Regulatory Interactions among Three Members of the Vertebrate Aryl Hydrocarbon Receptor Family: AHR Repressor, AHR1, and AHR2*

Sibel I. Karchner, Diana G. Franks, Wade H. Powell‡, and Mark E. Hahn§

From the Biology Department, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543

The effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds occur via the aryl hydrocarbon receptor (AHR), a member of the basic helix-loop-helix-Per-ARNT-Sim homology (bHLH-PAS) protein superfamily. A single AHR gene has been identified in mammals, whereas many fish species, including the Atlantic killifish (Fundulus heteroclitus) possess two distinct AHR genes (AHR1 and a novel form, AHR2). A mouse bHLH-PAS protein closely related to AHR and designated AHR repressor (AHRR) is induced by 3-methylcholanthrene and represses the transcriptional activity of the AHR. To determine whether AHRR is the mammalian ortholog of fish AHR2 and to investigate the mechanisms by which AHRR regulates AHR function, we cloned an AHR ortholog in F. heteroclitus with high sequence identity to the mouse and human AHRRs. Killifish AHRR encodes a 680-residue protein with a predicted molecular mass of 75.2 kDa. We show that in vitro expressed AHRR proteins from human, mouse, and killifish all fail to bind [3H]TCDD or [3H]β-naphthoflavone. In transient transfection experiments using a luciferase reporter gene under control of TCDD response elements, killifish AHRR inhibited the TCDD-dependent transactivation function of both AHR1 and AHR2. AHRR mRNA is widely expressed in killifish tissues and is inducible by TCDD or polychlorinated biphenyls, but its expression is not altered in a population of fish exhibiting genotoxic resistance to these compounds. The F. heteroclitus AHRR promoter contains three putative AHR response elements. Both AHR1 and AHR2 activated transcription of luciferase driven by the AHRR promoter, and AHRR could repress its own promoter. Thus, AHRR is an evolutionarily conserved, TCDD-inducible repressor of AHR1 and AHR2 function. Phylogenetic analysis shows that AHRR, AHR1, and AHR2 are distinct genes, members of an AHR gene family; these three vertebrate AHR-like genes descended from a single invertebrate AHR.

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The abbreviations used are: AHR, aryl hydrocarbon receptor; AHRE, aryl hydrocarbon (receptor) response element; AHRR, aryl hydrocarbon receptor repressor; ARNT, aryl hydrocarbon receptor nuclear translocator; bHLH, basic-helix-loop-helix; BNF, β-naphthoflavone; CYP1A, cytochrome P450 1A; Me2SO, dimethyl sulfoxide; 3MC, 3-methylcholanthrene; PAS, Per-ARNT-Sim homology; PCB, polychlorinated biphenyl; RT, reverse transcription; RACE, rapid amplification of cDNA ends; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TCFD, 2,3,7,8-tetrachlorodibenzofuran; aa, amino acid; DMEM, Dulbecco’s modified Eagle’s medium; UPL, unprogrammed lysate.

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‡ Present address: Biology Dept., Kenyon College, Gambier, OH 43022.
§ To whom correspondence should be addressed: Biology Dept., MS 32, Woods Hole Oceanographic Institution, Woods Hole, MA 02543-1049. Tel.: 508-289-3242; Fax: 508-457-2134; E-mail: mhahn@whoi.edu.

1 The binding site for the AHR-ARNT complex was originally named “xenobiotic response element” or “dioxin-response element” because of its role in mediating the response to dioxins and related xenobiotics. Recently, however, it has been shown that in some systems the AHR-ARNT complex can bind this sequence in the absence of dioxin or other xenobiotic ligand (77, 79, 80). Therefore, we use the term AHRE (aryl hydrocarbon response element or AHR response element) (81, 82).

2 The AHR2 is a ligand-activated transcription factor through which TCDD and other polyhalogenated and polycyclic aromatic hydrocarbons cause altered gene expression and toxicity (1–3). When activated by ligand binding, the AHR forms a complex with ARNT that regulates the expression of target genes by interacting with AHR response elements (AHREs; also known as xenobiotic response elements or dioxin response elements) (4). The AHR may also possess physiological functions that are independent of exogenous chemical exposure (5–7).

The AHR and ARNT belong to the bHLH-PAS superfamily of transcription factors, members of which play key roles in development and environmental sensing (8, 9). The vertebrate bHLH-PAS superfamily consists of at least nine “paralog groups,” each of which contains two or three closely related genes (paralogs) that arose by duplication from an ancestral invertebrate homolog (10, 11). Two AHR genes (12, 13) have been identified in vertebrate animals. The first AHR was originally identified in mice (1, 12, 13) and is now known to be present also in other vertebrates (14–16), including fish, where it is called AHR1 (10, 17, 18). A second, highly divergent AHR (AHR2) has been identified in fish (10, 17, 19–21) but not yet in mammals. Recently, an AHR-related gene designated AHR repressor (AHRR) was identified in mice (22) and humans (23, 24). The mouse AHRR (22) acts as a negative regulator of AHR function by competing with AHR for the available pool of ARNT and by forming AHR-ARNT complexes that bind to AHREs but are transcriptionally inactive (22). The expression of mouse AHRR mRNA is inducible by 3MC via AHR-dependent activa-

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tion of AHRRs in the AHRR gene promoter (22, 25).

A better understanding of the function and regulation of the AHRR signaling pathway requires a more complete characterization of the diversity of positive and negative regulatory factors and the interactions among them. The identification of AHRR in mice and humans raised several questions concerning the evolutionary and functional relationships between AHRR1, AHRR2, and AHRR. Are fish AHRR2 and mammalian AHRR orthologous genes (i.e. descended from a single ancestral gene in the last common ancestor of fish and mammals) or do fish also possess an AHRR gene? Is AHRR capable of repressing the function of both AHRR1 and AHRR2? Are both AHRR1 and AHRR2 involved in the regulation of AHRR expression? Is AHRR capable of high affinity binding to TCDD and other aromatic compounds that are known ligands for AHRR1 and AHRR2 (1, 17)? Does enhanced expression of AHRR occur in fish selected for genetic resistance to TCDD?

To understand the relationship between mammalian AHRR and fish AHRR and to investigate the mechanisms by which (i) AHRR regulates AHRR function and (ii) AHRR regulates AHRR expression, we cloned and characterized an AHRR homolog from the estuarine teleost Fundulus heteroclitus (mummichog or Atlantic killifish). F. heteroclitus is an early vertebrate model system for studying the function, evolution, and adaptive significance of AHRR signaling pathways. We have characterized previously other killifish bHLH-PAS proteins, including AHRR1 and AHRR2 (10, 17), ARNT2 (26), and HIF-2a (27), and their role in evolved resistance to dioxins (28, 29). We show here that F. heteroclitus expresses an AHRR homolog that is able to repress the transcriptional function of both AHRR1 and AHRR2 as well as the murine AHRR. We also show (i) that killifish AHRR expression is inducible by TCDD and by PCBs in a variety of tissues in vivo, (ii) that this occurs through AHRR sequences in its promoter and can be mediated by either AHRR1 or AHRR2, and (iii) that the killifish AHRR is able to repress the function of its own promoter. However, AHRR expression, like that of other AHRR-regulated genes such as CYP1A1, is not induced in fish selected for genetic resistance to TCDD. In addition, we demonstrate that neither killifish AHRR, mouse AHRR, nor human AHRR is able to support high affinity binding of the AHRR ligands [3H]TCDD or [3H]BNF, suggesting that the repressive function of this protein is ligand-independent. Finally, our findings reveal that AHRR is one of three members of the vertebrate AHRR gene family, which arose by duplication and divergence of a single ancestral AHRR gene.

MATERIALS AND METHODS

Chemicals—2,3,7,8-Tetrachloro[1,6-3H]dibenzo-p-dioxin ([3H]TCDD, 35 Ci/mmol; $>$99% radiochemical purity as assessed by high pressure liquid chromatography (30)) and [3H]BNF (5.5 Ci/mmol; naphthoflavone, 19 Ci/mmol; $>$98% radiochemical purity) were obtained from Chemgeny Science Laboratories (Lenexa, KS). [35S]Methionine was purchased from Amersham Biosciences. TCDD and TCDF were obtained from Ultra Scientific (Hope, RD). All other chemicals were from Sigma.

Fish Collection and RNA Isolation—F. heteroclitus were collected in salt marshes, Cape Cod, MA, or New Bedford Harbor, MA, as described earlier (17, 28). The dissected organs were immediately frozen in liquid nitrogen. Total RNA was isolated using RNA STAT-60 (Tel-Test B, Inc., Friendswood, TX). Poly(A)+ RNA was purified with oligo(dT) spin column preparation. Gene-specific primers were coupled with adaptor sequences (corresponding to the 5′-1414 human AHR) of killifish, mouse, and human AHRRs plus selected mammalian and fish AHRRs were used to construct phylogenetic trees using Maximum Parsimony and Distance criteria in PAUP*4.0b8 (33), as described previously (10).

Genome Walking—F. heteroclitus genomic DNA was isolated from testes by homogenizing the tissue in 0.2% Triton X-100, 1 M NaOH, followed by phenol/chloroform extractions and ethanol precipitation (34). The protocol for CLONTECH’s Genome Walking kit was followed for cloning the promoter region of the AHRR. Briefly, 2.5 μg of genomic DNA was digested overnight with four blunt-cutting restriction enzymes. The digests were extracted with phenol/chloroform and precipitated with ethanol. Adaptors (Marathon cDNA Amplification kit, CLONTECH) were coupled with the genomic DNA fragments. PCR was performed on the adaptor-ligated fragments with gene-specific primers (RR20r and nested primer RR1r) and adaptor primers using the Advantage polymerase mix (CLONTECH). The amplification parameters are as follows: 94 °C, 2 s/72 °C, 4 min for 7 cycles and 94 °C, 2 s/67 °C, 4 min for 32 cycles, followed by 67 °C, 4 min final extension.

Expression Constructs—Full-length AHR repressor cDNA was amplified with the primers Rf22 and Rf23 from the cDNA prepared for the RACE procedure above. The clones were digested with Kpn1 and Xba1 at the primer sites and ligated into the same sites in pcDNA 3.1/Zeo (+) vector (Invitrogen, Inc., Carlsbad, CA) to make pcDNA-FhAHR. Several full-length clones were sequenced to ensure accuracy of the sequence. The pcGL-RR reporter construct was made by inserting the 2358-bp AHR promoter fragment into pGL3-Basic vector (Promega) and inserting the luciferase reporter sequence. The mouse AHRR cDNA (plasmid pBSKmAHRR; a gift from Y. Fuji-Kuriyama, Tohoku University, Japan) (22) was inserted into the pcDNA 3.1 vector to allow for mammalian expression and renamed pcDNA-mAHR. The human AHRR cDNA (clone H08618 of KIAA1234; a gift from Dr. Takahiro Nagase, Kizussa DNA Research Institute, Chiba, Japan) (23) was inserted into pcDNA 3.1 and renamed pcDNA-hAHR. Mouse AHRR (pSportMAHR) (13) and human AHRR (pSportAHR2) and ARNT (pSportARN2) (14) expression vectors were generously provided by Dr. C. Bradford (University of Wisconsin, Madison, WI). The plasmid pGudLuc 6.1, which is derived from pGudLuc1.1 (36) and contains the firefly luciferase reporter gene under the control of an MMTV promoter regulated by four AHREs from the murine Cyp1A1 promoter (37), was a generous gift from Dr. M. Denison (University of California, Davis). The killifish AHRR expression plasmid pcDNA-FhAHR was prepared by subcloning the insert from pDPbhAHR2 (17) into pcDNA. The killifish AHRR expression plasmid pcDNA-FhAHR1 was a generous gift from Dr. S. I. Karchner, manuscript in preparation. 4

In Vitro Protein Synthesis and Ligand-binding Assay—To quickly couple Recitolucyte Lysate Systems (Promega) were used to synthesize [35S]methionine-labeled or unlabeled proteins following manu-
Characterization of a F. heteroclitus AHRR cDNA—Previously, we identified two highly divergent AHR homologs (AHR1 and AHR2) in F. heteroclitus (17). In light of the recent characterization of a mammalian AHR-related bHLH-PAS protein, AHRR (22), we sought to determine the relationship between mammalian AHR and fish AHRR by investigating the presence of an AHRR homolog in killifish and characterizing its interactions with AHR1 and AHR2. Degenerate PCR primers, designed to recognize regions that are conserved among mouse and human AHRR proteins but divergent from AHRs, were used in RT-PCR. The primer pair Qf/RR-8r amplified a 670-bp cDNA from gonad cDNA of F. heteroclitus, which was cloned into pcDNA3.1 (Invitrogen). Parental cell lines, COS-7 monkey kidney cells, were transfected with the cDNA using LipofectAMINE 2000 (Invitrogen) as described previously (17). The mixture was then added to cells in DMEM with serum. Cells were treated 5 h after transfection with either Me2SO or TCDD (10 nM) and incubated overnight at 4 °C. Cultures were incubated overnight at 4 °C and 37 °C. Under these conditions, the amount of PCR product amplified from gonad cDNA was linearly related to cycle number and amount of template. Ten-μl aliquots from each reaction were run on 1.5% agarose gels, followed by ethidium bromide staining. Band intensities were quantified using a ChemiImager 4000 low light imaging system (Alpha Innotech, San Leandro, CA) with automatic background subtraction.

RESULTS

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Cell Culture, Transfection, and Luciferase Assays—COS-7 monkey kidney cells were purchased from ATCC (Manassas, VA) and maintained in DMEM (Sigma) supplemented with fetal calf serum (10% final concentration) at 37 °C under 5% CO₂. Cells were plated at 10⁴ cells/well in 48-well plates. Transfections were carried out 24 h after plating in triplicate wells. DNA and LipofectAMINE 2000 (Invitrogen) were each diluted in serum-free DMEM. For each well, a total of 500 ng of DNA was complexed with 1 μL of LipofectAMINE 2000. The mixture was then added to cells in DMEM with serum. Cells were treated 5 h after transfection with either Me2SO or TCDD (10 nM) and incubated overnight at 4 °C and 37 °C. Under these conditions, the amount of PCR product amplified from gonad cDNA was linearly related to cycle number and amount of template. Ten-μl aliquots from each reaction were run on 1.5% agarose gels, followed by ethidium bromide staining. Band intensities were quantified using a ChemiImager 4000 low light imaging system (Alpha Innotech, San Leandro, CA) with automatic background subtraction.

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regions, whereas it shares 38 and 37% identity with killifish AHR1 and AHR2, respectively, in this region (Fig. 2). Thus, killifish AHRR is distinct from AHR1 and AHR2 and likely represents an ortholog of the mammalian AHRRs.

Phylogenetic analysis of AHRR and AHR amino acid sequences indicates that AHRRs from killifish, mouse, and human form a clade that is distinct from the previously identified AHR1 and AHR2 clades (17) but still within the larger AHR family, which also includes the invertebrate AHR homologs from *Drosophila melanogaster* and *Caenorhabditis elegans* (Fig. 3). The topology of the phylogenetic trees remains the same when the PAS-B domain is excluded from the analysis, demonstrating that the classification is not solely driven by the divergence in this region (data not shown). In addition, the clustering of the AHRRs with vertebrate and invertebrate AHRs is retained when sequences of other bHLH-PAS proteins are included in the analysis (data not shown). These analyses demonstrate that the AHRR is a third member of the vertebrate AHR family that is found in both mammals and fish, and is distinct from AHR/AHR1 and AHR2.

**AHRRs from Mammals and Fish Lack the Ability to Bind Traditional AHR Ligands**—The relatively high degree of sequence identity between AHRR and AHR proteins prompted us to examine the ability of fish and mammalian AHRRs to bind typical AHR ligands. Killifish AHRR, AHR1, and AHR2 proteins were expressed by *in vitro* coupled transcription and translation (TnT), and the integrity and efficiency of synthesis were verified by SDS-PAGE of [35S]methionine-labeled TnT products (Fig. 4A). Unlabeled TnT reactions were incubated with [3H]TCDD (8 nM), and specific binding was determined by a sucrose gradient velocity sedimentation assay. As shown earlier (17), killifish AHR1 and AHR2 exhibited specific [3H]TCDD binding, which was displaced by a 100-fold excess of unlabeled ligand (Fig. 4B). In contrast, no specific [3H]TCDD binding was detected with the killifish AHRR. To determine whether AHRR is capable of binding a non-halogenated AHR ligand, *in vitro* expressed proteins were incubated with [3H]BNF (10 nM). As observed for [3H]TCDD, AHR1 and AHR2 exhibited specific binding of [3H]BNF, whereas no specific binding of this compound was observed with AHRR (Fig. 4C).

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**Fig. 2. Alignment of AHRR and AHR amino acid sequences.** The N-terminal regions of AHRR and AHR deduced amino acid sequences were aligned using ClustalX. Amino acids that are identical in three or more sequences are boxed and shaded. The bHLH region and PAS domain are indicated by lines above the alignment. The ligand-binding domain of AHRs (67) is in brackets. See the legend to Fig. 3 for GenBank™ accession numbers of the sequences used. Prefixes used: Fh, *F. heteroclitus*; Mm, *Mus musculus*; Hs, *Homo sapiens*.

**Fig. 3. Phylogenetic analysis of AHRR and AHR proteins.** The phylogenetic tree was constructed using Maximum Parsimony in PAUP*+4.0b8 as described under “Materials and Methods.” The sequences used corresponded to the N-terminal halves of the proteins, excluding positions with gaps (200 informative characters). Because of the low sequence identity among AHRs and AHRRs within the distal half of the protein and the resulting uncertainties in the alignments in this region, comparisons of the N-terminal halves of the proteins are most accurate. A single most parsimonious tree was found using an exhaustive search; it composed 949 steps. AHR1, AHR2, and AHRR clades are indicated. A similar topology was obtained using the distance criterion; the only difference was that in the distance tree, zebrafish AHR1 was basal to a group containing *F. heteroclitus* AHR1 and the mammalian AHRs, all still within the AHR1 clade. GenBank™ accession numbers and amino acid (aa) residues of the sequences used are as follows: killifish AHRR (AF443441; aa 1–398), mouse AHR (L19872; aa 1–414), human AHR (AB015140; aa 1–390), human AHRR (AB033060; aa 1–421), human AHRR (L19872; aa 1–414), mouse AHR (M94623; aa 1–408), killifish AHR1 (AF024591; aa 1–409), killifish AHR2 (U29679; aa 1–406), zebrafish AHR1 (AF258854; aa 1–419), zebrafish AHR2 (AF063446; aa 1–419), *D. melanogaster* AHR (AF050630; aa 1–389), and *C. elegans* AHR (AP039970; aa 1–419).
To determine whether the lack of binding to AHR ligands is a property shared by fish and mammalian AHRRs, we evaluated the specific binding of \[^{3}H\]TCDD and \[^{3}H\]BNF to AHRs and AHRRs from mouse and human. As we observed for Fundulus, neither human nor mouse AHRR was capable of binding \[^{3}H\]TCDD or \[^{3}H\]BNF, whereas both human and mouse AHRs exhibited high affinity, specific binding of these ligands (Fig. 5). Together, these data reveal that the inability to bind planar aromatic hydrocarbons distinguishes AHRR from the vertebrate AHRs (AHR/AHR1 and AHR2).

Repression of AHR Transactivation Function by Killifish AHRR—Based on the reported inhibitory effect of mouse AHRR on the transactivation function of mouse AHR (22), we first determined whether killifish AHRR has a similar effect on the mouse AHR. Expression constructs for mouse AHR, human ARNT, and a luciferase reporter gene under control of AHREs (pGudLuc6.1) were transiently expressed in COS-7 cells. Luciferase expression was induced in cells treated with TCDD (10 nM) as compared with control cells treated with Me\(_2\)SO (Fig. 6A, lane 1). Cotransfection of either the mouse AHRR construct or the killifish AHRR construct abolished both basal and TCDD-induced expression of luciferase (Fig. 6A, lanes 2 and 3).

By using the same reporter system, we evaluated the ability of killifish AHRR to affect transcriptional activation mediated by killifish AHR1 or AHR2. Each killifish AHR activated transcription of the luciferase reporter in conjunction with killifish ARNT2 (Fig. 6B, lanes 5 and 9); TCDD treatment up-regulated the reporter 2.5-fold by AHR1 and 4-fold by AHR2. Cotransfection of increasing amounts of killifish AHRR with killifish AHR1 or AHR2 resulted in an AHRR concentration-dependent decrease in luciferase expression, which was reduced to background levels when 50 ng of AHRR was cotransfected (Fig. 6B, lanes 8 and 12). Luciferase expression was AHR- and ARNT-dependent, and no expression above background was observed.
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with killifish AHRR and ARNT2 in the absence of AHR. Thus, killifish AHRR was not itself transcriptionally active, but it was able to inhibit the transactivation function of both AHR1 and AHR2, as well as that of the mouse AHR.

**Induction of AHRR mRNA by TCDD and PCBs**—To assess the tissue-specific expression of the killifish AHRR and its inducibility by AHR agonists, we measured AHRR transcripts in samples of RNA prepared for a previous study in which the inducibility of killifish AHR1, AHR2, and CYP1A mRNA expression was measured (28). As assessed by semi-quantitative RT-PCR, AHRR mRNA was expressed in a variety of tissues from adult killifish (Fig. 7). Exposure to TCDD increased the expression of AHRR, most noticeably in spleen, heart, and gut. When killifish were treated with a mixture of PCBs, AHRR expression was elevated mainly in liver and heart. These results demonstrate that killifish AHRR, like CYP1A (28), is inducible by halogenated aromatic hydrocarbons, including the potent AHR agonist TCDD.

**Analysis of the F. heteroclitus AHR1 promoter**—To investigate the mechanisms underlying the induction of AHRR by TCDD, we cloned and sequenced the promoter region of killifish AHRR via a genome-walking approach. Alignment of the 2358-bp promoter fragment with the previously obtained 5’-RACE product revealed an intron of 387 bp within the 5’-upstream sequence, indicating that the first exon is non-coding (Fig. 8A), as reported for the murine AHR1 gene (25). Scanning of the promoter sequence for possible transcriptional regulatory elements revealed that the killifish AHRR gene appears to have a TATA-less promoter. Three AHRE core consensus sequences (GCGTG) are present at positions +2, −986, and −1177 in relation to the transcriptional start site (as determined from the longest 5’-RACE product), and a GC box is present at −1016 (Fig. 8A). We did not find any NFκB sites in this region, unlike the mouse AHR promoter (25).

In light of the three putative AHREs in the killifish AHRR promoter, we tested the ability of ligand-activated killifish AHR1 and AHR2 to regulate expression of a reporter gene under the control of this sequence. The entire 2358-bp promoter fragment was fused to the luciferase gene of pGL3-Basic to generate a reporter construct, pGL-RR. COS-7 cells were transfected with pGL-RR and killifish ARNT2 together with either killifish AHR1 or AHR2. Luciferase expression was up-regulated in an AHR-dependent manner when the cells were treated with TCDD (Fig. 8B). Both AHR1 and AHR2 supported TCDD-dependent induction of luciferase expression from pGL-RR, although AHR2 (5.2-fold induction) appeared to be more efficient at activating transcription through this promoter than AHR1 (1.8-fold induction). Cotransfection of killifish AHRR eliminated the TCDD-dependent up-regulation of pGL-RR reporter activity mediated by either killifish AHR1 or AHR2, demonstrating that AHRR is able to repress its own promoter. Together, these results show that the killifish AHRR gene

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**Fig. 5.** Velocity sedimentation analysis of ligand binding by human and mouse AHRRs in comparison to human and mouse AHRs. A, in vitro transcription/translation of mouse AHR (mAHR), mouse AHRR (mAHR), human AHR (hAHR), and human AHRR (hAHR1, pSportMAHR, pcDNA-mAHR, pcDNA-hAHR1, pSporthAHR2, and pDNA-hAHR2 constructs were expressed in TnT T7 or SP6 Quick-coupled Reticulocyte Lysate Systems to synthesize [35S]methionine-labeled proteins. The TnT reactions were electrophoresed on the same SDS-polyacrylamide gel, followed by fluorography. B, binding of [3H]TCDD to mouse AHR, mouse AHRR, human AHR, and human AHRR.
contains a TCDD-inducible promoter that can be activated by either AHR1 or AHR2 and can be repressed by its own gene product.

AHRR Expression in a Dioxin-resistant Population of F. heteroclitus—We have been investigating the molecular basis of dioxin resistance that has evolved in a population of killifish inhabiting a PCB-contaminated site, New Bedford Harbor, MA (28, 29, 39). Fish from New Bedford Harbor express low levels of CYP1A, despite having high tissue concentrations of PCBs, and experimental treatment of these fish with TCDD or other AHR agonists fails to induce CYP1A expression (29). To test the hypothesis that an alteration in AHRR expression contributes to the resistant phenotype, we compared tissue-specific AHRR mRNA expression in adult, dioxin-sensitive fish from a reference site (Scorton Creek) to that of adult, dioxin-resistant fish from the polluted site (New Bedford Harbor). Using semi-quantitative RT-PCR, we found no appreciable differences between populations in the amounts or pattern of AHRR mRNA expression (Fig. 9). Thus, despite the inducibility of AHRR by experimental exposure of reference site fish to TCDD or PCBs (Fig. 7), this gene is not induced or otherwise overexpressed in fish genetically selected for resistance to AHR agonists following chronic environmental exposure to high concentrations of PCBs.

**DISCUSSION**

The AHR is a key transcriptional regulatory protein involved in the altered gene expression and toxicity that results from exposure of vertebrate animals to chlorinated dioxins, PCBs, polynuclear aromatic hydrocarbons, and certain other classes of compounds (40–44). The AHR also possesses physiological functions that are independent of exogenous chemical exposure (5–7). Determining how the AHR signaling pathway is regulated is necessary for understanding both of these roles as well as for understanding the mechanisms underlying differences among individuals, populations, or species in the response to AHR agonists. Regulation of the AHR pathway occurs through a variety of mechanisms, including ligand binding (45), modulation of AHR expression and degradation (46–48), post-translational modification (37, 49), protein-protein interactions (50–56), and transcriptional repression (57, 58). Mechanisms of transcriptional repression may involve competition for coac-
tors (59–61), binding to negative regulatory elements (57, 62, 63), or interference with the AHRE binding of the AHR-ARNT complex (58, 64, 65). The recent cloning and characterization of the AHRR in mice (22) identified a novel autoregulatory loop involving the bHLH-PAS proteins AHR, ARNT, and AHRR. The data presented here demonstrate that this regulatory loop activates the AHR-ARNT complex, thereby increasing the expression of AHRR.

**Fig. 8.** Structural and functional analysis of the *F. heteroclitus* AHRR promoter. **A**, sequence of killifish AHRR upstream regulatory region as determined by genome walking. Three consensus AHRE sequences are boxed, and one GC box is underlined. The putative transcriptional start site based on the longest 5′ RACE product is labeled as 1. The lowercase region indicates the intron within the 5′-upstream sequence. The translation initiation codon ATG is in bold. The nucleotide sequence of the killifish AHRR promoter has been deposited in the GenBank™ data base with accession number AF443442. **B**, AHR-dependent transactivation via the killifish AHRR promoter. The entire promoter region depicted in A was ligated into the pGL3-basic vector to drive firefly luciferase expression (construct pGL-RR). COS-7 cells were transiently transfected with the pGL-RR and pRL-TK along with expression constructs for killifish AHR1 or AHR2, killifish ARNT2, and killifish AHRR as indicated in the figure and described under “Materials and Methods.” Cells were treated with Me 2SO (DMSO) or TCDD and luciferase activities measured after 18 h. Relative luciferase units were calculated by normalizing firefly luciferase activity to the transfection control Renilla luciferase. Each data point represents the mean of triplicate wells, and error bars represent S.D. Results shown are representative of two independent experiments. The prefix used is Fh for *F. heteroclitus*. 
mechanism is evolutionarily conserved and that it can also involve a fourth bHLH-PAS protein, AHR2. We show for the first time that mammalian and fish AHRRs are unable to bind the AHR ligands TCDD and BNF. We demonstrate also that AHR expression is inducible by halogenated AHR agonists such as TCDD and PCBs and that AHR2 is able to repress transcription induced via its own promoter.

Interactions among AHRR, AHR1, and AHR2—Fujiki, Kuriyama and colleagues (22) showed that the mouse AHRR is capable of forming a complex with ARNT and interacting with AHRs to inhibit AHR-mediated transcription of a reporter gene, thus defining AHRR as a repressor of AHR function. The results presented here demonstrate that the fish (F. heteroclitus) ortholog of the murine AHRR also is able to repress the function of the mouse AHR, demonstrating conservation in the function of the AHRR over more than 400 million years since the divergence of fish and mammalian lineages. In addition to its effect on the function of the mouse AHR, the killifish AHRR acts as a repressor of AHR1 (the fish ortholog of the mammalian AHR) as well as AHR2, which is possibly a fish-specific AHR form (17).

Other elements of the AHRR-AHR autoregulatory loop also are conserved. Mimura et al. (22) reported induction of mouse AHRR mRNA in vivo by the polynuclear aromatic hydrocarbon 3MC, a known AHR agonist. We have extended those results by showing that AHRR, like CYP1A, is inducible also by halogenated AHR ligands, including TCDD and a PCB mixture. Consistent with this, both fish (Fig. 8) and mouse (22) AHR promoters contain consensus AHRE sequences that function in transient transfection assays to mediate AHR-dependent induction of reporter gene expression in response to TCDD (this study) or 3MC (22). In the present study, both AHR1 and AHR2 were capable of supporting inducible transcription from the AHRR promoter (Fig. 6). In addition, killifish AHRR was shown to repress either AHR1- or AHR2-dependent transcription from its own promoter (Fig. 8) in addition to its repression of promoters regulated by enhancer elements from the murine Cyp1a1 gene (Fig. 6 and Ref. 22). This finding suggests that the autoregulatory loop involving AHRR and AHRs likely includes a variety of AHR-regulated genes that are first induced via the AHR and then subsequently repressed by the induced AHRR.

AHRR Function—Is Ligand-independent—Previous results (22) with the mouse AHRR had suggested that the ability of this protein to interact with ARNT and bind AHRE sequences was independent of ligand, but ligand binding had not been assessed directly. The data presented here reveal that neither the mouse, human, nor killifish AHRR is capable of specific, high affinity binding of [3H]TCDD or [3H]BNF. This finding, although not previously demonstrated, is consistent with the poor sequence conservation between AHRs and AHRRs in the PAS-B domain, which forms part of the ligand-binding domain of AHRs (13, 66–68). Thus, the function of AHRR proteins appears to be ligand-independent, unlike their expression, which is regulated by ligands acting through binding to AHRs.

AHRR: Role in Evolved Dioxin Resistance—F. heteroclitus is emerging as a valuable model vertebrate for studying the evolution and adaptive significance of AHR signaling pathways. This species is sensitive to dioxins (69) and possesses an inducible CYP1A1 (70), indicating the existence of a functional AHR signaling system. However, following multigenerational exposure to high levels of dioxins and PCBs, killifish develop heritable resistance to AHR agonists (29, 39, 71, 72). One hypothesis concerning the mechanism of resistance involves the increased expression of a repressor protein that is able to prevent AHR-dependent alterations in gene expression (29, 73). Analysis of a set of tissue RNAs from adult, dioxin-sensitive and dioxin-resistant fish showed no evidence for up-regulation of AHRR in the resistant fish (Fig. 9). This suggests that altered transcriptional regulation of AHRR is not associated with the resistance mechanism in adult fish. However, the possible role of such a mechanism in the resistance seen in killifish embryos and larvae (39) is not addressed by this experiment. Similarly, the possibility of mechanisms involving altered functional properties or post-transcriptional regulation of AHRR specific to dioxin-resistant fish remains to be evaluated.

AHRR, AHR1, and AHR2 Comprise Three Members of the Vertebrate AHR Gene Family—The identification and characterization of an AHR in a teleost fish illuminates not only the conserved nature of this gene but the evolutionary history of the AHR family as well. F. heteroclitus is the first species in which three AHR-related genes have been found. We have also identified an AHR homolog in the zebrafish (Danio rerio),5 a species that also possesses an AHR1 (18) and AHR2 (20). The zebrafish AHR gene maps6 to linkage group 24, which contains regions of conserved synteny (74, 75) with human chromosome 5, the location of the human AHRR gene (25). Together with the data presented here, this provides strong evidence for orthology between the fish and mammalian AHRRs.

Phylogenetic analysis of AHR and AHRR amino acid sequences from mammals and fish demonstrates that these genes segregate into three groups (clades): AHR/AHR1, AHR2, and AHRR (Fig. 3). The AHR2 clade is known so far only from fish, and we have suggested (17, 76) that AHRR was lost at some point in the lineage leading to mammals. Importantly, the present results show definitively that AHRR and AHR2 are distinct genes, a conclusion that is also supported by the mapping of zebrafish AHR1, AHR2, and AHRR to separate linkage groups (18, 76).8 In phylogenetic analyses, AHR/AHR1, AHR2, and AHRR all fall within a larger group that also includes the single AHR homolog identified in several invertebrate species (10, 77–79); we refer to this larger group as the "AHR family" within the bHLH-PAS protein superfamily (17, 79). The topology of the phylogenetic trees strongly suggests that the three vertebrate members of the AHR family arose and diverged following two duplications of an ancestral AHR gene, which is represented today by a single AHR-like gene in invertebrate animals. The trees, combined with the ligand-binding data presented here (Figs. 4 and 5) and elsewhere (77, 79), further imply that the ability to bind planar aromatic compounds first evolved in the vertebrate lineage and after the gene duplication that resulted in the ancestral AHR and AHRR genes. Thus,

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5 B. R. Evans and M. E. Hahn, unpublished data.
6 B. R. Evans, M. Ekker, and M. E. Hahn, unpublished data.
AHR and AHR2, which arose from a second gene duplication subsequent to the AHR/AHR2 split, are both capable of specific, high affinity binding of TCDD (1, 17, 19), whereas neither AHR (this study) nor the invertebrate AHR homologs (77, 79) possess this property. In summary, the AHR defines a third member of the AHR family in vertebrate animals and is part of an evolutionarily conserved autoregulatory loop that involves AHR1 and AHR2 in addition to ARNT or ARNT2. AHR does not itself bind aromatic hydrocarbons, but its expression can be induced by halogenated and nonhalogenated aromatic hydrocarbons acting through an AHR1- or AHR2-dependent mechanism. AHRR protein is capable of repressing AHRE-containing promoters, including its own. Further characterization of AHR function in fish and mammals may contribute to an understanding of mechanisms responsible for differences among species, populations, individuals, and cell types in the response to aromatic hydrocarbon exposure.

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Sibel I. Karchner, Diana G. Franks, Wade H. Powell and Mark E. Hahn

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