Isolation and identification of metallothionein isoforms (MT-1 and MT-2) in the rat testis

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It has been a long-lasting controversial issue as to whether or not the male genital organs, such as the testis and prostate, contain metallothioneins (MTs), a group of cysteine-rich heavy-metal-binding proteins that play a role in detoxifying heavy metals such as cadmium (Cd). Earlier studies reported that the rodent testis lacks MTs and concluded that this is why the testis is very susceptible to Cd, although other indirect experimental evidence suggests that MTs are present in this organ. A deficiency of MTs in the testis was originally suspected on the basis of amino acid composition analysis, since MT-like proteins isolated as Cd-binding proteins did not have a characteristic MT structure. In the present study, we demonstrate that the rat testis indeed expresses Cd-binding proteins with sequences identical to those previously described for MT-1 and MT-2, the major isoforms. To confirm that MT-1 and MT-2 are present in the rat testis, we purified and isolated Cd-binding proteins by homogenization using Cd-containing buffer, followed by sequential purification using Sephadex G-75 gel filtration chromatography and anion HPLC column chromatography, which yielded Cd-binding protein-1 (Cd-BP-1) and -2 (Cd-BP-2). After pyridylethylation, Cd-BP-1 and Cd-BP-2 were subjected to specific protein fragmentation by acids and endopeptidases, which revealed that these Cd-binding proteins have the same primary structures as MT-1 and MT-2 respectively. Thus we believe that the present results clearly resolve the long-standing debate about the presence of MTs in the testis, at least in the rodent.

INTRODUCTION

Metallothioneins (MTs) are classified as a family of stress proteins that are present ubiquitously in various animal and plant species. They are composed of 61 amino acid residues, one-third of which are cysteine, but there are no disulphide bridges within the molecule. The MTs play a role in detoxifying heavy metals by sequestration using cysteine residues [1]. However, the mammalian scrotal testis is known to be susceptible to cadmium (Cd) exposure, and minimal amounts of this metal, which do not produce any other significant effects, cause haemorrhage in the capillaries of the testis, followed by degeneration and necrosis of seminiferous tubule cells [2,3]. Subsequent studies have shown that Cd has a tumorigenic effect in the testis and prostate of rats injected with Cd solution. Earlier studies reported that the Cd- and zinc (Zn)-binding proteins purified as MTs differed from authentic MTs in their amino acid composition [4,5], and this was thought to explain the toxicity and carcinogenicity caused by Cd administration. On the other hand, immunohistochemical and biochemical studies have revealed the presence of MT protein and mRNA in rat and mouse testes, as well as in other auxiliary genital organs [6–10]. Thus whether or not MTs are present in male genital organs such as the testis and prostate is a long-standing issue of debate [11].

In the present study, we demonstrate that the rat testis contains mRNA sequences identical to those already described for MT-1 and MT-2, and that the testicular MT-like proteins purified as Cd-binding proteins are identical to MT-1 and MT-2.

MATERIALS AND METHODS

Animals

For the analysis of MT genes, male rats (Wistar strain; 7 weeks old) were purchased from Japan Clea (Tokyo, Japan). For preparation of a positive control specimen for MT mRNA by Northern blot analysis, several rats were subjected to 70% partial hepatectomy as described previously [12]. The testes and liver were removed under pentobarbital anaesthesia, quickly frozen and stored at −80 °C until analysis. For the isolation of testicular Cd-binding proteins, rat testis specimens were purchased from Pel-Freez Biologicals (Rogers, AK, U.S.A.) and stored at −80 °C until analysis.

Isolation of total RNA and Northern blot analysis

Total RNA was extracted from the rat tissues (testis and liver) by homogenization in guanidine isothiocyanate (BRL), followed by centrifugation in CsCl (BRL) solution. Extracted total RNA was quantified by absorbance measurements at 260 and 280 nm and stored at −80 °C. Total RNA (25 μg) was denatured with glyoxal and SDS, separated by 1.5% (w/v) agarose gel electrophoresis and analysed by Northern blot hybridization as described previously [12]. Mouse MT-1 cDNA {*Eco*RI/*Pst*I fragment of pT7/T3-MT1 [an *Ava*II fragment (225 bp) of m_1pEH.4 was subcloned to the pT7/T3α-18 vector, which was renamed pT7/T3-MT1]} that can hybridize equally well to both MT-1 and MT-2 mRNAs was used as probe for Northern analysis.

PCR primers

The oligonucleotide primers were synthesized using a DNA synthesizer (Pharmacia). The sequences of the sense and antisense primers for rat MT-1 were 5′-ACTGCCTTCTTGTCGCTTA-3′ and 5′-TGGAGGTGTACGGCAAGACT-3′ respectively. They spanned a 310 bp fragment. The sequences of the sense and antisense primers for rat MT-2 were 5′-CCAACTGCCGCCTCCATTCG-3′ and 5′-GAAAAAAGTGTGGAGAACCG-3′ re-

Abbreviations used: MT, metallothionein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription–PCR; Cd-BP-1(-2), Cd-binding protein-1(-2).

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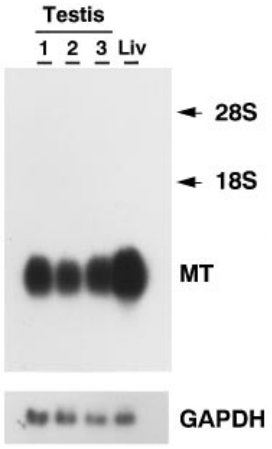

Figure 1 Northern blot analysis of MT mRNAs in the rat testis

Samples of total RNA (25 μg) isolated from rat testes (lanes 1–3) and from regenerating liver ('Liv') were separated on a 1.5% (w/v) agarose gel. Mouse MT-1 cDNA was used as a probe to detect both MT-1 and MT-2 mRNAs.

spectively, spanning a 297 bp fragment. The sequences of sense and antisense primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5′-TTCATTGACCTCAACTACATG-3′ and 5′-GTGGCAGTGATGGCATGGAC-3′ respectively, spanning a 443 bp fragment.

Reverse transcription–PCR (RT-PCR)

First-strand cDNA was synthesized using the procedure described by Suzuki and co-workers [13], and the cDNA products were stored at −20 °C until use.

PCR was carried out in a 25 μl reaction mixture containing 0.2 mM of each dNTP, 1 pmol of each sense and antisense primer and 0.625 unit of *Pfu* DNA polymerase (Stratagene) including 7.5 μl of cDNA products. The reaction was carried out for 30 cycles, each comprising 1 min at 95 °C, 1 min at 50 °C and 1 min at 75 °C. The PCR products were applied to electrophoresis using a 1.5% (w/v) agarose gel, which was stained with ethidium bromide and visualized under UV light. In order to confirm that there was no significant contamination in the total RNA preparation, we synthesized the first-strand DNA and performed control reactions in the absence of reverse transcriptase, and did not find any band on further PCR.

Southern blot analysis

After the PCR products had been separated by 1.5% (w/v) agarose gel electrophoresis, the gel was soaked in 200 mM NaOH/600 mM NaCl for 15 min to allow denaturation, rinsed with water and neutralized with 200 mM Tris/HCl, pH 7.5/600 mM NaCl. Then the separated products were transferred to a nylon membrane (Hybond N^+; Amersham) with 20 × SSC. The filter was probed with ^{32}P-labelled mouse MT-1 cDNA (*Eco*RI/*Pst*I fragment of pT7/T3-MT1).

Cloning of PCR products

PCR products were cloned using the pCR-Script SK(+) cloning kit (Stratagene). The constructed plasmids were transfected into XL1-Blue, positive colonies were selected and the DNA sequence was analysed using a DNA sequencer (Pharmacia).

Purification of testicular Cd-binding proteins

Testis specimens (approx. 50 g) were homogenized in 4 vol. of buffer (100 mM Tris/HCl, pH 7.5, containing 5 mM 2-mercaptoethanol) using a Polytron homogenizer. Unless specified, the procedure was performed at 4 °C. An appropriate volume of Cd solution was added to make the Cd concentration in the homogenate 0.05 mg/ml. The homogenate was centrifuged at 8900 ***g*** for 10 min, followed by heat treatment at 80 °C for 20 min. The resulting aggregated macromolecules were centrifuged at 40000 ***g*** for 20 min, and the supernatant was concentrated by ultrafiltration using an Amicon YM3 membrane.

The concentrated solution was applied to a Sephadex G-75 gel filtration column [50 mm (inner diam.) × 930 mm], and proteins were eluted with 2 mM potassium phosphate buffer, pH 7.5, containing 5 mM 2-mercaptoethanol at a flow rate of 1.0 ml/min with a fraction size of 20.0 ml. Peak fractions containing MT immunoreactivity and Cd were combined and subjected to ultrafiltration. The protein solution was applied to an anion-exchange HPLC column [Asahipak ES-502NP; 21.5 mm (inner diam.) × 100 mm; Asahi-kasei, Kawasaki, Japan], eluted with a linear gradient of 2–25 mM potassium phosphate buffer, pH 7.5, and washed with 250 mM potassium phosphate buffer, pH 7.5, at a flow rate of 5.0 ml/min with a fraction size of 2.5 ml. For protein sequence analysis we collected peak fractions that had the same retention times as rat liver MT-1 or MT-2 that were purified by essentially the same methods and were utilized as MT standards. To minimize oxidation during purification, all buffers were degassed by flushing with nitrogen or argon gas during chromatography steps.

Protein sequence analysis

Separated peak fractions 1 and 2 were lyophilized and subjected to pyridylethylation by essentially the same method as described in [14]. The protein specimen was then subjected to reverse-phase HPLC to isolate pyridylethylated proteins from unreacted substances as well as from possible contaminant proteins at a wavelength of 254 nm. Reverse-phase HPLC was performed using a Cosmosil $5C_{18}$-AR-300 column [10 mm (inner diam.) × 250 mm; Nakalai, Kyoto, Japan] and elution solutions A (0.06% trifluoroacetic acid) and B (100% acetonitrile containing 0.06% trifluoroacetic acid), with a linear gradient of 5% to 40% B over 90 min.

The alkylated proteins were digested in order to obtain peptide fragments by using either endopeptidases or acids. The testicular Cd-binding proteins could be successfully digested only after removing metals from the protein moiety. For performic acid digestion, the protein sample (50 μg) was digested with 70% performic acid at 40 °C for 72 h. For Asp-N digestion, the protein specimen (40 μg) was dissolved in 200 μl of 50 mM Tris/HCl, pH 8.0, containing 0.04 μg of endoproteinase Asp-N (Wako, Osaka, Japan) and incubated at 37 °C for 18 h. For lysine cleavage, the protein sample (50 μg) was dissolved in 200 μl of 100 mM Tris/HCl, pH 9.0, containing 0.25 μg of lysyl endopeptidase (Wako) and incubated at 37 °C for 30 min. For hydrolysis by HCl, the protein specimen (1 μmol) was dissolved in 1.0 ml of conc. HCl and kept at 25 °C for 24 h. The fragments obtained following the above treatments were separated under the same reverse-phase HPLC conditions as described above and analysed using a protein sequencer (model 476A; Perkin–Elmer).

Western blot analysis

SDS/PAGE and Western blot analysis were performed using the methods described previously [15,16]. In brief, proteins (5–10 μg

per lane) were transferred to a PVDF membrane (Immobilon P; Millipore) and probed with affinity-purified sheep anti-(rat MT-2) IgG. Bands were visualized using horseradish-peroxidase-labelled goat anti-(sheep IgG) IgG (Wako), with 4-chloro-1-naphthol (Bio-Rad) and hydrogen peroxide as substrates.

Other analyses

Cd and Zn were determined at each purification step by flame atomic absorption spectroscopy (Model 180–80; Hitachi, Tokyo, Japan). MTs and total proteins were determined by radioimmunoassay [9] and Bio-Rad protein assay respectively.

RESULTS

Northern blot hybridization analysis

As a positive control for the expression of MT mRNA, total RNA was extracted from the remaining liver of a rat at 6 h after partial hepatectomy and subjected to Northern blot analysis. A single band was visible on the gel (Figure 1, upper panel, lane 'Liv'). Total RNA extracted from the testis of each of three rats was found to contain a single band with a mobility corresponding to that of liver MT mRNA (Figure 1, upper panel, lanes 1–3). Northern blot analysis of GAPDH mRNA indicated that equivalent amounts of total RNA were applied to the agarose gel and that RNA integrity was maintained during electrophoresis (Figure 1, lower panel).

Gene expression of rat MT-1 and MT-2

The Northern blot analysis described above (Figure 1) was unable to identify which isoform of MT, i.e. MT-1 or MT-2, was expressed in the testis. We therefore utilized total RNA from the three rats and synthesized cDNA by RT using a random hexamer as primer. We then made primers specific to either rat MT-1 or MT-2, which were subjected to PCR in order to examine the expression of MT-1 and MT-2 mRNAs. Consequently, we were able to detect two separate bands, one covering 310 bp of the MT-1 coding and non-coding regions, and the other covering 297 bp of the MT-2 coding and non-coding regions (Figure 2, middle panels).

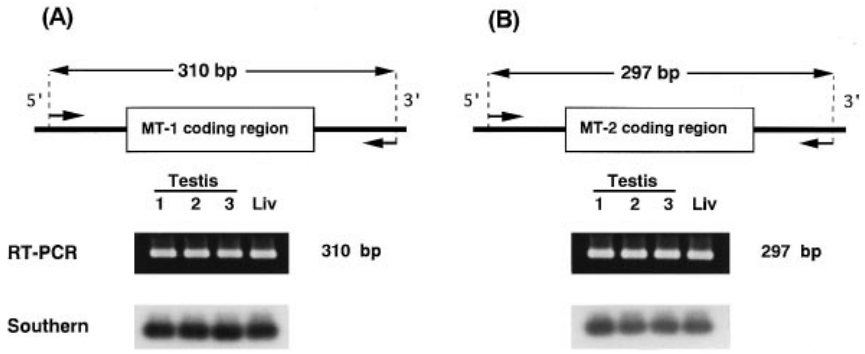

Figure 2 Detection of (A) MT-1 and (B) MT-2 mRNAs in the rat testis by RT-PCR and Southern blot analysis

Primers for PCR are indicated by the arrows. Liv, liver. See the text for details.

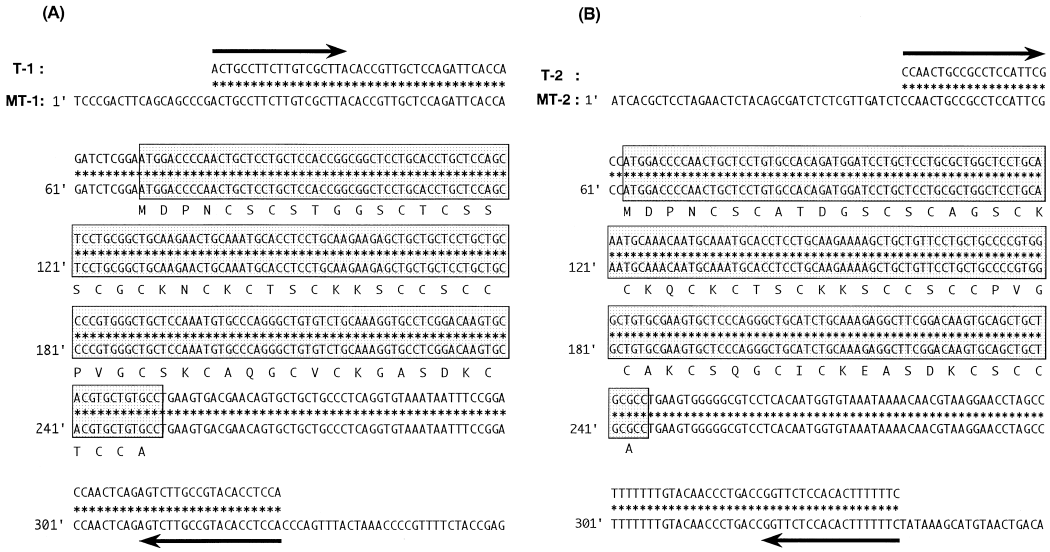

Figure 3 DNA sequences of RT-PCR products from the rat testis and comparison with rat MT-1 and MT-2 cDNAs

Specific primers for rat MT-1 or MT-2 are indicated by the arrows. Asterisks indicate sequence identity with MT-1 (**A**) or MT-2 (**B**) cDNA.

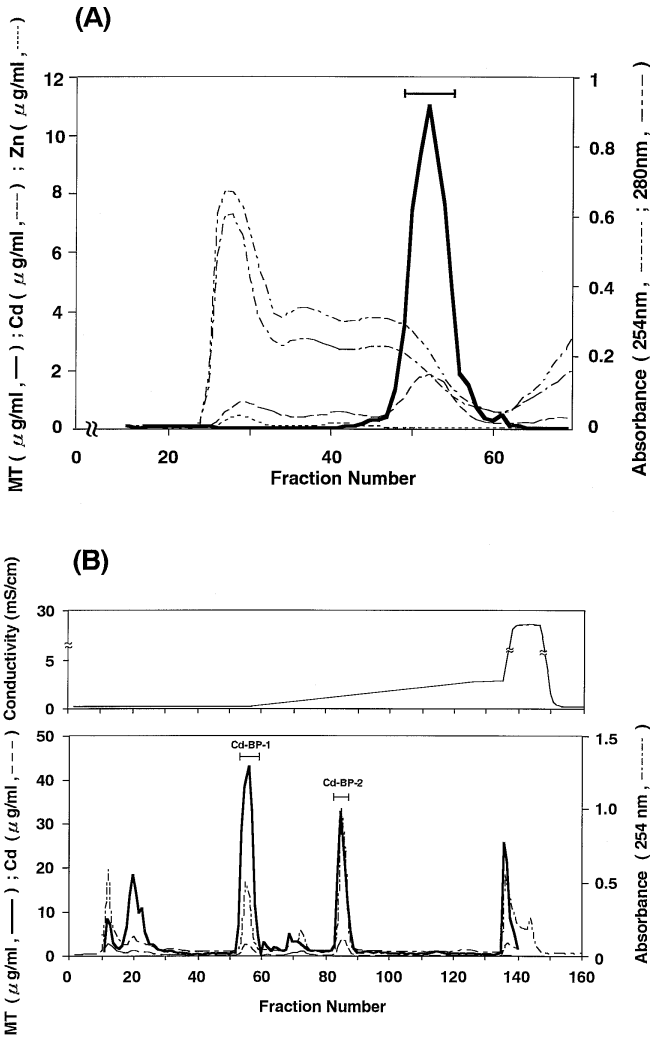

Figure 4 Purification of Cd-binding proteins from rat testis

The amount of MT was determined by radioimmunoassay. Cd and Zn were determined by atomic absorption spectroscopy. Absorbances at 280 nm and 254 nm were also determined. (**A**) Gel filtration column chromatogram. (**B**) Anion-exchange HPLC of Cd-binding proteins from the rat testis.

These products were separated by gel electrophoresis, transferred to a nylon membrane and hybridized with mouse MT-1 cDNA. The presence of a single band each for MT-1 and MT-2 (Figure 2, lower panels) strongly suggests that MT-1 and MT-2 were expressed in the rat testis.

DNA sequences of RT-PCR products

After cloning the RT-PCR products described above into a pCR-Script vector, we examined their DNA sequences. The sequences of the 310 bp and 297 bp bands were found to be identical with the sequences of rat MT-1 and MT-2 cDNAs respectively (Figure 3). These data clearly indicate that the MT-1 and MT-2 genes are constitutively expressed in the rat testis.

Purification of Cd-binding proteins from rat testis

To further confirm at the protein level whether rat testis contains MT-1 and MT-2, we purified the low-molecular-mass Cd-binding proteins and investigated their protein sequences. We first tried to purify MT-like proteins as Zn-binding proteins, but failed to obtain sufficient amounts due to aggregation, probably because of oxidation of the proteins during anion-exchange HPLC. We found that the addition of Cd to the homogenization buffer stabilized the target proteins by replacing Zn with Cd. Gel filtration using Sephadex G-75 yielded a single peak containing both MT immunoreactivity and Cd, but not Zn. The peak fractions (48–56 in Figure 4A) were combined and subjected to further purification by anion-exchange (Asahipak ES-502NP) HPLC. We detected several distinct peaks with MT immunoreactivity; these contained Cd, but no Zn. The two major peaks that showed the same retention times as authentic rat liver MT-1 and MT-2 were designated Cd-binding protein-1 (Cd-BP-1; fractions 52–59 in Figure 4B) and Cd-BP-2 (fractions 84–88 in Figure 4B) respectively, and these were subjected to protein sequence analysis.

A typical example of the purification of Cd-binding proteins from rat testis is shown in Table 1. Cd-BP-1 and Cd-BP-2 from anion-exchange HPLC were purified with yields of 4.3% and 12.0% respectively. The maximum metal-binding capacity of MTs is 7 atoms of Cd or Zn/mol of MT, but the present results showed 4.7 atoms of Cd/mol of Cd-BP-1 and 4.3 atoms/mol of Cd-BP-2. Since no Zn was detected in this preparation, it is speculated that some thiol groups of cysteine residues are free of the metal ions. This unsaturation may explain the polymerization of both Cd-BP-1 and Cd-BP-2 as purification proceeds. SDS-denatured and nascent MTs react with the Coomassie Brilliant Blue used in the Bio-Rad protein assay to an approx. 4 times lesser degree than does BSA used as a standard. This is why the amounts of protein obtained at the anion-exchange HPLC step are less than the MT amounts determined by radioimmunoassay.

SDS/PAGE analysis showed that several protein bands were revealed by Coomassie Brilliant Blue staining after each purification step from the supernatant after centrifugation (8900 ***g***) to the eluate of gel filtration chromatography (Figure 5A, lanes 1–3). Both the Cd-BP-1 and Cd-BP-2 fractions from anion-exchange HPLC showed a single band on Coomassie Brilliant Blue staining (Figure 5A, lanes 4 and 5). Western blot analysis of these specimens using an affinity-purified IgG raised to MT

Table 1 Purification of Cd-binding protein from rat testis

Purification step	Volume (ml)	Protein (mg)	MT (μg)	Cd (μg)	Cd/MT (mol/mol)	Yield (%)
Supernatant (8900 ***g***)	232	6360	5080	–	–	100
Heat-treated supernatant	199	263	4770	–	–	93.9
Gel filtration	187	9.70	1830	328	9.69	36.0
Anion-exchange HPLC						
Peak 1	2.93	0.09	220	19.1	4.68	4.33
Peak 2	2.98	0.16	611	48.3	4.27	12.0

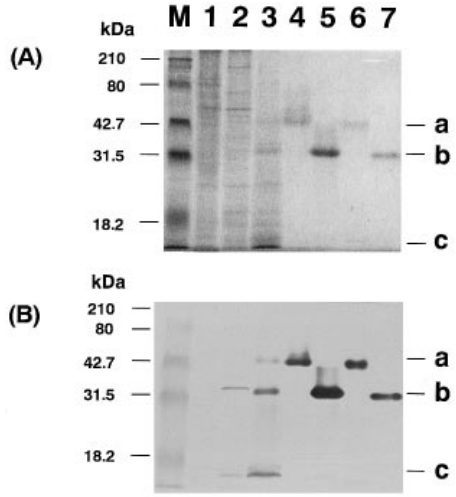

Figure 5 SDS/PAGE and Western blot analysis of Cd-binding proteins from rat testis

(**A**) SDS/PAGE stained with Coomassie Brilliant Blue. (**B**) Western blot analysis. Lanes: M, Kaleidoscope molecular mass markers (Bio-Rad); 1, supernatant after centrifugation at 8900 ***g***; 2, supernatant after heat treatment; 3, sample after gel filtration; 4, Cd-BP-1 from anion-exchange HPLC; 5, Cd-BP-2 from anion-exchange HPLC; 6, rat MT-1; 7, rat MT-2. The following are indicated on the right: a, polymerized form of MT-1; b, polymerized form of MT-2; c, monomeric form of MTs.

revealed bands corresponding to the polymerized forms of MT-1 and MT-2 (Figure 5B, lanes 6 and 7) in the eluates of Cd-BP-1 and Cd-BP-2 respectively. On Western blot analysis, the supernatant after heat treatment showed a monomeric form of MT and a polymerized form of MT-2, whereas the sample from gel filtration chromatography contained the monomeric form and polymerized forms of both MT-1 and MT-2 (Figure 5B, lanes 2 and 3); the staining intensity of Cd-BP-1 was less than that of Cd-BP-2. As the purification proceeds, monomeric MT-1 and MT-2 are thought to be oxidized to give polymerized MT-1 and MT-2; this is partly due to the fact that some thiol groups are unoccupied by metals (see above). It should be pointed out that this polymerization process might have occurred during electrophoresis. Since the addition of reducing reagent (2-mercaptoethanol or dithiothreitol) into the sample buffer of SDS/PAGE causes smearing of the protein bands [17], we did not use such a reagent in the sample buffer.

Pyridylethylated Cd-BP-1 and Cd-BP-2 protein samples, which contained small quantities of contaminant proteins, were successfully purified by reverse-phase HPLC. Peptide fragments were obtained from alkylated Cd-BP-1 and Cd-BP-2 following specific cleavage with formic acid at Asp–Pro, with conc. HCl at Ser and Thr, with endopeptidase Asp-N or with lysyl endopeptidase. The complete amino acid sequences of purified Cd-BP-1 and Cd-BP-2 are shown in Figure 6, based on fragment sequences that essentially cover the entire protein sequence. The Cd-BP-1 and Cd-BP-2 peptide sequences are identical with those of authentic MT-1 and MT-2 respectively [18,19] and with those deduced from the cDNA sequence (Figure 3), with the N-terminal amino acid being methionine.

DISCUSSION

MTs exist not only in tissues from various animal species, but also in bacteria and plants, and are thought to play essential, but as yet unknown, roles in cellular processes. MTs are known to detoxify heavy metals; however, the male genital organs, par-

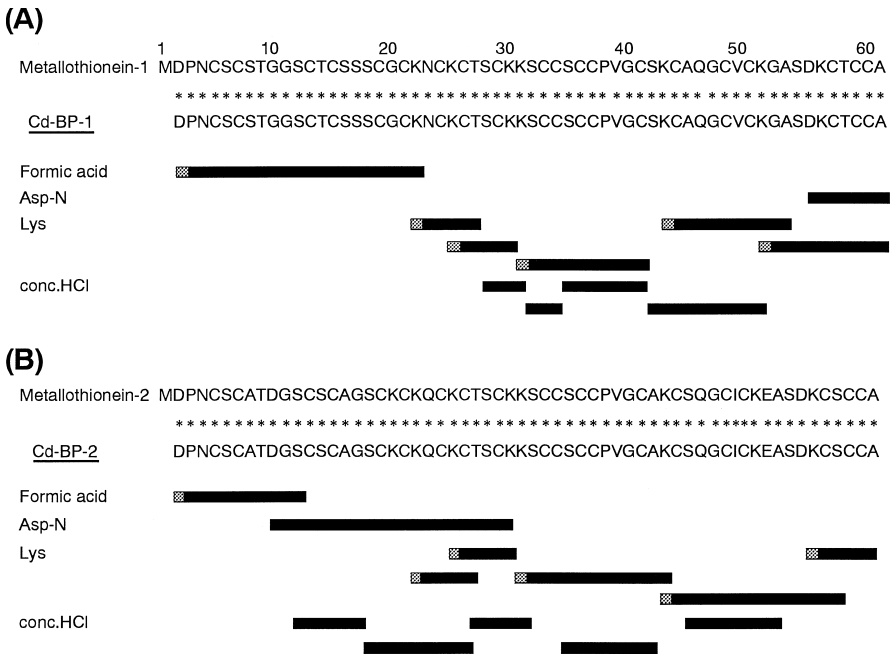

Figure 6 Amino acid sequences of testis Cd-binding proteins and their identity with rat MTs

Each bar represents the length of an analysed fragment, obtained using the treatments indicated on the left. Filled and shaded portions indicate peptide sequences unambiguously determined and those deduced from known specific proteolytic reactions respectively. The amino acid sequences obtained from these fragments were found to be identical with those of rat MT-1 and MT-2 respectively, which are shown for comparison [18].

ticularly the testis, are extremely susceptible to Cd. MTs cannot be induced in these tissues by various stimuli, including heavy metals. Thus it has been a long-standing issue as to whether or not MTs are present in the male genital organs, and we have previously summarized the major points of dispute arising from earlier studies which advocated either the presence or the absence of MTs in the testis [11]. Since these earlier studies utilized indirect experimental methods to characterize testicular Cd-binding proteins, we considered it essential to analyse directly the cDNA sequence as well as the protein sequence of the purified protein. We now clearly demonstrate by RT-PCR analysis and DNA sequence analysis of the cloned PCR products that the MT-1 and MT-2 genes are transcribed as their respective mRNAs, and by protein sequence analysis that these messages are translated as MT-1 and MT-2 proteins in the rat testis.

It is necessary to understand why the present study was able to show the existence of MTs in the testis, despite the fact that earlier investigations [4,20–23] had failed to do so. Waalkes and co-workers [4,5,24] and Deagen and Whanger [22] purified and characterized Cd-binding proteins from cytosolic fractions of rat testis. In brief, they found three Cd-binding protein peaks in the supernatant from the testes of rats that had been administered Cd [22], and also in a similar supernatant to which Cd had been added *in vitro* [24]. Both research groups found that two Cd-binding protein peaks eluted at the same conductance as rat liver MT-1 and MT-2 on anion-exchange column chromatography. They used the Cd-binding protein peaks for examining the purity of the proteins by SDS/PAGE, and also for amino acid analysis. Each preparation appeared to contain some other proteins that were visualized by dye staining [5,22]. In another study [4], a more simplified purification protocol was utilized to study a testicular Cd-binding protein purified by reverse-phase column chromatography under acidic conditions. The protein peaks that were eluted at retention times similar, but not identical, to those of MT-1 and MT-2 were assumed to be the corresponding testis metal-binding protein isoforms. Amino acid analysis of testicular Cd-binding proteins showed a marked variation in terms of the numbers of glutamate, glycine, cysteine and lysine residues among these studies [4,22,24], suggesting that the use of partially purified protein fractions may have yielded misleading data, probably due to contaminating proteins.

The present results show clearly that rat testis contains MT-1 and MT-2, the major isoforms of MT, thus supporting the results of earlier studies, not only in terms of the amounts of MT (-like) proteins estimated by the Cd-binding method in the rat testis [25,26], but also as regards the localization of MT in the male genital tissues. Even testes from untreated rats were found to have a relatively high level of MT in comparison with other tissues, and minimal induction was observed only when Zn was used. As we have reported previously, under physiological conditions MT is localized mainly in the spermatocytes, spermatids, spermatozoa and Sertoli cells of adult rats, but not in the interstitial cells [7]. This observation was further substantiated by *in situ* hybridization, which showed the presence of MT mRNA during spermatogenesis in mice [6] and rats [8]. In contrast, in the testes of Cd-administered rats, MT was induced and present in the Sertoli cells and Leydig cells, but not in the spermatogenic cells [3,27]. These results indicate that the spermatogenic cells, which constitutively express the MT gene, are so susceptible to Cd toxicity that they develop degenerative changes upon exposure to this metal at levels that can induce MT in less vulnerable cells such as Leydig and Sertoli cells. Overexpression of MT in the testes of transgenic mice was not sufficient to protect from Cd-induced testicular damage [28]. These observations raise a significant question in terms of the toxicological role of MTs, and in particular the susceptibility of the testis to Cd. The autosomal recessive *cdm* gene [29] may play an as yet unidentified role, more important than that of MT, in protection against Cd toxicity.

It is not clear why MT is not easily inducible in the testis by heavy metals and other stimulatory agents such as lipopolysaccharide. One plausible explanation could be that Cd cannot be easily transferred to this organ and is present at levels much lower (0.2 % of the dose) than in other tissues such as liver (45 % of the dose) after a single injection [30]. In addition, spermatogenic cells may show degeneration and necrosis before MT is induced in response to such small amounts of Cd. Another aspect, possibly related to the low inducibility of MT, may be the hypermethylation status of the MT gene in the rat testis [31], since DNA methylation controls MT-1 gene expression in murine lymphoid cells [32]. Nevertheless, if this is the case, it is unclear why large amounts of MT protein [7] and MT mRNA [6,32,33] are detected in the testis under physiological conditions. Consistent with these observations, transgenic mice carrying the interferon gene under the control of the MT promoter expressed high levels of interferon mRNA in the testes in the absence of an exogenous inducer such as Cd [34]. Although oestrogen, progesterone and testosterone were reported to be involved to various degrees in the regulation of MT gene expression [9,11,35], the mechanisms by which MT gene expression is regulated in the male genital organs remain to be studied.

The presence of MT in male auxiliary organs [7,10,36] and female reproductive tissues [37–39] has been suggested by reports from several laboratories. Interestingly, cell-specific expression of the MT gene has been demonstrated in the rat prostate: in the ventral lobe neither MT mRNA nor MT protein is detectable, whereas the dorsolateral lobe constitutively harbours both MT mRNA and protein [7–10]. In contrast, the amino acid compositions of Zn-binding proteins purified from the prostate of rodents and primates and from the ovary of Syrian hamsters are reported to be markedly different from that of MT [20,40,41]. Since the purification procedures used in these studies were essentially the same as that used for testicular Cd-binding protein, it is highly likely that MT also exists in these male and female reproductive organs.

From the protein purification results, it is plausible that other MT isoforms are present in the rat testis. Specifically, in addition to Cd-BP-1 and Cd-BP-2, other peaks that reacted with anti-MT antibody were present in the anion-exchange HPLC chromatogram (Figure 4). Although characterization of these peaks remains to be carried out, we suggest that there may be other isoforms of MT in the testis. This is consistent with our observation that testes from MT-null mice whose MT-1 and MT-2 genes were knocked out by homologous recombination contain other proteins that interact with a polyclonal anti-MT antibody that possibly reacts with MT-3 and other forms [9]. Various organs, including the testis, of rats and mice were found to express MT-3 by RT-PCR analysis using MT-3-specific primers and by the use of an anti-MT-3 antibody (J. S. Suzuki, N. Nishimura and C. Tohyama, unpublished work). MT-3 mRNA has been detected by Northern blot analysis and *in situ* hybridization not only in neural tissues but also in the testis and other male and female reproductive organs of the rat, mouse and human [42]. Thus the possibility exists that MT-3 is present in rat testis, in addition to other as yet unidentified forms of MT-like proteins.

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