ANALYSIS OF MEDAKA CYP3A GENE REGULATION, PROMOTER REGULATORY REGIONS AND CLONING OF THE ORPHAN NUCLEAR RECEPTOR PXR

Seth W. Kullman
Nicholas School of the Environment and Earth Sciences, Duke University, Durham, North Carolina 27708

In aquatic species, embryonic development, sexual maturation, and reproduction are complex biological processes that are regulated by endogenous substances (hormones), and synchronized by exogenous factors (photo period, temperature, feeding and social factors). Several reports have documented however, that many environmental pollutants are capable of modifying/disrupting normal endocrine function, thus influencing developmental and reproductive processes of oviparous organisms. One potential mechanism of action for these compounds is to alter normal hormonal homeostasis by disruption of hormone biosynthesis and/or metabolism. Recently we have been investigating the major metabolic enzymes associated with hormone metabolism in fish liver. Like in mammalian systems, cytochrome P450 isozymes are thought to be responsible for the initial hydroxylation/metabolism of these endogenous substrates and thus participate in the regulation of circulating levels of hormonal signal. In both mammalian and fish species cytochrome P450 3A (CYP3A) is an abundant, constitutively expressed enzyme found in liver and intestine. Numerous studies have shown CYP3A to have a broad substrate specificity with high affinities for both endogenous and exogenous substrates including steroids, cholic acids, retinoids, pesticides antibiotics and poly-aromatic hydrocarbons (PAH's) (Plant, NJ. et al. Curr. Opin. Drug. Discov. Devel. 6:50-6, 2003). Multiple forms of CYP3A have been identified in numerous teleost species. This is consistent with studies in mammalian species where multiple isozymes of gene family 3 have been identified.

Until recently, protein induction of CYP3A was thought to be somewhat paradoxical. Early studies demonstrated that both glucocorticoids and anti-glucocroticoids were highly effective at up regulating CYP3A message levels in select species. Additionally it has been demonstrated that estrogen and specific "estrogen like" xenobiotics including DDT, methoxychlor, endosulfan, and nonylphenol modulate CYP3A expression and subsequent 6-β hydroxylase activity in select mammalian and fish models (Dubios, MA. et al. Environ. Toxicol. Phamacol. 1:249-256, 1996, Lee, PC. et al. Xenobiotica. 26:831-838, 1996, Li, HC. et al. J. Biol. Toxicol. 10:51-61, 1995). It is now known that most CYP genes belonging to families 1-4 can be transcriptionaly activated by select xenobiotics (Akiyama TE et al. Biochim. Biophys. Acta. 1619:223-34, 2003). Studies have demonstrated that gene expression for CYP families 2, 3 and 4 are governed in part by specific orphan nuclear receptors (ONR's) CAR, PXR and PPAR respectively (Akiyama TE et al. Biochim. Biophys. Acta. 1619:223-34, 2003). These studies have helped to unravel some of the mystery behind the mechanisms in which foreign chemicals induce the expression of these drug-metabolizing enzymes. Little information is available however, regarding basic mechanisms governing CYP family 2, 3 and 4 gene regulation in teleost species. It is hypothesized that a similar regulatory mechanism involving CAR, PXR and

PPAR homologs are utilized; however, often fish species display dissimilar regulatory (induction and repression) patterns as those described in mammals.

To date few studies have demonstrated ONR mediated regulation of teleost CYP 2, 3 and 4 genes, and thus a substantial effort is underway to identify ONR's and their relation to CYP gene expression in teleosts and other freshwater/marine species. In an effort to elucidate the mechanisms associated with teleost CYP3A expression, we have conducted the following research: 1. Identify and characterize the 5' promoting regions for medaka CYP3A38 and CYP3A40 and 2. Characterize and clone the orphan nuclear receptor PXR from medaka.

In an initial round of studies, medaka were exposed to stereotypical PXR agonists (single i.p. injection 50mg/kg) and assayed for CYP3A expression 24-48 hours post exposure. In mammalian systems, CYP3A is highly regulated by PXR agonists including dexamethasone, PCN, and rifampicin. Analysis of medaka CYP3A38/40 (liver microsomal protein) expression revealed that these compounds were ineffective for CYP3A gene induction, suggesting that these compounds maybe poor agonists for teleost PXR activation. In subsequent studies, we examined a range of additional substrates known to activate PXR in several species. Of those tested, the androgens DHT (24 hours post exposure) and DHEA (48 hours post exposure) exhibited the greatest degree (~5 fold) of CYP3A protein induction. Other compounds, including the pharmaceuticals nifedipine, clotrimazole, phenobarbital and propyl-p-hydroxybenzoate exhibited minimal to no induction. Conversely, when microsomal CYP3A enzyme activity was examined using BFC, a CYP3A specific probe, activity was decreased in androgen-treated microsomes compared to controls, suggesting that some degree of enzymatic inhibition may be occurring.

Lack of CYP3A protein induction may be due to differences in PXR binding activity between species. PXR is demonstrated to exhibit broad differences in ligand binding across species and thus the potential for differences in CYP regulation is possible. The above results however, do not rule out that CYP3A gene expression is not governed by the binding activity of PXR in teleost species and induction by androgens is mediated by an alternate mechanism. To address this issue we have cloned approximately 2.5 Kb of the 5'promoting regions for both CYP3A38 and CYP3A40 from medaka genomic DNA. Promoter sequences were obtained by a combination of genome walking and screening of a 4X medaka BAC library. Comparisons of CYP3A promoter/enhancer regions have been made to mammalian CYP3A promoters and teleost CYP3A promoters identified from the Puffer Fish genome database. Multiple response and cis acting elements have been identified including those for basal transcription including CAAT, TATA, and SP1. Sequence analysis however, suggests that the medaka promoters do not contain the functional PXR binding motifs including DR3, 4 or ER6, 8 half sites within the 2.5 Kb region isolated. Puffer fish on the other hand, exhibited both imperfect DR3 or DR4 response elements associated with nuclear receptor binding, each deviating from the consensus sequences of (AG(T/G)CTA) by two to three nucleotides and located approximately 150 bp up-stream from the transcriptional start site of CYP3A47 and CYP3A50. Functional promoter sequences from mammalian CYP3A sequences exhibit

either a DR3 (4) or ER6 in the proximal promoter approximately 150-300 bp upstream of the transcriptional start site. Enhancer elements containing ONR half sites have additionally been described approximately 8 Kb upstream of human CYP3A4 (Kliewer et al. Endocr. Rev. 23:687-702, 2002). Other genes such as the rat MRP2 transporter contain putative PXR binding motifs 5-8 Kb upstream from the transcriptional start site. It is thus possible that we have not obtained a large enough piece of the medaka CYP3A promoter to identify the appropriate PXR binding motif.

In an additional component of this study, we have cloned the medaka homolog to PXR. We have developed a degenerate RT-PCR protocol that exploits regions of sequence conservation within the DNA (P-box) and ligand binding regions of this class of nuclear receptors. Alignments of multiple mammalian PXR/SXR sequences revealed several highly conserved target regions. To assume a bias for teleost species we additionally compared these alignments to a putative PXR sequence identified from the puffer fish genome database. Based on these sequence alignments, degenerate primers were designed and used for subsequent PCR reactions. Degenerate RT-PCR using medaka liver cDNA, resulted in the formation of a 750 bp product. This product was identical in size to that predicted by base number between primer sites. PCR products were gel excised and cloned into a TA vector and subsequently sequenced in both directions. Translation of the established DNA sequences demonstrated a partial protein sequence highly homologous to that of previously identified mammalian PXR. Homology comparisons show greatest similarity to the putative fugu PXR sequence and to a partial sequence reported for zebrafish.

In these studies we have identified homologous gene sequences for the nuclear receptor PXR in the medaka. PXR activation and subsequent gene induction of CYP3A has been identified as integral components of steroid and bile acid homeostasis. Cytochrome P450s play a major role in the transformation and elimination of numerous xenobiotics. More recently however, the role of these enzymes in maintaining/regulating endobiotic homeostasis has been established. P450's are now known to mediate cellular signaling through the production and metabolism of numerous cellular ligands including fatty acids, arachidonic acids, bile acids and certain steroids. Cellular mechanisms associated with sensing signaling ligands, often involve the binding activity orphan nuclear receptors, which in turn regulate P450 transcription. The degree to which teleost CYP3A gene transcription is governed by PXR activation is unknown. These studies have been initiated as an initial component of establishing the relationship between ONR's and P450 regulation in these species.

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