Metallothionein-2 gene from the mandarin fish Siniperca chuatsi: cDNA cloning, tissue expression, and immunohistochemical localization

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Abstract

The metallothionein-2 (MT-2) gene was isolated from the mandarin fish, one of the most important industrial aquatic animals in China, by using rapid amplification of cDNA ends (RACE). The deduced amino acid sequence of MT-2 comprised 60 amino acids and showed approximately 62.3% identity to human metallothionein. Its promoter region was amplified by thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR). The MT-2 gene consists of 3 exons and 2 introns, extending approximately 900 bp of genomic sequence. Phylogenetic analysis clearly demonstrated that MT-2 formed a clade with fish metallothionein. The promoter region contained 5 putative metal-regulatory elements (MREs) and 1 TATA box. Real-time quantitative RT-PCR analysis revealed that MT-2 transcripts were significantly increased in the brain and gills and were stable in the muscles, liver, and trunk kidney in Cd2+-stimulated fish. Western blotting analysis demonstrated that the protein of the MT-2 gene was expressed mainly in the gills, liver, heart, trunk kidney, muscle, and intestine; it was weakly detected in the brain and head kidney. Moreover, the MT-2 protein was immunohistochemically detected in the cytoplasm in the liver and trunk kidney. All the above results revealed that the mandarin fish MT-2 would be a useful biomarker for metal pollution.

1. Introduction

Metallothioneins (MTs) are cysteine-rich intracellular metal-binding proteins (molecular weight 6–7 kDa) (Kagi and Schaffer, 1988). MTs play multifunctional roles, including homeostasis of trace elements and detoxification of poisonous heavy metals. In metal homeostasis, MTs participate in a number of biochemical processes by providing a reservoir of Cu2+ and Zn2+ in the biosynthesis of metalloenzymes and metalloproteins within the cells (Kelly et al., 1996; Jiang et al., 1998). In metal detoxifications, MTs may reduce the toxic effects of metals by debasing the ratio of the uptake of heavy metal ions peroxides into cells (Kagi and Schaffer, 1988; Kagi, 1991; Roesijadi, 2000).

In fish, exposure to sub-lethal levels of heavy metals may result in the production of MT (Klaverkamp et al., 1984), e.g., in the winter flounder (Pleuronectes americanus) (Jessen-Eller and Crivello, 1998), common carp (Cyprinus carpio), gibel carp (Carassius auratus gibelio) (De Boeck et al., 2003) and gudgeons (Gobio gobio) (Knapen et al., 2007). Since fish are susceptible to metal contamination in water, MT can be used as a biomarker of metal ion contamination. Therefore, considerable efforts have been focused on the use of MT for monitoring metal contamination in water or in fish (Hermesz et al., 2001; Van Der Oost et al., 2003; Tom et al., 2004; Amado et al., 2006; Wu et al., 2007). However, tissue MT levels and metal contents in fish are influenced by age, season, sex, and species, e.g., in the flounder (Platichthys flesus), hepatic MT had the highest levels during the autumn in a year and increased significantly with age; in the mature female squ'lifish (Holocentrus adscensionis), the MT mRNA and protein level increased at the onset of sexual maturation compared to immature females and males; the milkfish (Chanos chanos) had a higher tolerance to Cd exposure than either tilapia (Oreochromis mossambicus) or Arososchelius paradoxus (Goksøyr et al., 1996; Hylland et al., 1998; Rotchell et al., 2001; Thompson et al., 2002; Wu et al., 2007). Even difference existed between isoforms and tissues, e.g., in the zebrafish (Danio rerio), the MT-2 compared to MT-1 was more expressed at the basal level in the liver than in brain, gills or skeletal muscles (Kagi and Schaffer, 1988; Kagi, 1991; Gonzalez et al., 2006). It is, therefore, a challenge to use fish MT as a biomarker of metal exposure in field studies.

In human and other mammals, 4 MT isoforms have been found and MT-1 and MT-2 are the major isoforms. Thus far, only 2 MT isoforms (MT-1 and MT-2) have been found and could be induced by exposure to metal ions in many species of fish such as the common carp (C. carpio) (Hermesz et al., 2001), icefish (Chionodraco hamatus) (Scudiero et al., 2001), and pike (Esox lucius) (Kille et al., 1993). In mammals, MT-1 is polypeptides with a higher degree of cysteine conservation compared to MT-2 (Miles et al., 2000). The differences...
between MT-1 and MT-2 could be detected by many methods, e.g., in capillary electrophoresis, MT-1 had a different migration in comparison with MT-2 (Richards and Beattie 1995). In common, lysine instead of threonine at position 26 simply distinguishes MT-1 from MT-2 in the metallothionein gene structures of many species of fish (Carginale et al., 1998; Scudiero et al., 2001; Yan and Chan, 2004). Even if the amino acid sequence of MT isoforms might differ, in general, their cysteine content and metal-binding capacity does not vary (Winge et al., 1984; Ebadi and Babin, 1989).

In China, the mandarin fish Siniperca chuatsi has a relatively high market value and is widely cultured throughout the country; it is also important in stocking fisheries in lakes and reservoirs (Liu and Cui, 1998). However, it is susceptible to aquatic environmental changes and has a low survival in polluted water (Zhang and Zhao, 1999). Despite the economic importance of the fish and the demands of the aquaculture environment, little research has been carried out on the relationship of the mandarin fish and water pollution, for example, heavy metal contamination. Here, we report the isolation of MT cDNA clones from stimulated liver of mandarin fish, using rapid amplification of cDNA ends (RACE) according to cDNA sequences from other Perciformes fish. The gene and promoter were successfully obtained using polymerase chain reaction (PCR) and thermal asymmetric interlaced PCR (TAIL-PCR) (Liu et al., 1995; Liu and Whittier, 1995) respectively. Further, changes in the expression levels of transcripts after exposure to CdCl2 were examined, and the distribution of MT-2 respectively. Changes in the expression levels of transcripts in different tissues was also analyzed by Western blotting and immunohistochemical staining.

2. Materials and methods

2.1. Fish RNA extraction, reverse transcription, PCR amplification, and cDNA cloning

Prior to stimulation, mandarin fish weighing approximately 200 g each were maintained for 2 weeks in an aquarium containing aerated water at about 25 °C (water hardness was 126 mg/L of calcium carbonate, pH 6.85, and turbidity 0.38 NTU). After the fish were exposed in 1 mg/L Cd2+ (2.03 mg/L CdCl2×2H2O) for 7 days, the fish were anesthetized by MS-222 (Sandoz, Basel, Switzerland) and sacrificed. Total RNA from the liver tissue was extracted with Trizol (Invitrogen, Carlsbad, USA), according to the manufacturer's instruction. Approximately 5 µg RNA was reverse transcribed by PowerScript™ reverse transcriptase with coding sequences (CDS) and SMART II primer (SMART RACE cDNA Amplification Kit, Clontech, Mountain View, CA, USA) at 42 °C for 70 min, followed by treatment at 72 °C for 15 min. Next, first-strand cDNA was used as a template for amplifying the internal region by PCR with the special primers that were designed by Primer 5.0 software based on the conserved sequences of MT homologues from Dicentrarchus labrax (GenBank accession no. AF199014) and Morone saxatilis (CA969561) (Table 1). The PCR cycling conditions were 94 °C for 5 min; 30 cycles at 94 °C for 40 s, 62 °C for 40 s, and 72 °C for 40 s; followed by 1 cycle at 72 °C for 10 min. The products were isolated using the Gel Extraction Kit (Omega, Stamford, USA), cloned into the pMD18-T vector (Takara, Tokyo, Japan), and transformed into Escherichia coli strain DH5α-competent cells, according to the manufacturer's instruction. Putative clones were screened by PCR using the above primers under the same conditions, and the selected clones were sequenced using the dyeodeoxy chain-termination method on an automatic 3730xl DNA Analyzers (ABI, Foster City, CA, USA). To obtain the full-length CDS sequence, 5’ RACE and 3’ RACE were performed using the genespecific primers and adaptor primers (UPM) listed in Table 1. The PCR cycling conditions were 1 cycle at 94 °C for 5 min; 10 cycles at 94 °C for 40 s, 67 °C for 40 s, and 72 °C for 40 s; 25 cycles at 94 °C for 40 s, 62 °C for 40 s, and 72 °C for 60 s; 25 cycles at 94 °C for 40 s, 62 °C for 40 s, and 72 °C for 60 s; followed by 1 cycle at 72 °C for 10 min. The purified fragments were then cloned and sequenced.

2.2. Sequence analysis

Protein prediction was performed using the software at the ExPASy Molecular Biology Server (http://expasy.org/). The putative CDS were analyzed for the presence of signal peptides, using the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). Multiple sequence alignments were performed using the CLUSTALW 1.83 program. A phylogenetic tree was constructed based on the CDS covering all the major groups of fish MTs, using the Neighbor-Joining (N-J) algorithm of PHYLIP version 3.66. The reliability of the tree was assessed by 1000 bootstrap repetitions.

Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Application</th>
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<tbody>
<tr>
<td>Upm (long)</td>
<td>CTAATAGCACTAGTATAGGCTGGTATCAACGCGAGGAGT</td>
<td>Race-PCR</td>
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<tr>
<td>Upm (short)</td>
<td>CTAATAGCACTAGTATAGGCG</td>
<td>Universal primers mix</td>
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<tr>
<td>MTF1</td>
<td>TGGACACCTGAACTGCGATC</td>
<td>Conserved region cloning</td>
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</tr>
<tr>
<td>MNSP5</td>
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</tr>
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<td>MNPS50720</td>
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<td></td>
</tr>
<tr>
<td>MTR2</td>
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</tr>
<tr>
<td>NGSP3</td>
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</tr>
<tr>
<td>NGSP3</td>
<td>AGCTGGTCGTGATGCTGCTCGT</td>
<td>5’ RACE 1st round PCR</td>
</tr>
<tr>
<td>NGSP3</td>
<td>AGCTGGTCGTGATGCTGCTCGT</td>
<td>5’ RACE 2nd round PCR</td>
</tr>
<tr>
<td>NGSP3</td>
<td>AGCTGGTCGTGATGCTGCTCGT</td>
<td>3’ RACE 1st round PCR</td>
</tr>
<tr>
<td>NGSP3</td>
<td>AGCTGGTCGTGATGCTGCTCGT</td>
<td>3’ RACE 2nd round PCR</td>
</tr>
<tr>
<td>NGSP3</td>
<td>AGCTGGTCGTGATGCTGCTCGT</td>
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<td>Reverse0822</td>
<td>ATTCAGCTAGTGGCACAGAG</td>
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<td>TAIL-PCR arbitrary primer</td>
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<tr>
<td>AD2</td>
<td>NGTCGAGC/T(A/T)/CGA/GA/T</td>
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</tr>
<tr>
<td>AD3</td>
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<tr>
<td>F80501</td>
<td>CCAGTACATGACCTCTCCAC</td>
<td>TAIL-PCR 2nd round</td>
</tr>
<tr>
<td>F80501</td>
<td>CCAGTACATGACCTCTCCAC</td>
<td>Expression in E. coli</td>
</tr>
<tr>
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</tr>
<tr>
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<td>Real-time PCR</td>
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<tr>
<td>realF2</td>
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</tr>
</tbody>
</table>

Note. a Fragment obtained from the two reactions compose the entire 3’ sequence.
2.3. Cloning of the MT genomic sequence and promoter region

Genomic DNA was purified from muscle by the phenol–chloroform method (Sambrook and Russell, 2001). Based on the resultant MT-2 mRNA sequence, primers were designed to yield the full sequence of the corresponding DNA. The 5′ flanking region was obtained using a TAIL-PCR approach (Liu et al., 1995; Liu and Whittier, 1995). All primers are shown in Table 1, and the PCR cycling conditions are listed in Table 2. The sequence of the 5′ flanking region was analyzed in relation to the characters of MT promoters from other fish, as reported in previous articles (Ghoshal and Jacob, 2001; Scudiero et al., 2001; Mayer et al., 2003; Chen et al., 2004; Yan and Chan, 2004; Ren et al., 2006; He et al., 2007).

2.4. Real-time quantitative RT-PCR

Apparantly healthy mandarin fish, weighing approximately 150 g, were acclimatized for 10 days in an automatic aerated water-circulating system at room temperature. Subsequently, they were divided into 2 groups (3 fish per group), with 1 group exposed in 1 mg/L Cd^{2+} (2.03 mg/CdCl_{2}×CdCl_{2}·2.5H_{2}O) and another group as the control. Fish were sampled on day 8. Total RNA from the brain, gill, liver, trunk kidney, and muscle from both groups was extracted using Trizol reagent, as described by the manufacturer (Invitrogen, Carlsbad, CA, USA). After treatment with RNa-seafree DNase, 1 μg RNA from different tissues was individually reverse transcribed with Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania). All cDNA samples were stored at −20 °C until used in real-time PCR assays. Quantitative real-time PCR was conducted on a Chromo4 Real-time Detection System (MJ Research, Waltham, MA, USA). Amplifications were carried out at a final volume of 20 μL, containing 10 μL of 50-fold diluted cDNA template, 10 μL SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, Japan), 1.0 μL of each primer, and 7.0 μL ddH_{2}O, following the manufacturer’s instructions. The β-actin and MT-2 Real-time PCR primers are listed in Table 1. PCR amplification was performed in triplicate wells, using the following conditions: 5 min at 94 °C, followed by 45 cycles of 20 s at 94 °C, 20 s at 58 °C, and 30 s at 72 °C. The 2 groups were analyzed in triplicate, and the results were expressed as the relative fold of the expression of the β-actin gene with the 2−ΔΔCT method (Livak and Schmittgen, 2001). The statistical analysis was based on comparisons of relative expression ratio of MT-2 gene to the β-actin gene between the control and induction groups by calculating 2−ΔΔCT. Mean±SD (standard deviation) represents relative expression ratio.

### Table 2

Using TAIL PCR to amplify the promoter sequence of mandarin fish MT-2 gene

<table>
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<th>Reaction</th>
<th>File no.</th>
<th>Cycle no.</th>
<th>Thermal settings*</th>
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</thead>
<tbody>
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<td>1</td>
<td>1</td>
<td>92 °C 3 min; 95 °C 1 min;</td>
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<tr>
<td></td>
<td>2</td>
<td>5</td>
<td>94 °C 30 s; 60 °C 1 min; 72 °C 2 min;</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1</td>
<td>94 °C 30 s; 25 °C 3 min; ramping to 72 °C</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>10</td>
<td>Over 3 min;</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>12^a</td>
<td>72 °C 3 min;</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>94 °C 30 s; 44 °C 1 min; 72 °C 2 min;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>94 °C 30 s; 60 °C 1 min; 72 °C 2 min;</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1</td>
<td>72 °C 3 min;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>94 °C 30 s; 60 °C 1 min; 72 °C 2 min;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>94 °C 30 s; 44 °C 1 min; 72 °C 2 min;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>72 °C 5 min.</td>
</tr>
<tr>
<td><strong>Secondary</strong></td>
<td>1</td>
<td>1</td>
<td>92 °C 3 min; 95 °C 1 min;</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>12^a</td>
<td>94 °C 30 s; 60 °C 1 min; 72 °C 2 min;</td>
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<td>94 °C 30 s; 60 °C 1 min; 72 °C 2 min;</td>
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<td>94 °C 30 s; 44 °C 1 min; 72 °C 2 min;</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1</td>
<td>72 °C 5 min.</td>
</tr>
</tbody>
</table>

Note: *The program files in each reactions were linked automatically. ^These are nine-thermal-segment super cycles consisting of two high-stringency and one reduced-stringency.

Student’s t-test was performed using Microsoft® Excel 2003 with *P<0.05 as the significance level.

2.5. Production of fusion proteins and polyclonal antibodies

The expression primers were designed to include a HindIII site within the sense primer and a KpnI site within the antisense primer and used to amplify all the 60 amino acids. The primers are listed in Table 1. PCR amplifications were performed using 1 cycle at 94 °C for 5 min; 10 cycles at 94 °C for 45 s, 53 °C for 45 s, and 72 °C for 45 s; 25 cycles at 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 50 s; followed by 1 cycle at 72 °C for 10 min. The purified fragment was digested with KpnI and HindIII, ligated to the pET-32a expression vector for constructing recombinant proteins, and transformed into DH5α competent cells. After sequencing the positive clones to ensure in-frame insertion, the pET-32a-MT-2 construct was transformed into E. coli BL21 (DE3) strain for protein expression. The fusion protein was expressed by isopropyl-beta-D-thiogalactopyranoside (IPTG) induction and analyzed with 15% SDS-polycrylamide gel (SDS-PAGE).

To prepare the polyclonal antibodies, IPTG was added in a final concentration of 1 mM when the culture reached OD_{600}=1.0. After culturing for 12 h at 28 °C, the cells were harvested by centrifugation and disrupted by sonication. The recombinant protein was purified by affinity chromatography in a column of Ni^{2+}-charged resin (Novagen, Darmstadt, Germany). Recombinant Hisş-MT-2 protein was eluted from the resin containing 20 mM Tris–HCl (pH 7.9), 300 mM imidazole, and 0.5 M NaCl. The purity and concentration of the recombinant protein were assessed on a 12% SDS-PAGE gel. We used 3 mg recombinant protein to immunize rabbits and mice in order to generate polyclonal antibodies. The specificity of the polyclonal antibodies was evaluated by Western blotting analysis and immunohistochemical detection.

2.6. Western blotting analysis

For identifying the protein expression of MT-2, 100 mg tissue samples from the gill, liver, heart, trunk kidney, muscle, head kidney, brain, spleen, and intestine of the mandarin fish were washed with phosphate-buffered saline (PBS) and then homogenized with ice-cold extraction buffer [MEB, 100 mM sodium β-glycerophosphate, 15 mM MgCl_{2}, 20 mM EGTA, 1 mM dithiothreitol (DTT), 2 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM HEPES, 2.5 μg aprotinin, and 2.5 μg leupeptin; pH 7.5]. The homogenate was centrifuged at 120,000 g for 30 min, and the supernatant containing the proteins was retained. Protein concentrations were determined using the Bradford protein assay (Biorad, Hercules, CA, USA). Each sample, equivalent to 5 μg total protein, was run on 16.5% Tricine–SDS-PAGE gel electrophoresis and subsequently transferred to a 0.22-μm PVDF membrane (Millipore, Temecula, CA, USA) (Schagger and Von Jagow, 1987; Mizzen et al., 1996; Schagger, 2006). Following transfer, the primary membrane was incubated in 2.5% glutaraldehyde in water for 1 h and then washed 3 times in PBS for 5 min. The blotting membrane was blocked with 5% dry milk for 2 h and then incubated overnight at room temperature in TBST buffer containing 5% milk with rabbit antisera (1: 500). After washing 3 times for 10 min, the membrane was further incubated for 2 h with 1:2000 diluted alkaline phosphatase-conjugated goat anti-rabbit IgG (Sino-American Biotechnology Company, Beijing, China). After washing 3 times for 10 min in TBST buffer, detection was performed using BCIP/NBT staining. Samples from different organs were divided into 2 groups: control and positive.

2.7. Immunohistochemical identification of MT-2 using mouse antiserum

Paraffin-embedded, 4-μm-thick sections of the liver and trunk kidney were obtained by the standard techniques. Sections were mounted on aminopropyltriethoxysilane-treated slides. The slides...
were routinely deparaffinized with xylene and rehydrated with a series of ethanol washes. Endogenous peroxidase was blocked by incubation with 0.3% hydrogen peroxide in absolute methanol. After washing with PBS, the sections were subjected to antigen retrieval in boiling sodium citrate buffer (0.01 mM; pH 6.0) for 10 min in a microwave oven set at 95 °C. After cooling for 20 min and washing 3 times with 0.1 M Tris–HCl, 5% BSA was added, and the sections were incubated for 20 min. Mouse antiserum was diluted 1:200 and added to slides. The slides were then maintained at 4 °C overnight, following which they were again washed 3 times. The slides were further incubated with a biotinylated anti-IgG antibody – with streptavidin (DAB, KPL) was added to the organ sections, followed by incubation for 10 min without direct light. The slides were washed 3 times with reagent quality water and then counterstained with Mayer’s hematoxylin (Boster) for 10 min and washed in running water.

Gradient ethanol was utilized for dehydration, and dimethylbenzene, for clearing the sections, which were then mounted in neutral resin. The slides were observed under a Zeiss Axioplan 2 imaging microscope (Carl Zeiss MicroImaging, Inc. Thornwood, NY, USA). The controls were treated with non-immune serum instead of antiserum.

3. Results

3.1. Cloning and characterization of the mandarin fish MT-2 gene

The mandarin fish MT mRNA is 388 nt in length (GenBank accession no. EU258624), containing a 183-nt CDS. The 5′ untranslated region (UTR) and 3′ UTR are 49 and 156 nt respectively. The polyadenylation signal (AAATAA) is found starting at 18 nt upstream from the polyA tail. The putative MT is predicted to be a peptide of 60 amino acids, with a calculated molecular weight of 5.982 kDa and an isoelectric point of 8.24. The SignalP 3.0 software did not predict any signal peptide. The putative MT presented a vertebrate MT signature as determined by the ScanProsite programs in the Prosite database (http://ca.expasy.org/prosite). The deduced amino acid sequence of the MT is 83.61% and 85% identical to trout MT1 and MT2 (P52722 and NP_919249). The identities with other reported MTs are 54.84% to frog MT9A, 65.57% and 56.02% to mouse MT1 and MT2 respectively, 63.93% to pig MT1 and MT2, 62.3% to human MT1 and MT2, and 63.45% and 57.14% to chicken MT1 and MT2 respectively, as determined via alignments of amino acid sequences (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=NPSA/npsa_clustalw.html) (Fig. 1). Moreover, the total number of cysteine residues (20; 33.33%) and their locations were highly conserved.

In the phylogenetic tree based on the MT nucleotide sequences from 26 fish species, the mandarin fish MT was clustered with those of S. saali and D. labrax MTB (AF091100 and AF199014). Meanwhile, mandarin fish MT was considerably nearer to that of the Perciformes fish than to those of fishes in other Orders (Fig. 2). Moreover, as one kind of Perciformes fish species the presence of threonine instead of lysine at position 26 suggested that this MT gene expressed the MT-2 isoform (Cargnale et al., 1998; Scudiero et al., 2001; Yan and Chan, 2004).

**Fig. 1.** Alignment of mandarin fish MT amino acid sequences with pig, human, mouse, chicken, frog, trout and zebrafish. *Represents the complete sequence identity, shaded black indicates the conserved cysteine residues. GenBank accession numbers for the sequences are as follows: zebrafish: MT1: P52722, MT2: NP_919249; trout MTA: P68503, MTE: P68501; frog: MTA: AAB60616; chicken: MTA: BAF51974, MT2: NP_590606; human: MTA: NP_005937, MT2: NP_005944; mouse MT1: P02602, MT2: P02798; pig MTA: PA8068, MTE: P66879.
3.2. Gene structure and promoter region of MT

The amplified MT-2 genomic sequence is 897 bp in length (EU258623). From the first transcription–initiation site, the nt of the mandarin fish MT-2 gene extends up to the end of the 3′ UTR. Sequence analyses of the zebrafish MT-2 gene and its cDNA revealed that the coding region is interrupted by 2 introns of 129 and 454 nt at nucleotide positions 26 and 222, respectively. Intron 1 is 38.76% GC-rich and intron 2 is 39.21% GC-rich. The promoter region upstream of the mandarin fish MT-2 gene with nt extending to the end of the 5′ UTR, contains a typical TATA box (TATAAA) located from −92 to −97 bp upstream of the transcription-starting site. Five putative MREs were observed; 3 of which (a, b, and d) contained the motif TGCRCNC consensus core sequence in forward orientation, and 2 (c and e) contained the GNGYGCA sequence in reverse orientation (Fig. 3).

3.3. Expression of MT-2 mRNA following stimulation by Cd2+

In response to CdCl2×2.5H2O, the mRNA expression level significantly increased in the gill (4.18±0.13-fold, P<0.05) and brain (8.36±1.82-fold, P<0.05), while it did not significantly change in the muscle (1.20±0.41-fold, P>N0.05), liver (2.05±0.25-fold, P>0.05), and trunk kidney (0.54±0.16-fold, P>0.05) (Fig. 4).

3.4. Western blot detection of protein expression in organs

Distribution of MT-2 protein in different organs was examined by Western blotting analysis. As shown in Fig. 5(A), the 27 kDa purified recombinant protein was detected by the antibody. Further, as seen in Fig. 5(B), an immunoreactive band at approximately 14 kDa was detected with gill, liver, heart, trunk kidney, muscle, and intestine...
specimens; weakly detected with the brain and head kidney specimens; and hardly detected in the spleen specimens.

3.5. Immunohistological localization of MT in the liver and trunk kidney of fish

Immunohistochemical staining of the liver and trunk kidney of mandarin fish revealed MT-2 immunoreactivity. On staining the liver, MT was observed in the cytoplasm of hepatocytes, where MT-2 distribution was not uniform and diffused [Fig. 6(A)]. Meanwhile, immunoreactivity was also detected in the acinar cells of the pancreas, though these cells were stained slightly. In the trunk kidney, positive staining for MT-2 was detected in the cytoplasm of the collecting duct epithelium and the proximal and distal tubular epithelium [Fig. 6(B)]. No staining was observed in the nuclei of these cells. The glomeruli and vascular endothelial cells stained negatively for MT. The controls did not show a positive reaction [Fig. 6(C, D)].

4. Discussion

The present study is the first to describe the MT cDNA sequence of the mandarin fish. The mandarin fish MT-2, isolated from the liver, was highly similar to the *M. saxatilis* and *D. labrax* MTs. Comparison of the deduced amino acid sequence with MT sequences from other vertebrates revealed a conserved cysteine-rich protein, with no change in any of the 20 cysteine residues. Moreover, it showed a typical characteristic of the MT-2 gene, that is, threonine substituting lysine at amino acid position 26 (Carginale et al., 1998; Yan and Chan, 2004). In the present study, MT-1 was not found in the mandarin fish. Thus far, the function of the 2 MTs with threonine or lysine in the middle hinge of the 2 metal-binding domains remains unknown.

The gene structure and promoter region of the MTs from other vertebrates, including some species of fish have been reported (Samson et al., 2001; Scudiero et al., 2001; Chan et al., 2004; Chen et al., 2004; Yan and Chan, 2004; En et al., 2006; He et al., 2007). In the MT-2 gene of mandarin fish, 2 introns interrupted 3 exons at positions that were precisely homologous to those in the case of zebra fish (Chen et al., 2004), common carp (Chan et al., 2004) and crucian carp (Ren et al., 2006; He et al., 2007), etc. The promoter region possessed a TATA box, which was similar to that of icefish MTs (Scudiero et al., 2001), but it lacked a canonical GC box compared with the zebra fish MT-2 (Yan and Chan, 2004). In addition, all vertebrate MT promoter regions have been characterized as containing 2 or more MREs, which shows high level of conservation to the core consensus sequence “TGCRCNC” (Ghoshal and Jacob, 2001). The MT-2 promoter contains 5 MREs distributed in 2 clusters. Starting from the transcription point, the proximal cluster that is less than 200 bp includes 3 MREs, and the distal cluster that ranges between 570 and 610 bp includes 2 MREs. This promoter shares the same unique feature of the fish MT genes, e.g., icefish MTII (Scudiero et al., 2001), rainbow trout MTI (Mayer et al., 2003), crucian carp MTI (He et al., 2007) and MTII (Ren et al., 2006), whose MREs were also divided two clusters at the at the promoter region.

In previous reports, MT gene mRNA expression has often been proposed as a sensitive and efficient biomarker for evaluating the cumulative biological effects of metal exposure in the tissues of a wide

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**Fig. 6.** Photomicrograph of MT-2 immunohistological staining in normal mandarin fish liver (A) and trunk kidney (B). Negative controls (C) (liver) and (D) (trunk kidney) using the non-immune serum reveal no immunoreactivity. A: Positive hepatocyte and acinar cells in pancreas tissue. (Cy) cytoplasm; (Ac) acinar cells. B: Positive proximal and distal tubules and negative glomeruli. (Pt) proximal tubule; (Dt) distal tubule; (Gl) glomeruli. Scale bars: 50 μm.
range of aquatic species, e.g., the common carp (C. carpio) (Herzmes et al., 2001), brown trout (Salmo trutta) (Linde et al., 1999), and gudgeons (G. gobio) (Knapen et al., 2007). Further, it was considered useful for evaluating metal exposure in both laboratory and field studies (Viaengo et al., 1999; Fernandes et al., 2008) by using either common RT-PCR or real-time PCR. In the present study, mandarin fish MT-2 mRNA was significantly elevated in the gill and brain after induction by CdCl₂, which is similar to the findings of previous researches (Rising et al., 1995; Tom et al., 1998; Hansen et al., 2006; Knapen et al., 2007). Regarding other organs or tissues, the mRNA expression level slightly increased in the muscle and liver, though the differences were not statistically significant; it slightly decreased in the trunk kidney; and it did not change markedly in the liver. In general, the liver and trunk kidney are considered major organs that protect against Cd²⁺-induced injury (Hamer, 1986; Henry et al., 1994). However, in the present study, the mRNA expression level was relatively stable in the liver and trunk kidney. The possible reason is that some other factors, including gender, age, heavy metal concentration, induction time, exposure route (injection or natural exposure) and accumulated metal concentrations in tissues after fish were fed also play important roles in determining Cd²⁺ toxicity (Goksøyr et al., 1998; Hylland et al., 1998; Rotchell et al., 2001; Serafin and Biebanno, 2001; Vasconcelos et al., 2002; Knapen et al., 2007). More studies are required in order to acquire further evidence that explains the influence of all these factors.

The mandarin fish MT-2 had a ubiquitous tissue distribution with particular abundance in the gill, liver, heart, trunk kidney, and intestine; relatively low distribution in the brain and head kidney, and it was hardly detectable in the spleen. The expression differences of protein level in various tissues are possibly resulted by the variable synthesis ability of tissues induced by accumulated different metal concentrations in tissues (Knapen et al., 2007). In addition, the predicted molecular size of the mature MT-2 was approximately 6 kDa, but the actual size of the MT was approximately 14 kDa, as determined by SDS-PAGE under reducing conditions [Fig. 5B], and the actual value was more than twice the predicted value. The 14-kDa protein is probably a dimer, which is common in SDS-PAGE electrophoresis (Chatterjee and Maiti, 1987; Carpenne et al., 2007). However, more possible reason might be that the retarded migration caused by the status of MTs binding metal ions (Chyan et al., 2005). The similar molecular weight of MT-like proteins also have been reported in other fish, e.g., the channel catfish (Ictalurus punctatus) (Chatterjee and Maiti, 1987), killifish (Heterandria formosa) (Xie and Klers, 2004) and the mollusk, for example, the zebra mussel (Dreissena polymorpha) (High et al., 1997). Different expression patterns between the level of mRNA and the level of protein were observed in present study, and these may not be mutually exclusive. On the one hand, there are many complicated and varied posttranscriptional mechanisms involved in turning mRNA into protein that are not yet sufficiently well defined to be able to compute protein concentrations from mRNA; on the other hand, proteins may differ substantially in their in vivo half lives (Greenbaum et al., 2003).

Positive immunohistochemical staining for MTs has been reported in the liver and trunk kidney of a few species of fish, e.g., the carp and turbot (Kito et al., 1986; Amaral et al., 2002). The immunoreactivity findings in the mandarin fish are similar to those in the carp and turbot, but the extent and intensity of the immunohistochemical staining are lower in the mandarin fish. The less significant effect could also be due to the fact that different antibodies were used.

In conclusion, the present results suggest that the cloned MT-2 gene and its promoter structure are similar to the MT genes in other fish species. The level of transcribed mRNA increased markedly in some organs after exposure to CdCl₂, and protein expression was detected in most of the organs. Meanwhile, positive immunohistochemical staining was seen mainly in the cytoplasm in the liver and trunk kidney specimens. All these characters suggest that mandarin fish MT-2 would be a useful biomarker of metal exposure, and that MT-2 functions merit in-depth investigation.

Acknowledgments

The present study was financially supported by the Hubei Gongguan Project of Science and Technology through the grant 2007AA203A01, and by the National Basic Research Program by the project No. 2002CB412308.

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