Difference in hepatic metallothionein content in Antarctic red-blooded and haemoglobinless fish: undetectable metallothionein levels in haemoglobinless fish is accompanied by accumulation of untranslated metallothionein mRNA

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Icefish (family Channichthyidae, suborder Nothothenioidei) are a group of Antarctic fish that have evolved unique phenotypes in order to adapt to the environment in which they live. Besides the lack of haemoglobin and the drastic reduction in the number of erythrocyte-like cells, another striking feature of the icefish is that their liver is devoid of metallothionein. These cysteine-rich heavy-metal-binding proteins are usually present in large amounts in a large variety of organisms, from bacteria to mammals. Despite the failure to detect appreciable levels of metallothionein in icefish liver, a cDNA encoding metallothionein was produced from total RNA by reverse transcriptase PCR. The icefish metallothionein showed high percentage identity with metallothionein from *Trematomus bernacchii*, a red-blooded Antarctic fish in which a normal content of hepatic metallothionein was found. Steady-state mRNA levels were assessed in fish liver by high-stringency hybridization of the metallothionein probe with total RNA. The results showed that icefish livers retain large amounts of untranslated metallothionein mRNA. The stability of the icefish transcript might be correlated with the lack of specific motifs in the untranslated 3′ ends of mRNA.

INTRODUCTION

Metallothioneins (MTs) are low-molecular-mass cysteine-rich metal-binding proteins with high affinity for heavy metal ions, found in a large variety of organisms [1,2]. Although the biological functions of MTs have not been fully elucidated, they are thought to play an important role in detoxification of toxic elements such as cadmium and mercury [3–5].

MTs have been detected and characterized in a large number of fish, including trout [6–8], goldfish [9,10], flounder [11] and carp [5]. As with many other vertebrate species, resistance to heavy-metal toxicity in fish is related to their ability to overexpress MT genes after exposure to metal ions [12–18].

The Antarctic family Channichthyidae (suborder Notothenioidei) comprises 15 species of haemoglobinless fish, also known as icefish. From our earlier studies on one of these icefish species, *Chionodraco hamatus*, we reported that the liver is devoid of MT, the only metal-binding protein present in the liver being a low-molecular-mass zinc protein with characteristics different from MTs [19]. In the present report, we have studied MT in the livers of three Antarctic fish species, the icefish *Chionodraco rastrospinosus* and *Chaenocephalus aceratus* (family Channichthyidae) and in the red-blooded species *Trematomus bernacchii* (family Nototheniidae). The results show that of the three species examined, only *T. bernacchii* contains appreciable amounts of MT in the liver.

To determine the level of MT transcripts in the three fish, we generated MT cDNAs from their liver tissue by reverse transcriptase PCR (RT-PCR) using as primers poly(T) and an oligonucleotide derived from the N-terminal sequence of piscine MT. Nucleotide sequence determination of cDNA clones showed that all the PCR products contained sequences encoding MT. Using one of these cDNAs as a probe in Northern blots, we estimated the amounts of MT mRNA accumulated in the livers of the three species examined.

The DNA sequences which we present in this report have been submitted to EMBL GenBank and DDBJ Nucleotide Sequence Databases, and have the following accession numbers: *C. aceratus* MT, Z72483; *Ch. rastrospinosus* MT, Z72484; *T. bernacchii* MT, Z72485.

EXPERIMENTAL

Fish

Adult specimens of *T. bernacchii*, *Ch. rastrospinosus* and *C. aceratus* were collected in the proximity of Palmer Station (U.S.A.), Antarctic Peninsula. The fish were maintained in sea water aquaria at temperatures ranging from -1 to $+1$ °C.

Livers were quickly removed; the tissue used for RNA extraction was frozen in liquid nitrogen and stored at -70 °C. Livers to be used for protein extraction were converted into acetone powder by a previously described procedure [19]; 1 g of wet tissue yielded 185 mg of acetone powder.

Abbreviations used: MT, metallothionein; DTT, dithiothreitol; PEG, poly(ethylene glycol); RT-PCR, reverse transcriptase PCR; TFA, trifluoroacetic acid; UTR, untranslated region.

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The nucleotide sequences of *Chaenocephalus aceratus*, *Chionodraco rastrospinosus* and *Trematomus bernacchii* have been submitted to the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under accession numbers Z72483, Z72484 and Z72485 respectively.

Determination of MT

MT concentration in liver tissues was determined by the silver saturation assay as described by Scheuhammer and Cherian [20], and ELISA [21] using an antibody raised against trout MT. The presence of MT in an oxidized form was tested as described by Klein et al. [22].

Gel-permeation chromatography

Extracts were prepared as follows. Acetone powder (1 g) was homogenized in 5 ml of Tris/dithiothreitol (DTT)/PMSF buffer containing 50 mM Tris/HCl, pH 8.6, 2 mM DTT and 0.1 mM PMSF. The extracts were centrifuged at 10000 ***g*** for 30 min and the supernatant was centrifuged again at 100000 ***g*** for 1 h. Each supernatant (8 ml; 200 mg of protein) was loaded on a Sephadex G-75 column (2.6 cm × 35 cm) equilibrated and eluted with Tris/DTT buffer containing 10 mM Tris/HCl, pH 8.6, and 2 mM DTT. Fractions were collected and monitored for A_{280} and zinc content.

MT purification

The low-molecular-mass zinc-containing peak obtained by gel-filtration chromatography of *T. bernacchii* extract was pooled and directly loaded on a DEAE-cellulose DE-52 column equilibrated with 20 mM Tris/HCl, pH 8.6. The column was developed with a linear gradient from 0 to 400 mM NaCl in equilibration buffer. Fractions were monitored for A_{280} and zinc content. The zinc-containing peak from the anion-exchange chromatography step was concentrated with poly(ethylene glycol) (PEG) 35000 and chromatographed on a TSK-ODS C_{18} HPLC column (Pharmacia Biotech.). The column was eluted using as solvent A 0.1% trifluoroacetic acid (TFA) and as solvent B 60% acetonitrile in 0.1% TFA in the following conditions: 0% of solvent B for 20 min followed by a gradient from 1 to 100% solvent B in 40 min at a flow rate of 1 ml/min. A_{220} was monitored.

Amino acid analysis

Amino acid analysis was performed on an automatic analyser (model 3A30; Carlo Erba). Cysteine was determined as cysteic acid after performic acid oxidation [23]. Recovery was determined by adding norleucine as internal standard.

Determination of metals

Zinc content in chromatographic eluates was estimated by flame absorbance atomic spectrometry. Silver was determined with a graphite-furnace-equipped atomic spectrometer (Perkin–Elmer).

Preparation and sequence analysis of MT cDNA

Total RNA was isolated from frozen fish livers as described by Chomczynski and Sacchi [24]. First-strand cDNA synthesis was performed by heating 5 μg of total RNA to 70 °C for 3 min. The denatured RNA was mixed with 15 pmol of dNTPs, 20 units of RNasin (Promega), 50 pmol of oligo(dT)-adaptor primer [5′-CGGAGATCTCCAATGTGATGGGAATTC$(T)_{17}$-3′] (synthesized by Pharmacia Biotech.) and 200 units of Moloney murine leukaemia virus reverse transcriptase (Promega), and incubated for 2 h at 42 °C under the buffer conditions described by the manufacturer. The reaction was stopped by heating at 65 °C for 5 min. The reverse transcription mixture was amplified by PCR using as primers a 20-mer N-terminal primer 5′-AAATGGATCCCTGCGAITGY-3′ (where I is inosine and Y is C + T) derived from the N-terminal amino acid sequence of piscine MT, and the adaptor primer described above. Amplification was performed with *Taq* DNA polymerase using a reaction mixture containing single-stranded cDNA derived from 0.5 μg of total RNA, *Taq* polymerase (5 units), 50 pmol of each of the above primers and 0.25 mM dNTPs (final concentration) buffered with 5 mM KCl/10 mM Tris/HCl, pH 8.3, containing 2.5 mM $MgCl_2$. The PCR program, performed on a Cetus thermocycler model 420 (Perkin–Elmer), consisted of 30 cycles of 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C, ending with a single cycle of 15 min at 72 °C. RT-PCR product was purified using the Qiaquick gel-extraction kit (Qiagen) and ligated in the pGEM-T vector (Promega) with T4 DNA ligase. *Escherichia coli* (strain TG2) cells were transformed with the ligation mixture. Plasmid DNA was denatured and the cloned cDNA fragment sequenced on both strands by the dideoxy method [25] using the T7 sequencing kit (Pharmacia Biotech.).

Northern-blot analysis of RNAs

Total RNA from the fish was electrophoresed through a 1.2% agarose gel containing 2.2 M formaldehyde [26]. RNA was blotted on Hybond-N nylon membrane (Amersham), and probed for MT mRNA by hybridization to *Ch. rastrospinosus* MT cDNA that had been ^{32}P-labelled by random priming [26]. Prehybridization and hybridization of the membrane was performed in solution containing 5 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), 50% formamide, 5 × Denhardt's reagent (where 1 × Denhardt's reagent is 0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.02% BSA) and 200 μg/ml fragmented calf thymus DNA at 42 °C for 2 h and 20 h respectively. The membrane was washed (final stringency 0.5 × SSC; 50 °C; 15 min), and hybridization to target RNA was detected by autoradiography.

RESULTS

MT in haemoglobinless and red-blooded Notothenioids

Hepatic MT content was estimated by the silver saturation assay and ELISA. The results reported in Table 1 show that an appreciable amount of MT is present in the red-blooded fish, whereas no MT is detectable in the livers of the two icefish. These results are further supported by the chromatographic analyses carried out on tissue extracts of the three fish. Figures 1(a) and 1(b) show the elution profiles from gel-permeation chromatography columns of icefish liver extracts: no zinc is present corresponding to the V_e/V_o of standard MT (indicated by the arrow). A search for the presence of oxidized MT gave negative

Table 1 Quantification of MT content in Antarctic fish livers

MT content was determined by the silver saturation assay and ELISA as described in the Experimental section. Quantification of MT was performed on the basis of standard curves obtained with rabbit MT in the silver saturation assay and trout MT in ELISA. Assays were run in triplicate, and results are means ± S.E.M.

	MT (μg/g wet tissue)	
	Silver assay	ELISA
Ch. rastrospinosus	0.15 ± 0.03	0.13 ± 0.05
C. aceratus	0.07 ± 0.02	0.10 ± 0.04
T. bernacchii	37.5 ± 1.5	52.8 ± 2.8

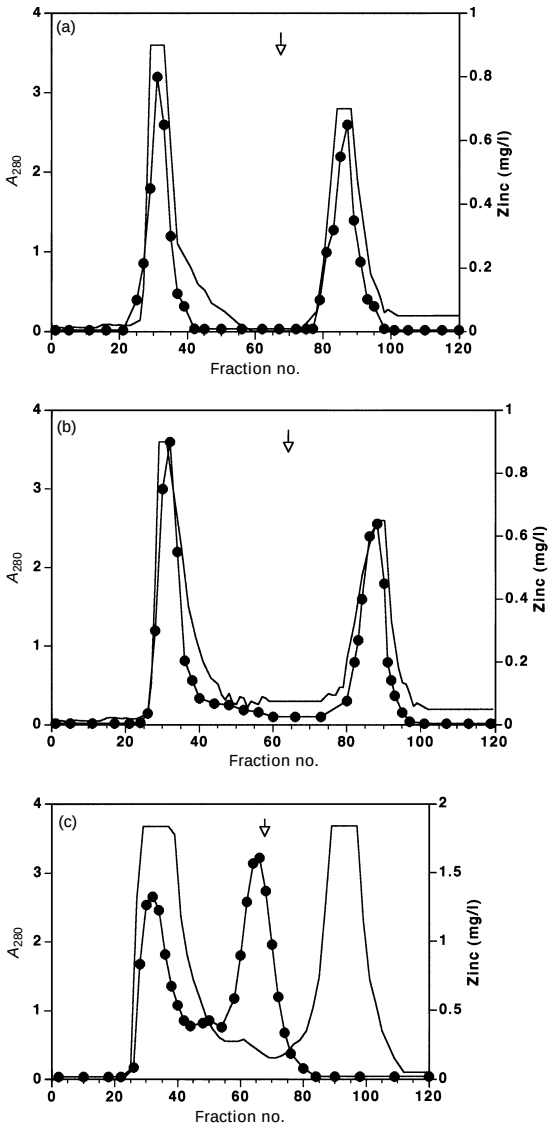

Figure 1 Elution profile from a gel-permeation chromatography column of extracts from livers of haemoglobinless and red-blooded fish

About 1 g of acetone powder prepared from frozen livers was homogenized in Tris/DTT/PMSF buffer and the homogenates were centrifuged for 30 min at 10000 ***g*** and 1 h at 100000 ***g***. About 200 mg of protein from the final supernatants was loaded on a Sephadex G-75 column (2.6 cm × 35 cm) which was equilibrated and eluted with Tris/DTT buffer at a flow rate of 1 ml/min. Fractions (2 ml) were collected and monitored for zinc content (●) and A_{280} (——). The three chromatographic profiles were obtained for *Ch. rastrospinosus* (**a**), *C. aceratus* (**b**) and *T. bernacchii* (**c**) extracts. The arrow indicates the elution volume of rabbit liver MT used as standard.

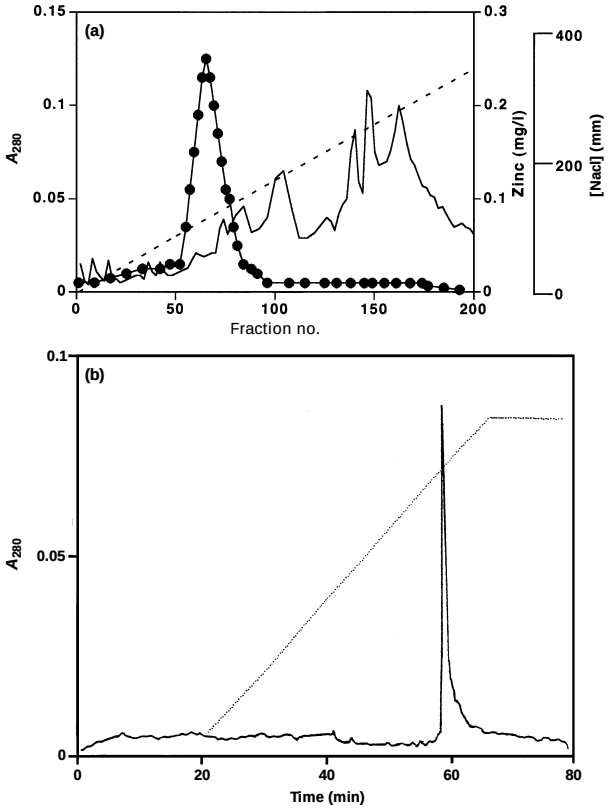

Figure 2 Purification of the *T. bernacchii* MT by anion-exchange chromatography and HPLC

The zinc-containing low-molecular-mass peak from the column in Figure 1(c) was loaded on a DEAE-cellulose column (1.8 cm × 25 cm) equilibrated with Tris buffer (**a**). The column was eluted at a flow rate of 3 ml/min with a linear gradient formed by mixing 600 ml of 20 mM Tris/HCl, pH 8.6 and 600 ml of 400 mM NaCl in Tris buffer. Fractions (5 ml) were monitored for zinc content (●), A_{280} (——) and NaCl concentration (– – – –). The latter was determined by measuring electric conductivity of the eluate. The peak of zinc eluted with the gradient was pooled and concentrated with PEG. Aliquots (200 μg) were loaded on a C_{18} reverse-phase HPLC column (0.78 cm × 30 cm) (**b**), and the column was developed at a flow rate of 1 ml/min with a 20 min step of 0.1% TFA, followed by a 40 min gradient from 0.6 to 60% acetonitrile in 0.1% TFA. A_{220} was recorded.

results. The low-molecular-mass zinc peak is eluted at the same position of the non-MT zinc-containing protein described previously in *Ch. hamatus* [19].

In contrast, the elution profile obtained with the extract from *T. bernacchii* shows the presence of a zinc peak eluted very close to the V_e/V_o of standard MT (Figure 1c). This peak was pooled and subjected to anion-exchange chromatography. The elution profile of zinc from this column shows the presence of a single peak of metal (Figure 2a). This material was further purified by HPLC (Figure 2b). The major peak of absorbance eluted with the gradient was characterized by SDS/PAGE and analysis of amino acid composition. The molecular mass of this component was estimated to be about 6 kDa (results not shown). The amino acid composition showed an abundance of cysteine (30% of the total residues) and absence of aromatic and histidine residues, characteristics that are typical of MT.

Fish MT cDNA

MT cDNAs were generated by RT-PCR using RNA extracted from fish livers. Electrophoresis of PCR mixtures reported in Figure 3 shows bands of about 350 bp; the lower band can be attributed to the oligonucleotides used as primers. No band was obtained with mixture from a control reaction carried out in the absence of single-stranded cDNA. The 350 bp fragments from the gel were ligated into the plasmid vector pGEM-T. The constructs were cloned in *E. coli* in the presence of ampicillin. Sequencing was performed on six *T. bernacchii* cDNA clones, ten *Ch. rastrospinosus* cDNA clones and eight *C. aceratus* cDNA clones.

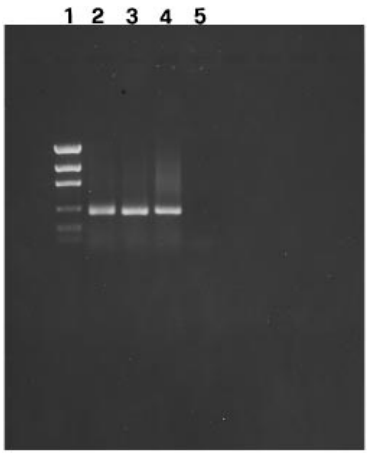

Figure 3 RT-PCR products obtained from liver RNA of haemoglobinless and red-blooded fish

Single-stranded cDNA preparation and MT cDNA amplification reactions were carried out as described in detail in the Experimental section. The products were analysed by electrophoresis on 1.5% agarose gel stained with ethidium bromide. Lane 1, low DNA mass ladder (Gibco Life Technologies); lanes 2–4, single-stranded cDNA from *T. bernacchii*, *Ch. rastrospinosus*, *C. aceratus* respectively; lane 5, negative control reaction without single-stranded cDNA.

DNA sequence analysis

Nucleotide sequences of *T. bernacchii* and of the two icefish cDNAs are reported in Figure 4. The sequences encode proteins of 60 amino acids with 20 cysteines arranged in a fashion typical of class-I MTs. *T. bernacchii* MT shows 98 % amino acid sequence identity with the icefish MTs. The 3′-untranslated regions (3′-UTRs) of all nucleotide sequences carry the AATAAA poly-adenylation signal 12 bases upstream of the last base before the poly(A) tail. There is a high level of identity also at the level of the 3′-UTRs, except that in the icefish there are two deletions within the 3′-UTR.

Transcriptional status of MT genes in *T. bernacchii* and in the icefish *Ch. rastrospinosus* and *C. aceratus*

The production of a cDNA encoding MT in the two icefish could be attributed to the ability of the PCR technique to amplify a rare mRNA possibly present in fish liver. MT mRNA expression was therefore tested by Northern blotting of total RNA using *Ch. rastrospinosus* MT cDNA as probe. The results depicted in Figure 5 show that the amounts of mRNA in icefish livers are markedly higher than those in the red-blooded species.

DISCUSSION

The presence of MT has been demonstrated in several piscine species [6,11,27–29]. Liver, in particular, is one of the primary sites for MT production especially during reproduction and development [30]. In general, high levels of MT are correlated with high levels of MT mRNA [12].

In the present study, we have detected and isolated an MT in the liver of the red-blooded Notothenioid *T. bernacchii*. In contrast, we could not find any trace of MT in the liver of the icefish *Ch. rastrospinosus* and *C. aceratus*. However, using liver RNA from the three fish, we have been able to generate by RT-PCR cDNAs encoding MTs, which display a high degree of similarity to other fish MTs. When Northern-blot analysis of total RNA was performed with one of these cDNAs, we detected

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                1                                                          60
C.aceratus      ATGGATCCCTGCGAGTGCACCAAAAGTGGGACCTGCAACTGCGGAGGATCCTGCACTTGC
C.rastrospinosus ATGGATCCCTGTGAGTGCTCCAAAAGTGGGACCTGCAACTGCGGAGGATCCTGCACTTGC
T.bernacchii    ATGGATCCCTGCGAGTGCTCCAAAAGTGGGACCTGCAACTGCGGAGGATCCTGCACTTGC
                *********** ****** ******************************************

               61                                                          120
C.aceratus      ACAAACTGCTCCTGCACCAGCTGCAAGAAGAGCTGCTGCCCATGCTGCCCATCCGGCTGC
C.rastrospinosus ACAAACTGCTCCTGCACCAGCTGCAAGAAGAGCTGCTGCCCATGCTGCCCATCCGGCTGC
T.bernacchii    ACAAACTGCTCTTGCACCAGTTGCAAGAAGAGCTGCTGCCCATGCTGCCCATCCGGCTGC
                *********** ******** ******************************************

              121                                                          180
C.aceratus      ACCAAATGCGCCTCTGGCTGCGTGTGCAAAGGGAAGACTTGTGACACAAGCTGCTGTCAG
C.rastrospinosus ACCAAATGCGCCTCTGGCTGCGTGTGCAAAGGGAAGACTTGTGACACAAGCTGCTGTCAG
T.bernacchii    ACCAAATGCGCCTCTGGCTGTGTGTGCAAAGGGAAGACTTGTGACACAAGTTGCTGTCAG
                ******************** ***************************** *********

              181                                                          240
C.aceratus      TGAAGAAC-------TCAGCCGCTTCTGCTCTTGGAATGGAGCCTTTGTGAACTACTTTG
C.rastrospinosus TGAAGACC-------TCAGCCGCTTCTGCTCTTGGAATGGAGCCTTTGTGAACTACTTTG
T.bernacchii    TGAAGAGCCTGACCTCCAGTCGCTTCTGCTCTTGGAATGGAGCCTTTGTGAACTACTTTA
                ****** *        *** ****************************************

              241                                                          300
C.aceratus      AATACATTCCTGTTCGAAATGTCTACAGAGAATTGTGTACTTGTTTACT---------AT
C.rastrospinosus ACTACATTCCTGTTGCAAATGTCTACAGAGAATTGTGTACTTGTTTACG---------AT
T.bernacchii    ACTACATTCCTGTTGCCAATGTCTACAGAGAATGGTGAATTTTTGTACTTGTTTATGAAT
                * ************   ***************** *** * ** * ***         **

              301                  323
C.aceratus      GTTGA AATAAA TGCAGTTCCTTG
C.rastrospinosus GTTGA AATAAA TGCAGTTACTTG
T.bernacchii    GTTGA AATAAA TACAGTTCCTTG
                ***** ****** * ***** ****

                1       10        20        30        40        50        60
C.aceratus      DPCECTKSGTCNCGGSCTCTNCSCTSCKKSCCPCCPSGCTKCASGCVCKGKTCDTSCCQ

C.rastrospinosus DPCECSKSGTCNCGGSCTCTNCSCTSCKKSCCPCCPSGCTKCASGCVCKGKTCDTSCCQ

T.bernacchii    DPCECSKSGTCNCGGSCTCTNCSCTSCKKSCCPCCPSGCTKCASGCVCKGKTCDTSCCQ

Rabbit          DPNCSCATGNSCTCASSCKCKECKCTSCKKSCCSCCPAGCTKCAQGCICKGASDKCSCCA
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Figure 4 Nucleotide sequences of MT cDNAs from *C. aceratus*. *Ch. rastrospinosus* and *T. bernacchii* and primary amino acid sequences of the encoded MTs

Nucleotide sequences (top) were determined by double-strand sequencing using not less than six distinct clones for each sequence. The sequences were aligned by means of program PileUp (GCG-Wisconsin). The coding sequence is underlined; the consensus sequence for poly-adenylation is shown in the box. The missing nucleotide stretches in the 3′-UTR of the two icefish are represented by gaps filled with dashes. The asterisks indicate identity between the sequences. Primary sequences (bottom) were deduced from the coding regions of the nucleotide sequences reported above. The amino acid sequence of rabbit MT is reported to allow a comparison with mammalian MT.

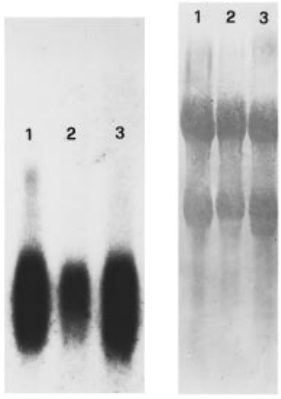

Figure 5 Expression of MT transcripts in *Ch. rastrospinosus*, *T. bernacchii* and *C. aceratus*

Northern blots (left) of total RNA from liver tissues were probed with *Ch. rastrospinosus* cDNA for MT. Some 30 μg of each RNA sample was loaded on the gel, and prehybridization was performed in 5 × SSC/50% formamide/5 × Denhardt's reagent for 2 h. Hybridization was carried out under high-stringency conditions in 200 μg/ml fragmented calf thymus DNA at 42 °C for 20 h: the filter was washed in 0.5 × SSC at 50 °C for 15 min before autoradiography. Visualization of amounts of RNA loaded was achieved by staining the filter with Methylene Blue (right). Lane 1, *Ch. rastrospinosus*; lane 2, *T. bernacchii*; lane 3, *C. aceratus*.

the presence of MT mRNA in the liver of the two icefish as well as in the liver of the red-blooded fish. From these results we conclude that the lack of MT in icefish liver is not a consequence of evolutionary loss or transcriptional inactivation of MT genes as in the case of globin genes [31].

On the other hand, accumulating evidence shows that control of gene expression occurs not only at transcriptional level but also at translational level, and a number of studies indicate the existence of factors acting on mRNA stability and translation. Masked or translationally inactive mRNAs are commonly present in oocytes and eggs [32]; translational activation of these transcripts usually occurs after fertilization. One of the best characterized systems in adult tissues is that of ferritin mRNA, the translation of which is down-regulated by an RNA-binding protein which interacts with a hairpin motif in the 5′-UTR [33]. In other cases, translational regulation may be mediated by proteins that specifically bind to motifs at the 3′ end of mRNAs [34–36]. Although normally observed in developing systems, the regulation of mRNA translation by motifs in the 3′-UTR may occur in somatic cells as well. It is noteworthy that in the two icefish, the 3′-UTRs lack two-nucleotidic stretches, which are present in the *T. bernacchii* mRNA. It is possible that mRNA accumulated in icefish liver is made more stable by the absence of specific motifs in the 3′-UTR. Evidence exists of high levels of MT mRNA in rats accompanied by low levels of MT protein [37–39]. It has been inferred that MT synthesis can be controlled not only at the transcriptional, but also at the translational level [40–42].

Is lack of MT of any adaptive advantage to icefish? One of the proposed roles for MTs is protection of cells from the oxidative stress brought about by the toxic effects of reactive oxygen species [1,43–45]. Since icefish are devoid of haemoglobin, the only oxygen present is that physically dissolved in the blood. Owing to the reduced oxygenation, it is conceivable that formation of free radicals in icefish occurs at a lower rate, and this may explain the complete suppression of MT production. Such a suppression, however, does not seem to be permanent, as we have been able to induce MT production in the icefish *Ch. hamatus* by treatment with cadmium salt (R. Scudiero, M. Riggio, A. Capasso, V. Carginale, G. di Prisco, P. Kille and E. Parisi, unpublished work). This suggests that an MT mRNA can be produced and/or made translationally active by exposure to metal. The reason for such an activation is still unclear, but these studies should help us to understand the mechanisms responsible for translational control of mRNA in eukaryotes.

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