MARINE BIOTECHNOLOGY

Shark (*Scyliorhinus torazame*) Metallothionein: cDNA Cloning, Genomic Sequence, and Expression Analysis

Young Sun Cho,[1] Buyl Nim Choi,[1] En-Mi Ha,[1] Ki Hong Kim,[2] Sung Koo Kim,[3] Dong Soo Kim,[1] Yoon Kwon Nam[1]

[1]Department of Aquaculture, Pukyong National University, Busan 608-737, South Korea
[2]Aquatic Life Medicine, Pukyong National University, Busan 608-737, South Korea
[3]Biotechnology & Bioengineering, Pukyong National University, Busan 608-737, South Korea

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Abstract

Novel metallothionein (MT) complementary DNA and genomic sequences were isolated from a cartilaginous shark species, *Scyliorhinus torazame*. The full-length open reading frame (ORF) of shark MT cDNA encoded 68 amino acids with a high cysteine content (29%). The genomic ORF sequence (932 bp) of shark MT isolated by polymerase chain reaction (PCR) comprised 3 exons with 2 interventing introns. Shark MT sequence shared many conserved features with other vertebrate MTs: overall amino acid identities of shark MT ranged from 47% to 57% with fish MTs, and 41% to 62% with mammalian MTs. However, in addition to these conserved characteristics, shark MT sequence exhibited some unique characteristics. It contained 4 extra amino acids (Lys-Ala-Gly-Arg) at the end of the β-domain, which have not been reported in any other vertebrate MTs. The last amino acid residue at the C-terminus was Ser, which also has not been reported in fish and mammalian MTs. The MT messenger RNA levels in shark liver and kidney, assessed by semiquantitative reverse transcriptase PCR and RNA blot hybridization, were significantly affected by experimental exposures to heavy metals (cadmium, copper, and zinc). Generally, the transcriptional activation of shark MT gene was dependent on the dose (0–10 mg/kg body weight for injection and 0–20 μM for immersion) and duration (1–10 days); zinc was a more potent inducer than copper and cadmium.

Key words: metallothionein — tiger shark *Scyliorhinus torazame* — heavy metals — gene expression

Correspondence to: Yoon Kwon Nam; E-mail: yoonknam@pknu.ac.kr

Introduction

Metallothioneins (MTs) are low molecular weight (6–7 kDa) cytoplasmic heavy-metal-binding proteins. These cysteine-rich proteins have several biological functions in eukaryotic organisms, including (1) metal ion homeostasis, (2) detoxification of excess reactive heavy metal ions, and (3) providing a reserve of essential metals for other metalloproteins (Hamer, 1986; Muto et al., 1999). Owing to their highly inducible expression (transcriptional activation), MTs have also been used as molecular bioindicators to monitor the heavy metal pollution of aquatic or marine ecosystem, and to investigate the adaptive response of aquatic animals to metal-induced stresses (Hamilton and Mehrle, 1986; Roesijadi, 1994; Olsson, 1996; Langston et al., 2002).

MT genes are known to be evolutionarily conserved in most vertebrates because of their key roles in a variety of enzymatic reactions (Olsson, 1993; Binz and Kagi, 1999). With interests in the molecular evolution of the MT genes among vertebrates numerous studies have been made on the structure and function of MT genes from fish, the evolutionarily lowest vertebrates. These include MT cDNA or genomic DNA genes from common carp (Hermesz et al., 2001; Chan et al., 2004), crucian carps (Ren et al., 2000), goldfish (Chan 1994), plaice (George et al., 1990), rainbow trout (Bonham et al., 1987), and ayu (Lin et al., 2004). Despite the numerous studies on fin-rayed bony fish MTs, there are few reports on the MT gene of cartilaginous fish species. The evolutionary position of these elasmobranch fishes relative to other vertebrates including advanced bony fish and mammals makes them useful model systems for studying the molecular evolution of vertebrate genes (Cho and Kim, 2002; Nam et al., 2002). Tiger shark (*Scyliorhinus torazame*) resides in the

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waters of East Asia including coastal areas of the Korean peninsula. Increasing pollution in coastal areas of South Korea caused by industrial activities gives rise to concern about the bioaccumulation of toxicants such as heavy metals in marine inhabitants, of which this shark species is thought to be one of main recipients. The objective of this study was to isolate and characterize the MT gene from tiger shark, and to examine its transcriptional responses to heavy metal exposure.

Materials and Methods

Fish Samples, RNA Isolation, and cDNA Library Construction. Live shark specimens were purchased from a local fish market and transferred to the laboratory, where tissue samples were surgically removed. Liver tissues from 3 males and 3 females were pooled, and total RNAs were extracted using the TriPure Isolation Kit (Roche Molecular Biomedical). From total RNA, the poly $(A)^+$ RNAs were purified using biotin-labeled oligo-$d(T)_{20}$ and streptavidin-coated magnetic particles (Promega) according to the manufacturer's instructions. Five micrograms of poly $(A)^+$ RNA was used as template for cDNA synthesis. All the procedures for cDNA library construction including cDNA synthesis, ligation, and packaging were performed following the protocol of λ Zap cDNA Synthesis Kit (Stratagene). The primary library (1.5×10^6 pfu/ml) was amplified to 1.0×10^{11} pfu/ml, and an aliquot of phage (5×107 pfu) was excised into plasmid pBluescript SK vector in *Escherichia. coli* SOLR cells.

Isolation of Shark MT cDNA and Genomic ORF Sequence. Of our expressed sequence tag (EST) clones identified in the liver of this species, SL0262 showed higher similarity (e-value of 1E-71) with previously known MT sequences (unpublished data) based on the BLASTx search against NCBI GenBank. A total of 6144 random bacterial clones from the liver cDNA library were arrayed on 4 nylon membranes (1536 clones per membrane) and hybridized with the digoxygenin-11-dUTP-labeled insert from EST clone SL0262 prepared using the DIG DNA Labeling and Detection Kit (Roche Applied Biosciences). Hybridization, washing, and signal detection were performed according to the manufacturer's recommendations. The clones showing hybridization-positive signals were selected and sequenced using an ABI 377 automatic sequencer (Applied Biosystems). The raw sequence data collected were analyzed with the sequence editing software, Sequencher (Version 4.0; Gene Codes). The trimmed sequences were subjected to similarity search against GenBank database (http://ncbi.nlm.nih.gov/BLAST).

Genomic open reading frame (ORF) sequence of shark MT was isolated by PCR using 2 specific primers (sMT-1F, 5′-ATGTCTGACACGAAGCCCTGTG-3′; and sMT-1R; 5′-CTGAAACATCCAGTGTGTGG-3′) designed based on the shark cDNA sequence. Genomic DNA was purified from the whole blood using the conventional sodium dodecyl sulfate (SDS) and proteinase K method (Nam et al., 2002). One microgram of genomic DNA was subjected to PCR containing 20 pmol of each primer and 0.5 U *Taq* DNA polymerase (Takara). Thermal cycling condition (30 cycles) was 94°C for 45 seconds, 58°C for 1 minute, and 72°C for 1 minute with an initial denaturation step at 94°C for 3 minutes. Reaction volume was 50 μl. The amplified product was purified using spin column (Qiagen) and cloned into pGEM-T easy vector (Promega). The recombinant clones of correct size were selected, and the inserts were sequenced using ABI377 automatic sequencer (Applied Biosystems).

Phylogenetic Analysis of Shark MT. The amino acid sequence deduced from the identified shark MT, cDNA was aligned with other MT sequences. For phylogenetic analysis a total of 119 unique MT sequences from teleosts (17 sequences for MT-A, 16 for MT-B, and 22 for unclassified MTs) and mammals (37 sequences for MT-I, 17 for MT-II, 7 for MT-III, and 3 for MT-IV) were obtained from GenBank (Table 1). Multiple alignment was carried out using CLUSTAL W (Thompson et al., 1994). Gap open penalty and gap extension penalty were set to 10 and 0.05, respectively. The weight matrix was BLOSUM (for protein). The output alignment was manually edited using the GeneDoc program (http://www.psc.edu/biomed/genedoc/) for optimum alignment. Identities between shark MT and other MTs were also calculated as percentages using the same program. The edited alignment was subjected to distance and parsimony analyses to evaluate the phylogenetic relationship of shark MT with other MT orthologues. Distance analysis was performed using CLUSTAL W or PAUP* (Version 4.0b). Unrooted phylogenetic trees were calculated using the neighbor-joining (NJ) method. Bootstrap replications (1000) were performed to obtain confidence estimates for each node in the tree. Distance was measured by mean character difference. The maximum parsimony (MP) analysis was carried out using PAUP*. Of 81 total characters (number of positions in alignment including gaps), 40 characters were parsimony-informative and gaps were treated as missing. Heuristic search was performed based on

Table 1. Metallothionein Protein Sequences used in Phylogenetic Analyses

Mammals				*Fishes*			
Label	*Species*	*MT protein*	*Accession no.*	*Label*	*Species*	*MT protein*	*Accession no.*
M1-1	*Homo sapiens*	MT-IA	NP_005937	Shark	*Scylorhinus orazame*	MT (present study)	AY605090
M1-2	*Homo sapiens*	MT-IB	NP_005938	A1	*Chaenocephalus aceratus*	MT-A	O93593
M1-3	*Homo sapiens*	MT-IE	P04732	A2	*Chionodraco hamatus*	MT-A	O13258
M1-4	*Homo sapiens*	MT-IF	NP_005940	A3	*Cyprinodon* sp.	MT-A	Q92044
M1-5	*Homo sapiens*	MT-IG	NP_005941	A4	*Cyprinus carpio*	MT-A	O13269
M1-6	*Homo sapiens*	MT-IH	NP_005942	A5	*Danio rerio*	MT-A	NP_571150
M1-7	*Homo sapiens*	MT-IJ	NP_783321	A6	*Notothenia coriiceps*	MT-A	P62339
M1-8	*Homo sapiens*	MT-IK isoform 1	P80296	A7	*Parachaenichthys charcoti*	MT-A	O93450
M1-9	*Homo sapiens*	MT-IK isoform 2	AAH28280	A8	*Oncorhynchus mykiss*	MT-A	P09861
M1-10	*Homo sapiens*	MT-IK isoform 3	NP_789846	A9	*Salmo salar*	MT-A	CAA65929
M1-11	*Homo sapiens*	MT-IL	P80297	A10	*Salvelinus alpinus*	MT-A	AAB66342
M1-12	*Homo sapiens*	MT-IM	AAL83902	A11	*Sparus aurata*	MT-A	P52727
M1-13	*Homo sapiens*	MT-IQ	AAO49186	A12	*Thermarces Cerberus*	MT-A	P52721
M1-14	*Homo sapiens*	MT-IR	Q93083	A13	*Trematomus bernacchii*	MT-A	O93609
M1-15	*Homo sapiens*	MT-IS	AAK26162	A14	*Chionodraco rastrospinosus*	MT-A	CAA09714
M1-16	*Homo sapiens*	MT-IX isoform 1	NP_005943	A15	*Gymnodraco acuticeps*	MT-A	CAA07555
M1-17	*Homo sapiens*	MT-IX isoform 2	AAH18190	A16	*Pagothenia borchgrevinki*	MT-A	CAA07558
M1-18	*Equus caballus*	MT-IA isoform 1	P02800	A17	*Carassius cuvieri*	MT-A	AAN85819
M1-19	*Equus caballus*	MT-IB isoform 1	P02801	B1	*Chaenocephalus aceratus*	MT-B	P52724
M1-20	*Equus caballus*	MT-IA isoform 2	SMHO1A	B2	*Chionodraco hamatus*	MT-B	P62711
M1-21	*Equus caballus*	MT-IB isoform 2	SMHOB	B3	*Chionodraco rastrospinosus*	MT-B	P62679
M1-22	*Sus scrofa*	MT-IA	P49068	B4	*Cyprinus carpio*	MT-B	Q9I9I0
M1-23	*Sus scrofa*	MT-IC	P79376	B5	*Danio rerio*	MT-B	NP_919249
M1-24	*Sus scrofa*	MT-ID	P79377	B6	*Dicentrarchus labrax*	MT-B	Q9PTG9
M1-25	*Sus scrofa*	MT-IE	P79431	B7	*Gymnodraco acuticeps*	MT-B	P62713
M1-26	*Sus scrofa*	MT-IF	P79378	B8	*Morone saxatilis*	MT-B	P62712
M1-27	*Ovis aries*	MT-IA	S00808	B9	*Notothenia coriiceps*	MT-B	P62680
M1-28	*Ovis aries*	MT-IB	P09577	B10	*Oncorhynchus mykiss*	MT-B	P09862
M1-29	*Ovis aries*	MT-IC	P09578	B11	*Pagothenia borchgrevinki*	MT-B	P62681
M1-30	*Bos taurus*	MT-I isoform 1	P58280	B12	*Parachaenichthys charcoti*	MT-B	P62682
M1-31	*Bos taurus*	MT-I isoform 2	P55942	B13	*Salmo salar*	MT-B	CAA65930
M1-32	*Canis familiaris*	MT-I	O19000	B14	*Salvelinus alpinus*	MT-B	AAB66343
M1-33	*Cercopithecus aethiops*	MT-I	P02797	B15	*Trematomus bernacchii*	MT-B	P62678
M1-34	*Cricetulus griseus*	MT-I	P02804	B16	*Carassius cuvieri*	MT-B	AAN85820
M1-35	*Mus musculus*	MT-I	P02802	C1	*Barbatula barbatula*	MT	P25128
M1-36	*Oryctolagus cuniculus*	MT-I	AAA31147	C2	*Carassius auratus*	MT	P52723
M1-37	*Rattus norvegicus*	MT-I	P02803	C3	*Carassius auratus*	MT	JC2419
M2-1	*Bos taurus*	MT-II isoform 1	P09579	C4	*Esox lucius*	MT	P25127
M2-2	*Bos taurus*	MT-II isoform 2	P55943	C5	*Gadus morhua*	MT	P51902
M2-3	*Bos taurus*	MT-II isoform 3	SMBO2	C6	*Gadus morhua*	MT	CAA65924
M2-4	*Mus musculus*	MT-II isoform 1	P02798	C7	*Gobiomorphus cotidianus*	MT	AAO89258
M2-5	*Mus musculus*	MT-II isoform 2	SMMS2	C8	*Ictalurus punctatus*	MT	O93571
M2-6	*Homo sapiens*	MT-II isoform 1	P02795	C9	*Lithognathus mormyrus*	MT	AAL37187
M2-7	*Homo sapiens*	MT-II isoform 2	P80295	C10	*Liza aurata*	MT	O13257
M2-8	*Sus scrofa*	MT-IIA	P79379	C11	*Oreochromis aureus*	MT	AAP14677

continues

Table 1. Continued

Mammals				*Fishes*			
Label	*Species*	*MT protein*	*Accession no.*	*Label*	*Species*	*MT protein*	*Accession no.*
M2-9	*Sus scrofa*	MT-IIB	P79380	C12	*Oreochromis mossambicus*	MT	AAP14678
M2-10	*Canis familiaris*	MT-II	Q9XST5	C13	*Oreochromis mossambicus*	MT	P52726
M2-11	*Cercopithecus aethiops*	MT-II	P02796	C14	*Oryzias latipes*	MT	AAR30249
M2-12	*Cricetulus griseus*	MT-II	P02799	C15	*Pagrus major*	MT	Q9IB50
M2-13	*Cricetulus longicaudatus*	MT-II	I48116	C16	*Perca fluviatilis*	MT	P52725
M2-14	*Mesocricetus auratus*	MT-II	P17808	C17	*Plecoglossus altivelis*	MT	AAP43669
M2-15	*Ovis aries*	MT-II	S00811	C18	*Pleuronectes platessa*	MT	S30567
M2-16	*Rattus norvegicus*	MT-II	P04355	C19	*Pseudopleuronectes americanus*	MT	P55945
M2-17	*Stenella coeruleoalba*	MT-II	P14425	C20	*Pseudopleuronectes americanus*	MT	CAA31930
M3-1	*Rattus norvegicus*	MT-III	P37361	C21	*Rutilus rutilus*	MT	P80593
M3-2	*Ovis aries*	MT-III	AAM21134	C22	*Zoarces viviparous*	MT	P52728
M3-3	*Bos taurus*	MT-III	P37359				
M3-4	*Equus caballus*	MT-III	P37360				
M3-5	*Homo sapiens*	MT-III	P25713				
M3-6	*Mus musculus*	MT-III	P28184				
M3-7	*Sus scrofa*	MT-III	P55944				
M4-1	*Canis familiaris*	MT-IV	Q9TUI5				
M4-2	*Homo sapiens*	MT-IV	P47944				
M4-3	*Mus musculus*	MT-IV	P47945				

tree-bisection-reconnection (TBR) branch-swapping algorithm. Bootstrap analyses were carried out using both fast stepwise-addition search (1000 replications) and full heuristic search (100 replications). The bootstrap 50% majority-rule consensus trees from both NJ and MP analyses were visualized using the TreeView (Win32 1.52) program (*http://taxonomy.zoology.gla.ac.uk/rod/treeview*).

Experimental Exposures to Heavy Metals. To examine transcriptional induction of the shark MT gene by heavy metal ions, 3 experimental exposures to heavy metals were conducted. First, sharks (average body weight, 320 ± 38 g) were given an intraperitoneal injection of $CdCl_2$ (Sigma) at different dose levels (2.5, 5.0, and 10.0 mg/kg body weight). A control group injected with saline containing no cadmium. The changes of MT mRNA levels in liver and kidney were monitored for 7 days. The injected fish (n = 16 per dose) were transferred to a 150-L well-aerated tanks and individuals (n = 4) were sampled from each group at 2, 4, and 7 days after injection. Second, the sharks were exposed to equal molar concentrations of cadmium, copper, and zinc in order to examine which heavy metal was the most potent inducer for shark MT. Fish were immersed in seawater (150 L) containing 0, 5, 10, or 20 μM of each heavy metal for 24 hours. The effect of extended durations (48 and 96 hours) on MT expression was also examined with a fixed dose (10 μM) of cadmium, copper, or zinc. Livers were sampled from 4 fish belonging to each treatment group. Third, the time course of MT expression during the zinc exposure was examined up to 10 days. Fish were immersed in seawater containing 0 or 10 μM of zinc, and 3 individuals were randomly chosen from each tank at 1, 4, 7, and 10 days after exposure. The starting level of MT mRNA was also examined at day 0. Liver and kidney samples were subjected to RNA analysis. Water temperature was adjusted at 13° ± 1°C throughout the experiments.

RNA Blot Analysis. Total RNA was isolated using TriPure Isolation Kit (Roche Applied Biosciences) and treated with DNase I (10U/p μg total RNA) for 30 minutes at 37°C in order to remove possible contaminating DNA. DNase I was inactivated by incubating the reaction at 90°C for 15 minutes. One microgram of resulting total RNA was spotted onto a positively charged nylon membrane (Roche Applied Biosciences) in a volume of 1 μl. The membrane was processed according to the manufacturer's instructions and hybridized with digoxygenin-11-dUTP-labeled full-length shark MT

cDNA. Labeling, hybridization, washing, and detection were performed as described above. The hybridized signal was analyzed using Quantity-One software (BioRad) to evaluate the relative intensity of the hybridized signal. Arbitrary values for intensity (INT/mm^2) generated from the software were used for the comparative analysis of hybridization signals among experimental groups. For Northern blot analysis, 10 μg of purified total RNA was separated by electrophoresis in a MOPS-formaldehyde agarose gel (1.2%). The RNA was transferred to a nylon membrane using the capillary method (Sambrook et al., 1989), processed according to the manufacturer's instructions (Roche), and hybridized with DIG-labeled shark MT cDNA. The membranes (dot blot and Northern blot) were stripped and reprobed with shark actin cDNA insert (EST clone; unpublished data) in order to normalize the MT mRNA levels.

Semiquantitative RT-PCR Analysis. The differential change of MT transcripts was examined with semiquantitative reverse transcriptase PCR. Prior to semiquantitative RT-PCR analysis, optimal conditions were established regarding the range of input total RNAs (0.2–2 μg), the number of cycles (12–30 cycles), and thermal cycling conditions for MT gene and actin gene (normalization control). The numbers of cycles were kept to a minimum and the RT-PCRs were linear in the range of input total RNA tested. As a negative control for each set of primers, RT-PCRs were performed in the absence of RT and RNA (data not shown). First-strand cDNA using Superscript II Reverse Transcriptase (Invitrogen) was generated from 1 μg of total RNA (DNase-treated) with oligo(dT)$_{18}$ primers. For PCR reactions, 0.5 U ExTaq DNA polymerase (Takara) and 2 μl cDNA were used in 50 μl of amplification buffer containing 30 pmol of primers. The primer pair specific for shark MT cDNA was sMT-1F and sMT-1R as described above. The primer pair specific for shark β-actin was sACT 1F 5′-CTGTGCCCATCTAC GAAGGT-3′ and sACT 1R 5′-AGAGCGGTGATCTCCTT CTG-3′. PCR was performed using the iCycler (BioRad) under the following conditions: 94°C for 2 minutes (initial denaturation), 94°C for 1 minute, 58°C for 1 minutes, and 72°C for 1 minute. Numbers of cycles for MT and actin genes were 25 and 20, respectively. PCR reactions were repeated 3 times for each cDNA sample. Expected sizes of PCR products of MT and actin are 280 and 475 bp, respectively. PCR products were electrophoretically separated on 2.0% agarose gels, and the ethidium-bromide-stained bands were analyzed by densitometry using Quantity-One software to determine the relative mRNA levels.

Results and Discussion

Isolation and Characterization of Shark MT cDNA and Genomic Sequences. From 4 arrays, each containing 1536 randomly selected clones, 2 clones showed a positive signal with the MT probe in the filter hybridization. Both clones contained full-length ORFs corresponding to shark MT mRNA, and had the identical sequence composed of a 5′-untranslated region (UTR) of 38 bp, a single ORF (204 bp) encoding 68 amino acids, and 3′-UTR of 183 bp excluding 71 bp of poly (A)$^+$ tail. The consensus sequence for polyadenylation was also found 19 bp upstream of the poly (A)$^+$ tail (Figure 1, A). The genomic fragment isolated by PCR using sMT 1F and 1R primers was 923 bp in length. It consisted of 3 exons, 2 introns, and partial 3′-UTR: exons I (34 bp), II (78 bp), and III (92 bp) were separated by introns I (203 bp) and II (449 bp). Consensus exon-intron boundary sequence (GT-AG) was clearly conserved (Figure 1, B).

Multiple Sequence Alignment Analysis. The amino acid sequence of the putative shark MT deduced from the cDNA sequences shared relatively high similarity with other previously known MT sequences from vertebrates including mammals and bony fishes. Overall amino acid identities of shark MT ranged from 41% to 62% with other MTs: average identities were 57% ± 3% (range, 52%–62%) with mammalian MT-Is (37 sequences from 11 species), 58% ± 2% (55%–61%) with mammalian MT-IIs (17 sequences from 12 species), 47%–3% (41%–48%) with mammalian MT-IIIs (7 sequences from 7 species), 50% ± 1% (50%–52%) with mammalian MT-IVs (3 sequences from 3 species), 53% ± 2% (49%–56%) with teleost MT-As (17 sequences from 17 species), 54% ± 2% (47%–55%) with teleost MT-Bs (16 sequences from 16 species) and 52%–3% (47%–57%) with teleost MTs that had not been yet classified as MT-A or MT-B (tables not shown).

Optimized multiple alignment using shark MT and 119 orthologues generated 81 positions including gaps (only the selected sequences are shown in Figure 2). In mammalian MTs most MT-Is and IIs comprised 61 amino acids. Two exceptional MT-I sequences were human MT-IK (M1-8 in Figure 2) and horse MT-IA (M1-20 in Table 1; not shown in Figure 2), containing 62 and 60 amino acids, respectively. Human MT-IK (M1-8) had an exceptional insertion of Ala at the 14th position, which cannot be found in any other vertebrate MTs. Mammalian MT-IIIs consisted of 65 to 68 amino acids with additional sequences in both β-domain (Thr or Ala at the 8th position) and α-domain (from

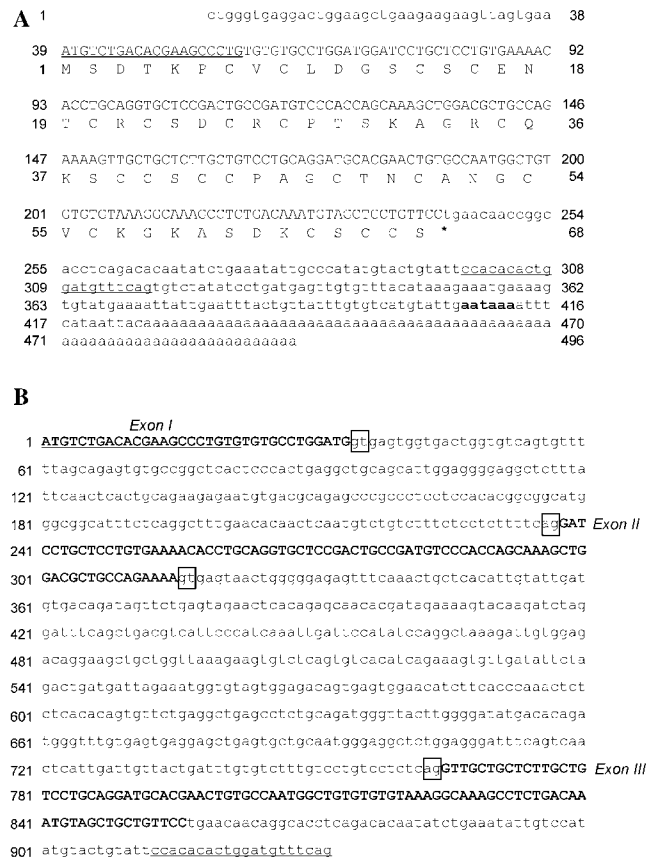

Fig. 1. **A**: Nucleotide sequence of shark MT cDNA. Coding sequence is represented by uppercase type and noncoding sequence by lowercase. The deduced amino acid sequence is indicated below the nucleotide sequence in single letter code. The termination codon is indicated by an asterisk, and the putative polyadenylation signal (aataaa) is in boldface. Sequences of primers sMT-1F and sMT-1R are underlined. **B**: Genomic sequence of shark MT gene isolated by PCR using sMT-1F and sMT-1R primers (underlined). Coding sequence is represented by uppercase boldface. Exon-intron boundary sequences (gt-ag) are boxed.

the 64th to 71st positions): the consensus sequence (G/E)EGAEAE(A/E) and its modified forms inserted in α-domain were found in all of the previously known mammalian MT-IIIs, but in no other MTs. Mammalian MT-IVs had 62 amino acids with an insertion of Glu at the 8th position. Compared with the multiplicity of mammalian MTs, teleosts have been known to possess 2 distinct MTs; MT-A and MT-B. All the fish MT-Bs had 60 amino acids without variation. Most, but not all, teleost MT-As also comprised 60 amino acids. Three salmonid MT-As from *Oncorhynchus mykiss* (A-8 in Figure 2), *Salmo salar* (A-9), and *Salvelinus alpinus* (A-10) had an additional Ala residue at the border between β-domain and α-domain, which was not seen in MT-Bs from the same species. The insertion at the border region has recently been reported in a nonsalmonid fish, ayu (*Plecoglossus altivelis*); however, the inserted amino acid was Thr (C-17 in Table 1; Lin et al., 2004).

Like many other MT sequences, shark MT contained a high proportion of Cys residues (20 [29.4%] of 68) as Cys-X-Cys or Cys-Cys forms. The positions of most, but not all Cys residues in shark MT sequences were well conserved when aligned to other fish and mammalian sequences. In addition to these conserved cysteine residues, shark MT shared several identical residues with other MTs. In β-domain, Asp (the 3rd position), Pro (the 6th position), and also Lys (the 41st position) were conserved in all the fish and mammalian MTs. Serine at the beginning of α-domain (the 43rd position; except human MT-IB) and Pro (49th position) were conserved in most vertebrate MTs. However, shark MT had some unique features, including 4 extra amino acids (Lys-Ala-Gly-Arg; 35th to 38th positions) which have not been reported in any other vertebrate MTs. The position of these additional amino acids was close to the end of β-domain (see Olsson, 1993). Analysis of genomic sequence indicated that these amino acids should be encoded by exon II of shark MT (see also Figure 1). However, the physiological function remains to be studied. On the one hand, further comparisons of shark MT with other teleost MTs based on this multiple alignment showed that shark MT had unique insertions in β-domain which were missing in other MTs: Ser (the 2nd position), Thr-Lys (the 4th and 5th positions), and Pro (the 32nd position). On the other hand, there was a gap in shark MT at the 15th position where many mammalian MTs had Gly or Asp, while most teleosts had Ser or Thr. Shark MT also had Gln at the 40th position (close to the end of β-domain), while most fish and mammalian MT-I, MT-II and MT-IIIs had Lys (mammalian MT-IV had Arg). Similarly, the amino acid at the 54th position was Asn in shark MT, while most mammalian and all the teleost MTs had Lys at this position. The last amino acid residue at the C-terminus in shark MT was Ser, which has not been reported in any other vertebrate MTs: all the teleost MTs and mammalian MT-IIIs had Gln, while most mammalian MT-Is and MT-IIs showed Ala at the C-terminus.

Shark MT represented intermediate characters between teleost and mammalian MTs, suggesting that the shark MT might be an ancestral form of vertebrate MTs. Shark MT shared homology with fish MTs, including the gaps at the 7th and 8th positions in β-domain. Similarity between shark and teleost MTs was also found at the 72nd position: the Lys residue was conserved in the majority of fish

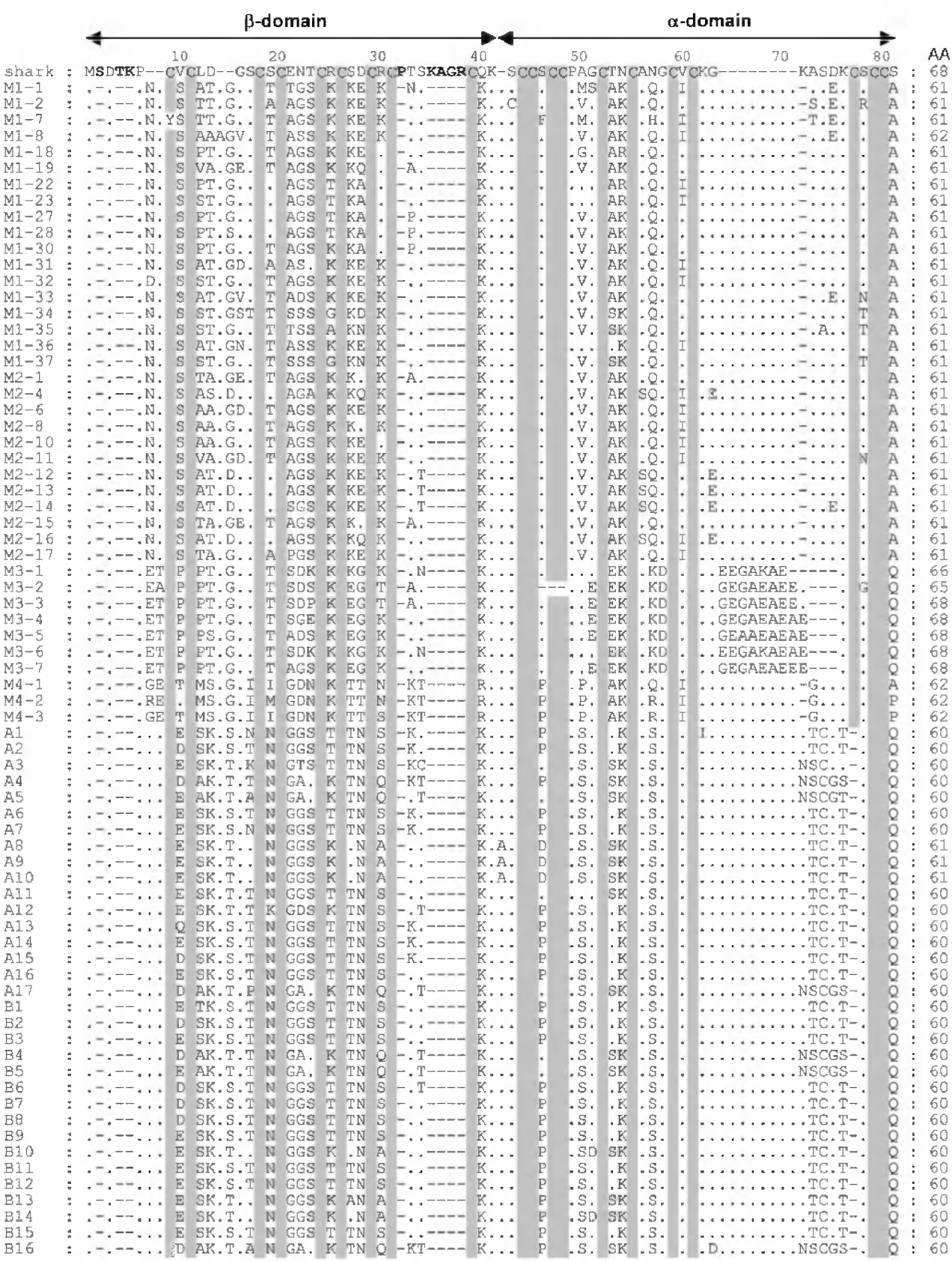

Fig. 2. Alignment of shark MT polypeptide sequences with mammalian MTs (M1-X for MT-I, M2-X for MT-II, M3-X for MT-III, and M4-X for MT-IV) and teleost MTs (A1 to A17 for MT-A and B1 to B16 for MT-B). Multiple alignment was performed with 120 unique sequences indicated in Table 1, but only representative sequences are shown here. For the species name and GenBank accession number of each sequence, see Table 1. Dots indicate the identities with shark MT. Hyphens represent gaps introduced for optimal alignment, and letters represent amino acids where substitutions occur. Conserved cysteine residues are boxed, and extra amino acids found only in shark MT are in boldface. The number of amino acids is noted at the right of each sequence.

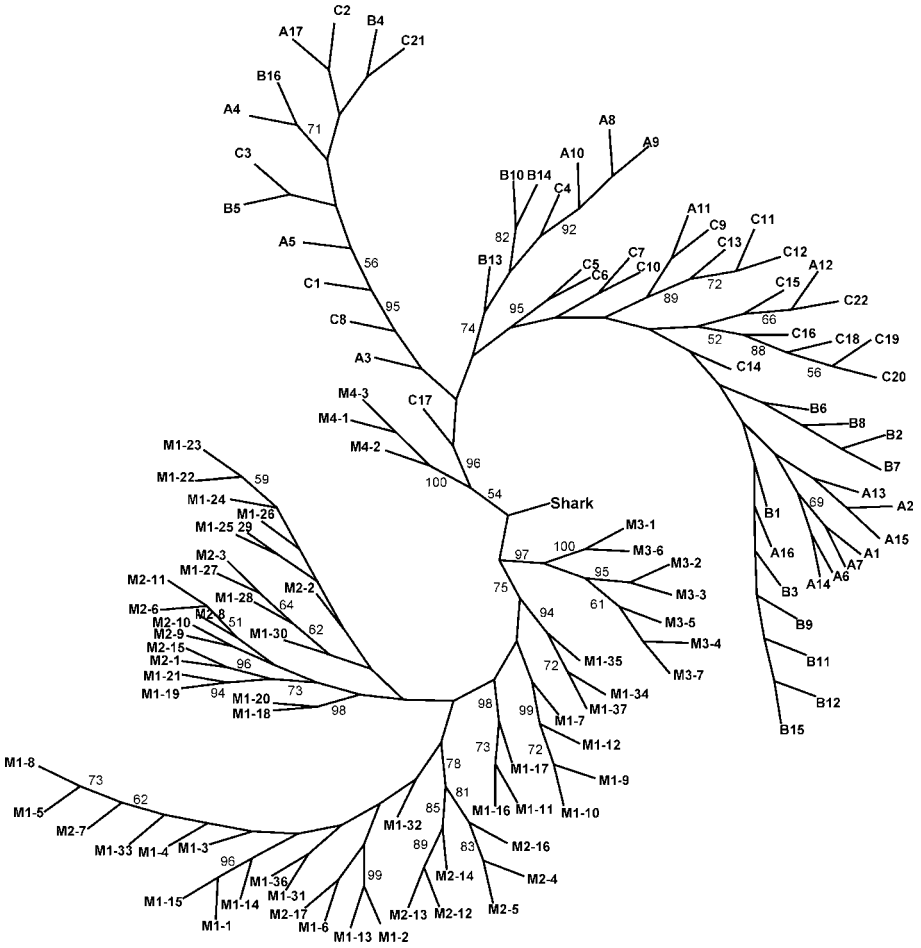

Fig. 3. Phylogenetic tree of MT inferred using NJ method. The 120 unique MTs from fish and mammalian species (shown in Table 1) were used to construct the tree. The bootstrap values as percentages shown at the nodes of the tree are based on 1000 replications. Only values greater than 50% are shown. The GenBank accession number of each sequence is shown in Table 1.

MTs but was found in only 2 mammalian MT-IIIs. In addition to the similarity with teleost MTs, shark shared homology with mammals in their MT sequences. First, Arg at the 31st position was found in many mammalian MT-Is and MT-IIs, but was in none of the fish MTs. Second, shark shared Arg-Ser at 73rd-74th positions with most mammalian MT-Is and MT-IIs, but the consensus sequence at this position in fish MTs was Thr-Cys. Third, the third Cys from the C-terminus (the 77th position) was conserved in shark and mammalian MTs, but was missing in all of the teleost MTs.

Construction of Phylogenetic Trees. The phylogenetic relationships among 120 MT sequences including shark MT inferred using NJ and MP methods are shown in Figure 3 and Figure 4, respectively. In both NJ and MP analyses, shark formed a unique branch. The NJ and MP analyses yielded similar phylogenetic hypotheses, with the same nodes receiving bootstrap support higher than 80% replicates in most cases. In the NJ tree the teleost MT, mammalian MT-IV, and mammalian MT-III groups were supported by the bootstrap values higher than 90% replicates. However, mammalian MT-I and MT-II groups were not clearly divided from each other, and the large group consisting of both MT-I and MT-II was characterized by 75% bootstrap support. Of a total of 16 highly supported nodes in NJ tree (>90% of replicates), 8 nodes belonged to subgroups of mammalian MT-I/II. Within the teleost group, Cypriniformes containing 10 MT sequences was characterized by 95% bootstrap support, 3 salmonid MT-As (A8, A9, and A10) by 92%, and 2 isoforms of *G. morhua* (C5 and C6) by 95%. Further insights into teleost MTs have shown that the MT-A and MT-B isoforms from the group containing Salmoniformes (A8, A9, A10, B10, B13, and B14) appear to have originated before the species separations, because the clades within Salmoniformes are characterized by MT isoforms (MT-A or MT-B) rather than by species: e.g., arctic char MT-A (A10) is more closely related to rainbow trout MT-A (A8) than to arctic char MT-B (B14) (see also Bargelloni et al., 1999). A similar phenomenon is found in tilapia species (see C11, C12, C13): *Oreochromis mossam-*

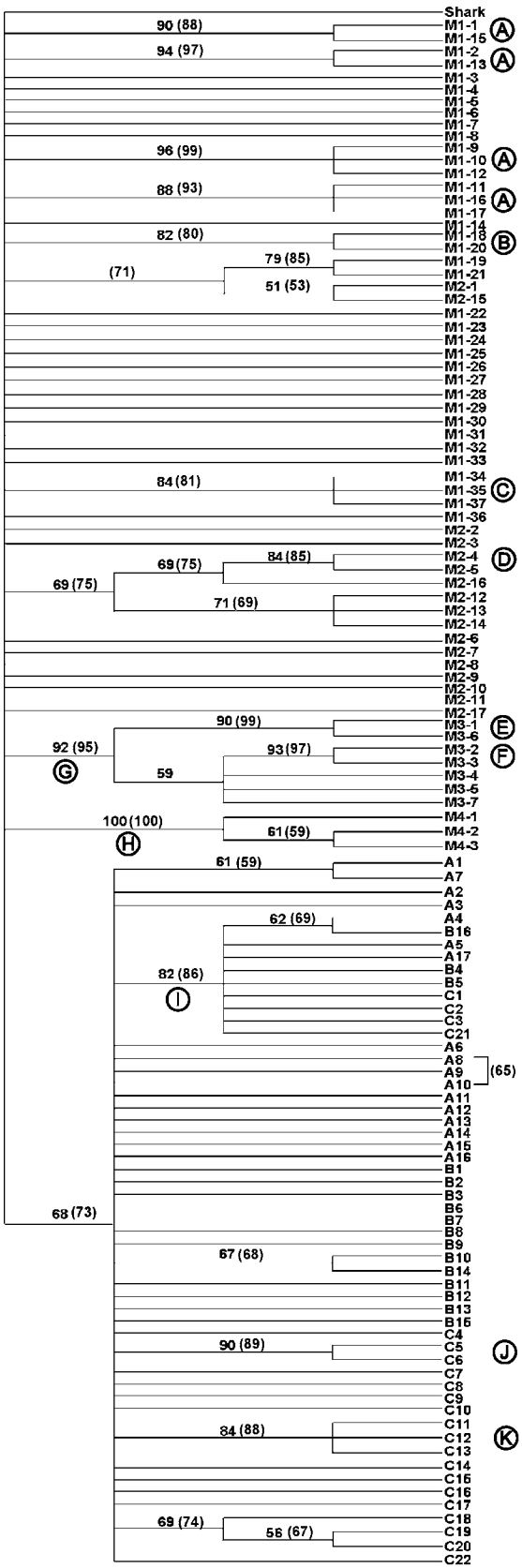

Fig. 4. Consensus tree summarizing results from parsimony analyses using PAUP program under TBR algorithm (40 parsimony-informative sites; tree length, 302; consistency index, 0.5497; retention index, 0.8967; gaps treated as missing). Numbers on branch nodes indicate bootstrap values (%) after 1000 replications using fast stepwise-addition search or 100 replications using full heuristic-search (in parentheses). Only values greater than 50% are shown. Uppercase labels (A to K) in circles represent distinct clades supported by bootstrap values of 80% or higher in both searches. The GenBank accession number of each sequence is shown in Table 1.

bicus MT isoform (C12) is more closely related to *O. aureus* MT (C11) than is *O. mossambicus* MT isoform (C13) (Figure 3).

The consensus tree summarizing results from MP analysis is shown in Figure 4. The general topology of the MP tree was similar to that of the NJ tree. The nodes receiving strong bootstrap support (≥80% in both fast stepwise-addition and full heuristic searches) characterized 14 clades. Clades As are all isoforms of human MT-Is. Clades B and D are 2 isoforms of horse (*Equus caballus*) MT-I and mouse MT-II, respectively. Clade C represents the MT-Is from 3 Muridae families. Clade G covers all of mammalian MT-IIIs including 2 highly supported clades, E and F. Clade H is a distinct group composed exclusively of previously known mammalian MT-IVs. The separation of MT-III and MT-IV groups at high confidence levels is similar to the finding in NJ analysis. Unlike the NJ tree, the node for the teleost group is supported by relatively lower bootstrap values (68% in fast search and 73% in full search). Within this clade the formations of nodes are generally in agreement with the expected taxonomic placements. Fish species belonging to Cypriniformes formed a distinct clade I supported by 82% to 86% bootstrap values. Although the salmonid species (A8, A9, and A10) formed a distinct group in the NJ tree, they did not receive high bootstrap support in MP analysis: only full heuristic search resulted in a node with 65% bootstrap support. Clade J represents 2 isoforms of MT from *G. morhua*, and tilapia MT isoforms also formed a distinct clade K, as in NJ analysis (Figure 4).

Transcriptional Activation of Shark MT Gene by Intraperitoneal Injection of Cadmium. The concentration of cadmium injected affected the induction of shark MT transcripts. On the RNA blot assay, the basal level of MT expression was significantly higher in liver than in kidney. The MT mRNA level of nonexposed fish was not significantly changed in either tissue throughout the experiment. In kidney, all the dose levels (2.5–10 mg/kg body weight) of

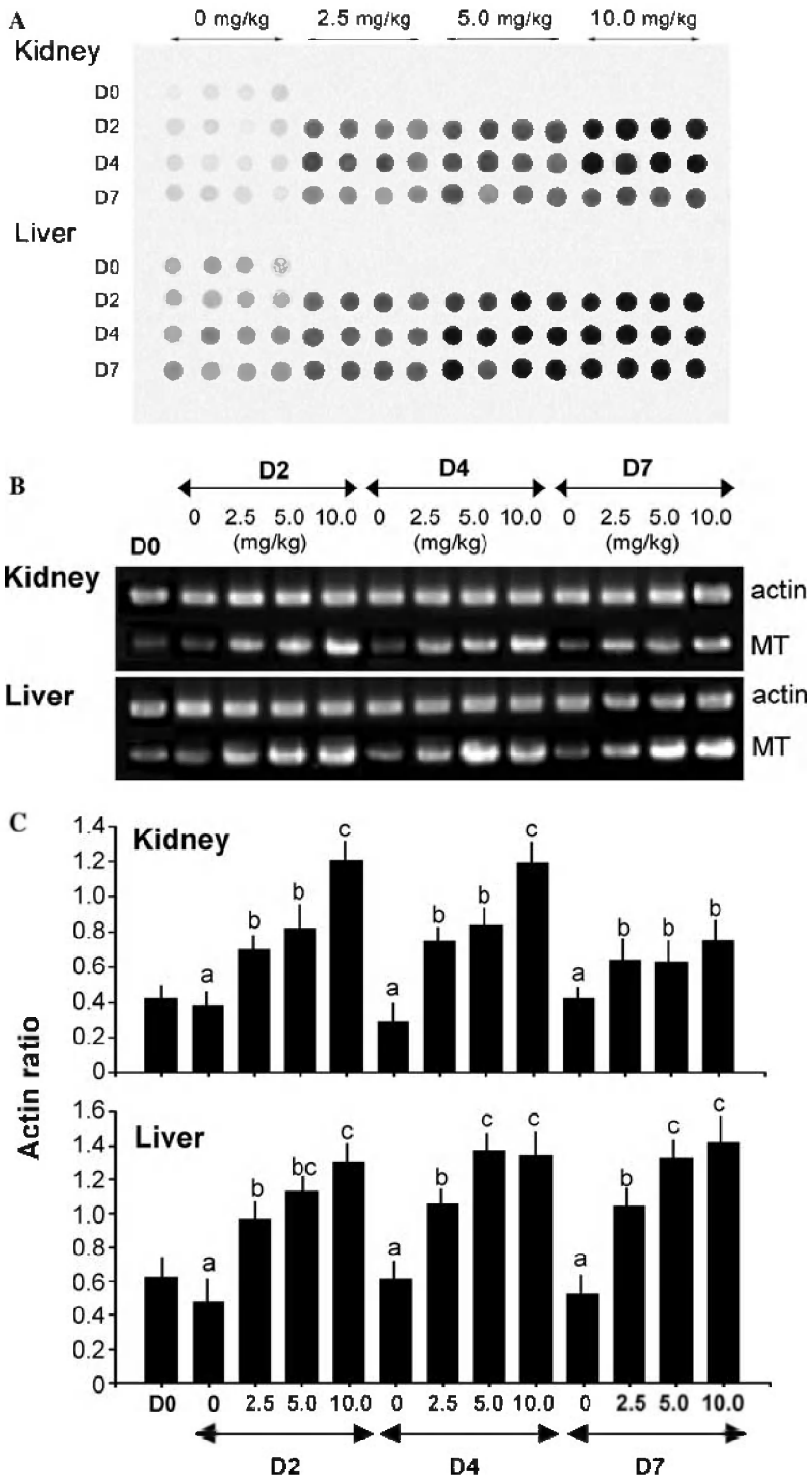

Fig. 5. Levels of shark MT transcripts in kidney and liver induced by cadmium injection. **A**: RNA dot blot hybridization probed with shark MT cDNA. MT mRNA was induced by intraperitoneal injection of different doses of cadmium (0–10 mg/kg body weight). The MT mRNA levels were assayed at 0, 2, 4, and 7 days after injection. **B**: Representative gel showing the RT-PCR products of the expected sizes (475 bp for actin and 280 bp for MT) fractionated in 2% agarose gel electrophoresis and stained with ethidium bromide. **C**: Densitometric analysis (actin ratio) representing the means of 3 independent PCR amplifications of a cDNA (see "Materials and methods"). Signal intensities (INT/mm^2) of the bands were assigned by image analysis software (Quantity One). Standard deviations are indicated by bars on the histograms. Means with the same letters on each histogram within a day were not statistically different at $P < 0.05$ based on analysis of variance.

cadmium injection revealed significant dose-dependent increases in MT transcripts at 2 days after injection. A transient increase in MT transcripts was observed up to day 4 after injection, which dropped to a lower level at day 7, although the level was still elevated as compared to that in the control fish (Figure 5, A). The transient response of MT transcript in kidney to heavy metal exposure has already been reported in rainbow trout (Norey et al., 1990). Liver also displayed a sharp increase of MT mRNA

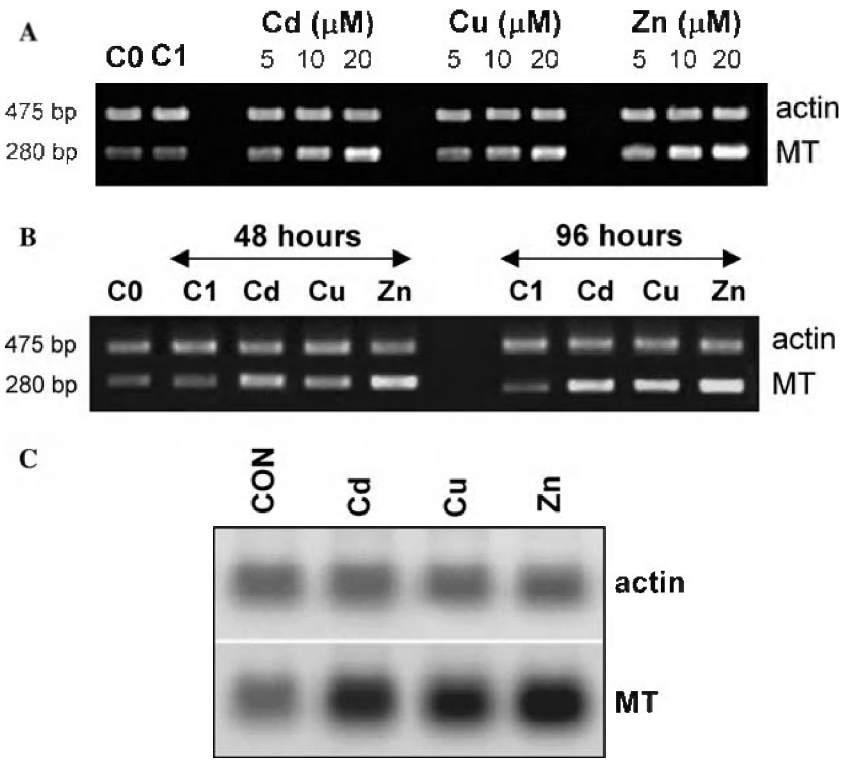

Fig. 6. Semiquantitative RT-PCR analysis of RNA from fish exposed to cadmium, copper, and zinc. **A**: Induction of hepatic MT mRNA by immersing sharks in equal molar concentrations (5, 10, or 20 μM) of 3 heavy metals for 24 hours. C0 indicates the starting level of MT transcripts assayed at day 0. C1 is the nonexposed control assayed at 24 hours after immersion (immersion in water containing no heavy metal). **B**: MT mRNA levels induced by exposures to heavy metals at 10 μM for 48 and 96 hours. C0 is the starting level of MT transcripts and C1 lanes are nonexposed controls assayed at 48 and 96 hours after immersion. **C**: Northern blot hybridization to show MT mRNA levels in shark liver induced by exposure to cadmium, zinc, or copper at 10 μM for 96 hours. Ten micrograms of liver total RNA was transferred to nylon membrane and probed with digoxygenin-labeled full-length shark MT cDNA probe. Control hybridization was a with shark β-actin cDNA fragment.

levels by cadmium injection; however, the expression pattern in liver was different from that in kidney. The maximum MT mRNA level was attained in liver at day 4 and retained up to day 7, in contrast to the rapid drop of MT mRNA level at day 7 in kidney (Figure 5, A). A similar pattern of MT induction at mRNA level was observed in semiquantitative RT-PCR analysis (Figure 5, B and C). Actin gene showed steady-state expression without significant variations in kidney or liver tissues. However, MT transcripts were clearly induced by cadmium injection, and the increases in these tissues were dose-dependent, even though there was not a linear relationship between doses and mRNA levels. As in RNA blot analysis, the induction of MT mRNA in kidney was transient; however, elevation was retained in liver (Figure 5, B and C). The view that differential regulation of MT expression in different organs might be due to the different rates of metal-ion uptake or excretion in the organs, which can also be affected by other metal-binding proteins existing in different organs, has been widely accepted (see Gedamu and Zafarullah, 1993).

Induction of MT mRNA by Immersion Exposure to Cadmium, Copper, and Zinc. Sharks were exposed to equal molar concentrations (0, 5, 10, and 20 μM) of 3 different heavy metal ions (Figure 6, A). Actin transcript was not changed during immersion, but significant increases were detected in fish subjected to all the doses except nonexposed fish. According to the scanning densitometry, zinc was the more potent inducer than cadmium and copper: MT mRNA levels of fish exposed to zinc were always higher than those of fish exposed to cadmium and copper (pixel data not shown). The extended exposures using a single dose (10 μM) also showed similar patterns of increase (Figure 6, B). The MT mRNA level induced by zinc was more than 2-fold that of nonexposed fish at 48 hours after immersion. Cadmium induced slightly more MT mRNA in liver than copper, but the difference was not significant. Further extension of duration up to 96 hours decreased the difference in the induced mRNA levels by the 3 heavy metals. Although the MT transcript level induced by zinc was still slightly higher than those by cadmium and copper, the difference between cadmium and copper was diminished at 96 hours (Figure 6, B). Northern blot analysis using the RNA from the fish exposed for 96 hours showed similar results, but the difference was less compared to that on RT-PCR (Figure 6, C). This result was similar to the findings of a previous report on rainbow trout in which higher induction was detected by exposures to zinc and cadmium than to copper (Gedamu and Zafarullah, 1993). Olsvik et al. (2001) also reported that brown trout from a stream contaminated with cadmium and zinc showed signifi-

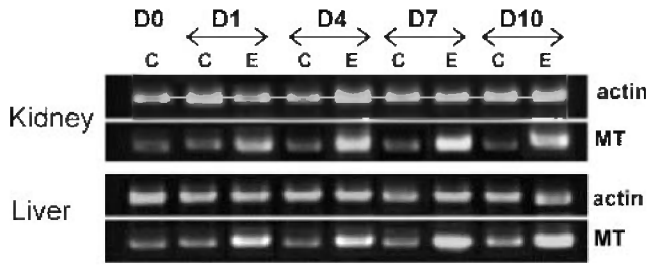

Fig. 7. Time course expression of MT transcripts assessed by semiquantitative RTPCR with the RNAs from fishes exposed to 0 (C) or 10 μM (E) of zinc up to 10 days. RT-PCR products were analyzed by electrophoresis onto a 2.0% agarose gel followed by ethidium bromide staining.

cantly higher MT level than fish from a copper-polluted river. The differential response of the MT gene to different kinds of metal inducers might be due to not only the differential affinity of the heavy metals for a metal-binding transcription factor but also the different rates of metal flux and availability (Gedamu and Zafarullah, 1993). However, Boeck et al. (2003) proposed that there also might be significant variations among fish species in the regulatory capacity for a specific metal homeostasis: for example, cyprinid fish species exhibited higher tolerance for copper exposure and showed much more positive correlation between tissue copper levels and tissue MT levels than salmonid fish.

Using the most potent inducer, zinc, the time course of MT expression was monitored up to 10 days (Figure 7). In both kidney and liver, significant increase of MT transcripts was detectable at day 1 by semiquantitative RT-PCR. Although there was a trend toward higher induction with longer duration of zinc exposure, the level of MT transcripts rapidly reached its maximum at day 4 (kidney) and at day 7 (liver). Unlike the transient pattern of expression in kidney by cadmium injection, however, there was no evidence that the MT mRNA dropped rapidly after reaching maximum induced level: induced MT transcripts remained elevated up to 10 days without any significant decrease. Positive correlations between MT levels and periods of exposure to heavy metals have also been observed in other teleost species at mRNA or protein levels (George et al., 1996; Boeck et al., 2003; Lin et al., 2004). The short period required to reach maximum induction observed in this study might be due to the relatively high concentration of doses (10 μM). To consider the environmentally realistic doses, further experiments should be conducted using lower concentrations and more extended periods. It also would be valuable to examine the expressed profile of other stress-responsive genes together with MT transcripts during heavy metal exposures, because stress factors other than metal ions also could affect MT mRNA levels.

In summary, a novel MT cDNA and genomic sequence was isolated from a cartilaginous shark species, *Scyliorhinus torazame*. The shark MT shared homologous traits with MTs from both advanced bony fishes and mammalian species, suggesting that the shark MT may be the ancestral form of vertebrate MTs. However, it is not clearly understood yet whether or not the present type of shark MT gene belongs to subtype MT-A or MT-B. Further experiments on the genomic structure of shark MT gene including genomic Southern blot hybridization and inverse PCR will be useful for better understanding the origin of ancestral MT gene of vertebrates. The highly inducible expression of shark MT gene by various metal ions may also allow the use of MT transcripts as molecular biomarkers to address environmental contamination by heavy metals.

Acknowledgments

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