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Characterization of an Aryl Hydrocarbon Receptor Repressor (AHRR) from the Frog Xenopus laevis

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Characterization of an Aryl Hydrocarbon Receptor Repressor (AHRR) from the Frog *Xenopus laevis*

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Questions

- Does the lack of TCDD toxicity and CYP1A inducibility during early frog life stages result from constitutive AHRR expression?
- Does AHRR repress transcriptional activity of the two *X. laevis* AHR proteins: AHR1α and AHR1β?
- What is the temporal and TCDD inducible expression pattern of AHRR mRNA?

Abstract

Xenopus laevis and other frogs are extremely insensitive to the toxic effects of xenobiotic ligands of the aryl hydrocarbon receptor (AHR), including 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD). Embryos and premetamorphic life stages are especially insensitive, and they are refractory to induction of *Cytochrome P4501A6* (*CYP1A6*), the most highly induced target gene in older tadpoles. The aryl hydrocarbon receptor repressor (AHRR) is a member of the AHR gene family. AHRR expression is ordinarily induced by TCDD in an AHR- and ARNT-dependent fashion. The AHRR protein then binds ARNT and represses AHR transcriptional activity in an apparent negative feedback loop. In this study, we sought to test the hypothesis that constitutive AHRR expression underlies the lack of CYP1A6 induction and TCDD toxicity in early frog life stages. Using an RT-PCR approach, we determined the sequence of a single AHRR cDNA encoding a protein of 85.3 kDa sharing 52-55 percent identity with the aligned bHLH/PAS domains of AHRRs from mammals and fish. In transient transfection assays, *X. laevis* AHRR inhibited TCDD-induced reporter gene expression mediated by either *X. laevis* AHR paralog, AHR1α or AHR1β. AHRR mRNA was not detectable by quantitative RT-PCR in *X. laevis* embryos (Nieuwkoop-Faber stage 33-38; approximately 52 h.p.f.), even following overnight exposure to 3H-TCDD (59.4 ng/g wet weight). In contrast, AHRR was induced 2- to 4-fold in whole tadpoles at stages 52-55 (prometamorphic; ~4 weeks post-fertilization) and in isolated viscera of stage 62 tadpoles (in the metamorphic climax; ~ 7 weeks post-fertilization), even at much smaller TCDD body burdens (3-8 ng/g). Although the magnitude of induction was much smaller, the temporal pattern of AHRR expression and inducibility resembled that previously observed for *CYP1A6*. Thus, attenuated transcriptional activation of AHR target genes and low TCDD toxicity in *X. laevis* embryos cannot be explained by constitutive, high-level expression of AHRR.

Dioxin Toxicity

•Dioxin-like compounds, including polychlorinated biphenyls (PCBs) and 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD), comprise a major class of environmental toxins and have a widespread impact on the health of ecosystems.
•These contaminants are released by industrial processes including paper bleaching, metal refining, and waste incineration (1).
•Dioxins can accumulate in human and animal tissues and persist up the food chain.
•Exposure to dioxin-like compounds leads to numerous pathologies in most vertebrates, including wasting, thymic atrophy, skin lesions, immunological effects, reproductive and developmental abnormalities, and death (1,2).
•Unusually, *X. laevis* and other frogs are 100 to 1000-fold less sensitive to dioxin-induced developmental toxicity than fish (3).
•Insensitivity of frog embryos to a major class of developmental toxicants suggests the need to re-evaluate the use of *Xenopus* as a model organism for developmental toxicity testing.

AHR Pathway & AHRR

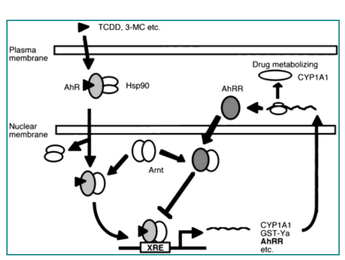

Figure from Mimura *et al.* (1999)

•AHR pathway is activated by dioxin exposure and mediates toxicity by altering expression of genes responsible for cell division and detoxification (4) .
•After binding TCDD, AHR enters the nucleus, and forms a dimer with the ARNT protein (AhR nuclear translocator).
•AHR-ARNT binds to xenobiotic response elements and upregulates transcription of target genes, including CYP1A6 and AHRR (AHR Repressor). (5)
•AHRR protein is a member of AHR family, documented in mammals and fish (4, 6).
•AHRR protein competes with AHR for binding ARNT and XREs and represses the transcriptional activity of AHR (2, 6).

Results

Cloning the AHRR Open Reading Frame (ORF)

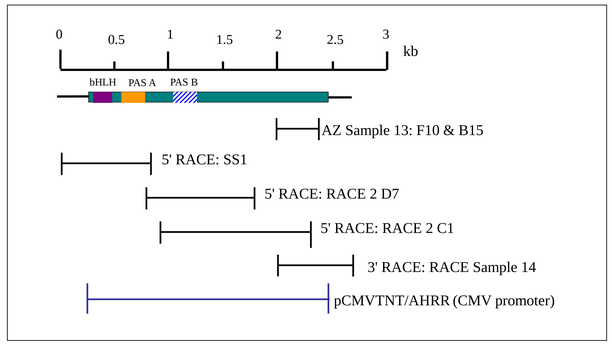

Figure 2. *X. laevis* AHRR opening reading frame (ORF) cDNA sequence was determined via reverse transcriptase PCR and RACE (Rapid Amplification of cDNA Ends). Partial cDNA encoding AHRR (AZ Sample 13) was amplified from stage 62-64 TCDD-dosed total RNA using degenerate primers designed based on a putative AHRR sequence from the *Xenopus tropicalis* genome database. From total RNA, RACE was used to synthesize cDNA with adaptor sequences on each 5' end. Primers designed to bind to my partial cDNA sequence and primers specific to the adaptor sequences provided by Clontech were used to extend the 5' and 3' ends of the DNA. Sequence for the 5' end of the *X. laevis* AHRR gene was determined by Shana Scogin using the GeneRacer kit (5' RACE: SS1). PCR Conditions: 95˚ /10 min; [94˚ /30s; 60˚ /30s; 72˚ /90s] x 5 cycles; [94˚ /30s; 58˚ /30s; 72˚ /90s] x 5 cycles; [94˚ /30s; 55˚ /30s; 72˚ /90s] x 27 cycles; 72˚ /7 min. RACE PCR: [94˚ /5s; 72˚ /3 min] x 5 cycles; [94˚ /5s; 70˚ /10s; 72˚ /3 min] x 3 cycles; [94˚ /5s; 67˚ /10s; 72˚ /3 min] x 35 cycles. Nested RACE PCR: [94˚ /5s; 66˚ /10s; 72˚ /3 min] x 25 cycles; 72˚ /4 min.

Conclusions: *X. laevis* AHRR cDNA is 2769 bp long, including ~220 bp 5' UTR and ~280 bp 3' UTR. AHRR cDNA encodes for a protein of 85.3 kDa (754 amino acids). The full length AHRR ORF was synthesized and cloned into the pCMVTNT expression vector by Epoch Biolabs (Texas).

Phylogenetic Analysis of AHRR & AHR Proteins

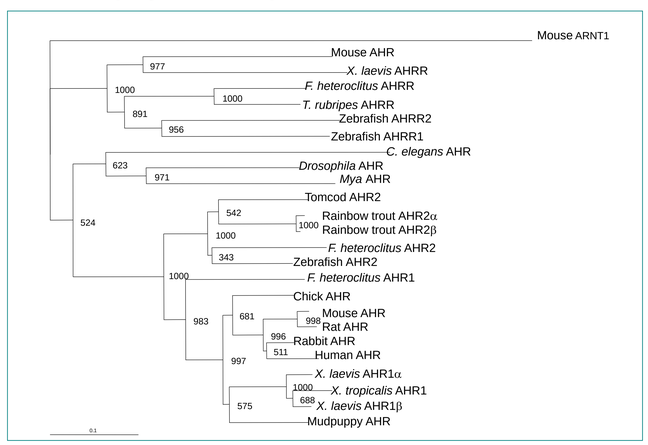

Figure 2. The PAS domains of AHR and AHRR proteins were aligned in ClustalX and a phylogenetic tree was constructed using the Neighbor-Joining approach. Numbers at the branch point represent bootstrap values based on 1000 samplings. Mouse (Mus musculus) ARNT1 (GenBank accession number U14333) was used as an outgroup. All other sequences obtained from GenBank.

Conclusions: Analysis places *X. laevis* AHRR in the AHRR clade, distinct from AHR proteins. An alignment of the deduced *X. laevis* AHRR amino acid sequence with AHRR amino acid sequences from other vertebrates demonstrates high levels of conservation in the N-terminal regions, particularly in the bHLH and PAS-A domains and a region following the PAS-A domain (data not shown). The 85.3 kDa *X. laevis* AHRR protein shares 52-55 percent identity with the aligned bHLH/PAS domains of AHRRs from mammals and fish.

Repression of AHR-dependent transactivation by AHRR

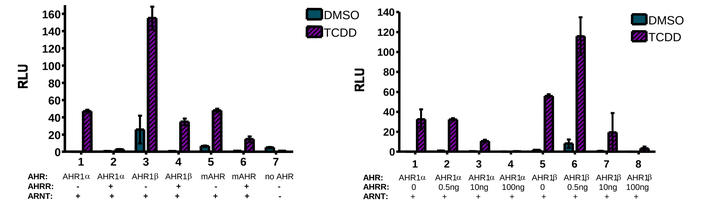

Figure 3. A) Cos-7 cells were transfected with 50ng AHRR (or 50ng pCMVTNT), 50ng AHR1α or AHR1β, 50ng ARNT1, pGudLuc6.1, pRL-TK, and pCMTVTNT to a total of 300ng DNA. Cells were treated with DMSO or 50nM TCDD. Relative luciferase units are given as a ratio of firefly to Renilla luciferase activity. mAHR = mouse AHR **B)** COS-7 cells were transfected with 0-100ng of AHRR, 50 ng ARNT1, 50 ng AHR1α or AHR1β, pGudLuc6.1, pRL-TK, and pCMTVTNT to a total of 300ng DNA. Cells dosed and luciferase activity measured as in A. All plasmid constructs are made from *X. laevis* unless noted. Error bars = Standard error.

Conclusions: In transient transfection assays, *X. laevis* AHRR inhibited constitutive and TCDD-induced reporter gene expression mediated by either *X. laevis* AHR paralog, AHR1α or AHR1β. Repression of AHR1α and AHR1β transactivation by AHRR depends on the amount of AHRR used in the transfection, as previously shown in other organisms (4, 6).

Expression of AHRR and CYP1A6 at Sequential Life Stages

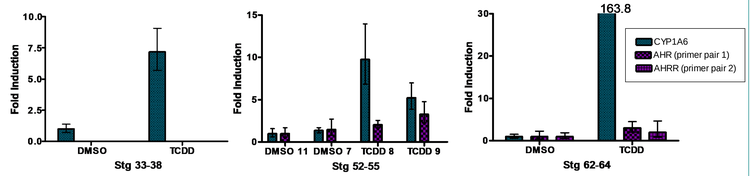

Figure 4. Expression and TCDD inducibility of AHRR and CYP1A6 mRNA in *X. laevis*. Total RNA was extracted from *X. laevis* whole tadpoles at stage 33-38, stage 52-55, and isolated viscera of stage 62-64 tadpoles following overnight exposure of tadpoles to 3H-TCDD or DMSO control. RNA was treated with DNase to remove contaminating DNA. cDNA was synthesized from 1μg total RNA using random hexamers and TaqMan Reverse Transcriptase kit. Primers for AHRR PCR were designed based on a deduced *X. laevis* partial consensus sequence for AHRR. PCR reactions consisted of: 10.25 μl H20, 12.5 μl SYBR Green Master Mix, 0.125 μl 5' primer (10 μM), 0.125 μl 3' primer (10μM), and 2 μl cDNA. PCR conditions: 95˚ /10 min; [95˚ /15s; 60˚ /1 min] x 45 cycles. Error bars = standard error.

Conclusions: The lack of detectable AHRR mRNA in stage 33-38 *Xenopus* did not support my hypothesis that low levels of dioxin sensitivity early in frog development results from high level, constitutive AHRR expression. Although the magnitude of induction is much smaller, the temporal pattern of AHRR expression and inducibility resembles that observed for *CYP1A6*. AHRR and CYP1A expression appear to be modulated by a common pathway in frogs and other organisms. (7)

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Acknowledgements

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