Vol. 205, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1994 Pages 368-374

PCR-CLONING OF GOLDFISH AND TILAPIA METALLOTHIONEIN COMPLEMENTARY DNAs

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Received October 3, 1994

Summary Metallothionein (MT) is believed to be a sensitive and effective "biomarker" for monitoring metal contamination in fish. Comparison of amino acid sequences between flounder MT and trout MT revealed that there is a conserved region at their N-terminals. Using oligonucleotides derived from this conserved region, reverse transcription- polymerase chain reaction (RT-PCR) was performed to obtain MT complementary DNAs (cDNAs) from goldfish, *Carassius auratus*, and tilapia, *Tilapia mossambica*. These cDNA probes would be useful in developing sensitive PCR-based methods to detect MT gene expression for monitoring metal pollution in local waters.

Metallothionein (MT) is a family of low molecular weight, cysteine-rich proteins that have a high affinity for divalent cations. Because MT has a high cysteine content (30%) and is found to have extensive homology among amino acid sequences of MTs isolated from diverse species from mushroom, crab, and mammals, therefore it is suggested that there has been a conservation of heavy-metal binding function throughout evolution [1, 2]. Although the physiological functions of MT remain obscure, it is generally accepted that MT plays important roles in the regulation and metabolism of the essential trace metals such as zinc and copper and in the detoxification of heavy metal cadmium and mercury [3, 4]. However, there is a paucity of information on the interactions among metal ions, MT and other metal binding proteins and thus the precise *in vivo* mechanism of action of MT is not yet known [1, 2, 3, & 4]. Recently, considerable efforts have been made on studying the role of MT in cancer chemotherapy [5], nephrotoxicity [6, 7], zinc regulation in brain [8, 9], transgenesis [10, 11], and the use of MT for bio-monitoring of metal contamination in waters [2, 12, 13, & 14].

Research on MT in fish is still in its infancy with almost all of the studies following up on the MT detoxification hypothesis forwarded for mammals. Fish is one of

0006-291X/94 $5.00

the most widely distributed organisms in aquatic environment, suspectible to metal contamination and could reflect the extent of biological effects of metal pollution in waters. In this regard, it is interesting to know the role of MT plays in fish for normal metal physiology or detoxification. In fish, preexposures to sublethal levels of metals result in the induction of MT and can result in an acclimation to a potentially toxic level of these metals in the waters [15]. MTs have been characterized from various fish, including flounder, trout, perch, and carp [16, 17, 18, & 19]. Recently, MT gene expression in caged fish exposed in the field is believed to be a sensitive and reliable biomarker for monitoring of metal pollution in waters [Wirgin, New York, personal communication]; similar approach has been used for monitoring of organic pollution using fish cytochrome P450Ia1 levels as biomarker [20].

From our earlier studies on flounder MT cDNA [21], we reported the use of a MT oligonucleotide probe derived from the N-terminal amino acid sequence of purified flounder MT. This N-terminal amino acid sequence seems to be highly conserved in teleost as shown from the alignment of the Salmonidae and Pleuronectidae MTs [21, 22] (Figure 1). Using oligonucleotide derived from this conserved domain, RT-PCR was performed to obtain MT cDNAs from liver tissues of goldfish (Cyprinidae) and tilapia (Cichlidae). These cDNAs are useful probes in detection of MT mRNA levels and the cDNA sequences will be useful in designing specific MT primers for RT-PCR assay of fish MT mRNA levels.

MATERIALS AND METHODS

Design of Oligonucleotides

From the N-terminal decapeptide region as a conserved domain in fish MTs (Figure 1) the fish MT oligonucleotide is designed as 5'ATG GAT CCN TGC GAA TG3', with minimized of mixed sequences. The RACE-T primer and adaptor sequences are adapted from Frohman *et al.*, 1988 [23].

RT-PCR Primers for cloning:

- RACE-T primer for reverse transcription: 5'ccgaa ttctc gagat cgatt ttttt ttttt tt3'
- PCR 5'-primer (Fish MT-oligonucleotide): 5'atgga tccnt gcgaa tg3'
- PCR 3'-adapator primer: 5'ccgaa ttctc gagat cga3'

From the cloned MT cDNA sequences, a new specific 3'-reverse primer (5'CTCCTCACTGGCAGCAGCT3'; nucleotide number 169-187, Figure 4) was then designed from the coding region encoding for the C-terminal of tilapia MT.

Oligonucleotides were ordered from Bio-synthesis, Inc., Texas, USA.

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N-TERMINAL  SEQUENCES:
        10        20        30    ,etc.
MDPCECSKTGTCNCGGSCTCKNCSCTTCNKSCCP....(FLOUNDER)
----------S-------K-S--A--S-K---------....(TROUT-B)

N-TERMINAL  CONSERVED  SEQUENCE:
MDPCECSKTG--
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Figure 1. Alignment of flounder MT and salmonidae MT amino acid sequences (partial).

RNA Extraction and Reverse Transcription- Polymerase Chain Reaction (RT-PCR)

This method is also known as RACE, rapid amplification of cDNA ends as developed by Frohman *et al.* [23]. Total RNA was prepared from livers of tilapia and goldfish using the Acid-Guanidinium-Thiocyanate-Phenol-Chloroform method [24]. One microgram RNA was used for reverse transcription in a 50 microlitre reaction and was incubated at 42 C for one hour. The reverse transcription reaction contains 10 units of ribonuclease inhibitor (Pharmacia), 10 units of avian myeloblastosis virus reverse transcriptase (Promega), 2mM of each dNTP, 10 ng of RACE-T primer, and buffer supplied from Promega. The reaction was then diluted to a final volume of 200 microlitres at the end and then two microlitres of the first strand cDNA pool were used for PCR (94 C, 1 min.; 50 C, 2 min.; 72 C, 3 min.; 25 cycles), using a PTC-100 thermal cycler, MJ Research, Inc. Enzymes and reagents for PCR were purchased from Promega. Five units of *Taq* polymerase, 100 ng of each primer and 1.25 mM of each dNTP were contained in a reaction of 50 microlitres. The primers for the PCRs were the fish MT oligonucleotide, with the 3'-adaptor primer or the 3'-reverse primer. A set of β-actin primers designed by Marsh and Chen (Centre of Marine Biotechnology, University of Maryland, unpublished data) was used as control primers for PCRs (Fig. 2).

Eight microlitres of the PCR products were fractionated on a 2% agarose gel with Tris-EDTA-acetic acid buffer for electrophoresis[25]. The desire PCR products were purified using glass-milk/ sodium iodine method after agarose gel electrophoresis [26]. The purified DNA samples were then phosphorylated with ATP using polynucleotide kinase (Promega), filled-in with Klenow polymerase and dNTPs (Pharmacia), and blunt-end ligated to *Sma* I digested pUC 18 plasmid (Pharmacia) for nucleotide sequence determination using a T7 DNA polymerase sequencing kit (Pharmacia).

Fish Experiment

Tilapia (*Tilapia mossambica,* also known as *Sarotherodon mossambicus* or *Oreochromis mossambicus*) was collected from a land-locked pond on the campus of the

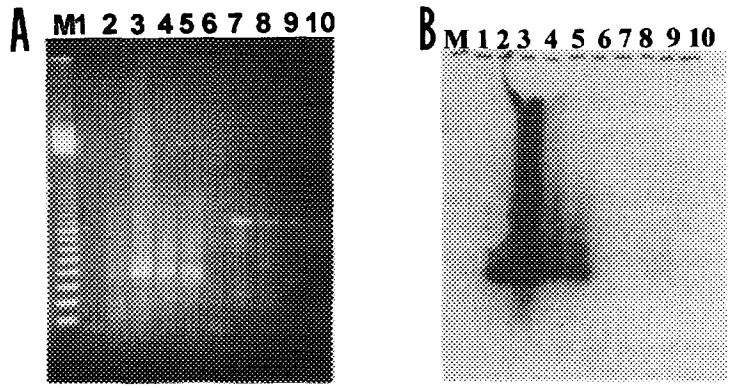

Figure 2. RT-PCR products probed with ^{32}P-labelled flounder MT cDNA. Panel A is the ethidium bromide stained gel (2% agarose) before blotting. Panel B is an autoradiogram of Southern blot analysis of DNA samples hybridized with a ^{32}P-labelled flounder MT cDNA (labelled by nick translation) after low stringency wash at 2X SSC, 65 C. LaneM contains 100 bp markers (Pharmacia). Lane 1 to 10 are loaded with PCR products, lane 1 to 5 are loaded with reactions using the universal MT oligonucleotide and the 3'-adaptor primer, whereas lane 6 to 10 are loaded with reactions using the actin primers. Lane 1 and 6 are negative control reactions without any cDNA pools; lane 2 and 7 are reactions with tilapia liver cDNA pools; lane 3 and 8 are reactions with liver cDNA pools from zinc-treated tilapia; lane 4 and 9 are reactions with goldfish liver cDNA pools; lane 5 and 10 are liver cDNA pools from estrogen treated goldfish. Exposure time for autoradiography was 12 hours using Kodak X-OMAT film.

Chinese University. Golfish (*Carassius auratus*) was obtained from local fish farm. The fish were kept in freshwater aquaria and maintained at around 20 C in the laboratory. Multiple injections of zinc ions were done as described [21].

RESULTS AND DISCUSSION

RT-PCR were performed using the fish MT oligonucleotide and the 3'-adaptor primer; actin primers were also used as control for PCRs. Figure 3 shows the results of DNA samples of PCRs resolved on a 2% agarose gel. Endogenous expression of MT was detected in goldfish and tilapia, however the PCR products found in zinc treated tilapia (total dosage of 20 mg Zn^{2+}/ kg body weight) were much more than that of the un-treated control. Estrogen treatment did not show any enhancement of PCR products indicating the lack of induction of MT mRNA level in the liver of goldfish. The desire PCR products were then excised out from the agarose gel and cloned into plasmid for nucleotide sequence determination to confirm if they are MT sequences. From the normal goldfish sample, eight clones were isolated and found to have inserts of about 400 bp, however only one of these clones hybridized to the flounder MT cDNA probe (data not shown). From the tilapia samples (zinc- treated) six clones were obtained and they all hybridized with the flounder MT cDNA probe (data not shown).

Nucleotide sequence determination of one goldfish MT cDNA clone (Figure 4) and one tilapia MT cDNA clone (Figure 5) further confirmed that the PCR products contain MT sequences. They both encode for 60 amino acids with 20 cysteine residues, and are Class I MTs. The tilapia MT shows 83% amino acid sequence identity with the flounder MT, whereas the goldfish MT shows 76% amino acid sequence identity with the flounder MT. Their locations of cysteines are highly conserved in fish MTs (Figure 5).

Tilapia, *Tilapia mossambica*, is chosen as a model fish in Hong Kong as it is the most commonly found euryhaline fish in local waters. This tropical fish also distributes world-wide and therefore is an important fish to be used for bio-monitoring of metal pollution. Tilapia is also easy to be kept in aquaria, with total seawater or freshwater, for experimental purposes. The tilapia MT cDNA has been cloned as a first step towards the development of RT-PCR to assay for MT mRNA levels in tilapia. From the cloned tilapia MT cDNA sequences, a new specific 3'-reverse primer was then designed from the coding region coding for the C-terminal of tilapia MT. I have also used this specific 3'- reverse primer and the universal fish MT oligonucleotide to carry out the RT-PCRs (Figure 6). Significant induction of hepatic MT mRNA following administration of cadmium chloride ions, with a minimal background in the liver of tilapia. More careful experiments would be carried out to standardize the RT-PCR protocol to assay for MT mRNA levels with precision and accuracy. Moreover, different tissues such as gill filament should be tested for their suitability as tissue to be assayed for MT mRNA level as biomarker, recently non-invasive sampling technique using gill filament has become a common method for toxicological testing.

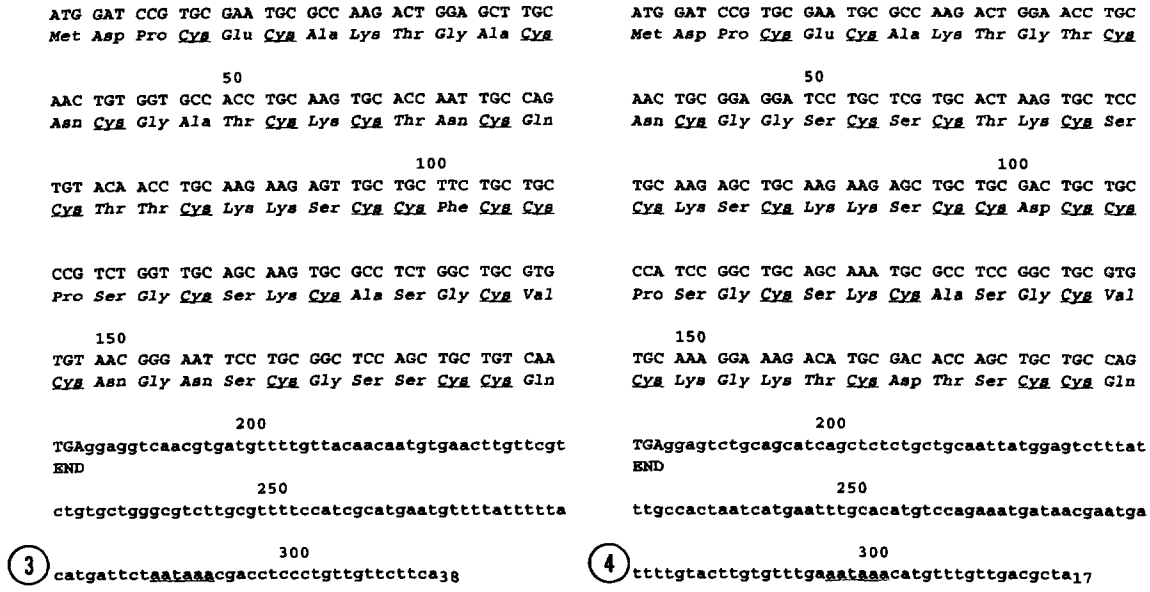

Figure 3. Nucleotide sequence of goldfish (*Carassius auratus*) metallothionein cDNA. The complete coding region and the predicted amino acid sequence of a cloned MT cDNA are shown. Lower-case letters are used to denote the untranslated region; the last "a" represents the first A of 38 A residues.

Figure 4. Nucleotide sequence of tilapia (*Tilapia mossambica)* metallothionein cDNA. The complete coding region and the predicted amino acid sequence of a cloned MT cDNA are shown. Lower-case letters are used to denote the untranslated region; the last "a" represents the first of 17 A residues.

In conclusion, this communication reports the use of a universal fish MT oligonucleotide to perform PCR-cloning of two MT cDNA from two different fish species, namely from the Cyprinidae (goldfish) and Cichlidae (tilapia). This PCR cloning strategy can be applied to obtain other fish MT cDNA sequences, Kille *et al*, 1991 [27]

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1        10        20        30        40        50        60
MDPCECSKTGTCNCGGSCTCKNCSCTTCNK SCCPCCPSGCPKCASGCVCKGKTCDTTCCQ (1)
----------S-------K-S--A--S-K-A---D------S---------------S--- (2)
----------S-------K-S--A--S-K- --------D-S---------------S--- (3)
---------------AT-K-T--Q----K- ----------S----------NS--SS--- (4)
------A--------AT-K-T-------K- ---F------S--------N-NS-GSS--- (5)
------A-----------S-TK---KS-K- ---D------S---------------S--- (6)

(1)  WINTER FLOUNDER MT, adapted from [21].
(2)  RAINBOW TROUT MT-A, adapted from [22].
(3)  RAINBOW TROUT MT-B, adapted from [22].
(4)  STONE- LOACH MT, adapted from [27].
(5)  GOLDFISH MT, PRESENT STUDY.
(6)  TILAPIA MT, PRESENT STUDY.
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Figure 5. Alignment of fish MT amino acid sequences. Metallothioneins are highly conserved in Teleosts and the 20 cysteine residues remain unchanged.

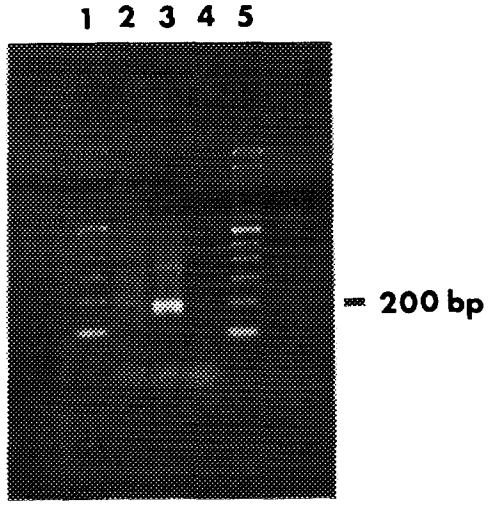

Figure 6. RT-PCR products resolved by agarose (2%) gel electrophoresis and visualized after ethidium bromide staining. Lane 1 and 5 are loaded with 100 bp markers (GibcoBRL), lane 2, 3, and 4 are loaded with PCR products using the universal MT oligonucleotide and the tilapia MT 3'-specific primer. Lane 2 is loaded with reaction using tilapia liver (normal) cDNA pools as templates; lane 3 is loaded with reaction using liver cDNA pools from zinc-treated tilapia; and lane 4 is loaded with reaction without the addition of templates (negative control).

also described a similar method to clone Salmonidae (trout and pike) MT cDNAs. From the cloned tilapia MT cDNA sequence, specific 3'-reverse primer was further designed and together with the universal MT oligonucleotide, specific MT mRNA could be amplified by PCR. RT-PCR assay will be a very sensitive method and can be adopted to perform quantitative work [28].

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