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CYP3C1, the first member of a new cytochrome P450 subfamily found in zebrafish (*Danio rerio*)

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Abstract

We report a new cytochrome P450 (CYP) subfamily CYP3C and the cloning through PCR from zebrafish (*Danio rerio*) of the first member, CYP3C1 (AY499002). The *CYP3C1* gene is on Chromosome 3 with 13 ORF exons encoding a 505 amino acid protein which has 44–54% identities with mammalian and teleost CYP3A and CYP3B forms. As evidenced by spectral analysis, the CYP3C1 protein heterologously expressed in yeast is functional. In silico analysis identified, on the same region of the chromosome, three more genes encoding CYP3C1-like proteins that formed a clade with CYP3C1 in a phylogenetic tree. Using RT-PCR, the CYP3C1 mRNA was detected in 1–6 dpf embryo/larvae and in adult fish liver and seven extrahepatic tissues. Whole-mount in situ hybridization using a riboprobe demonstrated expression in the brain during 12–120 hpf. At the 120 hpf larval stage, CYP3C1 mRNA was also detected in the pharynx and gastrointestinal tract. TCDD, dexamethasone, and rifampicin, which up-regulated CYP3A65 mRNA in zebrafish larvae, did not alter the CYP3C1 transcript levels suggesting regulatory differences between CYP3A and CYP3C enzymes in this species.

Keywords: Cytochrome P450; CYP3C1; AY499002; Zebrafish; Phylogenetic tree; Embryo; Tissues; Yeast

Cytochrome P450 3A (CYP3A) enzymes, currently with 70 subfamily members [1], are the most abundant enzyme among all the CYPs in vertebrates and are responsible for the metabolism of a large number of physiologically, clinically, and toxicologically important chemicals and drugs [2,3]. The zebrafish (*Danio rerio*) is a popular model for studying embryogenesis and developmental genetics [4,5], and this fish species also has been extensively used as an animal model to investigate toxicity [6] and physiological processes [7] and the mechanisms for chemical carcinogenesis [8–10]. Therefore, it was important to identify the CYP3A orthologs that are present in zebrafish. Previously we have identified a member of CYP3A subfamily, CYP3A65, from zebrafish, which shares significant identity with mammalian, avian, and teleost CYP3A members and is extensively expressed in gastrointestinal tract after hatching [11]. In this study, we used two GenBank registered zebrafish ESTs, AI332015 and AI330590 that were predicted [1] based on protein sequence similarity to belong to the CYP3A subfamily, as starting sequences to search for the full-length coding cDNA (fl-CDS) for zebrafish CYP3A orthologs. We serendipitously discovered, instead, the first member of a new cytochrome P450 CYP3C subfamily. Here we describe the molecular cloning of CYP3C1, determination of its primary structure, the in silico analysis of its genomic organization, and the mining for more CYP3C1-like genes. We also constructed a phylogenetic tree to evaluate its evolutionary relationships with other mammalian

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and teleost CYP3A and CYP3B proteins. Whole-mount in situ hybridization and the RT-PCR techniques were applied for establishing the embryonic expression and adult tissue distribution pattern of CYP3C1. We carried out heterologous expression in yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) to demonstrate its functionality by spectral analysis. Finally, modulation of CYP3C1 mRNA expression was attempted by treatment of zebrafish larvae with TCDD, dexamethasone, and rifampicin. Our report opens the door for further studies on this new zebrafish CYP gene subfamily.

Materials and methods

Fish and embryo maintenance. Zebrafish (*Danio rerio*) AB strain wild-type embryos and fish were maintained at 28.5 °C and were staged by hours post-fertilization (hpf) or days post-fertilization (dpf) as described [12,13]. Adult fish of 6 month age were used for the RT-PCR of tissues.

Molecular cloning by polymerase chain reaction (PCR). Two zebrafish ESTs from the Washington U. St. Louis zebrafish Genomic Project, AI332015 (447 bp) and AI330590 (442 bp), were identified as CYP3A subfamily members on David Nelson's Cytochrome P450 web site (http://drnelson.utmem.edu/CytochromeP450.html) [1]. The nucleotide sequences were translated into amino acids (aa) using a 6-frame translation program through BCM (Baylor College of Medicine) web site (http://searchlauncher.bcm.tmc.edu/seq-util/seq-util.html) and then using NCBI's BLASTp to determine which frame yielded a P450 match. We then obtained the untrimmed sequence for these ESTs through a website link kindly provided by Dr. Steve Johnson, Washington University (http://www.genetics.wustl.edu/fish_lab/cgi-bin/display.cgi?type=wz&value). These untrimmed 5′ and 3′ EST nucleotide sequences, fa96d12.y1 (953 bp) and fa96d12.x1 (1409 bp), respectively, overlapped in contig alignment. However, the overlap was in area of poor sequence quality and many uncertainties in nucleotide sequence occurred for hundreds of bases in the middle of this putative full-length coding consensus sequence (fl-CDS). In order to verify and to fill in the uncertain gaps, we carried out PCR, 5′-, 3′-RACE PCR, RT-PCR, and primer-walking for every 300–500 nucleotide (nt) along the assembled sequence. The amplification template used was either a zebrafish cDNA library or cDNA from total RNA. The cDNA library was obtained from Dr. Koichi Kawakami (the KK library), National Institute of Genetics, Japan, through the I.M.A.G.E. Consortium [14]. 5′RACE and the 3′RACE PCRs were carried out using the vector (pME18S-FL3) primers (forward, z161pME738PCRf: 5′-tgttgcctttacttctaggcctgtacg-3′ and reverse, z164pME1271PCRr: 5′- tgtttcaggttcaggggaggtgt-3′), various gene-specific primers, and the KK library as a template. After confirming the 5′-untranslational region (UTR) close to the translation start codon and the 3′UTR near the stop codon, an RT-PCR (using total RNA from adult zebrafish as described [15,16] and the primers designed from the UTRs: forward/ reverse, 5′gcattagtgtacatgagcatttg3′/5′tgaaccacgaaacaacg agc3′) was performed to obtain the fl-CDS for the CYP isoform for which we were searching. The RT-PCR product was cloned into pGEM-T Easy (Promega) for later in vitro transcription synthesis of a riboprobe. Regular PCR using primers including the translation start codon (z130-15-590Fatg2: 5′-cacc**atg**tttgatctctcgtctctgtctgtgacctggacc-3′) and the stop codon (z131-15-590Rstop: 5′-**tta**ttgtttttccttgtgagaccttggtattaacttcagtgtga tgg-3′) and the KK library was also carried out to confirm the nt sequence involving the open-reading frame (ORF). Our eventual goal was to heterologously express this gene for further characterization studies hence an accurate sequence in the coding region was important. Sequencing for both strands was carried out by the Central Service Lab of the Center for Genome Research and Biocomputing at Oregon State University and by commercial sequencing services in Taiwan, Republic of China.

In silico gene analysis (computer gene analysis). NCBI BLAST programs [17] were used for gene analysis to obtain gene comparisons among different species. Chromosome localization of the cloned CYP was determined using the *Danio rerio* sequencing project database of the Sanger Institute (http://www.sanger.ac.uk/Projects/D_rerio/). The Spidey program [18] (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/), an mRNA-to-genome alignment program from NCBI, was utilized to determine the genomic exon and intron organization. Exon and intron junctions were also ascertained by alignment of the cloned CYP cDNA to zebrafish version 5 genomic DNA sequence using Ensembl BLASTVIEW (http://www.ensembl.org/Multi/blastview?species=Danio_rerio/) and SSA-HA (Sequence Search and Alignment by Hashing Algorithm). The alignment was performed with the BioEdit Sequence Alignment Editor version 6.0.7 BioEdit (1997–2004 Tom Hall, Iris Pharmaceuticals, Inc.) by insertion of gaps into the cDNA sequence to bring about alignment with the genomic sequence. Gaps were manually adjusted to maintain exact matches in exons and to have introns with canonical dinucleotides [19,20] "gt" at the 5′ end and "ag" at the 3′ end of introns.

Phylogenetic tree. The amino acid sequences, a total of 32 involving mammalian and fish CYP3A and CYP3B subfamily members, used in constructing the tree are listed in Table 1. Sequence alignment was performed using ClustalX ver 1.85 (www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html), and non-aligning sequence segments were masked off prior to constructing the tree. The rooted tree was prepared using the Neighbor Joining method of Saitou and Nei [21] with Bootstrap values of 1000 replicates.

RNA extraction and RT-PCR for developmental and tissue-specific expression studies. Total RNA extraction from adult fish tissues and RT-PCR were performed as described previously [15,16]. Tissues examined in this study included brain, eye, gill, heart, liver, intestine, ovary, and skin. The primers used in RT-PCR to amplify the 1741 bp DNA fragment for CYP3C1 are the following: forward (5′gcattagtgtacat gagcatttg3′) and reverse (5′tgaaccacgaaacaacgagc3′). β-Actin was chosen as internal standard as previously described [20] using the degenerate zebrafish actin primers (forward 5′cttgggaygayatggaraa3′ and reverse: 5′atytcytgytcra-artc3′) in the RT-PCR. The expected size of the amplicon was 467 bp.

Whole-mount in situ hybridization. Whole-mount in situ hybridization was carried out as described [11,12]. The sense (as negative control) and the anti-sense riboprobes were DIG-UTP labeled and synthesized using the DIG RNA labeling kit (Sp6/T7) (Roche, Indianapolis, IN) with the prepared pGEM-T plasmid containing the desired CYP gene as a template. The hybridization was detected by anti-DIG antibody conjugated to alkaline phosphatase. Embryos were incubated in a 0.003% (0.2 mM) phenylthiourea (PTU) to block melanin production and hence extend the period that larva remained optically clear [13].

Modulation of gene expression. Three chemicals that induced zebrafish CYP3A65 [11] were tested for their ability to increase mRNA levels of our newly cloned P450 isoform. Fertilized zebrafish embryos were incubated at 28.5 °C in waterborne 10 or 100 μM dexamethasone, 10 or 100 μM rifampicin, or 1 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) as described previously [11]. Xenobiotics were dissolved in dimethylsulfoxide (DMSO) so that final solvent concentration was 0.03% for rifampicin and dexamethasone treatments and 0.003% for TCDD treatment. Whole-mount in situ hybridizations were performed to examine the effect of the treatments at various time point up to 120 h post-fertilization (hpf) as described [11].

Heterologous expression in yeast. The Gateway Cloning Technology from Invitrogen (Carlsbad, CA) was employed in our heterologous expression study similar to our previous expression studies with zebrafish CYP1A [22]. The full-length ORF was amplified by PCR using a forward primer, 257-3C1flF: 5′cacc**atg**tttgatctctcgtctctgtctgtgac3′ (the start codon is shown as bold and underlined), a reverse primer, 269-3C1flRstop: 5′tgctccacatttccatt**att**gtttttccttgtgag3′ (the antisense of the stop codon is shown as bold and underlined), Vent DNA polymerase (NEB, Ipswich, MA), and the KK library (as a template) to generate a blunt-ended product for constructing an entry clone with entry vector, pENTR/SD/D TOPO. Subsequent transformation into TOP10 *Escherichia coli* cells, selection and analyzing colonies, isolating plasmid, sequence confirmation, and eventually proceed to the recombination reaction to construct an "expression clone" through LR clonase with a destination vector, pYES-DEST52, were all according to the Manufacturer's Instruction Manuals.