Published in final edited form as:

Aquat Toxicol. 2013 October 15; 0: . doi:10.1016/j.aquatox.2013.09.014.

Functional Characterization of a Full Length Pregnane X Receptor, Expression in vivo, and Identification of PXR Alleles in Zebrafish (*Danio rerio*)

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Abstract

The pregnane X receptor (PXR) (nuclear receptor NR1I2) is a ligand activated transcription factor, mediating responses to diverse xenobiotic and endogenous chemicals. The properties of PXR in fish are not fully understood. Here we report on cloning and characterization of full-length PXR of zebrafish, *Danio rerio*, and *pxr* expression in vivo. Initial efforts gave a cDNA encoding a 430 amino acid protein identified as zebrafish *pxr* by phylogenetic and synteny analysis. The sequence of the cloned Pxr DNA binding domain was highly conserved, with 74% identity to human PXR, while the ligand-binding domain (LBD) of the cloned sequence was only 44% identical to human PXR-LBD. Sequence variation among clones in the initial effort prompted sequencing of multiple clones from a single fish. There were two prominent variants, one sequence with S183, Y218 and H383 and the other with I183, C218 and N383, which we designate as alleles *pxr*1* (*nr1i2*1*) and *pxr*2* (*nr1i2*2*), respectively. In COS-7 cells co-transfected with a PXR-responsive reporter gene, the full-length Pxr*1 (the more common variant) was activated by known PXR agonists clotrimazole and pregnenolone 16 α -carbonitrile but to a lesser extent than the full-length human PXR. Activation of full-length Pxr*1 was only 10% of that with the Pxr*1 LBD. Quantitative real time PCR analysis showed prominent expression of *pxr* in liver and eye, as well as brain and intestine of adult zebrafish. The *pxr* was expressed in heart and kidney at levels similar to that in intestine. The expression of *pxr* in liver was weakly induced by ligands for mammalian PXR or CAR (NR1I3). The results establish a foundation for PXR studies in this vertebrate model. PXR allelic variation and the differences between the full-length PXR and the LBD in reporter assays have implications for assessing the action of PXR ligands in zebrafish.

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Introduction

The NR1I subfamily of nuclear receptors includes three ligand activated transcription factors, the vitamin D receptor VDR (NR1I1), the pregnane X receptor (PXR; NR1I2; also known as the promiscuous xenobiotic receptor, or the steroid xenobiotic receptor, SXR), and the constitutive androstane receptor (CAR; NR1I3). Mammalian PXR¹ and CAR bind a great diversity of ligands including numerous steroids and xenobiotics (Ekins et al., 2008; Kretschmer and Baldwin, 2005; Waxman, 1999; Yasuda et al., 2008) and are referred to as xenobiotic sensors. In mammals, PXR and CAR regulate overlapping sets of genes for enzymes involved in metabolism or disposition of xenobiotics and various endogenous substances, including members of the cytochrome P450 (CYP) gene families 2 and 3, conjugating enzymes, and ATP-binding cassette (ABC) transporters and others (Goodwin et al., 2003; Kliewer et al., 2002; Kliewer et al., 1998; Moore et al., 2003; Park et al., 2012; Wang et al., 2012; Waxman, 1999).

PXR likely arose from the duplication of an ancestral chordate PXR/VDR, before vertebrate emergence (Ekins et al., 2008; Fidler et al., 2012). However, CAR diverged from PXR in or prior to the Sarcopterygian (lobe-finned fish) line, and was lost from or did not occur in the teleost line (Mathas et al., 2012). Thus, PXR in teleosts could represent a receptor with ligand-binding and/or regulatory properties serving functions that in mammals are served by both PXR and CAR. Ligands for mammalian PXR include such compounds as pregnenolone, pregnenolone-16 α -carbonitrile (5-pregnen-3 β -ol-one-16 α -carbonitrile; PCN), rifampicin (RIF), nifedipine (NIF), clotrimazole (CLO) and phenobarbital (PB) (Ekins et al., 2008; Moore and Kliewer, 2000; Watkins et al., 2001). CAR is activated by some of these same compounds (e.g., PB), though generally there are differences in ligand selectivity or efficacy [e.g., (Timsit and Negishi, 2007)]. PXR and CAR also bind bile acids and it has been suggested that the changing repertoire of cholesterol metabolites may have driven NR1I evolution (Krasowski et al., 2005b).

Here we report on PXR from zebrafish. This study began as a search for receptors (other than the aryl hydrocarbon receptor) that may regulate genes for xenobiotic metabolism in fish. Unlike mammals, teleost fish show little or no induction of microsomal enzyme activity or CYP protein by PB (Addison et al., 1987; Ankley et al., 1987; Elskus and Stegeman, 1989; Goksøyr et al., 1987; Kleinow et al., 1990). Whether this reflects differences in receptors or differences in the repertoire or regulatory mechanisms of CYPs still is unclear, although response of some *CYP2* genes to PB has been reported recently in zebrafish (Kubota et al., 2013). Knowledge of PXR in fish should help to discern the underlying mechanisms for the response to PB type inducers in fish, and the differences in such responses between fish and mammals.

As induction by PB is prominently mediated by CAR, our initial cloning effort was in search of a CAR homolog, which at the time was not known to be absent from teleosts; that effort repeatedly resulted in sequences that phylogenetic analyses indicated were PXR. Here we report on cloning and analysis of the full-length zebrafish *pxr* cDNA sequence, and provide evidence for *pxr* allelic variants in one strain of zebrafish. Expression of *pxr* in vivo, including responses to potential agonists, also was examined. Studies of ligand selectivity of

¹Gene and protein symbols in this paper follow standard zebrafish, human, and mouse nomenclature guidelines. We default to the human nomenclature when the species is not specified, when nomenclature has not been established for a species, or when referring to multiple species. In all cases reference to zebrafish cytochromes P450 follow human symbols, due to nomenclature precedence.

species / gene / protein
 zebrafish / *pxr* / Pxr
 human / PXR / PXR
 mouse / *Pxr* / PXR

PXR of zebrafish and other non-mammalian species most often examine reporter gene expression stimulated by activation of the PXR ligand-binding domain (LBD) fused to a Gal4 DNA-binding domain (DBD) [e.g., (Ekins et al., 2008; Fidler et al., 2012; Krasowski et al., 2005b; Milnes et al., 2008; Moore et al., 2002)]. Full-length PXR sequences are less commonly used in such reporter assays. A single study with full-length rainbow trout PXR (in HepG2 cells) showed that rainbow trout PXR is less responsive to prototypical mammalian PXR agonists (Wassmur et al., 2010). We expressed PXR sequences in reporter systems and compared activation of the full-length zebrafish Pxr with that of full-length human PXR, and compared activation of full length zebrafish Pxr with activation of the Pxr LBD, by the same ligands.

Material and methods

Chemicals

1,4-Bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) was kindly provided by Dr B.A. Diwan and Dr L. Anderson (National Cancer Institute, Frederick Cancer Research and Development Center, MD, USA). The product was checked for purity by elemental analysis, melting point, ¹H NMR and TLC, and was considered essentially pure. All ligands used were purchased from Sigma Aldrich (St. Louis, MO): clotrimazole (CLO) (Cat No: 23593), nifedipine (NIF) (Cat No: N7634), and 5-pregnen-3 β -ol-one-16 α -carbonitrile (pregnenolone 16 α -carbonitrile; PCN) (Cat No: P0543). All other reagents used also were purchased from Sigma Aldrich (St. Louis, MO). Chemicals were of the highest grades commercially available. The molecular biology kits used are indicated below.

RNA extraction and cDNA analysis

Sequences were cloned from liver of the Tüpfel long fin (TL/previously named Tuebingen long fin) strain of *D. rerio*. Fish were anesthetized, killed by cervical section and the liver was excised immediately and frozen in liquid nitrogen. The mRNA was isolated from liver of fish using the MicroPoly(A)Pure™ (Ambion). Oligo d(T)-primed cDNA synthesis was carried out using SMART™ PCR cDNA synthesis kit (Clontech). An initial fragment of the *pxr* was identified as an EST sequence (accession no. AI943313) and used for designing primers for amplification. The 5' and 3' ends of the cDNA were amplified using SMART™ RACE cDNA Amplification kit (Clontech). Initially, primers 5'-GCCTCAATGTAAAGTCTTTAGGGTAAGC-3' and 5'-TATGCGGCGACAAATCTACTGGC-3' were designed in order to amplify the internal region of the gene. A 986 bp product was obtained and the sequence used in a BLAST search of sequences in GenBank to confirm the similarity with other known PXR genes. Nested RACE was carried out using the primers 5'-TTGTGCCCATTTTCAGTCAGTTCCG-3' on the plus strand, and 5'-TCCTCCATAACCAGAGCCCTGAAC-3' on the minus strand, to obtain the 3' and 5' ends, respectively. Primers to amplify the full-length *pxr* coding sequence were designed on 5' and 3' untranslated regions, as 5'-CGTGTCTGCTCGTGATGAGGCA-3' and 5'-GAGTTGCACGTGCTTTGTGAGGAC-3', respectively. Touchdown PCR was carried out as follows: 5 cycles (94°C, 5 s; 72°C, 90 s); 5 cycles (94°C, 5 s; 70°C, 90 s); 30 cycles (94°C, 5 s; 59°C, 10 s; 72°C, 90 s). The PCR product was purified using GeneClean II (Bio 101, Inc.) and ligated to the pGEM®-T easy vector overnight at 4°C. Plasmids containing the insert were purified using Qiagen miniprep kit. The sequences were analyzed using ABI Prism DNA PE Applied Biosystems using BigDye™ Terminator for sequencing reactions. Sequence data were analyzed using the Sequencer software (Gene Codes Corporation, Ann Arbor, MI).

Sequence comparisons and alignments were carried out using MacVector (Cary, NC), ClustalX (Larkin et al., 2007), McClade (Sinauer Associates, Sunderland, MA), and BioEdit (Ibis Biosciences, Carlsbad, CA). Phylogenetic trees were constructed by analyzing cDNA sequences under maximum parsimony using MEGA5 (Tamura et al., 2011), or inferred or confirmed amino acid sequences under maximum likelihood using RAxML (v 7.2.6 (Stamatakis, 2006), with the WAG model of amino acid substitution (Whelan and Goldman, 2001).

Sampling of tissues

Adult male and female zebrafish were euthanized with MS222. A dissection protocol similar to Gupta and Mullins (Gupta and Mullins, 2010) was followed, and multiple tissues were obtained. Three replicates were collected for both males and females, resulting from four individuals pooled per replicate for each organ. The dissected organs were flash frozen in liquid nitrogen and were stored at -70°C until RNA isolation. These were the same samples as reported in our recent study (Kubota et al., 2013).

Chemical treatment

Sexually mature TL zebrafish were used to study response of zebrafish to potential agonists for PXR. We used the same samples as reported in our recent study (Kubota et al., 2013) for the analysis of *pxr* mRNA expression. Briefly, given the variation in *pxr* expression seen in females (see above), only males were used in order to avoid possible confounding effects of sex in the levels of transcription of selected genes. Individuals ($n=8$) were injected *i.p.* with TCPOBOP ($1.5\text{ }\mu\text{g/g}$) or PB ($70\text{ }\mu\text{g/g}$) dissolved in a solution of 15% dimethylsulfoxide (DMSO) and 85% saline. Fish from the vehicle control group were injected with the DMSO/saline solution. Three days after injection, fish were killed by cervical transection, liver was excised immediately and frozen in liquid nitrogen. This time point has been shown to be sufficient for significant levels of induction of other genes that are possible targets of Pxr (Kubota et al., 2013). Total RNA was isolated from the liver of individual fish using RNA STAT-60 (Tel-Test B, Inc., Friendswood, TX); cDNA was synthesized from $1\text{ }\mu\text{g}$ total RNA using the iScript kit (Bio-Rad, Hercules, CA).

Quantitative real time PCR

Quantitative real time PCR (qPCR) was performed using the iQ SYBR Green Supermix (Bio-Rad) in a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad), according to the manufacturer's instruction. Gene-specific primers for real time PCR were synthesized by Eurofins MWG Operon (Huntsville, AL, USA). Primer sequences for *arnt2* and *efl1a* have been published previously (Jonsson et al., 2007). A new primer pair was designed for *pxr*; F-GCATTTCGCGTCCATATCACAGAG and R-CTAACTAGGGCTCCACTTCCTGG, and for *CYP3A65*; F-ATGGTGCCGACCTACGCCCTC and R-GGGCCCAGACCGAACGGCAT (5' to 3' sequences). In each sample, the genes were analyzed in duplicate with the following protocol: 95°C for 3 min and 95°C for 15s / 62°C for 1 min (45 cycles). A melt curve analysis was performed at the end of each PCR run to ensure that a single product was amplified.

Sequencing of the amplicon generated with some tissue samples, including liver, eye and brain, confirmed that the right product (i.e. *pxr*) was amplified by qPCR with the new primer pair. Aliquots of qPCR amplicons randomly selected from wells of 96 well plates were ligated to pGEM-T Easy vector (Promega). One Shot Mach1 T1 Phage-Resistant Chemically Competent *E. coli* (Invitrogen) were transformed with the ligated plasmid. Plasmids containing the insert were purified using Qiagen Mini Prep kit (Qiagen) and then used for sequencing (Eurofins MWG Operon). Relative mRNA expression of each target gene was normalized to that of *arnt2* and *efl1a* ($E^{-\Delta\text{Ct}}$; where $\Delta\text{Ct} = [\text{Ct}(\text{target genes}) -$

Ct(geometric mean of *arnt2* and *ef1a*)). Relative changes due to treatment (TCPOBOP or PB) were determined by $E^{-\Delta\Delta Ct}$ ($E^{-\Delta Ct[\text{sample}]/\text{mean } E^{-\Delta Ct[\text{control}]}}$). PCR efficiencies (E) for within-experiment amplicon groups were determined by the LinRegPCR program (Ramakers et al., 2003; Ruijter et al., 2009).

Cell culture, transfection and reporter assays

Two different approaches were used in the analysis of ligand activation of the zebrafish Pxr. Both were carried out in COS-7 monkey kidney cells (ATCC, Manassas, Virginia, USA). Cells were maintained in DMEM (Sigma) supplemented with fetal bovine serum (FBS) (10% final concentration) (Sigma) at 37°C under 5% CO₂, with 4 mM L-glutamate, 1 mM sodium pyruvate, 100 U/mL penicillin and streptomycin.

In the first approach, cells were plated at 3.0×10^4 cells/well in 48-well plates. Transfection in triplicate wells was carried out 24 h after plating. DNA and Lipofectamine 2000 reagent (Life Technologies) were diluted in serum-free DMEM. For each well, a total of approximately 300 ng of DNA was complexed with 1 µl of Lipofectamine 2000. The mixture was then added to cells in DMEM with serum. Renilla luciferase (pRL-TK, Promega) was used as the transfection control.

The full-length zebrafish *pxr* cDNA was cloned into the expression vector pcDNA 3.1 Zeo (+) (Invitrogen) and transiently transfected into COS-7 cells along with a reporter construct (XREM-TK-Luc) bearing a CYP3A enhancer module (a gift from John Moore), and a transfection control construct (pRL-TK) (Promega). Transfected DNA amounts were 100 ng of either zebrafish Pxr or human full length PXR (a gift from Steven Kliewer, used for comparison and as a positive control), 50 ng of XREM-TK-Luc and 3 ng of pRL-TK. The total amount of transfected DNA was kept constant by addition of pcDNA 3.1 vector with no insert. Cells were treated 5 h after transfection with either dimethylsulphoxide (DMSO) (solvent control), or CLO, NIF or PCN. Cells were lysed at 24 or 48 h after dosing and luminescence was measured using the Dual Luciferase Assay kit (Promega) in a TD 20/20 Luminometer (Turner Designs, Sunnyvale, California, USA). The final luminescence values are expressed as a ratio of the firefly luciferase units to the Renilla luciferase units. The data obtained were analyzed by Student's t-test and the level of significance was $p < 0.05$.

Subsequent to the first assessment with the full-length *pxr*, luciferase reporter gene transactivation potentials of the full-length zebrafish *pxr* were compared to those obtained using a Gal4-DBD-zebrafish *pxr* LBD fusion protein. The full-length zebrafish *pxr* in the pcDNA3.1 expression vector used above was the template for amplification of an open reading-frame encoding the hinge and ligand-binding regions of PXR (AA111-430). Using the *EcoRI* and *BamHI* sites, the amplicon was cloned into a eukaryotic expression vector carrying the DNA binding region of the yeast transcription factor Gal4 (AA1-147) (Blumberg et al., 1998), resulting in a plasmid expressing a 467 amino acid long fusion protein consisting of the DNA-binding domain of the yeast Gal4 transcription factor and the LBD of zebrafish Pxr.

For the luciferase assays using the Gal4-DBD/*pxr*-LBD fusion, COS-7 cells were plated at 5×10^3 cells/well in 96-well plates and transiently transfected by the CaPO₄ method using 0.5 µg/plate of receptor plasmids and 10 µg/plate of luciferase reporter and β-galactosidase transfection control plasmid, essentially as described by Grün and coworkers (Grün et al., 2002). Cells were maintained as above. Receptors and β-galactosidase were expressed continuously from CMV-based plasmids. Activation assays with full-length zebrafish Pxr used the XREM-TK-Luc luciferase reporter plasmid regulated by a promoter region consisting of core and distal xenobiotic responsive enhancer modules of the human CYP3A4 gene (Goodwin et al., 1999), while LBD activations used tkx4(MH100)-luc reporter plasmid

with a herpes virus thymidine kinase promoter with four Gal4 upstream activation sequences (Gal4-UASs) (Harmon et al., 1995). Twenty-four hours post-transfection, the cells were exposed to the test compounds at 10^{-7} to 6×10^{-5} M. Ligands, dissolved in DMSO, were diluted in phenol-red free DMEM supplemented as during maintenance except that FBS was replaced by heat-inactivated, charcoal-stripped FBS.

Following exposures, cells were lysed, and assayed for transfection efficiencies and reporter gene transactivation measured by luminescence for luciferase and by absorbance at 420 nm for β -galactosidase (EnSpire multimode plate reader, Perkin Elmer, Waltham, Massachusetts, USA). Luciferase activities were normalized for variations in transfection efficiencies by dividing the luciferase activity from a lysate by β -galactosidase activity in the same lysate. Ligand activation was expressed as fold induction of luciferase activity relative to the DMSO solvent control. Experiments were performed in triplicate for each concentration and repeated in three independent experiments. Mean luciferase activities and standard deviations were determined and dose-response curves fitted by non-linear regression using the GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). The data obtained were analyzed by Student's t-test and the level of significance was $p < 0.05$.

Statistics

Messenger RNA expression levels of target genes are presented as mean $\pm$ SD for tissue distribution analysis and as range with 25th to 75th percentile for chemical effect analysis. Significant differences in the *pxr* and *CYP3A65* mRNA levels among 7 tissues were determined by one-way ANOVA, which if significant was followed by Tukey-Kramer test. Significance of differences in the *pxr* mRNA expression levels between control and treatment groups (i.e., TCPOBOP or PB) was determined by one-way ANOVA, which if significant was followed by Dunnett's test. The significant level was set at $p < 0.05$. All statistical analyses regarding the *pxr* expression in vivo in zebrafish were performed with GraphPad Prism 5.0.

Results

Cloning of the full length *pxr*

The initial full-length zebrafish *pxr* cDNA that was sequenced (GenBank Accession # DQ069792.1) contained 1290 nucleotides encoding a 430 amino acid protein. This sequence is 46 % identical to PXR in human, monkey, mouse and rat. Phylogenetic analysis was carried out with amino acid sequences for NR1I2 genes representing various chordate taxa including tunicate PXR/VDR (Ekins et al., 2008) (Fig. 1). The zebrafish full-length sequence clustered with other teleost PXR sequences. As expected, the PXR cluster forms a sub-group distinct from the NR1I2 (CAR) and NR1I1 (VDR) clades (Bertrand et al., 2004; Krasowski et al., 2011b). The percent identity with other (inferred) full-length PXR sequences from fish ranges from 42–57% (Supplemental Table 1). Shared microsynteny between zebrafish and other vertebrate sequences (Fig. 2) confirms this zebrafish sequence as the ortholog of mammalian PXR.

DNA and ligand binding domains were determined based on the NCBI conserved domains for PXR [cd07162 and cd06934; (Marchler-Bauer et al., 2013)]. The percent identities among the cloned zebrafish sequences and representative cloned or inferred DBDs and among the LBDs (Supplemental Tables 2 and 3) show that zebrafish PXR-DBD is 74% identical to the DBD of the human PXR, nearly 70% identical to rat and mouse PXR-DBDs, and 77% identical to frog PXR-DBD. By contrast, the zebrafish PXR-LBD is between 44–46% identical to the rodent, monkey, rabbit and human PXR-LBDs. The percent identities among the (inferred) fish PXRs also show greater identities in the DBD than in the LBD

(Supplemental Table 2). Molecular phylogenetic analyses of the two separate domains confirm the conservation of the DBD, and the diversity of the LBD (data not shown).

The initial sequence was obtained from liver tissue pooled from four fish. Multiple clones were sequenced, and sequence variation appeared in clones from that pool (Fig. 3, Table 1). This prompted us to prepare and sequence multiple clones from a single individual. The clones from the pooled sample and the single fish revealed several non-synonymous nucleotide substitutions. Most striking were the differences in amino acid residues at three positions, 183, 218 and 383, with substitutions that were consistently associated to form two distinct groups, one with S183, Y218 and H383, and the other with I183, C218 and N383. The consistent variation at these sites we conclude represents two different alleles of *pxr*, which we designate *pxr*, *allelic variant 1* and *pxr*, *allelic variant 2*, respectively (*pxr*1* and *pxr*2*; formally *nr1i2*1* and *nr1i2*2*). We note that the current zebrafish nomenclature scheme does not have guidelines for naturally occurring alleles (ZFIN-Community, 2013). Hence the allele nomenclature proposed here follows that of the HUGO Gene Nomenclature Committee (Antonarakis, 1998; den Dunnen and Antonarakis, 2000, 2001; Gray et al., 2013), and is in accordance with the scheme currently in use for *CYP* alleles (Sim and Ingelman-Sundberg, 2013) and other genes with naturally-occurring wild-type alleles (Nebert, 2002). This nomenclature scheme has the virtue of clarity and simplicity, and the additional value of facilitating comparisons to the deep literature on allelic differences in human drug and toxicant metabolism.

In addition to the differences between *pxr*1* and *pxr*2*, there were less frequent substitutions in other locations (Table 1). These other changes appeared to be associated with one or the other of the two common allelic variants. Thus, insertion of an N after N222 along with S232N and T234S was present in some fish with S183, Y218 and H383 residues (*pxr*1*). The changes associated with the other presumed allele (*pxr*2*: I183, C218 and N383) were primarily synonymous differences, mostly A309T (Table 1). The various substitutions are located in both the DNA and the ligand binding domains (Fig. 3A). Two distinct substitution patterns were obtained from one fish (*pxr*1* and *pxr*2*; Fig. 3A), and no distinctly different additional patterns were observed in clones from the pooled sample (4 fish). Although we only obtained two distinct variants in this cloning effort, conservatively 10 alleles are possible (2 per fish).

In vivo expression of *pxr* and CYP3A65

To assess regulation, we examined expression of *pxr* and of a putative PXR target gene, *CYP3A65*. A quantitative real time RT-PCR primer for *pxr* was designed in the 5'-UTR, which enabled us to detect both alleles of *pxr* (i.e., *pxr*1* and *pxr*2*). Quantitative real time PCR showed that *pxr* expression in male zebrafish was more prominent in liver and eye, followed by intestine, brain, heart, and kidney (Fig. 4A). Cloning and sequencing of the amplicon obtained from eye and brain confirmed that the sequence amplified from these organs was indeed *pxr*. A similar tissue distribution pattern was observed in females, although with relatively greater inter-individual variation (Fig. 4B). The *pxr* expression in gonad was the lowest among the tissues examined for both sexes.

Unlike *pxr*, *CYP3A65* mRNA was expressed principally in intestine, and to a lesser extent in the liver (Fig. 4C, 4D). Other tissues examined, including testes/ovary, heart, kidney, brain, and eye, had low levels of *CYP3A65* expression in adult zebrafish.

Response to NR agonists

An initial semi-quantitative analysis of *pxr* expression (data not shown) suggested an induction of *pxr* expression in liver of zebrafish treated with TCPOBOP and PB, typical

inducers of CYP2B and CYP3A expression in mammals. Subsequent qPCR analyses showed no significant increase in the transcript levels of *pxr* in liver of zebrafish treated with these chemicals (Fig. 5). However, there was large inter-individual variation in the expression level of *pxr* within a treatment group. This variation seemed to be more pronounced in zebrafish treated with TCPOBOP and PB than in fish treated with vehicle. Thus, some individuals in the treatment groups had 2-fold greater expression than that of the average value in the vehicle group.

Ligand activation assays

To better understand its functional properties, we analyzed the ability of full-length zebrafish Pxr to activate transcription of a reporter gene after transient transfection into COS-7 cells. The initial studies were with the reporter gene construct (XREM-Luc) that contained a luciferase gene under control of the CYP3A4 promoter. In this set of assays, we compared the ligand-dependent induction of reporter gene expression in cells transfected with the full-length zebrafish *pxr*, to the response of cells transfected with full-length human *pxr*. The zebrafish Pxr variant used in these experiments was Pxr*1 (with S183, Y218 and H383). Zebrafish Pxr*1 was activated 3.4-fold by CLO at 10 μ M, and 2.1-fold by PCN at 10 μ M, compared to DMSO-treated cells. NIF at 10 μ M did not activate the zebrafish Pxr. These responses differ from that in cells transfected with human *pxr*, and treated with the same concentrations of these compounds. CLO, NIF and PCN activated human PXR at 5.6-, 2.4- and 1.5-fold respectively (Fig. 6).

Studies of PXR activation in transient transfection assay frequently employ the LBD, rather than the full-length sequence. To compare the activation of the full-length zebrafish Pxr to that of the LBD, the LBD was cloned from the same sequence used above (*pxr*1*), and the activation of the LBD and the full-length sequences were compared, using CLO. There was a dramatic difference between the activation of the full length Pxr and the LBD, with the LBD giving between 30 to 40-fold induction of luciferase activity at the maximally effective dose of CLO, while the full-length Pxr was much less effective, giving between 2- and 3-fold induction of reporter gene activity at the same dose of CLO (Fig. 7). The level of induction of reporter gene activity by CLO with the full-length Pxr was similar in the two sets of experiments.

Discussion

In these studies we have cloned a full-length sequence from zebrafish, which was unambiguously identified as a zebrafish *pxr* and an ortholog of the human *pxr* (*sxr*) gene based on molecular phylogeny and shared microsynteny. There is substantial sequence variation among vertebrate PXR sequences sequenced to date. However, the vertebrate PXR DBDs tend to be much more highly conserved (averaging 68% identity) than are the LBDs, which exhibit only 50% to 65% identity. The percent identities we observed for the DBD and LBD of the cloned zebrafish Pxr were consistent with, and have been suggested before for zebrafish PXR based on amino acid sequences inferred from public genome databases (Krasowski et al., 2005b). By comparison, both the DBD and the LBD of some other nuclear receptors, e.g., the estrogen receptor β , are more highly conserved among vertebrates (Katsu et al., 2008). The sequence similarity among the DBDs suggests that the PXR in different species may recognize similar response elements in target genes, while variation in the LBDs is consistent with species differences in ligand specificity. Indeed, the selectivity for xenobiotic ligands differs sharply among mammalian PXR. As a well-known example, human PXR is strongly activated by rifampicin but not PCN, while rodent Pxr is strongly activated by PCN but not rifampicin (Jones et al., 2000). A narrower selectivity has been

reported for zebrafish and for tetraodon Pxr than seen with the mammalian orthologs (Ekins et al., 2008; Krasowski et al., 2011a; Milnes et al., 2008; Moore et al., 2002).

Full length vs. ligand binding domain in reporter systems

In the current study, ligand activation of zebrafish Pxr was measured using two related but different luciferase reporter gene assays, one based on full-length zebrafish *pxr* and XREM-reporter plasmid and one based on a receptor-chimera consisting of Gal4-DBD and zebrafish *pxr*-LBD and a tkx4(MH100)luc reporter plasmid. In both systems PXR is over-expressed to enhance assay sensitivity. The expression of luciferase in the XREM-based reporter plasmid (XREM-luc) is regulated by a native human CYP3A4 proximal promoter. This allows studies of ligand activation at close-to-native conditions that include heterodimerization with retinoid X receptor (RXR) and competition for response elements in the promoter by different nuclear receptors. Transcription mediated by the *CYP3A4*-promoter of the XREM-luc plasmid has been shown to be comparable to transcription of *CYP3A4* via endogenous PXR in HepG2 cells, with regard to both time- and dose-dependence and to magnitude of response (Goodwin et al., 1999).

When studying the activation of a receptor such as PXR, the use of a native promoter may reduce the response (measured as fold-change) as the promoter also contains response elements different from those typically recognized by PXR, and thus may exhibit some PXR-independent basal activity. Thus, the reporter plasmid may be transcribed via activation of other transcription factors, e.g., the growth hormone receptor. However, over-expression of the receptor of interest will probably reduce the interference from other transcription factors. The use of the tkx4(MH100)luc eliminates problems with cross-reactivity. Moreover, the use of an artificial promoter with multiple response elements enhances sensitivity of the assay (Paguio et al., 2010). Increased sensitivity has been reported for the Gal4 reporter assays for some nuclear receptors compared to using full-length receptors (Wilkinson et al., 2008). Hence, an increase in selectivity and sensitivity in the Gal4/UAS-MH100 system may be achieved at the expense of biological realism, e.g., heterodimerization of PXR and RXR is no longer necessary for transactivation.

While both systems are capable of delivering qualitatively reliable results, very large quantitative differences were observed in the responses. The level of activation of the full-length zebrafish protein by CLO relative to DMSO was similar in the results obtained in our separate laboratories. By comparison, activation of the LBD by CLO was much more efficient. There was a similar difference in the signal obtained with DMSO alone, suggesting that the difference reflects real features of the reporter systems. However, there are some uncertainties about the difference as observed. Thus, the basal activity of reporter alone, with no transfected PXR, could differ for the two reporters. This may be a greater problem with XREM-TK-Luc, which might be activated to some extent by endogenous PXR or other NR in the cells. The efficiency of the zebrafish Pxr to activate transcription by binding to the mammalian reporter construct could differ from activation via the zebrafish *CYP3A65* promoter. Such difference would not likely be great, however, due to the general conservation of vertebrate transcriptional machinery. Multiple PXR half-sites are present in the zebrafish *CYP3A65* promoter, although these have not been tested in a reporter system (Chang et al., 2013). This is an area that warrants further analysis.

There could be other differences in properties of the reporter plasmids XREM-TK-Luc and tkx4(MH100)-luc (Gal4-UAS), such as differences in transfection efficiency or copy number, that might affect the magnitude of the response to FL vs LBD PXR, independent of the PXR construct used. Sedlak et al (2011) identified some artifacts introduced by the Gal4/LBD reporter assays with the estrogen receptor (ER) in contrast to their full-length receptor reporter counterparts (Sedlak et al., 2011). However, it also may be true that the PXR-FL

has an inherently reduced responsiveness in the reporter systems, related for example, to the presence of functional domains in the full protein not present in the LBD construct, or differences in stability. This raises questions about inferences regarding *in vivo* responses, and possibly differences in ligand selectivity in screening compounds for agonist/antagonist activity. In a total of 6 experiments PCN did not activate the full-length zebrafish Pxr. Studies on activation of the Pxr-LBD by PCN and NIF are planned. Comparison of responses for these potential agonists may help to discern whether FL or LBD more accurately reflects *in vivo* responses. Future studies also could assess whether full-length zebrafish Pxr responds to zebrafish bile salts or other compounds such as androstanol (5 α -androstan-3 α -ol) shown to activate zebrafish Pxr-LBD constructs in other studies (Krasowski et al., 2005a, b; Moore et al., 2002).

PXR Expression

We observed high levels of *pxr* transcript expression, by qPCR, in a number of organs including eye and brain. The expression in eye and brain was confirmed by sequencing the amplicons from those tissues. Tissue distribution pattern of zebrafish *pxr* in four organs including liver, intestine, kidney and heart was similar to that in juvenile rainbow trout (Wassmur et al., 2010). While earlier reports noted PXR expression principally in liver or gastrointestinal tract in mammals, including humans (Kliwer et al., 1998; Lehmann et al., 1998; Zhang et al., 1999), more recently expression of PXR and the induction of ABC transporters in brain and retina indicate that this receptor is expressed in those organs in mammals as well (Bauer et al., 2004; Lamba et al., 2004; Zhang et al., 2012). Bertrand et al. (2007) reported spatiotemporal expression patterns of a full suite of nuclear receptors in developing zebrafish. That study indicated that *pxr* transcript first occurs in pituitary at 24 hpf, and then the expression is expanded in telencephalon and diencephalon by 36 hpf (Bertrand et al., 2007). At 48 hpf, the *pxr* expression in the central nervous system remains and the expression in intestine and liver occurs at relatively lower levels (Bertrand et al., 2007). Many of the other nuclear receptors including *rxr*, the heterodimerization partner of Pxr, were found to be expressed prominently in the retina and brain in developing zebrafish (Bertrand et al., 2007). Expression of NRs in adult tissues has not been examined thoroughly, so whether other NRs are expressed in the eye of adult zebrafish, as we saw here for *pxr*, is not known. While there is early expression of *pxr* in the central nervous system (Bertrand et al., 2007) and expression in the adult eye (the present study), the putative target gene for PXR, *CYP3A65*, (Chang et al., 2013), is expressed principally in intestine and liver. Prominent expression of *CYP3A65* in intestine and liver was reported also in larval zebrafish (Tseng et al., 2005). Thus, the patterns of tissue distribution of *pxr* and *CYP3A* genes are substantially different. This suggests that there may be previously unrecognized and perhaps novel functions or targets of PXR, including in eye and brain, not reflected in the expression of *CYP3A65*. Transcriptomic and phenotypic analyses using PXR knockdown or knockout could point to novel functions.

In a semi-quantitative PCR assay we detected a slight induction of *pxr* mRNA expression in liver of adult zebrafish treated with PB, suggesting a self-upregulation. A similar positive self-regulation was suggested in rats treated with PB (Zhang et al., 1999) and in liver of zebrafish treated with a mammalian PXR agonist PCN (Bresolin et al., 2005). In contrast, juvenile rainbow trout injected with mammalian PXR agonists, lithocholic acid or omeprazole, showed no clear change in the *pxr* transcript levels as compared to fish treated with vehicle control (Wassmur et al., 2010). Although, in primary cultures of hepatocytes from rainbow trout exposure to the mammalian PXR agonist dexamethasone resulted in a significant down-regulation of PXR mRNA that was not reflected on either CYP3A or ABC transporter (*i.e.* P-glucoprotein) mRNA levels (Wassmur et al., 2010). In the present study our initial results with semi-quantitative PCR suggested an increase in transcript levels of

pxr in liver of zebrafish treated with TCPOBOP. Earlier studies with in vitro cell-based reporter gene assays did not indicate activation of zebrafish PXR by TCPOBOP (Ekins et al., 2008; Moore et al., 2002). Those studies, and the absence of statistically significant increases in *pxr* mRNA expression in liver of zebrafish treated with PB or TCPOBOP when analyzed by qPCR, draw into question our initial results. However, the lack of significant increase could in part be due to an inter-individual variability in the *pxr* levels within the same treatment group; some fish showed no or weak response to these chemicals, but others showed a >2 fold increase as compared to the average values in the vehicle control group. There is a possibility that *pxr* alleles identified in the present study participate in the inter-individual differences in the PXR responses. We speculate that PXR alleles may vary in susceptibility to activation by the same ligands.

Allelic variation

While 40 human *CYP3A4* alleles have been identified, it has been estimated that 25% of inter-individual variability in *CYP3A* expression results from sequence variation in other genes, including variation in *PXR* (Istrate et al., 2010; Klein and Zanger, 2013; Lamba et al., 2010). The human *PXR* gene is located at chromosome 3 (3q13-21) and consists of nine coding and one non-coding exon (Zhang et al., 2001). In addition to several important splice variants (Lamba et al., 2004; Zhang et al., 2001), more than 70 single-nucleotide polymorphisms (SNPs) have been identified in human *PXR*, including in exons, introns, 5'- and in 3'-UTRs, in multiple sequencing initiatives on ethnic and national groups or populations [reviewed in (Zhou et al., 2009)]. Some of these human *PXR* SNPs have been associated with change in PXR expression levels [e.g. (Lamba et al., 2008)]; changes in PXR function, including the ability of DNA-binding, response element preference, basal *CYP3A* expression and the induction potential of the receptor (Doricakova et al., 2013; Hustert et al., 2001; Lamba et al., 2008; Oleson et al., 2010; Zhang et al., 2001); changes in the hepatic clearance of various pharmaceuticals (Schipani et al., 2010); and susceptibility to disease (Andersen et al., 2011; Dring et al., 2006).

The frequency of SNPs in human *PXR* varies greatly. The majority of the SNPs are relatively rare (frequency <0.03), while others are frequently observed (>0.5). The prevalence of SNPs varies with location in the *PXR* gene: while coding SNPs are relatively rare, SNPs in flanking introns, 3'- and 5'-UTRs are much more common (Hustert et al., 2001; Zhang et al., 2001). Non-coding SNPs have been associated with changes in *PXR* expression and mRNA stability, while coding SNPs have been associated with alterations in DNA-binding, response element preference and ligand activation [reviewed in (Zhou et al., 2009)].

In the present study, only the coding sequence of zebrafish *pxr* was analyzed, and thus changes in the non-coding 3'- and 5'-UTR regions have not been identified. We report here three coding SNPs that introduce amino acid substitutions, S183I, Y218C and H383N. Because these are non-synonymous coding rather than non-coding SNPs, effects are likely to be related to receptor function rather than expression levels. The position of substituted amino acids could give clues to potential functional effects. Of three variable codons identified, one was for an amino acid in the hinge region (S183I) and two coded amino acids in the ligand-binding region (Y218C and H383N; see Table 1). None of these substituted amino acids occur in a helix, based on a homology model of zebrafish PXR, nor do they form any part of the ligand-binding pocket (Supplemental Fig. 2). Y218C falls in a beta-sheet (sheet $\beta 1'$) but does not appear to interfere with receptor heterodimerization, or the binding of the small co-activator SRC-1, based on comparisons to the crystal structures of CAR/RXR heterodimers (Xu et al., 2004) and PXR-SRC1 tethered proteins (Wang et al., 2008). Any mechanistic effects of these three substitutions still remain to be elucidated.

Summary

In summary, this study reports cloning and analysis of full-length *pxr* sequences from zebrafish. The cloned PXR-DBD was highly conserved between zebrafish and human, while the zebrafish PXR-LBD was only 44% identical to human PXR-LBD. Sequence variation observed among clones indicates the presence of two prominent variants, S183I, Y218C and H383N, which we designate as alleles *pxr*1* (*nr1i2*1*) and *pxr*2* (*nr1i2*2*), respectively. The full-length Pxr*1 was activated by known PXR agonists CLO and PCN but to a lesser extent than the full-length human PXR. Activation of full-length Pxr*1 *1* was only 10% of that obtained with the Pxr*1 LBD. Quantitative real time PCR showed expression of *pxr* in all organs examined, including brain, eye and heart as well as the expected liver and intestine. Expression of *pxr* in liver was weakly induced by ligands for mammalian PXR or CAR (NR1I3). If the Pxr alleles have functional differences this would have important implications for determining the action and screening of potential PXR ligands in zebrafish.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Portions of this work were presented at the 12th International Symposium on Pollutant Response in Marine Organisms, 2003. We gratefully acknowledge the technical assistance of Bruce R. Woodin in early parts of this study. This work was supported in part by the U.S. National Institutes of Health grant P42ES007381 (Superfund Research Program at Boston University), by a grant from the Norwegian Research Council (MILJØ2105, 181888), and by a grant from the Swedish Research Council Formas (216-2007-468). A.C.D.B. was a recipient of Postdoctoral fellowship from CAPES, Ministry of Education, Brazil, and is a recipient of a CNPq productivity fellowship. Japan Society for the Promotion of Science Postdoctoral Fellowship and Postdoctoral Fellowship for Research Abroad to A.K. are acknowledged (nos. 4313 and 820, respectively). The sponsors had no involvement in performing or in the decision to publish this study.

ABBREVIATIONS

ABC transporters	ATP-binding cassette transporters
ARNT2	aryl hydrocarbon receptor nuclear translocator
CAR	constitutive androstane receptor
CLO	clotrimazole
CYP	cytochrome P450
DBD	DNA binding domain
DMSO	dimethylsulfoxide
EF1α	elongation factor 1α
LBD	ligand binding domain
NIF	nifedipine
NR	nuclear receptor
PB	phenobarbital
PCN	pregnenolone 16α-carbonitrile
PXR	pregnane X receptor
RACE	rapid amplification of cDNA ends

RIF	rifampicin
RXR	retinoid-X-receptor
SNPs	single-nucleotide polymorphisms
SXR	steroid xenobiotic receptor
TCPOBOP	1,4-bis [2-(3,5-dichloropyridyloxy)] benzene
UTR	untranslated region
VDR	vitamin D receptor

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PXR Highlights

1. Full-length *pxr* has been cloned from zebrafish.
2. Alleles of *pxr* were identified in zebrafish.
3. Full length PXR was activated less strongly than ligand binding domain in cell-based reporter assays.
4. High levels of *pxr* expression were found in eye and brain as well as in liver.
5. TCPOBOP and PB did not significantly alter expression of *pxr* in liver.

Figure 1. Maximum likelihood phylogenetic tree of some NR1I genes (PXR, CAR, VDR), including zebrafish and frog PXR (NR1I2). Phylogenetic tree was constructed using RAxML (see Methods). Gaps and ambiguously aligned characters were excluded from the analysis. Branch numbers represent bootstrap support (500 replicates). The low bootstrap values for frog PXR may reflect high positive evolutionary selection observed for this gene (Krasowski et al., 2005b).

Figure 2.

Gene order arrangement (synteny) of PXR genes in vertebrate genomes. Gene orders were obtained from ENSEMBL. Note that PXR appears to be missing from stickleback, but not from other surveyed teleost genomes. The tunicate genomes (*Ciona* spp.) have a MAATS1 ortholog, as depicted; however they have a non-syntenous ancestral PXR/VDR gene (Ekins et al, 2008).

Figure 3.

PXR variants in zebrafish. **(A)** Percent identity between zebrafish and human PXR, and the location of the amino acid substitutions between *pxr*1* and *pxr*2*. **(B)** Maximum parsimony tree for cDNAs obtained in the original cloning effort (see Results). Both complete and partial sequences were assembled from individual clones obtained from either a single fish (sequence IDs beginning “90-”) or from a pool of 4 fish (all other sequences).

Figure 4.

Tissue distribution of *pxr* (A, B) and *CYP3A65* (C, D) in adult zebrafish. Transcript levels of *pxr* and *CYP3A65* were determined by qPCR in seven tissues from males and females. Data were normalized by geometric mean of two separate reference genes, *ef1a* and *arnt2*.

Results are shown as values in each tissue relative to the average value in testis/ovary for *pxr* expression and in intestine for *CYP3A65* expression, as these organs showed one of the least inter-individual variability and sex-differences in the expression levels of respective genes. Statistical differences in transcript levels among tissues were determined by one-way ANOVA followed by Tukey's multiple comparisons test and are shown by different letters ($p < 0.05$). Outliers were excluded based on the Grubbs test.

Figure 5.

Effects of TCPOBOP and PB on mRNA expression level of *pxr*. The *pxr* transcript level was determined by qPCR in liver of adult male zebrafish treated with vehicle DMSO, TCPOBOP (1.5 μ g/g) or PB (70 μ g/g). Data were normalized by geometric mean of two separate reference genes, *ef1a* and *arnt2*. Results are shown as values in each treatment relative to the average value in DMSO control (fold-control). The whiskers show data range, while the box extends from the 25th to 75th percentile. No significant differences in transcript levels among treatments were observed by one-way ANOVA followed by Tukey's multiple comparisons test ($p > 0.05$). $N = 8$.

Figure 6.

Transfection assays were performed in COS-7 cells using 100 ng of zebrafish PXR or 100 ng of human PXR, 50 ng of XREM Luc and 3 ng of pRL-TK. The total amount of transfected DNA was kept constant by addition of pcDNA 3.1 Zeo (+) (Invitrogen) vector with no insert. Cells were treated 5 h after transfection with either dimethylsulphoxide (DMSO), clotrimazole (CLO), nifedipine (NIF) or pregnenolone 16 α -carbonitrile (PCN). The final luminescence values are expressed as a ratio of the firefly luciferase units to the Renilla luciferase units. *indicates statistical differences for $p < 0.05$.

Figure 7.

Luciferase reporter gene ligand activation assay in COS-7 cells. COS-7 were transiently transfected by the CaPO_4 method in 96-well plates (5×10^3 cells/well, 5 ng/well of Gal4-DBD-zfPXR-LBD plasmid, 50 ng/well of tkx4(MH100)luc and β -galactosidase plasmids) for 24 hours and exposed to test clotrimazole in concentrations ranging from 10^{-7} to 6×10^{-5} M for 24 hours. Luciferase activities were normalized by β -galactosidase activities and ligand activation expressed as the ratio of normalized luciferase values in treated cells over solvent-control cells. *indicates statistical differences for $p < 0.05$.

Table 1

List of variant residues. Only residues occurring in at least 3 different independent clones are included.

nucleotide	amino acid	Protein region
T189C ^b	-	-
G231A ^b	-	-
T309A ^b	-	-
A460T ^b	-	-
T551G	I184S ^a	hinge
C621T ^b	-	-
T622C ^b	S208P ^b	hinge
C653A	C218Y ^a	hinge
666AAT667 insertion ^b	N223 insertion ^b	hinge
G695A ^b	S232N ^b	LBD
A700T ^b	T234S ^b	LBD
C1104T ^b	-	-
A1130G ^b	K377R ^b	LBD
A1150C	N384H ^a	LBD

^a defines *pxr*1* versus *pxr*2* (see text).

^b only present in *pxr*1*