Cloning of cytochrome P450 1A (CYP1A) genes from the hermaphrodite fish *Rivulus marmoratus* and the Japanese medaka *Oryzias latipes*

Il-Chan Kim a, Young Ja Kim b, Yong-Dal Yoon c, Shoji Kawamura d, Yong-Sung Lee a, Jae-Seong Lee b, *

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*Department of Biochemistry, College of Medicine, Hanyang University, Seoul 133-791, Republic of Korea*

b *Department of Environmental Science, Graduate School, Hanyang University, Seoul 133-791, Republic of Korea*

c *Department of Life Science, College of Natural Sciences, Hanyang University, Seoul 133-791, Republic of Korea*

d *Department of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa, Chiba 277-8562, Japan*

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**Abstract**

To use two small fish *Rivulus marmoratus* (Cyprinodontiformes, Rivulidae) and the Japanese medaka *Oryzias latipes* (Beloniformes) as testing models in molecular ecotoxicology, we have cloned the cytochrome P450 1A (CYP1A) gene after screening of both genomic DNA libraries, and sequenced 11,863 and 7,243 bp including all the exons and introns with promoter regions, respectively. The *Rivulus* and the medaka CYP1A gene consisted of seven exons (including non-coding exons) with high homology to mammals. In the promoter region, *Rivulus CYP1A* gene has seven xenobiotic response elements (XREs) and two metal response elements (MREs), while the Japanese medaka CYP1A gene has six XREs and four MREs. Interestingly, medaka CYP1A gene has a number of MREs at the promoter, which may affect its response on metal exposure. We describe here the gene structure of both fish CYP1A genes.

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*Corresponding author. Tel.: +82-2-2290-0769; fax: +82-2-2294-6270.
E-mail address: jslee2@hanyang.ac.kr (J.-S. Lee).*

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The fish cytochrome P450 1A (CYP1A) gene has been cloned and sequenced from many organisms for use in assessing contamination in the aquatic environment (Fent, 2003; Meyer, Nacci, & Di Giulio, 2002 Moore et al., 2003; Williams, Lech, & Buhler, 1998). Recently, its utility in identifying the action of suspected endocrine-disrupting chemicals has been emphasized (Schlezinger & Stegeman, 2001). However, the sequence similarity between CYP1A genes is not very high, resulting in moderately low interspecies cross-reactivity between CYP1A antibodies (Cousinou, Nilsen, Lopez-Barea, & Dorado, 2000), although certain antibodies will bind to CYP1A proteins between different genera or orders. It may therefore be necessary to obtain the sequence of CYP1A to use a new testing organism for reverse transcriptase-polymerase chain reaction (RT-PCR) and/or Western blots for laboratory or cage-experimental CYP1A induction (Cousinou et al., 2000). In this connection, we cloned the CYP1A gene from the self-fertilizing fish, *Rivulus* and the Japanese medaka *Oryzias latipes* both of which are well-known species for environmental toxicology studies (Lee, Park, Choe, & Chipman, 2000, 2002; Rotchell, Lee, Chipman, & Ostrander, 2001). In this paper, we show the genomic structure of both fish CYP1A genes and suggest that the Japanese medaka would be useful for testing heavy metals as well as endocrine-disrupting chemicals based on the response elements in its promoter region.

To clone the CYP1A gene of both fish, we screened *Rivulus* λGEM-11 genomic DNA library (from Jae-Seong Lee’s lab) and medaka (HNI strain) λEMBL3 genomic DNA library (from Shoji Kawamura’s lab). About $3 \times 10^5$ genomic clones were screened by plaque hybridization using a radiolabelled *Rivulus* CYP1A partial probe according to the plaque hybridization method. Autoradiography was carried out with Kodak X-ray film and an intensifying screen at $-70 \, ^\circ C$ for 48 h. CYP1A positive clones were repurified. Ten *Rivulus* and eight medaka CYP1A clones were isolated from the primary, secondary and tertiary screening of these genomic DNA libraries. Using the consensus primers (Fish-CYP1A-F, 5'-CAT CCG TGA CAT CAC TGA CTC-3' and Fish-CYP1A-R, 5'-GCC GTA TTC TGG GGT CAT GTC-3') for CYP1A, we amplified both CYP1A genes and sequenced them. After obtaining the amplified internal sequences of both CYP1A genes by PCR, we were able to isolate the amplified long-PCR products of both CYP1A clones using additional primers (internal primers from the previous PCR products amplified using consensus primers and the Sp6/T7 primer from λphage multicloning site). This avoided time-consuming work in purifying *Rivulus* and medaka λCYP1A clones by a conventional method. We subcloned them to pCR2.1 vector (Invitrogen) and sequenced with an automated DNA sequencer. To analyze the gene structure of both CYP1A genes, we used the BLAST search program of NCBI and compared them to the existing CYP1A sequences of other species.

The sequences of both CYP1A genes were deposited in GenBank under the accession numbers of AY279213 (Rivulus CYP1A gene) and AY233000 (Japanese medaka CYP1A gene) (Fig. 1(a)). Both the *Rivulus* and the medaka CYP1A genes consisted of seven exons (including non-coding exons), and they spanned about 3 kb. The accepting and donor sequences of exon/intron boundary were according to the
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**Fig. 1.** (a) Schematic representation of the *Rivulus marmoratus* and the Japanese medaka *Oryzias latipes* CYP1A genes. The shadow and black boxes indicate a non-coding and the coding exons, respectively. X, Xenobiotic Response Element (XRE); M, Metal Response Element (MRE); A, Poly(A) signal sequence. (b) Dendrogram inferred from CYP1A in fishes. Numbers on clade indicate the bootstrap value.

GT/AG rule and were identical to those of other CYP1A genes. Therefore, *Rivulus* and medaka CYP1A genes are highly structurally conserved with those of other species. Poly(A) signal sequences of the CYP1A genes were located at 0.7 and 1.5 kb downstream for *Rivulus*, and there were five sites in 1 kb for medaka CYP1A gene. The 3'-untranslated region (UTR) of medaka CYP1A contained (ATCT)$_3$; repetitive sequences.

The phylogenetic tree relating to fish CYP1A was constructed after we applied them to the ClustalW program (www.ebi.ac.uk/clustalw/index.html) with TreeView of PHYLIP, based on similarity of amino acid residues of both CYP1A genes to those of other fishes; *Fundulus* (Morrison, Weil, Karchner, Sogin, & Stegeman, 1998; AF026800), European flounder (Williams, Lee, Sheader, & Chipman, 2000; AJ132353), eel (Aoki, Itakura, Kato, & Sato, 1999; AB020414), rainbow trout (AF015660), and zebrafish (AB078927). Both of our fish CYP1A genes showed generally high similarity but some regions were poorly matched, which may result in non-specific binding to CYP1A antibodies derived from other species, even though the monoclonal antibody from John Stegeman’s lab has had remarkable cross-reactivity with CYP1A from various species (personal communication). It may prove
necessary to clone each CYPIA gene and raise specific antibodies for each potential testing organism to obtain a good correlation of chemical-induced protein expression profiles by Western blotting for specific species.

In the CYPIA promoter region, Rutilus CYPIA gene has seven XREs consensus sequences in 7704 bp, which we expect to mediate the induction of this gene by polycyclic aromatic hydrocarbons, including some EDCs. We also found two MREs, giving rise to the possibility that there is some regulation of this gene by heavy metals (Fig. 1(a)). The European flounder CYPIA gene has two potential MREs (one is proximal and other is distal), this was the first report to date that a fish CYPIA gene promoter contained MRE sites (Williams et al., 2000).

However, the Japanese medaka CYPIA gene has four MREs and six XREs in a short sequence (2596 bp) of its promoter region (Figs. 1A and 2). It is interesting that the Japanese medaka CYPIA gene promoter included 4 MREs, which may potentially confer a response to metal exposure, as is found for the metallothionein gene. As shown in Fig. 1, we find that the XREs and MREs on the medaka CYPIA promoter are closely associated while other fish's CYPIA promoters have their response elements spread out over a longer sequence. Also, in the medaka CYPIA gene promoter, there are additional elements, a PRL motif and an Sp1 binding site (Fig. 2). Therefore, we may speculate that the Japanese medaka could be an ideal model to determine the relationships between environmental contaminant-mediated gene induction and the effects on the whole organism. To make use of these characteristics of the medaka CYPIA gene promoter, we are constructing a transgenic medaka line using compact chemical response elements in a luciferase reporter gene construct.

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**Fig. 2.** Nucleotide sequence of the Japanese medaka CYPIA 5'-flanking region, exon 1 and intron 1. Nucleotides are numbered to the putative transcriptional start site, with negative numbers representing the 5'-flanking region. Nucleotides in the exons are represented in bold type. Consensus transcription factor binding sites are underlined (see text). These were determined using MacDNASIS program.
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