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Cloning and characterization of metallothionein gene in ayu *Plecoglossus altivelis*

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Abstract

Metallothionein (MT) has been used widely as a potential molecular marker to detect the deleterious effects of heavy metals in aquatic ecosystem. Here we exposed ayu, *Plecoglossus altivelis*, to zinc (Zn) and tested the distribution as well as the induction of MT in various tissues such as liver, kidney, intestine and stomach. MT induction was significant in liver tissue, followed by kidney and intestine, whereas no induction was detected in stomach. The gene encoding ayu MT was successfully cloned and characterized. Complete nucleotide sequencing and analysis of the 4.5 kb DNA fragment containing the ayu MT gene revealed that the gene has three exons interrupted by two introns, a 5′-flanking region of about 2.5 kb and about 1.6 kb of 3′-flanking region. In grouper heart and kidney cells, the 2.5 kb promoter containing eight metal responsive elements (MREs), two hepatic nuclear factor 5 responsive elements (HNF5REs) and one cAMP responsive element (CRE) had the highest reporter activity.

Keywords: Ayu; cAMP responsive element (CRE); Hepatic nuclear factor 5 responsive element (HNF5RE); Metallothionein; Metal responsive element (MRE); Promoter

1. Introduction

Rivers and estuaries are considered to be major recipients of pollutants, especially with high content of heavy metals. Metal may cause serious consequences to fish species in terms of retarded growth, decreased reproduction rate and increased sensitivity to diseases (Langston et al., 2002). Zinc (Zn) is an essential heavy metal for gene expression and metabolic processes (MacDonald, 2000). However, excess Zn can be highly toxic and harmful to health (Hogstrand and Wood, 1996). Detection of sub-lethal changes at the molecular and cellular level using the metal-binding protein metallothionein (MT) has been proposed as a biological indicator of metal pollution in aquatic ecosystems (for example, Roesijadi, 1994; Langston et al., 2002).

MTs are a class of low molecular weight (6–7 kDa), cysteine-rich (20–33%), heavy metal-binding proteins, ubiquitously present in eukaryotes (Kägi et al.,

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1984; Hamer, 1986). Studies reveal that they play a variety of roles, such as metal ion homeostasis, detoxification and cytoprotection (Kägi and Schaffer, 1988; Schroeder and Cousins, 1990; Suzuki et al., 1993). MTs are regulated transcriptionally by metal responsive elements (MREs), which are *cis*-acting elements and present in multiple copies (Stuart et al., 1985). MREs consist of a highly conserved heptanucleotide core, TGCRCNC and less conserved flanking nucleotides (Searle et al., 1987; Culotta and Hamer, 1989). Although two MREs are necessary and sufficient for substantial induction by heavy metals, additional MREs are often present in the promoter region and can augment the activity of the MT promoter (Stuart et al., 1985; Searle et al., 1985, 1987; Maroni et al., 1986; Cserjesi et al., 1997).

Ayu, *Plecoglossus altivelis*, also called sweetfish, resides in fresh and brackish waters. It is cultured in East Asian countries, especially in China, Japan, Korea and Taiwan. Ayu is salmon-like and light yellow or olive colored. It is distinguished by unique characteristics such as a folded tongue, a sail-like dorsal fin and teeth arrangement on saw-edged plates at the sides of the jaws (Safra, 1998). As ayu spans its life cycle in river, brackish and seawaters, this would be a suitable fish model for studying the biological responses to heavy metals in aquatic animals.

A wealth of information has been acquired in recent years about the induction of MT promoter activity in fish, treated with different heavy metal ions (for example, Samson and Gedamu, 1995, 1998; Ren et al., 2000). Immortal cell lines have been used extensively, to study MT gene regulation by heavy metals (Price-Haughey et al., 1987; Zafarullah et al., 1989; Misra et al., 1989; Olsson and Kille, 1997). Primary culture techniques with fish cells have also been established to study the expression pattern of MT genes (Hyllner et al., 1989; Olsson et al., 1990). Rainbow trout MT-A promoter, when introduced into a cell-line derived from trout liver and fry of a fresh water fish medaka, was induced after exposure to zinc (Inoue et al., 1992). It has been suggested that MT promoters are useful for the regulation of transgenes in fish (Chan and Devlin, 1993). Therefore, considering all these information, the present study was aimed to investigate the structure of the ayu MT gene and its promoter activity.

2. Materials and methods

2.1. Northern blot hybridization

Total RNA was extracted from different tissues of ayu, viz. liver, kidney, intestine, and stomach using the RNAzol method (RNAzol^{TM}B REAGENT, TEL-TEST "B," INC.). The quantity and quality of RNA were determined by observing $OD_{260/280}$ and formaldehyde agarose gel electrophoresis, respectively. Twenty micrograms of total RNA was dissolved in 50 μl of loading buffer (50% formamide, 6.475% formaldehyde, 0.05% saturated bromophenol blue, 0.5 μg/μl ethidium bromide, and 4% glycerol in 1× 3-morpholinopropane sulfonic acid (MOPS, MERCK)). The mixtures were incubated at 65 °C for 15 min and loaded on 1% formaldehyde agarose gel (1 g agarose, Amersco Inc., in 100 ml DEPC-treated distilled water including 1× MOPS and 2% formaldehyde). The gel was transferred to Hybond-N nylon membrane using 20× SSC (3 M sodium chloride and 300 mM sodium citrate, pH 7.0) salt bridge overnight. Transferred membrane was rinsed with 2× SSC, air-dried, and cross-linked by UV Stratalinker 1800 (Stratagene, La Jolla, CA, USA). After 1 h pre-hybridization, the membrane was incubated overnight at 42 °C in 50 ml hybridization solution (0.25 M phosphate buffer, pH 7.2, 10% PEG 8000, 50% formamide, 0.25 M NaCl, 0.5 mM EDTA, and 7% SDS) containing 5×10^5 cpm of probe/ml. The membrane was washed with 0.1× SSC and 0.1% SDS of increasing temperature and air-dried for autoradiography.

2.2. Cosmid genomic library construction

Sperm DNA of ayu, *P. altivelis* was prepared with 1× genomic DNA extraction buffer (10 mM Tris–HCl, pH 8.5, 5 mM EDTA, 200 mM NaCl, 0.2% SDS and 200 μg/ml proteinase K). Briefly, 10 μl of ayu sperm was diluted in 90 μl of 1× PBS. An equal volume of 2× genomic DNA extraction buffer was added and mixed gently and inverted several times. The DNA was extracted with phenol/chloroform, precipitated with 2 vol. of 95% ethanol, and washed with 70% ethanol. The genomic DNA pellet was air-dried and dissolved in double distilled water. The ayu genomic DNA was partially digested with *Sau*3AI. The

DNA fragments with size between 45 and 55 kb were collected through a sucrose gradient and subjected to 0.4% agarose gel electrophoresis. Dephosphorylation and phenol extraction of the DNA were carried out using the recommendations of the supplier (Stratagene, La Jolla, CA, USA). Approximately, 2.5 μg of the purified DNA was ligated into 1 μg of *Xba*I-*Bam*HI digested SuperCos I cosmid vector arms and packaged with Gigapack®III XL packaging extract (Stratagene, La Jolla, CA, USA).

2.3. Ayu MT cDNA cloning

We compared the sequences of different fish MT cDNAs from gene bank and synthesized the following primers, MT(+)BI: 5′-CGG GAT CCA TGG A(C/T)C C(C/T)T G(C/T)G A(G/A/T)T GC(G/T) C(C/T)A A-3′ and MT(−)RI: 5′-GGA ATT CTT (A/G)CA CAC (A/G)CA GCC (A/T)CA (A/G)GC (A/G)CA-3′, where BI and RI represent *Bam*HI and *Eco*RI excision sites, respectively; + and − represent forward and reverse, respectively; and the underlines represent the enzyme sites. Complementary DNA was synthesized using the above primers by reverse transcriptase-polymerase chain reaction (RT-PCR) method. The reverse transcription contained 10 μg of total RNA, 1 mM methyl mercury hydroxide (CH_3HgOH), 5 mM β-mercaptoethanol, 400 μM dNTPs, 30 ng/μl oligo-dT, 0.5 U/μl RNAsin, and 1 U/μl moloney murine leukemia virus reverse transcriptase (MMLV-RT, Stratagene, La Jolla, CA, USA) in 70 μl of 1× first strand buffer at 37 °C for 1 h. The resulting products were amplified by adding 0.05 U/μl *Taq* DNA polymerase (Viogene, Shijr, Taipei, Taiwan) and 0.3 μg/μl each of the forward and reverse primers with a Perkin-Elmer Cetus DNA thermal cycler 480, using a program consisting of 1 cycle of 5 min at 95 °C, 35 cycles of 2 min at 95 °C (denaturation), 1 min at 80 °C (hot start), 1 min at 55 °C (annealing) and 2 min at 72 °C (extension). After 35 cycles, the reaction mixtures were incubated at 72 °C for an additional 10 min to allow complete synthesis. The cDNA thus produced was amplified by an additional PCR with a reaction mixture containing 5 μl of the RT-PCR product, 0.05 U/μl *Taq* DNA polymerase in 50 μl of 1× *Taq* DNA polymerase buffer, 0.3 μg/μl of each forward and reverse primers, 50 μM dNTPs and 150 μM $MgSO_4$. The thermal cycler was programmed as above but for the annealing temperature, which was elevated to 58 °C. This PCR product was treated with *Bam*HI and *Eco*RI, and subcloned into the same sites of pBluescript SK(±) vector (Stratagene, La Jolla, CA, USA) for sequencing. The MT cDNA fragment of 142 bp was sequenced and used as a probe to screen MT containing clones from the constructed cosmid genomic library by colony hybridization.

2.4. Screening of ayu cosmid genomic library

The probe was labeled with [α-^{32}P]dCTP and rediprime II kit (Amersham Biosciences, NJ, USA). The 3×10^5 colonies of ayu cosmid genomic library was plated onto 12 Hybond-N nylon membrane-plates (Amersham Biosciences, NJ, USA) and incubated at 37 °C overnight. The membranes were mirror-copied twice onto new nylon membranes and cultured for 3–4 h at 37 °C. Two sets of mirror-copied membranes were denatured in denaturing buffer (1.5 M NaCl and 0.5 M NaOH) for 5 min, and then neutralized in neutralizing buffer (1.5 M NaCl and 1 M Tris, pH 8.0) for 5 min. After air-dried, these membranes were cross-linked by UV Stratalinker 1800 and hybridized with the probe. The signal clones were screened twice with the same probe and finally, a single cosmid clone containing ayu MT gene was isolated.

2.5. Shot-gun ligation, DNA sequencing and analysis

Ayu MT cosmid clone was subcloned into pBluescript SK(±) vector following the shot-gun ligation method (Davis et al., 1994), by using *Sac*I and screened again by colony hybridization using the same MT cDNA probe. Nucleotide sequencing was performed by the commercially available dideoxynucleotide chain-termination method (ABI Prism™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase) using PRISM 377 DNA Sequencer (Perkin-Elmer, MA, USA). The ayu MT DNA was analyzed with the Web-based sequence analysis of GCG program (Wisconsin Package Version 10.0, Genetics Computer Group, Madison, WI) and searched for transcription factor binding sites with a Transcription Factor Search Program, http://www.cbrc.jp/research/db/TFSEARCH.html. The FASTA program

was used to search the homologous sequence of GenBank. The localization of exon and intron sequences was predicted using the BestFit program. The 142 bp cDNA contained partial sequences of exons I and III, and the complete sequence of exon II for MT. A phylogenetic tree was generated using the algorithm, http://www.ebi.ac.uk/clustalw/index.html.

2.6. Construction of recombinant plasmids

For qualitative analysis, the shot-gun ligated clone containing the ayu MT promoter region was digested with *Bsp*EI and blunted with Klenow and dNTPs. After phenol/chloroform extraction and ethanol precipitation, the dissolved DNA was digested again with *Sac*I. A fragment of 2.5 kb comprising the ayu MT promoter was eluted with the gel extraction kit (Viogene, Shijr, Taipei, Taiwan) and ligated with pEGFP-1 vector (Clontech, Palo Alto, CA, USA) which was previously digested with *Sac*I and *Sma*I. This pEGFP-a$MT_{2.5}$ plasmid construct was confirmed with restriction enzyme mapping and sequencing. For quantitative analysis, four plasmids (pSEAP2-a$MT_{0.2}$, pSEAP2-a$MT_{0.7}$, pSEAP2-a$MT_{1.0}$ and pSEAP2-a$MT_{2.5}$) containing 0.2, 0.7, 1.0 and 2.5 kb of the MT promoter 5′-flanking sequence was used to access MT regulation.

2.7. Transfection and Zn induction

The NIH 3T3 cell line (cultured in DMEM containing 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamate and 10% fetal bovine serum (FBS) at 5% CO_2 and 37 °C humidity incubator) and grouper cells, GL, GK, GF, GH, and GSB derived from liver, kidney, fin, heart, and swim bladder, respectively (cultured in Leibovitz's (L15) medium containing 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamate and 10% FBS at 28 °C) (Lai et al., 2000, 2003), were seeded at a density of 5×10^5 cells/well in a 6-well plate. Monolayer cells with 80% confluence were transfected with 2 μg of plasmid using lipofectamine plus™ reagent kit (Invitrogen, Carlsbad, CA, USA) and incubated for 24 h. The transfected cells were subjected to G418 selection for one month, to achieve stable expression. Briefly, the transfected NIH 3T3 cells were cultured in the presence of G418 at 600 μg/ml (for grouper cells, 800 μg/ml) for three days. This was repeated twice, and then the concentration of the drug was increased to 800 μg/ml (for grouper cells, 1000 μg/ml) and cultured for three days. Pools of thousands of clones were selected and cultured in the presence of the drug at the initial concentration for one month. The transfected cells were treated with 100 μM $ZnCl_2$ and green fluorescence signals were observed to assess expression after 24 and 48 h under a fluorescence microscope (Olympus, IX70, Tokyo, Japan).

For quantitative analysis, the GH and GK cells were transiently transfected with the ayu MT promoter deletion clones and pSEAP2-Basic vector (control). For induced expression, these cells were treated with a final concentration of 100 μM $ZnCl_2$ for 24 h and assayed using Great EscAPe™ SEAP Chemiluminescence Detection kit (Clontech, Palo Alto, CA, USA).

3. Results

3.1. Expression of MT in different tissues of ayu

The liver, kidney, intestine and stomach tissues were dissected out from ayu, which was previously cultured in 50 l of deionic water containing $ZnCl_2$ at 100 μM for 4 h or 10 μM for 96 h. Total RNA isolated from these tissues were subjected to Northern blot hybridization, to observe the induction of MT expression. In liver and intestine, 96 h exposure of Zn significantly induced MT mRNA expression, whereas 4 h of exposure to Zn failed to induce MT mRNA in intestine (Fig. 1). In kidney, MT induction was observed only at 10 μM $ZnCl_2$ for 96 h, while no increase of MT mRNA could be detected after 4 h. Stomach tissue showed no induction of MT expression.

3.2. Cloning of the gene encoding ayu MT

To understand the structure of the ayu MT gene, a genomic library of ayu, with inserts ranging from 45 to 55 kb was constructed in the SuperCos I cosmid vector. A MT cDNA fragment, from ayu liver total RNA, was prepared by RT-PCR using degenerate primers. After sub-cloning, the 142 bp of MT cDNA fragment was confirmed by sequencing and used to detect the MT gene from the cosmid genomic library. Subsequent restriction enzyme mapping and Southern

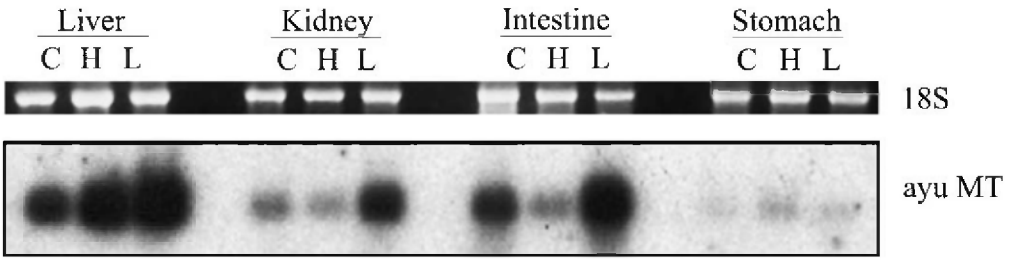

Fig. 1. Northern blot analysis for MT gene expression in different tissues from zinc-treated ayu. C: control, no $ZnCl_2$ treatment; H: 100 μM $ZnCl_2$ for 4 h; L: 10 μM $ZnCl_2$ for 96 h.

blot analysis (data not shown) identified a 4.6 kb-sized *Sac*I fragment which hybridized to the cDNA probe (Fig. 2A). Further restriction mapping was performed, to facilitate sub-cloning into pBluescript SK(±) vector, for sequence determination (Fig. 2B).

3.3. Sequence analysis of ayu MT gene

Fig. 2B shows the full-length nucleotide and the deduced amino acid sequences of ayu MT gene. The comparison of ayu MT genomic sequence with the ayu MT cDNA partial sequence revealed that the coding region of the ayu MT gene is interrupted by two introns of 97 and 153 nucleotides at nucleotide positions 26 and 192, respectively. Intron 1 was 69% AT-rich, and intron 2 was 59% AT-rich. The open reading frame (ORF) encodes 61 amino acids. Exon 1 encodes the first $8\frac{1}{3}$ amino acids (25 bp); exon 2 encodes 23 ($\frac{2}{3} + 22 + \frac{1}{3}$) amino acids (69 bp) and the exon 3 encodes the last $29\frac{2}{3}$ amino acids (89 bp).

The 5′-flanking region of the ayu MT gene features a typical TATA box. Eight MREs, with three MREs (1, 3 and 7) having the motif TGCRCNC consensus core sequence in forward orientation, and five MREs (2, 4, 5, 6 and 8) with the GNGYGCA sequence in reverse orientation were observed. Two hepatic nuclear factor 5 responsive elements (HNF5REs) with GCAAACA or TGTTTGT sequence and one cAMP responsive element (CRE) with the TGACGTCA sequence were also identified. Two additional HNF5REs, with ACAAATA or TATTTGT sequence, are also noted in the 3′-flanking region. Three possible polyadenylation signals with the AATAAA sequence were observed in the 3′-flanking region.

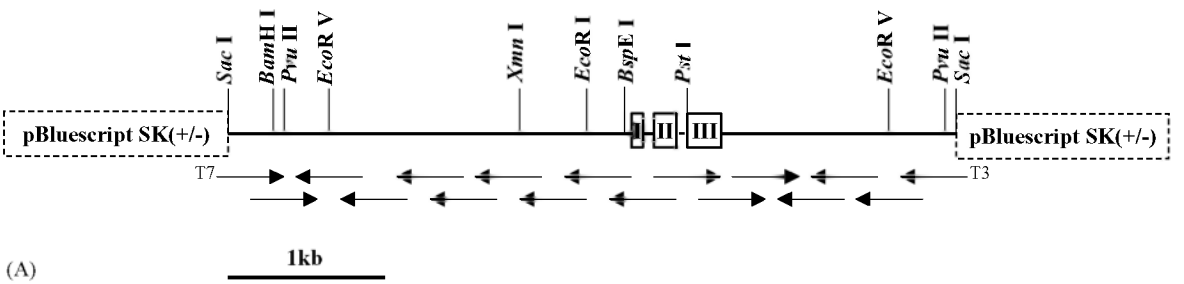

Fig. 2. Structure and nucleotide sequence of ayu MT gene. (A) Restriction enzyme mapping of MT gene. Only important enzyme sites are shown. Exons I, II and III are boxed. Dot-boxes indicate the vector region. Arrows indicate the sequencing strategy. (B) The nucleotide sequence of ayu MT gene. Exons and introns are shown in uppercase and lowercases, respectively. The introns are demarcated by consensus GT–AG splicing signals. The decoded amino acid sequence is shown in single letter code below the exons. The TATA box is boxed. The putative MREs (—►) and HNF5REs (—►) are shown. The arrows indicate the orientation with respect to the direction of transcription. CRE is underlined. The shaded regions indicate the polyadenylation signals. The ayu MT gene sequence was submitted to the GenBank database (accession number: AY208860).

Fig. 2. (*Continued*).

3.4. Phylogenetic analysis

The deduced amino acid sequence of ayu MT was subjected to phylogenetic analysis and that reveals the extensive evolutionary conservation of various fish MTs (Fig. 3). The figure shows that the MT-A and MT-B and MT-I and MT-II are structurally distinct. Based on phylogenetic analysis, this ayu MT gene was grouped to the MT-A clad.

3.5. Comparison of ayu and other fish MTs

The amino acid sequence of ayu MT shows a remarkably high order homology with other fish MTs.

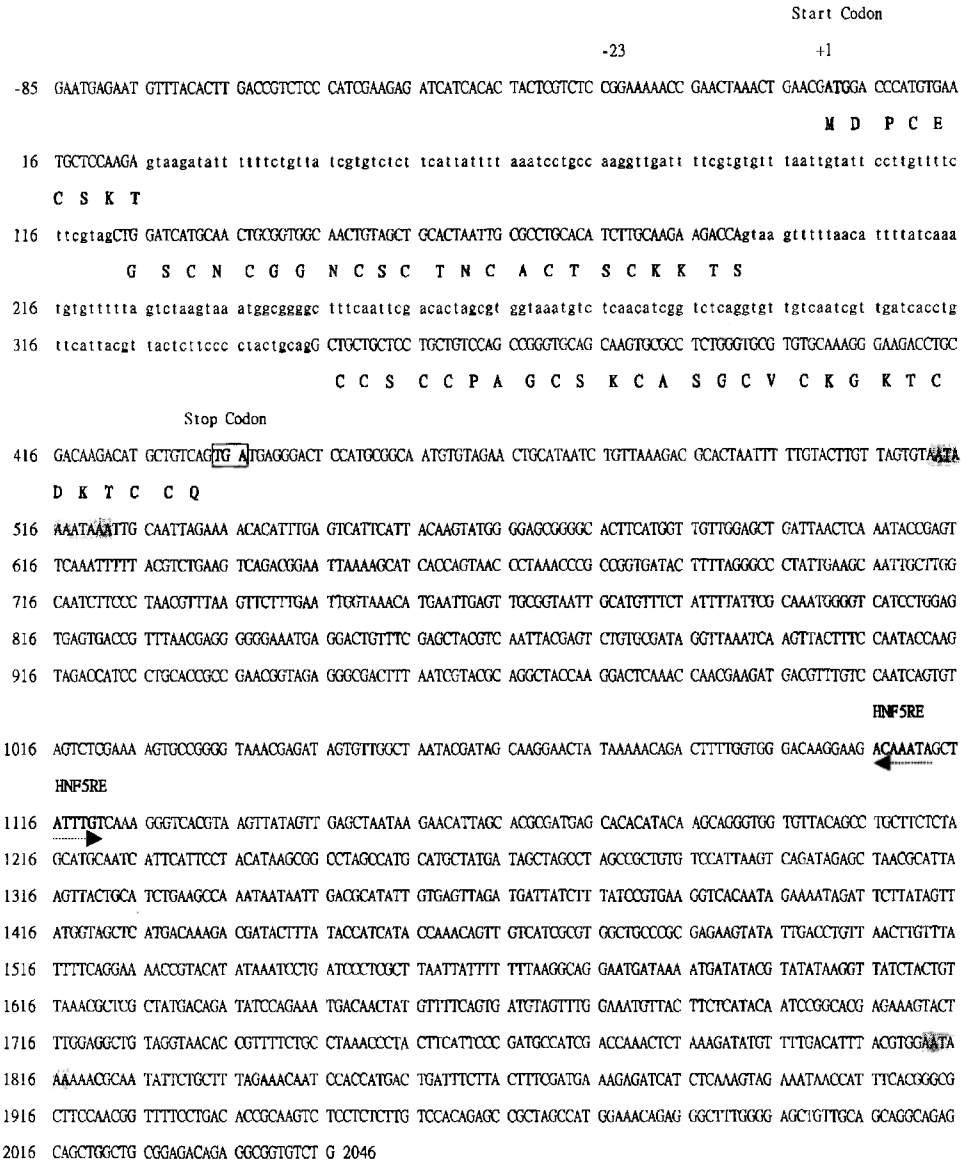

Fig. 2. (*Continued*).

Alignment of fish MT amino acid sequences shows the extent of homology with that of ayu MT ranging from 75 to 90% (Fig. 4). Invariant replacements were found at positions, 11 (S–A or K or T or N), 16 (G–T or A), 17 (N–S or T), 19 (S–T or K), 21 (T–A or S or K), 24 (A–Q or S), 27 (S–T or C), 29 (K–N), 31 (T–A), 35 (S–D or P or F), 39 (A–S), 40 (G–D), 42 (S–T or P), 49 (V–I), 53 (K–N), 54 (T–S), 57 (K–S or T) and 58 (T–S). Ayu MT gene encodes 20 cysteine residues (33%), and their locations were conserved among fish MTs (Fig. 4).

3.6. Promoter activity analyses

The promoter region of the ayu MT gene, spanning 2.5 kb was cloned in the pEGFP-1 vector and

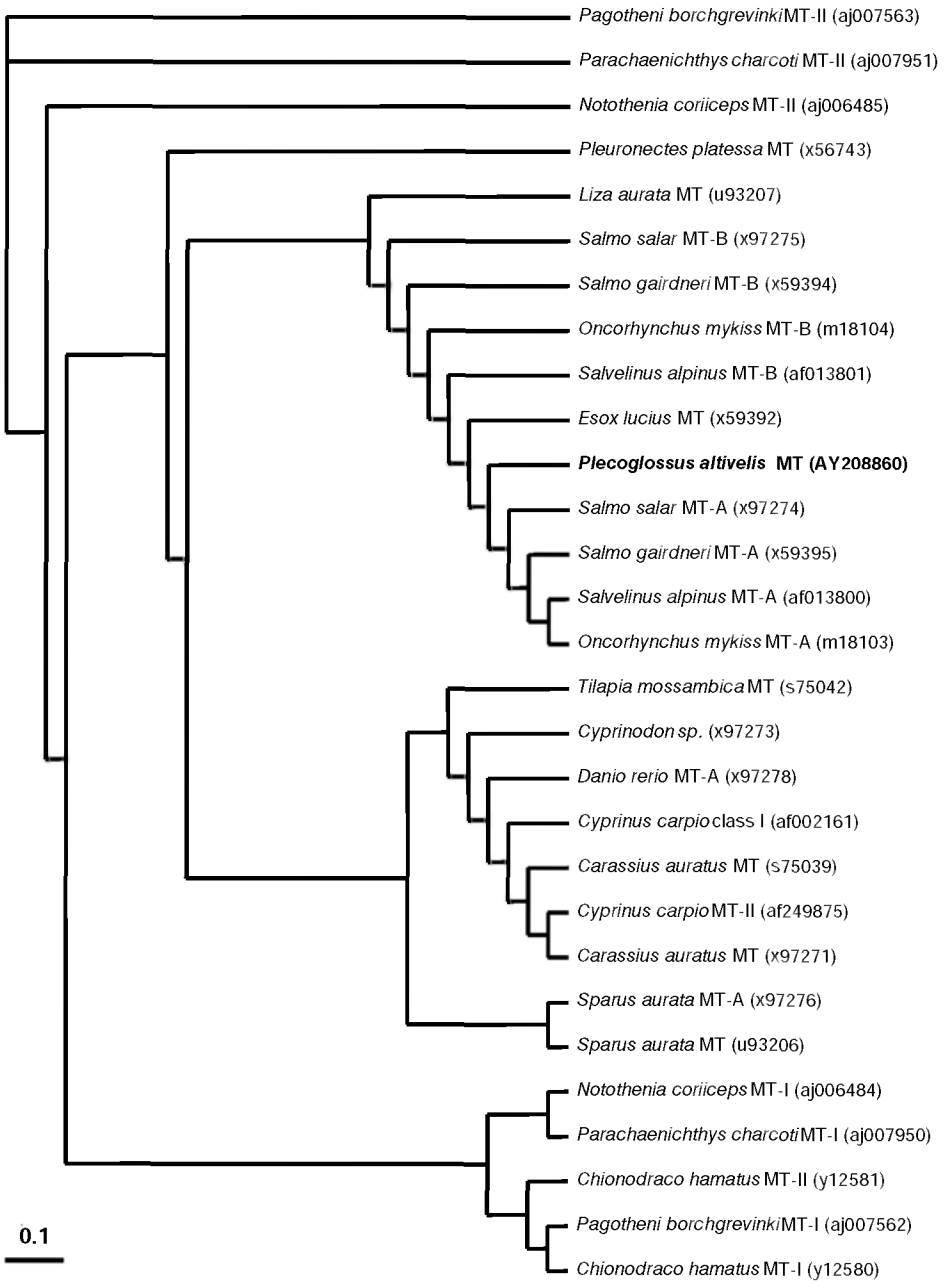

Fig. 3. Phylogenetic analysis of fish MTs. Accession number for each fish species is given in the parenthesis.

transferred into the NIH 3T3 and five grouper cell lines, GL, GK, GF, GH, and GSB. Green fluorescence signal was observed in all the transfected cells (Fig. 5). In NIH 3T3 cells, the pEGFP-a$MT_{2.5}$ showed stronger intensity. After the addition of $ZnCl_2$, relative fluorescence was greater. Similar phenomenon was also observed in grouper cell lines. The GL, GK, GF, and GH cells showed higher level of promoter activity than GSB cells. However, $ZnCl_2$ treatment induced the promoter activity

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                          1         11        21        31        41        51        61 Identity(%)
P. altivelis MT           MDPCECSKTGSCNCGGNCSCTNCACTSCKKTSCCSCCPAGCSKCASGCVCKGKTCDKTCCQ
S. aurata MT-A            ..........T.....S.T....S......-.........................TS...    90
S. salar MT-B             ................S.K.A.........-...P...S.................TS...    89
E. lucius MT              ................S.K.S.........-.......S.........I.......TS...    89
S. gairdneri MT-A         ................S.K.S.........A...D...S.................TS...    87
S. gairdneri MT-B         ................S.K.S.........-...P...SD................TS...    87
O. mykiss MT-A            ................S.K.S.........A...D...S.................TS...    87
O. mykiss MT-B            ................S.K.S.........-...P...SD................TS...    87
S. alpinus MT-A           ................S.K.S.........A...D...S.................TS...    87
S. alpinus MT-B           ................S.K.S.........-...P...SD................TS...    87
S. salar MT-A             ................S.K.S.........A...D...S.................TS...    87
L. aurata MT              ..........K.S...S.T....S......-...P...S.................TS...    85
N. coriiceps MT-II        ........S.T.....S.T....S......-...P...S..T..............TS...    84
P. borchgrevinki MT-II    ........S.T.....S.T....S......-...P...S..T..............TS...    84
P. charcoti MT-II         ........S.T.....S.T....S......-...P...S..T..............TS...    84
T. mossambica MT          ......A...T.....S....K.S.K....-...D...S.................TS...    84
C. hamatus MT-II          ....D...S.T.....S.T....S......-...P...S..T..............TS...    82
Cyprinodon sp. MT-A       ..........K....TS.T....S.KC...-.......S.............NS...S...    82
N. coriiceps MT-I         ........S.T.....S.T....S.K....-...P...S..T..............TS...    82
P. charcoti MT-I          ........S.N.....S.T....S.K....-...P...S..T..............TS...    82
C. hamatus MT-I           ....D...S.T.....S.T....S.K....-...P...S..T..............TS...    80
P. borchgrevinki MT-I     ....D...S.T.....S.T....S.K....-...P...S..T..............TS...    80
P. platessa MT            ..........T.....S.T.K..S..T.N.-...P...S..P..............TS...    80
D. rerio MT-A             ......A...A....AT.K....Q..T...-.......S.............NS.GTS...    79
C. auratus MT[a]          ....D.A...A....AT.K....Q..T...-.......S.............NS.GSS...    77
C. carpio MT-II           ....D.A...T....AT.K....Q..T...-.......S.............NS.GSS...    77
C. auratus MT[b]          ......A...A....AT.K....Q..T...-...F...S...........N.NS.GSS...    75
C. carpio MT-I            ....D.A...T....AT.K....Q..T...-...P...S.............NS.GSS...    75
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Fig. 4. Alignment of fish MT amino acid sequences. Dots demonstrate the homology. Hyphens fill the gaps for optimal alignment. Appropriate amino acid is shown, where substitutions occur. The a and b are different amino acid sequences of MT (accession numbers: X97271 and S75039, respectively), from same species.

to a significant level in all the grouper cell lines (Fig. 5).

A series of deletion mutants of the 5′-flanking promoter region were fused into pSEAP2-Basic vector (Fig. 6A) and transfected into GH and GK cell lines (Fig. 6B). The pSEAP2-aMT$_{2.5}$ construct containing 8 MREs showed significant induction in response to Zn and produced a stronger signal in GH cells, whereas

the extent of induction decreased as the fragment size of the promoter region was reduced. The smallest 0.2 kb fragment, pSEAP2-aMT$_{0.2}$ also showed activity, but the signal was less intensive than that of 2.5 kb fragment. After the addition of $ZnCl_2$, the expression increased significantly. In contrast, although the induction is very obvious, no difference of non-induced and induced expression was observed among different deletion clones in the GK cell line.

4. Discussion

MT has been used as a molecular marker to monitor exposure to heavy metals in aquatic animals (Benson et al., 1990; Couillard, 1997; AETE, 1999). Metals have been shown to induce MT in rainbow trout (*Salmo gairdneri*), flounder (*Platyichthys flesus*), Arctic char (*Salvelinus alpinus*) and Pacific oyster (*Crassostrea gigas*) (Roch and McCarter, 1984a,b; Sulaiman et al., 1991; Dallinger et al., 1997; Tanguy et al., 2001). MT mRNA synthesis in response to metal exposure is reported to be high in the liver of fish (George et al., 1996; Olsson, 1996). The hepatic MT level of ayu was also induced to a higher level following exposure to Zn (Fig. 1). It deserves mention that the ayu MT induction was not evident for short-term exposure of Zn, even at high concentration (100 μM), while the induction was more obvious for long-term exposure even at low concentration (10 μM). This indicates that the concentration as well as the duration of exposure influences the MT induction. The ayu MT expression was not significant even at induced condition in stomach revealed that, the expression might be tissue-specific.

In this report, we also described the molecular cloning, structure and promoter activity of ayu MT gene. Our results revealed that the ayu MT gene has a tripartite exon–intron similar to that of other fish MT genes, although minor differences may be found (Hamer, 1986). The splicing junctions follow the GT–AG rule (Breathnach and Chambon, 1981) as they are conserved among vertebrates (Hamer, 1986). Intron 1 of ayu MT is 69% AT-rich, which is same as that of rainbow trout MT-B gene and intron 2 of ayu MT and rainbow trout MT-B are 59 and 61% AT-rich, respectively (Zafarullah et al., 1988). The ayu MT gene encodes 20 cysteine residues (33%), which is a conserved property of MT proteins.

The 5′-flanking region of ayu MT gene contains proximal as well as distal clusters of forward and reverse putative MRE sequences. It has been reported that a pair of complementary MRE sequences are consistently located between 40 and 120 nucleotides prior to the transcription start point and are identified as the major promoter elements involved in MT gene expression (Stuart et al., 1985). Interestingly, the ayu MT gene contains two MREs at the proximal region of the ayu MT promoter and may be involved in activating transcription. However, additional MRE sequences, two in forward and four in reverse orientations were located distal from the TATA box. These elements may also be involved in enhancing the expression level. We also found two HNF5REs in 5′-flanking region and two HNF5REs in 3′-flanking region. These elements facilitate the binding of a liver-specific nuclear factor, HNF5 (Grange et al., 1991). But their functional significance on MT expression is yet to be confirmed. Only one CRE was identified in the 5′-flanking region. It has been demonstrated that innumerable cellular promoters are cAMP responsive, which usually contain CRE element within 100 bp of the TATA box and the CRE is less active if it present further upstream of the promoter (Lee and Masson, 1993; Mayr and Montminy, 2001). In ayu MT promoter, the CRE is located at −1051 bp from the TATA box. However, it deserves further investigation to figure out the influence of this element on MT expression.

Induction of fish MT promoter activity in response to heavy metals has been reported previously (Bonham et al., 1987; Samson and Gedamu, 1995, 1998). The activity of ayu MT promoter was assessed qualitatively, by constructing a plasmid containing a 2.5 kb fragment of the ayu MT promoter region linked to a jellyfish EGFP reporter gene. Transfection of this plasmid into NIH 3T3 and grouper cells, and treatment with $ZnCl_2$ induced expression of MT in all the cells. To appreciate the extent of promoter activity as well as the regulative participation of the different putative sequences in non-induced and induced expression, we constructed different recombinant plasmids, containing portions of 5′-flanking region of ayu MT gene, fused to the pSEAP2-Basic vector and transferred into GH and GK cells. In GH cells, the level of MT expression was decreased proportionally as the size of the

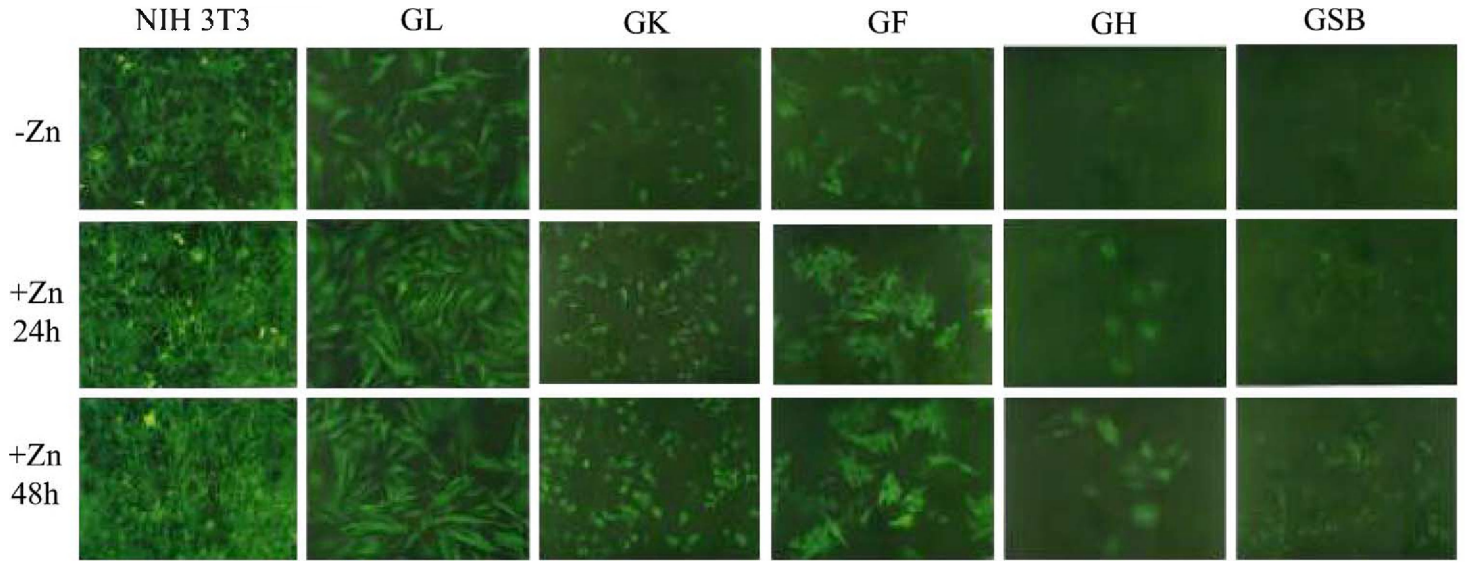

Fig. 5. Tissue-specific expression of ayu MT promoter. An EGFP reporter construct with 2.5 kb fragment of ayu MT promoter region was transfected into NIH 3T3 and grouper cell lines, GL, GK, GF, GH and GSB. Effect of Zn (100 μM) treatment on MT promoter activity is demonstrated.

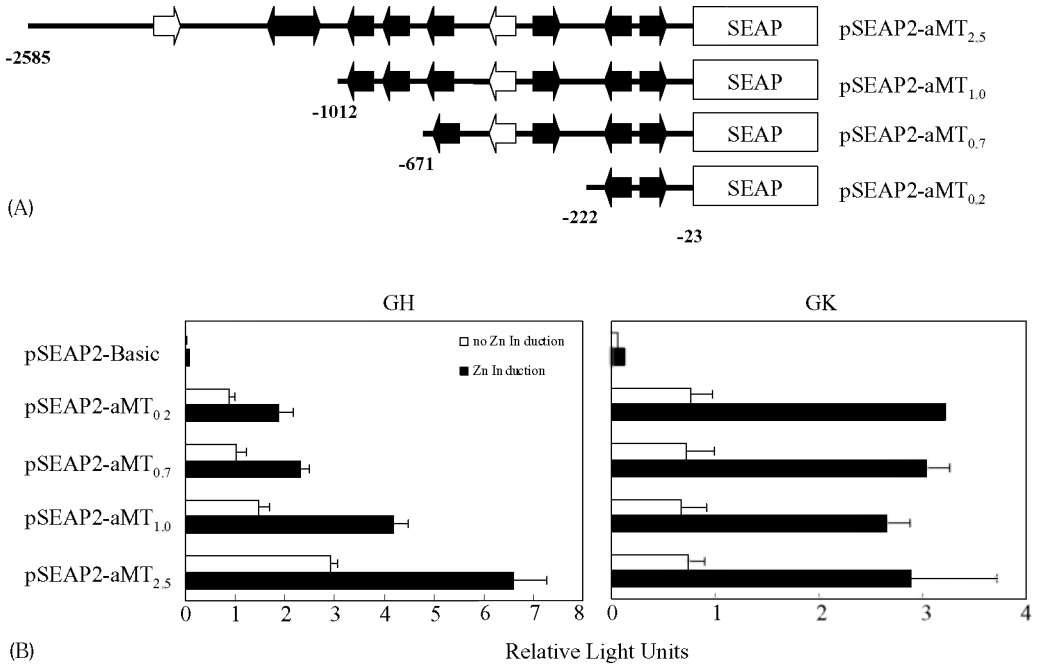

Fig. 6. Deletion analysis for ayu MT promoter activity. (A) pSEAP2-aMT$_{0.2}$, pSEAP2-aMT$_{0.7}$, pSEAP2-aMT$_{1.0}$ and pSEAP2-aMT$_{2.5}$ are a series of progressive 5′-deletion clones, constructed using a vector, pSEAP2-Basic with secreted alkaline phosphatase (SEAP) as reporter. The putative MREs (➡) and HNF5REs (⊣) are indicated. (B) Transfection of the deletion clones into GH and GK cell lines. MT promoter activity with and without Zn (100 μM) treatment is shown. Error bars indicate the standard deviations from triplicate estimations.

promoter region decreased. This shows that, all the putative elements are required for maximal MT expression. However, in GK cells, deletion of the promoter region does not influence the level of MT expression. Interestingly, the proximal two MREs are more than sufficient to produce MT expression.

MREs are highly conserved across vertebrates, indicating similar regulatory protein factors (for example, metal responsive transcription factor, MTF-1) are present in most species that bind to them (Cserjesi et al., 1992; Chen et al., 2002). The promoter of ayu MT gene was actively transcribed and induced in NIH 3T3 and grouper cells. This implies that, the ayu MT promoter could be used to express novel genes in transgenic fish.

In conclusion, the data presented in this report demonstrate the response of ayu MT to zinc in various tissues as well as in different cell lines. However, further study is essential to establish the use of this probe as a biomarker, including field studies to understand the extent of accumulation of various heavy metals in different tissues, their biological effect, and the target specificity of metals on the organism concerned.

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References

AETE, 1999. Aquatic Effects Technology Evaluation (AETE). Program Synthesis Report of Selected Technologies for Cost-Effective Environmental Monitoring of Mine Effluent Impacts in Canada. Prepared by ESG International Inc., Guelph, Ontario, p. 118.

Benson, W.H., Baer, K.N., Watson, C.F., 1990. Metallothionein as a biomarker of environmental metal contamination: species-dependent effects. In: McCarthy, J.F., Shugart, L.R. (Eds.), Biomarkers of Environmental Contamination. Lews Publishers, Florida, pp. 255–265.

Bonham, K., Zafarullah, M., Gedamu, L., 1987. The rainbow trout metallothioneins: molecular cloning characterization of two distinct cDNA sequences. DNA 6 (6), 519–528.

Breathnach, R., Chambon, P., 1981. Organization and expression of eukaryotic split genes coding for proteins. Annu. Rev. Biochem. 50, 349–383.

Chan, W.K., Devlin, R.H., 1993. Polymerase chain reaction amplification and functional characterization of sockeye salmon histone H3, metallothionein-B, and protamine promoters. Mol. Mar. Biol. Biotech. 2 (5), 308–318.

Chen, W.Y., John, J.A.C., Lin, C.H., Chang, C.Y., 2002. Molecular cloning and developmental expression of zinc finger transcription factor MTF-1 gene in zebrafish, *Danio rerio*. Biochem. Biophys. Res. Commun. 291, 798–805.

Couillard, Y., 1997. Technical evaluation of metallothionein as a biomarker for the mining industry. AETE Project 2.2.1. Natural Resources Canada, Centre for Mineral and Energy Technology, Ottawa, Ont., Canada, p. 191.

Cserjesi, P., Fairley, P., Brandhorst, B.P., 1992. Functional analysis of the promoter of a sea urchin metallothionein gene. Biochem. Cell Biol. 70, 1142–1150.

Cserjesi, P., Fang, H., Brandhorst, B.P., 1997. Metallothionein gene expression in embryos of the sea urchin *Lytechinus pictus*. Mol. Reprod. Dev. 47, 39–46.

Culotta, V.C., Hamer, D.H., 1989. Fine mapping of a mouse metallothionein gene metal response element. Mol. Cell. Biol. 9 (3), 1376–1380.

Dallinger, R., Egg, M., Köck, G., Hofer, R., 1997. The role of metallothionein in cadmium accumulation of Arctic char (*Salvelinus alpinus*) from high alpine lakes. Aquat. Toxicol. 38, 47–66.

Davis, L., Kuehl, M., Battey, J. (Eds.), 1994. Basic Methods in Molecular Biology, 2nd ed. Appleton and Lange, Norwalk, p. 777.

George, S.G., Todd, K., Wright, J., 1996. Regulation of metallothionein in teleosts: induction of MT mRNA and protein by cadmium in hepatic and extrahepatic tissues of a marine flatfish, the turbot (*Scophthalmus maximus*). Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 113 (2), 109–115.

Grange, T., Roux, J., Rigaud, G., Pictet, R., 1991. Cell-type specific activity of two glucocorticoid responsive units of rat tyrosine aminotransferase gene is associated with multiple binding sites for C/EBP and a novel liver-specific nuclear factor. Nucleic Acid Res. 19 (1), 131–139.

Hamer, D.H., 1986. Metallothionein. Ann. Rev. Biochem. 55, 913–951.

Hogstrand, C., Wood, C.M., 1996. The physiology and toxicology of zinc in fish. In: Taylor, E.W. (Ed.), Toxicology of Aquatic Pollution. Cambridge University Press, Cambridge, pp. 61–84.

Hyllner, S.J., Andersson, T., Haux, C., Olsson, P.E., 1989. Cortisol induction of metallothionein in primary culture of rainbow trout hepatocytes. J. Cell Physiol. 139 (1), 24–28.

Inoue, K., Akita, N., Shiba, T., Satake, M., Yamashita, S., 1992. Metal-inducible activities of metallothionein promoters in fish cells and fry. Biochem. Biophys. Res. Commun. 185 (3), 1108–1114.

Kägi, J.H.R., Schaffer, A., 1988. Biochemistry of metallothionein. Biochemistry 27 (23), 8509–8515.

Kägi, J.H.R., Vasak, M., Lerch, K., Gilg, D.E., Hunziker, P., Bernhard, W.R., Good, M., 1984. Structure of mammalian metallothionein. Environ. Health Perspect. 54, 93–103.

Lai, Y.S., Murali, S., Ju, H.Y., Wu, M.F., Guo, I.C., Chen, S.C., Fang, K., Chang, C.Y., 2000. Two iridovirus-susceptible cell lines established from kidney and liver of grouper, *Epinephelus awoara* (Temminck & Schlegel), and partial characterization of grouper iridovirus. J. Fish Dis. 23, 379–388.

Lai, Y.S., John, J.A.C., Lin, C.H., Guo, I.C., Chen, S.C., Fang, K., Lin, C.H., Chang, C.Y., 2003. Establishment of cell lines from a tropical grouper, *Epinephelus awoara* (Temminck & Schlegel), and their susceptibility to grouper irido- and nodaviruses. J. Fish Dis. 26, 31–42.

Langston, W.J., Chesman, B.S., Burt, G.R., Pope, N.D., McEvoy, J., 2002. Metallothionein in liver of eels *Anguilla anguilla* from the Thames Estuary: an indicator of environmental quality? Mar. Environ. Res. 53, 263–293.

Lee, K.A.W., Masson, N., 1993. Transcriptional regulation by CREB and its relatives. Biochim. Biophys. Acta 1174, 221–233.

MacDonald, R.S., 2000. The role of zinc in growth and cell proliferation. J. Nutr. 130 (Suppl. 5S), 1500S–1508S.

Maroni, G., Otto, E., Lastowski-Perry, D., 1986. Molecular and cytogenetic characterization of a metallothionein gene of *Drosophila*. Genetics 112 (3), 493–504.

Mayr, B., Montminy, M., 2001. Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat. Rev. Mol. Cell Biol. 2, 599–609.

Misra, S., Zafarullah, M., Price-Haughey, J., Gedamu, L., 1989. Analysis of stress-induced gene expression in fish cell lines exposed to heavy metals and heat shock. Biochim. Biophys. Acta 1007, 325–333.

Olsson, P.E., 1996. Metallothioneins in fish: induction and use in environmental monitoring. In: Taylor, E.W. (Ed.), Toxicology of Aquatic Pollution: Physiological, Molecular and Cellular Approaches. Cambridge University Press, Cambridge, pp. 187–203.

Olsson, P.E., Kille, P., 1997. Functional comparison of the metal-regulated transcriptional control regions of metallothionein genes from cadmium-sensitive and tolerant fish species. Biochim. Biophys. Acta 1359, 325–334.

Olsson, P.E., Hyllner, S.J., Zafarullah, M., Andersson, T., Gedamu, L., 1990. Differences in metallothionein gene expression in primary cultures of rainbow trout hepatocytes and the RTH-149 cell line. Biochim. Biophys. Acta 1049, 78–82.

Price-Haughey, J., Bonham, K., Gedamu, L., 1987. Metallothionein gene expression in fish cell lines: its activation in embryonic cells by 5-azacytidine. Biochim. Biophys. Acta 908 (2), 158–168.

Ren, H.W., Itoh, N., Kanekiyo, M., Tominaga, S., Kohroki, J., Hwang, G.S., Nakanishi, T., Muto, N., Tanaka, K., 2000. Two metallothioneins in the fresh-water fish, crucian carp (*Carassius cuvieri*): cDNA cloning and assignment of their expression isoforms. Biol. Pharm. Bull. 23 (2), 145–148.

Roch, M., McCarter, J.A., 1984a. Hepatic metallothionein production and resistance to heavy metals by rainbow trout (*Salmo gairdneri*)—I. Exposed to an artificial mixture of zinc, copper and cadmium. Comp. Biochem. Physiol. C 77 (1), 71–75.

Roch, M., McCarter, J.A., 1984b. Hepatic metallothionein production and resistance to heavy metals by rainbow trout (*Salmo gairdneri*)—II. Held in a series of contaminated lakes. Comp. Biochem. Physiol. C 77 (1), 77–82.

Roesijadi, G., 1994. Metallothionein induction as a measure of response to metal exposure in aquatic animals. Environ. Health Perspect. 102 (Suppl. 12), 91–96.

Safra, J.E., 1998. Sweetfish. The New Encyclopaedia Britannica 11, 441.

Samson, S.L.A., Gedamu, L., 1995. Metal-responsive elements of the rainbow trout metallothionein-B gene function for basal and metal-induced activity. J. Biol. Chem. 270 (12), 6864–6871.

Samson, S.L.A., Gedamu, L., 1998. Molecular analyses of metallothionein gene regulation. Prog. Nucleic Acid Res. Mol. Biol. 59, 257–288.

Schroeder, J.J., Cousins, R.J., 1990. Interleukin 6 regulates metallothionein gene expression and zinc metabolism in hepatocyte monolayer cultures. Proc. Natl. Acad. Sci. U.S.A. 87 (8), 3137–3141.

Searle, P.F., Stuart, G.W., Palmiter, R.D., 1985. Building a metal-responsive promoter with synthetic regulatory elements. Mol. Cell Biol. 5 (6), 1480–1489.

Searle, P.F., Stuart, G.W., Palmiter, R.D., 1987. Metal regulatory elements of the mouse metallothionein-I gene. Experientia (Suppl.) 52, 407–414.

Stuart, G.W., Searle, P.F., Palmiter, R.D., 1985. Identification of multiple metal regulatory elements in mouse metallothionein-I promoter by assaying synthetic sequences. Nature 317, 828–831.

Sulaiman, N., George, S., Burke, M.D., 1991. Assessment of sublethal pollutant impact on flounders in an industrialised estuary using hepatic biochemical indices. Mar. Ecol. Prog. Ser. 68, 207–212.

Suzuki, K.T., Imura, N., Kimura, M. (Eds.), 1993. Metallothionein III: biological roles and medical implications. In: Proceedings of the Third International Meeting on Metallothionein, Tsukuba, Japan, December 8–10, 1992. Birkhäuser, Basel.

Tanguy, A., Mura, C., Moraga, D., 2001. Cloning of a metallothionein gene and characterization of two other cDNA sequences in the Pacific oyster *Crassostrea gigas* (CgMT1). Aquat. Toxicol. 55, 35–47.

Zafarullah, M., Bonham, K., Gedamu, L., 1988. Structure of the rainbow trout metallothionein B gene and characterization of its metal-responsive region. Mol. Cell Biol. 8 (10), 4469–4476.

Zafarullah, M., Olsson, P.E., Gedamu, L., 1989. Endogenous and heavy-metal-ion-induced metallothionein gene expression in salmonid tissues and cell lines. Gene 83 (1), 85–93.