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Cloning of a river pufferfish (*Takifugu obscurus*) metallothionein cDNA and study of its induction profile in cadmium-exposed fish

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Abstract

We report here the full-length cDNA sequence of metallothionein (MT) gene from an anadromous river pufferfish, *Takifugu obscurus* (order: Tetradotiformes; family: Tetradontidae). Phylogenetic relationship analysis revealed that the identified MT has high sequence similarity with many Perciformes fish species. The tissue distribution and concentration- and time-dependent expression of MT mRNA were studied in fish exposed to cadmium. Liver showed the highest level of MT gene expression followed by other tissues (brain, gill and kidney) in response to cadmium exposure. Muscle showed a weak expression response of MT gene. Time-course study revealed highest early phase (at 6 h) expression in the brain and late persistence of induction in the intestine. MT mRNA expression showed a concentration-dependent expression in all the tissues. However, induction in brain and liver occurred at much lower concentrations as compared to other tissues. Our results demonstrate that MT in *T. obscurus* is induced by cadmium exposure which indicates that it plays a functionally conserved function of metal detoxification.

Keywords: *Takifugu obscurus*; Metallothionein; Cadmium; Detoxification; Biomarker

1. Introduction

Metallothioneins (MTs) are important low molecular weight cysteine-rich proteins found universally in almost all types of organisms (Theocharis et al., 2003; Vasak, 2005; Thirumoorthy et al., 2007). Although a variety of functions have been attributed to MTs, they are believed to be primarily involved in homeostasis of some essential metals such as copper and zinc and metal detoxification (Coyle et al., 2002; Vasak, 2005). Antioxidant role of MTs has also been described (Sato and Kondoh, 2002; Atif et al., 2006). MTs can be induced by a number of stressors and, therefore, they have been used as biomarkers of exposure, especially in aquatic species (Linde et al., 2001; Berthet et al., 2005; Amiard et al., 2006; Chu et al., 2006; Knapen et al., 2007). Studies on marine as well as freshwater fish MTs are mainly aimed at identification of their functional role or for their use in biomarker studies (Stien et al., 1998; Linde et al., 2001; Hayes et al., 2004). Amongst metals, cadmium is one of the strongest inducers of MT in fish as well as in mammals (Klaassen et al., 1999). To date, four isoforms of MT (MT-I–IV) have been described in vertebrates (Coyle et al., 2002; Thirumoorthy et al., 2007). Of these four isoforms, MT-I and MT-II are the best reported in most of the species studied so far (Andrews, 2000; Coyle et al., 2002). Recently, MTs have been suggested to be involved in diverse physiological processes other than detoxification such as inflammation, pregnancy,

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aging, etc (Coyle et al., 2002; Yang et al., 2006). Study of MTs in fish is an interesting and ever-expanding area of research as fish live in challenging environments and MT induction is recognized as one of the robust adaptive and stress responses to such challenges.

Takifugu species (order: Tetradotiformes; family: Tetradontidae) have attracted attention of scientists for their peculiar physiology, morphology and genomics (Venkatesh et al., 2000; Aparicio et al., 2002). The Japanese pufferfish *Takifugu rubripes* has the shortest known genome of any vertebrate species (Venkatesh et al., 2000; Aparicio et al., 2002). Although extensive research is reported on *T. rubripes*, other species of *Takifugu* have been rather ignored. Two species of *Takifugu* (*T. obscurus* and *T. ocellatus*) which are anadromous and commercially very important in the East China Sea, the South China Sea, and inland waters in China and the Korean Peninsula offer a good opportunity for study of stress responses (Kato et al., 2005). *T. obscurus* (common name-river puffer) is a popular fish among locals in Korea and fetches a premium price owing to its high-quality meat. However, wild populations of this species are currently declining because of overexploitation and due possibly to environmental pollution (Yang and Chen, 2004). It lives in the bottom layer of inshore and inland waters and most of the growth takes place in the sea but spawns in brackish and fresh water. During the spawning season, sexually mature fish run into the river estuaries and spawn in inland waters including rivers, lakes, and ponds. The fingerlings grow in the inland water and either return to the sea the next spring or remain there for a few months before returning to the sea (Kato et al., 2005). Such a dynamic reproductive behaviour might expose *T. obscurus* to both freshwater and saltwater environments and accordingly to the aquatic pollutants of a diverse kind. We report here the molecular characterization of one of the important stress and adaptive response genes, MT gene from *T. obscurus*. We also studied MT gene expression profile in *T. obscurus* exposed to a common marine and freshwater pollutant, cadmium.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals, reagents and kits used in this study were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA), Qiagen (Valencia, CA, USA), Invitrogen (Carlsbad, CA) or Promega (Madison, WI, USA). Oligonucleotide synthesis and DNA nucleotide sequencing were performed at the Bionics (Seoul, South Korea).

2.2. Fish

Juvenile river pufferfish, *T. obscurus* (body length, 12.5 ± 2.1 cm; body weight 37.2 ± 3.6 g) were procured from Yang-chon Fish Hatchery (Kimpo, Kyoung-gi, Korea) and transported to the laboratory without causing any physical stress. Fish were acclimated under laboratory conditions (photoperiod 12 h light, 12 h dark cycles, temperature 23 ± 1 °C) for two weeks in 100 L tank filled with filtered water (salinity, 15 ± 0.5‰, pH 7.81, dissolve oxygen, DO 5.3 mg/L). During the acclimation period fish were fed a commercial fish diet S7 (Higashimaru Foods, Inc., Kagoshima, Japan) twice a day to satiation.

2.3. RNA isolation, reverse transcription and first strand cDNA synthesis

Total RNA from the liver was isolated using Trizol® reagent (Molecular Research Center, Inc., Cincinnati, OH), purified and quantitated using standard procedure as described by Lee et al. (2005). cDNA was made using the kit (Invitrogen) following the manufacturers' instructions.

2.4. PCR, cloning, amplification of 3′ and 5′ ends and sequence analysis

The degenerative primers were designed using the conserved domains after multiple alignments of previously reported full-length cDNA sequences of teleost MT gene. The detail of primers, their location and PCR conditions for amplification of partial sequence are given in Table 1. The PCR product was eluted from the gel using kit (Quiagen) and subcloned into pCR2.1 TA vector using chemically-competent bacterial cells (Invitrogen). The plasmid DNA was extracted from the bacterial culture using kit (Promega) and incorporation and orientation of insert were verified by restriction analysis as described by Lee et al. (2005). The full-length sequence of *T. obscurus* MT was deduced using GeneRacer kit (Invitrogen). The 3′-rapid amplification of cDNA ends (3′-RACE) and 5′-RACE were performed using the primers and PCR conditions as detailed in Table 1 and following the manufacturers' instructions.

2.5. Phylogenetic relationship

To place the river pufferfish MT gene within a fish phylogenetic tree, we aligned diverse fish MT genes at the level of DNA sequences determined in this study and obtained from the DDBJ/EMBL/GenBank by Clustal X ver. 1.83 (Thompson et al., 1997). This was included at complete nucleotide sequences of open reading frame (ORF) excluding the flanking untranslated region (UTR). The alignment results were adjusted manually for obvious alignment errors. Only those positions that could be unambiguously aligned were used in the analysis. This resulted in 186 sites out of the 189 alignment positions for the subsequent analysis. The Bayesian analysis was implemented with MrBayes ver. 3.1.2 (Huelsenbeck and Ronquist, 2001) using the molecular model selected by Akaike information criterion (AIC), namely a generalized time reversible (GTR) nucleotide substitution model, with among-site rate variation

Table 1
Primer and PCR condition used in this study

Gene	Oligo name	Sequences (5′ → 3′)	Nucleotide position	Remarks	PCR condition
TO-MT	RT-F	AAGACTGGAAGCTGCAACTGTGG	22–44	cDNA amplification	95 °C/5 min:40 cycles of 98 °C/25 s, 55 °C/40 s, 72 °C/90 s: 72 °C/10 min
	RT-R	AATTCCCGTTACACACGCAGCC	136–157		
	3GSP1	ACTGGAAGCTGCAACTGCGGAG	25–46	3′-RACE	94 °C/3 min: 35 cycles of 98 °C/25 s, 55 °C/60 s, 72 °C/90 s: 72 °C/10 min
	3GSP2	TCCTGCACCACCTGCAAGAAGAG	70–92		
	5GSP1	CACATCACAAGGTCAG	208–223	5′-RACE	94 °C/2 min: 35 cycles of 94 °C/60 s, 55 °C/60 s, 72 °C/2 min: 72 °C/7 min
	5GSP2	AGCAGCTGGGGTCACATGTCTTC	153–175		
	5GSP3	TCTTCTTGCAGGTGGTGCAGGAAC	68–91		
	Met RT-F	CCGAGAGGATAGACGCCGACAG	75–96	Real-time PCR	94 °C/5 min: 35 cycles of 94 °C/30 s, 50 °C/30 s, 72 °C/30 s: 72 °C/7 min
	Met RT-R	GCAAGCTCTTCTTGCAGGTGGTG	−39–61		
TO-β-actin	RT-F	CATCACCATCGGCAACGAGAGG	721–743		
	RT-R	CGTCGCACTTCATGATGCTGTTG	817–840		

TO = *Takifugu obscurus.*

modeled with a proportion of sites being invariable. The rates for variable sites were drawn from a gamma distribution. The Markov chain Monte Carlo (MCMC), process was set to four chains and 1 000 000 generations were conducted. The sampling frequency was assigned as every 100 generations. After analysis, the first 1000 trees were deleted as burn-in and the consensus tree was subsequently constructed. Bayesian posterior probabilities (>50%) were indicated at each branch node. MT gene from the amphibian *Ambystoma mexicanum* (GenBank No. AF008583) was used as the out-group. The consensus tree was visualized with TreeView ver.1.6.6 (Page, 1996).

2.6. Tissue distribution

Real-time reverse transcription PCR (RT-PCR) was performed to study tissue distribution pattern of MT gene in brain, gill, intestine, kidney, liver, and muscle using oligo(dT)20 primer and SuperScript™ III reverse transcriptase (Invitrogen) according to the manufacturers' instructions. The PCR conditions are described in Table 1. The PCR products were separated on 1% TBE agarose gels containing ethidium bromide (EtBr, Sigma) and visualized on a Fluor-STM Multimager system (Bio-Rad).

2.7. Cadmium-induced expression of MT gene

Acclimatized fish ($n = 15$) were exposed to cadmium (5 ppm, $CdCl_2{\cdot}5H2O$, Sigma, purity 99% dissolved in ultra-pure distilled water). In control group ($n = 15$) fish tank was mixed with distilled water matching the level in exposed group. The cadmium concentration was selected based on the previous studies dealing with fish MT induction (Wu et al., 1999; Hermesz et al., 2001). Fish were randomly sampled at 0, 6, 12, 24, 48 and 96 h of exposure, anesthetized by immersion in buffered tricaine methanesulfonate (MS-222, Sigma; 200 mg/L, pH 7.0) and sacrificed. Brain, gill, intestine, kidney, liver, and muscle were dissected out for study of expression of MT using real-time RT-PCR. In order to study concentration-dependent effect of cadmium on MT gene induction, fish were exposed to cadmium in concentration range of 50–5000 ppb for 24 h. All the tissues as mentioned above were analyzed for MT mRNA expression. Fish were fasted for two days before the exposure and no food was provided during the exposure.

2.8. Real-time RT-PCR

Real-time RT-PCR was performed to study the tissue distribution and expression pattern of cadmium-induced MT mRNA. Primer and PCR detail are given in Table 1. SYBR® Green (Molecular Probe, Invitrogen) was used to detect specific PCR products. Amplification and detection of SYBR® Green were performed with the MyiQ detection system (Bio-Rad). All the data are expressed relative to *T. obscurus* β-actin, which was used as a house keeping reference to normalize the expression levels between the samples. Fold change in the gene expression relative to controls was determined by the standard $2^{-\Delta\Delta CT}$ method of Giulietti et al. (2001).

3. Results and discussion

The *T. obscurus* MT gene consisted of 183 bp of ORF encoding 60 amino acids of a putative protein, 73 bp of 5′-untranslated region (UTR) and 135 bp of long 3′-UTR (Fig. 1). It had 8.24 of theoretical *pI* and 5.96 kDa of molecular weight. The most characteristic features of MT are their high cysteine content and cysteine alignment. Cysteine accounts for 30% of the total amino acids in this protein and Cys–Cys motifs show three major variations, Cys–Cys, Cys–*X*–Cys, and Cys–*X*–*Y*–Cys, where *X* and *Y* are amino acids other than cysteine (Kojima et al., 1976). The *T. obscurus* MT had 20 cysteine residues among the

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-74                                                        AGCACAAACACAT
-61 CCGAGAGGATAGACGCCGACAGCTTCTGACGAAGAACCTGCAGATACTTACAGAACAGCA
  1 ATGGACCCTTGTGACTGCTCAAAGACTGGAAGCTGCAACTGCGGAGGATCCTGCGCTTGC
    M  D  P  C  D  C  S  K  T  G  S  C  N  C  G  G  S  C  A  C
 61 AAAAACTGTTCCTGCACCACCTGCAAGAAGAGCTGCTGCTCATGCTGCCCATCTGGCTGC
    K  N  C  S  C  T  T  C  K  K  S  C  C  S  C  C  P  S  G  C
121 AGCAAGTGCGCCTCTGGCTGCGTGTGCAAGGGGAAGACATGTGACCCCAGCTGCTGCCAG
    S  K  C  A  S  G  C  V  C  K  G  K  T  C  D  P  S  C  C  Q
181 TGAGGAGTTTGCAGCATCAATCATGCACTGACCTTGTGATGTGTCCTCATTTTCAAATGT
    *
241 TTGTAAAAATCTAATTTTACTGTTGAAATAAACCCCTTTCCCCTTGAAAAAAAAAAAAAA
301 AAAAAAAAAAAAAAAAAA
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Fig. 1. Representative DNA sequence of the *Takifugu obscurus* Metallothionein gene (GenBank accession no. EF622234). (∗) Indicates the position of termination codon. Poly(A) signal sequence was underlined.

60 amino acids (approximately 33.3%). Six Cys–*X*–Cys, three Cys–*X*–*Y*–Cys, and three Cys–Cys alignments can be found throughout the ORF. MTs have an unusual amino acid composition. Besides high cysteine content and characteristic arrangement of cysteinyl residues in Cys–*X*–Cys or Cys–*X*–*X*–Cys motifs, they lack aromatic residues (Capasso et al., 2003). All the cysteine residues can form metal thiolate clusters organized in two domains, the amino-terminal β-domain including nine cysteines and three metal equivalents, and the carboxy-terminal α-domain with 11 cysteines and four metal equivalents (Kagi and Schaffer, 1988; Capasso et al., 2003).

T. obscurus MT gene can be classified as class I because the locations of the cysteine residues are exactly the same as for mammal MT-I (Scudiero et al., 2001). The *X* and *Y* in Cys–Cys, Cys–*X*–Cys and Cys–*X*–*Y*–Cys sequences determine different structural MT classes where *X* and *Y* are amino acids other than cysteine (Amiard et al., 2006).

The phylogenetic relationship of the *T. obscurus* MT gene with other MT genes from various fish orders showed that the six orders included here were clustered according to each taxonomic level (Fig. 2). Within each clades of the six orders, the genes were generally separated into MT gene types such as MT-A, -B/or MT-I, -II. In a midpoint rooting, the Tetraodontiformes formed a branch of the Perciformes. On the clade, *T. obscurus* MT gene was clustered with the green pufferfish (*Tetraodon nigroviridis*), which was identified by BLAST search, with strong posterior probability (100%), a sister relationship with gilthead seabream (*Sparus aurata*) and striped seabream (*Lithognathus mormyrus*) with 91.2% and 89.6% of DNA sequence identity, respectively (Fig. 2). The conceptual translation of *T. obscurus* MT gene showed highest similarity (90%) to red seabream MT gene (Fig. 3). MTs have many conserved sequences and such a high sequence similarity with fish of rather distant orders is not surprising (Klaassen et al., 1999).

The tissue distribution pattern indicated highest level of MT mRNA expression in the liver followed by kidney and brain (Fig. 4). Gill and intestine showed a relatively lower level of expression compared to brain and liver. In muscle MT mRNA was barely detectable. In fish, liver is the main site of MT synthesis (Hogstrand and Haux, 1996; Hogstrand et al., 1996; Cheung et al., 2004; Long and Wang, 2005). Nevertheless, MT induction by cadmium in other

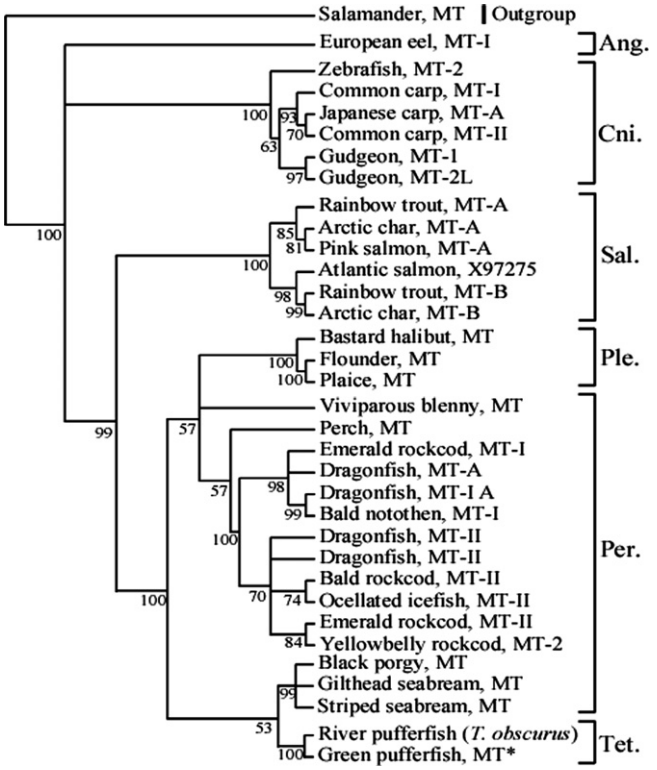

Fig. 2. A Bayesian tree inferred from MT DNA sequences from 33 taxa of fish, computed with MrBayes ver. 3.1.2. The numbers at the nodes are posterior probabilities, in which posterior probability values above 50% are indicated at each node. An amphibian species (*Ambystoma mexicanum*, AF008583) was used as the out-group. MT DNA sequences included here are as follows: seven Cypriniformes species (*Anguilla anguilla*, DQ493910; *Carassius cuvieri*, AY165047; *Cyprinus carpio*, AF001983; *C. carpio*, AF249875; *Danio rerio*, BC051612; *Gobio gobio*, AY953544; *G. gobio*, AY953546), five Salmoniformes species (*Oncorhynchus mykiss*, M81800; *O. gorbuscha*, DQ139338; *Salvelinus alpinus*, AY267819; *S. alpinus*, AF013801; *Salmo gairdneri*, X59394), three Pleuronectiformes species (*Paralichthys olivaceus*, EF406132; *Pleuronectes platessa*, X56743; *Pseudopleuronectes americanus*, X13594), 15 Perciformes species (*Acanthopagrus schlegelii*, EU126549; *Chionodraco rastrospinosus*, Z72484; *Gymnodraco acuticeps*, AJ007561; *G. acuticeps*, J007560; *Lithognathus mormyrus*, DQ850666; *Notothenia coriiceps*, AJ006485; *Pagotheni borchgrevinki*, AJ007563; *Pagothenia borchgrevinki*, AJ007562; *Parachaenichthys charcoti*, AJ007950; *P. charcoti*, AJ007951; *Perca fluviatilis*, X97272; *Sparus aurata*, X97276; *Trematomus bernacchii*, AJ011585; *T. bernacchii*, Z72485; *Zoarces viviparus*, X97270), and two Tetraodontiformes species (*Takifugu obscurus*, EF622234; *Tetraodon nigroviridis*, CR651750). (∗) represents a genome-based sequence identified by BLAST search, yet the gene was not annotated to MT gene. MT gene types such as MT-A, -B, -I, and -II, recorded in database are shown.

tissues such as brain and gills is reported in a number of fish species (Hermesz et al., 2001; Filipovic and Raspor, 2003; Bae et al., 2005; Chowdhury et al., 2005). Route of exposure also has effect on MT induction levels (Chowdhury et al., 2005). Additionally, differential MT induction has been reported in different species (De Boeck et al., 2003). MT expression is controlled mainly at the transcriptional level by several external agents. Based on studies in diverse species, metals have been observed as the most powerful inducers of MT (Durnam and Palmiter, 1981;

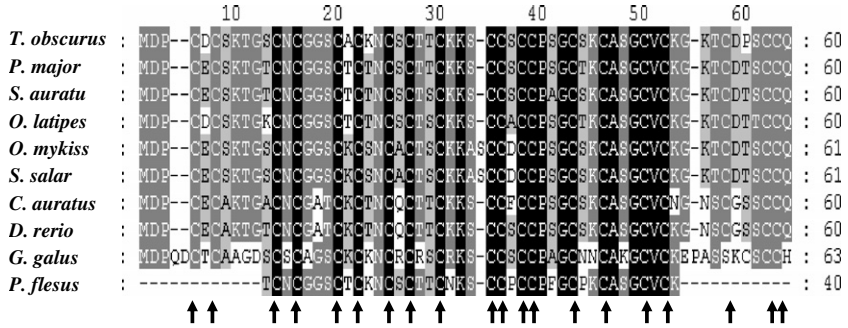

Fig. 3. Comparison of amino acid sequences of *Takifugu obscurus* MT with other species. River puffer (*Takifugu obscurus*; EF622234), flounder (*Platichthys flesus*; CAC28138), gilthead seabream (*Sparus aurata*; AAC32738), Japanese medaka (*Oryzias latipes*; AAR30249), rainbow trout (*Oncorhynchus mykiss*; CAA42038), Atlantic salmon (*Salmo salar*; CAA65929), goldfish (*Carassius auratus* ; AAB32777), zebrafish *Danio rerio*; AAS00514), chick (*Galus galus*; P68497), red seabream (*Pagrus major*; BAA92364). The sequences were taken from the public domain GenBank/EMBL/DDBJ sequence databases. Cysteine positions are marked by arrows.

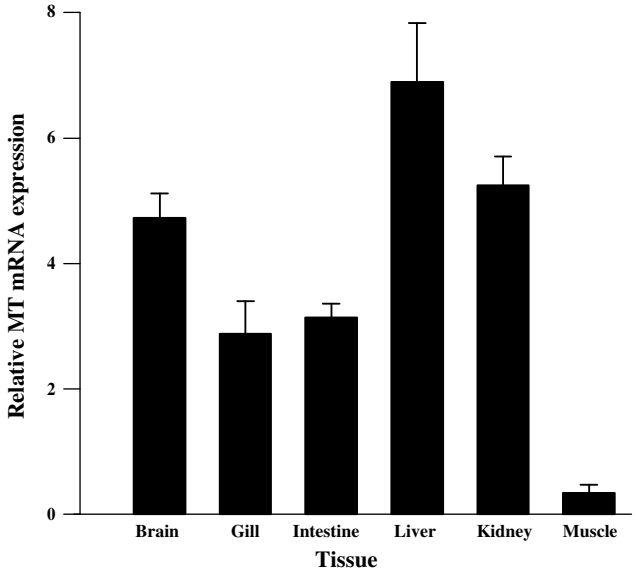

Fig. 4. Expression of *Takifugu obscurus* MT gene in different tissues (brain, gill, intestine, kidney, liver, muscle) in control fish. The expression of MT mRNA is relative to β-actin gene expression, which was used as a housekeeping reference gene. Values are means of triplicate samples for each fish and tissue.

Klaassen et al., 1999). Analysis of MT gene promoters show short *cis*-acting elements, metal responsive elements (MREs), in the 5′-flanking region of MT gene (Scudiero et al., 2001). Furthermore, an MRE-binding transcription factor (MTF) has been cloned from several species including fish (Bourdineaud et al., 2006). It has been observed that following stress stimulus MTF-1 translocates from the cytoplasm to the nucleus (Smirnova et al., 2000).

Time-course study revealed that cadmium induced MT mRNA expression in all the tissues (Fig. 5). It was expected as Cd is considered one of the strongest inducers of MT (Klaassen et al., 1999; Chowdhury et al., 2005). However, the level of expression was different in various tissues. While only brain showed strong induction at the initial (at 6 h), most of the other organs showed peak induction after 12 h and onwards in 96 h exposure study. In case of kidney the peak induction was at 48 h. Except in case of muscle at 6 h and 96 h and brain at 24 h and 48 h, induction in all other tissues at all the time intervals was significantly higher ($P < 0.05$) than that at 0 h. Cadmium is initially taken up by the liver, where it can bind with reduced glutathione (GSH) and be excreted into bile. Alternatively, it can bind to MT and subsequently stored (Klaassen et al., 1999). The distribution of Cd and consequently induction of MT are regulated by these factors. Chowdhury et al. (2005) observed a time-dependent induction of MT in liver, kidney and in fish exposed to Cd (3 μg/L through water) up to 28 days. They also observed no MT induction on fourth day. Cheung et al. (2004) observed more than 30-fold of MT mRNA induction in gill, 15-fold induction in liver and 2.5-fold induction in kidney in tilapia after intraperitonial injection of Cd (5 mg/kg) after 24 h.

MT gene showed a concentration-dependent expression in all the tissues, except kidney (Fig. 6). While gills and intestine showed no significant expression at 50 ppb of Cd, other tissues such as brain, liver and muscle showed a significantly ($P < 0.05$) greater level of MT gene expression as compared to controls at this concentration level. Similar to above mentioned findings, brain showed more pronounced expression as compared to other tissues. Finding by the other workers and those of the present study suggest that the sensitivity and organ specificity in MT gene induction by the specific heavy metal may be influenced by exposure condition including doses and manner of treatment and species involved. For example, De Boeck et al. (2003) showed that three fish species, rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*) and gibel carp (*Carassius auratus gibelio*) had different expression levels of MT in different tissues in response to copper exposure. Recently, Choi et al. (2007) also showed that cadmium chloride at different doses induced differential response in four tissues of goldfish. Brain at higher dose (100 μg/g body mass BW) showed lower MT gene expression at 36 h compared to level that was observed at 24 h. However, in case of liver at this dose a reduced level of

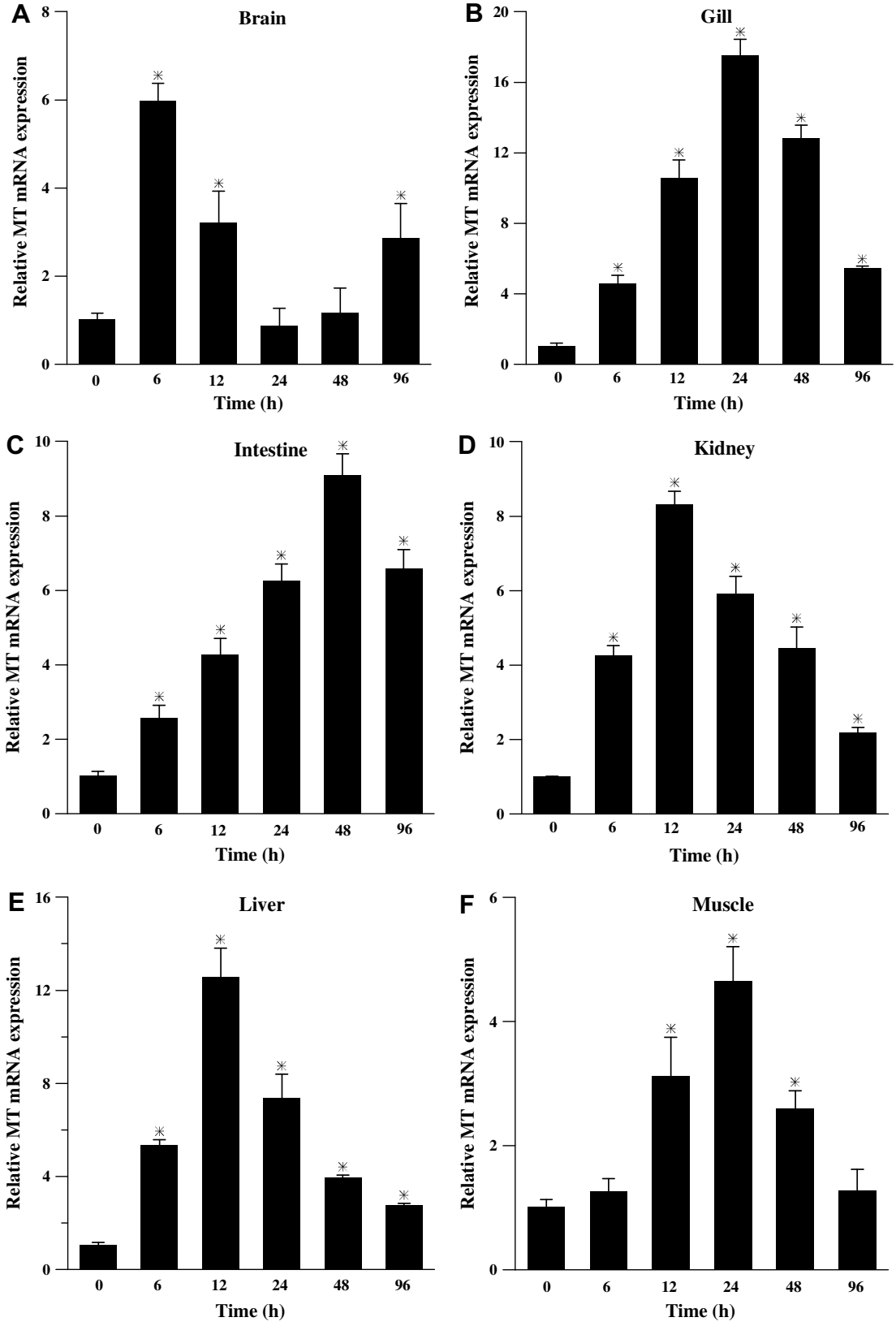

Fig. 5. Relative mRNA expression of *Takifugu obscurus* MT gene in different tissues (A: brain, B: gill, C: intestine, D: kidney, E: liver, F: muscle) after exposure to cadmium (5 ppm) for 96 h. (∗) Indicates significance ($P < 0.05$) change over control values (0 h group). The expression of MT mRNA is relative to β-actin expression, which was used as a housekeeping reference gene. Values are means of triplicate samples for each fish and tissue.

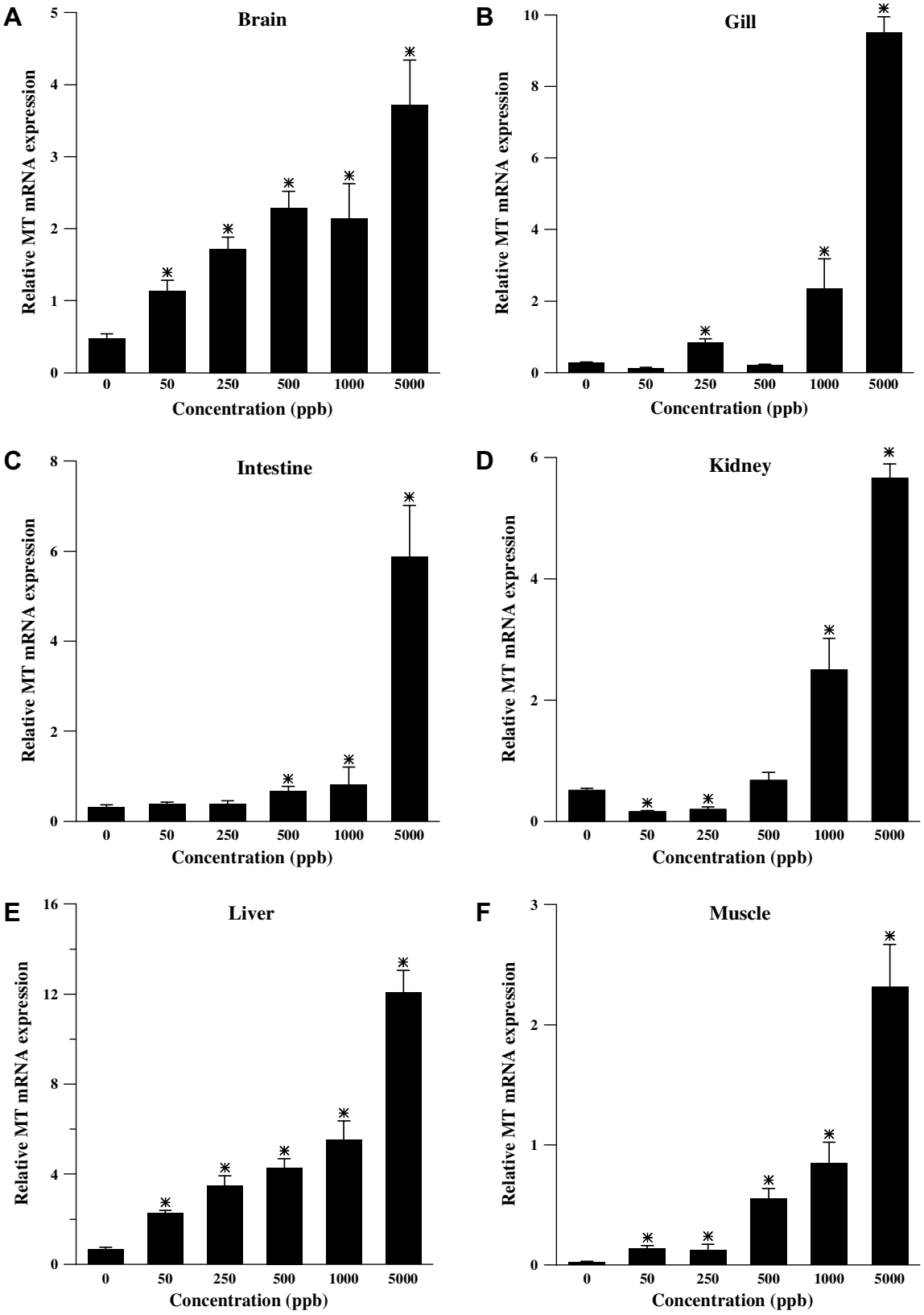

Fig. 6. Relative mRNA expression of *Takifugu obscurus* MT gene in different tissues (A: brain, B: gill, C: intestine, D: kidney, E: liver, F: muscle) after exposure to a range of cadmium concentrations (50 ppb to 5000 ppb) for 24 h. (∗) indicates significance ($P < 0.05$) change over control values (0 ppb cadmium). The expression of MT mRNA is relative to β-actin expression, which was used as a housekeeping reference gene. Values are means of triplicate samples for each fish and tissue.

MT gene expression was observed at even 24 h. These workers reported no such pattern of MT gene expression in case of kidney.

Overall, to our knowledge this is the first report of a full cDNA sequence of any MT gene from *T. obscurus*. Studies on other genes from *T. obscurus* involved in detoxification and antioxidant defense are underway in our laboratory. Because of anadromous behaviour of *T. obscurus* study of stress genes such as MTs can provide useful information on their adaptive strategies. Further studies are needed on MT expression at various stages of development and in different habitat characteristics to fully understand role of MTs in functions other than metal detoxification in *T. obscurus*.

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