of both mouse MT-I and MT-II [Glanville et al., 1981; Searle et al., 1984] genes, was synthesized for primer extension experiments. The primer was ^{32}P end labeled and 50,000 cpm of the primer was mixed with 50 μg of total RNA. The primer extension experiment was carried out as described by Ausubel et al. [1989].

Detection of MT

Leydig and Sertoli cell lines were cultured in the medium described above. Before conducting the experiments, the medium was replaced with a cysteine-deficient RPMI 1640 medium (GIBCO BRL) with various MT inducers. Two hours later, 5 μl of ^{35}S-cysteine (New England Nuclear, 1197.6 Ci/mmole) was added for a final radioactivity of 5 μCi/ml. Cells were harvested and collected into microcentrifuge tubes 12 hr later and resuspended in 100 μl of Tris-Cl (pH 7.4). The cells were lysed by the freezing–thawing method and the cytosolic fractions were obtained after centrifugation at 12,000 rpm for 5 min. Protein concentration in each sample was determined by the dye binding method of Bradford [1976]. An equal amount of protein from each sample was boiled for 2 min, and the soluble fraction was carboxymethylated according to Koizumi et al. [1982]. Proteins were separated by electrophoreses in a 10–18% linear gradient SDS polyacrylamide gel. The MT bands were identified after autoradiography.

Cell Survival Assay

Cell survival assay was carried out according to the procedure of Alley et al. [1988]. Briefly, 1×10^4 (STO) or 5×10^3 (Leydig and Sertoli) cells were seeded on a 24-well plate. Sixteen hours later, fresh medium with various concentrations of Cd was added. After incubation for 5 hr, the cells were washed three times with PBS and 1 ml of fresh medium was added. Two days later, 0.4 ml of fresh medium containing 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added to each well. Three hours later, the medium was replaced with 0.5 ml of dimethylsulfoxide. The final reaction product was then transferred to a 96-well plate and quantitated at 540 nm with a Microplate Autoreader (Bio-tek Instruments).

RESULTS

It has been speculated that Cd does not accumulate in testicular cells due to a defect of the transport system, and thus no MT is induced. To test this hypothesis, various concentrations of Cd were added to the medium and the amounts of cellular Cd were determined. As shown in Figure 1, Cd accumulated in Leydig and Sertoli cells in a dose-dependent manner. This indicates that metal transport is not blocked in these two testicular cell lines. After confirming that Cd can accumulate in these cells, the ability of Cd to stimulate the transcription of MT genes was examined. We first investigated the possibility of defective MT transcriptional factor(s) in testicular cells such that MT could not be expressed or induced. A plasmid containing CHO MTII promoter chloramphenicol acetyl transferase (CAT) gene was transfected into the cells, and the expressions of CAT after Cd challenge were measured. The results in Figure 2 show that CAT activities can be demonstrated in both cell lines transfected with pMTCAT but not with pCAT. Furthermore, CAT activities were 2.5- and 8.5-fold higher in Cd-treated TM3 and TM4 cells, respectively. The results indicate clearly that the *trans*-acting factor(s) for the MT promoter is present in the testicular cells and is functionally active. Next, we studied the expression of MT genes in the presence or absence of Cd. Figure 3 shows the steady state level of MT

mRNA in the two cell lines with and without Cd induction. There is a basal level of MT mRNA and the level increases in both Leydig and Sertoli cells upon Cd concentration elevation. The induction was blocked by Actinomycin D (data not shown), suggesting that Cd indeed stimulates the transcription of MT genes.

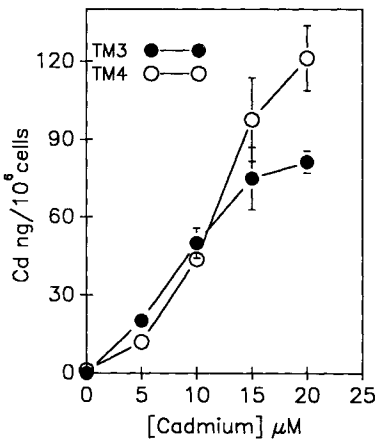

Fig. 1. Accumulation of Cd in testicular cells. Leydig (TM3) and Sertoli (TM4) cell lines were treated with various levels of Cd for 12 hr and cellular concentrations of Cd were determined by atomic absorption spectrophotometry. Each value represents a mean ± SD of three samples.

It is speculated that delay of MT mRNA expression in testicular cells may not sequester Cd in time, allowing it to cause damage to the tissue. Therefore, the kinetics of MT mRNA induction were investigated. Figure 4 shows MT gene expression at various time points after Cd administration. For both Leydig and Sertoli cells, MT genes respond to Cd stimulation within a very short time. These results are consistent with the findings in other cell types in which the responses were seen within 1–2 hr after metal exposure [Durnam and Palmiter, 1981].

Since MT is quite similar in physical and chemical characteristics to testicular metal binding proteins [Bhave et al., 1988], it is conceivable that the results obtained from Northern analyses are due to cross hybridization of MT cDNA with mRNAs of testicular metal binding proteins. The specificity of induction and the nature of the induced mRNA were studied to rule out this possibility. First, cells were exposed to various concentrations of zinc, copper, or dexamethasone, which are known to be able to induce MT. The results in Figure 5 show that MT accumulated in Leydig cells in a dose-dependent manner for all the inducers tested. The induction of MT was quite limited in the

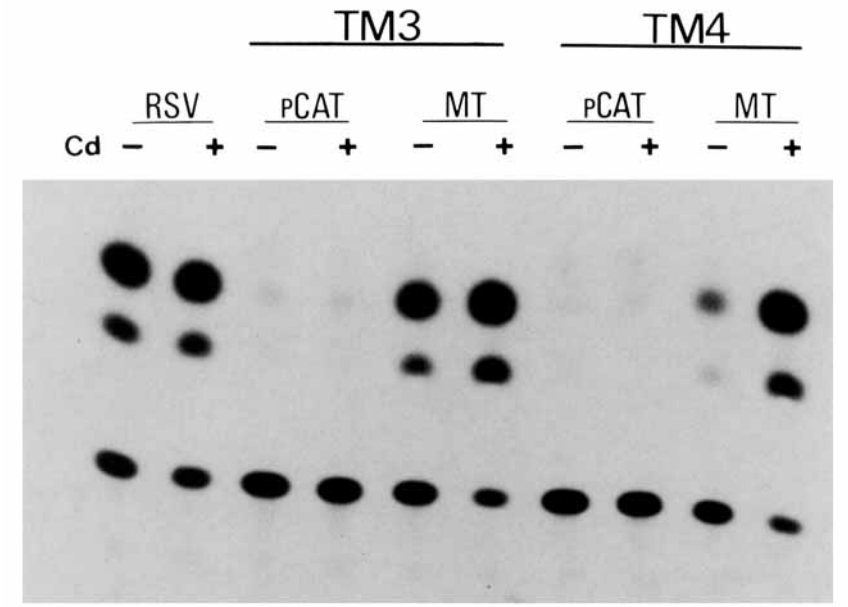

Fig. 2. Transcriptional factors analyses. Leydig (TM3) and Sertoli (TM4) cells were cotransfected with 5 μg of indicated plasmid and 5 μg of pSV-β-galactosidase vector. Ten hours before cell harvest, 10 μM of Cd was administered. Cytosols with equal β-galactosidase activity were reacted with ^{14}C-chloramphenicol and acetyl Co-A at 37°C for 1 hr. The products were analyzed by thin layer chromatography. Percent conversion was calculated by cutting out the acetylated and nonacetylated forms of ^{14}C-chloramphenicol and quantifying them in a scintillation counter. The percent conversion from left to right is 67, 75, 1, 1, 36, 89, 1, 1, 11, and 94, respectively. pCAT, plasmid without MT promoter; MT, plasmid with MT promoter inserted (pMTCAT); RSV, RSVCAT transfected into TM3 cells as a positive control. –, no Cd treated; +, 10 μM of Cd treated.

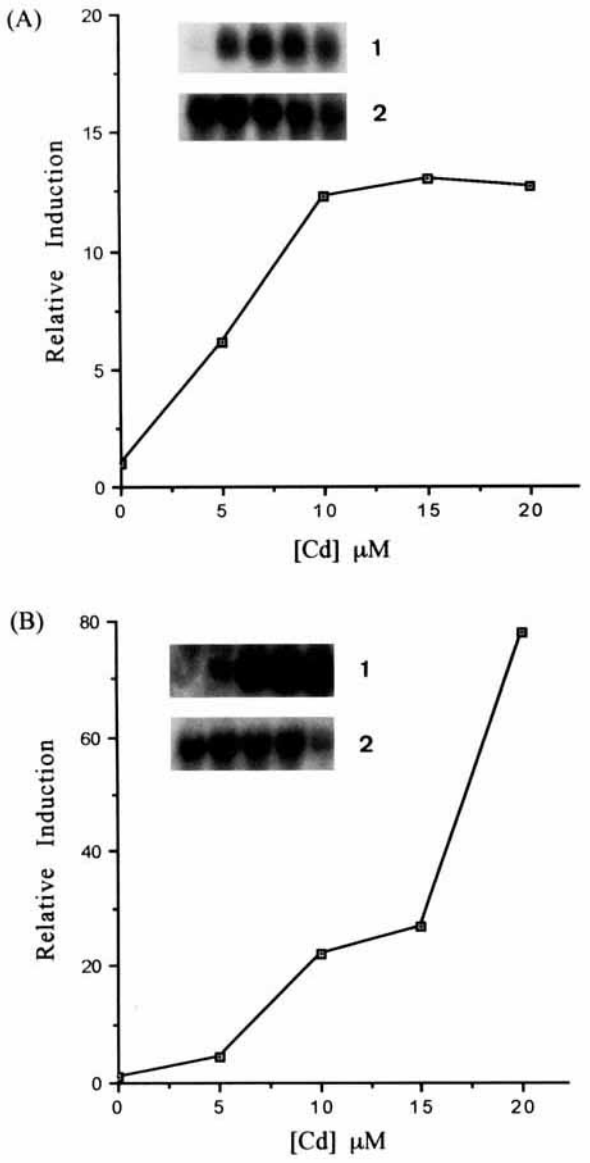

Fig. 3. Induction of MT mRNA after Cd administration. Leydig (A) and Sertoli (B) cells were treated with various concentrations of Cd for 12 hr and cytosolic RNAs were extracted for Northern analyses. The relative induction of MT mRNA was determined by densitometric scanning of the autoradiogram using α-tubulin as the internal standard. 1, MT mRNA; 2, tubulin mRNA.

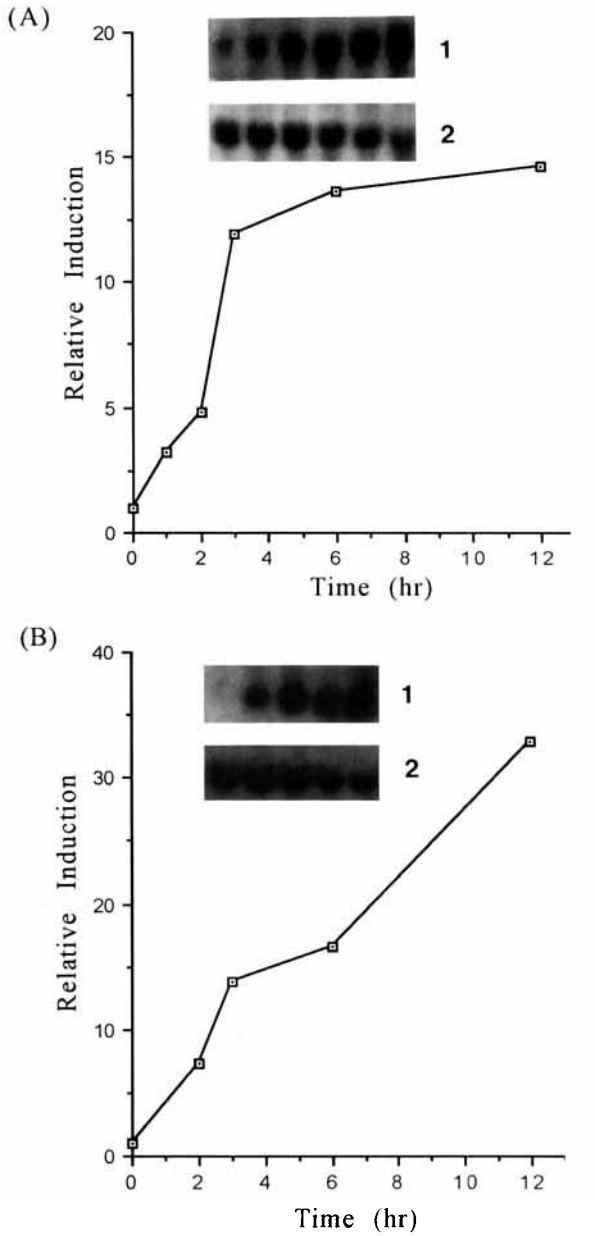

Fig. 4. Time course studies of MT mRNA expression in testicular cells. Leydig (A) and Sertoli (B) cells were treated with 20 μM of Cd and harvested at various time points. Cytosolic RNAs were extracted and analyzed by Northern hybridization. The relative induction of MT mRNA was determined by densitometric scanning of the autoradiogram using mouse α-tubulin as the internal control. 1, MT mRNA; 2, tubulin mRNA.

copper- and dexamethasone-treated Sertoli cells. Isoform-specific oligonucleotides were used to investigate the identity of induced mRNA. The sequences of the oligonucleotides were complementary to and specific for the 3′ untranslated region of mouse MT-I and MT-II genes, respectively [De et al., 1991]. Figure 6 shows that both MT-I and MT-II mRNAs are synthesized in Leydig and Sertoli cells after administration of Cd and Zn.

Since MT mRNA is inducible in these cells, we determined whether the newly synthesized RNAs are transcribed correctly by primer extension experiments. Due to the fact that the sequence of the first 20 nucleotides (see Materials and Methods) from the translation initiation site (ATG) for both mouse MT-I and MT-II genes is exactly the same [Glanville et al., 1981;

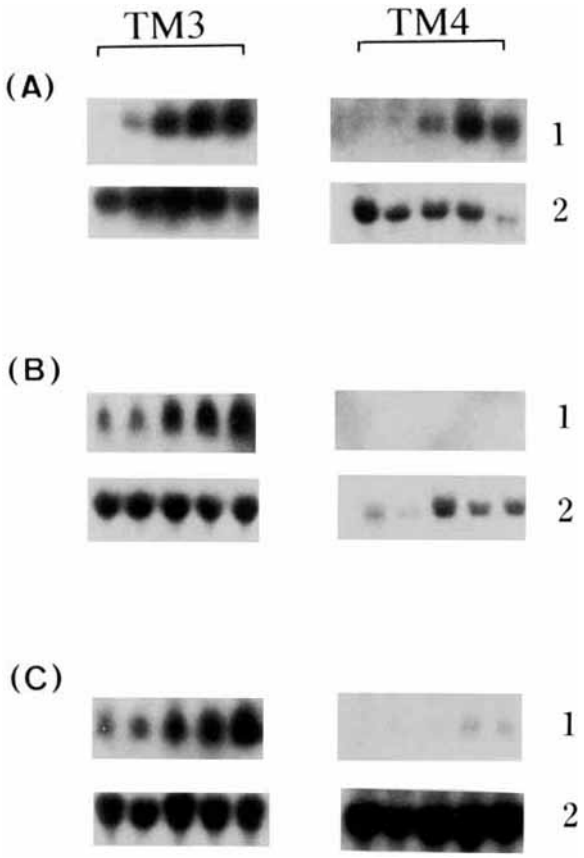

Fig. 5. Induction of MT mRNA by various inducers. Leydig (TM3) and Sertoli (TM4) cells were treated with various concentrations of zinc (A), copper (B), or dexamethasone (C) for 12 hr and cytosolic RNAs were extracted for Northern analyses. The dose (from left to right) used for induction was (A) 0, 20, 50, 80, 100 μM for TM3 and 0, 10, 20, 50, 70 μM for TM4; (B) 0, 20, 50, 80, 100 μM for both cells; (C) 0, 0.5, 1, 5, 10 μM for both cells. 1, MT mRNA; 2, tubulin mRNA.

Searle et al., 1984], an oligonucleotide complementary to this sequence was synthesized and used as a primer for analyzing RNAs isolated from metal-treated Leydig and Sertoli cells. The distance between the transcription and translation initiation sites of mouse MTI gene is six bases longer than that of MT-II [Glanville et al., 1981; Searle et al., 1984]. It is possible to identify each isoform on a sequencing gel. Figure 7 shows that both MT-I and MT-II mRNA of testicular cells were transcribed from the correct positions of their respective genes. The ladder shown in the metal-induced Leydig cells may be resulted from the incomplete extension.

After examining MT expression at the transcriptional level, we investigated the translation of MT mRNA into proteins. As a cysteine-rich protein, a high proportion of cysteine will be incorporated into MT during MT synthesis. Therefore, cells were cultured in a cysteine-deficient medium, and ^{35}S-cysteine was fed to allow incorporation of the isotope into MT after metal induction. The cells were then harvested and the cytosolic proteins were separated by electrophoreses. Mouse MTs are heat-stable proteins that can be identified in SDS-polyacrylamide gel only after carboxymethylation [Koizumi et al., 1982]. Taking advantage of these characteristics, the cytosolic proteins were heat treated, followed by chemical modification, and electrophoresed in 10–18% linear gradient gels. Figure 8 shows the autoradiograms of the gels. Intense bands were observed at low molecular weight regions of the induced samples. These

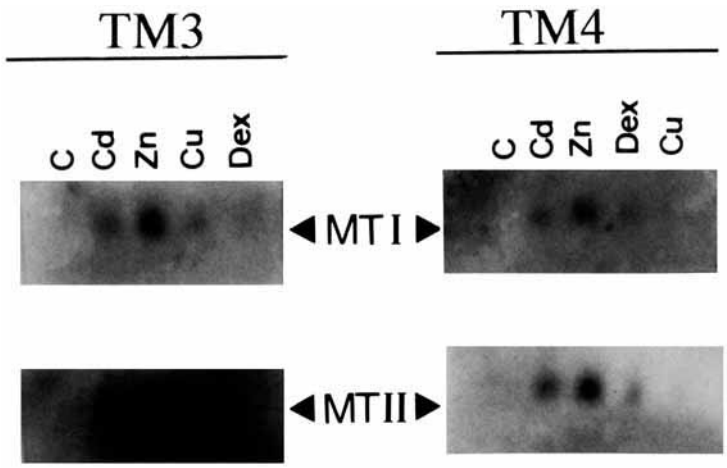

Fig. 6. Identification of MT mRNA isoforms in testicular cells after induction. Leydig (TM3) and Sertoli (TM4) cells were treated with Cd, Zn, Cu, or dexamethasone for 12 hr and cytosolic RNAs were extracted for Northern analyses. The nitrocellulose membrane was initially hybridized with MTI-specific probe. After autoradiography, it was washed and rehybridized with MTII-specific probe.

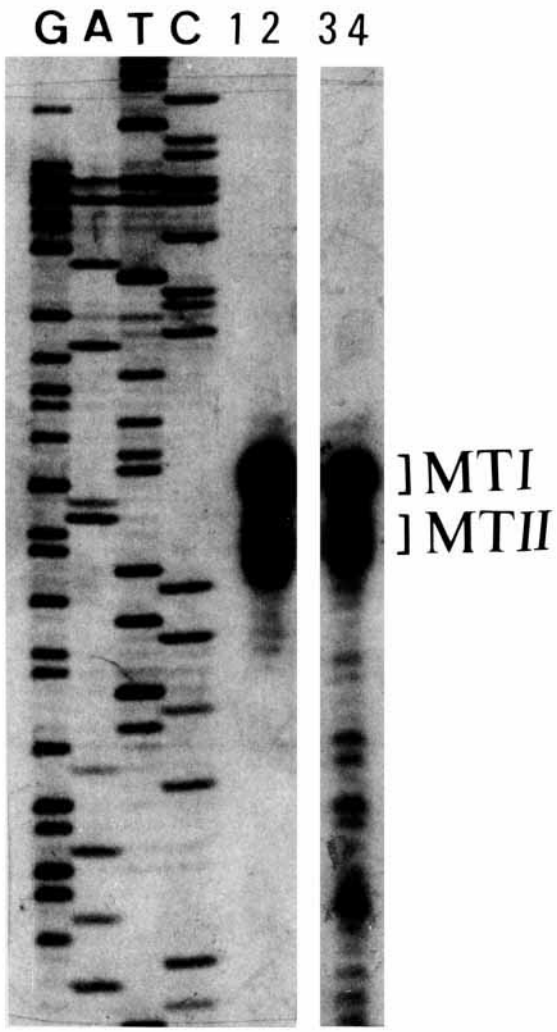

Fig. 7. Primer extension analyses of RNAs isolated from testicular cells. RNAs isolated from Leydig (TM3) and Sertoli (TM4) cells with or without metal treatment were analyzed by primer extension. **Lanes 1 and 2,** Sertoli cells without and with Cd treatment; **Lanes 3 and 4,** Leydig cell without and with Zn treatment.

bands migrated to a position similar to that of purified duck MT [Lin et al., 1990] and they cannot be visualized in samples without carboxymethylation (data not shown). The intensity of the MT bands increased upon metal administration. The results demonstrate that the translational systems are effective and MT proteins are synthesized in testicular cells.

All these studies indicate that MT is inducible in Leydig and Sertoli cells at both transcriptional and translational levels. We then address the question of whether these cells are more susceptible to Cd toxicity than other mouse cells. Thus, a mouse embryonic fibroblast (STO) cell line was used for comparison with testicular cells for metal tolerance. After exposure to various concentrations of Cd for 5 hr, the fraction of surviving cells was estimated. As shown in Figure 9, no obvious difference in metal tolerance among the cell types can be detected. This suggests that even at the cellular level, Leydig and

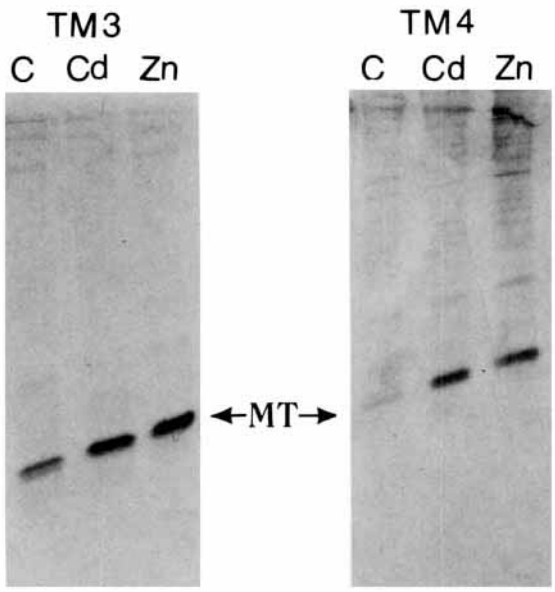

Fig. 8. Identification of MT protein by polyacrylamide gel electrophoresis. Leydig (TM3) and Sertoli (TM4) cells were cultured in media supplemented with ^{35}S-cysteine. After induction for 12 hr, the cells were harvested and cytosolic proteins were heat treated, carboxymethylated, and electrophoresed. The positions of MT bands were indicated. C, cells without induction; Cd, cells treated with 20 μM of Cd; Zn, cells treated with 70 μM of Zn.

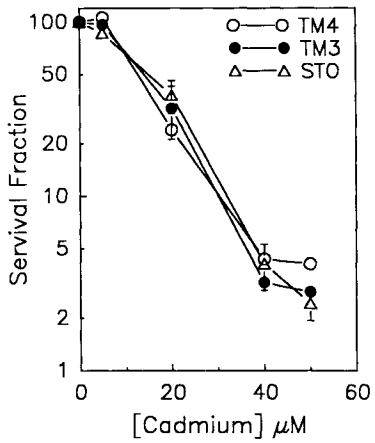

Fig. 9. Comparison of Cd susceptibility among cells. Mouse Leydig (TM3), Sertoli (TM4), and embryonic fibroblast (STO) cells were treated with various concentrations of Cd for 5 hr and survival fractions were determined by MTT assay. Each value represents a mean ± SD of three samples.

Sertoli cells respond to Cd challenge in a manner similar to fibroblast cells.

DISCUSSION

In this report, we present a detailed study on the induction of metallothionein in both Leydig and Sertoli cells. This study was initiated with the investigation of metal accumulation in testicular cells (Fig. 1). Once metal accumulation was demonstrated in the cells, the transcriptional system for metallothionein expression was examined. Data from transcriptional factor(s) and Northern analyses indicate that the machinery is active and MT mRNA can be synthesized in both Leydig and Sertoli cells by dose- and time-dependent manners after Cd induction (Figs. 2–4). To further confirm that the induced mRNA belongs to the MT family rather than to testicular metal binding proteins and to ensure MT is transcribed correctly, isoform-specific probe hybridization (Fig. 6) and primer extension analyses (Fig. 7) were performed. The results show clearly that MT mRNA was indeed synthesized without defect in the cells after induction. Following transcription, the translational mechanism was also examined. As shown in Figure 8, MT mRNA can be translated into proteins. Finally, the Cd susceptibilities of testicular cells were compared with cells from other tissues and no obvious differences were found among them. The results of this series of studies confirm that MT is inducible and expressed in Leydig and Sertoli cells.

By the absence or presence of testicular necrosis following exposure to Cd, strains of mice can be classified into "susceptible" and "resistant" to Cd toxicity. It may be argued that the cell lines used in this study are derived from a mouse strain (BALB/c) which is "resistant" to Cd-induced testicular toxicity [Gunn et al., 1964]. It has been proposed that the resistance to Cd toxicity is mainly due to hypomethylation of MT genes [Bhave et al., 1988]. The lower level of gene methylation may result in a more effective expression of MT in testicular cells and hence reduced Cd toxicity. Thus, the production of MT in the cell lines used is expected. However, no difference in testicular MT levels can be detected between "resistant" or "susceptible" strains of mice after Cd administration [Abel et al., 1991]. In addition, the basal level of MT expression is similar in both strains and is three- to sevenfold higher than those of liver and kidney. If MT genes in testes were methylated in one of the strains, there should be a difference in expression under either basal or induced situations. Thus, methylation of MT genes is not the major factor in determining the ineffectiveness of MT induction in testes, and the "resistance" to Cd toxicity in testes does not correlate with MT induction in the testicular cells.

The discrepancy between the induction of MT in the whole testicular tissue and isolated cells is difficult to explain. It may be speculated that long-term culture conditions have changed the physiological characteristics of the cells. However, studies by Abel et al. [1991] suggest that freshly isolated Leydig cells show the same properties as established cell lines in the synthesis of MT or MT-like proteins. We expect that the same conclusion is applicable to our studies here. Another explanation may be that the proportion of MT-synthesizing cells in testes is low enough that the amount of MT produced is masked by other cellular compartments. This may be true for Leydig cells that contribute only 3–4% of the total tissue volume [Mori and Christensen, 1980]. With the addition of Sertoli cells, the difference of MT content between Cd-treated and untreated testes should be measurable. Furthermore, high levels of MT mRNA have been found in the male germ cells of adult mice [Chen et al., 1974; De et al., 1991]. It thus becomes obvious that MT cannot be isolated successfully from testes of Cd-exposed animals [Waalkes et al., 1984a,b; Deagen and Whanger, 1985; Waalkes and Perantoni, 1986, 1988].

Parizek [1960] has shown that Cd exposure may cause male sterilization due to interference with Zn metabolism in testes and lead to atrophy of the cells lining the seminiferous tubules. Another study indicates that testicular injury is due to the binding of Cd to testicular capillary endothelium and an increase in its permeability [Chen et al., 1974]. Both may eventually result in necrosis of the tissue. Thus, cell damage may be the reason for the lack of MT induction in testes of Cd-treated animals. However, early changes in tubules and interstitial edema of testes were observed 6 hr after Cd injection [Parizek, 1960], while MT mRNA was synthesized in Leydig and Sertoli cells within 2 hr of metal exposure (Fig. 4). There should be time to observe the increase of MT in the testes. Nevertheless, the MT level did not change in mouse testes 3 hr after Cd injection [Durnam and Palmiter, 1981]. It is thus speculated that distribution of Cd in testes may be the critical factor in determining MT induction.

In summary, we used the established Leydig and Sertoli cell lines to investigate MT expression at transcriptional, translational, and cellular levels. All the results indicate that MT can be expressed in these cells. It is possible that other physiological factors are involved in the inability to measure MT accumulation in testes after Cd exposure.

ACKNOWLEDGMENTS

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