Published in final edited form as:
Toxicol Sci. 2006 October ; 93(2): 331–340.

CYP1C1 Messenger RNA Expression is Inducible by Benzo[*a*]pyrene in *Fundulus heteroclitus* Embryos and Adults

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Abstract

CYP1C is the newest member of the CYP1 family of P450s; however, its physiological significance, inducers, and metabolic functions are unknown. Two full-length alleles of *Fundulus heteroclitus* CYP1C1 complementary DNA were cloned. The 529 amino acid protein shared the highest amino acid identity with *Stenotomus chrysops* CYP1C1 (81%). To investigate whether the carcinogen benzo[*a*]pyrene (BaP) was a CYP1C1 inducer, we used real-time PCR to quantitatively measure tissue- and sex-specific expression of both CYP1C1 and CYP1A messenger RNAs (mRNAs) in BaP-exposed adult fish. CYP1C1 mRNA expression was constitutively higher than CYP1A in brain, spleen, eye, and gonad, while CYP1A was higher in gastrointestinal tract (GI), heart, gill, and liver. Kidney had equal but high expression of both CYP1s. There were sex differences in constitutive CYP1 expression in the GI, liver, gill, and eye. BaP exposure caused induction of CYP1C1 expression in female and male heart (31- and 17-fold), gill (seven- and four-fold), and liver (six- and five-fold), respectively. Embryo CYP1 expression was constitutively highest at 2 weeks posthatch, and whole embryos expressed 3- to 15-fold more CYP1C1 mRNA compared to CYP1A. BaP, 10 μg/l for 10 days, caused induction of both genes at 120 and 240 h postfertilization. Our results suggest that teleost CYP1C, in addition to CYP1A, is inducible by BaP, has a broad tissue distribution, and should be further investigated for its role in carcinogen bioactivation.

Keywords

CYP1C1; cytochrome P450; benzo[*a*]pyrene; killifish; embryos; *Fundulus*

Until the mid-1990s the CYP1 family of cytochrome P450 enzymes was believed to contain a single subfamily with two members, namely CYP1A1 and CYP1A2. In fish, this sub-family is generally recognized as CYP1A because mammalian CYP1A1 and CYP1A2 genes diverged less than 250 million years ago by a gene duplication event while fish diverged from land animals prior to that time (Morrison *et al*., 1995;Nebert and Gonzalez, 1987). However, in 1994, a second CYP1 subfamily was discovered when CYP1B1 was isolated from 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)–induced human keratinocyte cells (Sutter *et al*., 1994) and TCDD and polycyclic aromatic hydrocarbon (PAH)–induced mouse embryo fibroblast cells (Savas *et al*., 1994;Shen *et al*., 1994). The genes in the CYP1 family (CYP1A1, CYP1A2, and CYP1B1) are induced by environmental contaminants including PAHs and halogenated aromatic hydrocarbons (HAHs) (Murray *et al*., 2001) via the aryl hydrocarbon receptor (AhR) pathway. Both CYP1A1 and CYP1B1 are involved in carcinogen bioactivation of PAHs

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including benzo[*a*]pyrene (BaP) and 7,12-dimethyl-benzanthracene (Buters *et al*., 2003;Kim *et al*., 1998;Shimada *et al*., 1999a). In addition, the potential for CYP1A1 and CYP1B1 to be involved in estrogen genotoxicity and oxidative stress by generating the 2- and 4-hydroxyestradiol metabolites was recognized (Hayes *et al*., 1996).

The induction of CYP1A, particularly in fish, by PAHs has been used as a biomarker of exposure since the mid-1970s (Payne and Penrose, 1975). A number of studies have correlated CYP1A induction with biliary PAH metabolites, DNA adducts, immune suppression, and tumor formation in wild fish and following laboratory exposures (Carlson *et al*., 2004;Collier *et al*., 1992;Willett *et al*., 1995;Wirgin and Waldman, 1998). More recently a refractory CYP1A phenotype has been noted in fish living in highly PAH- or HAH-contaminated environments (Bello *et al*., 2001;Meyer *et al*., 2002;Nacci *et al*., 2002). The genetic and physiological significance of this lack of CYP1A induction in these populations is an area of ongoing research.

Another important recent finding in fish is that they also have CYP1B and now CYP1C genes. CYP1B was first cloned in plaice (*Pleuronectes platessa*) and was detected by Northern blot in gill tissue (Leaver and George, 2000). In both plaice and channel catfish only a single isoform of CYP1B has been identified, whereas both CYP1B1 and CYP1B2 genes have been cloned in carp (*Cyprinus carpio*). Carp exposed to 3-methylcholanthrene had CYP1B1 messenger RNA (mRNA) expression in liver, intestine, and gill, while CYP1B2 was only induced in the gills (El-kady *et al*., 2004a,b). Similarly, BaP-exposed catfish had significant CYP1B mRNA induction in blood, liver, and gonad tissues and high constitutive expression in gill (Willett *et al*., 2006).

The vertebrate CYP1C family was first described by Godard *et al*. (2005) when they identified a CYP1C1 and CYP1C2 in scup (*Stenotomus chrysops*) liver and head kidney. Carp express a CYP1C1 constitutively in gill tissue (Itakura *et al*., 2005). Analysis of sequence domains suggests that fish CYP1B and CYP1C enzymes will likely have unique catalytic functions or substrates; however, the function of these newly reported P450s is currently unknown. There has not been a CYP1C identified in mammals; however, because fish are extensively used in toxicology testing, biomonitoring, and as developmental biology and cancer models, it is important to understand the physiological roles, tissue distribution, and metabolic capacity of these CYP1 genes. It is possible that this new P450 subfamily has a role in endogenous compound metabolism, steroid metabolism, and/or xenobiotic metabolism and toxicity.

Fundulus heteroclitus (killifish or mummichog) are found in contaminated environments along the Atlantic coast of the United States with diseases including cancer and reproductive/developmental deficits and are used as a model for marine toxicology (Leblanc *et al*., 1997;Meyer *et al*., 2002;Vogelbein *et al*., 1990;Zhou *et al*., 2000). *Fundulus* embryos are also sensitive to classic CYP1A inducers, which can even be measured by *in ovo* ethoxyresorufin-*O*-deethylase activity (Nacci *et al*., 1998;Wassenberg and Di Giulio, 2004). The genes involved in CYP1 induction, including the AhR1 and AhR2 (Karchner *et al*., 1999), the aryl hydrocarbon repressor (Karchner *et al*., 2002), the aryl hydrocarbon nuclear translocator 2 (Merson *et al*., 2006;Powell *et al*., 1999), and several other CYPs from families 1 to 3, have been cloned (Celander and Stegeman, 1997;Morrison *et al*., 1998;Oleksiak *et al*., 2000). The *Fundulus* antioxidant defense system has also been described (Meyer *et al*., 2003).

Our aim was to characterize the expression of the newest CYP1 subfamily member CYP1C1 in *Fundulus* adult and embryos. We found that *in vivo* BaP exposure induced CYP1C1 differentially compared to CYP1A which warrants additional research into the possibility that CYP1C1 may contribute to the mechanism of PAH toxicity in the heart, gill, or liver.

Furthermore, embryos constitutively expressed higher levels of CYP1C1 than CYP1A suggesting a possible developmental role for this gene.

MATERIALS AND METHODS

Fish source, care and handling

A parental population of *F. heteroclitus* collected from an uncontaminated site at the New River inlet near Beaufort, NC was raised under the University Institutional Animal Care and Use Committee approved conditions. Sexually mature fish were bred and kept in salt water (20–25 parts per thousand [ppt]). The fish were maintained at 14:10 light-dark cycle in summer and 10:14 light-dark cycle in the winter. Adult fish were fed twice daily with tropical flake fish food (Tetramin, Tetra Werke, Germany) and live brine shrimp. First generation offspring, from wild parents, were used for the studies described here.

CYP1C1 cloning

Two full-length alleles of *Fundulus* CYP1C1 complementary DNA (cDNA) were cloned from 5 mg/kg BaP i.p. injected *Fundulus* liver and gill tissues. cDNA was synthesized from total RNA using oligo (dT) primers and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Because a partial *Fundulus* CYP1B mRNA sequence was available on GenBank (Accession #AF235140), we initially anticipated cloning a CYP1B, and primers were designed based on the CYP1B partial sequence available. The amplification of initial cDNA fragment was done with 10 μM of Primers 1 and 2 (Table 1) and an aliquot (10%) of the first-strand reaction and amplified with PCR Master mix (Roche Diagnostics Corporation, Indianapolis, IN). The 5′ and 3′ ends of the CYP1C1 cDNA were obtained by rapid amplification of cDNA ends (RACE) using the GeneRacer kit (Invitrogen) as per the supplier's protocol. 5′ RACE was done with Primer 3 initially and nested PCR was then done with Primer 4.3′ RACE done with Primer 5 and nested with Primer 6. Primers that ultimately provided the full-length CYP1C1 were Primers 7 and 8 or 9.

For cloning, DNA bands were excised from the gel and extracted using QIAquick gel extraction kit (Qiagen, Valencia, CA). pGEM-T Easy Vector System I (Promega, Madison, WI) was used for ligation. Ligated DNA was transformed into DH5α *Escherichia coli*–competent cells and then plated on the Luria-Bertani broth/ampicillin/Isopropyl-β-d-1-thiogalactopyranoside (IPTG)/5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal) plates. Seven white colonies from each of two separate PCR reactions were picked and miniprepped with a Qiaprep Spin miniprep kit (Qiagen). Sequencing was done by using the BigDye 3.1 sequencing kit from Applied Biosystems (Foster City, CA), except the reaction conditions were reduced to 5 μl using 0.25 μl of the BigDye mix. Sequencing reactions were analyzed on an ABI 3730Xl sequencer. Sequence homology searches were carried out using the BLAST (Basic Local Alignment Search Tool) program at http://www.ncbi.nlm.nih.gov/BLAST/, whereas sequence alignment was performed using the ClustalW program at http://workbench.sdsc.edu/ or with MegAlign in DNAStar. The final contiguous sequence of each of the 14 clones sequenced in both directions was arrived at using Lasergene sequence analysis software (DNAStar).

Adult BaP exposure

Fish were exposed in 5-l tanks containing one male and one female adult fish. Fish were kept in the tanks 7 days for acclimatization. The *Fundulus* (n = 20, 10 tanks) were exposed to the following treatment for 15 days (complete reproductive cycle): control (50 μl ethanol) or BaP 10 μg/l (Sigma, St Louis, MO; stock solution 1 mg/ml in 100% ethanol). Exposure conditions were 17.5–20.5°C, 14:10 light-dark period, and 20–25 ppt. Fish were fed Tetramin flakes twice daily. The tanks were checked twice every 24 h, and water was changed (85–100% static water renewal) daily at 15:00.

The fish were anaesthetized with 3-aminobenzoic acid ethyl ester (MS-222, Sigma), and their weight and length were recorded. Brain, gonad, liver, gill, gastrointestinal tract (GI), spleen, kidneys, heart, and eyes were dissected from each fish and frozen – 80°C in 1 ml RNALater (Ambion, Austin, TX). Tissues were disrupted and homogenized in 1 ml of TRIzol Reagent (Invitrogen) and extracted by the manufacturer's protocol. The total RNA was further cleaned by using an RNeasy Kit (Qiagen) with a DNAse I digestion. The RNA concentration was determined with an Agilent 2100 Bioanalyzer. RNA was stored at – 80°C.

Embryo BaP exposure

Pooled oocytes and sperm stripped from parental population of around 50 fish were mixed together for *in vitro* fertilization. Fertilized eggs were randomly sorted into three treatment groups, namely untreated, dimethyl sulfoxide (DMSO) control (1 μl/ml), or BaP (10 μg/l). Each egg was placed in 1 ml of water (~21 ppt) in an autosampler vial (Fisher C4013-1) with a Teflon lined cap (Fisher B7815-8) and exposure began at approximately 4.5-h postfertilization (hpf).

Each egg was inspected daily for normal development. Every other day water was changed and eggs were redosed. Exposure lasted 10 days at which time the eggs were placed in 96-well plates on a circle of damp filter paper. After 7 days, the eggs remaining from each treatment were pooled and hatched. Simultaneously hatched fish were raised an additional 2 weeks in small finger bowls. The fry were fed brine shrimp twice daily. Water was changed in finger bowls every 48 h. Ten embryos were pooled per time point and were collected for CYP mRNA analysis at 120 and 240 hpf, immediately following hatch, and at 2 weeks posthatch (ph). Embryos were stored in RNAlater at – 80°C until they were extracted with TRIzol and RNeasy kits as described above. The exposure was repeated four times.

Analysis of water samples from exposures

Water samples (200 ml) were collected 20 min after water dosing to confirm water control and BaP concentrations at least twice during each exposure. Water samples were passed over Water Sep-Pak Vac RC C18, 500 mg (#Wat 036945, Waters Corp., Milford, MA) columns prerinsed with methanol:water ratio of 3:1 (50 ml). Columns were extracted with 15 ml methylene chloride and samples were allowed to dry under a gentle stream of nitrogen. Control samples were suspended in 500 μl of *iso*octane and BaP samples were suspended in 1.5 ml of *iso*-octane and analyzed for BaP concentrations using gas chromatography/mass spectrometry in selected ion monitoring mode. BaP standards (0.1, 0.2, 0.5, 1, and 2 ppm) were prepared in *iso*-octane and used to develop a calibration curve. BaP was detected as an ion with molecular weight 252 g/mol (Patel *et al*., 2006). Actual BaP water concentration in tanks for the adult exposure averaged 13.1 ± 2.15 μg/l. The embryo exposure average concentration was 18.1 ± 1.29 μg/l.

Quantitative real-time reverse transcription PCR

Primers for quantitative real-time PCR were designed according to the requirements of Primer Express v2.0 software. Primers for CYP1A (Primers 12 and 13), CYP1C1 (Primers 10, 11), and 18S ribosomal RNA (rRNA) (Primers 14, 15) are shown in Table 1. Amplification efficiencies of all three primer pairs were determined and were not statistically different which allows for comparisons between genes (slopes = – 3.94, – 3.55, and – 3.80 for CYP1A, CYP1C1, and 18S rRNA, respectively, $p = 0.15$, data not shown). RNA from tissues or 10 pooled whole embryos was isolated as described above. Synthesis of cDNA was conducted with 250 ng total RNA using Taqman Reverse Transcription (RT) Reagents (Applied Biosystems): random hexamers, multiscribe RT, RNase inhibitor, deoxynucleotide triphosphate mix, 25mM $MgCl_2$, 10× RT buffer in a 25 μl reaction. cDNA corresponding to 20 ng for CYP1s and 10 pg for 18S of reverse-transcribed RNA served as templates for each duplicate 25 μl PCR reactions using SYBR Green PCR Master Mix (Applied Biosystems). The

PCR amplifications and fluorescence detection were performed with the GeneAmp 7500 Sequence Detection System (Applied Biosystems). Universal thermal cycling conditions according to the manufacturer were used. For quality control, no template controls and no RT controls were done, as well as melt curve analyses. Samples were amplified in duplicate in separate reactions on the same plate. The amount of CYP1 mRNA, normalized to 18S, was given by the formula = $2^{-\Delta\Delta C_T}$, where C_T is the threshold cycle indicating the fractional cycle number at which the amount of amplified CYP1 reached threshold. The ΔC_T value is determined by subtracting the average 18S C_T value from the average CYP1 C_T value. Then, the calculation of $\Delta\Delta C_T$ involves subtraction of the ΔC_T value of the calibrator (in our case the calibrator was average ΔC_T value of control fish response in the BaP studies or specific CYP1 form in the tissue comparisons) from the ΔC_T value of each sample (ABI PRISM 7700 Sequence Detection System User's Manual). Accordingly, CYP1 mRNA levels were reported as fold change in abundance relative to the average calibrator response. In the embryo experiments, CYP1 levels were reported relative to the untreated controls. The 18S C_T values were very consistent between adult tissues and various PCR runs and averaged 16.5 ± 0.038. For embryos, the 18S C_T values were 17.3 ± 0.064. Statistical differences between treatments, sexes, or genes in each tissue were determined with one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test ($p < 0.05$) using GraphPad Prism 4.0 version.

RESULTS

Cloning of Fundulus CYP1C1

Two full-length alleles of *Fundulus* CYP1C1 cDNAs were cloned from 5 mg/kg BaP i.p. injected *Fundulus* liver and gill tissues. Allele I (GenBank Accession #DQ133570) contained a 5′ noncoding region of 182 bp, an open reading frame (ORF) of 1590 bp, and a 3′ noncoding region of 988 bp to the polyA tail. Allele II (GenBank Accession #DQ133571) contained a 5′ noncoding region of 183 bp, an ORF of 1590 bp, and a 3′ noncoding region of 992 bp to the polyA tail. The two alleles shared 99% nucleotide identity and encoded for the same deduced 529 amino acid sequence. CYP1C1 shared the highest amino acid identity with *S. chrysops* 1C1 (81%, GenBank Accession #AAL78297) while exhibiting only 35% identity with the *Fundulus* CYP1A (GenBank Accession #AAD01809). Interestingly, the CYP1C1 was only 66% similar at the amino acid level with the *Fundulus* CYP1B partial sequence (Gen-Bank Accession # AAL78301) which was used to design the initial cloning primers.

Despite our efforts, we were not able to successfully generate a full-length CYP1B cDNA sequence with 5′ or 3′ RACE or by PCR with degenerate primers. Similarly, we did not isolate a *Fundulus* CYP1C2 from any of the PCR reactions or sequenced colonies. It is possible that *Fundulus* do have these other CYP1 isoforms based on the fact that zebrafish, scup, carp, and fugu have both CYP1C1 and CYP1C2 genes.

Previously, Gotoh (1992) had identified six regions on CYP2 that are putative substrate recognition sites (SRS). Godard *et al*. (2005) have applied these same regions for analysis of the functional divergence of CYP1s. Figure 1 highlights the six SRS of *Fundulus* CYP1C1 aligned with scup and zebrafish CYP1C1 and CYP1C2, *Fundulus* CYP1A, and the *Fundulus* CYP1B partial sequence. As expected *Fundulus* CYP1C1 is most similar to the other fish CYP1C1s especially in SRS 1, 4, and 5. The least conserved SRS was 2.

CYP1 Expression in Adult Tissues

Constitutive expression of CYP1C1 mRNA was significantly higher compared to CYP1A in brain, eye, gonad, and spleen tissues in both sexes (ANOVA, $p < 0.001$). Real-time PCR results for both CYP1 genes are plotted for the four tissues that had higher constitutive CYP1C1

expression in Figure 2. The data are presented as CYP1 C_T – 18S C_T. The 18S rRNA signal was used as a normalization standard for each sample. Therefore, the shorter bar height represents higher mRNA expression. Relative fold induction was calculated by the equation $2^{-\Delta\Delta C_T}$ as described above. The tissue with the most CYP1C1 expression as compared to CYP1A was the testis followed by ovary where there was 108- and 65-fold more CYP1C1 compared to CYP1A, respectively (Table 2). There was 39- and 81-fold more CYP1C1 than CYP1A in male and female spleen, respectively.

There were four tissues with significantly (ANOVA, $p < 0.01$) higher CYP1A expression, namely, GI, heart, gill, and liver (Fig. 3). Liver had ~30-fold more CYP1A relative to CYP1C1 followed by heart (~15-fold) (Table 2). In kidney there was equal expression of CYP1A and CYP1C1 (data not shown).

When sex differences in constitutive gene expression were compared, there was higher CYP1A mRNA expression in male GI (four-fold) and liver (3.5-fold) and the female gill (2.5-fold) (ANOVA, $p < 0.01$, Fig. 4). Constitutive CYP1C1 expression was significantly higher in male eye (2.5-fold) and liver (4.5-fold, $p < 0.01$) and female gill (3.5-fold, $p < 0.001$). For all other tissues, the expression of CYP1A or CYP1C1 was not significantly different between the sexes.

BaP Effects on CYP1 Expression in Adult Tissues

In male fish, 15 days of 10-μg/l BaP exposure caused CYP1A induction in every tissue analyzed except spleen and liver (Figs. 2 and 3, Table 2). In females, significant induction was noted in all but the GI and liver. The sex differences in CYP1A tissue expression were maintained following BaP exposure in GI, gill, and liver. There was significantly ~2- to 4-fold higher induction of CYP1A in female spleen, eye, and gill compared to male CYP1A induction. In BaP-treated fish, male CYP1A expression compared to female CYP1A expression was significantly higher in GI (19-fold) and liver (three-fold).

Compared to CYP1A, CYP1C1 was significantly induced by BaP in fewer tissues. The highest CYP1C1 induction was in male and female heart 17- and 31-fold, respectively. CYP1C1 induction ranged from four- to seven-fold in gill and liver tissues. Like CYP1A expression, the sex differences in constitutive CYP1C1 expression (i.e., higher expression in female gill and male liver) were maintained following BaP exposure with the exception of in the eye where there was no sex difference following BaP exposure.

CYP1 Expression in Embryos

RNA was isolated from embryos at 4.5, 120, 240 hpf, at hatch (17–18 days pf; 408–432 hpf), and 2 weeks ph. Constitutive CYP1 expression increased with age for both CYP1A and CYP1C1 (Fig. 5). In embryos between 120 hpf and freshly hatched, there was between 12- and 15-fold higher CYP1C1 expression compared to CYP1A. However, by 2 weeks ph embryos expressed only three-fold more CYP1C1 than CYP1A (Fig. 6).

BaP Effects on CYP1 Expression in Embryos

Both CYP1A and CYP1C1 were induced in embryos by exposure to 10 μg/l BaP for 10 days (Fig. 7). The highest induction of both genes corresponded with the end of exposure (240 hpf) and dropped off to control levels by hatch. CYP1A was much more highly induced relative to controls than CYP1C1 was (on average 330 vs. 18-fold, respectively).

DISCUSSION

The results of this study demonstrate that CYP1C1 mRNA is widely distributed in adult *Fundulus* tissues, is present in embryos through development, and it is inducible by BaP in

embryos and select tissues. Prior to this report, the distribution and inducibility of CYP1C was largely unknown. In untreated male scup liver, CYP1 expression was highest for CYP1A followed by CYP1C1 and CYP1C2 (Godard *et al*., 2005). We also found 27- to 33-fold higher liver CYP1A compared to CYP1C1 expression in control *Fundulus*. In contrast to scup, where liver expression of the CYP1s was higher than in head kidney, we found both CYP1A and CYP1C1 expressed more highly in kidney. In our studies the highest CYP1C1 constitutive expression was found in spleen, kidney, eye, gill, and gonad, respectively. In carp, CYP1C1 has been reported by Northern blot in gills but not kidney, liver, or intestine (Itakura *et al*., 2005).

No previous studies, to our knowledge, have shown either CYP1B or CYP1C expression in embryonic fish. With quantitative real-time reverse transcriptase–PCR we were able to measure both CYP1A and CYP1C1 expression as early as 4.5 hpf suggesting either a maternal contribution of these RNAs to the embryo or early synthesis of these mRNAs. Constitutive expression levels of both CYP1A and CYP1C1 were low and not increasing from 4.5 to 240 hpf (Fig. 5). In *Fundulus* development, 120 hpf (~stages 27 and 28) is after the onset of circulation and corresponds to when the ventricle of heart forms a definite chamber, the otoliths are dense concrete bodies and body movement increases. The heart chambers are differentiated and the liver and gut become apparent at about 168 hpf (stage 31). At 240 hpf (stages 34 and 35), the lower jaw forms and the mouth may begin to open (Armstrong and Child, 1965). In our experiments at 240 hpf, the eggs are removed from the exposure and placed in a well on filter paper to dry out prior to hatch. Hatch occurs on day 17 or 18, and by 2 weeks ph embryos are approximately 11 mm long. CYP1A and CYP1C1 expression increased dramatically at hatch (6- to 16-fold over the 4.5 hpf levels) and continued to increase by 2 weeks ph (25-to 37-fold). When the relative amounts of CYP1 were compared in the untreated embryos, we found that CYP1C1 expression was higher than CYP1A at all time points and the highest relative expression was between 120 hpf and hatch (Fig. 6). These studies suggest that CYP1C1 may be more important than CYP1A during development. We have ongoing studies using *in situ* hybridization to investigate the specific location of CYP1C1 expression because in the studies described here whole embryos were pooled and homogenized.

In fish and mammals, BaP exposure is associated with suite of toxicities including immunosuppression, oxidative stress, mutagenicity, and stable DNA adduct formation. Bay region diol epoxide metabolites are largely believed to be formed by the action of CYP1 enzymes and are associated with stable DNA adduct formation and cancer initiation. In most species and tissues, there are relatively low levels of CYP1 constitutively expressed, however, they can be highly induced by exposure to AhR-ligands including BaP. *Fundulus* exposed to BaP show dose-related increases in biliary PAH metabolites, CYP1A mRNA, EROD activity, CYP1A protein, and DNA adduct formation (Patel *et al*., 2006;Willett *et al*., 1995, 2001). We found CYP1A mRNA was significantly induced by BaP exposure in all tissues investigated except female GI, male spleen and livers. While the lack of the induction in the liver was unexpected, it may be because the long exposure time (15 days) may have lead to high CYP1A protein and thus rapid biotransformation of BaP in the liver lowering the concentrations in this tissue. The highest fold induction of CYP1A was in the heart, brain, and testis. Similar results were reported in zebrafish exposed to 1.5 and 3 μg/l waterborne BaP for 56 days. In zebrafish, there was a dose-related increase in CYP1A mRNA expression in heads but not in liver (Hoffman and Oris, 2006).

Understanding the inducers and target tissues of the newer fish CYP1s will be important for further understanding of the potential contribution of these CYP1s in bioactivating or inactivating environmental contaminants. In fish, there are somewhat mixed results concerning PAH induction of the more recently discovered CYP1B and CYP1C genes. In plaice, constitutive CYP1B expression was noted in gill, but it was not induced by 50 mg/kg i.p.

injection of β-naphthoflavone (Leaver and George, 2000). In contrast, 20 mg/kg BaP caused induction of CYP1B mRNA in channel catfish blood, gonad, and liver. Induction of CYP1B mRNA was also detected in primary cultured catfish gill cells when treated with BaP, TCDD, and polychlorinated biphenyls (PCBs) 77, 126, and 169 but not by treatment with PCB153 or 4,4′-dichlorodiphenyltrichloroethane (Willett *et al*., 2006). In carp, 100 mg/kg 3-methylcholanthrene caused induced expression of CYP1B1 in liver, intestine, and gills, but CYP1B2 expression was only induced in gills (El-kady *et al*., 2004a,b). The carp CYP1C1 was not induced in liver, intestine, gill, or kidney 24 h following a 100 mg/kg i.p. injection of β-naphthoflavone (Itakura *et al*., 2005). Our results represent the first evidence that CYP1C1 is inducible by PAH exposure. The difference between carp and *Fundulus* may be species related or may be due to less sensitivity in the Northern blot methods used in the carp study compared to the real-time PCR used here. CYP1C1 was significantly induced in adult *Fundulus* gill, liver, and heart following a 15-day waterborne 10-μg/l exposure. Highest induction was found in the heart, a known target organ for PAH toxicities in the early life stages.

To date, nothing is known about the protein expression or the metabolic substrates of the fish CYP1B and CYP1Cs. Hypotheses can be generated by comparing the amino acid sequences particularly in the substrate recognition sites. In their analysis of fish CYP1Cs, Godard *et al*. (2005) proposed that SRS1, SRS3, and SRS4 likely contribute the most to differences in substrate recognition between CYP1C1s and CYP1C2s. Particularly in SRS4, where the I-helix interacts with the heme-oxygen, there is a conserved threonine that is preceded by two amino acids (*Fig. 1) that are suggested to play a role in substrate specificity. In *Fundulus* CYP1C1, this residue is a glutamine, in scup and zebrafish CYP1C2s it is a leucine, in *Fundulus* CYP1A it is a phenylalanine, and in CYP1B it is a methionine. Differences in SRS2 and SRS6 have been suggested to be involved in differentiating substrate specificities between the CYP1Cs and the CYP1Bs (Godard *et al*., 2005). To better understand the differences in metabolic capacity of these CYP1s, the enzymes should be recombinantly expressed. In mammals, for example, ethoxyresorufin is a better substrate for CYP1A1 compared to CYP1B1. CYP1B1 is an estradiol hydroxylase primarily at the C-4 position compared to the C-2 position for CYP1A1 (Hayes *et al*., 1996). In some species, CYP1B1 is more active than CYP1A1 in metabolizing BaP to the proximate toxicant BaP-7,8-diol (Shimada *et al*., 1999b).

PAHs cause embryo toxicity with symptoms including cranial-facial malformations, yolk sac and pericardial edema, subcutaneous hemorrhaging, and reduced growth (Billiard *et al*., 1999;Incardona *et al*., 2004;Wassenberg and Di Giulio, 2004). Neither a BaP exposure 10 days at 10 μg/l (this study) nor BaP 7 days at 100 μg/l caused significant deformities in *Fundulus* embryos (Wassenberg and Di Giulio, 2004). However, when fish were cotreated with PAHs and classic CYP1A inhibitors including α-naphthoflavone and piperonyl butoxide, embryonic EROD activity was significantly decreased while the deformity index was dramatically increased (Wassenberg and Di Giulio, 2004). This study suggested that inhibiting CYP1A increased the toxicity of PAHs and could possibly implicate the involvement of another CYP in the toxicity. More recently, work in zebrafish using AhR2 and CYP1A morpholinos showed that knocking down AhR2 reduced cardiac toxicity of β-naphthoflavone and α-naphthoflavone cotreatments. However, when CYP1A was knocked down, it enhanced the toxicity of both β-naphthoflavone and the β-naphthoflavone and α-naphthoflavone cotreatment (Billiard *et al*., 2006). Therefore, the PAH-mediated toxicity appears to be AhR mediated, but CYP1A seems to be providing a protective role. In our embryo study, both CYP1A and CYP1C1 were induced by BaP. However, CYP1A was maximally induced relative to controls ~300-fold, whereas CYP1C1 was only induced about 18-fold. The extensive induction of CYP1A may be providing protection against BaP toxicity and further research is necessary to understand the significance of constitutively higher CYP1C1 expression in embryos and its induction by BaP. It is also unknown whether the classic CYP1A inhibitors will also inhibit CYP1C1 expression.

In summary, we have described a new CYP1C1 gene in *Fundulus* an important model species in marine environmental toxicology. CYP1C1 mRNA had a wide tissue distribution and was inducible in three toxicologically relevant tissues (heart, gill, and liver). Additional research may find that this enzyme is involved both in PAH bioactivation to carcinogenic intermediates and/or in the mechanisms associated with PAH embryo toxicity.

Acknowledgements

This work was supported by National Institute of Environmental Health Sciences grant R01ES012710 and instruments provided by National Center for Research Resources grant RR016476 from the MFGN INBRE program. We thank Annette Ford and Kate Argote for technical assistance with exposures and fish care.

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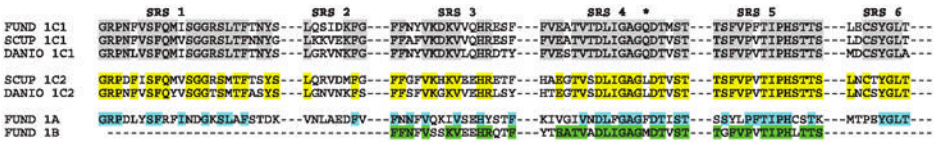

FIG 1.

Amino acid alignment of the six substrate recognition sites (SRS) as described in (Godard *et al*., 2005;Gotoh, 1992) of *Fundulus* CYP1C1 with scup and zebrafish CYP1C1 and CYP1C2s and *Fundulus* CYP1A and the *Fundulus* CYP1B. Gray and yellow shading indicate similar sites between *Fundulus* CYP1C1 and other fish CYP1C1s or CYP1C2s, respectively. Blue and green shading indicate similar sites between *Fundulus* CYP1C1 and *Fundulus* CYP1A or *Fundulus* CYP1B, respectively. *Indicates the position of the AA that is suggested to play a role in substrate specificity.

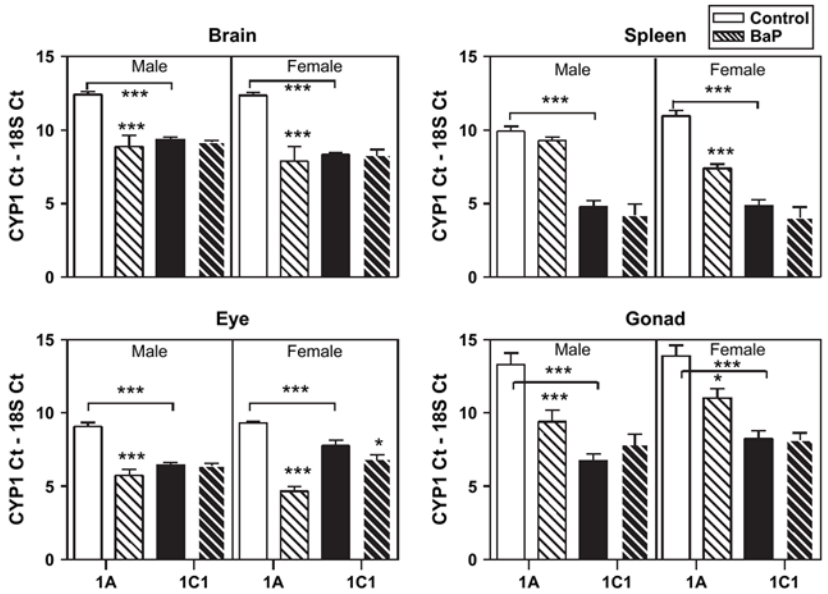

FIG 2.

CYP1A (white bars) and CYP1C1 (black bars) mRNA expression in control (solid bars) and BaP-exposed (thatched bars) adult *Fundulus*. Four tissues (brain, spleen, eye, gonad) had significantly higher constitutive CYP1C1 expression in both sexes. Bar height represents the threshold cycle number for the CYP1s minus the threshold cycle number for the 18S for each sample. Note that the shorter the bar, the higher the mRNA expression. See "Materials and Methods" for details of experiments. Significant differences between treatment and control or differences between CYP1 constitutive expression are noted. Bars represent mean and standard error of the mean (*$p < 0.05$; ***$p < 0.001$; ANOVA, $n = 3–5$).

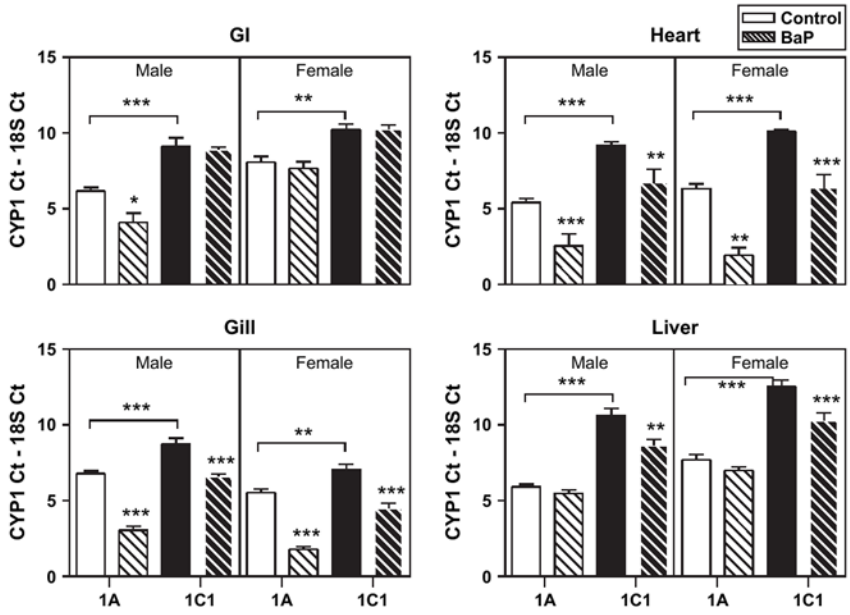

FIG 3.

CYP1A (white bars) and CYP1C1 (black bars) mRNA expression in control (solid bars) and BaP-exposed (thatched bars) adult *Fundulus*. Four tissues (GI, heart, gill, liver) had significantly higher constitutive CYP1A expression in both sexes. See Figure 2 for explanation of bars. Significant differences between treatment and control or differences between CYP1 constitutive expression are noted. Bars represent mean and standard error of the mean (*$p <$ 0.05; **$p < 0.01$; ***$p < 0.001$; ANOVA, $n = 3–5$).

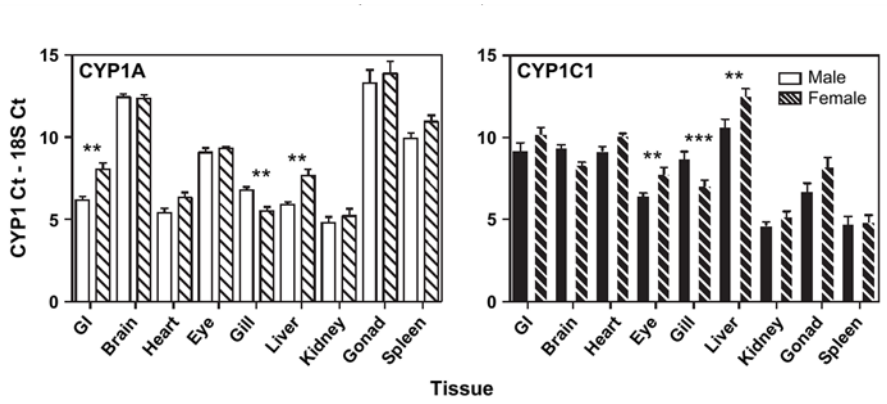

FIG 4.

Constitutive CYP1A (white bars) and CYP1C1 (black bars) mRNA expression in adult male (solid bars) and female (thatched bars) *Fundulus*. See Figure 2 for explanations of bars. Significant differences between males and females within a tissue are noted (**$p < 0.01$; ***$p < 0.001$; ANOVA, $n = 3–5$).

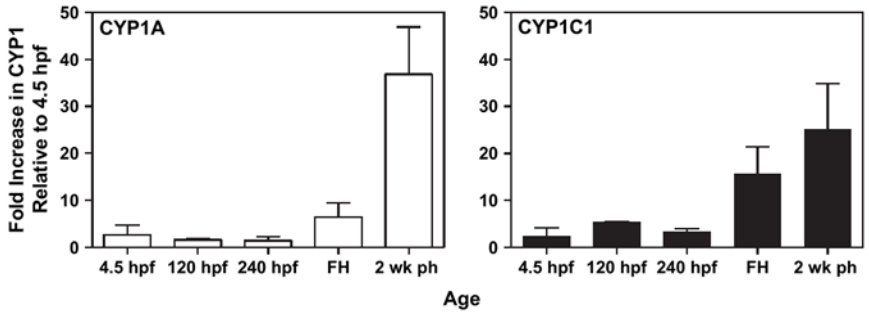

FIG 5.

Constitutive CYP1A (white bars) and CYP1C1 (black bars) mRNA level in a pool of 10 whole embryos ($n = 4$). See "Materials and Methods" for details of experiments. Each bar represents the mean and standard error of the mean of the fold increase in CYP1 expression at the different ages relative to 4.5 hpf (FH = at hatch).

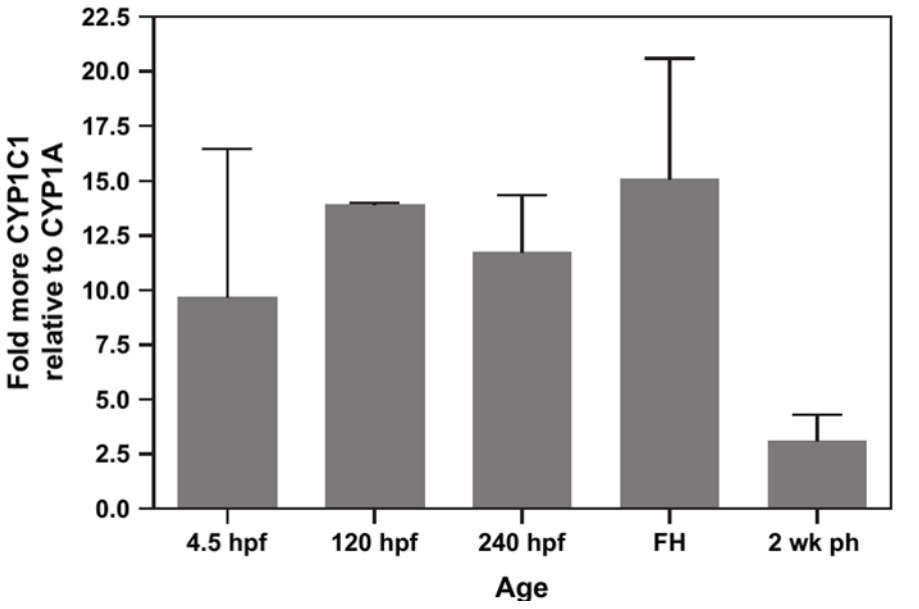

FIG 6.

Fold-higher CYP1C1 mRNA level expression relative to CYP1A in control whole embryos. Each bar represents fold increases in CYP1C1 expression relative to CYP1A within an age.

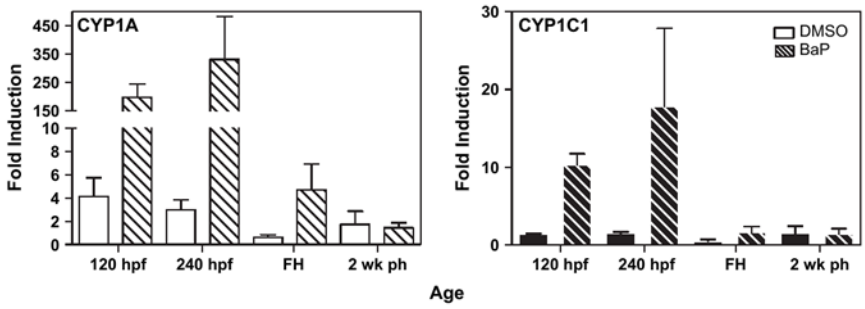

FIG 7.

BaP effects on CYP1A (white bars) and CYP1C1 (black bars) mRNA level in a pool of 10 whole embryos (n = 4). Each bar represents mean and standard error of the mean of the fold increases in CYP1 expression in DMSO control (solid bars) or BaP treatment (thatched bars) relative to the untreated group at each age.

TABLE 1

Primers for *Fundulus* CYP1C1 Cloning and Real-Time PCR

Oligo	Primer Sequence 5′–3′	Location	Function
1	AGTTTGGTTGATGTCTTGCC	897–916	1C1 forward
2	GTCAAAGGCTTGAGGGTCCT	1459–1478	1C1 reverse
3	CACCTCAGGGGGTCGTGATTGACAG	1432–1456	1C1 5′RACE reverse
4	GGGGGTCGTGATTGACAGACCACTG	1425–1449	1C1 5′RACE reverse
5	CGCTTCACCAGCTTTGTCCCATTCA	1323–1347	1C1 3′RACE forward
6	CCCAAGGACACGGTGGTCTTCATCA	1398–1422	1C1 3′RACE forward
7	CTTAAGCCTTAAATCTTGATGATAGAC	1–27	1C1 forward for full-length
8	CAGTATATAAAGGCTCAGTGGGACTC	2698–2723	1C1 reverse for full-length
9	AACACTAAAGCAACAACTCCAGATT	2672–2696	1C1 reverse for full-length
10	TCTGGACGCCTTCATCTACGA	1295–1315	1C1 real-time forward
11	GTGACGTCCGATGTGGTTGA	1357–1378	1C1 real-time reverse
12	CGAAGATTTTGTCCAGGTGACA	816–837	1A real-time forward
13	CATTGACTTGTTGGGCAGAAACT	874–897	1A real-time reverse
14	TGGTTAATTCCGATAACGAACGA	1319–1341	18S real-time forward
15	CGCCACTTGTCCCTCTAAGAA	1395–1415	18S real-time reverse

TABLE 2

CYP1 mRNA Expression in Adult *Fundulus* Exposed 15 Days to Ethanol Control or 10 μg/l BaP

	Males			Females		
	Fold BAP induction[a]		Control isoform[b]	Fold BAP induction[a]		Control isoform[b]
	CYP1A	CYP1C	Fold abundance	CYP1A	CYP1C	Fold abundance
Heart	**18.9 ± 9.70**	**16.8 ± 9.26**	**14.6 ± 2.77 more 1A versus 1C1**	**27.9 ± 10.6**	**30.5 ± 21.8**	**14.6 ± 3.64 more 1A versus 1C1**
Gill	**14.2 ± 2.16**	**4.47 ± 0.457**	**3.94 ± 0.478 more 1A versus 1C1**	**13.8 ± 1.47**	**6.60 ± 1.74**	**3.05 ± 0.447 more 1A versus 1C1**
GI	**6.75 ± 3.77**	1.20 ± 0.14	**8.05 ± 1.23 more 1A versus 1C1**	1.59 ± 0.46	1.03 ± 0.17	**5.12 ± 1.46 more 1A versus 1C1**
Liver	1.43 ± 0.25	**4.65 ± 1.26**	**27.4 ± 2.78 more 1A versus 1C1**	1.70 ± 0.28	**5.85 ± 1.73**	**33.1 ± 9.76 more 1A versus 1C1**
Brain	**23.6 ± 15.7**	1.14 ± 0.08	**8.63 ± 1.21 more 1C1 versus 1A**	**37.6 ± 20.5**	1.13 ± 0.32	**16.7 ± 1.63 more 1C1 versus 1A**
Eye	**12.3 ± 4.70**	1.08 ± 0.16	**6.27 ± 0.692 more 1C1 versus 1A**	**28.2 ± 6.52**	**2.06 ± 0.422**	**3.39 ± 0.870 more 1C1 versus 1A**
Gonad	**25.1 ± 11.2**	0.79 ± 0.40	**108 ± 24.6 more 1C1 versus 1A**	**9.71 ± 2.96**	1.26 ± 0.39	**65.3 ± 20.7 more 1C1 versus 1A**
Spleen	1.61 ± 0.28	1.90 ± 0.86	**39.2 ± 11.9 more 1C1 versus 1A**	**12.4 ± 2.59**	2.15 ± 0.75	**80.8 ± 22.6 more 1C1 versus 1A**
Kidney	**2.90 ± 0.653**	1.62 ± 0.37	1.14 ± 0.13 more 1C1 versus 1A	**3.00 ± 0.395**	2.30 ± 0.37	1.07 ± 0.20 more 1C1 versus 1A

[a] Calculated by $\Delta\Delta C_T$ approach. Data normalized to 18S expression and reported as fold induction relative to controls (n = 3–5).

[b] Calculated by $\Delta\Delta C_T$ approach. CYP1A and CYP1C1 expression in control animals was normalized to 18S expression and is shown as relative fold abundance.

Numbers in bold are significantly different, ANOVA $p < 0.05$, as analyzed on 18S corrected raw data.

Gray-shaded rows represent tissues where CYP1C1 expression was constitutively higher than CYP1A expression.