

Transactivation Activity of Human, Zebrafish, and Rainbow Trout Aryl Hydrocarbon Receptors Expressed in COS-7 Cells: Greater Insight into Species Differences in Toxic Potency of Polychlorinated Dibenzo-*p*-dioxin, Dibenzofuran, and Biphenyl Congeners

Christian C. Abnet, Robert L. Tanguay, Warren Heideman, and Richard E. Peterson¹

School of Pharmacy and Environmental Toxicology Center, University of Wisconsin, Madison, Wisconsin 53706

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Transactivation assays were used to compare the potency and efficacy of polychlorinated dibenzo-*p*-dioxin (PCDD), dibenzofuran (PCDF), and biphenyl (PCB) congeners in activating aryl hydrocarbon receptors (AhRs) from rainbow trout (rtAhR2 α and rtAhR2 β), zebrafish (zfAhR2), and human (huAhR), respectively. All AhRs were expressed with their species-specific AhR nuclear translocator (ARNT) in COS-7 cells. Transactivation activity was determined for two PCDD, two PCDF, and seven PCB congeners with each of the four AhR/ARNT pairs using prt1A*luc*, a luciferase reporter driven by two dioxin-responsive enhancer elements (DREs) from the rainbow trout *cyp1A* gene. Maximal-fold induction, EC50, and relative potency values were calculated for congeners that exhibited dose-related activity in the assay. Of the four AhR/ARNT pairs tested with PCDD, PCDF, and non-*ortho* PCB congeners, three exhibited high activity (rainbow trout AhR2 α , zebrafish AhR2, and human AhR), while rainbow trout AhR2 β had very weak or no activity. Comparisons between these AhRs showed that while mono-*ortho* PCBs were able to activate the human AhR, they were generally ineffective in activating rainbow trout and zebrafish AhR2s. This supports the hypothesis that structural differences between mammalian and fish AhRs may account for differences in relative potencies of the mono-*ortho* PCBs between mammals and fish. Another important finding was a significant difference in transactivation activity between the two rainbow trout AhR2 isoforms despite the fact that they are 95% identical at the amino acid level. For all PCDD, PCDF, and PCB agonists tested, rainbow trout AhR2 α was significantly more active than AhR2 β . However, rainbow trout AhR2 β is active as a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-activated transcription factor, with enhancer elements from the mouse *cyp1A* gene.

¹ To whom correspondence should be addressed at School of Pharmacy, 425 N. Charter Street, University of Wisconsin, Madison, WI 53706. Fax: (608) 265-3316; E-mail: repeterson@pharmacy.wisc.edu.

This suggests that AhR2 β may have evolved to serve a different physiological function than AhR2 α in salmonid fish species. © 1999

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Key Words: PCDDs; AhR agonist; zebrafish; rainbow trout; TCDD; dioxin.

The toxicity of a wide range of bioaccumulative compounds, including polychlorinated dibenzo-*p*-dioxins (PCDDs),² dibenzofurans (PCDFs), and biphenyls (PCBs) is mediated by a conserved signaling pathway. These compounds bind to and activate the aryl hydrocarbon receptor (AhR), which in turn mediates transcriptional responses to the compounds. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent activator of the AhR and is the prototype compound to which other PCDD, PCDF, and PCB congeners are compared. Despite the strong conservation of this pathway, wide differences in responsiveness to AhR agonists occur between different vertebrate classes and species of the same class (Peterson *et al.*, 1993; Walker and Peterson, 1994). Also, different strains of the same species, such as the C57BL/6 and DBA/2 mouse strains or the Sprague-Dawley and Han Wistar rat strains, show differences in responsiveness to AhR agonists (Poland *et al.*, 1994; Unkila *et al.*, 1994). The basis for these cross-species and cross-strain differences in toxicity of TCDD is complex and is only beginning to be understood.

Differences in sensitivity between classes of vertebrates are illustrated by comparing structure activity relationships for relative potencies (REPs) of AhR agonists between fish and mammals (Zabel *et al.*, 1995; Cook *et al.*, 1997). While, in general, REPs for PCDDs and PCDFs are similar in mammals

² Abbreviations used: rt, rainbow trout; zf, zebrafish; hu, human; AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PCDD, polychlorinated dibenzo-*p*-dioxin; PCDF, polychlorinated dibenzofuran; PCB, polychlorinated biphenyl; ARNT, aryl hydrocarbon receptor nuclear translocator; bHLH, basic helix-loop-helix; CYP1A, cytochrome P4501A mRNA; *cyp1A*, cytochrome P4501A gene; DRE, dioxin response element; RTG-2, rainbow trout gonadal cells; DMSO, dimethyl sulfoxide; REP, relative potency.

and fish, REPs for PCBs tend to be lower in fish than mammals. This is particularly true of mono-*ortho* PCBs, which are completely inactive in producing early life stage toxicity in rainbow trout (Zabel *et al.*, 1995) yet can elicit signs of TCDD toxicity in mammals (Van den Berg *et al.*, 1998). A similar result was found in an assay that measured the potency of these compounds in up regulating *cyp1A* mRNA in rainbow trout gonadal cells (Zabel *et al.*, 1996). Mono-*ortho* PCBs were found to have either very weak or no activity in this assay.

The mechanism by which the AhR mediates alterations in gene transcription has been studied extensively in mammals (Whitlock *et al.*, 1996). AhR exists as a multi-protein complex, which includes an hsp90 dimer and AhR interactive protein, in the cytoplasm in its quiescent state. Upon activation by AhR ligand, the receptor translocates to the nucleus and dimerizes with ARNT, forming a new complex that binds to specific transcriptional regulatory sequences called dioxin-response elements (DREs) (Safe and Krishnan, 1995). The mechanism whereby this activated complex up regulates the expression of genes, such as the xenobiotic metabolizing enzyme gene *cyp1A1*, involves alterations in chromatin structure and interactions with the basal transcription machinery (Okino and Whitlock, 1995; Rowlands *et al.*, 1996). These interactions increase the rate at which these genes are transcribed, and a concomitant increase in abundance of the particular protein occurs. The AhR/ARNT signaling pathway is presumed to operate in a similar manner in fish (Walker and Peterson, 1994).

The AhR signaling pathway has the potential for regulation at a number of different levels; however, the differences in sensitivity to AhR agonists among different rodent strains appear to involve differences within the AhR itself. Differences in susceptibility to TCDD between strains are associated with differences in the relative affinity of agonists for the AhR, as congenic mice, which differ only at the *AhR* locus retain differences in sensitivity to PCDD, PCDF, and PCB AhR agonists (Poland *et al.*, 1994). The difference between fish and mammals in relative potencies of the PCBs, particularly the mono-*ortho* PCBs, might be attributable to structural differences in the AhR proteins. AhRs, ARNTs, and dioxin-response elements (DREs) in the *cyp1A1* gene have been cloned from fish and mammals (Burbach *et al.*, 1992; Dolwick *et al.*, 1993; Berndtson and Chen, 1994; Pollenz *et al.*, 1996; Tanguay *et al.*, 1999a,b; Abnet *et al.*, 1999). We now know that fish and mammalian AhRs have significant structural differences both within the highly divergent C-terminal ends, as well as within the more conserved PAS and basic helix-loop-helix (bHLH) domains. Furthermore, alignment of several AhR gene fragments demonstrates that fish have at least two different types of AhR, designated as AhR1 and AhR2 (Hahn *et al.*, 1997).³ Recently, we identified two type 2 AhR isoforms in rainbow

trout, which we designated rtAhR2 α and rtAhR2 β (Abnet *et al.*, 1999). With these components of the AhR signaling pathway available, it is now possible to compare activities of the rainbow trout, zebrafish, and human AhRs in response to individual PCDD, PCDF, and PCB congeners that differ widely in AhR binding affinity and toxic potency.

The purpose of the present study was to compare the ability of rainbow trout, zebrafish, and human AhR/ARNT pairs expressed in COS-7 cells to induce luciferase activity in response to graded concentrations of individual PCDD, PCDF, and PCB congeners added to the media. The transactivation assay uses a luciferase reporter vector driven by a promoter fragment from the rainbow trout *cyp1A* gene to measure transactivation activity. This allows us to compare activity of the fish and mammal AhR/ARNT dimers to AhR agonists in a setting where the cellular background is constant, and only the receptor molecules vary. By using this approach, it was our objective to compare the ability of AhRs of mammalian and fish origin to induce transcription in response to PCDD, PCDF, or PCB congeners.

It is known that clear differences exist in the ability of mono-*ortho* PCBs to induce toxicity between fish and mammals (Walker and Peterson, 1991; Zabel *et al.*, 1995; Cook *et al.*, 1997; Van den Berg *et al.*, 1998). Rainbow trout are essentially nonresponsive to mono-*ortho* PCB-induced toxicity compared to mammals (Walker and Peterson, 1991, 1994; Zabel *et al.*, 1995). One hypothesis to explain this difference for rainbow trout is that it is related to structural differences between the rainbow trout AhRs and the mammalian AhR forms of the receptor. As an initial step towards testing this hypothesis, the ability of mono-*ortho* PCBs to induce luciferase activity with three different fish AhR2s (rtAhR2 α , rtAhR2, and zfAhR2) was compared to a mammalian AhR (huAhR). The present study will show that mono-*ortho* PCBs were essentially ineffective in activating fish AhR2s, but were able to activate the human AhR.

METHODS

Cell culture. COS-7 cells obtained from ATCC (Bethesda, MD) were cultured at 37°C in an atmosphere of 95% air and 5% CO₂. Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum served as a growth media. Cells were passed every 3 days in tissue-culture-treated polystyrene and split 1:6. For experiments with 24-well plates, cells were plated at 60,000 cells/well (approximately 70% confluence) the day before transfections.

Transfections. COS-7 cells were transfected with plasmids described below using SuperFect δ (Qiagen, Chatsworth, CA), essentially as described by the manufacturer, 24 h after seeding. Briefly, for each well of a 24-well plate a total of approximately 1 μ g plasmid DNA (475 ng AhR, 475 ng ARNT, 20 ng prt1Aluc, and 3 ng pRL-TK) was diluted to 60 μ l in serum-free media. SuperFect δ , 3 μ l, was added to each tube, vortexed, and incubated 10 min. During incubation medium was aspirated and cells were washed one time with 1 \times PBS. Serum-containing medium, 350 μ l, was added to the transfection solution before addition to cells and was incubated for 2.5 h. Serum-containing

³ The AhR1 and AhR2 nomenclature used to name AhRs is based on (Hahn *et al.*, 1997).

medium was then added so that the total volume of medium in each well was 1 ml.

Polychlorinated aromatic hydrocarbons and cell exposures. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) (>99% purity) was purchased from Chem-syn (Lenexa, KS) and 2,3,7,8-tetrachlorodibenzofuran (TCDF) and 2,3,4,7,8-pentachlorodibenzofuran (PCDF) (>99% purity) were provided by Dr. Linda Birnbaum (U.S. Environmental Protection Agency, Research Triangle Park, NC). 1,2,3,7,8-Pentachlorodibenzo-*p*-dioxin (PCDD); 3,3',4,4'-tetrachlorobiphenyl (PCB 77); 2,3,3',4,4'-pentachlorobiphenyl (PCB 105); 2,3',4,4',5-pentachlorobiphenyl (PCB 118); 3,3',4,4',5-pentachlorobiphenyl (PCB 126); 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153); and 2,3,3',4,4',5-hexachlorobiphenyl (PCB 156) (95–99% purity) were obtained from UltraScientific (North Kingstown, RI). For studies, an individual PCDD, PCDF, or PCB congener dissolved in *p*-dioxane was dried under N₂, resuspended in DMSO at the highest concentration desired, and serially diluted. Twenty-four hours after transfection, cells were exposed to either DMSO (control) or graded concentrations of an individual PCDD, PCDF, or PCB congener in DMSO. Each congener was added directly to existing media with a final DMSO concentration of 0.1%, except for higher concentrations of certain congeners, used to confirm that maximal luciferase induction had occurred, where the final DMSO concentration was 0.5%.

AhR and ARNT expression vectors. All AhR and ARNT expression vectors were constructed in pBK-CMV (Stratagene, La Jolla, CA) except rtARNTb that was in pRc/CMV (Invitrogen, Carlsbad, CA). The rainbow trout AhR expression vectors were constructed as follows. rtAhR2 α (GenBank Accession No. AF065137) and rtAhR2 β (GenBank Accession No. AF065138) were used as templates for PCR reactions to produce expression vectors that encoded full-length AhRs but eliminated the UTR sequences (Abnet *et al.*, 1999). The PCR products were blunted with DNA polymerase I Klenow fragment and ligated into pBK-CMV that had been blunt-digested with *Sma*I to produce pFFLrtAhR2 α and pFFLrtAhR2 β . The rtARNTb (GenBank Accession No. U73841) expression vector was supplied by Dr. Richard Pollenz (Medical University of South Carolina, Charleston, SC) (Pollenz *et al.*, 1996). zfAhR2 (GenBank Accession No. AF063446) was constructed by digesting the full-length AhR (Tanguay *et al.*, 1999a) with *Dra*I to release the coding sequences, and this fragment was ligated into pBluescriptII SK (Stratagene) that had been digested with *Eco*RV. This construct was digested with *Apa*I and *Bam*HI and was directionally cloned into pBK-CMV that had been digested with the same enzymes. A construct containing the human AhR coding sequence (GenBank Accession No. L19872), pSportAhR2, was obtained from Dr. Chris Bradfield (University of Wisconsin, Madison, WI). Although this construct is named pSportAhR2, this designation does not reflect the AhR classification system of Hahn (Hahn *et al.*, 1997). This construct was digested with *Pst*I and *Apa*I to release the coding sequence and ligated into pBK-CMV that had been digested with the same enzymes. The full-length zebrafish ARNT construct was obtained by 5' RT-PCR from adult zebrafish RNA, and subcloned into the *Nor*I site of pBK-CMV (Tanguay *et al.*, 1999b). To construct a human ARNT expression vector, a RT-PCR strategy was used. Total RNA isolated from LNCaP (human prostate) cells was used as a template for RT-PCR with primers designed to amplify the coding sequence of human ARNT (GenBank Accession No. M69238). The 2376-bp PCR product was TA-cloned into pGemT-Easy (Promega, Madison, WI). This plasmid was digested with *Nor*I and ligated into pBK-CMV that had been previously digested with the same enzyme. Orientation of the insert was verified by restriction digest analysis and sequencing.

Reporter vector. The prt1*Aluc* reporter vector was constructed in our laboratory as follows. PCR with rainbow trout genomic DNA was performed using PCR primers (forward = 5' AGGTTGGTTGAGTGAGATG 3', reverse = 5' TGCAGGGAGATCGAAGAAG 3') designed to amplify a 1540-bp portion of the 5' flanking region of the rainbow trout *cyp1A* gene promoter from base pair 139 to 1678 (GenBank Accession No. S69277). This region includes 2 DREs and the transcriptional start site (base pair 1594). The PCR product was TA-cloned into pGemT-Easy. The resulting plasmid was

digested in its multiple cloning region with *Sac*I and *Nco*I and ligated into pGL3-basic that had been digested with the same enzymes. This plasmid was named prt1*Aluc* and provided a TCDD-responsive, firefly luciferase reporter vector under control of the endogenous rainbow trout *cyp1A* gene promoter and enhancer. The integrity and orientation of the plasmid's promoter/enhancer sequence were confirmed by sequencing.

Reporter gene assays. Twenty-four hours after exposure to 0.1% or 0.5% DMSO (control) or to graded concentrations of a PCDD, PCDF, or PCB congener dissolved in the same volume of 0.1% or 0.5% DMSO reporter gene assays were performed. A Dual Luciferase Assay (Promega) was used to determine firefly (AhR agonist-dependent) and *Renilla* (transfection control) luciferase activity for each well. Media was removed by vacuum aspiration, each well was washed with 1 \times PBS, and 100 μ l passive lysis buffer was added. Plates were incubated 20 min at room temperature on an orbital shaker. Cell lysis was confirmed microscopically and a 10- μ l aliquot was transferred to a 96-well luminometer plate. Luminescence assays were completed using a Dynatech Laboratories ML-2250 luminometer (Chantilly, VA). Assays for luciferase activity were conducted; 50 μ l of Luciferase assay buffer II was injected into each well and incubated for 2 s, and the resulting luminescence was integrated over the next 10 s. After reading each plate, the assay buffer was changed to Stop & Glo, and identical assay conditions were used to measure *Renilla* activity in the same wells. Because the *Renilla* luciferase control vector is susceptible to induction by *trans* effects when a second reporter construct with a strong promoter is activated (Farr and Roman, 1992), the amount of control plasmid was reduced to 3 ng pRL-TK/ μ g DNA transfected in each well to avoid this problem.

In vitro transcription/translation. *In vitro* transcription/translation reactions were completed using a TNT kit from Promega essentially as directed by the manufacturer. For each reaction 1 μ g plasmid DNA was used in a 50 μ l total reaction volume. Amino acid mixture lacking methionine was added to each reaction, and reactions were supplemented with translational grade ³⁵S-methionine (New England Nuclear, Boston, MA). Ten microliters of each reaction was diluted with an equal volume of 2 \times loading dye and separated on 10% SDS-PAGE. Gels were fixed, dried onto filter paper, and exposed to phosphorimager screens for 4 h.

Statistical analysis. A normalized luciferase activity number was determined for each individual transactivation assay by dividing the firefly luciferase activity by *Renilla* luciferase activity. The fold induction (mean \pm SE) over DMSO control was plotted versus PCDD, PCDF, and PCB congener concentration on a log scale. The maximal-fold induction of luciferase activity caused by graded concentrations of each congener was determined. For those congeners that induced luciferase activity at least 4.0-fold, the EC50 of the congener was determined using nonlinear regression in the Statistica software package (StatSoft, Tulsa, OK). The potency of each congener for inducing luciferase activity relative to TCDD was determined by dividing EC50 of TCDD by EC50 of the congener. The relative potencies (REPs) of PCDD, PCDF, and PCB congeners for inducing luciferase activity with rainbow trout AhR2 α in COS-7 cells was further evaluated by linear regression using the Statistica software package with their REPs both for inducing CYP1A activity in a rainbow trout gonadal cell line (RTG-2; Zabel *et al.*, 1996) and for causing early life stage mortality in rainbow trout (Zabel *et al.*, 1995).

RESULTS

The structure activity relationship for *in vivo* toxicity of polychlorinated aromatic hydrocarbons varies between fish and mammals. One possible explanation for this difference is that the AhR/ARNT proteins from these vertebrate classes are intrinsically different in their recognition of agonists. To test this hypothesis, we compared the ability of different AhR/ARNT pairs from rainbow trout (rt), zebrafish (zf), and human

(hu) to induce reporter expression in a transactivation assay in response to individual PCDD, PCDF, and PCB congeners. This assay system uses transfected COS-7 monkey kidney cells to express the different AhR/ARNT proteins, and a luciferase reporter vector driven by a promoter fragment from the rainbow trout *cyp1A* gene to measure transactivation activity. This allowed us to compare activity of the different AhR/ARNT dimers to AhR agonists in a setting where the cellular background remained constant, and only the receptor molecules varied.

In these experiments, we compared the activity of two AhR isoforms from rainbow trout (rtAhR2 α and rtAhR2 β) to that of a human AhR (huAhR) and to zebrafish AhR (zfAhR2). The AhR cDNAs were transfected together with ARNT cDNAs from the same species. In the case of rainbow trout, the same form of ARNT (rtARNTb) was paired with both the α and β forms of the AhR. Transcription of the AhR and ARNT constructs was verified by Northern blot (results not shown). COS-7 cells do not express endogenous AhR, but do express a small amount of endogenous ARNT protein (Ema *et al.*, 1994). The COS-7 cells were transfected with the constructs, exposed to 0.1% DMSO (control) or to graded concentrations of PCDD, PCDF, or PCB AhR agonists dissolved in 0.1% DMSO, and assayed for luciferase activity as a measure of transactivation. The transfection efficiency in each assay well was monitored by inclusion of a control construct, pRL-TK, expressing *Renilla* luciferase activity in all transfections.

Since these experiments involve expression of proteins from species that inhabit very different environments, it was impossible to assay their activity at one set of conditions that resembled the native environments of all of the proteins. For example, the different proteins normally encounter very different temperatures. Trout are cold-water fish, zebrafish inhabit room temperature water, and the human receptor is normally found at 37°C. Another concern was that in the COS cell transfection system, we cannot determine the relative level of expression of the different proteins because no one antibody recognizes all of the proteins. As one measure of relative translatability and stability in a heterologous system, we translated all four forms of the AhR, and the three ARNT proteins *in vitro*, using a reticulocyte lysate system (Fig. 1A). This shows that each of the protein was made at roughly equal levels, and remained stable at 30°C. In addition, we have used these translated proteins at a wide range of temperatures in gel shift assays with similar results, and all of the receptor pairs were able to produce transactivation activity in the COS cell assay at 37°C. We therefore conclude that it is reasonable to expect that these different proteins were expressed at roughly similar levels in the transfection system.

We first tested the ability of the different AhR/ARNT combinations to respond to graded concentrations of TCDD (Fig. 1B). In this experiment, the human receptor (huAhR) produced a response with both a higher maximal effect and lower EC₅₀

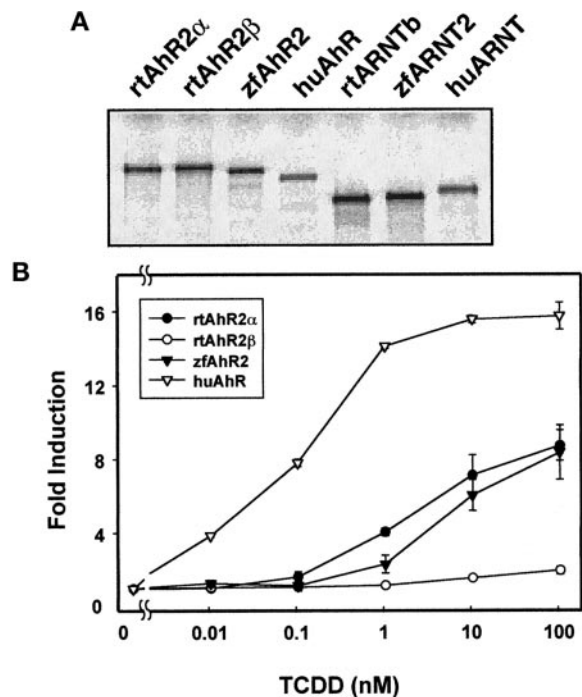


FIG. 1. (A) *In vitro* translation demonstrates that AhR and ARNT clones used in this study produce translation products at similar levels in a heterologous system. Equal quantities of DNA constructs encoding the proteins indicated were used to produce *in vitro* translated proteins using rabbit reticulocyte lysate as described in the methods. (B) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) dose-response curves for induction of luciferase activity from a firefly luciferase reporter gene, under control of the endogenous rainbow trout *cyp1A* gene promoter and DREs, with AhR/ARNT proteins from rainbow trout (rtAhR2 α /rtARNTb or rtAhR2 β /rtARNTb), zebrafish (zfAhR2/zfARNT), or human (huAhR/huARNT). COS-7 cells used were transfected with a single set of AhR/ARNT expression constructs, pRL-TK, and prt1A*luc*. Each point and associated vertical line on the TCDD dose-response curves represents fold induction of luciferase activity above the 0.1% DMSO control (mean \pm SE, $n = 3$ replicates/dose).

than the fish receptors. For this and other AhR agonists, maximal-fold induction and EC₅₀ are reported for each of the four AhR/ARNT proteins in Table 1. An EC₅₀ is only calculated for a congener if the maximal-fold induction with a particular AhR is 4.0-fold or greater. Of the two rainbow trout receptors, rtAhR2 α showed almost a 9-fold increase in transactivation, with an EC₅₀ for TCDD of approximately 2 nM, while rtAhR2 β produced little if any response. The response of the zebrafish receptor (zfAhR2) to TCDD was similar to rtAhR2 α .

This pattern, in which rtAhR2 β produces little or no response, was also seen in this assay with other AhR agonists. Dose-response curves for the induction of luciferase activity from prt1A*luc* by 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (PCDD) in cells transfected with different AhR/ARNT pairs are shown in Fig. 2. Maximal induction by PCDD depended on the AhR transfected. The greatest induction occurred in cells transfected with rtAhR2 α , in which PCDD induced luciferase activity

TABLE 1

Summary of Dose-Related Effects of Individual PCDD, PCDF, and PCB Congeners on AhR-Dependent Luciferase Induction from a Firefly Luciferase Reporter Gene, under Control of the Endogenous Rainbow Trout *cyp1A* Gene Promoter and DREs, with AhR/ARNT Proteins from Human, Rainbow Trout, and Zebrafish

Class	Congener	Maximal-fold induction ^{a,b}				EC50 (nM) ^b			
		huAhR	rtAhR2 α	rtAhR2 β	zfAhR2	huAhR	rtAhR2 α	rtAhR2 β	zfAhR2
PCDDs	2,3,7,8 TCDD	15.4 ± 0.7	8.7 ± 0.8	2.0 ± 0.2	8.4 ± 1.5	0.15 ± 0.03	2.0 ± 1.6	*	4.9 ± 5.4
	1,2,3,7,8 PCDD	16.0 ± 1.0	32.4 ± 0.5	4.3 ± 0.4	9.8 ± 0.6	1.4 ± 0.3	1.6 ± 0.6	2.0 ± 0.2	1.5 ± 0.2
PCDFs	2,3,7,8 TCDF	16.3 ± 1.7	10.7 ± 1.7	3.0 ± 0.8	15.2 ± 0.9	3.5 ± 3.1	3.7 ± 0.4	*	3.0 ± 6.4
	2,3,4,7,8 PCDF	15.7 ± 1.3	5.3 ± 0.9	1.4 ± 0.1	13.5 ± 0.5	16.0 ± 3.5	10.2 ± 1.7	*	13.1 ± 2.3
Non-ortho PCBs	PCB 126	16.0 ± 2.5	5.8 ± 0.4	3.4 ± 0.2	3.8 ± 0.1	25.0 ± 8.3	11.2 ± 4.8	*	*
	PCB 77	16.0 ± 0.7	5.8 ± 0.6	—	—	1306 ± 298	203 ± 195	—	—
Mono-ortho PCBs	PCB 156	5.8 ± 0.7	3.8 ± 0.6	—	—	6998 ± 881	*	—	—
	PCB 105	2.8	—	—	—	*	—	—	—
	PCB 118	2.8	—	—	—	*	—	—	—
Di-ortho PCB	PCB 153	—	—	—	—	—	—	—	—

Note. *, EC50 not calculated for dose-response curve with less than 4-fold maximal induction. —, inactive in assay.

^a Fold induction was determined by dividing activities at the maximally effective dose ($n = 3$) by mean activity of the 0.1% DMSO-treated control.

^b Numbers indicate means ± SE; $n = 3$.

32-fold. In this experiment and others, for some AhR/ARNT pairs with certain congeners, maximal induction was reached at only the highest possible concentration that could be used with 0.1% DMSO as vehicle. In these cases a plateau at the top of the dose-response curve was not evident. In order to assign EC50 values to such congeners, it was necessary to confirm

that maximal induction had indeed been reached at the highest concentration tested. To do so higher concentrations were tested to confirm that maximal induction had been reached. However, as these higher congener concentrations required a higher vehicle concentration (0.5% DMSO) the points are not included in figures. The asterisk at the top of the PCDD dose-response curve with rtAhR2 α (Fig. 2) signifies maximal induction, as higher PCDD concentrations failed to increase luciferase activity further. Compared to rtAhR2 α , PCDD induced low transactivation activity with rtAhR2 β and intermediate activity with the zebrafish and human AhRs.

Dose-response curves for two polychlorinated dibenzofuran congeners, 2,3,7,8-tetrachlorodibenzofuran (TCDF) and 2,3,4,7,8-pentachlorodibenzofuran (PCDF), with each of the four AhR/ARNT pairs, are presented in Fig. 3. As before, the asterisk denotes verified maximal induction values in curves that do not show a plateau. TCDF was quite potent in inducing luciferase activity with the huAhR, zfAhR2, and rtAhR2 α , while the β isoform of the rainbow trout receptor had only very weak activity at the highest dose. Essentially the same pattern of results was observed when PCDF was tested with the four AhR/ARNT pairs.

Two non-ortho PCBs, 3,3',4,4',5-pentachlorobiphenyl (PCB 126) and 3,3',4,4'-tetrachlorobiphenyl (PCB 77), were tested with each of the four AhR/ARNT pairs (Fig. 4). PCB 126 and PCB 77 produced a significant induction with huAhR and rtAhR2 α , but not with either the zfAhR2 or rtAhR2 β . For both PCB congeners the magnitude of the response in cells expressing rtAhR2 α was less than in those expressing huAhR.

A single di-ortho PCB and three mono-ortho PCBs were also tested in the transactivation assay (Fig. 5, top panel). All

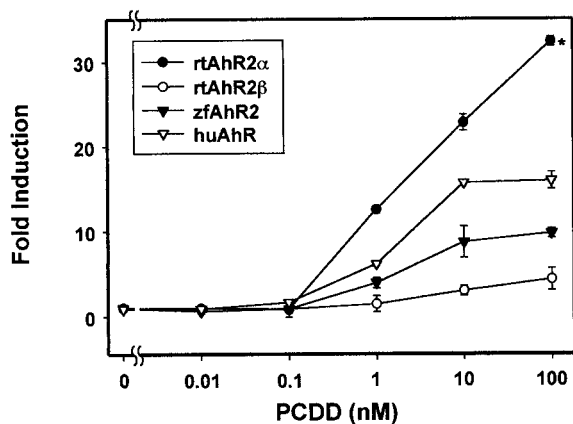


FIG. 2. 1,2,3,7,8-Pentachlorodibenzo-*p*-dioxin (PCDD) dose-response curves for induction of luciferase activity from a firefly luciferase reporter gene, under control of the endogenous rainbow trout *cyp1A* gene promoter and DREs with AhR/ARNT proteins from rainbow trout (rtAhR2 α /rtARNT α or rtAhR2 β /rtARNT β), zebrafish (zfAhR2/zfARNT), or human (huAhR/huARNT). COS-7 cells used were transfected with a single set of AhR/ARNT expression constructs, pRL-TK, and prt1*Aluc*. Each point and associated vertical line on PCDD dose-response curves represents the fold induction of luciferase activity above the 0.1% DMSO control value (mean ± SE, $n = 3$ replicates/dose). Asterisk indicates verified maximal-fold induction (i.e., fold induction over a 0.5% DMSO control at 500 nM PCDD with rtAhR2 α = 30.8-fold induction).

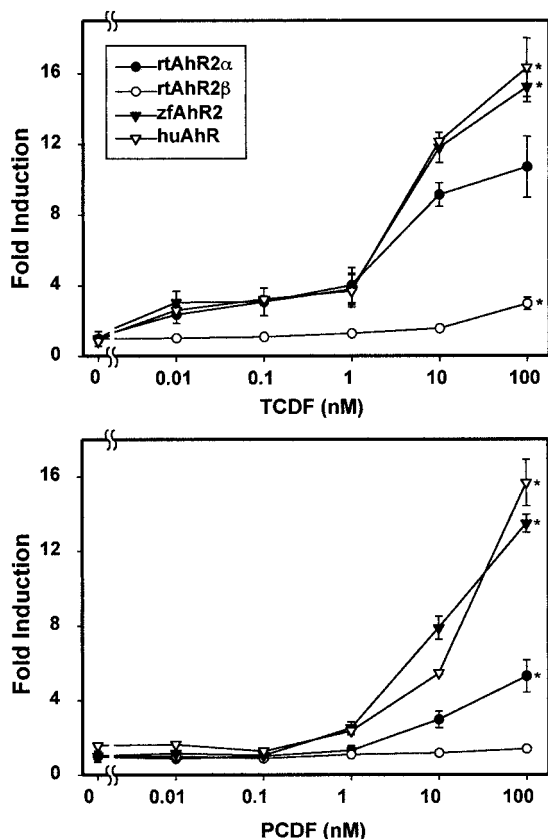


FIG. 3. 2,3,7,8-Tetrachlorodibenzofuran (TCDF, top panel) and 2,3,4,7,8-pentachlorodibenzofuran (PCDF, bottom panel) dose-response curves for induction of luciferase activity. All other conditions were the same as described in the Fig. 2 legend.

of these congeners were initially tested at a single, high concentration. The di-*ortho* compound, PCB 153, failed to induce luciferase activity with any of the four AhRs. However, all of the mono-*ortho* PCBs (PCB 118, 105, and 156) induced luciferase activity with the huAhR. For the fish receptors the response to mono-*ortho* PCBs depended on the congener tested. PCB 118 and 105 failed to induce luciferase activity with all three fish receptors. On the other hand, PCB 156 caused a significant induction when tested with rtAhR2 α , but not with rtAhR2 β or zfAhR2. Because of this result, PCB 156 dose-response curves were generated for the two AhRs activated by this congener (Fig. 5, bottom panel). Maximal induction caused by PCB 156 with rtAhR2 α was weak and less than that for the huAhR. The EC₅₀ of PCB156 with rtAhR2 α was approximately four times higher than with the huAhR.

Relative potencies (REPs) of PCDD, PCDF, and PCB congeners for eliciting each of three distinct responses mediated by rainbow trout AhRs are shown in Table 2. REPs are defined as EC₅₀ (or LC₅₀) of TCDD divided by EC₅₀ (or LC₅₀) of the congener. The rainbow trout AhR-mediated responses for which REPs were determined were: (1) rtAhR2 α -mediated induction of luciferase (*Luc*) activity from a reporter controlled

by the endogenous rainbow trout *cyp1A* gene promoter and enhancer in COS-7 cells (Abnet *et al.*, 1999); (2) induction of CYP1A mRNA abundance in RTG-2 cells, a rainbow trout gonadal cell line (Zabel *et al.*, 1996); and (3) early life stage mortality in rainbow trout exposed as embryos to graded concentrations of individual PCDD, PCDF, and PCB congeners (Zabel *et al.*, 1995). Inspection of the REPs in Table 2 for luciferase induction in the COS-7 cell transactivation assay shows that they are more similar to REPs based on CYP1A mRNA induction than REPs based on rainbow trout early life stage mortality. The lower REP for TCDF, based on early life stage mortality in rainbow trout, may be caused by greater metabolism and elimination of TCDF from trout (Zabel *et al.*, 1995) than from COS-7 and RTG-2 cells. Overall, the REPs for these three endpoints mediated by rainbow trout AhRs are similar to one another.

This is further illustrated in Fig. 6 by the highly significant correlation between REPs for luciferase induction in COS-7 cells and REPs for CYP1A induction in RTG-2 cells ($R^2 = 0.98$, $p < 0.01$, top panel). The correlation between REPs for luciferase induction and REPs for early life stage mortality in

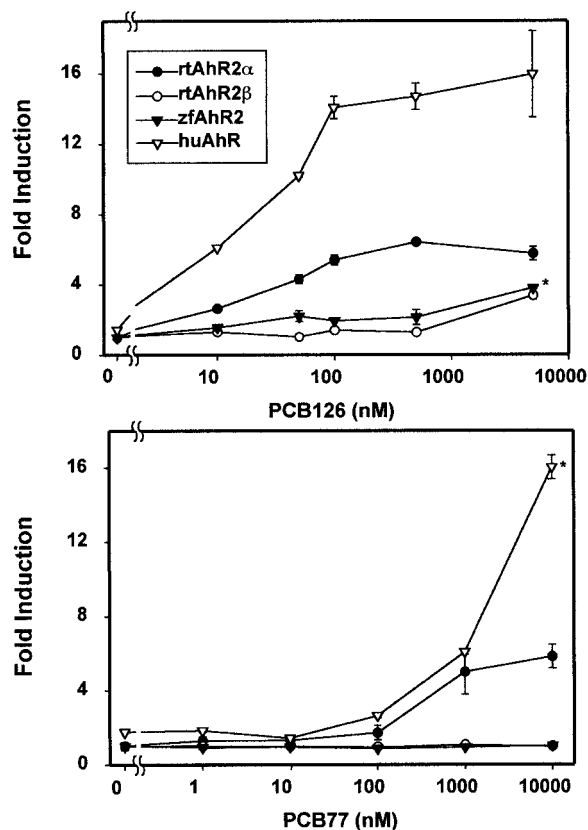


FIG. 4. 3,3',4,4',5-Pentachlorobiphenyl (PCB 126, top panel) and 3,3',4,4'-tetrachlorobiphenyl (PCB 77, bottom panel) dose-response curves for induction of luciferase activity. All other conditions were the same as described in the Fig. 2 legend.

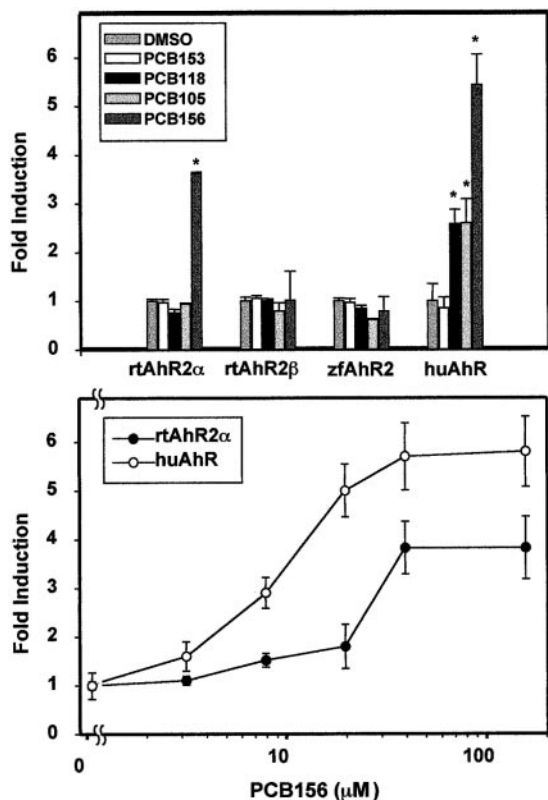


FIG. 5. Effect of mono-ortho and di-ortho PCB congeners on AhR-dependent transactivation with AhRs from fish and human. Top: Effects of a single, high dose of a di-ortho PCB congener (PCB 153) and three mono-ortho PCB congeners (PCB 118, 105, and 156) on induction of luciferase activity from a firefly luciferase reporter gene, under control of the endogenous rainbow trout *cyp1A* gene promoter and DREs, with AhR/ARNT proteins from rainbow trout (rtAhR2 α /rtARNTb or rtAhR2 β /rtARNTb), zebrafish (zfAhR2/zfARNT), or human (huAhR/huARNT). COS-7 cells were transfected with a single set of AhR/ARNT expression constructs, pRL-TK, and prt1Aluc. Concentrations of PCB congeners used for experiments shown in the top panel were PCB 153 (2,2',4,4',5,5'-hexachlorobiphenyl, 38.9 μ M), PCB 118 (2,3',4,4',5-pentachlorobiphenyl, 20.6 μ M), PCB 105 (2,3,3',4,4'-pentachlorobiphenyl, 23.9 μ M), and PCB 156 (2,3,3',4,4',5-hexachlorobiphenyl, 157.6 μ M). Height of each bar and associated vertical line is fold induction over 0.5% DMSO control (mean \pm SE, $n = 3$ replicates/dose). *Significantly higher induction than control at $p < 0.05$. Bottom: PCB 156 dose-response curves for luciferase induction using the same transactivation assay but with only two pairs of AhR/ARNT proteins, rtAhR2 α /rtARNTb and huAhR/huARNT. Each point and associated vertical line on PCB 156 dose-response curves represent fold induction of luciferase activity above the 0.5% DMSO control (mean \pm SE, $n = 3$ replicates/dose).

rainbow trout is not as strong ($R^2 = 0.69$, $p < 0.05$, bottom panel).

Maximal-fold inductions and EC50s are summarized for all PCDD, PCDF, and PCB congeners tested with each AhR in Table 1. AhRs that showed the greatest maximal-fold induction in response to PCDD, PCDF, and non-ortho PCB congeners were huAhR, rtAhR2 α , and zfAhR2. In contrast, rtAhR2 β consistently exhibited weak activity with these congener

classes. EC50s of PCDD and PCDF congeners with the two fish AhR2s, rtAhR2 α and zfAhR2, were similar. However, the non-ortho PCBs were more efficacious with rtAhR2 α . The mono-ortho PCBs were inactive with both of these fish AhR2s. The EC50s for PCDD and PCDF congeners with the huAhR were similar to their EC50s with fish AhR2s with the notable exception that TCDD appeared to be more potent with the huAhR.

The three mono-ortho PCBs tested, PCB 105, 118, and 156, all activated the human form of the receptor. The only fish AhR activated by a mono-ortho PCB was rtAhR2 α and it responded only weakly to PCB 156 (Table 1). rtAhR2 β and zfAhR2 failed to be activated by the high concentration of PCB 156 that was tested, and none of the three fish AhR2s responded to the other mono-ortho PCBs—PCB 105 and 118. Taken together, these findings support the hypothesis that the AhR form in mammalian vertebrates is more active in mediating transactivation in response to mono-ortho PCBs than the AhR2 forms found in fish.

DISCUSSION

Differences in transactivation potency of PCDDs, PCDFs, and PCBs with AhRs of mammalian) and fish origin. A comparison of the structure activity relationship for toxicity of PCDDs, PCDFs, and PCBs between fish and mammalian species shows that relative potencies (REPs) of the PCBs are far lower in fish than mammals (Zabel *et al.*, 1995; Cook *et al.*, 1997). This is illustrated by mono-ortho PCBs being completely inactive in producing early life stage toxicity in rainbow trout (Zabel *et al.*, 1995) and having either very weak or no activity in up regulating CYP1A mRNA abundance in a rainbow trout gonadal cell line (Zabel *et al.*, 1996). Similar results have been more recently obtained using a reporter system transfected into rainbow trout hepatoma cells to measure endogenous AhR/ARNT activity (Villeneuve *et al.*, 1999). A simple explanation for this difference is that AhR2s from rainbow trout and AhR from mammals differ in their ability to bind and be activated by mono-ortho PCBs. The present study represents the initial, first step in elucidating the molecular basis for the structure activity difference for polychlorinated aromatic hydrocarbons between these vertebrate classes. We utilized an assay system, transfected COS-7 monkey kidney cells, to measure AhR/ARNT-mediated responses to PCDD, PCDF, and PCB congeners in a setting where the cellular background remains constant, and only the AhR/ARNT molecules vary. This allowed us to compare transactivation activity of four different AhRs.

Based on the EC50s obtained for PCDD, PCDF, and PCB congeners with the four pairs of AhR/ARNT proteins (huAhR/huARNT, rtAhR2 α /rtARNTb, rtAhR2 β /rtARNTb, and zfAhR2/zfARNT), the following generalizations can be made. First, the rank order potency for congener classes tested with huAhR,

TABLE 2

Relative Potencies of PCDD, PCDF, and PCB Congeners Based on Rainbow Trout AhR2 α -Mediated Luciferase Induction from a Firefly Luciferase Reporter Gene, under Control of the Endogenous Rainbow Trout *cyp1A* Gene Promoter and DREs in COS-7 Cells, CYP1A Induction in Rainbow Trout Gonadal Cells (RTG-2) and Early Life Stage Mortality in Rainbow Trout

Class	Congener	rtAhR2 α Luc induction COS-7 cells ^a	CYP1A induction rainbow trout RTG-2 cells ^b	Early life stage mortality rainbow trout sac fry ^c
PCDDs	2,3,7,8 TCDD	1.0	1.0	1.0
	1,2,3,7,8 PCDD	1.3	1.6	0.7
PCDFs	2,3,7,8 TCDF	0.5	0.5	0.03
	2,3,4,7,8 PCDF	0.2	0.1	0.3
Non-ortho PCBs	PCB 126	0.2	0.06	0.005
	PCB 77	0.01	0.009	0.0002
Mono-ortho PCBs	PCB 156	0.00007	0.000009	—
	PCB 105	—	—	—
	PCB 118	—	—	—
Di-ortho PCB	PCB 153	—	—	—

Note. Relative potency was determined for each congener for enzyme induction by dividing EC50 of TCDD by EC50 of congener. Relative potency for rainbow trout early life stage mortality was based on LC₅₀s. —, inactive at highest concentration tested.

^a Relative potencies were determined for each congener based on results with rtAhR2 α in Table 1.

^b From Zabel *et al.* (1996).

^c From Zabel, Cook *et al.* (1995).

rtAhR2 α , and zfAhR2 was PCDDs > PCDFs > non-ortho PCBs. Second, all fish AhR2s tested expressed with their species-specific ARNT in the transactivation assay were either less responsive or nonresponsive to the mono-ortho PCBs when compared to the mammalian AhR form of the receptor. Of the three mono-ortho PCBs tested with fish AhR2s, PCB156 weakly induced luciferase activity with rtAhR2 α , but the other two mono-ortho PCBs were inactive with this form of the receptor. The other two fish AhR2s tested with their corresponding ARNT, rtAhR2 β and zfAhR2, failed to respond to any of the mono-ortho PCBs. In sharp contrast, the huAhR, responded to all mono-ortho PCBs tested. Taken together, these findings support the hypothesis that the lower toxic potency of the mono-ortho PCBs in fish than mammals may arise at the molecular level from structural differences between the fish and mammalian AhR proteins, with huAhR being more responsive to mono-ortho PCBs than the fish AhR2s.

Clearly, the basis for the differences in responsiveness between the human and fish receptors must lie in differences in the primary sequences of these proteins. As shown in Fig. 7, the C-terminal portions of the receptor molecules contain the most differences. The function of this region is poorly characterized, and the C-terminal portion could influence responsiveness to ligand. On the other hand, differences between the fish and mammalian proteins extend throughout even the more highly conserved bHLH and PAS domains. In particular, the PAS B domain functions in ligand binding and activation of the receptor (Fukunaga *et al.*, 1995; Schmidt and Bradfield, 1996; Rowlands and Gustafsson, 1997). This region of the protein also contains the sequence differences first noted between the

type 1 and type 2 receptors by Hahn and coworkers. We do not know whether the type 1 and type 2 receptors differ functionally, and if so whether the properties of the human receptor more closely resemble type 1 or type 2. However, one possibility is that the differences that we observed between fish type 2 receptors and the human AhR will also be seen between the fish type 2 and type 1 AhRs. Further work, including functional studies using chimeric AhRs will be needed to test this hypothesis.

We also cannot exclude the possibility that some of the differences that we observe are due to interspecies differences in the ARNT proteins. Such differences might be identified by further experiments with different AhR/ARNT pairs, combining AhR and ARNT proteins from the different species. We have not yet investigated this line of inquiry.

Difference in responsiveness between rainbow trout AhR2 α and AhR2 β to PCDDs, PCDF, and PCB congeners. We have recently discovered differences in the transactivation properties of the α and β isoforms of AhR2 in rainbow trout (Abnet *et al.*, 1999). As in the present study, we transfected COS-7 cells with cDNAs for one of two rainbow trout AhR/ARNT pairs (rtAhR2 α /rtARNTb or rtAhR2 β /rtARNTb) along with the reporter plasmid (prt1Aluc). The prt1Aluc plasmid provided a TCDD-responsive firefly luciferase reporter under control of the rainbow trout *cyp1A* gene promoter and enhancer. Using graded doses of TCDD, we showed that the magnitude of luciferase induction by the α form of the receptor was strikingly greater than the β form, which was barely detectable (Abnet *et al.*, 1999).

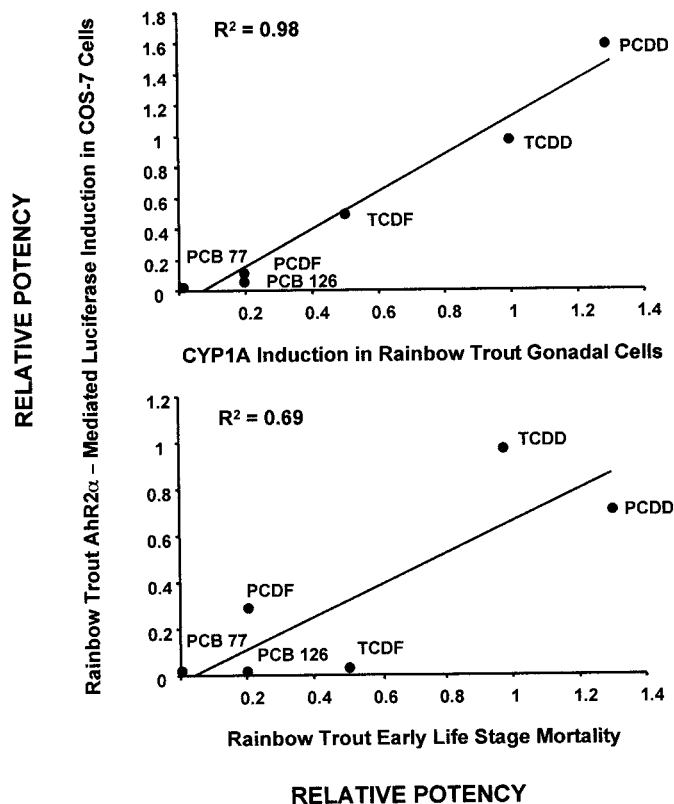


FIG. 6. Correlation between relative potencies (REPs) of PCDDs, PCDFs, and PCBs for: (1) inducing luciferase activity from a firefly luciferase reporter gene under control of the endogenous rainbow trout *cyp1A* gene promoter and DREs with rtAhR2 α /rtARNTb in transfected COS-7 cells, (2) inducing CYP1A mRNA abundance in rainbow trout gonadal cells (RTG-2, top panel), or (3) causing rainbow trout early life stage mortality (bottom panel). Both R^2 values are significant at $p < 0.05$.

The finding that rtAhR2 β showed nonexistent to weak transactivation activity in response to AhR agonists when tested with the prt1Aluc reporter might tend to lead to the conclusion that rtAhR2 β is inactive as a ligand-activated transcription factor, but this is not the case. We have shown that rtAhR2 β binds TCDD specifically and with high affinity, binds DREs in gel shift assays, and has significant TCDD-dependent transactivation activity when assayed with a different reporter containing mouse (rather than rainbow trout) *cyp1A1* enhancer DREs (Abnet *et al.*, 1999). This may indicate that rtAhR2 β recognizes response elements that are not found in the rainbow trout *cyp1A* promoter. If this is the case, rainbow trout genes, regulated by rtAhR2 β , may exist that have yet to be identified.

The present study extends this finding and demonstrates that the differences in transactivation activity between rtAhR2 α and rtAhR2 β are also observed with several other classes of polychlorinated aromatic hydrocarbons, namely the PCDDs, PCDFs, non-*ortho* PCBs, and mono-*ortho* PCBs. For all of the representative AhR agonists tested in each class, it was found that transactivation with rtAhR2 α was consistently greater than

rtAhR2 β . In fact, activation of the β isoform of rtAhR2 by most of these congeners, with the prt1Aluc reporter, was so weak it was of questionable biological significance. The only congeners that produced more than a 3.0-fold induction of luciferase activity with rtAhR2 β were PCB 126 and 1,2,3,7,8-PCDD, and they caused only 3.4- and 4.3-fold maximal induction, respectively.

Correlation between relative potencies of PCDD, PCDF, and PCB congeners in the COS-7 cell transactivation assay mediated by rtAhR2 α and their relative potencies for inducing AhR-mediated biochemical and toxic responses in rainbow trout. The relative potencies (REPs) of PCDD, PCDF, and PCB congeners for rtAhR2 α -dependent activation of a luciferase reporter gene, driven by DREs from the endogenous rainbow trout CYP1A promoter, correlated very well with their REPs for CYP1A induction in the RTG-2 rainbow trout gonadal cell line. This occurred even though COS-7 cells were maintained at 37°C while RTG-2 cells were cultured at 21°C. The finding suggests that rtAhR2 α may mediate induction of CYP1A in the RTG-2 cell line.

A significant correlation was also observed for REPs of PCDD, PCDF, and PCB congeners for rtAhR2 α -dependent activation in the COS cell assay and their REPs for causing early life stage mortality in rainbow trout. However, the correlation was not as high as for CYP1A induction in RTG-2 cells. This may reflect the complexity of the *in vivo* response. Since we now know that rainbow trout have at least two forms of AhR, with the possibility of an undiscovered rtAhR1, toxic responses are likely to involve multiple AhRs. As such, the REPs for toxic responses would be expected to correlate not with the REPs for just rtAhR2 α , but rather with a more complex function involving transactivation REP values of all of the receptor types involved in toxicity. This type of analysis awaits the characterization of all of the AhR isoforms in the species.

Information gaps in our understanding of AhR agonist-induced early life stage toxicity in fish and a role for in vitro approaches. Fish exposed to TCDD and related compounds as newly fertilized embryos develop an early life stage toxicity syndrome that can first be detected near the time of hatching. The syndrome is characterized by yolk sac, pericardial and meningeal edema, craniofacial malformations, impaired cardiovascular development and function, uninflated swim bladder, exophthalmia, and retarded growth, which culminates in mortality (Spitsbergen *et al.*, 1991; Walker *et al.*, 1991; Henry *et al.*, 1997; Cantrell *et al.*, 1998; Elonen *et al.*, 1998; Johnson *et al.*, 1998; Hornung *et al.*, 1999). A major unknown in understanding the mechanism of action of TCDD in causing these effects are the identities of AhR-regulated genes that play primary roles in causing the syndrome. The TCDD dose-response relationship for CYP1A induction in the vascular endothelium of trout early life stages is similar to that for trout early life stage mortality (Guiney *et al.*, 1997). However, this does

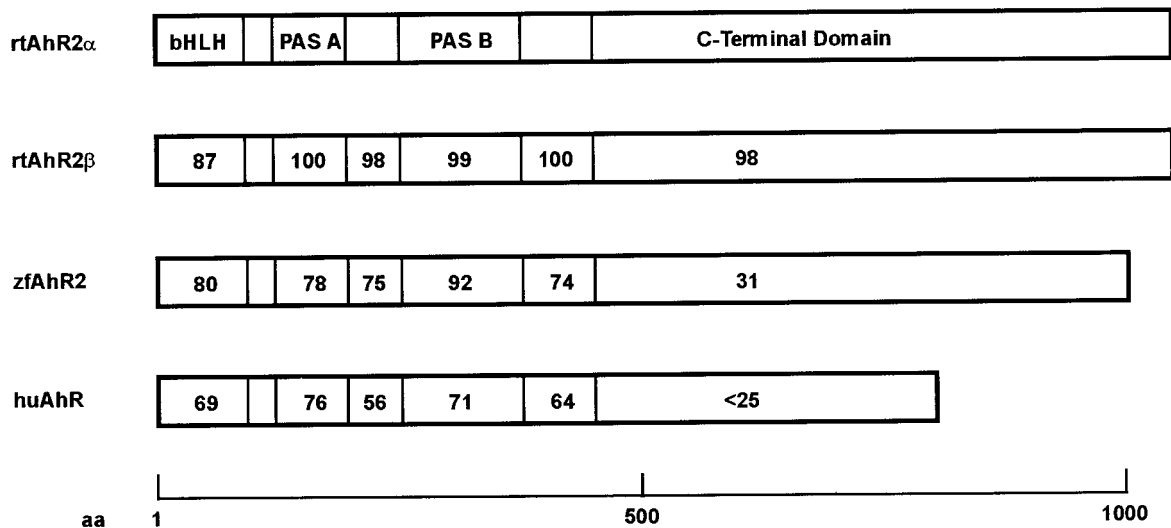


FIG. 7. Diagram showing levels of amino acid sequence homology between different domains of huAhR, zfAhR2, rtAhR2 α , and rtAhR2 β . Numbers reflect percent sequence identity between the indicated domains and the corresponding domains in rtAhR2 α .

not prove that increased expression of the CYP1A gene triggers the profile of toxic responses in trout embryos. It is possible that some of genes regulated by AhR agonists are not involved in causing the early life stage toxicity syndrome. It is also possible that a subset of toxic responses to AhR agonists are mediated by one isoform of AhR2, while other responses are mediated by the other receptor isoform. To date, little is known about the structure and function of the other major class of AhRs in fish—AhR1 (Hahn *et al.*, 1997). Thus, to understand the risk which bioaccumulative AhR agonists pose to feral fish populations, far greater insight into the mechanism of action of these toxicants is needed at the molecular level. We need to know the identities of genes downstream from rtAhR2 α and rtAhR2 β , respectively, which may mediate certain signs of toxicity, and which AhR2 isoforms regulate these genes. Furthermore, this same kind of information will be needed for AhR1 in rainbow trout if discovered. Development of *in vitro* methods to measure the activity of different AhR/ARNT pairs with specific enhancer elements in a controlled setting represents an important first step in obtaining this information.

The results of the present study are important because they provide the first clue that mono-*ortho* PCBs may be less toxic in fish because of structural and functional differences between the AhRs in fish and the AhRs found in mammals.

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