AHR1B, a new functional aryl hydrocarbon receptor in zebrafish: tandem arrangement of *ahr1b* and *ahr2* genes

Sibel I. KARCHNER¹, Diana G. FRANKS and Mark E. HAHN

Biology Department, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, U.S.A.

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that regulates gene expression following activation by TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) or a variety of other synthetic and natural compounds. Previous studies have identified two AHR genes, AHR1 and AHR2, in zebrafish (*Danio rerio*), a widely used model species for studying vertebrate development and an emerging model in developmental toxicology. Zebrafish AHR2 binds TCDD with high affinity, is transcriptionally active and has a major role in mediating the developmental toxicity of TCDD. Zebrafish AHR1 lacks the ability to bind TCDD and activate transcription, and has no known function. In the present study, we report a new zebrafish AHR, designated AHR1B, which shares 34% amino acid sequence identity with AHR1 (AHR1A). The *ahr1b* gene resides on chromosome 22, adjacent to *ahr2*, whereas the *ahr1a* gene is located on chromo-

some 16. AHR1B is expressed in embryos as early as 24 hours post-fertilization and increases through the next 2 days, but expression is not inducible by TCDD. In contrast with the previously identified AHR1A, *in vitro*-expressed AHR1B protein exhibits specific, high-affinity binding of [³H]TCDD. Furthermore, AHR1B is able to activate the transcription of a reporter gene under the control of AHR response elements with an efficacy comparable with that of AHR2, but with a higher EC₅₀. We speculate that AHR1B may have a physiological role, such as in embryonic development, whereas AHR2 mediates the response to xenobiotics.

Key words: aryl hydrocarbon receptor (AHR), dioxin, gene duplication, pufferfish, toxicology, zebrafish.

INTRODUCTION

TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) is a potent member of a group of toxic compounds found ubiquitously in the environment. The toxic effects of TCDD are similar among vertebrate species, and are mediated by a ligand-activated transcription factor, AHR (aryl hydrocarbon receptor) [1–3]. Upon ligand binding, the AHR translocates to the nucleus and forms a heterodimer with ARNT (AHR nuclear translocator). The AHR/ARNT heterodimer can regulate gene expression by binding to AHREs (AHR response elements) in the promoters of target genes, such as CYP1A (cytochrome P4501A) [4].

Fish are highly sensitive to TCDD, especially during early development [5–7], suggesting that they may be valuable models for elucidating mechanisms of altered gene expression and toxicity caused by dioxin-like compounds. The zebrafish (*Danio rerio*), in particular, has become a widely used model for studying multiple aspects of vertebrate development [8], including developmental toxicology [9–11]. The externally developing, transparent zebrafish embryos facilitate mechanistic studies involving environmental toxicants, such as TCDD. In addition, extensive molecular and genetic tools have been developed for use in zebrafish, including mapping panels, cDNA and genome sequencing projects (http://www.ensembl.org/Danio_rerio), and antisense knockdown technology.

Characterization of piscine AHR pathway members is essential for our ability to use fish as models in toxicology. AHRs have been identified in several fish species [12–17]. An interesting feature of the fish AHR pathway is the presence of multiple AHR forms

(2–6 per species), in contrast with the single mammalian AHR. Fish AHRs occur as two clades, designated AHR1 and AHR2 [13]. Unlike many other sets of paralogous genes in fish [18,19], AHR1 and AHR2 did not originate in the genome duplication specific to ray-finned fishes, but rather arose in an earlier duplication event that occurred before the divergence of ray-finned and lobe-finned fishes [20]. The functional significance of possessing two AHRs is not yet clear, but it has been suggested that the pleiotropic AHR functions have been partitioned among multiple fish AHRs [21].

Several members of the zebrafish AHR signalling pathway have been characterized, including an AHR1 [22,23], AHR2 [24], ARNT2 [25], ARNT1 [26] and two AHR repressors (AHR1 and AHR2) [27]. Zebrafish AHR2 exhibits high-affinity TCDD binding and can support transactivation of a reporter gene in conjunction with the zebrafish ARNT2b [23,24,28]. Knocking down AHR2 expression in zebrafish embryos provides complete or partial protection from certain toxic endpoints of TCDD exposure, indicating its important role in mediating TCDD developmental toxicity [3,29]. In contrast, the previously identified zebrafish AHR1 lacks the ability to bind TCDD or β -naphthoflavone *in vitro* and also is inactive in transactivation assays [23].

In the present study, we describe a new zebrafish AHR, namely AHR1B, which belongs to the AHR1 clade but is distinct from the previously identified AHR1 (AHR1A). Interestingly, the *ahr1b* gene is located on chromosome 22, adjacent to *ahr2*, whereas *ahr1a* maps to chromosome 16. We demonstrate that zebrafish *ahr1b*, unlike the previously identified *ahr1a*, encodes a functional AHR protein, is expressed during development and thus may play an important physiological role.

Abbreviations used: AHR, aryl hydrocarbon receptor; AHRE, AHR response element; ARNT, AHR nuclear translocator; CYP, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium; hpf, hours post-fertilization; MO, morpholino-modified oligonucleotide; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; UTR, untranslated region.

¹ To whom correspondence should be addressed (email skarchner@whoi.edu).

The nucleotide sequence for zebrafish AHR1B reported in this paper has been deposited in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under accession no. DQ016494.

MATERIALS AND METHODS

Chemicals

[³H]TCDD (2,3,7,8-tetrachloro[1,6-³H]dibenzo-*p*-dioxin; 35 Ci/mmol, purified to ≥ 95 % by HPLC) was obtained from Chemsyn Science Laboratories (Lenexa, KS, U.S.A.). [³⁵S]Methionine was purchased from Amersham Biosciences. TCDD was obtained from Ultra Scientific (Hope, RI, U.S.A.). All other chemicals were from Sigma (St. Louis, MO, U.S.A.).

Tissues and RNA isolation

Adult zebrafish (TL strain) were generously provided by Dr M. Fishman (Massachusetts General Hospital, Charlestown, MA, U.S.A.). Fish were maintained according to the guidelines in the zebrafish handbook [30]. Total RNA was isolated using RNA STAT-60 (Tel-Test B, Inc., Friendswood, TX, U.S.A.). Poly(A)⁺ (polyadenylated) RNA was isolated with the MicroPoly(A)Purist kit (Ambion, Austin, TX, U.S.A.). Zebrafish embryos at 24 hpf (hours post-fertilization) were obtained from Scientific Hatcheries (Huntington Beach, CA, U.S.A.). Total RNA was isolated from two groups of 100 untreated embryos at 24 hpf. Groups of 100 embryos were exposed to DMSO (0.1 %) or TCDD (2 nM) for 4 h at 28 °C. Total RNA was isolated from groups of 100 embryos at 48 and 72 hpf (three groups of embryos per chemical at each time) and used for real-time RT (reverse transcriptase)-PCR as described below.

Oligonucleotide primers

Primers were synthesized by the Midland Certified Reagent Co., Inc. (Midland, TX, U.S.A.) and used as described below. Primer sequences were: Dr1r1, 5'-AGAGACATATTTGGGGCAGGATCTCC-3'; Dr1r2, 5'-GGCCAGTGTAATTCGCGCTGAAGG-3'; Dr1f1, 5'-AGGGAGCTATGGCATCATCTTCGTCC-3'; Dr1f2, 5'-CACCCACTTATTACGCCTCCTATACC-3'; Dr1ex4F, 5'-AATGGCTTTGTGTTGGTGGTCACAGC-3'; Dr1ex10R, 5'-CATGGGTCAACTCAGAGCCATAATCC-3'; Dr1Met, 5'-GACTTG-GACTCCTCATATTGACG-3'; Dr1End, 5'-ATGGATGGCATC-TCTTCGGATGG-3'; qfex5, 5'-GGACTACTTGGGGTTCCAT-CAGTCG-3'; ex6-3', 5'-ACTGTAGCACGTCCTTGATGCATC-AG-3'; qfex4-5, 5'-CTACTTGGGCTTCCATCAGTCG-3'; qr ex5-6, 5'-ATCACTGCTGCTCTGCATTGCCTC-3'; DrAHR1Af, 5'-TTGTTCTTGGCTACACAGAGGCAGAGC-3'; DrAHR1Ar, 5'-CCCAGTTTCACCGGTCCTCATCATCC-3'; DrAHR1Bf, 5'-GGTTTGTGCTCAAAACAACAGTAACCACG-3'; DrAHR1Br, 5'-CCACCAACACAAAGCCATTAAGAGCCTG-3'; zfAHR2qf, 5'-CTACTTGGGCTTCCATCAGTCG-3'; zfAHR2qr, 5'-GTCA-CTTAGGGATTGAGAGCG-3'; zBacqf, 5'-CAACAGAGAG-AAGATGACACAGATCA-3'; zBacqr, 5'-GTCACACCATCAC-CAGAGTCCATCAC-3'.

5'- and 3'-RACE (5'- and 3'-rapid amplification of cDNA ends)

Double-stranded zebrafish cDNA was synthesized using a Marathon cDNA Amplification kit (BD Biosciences, Palo Alto, CA, U.S.A.). Adaptors were ligated to both ends of the cDNAs. Gene-specific primers were coupled with adaptor primers in the PCR using Advantage DNA polymerase mix 2 (BD Biosciences). For 5'-RACE, primer Dr1r1 and nested primer Dr1r2 were used. For 3'-RACE, primer Dr1f1 and nested primer Dr1f2 were used. The PCR parameters for the first round amplification were: 94 °C, 5 s/72 °C, 2 min for 5 cycles; 94 °C, 5 s/70 °C, 2 min for 5 cycles; and 94 °C, 5 s/68 °C, 2 min for 25 cycles. The nested PCR parameters were 94 °C, 5 s/72 °C, 2 min for 5 cycles; 94 °C, 5 s/70 °C, 2 min for 5 cycles; and 94 °C, 5 s/68 °C, 2 min for 20 cycles.

RT-PCR

An Omniscript cDNA synthesis kit (Qiagen, Valencia, CA, U.S.A.) was used to synthesize cDNA from zebrafish total RNA. Full-length AHR1B was amplified from a single whole female fish cDNA using Advantage DNA polymerase mix 2 (BD Biosciences) with the following PCR conditions: 94 °C, 1 min; 94 °C, 5 s/72 °C, 3 min for 5 cycles, 94 °C, 5 s/70 °C, 3 min for 5 cycles and 94 °C, 5 s/68 °C, 3 min for 30 cycles; and 68 °C, 5 min. AHR1A (primer pair qfex5/ex6-3'), AHR1B (primer pair Dr1ex4F/Dr1r1) and AHR2 (primer pair qfex4-5/qr ex5-6) fragments were amplified with AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, U.S.A.) from zebrafish embryo cDNA. The amplification parameters were: 94 °C, 10 min and 94 °C, 15 s/65 °C, 30 s for 35 cycles.

Real-time RT-PCR

cDNA was synthesized from 2 µg of total RNA using random hexamers and the Omniscript cDNA Synthesis kit (Qiagen). Quantitative PCR was performed using the iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, U.S.A.) in an iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories). Primers for AHR1B (DrAHR1Bf/DrAHR1Br), AHR1A (DrAHR1Af/DrAHR1Ar), AHR2 (zfAHR2qf/zfAHR2qr) and β-actin (zBacqf/zBacqr) were designed with one primer spanning an exon-exon junction to avoid amplification of genomic DNA. The PCRs consisted of the following: 11 µl of water, 12.5 µl of iQ SYBR Green Supermix, 0.25 µl of 5'-primer (10 µM), 0.25 µl of 3'-primer (10 µM) and 1 µl of cDNA. The PCR conditions were: 95 °C for 3 min, 95 °C for 15 s/65 °C for 1 min (40 cycles) for all samples, except that the annealing temperature was 66 °C for the AHR1A amplification. At the end of each PCR run, the PCR products were subjected to melt curve analysis to ensure that only a single product was amplified. There were three biological replicates (pools of embryos) and three technical replicates of each RNA sample. Expression data were quantified based on threshold cycle (*C_t*) values. For each gene, *C_t* values for each sample were averaged and normalized to values of β-actin. Relative mRNA expression for each gene was calculated as the fold change compared with the 24 hpf (untreated) control, according to the following equation [31,32]:

$$\text{Relative mRNA expression} = 2^{-\Delta\Delta C_t}$$

$$\text{where } \Delta\Delta C_t = [C_{t(\text{AHR})} - C_{t(\text{actin})}]_{48 \text{ hpf or } 72 \text{ hpf}} - [C_{t(\text{AHR})} - C_{t(\text{actin})}]_{24 \text{ hpf}}$$

DNA sequence and phylogenetic analysis

All PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI, U.S.A.) and sequenced on an ABI 373A Stretch DNA sequencer (University of Maine DNA Sequencing Facility, Orono, ME, U.S.A.). Several full-length clones were sequenced to maximize accuracy. DNA sequences were assembled and translated using MacVector and AssemblyLign sequence analysis software package (Oxford Molecular Group, Madison, WI, U.S.A.). Multiple sequence alignments were performed using CLUSTAL X [33]. The aligned amino acid sequences were used to create phylogenetic trees using maximum parsimony and distance (minimum evolution) criteria in PAUP*4.0b10 [34], as described previously [13,35].

Expression constructs

The full-length AHR1B cDNA was cut out of pGEM-T Easy vector with restriction enzymes EcoRI and NotI, and inserted into the same sites in pcDNA3.1 (Invitrogen, Carlsbad, CA,

U.S.A.). Expression constructs for the zebrafish AHR1A, AHR2 and ARNT2b (pBKCMV-zfAHR1, pBKCMV-zfAHR2 and pBKCMV-zfARNT2b) were generously provided by Dr R. Tanguay (Oregon State University, Corvallis, OR, U.S.A.) and Dr R. E. Peterson (University of Wisconsin, Madison, WI, U.S.A.). The pcDNA-zfARNT2b expression construct was made by transferring the insert from the pBK-CMV construct. The plasmid pGudLuc6.1, which contains the firefly luciferase reporter gene under the control of an MMTV (murine-mammary-tumour virus) promoter regulated by four AHREs from the murine *Cyp1A1* promoter [36], was a gift from Dr M. Denison (University of California, Davis, CA, U.S.A.).

In vitro protein synthesis and ligand-binding assay

TnT-Quick Coupled Reticulocyte Lysate System (Promega) was used to synthesize ³⁵S-methionine-labelled or -unlabelled proteins according to the manufacturer's instructions. Then, 5 µl of the ³⁵S-labelled TnT reaction mixtures was subjected to SDS/PAGE, followed by fluorography. AHR ligand binding was determined using unlabelled proteins by velocity sedimentation on sucrose gradients in a vertical tube rotor as described in detail earlier [14] with the following modifications. The TnT reaction mixtures were incubated overnight at 4°C with [³H]TCDD (2 or 8 nM). Non-specific binding was determined by reactions containing an empty vector (unprogrammed lysate).

Transfection and luciferase reporter assay

COS-7 cells were purchased from A.T.C.C. (Manassas, VA, U.S.A.) and maintained in DMEM (Dulbecco's modified Eagle's medium; Sigma) supplemented with fetal calf serum (10% final concentration) at 37°C under 5% CO₂. Cells were plated at 3 × 10⁴ cells/well in 48-well plates. Transfections were carried out in triplicate wells 24 h after plating. DNA and also Lipofectamine™ 2000 reagent (Invitrogen) were diluted in serum-free DMEM. For each well, a total of approx. 300 ng of DNA was complexed with 1 µl of Lipofectamine™ 2000. The mixture was then added to cells in DMEM with serum. Cells were dosed 5 h after transfection with either DMSO or TCDD at 0.5% final DMSO concentration. *Renilla* luciferase (pRL-TK; Promega) was used as the transfection control. Transfected DNA amounts were 5 ng each (unless noted otherwise) of the AHR expression constructs, 25 ng of ARNT2b, 20 ng of pGudLuc6.1 and 3 ng of pRL-TK. The total amount of transfected DNA was kept constant by the addition of pcDNA vector with no insert. Cells were lysed 18 h after dosing and luminescence was measured using the Dual Luciferase Assay kit (Promega) in a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA, U.S.A.). The final luminescence values were expressed as a ratio of the firefly luciferase units to the *Renilla* luciferase units. EC₅₀ values for induction of luciferase were determined by nonlinear regression using Prism version 3 software (GraphPad, San Diego, CA, U.S.A.). The data were fitted to the equation $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / \{1 + 10^{[(\log \text{EC}_{50} - X) \times \text{Hill slope}]}\}$, where X is the logarithm of TCDD concentration and Y is the normalized luciferase activity, and the slope was allowed to vary.

RESULTS

Discovery of a new zebrafish AHR gene

A survey of the zebrafish genome database (http://www.ensembl.org/Danio_rerio; assembly version 4) for sequences sharing significant identity with the killifish (*Fundulus heteroclitus*) AHR1

Figure 1 Phylogenetic analysis of AHR proteins

AHR amino acid sequences were aligned using CLUSTAL X and the alignments were used to construct phylogenetic trees using PAUP*4.0b10. Sequences aligned through amino acid 892 of zebrafish AHR1B (567 total characters including gaps but excluding poorly aligned regions) were used to construct a distance (minimum evolution) tree. The neighbour-joining tree was used as the starting tree and the optimal tree was found using a heuristic search with tree-bisection-reconnection as the branch-swapping algorithm. Numbers at nodes represent bootstrap values out of 100 replicates. Additional trees constructed using alignments of only AHR1 sequences (zebrafish, pufferfish, killifish and human) and either minimum evolution or maximum parsimony as optimality criterion showed the same topology, with strong bootstrap support for the DreAHR1B/TruAHR1B clade (92% in both cases). The prefixes used are Dre (*D. rerio*), Fhe (*F. heteroclitus*), Tru (*Ta. rubripes*), Ola (*Oryzias latipes*), Mmu (*Mus musculus*), Hsa (*Homo sapiens*) and Dme (*Drosophila melanogaster*). The GenBank® accession numbers of the sequences used in Figures 1 and 2 are: DQ016494 (DreAHR1B), AF258854 (DreAHR1A), AF063446 (DreAHR2), DQ088137 (TruAHR1A), DQ088138 (TruAHR1B), DQ088139 (TruAHR2A), DQ088140 (TruAHR2B), DQ088141 (TruAHR2C), AF024591 (FheAHR1), U29679 (FheAHR2), AB065094 (OlaAHR1), L19872 (HsaAHR), M94623 (MmuAHR) and AF050630 (DmeAHR).

(GenBank® accession no. AF024591) and AHR2 (GenBank® accession no. U29679) led to the detection of a previously unidentified AHR coding sequence. The transcript predicted by the database (ENSDART00000028787, derived from the predicted gene ENSDARG00000023537), contained a partial coding sequence for an AHR, corresponding to exons 4–11 of the human AHR. We completed the coding sequence at the 5'-end by RACE and obtained the sequence for exons 1–3. We also confirmed the 3'-end of the cDNA by RACE, and discovered that exon 11 of the predicted transcript was assembled incorrectly. (Subsequently, the ENSDART00000028787 cDNA in the database was updated with the correct exon 11; however, at the time of submission of the present paper, the 5'-end was still missing.) Using the 5'- and 3'-sequences determined by RACE, we amplified a full-length AHR cDNA of 4081 bp, including 467 and 794 bp of 5'- and 3'-UTRs (5'- and 3'-untranslated regions) respectively. The deduced AHR protein has 940 amino acids with a predicted molecular

Figure 2 Alignment of fish AHR1 and mammalian AHR protein sequences

The zebrafish AHR1B deduced amino acid sequence was aligned with other fish AHR1s and mammalian AHRs using CLUSTAL X. Shading and boxing indicates amino acid residues where three or more sequences are identical. The functional domains are indicated by solid lines and labelled above each solid line. Brackets indicate the ligand-binding domain. The prefixes and the accession numbers of sequences used in the alignment are listed in the legend to Figure 1.

mass of 104.8 kDa. It is distinct from the previously identified zebrafish AHRs, sharing 64 and 60% identity with AHR1 (GenBank® accession no. AF258854) and AHR2 (GenBank® accession no. AF063446) respectively when the conserved

N-terminal halves of the proteins are compared. The nucleotide sequence has been deposited in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under accession number DQ016494.

Figure 3 Chromosomal locations and gene structures of the zebrafish AHR genes

The gene structures of the zebrafish AHR1B and AHR2 on chromosome 22 (scaffold1810.3) and the zebrafish AHR1A on chromosome 16 were drawn to scale, as obtained from the Zv4 release of the zebrafish genome project (http://www.ensembl.org/Danio_rerio). Exons are shown as black boxes and numbered on top. UTRs are indicated as white boxes. The question marks indicate the introns for which size is not known. The arrows indicate the antisense strand (5'–3') direction. Owing to a possible misassembly in the genome database, the 5'-UTR of *ahr1b* and exons 4–11 are situated in opposing 5'–3' directions. Exons 1–3 of *ahr1b* are not included in the latest release of the genome project, and are therefore omitted from this diagram.

Phylogenetic analysis

Alignment of the new zebrafish AHR with mammalian and other fish AHRs followed by phylogenetic analysis revealed that it groups with fish AHR1s, most closely related to the pufferfish (*Takifugu rubripes*) AHR1B [37] (Figure 1). We chose to name this zebrafish gene *ahr1b* and designate the previously described AHR1 gene [23] as *ahr1a*. Alignment of full-length amino acid sequences of various fish and mammalian AHR1s shows a high degree of conservation in bHLH (basic helix–loop–helix) and PAS (PER-ARNT-SIM) domains (Figure 2). However, as compared with the other fish AHR1s, the zebrafish AHR1A has a noticeable degree of sequence divergence within these functional domains. This is also reflected in the tree (Figure 1), in which AHR1A appears as a sister group to the other AHR1 sequences.

Gene structure

The overall gene structure of *ahr1b* is similar to that of mammalian AHRs and other fish AHR1s with regard to the number and size of exons (Figure 3). The exon–intron boundaries were determined and found to be well conserved with the mammalian and other fish AHRs. Because exons 1–3 are not yet assembled in the latest release of the genome project (Zv4), the boundaries of these exons were determined from the trace archives data (<http://www.ncbi.nlm.nih.gov/Traces>); the sizes of introns 1–3 are not known. Interestingly, *ahr1b* is located on chromosome 22, adjacent to *ahr2*, whereas *ahr1a* is found on chromosome 16. The distance between the stop codon of AHR2 and the beginning of the 5'-UTR of AHR1B is approx. 6.3 kb. The tandem positioning of zebrafish *ahr2* and *ahr1b* is similar to the tandem arrangement of AHR2 and AHR1 genes in the *Ta. rubripes* genome [37].

Developmental expression of the zebrafish AHR1B

Previous studies have shown that members of the zebrafish AHR pathway (AHR1A, AHR2, ARNT2b and CYP1A) are expressed in embryos as early as 24 hpf, and CYP1A is inducible by TCDD at 24 hpf [23,24,38]. As a first step in investigating the potential contribution of AHR1B to the AHR pathway during development, we determined the mRNA expression in embryos (Figure 4). AHR1B transcripts, along with those for AHR1A and AHR2, were detectable in 24 hpf embryos (Figure 4a). Analysis by real-time RT–PCR showed that the expression of AHR1B increased at 48 and 72 hpf (Figure 4b). To determine whether AHR transcript levels are inducible by AHR ligands, we exposed

embryos to TCDD at 24 hpf and measured AHR expression at 48 and 72 hpf. AHR1A (Figure 4c) and AHR2 (Figure 4d) both were induced by TCDD at 72 hpf, similar to previous results showing induction of AHR1A (120 hpf [23]) and AHR2 (48 hpf [24,38]) in zebrafish embryos. In contrast, the expression of AHR1B was not significantly different in DMSO- and TCDD-exposed embryos (Figure 4b).

Functional characterization of the zebrafish AHR1B

In light of the fact that the AHR1A identified earlier is non-functional [23], we sought to determine whether the new AHR1B protein is capable of high-affinity binding and activation by TCDD. We synthesized AHR1A, AHR1B and AHR2 proteins by *in vitro* transcription and translation in the presence of [³⁵S]methionine to confirm expression (Figure 5a). We then synthesized unlabelled proteins and evaluated their [³H]TCDD-binding activities by velocity sedimentation analysis (Figures 5b and 5c). AHR1B exhibited high-affinity ligand binding at both 2 and 8 nM [³H]TCDD, with a specific binding peak comparable with that of AHR2. AHR1A failed to bind [³H]TCDD, as shown previously [23]. In an additional experiment, we used saturation-binding analysis to estimate the relative [³H]TCDD-binding affinities of AHR1B and AHR2. The *K_d* for specific binding of [³H]TCDD to AHR2 was 1.1 nM. AHR1B exhibited substantial specific binding even at low concentrations of [³H]TCDD, but binding was not saturated under the conditions of the assay, suggesting a higher *K_d* (lower affinity) for [³H]TCDD (results not shown).

We then assessed the ability of AHR1B to bind to AHREs and activate transcription after transient transfection into cultured cells. We chose COS-7 cells because these cells have been widely used to characterize AHR proteins from a variety of species [14,28,35,39] and were used to characterize the original zebrafish AHRs [23,24]. AHR1B was transiently expressed in COS-7 cells along with the zebrafish ARNT2b, and a luciferase reporter gene under the control of AHREs. In DMSO-treated cells, transfection of 5 or 50 ng of AHR1B expression construct resulted in an increase in the basal level of luciferase expression (Figure 6a), as seen with other AHRs [33]. Exposure of the cells to TCDD (final concentration, 10 nM) caused an approx. 5-fold increase in luciferase expression in AHR1B-transfected cells. AHR1B and AHR2 (5 ng each) supported comparable levels of TCDD-induced luciferase activity. Co-transfection of AHR1A (up to 50 ng) with ARNT2b did not activate luciferase expression above

Figure 4 AHR mRNA expression in zebrafish embryos

Zebrafish embryos (24 hpf) were either sampled untreated or exposed to DMSO (0.1%, v/v) or TCDD (2 nM) for 4 h and then sampled at 48 and 72 hpf. Total RNA was isolated from zebrafish embryos at the indicated ages, and used for cDNA synthesis. (a) Small fragments of the zebrafish AHR cDNAs (238, 180 and 158 bp fragments of AHR1B, AHR1A and AHR2 respectively) were amplified by PCR and resolved on agarose gels stained with ethidium bromide. An inverted (black-on-white) image is shown. (b–d) Real-time RT–PCR was performed as described in the Materials and methods section. Results for each AHR gene were normalized to those for β -actin. Relative mRNA expression for each gene was calculated as the fold change compared with the 24 hpf (untreated) control. Error bars indicate standard deviation ($n = 3$). For the 24 hpf samples, $n = 2$. (b) AHR1B, (c) AHR1A, (d) AHR2. * $P < 0.05$, statistical significance versus DMSO-treated embryos (t test). The P values for differences between DMSO and TCDD groups were: (b) AHR1B, 48 hpf ($P = 0.588$) and 72 hpf ($P = 0.145$); (c) AHR1A, 48 hpf ($P = 0.219$) and 72 hpf ($P = 0.00006$); (d) AHR2, 48 hpf ($P = 0.052$) and 72 hpf ($P = 0.002$).

the ‘no AHR’ control in either the DMSO- or TCDD-treated cells (Figure 6a). To determine the sensitivity of AHR1B in relation to AHR2, we compared the transactivation abilities of AHR1B and AHR2 (5 ng of each plasmid) in COS-7 cells exposed to increasing concentrations of TCDD (0.01–10 nM; Figure 6b). The maximum levels of luciferase activity for AHR1B and AHR2 were similar, but AHR1B appeared to be less sensitive to activation by TCDD,

with apparent EC_{50} values of 5.9 and 0.7 nM for AHR1B and AHR2 respectively.

DISCUSSION

The zebrafish is an established model in developmental biology and an emerging model in developmental toxicology. The optimal use of this model requires an understanding of the similarities and differences in signalling pathways involved in developmental or toxicological processes. The AHR signalling pathway, which has both developmental and toxicological roles [1,40,41], has been studied extensively in both mammalian and fish model systems, including zebrafish. We report in the present study a new zebrafish AHR, namely AHR1B, which, unlike a previously cloned zebrafish AHR1, exhibits high-affinity binding of TCDD and is transcriptionally active. These results demonstrate that zebrafish have at least two functional AHR paralogues, AHR1B and AHR2, which are expressed in embryos and are potentially involved in developmental signalling and in mediating the developmental toxicity of dioxin-like compounds.

Evolutionary implications of the zebrafish AHR1B

The identification and chromosomal location of AHR1B provide insights into the evolution of vertebrate AHR genes. Previous studies in killifish (*F. heteroclitus*) and other fish species, including zebrafish, revealed that most fish possess at least two AHR genes (AHR1 and AHR2) as opposed to the single AHR (AHR1) in mammals [13,14,17,23,24]. The existence of an AHR2 gene in cartilaginous fishes shows that the gene duplication event that gave rise to AHR1 and AHR2 occurred before the divergence of bony and cartilaginous fishes, and therefore before the separation of fish and mammalian lineages [13]. The newly identified *ahr1b* is adjacent to the *ahr2* gene on zebrafish chromosome 22. Similarly, there is emerging genomic evidence for a tandem arrangement of AHR1 and AHR2 genes in pufferfish (*Ta. rubripes* [37] and *Tetraodon nigroviridis*), medaka (*Oryzias latipes*) and chicken (J. M. Lapsieritis, S. I. Karchner and M. E. Hahn, unpublished work). These results suggest that AHR1 and AHR2 arose by a tandem gene duplication, followed by sequence divergence. In addition, some fish species (*Takifugu*, *Tetraodon* and medaka) have two sets of AHR1–AHR2 tandem pairs, most probably a result of the genome duplication that occurred in the ray-finned fish lineage after the divergence of ray-finned and lobe-finned fishes (leading to mammals) [18,19].

In zebrafish, the genes that code for functional AHRs, *ahr1b* and *ahr2*, reside on chromosome 22, whereas *ahr1a* is located on chromosome 16. The AHR1A protein appears to be functionally inactive and exhibits sequence divergence in the domains normally associated with AHR function [23]. Because of this, we suggest that *ahr1a* is an incipient pseudogene. There is currently no evidence for an additional *ahr2* gene adjacent to *ahr1a* on chromosome 16.

The phylogenetic relationships of AHRs in pufferfish, zebrafish and killifish (Figure 1) provide evidence for the role of lineage-specific gene loss in shaping AHR diversity in fishes. Early ray-finned fishes (i.e. after the fish-specific whole-genome duplication) most likely possessed four AHR genes, organized in two tandem pairs: AHR1A–AHR2A and AHR1B–AHR2B. These four AHRs have been retained in the pufferfish [37] (Figure 1). In the zebrafish lineage, however, AHR2A has been lost, and AHR1A appears to be non-functional. In contrast, in killifish, the AHR1A and AHR2A genes have been retained, whereas AHR1B and AHR2B most likely are not present. This might be

Figure 5 Analysis of ligand binding by the zebrafish AHRs

(a) Autoradiogram of *in vitro* translated zebrafish AHR expression constructs in the presence of $[^{35}\text{S}]\text{methionine}$. (b, c) The zebrafish AHRs were expressed by *in vitro* transcription and translation, incubated with (b) 2 nM and (c) 8 nM $[^3\text{H}]\text{TCDD}$, and analysed on sucrose gradients (■). Non-specific binding (□) was measured as the binding of $[^3\text{H}]\text{TCDD}$ to unprogrammed lysate containing an empty pcDNA3.1 vector. Fraction number 1 corresponds to the top of the gradient.

considered as an example of ‘divergent resolution’ or ‘reciprocal silencing’ [42] at the species level. The important point is that although zebrafish and killifish each has retained a functional AHR1–AHR2 pair, the zebrafish AHR1(B) and killifish AHR1(A) are paralogues rather than orthologues (and each is a co-orthologue of the mammalian AHR). Similarly, zebrafish AHR2(B) and killifish AHR2(A) are paralogues, not orthologues. Understanding the functional consequences of these relationships will require further investigation.

Functional implications of the zebrafish AHR1B

The physiological or toxicological roles of AHR1B, and whether they are distinct from those of AHR2, are not yet known. Recent studies have demonstrated a fundamental role for zebrafish AHR2 in mediating CYP1A induction and the developmental toxicity of TCDD in zebrafish embryos. Targeted knock-down of AHR2 expression in embryos using an antisense MO (morpholino-modified oligonucleotide) provided partial or complete protection from many of the toxic endpoints of TCDD exposure, such as oedema, reduction in blood flow and lower jaw malformation [3]. The AHR2-MO also prevented induction of CYP1A between 24 and 96 hpf, suggesting that AHR2 expression was eliminated or reduced during this period, and that AHR2 regulates the induction of CYP1A. The AHR2-MO had no effect on the low expression of CYP1A in DMSO-treated embryos, however, and the untreated AHR2-morphant embryos did not have any visible developmental defect. This suggests that AHR2 does not regulate constitutive expression of CYP1A and that AHR2 expression is not essential in the normal progression of early developmental events (24 to ~96 hpf). Alternatively, low-level AHR2 expression, such as may occur in MO-treated embryos, may be sufficient for these events to occur.

Other studies suggest that there may be cell-type-specific regulation of CYP1A by different AHRs. Andreassen et al. [38] found that the expression of AHR2 and induced CYP1A mRNAs co-localized in many cell types of developing zebrafish embryos after exposure to TCDD. Haematopoietic tissues and renal tubules, however, expressed induced CYP1A mRNA but lacked detectable expression of AHR2. CYP1A induction in these cells could be regulated by AHR1B. Taken together, these and the AHR2 knockdown studies suggest possible roles of AHR1B in normal development and in regulating constitutive expression of CYP1A or cell-specific CYP1A induction.

To assess the functional properties of AHR1B, we chose two expression systems, (i) *in vitro* transcription and translation using rabbit reticulocyte lysate and (ii) transient transfection of COS-7 cells, that have been widely used to characterize AHRs from a variety of mammalian and non-mammalian species [14,28,35,39,43], including zebrafish [23,24,28]. As with any heterologous system, and despite the fundamental conservation of transcriptional and translational machinery in eukaryotes, it is formally possible that the results obtained do not precisely reflect the true function that would be found using a species-specific background. Thus our results, while highly suggestive, will require confirmation in zebrafish-specific systems.

Unlike the previously identified zebrafish AHR1A, AHR1B appears to be fully functional, at least as assessed *in vitro* and in a heterologous cell system. It exhibits specific binding of TCDD and is competent in activating transcription of a reporter gene under the control of AHREs, with an efficacy comparable with that of AHR2 but a sensitivity that appears to be approx. 8-fold lower. Saturation-binding analysis provides additional evidence that AHR1B has a somewhat lower TCDD-binding affinity than AHR2, suggesting that AHR2 may have greater relevance at low exposure concentrations of TCDD. It is not yet known whether this

Figure 6 Transcriptional activation by the zebrafish AHRs

(a) COS-7 cells were transfected with expression constructs for the zebrafish ARNT2b (25 ng), pGudLuc6.1 (a luciferase reporter construct under the control of AHREs), pRL-TK (transfection control) and the indicated AHR expression constructs. 'No AHR' indicates transfection with only the reporter construct and the transfection control. The cells were exposed to DMSO or TCDD (10 nM). The luciferase activity was measured in a luminometer and the relative luciferase units were calculated by normalizing the firefly luciferase activity to the transfection control *Renilla* luciferase activity. (b) COS-7 cells were transfected with the zebrafish ARNT2b (25 ng), pGudLuc6.1, pRL-TK and either the zebrafish AHR1B (5 ng) or AHR2 (5 ng) expression constructs. The cells were exposed to increasing concentrations of TCDD (0–10 nM) and the relative luciferase units were determined as in (a). EC₅₀ values were obtained by nonlinear regression.

apparent difference in affinity for TCDD extends to other AHR ligands, such as non-halogenated polynuclear aromatic hydrocarbons, which are also known to exhibit dioxin-like toxicity in fish embryos [44,45]. It is possible that AHR1B plays a role in development, in regulation of CYP1A and/or in some aspects of TCDD toxicity.

The mammalian AHR has both physiological and toxicological/adaptive functions [46–48]. The duplication/degeneration/complementation model of evolution after gene duplication predicts that, under some conditions, duplicate genes may partition the subfunctions of their pro-orthologue [49,50]. While AHR1B and AHR2 are not strictly co-orthologues of the mammalian AHR (AHR1B is an orthologue and AHR2 is a paralogue), it is possible that they have distinct functions that together resemble those of the mammalian AHR. We suggested earlier [21] that fish AHR2s may have a more prominent role in the response to xenobiotics, whereas AHR1s may serve other, more physiological, functions. AHR1B mRNA is expressed in zebrafish embryos as early as 24 hpf, consistent with a potential role in normal development. The apparently reduced TCDD sensitivity of AHR1B as compared with AHR2 might also be taken as indirect evidence for a more physiological role for AHR1B.

However, it will be important to determine the relative abilities of natural ligands of potential physiological relevance [51–54] to interact with these two proteins *in vitro* and *in vivo*. In addition, morpholino experiments to knock down the expression of AHR1B and AHR2 individually or simultaneously will be valuable in revealing their possible distinct versus overlapping roles.

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