ELSEVIER

Biochemical Pharmacology 64 (2002) 49–60

Biochemical Pharmacology

Analysis of rainbow trout Ah receptor protein isoforms in cell culture reveals conservation of function in Ah receptor-mediated signal transduction

Richard S. Pollenz[a,*], Brian Necela[b,1], Katherine Marks-Sojka[b,2]

[a]*Department of Biology, University of South Florida, 4202 East Fowler Avenue, SCA 110, Tampa, FL 33620-5200, USA*
[b]*Medical University of South Carolina, Charleston, SC 29425, USA*

Received 29 June 2001; accepted 10 January 2002

Abstract

Two distinct aryl hydrocarbon receptor (AHR) cDNAs have been isolated from rainbow trout. The encoded receptor protein products termed rtAHR2α and rtAHR2ß are 97% identical at the amino acid level but are reported to have distinct functions with regard to AHR-mediated gene regulation. To test this hypothesis, the two proteins were evaluated functionally both *in vitro* and in a Chinese hamster lung cell line, E36. To facilitate analysis, both rtAHR2 isoforms were tagged with the FLAG peptide and could be expressed and quantified in a rabbit reticulocyte lysate. However, both proteins failed to form functional complexes with mammalian or rainbow trout AHR nuclear translocator protein (ARNT) that could associate with xenobiotic response elements (XREs) in a ligand-dependent manner *in vitro*. In contrast, both proteins exhibited positive function on AHR-mediated signaling when expressed in the E36 cell line. Both rtAHR2 isoforms showed a cytoplasmic distribution in the unliganded state and could drive the expression of a reporter gene under control of the trout *CYP1A3* promoter. Although both proteins induced reporter gene activity to the same magnitude, the EC_{50} values of the two isoforms for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) differed by an order of magnitude, with the rtAHR2ß isoform less responsive to TCDD. When the functions of the rtAHR2 isoforms were tested in the context of the dominant negative $rtARNT_a$ protein, TCDD-mediated induction of reporter gene activity was reduced as the level of $rtARNT_a$ protein increased. In summary, both rtAHR2 isoforms appear to exhibit positive function in AHR-mediated signaling, suggesting conservation of function.

Keywords: Rainbow trout; Aryl hydrocarbon receptor; ARNT; TCDD; Cell culture; Gene regulation

1. Introduction

Fish are one of the most sensitive vertebrates to TCDD and exhibit a wide range of developmental defects following embryonic exposure. The observed defects include craniofacial malformations, growth arrest, edema, and cardiovascular dysfunctions [1–5]. The mechanisms through which TCDD mediates these effects are unclear but are likely to involve the AHR signaling pathway. Support for involvement of the AHR comes primarily from the fact that the structure–activity relationships of polychlorinated dibenzodioxins for producing many of the toxic responses in fish are similar to what has been shown in better characterized mammalian models [2,6–8]. In addition, over the past few years the AHR signaling pathway has been shown to be functional in fish, and numerous AHR and ARNT genes have been characterized [9–18].

* Corresponding author. Tel.: +1-813-974-1596; fax: +1-813-974-3263.
E-mail address: pollenz@chuma1.cas.usf.edu (R.S. Pollenz).

[1] Current address: Laboratory of signal transduction, NIEHS, 111 Alexander Drive, Research Triangle Park, NC 27705, USA.

[2] Current address: DAKO Corp., 6392 Via Real, Carpinteria, CA 93013, USA.

Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AHR, aryl hydrocarbon receptor; rtAHR, rainbow trout AHR; ARNT, aryl hydrocarbon receptor nuclear translocator; rtARNT, rainbow trout ARNT; mARNT, mouse ARNT; bHLH, basic helix-loop-helix; PAS, PER-ARNT-SIM; GAR-HRP, goat anti-rabbit antibodies conjugated to horseradish peroxidase; GAR-TR, goat anti-rabbit IgG conjugated to Texas red; TBS, Tris-buffered saline; TTBS, Tris-buffered saline with Tween 20; TBE, Tris, boric acid, EDTA; FBS, fetal bovine serum; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; PMSF, phenylmethylsulfonyl flouride; TNT, transcription and translation; XRE, xenobiotic response element; ß-gal, ß-galactosidase; and EC_{50}, effective concentration for 50% activity.

0006-2952/02/$ – see front matter
PII: S0006-2952(02)01061-4

At present, full-length AHR sequences have been isolated from Atlantic tomcod [12], *Fundulus heteroclitis* (killifish, [13]), *Oncorhynchus mykiss* (rainbow trout, [11]), and *Danio rerio* (zebrafish, [15]). The most striking findings from these studies have been the isolation of multiple cDNAs that encode distinct AHR proteins in each of the fish. In killifish, for example, two highly divergent AHRs were characterized that exhibit only 40% amino acid identity to each other [13]. These proteins, termed fhAHR1 and fhAHR2, were shown to bind TCDD *in vitro*, but the genes showed distinct patterns of expression in *Fundulus* tissue.

In contrast, two distinct AHR cDNAs have been isolated from rainbow trout that both segregate with the AHR2 clade [11]. These proteins termed rtAHR2α and rtAHR2ß are 97% identical at the amino acid level and appear to have similar patterns of expression in trout tissues. However, despite the high level of identity between the two proteins, recombinant expression in the mammalian COS-7 cell line suggests that the two receptors show distinct preferences for trout or mammalian enhancer sequences [11,19]. Thus, it has been hypothesized that the two isoforms may play different roles in the biological responses produced by AHR agonists such as TCDD or to endogenous ligands [11,19]. To test the validity of this hypothesis, studies were carried out *in vitro* and in mammalian cell culture lines to directly assess the function of the rtAHR2 proteins in the context of mammalian and rainbow trout ARNT protein. In contrast to previous reports, the results presented here indicate that both rtAHR2α and rtAHR2ß have positive function in AHR-mediated signaling in a Chinese hamster lung cell line (E36) and can effectively drive a reporter gene construct under the control of the trout *CYP1A3* promoter. In addition, studies revealed that the concentration–response curve for TCDD-mediated induction of *CYP1A3* driven reporter gene activity was shifted nearly an order of magnitude to the right when rtAHR2ß was used in the assay. Importantly, the current studies also show that while both rtAHR2 proteins functioned in cell culture assays, neither protein functioned in *in vitro* assays when compared with mammalian AHR proteins.

2. Materials and methods

2.1. Antibodies

Specific antibodies against either the mouse AHR (A-1, A-1A) or mouse ARNT protein (R-1) are identical to those described previously [20,21]. Specific antibodies against rainbow trout ARNT (rt84) are identical to those described previously [10]. For western blot analysis, GAR-HRP was utilized. For immunohistochemical studies, GAR-TR was used. Both of these reagents were purchased from Jackson Immunoresearch. Polyclonal rabbit ß-actin antibodies and polyclonal rabbit antibodies against FLAG were purchased from the Sigma Chemical Co.

2.2. Buffers

PBS: 0.8% NaCl, 0.02% KCl, 0.14% Na_2HPO_4, 0.02% KH_2PO_4 (pH 7.4).
2X Gel sample buffer: 125 mM Tris (pH 6.8), 4% SDS, 25% glycerol, 4 mM EDTA, 20 mM dithiothreitol, 0.005% bromphenol blue.
TBS: 50 mM Tris, 150 mM NaCl (pH 7.5).
TTBS: 50 mM Tris, 0.2% Tween 20, 150 mM NaCl (pH 7.5).
TTBS+: 50 mM Tris, 0.5% Tween 20, 300 mM NaCl (pH 7.5).
BLOTTO: 5% dry milk in TTBS.
2X Lysis buffer: 50 mM HEPES (pH 7.4), 40 mM sodium molybdate, 10 mM EGTA, 6 mM $MgCl_2$, 20% glycerol.
5X Gel shift buffer: 50 mM HEPES (pH 7.5), 15 mM $MgCl_2$, 50% glycerol.
0.5X TBE: 45 mM Tris-borate, 1 mM EDTA.
MENG: MOPS, EDTA, NaN_3, glycerol.

2.3. Cells and growth conditions

Hepa-1 and Type I Hepa-1 variants (LA-I) were gifts from Dr. Jim Whitlock, Jr. (Department of Pharmacology, Stanford University). E36 cells were a gift from Dr. Alan Schwartz (Washington University). Hepa-1 cells were propagated at 37° in Dulbecco's Minimum Essential Medium containing 5% FBS. E36 cells were propagated at 30° in α-Minimum Essential Medium containing 10% FBS. All cells were passaged at 1-week intervals and used in experiments during a 2-month period.

2.4. Generation of expression constructs

Full-length rtAHR2α and rtAHR2ß cDNA clones were gifts from Dr. Richard Peterson (University of Wisconsin). The coding regions for each gene were amplified by PCR using a gene specific 5′ primer that spanned the ATG start site and a 3′ primer unique to the last 8 codons of each rtAHR2 that was in frame with the coding sequence for the eight amino acid FLAG sequence followed by a translation termination codon. The resulting full-length rtAHR2 cDNAs were ligated into pcDNA3.1 (Clontech) to produce FLAG-rtAHR2α and FLAG-rtAHR2ß. Murine AHR cDNA was amplified from pSPORT'M'AHR (gift from Dr. Chris Bradfield, University of Wisconsin) by PCR with a 3′ primer that contained the coding sequence for the eight amino acid FLAG peptide and a 5′ primer that spanned the start codon. The resulting fragment was ligated onto the mammalian expression vector pcDNA3.1 to produce FLAG-mAHR. All constructs were sequenced prior to use. The rtARNT and mARNT expression

constructs used in these studies have been detailed previously [10,17,18].

2.5. In vitro expression of protein

Recombinant protein was produced from expression constructs using the TNT™ Coupled Rabbit Reticulocyte Lysate Kit essentially as detailed by the manufacturer (Promega). Upon completion of the 90-min reaction, samples were either combined with an equal volume of 2X gel sample buffer and boiled for 5 min, or stored at −80° for use in functional studies.

2.6. Production of total cell lysates and cytosol

Total cell lysates for western blot analysis were prepared by sonicating cell pellets in 1X lysis buffer containing 0.5% Nonidet P-40 as detailed previously [10,20,21]. Cytosol was prepared from cells by homogenization in MENG buffer supplemented with leupeptin (10 μg/mL), aprotinin (20 μg/mL), and PMSF (100 μM). Homogenates were centrifuged at 22,000 *g* for 15 min in a refrigerated microcentrifuge, and aliquots of the supernatant were stored at −70° prior to use in EMSA. Protein concentrations were determined by the Coomassie Blue Plus assay (Pierce), using BSA as the standard.

2.7. Western blot analysis and quantification of protein

Protein samples were resolved by denaturing electrophoresis on discontinuous polyacrylamide slab gels (SDS–PAGE) and were transferred electrophoretically to nitrocellulose. Immunochemical staining was carried out with various concentrations of primary antibody (see text and figure legends) in BLOTTO buffer supplemented with DL-histidine (20 mM) for 1–2 hr at 22°. Blots were washed with three changes of TTBS+ for a total of 45 min. The blot was then incubated in BLOTTO buffer containing a 1:10,000 dilution of GAR-HRP for 1 hr at 22° and washed in three changes of TTBS+ as above. Prior to detection, the blots were washed in TBS for 5 min. Bands were visualized with the enhanced chemiluminescence (ECL) kit as specified by the manufacturer (Amersham). Multiple exposures of each set of samples were produced. The relative concentration of target protein was determined by computer analysis of the autoradiographs as detailed previously [10,17,18,20].

2.8. In vitro activation of AHR:ARNT complexes and EMSA

For EMSA, a double-stranded fragment corresponding to the consensus XRE-1 of the murine *CYP1A1* promoter (mXRE) has been described previously [22]. A double-stranded fragment corresponding to the consensus XRE2 of the trout *CYP1A3* promoter also has been described previously [9]. For *in vitro* activation, approximately 25 ng of recombinant AHR and ARNT protein or 50 μg of cytosol were combined with MENG buffer in a 60-μL reaction. Each sample was then supplemented with TCDD (16 nM) or DMSO (0.5%) and incubated at 30° for 2 hr. The activated samples (15 μL) were then incubated at 22° for 15 min in 1X gel shift buffer supplemented with KCl (80 mM) and poly(dIdC) (0.1 mg/mL). Approximately 4 ng of ^{32}P-labeled XRE was added to each sample, and the incubation was continued for an additional 15 min at 22°. The samples were resolved on 5% acrylamide/0.5% TBE gels, dried, and exposed to film. In some instances, activated samples were analyzed by western blotting to assess expression of AHR and ARNT.

2.9. Transfection and reporter gene assay

The reporter gene construct used in these studies was p–1897*Om*1A3*luc* that contains 1897 bp of promoter sequence from the rainbow trout *CYP1A3* gene [23]. This sequence contains five putative XREs. A mixture was generated that contained various combinations of the AHR or ARNT expression plasmids indicated in the text or figure legends, as well as a constitutive ß-gal expression vector (pSV-ß-gal) and LipofectAMINE (Gibco) transfection reagent. The volume of the mixture was sufficient to transfect 6–8 dishes (35 mm) of cells and assured that 3–4 control and 3–4 treatment samples were all transfected with the exact same solution of DNA. In all cases, the total amount of DNA transfected in the different test groups was constant. After a 6- to 8-hr transfection period, cells were treated with TCDD or DMSO for 16 hr, or allowed to recover for 16 hr and then treated for 8 hr. Cells were then scraped from plates in 1X Reporter Gene Buffer (Promega), and luciferase and ß-gal activity was quantified as detailed by the manufacturer. The relative luciferase units (RLU) were then divided by the ß-gal activity to control for transfection efficiency. In all experiments, transfection efficiency was generally consistent between all samples; thus, the overall trend of the data was never changed by the normalization procedure. The mean ± standard deviation is reported for three independent samples. The EC_{50} for TCDD was determined using nonlinear regression in the Statistica software package (StatSoft).

2.10. Immunofluorescence staining and microscopy

All immunocytochemical procedures (cell plating, fixation, staining, and photography) were carried out as previously described [20,21]. Cells were observed on an Olympus microscope using the 568 nm filter. On the average, 15–20 fields (5–20 cells each) were evaluated on each coverslip, and 3–4 fields were photographed to generate the raw data. Experiments were repeated at least two times.

2.11. Gal4 transactivation assays

For the generation of Gal4/rtAHR2 fusions, PCR was used to amplify 200 and 300 nucleotide fragments from the most 3′ coding regions for both rtAHR2α (corresponding to amino acids 759-1059 and 859-1059) and rtAHR2ß (corresponding to amino acids 758-1058 and 858-1058). Fragments were ligated into pc424 [17,18], immediately downstream of the Gal4 DNA binding domain. To evaluate the transcriptional activity of the chimeric proteins, each construct was cotransfected into the Hepa-1 cells with the reporter gene, pFR-LUC [17,18], and pSV-ß-gal. Luciferase and ß-gal assays were performed as described above. In some experiments, equal amounts of total cell lysate, prepared from cells transfected with the identical DNA transfection mixture used to generate the luciferase data, were evaluated for the expression of the Gal4/rtAHR2 chimeras with an anti-Gal4 antibody.

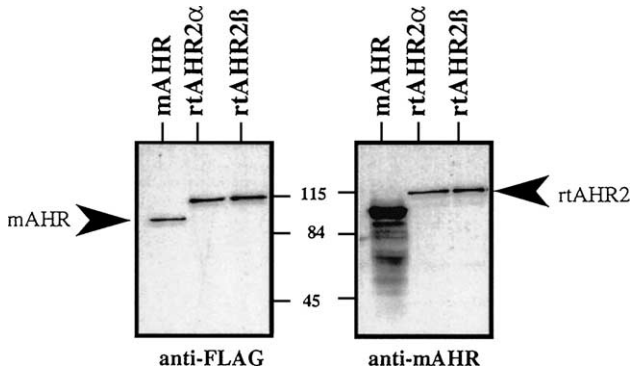

Fig. 1. Western blot analysis of AHR proteins expressed *in vitro*. FLAG-rtAHR2α, FLAG-rtAHR2ß, and FLAG-mAHR proteins were expressed *in vitro* and analyzed by western blotting using 1 μg/mL of M5 anti-FLAG IgG (shown on the left) or 1 μg/mL of A-1A IgG (shown on the right), followed by GAR-HRP (1:10,000). The molecular mass of standard proteins is indicated in the center in kilodaltons. Note that the level of protein expression in all lanes is the same when using the M5 antibody. All experiments were performed 2–3 times.

3. Results and discussion

3.1. rtAHR2α and rtAHR2ß activity in vitro

Previous studies suggest that the rtAHR2α and rtAHR2ß proteins have distinct functionality in AHR-mediated signaling [11,19]. However, the molecular nature of the differences between these two proteins is unclear. In mammalian systems, a great wealth of functional information on the AHR and ARNT proteins has been obtained from the expression and activation of the proteins *in vitro*. Therefore, studies were performed to determine whether *in vitro* analysis would provide insight into the functional differences between the rtAHR2 proteins. Since an important aspect of the *in vitro* analysis is the ability to directly assess the level of recombinant protein expression, each rtAHR2 cDNA was ligated into an expression plasmid so that the eight amino acid FLAG™ epitope was expressed at the C-terminus of each protein to produce FLAG-rtAHR2α and FLAG-rtAHR2ß. The FLAG tag was also expressed on the C-terminus of the mouse AHR (FLAG-mAHR).

Fig. 1 shows that both FLAG-mAHR and FLAG-rtAHR2 proteins were detected by western blot analysis with the M5 anti-FLAG antibody and that each protein was expressed at the same level in the *in vitro* TNT reactions. As expected, both rtAHR2 proteins migrated with a molecular mass of approximately 115 kDa compared with 95 kDa for the mAHR. In addition, both rtAHR2 isoforms could be detected with antibodies produced against mouse AHR, although the sensitivity of detection was much less than that for the mAHR due to the sequence divergence of the AHRs (Fig. 1).

Having shown that both rtAHR2 proteins could be expressed and quantified, experiments next focused on the ability of the proteins to dimerize with ARNT and bind murine (mXRE) or trout (rtXRE) DNA sequences *in vitro*. Therefore, equal amounts of recombinant AHR and ARNT were combined and activated in the presence or absence of TCDD for 2 hr at 30°. The activated samples were then analyzed by (i) EMSA with the mXRE, (ii) EMSA with the rtXRE, and (iii) western blotting to confirm the level of AHR and ARNT protein expression. Fig. 2 shows a representative experiment. FLAG-mAHR formed complexes with mARNT or the $rtARNT_b$ isoform that associated equally well with either the rtXRE or mXRE oligonucleotide in a TCDD-dependent manner (Fig. 2A, lanes 2, 4, 8, and 10). Since both XREs were the same length and labeled to the same specific activity, the intensities of the shifted bands are directly comparable. In contrast, when the FLAG-rtAHR2α was used in the experiments, there were no detectable shifts of either XRE sequence (Fig. 2A, lanes 5, 6, 11, and 12), even following exposure of the gel for the equivalent of 168 hr (data not shown). The FLAG-rtAHR2ß protein was also evaluated for DNA binding in the presence mARNT or $rtARNT_b$ and failed to produce any detectable gel shifts (data not shown). To confirm that the lack of DNA binding of the FLAG-rtAHR2α protein was not due to differences in the amount of AHR used in the assay, western blot analysis was carried out on the identical samples used in lanes 1–6 of Fig. 2A. Fig. 2B shows that the amounts of FLAG-mAHR and FLAG-rtAHR2α are essentially identical.

Previous studies have shown that rainbow trout gonad cells (RTG-2) express rtAHR protein capable of binding TCDD and driving gene expression and that AHR·ARNT complexes can be isolated that bind DNA *in vitro* [8,9]. Thus, possible explanations for the reduced function of the FLAG-rtAHR2 proteins expressed *in vitro* include the need for endogenous fish proteins to stabilize the rtAHR2, the need for additional proteins for the DNA binding interaction in fish, or the temperature of the assay. To test some of these possibilities, cytosol was produced from RTG-2 cells

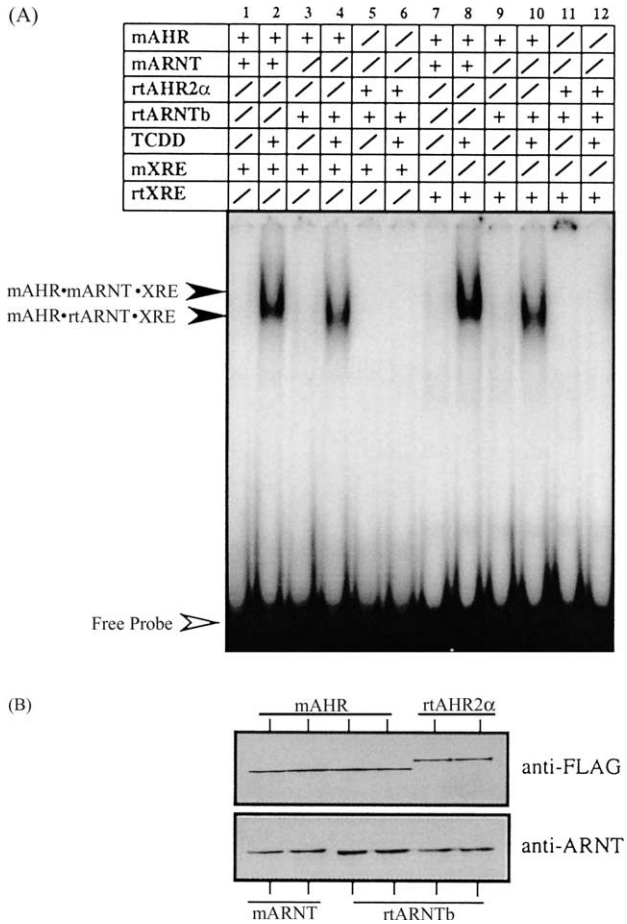

Fig. 2. EMSA of recombinant AHR and ARNT proteins. (A) FLAG-mAHR, mARNT, FLAG-rtAHR2α, and FLAG-rtARNT$_b$ proteins were expressed *in vitro* combined as indicated and incubated with TCDD (16 nM) or DMSO (0.5%) for 2 hr at 30°. Activated samples were then mixed with ^{32}P-labeled mXRE or rtXRE and analyzed by EMSA as detailed. Specifically shifted and free XRE are indicated. (B) Aliquots from samples 1–6 were analyzed by western blotting with M5 anti-FLAG (1 μg/mL) or anti-ARNT R-1 antibody (1 μg/mL) followed by GAR-HRP (1:10,000) to demonstrate equal expression of protein. All experiments were performed 2–3 times.

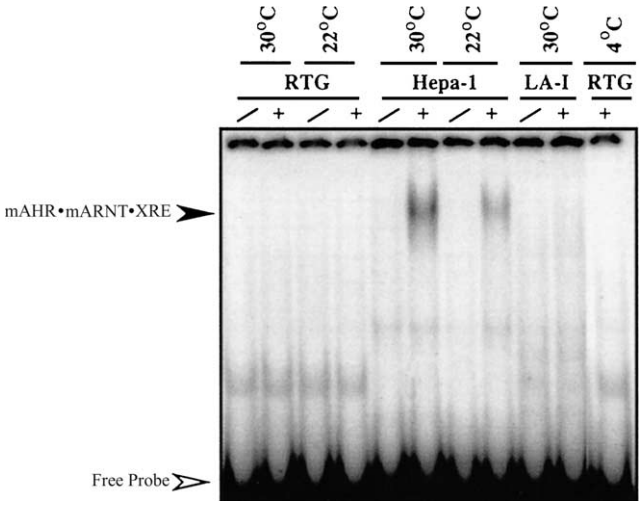

Fig. 3. EMSA of cell culture cytosol. Cytosol was prepared from rainbow trout gonad (RTG), murine Hepa-1 cells (Hepa-1), and the Hepa-1 variant (LA-I) as detailed. Fifty micrograms of cytosol was incubated with TCDD (16 nM) or DMSO (0.5%) for 2 hr at 4°, 22° or 30°. Activated samples were then mixed with ^{32}P-labeled mXRE and analyzed by EMSA as detailed. Specifically shifted and free XRE are indicated. All experiments were performed 2–3 times.

and activated *in vitro* with TCDD at various temperatures in the presence or absence of FLAG-rtAHR2α. A representative study is shown in Fig. 3. Control cytosol from the mouse Hepa-1 cell line produced a TCDD-dependent gel shift when activated at 22 or 30°, while cytosol isolated from the RTG-2 cells or the Hepa-1 variant (LA-I) was unable to produce a DNA binding complex at any of the assay temperatures (Fig. 3). When RTG-2 cytosol was spiked with recombinant FLAG-rtAHR2α protein, the sample still did not produce any detectable gel shift (data not shown). Thus, the inability to produce functional rtAHR2 protein *in vitro* may be related to the stability of the protein and its interactions with mammalian hsp90 or the ARA9 protein at the assay temperatures. Trout usually live at temperatures below 15°, but recombinant proteins are produced at 30° in the TNT assay and this may affect the folding of the rtAHR2 or its interaction with other proteins *in vitro*. Indeed, previous studies utilizing ligand binding assays on trout AHR have noted the difficulty of working with these proteins in *in vitro* assays [24,25]. In addition, similar difficulties have been described with the *in vitro* expression and analysis of the *F. heteroclitis* AHR isoforms [13]. Thus, the results presented here clearly show that the function of the rtAHR2 protein is compromised *in vitro*, and alternate methods are needed for functional analysis.

3.2. Transactivation potential of rtAHR2α and rtAHR2β

Since differences in the function of rtAHR2α and rtAHR2ß could not be evaluated effectively *in vitro*, studies turned to the analysis of these proteins in a cell culture model system. Previous studies suggest that rtAHR2ß has reduced transactivation potential when evaluated in the COS-7 cell culture model [11,19]. However, molecular analysis has not been used to directly test the rtAHR2 isoforms for transactivation function. To carry out such an analysis, PCR was used to generate C-terminal fragments from both rtAHR2α and rtAHR2ß. The fragments were ligated downstream of the GAL4 DNA binding domain and analyzed for the ability to drive luciferase expression from a Gal4-dependent reporter construct (transactivation potential) as detailed previously [17,18]. Fig. 4 shows that all of the rtAHR2 C-terminal constructs generated contain potent transactivation domains. In addition, there were no differences in the levels of activity between the domains isolated from rtAHR2α and rtAHR2ß. The finding that the C-terminal domains of the rtAHR2 isoforms possess potent transactivation function are consistent with studies of mammalian AHR [26–28] and suggest that both proteins have the potential for positive action as transcription factors.

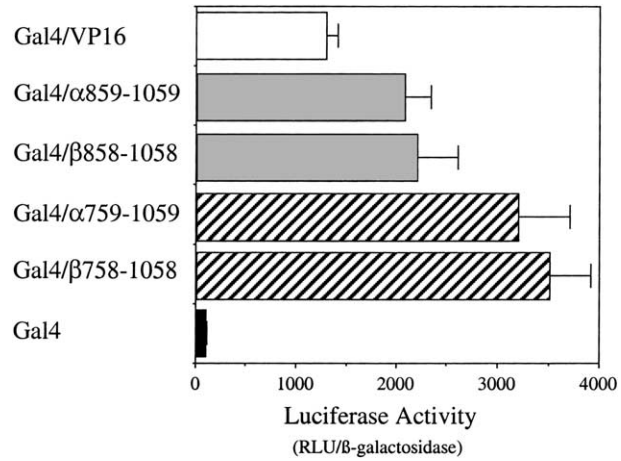

Fig. 4. Transactivation assay of rtAHR isoforms. The indicated C-terminal domains of rtAHR2α and rtAHR2ß were ligated to the Gal4 DNA binding domain as indicated in Section 2. Each construct was transfected into three plates of Hepa-1 cells along with pFR-LUC and pSV-ß-gal, and cells were propagated for 16 hr. Cells were scraped from plates and analyzed for luciferase and ß-gal activity. The level of luciferase activity (RLU) was divided by the level of ß-gal activity to normalize for transfection efficiency. Each bar represents the mean ± SD of three independent samples. The Gal4/VP-16 construct was used as a positive control.

3.3. *rtAHR2α and rtAHR2ß activity in cell culture*

Having established that both rtAHR2s contained functional transactivation domains, studies focused on whether rtAHR2α and rtAHR2ß exhibited differences in the ability to drive reporter constructs containing the trout *CYP1A3* promoter. The *CYP1A3* reporter gene utilized was 1897 bp in length and contained five xenobiotic response elements [23]. The cell line used in these studies was a Chinese hamster lung cell line, E36. The choice of this cell line was based on several criteria. First, this cell line has been very effective in the evaluation of the subcellular trafficking and function of the murine AHR [29,30]. Second, E36 cells express low levels of endogenous AHR and ARNT protein; thus, exogenous expression of recombinant AHR or ARNT protein can be quantified effectively [29,30]. Finally, E36 cells can be propagated at 30°, and this temperature may be more appropriate for the analysis of fish proteins than 37°.

In the first set of studies, different combinations of FLAG-rtAHR2 and rtARNT$_b$ expression plasmids were analyzed for their ability to drive luciferase activity by the trout *CYP1A3* promoter in a TCDD-dependent manner. A representative experiment is shown in Fig. 5. In the absence of any rtAHR2 DNA, luciferase activity was low and showed no significant induction in the presence of TCDD (lanes 1 and 2). These results are consistent with the low level of AHR expressed in the cells [20,29]. In contrast, E36 cells transfected with either of the FLAG-rtARH2 constructs in the presence or absence of rtARNT$_b$ showed a 5- to 6-fold induction of luciferase activity in a TCDD-dependent manner. The overall level of luciferase activity was increased by approximately 6-fold when rtARNT$_b$ was included in the transfection mixture. Importantly, these studies show that *both* FLAG-rtAHR2α and FLAG-rtAHR2ß can drive the expression of luciferase from the *CYP1A3* promoter in a TCDD-dependent manner. In addition, the magnitude of luciferase activity and the overall level of induction were essentially identical between the

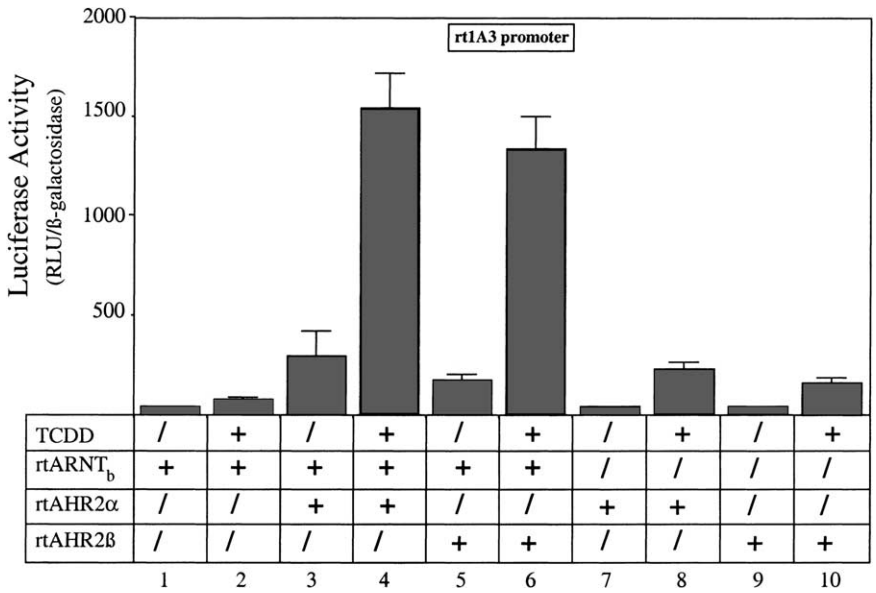

Fig. 5. TCDD-induced reporter gene activation in E36 cells transfected with rtAHR2α or rtAHR2ß. E36 cells were cotransfected with different combinations of rtARNT$_b$, FLAG-rtAHR2α, and FLAG-rtAHR2ß, as indicated below each bar, along with p–1897*Om*1A3*luc* and pSV-ß-gal and propagated for 16 hr. The total amount of DNA used in each transfection was constant. Cells were then treated with TCDD (10 nM) or DMSO (0.5%) for 8 hr and evaluated for luciferase and ß-gal activity. The relative luciferase units (RLU) were divided by the ß-gal activity to control for transfection efficiency. Data are presented as the means ± SD for three samples.

two rtAHR2 isoforms, a finding that was consistent and repeatable in the E36 cell line. In addition, similar data sets were obtained when reporter genes that contained promoter sequences derived from the human *CYP1B1* promoter were used (data not shown).

To confirm that the results were not influenced by differences in the amount of rtAHR2 protein expressed in the transfected cells, samples of cell lysates were analyzed for rtAHR2 expression by western blotting with anti-FLAG and anti-mAHR antibodies. Unfortunately, both antibodies were unable to detect rtAHR2 expression due to low sensitivity to the expressed proteins (data not shown). Thus, expression of the two rtAHR proteins was evaluated by indirect immunofluorescence microscopy with the anti-FLAG antibodies. E36 cells were propagated on coverslips and transfected with an aliquot from the identical transfection mixture used to generate the luciferase data sets shown in Fig. 5 (lanes 3 and 6). Cells were then fixed and stained with anti-FLAG IgG. Representative fields are shown in Fig. 6. The data clearly show that both

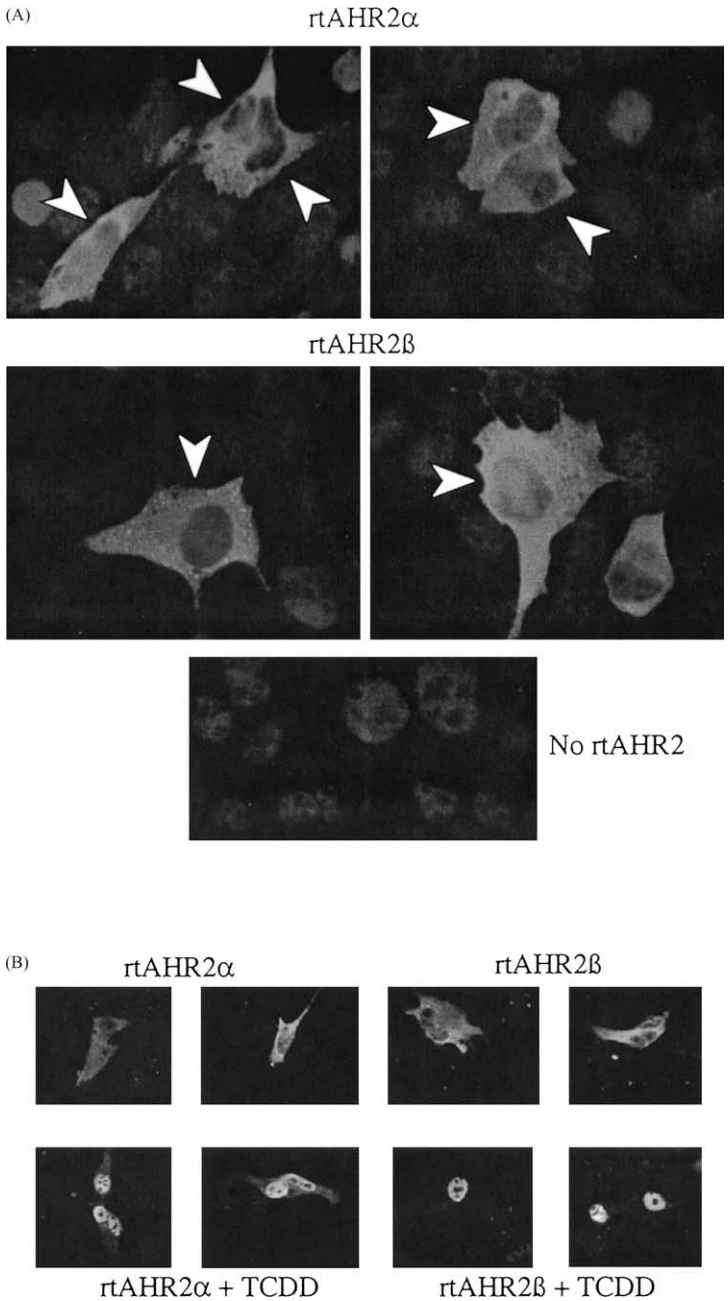

Fig. 6. Subcellular localization of FLAG-rtAHR2α and FLAG-rtAHR2ß transfected into E36 cells. (A) The same transfection mixture used to generate the data in Fig. 5 (lanes 3 and 5) was used to transfect E36 cells with FLAG-rtAHR2α or FLAG-rtAHR2ß. Following incubation at 30° for 16 hr, cells were fixed and stained with 1 μg/mL of anti-FLAG IgG followed by GAR-TR (1:750). (B) E36 cells were transfected with either FLAG-rtAHR2α or FLAG-rtAHR2ß. Following a 24-hr recovery period, cells were treated with TCDD (5 nM) or DMSO (0.5%) for an additional 3 hr and then fixed and stained with anti-FLAG as detailed above. All photos represent identical exposure times. These studies were performed two times.

rtAHR2 proteins can be detected and are expressed predominately within the cytoplasmic compartment of untreated E36 cells. Since the pictures represent identical exposure times, it can be observed that both sets of cells show similar levels of fluorescence intensity, and thus are likely expressing similar levels of rtAHR2 protein (Fig. 6A). In addition, the absolute number of AHR reactive cells in each field was quantified for both FLAG-rtARNT2α and FLAG-rtAHR2ß and found to be essentially the same (data not shown). This finding was consistent with the ß-gal transfection control data that showed similar levels of activity in the cells transfected with either FLAG-rtAHR2α or FLAG-rtAHR2ß. Taken together, these studies indicate that the unliganded rtAHR2s are localized to the cytoplasm and appear to be expressed at similar levels, and that the normalized reporter gene values reflect similar functionalities of these proteins under the conditions of the assay. To further evaluate the function of these rtAHT2 proteins, E36 cells were propagated on coverslips and transfected with either FLAG-rtAHR2α or FLAG-rtAHR2ß expression constructs. Cells were treated with TCDD (5 nM) or DMSO for 3 hr, and the cells were fixed and stained with anti-FLAG IgG. Panel B of Fig. 6 shows that both rtAHR2 proteins are capable of translocating to the nucleus following TCDD exposure. These results are consistent with the fact that both proteins transactivate reporter gene activity (Fig. 5).

3.4. EC_{50} of rtAHR2ß and rtAHR2α in cell culture

Having established that the E36 cell culture model allowed functional analysis of the rtAHR2 proteins, studies next focused on whether the two isoforms showed similar responses to TCDD. FLAG-rtAHR2 and $rtARNT_b$ expression plasmids, along with the *CYP1A3* reporter and pSV-ß-gal construct were transfected into triplicate wells of E36 cells and evaluated for luciferase activity following incubation with graded concentrations of TCDD. A representative experiment is shown in Fig. 7. Consistent with the data presented in Fig. 5, each rtAHR2 induced luciferase activity above baseline; however, the concentration–response curve for FLAG-rtAHR2ß was shifted to the right by an order-of-magnitude. The EC_{50} value for TCDD-mediated induction of the *CYP1A3* driven reporter gene was 100 ± 15 pM for FLAG-rtAHR2α, while the EC_{50} was 1050 ± 150 pM for FLAG-rtAHR2ß. Thus, FLAG-rtAHR2α exhibits an EC_{50} value for TCDD that is within the same range determined for mammalian AHRs [31–33], while FLAG-rtAHR2ß appears to be less responsive. This observed shift in concentration response for FLAG-rtARNT2ß is consistent with previous data that evaluated the ability of the rtAHR2 proteins to drive a reporter gene under control of the murine *CYP1A1* promoter [11]. Thus, the results presented here suggest that there are some functional differences in the two rAHR2 isoforms. This

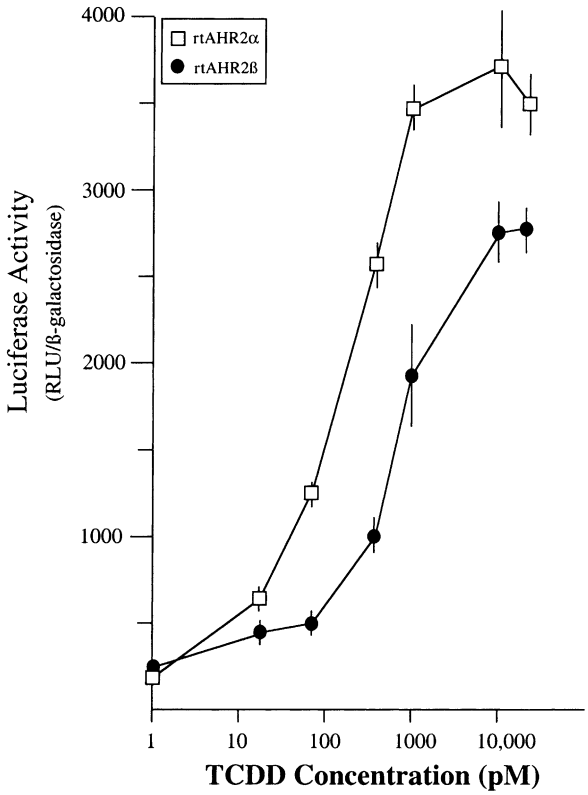

Fig. 7. Concentration–response curve for TCDD-induced reporter gene activation in E36 cells transfected with rtAHR2α or rtAHR2ß. E36 cells were cotransfected with a mixture of either $rtARNT_b$ and FLAG-rtAHR2α, or $rtARNT_b$ and FLAG-rtAHR2ß along with p–1897*Om*1A3*luc* and pSV-ß-gal. Sixteen hours after transfection, cells were incubated with 1, 40, 100, 400, 1000, 10,000, and 20,000 pM TCDD (final concentration) for 8 hr and then evaluated for luciferase and ß-gal activity. The relative luciferase units (RLU) were divided by the ß-gal activity to control for transfection efficiency. Data are presented as the means ± SD for three independent samples. Key: (□) FLAG-rtAHR2α, and (●) FLAG-rtAHR2ß.

indicates that it would be possible for a cell expressing a given amount of rtAHR2ß protein to be non-responsive to ligand exposure while a cell expressing a similar amount of rtAHR2α protein would respond at that concentration. It is important to note, however, that since both proteins can induce luciferase activity to the same overall magnitude within the assays performed in this study, rtAHR2ß appears unlikely to exert a negative function on *CYP1A* gene regulation as suggested [11,19].

3.5. Dominant negative activity of $rtARNT_a$

Studies next focused on the function of the rtAHR2α in the presence of the two rtARNT protein isoforms. Previous studies have demonstrated that the $rtARNT_a$ protein can exert dominant negative activity of AHR-mediated signaling both *in vitro* and in cell culture [10,17,18]. However, the function of the two rtARNT isoforms has always been evaluated in the context of the mouse AHR protein. Thus, to determine whether the $rtARNT_a$ protein exhibited negative activity in the context of rtAHR2, expression plasmids

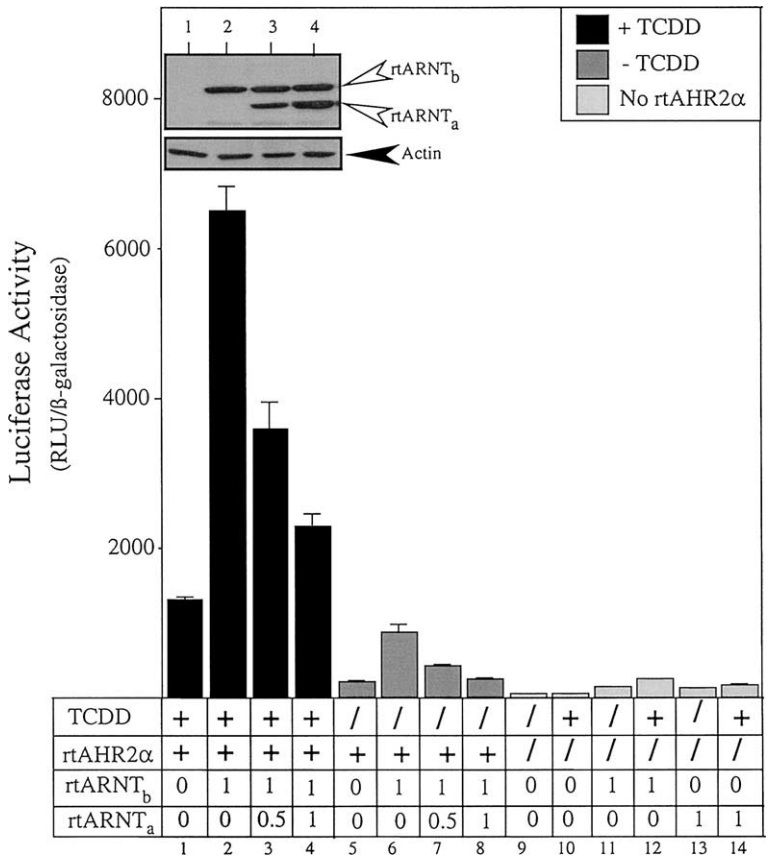

Fig. 8. TCDD-induced reporter gene activation and rtARNT expression in E36 cells transfected with rtAHR2α and $rtARNT_a$ or $rtARNT_b$. E36 cells were cotransfected with different combinations of $rtARNT_b$, $rtARNT_a$, and FLAG-rtAHR2α as indicated below each bar, along with p–1897*Om*1A3*luc* and pSV-ß-gal. The amounts of $rtARNT_b$ and $rtARNT_a$ plasmid used for transfection are indicated (μg); the total amount of DNA transfected in each sample was constant. Following 8 hr, cells were treated with TCDD (10 nM) or DMSO (0.5%) for an additional 16 hr and evaluated for luciferase and ß-gal activity or harvested for western blot analysis. The relative luciferase units (RLU) were divided by the ß-gal activity. Data are presented as the means ± SD for three samples. A western blot is shown in the inset. For these data, cell lysates were generated from plates of cells transfected with the exact DNA mixture used to generate the reporter gene data in samples 1–4. Total lysates were resolved on a western blot and stained with 1 μg/mL of anti-rtARNT IgG (rt-84) followed by GAR-HRP (1:10,000). Note that the expression of the $rtARNT_b$ isoform is consistent in samples 2–4, while $rtARNT_a$ is increased in proportion to the amount of DNA utilized.

were transfected into the E36 cell line along with the *CYP1A3* reporter and pSV-ß-gal. Samples contained FLAG-rtAHR2α alone, FLAG-rtAHR2α with $rtARNT_b$, and FLAG-rtAHR2α with $rtARNT_b$ and increasing amounts of $rtARNT_a$. Luciferase activity was measured following a 16-hr exposure to TCDD. A representative data set for the FLAG-rtAHR2α protein is shown in Fig. 8.

As shown in Fig. 5, expression of FLAG-rtAHR2α in the E36 cell line resulted in induction of luciferase activity in a TCDD-dependent manner (Fig. 8, lanes 1 and 5). When the $rtARNT_b$ expression plasmid was included in the transfection mixture, the overall level of luciferase activity was increased 3.8-fold compared with that found using FLAG-rtAHR2α alone (Fig. 8, lanes 2 and 6). However, when increasing amounts of the $rtARNT_a$ expression plasmid were included in the transfection mixture, the overall level of TCDD-mediated luciferase activity was reduced by 50 and 65% when the ratios of $rtARNT_a$/$rtARNT_b$ DNA were 0.5:1 and 1:1, respectively (Fig. 8, lanes 3, 4, 7, and 8). To confirm that the reduced level of luciferase activity was the result of increased levels of $rtARNT_a$ protein expression, additional plates of cells were transfected with the identical DNA solutions used to generate the luciferase data presented in Fig. 8 (lanes 1–4). Cells were then harvested and evaluated by western blot analysis with anti-rtARNT antibodies (Fig. 8, inset). The results show that the level of $rtARNT_b$ protein is constant in samples 2–4, but the level of $rtARNT_a$ protein increases with increasing levels of DNA. Thus, when the levels of $rtARNT_a$ and $rtARNT_b$ protein are similar in the transfected cells, the magnitude of TCDD-mediated gene induction is reduced significantly compared with samples that do not express $rtARNT_a$ (compare lane 2 to lanes 3 and 4). These results are consistent with previous studies of the $rtARNT_a$ protein that were carried out in the context of the mouse AHR [10,17,18] and indicate that $rtARNT_a$ exhibits activity consistent with a dominant negative protein in the context of FLAG-rtAHR2α. Similar results were observed when the FLAG-rtAHR2ß was evaluated in the presence of $rtARNT_a$ (data not shown).

3.6. Conclusions and implications

In this study, rtAHR2α and rtAHR2ß proteins induced a reporter construct under control of the trout *CYP1A3* promoter to a similar magnitude in the presence of 10 nM TCDD. These results are in sharp contrast to previous reports where rtAHR2ß showed reduced function compared to rtAHR2α in the ability to drive gene expression from a reporter construct under control of the trout *CYP1A* promoter [11,19]. The differences in the conclusions drawn by the three studies may be found in the distinct experimental designs that were used. First, the studies reported by Abnet *et al.* [11,19] utilized the COS-7 cell culture model and did not test for the expression or distribution of the rtAHR2 proteins within the transfected cells. In contrast, the current study utilized the E36 Chinese hamster lung cell line. This is an important difference because the COS-7 cell line has been shown recently to exhibit nuclear localization of transiently expressed mammalian AHR in the absence of ligand due to limiting levels of the ARA9 protein [34]. Reduced levels of ARA9 may, in fact, compromise the stability of the AHR and its ability to function in the signal transduction pathway [34]. On the other hand, the expression, localization, and function of the AHR in the E36 model have been well characterized [29,30], and the studies here demonstrate that the unliganded rtAHR isoforms were uniformly expressed in these cells in a pattern consistent with previous studies of mAHR [20,21,26,27,31].

Second, the COS-7 and E36 cell culture systems have slightly different propagation temperatures; E36 cells are grown at 30°, while COS-7 cells are grown at 37°. Since rainbow trout live at temperatures below 15°, a lower temperature may be more physiological for the analysis of the rtAHR. Third, in the reports of Abnet *et al.* [11,19], the investigators utilized a luciferase reporter gene driven by the rainbow trout *CYP1A* promoter. This reporter gene construct contains 1540 bp of upstream sequence (−1678 to −139) from the *rtCYP1A* promoter and only contains two XREs. The reporter used in the current study was driven by 1897 bp of upstream sequence of the *rtCYP1A3* gene, which is thought to be the predominant TCDD-induced CYP1A in the trout [23,35]. In addition, the *rtCYP1A3* sequence contains the two identical XREs found in the *rtCYP1A* sequence used in the studies of Abnet *et al.* but has an *additional three* XREs located upstream of nucleotide −1678 [23]. The number of XREs in the promoter of TCDD-inducible genes has been shown to impact the magnitude of AHR-mediated signal transduction [22,36].

Finally, the luciferase reporter gene data of Abnet *et al.* [11,19] were reported as fold-increase above control as opposed to absolute luciferase units. Therefore, it is not possible to conclude that the activities of each rtAHR2 isoform are distinct since an induction that went from 1–10 would be scored the same as an induction that went from 10–100, even though one protein would induce 10-fold *less* luciferase than the other. In addition, a protein that resulted in a high level of luciferase activity that also exhibited a high level of basal induction, would clearly be active, yet would not be scored as showing a high level of activity. These are key issues, given the hypothesis that the rtAHR2ß may function as a dominant negative factor (like the $rtARNT_a$) to affect the function of the rtAHR2α [11,19]. Thus, the key finding of the data reported here is that rtAHR2ß was able to function in a TCDD-dependent manner to essentially the same magnitude as rtAHR2α (see Figs. 5 and 7). These results suggest that rtAHR2ß has a positive action on AHR-mediated signaling, making it unlikely that the protein would directly impact the function of the rtAHR2α in a negative manner.

It is important to note, however, that the results presented in this report also show that the rtAHR2ß concentration response to TCDD was shifted to the right of rtAHR2α. This finding was also noted previously [11] where rtAHR2ß showed little to no induction of the *rtCYP1A* reporter gene yet produced a concentration–response curve that was shifted to the right of the rtAHR2α when a reporter was used that was driven by a portion of the murine *CYP1A1* promoter (containing four XREs). Since the absolute affinities of each AHR for TCDD have not been determined, these findings suggest that the differences in rtAHR2 function could be due to variations in the affinity of the rtAHR proteins for TCDD, rtARNT, DNA, or other proteins that may be participating in DNA binding. Thus, it would be possible for a cell expressing rtAHR2ß to be non-responsive to ligand exposure while a cell expressing an identical amount of rtAHR2α protein would respond. This is a very different scenario from one where a true dominant negative would bind to a dimerization partner and prevent gene regulation, thereby removing that partner from the pool of available proteins and ultimately decreasing the level of gene induction as shown for the $rtARNT_a$ isoform [10,17,18]. Thus, the reduced responsiveness of rtAHR2ß to TCDD is absolutely distinct from that of a dominant negative regulator.

In summary, both rtAHR2 isoforms appear to exhibit positive function in AHR-mediated signaling in the context of the experiments carried out in this study. In addition, the negative action of $rtARNT_a$ on AHR-mediated signaling was conserved when rtAHR2 was used as the dimerization partner. Finally, the inability to carry out studies on *in vitro* expressed rtAHR2 proteins indicates that methods need to be developed to allow molecular analysis of these proteins and their binding interactions so that direct comparisons can be made to mammalian bHLH/PAS proteins.

Acknowledgments

Special thanks to Dr. R.E. Peterson and Dr. R. Tanguay who supplied the rtAHR2 expression cDNAs and shared

unpublished data. Dr. Mark Hahn is also acknowledged for helpful discussions of these results. Thanks is given to Dr. Chris Bradfield for the murine AHR cDNA and Dr. Michael Carvan for the p–1897*Om*1A3*luc* expression vector. This work was supported, in part, by National Institute of Health Grants ES-08980 and ES-10410 to R.S.P.

References

[1] Walker MK, Spitsbergen JM, Olson JR, Peterson RE. Tetrachlorodibenzo-p-dioxin (TCDD) toxicity during early life stage development of lake trout (*Salvelinus namaycush*). Can J Fish Aquat Sci 1991;48:875–83.

[2] Walker MK, Peterson RE. Potencies of polychlorinated dibenzo-p-dioxin, dibenzofuran and biphenyl congeners relative to 2,3,7,8-tetrachlorodibenzo-p-dioxins, for producing early life stage mortality in rainbow trout (*Onchorhynchus mykiss*). Aquat Toxicol 1991;21:219–38.

[3] Walker MK, Peterson RE. Aquatic toxicity of dioxins and related chemicals. In: Schecter A, editor. Dioxins and health. New York: Plenum Press, 1994. p. 347–87.

[4] Elonen GE, Sphear RL, Holcombe GW, Johnson RD. Comparative toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin to seven freshwater species during early life stage development. Environ Toxicol Chem 1998;17:472–83.

[5] Hornung MW, Spitsbergen JM, Peterson RE. 2,3,7,8-Tetrachlorodibenzo-p-dioxin alters cardiovascular and craniofacial development and function in sac fry of rainbow trout (*Onchorhynchus mykiss*). Toxicol Sci 1999;47:40–51.

[6] Cook PM, Zabel EW, Peterson RE. The TCDD toxicity equivalence approach for characterization of trout early life stage mortality risks associated with exposures to TCDD and related chemicals. In: Rolland RM, Gilbertson M, Peterson RE, editors. Chemically-induced alterations in functional development and reproduction of fishes. SETAC Technical Publications Series. Pensacola: SETAC Press, 1997. p. 9–27.

[7] Zabel EW, Cook PM, Peterson RE. Toxic equivalency factors of polychlorinated dibenzo-*p*-dioxin, dibenzofuran and biphenyl congeners based on early life stage mortality in rainbow trout (*Onchorhynchus mykiss*). Aquat Toxicol 1995;31:315–28.

[8] Zabel EW, Pollenz RS, Peterson RE. Relative potencies of individual polychlorinated dibenzo-p-dioxin, dibenzofuran and biphenyl congeners and congener mixtures based on induction of cytochrome P4501A mRNA in a rainbow trout gonadal cell line (RTG-2). Environ Toxicol Chem 1996;15:2310–8.

[9] Pollenz RS, Necela B. Characterization of two continuous cell lines derived from *Oncorhynchus mykiss* for models of aryl-hydrocarbon-receptor-mediated signal transduction. Direct comparison to the mammalian Hepa-1c1c7 cell line. Aquat Toxicol 1998;41:31–49.

[10] Pollenz RS, Sullivan HR, Holmes J, Necela B, Peterson RE. Isolation and expression of cDNAs from rainbow trout (*Oncorhynchus mykiss*) that encode two novel basic-helix-loop-helix/PER-ARNT-SIM (bHLH/PAS) proteins with distinct functions in the presence of the aryl hydrocarbon receptor. Evidence for alternative mRNA splicing and dominant negative activity in the bHLH/PAS family. J Biol Chem 1996;271:30886–96.

[11] Abnet CC, Tanguay RL, Hahn ME, Heideman W, Peterson RE. Two forms of aryl hydrocardon receptor type 2 in rainbow trout (*Oncorhynchus mykiss*). Evidence for differential expression and enhancer specificity. J Biol Chem 1999;274:15159–66.

[12] Roy NK, Wirgin I. Characterization of the aromatic hydrocarbon receptor gene and its expression in Atlantic tomcod. Arch Biochem Biophys 1997;344:373–86.

[13] Karchner SI, Powell WH, Hahn ME. Identification and functional characterization of two highly divergent aryl hydrocarbon receptors (AHR1 and AHR2) in the teleost *Fundulus heteroclitis*. Evidence for a novel subfamily of ligand-binding basic helix loop helix-Per-ARNT-Sim (bHLH-PAS) factors. J Biol Chem 1999;274:33814–24.

[14] Hahn ME, Karchner SI, Shapiro MA, Perera SA. Molecular evolution of two vertebrate aryl hydrocarbon (dioxin) receptors (AHR1 and AHR2) and the PAS family. Proc Natl Acad Sci USA 1997;94:13743–8.

[15] Tanguay RL, Abnet CC, Heideman W, Peterson RE. Cloning and characterization of the zebrafish (*Danio rerio*) aryl hydrocarbon receptor. Biochim Biophys Acta 1999;1444:35–48.

[16] Tanguay RL, Abnet CC, Heideman W, Peterson RE. Identification and expression of alternatively spliced aryl hydrocarbon nuclear translocator 2 (ARNT2) cDNAs from zebrafish with distinct functions. Biochim Biophys Acta 2000;1494:117–28.

[17] Necela B, Pollenz RS. Functional analysis of activation and repression domains of the rainbow trout aryl hydrocarbon receptor nuclear translocator (rtARNT) protein isoforms. Biochem Pharmacol 1999; 57:1177–90.

[18] Necela B, Pollenz RS. Identification of a novel C-terminal domain involved in the negative function of rainbow trout Ah receptor nuclear translocator protein isoform a ($rtARNT_a$) in Ah receptor-mediated signaling. Biochem Pharmacol 2001;62:307–18.

[19] Abnet CC, Tanguay RL, Heideman W, Peterson RE. 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. Toxicol Appl Pharmacol 1999;159: 41–51.

[20] Pollenz RS, Sattler CA, Poland A. The aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator protein show distinct subcellular localizations in Hepa 1c1c7 cells by immunofluorescence microscopy. Mol Pharmacol 1994;45:428–38.

[21] Holmes JL, Pollenz RS. Determination of aryl hydrocarbon receptor nuclear translocator protein concentration and subcellular localization in hepatic and nonhepatic cell culture lines: development of quantitative western blotting protocols for calculation of aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator protein in total cell lysates. Mol Pharmacol 1997;52:202–11.

[22] Shen ES, Whitlock Jr JP. Protein-DNA interactions at the dioxin-responsive promoter. J Biol Chem 1992;267:6815–9.

[23] Carvan MJ, Ponomareva LV, Solis WA, Matlib RS, Puga A, Nebert DW. Trout CYP1A3 gene: recognition of fish DNA motifs by mouse regulatory proteins. Mar Biotechnol 1999;1:155–66.

[24] Lorenzen A, Okey AB. Detection and characterization of [^{3}H]2,3,7,8-tetrachlorodibenzo-*p*-dioxin binding to Ah receptor in a rainbow trout hepatoma cell line. Toxicol Appl Pharmacol 1990;106:53–62.

[25] Swanson HI, Perdew GH. Detection of the Ah receptor in rainbow trout: use of 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-p-dioxin in cell culture. Toxicol Lett 1991;58:85–95.

[26] Jain S, Dolwick KM, Schmidt LV, Bradfield CA. Potent transactivation domains of the Ah receptor and the Ah receptor nuclear translocator map to their carboxy termini. J Biol Chem 1994;269:31518–24.

[27] Li H, Dong L, Whitlock Jr JP. Transcriptional activation function of the mouse Ah receptor nuclear translocator. J Biol Chem 1994;269: 28098–105.

[28] Whitelaw ML, Gustafsson JA, Poellinger L. Identification of transactivation and repression functions of the dioxin receptor and its basic helix-loop-helix/PAS partner factor Arnt: inducible versus constitutive modes of regulation. Mol Cell Biol 1994;14:8343–55.

[29] Davarinos NA, Pollenz RS. Aryl hydrocarbon receptor imported into the nucleus following ligand binding is rapidly degraded via the cytoplasmic proteasome following nuclear export. J Biol Chem 1999;274:28708–15.

[30] Pollenz RS, Barbour ER. Analysis of the complex relationship between nuclear export and Ah receptor-mediated gene regulation. Mol Cell Biol 2000;20:6094–105.

[31] Pollenz RS. The aryl-hydrocarbon receptor, but not the aryl-hydrocarbon receptor nuclear translocator protein, is rapidly depleted in hepatic and nonhepatic culture cells exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Mol Pharmacol 1996;49:391–8.

[32] Bradfield CA, Kende AS, Poland A. Kinetic and equilibrium studies of aryl hydrocarbon receptor ligand binding. Mol Pharmacol 1998;34: 229–37.

[33] Okey AB, Riddick DS, Harper PA. The Ah receptor: mediator of the toxicity of 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) and related compounds. Toxicol Lett 1994;70:1–22.

[34] LaPres JJ, Glover E, Dunham EE, Bunger MK, Bradfield CA. ARA9 modifies agonist signaling through an increase in cytosolic aryl hydrocarbon receptor. J Biol Chem 2000;275:6153–9.

[35] Cao ZJ, Hong J, Peterson RE, Aiken J. Characterization of CYP1A1 and CYP1A3 gene expression in rainbow trout (*Oncorhynchus mykiss*). Aquat Toxicol 2000;49:101–9.

[36] Fujisawa-Sehara A, Sogawa K, Yamane M, Fujii-Kuriyama Y. Characterization of xenobiotic responsive elements upstream from the drug-metabolizing cytochrome P450c gene: a similarity to glucocorticoid regulatory elements. Nucleic Acids Res 1987;15:4179–91.