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Biochimica et Biophysica Acta 1444 (1999) 35–48

BIOCHIMICA ET BIOPHYSICA ACTA

BBA

Cloning and characterization of the zebrafish (*Danio rerio*) aryl hydrocarbon receptor¹

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Received 3 November 1998; accepted 12 November 1998

Abstract

The aryl hydrocarbon receptor (AhR) mediates the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds in vertebrates. To further establish zebrafish as a vertebrate model to study the molecular mechanism of TCDD toxicity, we have isolated and characterized the cDNA encoding the zebrafish aryl hydrocarbon receptor (zfAhR2). Analysis of the deduced protein sequence revealed the 1027 amino acid protein is approximately 200 amino acids longer than previously isolated receptors. zfAhR2 is homologous to previously cloned PAS proteins within the basic helix-loop-helix and PAS domains. The C-terminal domain of zfAhR2 diverges from the mammalian AhR at position 420, and does not contain a Q-rich domain. zfAhR2 mRNA is first detected by Northern blot analysis at 24 h post fertilization, and expression increases throughout early development. Treatment of zebrafish embryos and zebrafish liver cells with graded doses of TCDD results in a dose-dependent increase in zfAhR2 mRNA. The time course for zfAhR2 and cytochrome P4501A mRNA induction by TCDD are similar. In vitro produced zfAhR2 protein dimerizes with the rainbow trout aryl hydrocarbon receptor nuclear translocator (rtARNTb) and binds dioxin response elements derived from the rainbow trout *CYP1A* gene. Finally, transient coexpression of zfAhR2 and rtARNTb in COS-7 cells results in a TCDD dose-related increase in transcription driven by the rainbow trout *CYP1A* promoter and enhancer. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Aryl hydrocarbon receptor; 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin; (Zebrafish)

Abbreviations: AhR, aryl hydrocarbon receptor; PCDD, polychlorinated dibenzo-*p*-dioxin; PCDF, polychlorinated dibenzofuran; PCB, polychlorinated biphenyl; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; ARNT, aryl hydrocarbon receptor nuclear translocator; bHLH, basic helix-loop-helix; HIF-1 α , hypoxia inducible factor-1 α ; SIM, single minded; AIP, AhR interacting protein; P4501A, cytochrome P4501A protein; *CYP1A*, cytochrome P4501A gene; DRE, dioxin response element; ZF-L, zebrafish liver cells; GCG, Genetics Computer Group; UTR, untranslated region; hpf, hours post fertilization; EC₅₀, effective concentration causing a half maximal response; DMSO, dimethyl sulfoxide; zf, zebrafish; rt, rainbow trout; luc, luciferase

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¹ GenBank accession number: AF063446.

1. Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs) and biphenyls (PCBs) are global environmental contaminants that bioaccumulate in the food chain and produce toxicity by a common aryl hydrocarbon receptor (AhR) mediated mechanism at body burdens in the parts per trillion range posing a risk to fish, wildlife and humans (reviewed in [1]). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent and thoroughly investigated of all PCDD, PCDF and PCB congeners in causing toxicity to vertebrates and is the prototype

compound used to study the mechanism of action of these compounds [2].

The AhR is a member of the basic helix-loop-helix (bHLH) PAS family of transcription factors that include ARNT, ARNT2, HIF-1 α , SIM, and others (reviewed in [3]). The mechanism by which the AhR alters gene transcription in mammals has been well studied [3,4]. In the cytoplasm, unliganded AhR is associated with two molecules of the chaperonin hsp90 and AhR interacting protein (AIP). TCDD binding activates AhR, allowing translocation into the nucleus where aryl hydrocarbon receptor nuclear translocator (ARNT) dimerization occurs [5,6]. AhR/ARNT heterodimers recognize and bind dioxin response elements (DREs) found in promoters of responsive genes to alter gene expression [7–9]. The mammalian DRE consensus core TNGCGTG [10,11] has been identified in the 5' flanking region of the genes encoding the cytochrome P4501A1, P4501A2 and P4501B1 monooxygenases and the well characterized TCDD-induced expression of CYP1A mRNA and protein is often used as an indicator of AhR activity. In addition to the cytochrome P450 enzymes, glutathione *S*-transferase Ya subunit, class 3 aldehyde dehydrogenases, and NAD(P)H:quinone reductase 1 and 2 genes are TCDD regulated (reviewed in [4]).

Although the fish AhR signaling pathway is not well understood, there is considerable evidence that toxicity caused by TCDD in fish is AhR mediated. PCDDs, PCDFs and PCBs induce cytochrome P4501A enzymes in fish cells [12–14] and the DRE core consensus sequence has been identified in the 5' flanking region of the rainbow trout and tomcod *CYP1A* genes [15,16]. For PCDDs and PCDFs the structure-activity relationship for producing toxicity in mammals and fish is similar [14,17]. AhR and ARNT proteins have been identified in fish species. Full-length AhR cDNAs have been isolated from tomcod [16] and two distinct rainbow trout AhRs have been isolated in our laboratory [18]. Partial AhR sequences have been identified in several fish ([19,20], reviewed in [21]). Two full-length forms of rainbow trout ARNT [22], and a partial zebrafish ARNT sequence have also been isolated [23].

The rationale for studying the AhR signaling pathway in fish is that fish are the most sensitive vertebrates to the developmental toxicity of TCDD. When

exposed to TCDD as embryos, fish are up to 100 times more sensitive to TCDD lethality than when exposed as adults, suggesting that TCDD has the ability to perturb critical developmental events [17,24]. TCDD exposure to developing fish embryos of various species results in cardiovascular dysfunction, edema, hemorrhages, craniofacial malformations, growth arrest and mortality [17,25,26]. The cardiovascular system is the key, primary target for TCDD developmental toxicity in fish [27,28]. Although the signs of TCDD embryo toxicity are generally similar between fish species, embryo lethal potency between fish species varies greatly. For example, the TCDD LC_{egg50} in zebrafish is approximately 40-fold higher than that for lake trout, the most sensitive fish species [29]. The basis for this difference is unknown.

Despite extensive efforts to characterize TCDD toxicity in fish, there is a meager understanding of the underlying molecular mechanism of toxicity. To further this effort, it is necessary to characterize the AhR signaling pathway in an experimentally advantageous fish species.

A major goal of our laboratory has been to establish zebrafish as a vertebrate model for investigating the developmental toxicity of TCDD. Zebrafish embryos exposed to TCDD are responsive to the toxicant and develop essentially the same signs of developmental toxicity as other fish species. TCDD exposure results in impaired cardiovascular development and function in zebrafish [26]. Zebrafish also have a functional AhR signaling pathway as evidenced by AhR agonists inducing CYP1A activity in cultured zebrafish cells [30]. Furthermore, zebrafish embryogenesis is well studied and they are amenable to molecular and genetic techniques [31,32]. Embryos are transparent, develop externally allowing observations at any developmental stage, and mature rapidly requiring only 96 h to advance from a single cell to a free swimming fish. Juvenile zebrafish also reach sexually maturity rapidly and females produce hundreds of eggs weekly. The practical advantages of zebrafish make it possible to perform saturation mutagenesis screens in a vertebrate, similar to those done in invertebrates such as *Caenorhabditis elegans* and *Drosophila*. To date, large-scale zebrafish mutant searches have led to the identification of hundreds of mutants with unique embryonic and larval develop-

ment defects [33–37]. Large-scale mapping and cloning of the mutations are under way [38]. The ability to identify genes involved in TCDD toxicity using zebrafish mutants will further our understanding of the mechanism of TCDD toxicity.

Here we describe cloning of the full-length zebrafish AhR2 cDNA, the developmental and TCDD regulated expression of the mRNA, and some of the biochemical properties of the encoded protein. Recently a partial zebrafish AhR-like sequence, distinct from zfAhR2, was reported [23]. For comparisons, this partial sequence is referred to as zfAhR1.

2. Materials and methods

2.1. Cell culture

Zebrafish liver cells (ZF-L) obtained from David Barnes (Oregon State University, Corvallis, OR) were maintained at 27°C in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 5% fetal bovine serum and 0.5% rainbow trout serum [39]. Monkey kidney COS-7 cells (ATCC) were maintained in DMEM supplemented with 10% fetal bovine serum incubated in a 100% humidity 5% CO₂ incubator at 37°C. For TCDD exposures, DMSO or TCDD previously dissolved in DMSO (0.1% medium volume) was added to the medium and incubated overnight before cell harvest.

2.2. Oligonucleotides

All primers are written 5' to 3'. zfAHR-F1 (position 2159): ACGGTGAAGCTCTCCCATATA; zfAhR-R2 (position 2636): GAGTGTGGAGAGAAA-CTG; rtCYP1A-F: AGGTTGGTTGAGTGAGATG; rtCYP1A-R: TGCAGGGAGATCGAAGAAG. Primers for DNA gel shift experiments (the DRE consensus core is indicated by underline and mutated bases are in bold): wt rtDRE1: ACCTTTG**CACG**-**CTATCG**AAAT; wt rtDRE2: ATTT**CGATAG**-**CGTG**CAAAGGT; mut rtDRE1: ACCTTTG**C**-**GCGCTATCG**AAAT; mut rtDRE2: ATTT**CG**-**ATAGCGCG**CAAAGGT; wt mouse DRE1: GGCTCGGAGTT**GCGTG**GAGAAGAG; wt mouse DRE2: GGCTCTT**CTCACG**CAACTCCGAG; mut mouse DRE1: GGCTCGGAGTT**GCGCG**GAGAA-

GAG; mut mouse DRE2: GGCTCTT**CTCGC**-**GCAACTCCG**AG.

2.3. RT-PCR

First strand cDNA synthesis using 200 ng total RNA primed with oligo dT was amplified using degenerate primers fPAS-1 and rPAS-1 (Table 1) each at 1.0 μM. Amplification for 35 cycles was as follows: 30 s 94°C, 30 s 50°C, and 1 min 72°C. A final 72°C, 7 min extension followed the last cycle. Aliquots (0.1 μl) from the original PCR reactions were reamplified under the same conditions as above using nested fPAS-2 and rPAS-2 primers. Amplified products were visualized by ethidium bromide staining and subcloned into PCRII (Invitrogen).

2.4. Library construction, screening and DNA sequencing

A cDNA library was constructed in λZAP Express (Stratagene) using poly(A)⁺ RNA from zebrafish liver cells. The cDNAs were synthesized using a cDNA cloning kit (Stratagene) and subsequently inserted into λZAP arms using *EcoRI-XhoI* adapters. Four positive clones out of approximately 2 × 10⁶ recombinant plaques were isolated using the ³²P-labeled 500 bp partial zebrafish AhR cDNA obtained by degenerate primer RT-PCR. Putative zebrafish AhR clones were sequenced using fluorescent dye-labeling cycle sequencing (Applied Biosystems, UW Madison Biotech Center) stepwise using gene specific primers. Both strands of each clone were sequenced at least three times before GenBank submission. Genetics Computer Group (GCG) [40] software and the Baylor College of Medicine Human Genome Center search launcher (web site <http://kiwi.bcm.edu:8088/search-launcher/launcher.html>) were used for sequence analysis.

2.5. Plasmid construction for in vivo transactivation and in vitro translation

A 3.3 kb zfAhR2 *DraI* fragment from the original 7.2 kb zfAhR2 clone containing a 66 bp 5' UTR, the entire coding region and a 234 bp 3' UTR was subcloned into the *EcoRV* site of pBluescript (Stratagene). For expression studies, the zebrafish AhR

fragment was excised from pBluescript by *ApaI* and *BamHI* digestion (sites located in the polylinker) and inserted into the corresponding sites in pBK-CMV expression vector (Stratagene). Rainbow trout pMVrtARNTb was generously provided by R. Pollenz, Medical University of South Carolina, Charleston, SC, and has previously been described [22]. The rainbow trout dioxin responsive reporter construct, prt1Ahuc, was constructed as follows. A 1540 bp portion of the rainbow trout *CYP1A* promoter and enhancer, containing two dioxin responsive elements, from position 139 to 1678 (GenBank accession number S69277) was amplified from rainbow trout genomic DNA using oligonucleotides rtCYP1A-F and rtCYP1A-R. The amplicon was subcloned into PGEM-T Easy Vector (Promega) and sequenced. The 1540 bp insert was excised by *SacI* and *NcoI* digestion and subcloned into corresponding sites in pGL3basic vector (Promega). pRL-TK (Promega) was used as an internal *Renilla* luciferase reporter control.

2.6. Static water exposure of zebrafish embryos to TCDD

Within 3 h of spawning, fertilized eggs were exposed to 20 ml water containing 0.2% acetone or graded concentrations of TCDD previously dissolved in acetone. After 1 h, embryos were rinsed several times with TCDD-free water and allowed to develop in 150 mm petri dishes. Embryos were maintained at 28°C and dead embryos were removed from the dishes daily. Culture water was replaced daily for the duration of the study. Embryos allowed to develop past 96 h were fed live paramecia once per day. Twenty embryos from each dosing group were raised in individual 400 ml beakers for 10 days to assess mortality. For dose-response experiments, embryos were terminated for RNA isolation at 48 h post fertilization (hpf). For time course experiments, embryos were terminated at 12, 24, 48, 72 and 96 hpf.

2.7. RNA isolation and Northern analysis

Total RNA was isolated from ZF-L cells after TCDD exposure using RNeasy extraction kits (Qiagen), by directly adding lysis buffer to the plates after medium removal. To obtain RNA from whole adult

zebrafish, 1 year old fish anesthetized with Tricane were frozen in liquid nitrogen and pulverized. RNA was isolated from zebrafish adults and embryos using TRI Reagent (Molecular Research Center). Poly(A)⁺ RNA was isolated using the PolyAtract system (Promega). 10–20 µg RNA was electrophoresed on a 1.2% agarose formaldehyde gel and transferred to Hybond⁺ (Amersham). Membranes were prehybridized and hybridized at 42°C in 6×SSC, 0.1% SDS and 50% formamide with random primed ³²P-labeled cDNA probes. The C-terminal specific zfAhR2 cDNA probe was made by PCR amplification from cDNA using zfAhR-F1 and zfAhR-R1 primers. The resulting 480 bp product was subcloned into the PGEM-T Easy Vector (Promega). The rainbow trout *CYP1A* and rat β-actin cDNAs were described previously [14]. Blots were sequentially probed with random primed zfAhR2, rainbow trout *CYP1A*, and rat β-actin probes and analyzed by phosphorimaging (Molecular Dynamics). The intensity of each band was normalized to actin mRNA levels. For TCDD dosing experiments, the half maximal response (EC₅₀) for induction of both AhR and *CYP1A* mRNAs was calculated by non-linear regression using the Statistica software package (StatSoft).

2.8. In vitro translation and DNA binding assay

zfAhR2 and rtARNTb proteins were produced from vectors pBKCMVzfAhR2 and pMVrtARNTb in TNT rabbit reticulocyte lysates as recommended by the supplier (Promega). Side reactions containing [³⁵S]methionine were performed to assess relative protein production. After the 90 min incubation at 30°C, radioactive translation products were resolved on a 8% SDS polyacrylamide gel, dried and phosphorimaged.

Oligonucleotide wt rtDRE1 was 5' end labeled with ³²P using T4 polynucleotide kinase and annealed to a 3-fold molar excess unlabeled wt rtDRE2 oligonucleotide followed by probe purification. Unlabeled competitor DNAs were similarly produced by annealing unlabeled wt rtDRE1:wt rtDRE2 and mut rtDRE1:mut rtDRE2 oligonucleotides. The wt mouseDRE1:wt mouseDRE2 and mouse mut mouseDRE1:mut mouseDRE2 were annealed and filled in by Klenow [41]. For in vitro DNA binding

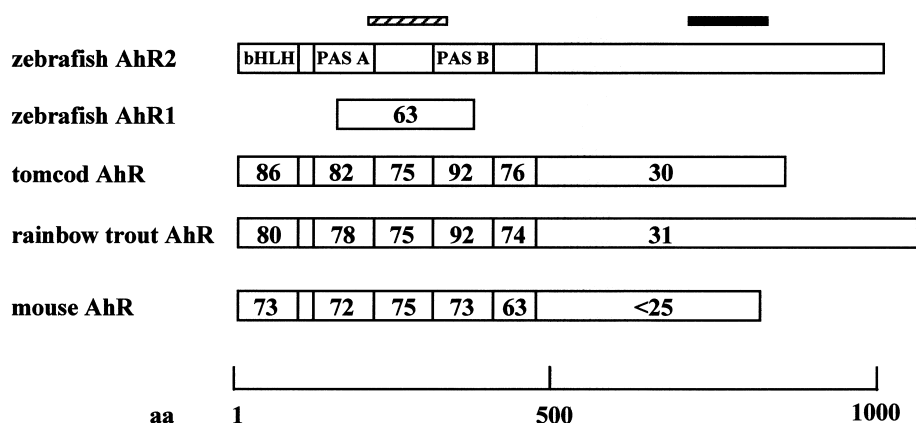


Fig. 2. Schematic representation and gap-free BLASTP alignment of zfishAhR2 and other AhRs. The indicated regions of zfishAhR2 were separately compared to the other AhRs. The numbers within the boxes indicate percent identity with the zebrafish AhR2 deduced amino acid sequence. The position of the probe derived by degenerate PCR is indicated by the hatched bar, and the probe used for Northern analysis is indicated by the solid bar. GenBank accession numbers for the sequences used are the same as in Fig. 1.

assays, approximately equal amounts of *in vitro* produced zfishAhR2 and/or rtARNTb proteins were incubated in the presence of 10 nM TCDD in 0.2% DMSO or DMSO alone for 90 min at 22°C. Following incubation, 1.5 µg poly dI-dC and binding buffer (20 mM HEPES, pH 7.9, 100 mM NaCl, 1 mM DTT, 6% glycerol) were added and the incubation continued for an additional 20 min at 22°C before the addition of 100 000 cpm of the rtDRE probe (core sequence TAGCGTG) and 10-fold molar excess unlabeled wild type rtDRE, mutated rtDRE (TAGCGCG; mutated base underlined), wild type mouse DRE (core sequence TTGCGTG) or mutated mouse DRE (TTGTGTG; mutated base underlined) competitor DNAs. After 20 min incubation at 22°C, complexes were resolved on a 0.5×TBE (90 mM Tris, 64.6 mM boric acid, and 2.5 mM EDTA, pH 8.3) 4.5% acrylamide gel at 4°C. The dried gels were exposed to a phosphor screen overnight before analysis.

2.9. Transient transfection of COS-7 cells

prt1Aluc was cotransfected with pRL-TK, and the zfishAhR2 and rtARNTb expression vectors using the SuperFect protocol (Qiagen). Briefly, 450 ng of AhR and/or ARNT expression vectors were mixed with 100 ng prt1Aluc and 3 ng pRL-TK vector and incubated with 60 000 cells in 24 well plates for 2.5 h at 37°C in 400 µl serum containing medium. The

amount of DNA in each well was adjusted to 1 µg using empty pBK-CMV expression vector. Following incubation, 600 µl fresh serum containing medium was added and the cells were incubated for 20 h at 37°C. Following incubation, DMSO or TCDD previously dissolved in DMSO (0.1% medium volume) were added to the cells. Cells were incubated at 37°C for 20 h before cell harvest. Cell extraction and luciferase assays were performed according to the manufacturer's protocol (Dual-Luciferase assay system, Promega).

3. Results

3.1. Cloning of zebrafish AhR cDNA

Degenerate PCR primers designed using the mouse AhR amino acid sequence (Table 1) were used to amplify partial zebrafish AhR cDNA sequences. A 500 bp product was RT-PCR amplified using RNA isolated from ZF-L cells and whole adult zebrafish (data not shown). Ten subclones derived from adult and ZF-L RNA were sequenced and found to be identical. The zebrafish amplicon was 74% identical to the corresponding mouse AhR region and the predicted amino acid sequence shares 72% identity with mouse AhR. To obtain the full-length zebrafish AhR cDNA, a poly(dT) primed cDNA library was prepared from zebrafish liver cells. The ZF-L cells

Table 1
Degenerate oligonucleotides used to amplify partial zebrafish AhR cDNA

Oligo	Sequence	Amino acid target (mouse)	Amino acid position (mouse)
fPAS-1	CARGCICTSAAYGGITT	QALNGF	118–123
rPAS-1	CATCATRTCIGCIGCRTG	HAADILH	320–325
fPAS-2	GAYTAYCTIGGITTTCARCA	DYLGFFQQ	142–148
rPAS-2	AGCTCIATYTCIGTRTAICC	GYTEVEL	303–309

were used since CYP1A induction has been shown to follow TCDD exposure in this cell line [30,42]. A total of four overlapping clones were obtained after screening approximately 2×10^6 recombinant plaques with the 500 bp zebrafish AhR PCR fragment. The two largest clones result from alternative polyadenylation and have identical open reading frames with translational initiation codons that fall within the Kozak consensus sequence [43]. The 7.2 kb and 4.3 kb clones have 229 bp and 245 bp 5' UTRs respectively, identical 3082 bp coding regions, and terminate in poly(A) tails. The resulting 3' UTRs are 2797 bp and 998 bp, respectively. Both transcripts are detected by RNase protection assays (data not shown), however, only a single 7.2 kb message is detected by Northern analysis and is the focus of this work.

There are several interesting zfAhR2 characteristics worth noting (Fig. 1). The 1027 amino acid protein with a predicted molecular weight of 113 kDa is approximately 200 amino acids longer than most other AhRs, including the mouse and tomcod AhRs, and it lacks the Q-rich domain, shown to be important for transactivation in the mammalian AhR [44]. Alignment of zfAhR2 with the partial zfAhR1, Atlantic tomcod, rainbow trout α and mouse AhRs illustrates that the greatest similarity lies within the bHLH and PAS domains (Figs. 1 and 2), regions important for DNA and ligand binding and protein dimerization [45,46]. Two putative AhR partial cDNA sequences from several fish species were recently isolated and have been divided into two distinct classes, AhR1 and AhR2, based on sequence differences [20]. Corresponding sequences from the zfAhR2 described here are most similar to the dogfish AhR2 (74%) and *Fundulus* AhR2 (72%) receptors over the 200 amino acid overlap. zfAhR2 is 70% and 67% identical to dogfish AhR1 and *Fundulus* AhR1, respectively, over this same region. The recently described 184 amino acid partial zfAhR1

sequence is most similar to the dogfish AhR1 sequence (71%) and only 63% identical to zfAhR2 (Figs. 1 and 2) indicating there are at least two zebrafish AhRs [23]. Noteworthy, the 108 amino acids between the zfAhR2 PAS domains are 75% identical to the tomcod, trout and mouse AhRs. The 75 amino acids just C-terminal to the PAS B domain are also well conserved. The C-terminal domain of zfAhR2 is only 31% and 30% identical to the rainbow trout and tomcod AhRs, respectively, and is not homologous to the mouse AhR.

3.2. Developmental expression and TCDD induction of zebrafish AhR mRNA

To determine the expression of zfAhR2 mRNA during development, RNA was isolated from zebrafish embryos at different hours post fertilization, from whole adult zebrafish and from ZF-L cells for Northern blot analysis. Northern blots were probed with a random primed zfAhR2 probe (Fig. 3). A single 7.2 kb transcript was detected in all samples.

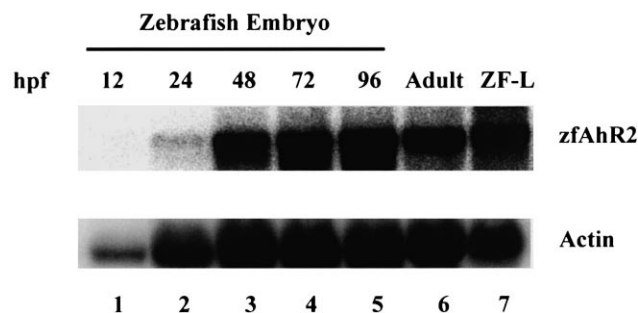


Fig. 3. Developmental expression of zebrafish AhR2 mRNA. Each lane consists of 20 μ g total RNA prepared from zebrafish embryos collected at the indicated hours post fertilization (hpf), from whole adult zebrafish and zebrafish liver cell (ZF-L) cells. The blots were probed sequentially with random primed cDNA fragments for zfAhR2 and rat β -actin as a loading control and exposed to a phosphor screen for 4 days and 1 day, respectively.

Overall expression of zfAhR2 mRNA was low, requiring 2–4 day phosphor screen exposures, and is first detected at 24 hpf with elevated levels at 48, 72 and 96 hpf (Fig. 3). zfAhR2 transcripts are detected as early as 5 hpf by RT-PCR (data not shown) and expression levels of zfAhR2 are similar in adult and ZF-L cells (Fig. 3, lanes 6 and 7).

To evaluate the expression of zfAhR2 mRNA and function of the protein in ZF-L cells, RNA was isolated from ZF-L cells exposed to graded concentra-

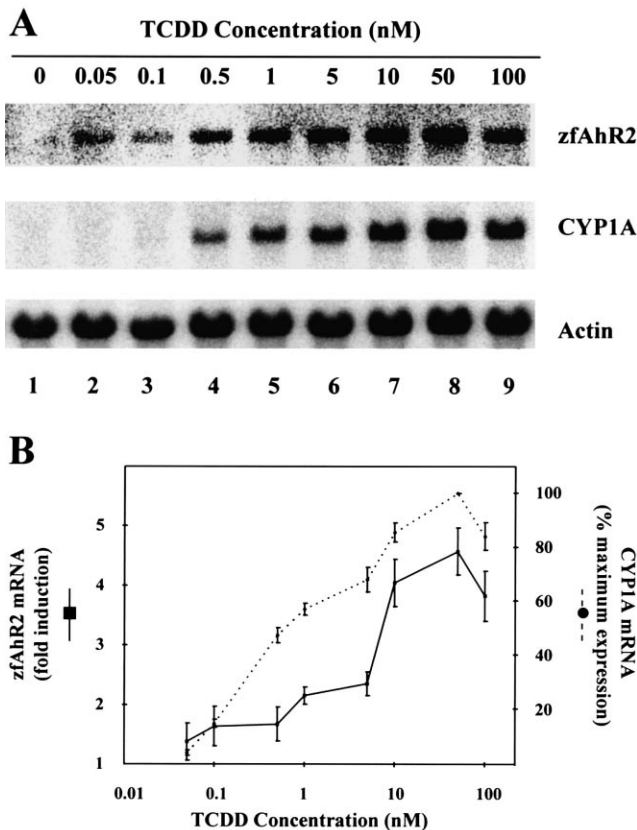


Fig. 4. TCDD induces zfAhR2 and CYP1A mRNAs in zebrafish liver cells. (A) Representative Northern blot of total RNA from ZF-L cells after a 24 h treatment with DMSO vehicle or graded concentrations of TCDD. Each lane consists of 15 μ g RNA. The blots were probed sequentially with random primed cDNA fragments for zfAhR2, rainbow trout CYP1A, and rat β -actin as a loading control; phosphor screen exposure times were 4 days, 2 days, and 12 h respectively. (B) Graphical representation ZF-L TCDD dose-response experiments (mean \pm S.E.M., $n=3$). The fold zfAhR2 mRNA induction (\blacksquare ; solid line) was calculated relative to the mean of DMSO treated cells. Percent maximum CYP1A expression (\bullet ; dashed line) was calculated based on the maximum expression after 50 nM TCDD exposure.

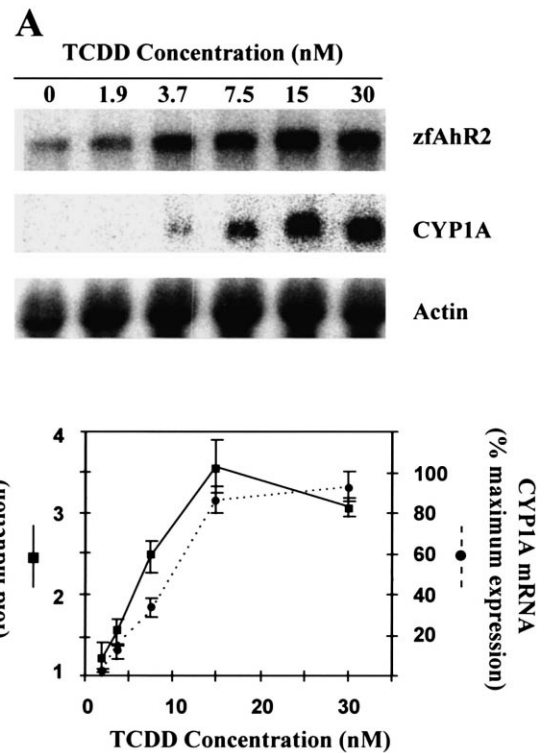


Fig. 5. Dose dependent induction by TCDD of zfAhR2 and CYP1A mRNAs in zebrafish embryos 48 h post fertilization. (A) Newly fertilized zebrafish eggs were statically exposed for 1 h to waterborne acetone (control) or to graded concentrations of TCDD, and allowed to develop in TCDD-free water for 48 h before termination, RNA isolation and Northern blot analysis. Each lane of the representative Northern blot consists of 20 μ g RNA. The blots were probed sequentially with zfAhR2, rainbow trout CYP1A, and rat β -actin probes; phosphor screen exposure times were 4 days, 2 days, and 12 h respectively. (B) Graphical representation of independent experiments (mean \pm S.E.M., $n=3$). The fold zfAhR2 mRNA induction (\blacksquare ; solid line) was calculated relative to the mean of embryos exposed to acetone. Percent maximum CYP1A expression (\bullet ; dashed line) was calculated based on the maximum expression after TCDD exposure.

tions of TCDD for 24 h followed by Northern analysis. Northern blots were sequentially probed with random primed zfAhR2, rainbow trout CYP1A, and rat β -actin probes (Fig. 4). The intensity of each band was normalized to β -actin mRNA levels. A clear dose response is observed as the concentration of TCDD increases. zfAhR2 expression in the absence of TCDD was low, and increased up to 5-fold over DMSO controls after 50 nM TCDD exposure, with an EC_{50} of 6.8 nM TCDD. CYP1A mRNA is not detectable in the absence of TCDD,

and is induced to a maximum level after 50 nM TCDD exposure with an EC_{50} of 1.1 nM TCDD.

The earliest overt sign of TCDD developmental toxicity in zebrafish is a reduction in blood flow to the trunk detected at 48 hpf ([26] and Tanguay and Peterson, unpublished results). To determine if zfAhR2 mRNA is expressed and functional (using AhR dependent CYP1A mRNA expression as a marker) at this critical time point, zebrafish embryos were statically exposed for 1 h as newly fertilized eggs to graded concentrations of waterborne TCDD and allowed to develop in TCDD-free water. Northern analyses using RNA isolated from embryos 48 hpf were performed (Fig. 5). In the absence of TCDD, zfAhR2 mRNA abundance is low, and increases in a dose-related manner to 4-fold that of the DMSO control after TCDD treatment. The EC_{50} s of TCDD for zfAhR2 and CYP1A mRNA induction are 2.7 and 7.9 nM TCDD, respectively.

To determine if zfAhR2 mRNA is induced and if the AhR protein is functional at other developmental stages, zebrafish embryos were statically waterborne exposed for 1 h as newly fertilized eggs to 15 nM TCDD. At this TCDD concentration 100% mortality occurs at 9 days post fertilization, therefore zfAhR2 and CYP1A mRNA levels were measured at earlier developmental time points using Northern blot analysis (Fig. 6A). In the absence of TCDD zfAhR2 mRNA levels increase from 24 to 96 hpf, but CYP1A mRNA is not detected at any time. In the presence of TCDD, zfAhR2 mRNA is elevated at 24, 48, 72 and 96 hpf, 1.4-, 1.9-, 2.1- and 1.9-fold respectively over controls (Fig. 6B). CYP1A mRNA is induced as early as 24 hpf, coincidental with zfAhR2 mRNA expression, with maximal induction occurring at 96 hpf.

3.3. Binding of zebrafish AhR to the dioxin response element

To begin to characterize the zebrafish AhR2 protein, zfAhR2 and ARNTb from rainbow trout (rtARNTb) were produced in reticulocyte lysate for in vitro DNA binding assays (Fig. 7A). Rainbow trout ARNTb was used because it was the only full-length fish ARNT sequence available at the time, and had been shown to dimerize and transactivate with mammalian AhR [22]. In vitro translated

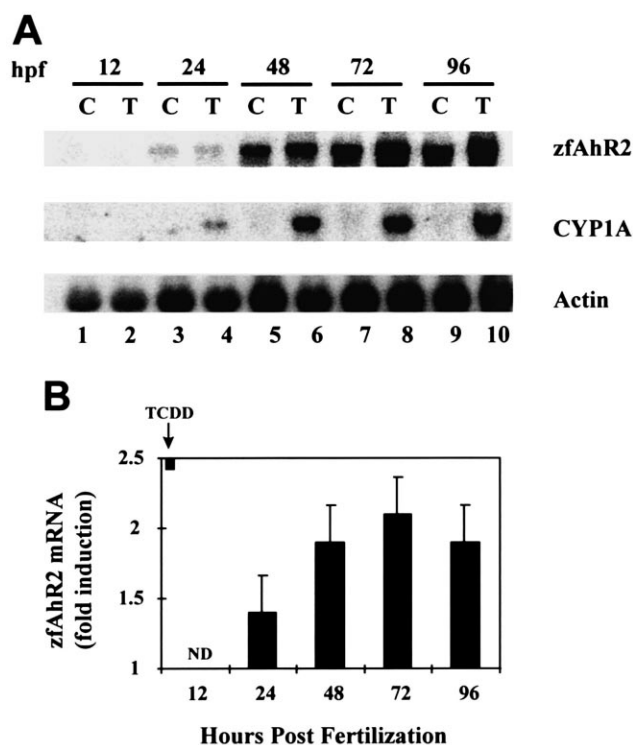


Fig. 6. Induction of AhR and CYP1A mRNAs by TCDD during zebrafish development. Newly fertilized zebrafish eggs were statically exposed for 1 h to waterborne acetone (C) or TCDD (T) at a concentration of 15 nM TCDD. The embryos were then transferred to acetone- and TCDD-free water before termination at the indicated hours post fertilization (hpf). (A) Each lane of the representative Northern blot consists of 20 μ g RNA. The blot was probed sequentially with zfAhR2, rainbow trout CYP1A, and rat β -actin probes; phosphor screen exposure times were 4 days, 2 days, and 12 h respectively. (B) Graphical representation of the independent experiments (mean \pm S.E.M., $n=3$). The fold induction zfAhR2 mRNA abundance after TCDD exposure was calculated at each developmental time point relative to control embryos exposed to acetone. Arrow indicates when TCDD or vehicle were added; ND, not determined.

zfAhR2 has an apparent molecular weight of approximately 123 kDa. In preliminary experiments, zfAhR2/rtARNTb proteins failed to significantly bind DRE sequences derived from the mouse *cyp1a1* promoter (data not shown). One possible explanation is that fish DREs differ from mammalian DREs. The rainbow trout *CYP1A* promoter contains two DRE elements necessary for TCDD regulation [15]. DRE-1, which lies 1350 bp upstream of the transcription site, was used as a fish specific probe to study AhR/ARNT interactions. A TCDD induced

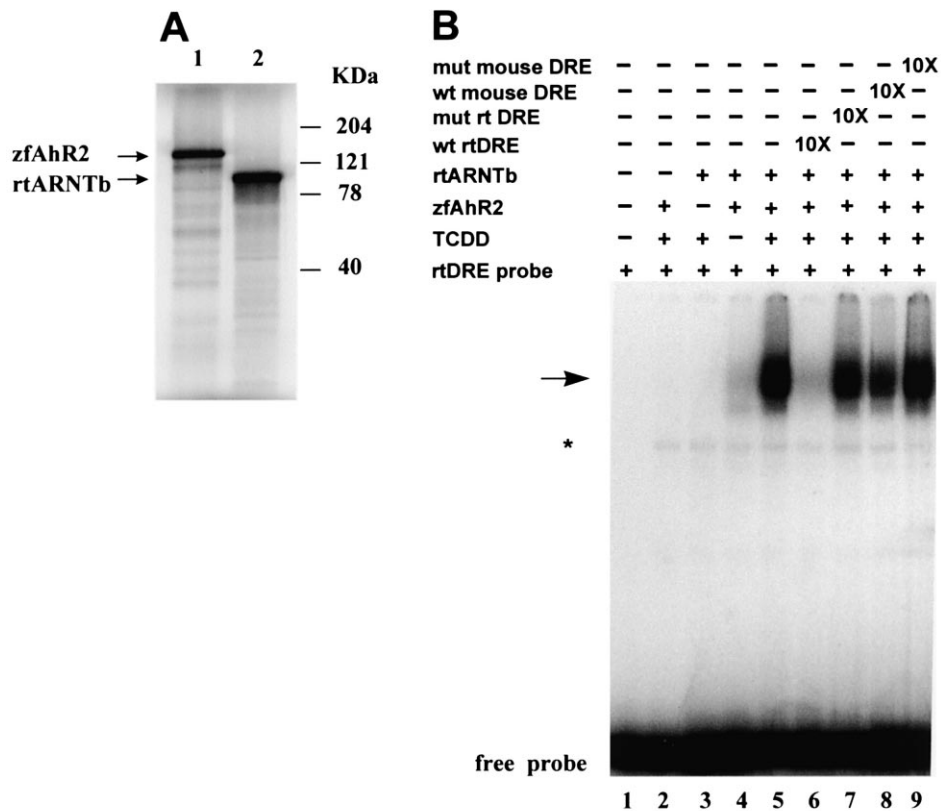


Fig. 7. Gel shift analysis of zfAhR2 and rtARNTb interactions in vitro. (A) [³⁵S]Methionine-labeled in vitro translated zfAhR2 and rtARNTb proteins resolved on an 8% SDS polyacrylamide gel followed by 12 h phosphorimage analysis. Arrows indicate position of the full-length proteins. (B) zfAhR2 and/or rtARNTb proteins were incubated with or without 10 nM TCDD, 10-fold molar excess of the unlabeled competitor oligonucleotides and ³²P-labeled oligonucleotides derived from a dioxin responsive element (DRE) in the rainbow trout *cyp1a* enhancer as indicated. The arrow indicates the position of specific zfAhR2/rtARNTb-DRE complexes. The asterisks indicate the positions of a nonspecific band.

zfAhR2/rtARNTb complex is formed using the rtDRE probe (Fig. 7B, lane 5). A complex is not formed when zfAhR2 or rtARNTb proteins are added separately (Fig. 7, lanes 2 and 3). DNA binding is specific; the complex is competed by a 10-fold molar excess of unlabeled rtDRE (Fig. 7, lane 6), but rtDREs with a single base change in the core sequence fails to compete (Fig. 7, lane 7). The complex is also partially inhibited by the mouse DRE sequence (Fig. 8, lane 8), but not the mouse DRE with a single base substitution (Fig. 7, lane 9) suggesting there is weak affinity for the mouse DRE.

3.4. Zebrafish AhR transactivation activity in COS-7 cells

Since zfAhR2 heterodimerizes with rtARNTb and

binds DRE elements, the ability of these proteins to increase transcription of a luciferase reporter gene under control of a dioxin responsive element was determined. Since COS-7 cells have undetectable levels of endogenous AhR and only low levels of ARNT, they were selected as a null AhR signaling pathway cell line. TCDD responsiveness is reconstituted in COS-7 cells by transfection of AhR and ARNT containing plasmids [47]. zfAhR2 and rtARNTb expression plasmids were transfected separately or together in COS-7 cells with a luciferase reporter under control of the rainbow trout *CYP1A* promoter and DRE containing enhancer. Transfected cells were incubated for 20 h in medium with 0.1% DMSO, or with 10 nM TCDD dissolved in DMSO (Fig. 8). Transient expression with the reporter vector alone, rtARNTb or zfAhR2, in the

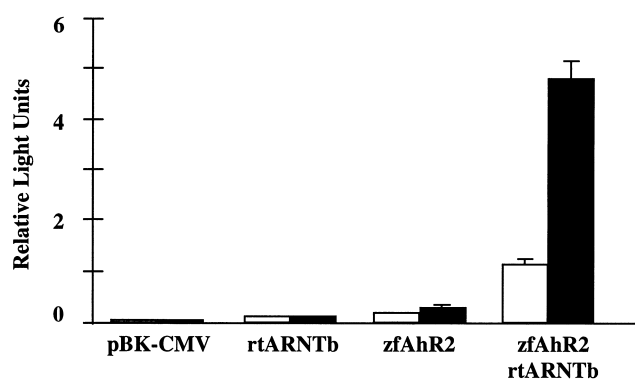


Fig. 8. Transactivation activity of zfAhR2 in COS-7 cells. COS-7 cells were transiently cotransfected with pBK-CMV, rtARNTb, zfAhR2 or zfAhR2 and rtARNTb expression plasmids with prt1A*luc* encoding the firefly luciferase gene under control of the rainbow trout *cyp1a* promoter and enhancer (see Section 2). PRL-TK encoding the *Renilla* luciferase reporter gene was also cotransfected as an internal control. Transfected cells were incubated for 20 h in media with DMSO (□) or with 10 nM TCDD dissolved in DMSO (■) before luciferase assay analysis. *Renilla* luciferase normalized activities are represented in arbitrary units. The results are means of three independent triplicate assays \pm S.E.M.

absence or presence of TCDD, results in low reporter activity. This demonstrates that background activity in this system is low. There is a slight TCDD-induced increase in reporter activity in cells transfected with zfAhR2, suggesting that the zfAhR2 can dimerize and function with the low endogenous mammalian ARNT in this cell line. This is not unexpected since it has been shown that the rtARNTb protein can dimerize and transactivate with mammalian AhR [22]. Reporter activity was always observed when both proteins were cotransfected, and TCDD exposure enhanced the reporter response approximately 4-fold. These results demonstrate that the zfAhR2 is active in mammalian cells and able to activate transcription driven by DREs.

4. Discussion

Previously we have described the signs of TCDD developmental toxicity in zebrafish [26]. However, to take advantage of the genetic methods in zebrafish, it is necessary first to characterize the AhR signaling pathway in this fish species. This study was initiated to characterize the zebrafish AhR. Comparison of

the predicted amino acid sequence of the zebrafish AhR reveals several notable features. The 1027 amino acid zfAhR2 is approximately 200 amino acids longer than the previously described receptors. The mammalian receptors range from 800 to 880 amino acids in length. The rainbow trout AhR α and AhR β cDNAs recently isolated in our laboratory [18] consist of 1058 and 1059 amino acid residues respectively. It is possible that fish AhRs may have extended C-terminal domains, however, the recently published tomcod AhR sequence consist of only 823 amino acids. The zfAhR2 bHLH and PAS domains share high identity to the previously identified AhRs suggesting that the DNA and ligand binding properties of the fish and mammalian AhRs may be similar. zfAhR2 shares 71% identity within the sequences defined as the mouse AhR ligand binding domain (residues 230–397) [48]. It is important to note, however, that single amino acid polymorphism within the mammalian AhR ligand binding domain affects TCDD responsiveness. More specifically, amino acid 375 in the mouse AhR is critical. The mouse AhR^b allele has a 4-fold higher TCDD binding affinity and is at least an order of magnitude more responsive to TCDD than the AhR^d allele. Sequence comparisons reveal there is a Ala³⁷⁵ to Val³⁷⁵ substitution in the AhR^d allele [47,49]. Interestingly, the human AhR contains a Val at this position [47]. zfAhR2 has an alanine at this critical position. Further analysis of the fish ligand binding domain may provide insight into the species differences in structure activity relationships of the mono-*ortho* PCBs. The failure of mono-*ortho* PCBs to elicit toxic responses in fish, for example, may be due to structural constraints of the fish AhR ligand binding domains.

The C-terminal domain of zfAhR2 diverges from the mammalian AhR at position 420, and does not contain a Q-rich domain shown to be important for mammalian AhR transactivation [48]. The mouse AhR has 17 glutamines, organized in stretches, between residues 594 and 640. The corresponding region of zfAhR2 is devoid of stretches of Q, containing a total of only five glutamine residues. The tomcod and rainbow trout receptors are also devoid of Q-rich sequences (Fig. 1). Clearly, the Q-rich sequence is not required for AhR mediated transactivation in fish. Overall, the C-terminal domains of the fish AhRs are dissimilar, with few stretches of con-

served sequences. Further analysis of the zfAhR2 C-terminus will allow elucidation of the functional transactivation domain.

ZfAhR2 mRNA is expressed, induced by TCDD, and functional in early zebrafish development. This is consistent with the time course for TCDD toxicity observed in zebrafish. Reduced caudal blood flow in the trunk 48 hpf is the first observable sign of TCDD toxicity in zebrafish embryos [26]. Northern blot analysis reveals zfAhR2 mRNA is first detectable at 24 hpf (Fig. 3). The zfAhR1 mRNA is also detected at this developmental stage [23]. Basal and TCDD induced AhR mRNA levels increase as the embryo develops. CYP1A mRNA is induced with a similar time course indicating that the AhR is functional in the early embryo, reaching maximal expression levels at 72 hpf. Toxic responses including pericardial edema, craniofacial malformations and yolk sac edema become evident at 96 hpf.

There is little evidence for TCDD directly regulating AhR levels in mammals. A dose dependent increase in TCDD specific binding is observed in the liver of rats chronically exposed to TCDD [50]. It is unknown whether the presumed increased in AhR protein in this study results from a change in mRNA level. TCDD exposure also down regulates AhR mRNA and protein throughout the palatal shelf in mouse embryo during gestation [51]. The liganded AhR is also rapidly depleted in several cell lines and organs without affecting AhR mRNA levels [52,53]. The depletion appears to result from a regulated and selective protease in Hepa 1c1c7 cells [54].

Rainbow trout AhR β and AhR α mRNA levels are elevated in rainbow trout gonadal cells exposed to TCDD [18]. The tomcod AhR message has not been shown to be TCDD regulated, however, TCDD dose-response studies with tomcod have not been completed. One possibility is that rainbow trout and zebrafish AhR promoters may contain DREs conferring TCDD responsiveness. Genomic zebrafish AhR clones have yet to be characterized. Promoter analysis of the human, mouse, and tomcod AhRs did not identify DREs [16,55,56]. It is intriguing to speculate that the sensitivity of fish to TCDD may be related, at least in part, to the induction of AhR. At this time it is not possible to determine if elevated mRNA levels result in elevated zfAhR2 protein since

mammalian AhR specific antibodies fail to recognize zfAhR2 proteins (data not shown).

Initial characterization of zfAhR2 protein indicates a difference in the DRE recognition sequences. Gel shift experiments using in vitro produced zfAhR2 and rainbow trout ARNTb proteins failed to form a significant complex with the murine DRE containing the core TTGCGTG sequence. This was somewhat surprising since extracts from a number of mammalian tissues form complexes with this DRE [57]; however, extracts from rainbow trout hepatic cytosol fail to bind the murine DRE [58]. The rainbow trout ARNTb protein dimerizes with the mammalian AhR to gel shift the murine DRE probe [22]. The observation that zfAhR2/rtARNTb form specific complexes with the rainbow trout DRE (core TAGCGTG) confirm zfAhR2/rtARNTb-DRE interactions suggesting that the AhRs from different vertebrate classes may have slightly different AhR DRE half site recognition sequences. The mammalian AhR half site is TNGC [10] (competitive DNA used here N = T). The rtDRE probe has TAGC as the AhR half site suggesting that an adenosine at the N position in the AhR half site is important for efficient zfAhR2/rtARNTb binding. Further analysis of the fish AhR/ARNT dimer specificity for DRE sequences is required to confirm this observation and to also investigate the possibility that sequences outside of the core contribute to the selective zfAhR2 DNA binding. Taken together, these results indicate that although the murine and zfAhR2 DNA binding domains and DRE core sequences are similar, differences exist limiting cross-species interactions.

Consistent with the DRE gel shift results, a reporter (pGudLuc1.1) consisting of four mouse DREs upstream of a MMTV-LTR viral promoter [59] was not activated by TCDD when zfAhR2 and rtARNTb were coexpressed in COS-7 cells (data not shown). To demonstrate zfAhR2 function, a fish specific DRE reporter was used. TCDD responsive gene promoters have yet to be isolated from zebrafish. Two DRE elements have been identified in the 5' flanking region of the rainbow trout *CYP1A* gene [15]. Transient coexpression of zfAhR2 and rtARNTb in COS-7 cells results in a TCDD responsive increase in transcription driven by the rainbow trout *CYP1A* promoter and enhancer. These results indicate that

zfAhR2 and rtARNTb recognize the rtDRE and are functional in mammalian cells.

Taken together, these findings will allow molecular, biochemical and genetic mechanistic studies on the AhR to be conducted in zebrafish. Functional comparisons of AhRs from different species may reveal the basis for the different potencies of halogenated aromatic hydrocarbons between vertebrate classes, and provide insight into the differences in TCDD potency between fish species.

Acknowledgements

We thank Dr. Richard Pollenz for providing the rainbow trout ARNTb expression plasmid, Dr. Tala Henry for assistance with TCDD exposure of ZF-L cells, and Dorothy Nesbit for excellent technical assistance. This is contribution number 320 of the University of Wisconsin Environmental Toxicology Center. This work was supported by the University of Wisconsin Sea Grant Institute under grants from the National Sea Grant College Program, National Oceanic and Atmospheric Administration, US Department of Commerce and the National Institutes of Health. Sea Grant Project numbers R/MW-58 and R/M-58 (R.E.P. and W.H.) and the National Institute of Environmental Health Sciences Individual National Research Award number F32 ES05786-01 (R.L.T.).

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