

# Identification of a Critical Amino Acid in the Aryl Hydrocarbon Receptor\*

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Two aryl hydrocarbon receptors (rtAHR2 $\alpha$  and rtAHR2 $\beta$ ) have been identified in the rainbow trout (*Oncorhynchus mykiss*). These receptors share 98% amino acid identity, yet their functional properties differ. Both rtAHR2 $\alpha$  and rtAHR2 $\beta$  bind 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), dimerize with rainbow trout ARNTb (rtARNTb), and recognize dioxin response elements *in vitro*. However, in a transient transfection assay the two proteins show differential ability to recognize enhancers, produce transactivation, and respond to TCDD. To identify the sequence differences that confer the functional differences between rtAHR2 $\alpha$  and rtAHR2 $\beta$ , we constructed chimeric rtAHRs, in which segments of one receptor form was replaced with the corresponding part from the other isoform. This approach progressively narrowed the region being examined to a single residue, corresponding to position 111 in rtAHR2 $\beta$ . Altering this residue in rtAHR2 $\beta$  from the lysine to glutamate found in rtAHR2 $\alpha$  produced an rtAHR2 $\beta$  with the properties of rtAHR2 $\alpha$ . All other known AHRs resemble rtAHR2 $\alpha$  and carry glutamate at this position, located at the N terminus of the PAS-A domain. We tested the effect of altering this glutamate in the human and zebrafish AHRs to lysine. This lysine substitution produced AHRs with transactivation properties that were similar to rtAHR2 $\beta$ . These results identify a critical residue in AHR proteins that has an important impact on transactivation, enhancer site recognition, and regulation by ligand.

The aryl hydrocarbon receptor (AHR)<sup>1</sup> and its associated dimerization partner ARNT are members of the basic helix-

loop-helix (bHLH) PAS family of proteins. These proteins transduce signals generated by environmental stresses into transcriptional responses. These stresses range from hypoxia to xenobiotic compounds (1, 2). The AHR is activated by a structurally broad range of ligands. Among these, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is one of the most potent and well-studied agonists (3). A broad spectrum of environmental contaminants, including TCDD, can produce toxic responses through activation of the AHR. Two hybrid and coprecipitation studies have revealed the presence of proteins that interact with AHR, including HSP90 and ARA9/AIP/XAP2 (4–7). These chaperone proteins stabilize and hold AHR in a conformation that is better able to bind ligand (8). TCDD binding causes the AHR protein to dissociate from cytosolic HSP90 and move into the nucleus where it forms a functional dimer with ARNT. This dimer then binds DNA to regulate the transcription of target genes. The AHR·ARNT dimer binds to specific enhancer elements that are often referred to as Dioxin Response Elements, or DREs. The best-characterized DREs lie upstream of genes encoding cytochrome P450s (CYP450s) (9, 10). Following nuclear localization and DNA binding, AHR exits the nucleus and is then degraded by the proteasome pathway (11–14).

The AHR protein is composed of several functional domains. The N terminus contains a domain rich in basic amino acids followed by a helix-loop-helix domain that is conserved among a variety of DNA binding proteins. The basic domain is required for DNA binding, whereas the helix-loop-helix domain is involved in dimer formation with ARNT. The N terminus also contains nuclear localization (NLS) and export (NES) domains (15, 16). C-terminal to the bHLH domain is a pair of PAS domains, PAS-A and PAS-B, that are conserved among a family of proteins. The PAS domains are named for several founding members of this protein family, *Per*, *ARNT*, and *Sim* (1). PAS domains act as regulated protein interaction surfaces and are involved in a wide variety of sensory/signaling processes in both eukaryotes and prokaryotes. These domains are involved in ligand binding to AHR, and the subsequent change in protein associations, subcellular location, and activity. The ligand-binding domain encompasses the PAS-B domain whereas HSP90 is thought to interact with the bHLH and PAS domains (17, 18). ARA9/AIP interacts with the PAS-B/ligand-binding domains (19, 20). Potential retinoblastoma protein binding sites have also been identified (21). The C-terminal domain is necessary for transcriptional activation and is the least conserved among AHR proteins (17).

Developing fish are especially sensitive to the toxic effects of TCDD (22). AHR and ARNT proteins have been identified in a variety of fish species and presumably mediate these effects. The ability of ligands to activate the AHR pathway is similar to their ability to cause TCDD-like toxicity (23–25). In contrast to mammals, most fish species appear to have at least two AHR

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<sup>1</sup> The abbreviations used are: AHR, aryl hydrocarbon receptor; hAHR, human AHR; rtAHR, rainbow trout AHR; ARNT, Aryl Hydrocarbon Receptor Nuclear Transporter; bHLH, basic helix-loop-helix; PAS, *Per*, *ARNT*, and *Sim* protein family; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; HSP90, heat-shock protein 90; DRE, dioxin response element; CYP450, cytochrome P450; NLS, nuclear localization signal; NES, nuclear export signal; CMV, cytomegalovirus; ORF, open reading frame; MOPS, 4-morpholinepropanesulfonic acid; PYP, photoactive yellow protein.

genes. Generally, one AHR (AHR1) is more similar to the mammalian AHR and a second (AHR2) is fish-specific (26). Full-length AHRs have been cloned in tomcod and two each in rainbow trout, *Fundulus heteroclitus*, and zebrafish (27–30). In addition, partial AHR sequences have been cloned from several fish (26). ARNT isoforms have been cloned from *Fundulus* (ARNT2) rainbow trout (rtARNTa and b) and zebrafish (zfARNT2a, b, and c) (31–33). No ARNT1 has been identified in fish.

To date, salmonids are the group of fish species that are most sensitive to the effects of TCDD. Two AHR genes encoding rtAHR2 $\alpha$  and rtAHR2 $\beta$  have been identified in rainbow trout. These two AHR isoforms are ~98% identical in primary sequence. Despite this similarity in structure, the two proteins have distinct properties. In general, rtAHR2 $\alpha$  has stronger transactivation properties than rtAHR2 $\beta$ . This is somewhat surprising in light of the fact that these two proteins are identical in sequence in the C-terminal domain that is thought to mediate transcriptional activation. In addition, rtAHR2 $\alpha$  and rtAHR2 $\beta$  have different enhancer sequence requirements. rtAHR2 $\beta$  appears to be active with a more limited set of enhancer sequences than rtAHR2 $\alpha$ . To explore the structural nature of these differences, we constructed a set of chimeric proteins in which segments of rtAHR2 $\beta$  were exchanged with the cognate sequence from rtAHR2 $\alpha$ . These experiments indicate that the functional differences between rtAHR2 $\alpha$  and rtAHR2 $\beta$  are conferred by a single amino acid difference corresponding to position 111 in rtAHR2 $\beta$ .

#### EXPERIMENTAL PROCEDURES

**Cell Culture**—COS-7 (monkey kidney epithelial) cells, obtained from ATCC (Manassas, VA), were maintained in 100% humidity in Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum in an atmosphere of 5%CO<sub>2</sub> at 37 °C. Cells were split and plated at a density of  $6.0 \times 10^4$  cells/well of a 24-well plate 1 day prior to transfections. Y1 cells (mouse adrenal cortex cells) generously provided by Dr. Collin Jefcoate (University of Wisconsin) were maintained in Ham's F-10 media supplemented with 15% horse serum and 2.5% fetal bovine serum. Cells were plated at a density of  $8.0 \times 10^4$  cells/well of a 24-well plate one prior to transfections.

**Oligonucleotides**—Primers are displayed 5' to 3' in the following. Positions of the primers are relative to the initiation codon in rtAHR2 $\beta$ , hAHR, or zfAHR2. Mutated bases are in lowercase letters. Added restriction sites are underlined. 3P-Xba (position 3189), CTAGTCTAGACAATGTGACGCATGTTTAC; 5P-Sal (position -74), GATCGTCGACTGAGGAAGACAGTGGATGT; EA17f (position 254), CCATGAAGAAAAGCAGTGTCTCTTTCC; EA17r (position 281), GGAAACAGGACACTGCTTTTCTTCATGG; UC6f (position 304), AATGGGATGaAcGCCACAACC; UC6r (position 324), GGTTGTGGCgTtCATCCCCATT; UC7f (position 310), ATGGAAGCCcCAACCTTCTCC; UC7r (position 330), GGAGAAGGTTGgGGCTTCCAT; UC14f (position 308), GGATGaAcGCCcCAACCTTCTCCgAGGG; UC14r (position 335), CCCTcGGAGAAGGTTGgGGCgTtCATCC; K111Ef (position 320), CAACCTTCTCCgAGGGGACC; K111Er (position 339), GTCCCCCTcGGAGAAGGTTG; K111Af (position 320), CAACCTTCTCCgGGGGACC; K111Ar (position 340), GGTCCCCcGGAGAAGGTTG; P208r (position 859), GTCGAGCTTGTTTGGTCTG; af (position -25), CAACTAATACAGCAGAAGCG;  $\beta$ f (position -27), TAGCCGATTTACAGCAGAAG; rtAHRsFLAGr (position 3156), CTAGTCTAGATCACTTGTTCATCGTCGTCCTTGATGTCGAAGTTGAAAAGTGATTTGG; EA-15f (position 2474), TCTAACCCAGCCACCTCCACAG; CMV-F (CMV-specific), AGCTATGACCTTGATTACGC; zf2E118Kf (position 341), CAACCTTCTCAaAAGGGGAGC; zf2E118Kr+5 (position 360), GCTCCCCTTtTGAGAAGTTGACTCC; RT-19 (position 982), TGATACCCAGAGCCTCTCAT; hE114Kf (position 325), GGCCTGAACCTTACAAaAAGGAGAATTC; hE114Kr (position 351), GAATTCTCCTTtTTGTAAGTTCAAGGCC; EA-20 (position 567), TCCTTGTCAGACTTTGTAC; T3 (CMV-specific), AATTAACCCCTACTAAAGGG.

**Rainbow Trout RNA Purification**—Juvenile trout obtained from Ennis National Fish Hatchery (Ennis, MT) were euthanized with tricaine methanesulfonate (Argent, Redmond, WA) and frozen in liquid nitrogen. Whole fish were pulverized and total RNA was extracted using TRI reagent (Molecular Research Laboratories, Cincinnati, OH) as directed

by the manufacturer. Total RNA was isolated from RTG cell lysates using Qiashredder homogenizers (Qiagen, Chatsworth, CA).

**Cloning of Rainbow Trout AHR N-terminal Fragments**—Oligo(dT)-primed cDNA was synthesized using 500 ng of total whole juvenile rainbow trout RNA or RTG cell RNA. Alpha- and beta-specific rtAHR sequences were amplified using  $\alpha$  and  $\beta$  primers, respectively, and the P208 reverse primer. Amplification was conducted for 35 cycles under the following conditions: 30 s at 95 °C, 30 s at 56 °C, and 4 min at 72 °C, followed by a 7-min extension after the last cycle. Amplified products were visualized by ethidium bromide staining and subcloned into pGEM-T Easy (Promega, Madison, WI) and sequenced.

**Expression Vectors**—The rtAHR2 $\alpha$  and rtAHR2 $\beta$  expression vectors (rtAHR2 $\alpha$ ORF and rtAHR2 $\beta$ ORF, respectively) were created by amplifying the open reading frame of each gene using primers 5P-Sal and 3P-Xba with *Pfu* polymerase (Promega, Madison WI). The products of these reactions were digested with *Sal*I and *Xba*I and subcloned into pBK-CMV previously digested with the same enzymes. The expression vector for ARNTb was supplied by Dr. Richard Pollenz (31). Zebrafish AHR2 and zebrafish ARNT2b expression constructs both in pBK-CMV have been previously reported (30, 33). A human AHR construct was made by digesting pSportAHR2 supplied by Dr. Chris Bradfield (University of Wisconsin, Madison, WI) with *Sma*I to release the coding sequence. This fragment was then ligated into pBK-CMV similarly digested with *Sma*I. The prt1Aluc luciferase reporter vector has been previously reported (27). Dr. Michael Denison (University of California, Davis, CA) generously provided the pGudLuc1.1 luciferase reporter (34). pBK-CMVrtAHRsrtAHR2 $\alpha$ , pBK-CMVrtAHR2 $\beta$ , and pBK-CMVrtAHR2 $\beta$ K111E were used as templates for PCR to make C-terminal FLAG-tagged proteins for Western analysis. Each template was amplified using *Pfu* DNA polymerase with EA-15f and rtAHRsFLAGr primers. PCR products were digested with *Bgl*II and *Xba*I and ligated into their corresponding pBK-CMVrtAHR2 vectors that were similarly digested. The sequence was verified for each clone.

**Chimeric and Mutant rtAHR Expression Constructs**—Plasmids rtAHR2 $\alpha$ ORF and rtAHR2 $\beta$ ORF were digested with *Sal*I and *Xba*I and temporarily subcloned into pBluescript II SK (Stratagene, La Jolla CA) previously digested with *Sal*I and *Xba*I. Six chimeric rtAHR2s were constructed by gel-purifying restriction digestion products of the pBluescript II SK rtAHR2 clones with a particular combination of *Sal*I, *Sph*I, *Bgl*II, and or *Xba*I (Fig. 1). Exchanging the *Bgl*II/*Xba*I digestion products produced Chimeras A and B. Chimeras C and D were constructed by switching the *Sal*I/*Sph*I digestion products. Chimeras E and F were constructed by exchanging the *Sph*I/*Bgl*II digestion products. Chimera G was made as follows. The bHLH domain of rtAHR2 $\alpha$  was amplified by PCR using *Pfu* DNA polymerase (Promega, Madison, WI) with EA17r and 5P-Sal for 25 cycles as follows: 30 s at 94 °C, 30 s at 68 °C, and 3 min at 72 °C. The product was then gel-isolated. The PAS-A domain of rtAHR2 $\beta$  was similarly amplified using EA17f and P208r and gel-isolated. The products of the preceding reactions were then combined and amplified under the same conditions for five cycles. Then primers 5P-SalI and P208r were added, and another 25 cycles of amplification were conducted. The product of this reaction was then digested with *Sal*I and *Sph*I and ligated into rtAHR2 $\beta$  in pBluescript II SK that was similarly digested. Chimera H was constructed similarly to Chimera G except the template rtAHR2s were reversed. The bHLH domain was amplified from rtAHR2 $\beta$ , and the PAS-A domain was amplified from rtAHR2 $\alpha$ . The resulting product was ligated into the *Sal*I/*Sph*I sites of rtAHR2 $\beta$  in pBluescript II SK.

Point mutations were made in the rtAHR2 $\beta$  expression vector by site-directed mutagenesis as follows. The template AHR was amplified by PCR using *Pfu* DNA polymerase in two separate reactions. The forward mutant primer and the external reverse primer were used in one reaction and the forward external primer with the reverse mutant primer in another. The conditions for these reactions is as follows: 30 s at 94 °C, 30 s at 58 °C, and 90 s at 72 °C for 25 cycles. The products were gel-isolated and combined for five cycles: 30 s at 94 °C, 30 s at 62 °C, and 90 s at 72 °C. External primers were then added to the reaction, and 25 more cycles were conducted under the same conditions. The amplified product was then gel-isolated and digested with *Sal*I and *Sph*I and ligated into rtAHR2 $\beta$  in pBSKII similarly digested. rtAHR2 $\beta$  chimeras and the point mutant clones were all subcloned into the *Sal*I/*Xba*I site of pBK-CMV. The human AHRE114K and zebrafish AHR2E118K mutants were made under the same conditions as the trout mutants using species-specific primers. All chimeric and mutated AHRs were confirmed by restriction digestion and sequence analysis. Each modified AHR could be translated *in vitro* and produced a band of similar size and intensity as the wild-type AHR.

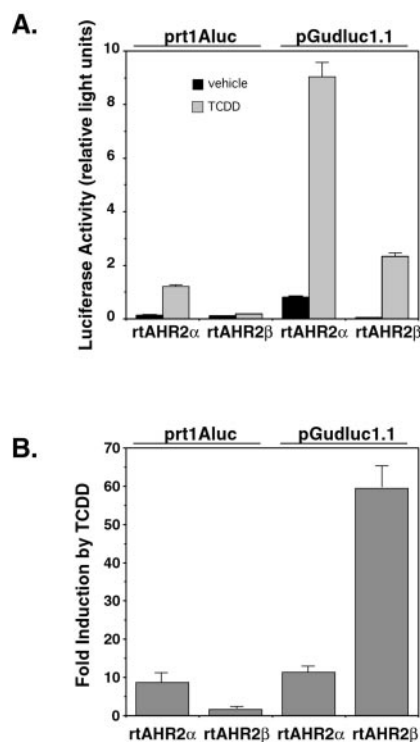
**Transient Transfection**—COS-7 cells were plated on 24-well plates at

a density of  $6 \times 10^4$  cells per well 1 day prior to transfection. Transient transfection was conducted using SuperFect (Qiagen, Chatsworth, CA). Each well was cotransfected with 400  $\mu$ l of serum containing media, including wild-type or mutant AHR (450 ng), a species-specific ARNT (450 ng) expression vector, 100 ng of a luciferase reporter (prt1Aluc or pGudluc1.1), and a  $\beta$ -galactosidase CMV reporter (50 ng) for estimation of transfection efficiency. Y1 cells were plated at  $8 \times 10^4$  cells per well and transfected with the indicated AHR (250 ng/well), luciferase reporter (200 ng/well), and  $\beta$ -galactosidase CMV reporter (100 ng/well). Following a 2-h incubation at 37 °C, 600  $\mu$ l of fresh serum-containing media was added to each well. After 20-h incubation, cells were exposed to Me<sub>2</sub>SO vehicle control or TCDD previously dissolved in Me<sub>2</sub>SO (0.1% media volume). Cells were harvested after a 20-h incubation. Media were aspirated, and each well was washed with 0.5 ml of phosphate-buffered saline. 100  $\mu$ l of lysis buffer was added to each well (100 mM KPO<sub>4</sub>, pH 7.8, 6 mM MgSO<sub>4</sub>, 0.1% Triton X-100, 1 mM dithiothreitol, and 4 mM ATP trihydrate), 10- $\mu$ l aliquots of cell lysate were transferred to a 96-well luminometer, and 50  $\mu$ l of luciferase assay buffer (Promega, Madison, WI) was injected into each well, incubated for 2 s, with the luminescence integrated over 10 s. Luciferase assays were completed using a Dynatech Laboratories ML-2250 luminometer (Chantilly, VA).  $\beta$ -Galactosidase activity was determined for each well as follows. 15  $\mu$ l of cell lysate was aliquoted to a 96-well plate. 200  $\mu$ l of reaction buffer (0.1 M NaPO<sub>4</sub>, 10 mM KCl, 1 mM MgCl<sub>2</sub>, 0.385%  $\beta$ -mercaptoethanol) was added to each well followed by the addition of 40  $\mu$ l of *o*-nitrophenyl- $\beta$ -D-galactopyranoside (4 mg/ml). The reaction was then incubated at 37 °C for 2–4 h. Plates were read at 405 nm using a Bio-Tek Instruments ELx800 plate reader (Winooski, VT). Results are expressed as luciferase activity normalized to the  $\beta$ -galactosidase activity in each sample. The -fold induction was calculated by dividing the relative luciferase activity measured in the presence of TCDD by the activity measured in the corresponding vehicle-treated sample.

**Western Blots**—COS-7 cells at 70% confluency were transfected with 5  $\mu$ g of either pBK-CMV empty vector, rtAHR2 $\alpha$ FLAG, rtAHR2 $\beta$ FLAG, or rtAHR2 $\beta$ K111EFLAG as described above. Whole cell lysate was harvested 20 h later. Briefly, the cells were rinsed two times with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM Na<sub>2</sub>PO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) containing EDTA and EGTA (1 mM each) and removed from the dish using a Teflon spatula, rinsed with 300  $\mu$ l of extraction buffer (25 mM MOPS, pH 7.5, containing 1 mM EDTA, 5 mM EGTA, 0.02% NaN<sub>3</sub>, 20 mM Na<sub>2</sub>MoO<sub>4</sub>, 10% (v/v) glycerol, 1 mM dithiothreitol, 5  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml aprotinin, and 5  $\mu$ g/ml pepstatin A), and transferred to a 1.5-ml centrifuge tube on ice. Cells were sonicated three times on ice and homogenized using a Dounce homogenizer. Debris was pelleted by centrifugation at 22,000  $\times g$  for 30 min. 20  $\mu$ g of lysate was resolved by SDS-PAGE on an 8% gel and transferred to nitrocellulose. Blots were blocked with 5% dry milk in TBS-T (25 mM Tris (pH 7.6), 150 mM NaCl, 0.1% Tween 20) for 1 h then washed three times with TBS-T. The FLAG epitope was then detected by incubation with anti-FLAG monoclonal antibody (Sigma Chemical Co., St. Louis, MO) diluted in TBS-T (2  $\mu$ g/ml) containing 1% dry milk. The antibody was removed after 2 h, and blots were washed with TBS-T three times. Horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Inc., Chicago, IL) diluted 1:4000 in TBS-T containing 5% dry milk was added for 1-h incubation. Blots were washed three times in TBS-T prior to chemiluminescence detection (Amersham Biosciences, Inc.).

## RESULTS

The rtAHR2 $\alpha$  and rtAHR2 $\beta$  proteins are almost 98% identical in primary sequence yet have distinctly different properties. We used transient transfection assays in COS-7 cells to measure transactivation properties of these proteins, because these cells are devoid of endogenous AHR and express only a low amount of ARNT (35). When assayed for reporter activation in a transient transfection assay using COS-7 cells and DRE-containing luciferase reporters, these proteins gave different results. When we used the prt1Aluc reporter construct, in which sequences from the rainbow trout *CYP1A* promoter are used to drive the luciferase reporter (36), rtAHR2 $\alpha$  produced a luciferase signal that was readily detected and sensitive to TCDD induction. In contrast, rtAHR2 $\beta$  produced only a low level of reporter activity, which was not increased by the addition of TCDD (Fig. 1). However, when we used pGudluc1.1, a reporter driven by mammalian sequences derived from the



**FIG. 1. Activity of rtAHR2 $\alpha$  and rtAHR2 $\beta$  in a transient transfection assay.** COS-7 cells were transiently transfected with expression vectors for the indicated rtAHR2 and rtARNTb along with a DRE containing firefly luciferase reporter construct, either prt1Aluc or pGudluc1.1 as indicated. A  $\beta$ -galactosidase expression vector was also included as a control for transfection efficiency. Cells were exposed to Me<sub>2</sub>SO as a vehicle control or to TCDD (10 nM in Me<sub>2</sub>SO) for 20 h prior to assay. A, data are expressed as  $\beta$ -galactosidase normalized relative light units. Light bars, TCDD exposed; dark bars, vehicle control. B, results are expressed as -fold induction by TCDD. The results are expressed as the means of three independent replicates  $\pm$  S.E.

mouse *cyp1A1* gene (34), we observed a different response. In this case, both AHR proteins were able to produce a readily detected reporter signal that was induced by TCDD. Although rtAHR2 $\alpha$  produced stronger transactivation with the pGudluc1.1 reporter than rtAHR2 $\beta$ , rtAHR2 $\beta$  was more responsive to TCDD, as indicated in the bottom right panel of Fig. 1. In this assay, rtAHR2 $\alpha$  was induced ~10-fold by TCDD, whereas the activity of rtAHR2 $\beta$  was induced by more than 50-fold, owing to the very low activity in the absence of TCDD. This rtAHR2 $\beta$  basal activity is close to the limit of detection, making calculated values for -fold TCDD induction of rtAHR2 $\beta$  somewhat variable. However, both the low basal activity and the high -fold induction by TCDD were consistently observed with this receptor. These results demonstrate several different properties of these receptor molecules: First, rtAHR2 $\alpha$  appears to have stronger transactivation properties than rtAHR2 $\beta$ , producing more luciferase expression with either reporter construct. Second, when assayed with the pGudluc1.1 reporter, rtAHR2 $\beta$  is more tightly regulated by TCDD, owing to the very low basal activity. Finally, the two AHR proteins appear to have different requirements for DNA target sequences.

To identify the domain that confers these differences in activity, we constructed chimeric rtAHR2 proteins by exchanging similar domains between rtAHR2 $\alpha$  and rtAHR2 $\beta$  and measured the activities of these chimeras with prt1Aluc and pGudluc1.1. We then attempted to correlate the presence of a region from the  $\alpha$  or  $\beta$  receptor isoforms with the different receptor characteristics. The C-terminal portions of the rtAHR2s are entirely conserved (Fig. 2), so our chimeras concentrated on the N-terminal half of the AHR proteins. The first set of chimeric

	<u>basic</u>		<u>basic</u>	<u>Helix-Loop-Helix</u>		<u>Oligo</u> ↓	
rtAHR2α	M-LSN-TGVYAVKKR	KKPVQTKKSPAPDV	VKSNPSKRHRDLNG	ELDLRTGLLPFPEDV	RSRLDKLSVLRISVG	YLKVKSPFKTTMKKS	88
rtAHR2β	*-***NA*****	*****T***E*	*****	*****	C*****L***	*****A*****	89
humanAHR	*NS*SANIT**SR**	R*****V*PIPAEG	I*****T	*****AS*****Q**	INK*****L**S	**RA****DVAL*S*	90
<b>PAS-A</b>							
rtAHR2α	SVLFPGG--GGLNMN	<u>GMNAPITFSE</u> EGDLLLQ	ALNGFVLVVTAEGHV	FYSSPTIQDYLGPHQ	SDVVHQSVFELIHTD	DRATFRRLQHLFALNP	176
rtAHR2β	*****S--N***I*	**E**T**K*****	*****	**A*****	*****	*****	177
humanAHR	PTERN**QDNCRAA*	<u>FREGLNLQ*</u> EF**	*****TDAL*	**A*S*****Q*	***I***Y***E	***E*Q***W***	180
↓ <i>Sph</i> I							
rtAHR2α	KPFDPEQGGDMASS	SDITRNIVTYNPEQL	PPENSSFLERNFVCR	FRCLLDNSSGFLALN	FQGRKFLHCQSMGLG	DDGTHSQPRLGLFTI	266
rtAHR2β	*****	*****	*****	*****	*****A*****	****R***N*****	267
humanAHR	S--QCTES*Q*IEEA	TGLPQTV*C**D**	****PLM**C*I**	L*****M*	***K**Y**G*KKK*	K**SILP*Q*A**A*	268
<b>PAS-B</b>							
rtAHR2α	ATPVHTPSILEIRNK	TIFFQTKHKLDFTPT	GVDARGKVVLYGSEI	ELCMRGSYGQFIHAA	DMMYCADSHVRMIKT	GESGLTTFRLLQKTG	356
rtAHR2β	***QN*****T*	*****M	*****M	*****	*****N*****	*****	357
humanAHR	***LQP*****T*	NFI*R*****I	*C**K*RI***T*A*	**T*****	**L***E**I****	***MIV***T*N	358
rtAHR2α	CWVWVQANARLVYKG	GRPDFI IARQRALLN	SEGEEHLRQRKMELP	FSFTTGEALLYETG-	---PTLDATEFQTN-	-----SPKIRKV	433
rtAHR2β	*****	*****	*****	*****-	-----	-----	434
humanAHR	R*T***S***L*N	***Y**VT**P*TD	E**T***K*NTK**	*M*****V***ATN	PPPAIM*PLPLR*KN	GTSGKDSATTSTLSK	448
rtAHR2α	ESLDPQSLGSLMNQ	DESIYTPQPEPQLPI	DQAFMDSRALTNVAC	NSWQSSMEPQGPDGD	DDGDGPSEVKQKQAV	VAMIDALEKMARDGD	523
rtAHR2β	*****	*****	*****	*****	*****	*****	524
humanAHR	D*N*S***AAM*Q*	****LY*ASSTST	APFENNFNEMNE*	RN**DN*TA*M*N*TI	LKHEQIDQPQDVNSF	AGGHPG*FQDSKNS*	538
rtAHR2α	LCEALQGLDVDEAEL	MEWESALLRLSQESN	GTGGGDTSPELDIDM	TNDIFSIVEEALFKE	SSESGNQPNCSIMV	NNPNLNLFTVEFNN	613
rtAHR2β	*****	*****	*****	*****	*****	*****	614
humanAHR	LYSIMKNLGIIDFEDI	RHMQNEKFFRNDFSG	-----EVDPRDIDL	TDEILTYVQDSLKSK	PFIPSDYQQQSLAL	N-----	608
rtAHR2α	NNQDGPFPGMVSPGT	VGQCKPGLLDSRSFI	HNGSPVNSLNGQVTG	NGPDGLAGQNQAGPH	QVFNSTQRLSHFGPQ	IPQMDLNIPTLQQLQ	703
rtAHR2β	*****	*****	*****	*****	*****	*****	704
humanAHR	-----	-----	-----	---SSCMV*EHLHLE	*QQQH*QVVVE**	Q---QLCQKMKHM*	646
↓ <i>Bgl</i> II							
rtAHR2α	LNDIFTPSLELPELS	IPHSSGQNGAVTFTCT	NMAGSCAQAPNNHMG	SPQGITGRVHSNQPP	PQFFTHNGLPATMAS	NGPQQISVPQSNHVA	793
rtAHR2β	*****	*****	*****	*****	*****	*****	794
humanAHR	V*GM*EN-----	-----WNSNQ	FVFPFN*P*QDPQYQ	VFTDLH*---IS*EF	*YKSEMDSM*Y*QNF	ISCN*PVL**HSKCT	715
rtAHR2α	PSLVDGWASMIIPNSA	FVSPQIESSNLNLSN	PLPTACLQGNAPFQ	SLKIQRVLQWPQNQQ	QLPPPASTIQNGIMA	NGHTFIPDCHSQDSE	883
rtAHR2β	*****	*****	*****	*****	*****	*****	884
humanAHR	-----	-----E*DY	*MGSFEPSPYPTTSS	LEDFVTC**I,*E**K	HGLN*Q*----A*IT	-----PQT--CY	765
rtAHR2α	TQRVPLTGIWPQNPN	RLYHQYQHGGGLANGQ	PAPSSSCMFENISPH	LPNGNSHVDGTRLAS	TLSVCQSRMVDPODQ	SPPKGCYFQWGPSE	973
rtAHR2β	*****	*****	*****	*****	*****	*****	974
humanAHR	AGA*SMYQCQ*EPQH	THVG*M*YNPVLP**	QAFLNKFQNGVLNET	Y*AELNNIN-----	-----NT-	-----	821
rtAHR2α	PVVGTSAVIQDSTST	SPPSRPLVANITPPE	GLLAMQQYLACGCSGV	GQTQIPSLPVIDSNG	ILSLPPLVNGSMCFT	EHNQINYNCF	1058
rtAHR2β	*****	*****	*****	*****	*****	*****	1059
humanAHR	-----QTTTHL	Q*LHH*SE*RP----	-----	-----F*DLT*S*	F*-----	-----	848

FIG. 2. Amino acid sequence alignment of rtAHR2α, rtAHR2β, and the human AHR. Sequence alignment of rtAHR2α (GenBank™ accession number AF065137), rtAHR2β (AF065138), and human AHR (L19872) was done using ClustalW1.8. Asterisks indicate perfect identity to rtAHR2α, and dashes indicate gaps in sequence alignment. The basic, HLH, and PAS domains are indicated by lines above the sequence. The ligand-binding domain is indicated by underlining (17, 18). The nuclear localization signal and nuclear export signal (16) are shaded.

proteins were made by taking advantage of conserved *Sph*I and *Bgl*II restriction sites found in both rtAHR2α and rtAHR2β. These were used to transfer domains from rtAHR2α to rtAHR2β and vice versa (Fig. 3, and see Fig. 2). In chimeras A through F, the ability to produce a robust transactivation signal with the prt1Aluc reporter, a characteristic of rtAHR2α, was observed only in chimeras in which the N-terminal 250 amino acids were from rtAHR2α. Chimeras carrying rtAHR2β sequence in this region were relatively inactive with the prt1Aluc reporter. Similarly, the tight regulation by TCDD observed in rtAHR2β, characterized by very low basal expres-

sion and resulting high -fold induction by TCDD with the pGuduc1.1 reporter, correlated with the presence of the rtAHR2β sequence in this N-terminal *Sph*I portion of the protein. In addition, the marked preference for pGuduc1.1, which characterizes rtAHR2β, was also conferred by this part of the protein.

This N-terminal *Sph*I fragment encoding the first 250 amino acids contains the majority of the differences between the rtAHR2α and rtAHR2β sequences. To narrow down the residues responsible for the functional differences between rtAHR2α and rtAHR2β and to possibly dissociate these func-

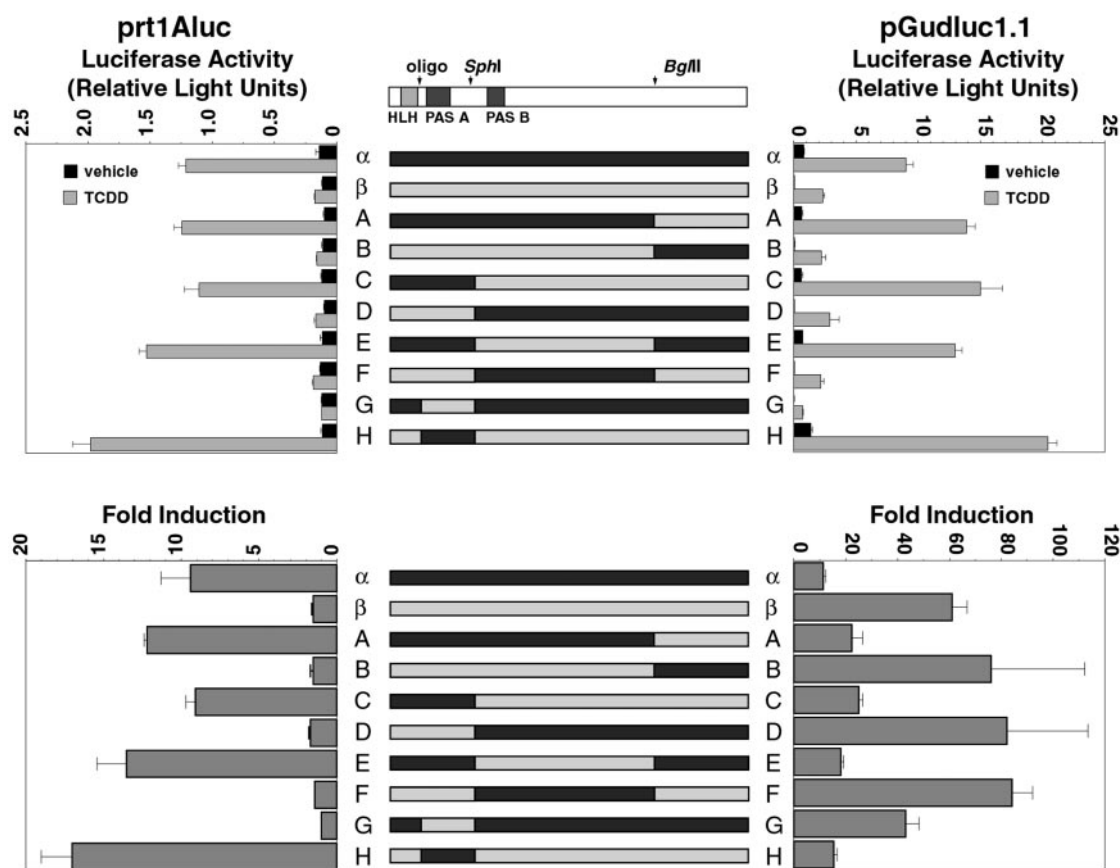


FIG. 3. **TCDD responsiveness of rtAHR2 chimeras in a transient transfection assay.** COS-7 cells were transiently transfected with expression vectors for the indicated rtAHR2 chimeras as described for Fig. 1. Maps of the AHR open reading frames indicate the positions of the chimera junctions. *Dark bars* indicate rtAHR2 $\alpha$  sequence, and the *light bars* represent rtAHR2 $\beta$  sequence. Data are expressed in the *upper panels* as  $\beta$ -galactosidase normalized relative light units: *light bars*, TCDD-exposed; *dark bars*, vehicle control. Results are expressed as -fold induction by TCDD in the *lower panels*. The results are expressed as the means of three independent replicates  $\pm$  S.E.

tional differences, we made an additional set of chimeras, G and H. In these chimeras the regions between position 86 and the *SphI* site, containing the PAS-A domain, are swapped. The designation of the junction at position 86 is arbitrary, because this junction occurs in a region of sequence identity; the actual position of the junction can be considered anywhere between positions 84 and 93 on rtAHR2 $\alpha$ . The ability to produce a robust transactivation signal with the prt1Aluc reporter was observed in chimera H, which carried rtAHR2 $\alpha$  sequence between positions 86 and 250, but not in the chimera containing rtAHR2 $\beta$  sequence in this region (Fig. 3). As observed with the previous set of chimeras, the tight regulation by TCDD observed with rtAHR2 $\beta$  and the pGudluc1.1 reporter was also conferred by this region of the protein. The chimera G, containing rtAHR2 $\beta$  sequence between positions 86 and 250, showed low activity with prt1Aluc and high -fold induction with pGudluc1.1. Thus, this portion of the protein confers both the rtAHR2 $\alpha$ - and rtAHR2 $\beta$ -specific characteristics.

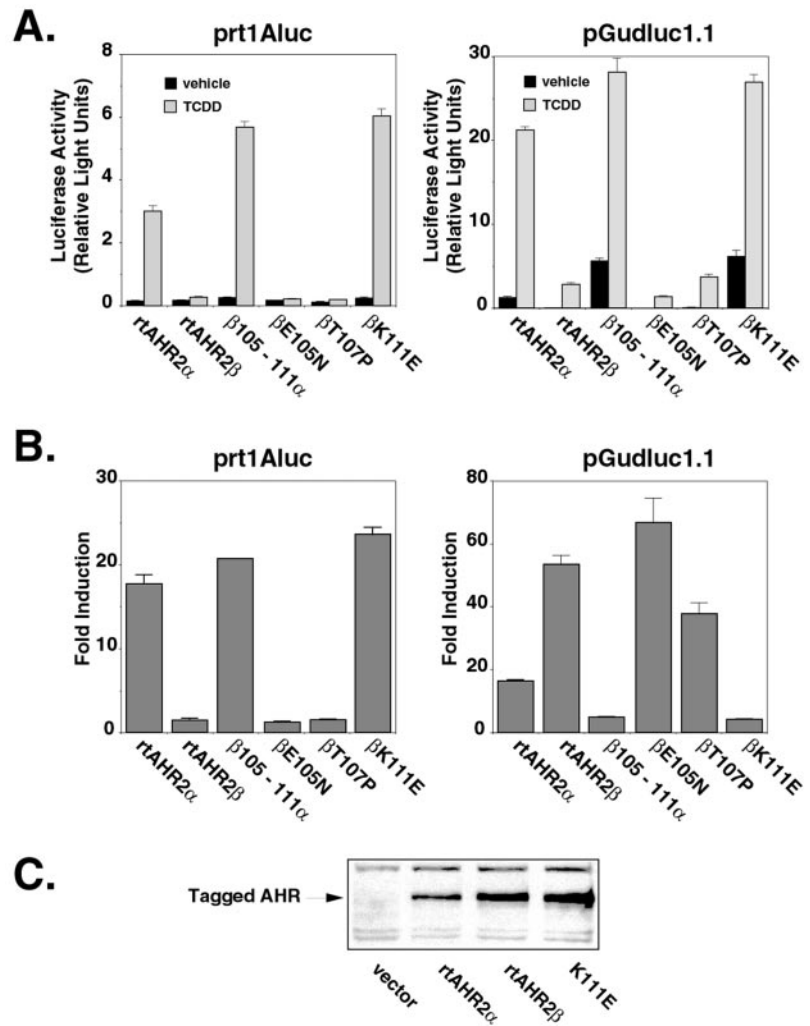
The domain from residues 86 to 250 found to be responsible for the differential transactivation properties of the two rtAHR2s is differentiated by 8 residues. Seven of these differences lie near the N terminus of the PAS-A domain (Fig. 2). To identify specific residues that confer the differential transactivation properties, we made a chimera in which the residues in rtAHR2 $\beta$  from position 105 through 111 were converted to those found in the rtAHR2 $\alpha$  sequence. This involves three alterations: glutamate 105 was converted to asparagine, threonine 107 was converted to proline, and lysine 111 was changed to glutamate. This produced a chimera that behaved like rtAHR2 $\alpha$  (Fig. 4). Activity with prt1Aluc was markedly in-

creased, while the -fold stimulation by TCDD measured with the pGudluc1.1 reporter was decreased. Single substitutions demonstrated that a single residue conveyed much of the differential properties of the rtAHR2s: conversion of the rtAHR2 $\beta$  lysine at position 111 to the corresponding glutamate from rtAHR2 $\alpha$  changed the properties of rtAHR2 $\beta$  to those of rtAHR2 $\alpha$ . The other single residue changes had no effect nor did altering rtAHR2 $\beta$  isoleucine 101 to methionine or alanine 135 to serine (not shown). This single-residue corresponding to position 111 in rtAHR2 $\beta$  therefore has an effect on transactivation, -fold regulation by ligand, and DNA sequence preference.

With both the prt1Aluc and pGudluc1.1 reporters, rtAHR2 $\alpha$  consistently produced a higher level of transactivation than rtAHR2 $\beta$ . This might have been caused by a difference in the level of expression between the two different receptor types. To test this we transfected epitope-tagged rtAHR2 $\alpha$  and rtAHR2 $\beta$  into COS-7 cells and used Western blotting to determine the relative levels of the tagged proteins (Fig. 4, *bottom*). We observed that the two receptors were expressed at comparable levels. In addition, the rtAHR2 $\beta$  K111E mutant was also expressed at levels similar to rtAHR2 $\alpha$  and rtAHR2 $\beta$ . This indicates that the stronger transactivation signal produced by rtAHR2 $\alpha$  and the rtAHR2 $\beta$  K111E mutant is not due to higher levels of expression.

The glutamate at position 110 in rtAHR2 $\alpha$  corresponding to lysine 111 in rtAHR2 $\beta$  is conserved among the known AHRs that bind TCDD. To be certain that the lysine identified at residue 111 in rtAHR2 $\beta$  was not an allelic variant or cloning artifact, RNA was isolated from four separate hatchery strains:

**FIG. 4. TCDD responsiveness of rtAHR2 chimeras created by point mutations in rtAHR2 $\beta$ .** COS-7 cells were transiently transfected with expression vectors for the indicated rtAHR2 chimeras as described for Fig. 1. The change in amino acid altered in rtAHR2 $\beta$  to that found in rtAHR2 $\alpha$  is labeled as the rtAHR2 $\beta$  residue followed by the position number and corresponding altered amino acid. *A*, data are expressed in the upper panels as  $\beta$ -galactosidase normalized relative light units. Light bars, TCDD-exposed; dark bars, vehicle control. *B*, data are expressed as -fold induction by TCDD. The results in both *A* and *B* are expressed as the means of three independent replicates  $\pm$  S.E. *C*, Western blot of the transfected FLAG epitope-tagged rtAHR2 $\alpha$ , rtAHR2 $\beta$ , and the rtAHR2 $\beta$  K111E substitution chimera proteins.



Arlee, Eagle Lake, McConoughy, and Shasta ( $n = 3$  for each strain). Amplified cDNAs for *rtAHR2 $\alpha$*  and *rtAHR2 $\beta$*  were generated. In addition, clones were obtained from cDNA prepared from a rainbow trout gonadal cell line (RTG-2). All samples yielded cDNAs for both *rtAHR2 $\alpha$*  and *rtAHR2 $\beta$* , and all of the *rtAHR2 $\beta$*  cDNAs encoded lysine at position 111 (not shown).

To determine whether the characteristics of rtAHR2 $\beta$  are determined by the presence of lysine at position 111 or the absence of the glutamate residue found at this position in all other AHRs, an rtAHR2 $\beta$  mutant was constructed in which the lysine at position 111 was replaced by alanine (rtAHR2 $\beta$ K111A). This produced a receptor with characteristics that were somewhat intermediate between rtAHR2 $\alpha$  and rtAHR2 $\beta$  (Fig. 5). This protein was a stronger transactivator than rtAHR2 $\beta$ , but weaker than rtAHR2 $\alpha$ . Similarly, when assayed with the pGudluc1.1 reporter, the rtAHR2 $\beta$ K111A mutant had a higher -fold induction by TCDD than the K111E mutant, but lower -fold induction than the normal rtAHR2 $\beta$ . This suggests that the glutamate in rtAHR2 $\alpha$  and the corresponding lysine in rtAHR2 $\beta$  both play important roles in determining the characteristics of their receptors.

We also determined whether the presence of this lysine at the corresponding position in other AHR proteins would produce characteristics of rtAHR2 $\beta$  in these AHRs. When the zebrafish AHR2 was mutated from glutamate to lysine at position 114 (corresponding to position 111 in rtAHR2 $\beta$ ) the mutated *zAHR2* displayed the characteristics of rtAHR2 $\beta$ . These included decreased transactivation, a requirement for prtGudluc1.1 as a reporter, and a low basal level of activity

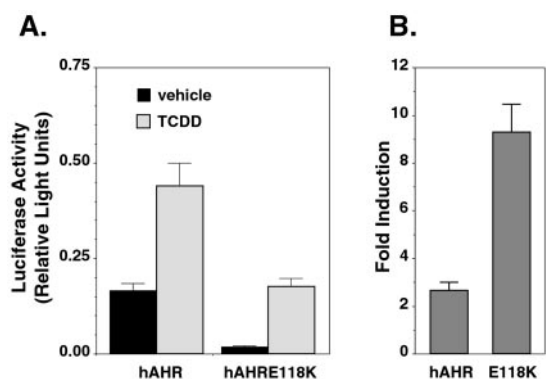
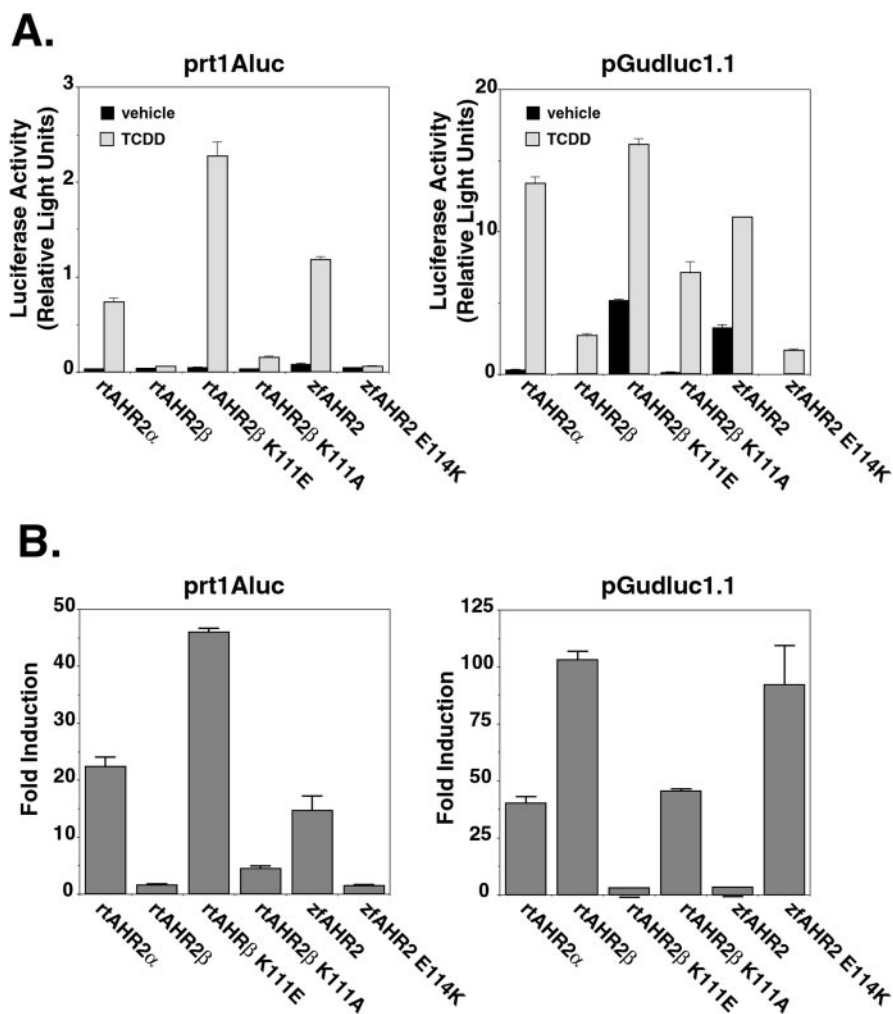
with a corresponding increase in -fold responsiveness to TCDD (Fig. 5).

A similar mutation was made in the human AHR (hAHR) in which glutamate 118 corresponding to K111 in rtAHR2 $\beta$  was mutated to lysine (hAHR118K) (Fig. 6). This also produced a decrease in transactivation and an increase in TCDD responsiveness characteristic of rtAHR2 $\beta$ . These results underscore the importance of this residue, which is consistently conserved among all known AHRs, other than rtAHR2 $\beta$ .

#### DISCUSSION

Our results indicate that the lysine at position 111 in rtAHR2 $\beta$  and the corresponding glutamate at position 110 in rtAHR2 $\alpha$  play important roles in the functions of the proteins that they reside in. The residue at this position affects transactivation, the degree of activation by ligand, and enhancer specificity. In rtAHR2 $\alpha$  the glutamate confers increased transactivation function at either of the two reporters used in our experiments. Substitution of glutamate for the lysine at position 111 on rtAHR2 $\beta$  produced a mutant rtAHR2 $\beta$  with increased transcriptional activity, similar to that of rtAHR2 $\alpha$ . Although the rtAHR2 $\beta$  K111E mutant gained these rtAHR2 $\alpha$ -like properties, it lost other properties, *i.e.* high -fold induction by TCDD with pGudluc1.1 and enhancer specificity, that are characteristic of rtAHR2 $\beta$ . These changes in receptor properties are apparently entirely the result of the lysine at position 111. The residues that differ at this position between rtAHR2 $\alpha$  and rtAHR2 $\beta$  (glutamate in rtAHR2 $\alpha$  and lysine in rtAHR2 $\beta$ ) are substantially different in charge and could therefore be

**FIG. 5. TCDD responsiveness of rainbow trout and zebrafish AHR2 chimeras created by site-directed point mutations.** COS-7 cells were transiently transfected with expression vectors for the indicated chimeras as described for Fig. 1. *A*, data are expressed as  $\beta$ -galactosidase normalized relative light units and -fold induction by TCDD. *Light bars*, TCDD-exposed; *dark bars*, vehicle control. *B*, the results are expressed as the means of three independent replicates  $\pm$  S.E.



**FIG. 6. TCDD responsiveness of human AHR carrying an E to K substitution at the position corresponding to rtAHR2 $\beta$  K111.** Y-1 cells were transiently transfected with expression vectors for the indicated AHR as described in the legend. Data are expressed as (A)  $\beta$ -galactosidase normalized relative light units and (B) -fold stimulation by TCDD. *Light bars*, TCDD-exposed; *dark bars*, vehicle control. The results are expressed as the means of three independent triplicates  $\pm$  S.E.

expected to produce different conformations in the respective proteins.

With the exception of rtAHR2 $\beta$ , all other known AHRs have a glutamate at the residue corresponding to rtAHR2 $\beta$ 's lysine 111. This complete conservation in sequence implies a conserved function among these AHR proteins. Because rtAHR2 $\beta$  does not share this conserved residue, our results suggest that rtAHR2 $\beta$  diverges in function in some way from these other AHRs.

Our results complement a body of work that has identified distinct functional domains in the AHR protein. A number of laboratories have identified residues that are required for transactivation, ligand binding, protein-protein interactions, and DNA binding. In addition to identifying the C terminus as a transactivation domain (17, 37–39) specific subdomains within the C terminus that are necessary for transactivation have been identified (40, 41). Mutation of residue 678 in hAHR blocks transactivation without affecting DNA binding (41). The domain spanning residues 230–421 containing the PAS-B domain has been shown to bind ligand, and residue 381 in hAHR is important for ligand binding (35). Alterations at positions 78 and 216 in the mouse AHR affect DNA binding (42, 43). Substitutions in the basic domain alter DNA binding activity (44). Differences in sensitivity to TCDD toxicity among rodent species have been attributed to differences in the C-terminal sequences of the respective AHRs (45, 46). Our results are significant, because they identify a part of the AHR protein that appears to play a role in the ligand-regulated interactions between these previously identified domains. This suggests that the residue at this position in AHR is in communication with domains responsible for these different processes.

Position 111 in rtAHR2 $\beta$  corresponds to the N-terminal border of the PAS-A domain that is strongly conserved in AHR proteins (Fig. 2). This position lies between the bHLH and PAS domains. Thus, it is close to regions involved in DNA binding, dimerization with ARNT and other proteins, as well as nuclear import and export signals. In addition, ligand binding in the PAS-B region must in some way transmit conformational

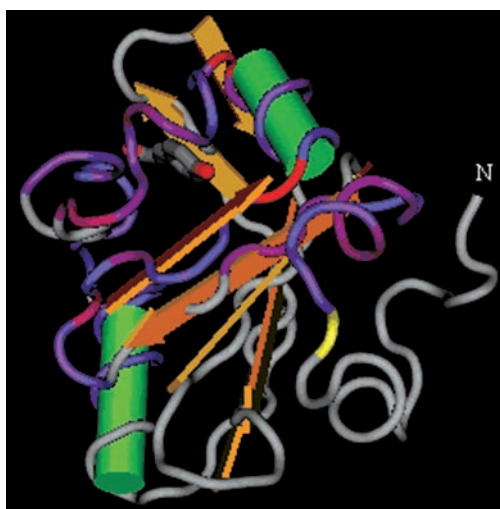


FIG. 7. Location of rtAHR2 $\beta$  residue K111 on a model of PAS domain structure. The amino acid sequence for the PAS-A domain from rtAHR2 $\beta$  was aligned by RPS-BLAST to the consensus PAS domain sequence in the NCBI data base. The structure model was produced by the Cn-3D 3.0 structure viewer ([www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml](http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml)). Red indicates strongly conserved PAS domain sequences. The position of the K111 residue is indicated in yellow.

changes to the PAS-A and helix-loop-helix domains. The residue at position 111 may be in a position to affect these processes. Clearly, a difference in residue polarity at such a central location could alter the function of the rtAHR2s.

The crystal structure for the PAS domain of photoactive yellow protein (PYP) from *Halorhodospira halophila* has been determined (47). This structure has been proposed as a model for PAS domains in eukaryotic proteins such as ARNT and AHR (48). Fig. 7 shows the structure of the PYP PAS domain with the position corresponding to the location of the rtAHR2 $\beta$  K111 highlighted in yellow on the polypeptide backbone (in PYP, this residue is methionine). Although lysine in position 111 in rtAHR2 $\beta$  is unique among AHRs, this region is not strongly conserved among all PAS proteins, and lysine is found at this position in some bacterial PAS family proteins. The degree of conservation between PAS domains from different proteins is indicated by color as well, with warmer colors indicating higher conservation (Fig. 7). It can be seen that the residue in question lies at the junction between the conserved PAS-A domain and the remainder of the N terminus of the protein. In PYP, this segment is referred to as the N-terminal cap, a part of the structure that helps enclose the hydrophobic portion of the PAS core (48). PAS domains are thought to function as protein interaction domains that can be regulated by signals. In the case of PYP, the signal is produced by isomerization of bound chromophore, a *p*-hydroxycinnamoyl anion. In the case of AHR, the signal is presumably generated by occupancy of the ligand-binding domain. Lysine 111 is situated at the edge of the PAS-A domain, in close proximity to the basic and helix-loop-helix domains. This residue is therefore in a position that might impact the interaction between these domains and PAS-A. Such a central position is consistent with the finding that this residue affects transactivation, choice of enhancer elements, and response to agonist.

Whereas our experiments identify a critical residue in AHR proteins and suggest divergent functions for rtAHR2 $\alpha$  and rtAHR2 $\beta$ , they do little to explain the functions of rtAHR2 $\alpha$  and rtAHR2 $\beta$  in rainbow trout. Our results suggest that rtAHR2 $\alpha$  is a more powerful transactivator than rtAHR2 $\beta$  and as such might be more suitable for the regulation of genes that are more highly expressed than those controlled by rtAHR2 $\beta$ .

On the other hand, with some enhancers rtAHR2 $\beta$  appears to be more tightly regulated by TCDD than rtAHR2 $\alpha$ . This tight control is produced primarily by virtue of extremely low basal activity in the absence of TCDD. This would suggest that rtAHR2 $\beta$  might regulate genes that must be very closely controlled. It is interesting that this high -fold induction by rtAHR2 $\beta$  is reporter-specific. This suggests that TCDD is regulating more than the process of translocation to the nucleus and dimerization with ARNT, processes that by themselves should be DNA-independent and unaffected by the enhancers.

As noted previously by Whitelaw *et al.* (37) transcriptional activation and TCDD responsiveness are substantially influenced by the enhancer elements used in the experiments. rtAHR2 $\alpha$  is more responsive to TCDD than rtAHR2 $\beta$  with prt1Aluc, but this is reversed with pGudluc1.1. Although rtAHR2 $\alpha$  appears to be a stronger transactivator than rtAHR2 $\beta$ , we cannot rule out the possibility that there might be promoter sequences with which rtAHR2 $\beta$  is the stronger transactivator. Thus, using the *in vitro* activities of these proteins alone to draw conclusions about function is limited by our current uncertainty about the gene targets for these receptors. Identifying these targets will be an important step forward in understanding AHR function in fish.

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