Identification of metallothionein gene structure in sterlet (*Acipenser ruthenus)*

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Abstract

Aquatic organisms present, not only simple sources of accumulated metal, but can interact with metals, altering their toxicity. Due to exposition of biosphere with metals, organisms have developed various defense mechanisms to protect themselves against adverse effects of these ions and their compounds. Metallothionein (MT) is one of that which represents a critical mechanism for detoxification of metals.The sterlet (*Acipenser ruthenus*) is a bottom feeding sturgeon specie and because the fish are dependent on invertebrate species for food throughout their life cycle, the sterlet could be a good indicator of the quality of the state of water ecosystem .Addition of copper to water leads to the induction of MT. The present study analyzed MTgene that was excreted from the liver of sterlet exposed to sub-lethal copper concentrations (0.075 mgL^{-1}). To begin to elucidate the molecular mechanism(s) of sensitivity of sturgeons to metals, a RNA encoding MT was purified from livers of sterlet, then a cDNA was synthesized and the MTgene was amplified. The primary structure of sterlet metallothionein (S-MT) contained 20 cysteine residues, which is the same as MTs of teleost fishes. However, the primary structure of S-MT contained 63 amino acids, which is longer than any MT identified in teleost fishes but similar to Lake sturgeon and White sturgeon. The complete nucleotide sequence of the S-MTgene has been detected. We have determined the structure of the fish copper-binding protein by DNA sequence analysis of the gene.

Keywords: Metallothionein, Sterlet, Copper, Gene structure.

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Introduction

Metallothionein (MT) plays an important role in maintaining metal homeostasis in animals. MT genes are readily induced by various physiologic and toxicologic stimuli. The amino acid sequences of MTs from many mammalian sources show that they all contain approximately 61 amino acids of remarkably similar composition. MTs are a group of low-molecular-weight (2 to 16 kDa), cysteine-rich and single-chain metal-binding proteins and synthesized in response to heavy metal ions. MTs have been found throughout the animal kingdom, in higher plants, in eukaryotic microorganisms, and in many prokaryotes (Kojima and Hunziker, 1991). MT has an unusual amino acid composition: It does not contain aromatic amino acids, and most important, one third of its residues are cysteines. Recent interest in MT has focused on the role they play in heavy metal detoxification. The metal binding domain of MTs includes 20 cysteine residues juxtaposed with basic amino acids (lysine and arginine) arranged in two thiol-rich sites (Eckschlager *et al*., 2009). Based on their affinity to metals, these proteins are able to transport essential metals to place of need or detoxify toxic metals to protect cells (Bortleson *et al*., 2001). Because the cysteines in MT are absolutely conserved across species, it was suspected that the cysteines are necessary for proper cell function and MT is essential for life.

Sturgeons (Acipenseridae) are ancient species of fishes with recognizable fossils dating back to more than 65 million years ago (Wilimovsky, 1956). Today, all of the 24 species of sturgeons found in Europe, Asia, and North America are protected under the Convention on the International Trade of Endangered Species of Wild Fauna and Flora (Cakic *et al*., 2004). Sterlet (*A. ruthenus*) is a bottom feeding sturgeon specie that exclusively inhabits freshwater habitats. The main sterlet habitat is Volga and waters with 5 ppt salinity are most suitable for this species. The maximum age reported for this species is 27 years, sexual maturity in the Volga region is reached at 4-5 years in male and 7-9 years in females. The main food of the sterlet in all rivers is benthic organisms. Sturgeons are generally considered to be highly susceptible to bioaccumulation of persistent environmental contaminants, mostly due to both high lipid content in their body and their specific life history, such as a long time to maturity, prominent longevity and benthivorous diet (Kruse and Scarnecchia, 2002; Stanic *et al*., 2006; Webb *et al*., 2006). Benthivorous fish are generally considered to be prone to higher levels of heavy metal accumulation (Dural *et al*., 2006; Stanic *et al*., 2006; Gupta *et al*., 2009).

In this study, MT in sterlet *(A. ruthenus*) was characterized to begin elucidating mechanism(s) of sensitivity of sturgeons to Cu. There is currently no information regarding the gene structure of MT of this specie. Because a limited number of individuals of this specie were available, an *in vitro* liver explant assay was utilized as an initial step to induce expression of MT in liver exposed to a range of concentrations of Cu. The sterlet was exposed to sublethal concentration of Cu to purposely induce MT expression and amplify the full-length coding sequence of S-MT. Full-length cDNAs of starlet-MT were amplified and the polymerase chain reaction (PCR) products were purified. The full-length MT genes had been identified for sterlet for the first time.

Materials and methods

Sample preparation

MT genes are expressed in most tissues of organisms like liver. However, in some cells, they are transcriptionally inactive; therefore, we need the liver tissue of the fish.

Liver explants were used to identify the structure of S-MT following exposure to copper.

First the sterlet specimens were exposed to a high concentration of sublethal copper, extra copies of the MT genes can be produced by exposing sterlet specimens to toxic concentrations of copper.

Sampling was carried out in February 2015. The total mass (g) and total standard body length (cm) of each individual were measured. Juvenile sterlets, ranging in mass from 18 to 38.50 g, were randomly selected from the well in which they were exposed to 17.5 μgL^{-1} of $CuSO_4$ for 14 days. Upon termination of the exposure, The sterlet specimens were dissected and samples of their liver were quickly removed and snap frozen at −80°C. Samples of liver were taken and stored at -80°C before analysis.

Total RNA isolation and MT PCR

Total RNA was isolated from liver of sterlet using TRIzol reagent (Invitrogen, Life Technologies) according to the manufacture's instruction. RNA integrity was assessed through agarose gel electrophoresis and RNA concentration and purity were determined spectrophotometrically using A260 and A280 measurements. Reverse transcription (RT) reactions (20 μL) consisted of 1 μg total RNA, 20 U of an RNAse inhibitor (Promega), 10 mmol dNTPs (Sigma), 4.0 μL of 5×M-MLV RT reaction buffer (Promega), 100 U MMLV transcriptase (Promega), 1.0 μL Oligo (dT) 12-18 (Promega). Cycle parameters for the RT procedure were 1 cycle of 20°C, 5 min; 1 cycle of 42°C, 60 min; 1 cycle of 70°C, 5 min; reaction was stopped by putting on ice. The RT products (cDNA) were stored at -20°C for PCR.

The PCR was carried out with the system (ABI 2720) and the PCR reaction (20 μL) contained 10 μL (GTP PCR Master Mix), and 2.0 μL primer (1.0 μL forward and 1.0 μL reverse), (Table 1: list of primer sequence), and 1.0 μL cDNA template, and 7 μL sterile super-stilled water. For the PCR reaction, the experimental protocol was as follows: denaturation program (95°C for 5 min), amplification and abundance program repeated 30 times (93°C for 30 s, 60°C for 30 s, 72°C for 40 s, and finally extension at 72°C for 10 min). The PCR product was determined with 1-5% agarose gel electrophoresis.

Sequences of sterlet oligonucleotide primers used in amplification of cDNA ends PCR, in sequencing of full-length cDNA.

Sequencing of MT

Full-length of S-MT gene was amplified, the PCR product was purified then sent to the Shine Gene Company for sequencing by Sanger method. Data is shown in Fig. 2.

Phylogenetic tree and multiple sequence alignment

The phylogenetic tree was done and the relationship of S-MT to MTs from other species of fishes, mammals, amphibians, and birds was shown. Alignments of the sequences for this gene region were analyzed in a two-step process. First, Clustal W was created (Besser *et al*., 2007) and then adjusted by eye to make the final alignments. The alignment were subjected to maximum likelihood (Felsenstein, 1981) as applied in Version 4.0 b10 of PAUP.

The construction of phylogenetic hypotheses from the data set was carried out using the maximum likelihood (ML).

Accession numbers of MTs used for these analyses are: *Homo sapiens* MT1 (CAA45516), MT2 (CAA65915), MT3 (AAH13081), and MT4 (AAI13445); *Canislupus familiaris* MT1 (NP_001003173), MT2 (NP_001003149), MT3 (AB001388), and MT4 (NP_001003150); *Rattus norvegicus* MT1 (NP_620181), MT2 (NP_001131036), MT3 (NP_446420), and MT4 (NP_001119556); *Xenopus laevis* MT4 (NP_001081042); *Gallus gallus* MT(BAF51974); *Danio rerio* MT1 (NP_571150) and MT2 (NP_001124525); *Cyprinus carpio* MT1 (AAV52385) and MT2 (AAV52384); *Salmo salar* MTA (NP_001117149) and MTB (NP_001117141); *Oncorhynchus mykiss* MTA (CAA42038) and MTB (CAA42037); *Esox lucius* MT (CAA42035); *A. transmontanus* MT (KP164836); *A. fulvescens* MT(KP164837).

Results

Identification of MT from sterlet

Isolating MT of sterlet by the degenerate primers designed by aligning nucleotide sequences of MT from teleost fishes were unsuccessful just like past efforts in previous researches for isolating MT gene from white sturgeon and lake sturgeon fishes by degenerate primers. A full-length MT gene was later identified in cDNA of the liver of sterlet. Gene specific primers were designed (Table 1) and used to amplify the full-length coding sequence of MT from sterlet (S-MT). Full-length cDNA of S-MT was amplified according to the method earlier mentioned. The PCR product was determined with 1-5% agarose gel electrophoresis (Fig. 1). And as earlier mentioned, the PCRproduct was purified and sent to the Shine Gene Company for sequencing by Sanger method.

Table 1: Sequences of sterlet oligonucleotide primers used in amplification of cDNA ends PCR, in sequencing of full-length cDNA.

Primer name		Primer Sequence(5′-3′)	length
MT1	F	5 ATGGATCCGCAATCTTGCACG 3	192 NT
	R	5 TCACTTGCAGCAGCCGGTGTC 3	
MT2	F	5ACTCGTCACCGGGAAACAAAGC 3	205 NT
	R	5 CGTTTGCCTCCAGACATAGGGG 3	

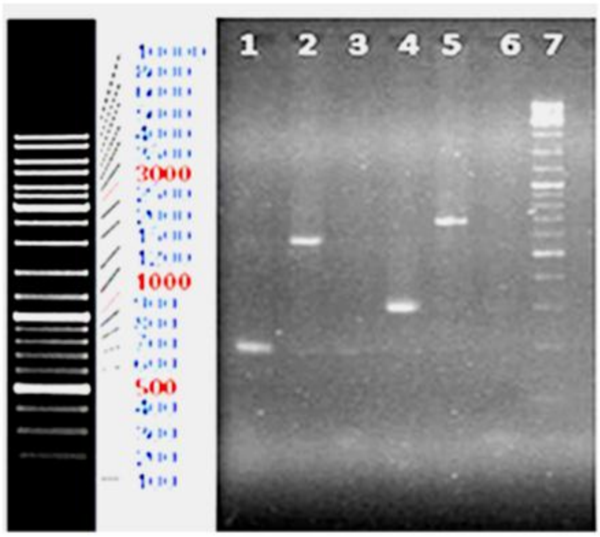

Figure 1: PCR product with 1% agarose gel. Line 1: MT 1 cDNA, line 2: positive control, line 3: negative control, line 4: MT 2 cDNA, line 5: positive control, line 6: negative control, line 7: ladder.

Results presented in this study demonstrate for the first time that sterlet expresses MT during exposure to copper. Full-length sequences of MT were identified in livers of sterlet. A cDNA encoding MT was amplified from livers of sterlet (S-MT). The sequences of nucleotides and the supposed sequence of amino acids are shown in Fig. 2.

The average total mass, total body length and standard body length of analyzed sterlet specimens were 27, 521 g and 21.028 cm, respectively. Cu concentrations were below the sublethal concentration in all analyzed samples.

The coding regions of (S-MT) are 192 nucleotides which encode 63 amino acids. The molecular mass of (S-MT) is predicted to be 6304 g/mol.

MT contain 20 C residues, a characteristic that is typical of MT identified up to now (Capasso *et al*., 2003).

ATG	GAT	CCG	CAA	TCT	TGC	ACG	TGC	GCT	CAG	GGT	GGT	TCA
Met	D	P	Q	S	C	T	C	*A*	Q	G	G	S
TGC	AGC	TGC	GGT	GAT	AAC	TGC	AAA	TGC	ACG	GAC	TGC	AAA
C	S	C	G	D	N	C	K	C	T	D	C	K
TGC	AAA	ACT	TGC	AAG	AAA	AGC	TGC	TGC	TCC	TGT	TGT	CCC
C	K	T	C	K	K	S	C	C	S	C	C	P
ACC	GAC	TGC	AGC	AAA	TGT	GCC	CAG	GGC	TGC	GTC	TGC	AAA
T	D	C	S	K	C	A	Q	G	C	V	C	K
GGG	GGA	GCC	ACC	TGC	GAC	ACC	GGC	TGC	TGC	AAG	TGA	AAAC
G	G	A	T	C	D	T	G	C	C	K	Stop	K

Figure 2: Nucleotide sequence of the protein coding region of S-MT. Amino acids is shown. Dissimilarity in amino acids is point to italics. The CXXXCC motif which is a typical of MTs from fishes is highlighted.

Discussion

Sequence comparison

The distinction in the primary structure of (S-MT) and other homologous fish like white sturgeon and lake sturgeon are at position 11 where WS-MT had an alanine (A) but S-MT and LS-MT have glycine (G) residue, and at position 9 where S-MT has a (A) residue but LS-MT and WS-MT have a (T) residue; also at position 50, where S-MT has a (V) residue but LS-MT and WS-MT have (A) residue (Fig. 3).

Sturgeons were separated from the lineage leading to teleost fishes in the neighborhood of 300 million years ago following divergence of the Actinopterygii (which includes both sturgeons and teleost fishes) from the Sarcopterygii (which includes the tetrapods) (Kumar and Hedges, 1998; Blair and Hedges, 2005)

The primary structure of S-MT that is from common ancestry of MTs from fishes support this conclusion that the S-MT was distinguished as being most closely related to MTs of fishes (Fig. 4). For example, the presence of a CXXXCC motif in the α-domain of S-MT (Fig. 2), resulting from a shift in the position of the ninth C residue of the α-domain, is a characteristic of MTs from fishes, whereas MTs from other clades contain a CXCC motif in this region (Saydam *et al*., 2002; Capasso *et al*., 2003).

There are some elements of the reported primary structure of S-MT proteins that are distinct from MTs in species of teleost fishes. For example, the number of cysteine–lysine (CK) or lysine–cysteine (KC) amino acid pairs is the distinction between MTs from fishes and mammals, the number of these pairs in MTs from mammals is 5 to 7, which is greater than in fishes (Dauria *et al*., 2001; Storelli *et al*., 2007).

A

ATG GAT CCG CAA TCT TGC ACG TGC GCT CAG GGT GGT TCA
Met D P Q S C T C *A* Q *G* G S

TGC AGC TGC GGT GAT AAC TGC AAA TGC ACG GAC TGC AAA
C S C G D N C K C T D C K

TGC AAA ACT TGC AAG AAA AGC TGC TGC TCC TGT TGT CCC
C K T C K K S C C S C C P

ACC GAC TGC AGC AAA TGT GCC CAG GGC TGC GTC TGC AAA
T *D* C S K C A Q G C *V* C K

GGG GGA GCC ACC TGC GAC ACC GGC TGC TGC AAG TGA AAAC
G G A T C D T G C C K *

B

ATG GAT CCG CAA TCT TGC ACG TGC ACT CAG GCT GGC TCG
M D P Q S C T C *T* *Q* *A* G S

TGC AGC TGT GGT GAT AAC TGC AAG TGC ACA GAC TGC AAA
C S C G D N C K C T D C K

TGC AAA ACT TGC AAG AAA AGC TGC TGC TCC TGT TGT CCC
C K T C K K S C C S C C P

ACC GGC TGC AGC AAG TGT GCC CAG GGC TGC GCC TGC AAA
T *G* C S K C A Q G C *A* C K

GGG GGA GCC ACC TGC GAC ACC GGC TGC TGC AAG TGA
G G A T C D T G C C K *

C

ATG GAT CCG CAA TCT TGC ACG TGC ACC CAG GGT GGT TCG
M D P Q S C T C *T* *Q* *G* G S

TGC AGC TGT GGT GAT AAC TGC AAG TGC ACG GAC TGC AAA
C S C G D N C K C T D C K

TGC AAA ACT TGC AAG AAA AGC TGC TGC TCC TGT TGT CCC
C K T C K K S C C S C C P

ACA GAC TGC AGC AAG TGT GCC CAG GGC TGC GCC TGC AAA
T *D* C S K C A Q G C *A* C K

GGG GGA GCC ACC TGC GAC ACC GGC TGC TGC AAG TGA
G G A T C D T G C C K *

Figure 3: Nucleotide sequence of the protein coding region of S- MT (A), W-MT (white sturgeon) (B) and L-MT(lake sturgeon) (C). Differences in amino acids are indicated in italics. The stop codon is advisable by an asterisk.

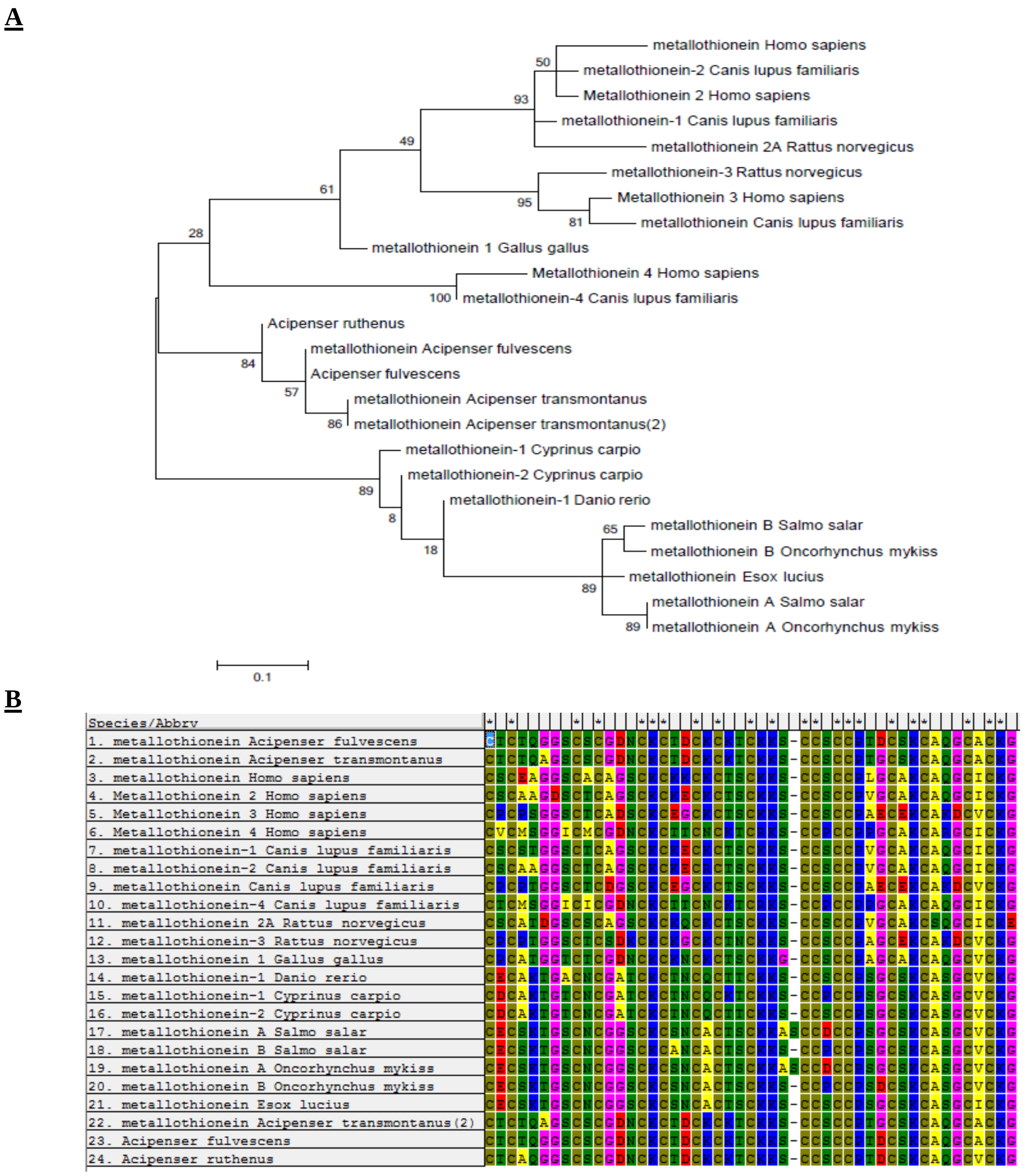

Figure 4: Correlation of sequences of amino acids of MTs with different species of vertebrates. Phylogenetic tree for correlation of MT with mammals, birds, amphibians, teleost fishes and two members of sturgeons (A). Branch lengths represent bootstrap values based on 1000 samplings. Alignment of sequence of MTs from teleost fishes (B). Accession numbers of MTs are specified in the Materials and methods section.

There are 4 CK (KC) pairs in each MT shown in the alignment (Fig. 4B). The exception to this is the MT from common carp (*C. carpio*), which has 5 pairs. However, 7 CK (KC) pairs are present in S-MT and also in WS-MT and LS-MT, and these extra pairs are located at positions 25–26, 27–28, and 62–63. One more difference between S-MT and MTs of other species of teleost fishes which is similar to L-MT and W-MT is the length of the protein. MTs of teleost fishes contain 60 amino acids, except for metallothionein A (MTA) from rainbow trout (*O. mykiss*) and Atlantic salmon *(S. salar*) that contain

61 amino acids (Erdogrul *et al*., 2007). However, S-MT and also WS-MT and LS-MT have 63 amino acids, which is longer than any MT identified in teleost fish.

Compared with other species of fishes, the S-MT, WS-MT and LS-MT, (three members of sturgeon fish, asipenceridae), have amino acids inserted at positions 4 (glutamine; Q), 5 (serine; S). An MT identified in the cloudy catshark (*Scyliorhinus torazame*), which is a cartilaginous fish, contains 68 amino acids (Erdogrul *et al*., 2007). In mammals, most MT1 and MT2 contain 61 amino acids, while MT3 consists of 65 to 68 amino acids, and MT4 of 62 amino acids (Kille *et al*., 1992). The length of S-MT is identical to that of MTs from several species of birds (McGeer *et al*., 2000; Keskin *et al*., 2007; Hollis *et al*., 2001; Yilmaz *et al*., 2007; Poleksic *et al*., 2010).

Liver is an important organ for accumulation of some metals and expression of MT, thus liver explants were used to identify S-MT following exposure to copper. The aim of this study was to identify MT genes for the first time in one Member of the Acipenseridae: sterlet (*A. ruthenus*). We have isolated this gene and analyzed its structure. The S-MT gene was extracted and its complete nucleotide sequence was reported. Enriched MT mRNA was used as a template for cDNA synthesis, primed by a MT-specific, synthetic nucleotide. The reported primary structures of S-MT were like MTs from other fishes and they have special structure for binding to metals.

As a suggestion, future studies should inquire other heavy metals compensatory responses in sterlet, responses in other target organs like gills or kidney, and gene expression of MT in response to copper exposure.

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