Cadmium-induced differential accumulation of metallothionein isoforms in the Antarctic icefish, which exhibits no basal metallothionein protein but high endogenous mRNA levels

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Reverse transcriptase-mediated PCR has been used to isolate two distinct metallothionein (MT) cDNA species from RNA extracted from icefish liver, namely MT-I and MT-II. Northern blot analysis with these cDNA species revealed that significant endogenous levels of MT mRNA were present in liver tissues of normal animals despite the fact that no MT protein could be found accumulating in the same tissue. However, multiple injections of $CdCl_2$ induced high levels of both MT mRNA and MT protein. Sequence analysis of the cDNA species that were present after cadmium injection revealed the presence of both isoforms. Quantification of the MT-I and MT-II transcripts from normal and heavy-metal-treated fish showed an alteration in the ratio of the MT isoform transcripts. Endogenous transcripts consisted mostly of MT-II, whereas the MT-I transcript was preferentially accumulated only in response to the cadmium salt. The protein encoded by each cDNA isoform was isolated from the heavy-metal-treated fish and the availability of the specific MT mRNA for translation was demonstrated by translation *in vitro*. These results show that: (1) there is a discrepancy between the significant endogenous levels of MT mRNA and the absence of MT protein; (2) the accumulation of MT in icefish liver can be triggered by heavy metals; (3) genes encoding distinct MT isoforms are differentially regulated by heavy metals.

INTRODUCTION

Metallothionein (MT) is a family of low-molecular-mass cysteine-rich proteins with a high affinity for divalent cations [1–3]. Although the physiological functions of MT have not been fully elucidated, it has been proposed that MTs have a major role in the control of intracellular metal concentration and detoxification [4–7]; in addition, there is increasing evidence for their antioxidant function [8,9].

It has been observed in many species that exposure to heavy metals leads to a rapid increase in MT levels [10,11]. The regulation of MT biosynthesis occurs predominantly at the level of the initiation of transcription. The analysis of the DNA sequences in the 5′ upstream regions of MT genes have shown promoter elements, termed metal-regulatory elements, responsible for the metal-activated expression of MT genes [12–15].

Most of the present knowledge of the MT gene structure and its regulatory mechanisms is derived from studies performed on mammals. Despite the importance that fish have in evolution, piscine MTs have been less extensively studied than those of other taxonomic groups [16–22].

Alone among piscine taxa, Antarctic icefish (Family Channichthyidae, Suborder Notothenioidei) have evolved unique phenotypes that allow them to maintain normal metabolic functions in the absence of both erythrocytes and the oxygen transporter haemoglobin [23]. Another striking feature of the icefish is the lack of endogenous MT in liver [24]. By taking advantage of the ability of $CdCl_2$ to induce MT synthesis, we have investigated MT gene expression in icefish treated with sublethal doses of $CdCl_2$. The objectives of the present study were: (1) to ascertain the presence of MT isoforms both in control and cadmium-treated fish; (2) to study synthesis *de novo* of MT mRNA in liver after multiple injections of metal ions; and (3) to investigate the possible differential expression of distinct MT isoforms in response to induction by metal ions.

EXPERIMENTAL

Fish

Adult specimens of *Chionodraco hamatus* (0.5–1.2 kg) were collected in the proximity of Terra Nova Bay Station in Antarctica (Italy, 74° 42′ S, 164° 07′ E) and kept in aquaria supplied with aerated seawater at approximately −1 °C. The fish were injected intramuscularly with $CdCl_2$ in 1.1% (w/v) NaCl (10 mg of $CdCl_2$/kg of fish) on alternate days for 7 days. At the end of the treatment, livers were quickly removed and frozen at −70 °C until use. Acetone-dried powder was prepared from frozen livers as described previously [25]; 1 g of wet tissue yielded approx. 180 mg of acetone-dried powder.

RNA isolation

Total RNA was isolated from frozen fish livers by the method of Chomczynski and Sacchi [26]. The concentration and purity of samples were analysed spectrophotometrically.

Abbreviations used: CE, capillary electrophoresis; DTT, dithiothreitol; MT, metallothionein; RACE, rapid amplification of cDNA ends; RT–PCR, reverse transcriptase-mediated PCR; UTR, untranslated region.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number Y12580 (MT-I) and Y12581 (MT-II).

Reverse transcription of RNA, and PCR amplification of MT cDNA

First-strand cDNA was synthesized from 5 μg total RNA. In brief, RNA was denatured at 70 °C for 3 min, mixed with 10 pmol of dNTPs, 20 units of RNasin (Promega), 50 pmol of oligo(dT)-adaptor primer (5′-CGGAGATCTCCAATGTGAT-GGGAATTC$(T)_{17}$-3′) (synthesized by Pharmacia Biotechnology) and 200 units of Moloney murine leukaemia virus reverse transcriptase (Promega), and incubated for 2 h at 42 °C. (All enzyme units are defined as certified by the manufacturers.) The reaction was stopped by heating at 65 °C for 5 min. The reverse transcription mixture was amplified by PCR, using as primers the 20-mer N-terminal primer PK70 (5′-AAATGG-ATCCCTGCGAITGY-3′), derived from the N-terminal amino acid sequence of piscine MT, and the adaptor primer described above. Amplification of the reverse transcriptase mixture, containing single-stranded cDNA derived from 0.5 μg of total RNA, was performed with *Taq* DNA polymerase (5 units), 50 pmol of each the above primers and 0.2 mM dNTPs (final concentration) in 10 mM Tris/HCl, pH 8.3, containing 5 mM KCl and 2.5 mM $MgCl_2$. After denaturation for 3 min at 95 °C, the PCR steps consisted of 1 min at 95 °C, 1 min at 55 °C, 1 min at 72 °C for 30 cycles, followed by 15 min at 72 °C and a 4 °C hold.

Rapid amplification of 5′-cDNA ends (5′-RACE)

A RACE protocol was performed with a Marathon cDNA amplification kit (Clontech Laboratories). Single-stranded cDNA was obtained as described above and second-strand synthesis was performed in accordance with the conditions described by the kit manufacturer. The resulting double-stranded cDNA was ligated with the 'Marathon adaptor' overnight at 16 °C by T4 DNA ligase. A portion of the ligated cDNA was then used as a template for PCR amplification, using as forward primer the Marathon AP1 primer and as reverse primer the MT-specific primer IN2 (5′-GCAGGAGCAGTTTGTGCAAGT-GCA-3′), which is complementary to bases 52–75 of the MT coding sequence. The conditions for PCR amplification were essentially identical with those described in the Marathon kit protocol.

Primer extension analysis

Primer extension analysis was performed on 90 μg of total RNA with 100 units of Moloney murine leukaemia virus reverse transcriptase (Promega) at 42 °C for 1 h. As a primer 50 pmol of the IN2 oligomer described above was used, which was labelled at the 5′ end with 10 units of T_4 polynucleotide kinase (Promega) and 40 μCi of [γ-^{32}P]dATP (Amersham) for 30 min at 37 °C. The extended products were analysed on a 6 % (w/v) denaturing polyacrylamide gel containing 7M urea.

Cloning and sequencing of PCR-amplified cDNA

The PCR fragments were purified with the Geno-Bind gel extraction kit (Clontech Laboratories). After purification, the PCR-amplified cDNA was ligated in the pGEM-T vector (Promega) with T4 DNA ligase. *Escherichia coli* (strain TG2) cells were transformed with the ligation mixture. The recombinant clones were used for nucleotide sequencing by the method of Sanger [27] with T7 DNA polymerase. All sequences were determined on both strands.

Northern analysis of RNA species

Total RNA (20 μg) from untreated and cadmium-treated fishes was size-fractionated by electrophoresis through a denaturing 2.2 M formaldehyde/1.2 % (w/v) agarose gel [28], transferred by capillary blotting to a nylon membrane (Amersham) and probed for MT mRNA by hybridization to *C. hamatus* MT-II-cDNA that had been labelled with ^{32}P by random priming [28]. Membrane was prehybridized for 3 h at 42 °C in 5 × SSC/50 % (v/v) formamide/5 × Denhardt's reagent/200 μg/ml fragmented calf thymus DNA. The labelled probe was then added and hybridization was performed at 42 °C for 16 h. The membrane was washed to remove non-specifically bound DNA as follows: 6 × SSC/0.1 % SDS for 15 min at room temperature, followed by 2 × SSC/0.1 % SDS at 50 °C, twice for 15 min, and a final wash with 1 × SSC/0.1 % SDS at 50 °C for 15 min. Specific hybridization to target RNA was determined by densitometric scanning of autoradiographs with a Foto/Eclipse apparatus (Fotodyne) and the Image Quant Software (Molecular Dynamics).

Relative quantification of MT mRNA isoforms

For the determination of the relative amounts of the two MT mRNA isoforms, reverse transcriptase-mediated PCR (RT–PCR) reactions were performed as described above except for the presence of 5 μCi of [α-^{32}P]dCTP in the master mix, and the use, as forward primer, of the more specific primer AF1 (5′-ACA-TGGACCCITGCGACTGC-3′) designed on the basis of *C. hamatus* MT N-terminal sequences established by 5′-RACE. To use one primer for the amplification of both MT mRNA species, inosine was placed at position 11 of the primer instead of the nucleotides C or T present in the cDNA sequences. In some experiments, PCR products (0.5 μg) were digested with 20 units of *Stu*I restriction enzyme for 1 h at 37 °C before being size-fractionated on a 2 % (w/v) agarose gel. The expected 360 and 187 bp bands were excised, placed in a scintillation cocktail (Ecolite) and counted in a Packard scintillation counter.

Translation *in vitro*

The capability of the specific MT transcripts to act as a template for transcription was tested by mixing 0.9 μg of mRNA from control and cadmium-treated fish with rabbit reticulocyte lysate (Promega) in the presence of 20 μCi of [^{35}S]cysteine (Amersham), 0.02 mM amino acid mixture minus cysteine, 0.07 mM KCl, 2 mM dithiothreitol (DTT) and 40 units of RNasin. Translation proceeded at 30 °C for 90 min. An aliquot (15 μl) of the translation reaction mixture was heated at 80 °C for 15 min and centrifuged at 20000 ***g*** for 20 min; the supernatant was analysed by size fractionation by 20 % (w/v) SDS/PAGE by the method of Laemmli [29]. For detection of the proteins containing cysteine the gel was dried on Whatman 3MM paper under vacuum and exposed to X-ray film for autoradiography.

Gel-permeation chromatography

The tissue extracts were prepared as follows. Acetone-dried powders (3 g) were homogenized in 20 ml of 50 mM Tris/HCl, pH 8.6, containing 2 mM DTT and 0.1 mM PMSF. The extracts were centrifuged for 30 min at 15000 ***g*** and the supernatants were centrifuged again for 60 min at 100000 ***g***. Each supernatant (15 ml, 300 mg of protein) was loaded on a Sephadex G-75 column (2.6 cm × 35 cm) previously equilibrated with Tris buffer containing 10 mM Tris/HCl, pH 8.6, and 2 mM DTT, and eluted with the same buffer. Column eluate was collected in 4 ml fractions and monitored for absorbance at 280 nm and for zinc and cadmium contents.

MT purification

MT from cadmium-treated icefish liver was purified by using a procedure described previously [24].

Determination of metal content

Zinc content in chromatographic eluates was estimated by flame absorbance atomic spectrometry. Cadmium was determined by a graphite-furnace-equipped atomic absorption spectrophotometer (Perkin Elmer).

Amino acid composition

Amino acid analyses were performed on an automatic analyser (Carlo Erba model 3A30). Cysteine was determined as cysteic acid after oxidation with performic acid [30]. After oxidation, samples were hydrolysed under N_2 in 6 M HCl for 1 h at 150 °C. Recovery was determined by means of norleucine added as an internal standard to all samples.

Capillary electrophoresis (CE)

Free-zone CE was performed on P/ACE 2000 equipment (Beckman) at 23 °C using as the run buffer a solution made from 0.4 % boric acid and 0.3 % sodium borate. Electrophoresis was performed in a 50 cm × 75 μm fused-silica capillary cartridge in accordance with the instructions given by the manufacturer.

RESULTS

RT–PCR amplification and cloning of MT-specific cDNA species

RNA preparations from livers of untreated and cadmium-injected fish were reverse-transcribed with the oligo-dT adaptor primer described in the Experimental section. The single-stranded cDNA obtained was then used as a template for PCR amplification with the primer encoding the conserved N-terminal region of piscine MTs (PK70) as forward primer, and an adaptor sequence added 5′ to the poly(A) tail during reverse transcription (as described in the Experimental section). The reaction mixtures, analysed on a 1.4 % (w/v) agarose gel stained with ethidium bromide, showed a single product band with a size of 360 bp. The DNA fragments were eluted from the agarose gel, ligated and subcloned into a pGEM-T plasmid. Double-stranded sequencing was performed on 13 positive clones derived from control fish and 13 from cadmium-treated fish. From the analysis of these sequences, two cDNA species encoding distinct MT isoforms, termed MT-I and MT-II, were identified in both control and cadmium-treated fish. The translated amino acid sequences of these two MT isoforms differed in a single residue substitution at position 26 (Lys → Thr) (Figure 1). Such a substitution would be expected to produce a change in the net charge of MT-I. The sequences at the 5′ end of the cDNA species were obtained by following the 5′-RACE protocol described in the Experimental section. The fragments obtained by 5′-RACE included the first 51 nt of the coding sequence, which allowed the proper attribution of the 5′-end to the MT-I and MT-II isoforms. The full nucleotide sequences reported in Figure 1 show marked differences at the level of the 5′- and 3′-untranslated regions (UTRs). At the level of the 3′-UTR, the nucleotide sequence of MT-II differs from MT-I by the absence of three short nucleotide stretches: the first from positions 185 to 189, the second from positions 271 to 280, and the third from positions 327 to 331. The 5′-UTR of MT-II contains a single gap of five nucleotides between positions −71 and −67.

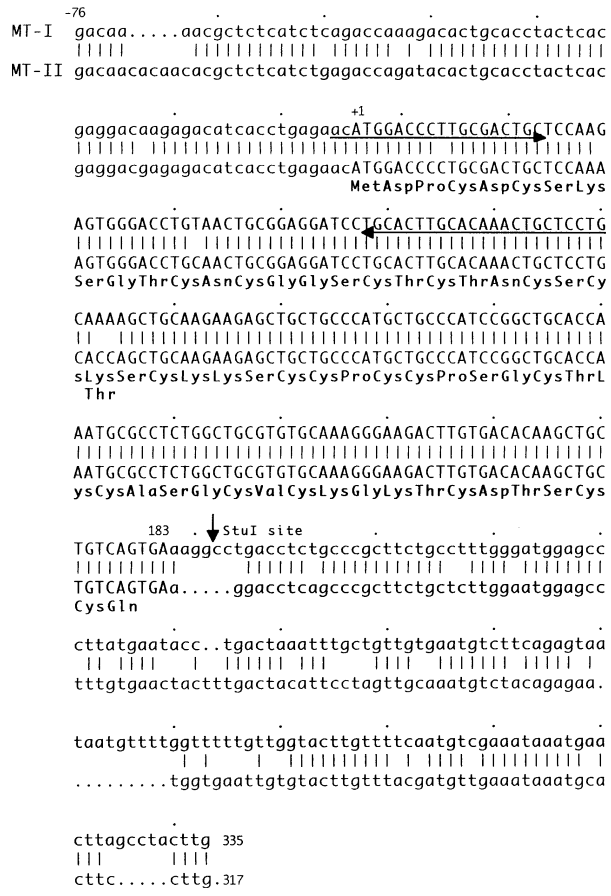

Figure 1 Aligned polynucleotide sequences of the two MT isoforms from *C. hamatus*

Sequences were analysed with the program GAP (GCG-Wisconsin). The region written in capital letters corresponds to the coding sequence, those in low case letters are the 5′- and 3′-UTRs. The translated amino acid sequence of the MT-I isoform is shown beneath the nucleotide sequence; the sequence of MT-II differs from MT-I by a single amino acid substitution. The PCR specific primer, the 5′-RACE primer and the *Stu*I site are indicated by arrows (PCR, arrowhead to right; 5′-RACE, arrowhead to left; *Stu*I, vertical arrow).

The difference in lengths of the 5′-UTRs of the MT-I and MT-II transcripts was also confirmed by the results of the primer extension analysis performed as described in the Experimental section. The PAGE of the reverse-transcribed RNA from cadmium-treated icefish showed the presence of two bands of sizes 151 and 146 bp, corresponding to the sizes of the 5′ leaders of MT-I and MT-II respectively plus the portion of coding sequence starting from bp 75 (Figure 2).

The MT-I and MT-II isoforms found in cadmium-treated fish were the same as those found in control fish. The only difference was in the frequency of each individual form: in control fish 1 out of 13 of the analysed clones carried the MT-I sequence, whereas in cadmium-treated fish 4 out of 13 clones contained MT-I.

Expression of MT mRNA in control and cadmium-treated fish

The levels of MT expression were determined by Northern blot analysis with RNA from control and cadmium-treated icefish (Figure 3) hybridized with *C. hamatus* MT-II cDNA probe radiolabelled with ^{32}P. Equal loading of RNA was verified by hybridization with an 18 S ribosomal RNA probe. The results of this analysis show the presence of appreciable levels of MT

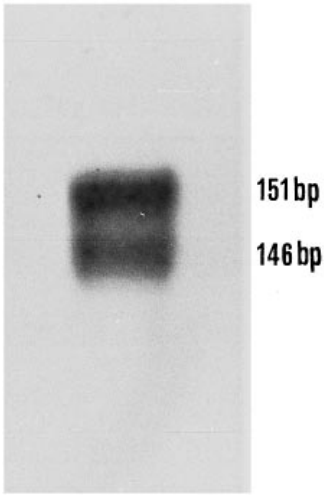

Figure 2 PAGE of the fragments obtained by primer extension reaction

Primer extension was performed as described in the Experimental section by using total RNA from cadmium-treated icefish liver. The sizes of the DNA fragments were determined by comparison with the sequence ladder of icefish MT cDNA (results not shown).

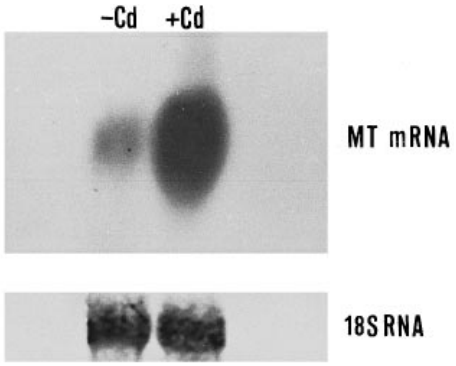

Figure 3 Northern blot analysis of RNA from control and cadmium-treated icefish

A Northern blot of total RNA from liver tissues was probed with homologous MT cDNA as described in the Experimental section. The amount of RNA loaded per lane was assessed by hybridization with a *C. hamatus* 18 S cDNA probe.

Table 1 MT mRNA levels in *C. hamatus*

Total MT mRNA levels were obtained by densitometric analysis of the Northern blot shown in Figure 3. The ratios between MT-I and MT-II transcripts were obtained from the experiments described in Figure 5. The MT-I and MT-II mRNA levels were calculated by the following relations: total MT mRNA = (MT-I mRNA) + (MT-II mRNA); ratio = (MT-I mRNA)/(MT-II mRNA).

	mRNA level (arbitrary units)		
mRNA species	Control	Cadmium-treated	Increase (fold)
Total MT	13488	141964	10.5
MT-I mRNA/MT-II mRNA	0.056	0.4	7
MT-I mRNA	716	40564	56.6
MT-II mRNA	12772	101400	7.9

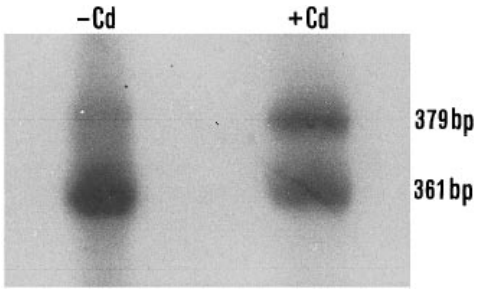

Figure 4 PAGE of ^{32}P-labelled RT–PCR products

RT–PCR was performed in the presence of [^{32}P]dCTP with total RNA from livers of control and cadmium-treated icefish. The reaction products were analysed by electrophoresis on a 6% (w/v) polyacrylamide gel containing 7 M urea. The size of the PCR products was established as in Figure 2.

mRNA in untreated fish and an increment in band intensity in cadmium-treated fish, suggesting the induction of MT mRNA expression by heavy metals. The MT mRNA levels were estimated by densitometric analysis of the Northern blot; the results are reported in Table 1.

To establish the relative abundances of the MT-I and MT-II transcripts, RT–PCR reactions were performed in the presence of [^{32}P]dCTP with a 20-nucleotide primer encoding the first six residues of the N-terminal sequences of both MT-I and MT-II and the adaptor primer described in the Experimental section. An exact nucleotide match was accomplished with both isoforms by introducing an inosine residue at position 11 in place of a C/T present in the coding sequences of MT-I and MT-II. The resulting PCR products were analysed by PAGE. Amplification products from control fish consisted of a major 379 bp band and a minor 361 bp band (Figure 4). However, although identically sized bands were produced on amplification from cDNA made from cadmium-treated fish, the relative intensities of these two bands changed appreciably (Figure 4).

The two MT isoforms could be distinguished by exploiting the presence of a *Stu*I site at position 187 in the MT-I cDNA, which was absent from the MT-II counterpart. The cleavage with *Stu*I generated from the MT-I cDNA two fragments of 187 and 192 bp, which co-migrated on agarose gel and could be separated from the uncleaved MT-II cDNA (Figure 5). The electrophoretic

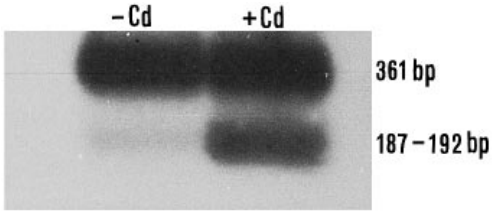

Figure 5 Agarose gel electrophoresis of ^{32}P-labelled RT–PCR products digested with *Stu*I restriction enzyme

RT–PCR was performed as described in the Experimental section. The reaction products were digested with *Stu*I and then analysed on a 2% (w/v) agarose gel. For further details see the Experimental section.

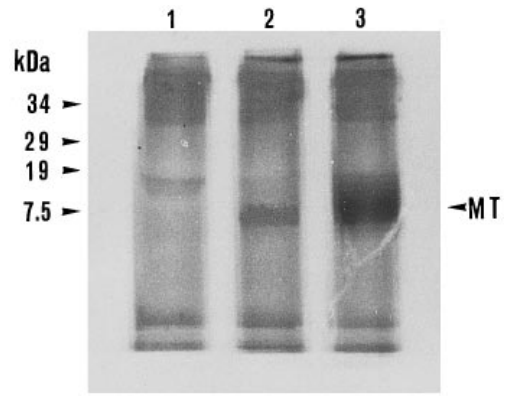

Figure 6 SDS/PAGE of proteins ^{35}S-labelled *in vitro*

Translation *in vitro* was performed as described in the Experimental section, in the absence of template mRNA (lane 1) and in the presence of mRNA from control (lane 2) and cadmium-induced fish (lane 3). The arrow indicates the band exhibiting the expected size of MT.

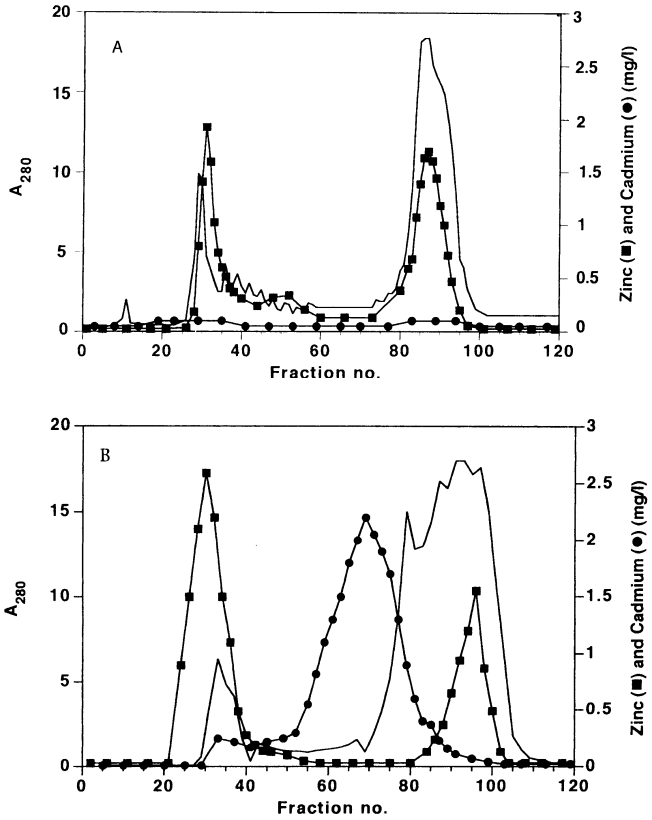

Figure 7 Elution profile from a gel-filtration chromatography column of hepatic extracts from control (A) and cadmium-treated (B) icefish

Extracts were prepared from acetone-dried powders obtained from frozen livers as described in the Experimental section. Approx. 150 mg of protein from the 100000 ***g*** supernatant was loaded on a Sephadex G-75 column (2.7 cm × 35 cm), which was equilibrated and eluted with Tris/DTT buffer at a flow rate of 1 ml/min. The eluates were monitored for heavy metal content and absorbance at 280 nm. The two chromatographic profiles were obtained for control (**A**) and cadmium-treated (**B**) icefish.

Table 2 Amino acid composition of icefish MT

A purified protein sample (approx. 100 μg of protein) was dried under vacuum and treated with 200 μl of performic acid for 15 min at room temperature. The sample was dried under vacuum and hydrolysed with 6 M HCl/1% (w/v) phenol/2.5% (v/v) 2-mercaptoethanol by heating for 1 h at 150 °C under N_2. After hydrolysis the sample was dried under vacuum and dissolved in 250 μl of 0.15 M sodium citrate, pH 2.2, containing 2% (v/v) thiodiglycol and 0.01% (v/v) pentachlorophenol. Amino acids were separated on an ion-exchange column before derivatization with ninhydrin. Cysteine was determined as cysteic acid.

Amino acid	Proportion (%)
Cys	30.2
Asp	8.8
Thr	10.7
Ser	13.2
Glu	3.5
Pro	7.5
Gly	9.3
Ala	2.7
Val	1.9
Met	0
Ile	0
Leu	0
Tyr	0
Phe	0
His	0
Lys	12.2
Arg	0

bands obtained by this analysis were excised from the gel and the radioactivity was measured by liquid-scintillation counting. The relative amounts of the two transcripts in control and cadmium-injected icefish were determined on the basis of the ratio of the radioactivity recovered in each band. The ratio of the intact MT-II 360 bp band over the 190 bp band, representing the cleaved MT-I, changed from 0.05 in control fish to 0.4 after treatment with cadmium (Table 1). By combining the values of these ratios with the estimated amounts of MT mRNA it was possible to calculate the respective levels of the MT-I and MT-II transcripts. The results in Table 1 show that, after treatment with cadmium, the amount of MT-I mRNA increased approx. 50-fold over the value of the control, whereas the amount of MT-II mRNA increased only 8-fold.

Translation *in vitro* of MT mRNA species in control and cadmium-treated fish

The capability of the specific MT transcripts to act as a template for transcription was demonstrated by translation of mRNA *in vitro* in the presence of [^{35}S]cysteine. On size fractionation of the heat-stable proteins produced by the translation of both control and cadmium-treated fish, a radiolabelled band of the predicted molecular mass for MT could be identified (Figure 6). The intensity of the ^{35}S-labelled protein band was significantly higher in the sample prepared from the cadmium-treated fish, paralleling the elevated level of MT mRNA previously detected in this sample.

Accumulation of MT protein in cadmium-treated fish

Hepatic extracts from *C. hamatus* fractionated by gel-permeation chromatography showed no zinc-thionein at the V_e/V_0 of standard MT (Figure 7A). However, a marked increase in hepatic MT accumulation was observed in extracts of fish treated with various doses of $CdCl_2$. The elution profile from gel-permeation chromatography showed the presence of a peak of cadmium

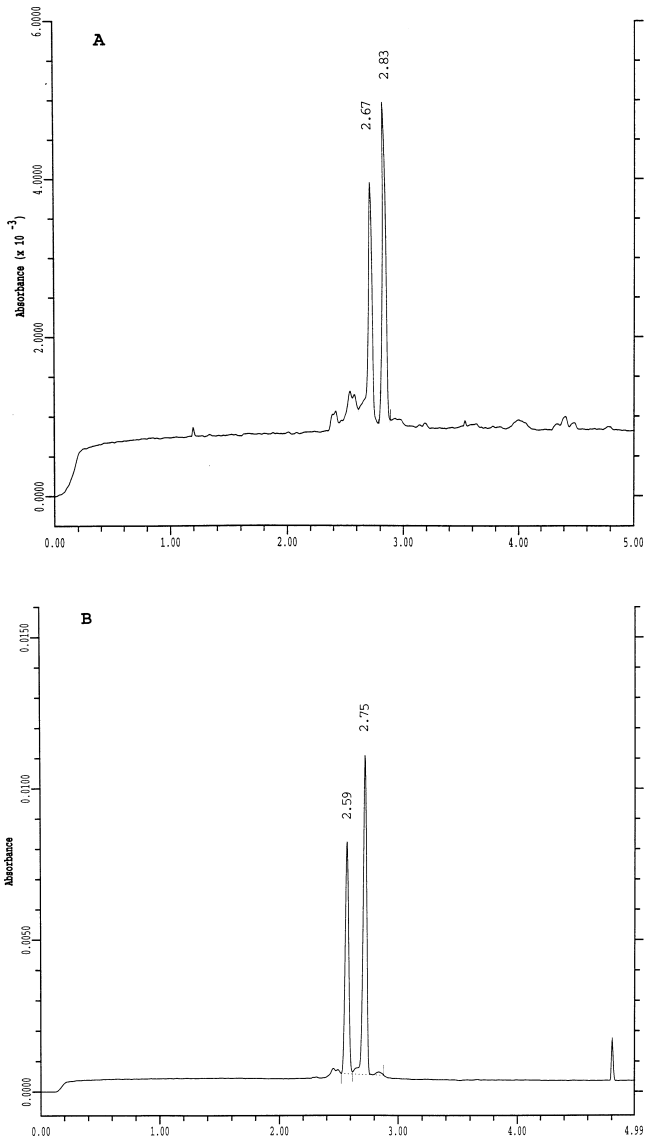

Figure 8 CE of MT isoforms

CE was performed as described in the Experimental section with a 50 cm × 75 μm fused-silica capillary. Absorbance was measured at 214 nm. (**A**) MT from liver of cadmium-treated icefish: the retention times were 2.67 min for MT-I and 2.83 min for MT-II. (**B**) MT from rabbit liver: the retention times were 2.59 min for MT-I and 2.75 min for MT-II.

eluting at the same position of standard MT (Figure 7B). This cadmium-containing peak was pooled and further purified by anion-exchange chromatography followed by reverse-phase HPLC. The fraction after the HPLC step was identified as an MT by SDS/PAGE and amino acid composition (Table 2). This fraction was resolved into two components by capillary zone electrophoresis. On the basis of the net charge calculated from the MT-I and MT-II sequences, the faster CE component was identified as the isoform MT-I, and the slower as MT-II (Figure 8A). For comparison, a purified mixture of MT isoforms from rabbit liver was analysed by CE in a similar manner to the *C. hamatus* MTs (Figure 8B) and clearly showed a resolution of the two inherent isoforms.

DISCUSSION

As in other vertebrates, resistance to heavy metal toxicity in fish is related to their ability to overexpress MT genes after exposure to metal ions [31–35]. The results of our previous studies showed an almost undetectable steady-state concentration of endogenous MT in icefish liver [25]. Here we demonstrate that the observed lack of MT in icefish is not the consequence of a genetic defect as for the globin gene [36], because the accumulation of a cadmium-thionein can be obtained after multiple injections of metal ions. It is intriguing that the MT thus induced can be further separated into two components with different net charges.

By using RT–PCR amplification specific for piscine MT transcripts, we have cloned and sequenced two cDNA species encoding distinct MT isoforms, MT-I and MT-II. These isoforms differ in a single amino acid substitution, which confers a difference in net charge of one negative unit. The analysis of the nucleotide sequences of the MT-I and MT-II transcripts shows significant differences at the level of the 5′- and 3′-UTRs, thus confirming that the two transcripts are products of two distinct gene loci. Both transcripts could be identified in livers of control and cadmium-treated fish. It should be noted that, in all the perciforms studied so far, only one MT isoform could be identified [19,22,37]; in contrast, salmonids show two distinct MT isoforms [19,38].

The MT mRNA levels, measured by Northern blot hybridization, increased approx. 10-fold in cadmium-treated fish over the basal level. However, uniquely in *C. hamatus*, the significant basal level of MT mRNA found also in untreated fish is not represented by an equivalent basal MT protein production. MT-I and MT-II mRNA levels, estimated by combining Northern blot data with the measured ratios of the MT-I over the MT-II transcript, were markedly different in untreated and in cadmium-injected fish. From this estimate, in untreated fish, the MT-II transcript was calculated to be approx. 18-fold more abundant than MT-I mRNA; after treatment with cadmium the relative abundance of the MT-II mRNA was only 2.5-fold that of the MT-I transcript. This demonstrates that the promoter regions of the MT-I and MT-II genes are differentially responsive to metal ions, perhaps as a function of differing numbers and/or sequences of metal-regulatory elements [39,40]. Confirmation that the MT transcript is available *in vitro* for translation suggests the presence of translationally inactive MT mRNA (mostly MT-II) in the liver of untreated icefish. This phenomenon might be due to the differences observed at the level of the 5′- and 3′-UTRs in the MT-I and MT-II transcripts, causing a physical perturbation of MT mRNA translation or a facilitated interaction with a specific ‘translation factor’ in the control icefish that is responsible for repressing translation. There is evidence of post-transcriptional control of MT gene expression in mammals [41,42].

The lack of an endogenous MT in the liver is an unusual finding in fish, which, like other vertebrates, generally have high hepatic MT levels [16,43]. Because it has been shown that the MT half-life ranges from 15 to 80 h depending on the species of metal bound to it [44], it seems unlikely that the small amount of MT found in icefish is the result of a fast MT turnover.

Although several lines of evidence are in favour of a detoxifying or regulatory role of MT against heavy metals and free radicals, the apparent normality of mice carrying null MT alleles [45,46] suggests that the presence of MT is not strictly required in the absence of particular stress conditions. From this point of view, icefish represent a good example of ‘life without MT’. These unusual organisms have evolved astonishing adaptation mechanisms, turning into an advantage genetic defects otherwise

incompatible with the life of other species. For example, by eliminating haemoglobin and erythrocytes, these fish have achieved a marked decrease in blood viscosity, which results in an advantage at a temperature of −1.9 °C. Icefish probably do not need to have a large store of MT, although they maintain the ability to synthesize MT in response to internal or external stimuli. As Antarctica is an environment with a very low pollution level, it is feasible that the main roles of MT in Antarctic organisms is the homeostasis of zinc or the scavenging of free radicals physiologically generated from reduced oxygen species. There is good evidence that both these roles can be performed by factors other than MT. Such a line of reasoning would lead to the conclusion that, whatever the role of MT, this protein might constitute an emergency tool that organisms manage without under 'normal' life conditions.

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