

# Identification and expression of alternatively spliced aryl hydrocarbon nuclear translocator 2 (ARNT2) cDNAs from zebrafish with distinct functions

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Received 19 June 2000; received in revised form 31 August 2000; accepted 31 August 2000

## Abstract

In order to further establish zebrafish as a vertebrate model for studying the mechanism of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) toxicity it is necessary to characterize the aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator (AhR/ARNT) signaling pathways in this species. In this study, three zfARNT2 cDNAs were isolated, expressed, and characterized and named zfARNT2b, zfARNT2c, and zfARNT2a. zfARNT2b, zfARNT2c, and zfARNT2a encode proteins with theoretical molecular weights of 81, 79, and 45 kDa, respectively. zfARNT2b and zfARNT2a proteins are identical over the first 403 amino acids but differ in their C-terminal domains as a result of alternative mRNA splicing. zfARNT2c is nearly identical to zfARNT2b, with the exception of an in frame 15 amino acid deletion adjacent to the basic region of zfARNT2c. Using quantitative RT-PCR methods the tissue distribution of each zfARNT2 isoform was determined. In COS-7 cells expressing zfARNT2b and zfAhR2, 10 nM TCDD causes a nine-fold induction of a dioxin responsive reporter gene. In COS-7 cells expressing zfARNT2a or zfARNT2c, TCDD does not induce reporter gene expression. In contrast, all three zfARNT2 proteins induce reporter gene activity under control of hypoxia responsive elements when cotransfected with the zebrafish endothelial specific PAS protein 1. DNA gel shift analysis suggests that the decreased function of zfARNT2a is due to inefficient binding of zfARNT2a/zfAhR2 complexes to dioxin responsive elements. These results also indicate that alternative mRNA splicing results in formation of ARNT proteins with distinct functional properties. © 2000 Elsevier Science B.V. All rights reserved.

**Keywords:** Aryl hydrocarbon receptor; Aryl hydrocarbon receptor nuclear translocator; Zebrafish; Alternative splicing

## 1. Introduction

The aryl hydrocarbon receptor nuclear translocator (ARNT) is a member of the PAS family of transcription factors, a growing family of basic helix-loop-helix (bHLH)

proteins that include the aryl hydrocarbon receptor (AhR), ARNT2, ARNT3, hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), endothelial specific PAS protein-1 (EPAS-1, also named HIF-2 $\alpha$ ), single minded (SIM), and others (reviewed in [3]). ARNT proteins can dimerize with a number of binding partners to regulate transcription. The AhR, a ligand activated transcription factor, is among these partners. The mechanism by which the AhR alters gene transcription in mammals has been well studied [1,2]. The unliganded AhR is associated with two molecules of the chaperonin hsp90 [3] and AhR interacting protein (AIP [4], also named XAP2 [5] and ARA9 [6]) in the cytoplasm. The binding of agonists such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) activates AhR, allowing translocation into the nucleus where ARNT dimerization occurs [7,8]. AhR/ARNT heterodimers recognize and bind dioxin response elements (DREs) found in promoters of responsive genes to alter gene expression [9]. Functional

Abbreviations: ARNT, aryl hydrocarbon receptor nuclear translocator; AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8 tetrachlorodibenzo-*p*-dioxin; bHLH, basic helix-loop-helix; HIF-1 $\alpha$ , hypoxia inducible factor-1 $\alpha$ ; SIM, single minded; AIP, AhR interacting protein; P4501A, cytochrome CYP4501A protein; *CYP1A*, cytochrome P4501A gene; DRE, dioxin response element; ZF-L, zebrafish liver cells; GCG, Genetics Computer Group; UTR, untranslated region; DMSO, dimethyl sulfoxide; zf, zebrafish; *luc*, luciferase; EPAS-1, endothelial specific EPAS-1; rt, rainbow trout; fh, *Fundulus heteroclitus*

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DRE sequences have been identified in the 5' flanking regions of the genes encoding cytochrome P4501A1, P4501A2 and P4501B1 monooxygenases, glutathione *S*-transferase Ya subunit, class 3 aldehyde dehydrogenases, and NAD(P)H:quinone reductase 1 and 2 (reviewed in [2]). Despite extensive effort to characterize the AhR signaling pathway in eukaryotes, the underlying molecular mechanism leading to toxicity is poorly understood.

The genes encoding AhR and ARNT proteins have been identified in several fish species. Full-length AhR cDNAs have been isolated from the Atlantic tomcod [10], two from *Fundulus* (FhAhR1, FhAhR2) [11], two from the rainbow trout (rtAhR2 $\alpha$  and rtAhR2 $\beta$ ) [12] and one from zebrafish AhR (zfAhR2) [13]. Partial AhR sequences have been identified in several fish [14,15], reviewed in [16]. Two forms of rainbow trout ARNT (rtARNTa, rtARNTb) [17], a *Fundulus heteroclitus* ARNT2 (fhARNT2), and a partial zebrafish ARNT sequence have also been isolated [18]. Zebrafish are ideal for studying the toxicity of these compounds and for developmental biology [19]. They are hardy, easy to work with vertebrates that produce a regular and plentiful supply of transparent eggs and embryos. A wealth of information about zebrafish development has been accumulated. Furthermore, we have shown that the signs of developmental toxicity produced in other fish species by dioxin also occur in zebrafish embryos and larvae. Large-scale efforts in a number of labs have led to the establishment of genetic techniques and resources that allow the isolation of genes associated with different developmental processes [20–24]. Identification of genes involved in TCDD toxicity in mutant zebrafish will increase our understanding of the mechanism(s) of TCDD toxicity. Here we describe cloning, expression and characterization of three zebrafish ARNT2 cDNAs that appear to arise from alternative mRNA splicing. The zfARNT2 isoforms have distinct functional properties.

## 2. Materials and methods

### 2.1. Cell culture

Monkey kidney COS-7 cells (ATCC, Rockville, MD, USA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum incubated in a 100% humidity, 5% CO<sub>2</sub> incubator at 37°C. For TCDD exposures, dimethyl sulfoxide (DMSO) or TCDD previously dissolved in DMSO (0.1% medium volume) was added to the media and incubated overnight before cell harvest.

### 2.2. Oligonucleotides

All oligonucleotide primers were synthesized by the University of Wisconsin Biotech Center or Gibco BRL

(Rockville, MD, USA) and are written 5' to 3'. F indicates forward primers corresponding to sense strands and anti-sense reverse primers are designated with an R. Initiation ATG codons are underlined and the sequence encoding the Flag epitope is indicated by dotted lines. Standard codes are indicated for degenerate oligonucleotide: Y=C+T, I=inosine, W=A+T, S=C+G and R=A+G. F1, GTAARCCIGAYAARYTIAC; F2, ATGGCIGT-IWSICAYATGAAR1; R1, TCISWRAAIGGIGGRAACAT; R2, GTIGGRTTYTGRAIGT; F3, GGAAACACCTCAACCGACG; F4, GGAGCTGAAGCACTTGATTCTGGA; R3, GGAAGCGGTACATCACTGAG; R4, GGGTGGCAGAACTCCAGAATGTCT, F5, CAGGCAATATGGCAACACC, R5, GTGATGTGCGTCAGGTGTGAGATG; R6, CACAGTGAAATATTCCTTGATC; F6, GACTGAATTCCTTTCACGCCAC; R7, CTGGAGCTGCTTGACGTTG; zfFLAG, CCGGACATGGACTACAAGGACGATGACGACAAGATGGCAACACCAGCCGCTG; Factivin, AAGCAGGAGTACGATGAGTC; and Ractin, TGGAGTCCTCAGATGCAATTG. Oligonucleotides for DNA gel shift experiments: the DRE consensus core is indicated by underline and mutated bases are in bold. Wild type rainbow trout DRE1 (wt rtDRE1): ACCTTTGCACGCTACGAAAT; wt rtDRE2: ATTTTCGATAGCGTGCAAAGGT; mutated rainbow trout DRE1 (mut rtDRE1): ACCTTTGCGCGCTATCGAAAT; mut rtDRE2: ATTTTCGATAGCGCGCAAAGGT.

### 2.3. RT-PCR and 5'-3' RACE

To obtain internal zebrafish ARNT sequences, oligo(dT) primed cDNA was synthesized using 200 ng total whole adult RNA. ARNT sequences were amplified using degenerate primers F1 and R1, each at 1.0  $\mu$ M, designed using aligned mammalian ARNT1 sequences. Amplification for 35 cycles was as follows: 30 s 94°C, 30 s 50°C, and 1 min 72°C. A final 72°C, 7 min extension followed the last cycle. Aliquots from the original PCR reactions were reamplified under the same conditions as above using nested F2 and R2 primers. Amplified products were visualized by ethidium bromide staining and subcloned into PCRII (Invitrogen, Carlsbad, CA, USA) and sequenced. A PCR-based approach was used to obtain the 5' and 3' ends of zfARNT2 using the 5'-3' RACE Kit essentially as detailed by the manufacturer (Clontech, Palo Alto, CA, USA). Briefly, whole adult poly(A) RNA was isolated using Tri Reagent (Molecular Research Center) as previously described [13] and reverse transcribed using AMV reverse transcriptase and the Marathon cDNA synthesis primer followed by second strand synthesis. After universal adapter ligation to the cDNA ends, the adapted cDNA was used as a template for 5' and 3' RACE using zfARNT2 specific primers F3 and R3 with the supplied adapter specific primer using the following conditions: 30 s 94°C, 30 s 58°C, 4 min 72°C (Fig. 1A). 5  $\mu$ l aliquots,

diluted 1:200 from the original PCR reactions, were reamplified under the same conditions as above using nested F4, R4, and adapter primers. Amplified products were visualized by ethidium bromide staining and subcloned into pGEM-T Easy (Promega, Madison, WI, USA). Following DNA sequencing, gene specific primers corresponding to sequences within the 5' (primer F5) and 3' untranslated regions (UTRs, primers R5 and R6) were designed to allow amplification of full-length cDNAs from poly(A) RNA.

#### 2.4. cDNA constructs for functional studies

cDNA (200 ng) derived from whole adult zebrafish was used as a template in PCR reactions with *zfARNT2* specific primer pairs (F5 with R5) and (F5 with R6) using the following conditions: 30 s 94°C, 30 s 58°C, 3 min 72°C (Fig. 1B) using the high fidelity *pfu* polymerase (Promega, Madison, WI, USA). Amplified products were visualized by ethidium bromide staining, and subcloned into pGEM-T EASY (Promega, Madison, WI, USA). Three distinct clones were isolated after restriction digestion and DNA sequence analysis of several clones and were named *zfARNT2a*, *zfARNT2b*, and *zfARNT2c*. To generate eukaryotic expression vectors, the inserts were excised from the pGEM-T EASY by *NotI* digestion and cloned into

pBKCMV previously cut with *NotI*. Restriction digestion and DNA sequencing verified the resulting clones, pBKCMV-*zfARNT2a*, pBKCMV-*zfARNT2b*, and pBKCMV-*zfARNT2c*. To generate N-terminal Flag-tagged *zfARNT2* proteins for functional studies, pBKCMV-*zfARNT2a*, pBKCMV-*zfARNT2b*, and pBKCMV-*zfARNT2c* plasmid DNAs were used as templates for PCR with the forward *zfFLAG* and the reverse R5 and R6 using *pfu* polymerase. After subcloning into pGEM-T Easy, the inserts were excised with *NotI* and inserted into the *NotI* site of pBK-CMV to create pBKCMV-Flag-*zfARNT2a*, pBKCMV-Flag-*zfARNT2b* and pBKCMV-Flag-*zfARNT2c*. Restriction analysis and DNA sequencing verified all clones. The rainbow trout dioxin responsive reporter, *prtIAluc*, and *zfAhr2* expression vector (pBKCMV-*zfAhr2*) were previously described [13]. The hypoxia responsive luciferase reporter construct pGLHIF $\beta$  was generously provided by Roland Wenger (Physiology Institute, University of Zurich, Switzerland) and previously described [25], and Len Zon (Children's Hospital, Boston, MA, USA) generously provided the expression plasmid encoding EPAS-1. pRL-TK (Promega, Madison, WI, USA) was used as an internal *Renilla* luciferase reporter control in the transient transfection experiments.

#### 2.5. Tissue specific mRNA expression

Tissues were dissected from 6 month old adult male zebrafish. Organs from six fish, representing one replicate, were pooled for RNA isolation and a total of five replicates were analyzed. Total RNA was isolated with Tri reagent (Molecular Research Laboratories, Cincinnati, OH, USA) according to the manufacturer's instructions from the brain, eye, fins (caudal, pectoral, pelvic and dorsal), gills, heart, kidney, liver, skeletal muscle, skin and swim bladder. cDNA was produced from 2  $\mu$ g of each RNA pool using Superscript II (Life Technologies, Gaithersburg, MD, USA) and the oligo(dT) primer in a 20  $\mu$ l volume. The Light Cycler (Roche, Indianapolis, IN, USA) was used for quantitative PCR using *ARNT2* splice variant specific primers F6, R7, and R6. 1  $\mu$ l of each cDNA pool was used for each PCR in the presence of SYBR Green, according to the manufacturer's instructions ([http://biochem.roche.com/prodinfo\\_fst.htm?light\\_cycler/WhatIs.htm](http://biochem.roche.com/prodinfo_fst.htm?light_cycler/WhatIs.htm)). Agarose gel electrophoresis and thermal denaturation were used to demonstrate the formation of a specific product.

#### 2.6. In vitro expression of *zfARNT2* proteins

Recombinant *zfARNT2* proteins were produced from plasmids pBKCMV-Flag-*zfARNT2a*, pBKCMV-Flag-*zfARNT2b*, pBKCMV-Flag-*zfARNT2c*, and *zfAhr2* protein was produced from plasmid pBKCMV-*zfAhr2* using the TNT Coupled Rabbit Reticulocyte Lysate Kit essentially as described by the manufacturer (Promega, Madi-

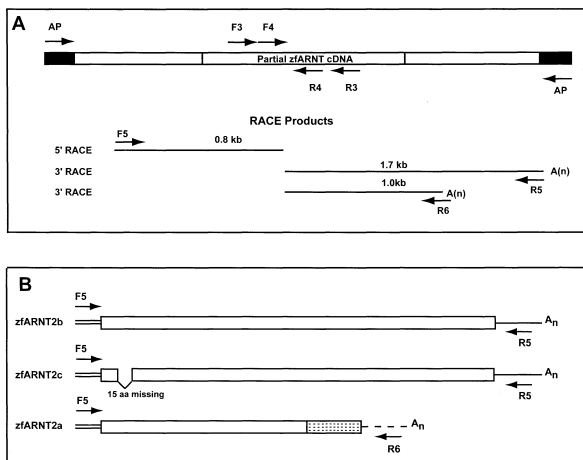


Fig. 1. RT-PCR amplification of zebrafish *ARNT2* cDNAs from whole adult zebrafish RNA. (A) 5' and 3' RACE was used to obtain ends of *zfARNT2* cDNA. RNA from whole adult zebrafish was converted into cDNA followed by adapter (dark boxes) ligation. Arrows indicate position of gene specific and adapter primers (AP). Primary 3' and 5' RACE reactions utilized primers F3 and R3, respectively, with AP. The nested 3' and 5' RACE reactions utilized primers F4 and R4 with AP. Thin lines illustrate the length of the subcloned RACE products. Following DNA sequencing, UTR specific primers were designed to allow amplification of complete open reading frames (F5, R5, and R6). (B). Poly(A)<sup>+</sup> RNA isolated from whole adult zebrafish was used as a template for RT-PCR. Gene specific primers F5, R5, and R6 were used to amplify full-length zebrafish *ARNT2* cDNAs. Arrows indicate locations of primers. Bars represent continuous open reading frames, double lines represent common 5' UTRs and single lines represents 3' UTRs. Triangles illustrate positions of alternative mRNA splicing and shaded bar represents unique C-terminal amino acids.

son, WI, USA). Side reactions containing [<sup>35</sup>S]methionine were performed to assess relative protein production. After the 90 min incubation at 30°C, radioactive translation products were resolved by 8% SDS polyacrylamide gel electrophoresis (SDS-PAGE), dried and phosphorimaged. Unlabeled reactions were stored at -70°C prior to functional studies.

### 2.7. Production of total COS-7 cell lysates and detection of Flag-tagged constructs

COS-7 cells at 60% confluency in 60 mm petri dishes were transfected with 5 µg of either pBKCMV-FlagzfARNT2a, pBKCMV-FlagzfARNT2b, pBKCMV-FlagzfARNT2c, or empty pBKCMV DNA using Superfect (Qiagen, Chatsworth, CA, USA) as detailed by the manufacturer. After 30 h, whole cell extracts were prepared essentially as previously described [26]. 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 µl 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 (DTT), 5 µg/ml leupeptin, 1 µg/ml aprotinin, and 5 µg/ml pepstatin A) and transferred into a 1.5 ml centrifuge tube on ice. Cells were sonicated three times on ice, homogenized using a Dounce homogenizer and debris was pelleted by centrifugation at 22 000 × g for 30 min. Protein concentrations were determined by the method of Bradford [27] using bovine serum albumin as a standard. To evaluate Flag-tagged zfARNT2 protein expression, 20 µg of each COS-7 cell lysate was resolved by SDS-PAGE on a 8% gel, transferred to nitrocellulose as previously described [28]. Immunochemical detection was carried out by blocking the blot with 5% dry milk in TBS-T (25 mM Tris (pH 7.6), 150 mM NaCl, 0.1% Tween-20) for 1–2 h followed by two TBS-T washes before the addition of the anti-Flag monoclonal antibody (Sigma, St. Louis, MO, USA) diluted in TBS-T (2 µg/ml) containing 1% dry milk. The antibody was removed after 2 h and the blot was washed two times with TBS-T, then the horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham, Chicago, IL, USA) diluted 1:5000 in TBS-T containing 1% dry milk was added. After 2 h, blots were washed three times with TBS-T before chemiluminescence detection (Amersham). The X-ray film was developed, digitally scanned and printed from PhotoShop 4.0 (Adobe, Seattle, WA, USA).

### 2.8. Nucleotide and amino acid sequence analysis

Both strands of each clone were sequenced at least three times using fluorescent dye-labeling cycle sequencing (Applied Bio Systems, UW Biotech Center, Madison, WI,

USA) stepwise using gene specific primers before GenBank submission. Genetics Computer Group (GCG) [29] software and the Baylor College of Medicine Human Genome Center search launcher (web site <http://kiwi.bcm.edu:8088/search-launcher/launcher.html>) and the National Center for Biotechnology Information (NCBI, web site <http://www.ncbi.nlm.nih.gov>) were used for sequence analysis.

### 2.9. In vitro DNA binding assay

In vitro gel shift analysis was performed as previously described [13]. Briefly, oligonucleotide wt rtDRE1 was 5' end labeled with <sup>32</sup>P using T4 polynucleotide kinase and annealed to a three-fold molar excess of unlabeled wt rtDRE2 oligonucleotide followed by probe purification. Unlabeled competitor DNAs were similarly produced by annealing unlabeled wt rtDRE1:wt rtDRE2 and mut rtDRE1:mut rtDRE2 oligonucleotides. For in vitro DNA binding assays, approximately equal amounts of in vitro produced zfAhR2 and one of the FlagzfARNT2 proteins were incubated in the presence of 10 nM TCDD in 0.2% DMSO or DMSO alone for 60 min at 22°C. Following incubation, 1.5 µg poly(dI-dC) and binding buffer (20 mM HEPES, pH 7.9, 100 mM NaCl, 1 mM DTT, 6% glycerol) were added and the incubation continued for an additional 20 min at 22°C. In some reactions 1 µg affinity purified monoclonal mouse anti-Flag or mouse anti-smooth muscle actin antibodies was added before the addition of 100 000 cpm of the rtDRE probe and 10-fold molar excess unlabeled wild type rtDRE or mutated rtDRE competitor DNAs. After 20 min incubation at 22°C, complexes were resolved on a 0.5 × TBE (90 mM Tris, 64.6 mM boric acid, and 2.5 mM EDTA, pH 8.3) 4.5% polyacrylamide gel at 4°C. The dried gels were exposed to a phosphor screen for 2 days before analysis.

### 2.10. Transactivation assay in transient transfection of COS-7 cells

prt1Aluc or pGLHIFluc were cotransfected with pRL-TK, FlagzfARNT2 and either zfAhR2 or EPAS-1 expression vectors using the SuperFect protocol (Qiagen, Chatsworth, CA, USA). The type and amount of DNA added to each well is indicated in Figs. 7 and 8. Briefly, zfAhR2, zfEPAS-1, and zfARNT2 expression vectors were mixed with 100 ng prt1Aluc or pGLHIFluc and 3 ng pRL-TK vector and incubated with 60 000 cells in 24 well plates for 2.5 h at 37°C in 400 µl serum containing medium. The amount of DNA in each well was adjusted to 1 µg using empty pBK-CMV expression vector. Following incubation, 600 µl fresh serum containing medium was added and the cells were incubated for 20 h at 37°C. Following the incubation, DMSO or TCDD previously dissolved in DMSO (0.1% medium volume) were added to the cells. Cells were incubated at 37°C for 20 h before cell harvest.

Cell extraction and luciferase assays were performed according to the manufacturer's protocol (Dual-Luciferase assay system, Promega, Madison, WI, USA). Briefly, a 10 µl aliquot was transferred to a 96 well luminometer plate and luciferase assays were completed using a Dynatech Laboratories ML-2250 luminometer (Chantilly, VA, USA). Assays for luciferase activity were conducted as follows: 50 µl of luciferase assay buffer II was injected into each well, incubated for 2 s, and the resulting luminescence integrated over the next 10 s. After reading each plate the assay buffer was changed to Stop and Glo and identical assay conditions were used to measure *Renilla* activity in the same wells. Because the *Renilla* luciferase control vector is susceptible to induction by *trans* effects when a second reporter construct with a strong promoter is activated, the amount of transfected control plasmid was reduced to 3 ng pRL-TK/µg DNA in each well in order to avoid this problem.

2.11. Statistical analysis

For TCDD-mediated transactivation experiments in transiently transfected COS-7 cells, 10 nM TCDD was used to produce maximal response and each experiment was repeated three times. A normalized relative luciferase activity was determined for each assay well by dividing the firefly luciferase activity by the *Renilla* luciferase activity. Results are expressed as mean ± S.E.M.

3. Results

A partial zebrafish ARNT cDNA was obtained via RT-PCR using degenerate PCR primers designed using the mouse ARNT1 amino acid sequence. A 974 bp product was amplified using RNA isolated from zebrafish liver (ZF-L) cells and whole adult zebrafish (data not shown). Ten clones were sequenced and found to be identical. The predicted amino acid sequence of the zebrafish amplicon

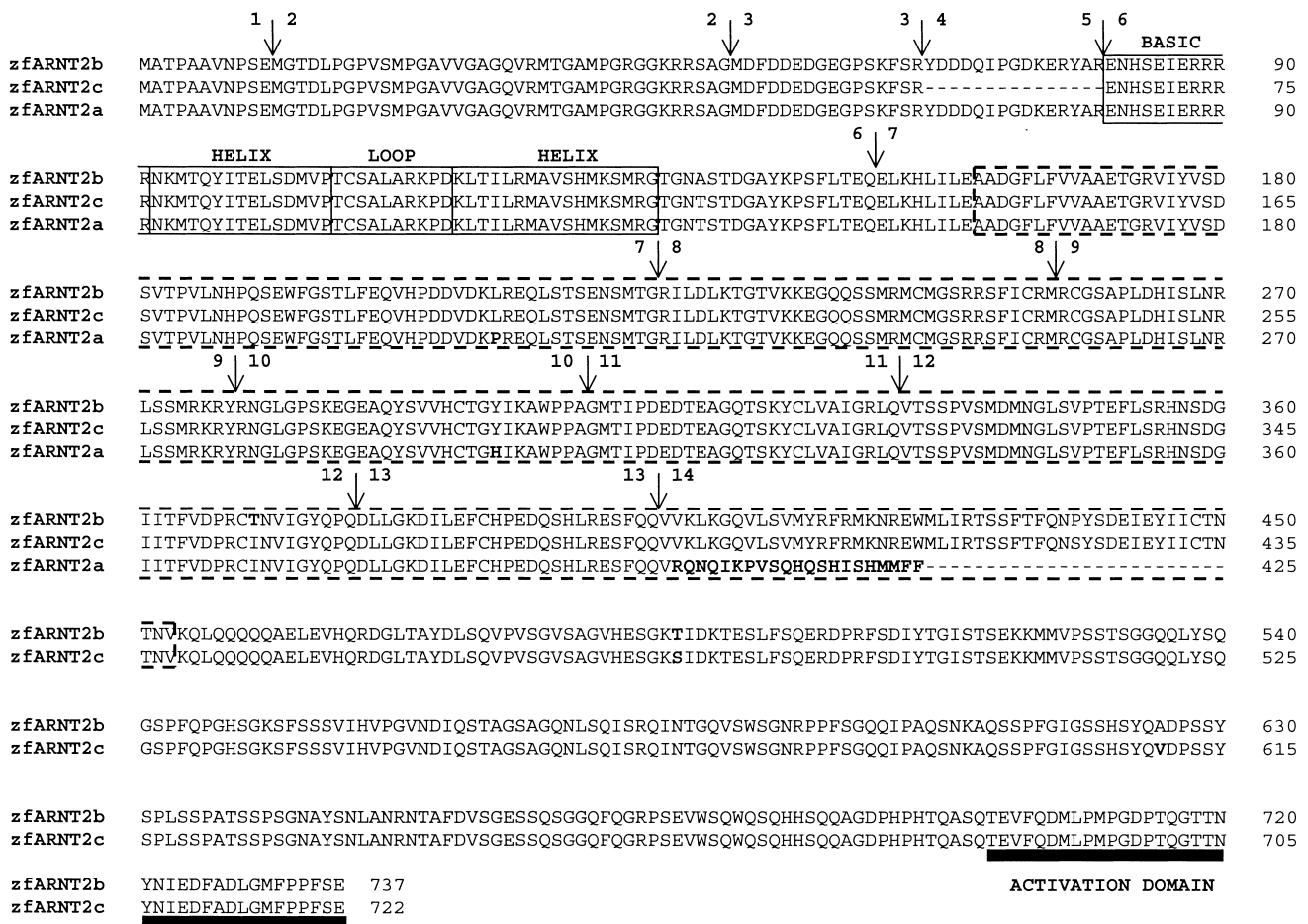


Fig. 2. Predicted amino acid sequence of the three alternatively spliced zARNT2 cDNAs. ClustalW sequence alignment of the three zARNT2 deduced amino acid sequences. Alignment gaps are represented by dashes and bold letters indicate non-identical amino acids. Location of bHLH domain is shown by solid lines, the highly conserved PAS domains are indicated by bold dashed lines, and the activation domain is indicated by the solid bar. The number and position of mouse ARNT1 derived exon-exon boundaries are indicated by arrows.

was 83% identical to the mouse ARNT2. This region was used as a probe to screen cDNA libraries derived from ZF-L cells, embryonic tissue, and whole adult zebrafish, and failed to detect a single clone. This suggests that our ARNT cDNA corresponds to a message that is not abundant in zebrafish. To obtain the full-length zebrafish ARNT cDNA, we used a nested PCR-based 5' and 3' RACE approach using gene specific primers F3, R3, F4, and R4 (Fig. 1A). A single 0.8 kb RACE product was obtained from the 5' primers, and two distinct RACE products of 1.7 and 1.0 kb were obtained from the 3' primer set (Fig. 1A). Both clones have poly(A) sequences, and the 1.0 kb clone has a polyadenylation signal (AAUAA) 19 bases upstream of the poly(A) tail. Although the 1.7 kb clone terminates in a poly(A) tract, an upstream poly(A) signal is absent suggesting this clone may not represent a full-length cDNA.

Following DNA sequencing, a primer including the predicted initiation ATG (F5) and two different reverse primers located just upstream of the poly(A) tracts (R5, and R6) were designed to allow amplification of complete open reading frames from zebrafish mRNA (Fig. 1B). 10 clones derived from each primer set were isolated and sequenced. A single 1311 (zfARNT2a) bp product was obtained using the R5-R6 primers encoding a 425 amino acid protein with a theoretical molecular weight of 45 kDa. For the R5-R6 reaction, two distinct products of 2298 (zfARNT2b) and 2253 (zfARNT2c) bp were obtained. The zfARNT2b clone encodes a 737 amino acid polypeptide with a predicted molecular weight of 81 kDa. The predicted amino acid sequence for zfARNT2c is 722 amino acids long and has a predicted mass of 79 kDa. The nucleotide sequence for zfARNT2a, zfARNT2b, and zfARNT2c have been deposited in the GenBank database under GenBank accession numbers AF219987, AF219988, and AF219989, respectively.

### 3.1. Cross species comparison of zebrafish ARNT2 predicted amino acid sequences

Alignment of the deduced amino acid sequences of the three zfARNT2 proteins reveals that the sequences are highly conserved throughout their lengths (Fig. 2). Sequence comparison reveals that the zfARNT2 proteins also share significant homology with previously cloned ARNT cDNAs (Fig. 3). zfARNT2b is most similar to the mouse and *Fundulus* ARNT2s with 82 and 80% identity over the entire protein, respectively. zfARNT2b is only 52% identical to the previously cloned rainbow trout ARNTb (rtARNTb) sequence and only 40% identical to mouse ARNT3 [30]. The most obvious difference between the zfARNT2 sequences is that zfARNT2a is truncated compared to zfARNT2b and zfARNT2c. The zfARNT2a sequence is identical to zfARNT2b over the first 403 amino acids, after which zfARNT2a diverges and terminates with 21 unique amino acids. These 21 amino acids are not

similar to any previously isolated proteins (Fig. 2). The shorter zfARNT2a therefore lacks the transcriptional activation domain that has been identified in other ARNT proteins [31,32] and is found in the other two zfARNT2s. The other major difference between the sequences is a 15 amino acid segment that is found in zfARNT2a and zfARNT2b between positions 65 and 79, but is not present in zfARNT2c.

The zfARNT2 cDNAs could arise from separate genes or from alternative mRNA splicing. Although it is apparent in mammals that different ARNT forms can arise from separate genes including ARNT1 [33], ARNT2 [34], and recently ARNT3 [30], the sequence differences between these ARNT forms are found scattered throughout the cDNAs, and are therefore unlikely to be produced by alternative splicing. In contrast, the differences between the zfARNT2 cDNAs are localized to distinct locations and could readily result from alternative mRNA splicing. At this time, there is no available zebrafish ARNT2 genomic structural information. Since ARNT genomic structure appears to be well conserved, we have placed the intron–exon boundaries derived from the mouse ARNT gene [35] onto the predicted amino acid sequence of the zebrafish ARNT2 sequences (Fig. 2). The differences between zfARNT2a, zfARNT2b, and zfARNT2c occur at predicted splice sites. The truncated zfARNT2a would result from alternative mRNA splicing between exons 13 and 14, terminating in a distinct 22 amino acid C-terminus, before reaching a stop codon. The 15 amino acids absent in zfARNT2c, but present in zfARNT2a and zfARNT2b, would occur by deletion of exons 4 and 5. Splice variation at this N-terminal location has previously been reported in mammalian ARNT proteins, however, the functional significance of alternative ARNT protein expression is poorly understood. In the human, alternative splicing at a location corresponding to the putative N-terminal zfARNT2 splice site results in an in frame addition of 15 amino acids [33]. In the rainbow trout, alternative mRNA splicing leads to the production of proteins with distinct C-terminal domains with unique functional properties [17]. Expression of a variant ARNT protein missing amino acids 330–789 is associated with AhR non-

	Fundulus ARNT2	Mouse ARNT2	Rat ARNT1	Mouse ARNT1	Rainbow Trout ARNTb
Zebrafish ARNT2b	80	82	59	59	52
Fundulus ARNT2		83	63	63	54
Mouse ARNT2			64	63	53
Rat ARNT1				91	51

Fig. 3. Amino acid conservation between aligned vertebrate ARNT sequences. Percent identities were calculated from full-length proteins. Gaps were excluded in the alignment calculations. Accession numbers used were: *Fundulus* ARNT2, AF79311; mouse ARNT2, D63644; rat ARNT1, U61184; mouse ARNT1, U10325; rainbow trout ARNTb, U73841.

responsiveness in MDA-MB-231 human breast cancer cells [36]. To be consistent with the nomenclature, we have adopted the conventions described by Pollenz et al. [17] to describe the *zfARNT2* isoforms in this study and therefore designate the truncated isoform *zfARNT2a* [17].

### 3.2. Tissue specific expression of *zfARNT2* mRNA isoforms

Early experiments using Northern blots demonstrated that all three forms of the *zfARNT2* mRNA are present at very low levels, making detection impractical with the small tissue samples available from zebrafish. To overcome this difficulty, we used real-time PCR to measure relative message concentrations of *zfARNT2* mRNAs in different tissues from adult zebrafish. The cDNAs for *zfARNT2* were then quantitatively amplified using a Light Cycler instrument, which measures product formation after each amplification cycle. This instrument allows real-time monitoring of the PCR reaction progress along with the simultaneous generation of a linear standard curve for template concentration using standards of known dilution. The standard curves had *R* values of greater than 0.95, and were used to quantify reactions using samples prepared from zebrafish organs. Two sets of primers were used: one that amplifies only *zfARNT2a* and another that amplifies both *zfARNT2b* and *zfARNT2c* (Fig. 4A). The standard curve allowed assignment of relative concentration values for mRNAs from each tissue type, and these values were normalized using

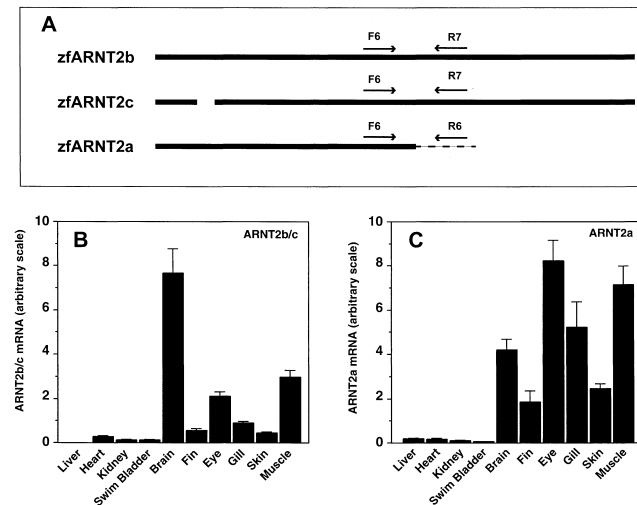


Fig. 4. Tissue specific expression of *zfARNT2* mRNA isoforms. (A) Two sets of primers, one specific for *zfARNT2a* and another for both *zfARNT2b* and *zfARNT2c*, were used to quantify *zfARNT2* mRNAs levels in several adult organs. (B) mRNA expression levels of *zfARNT2b* and *zfARNT2c* in adult zebrafish organs. Standard curves for each template were produced using standards of known dilution. The standard curve allowed assignment of relative concentration values for mRNAs from each tissue type, and these values were normalized to actin mRNA expression levels. (C) Expression levels of *zfARNT2a* in zebrafish organs. Bars represent normalized relative mRNA expression (mean  $\pm$  S.E.M.,  $n = 5$ ).

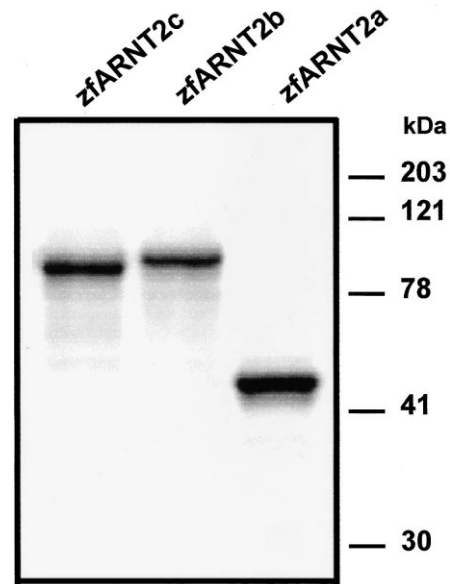


Fig. 5. In vitro expression of *zfARNT2* proteins. Flag*zfARNT2a*, Flag*zfARNT2b* and Flag*zfARNT2c* proteins were produced in a coupled TNT reaction with [<sup>35</sup>S]methionine incorporation, resolved on an 8% SDS polyacrylamide gel followed by phosphorimage analysis. Exposed to phosphor screen for 10 h.

zebrafish  $\beta$ -actin mRNA concentrations as a control for mRNA recovery and reverse transcription efficiency. As with Northern blots, the values produced are on an arbitrary scale and measurements for one mRNA species cannot be directly compared with another. All forms of *zfARNT2* are expressed in a tissue specific manner. The highest level of combined *zfARNT2b* and *zfARNT2c* expression is in the brain, eye and skeletal muscle with low or undetectable levels in the liver, heart, kidney and swim bladder (Fig. 4B). *zfARNT2a* is most highly expressed in the eye, skeletal muscle, gills and brain and to a lesser extent in fins and skin. *zfARNT2a* is also only barely detectable in the liver, heart, kidney and swim bladder (Fig. 4C).

### 3.3. In vitro expression of *zfARNT2a*, *zfARNT2b*, and *zfARNT2c* proteins

All three *zfARNT2* cDNAs produced proteins of the expected molecular weights when expressed in vitro using a coupled transcription/translation system (Fig. 5). To allow for convenient protein detection we added the nucleotides encoding the eight amino acid Flag epitope to the 5' end of each *zfARNT2* cDNA. We chose an N-terminal Flag fusion since this modification has not disrupted the function in other ARNTs [5]. Flag*zfARNT2* proteins with apparent molecular weights of 88, 90, and 49 kDa were produced in rabbit reticulocyte lysate from plasmids pBKCMV-Flag*zfARNT2c*, pBKCMV-Flag*zfARNT2b*, and pBKCMV-Flag*zfARNT2a* respectively. All forms appeared to be expressed at similar levels in this in vitro

system. zfAhR2 was produced following the same procedure using pBKCMV-zfAhR2 [13].

### 3.4. In vitro DNA binding assay

To determine if the alternatively spliced zfARNT2 cDNAs encode functional proteins, in vitro translated FlagzfARNT2b, FlagzfARNT2c, FlagzfARNT2a, and zfAhR2 proteins were used in DNA binding assays (Fig. 6). For these experiments we tested the ability of the three FlagzfARNT2 proteins to interact with zfAhR2 in binding to a radiolabeled probe containing DREs derived from the rainbow trout *cyp1a1* gene [13]. In the absence of TCDD, some specific zfAhR2/FlagzfARNT2b and zfAhR2/FlagzfARNT2c complexes were formed using the rtDRE probe (Fig. 6, lanes 3 and 10). However, this was enhanced when TCDD was present (Fig. 6, lanes 4 and 11). Similar binding properties have been observed with other fish AhR/ARNT combinations [11–13]. Interestingly, the specific complexes migrate as doublets, both of which are enhanced by ligand. At this time the nature of the doublet is unknown. The bands could arise from partial protein

degradation or perhaps from interactions with additional lysate proteins. This binding was specific since the complexes were effectively competed by a 10-fold molar excess of unlabeled rtDRE (Fig. 6, lanes 5 and 12), while rtDREs with a single base change in the core sequence failed to compete (Fig. 6, lanes 6 and 13). We observed no specific complexes when zfAhR2, FlagzfARNT2b, or FlagzfARNT2c proteins were added separately; however, there are lysate derived nonspecific bands with a higher mobility (Fig. 6, lanes 1, 2 and 9). To further demonstrate specificity, the complexes were supershifted using the monoclonal anti-Flag antibody (Fig. 6, lanes 7 and 14). Both of the specific complexes contain zfARNT2 and the N-terminus of the protein is intact since both complexes are supershifted using the Flag antibody. The anti-smooth muscle actin monoclonal antibodies used in control lanes did not affect the complexes (Fig. 6, lanes 8 and 15). The truncated FlagzfARNT2a protein with the unique 21 amino acids failed to form a specific complex with the rtDRE probe (data not shown). This was somewhat surprising since the expected DNA binding domain of zfARNT2a is identical to that of zfARNT2b. However, similar results have been reported with an alternatively spliced form of ARNT (rtARNTa) from rainbow trout [17]. In these studies, the region of homology between the ARNT forms extends into the C-terminal domain, to amino acid 533, and therefore the bHLH and PAS domains are conserved. Despite this, rtARNTa, when used with the murine AhR, only weakly interacts with mouse DRE sequences while rtARNTb readily binds to this probe [17]. This, along with our results, demonstrates that C-terminal sequences can affect formation of protein/DNA complexes in vitro. C-terminal deletion analysis of fish ARNT sequences is necessary to localize residues influencing complex formation or stability.

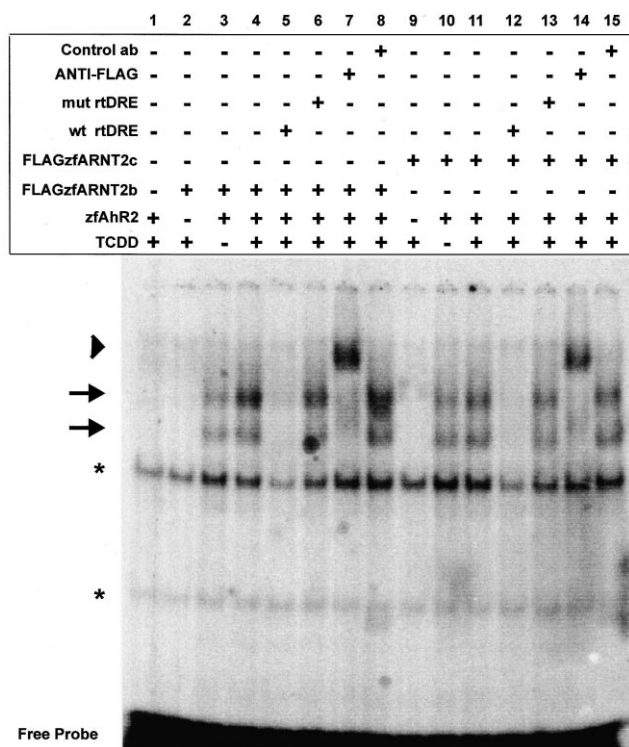


Fig. 6. Gel shift analysis of zfAhR2 and zfARNT2 protein interactions in vitro. Unlabeled, TNT produced zfAhR2 and FlagzfARNT2b and FlagzfARNT2c proteins were incubated with 10 nM TCDD in 0.2% DMSO or 0.2% DMSO alone for 1 h at 22°C. Gel shift analysis was performed as described in Section 2 using <sup>32</sup>P-labeled oligonucleotides derived from a DRE found in the rainbow trout *cyp1a* enhancer. The two arrows indicate positions of specific zfAhR2/FlagzfARNT2–DRE complexes. The arrowhead indicates the position of a complex supershifted with the anti-Flag antibody. The two asterisks indicate the positions of nonspecific bands.

### 3.5. Zebrafish AhR2 and ARNT2 transactivation activity in COS-7 cells

Since zfAhR2 heterodimerizes with zfARNT2b and zfARNT2c and binds rtDRE elements, the ability of these proteins to increase transcription of a luciferase reporter gene under control of a DRE was determined. COS-7 cells are easily transfected, have undetectable levels of endogenous AhR and only low levels of ARNT and are often used as a null AhR signaling pathway cell line [37]. zfAhR2 and zfARNT2 expression plasmids were transfected separately or together in COS-7 cells with prt1aluc, a luciferase reporter under control of the rainbow trout *cyp1a1* promoter and DRE containing enhancer. Transfected cells were incubated for 20 h in medium with 0.1% DMSO, or with 10 nM TCDD dissolved in DMSO (Fig. 7). Transient expression with the reporter vector alone, or any of the individual expression plasmids with the reporter vectors, resulted in low basal or TCDD induced reporter activity. On the other hand, when zfAhR2

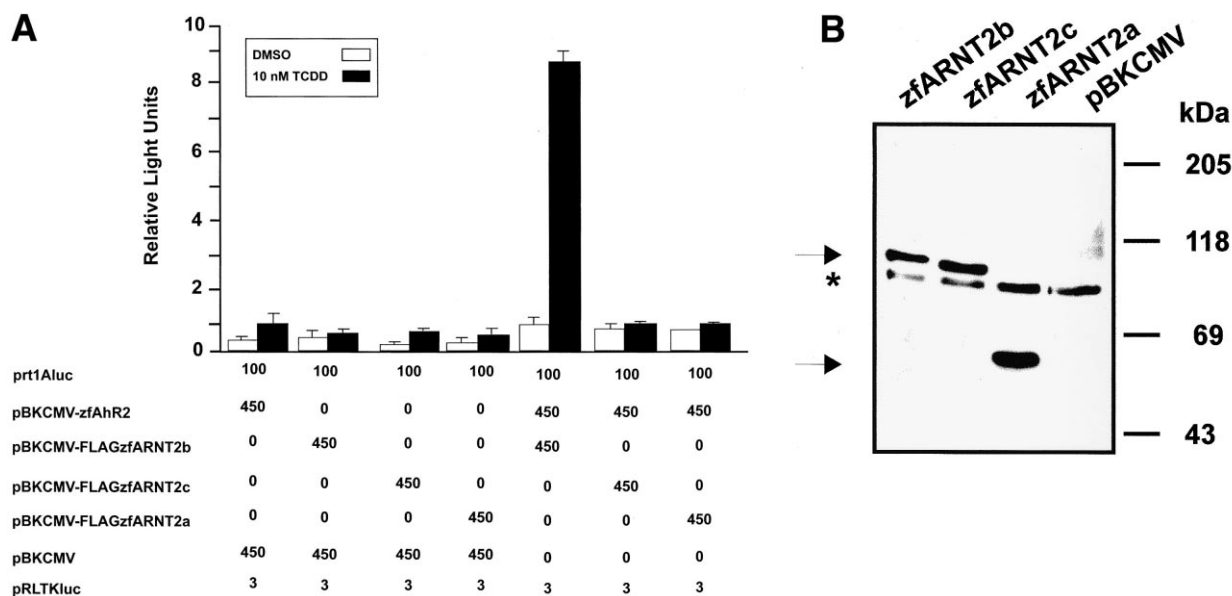


Fig. 7. zfARNT2b functions with zFAhR2 to induce dioxin responsive transcription in COS-7 cells. (A) Transactivation activity of the three FlagzfARNT2 proteins with zFAhR2 using a dioxin responsive *luciferase* reporter derived from the rainbow trout CYP1A promoter and enhancer (prt1Aluc). COS-7 cells were transiently transfected with the indicated expression constructs, along with prt1Aluc and pRL-TK (*Renilla luciferase*) as an internal transfection control and exposed to 0.1% DMSO or 10 nM TCDD in 0.1% DMSO. Numbers represent the amount of each DNA construct (ng) in each well of a 24 well plate. Bars represent normalized relative luciferase activity (mean  $\pm$  S.E.M.,  $n = 3$ ). (B) Western blot of COS-7 cell lysates. COS-7 cells were transiently transfected with expression plasmids encoding FlagzfARNT2a, FlagzfARNT2b, FlagzfARNT2c, and empty vector (pBKCMV). Cells were harvested 30 h after transfection and cell extracts were prepared as described in Section 2. 20  $\mu$ g of each sample were subjected to SDS-PAGE analysis. The gel was transferred to nitrocellulose and incubated with (2  $\mu$ g/ml) anti-Flag antibody followed by goat anti-mouse HRP (1:5000). ECL was used to visualize the bands. The positions of the zfFlagARNT2 and nonspecific proteins are indicated by arrows and the asterisk, respectively. The molecular weight of standard proteins is indicated.

was cotransfected with an equal amount of zfARNT2b we observed significant TCDD induced luciferase activity. These results demonstrate that the zfARNT2b dimerizes with zFAhR2 and is able to activate DRE dependent transcription. This is in marked contrast to the other zFAhR2/zfARNT2 combinations in which zfARNT2a and zfARNT2c failed to activate the reporter. The observation that zfARNT2a is transcriptionally inactive is consistent with the DNA gel shift data. At this time it is not possible to completely explain the lack of transactivation activity with zfARNT2c. zfARNT2c can dimerize with zFAhR2 and binds to DRE elements *in vitro*, but these interactions do not lead to transcriptional activity in COS-7 cells. There are several possible explanations for this lack of activity. First, the three ARNT2 expression plasmids may not be equally expressed in transiently transfected COS-7 cells, or perhaps the half-lives of the expressed proteins are different. To investigate these possibilities, extracts were prepared from COS-7 cells transfected with the FlagzfARNT2 expression plasmids and analyzed by Western blot (Fig. 7B). Monoclonal anti-Flag antibodies reveal similar levels of expression for all three zfARNT2 isoforms in COS-7 cells (Fig. 7B, lanes 1–3). In addition to the specific band, an additional nonspecific band is detected in all samples including the extracts prepared from pBKCMV only transfected cells (Fig. 7B, lane 4). This band has been previously observed in COS-7 cells [5]. These results demonstrate that differences in transcrip-

tional activity are not due to dissimilar zfARNT2 protein levels in COS-7 cells.

It is also possible that zFAhR2 and zfARNT2c fail to interact when overexpressed in COS-7 cells, or perhaps the 15 amino acids absent from zfARNT2c are required for proper protein folding. Changes in overall protein structure could interfere with necessary interactions with other endogenous proteins such as basal transcriptional machinery or coactivators. It is interesting to note that the *Fundulus* and murine ARNT2 proteins resemble zfARNT2c in that they also lack the 15 amino acids absent in zfARNT2c (Fig. 9). These proteins also interact with the AhR and recognize DREs [34,38]; however, the murine ARNT2 only partially rescued ARNT dependent reporter gene activity in the c4 ARNT deficient Hepa-1 cell line [34]. Transcriptional activity of the *Fundulus* ARNT2 has not been reported. The results presented here suggest that this region, adjacent to the basic region, may affect the transcriptional competence of ARNT2 proteins.

### 3.6. zfARNT2a, zfARNT2b, and zfARNT2c function with another PAS family member

There is considerable evidence that ARNT can form important heterodimers with PAS proteins other than AhR (reviewed in [1]). HIF-1 $\alpha$  must interact with ARNT before it can bind hypoxia response elements (HREs) found in the regulatory regions of hypoxia regu-

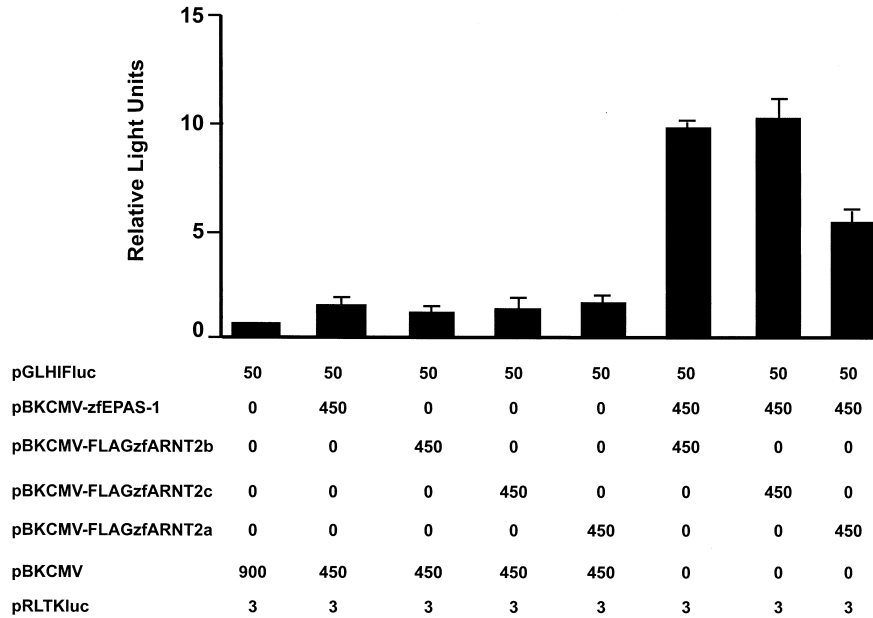


Fig. 8. *zfARNT2a*, *zfARNT2b*, and *zfARNT2c* interact with *zfEPAS-1* to increase transcription driven by hypoxia response elements. A hypoxia responsive *luciferase* reporter vector (pGLHIFluc) derived from the human erythropoietin enhancer was transiently transfected with the indicated expression constructs along with pRL-TK in COS-7 cells. Numbers represent the amount of each DNA construct (ng) in each well of a 24 well plate. Bars represent normalized relative luciferase activity (mean  $\pm$  S.E.M.,  $n=3$ ).

lated genes such as erythropoietin ([39] and reviewed in [40]). EPAS-1 (also called HIF-2 $\alpha$ ) is one of several proteins related to HIF-1 $\alpha$  that have been identified [41–43]. It is possible that ARNT2, although able to function with AhR in experimental settings, may be most important as a partner for proteins such as EPAS-1, or other yet unidentified PAS proteins. Similarly, the splice isoforms may have different repertoires of binding partners. To investigate this possibility, we performed transactivation assays to determine if the *zfARNT2* proteins can function with the zebrafish EPAS-1 (*zfEPAS-1*) in COS-7 cells. Under our experimental conditions hypoxia or CoCl<sub>2</sub> exposure was not required to activate *zfEPAS-1* in COS-7 cells (data not shown). COS-7 cells were transfected with combinations of *zfEPAS-1*, pGLHIFluc, Flag*zfARNT2b*, Flag*zfARNT2c*, and Flag*zfARNT2a* as indicated in Fig. 8. After 36 h luciferase activities were determined. When the reporter pGLHIFluc or *zfEPAS-1* and any of the *zfARNT2* expression constructs with pGLHIFluc were cotransfected, there was only low reporter gene activity illustrating low background in this system. When *zfEPAS-1*

and either of the *zfARNT2* isoforms were cotransfected, reporter gene activity increased significantly. *zfARNT2b* and *zfARNT2c* induced luciferase activity 10-fold and *zfARNT2a* caused a five-fold induction. These results demonstrate that all three *zfARNT2* proteins are functional with respect to EPAS-1 dimerization and transactivation and illustrate the point that when assigning ARNT activity, one must consider the protein partner involved.

#### 4. Discussion

We have identified cDNAs encoding three distinct forms of *zfARNT2*. Our results indicate that the ARNT isoforms have distinct activities. The transactivation results with *zfARNT2a* indicate that the C-terminal domain containing the transcriptional activation domain [44] in *zfARNT2a* is not required to reconstitute functional transcriptional activity with EPAS-1. However, this domain is essential for formation of functional complexes with *zfAhR2* and DREs. The gel shift experiments suggest

<i>zfARNT2b/a</i>	MATPAAVNPSEMGTDLDP--GPVSMGPVAV-VGAGQVRMTGAMPGRGGKRRSAGMDFDDEDGEGPSKFS-----RYDDDOI <del>PGDKERYA</del> RENHSEIER	88
<i>zfARNT2c</i>	MATPAAVNPSEMGTDLDP--GPVSMGPVAV-VGAGQVRMTGAMPGRGGKRRSAGMDFDDEDGEGPSKFS-----RENHSEIER	73
<i>Fundulus ARNT2</i>	MATPAAVNPSEMASELDP--GPVAMSGG--VGTGQVRMGGAVSGRGGKRRSAGMDFDDEDGEGPSKFS-----RENHSEIER	73
<i>rat ARNT2</i>	MATPAAVNPPEMADIP--GSVTLVPVAPMAATGQVRMAGAMPARGGKRRS--GMDFDDEDGEGPSKFS-----RENHSEIER	73
<i>mouse ARNT2</i>	MATPAAVNPPEMADIP--GSVALPVAPMAATGQVRMAGAMPARGGKRRS--GMDFDDEDGEGPSKFS-----RENHSEIER	73
<i>mouse ARNT1</i>	MAATTA-NP-EMTSDVPSLGPTIASGNP--G-PGIQGGGAVVQRAIKRRS-GLDFDDE-GEVNSKFLRCDDDEQMCNDKERFAR-----RENHSEIER	84
<i>mouse ARNT1+</i>	MAATTA-NP-EMTSDVPSLGPTIASGNP--G-PGIQGGGAVVQRAIKRRS-GLDFDDE-GEVNSKFLRCDDDEQMCNDKERFAR <u>SDDEOSSADKERL</u> ARENHSEIER	100
<i>rat ARNT1+</i>	MAATTA-N-PEMTSDVPSLGPTIASGNP--G-PGIQGGGAVVQRAIKRRS-GLDFDDE-GEVNSKFLRCDDDEQMCNDKERFAR <u>SDDEOSSADKERL</u> ARENHSEIER	100
<i>human ARNT1+</i>	MAATTA-N-PEMTSDVPSLGPTIASGNP--G-PGIQGGGAVVQRAIKRRS-GLDFDDE-GEVNSKFLRCDDDEQMCNDKERFAR <u>SDDEOSSADKERL</u> ARENHSEIER	100
<i>trout ARNTb</i>	MDSSTPDI-PDSSLGLGA-GGAQASSACAKRVNKRRAAP-DFDDEDGSGKLF-RCDDDDGGGDKERFA-----RENHSEIER	75

Fig. 9. ClustalW amino acid sequence alignment of the N-terminus of the *zfARNT2* proteins with *Fundulus ARNT2*, AF79311; *rat ARNT2*, U61405; *mouse ARNT2*, D63644; *mouse ARNT1*, U10325; *rat ARNT1*, U61184; *human ARNT1*, M69238; and rainbow trout ARNTb, U73841. Alignment gaps are represented by dashes and the + designates sequences containing the alternative spliced exon sequences are underlined.

that the lack of zfAhR2/zfARNT2a activity result from inefficient dimerization or DNA binding. Alternatively, the 21 C-terminal amino acids may actively prevent dimerization or DNA binding.

The results with zfARNT2c were somewhat surprising. How can we explain the observation that zfARNT2c interacts with zfAhR2 in vitro and binds DRE elements, yet this complex is not active in producing transactivation with a DRE containing reporter in COS-7 cells? This is more puzzling still in light of the fact that with EPAS-1 zfARNT2c is as active as zfARNT2b in stimulating transcription from HREs. Since the sequences of zfARNT2c and zfARNT2b are nearly identical, the 15 amino acid region missing in zfARNT2c must be essential for formation of transcriptionally competent complexes with zfAhR2. EPAS-1 is able to form competent complexes regardless of whether the 15 amino acids are present. Alignment of the amino acids N-terminal to the basic region of several ARNT1 and ARNT2 proteins reveals that alternative splicing occurs frequently in this region (Fig. 9). With the exception of zfARNT2a and zfARNT2b, all ARNT2 proteins, including zfARNT2c, contain gaps of 15 amino acids relative to the ARNT1s. In the ARNT1 proteins containing the alternative exon (underlined in Fig. 9) the gap is 30 amino acids. The functional significance of this exon is currently unknown. Prior to this study, an ARNT2 protein had not been identified that contained the alternative exon in this location as in zfARNT2b. Although zfARNT2b is clearly most similar to the ARNT2 protein family (Fig. 3), within this small N-terminal domain it is more similar to the ARNT1s. One interpretation of these results is that the addition of the 15 ARNT1-like residues allows zfARNT2b to form a *functional* dimeric complex with zfAhR2. Deletional analysis with ARNT1 from other species is required to more thoroughly investigate this possibility.

The physiological function of ARNT2 is beginning to be understood. Using in vitro approaches, all full-length ARNT2 proteins dimerize with AhRs and bind to DRE elements ([33,34,38,45] and Fig. 5); however, there is little evidence supporting a functional role for ARNT2/AhR dimers in vivo. The observation that ARNT2 only partially restored AhR signaling when expressed in ARNT deficient cell lines suggests that ARNT2 function may not be AhR dependent [34]. Furthermore, there are only limited data to indicate that AhR and ARNT2 are found together in the same cell types. ARNT2 is expressed in the brain ([45,46], E. Andreasen, R.L. Tanguay, W. Heideman, J.M. Spitsbergen, R.E. Peterson, unpublished data), whereas ARNT1 and AhR mRNAs appear to be expressed at only low levels in the brain [34,46,47]. If ARNT2 does not function with AhR, what is its role? Recent yeast two-hybrid experiments suggest that SIM may be an endogenous partner for ARNT2 [34]. The functional significance of this interaction is unclear at this time, but may be important given that SIM and ARNT2 mRNA expression

patterns are similar [34,48,49]. There is also increasing evidence that ARNT2 plays an important role in neuronal development. In the mouse, ARNT2 expression is associated with preventing neuronal cell death during development [50]. Furthermore, it has recently been reported that ARNT2 and SIM are coexpressed in the paraventricular and supraoptic nuclei and that loss of function of either affects the development of similar neuroendocrine cell types [51]. If ARNT2 has multiple dimeric partners, the potential for ARNT2 mediated cross talk between the AhR and other signaling pathways is an intriguing possibility. For example, in bird species, there is evidence that in ovo TCDD exposure results in the development of grossly asymmetric brains [52]. To further elucidate the role of ARNT2 and investigate the possibility for signal transduction cross talk, it will be necessary to identify protein partners that are coexpressed with ARNT2 in eukaryotic cells. Molecular and genetic approaches are needed to precisely determine in vivo ARNT2 function.

Taken together, these data illustrate that alternative ARNT2 mRNA splicing influences the functions of the resulting proteins. Furthermore, we suggest that at least some isoforms of ARNT2 may not be important components of the AhR2 signaling pathway. Since zebrafish clearly have a functional AhR pathway, a functional ARNT must be present, but whether it is zfARNT2b or an as yet unidentified zfARNT1 remains to be determined.

### Acknowledgements

We thank Dr. Roland Wenger for providing pGLHI-Fluc, Len Zon for providing the pBKCMV-EPAS-1 construct and especially Dorothy Nesbit for her excellent technical assistance. This work was supported by the University of Wisconsin Sea Grant Institute under grants from the National Sea Grant College Program, National Oceanic and Atmospheric Administration, US Department of Commerce and the National Institutes of Health. Sea Grant Project numbers R/MW-58 and R/BT-12 (R.E.P. and W.H.) and the National Institute of Environmental Health Sciences Individual National Research Award number F32 ES05786-01 (R.L.T.) and Institutional Award number 2 T32 ES07015-21. This is contribution number 330 of the University of Wisconsin Environmental Toxicology Center.

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