Molecular cloning and characterization of the aryl hydrocarbon receptors and aryl hydrocarbon receptor nuclear translocators in the American alligator

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Abstract (176 words)

Aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor, binds to a variety of chemical compounds including various environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. This receptor regulates expression of target genes through dimerization with the AHR nuclear translocator (ARNT). Since AHR-ARNT signaling pathways differ among species, characterization of AHR and ARNT is important to assess the effects of environmental contamination and for understanding the molecular mechanism underlying the intrinsic function. In this study, we isolated the cDNAs encoding three types of AHR and two types of ARNT from a reptile, the American alligator (*Alligator mississippiensis*). *In vitro* reporter gene assays showed that all complexes of alligator AHR-ARNT were able to activate ligand-dependent transcription on a xenobiotic response element. We found that AHR-ARNT complexes had higher sensitivities to a ligand than AHR-ARNT2 complexes. Alligator AHR1B showed the highest sensitivity in transcriptional activation induced by indigo when compared with AHR1A and AHR2. Taken together, our data revealed that all three alligator AHRs and two ARNTs were functional in the AHR signaling pathway with ligand-dependent and isoform-specific transactivations *in vitro*.

1. Introduction

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that mediates the metabolism of xenobiotic chemicals (Denison and Nagy, 2003). Environmental xenobiotic toxic compounds, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 3-methylcholanthrene (3-MC), and poly chlorinated biphenyls (PCBs), stimulate the transcriptional activity of AHR and cause developmental disorders and reproductive abnormalities (Guillette et al., 2000; Watanabe et al., 2005; Bunger et al., 2008; King-Heiden et al., 2012). Recently, it has been reported that the AHR also binds to intrinsic chemicals and is involved in various endogenous events, such as carcinogenesis, homeostasis, and immune responses (Opitz et al., 2011; Julliard et al., 2014; Bock, 2014).

The AHR is a member of the basic helix-loop-helix-Per-ARNT-Sim (bHLH-PAS) gene family (Sogawa and Fujii-Kuriyama, 1997). It binds to exogenous or endogenous ligands, and then translocates into the nucleus where it dimerizes with another member of the bHLH-PAS proteins, the AHR nuclear translocator (ARNT). The ligand-bound AHR-ARNT complexes regulate the transcription of target genes through the binding to xenobiotic responsive elements (XREs) which are located upstream of AHR-responsive genes such as the cytochrome P450 subfamily 1A (CYP1A)

genes, or interacting with other transcriptional factors (Mahajan and Rifkind, 1999; Hankinson, 2005; Lee et al., 2006; Rüegg et al., 2008; Lo and Matthews, 2012).

Both AHR and ARNT are evolutionarily conserved from invertebrates to mammals. The molecular cloning and characterization of AHR has been performed in many species including mammals, birds, teleost fishes, and some amphibians (Jensen and Hahn, 2001; Lavine et al., 2005; Yasui et al., 2007). Mammals have a single AHR gene, whereas non-mammalian species normally have two or more AHRs (Hahn et al., 2006). AHR binding specificity is different for each isoform of each species, leading to various susceptibilities to endocrine disrupting contaminants (EDCs) in a species-specific manner (Ema et al., 1994; Abnet et al., 1999; Yamauchi et al., 2005; Karchner et al., 2006; Lee et al., 2009; Doering et al., 2014). In addition, there are two isoforms of ARNT, ARNT1 and ARNT2 in vertebrates, and their functions are distinct. In mammals and zebrafish, it has been reported that ARNT1 is involved in AHR-mediated toxicity (Prasch et al., 2004, 2006; Sekine et al., 2006; Dougherty and Pollenz, 2008), whereas ARNT2 induced CYP1A5 promoter activity with AHR *in vitro* in chickens (Lee et al., 2011). In reptiles, full and partial sequences of AHR from two turtles (*Trachemys scripta* and *Chrysemys picta*) have been reported (Marquez et al., 2011). However, they have not been characterized, and the ARNTs in any reptile have not been reported. Understanding of the physiological role of AHR-ARNT in reptiles is important for evaluating the toxic potential of many xenobiotic chemicals.

In the present study, we isolated cDNAs encoding AHR and ARNT orthologs from the American alligator (*Alligator mississippiensis*). We identified three types of AHR and two types of ARNT. Furthermore, the transcriptional activities of alligator AHR-ARNT complexes were analyzed using reporter gene assays in transiently transfected cells with different ligands and various combinations of the isoforms.

2. Materials and methods

2.1. Animals

All experiments in this study involving alligators were carried out under the guidelines specified by the Institutional Animal Care and Use Committee at the Medical University of South Carolina (USA), and the Institutional Animal Care and Use Committee at the National Institutes for Basic Biology (Japan). All fieldwork was conducted under permits from the Florida Fish and Wildlife Conservation Commission and the U.S. Fish and Wildlife Service. Juvenile American alligators (*Alligator mississippiensis*) were caught by hand from airboats from Lake Woodruff National Wildlife Refuge, Volusia County, Florida, USA. Animals were euthanized with a lethal

dose of Nembutal, and tissues were collected. Tissues were incubated in RNAlater (Ambion, Austin, TX) at 4°C overnight, and then stored at -20°C until use.

2.2. Chemical reagents

Indigo and 3-methylcholanthrene (3-MC) were purchased from Wako Pure Chemicals (Osaka, Japan) and Enzo Biochem (New York, NY), respectively. All chemicals were dissolved in dimethylsulfoxide (DMSO). The concentration of DMSO in the culture medium did not exceed 0.1%.

2.3. Molecular cloning of AHRs and ARNTs

Two conserved amino acid regions of vertebrate AHR were selected to design degenerate oligonucleotide primer for PCR (QDYLGFFQQ and YKNGRPDY for AHR1A, QDYLGFFQQ and CQKMKHMQ for AHR1B, and QDYLGFFQQ and HADNM/VPQ for AHR2). As a template for PCR, cDNA was synthesized from 2 µg of total RNA isolated from the liver of juvenile alligators. After the first PCR, additional primer sets (QDYLGFFQQ and YKNGRPDY for AHR1A; QDYLGFFQQ and QFIHAADM for AHR1B and AHR2) were used for the second PCR. For ARNTs, two conserved amino acid regions (NDKERFA and HPEDQQL for ARNT1 and PAAVNP and GMFPPF for ARNT2) of vertebrate ARNTs were selected to design degenerate oligonucleotide primer for PCR. After the first amplification, nested primer sets (NKMTAYI and ATVGYYQ for ARNT1; GMDFFD and TQGTGNY for ARNT2) were used for the second PCR.

The amplified DNA fragments were subcloned into the TA-cloning plasmid pCR2.1 vector (Invitrogen, Carlsbad, CA) or pGEM-T Easy vector (Promega, Madison, WI). The DNA sequences of amplified fragments were analyzed using a BigDye terminator Cycle Sequencing-kit (Applied Biosystems, Foster City, CA) with T7, SP6, M13 forward and M13 reverse primers on an Applied Biosystems 3130 Genetic Analyzer and an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems). The 5'- and 3'-ends of the AHRs and ARNTs cDNAs were amplified and analyzed by using a SMART RACE cDNA Amplification kit (BD Biosciences Clontech, Palo Alto, CA).

2.4. Construction of plasmid vectors

The full-coding regions of the alligator AHR1A, alligator AHR1B, alligator AHR2, alligator ARNT1, alligator ARNT2, human AHR (accession number: NM_001621), human ARNT1 (accession number: NM_001668) and human ARNT2 (accession number: NM_014862) were amplified by PCR with KOD DNA polymerase (TOYOBO Biochemicals, Osaka, Japan). The PCR products were ligated into the

pcDNA3.1 vector (Invitrogen). A dioxin-regulated reporter vector with four xenobiotic-response elements (XREs, CACGCNA/T) was prepared by subcloning of oligonucleotides containing 4 x XRE into pGL4.23 vector (Promega) at the *KpnI-HindIII* site.

2.5. RNA isolation and RT-PCR

Total RNA was prepared from tissues of both female and male alligators using RNeasy (Qiagen, Venlo, Netherlands). For RT-PCR, 2 µg of total RNA was reverse-transcribed using SuperScript III transcriptase (Invitrogen) and oligo (dT) primer. The PCR amplifications were performed with gene-specific primers (Table1). For AHRs and ARNT, thirty cycles of amplification were carried out under the following conditions: denaturing at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min. At completion of the PCR, DNA fragments were resolved on 2% Metaphor agarose gel (BMA, Rockland, ME). For β-actin, the amplification condition was as follows: preheating at 95°C for 10 min, 28 cycles of PCR at 94°C for 30 sec, 58°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 7 min. The resulting PCR products were run on a 1.2% agarose gel.

2.6. Transactivation assays

Chemical-induced transcriptional activity was measured by a standard reporter gene assay with COS-7 cells, which were seeded in 24-well plates at 5x10⁴ cells/well in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum (Hyclone, South Logan, UT) and 1 mM sodium pyruvate. After 24 hours, the cells were transfected with DNAs using polyethylenimine "Max" (Polysciences, Inc., Warrington, PA) (Reed et al., 2006). Three hours after transfection, test chemicals were added to the media. After 21 hours of dosing test chemicals, the cells were lysed, and the luciferase activity was measured by a chemiluminescence assay using the Dual-Luciferase Reporter Assay System (Promega). Luminescence was measured using a Turner Designs Luminometer TD-20/20 (Promega). Promoter activity was expressed as a ratio of firefly (*Photinus pyralis*) luciferase activity versus sea pansy (*Renilla reniformis*) luciferase activity. All reporter gene assays were performed in triplicate or more.

2.7. Database and sequence analysis

All sequences generated were searched for similarity using Blastn and Blastp at web servers of the National Center of Biotechnology Information (NCBI). Phylogenetic analysis was conducted using the maximum-likelihood analysis method implemented in

MEGA5 (Tamura et al., 2011). Amino acid sequences of AHR and ARNT were aligned using ClustalW (Thompson et al., 1994) under default settings and removed the regions of ambiguous alignment and gaps. The phylogeny of AHRs and ARNTs were estimated by using the maximum-likelihood method based on the JTT+G model. Aryl hydrocarbon receptor repressors (AHRs) and invertebrate ARNT homologs were included in each phylogenetic analysis as outgroups. Bootstrap tests with 1,000 times replicates estimated the confidence in each branch point. Accession numbers for amino acid sequences used are as follows: chicken AHR (AF192502), chicken AHR1b (KM289155), chicken AHR2 (KM289156), cormorant AHR1 (AB109545), cormorant AHR2 (AB287294), turtle AHR (XP_005306718), turtle AHR-like (XP_005311552), turtle AHR2 (GQ141218), platypus AHR (XP_007670462), platypus AHR-like (XP_007659179), frog AHR1a (AY635782), frog AHR1b (AY635783), zebrafish AHR1a (NM_131028), zebrafish AHR1b (NM_001024816), zebrafish AHR2 (NM_131264), puffer AHR1A (DQ088137), puffer AHR1B (DQ088138), puffer AHR2A (DQ088139), puffer AHR2B (DQ088140), puffer AHR2C (DQ088141), mummichog AHR1 (AF024591), mummichog AHR2 (FHU29679), shark AHR1 (AFR24092), shark AHR2a (JN857973), chicken ARNT (AF348088), chicken ARNT2 (XP_413854), cormorant ARNT1 (AB264539), cormorant ARNT2 (AB264540), turtle ARNT1 (XP_008168535), turtle ARNT2 (XP_005301757), frog ARNT (NM_001123453), frog ARNT2 (NM_001100216), zebrafish ARNT1c (AAZ67070), zebrafish ARNT2 (NM_131674), human AHRR (AAI52407), chicken AHRR (ADQ38959), nematode ARNT (AAB99999), fly ARNT (AAD38396).

2.8. Statistical methods

Results are presented as mean $\pm$ SEM. Comparisons between two groups were performed using Student's *t*-test, and all multi-group comparisons were performed by ANOVA followed by Bonferroni post hoc analyses using GraphPad Prism (version 4.0c; GraphPad Software, Inc., San Diego, CA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cloning of alligator AHR cDNAs

Using standard PCR techniques described above, partial DNA fragments were amplified from RNA obtained from alligator liver. DNA fragments were gel purified, and sequence analysis revealed that these fragments had strong similarity to the AHR (data not shown). Using RACE, we obtained full-length sequences of three types of

alligator AHR cDNA, aAHR1A (accession number: LC032362), aAHR1B (accession number: LC032363), and aAHR2 (accession number: LC032364). The cDNAs for alligator AHR, aAHR1A, aAHR1B, and aAHR2 are composed of a predicted 858 amino acids with a calculated molecular mass of 96.5 kDa, a predicted 1009 amino acids with a calculated molecular mass of 114.1 kDa, and a predicted 1104 amino acids with a calculated molecular mass of 122.8 kDa, respectively (Supplementary Fig. 1). The amino acid sequences of alligator AHRs showed overall identity of 46% (AHR1A vs AHR1B), 34% (AHR1A vs AHR2), and 36% (AHR1B vs AHR2). Alligator AHRs have conserved domain structures: an N-terminal bHLH domain, followed by a PAS domain (including PAS-A and PAS-B domains) like AHR from other vertebrate species, and a ligand-binding domain (LBD) partially overlapping the PAS domain (Figure 1). The three amino acid sequences of alligator AHRs shared 71-82% of identity in the bHLH domain, 72-77% in the PAS domain and 78-86 % in the LBD. Thus, the bHLH-PAS domain (involved in DNA-binding and heterodimerization) and the LBD are highly conserved among alligator AHRs. To characterize the relationship of AHRs between alligator and various vertebrate species, we conducted phylogenetic analysis of AHR amino acid sequences. Based on a phylogenetic tree using the maximum-likelihood method, vertebrate AHRs were classified into three groups, tetrapod AHR, non-mammal AHR1, and AHR2. The alligator AHR1A was classified in the tetrapod AHR group, whereas aAHR1B was related to the non-mammal (teleost) AHR1s, and aAHR2 to the AHR2 group (Figure 2). Supplementary Fig. 2 shows the amino acid sequence identities of alligator AHRs with other vertebrates AHRs. Alligator AHR1A shared a higher amino acid sequence identity with chicken AHR (84%), cormorant AHR1 (86%), and the predicted sequence of turtle AHR (84%). Alligator AHR1B and AHR2 are more similar to turtle AHR-like (77%) and turtle AHR2 (61%), respectively when compared with other vertebrate AHR amino acid sequences. However, in the sequence comparison of the bHLH domain to the LBD, all alligator AHRs shared high identities with other AHRs, suggesting that the functional domains in half of the N-terminal sites were conserved in either. In contrast, the amino acid sequences in half of the C-terminal sites showed diversity among species.

3.2. Cloning of alligator ARNT cDNAs

Using standard PCR techniques, we determined the full-length cDNA for alligator ARNTs, ARNT1 (accession number: LC032365) and ARNT2 (accession number: LC032366). Alligator ARNT1 (aARNT1) and ARNT2 (aARNT2) encode 785 amino acids with a molecular mass of 86.0 kDa and 716 amino acids with a molecular

mass of 78.8 kDa, respectively (Supplementary Fig. 3). Alligator ARNTs contain the bHLH-PAS domain structure in the N-terminal region like AHR, based on those sequence homologies with other vertebrates ARNT amino acid sequences (Figure 3). The overall identity between alligator ARNT1 and ARNT2 was 58%, and the identities were 96% in the bHLH domain and 78% in the PAS domain. Amino acid sequences of the alligator ARNTs were compared with other vertebrates ARNTs (Supplementary Fig. 4). The overall identities of alligator ARNT1 with human, chicken, cormorant, turtle, frog, or zebrafish ARNT1s were 82, 85, 87, 87, 72, or 50%, respectively. Alligator ARNT1 showed a high similarity to avian ARNT1 however, alligator ARNT1 did not contain the avian specific exon in the PAS domain (Lee et al., 2007). The overall identities of alligator ARNT2 to human, chicken, cormorant, turtle, frog, or zebrafish ARNT2s were 96, 97, 98, 98, 89, or 84%, respectively. The bHLH-PAS domain of alligator ARNT1 shared 95-79% identities with ARNT1 from other species, whereas the bHLH-PAS domain of alligator ARNT2 exhibited 100-94% identity with the other species' ARNT2s. The similarities of ARNT2s were higher than those of ARNT1s among vertebrates. A phylogenetic analysis indicated that the alligator ARNTs were closely related to those of birds and turtles rather than those of mammals, amphibians and teleost fishes (Figure 4).

3.3. Tissue distribution of AHR and ARNT mRNAs

The relative expressions of AHRs and ARNTs mRNAs in various tissues from both female and male alligators were determined by RT-PCR (Figure 5). Alligator AHR1A and AHR2 were expressed in all tissues examined (gonad, kidney, liver, pancreas, spleen, and thyroid). The expression of aAHR1B mRNA was found in the gonad, thyroid, liver and pancreas of both sexes, but not in kidney and spleen. We found that aARNT1 mRNA was expressed in all tissues examined. Further investigations on the tissue distribution of AHRs and ARNTs in the alligator would be required.

3.4. Transcriptional activation of alligator AHR-ARNT complexes

A transactivation assay was conducted to determine whether aAHR-aARNT complexes display a xenobiotic-dependent transcriptional activity. AHR-ARNT complexes recognize a response element named XRE, containing a CGCAT site, and induce a transcript of its downstream region (Kubota et al., 1991). We examined the transcriptional activity of aAHR-aARNT complexes using a reporter gene assay with XRE-driven reporter construct. We selected two chemicals as test ligands, one was indigo as an endogenous ligand (Adachi et al., 2001; Peter Guengerich et al., 2004), and the other was 3-MC as an exogenous ligand (Riddick et al., 1994; Swedenborg et al.,

2012). aARNTs without aAHRs could not activate transcription via the XRE-driven reporter gene in the presence of 3-MC and indigo, whereas transfection of aAHR without aARNT displayed slight xenobiotic-induced transcription, likely due to low levels of endogenous ARNT protein in COS-7 cells (Figure 6). Co-transfection of aAHR with aARNT markedly enhanced transactivation by 3-MC and indigo (Figure 6). 3-MC and indigo equally stimulated the transcriptional activity of aAHR-aARNT1 complexes, whereas indigo weakly induced the transactivity of aAHR1A-aARNT2 and aAHR2-aARNT2 complexes. Further, we detected the ligand-independent transactivation of aAHR-aARNT complexes; especially complexes with aARNT1 had high activities of transcription via the XRE-driven reporter gene. The human AHR-ARNT complex also possessed the ligand-independent transactivity (Supplementary Fig. 5). Further study is necessary to clarify the mechanisms of ligand-independent activation of aAHR-aARNT complexes.

To compare ligand sensitivities of aAHR-aARNT complexes in a dose-dependent manner, transactivation was examined with increasing concentrations of 3-MC and indigo (Figure 7). Almost all combinations of aAHR-aARNT complexes were activated in a dose-dependent manner, and weaker inductions of aAHR1A-aARNT2 and aAHR2-aARNT2 complexes were found in the presence of indigo versus 3-MC. aAHR-aARNT1 complexes were more sensitive to both 3-MC and indigo than aAHR-aARNT2 complexes, when EC₅₀ values were compared (Table 2). The same tendency of efficiency for aAHR-aARNT complex was detected in human AHR-ARNT complexes (Supplementary Fig. 6). In contrast, the efficacies (fold activations) of aAHR-aARNT complexes were higher with the aAHR-aARNT2 complex when compared with the aAHR-aARNT1 complex. Further, aAHR1B-aARNT complexes, but neither the aAHR-aARNT complexes nor the human AHR-ARNT complexes, were more sensitive to indigo than 3-MC. This result indicates that aAHR1B could be functionally differentiated from aAHR1A and aAHR2 in regards to ligand selectivity.

4. Discussion

Environmental xenobiotic compounds can induce developmental abnormalities and reproductive disorders in aquatic species. In the aquatic reptiles like alligators and turtles, it has been reported that exposures to environmental contaminants can lead to developmental abnormalities (Guillette et al., 1995, 1999; Hamlin and Guillette, 2011; Storelli and Zizzo, 2014). AHR signaling potentially mediates these effects via interaction with many chemicals and its relationship to the sex hormone signaling pathway (Beischlag and Perdew, 2005; Endler et al., 2012; Sato et al., 2013). Although

Marquez et al. (2011) reported full and partial sequences of AHR from a turtle, functional information of the AHR/ARNT in reptiles did not exist prior to this study. Here, we have cloned alligator cDNAs for three AHR isoforms and two ARNT isoforms. Phylogenetic analysis using AHRs from several vertebrate species showed that teleost AHR1s are clustered into the same clade as non-mammalian AHR1s (non-mammal AHR1), supporting the hypothesis that two teleost AHR1 genes arose by teleost-specific whole genome duplication.

Many *in vitro* assay systems for AHR-mediated effects including AHR-ligand binding affinity and AHR-mediated reporter gene expression are available (Priskorska-Pliszczyńska et al., 1986; Sanderson et al., 1996; Machala et al., 2001). Because it is useful for investigating both chemical responsibility and the complex dependency of AHR-ARNT heterodimers, we applied a reporter gene assay using an XRE (xenobiotic-response element)-driven reporter construct. We demonstrated that all aAHRs acted in the aAHR-aARNT complex in a chemical-dependent and concentration-dependent manner. We found that the transactivations of aAHRs-aARNT1 complexes were more sensitive to indigo and 3-MC than aAHRs-aARNT2 complexes. aAHR1B-aARNTs complexes are more sensitive to indigo than 3-MC, whereas aAHR1A-aARNTs and aAHR2-aARNTs are more sensitive to 3-MC than indigo.

The avian AHR2 genes are categorized into three types according to the amino acids corresponding to 324th and 380th amino acids of chicken AHR1, type 1 (Ile324/Ser380, high sensitivity), type 2 (Ile324/Ala380, moderate sensitivity), and type 3 (Val324/Ala380, low sensitivity). This categorization is suitable for comparison of ligand-sensitivity between AHR1 and AHR2 at least in some avian species (Yasui et al., 2007; Mol et al., 2012). In alligator, aAHR1A and aAHR2 belong to type 3 and aAHR1B is type 2 based on their amino acid sequences. The ligand sensitivity of aAHR1A and aAHR2 to 3-MC and indigo is consistent within type 3 AHRs. The aAHR1B is categorized as type 2 and is supposed to be more sensitive based on the avian classification of AHRs. However, aAHR1B exhibits a high sensitivity to indigo, but not to 3-MC. These results suggest that this classification based on the avian AHR is not suitable for responsiveness to 3-MC in the American alligator, although the alligator and birds both belong to the archosaurian lineage. Teleost AHR1 and AHR2 that show the type 2 amino acid residues (Ile/Ala) often display different sensitivities to TCDD, and the key amino acids for ligand-sensitivity of teleost AHRs are still unknown (Hansson et al., 2008; Bak et al., 2013; Doering et al., 2014). A previous study using homology modeling indicated that a small group of internal residues in the ligand-binding cavity of

zebrafish AHR1b lies in different positions and results in a slightly reduced space available for the ligand when compared with zebrafish AHR2 (Fraccalvieri et al., 2013). These residues are well conserved between aAHR1B and zebrafish AHR1b, but an alternative structure in the ligand-binding cavity could occur among aAHRs due to amino acid conversions in aAHR1B (Leu234, Ser264, Met280, Met 293, Arg312, Ile320, His327, and Lys370), which is different from those in aAHR1A and aAHR2. These substitutions might affect the ligand-sensitivity, although involvement of these residues in ligand-binding has not been reported.

The transactivation of AHR complexes incorporating aARNT1 are more sensitive than those incorporating aARNT2. Although both aARNT1 and aARNT2 induced transcriptional activation of the XRE-reporter gene with aAHR, the complexes of aAHR-aARNT1 exhibited higher sensitivity to a ligand than aAHR-aARNT2 complexes, similar to observation using human ARNT genes (Supplementary Fig. 6). In mammals, expression of ARNT2 is restricted to kidney and neural tissues, and AHR-ARNT2 complex does not induce CYP1A1 promoter activity due to reduced dimerization affinity (Sekine et al., 2006). Since aARNT2 shows high-identity with mammalian ARNT2 in the amino acid sequence, reduced sensitivities of aARNT2 complexes might be caused by defective interaction between aAHR and aARNT2 as in mammals. Furthermore, the transactivational response to indigo via aAHR1A and aAHR2 with aARNT2 was significantly lower than it with aARNT1. In addition to the differences in the sensitivity to ligands, the efficacy (maximum induction) of transcriptional activation was significantly different depending on which isoform of aARNT is transfected. The transcriptional activity of aAHR1A with aARNT2 was markedly lower than that with aARNT1 at 10^{-6} M 3-MC treatment. These ligand- and isoform-dependent differences for transactivation suggested that not only the ligand-binding affinity but also ligand-specific modification such as conformational changes to each aAHR could be responsible for differential transcriptional activity through changes in protein-protein interaction, protein stability, and DNA binding ability (Murray et al., 2010; Backlund and Ingelman-Sundberg, 2004).

In summary, we have isolated cDNAs encoding AHRs and ARNTs from the alligator. This is the first report of the full-length sequence for AHR1A, AHR1B, ARNT1 and ARNT2 in the reptiles. Further, *in vitro* reporter gene assay demonstrated that aAHR-aARNT complexes can activate the transcription of an XRE-driven reporter construct in an isoform-specific and ligand dose-dependent manner. Although their physiological roles remains unknown, all AHR-ARNT isolated in this study could be relevant to the AHR signaling pathway in the alligator. Our results provide the basic

molecular information useful in examining the role of AHR and ARNT in the endocrine system of reptiles in response to endogenous and exogenous chemicals. Further investigations on the sensitivities to various chemicals with aAHRs and analyses of the regulation of endogenous target genes are necessary for an understanding of the effects of environmental xenobiotic compounds via AHR/ARNT signaling in the alligator as well as the evolution of AHR/ARNT signaling in vertebrates.

Conflict of interest statement

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no signify financial support for this work that could have influenced its outcome.

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Figure Legends

Table 1. Primers for RT-PCR.

Table 2. Gene transcriptional activities of chemicals mediated by alligator and human AHR-ARNT complexes.

Figure 1. **Amino acid sequence comparison of AHR proteins from alligator**

The amino acid sequences of alligator AHRs were aligned with ClustalW. Identical amino acids are shaded. Gray background indicates the residue not identical but similar to the column-consensus. The ligand-binding domain is in bracket, and the bHLH and PAS domains are indicated with solid lines. BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html) was used to make this figure.

Figure 2. **Phylogeny of AHR sequences in selected vertebrate species**

The phylogenetic tree was constructed using the maximum likelihood analysis with JTT+G model. The scale bar shows 0.2 expected amino acid substitutions per site. The number on each branch point represents the bootstrap value. Accession numbers for sequences used were indicated in materials and methods.

Figure 3. **Comparison of amino acid sequence for alligator ARNTs**

The amino acid sequences of alligator ARNTs were aligned with ClustalW. Identical amino acids are shaded. Gray background indicates the residue not identical but similar to the column-consensus. The bHLH, PAS A, and PAS B domains are marked by solid lines. BOXSHADE was used to make this figure.

Figure 4. **Phylogeny of ARNT sequences in selected animal species**

The tree was constructed using the maximum likelihood analysis with JTT+G model. The scale bar shows 0.2 expected amino acid substitutions per site. The number on each branch represents the bootstrap value. Accession numbers for sequences used were indicated in materials and methods.

Figure 5. Tissue distribution of alligator AHRs and ARNTs mRNAs

Total RNA was prepared from gonad (G), kidney (K), liver (L), pancreas (P), spleen (S), and thyroid (T) from both female and male alligator. β -actin was used as a positive control. Expression of mRNA was detected by RT-PCR (see Materials and Methods).

Figure 6. Transcriptional activities of alligator AHR-ARNT complexes

COS-7 cells were transfected with AHR alone, ARNT alone, or AHR + ARNT as indicated. Cells were treated with 3-MC or indigo at 10^{-6} M, or vehicle alone (0.1% DMSO). Data are represented as the ratio of experimental well to control well (no alligator AHR and ARNT, and treated with DMSO). Each bar represents the mean of triplicate determinations, and vertical bars represent the means $\pm$ SEM.

Figure 7. Concentration-response profiles for alligator AHR-ARNT complexes

Alligator AHRs were transfected into COS-7 cells with alligator ARNT1 (A-C) or alligator ARNT2 (D-F). Cells were incubated with increasing concentrations of 3-MC and indigo. Each bar represents the mean of the quadruplicate or quintuplicate determinations, and vertical bars present the mean $\pm$ SEM. The y-axis indicates fold activation compared with the activity of minimal concentration treatment.

Table 1

Primers for RT-PCR.

Primer	Sequence
β -actin-F	5'-CACGCCATCCTGCGTCTGGACTTG-3'
β -actin-R	5'-ATTCTGTCGGCGATGCCAGGGTAC-3'
AHR1A-F	5'-GCCATCAGCAACAGCCCATGGTTC-3'
AHR1A-R	5'-GGGTTGAGTTTCTGGTGGGCACTG-3'
AHR1B-F	5'-AAGGGGACCCGCTGTTATCTACCC-3'
AHR1B-R	5'-GACCTCCACTGCAGACCATTATGC-3'
AHR2-F	5'-ATGGATGTTGATGAGCCAGTGGCC-3'
AHR2-R	5'-CCTGAGGGTCAGAGCCTAATGTGC-3'
ARNT1-F	5'-CAGCGCAGCTGGCATCAAGACAGC-3'
ARNT1-R	5'-GCCAGTTGGTTCCGCTGACCTGAG-3'
ARNT2-F	5'-GATGGCCTCAGCATCCACTGCAGG-3'
ARNT2-R	5'-GTCTGGCTGGGCTGCTGGTGAGAG-3'

Table 2

Gene transcriptional activities of chemicals mediated by alligator and human AHR-ARNT complexes.

AHR	ARNT	ligand	EC50 [M]	95% Confidence Intervals [M]
alligator AHR1A	alligator ARNT1	3-MC	9.5×10^{-11}	1.799×10^{-11} to 4.976×10^{-10}
		Indigo	3.1×10^{-9}	8.893×10^{-10} to 1.076×10^{-8}
	alligator ARNT2	3-MC	1.2×10^{-9}	3.354×10^{-10} to 4.639×10^{-9}
		Indigo	-	-
alligator AHR1B	alligator ARNT1	3-MC	9.3×10^{-10}	4.478×10^{-10} to 1.944×10^{-9}
		Indigo	1.7×10^{-10}	3.913×10^{-11} to 7.474×10^{-10}
	alligator ARNT2	3-MC	6.8×10^{-8}	2.311×10^{-8} to 1.987×10^{-7}
		Indigo	9.4×10^{-9}	3.175×10^{-9} to 2.805×10^{-8}
alligator AHR2	alligator ARNT1	3-MC	2.7×10^{-11}	6.295×10^{-12} to 1.180×10^{-10}
		Indigo	2.9×10^{-9}	1.209×10^{-9} to 7.122×10^{-9}
	alligator ARNT2	3-MC	6.8×10^{-10}	2.005×10^{-10} to 2.275×10^{-9}
		Indigo	7.5×10^{-6}	1.132×10^{-6} to 4.952×10^{-5}
human AHR	human ARNT	3-MC	7.6×10^{-10}	1.839×10^{-10} to 3.173×10^{-9}
		Indigo	5.3×10^{-9}	2.769×10^{-9} to 1.012×10^{-8}
	human ARNT2	3-MC	3.6×10^{-9}	8.313×10^{-10} to 1.535×10^{-8}
		Indigo	2.0×10^{-7}	1.145×10^{-7} to 3.478×10^{-7}

Figure 1

Figure 2

Figure 4

Figure 5

Figure 6

A: AHR1A-ARNT1

D: AHR1A-ARNT2

B: AHR1B-ARNT1

E: AHR1B-ARNT2

C: AHR2-ARNT1

F: AHR2-ARNT2

Figure 7