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RESEARCH COMMUNICATION

Evolutionary conservation of the vertebrate Ah (dioxin) receptor: amplification and sequencing of the PAS domain of a teleost Ah receptor cDNA

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The PAS domain of a teleost Ah receptor was amplified using reverse transcription-PCR with degenerate primers containing inosine. The deduced amino acid sequence of the amplified cDNA fragment was 62–64 % identical with the PAS domains of mammalian Ah receptors. These data demonstrate the homology of Ah receptors in mammals and fish, and reveal regions of this protein that are highly conserved between these diverse vertebrate groups.

INTRODUCTION

The Ah receptor (AhR) is a ligand-activated transcription factor that mediates many of the biological effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related planar halogenated aromatic hydrocarbons [1–3]. The mammalian AhR is bound and activated by a variety of xenobiotic and some natural compounds, but an endogenous ligand has not yet been identified. The recent cloning of AhR cDNAs from mouse [4,5], human [6] and rat [7] has revealed that this protein belongs to a new class of transcription factors that also includes the mammalian AhR nuclear translocator protein (ARNT; [8]) and the *Drosophila* proteins Per and Sim [5,8,9]. These proteins possess a homologous region of approx. 260–310 amino acids encompassing two imperfect 50-amino-acid 'repeats' and designated the PAS domain [9]. This domain has been shown to be involved in ligand binding to the AhR [5,10–12] as well as in protein–protein interactions among members of the PAS family [11,13,14].

Ligand binding and photo-affinity labelling studies suggest that a protein with characteristics of the mammalian AhR is present in most vertebrate classes, including teleost fish [15,16]. However, nothing is known about the structural similarities between mammalian AhRs and those in lower vertebrates. The objectives of the present work were to determine whether the AhR expressed in liver of teleost fish is homologous to the mammalian AhR and to identify conserved regions among the mammalian and fish AhR proteins that might be important for AhR function. An additional objective was to evaluate a reverse transcription-PCR (RT-PCR) approach using mixed oligonucleotides containing inosine as a means to obtain AhR sequences from non-mammalian vertebrates.

In order to identify and amplify a portion of a teleost AhR, pairs of degenerate oligonucleotides targeting residues at both ends of the PAS domain were designed, synthesized and used as primers in the PCR to amplify hepatic cDNA from the teleost *Fundulus heteroclitus*. Sequencing of the amplified product revealed the homologous relationship between mammalian and piscine AhRs and identified regions of the AhR PAS domain that are highly conserved throughout vertebrate evolution. These results demonstrate the usefulness of this RT-PCR approach for obtaining AhR sequences from diverse vertebrate species.

EXPERIMENTAL

Animals and RNA isolation

Killifish *F. heteroclitus* were chosen for these studies because they express a 116 kDa hepatic protein (putative AhR) that is strongly and specifically labelled by the AhR photo-affinity ligand 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin [16]. Male *F. heteroclitus* were captured by minnow trap in salt marshes on Cape Cod, MA, U.S.A. Total RNA was isolated from pooled livers of 13 fish using the method of Chomczynski and Sacchi [17]. Poly(A)$^+$ RNA was isolated by one pass over a column of oligo(dT) cellulose (New England Biolabs) as described by Farrell [18]. Yields and purity were determined spectrophotometrically.

Oligonucleotides

Oligonucleotides targeted to near the 5′-end of the first PAS repeat (PAS 'A') and the 3′-end of the second PAS repeat (PAS 'B') were designed based on the sequence of the mouse and human AhR cDNAs [5,6]. In designing these primers, consideration was given to: (i) amino acids conserved in both AhR proteins as well as in the other PAS proteins (ARNT, Per, Sim); (ii) amino acids encoded by only 1 or 2 possible codons; (iii) where multiple codons were possible, a fish codon usage table [19] was used to reduce degeneracy; and (iv) use of inosine at positions where 3- or 4-fold degeneracy remained [20]. In addition, the 3′-terminus of both primers targeted an amino acid conserved in three or four of the known PAS proteins. The

Abbreviations used: AhR, Ah receptor; ARNT, Ah receptor nuclear translocator; bHLH, basic helix-loop-helix; RT-PCR, reverse transcription-PCR.
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The nucleotide sequence reported in this paper has been deposited in the GenBank data base (accession number U29679).

Table 1 Degenerate oligodeoxynucleotides used for PCR amplification of a fish AhR cDNA

Lower case nucleotides represent added restriction sites or linker sequences. Numbering is based on the amino acid sequence deduced from the mouse AhR cDNA sequence [5].

Oligonucleotide	Sequence	Target amino acid sequence	Amino acid no.
AhR-A1	5′-cgggatcCARGCICTSAAYGGITT-3′	QALNGF	118–123
AhR-B1	5′-gctctagaCATICCRCTYTCICCIGTYTT-3′	KTGESGM	336–342
AhR-A2	5′-cgggatccGAYTAYCTIGGITTYCARCA-3′	DYLGFQQ	142–148
AhR-B2	5′-gctctagAGCTCIRCYTCIGTRTAICC-3′	GYTEVEL	303–309

upstream primer, AhR-A1, was 8-fold degenerate and contained two inosines (Table 1). The downstream primer, AhR-B1, was 8-fold degenerate and contained three inosines. A second set of degenerate oligonucleotides internal to AhR-A1 and AhR-B1 was also designed using the same criteria (Table 1). All oligonucleotides were synthesized by National Biosciences (Plymouth, MN, U.S.A.).

RT-PCR and DNA sequencing

First-strand cDNA synthesis and subsequent amplification were performed using the Gene-Amp RNA-PCR kit (Perkin–Elmer) according to the manufacturer's instructions. Poly(A)$^+$ RNA (1 μg) was reverse transcribed with priming by random hexamers. Amplification was performed in the same tube with primers AhR-A1 and AhR-B1, each at 1 μM. PCR conditions were as follows: 2 min at 95 °C followed by 35 cycles of melting (1 min, 95 °C), annealing (1 min, 50 °C) and extension (1 min, 72 °C). The last cycle was followed by extension for 7 min at 72 °C. Aliquots (1 μl) of several dilutions (10^{-2}–10^{-5}) of the original PCR reaction were re-amplified using oligonucleotides AhR-A2 and AhR-B2. Conditions were as described above except that annealing was at 60 °C. PCR products were revealed by ethidium bromide staining after separation on 2 % agarose. The band of expected size was excised, purified (Geneclean II; Bio 101, La Jolla, CA, U.S.A.) and directly sequenced from both ends using oligonucleotides AhR-A1 and AhR-B1. The sequence closest to the PCR primers was obtained after cloning of the PCR fragment into pBluescript II SK – (Stratagene). Sequencing of both strands was performed using Sequenase (version 2.0; US Biochemical) with modifications for direct sequencing of PCR products as described by Bachmann et al. [21]. The sequence was confirmed by cycle-sequencing (SequiTherm long-read cycle sequencing kit; Epicentre Technologies, Madison, WI, U.S.A.) using an automated DNA sequencer (LI-COR, Inc., Lincoln, NE, U.S.A.). Multiple alignment of the deduced amino acid sequences was performed using Clustal W [22,23].

Southern blotting

Genomic DNA was isolated from the liver of *F. heteroclitus* by the method of Wirgin et al. [24], digested with *Bam*HI, *Eco*RI and *Hin*dIII, separated on a 0.8 % agarose gel and transferred to Hybond (Amersham). Membranes were prehybridized in 6 × [0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA]/0.5 % SDS/100 μg/ml calf thymus DNA for 2 h at 65 °C. Hybridization with the 690 bp PCR product (labelled with [^{32}P]dCTP) was performed in 50 % formamide/5 × [0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA]/0.1 % SDS overnight at 42 °C. Membranes were washed at 65 °C in 2 × (0.15 M NaCl/0.015 M sodium citrate)/0.1 % SDS for 30 min followed by fluorography with Kodak XAR-5 film and one intensifying screen.

RESULTS AND DISCUSSION

Amplification and sequencing of a *Fundulus* AhR

Two pairs of mixed oligonucleotide primers were designed to target amino acids flanking the PAS domain of mammalian AhRs (Table 1). The degeneracy of these oligonucleotides was minimized by employing codon usage tables and by incorporating inosine where necessary. Reverse transcription of *F. heteroclitus* poly(A)$^+$ RNA and subsequent amplification using primers AhR-A1 and AhR-B1 produced a single band of the expected size (690 bp, including 15 bp of restriction sites) (Figure 1). To examine the specificity of this product, several dilutions (10^{-2}–10^{-5}) of the original PCR reaction were re-amplified using oligonucleotides AhR-A2 and AhR-B2. A single band of approx. 500 bp (expected size: 520 bp) was observed, even when the annealing temperature was raised to 60 °C (Figure 1).

Most of the original 690 bp PCR product was directly sequenced using AhR-A1 and AhR-B1 as sequencing primers; the sequence closest to the PCR primers was obtained after cloning of the PCR fragment into Bluescript. The nucleotide and deduced amino acid sequence of the amplified product are shown in Figure 2. The BLAST algorithm [25] was used to find gap-free alignments of the *Fundulus* sequence with other sequences in the non-redundant Genbank data base. The *Fundulus* deduced amino acid sequence was most closely related to the PAS regions of AhRs from mouse, rat, and human, with identities of 64, 64 and 62 % respectively. If conservative substitutions are included, the *Fundulus* sequence shared 78–80 % similarity with the mammalian AhRs. At the nucleotide level, there was 63–67 % identity between the *Fundulus* and mammalian sequences. Lesser and more restricted sequence relatedness was found between the *Fundulus* amino acid sequence and those of the human ARNT

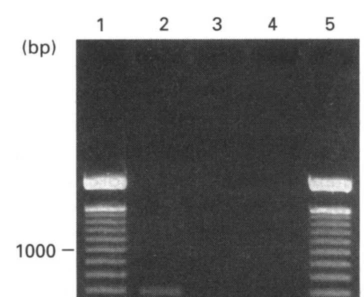

Figure 1 Ethidium bromide-stained gel showing PCR products resulting from amplification of *Fundulus* hepatic poly(A)$^+$ RNA with the degenerate primers listed in Table 1

Lanes 1 and 5, 100 bp ladder (Gibco/BRL); lane 2, 10 μl of PCR using primers A1 and B1; lane 3, 10 μl of PCR using primers A1 and B1, in the absence of reverse transcriptase; lane 4, 10 μl of PCR using primers A2 and B2 to amplify 1 μl of a 1:100 dilution of the reaction using A1 and B1.

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1/1                                     31/11
GTG CTG GTG GTC ACG TCT GAA GGA ATG GTC TTC TAC GCC TCT CCT ACG ATC AAA GAT TAC
val leu val val thr ser glu gly met val phe tyr ala ser pro thr ile lys asp tyr

61/21                                   91/31
CTG GGC TTC CAT CAG TCA GAC GTG GTC CAT CAG AGC GTG TTT GAG CTC ATC CAC ACT GAT
leu gly phe his gln ser asp val val his gln ser val phe glu leu ile his thr asp

121/41                                  151/51
GAC CGA GCG ATG TTc AGA GAG CAG CTC CAT TTT GCT TTA AAC CCT CCT CCA GTC GCC TCA
asp arg ala met phe arg glu gln leu his phe ala leu asn pro pro pro val ala ser

181/61                                  211/71
GAT GCA GAA TTC TCT CAG GGC TGT GCT AAA GCA GTG ATG TAC AAC CCT GAG CAG CTC CCA
asp ala glu phe ser gln gly cys ala lys ala val met tyr asn pro glu gln leu pro

241/81                                  271/91
CCG GAC AGC TCA TCC TTC CTG GAG AGA AGC TTT GTG TGT CGC TTC CGA TGT CTC CTG GAC
pro asp ser ser ser phe leu glu arg ser phe val cys arg phe arg cys leu leu asp

301/101                                 331/111
AAC TCC TCC GGC TTC CTG GCA CTG AAG TTC CAC GGG CGA CTA AAG TAC CTC CAA GGC CAG
asn ser ser gly phe leu ala leu lys phe his gly arg leu lys tyr leu gln gly gln

361/121                                 391/131
AAC CTT TGC AAG GAC ATT GAG ACG TGT AAA AAG GTT CAG CTG GCT CTG TTT GCC ATC GCC
asn leu cys lys asp ile glu thr cys lys lys val gln leu ala leu phe ala ile ala

421/141                                 451/151
ATG CCT GTC CAG CCT CCA TCC ATC GTG GAG ATC AGA GCC AAA ATG CTC CTT TTC CAA ACC
met pro val gln pro pro ser ile val glu ile arg ala lys met leu leu phe gln thr

481/161                                 511/171
AGG CAC AAG CTG GAC TTC ACA CCA ACA GGC GTT GAT ACC AGG GGG AAA GCC ATT CTG GGT
arg his lys leu asp phe thr pro thr gly val asp thr arg gly lys ala ile leu gly

541/181                                 571/191
TAC ACC GAG ATT GAA CTG TGT ATG AAA GGC TCG GGC TAC CAG TTC ATC CAT GCT GCC GAC
tyr thr glu ile glu leu cys met lys gly ser gly tyr gln phe ile his ala ala asp

601/201                                 631/211
ATG ATG TAC TGC GCT GAC AAC CAC ATC CGC ATG ATC
met met tyr cys ala asp asn his ile arg met ile
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Figure 2 Nucleotide and deduced amino acid sequence of the PAS domain of the *F. heteroclitus* AhR

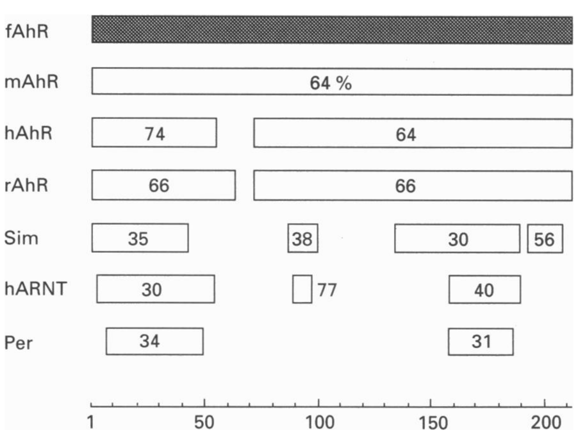

Figure 3 Results of a BLAST [25] search for gap-free alignments of the *Fundulus* sequence with other sequences in the non-redundant composite of protein data bases, using the BLOSUM62 scoring matrix [30]

Numbers within or next to boxes indicate percentage identity with the *Fundulus* AhR (fAhR) deduced amino acid sequence. GenBank accession numbers for the sequences used are as follows: mouse AhR b-1 allele (mAhR [5]; M94623), human AhR (hAhR [6]; L19872), rat AhR (rAhR [7]; U09000), Sim ([31]; A29945), human ARNT (hARNT [8]; P27540), and Per ([32]; A26427).

protein and the *Drosophila* proteins Per (several forms) and Sim (Figure 3; also see below). Together, these results indicate that we have identified a teleost homologue of the mammalian AhR.

Southern blotting and hybridization of restriction-digested *Fundulus* genomic DNA using the 690 bp PCR product as a probe provided evidence for *AHR* gene sequences in the *Fundulus* genome (Figure 4). The PCR product was also used to isolate clones from a *Fundulus* genomic DNA library; sequencing of these confirmed the sequence of the PCR product. Sequencing of additional clones suggested that a second *AHR* gene may be present in this species; this possibility is currently under investigation (S. I. Karchner and M. E. Hahn, unpublished work).

Conservation of AhR sequences among vertebrates

Overall, 129 amino acid residues out of 212 (61 %) were identical in all four of the vertebrate AhR PAS sequences (Figure 5). The identities were clustered within four regions, two of which contain the PAS-A and PAS-B repeats identified by other investigators [5,8,9]. Two regions between the PAS-A and PAS-B repeats were also highly similar; one of these contained a sequence of 12 amino acids (RCLLDNSSGFLA) that was perfectly conserved between the *Fundulus* AhR sequence and the three mammalian AhR sequences, but not ARNT, Sim, or Per.

The results obtained through amplification and partial sequencing of a *Fundulus* AhR provide evidence for the highly conserved nature of the AhR PAS domain between mammals and teleost fish, vertebrate classes separated by over 400 million years of evolution. The PAS domain is found in four proteins (Per, ARNT, AhR and Sim), three of which (ARNT, AhR and Sim) also possess an adjacent basic helix-loop-helix (bHLH) dimerization and DNA-binding motif. The PAS domain has been shown to be involved in the binding of ligands and a 90 kDa

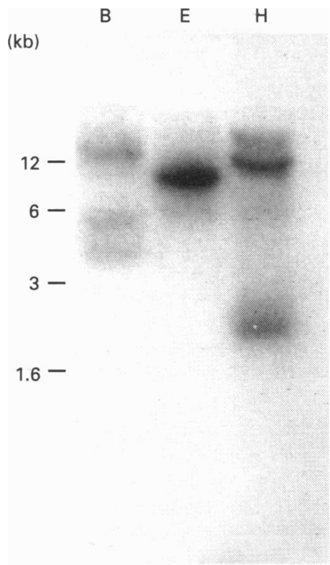

Figure 4 Southern blot of *Fundulus* genomic DNA

DNA was digested with *Bam*HI (B), *Eco*RI (E), and *Hin*dIII (H), separated, blotted and hybridized using the 690 bp PCR product as a probe as described in the Experimental section.

heat-shock protein to the AhR [5,10–12] as well as in the formation of Per–Per, Per–Sim, Per–ARNT and AhR–ARNT homo- and hetero-dimers [11,13,14]. In addition, analysis of *tim* mutants of *Drosophila* has implicated a region of PER containing the PAS domain in the *tim*-dependent nuclear localization of this protein [26]. Interestingly, a putative nuclear localization signal sequence (N/KKKGK) identified in the PAS domain of mammalian AhR proteins [6] is not well conserved in this *Fundulus* AhR (NLCKD), despite evidence that the AhR of other fish can undergo ligand-dependent cytosol-to-nuclear 'translocation' [15].

Phylogenetic relationship of PAS proteins

The phylogenetic relationship of the PAS domains of the known PAS proteins Per, Sim, ARNT (three species) and AhR (four species) was inferred using the Neighbor-Joining method [27] (Figure 6). The grouping of the four AhR sequences (including *Fundulus*) was strongly supported (bootstrap value = 100), as was the clustering of all ARNT sequences. Sim grouped with the AhR in 95 out of 100 bootstrap samplings. These relationships will require further analysis, but the topology of this tree suggests that the vertebrate AhR proteins are at least as closely related to the *Drosophila* protein Sim as they are to the mammalian ARNT proteins. The relationship of AhR to Sim is further supported by comparisons of the bHLH regions of both proteins [4,5]. In addition, the tree shown in Figure 6 suggests that the AhR–ARNT divergence is ancient and that homologues of ARNT will probably be found in fish and other 'early' vertebrates. Comparison of AhR, ARNT and Sim sequences (DNA and protein) from additional species will provide further insight into their phylogenetic relationships.

In summary, the present data provide strong evidence for an AhR homologue in teleosts, a conclusion consistent with our earlier results showing the presence of proteins specifically labelled by the photo-affinity ligand 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin in teleost and elasmobranch fish [16].

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Fundulus AhR  (1)  VLVVTSEGMVFYASPTIKDYLGFHQSDVVHQSVFELIHTDDRAMFREQLH  (50)
   mouse AhR  124  VLVVTADALVFYASSTIQDYLGFQQSDVIHQSVYELIHTEDRAEFQRQLH  173
   human AhR  126  VLVVTTDALVFYASSTIQDYLGFQQSDVIHQSVYELIHTEDRAEFQRQLH  175
     rat AhR  124  VLVVTADALVFYASSTIQDYLGFQQSDVIHQSVYELIHTEDRAEFQRQLH  173
                   *****.. .***** **.*****.****.****.*****.*** *. ***
                                             PAS-A

Fundulus AhR (51)  FALNPP---PVASDAEFSQG-CAKAVMYNPEQLPPDSSSFLERSFVCRFR  (96)
   mouse AhR  174  WALNP----DSAQGVDEAHGPPQAAVYYTPDQLPPENASFMERCFRCRLR  219
   human AhR  176  WALNPSQCTESGQGIEEATGLPQTVVCYNPDQIPPENSPLMERCFICRLR  225
     rat AhR  174  WALNPSQCTDSAQGVDETHGLPQPAVYYTPDQLPPENTAFMERCFRCRLR  223
                    ****          . . *     * *.*.*.**. .  .**.* ** *

Fundulus AhR (97)  CLLDNSSGFLALKFHGRLKYLQGQNLCKDIETCKKVQLALFAIAMPVQPP  (146)
   mouse AhR  220  CLLDNSSGFLAMNFQGRLKYLHGQNKKGKDGALLPPQLALFAIATPLQPP  269
   human AhR  226  CLLDNSSGFLAMNFQGKLKYLHGQKKKGKDGSILPPQLALFAIATPLQPP  275
     rat AhR  224  CLLDNSSGFLAMNFQGRLKYLHGQNKKGKDGALLPPQLALFAIATPLQPP  273
                   ***********. *.*.****.**         .     ******** *.***

Fundulus AhR (147) SIVEIRAKMLLFQTRHKLDFTPTGVDTRGKAILGYTEIELCMKGSGYQFI  (196)
   mouse AhR  270  SILEIRTKNFIFRTKHKLDFTPIGCDAKGQLILGYTEVELCTRGSGYQFI  319
   human AhR  276  SILEIRTKNFIFRTKHKLDFTPIGCDAKGRIVLGYTEAELCTRGSGYQFI  325
     rat AhR  274  SILEIRTKNFIFRTKHKLDFTPIGCDAKGQLILGYTEVELCNKGSGYQFI  323
                   **.***.*  .*.*.******* * *..*. .***** *** .*******
                                                  PAS-B

Fundulus AhR (197) HAADMMYCADNHIRMI  (212)
   mouse AhR  320  HAADILHCAESHIRMI  335
   human AhR  326  HAADMLYCAESHIRMI  341
     rat AhR  324  HAADMLHCAESHIRMI  339
                   ****.. **. *****
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Figure 5 Comparison of deduced amino acid sequences encompassing the PAS domain of *Fundulus* and mammalian AhRs

The mammalian sequences were from Burbach et al. [5], Dolwick et al. [6] and Carver et al. [7]. (For accession numbers, see the legend to Figure 3.) Alignment was performed using Clustal W [22,23]. Asterisks under the sequence represent residues that are identical in all four of the AhR sequences; periods indicate similar amino acids. The PAS 'A' and 'B' boxes are underlined.

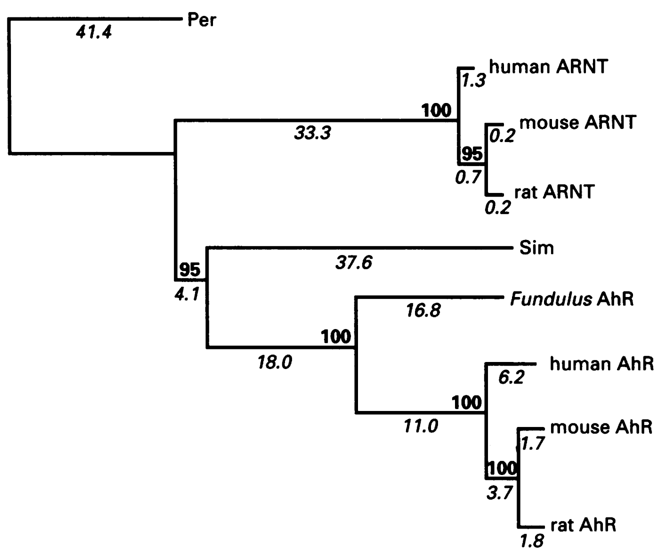

Figure 6 Phylogeny of the PAS proteins

The tree was inferred from aligned amino acid sequences (PAS domain only) using the Neighbor-Joining method [27] within CLUSTAL W [23]. Gaps were included and no corrections were made for multiple substitutions. For illustration purposes, the root for the tree was placed halfway along the longest branch (between Per and the other sequences). Numbers in italics under the horizontal lines represent percentage sequence divergence; the distance between proteins is the sum of the horizontal distances separating them. Bold numbers next to branch points are bootstrap values (a measure of confidence in that grouping) based on 100 samplings.

The sequence data reported here, together with data on AhR function in fish (reviewed in [28]), support the hypothesis [16] that a functional AhR signal transduction pathway appeared early in vertebrate evolution. The normal physiological function of the AhR is not yet understood, but the conservation of AhR sequences in diverse groups of vertebrate animals suggests an essential role for this ligand-activated transcription factor, as recently confirmed in AhR-deficient mice [29]. Further studies of AhRs in 'primitive' vertebrates, including bony, cartilaginous and jawless fish, may illuminate that role and its evolutionary origin. The method of RT-PCR using the mixed oligonucleotide primers described in this study may be useful in such a phylogenetic approach.

Note added in proof (received 19 July 1995).

A fifth eukaryotic PAS protein, hypoxia-inducible factor 1-α (HIF-1α) has recently been described [33]. The relationship of the *Fundulus* AhR sequence to HIF-1α is similar to the relationship of the *Fundulus* Ahr to Per, as shown in Figures 3 and 6.

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