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Towards molecular understanding of species differences in dioxin sensitivity: initial characterization of Ah receptor cDNAs in birds and an amphibian

S.I. Karchner [a], S.W. Kennedy [b], S. Trudeau [b], M.E. Hahn [a,*]

[a] *Biology Department, MS#32, Woods Hole Oceanographic Institution, Woods Hole, MA 02543-1049, USA*
[b] *Environment Canada, CWS/NWRC, Hull, Quebec, Canada*

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Abstract

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related planar halogenated aromatic hydrocarbons (PHAHs) are highly toxic to most vertebrate animals, but there are dramatic species differences in sensitivity, both within and among vertebrate classes. For example, studies in cultured avian hepatocytes have revealed differential sensitivity of birds to PHAHs [Kennedy et al. (1996). *Toxicol. Appl. Pharmacol., 141*, 214–230]. Differences in the characteristics or expression of the aryl hydrocarbon receptor (AHR) could contribute to these species differences in PHAH responsiveness. To investigate the molecular mechanism of differential PHAH sensitivity, we have begun to characterize the AHR in white leghorn chicken (*Gallus gallus*), Pekin duck (*Anas platyrhynchos*), and common tern (*Sterna hirundo*), as well as an amphibian, mudpuppy (*Necturus maculosus*). Partial AHR cDNAs encompassing the helix-loop-helix and PAS domains were cloned and sequenced. Comparison of amino acid sequences in this region indicated a high degree of sequence conservation among the bird species (97% amino acid identity). The percent identity between bird sequences and either mouse or mudpuppy was lower (79%); the mudpuppy AHR was 74% identical to the mouse AHR. Phylogenetic analysis of these and other AHR amino acid sequences showed that the bird and mudpuppy AHRs were more closely related to mammalian and fish AHR1 forms than to fish AHR2. Future studies include the in vitro expression and functional characterization of AHRs from these and other non-mammalian vertebrates.

Keywords: Aryl hydrocarbon receptor; Dioxins; Differential sensitivity; Common tern; Mudpuppy; Chicken; Birds

* Corresponding author. Tel.: +1-508-289-3242; fax: +1-508-457-2134.
E-mail address: mhahn@whoi.edu (M.E. Hahn).

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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related planar halogenated aromatic hydrocarbons (PHAHs) are common contaminants in terrestrial and aquatic environments. Exposure of some species of birds (Giesy, Ludwig & Tillitt, 1994) and amphibians (Bonin, Des Granges, Bishop, Rodrigue, Gendron & Elliott, 1995) to high levels of PHAHs has been documented. The toxicity of PHAHs to mammals is well known, but effects in non-mammalian vertebrates are less well understood. Large differences in sensitivity to these compounds among and within vertebrate classes have been observed, but the mechanisms underlying such differences are not well understood (Hahn, 1998b).

Most effects of PHAHs in vertebrate animals occur through activation of the aryl hydrocarbon receptor (AHR), a ligand-dependent transcription factor and member of the bHLH-PAS gene family (Hahn, 1998a; Schmidt & Bradfield, 1996). Differences in the characteristics or expression of the AHR could contribute to differences in PHAH responsiveness, as suggested by studies in inbred mice (Poland, Palen & Glover, 1994). Upon PHAH binding, the AHR forms a transcriptionally active protein complex that also includes the AHR nuclear translocator (ARNT) protein. The AHR–ARNT complex is able to alter the expression of several genes, such as cytochrome P450 1A1 (CYP1A1).

CYP1A1 induction has been utilized as a sensitive marker for the activation of AHR-dependent pathways. Earlier, we showed that induction of CYP1A1 in cultured avian embryo hepatocytes could be used to determine the sensitivity of several bird species to PHAHs (Kennedy, Lorenzen, Jones, Hahn & Stegeman, 1996) In those studies, the sensitivities to TCDD differed by orders of magnitude among species. For example, when EC_{50} values for CYP1A induction by TCDD in bird hepatocytes were expressed relative to that of white leghorn chicken (taken as 1.0), the relative sensitivities were 0.008 for Pekin duck, and 0.01 for common tern (Kennedy et al., 1996; Lorenzen, Shutt & Kennedy, 1997).

To understand the mechanistic basis for differential PHAH sensitivity, we have begun to characterize the AHR in several species of birds (chicken, duck, common tern) and an amphibian (mudpuppy). We utilized the approach of reverse transcription–polymerase chain reaction (RT–PCR) with degenerate primers (Karchner & Hahn, 1996) for obtaining partial AHR sequences. Our main objectives were to assess the degree of conservation of AHR amino acid sequences from mammalian and non-mammalian species, to determine whether avian and amphibian AHRs are more closely related to AHR1 or to AHR2 forms identified in fish (Hahn, Karchner, Shapiro & Perera, 1997; Karchner, Powell & Hahn, 1999), and to provide tools for future studies on the mechanistic basis of species differences in TCDD sensitivity.

Total RNA was isolated from livers of white leghorn chicken, Pekin duck, and common tern embryos, and adult mudpuppy using RNA Stat 60 solution and protocol (Tel-TestB, Inc., Friendswood, TX, USA). PolyA$^+$ RNA fraction was purified with oligo-dT spin columns (5′–3′, Inc., Boulder, CO, USA). Random hexamers were used to prime cDNA synthesis (Gene-Amp RNA PCR kit, Perkin–Elmer). Degenerate PCR primers were designed targeting conserved regions of the AHR, the helix-loop-helix and PAS domains, which are involved in ligand-binding and protein–protein interactions (Fig. 1, see also Karchner & Hahn, 1996). The annealing

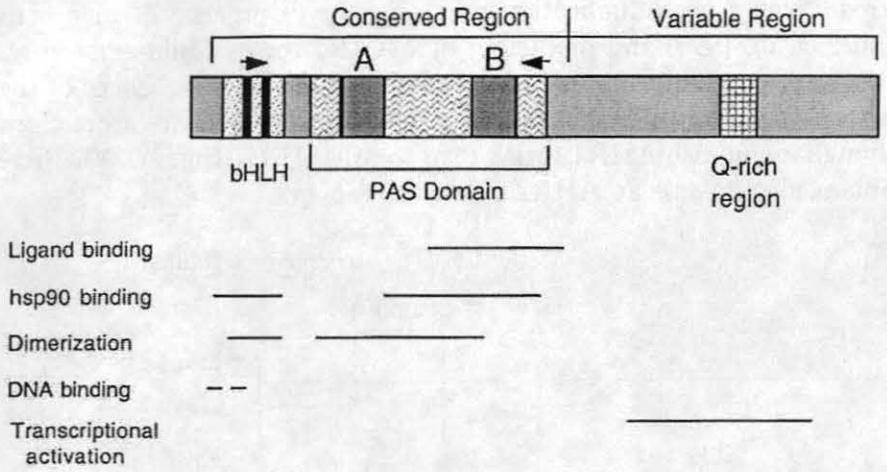

Fig. 1. Domain structure of the aryl hydrocarbon receptor (AHR) showing conserved regions: bHLH (basic helix-loop-helix), Per-Arnt-Sim (PAS) A and B imperfect repeats, and glutamine-rich regions. In this study, AHR cDNAs were amplified by reverse transcription–polymerase chain reaction (RT–PCR) using primers Qf and B1 targeted to the helix-loop-helix and the PAS domains, respectively (see arrows). The target of primer Qf (NPSKRHR) corresponds to amino acids 33–39 of the mouse AHR; the primer sequence is 5′-AACCCITCIAAGMGICAYMG-3′ (Karchner et al., 1999). The target of primer B1 (KTGESGM) corresponds to amino acids 336–342 of the mouse AHR; the primer sequence is 5′-GCTCTAGACATICCRCTYTCICCIGTYTT-3′ (Hahn & Karchner, 1995).

temperature for PCR was 52°C. PCR products were analyzed on agarose gels and cDNA fragments of the expected size were purified and subcloned into a plasmid vector (pGEM-T, Promega). DNA samples were sequenced with the SequiTherm Excel long-read cycle sequencing kit (Epicentre Technologies, Madison, WI, USA) and an automated DNA sequencer (LI-COR, Inc., Lincoln, NE, USA). DNA sequences were assembled and translated using MacVector/AssemblyLign sequence analysis software (Oxford Molecular Company, Madison, WI, USA). Multiple sequence alignments were performed using CLUSTALW version 1.7. The aligned amino acid sequences, including a sampling of mammalian and fish AHRs, were used to construct phylogenetic trees using the Neighbor-Joining algorithm, as described earlier (Hahn et al., 1997).

PCR-amplified cDNA fragments of the expected size (~950 bp) were obtained for chicken, duck, tern, and mudpuppy. Multiple clones from each fragment were sequenced; the translated sequences showed strong identities with the bHLH-PAS domains of known AHR sequences. Multiple alignment of the amino acid sequences of these and other AHRs indicated a high degree of sequence conservation among the bird species (97% amino acid identity). The percent identity between bird sequences and either mouse or mudpuppy was lower (79%); the mudpuppy AHR was 74% identical to the mouse AHR.

Earlier, we identified a second form of AHR (AHR2, see Hahn et al., 1997; Karchner et al., 1999) and showed that AHR2 is the predominant form in many fish (Hahn, 1998a; Karchner et al., 1999). AHR1 and AHR2 are highly divergent (~40% overall amino acid identity; ~60% identity in the N-terminal half), suggesting that

they arose from a gene duplication predating the divergence of mammals and fish (Karchner et al., 1999) and predicting that AHR2 forms could exist in other vertebrate taxa. Phylogenetic analysis of the partial amino acid sequences of the bird and mudpuppy AHRs identified here showed that these proteins are more closely related to mammalian and fish AHR1 forms than to fish AHR2 (Fig. 2). Whether birds and amphibians also possess an AHR2 is not yet known.

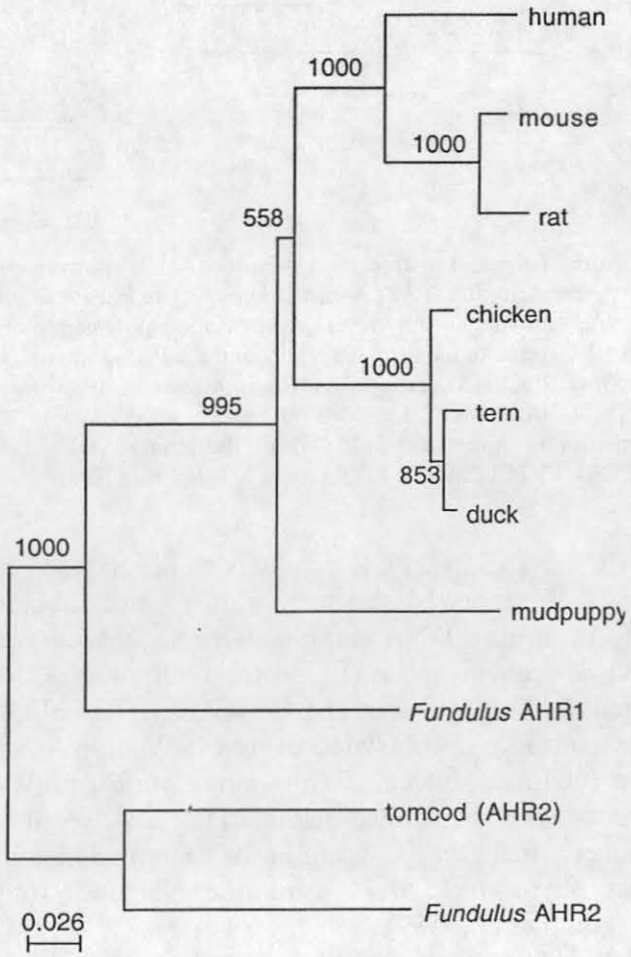

Fig. 2. Phylogenetic analysis (neighbor-joining method) of aryl hydrocarbon receptor (AHR) amino acid sequences. The tree was constructed from an amino acid alignment performed by CLUSTALW, version 1.7, and drawn using the NJPlot program. The alignment included an approximately 300-residue region amplified by the polymerase chain reaction (PCR) primers (Fig. 1). Bootstrap values based on 1000 samplings are shown above each branch. Positions with gaps are excluded and corrections were made for multiple substitutions. The scale bar indicates a distance corresponding to 26 amino acid residue differences out of 1000. Accession numbers for the analyzed sequences: human L19872 (Dolwick, Schmidt, Carver, Swanson & Bradfield, 1993), mouse M94623 (Burbach, Poland & Bradfield, 1992), rat U09000 (Carver, Hogenesch & Bradfield, 1994), *Fundulus* AHR1 AF024591 (Hahn et al., 1997; Karchner et al., 1999), *Fundulus* AHR2 U29679 (Hahn & Karchner, 1995; Karchner et al., 1999), tomcod S1079286 (Roy & Wirgin, 1997). The bird and amphibian AHR sequences reported here have been deposited in the GenBank database, with accession numbers: AF192502 (chicken), AF192503 (common tern), AF192501 (duck), and AF192500 (mudpuppy).

These results demonstrate the successful use of degenerate primers in RT–PCR to obtain initial, partial sequences of AHRs from non-mammalian vertebrates. Obtaining full-length AHR coding sequences for these species will allow complete characterization of the primary structure and better comparison of all functional regions, including the variable C-terminal transactivation domain (Fig. 1). In addition, the full-length coding sequence can be used to investigate the properties, including ligand-binding affinity, of each AHR by in vitro transcription and translation of the cDNAs, followed by equilibrium binding studies (e.g. Karchner et al., 1999). Determining levels of AHR mRNA and protein in several tissues could provide further insight into tissue and species differences in response to PHAHs. Characterization of other components of the AHR signaling pathway (e.g. CYP1A and the upstream dioxin response element sequences or AHR/ARNT interactions) in birds and amphibians might provide additional information on mechanisms underlying species differences in PHAH sensitivity.

Ignorance about species differences in sensitivity and their mechanistic basis is a major source of uncertainty in assessing the possible effects of PHAH in marine, estuarine, and freshwater environments. A molecular understanding of differential sensitivity will contribute to ecological and human risk assessments by providing a mechanistic foundation for extrapolation among species and by facilitating predictions of those species in an ecosystem that may be most at risk.

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