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Molecular Evidence Predicts Aryl Hydrocarbon Receptor Ligand Insensitivity in the Peregrine Falcon (*Falco peregrines*)

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Abstract

Some wild populations of fish-eating birds and raptors are exposed to high concentrations of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds such as other 2,3,7,8-substituted polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans and coplanar polychlorinated biphenyls, resulting in accumulation in their tissues. It has been demonstrated that TCDD-like chemicals cause toxic effects via aryl hydrocarbon receptor (AHR)-mediated signaling pathways. The aim of this study was to characterize the AHR from the peregrine falcon (*Falco peregrines)* to predict its sensitivity to TCDD-like chemicals. The AHR1, AHR2, and AHR nuclear translocator (ARNT) 1 of the peregrine falcon are more similar in amino acid sequence to avian species less sensitive to TCDD-like chemicals such as the cormorant (95%) than to more sensitive species such as the chicken (90%). From the amino acid sequence, it is likely that the ligand-binding affinity of peregrine falcon AHR1 and AHR2 would be very low compared with the chicken or other sensitive species, and it was actually proved by an *in vitro* reporter gene assay. We concluded that the peregrine falcon, one of raptor species, may be relatively resistant to TCDD-like chemicals.

Keywords: Avian; dioxin; raptor; falcon; aryl hydrocarbon receptor

Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), halogenated aromatic hydrocarbons (HAHs), and polynuclear aromatic hydrocarbons (PAHs) have garnered attention as environmental pollutants, and are known to be teratogenic, immunotoxic, and toxic to the reproductive system of birds (Walker *et al.* 1996; Bird *et al.* 1983; Blankenship *et al.* 2003; Larson *et al.* 1995; Peden-Adams *et al.* 1998). Dwelling at the top of the food chain, raptors tend to concentrate TCDD-like chemicals in body tissues (Clark *et al.* 2001; Gill *et al.* 2002; Elliot *et al.* 2001). Because avians are hatched from eggs rather than by live birth as for mammals, this process provides an additional high-risk step for exposure to environmental pollutants, which makes it more difficult for birds to live in highly polluted areas and results in reductions in their populations. Indeed, environmental pollutants including TCDD, polychlorinated biphenyls (PCBs) and 1,1'-(dichloroethylidene) bis [4-chlorobenzene] (DDE) have been reported to be concentrated in eggs and chicks of raptors (Clark *et al.* 2001; Gill *et al.* 2002; Elliot *et al.* 2001). From these reports, TCDD and TCDD-like compounds have been found to be highly magnified from fish to osprey eggs (over 30-fold). Previous reports that have indicated the effects of TCDD and TCDD-like chemicals on raptors include studies on osprey chicks in contaminated areas that seem to exhibit signs of wasting syndrome and growth inhibition (Woodford *et al.* 1998). In addition, PCBs have been reported to decrease sperm concentration and semen volume in male American kestrels (Bird *et al.* 1983). However, from work done in the laboratory, bald eagle hepatocyte cultures were the least sensitive based on TCDD induction of ethoxyresorufin-O-deethylase (EROD) induction among avian species examined to date, in terms of both the EC_{50} and the

concentration required to cause a modest increase in EROD activity (Kennedy *et al.* 2002). At both field-observed concentraions and at the concentration that is lethal to chicken eggs, 2,3,7,8-TCDD does not have an effect on the hatching success of osprey chicks (Elliott *et al.* 2001).

For these reasons, it is possible that one of the raptors, peregrine falcon (*Falco peregrines*), is one of the avian species at the highest risk of poisoning by TCDD-like chemicals. From the phylogenetic study, most of raptors are more similar to the TCDD-resistant species, including fish-eating birds, than the dioxin-sensitive birds, including chicken (Hackett *et al.* 2008). However, little information has been reported from the viewpoint of molecular biology. To better understand the sensitivity of raptor species to chronic exposure to TCDD-like chemicals, the characteristics, or the ligand binding affinity, of the raptor aryl hydrocarbon receptor (AHR) need to be better defined.

The AHR is a basic-helix-loop-helix/PAS family protein and a transcription factor activated by ligand binding (Burbach et al. 1992). When not bound to ligands, the AHR stays in the cytosol, forming a complex with heat shock protein 90 (HSP90), the AHR associated protein (XAP2 or ARA9), and p23 (Denis *et al.* 1988; Perdew 1998). Once bound with ligand, the AHR is translocated to the nucleus (Whitelaw *et al.* 1993), and constructs a heterodimer with AHR nuclear translocator (ARNT), which then binds to xenobiotic responsive element (XRE) (Matsushita *et al.* 1993; Reyes *et al.* 1992). After binding with XRE, transcription of many genes, such as CYP1A1, CYP1A2, and AHR repressor (AHRR), are activated.

It is known that avian species have two AHR isoforms, AHR1 and AHR2 (Yasui *et al.* 2007; Yasui *et al.* 2004), while most mammals possess only one. However, the

dominant isoform of AHR differs among species (Kim *et al.* 2008). There are large differences in function even within the same AHR isoform. For example, although avian AHR1s are highly conserved (more than 90%) among species, there are large interspecies differences in sensitivity to TCDD-like chemicals, which can be explained by differences in their ligand-binding affinities (Karchner *et al.* 2006) and transactivation abilities. It is reported that TCDD sentivity of avian species are predicted from the two amino acids at positions 325 and 381 of AHR1 (Karchner *et al.* 2006). In addition to the sequence information for AHR, *in vitro* reporter assays can confirm the prediction of AHR ligand-binding affinities. An EROD *in vitro* bioassay is also reported to be a useful method to evaluate the sensitivity to TCDD-like chemicals in avian species (Head *et al.* 2010; Kennedy *et al.* 1996; Brunstom *et al.* 1998), and thus, CYP1A transactivation ability is often used for this reason.

The objective of this study is to evaluate components of the AHR pathway to predict sensitivity of the peregrine falcon, a raptor species, to an AHR ligand. This is the first report that elucidates the characteristics of the AHR in a raptor species.

Materials and Methods

Animals and cDNA Cloning.

A liver from an 8-year-old female peregrine falcon was provided by Maruyama Zoo (Sapporo, Japan) in 2008. 2-month-old female white Leghorn chickens obtained from Hokkaido Central Chicken Farm to take their livers, and were housed in steel cages and fed a standard diet (Nihon Nosan Kogyo, Yokohama, Japan) and water *ad libitum*. Animals were maintained at 23°C on a 12 hour dark/light cycle (starting at 7:00). Treatment of all animals were performed according to the policies of the Institutional Animal Care and Use Committee of Hokkaido University. Animals were sacrificed with CO_2, and the livers were quickly removed, rinsed, weighed and then immediately frozen in liquid nitrogen. All frozen samples were stored at -80°C until use.

cDNA Cloning and Sequencing.

Hepatic total RNA was isolated by TRI Reagent (RNA/DNA/Protein isolation reagent: Sigma-Aldrich, St. Louis, USA). Peregrine falcon and chicken hepatic RNA was reverse-transcribed to cDNA primed by Oligo(dT). cDNA was synthesized as follows: 2 μg total RNA and 2.5 pmol Oligo(dT) primer was incubated in a total volume of 3.5 μl diethylpyrocarbonate (DEPC) water at 70°C for 10 min. This mixture was then made up to 10 μl with 2 μl of (5x) reverse transcription buffer (RT-buffer), 8ul of 10 mM deoxynucleotide triphosphates (dNTP), and 0.5 μl of reverse transcriptase (TOYOBO, Osaka, Japan). The mixture was incubated at 30°C for 10 min, 42°C for 1h and 90°C for 10 min. Partial or full-length peregrine falcon AHR1, AHR2, and ARNT1 were cloned by RT-PCR using the primers listed in Table 1. These primers were designed with

reference to sequences of other avian AHRs and ARNTs. Full-length falcon AHR1 was cloned by primers including start/stop codon, and the positions of primers were 4 bases before the start codon, or a base after the stop codon. Polymerase chain reaction (PCR) parameters were as follows: AHR1 94°C for 2 min, 94°C for 30 s / 66°C for 45 s / 72°C for 3 min for 35 cycles, and 72°C for 5 min. PCR products were direct-sequenced using primers Avian AHR1-1 to 4, annealing temperature at 50°C; AHR2. Primers were used as pairs, and the annealing temperatures were 61°C and 56°C; ARNT1 touchdown PCR was performed with conditions of 94°C for 2 min, 94°C for 30 s / 68-66°C decreased by 2°C for each of 3 cycles, 45 s/72°C for 3 min for 9 cycles, 94°C for 30 s / 62°C for 45 s / 72°C for 3 min for 25 cycles, and 72°C for 5 min. These PCR products then were used in nested PCR, with forward and reverse primer pairs avian ARNT1-1 to 4. The annealing temperature of these primers was 65°C. Finally, these nested PCR products were direct-sequenced using inner primers of avian ARNT1-1 to 4.

The partial sequence of AHR2 was cloned by AHR-A1 and AHR-B1 (Table 1), primers that were previously reported (Karchner *et al.* 1995). For obtaining DNA sequence information of the ligand-binding domain, we used AHR2-LBD-F and AHR2-LBD-R (Table 1).

Expression Constructs

The PCR products of full length AHR1s from chicken and falcon were ligated into pcDNA3.1/V5-His-TOPO vector, respectively (Invitrogen, Inc., Carlsbad, USA). Cormorant ARNT1 (pcDNA-ccARNT1) (Lee *et al.* 2007), CYP1A5 promoter containing 6 XREs (pGL4-ccCYP1A5-6XREs) (Lee *et al.* 2009), and pRL-SV40 (Promega, Madison, USA) were also used for the reporter assay.

Cell Culture

COS-7 cells were from Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, USA) with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, USA) at 37°C, 5% CO_2. These cells were plated on 24-well plates. The medium was changed 24 hours after plating to serum-free medium (VP-SFM, Invitrogen, Inc.).

Transfections and Luciferase Assays

Transfections were carried out 24 hours after changing the medium. Approximately 30 ng of AHR1, 300 ng of pcDNA-ccARNT1, 120 ng of pGL4-ccCYP1A5-6XREs, and 18 ng of pRL-SV40 were transfected per well. The total amount of transfected DNA was kept at 500 ng by the addition of empty pcDNA vector. The composition of DNA was determined by reference to previous studies (Yasui *et al.* 2007; Karchner *et al.* 2006). We first performed conditioning, and found this expression construct could highly induce the transactivation in AHR transfected cells. The DNAs were diluted with OPTI-MEM medium (Invitrogen, Inc.), and mixed with 1.25 µl per well of FuGENE HD Transfection Reagent (Roche, Basel, Switzerland). A total of 4 hours after transfection, cells were exposed to 0.1% dimethyl sulfoxide (DMSO), or SudanIII (10 µM final concentration) diluted with virus production-serum free medium (VP-SFM). Cells were lysed after 18 hours of dosing, and the luminescence was measured using Dual-Glo Luciferase Assay System (Promega, Madison, USA). Final luminescence values are expressed as a ratio of the firefly luciferase units to the renilla luciferase units.

In this assay, the same ARNT and XREs were used among chicken and falcon transfected cells, and the result was compared among those two species.

Phylogenetic Tree

DNA sequences of AHRs, ARNTs and AHRRs were aligned using ClustalX2 (Larkin *et al.*, 2007). Accession numbers for those sequences are: common cormorant AHR1 (AB109545), black-footed albatross AHR1 (AB106109), common tern AHR1 (AF192503), chicken AHR (AF192502), mouse AHR (M94623), human AHR (L19872), *Xenopus laevis* AHR1a (AY635782), *Xenopus laevis* AHR1b (AY635783), killifish AHR1 (AF024591), killifish AHR2 (U29679), black-footed albatross AHR2 (AB106110), common cormorant AHR2 (AB287294), *Xenopus laevis* ARNT1 (NM001088661), *Xenopus laevis* ARNT2 (NM001090153), human ARNT1 (BC041121), mouse ARNT TV1 (NM001037737), mouse ARNT TV2 (NM009709), chicken ARNT (AF348088), common cormorant ARNT1 (AB264539), killifish AHRR (AF443441), human AHRR (AB033060), mouse AHRR (AB015140). The *Drosophila melanogaster* spineless (AF050630) was added as an outgroup. We performed alignment only for about 300 amino acids, including the PAS-A and PAS-B domains, and excluded the areas with gaps. We constructed the phylogenetic tree with bootstrap N-J tree, and viewed it by NJ plot (Perrière *et al.*, 1996).

AHR1 and AHR2 Expression ratio

After cloning cDNAs of AHRs (full length for AHR1 and PAS-A, PAS-B domain for AHR2), the PCR products were purified with QIAquick PCR Purification Kit (QIAGEN, CA, USA). These purified PCR products were used as the standards. The

cDNA described previously were used in this experiment.

Quantitative real-time RT-PCR for AHR1 and AHR2 mRNA levels was performed using the Step One Plus Real-Time PCR System (Applied Biosystems LLC, Foster City CA, USA) and reagent for THUNDERBIRD SYBR qPCR Mix (TOYOBO). The primers used are listed in Table 1: real-time AHR1 F, real-time AHR1 R, real-time AHR2 F, and real-time AHR2 R.

The content of the mixture for PCR was 1x Master Mix, 0.3 μM of each primer, 100 ng of cDNA and 1x ROX reference dye. The total volume of the reaction mixture was kept constant at 10 μl by addition of RNase-free water. The reaction was performed as follows: 95°C for 60 sec, 40 cycles of 95°C for 15 sec, 62°C for 15 sec, and 72°C for 45 sec, and data collection was done at the 72°C step. The measurements were performed in duplicate. Approximate logarithmic curves were made from the threshold cycle of several concentrations of standards. Absolute copy numbers of each AHR were obtained from the standard curve, and an AHR1:AHR2 expression construct was calculated.

Statistical analyses

Data were analyzed using Tukey's HSD (Honestly Significant Difference) test with a significance level of $p < 0.05$ using JMP software (version 7.0; SAS Institute, Cary, NC, USA). The values are shown as the mean ± SD (standard deviation).

Results

Peregrine Falcon AHR1, AHR2 and ARNT Sequences

We cloned the full length AHR1 cDNA (AB560859). The sequence of peregrine falcon AHR1 encodes 863 amino acid residues, and it shares high degrees of sequence homology with fish-eating birds such as the black-footed albatross (95%) and the common cormorant (95%). The ligand-binding domain of the peregrine falcon AHR1 was the same as that of the common tern and the common cormorant (Figure 1). However, the full-length peregrine falcon AHR1 was less similar to the chicken than to the species mentioned above, with three major differences in the amino acid sequence of the ligand binding domain between the peregrine falcon and chicken. These three amino acid residues were Thr-258, Val-325, and Ala-381 in peregrine falcon AHR1 (Figure 1).

We also determined the partial sequence of AHR2 (AB560860), at the region around the ligand-binding domain. By comparing two important amino acids to determine the ligand affinity, aa 325 and 381 (Karchner *et al.* 2006), the ligand-binding affinity of falcon AHR2 was the same as that of the cormorant. However it was not indicated if these two amino acids are important in tems of ligand-binding affinity of the AHR2. Compared to the falcon AHR1, the homology of the amino acid sequence of the ligand-binding domain of the falcon AHR2 in comparison to other avian species was lower (90% for black-footed albatross, 89% for common cormorant) (Figure2).

Peregrine falcon ARNT1 cDNA sequence encoded 807 amino acid residues (AB560861), which is the same length as that of common cormorant, while chicken ARNT1 cDNA encodes 805 amino acid residues. Peregrine falcon ARNT1 and common cormorant ARNT1 were highly similar with 94% amino acid identity. The amino acid

sequence of the ARNT1 functional domain, such as bHLH, PAS-A, PAS-B, and the transcriptional activation domain were completely identical between the peregrine falcon and common cormorant ARNT1. In addition, chicken and falcon ARNT1 were similar, particularly for the functional domain. There were only two amino acid residues that were different in the activation domain between these two ARNTs.

Phylogenetic analyses of AHRs and ARNT

To confirm the distinction of AHR1, AHR2, and ARNT1, a phylogenetic tree was constructed (Figure 3). The AHR1, AHR2, and ARNT1 of the peregrine falcon are referred to as several independent AHR1, AHR2, and ARNT1 clades, even including those of fish or mammals. As an aside, when we performed alignment in full-length, killifish, albatross, and cormorant formed a single individual clade (data not shown). In a narrow portion of the tree that emphasizes avian species, the peregrine falcon is closer to fish-eating birds, known as species to be less sensitive to TCDD-like chemicals than the chicken, which is a highly sensitive species.

AHR1 and AHR2 Expression Ratio.

We investigated which AHR was dominantly expressed in peregrine falcon. Similar to chicken or the common cormorant, falcon AHR1 was expressed in a high percentage, 85%.

Luciferase Assay.

To compare the AHR1 transcriptional activity of the peregrine falcon with other avian species, we performed a luciferase reporter assay. COS-7 cells were transfected with

falcon or chicken AHR1. “No AHR” cells were transfected with the same amount of empty pcDNA vector. Each type of cells was exposed to SudanIII (10 μM final concentration), which was used as the AHR ligand (Lubert *et al.* 1983; Refat *et al.* 2008).

Compared with cells exposed to DMSO, SudanIII treated cells had the same level of luciferase activity as mock transfected cells, whereas they were1.3-fold higher in falcon cells, and 3.5-fold higher in chicken cells (Figures 4). Luciferase activity in chicken AHR transfected cells was significantly higher than that of falcon AHR or mock plasmid transfected cells. Comparing the relative luciferase activity in mock transfected cells and AHR1 transfected cells from each avian species exposed to 10 μM SudanIII, chicken AHR exhibited 3.6-fold greater activity than mock control, while falcon AHR showed only a 2-fold increase in activity compared with control plasmid (Figure 4). In addition, 3-methylcholanthrene (3MC) was also used as a typical AHR ligands, and we found that 3MC transactivation ability of falcon AHR was same as the activity of SudanIII (2.3-fold i chicken AHR transfected cells compared to control plasmid: data not shown). Other ligands, such as β-naphthoflavone was also used, and showed lower transcriptional activity compared to that of SudanIII, 1.02 fold in chicken AhR transfected cells compared to mock transfected cells (data not shown). Therefore, the transcriptional activity of falcon AHR1 was much lower than that of chicken AHR, a species which is known to be highly sensitive to TCDD-like chemicals.

Discussion

It is reported that many effects induced by TCDD-like chemicals are related to the function of the AHRs. Heart defects are the typical TCDD-induced effect and they are believed to be caused via the AHR because of the high expression of AHR in cardiac myocytes during cardiogenesis (Walker *et al.* 1996). Expanding the case to mammals, it is reported that AHR-null mice do not exhibit immune suppression or teratogenicity as a result of exposure to TCDD-like chemicals. In mice, the classical teratogenicities caused by TCDD-like chemicals are cleft palate and hydronephrosis, but neither pathology was seen in TCDD-treated AHR-null mice (Mimura *et al.* 1997, Fernandez-Salguero *et al.* 1996). Similarly, because AHR-null mice did not develop immunosuppression, the AHR is presumed to play an important role in this type of toxicity (Vorderstrasse *et al.* 2001).

From the phylogenetic tree containing AHR, AHRR, or ARNT of several animal species, AHR1, AHR2, and ARNT1 of the peregrine falcon belong to the AHR1, AHR2 and ARNT1 clades, respectively. Also, these three proteins of the peregrine falcon are more similar to those of fish-eating birds such as the common cormorant or the black-footed albatross than to the chicken or other animals. Based on the amino acid sequence in the ligand-binding domain, the sensitivity of the peregrine falcon to TCDD-like chemicals is predicted to be low (Yasui *et al*. 2007; Karchner *et al.* 2006). Peregrine falcon ARNT1 had a similar functional domain as that of the common cormorant, an avian species that is less sensitive to TCDD-like chemicals, while the peregrine falcon and chicken differ in their transcriptional activation domain. Measuring transcriptional activity is one of the primary methods to evaluate the function of AHRs (Yasui *et al.*

2007; Karchner *et al*. 2006). Using embryo hepatocyte culture, EROD EC_{50} values (or CYP1A induction) are reported to be correlated with LD_{50} values from egg injection studies (Head *et al*. 2010; Kennedy *et al*. 1996). For this reason, an interspecies comparison of sensitivity to TCDD-like chemicals and in ovo lethality can be done by determining the CYP1A induction ability of hepatocyte cultures. That means that the CYP1A transactivation ability would infer its AHR transcriptional activity and hence, the species' comparative sensitivity. Thus, we performed a reporter assay to evaluate AHR1 transcriptional activity. In our present study, SudanIII was used as the AHR ligand (Lubet *et al.* 1983; Refat *et al*. 2008). The AHR1 of peregrine falcon was highly similar to other lower sensitivity species in terms of its functional domain, bHLH, PAS A, and PAS B. Similar data have been obtained with 3MC.

On the other hand, the homology of the Q-rich domain was relatively low (albatross: 83%, chicken: 85%, cormorant: 90%). This suggests that the transactivation ability would vary among these species. Nevertheless, the amino acid sequence of ARNT1 was similar among avian species in terms of its AHR/ARNT heterodimer formation or binding to XREs, especially for the peregrine falcon and the common cormorant (Lee *et al.* 2006). Heart defects, for instance, are thought to be mediated by AHR/ARNT dimerization (Walker *et al*. 1996). Studying ARNT, in addition to AHRs, should provide valuable insights into the sensitivity of avian wildlife to TCDD-induced teratogenic effects.

Although all vertebrates are reported to possess AHR1, eutherian mammals are known not to have AHR2. The species reported to have the AHR2 are fish and birds, while mammals possess a single AHR (Hahn *et al.* 2006). A few avian species are reported to have AHR2, and among those that do, their amino acid sequences differ greatly. For

example, the cormorant and albatross AHR2 share 77% amino acid homology while their AHR1s are 96% similar (Yasui *et al.* 2007). Because the AHR2 plays a dominant role in some species (Kim *et al.* 2008), it is important for its function to be well defined. The cormorant, albatross, and chicken have an AHR2 that has low transactivation ability, while the crow AHR2 is reported to have high transactivation ability (Kim *et al.* 2008). The dominant AHR in the peregrine falcon is predicted to be the AHR1, as shown in Figure 3. As previously reported, differences in the transactivation region are predicted to determine the role of the AHR2 (Kim *et al.* 2008). Based on the two amino acids, Val-325 and Ala-381,that are reported to play a role in TCDD toxicity, the ligand-binding affinity of AHR1 is inferred to be low (Karcher *et al.* 2006). It is reported that AHR1 of avian species could be categorized into three groups, sensitive (Ile-325 and Ser-381), moderately sensitive (Ile-325 and Ala-381), and relatively insensitive (Val-325 and Ala-381) (Karchner *et al.* 2006). The peregrine falcon revealed to harbor AHR1 of relatively insentive from this study. However, aside from those two amino acids, in the case of the peregrine falcon AHR2, several amino acids in the ligand-binding domain are different from the AHR2s of other reported avian species. These two amino acids are believed not to be critical in the AHR2 sequence, because the ligand-binding affinities of the cormorant, albatross, and crow AHR2 were very different even though they have the same amino acids at positions 325 and 381 (Kim *et al.* 2008).

In this study, sensitivity of the falcon to TCDD-like chemicals was determined to be relatively insensitive by molecular biological assessment of AHR1, AHR2 and ARNT1. In fact, a high LD_{50} for raptors was indicated by the low transcriptional activity of AHR1, and the sensitivity of the species to TCDD-like chemicals were predicted by its

AHR activity. This is the first report on molecular-based functional analyses of the raptor AHR and ARNT.

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Table 1. Primers used for RT-PCR

	Forward 5'- sequence -3'	Reverse 5'- sequence -3'
AHR1 cloning		
Avian AHR Full	CAGGATGAACCCCAATGTCAC	GTCACATAAATCCACTAGATGCCAAA
Avian AHR1-1	GGATGAACCCCAATGTCACCTA	ATCGTCCTTGAAAATTCATA
Avian AHR1-2	TCATCTGCAGGTTACGATGCCT	ACACAGACTCATCTTGCCTTA
Avian AHR1-3	TGCCCTTCATGTTTGCCACTGGTGA	TCCAATTTGTGAACATCCCAT
Avian AHR1-4	CAGCTCTGTCAAAAGATGAAA	TTACATAAATCCACTAGA
AHR2 cloning		
AHR-A1	CGGGATCCARGCICTSAAYGGITT	
AHR-B1		GCTCTAGACATICCRCTYTCICCIGTYTT
AHR2-LBD	TCTCCAGACAAAGCACAAGCTGGAC	GTACAGGACTGCTTCCCCCGTG
Avian AHR2-1	ATGTACGCCGGGAGGAAGAGGAAG	CCGACTGATGAAAGCCCAGGTA
Avian AHR2-3	GGGTACACGGAAACGGAGCTGTG	CAGCTCTGCATTGTCCATGTTGAG
ARNT1 cloning		
Avian ARNT1 Full	ATGGCAGCCACCGCCGCCAACCCGGAA	TTTTACTCTGAAAAAGAAGGGAATATGTTCA
Avian ARNT1-1	CATGGCTGTCTCTCACA	AGCCCAGTTGGTTCCGCT
Avian ARNT1-2	ATCCAAAAACCGGGAAT	GTTCTTGACGTTGGTGTT
Avian ARNT1-3	AGCAGCATGGTACCTC	ATGTAACCCGTGCAGT
Avian ARNT1-4		GGAGGGTTTGTAGG
Real-time RT-PCR		
real-time AHR1	AGAAAGGGAAGGATGGCACT	CTGGTATCCCGTTCCTCTCA
real-time AHR2	AAGCCCTCCTTTGTGGAGAGG	TCCCGAGGAGTTATCCAGCAA

FIGURE LEGENDS

Fig. 1. Amino acid sequence alignment of avian AHR1 from four species.

The amino acid sequences of AHR1s were aligned using ClustalX2, and the accession numbers are listed in the Materials and Methods. PAS-A and PAS-B domains are shown by black bars. Boxes indicate the two critical amino acids at positions 325 and 381. The arrows indicate the ligand-binding domain. The identity percentage among falcon and each avian species were 95% for the black-footed albatross and common cormorant, and 90% for the chicken.

Figure 2. Amino acid sequence alignment of four species of avian AHR2.

The amino acid sequences of AHR2s were aligned using ClustalX2, and the accession numbers are listed in the Materials and Methods. PAS-A and PAS-B domains are shown by black bars. The arrows indicate the ligand-binding domain. The identity percentage among falcon and each avian species were 90% for black-footed albatross, and 89% for common cormorant.

Fig. 3. Phylogenetic Tree.

DNA sequences of AHRs, ARNTs and AHRRs were aligned using ClustalX2. *Drosophila melanogaster* spineless (AF050630) was added as an outgroup. Alignment was performed at a length of about 300 amino acids, including the PAS-A and PAS-B domains, and excluded positions with gaps. The phylogenetic tree was constructed with bootstrap N-J tree, and viewed by NJplot. The numbers are indicating the bootstrap values.

Fig. 4. Transcriptional activity of AHR1.

COS-7 cells were transfected with pcDNA-ccARNT1, pGL4-ccCYP1A5-6XREs, pRL-SV40, and AHR1. For AHR (-), an empty pcDNA vector was transfected instead. Cells were exposed to 0.1% DMSO, or SudanIII (10 μM final concentration). A) Relative luciferase units are expressed as a ratio of the firefly luciferase units to the renilla luciferase units. The result are expressed as the median value ± SD, n=4. B) The luciferase activity of the AHR ligand exposed cells that were divided by that of cells which were not exposed. The letters a and b indicate significant differences between the cell groups ($p < 0.05$).

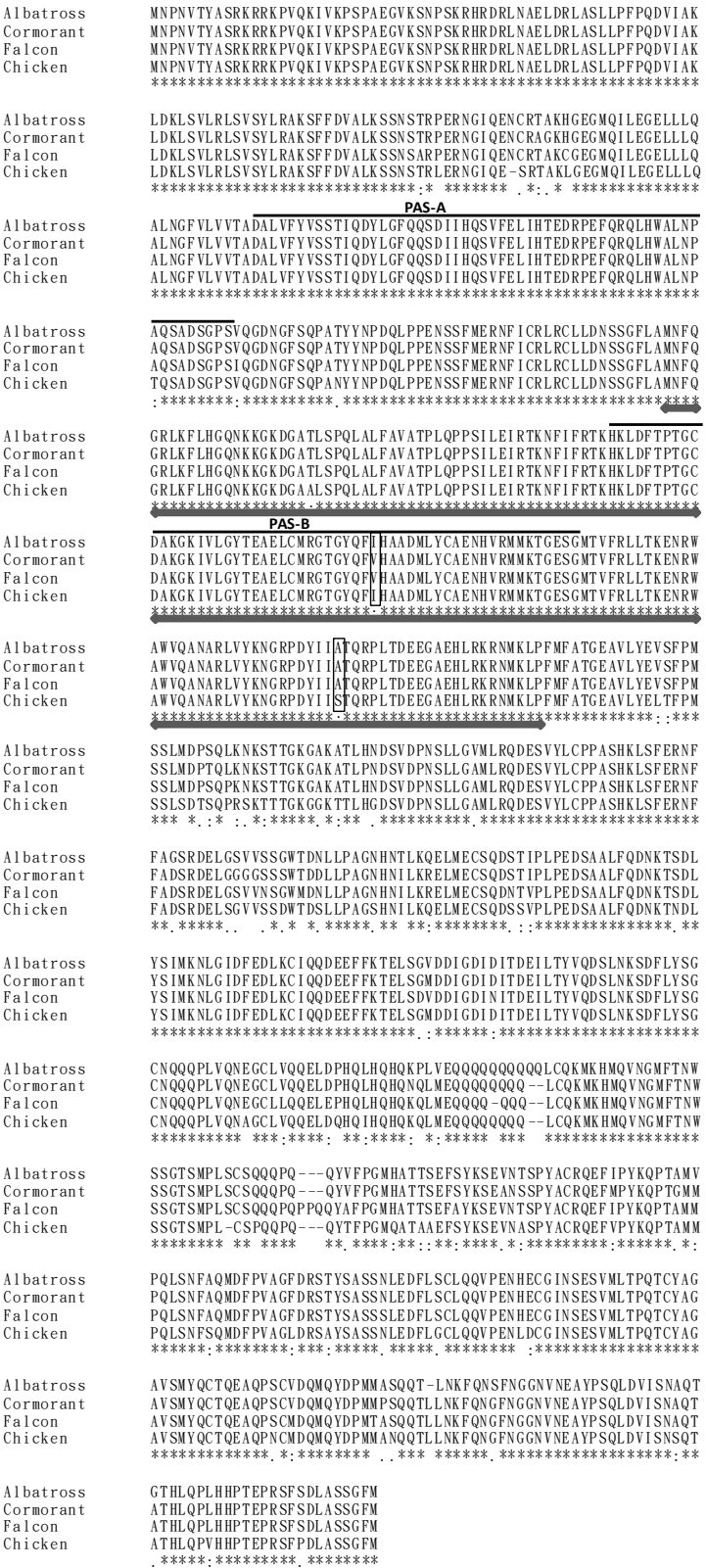

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Falcon MNPNVTYASRKRRKPVQKIVKPSPAEGVKSNPSKRHRDRLNAELDRLASLLPFPQDVIAK
Chicken MNPNVTYASRKRRKPVQKIVKPSPAEGVKSNPSKRHRDRLNAELDRLASLLPFPQDVIAK
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Cormorant LDKLSVLRLSVSYLRAKSFFDVALKSSNSTRPERNGIQENCRAGKHGEGMQILEGELLLQ
Falcon LDKLSVLRLSVSYLRAKSFFDVALKSSNSARPERNGIQENCRTAKCGEGMQILEGELLLQ
Chicken LDKLSVLRLSVSYLRAKSFFDVALKSSNSTRLERNGIQE-SRTAKLGEGMQILEGELLLQ
PAS-A
Albatross ALNGFVLVVTADALVFYVSSTIQDYLGFQQSDIIHQSVFELIHTEDRPEFQRQLHWALNP
Cormorant ALNGFVLVVTADALVFYVSSTIQDYLGFQQSDIIHQSVFELIHTEDRPEFQRQLHWALNP
Falcon ALNGFVLVVTADALVFYVSSTIQDYLGFQQSDIIHQSVFELIHTEDRPEFQRQLHWALNP
Chicken ALNGFVLVVTADALVFYVSSTIQDYLGFQQSDIIHQSVFELIHTEDRPEFQRQLHWALNP
Albatross AQSADSGPSVQGDNGFSQPATYYNPDQLPPENSSFMERNFICRLRCLLDNSSGFLAMNFQ
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Falcon AQSADSGPSIQGDNGFSQPATYYNPDQLPPENSSFMERNFICRLRCLLDNSSGFLAMNFQ
Chicken TQSADSGPSVQGDNGFSQPANYYNPDQLPPENSSFMERNFICRLRCLLDNSSGFLAMNFQ
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Falcon GRLKFLHGQNKKGKDGATLSPQLALFAVATPLQPPSILEIRTKNFIFRTKHKLDFTPTGC
Chicken GRLKFLHGQNKKGKDGAALSPQLALFAVATPLQPPSILEIRTKNFIFRTKHKLDFTPTGC
PAS-B
Albatross DAKGKIVLGYTEAELCMRGTGYQFIHAADMLYCAENHVRMMKTGESGMTVFRLLTKENRW
Cormorant DAKGKIVLGYTEAELCMRGTGYQFVHAADMLYCAENHVRMMKTGESGMTVFRLLTKENRW
Falcon DAKGKIVLGYTEAELCMRGTGYQFVHAADMLYCAENHVRMMKTGESGMTVFRLLTKENRW
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Cormorant FADSRDELGGGGSSSWTDDLLPAGNHNILKRELMECSQDSTIPLPEDSAALFQDNKTSDL
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Albatross CNQQQPLVQNEGCLVQQELDPHQLHQHQKPLVEQQQQQQQQQQLCQKMKHMQVNGMFTNW
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Falcon CNQQQPLVQNEGCLLQQELEPHQLHQHQKQLMEQQQQ-QQQ--LCQKMKHMQVNGMFTNW
Chicken CNQQQPLVQNAGCLVQQELDQHQIHQHQKQLMEQQQQQQQQQ--LCQKMKHMQVNGMFTNW
Albatross SSGTSMPLSCSQQQPQ---QYVFPGMHATTSEFSYKSEVNTSPYACRQEFIPYKQPTAMV
Cormorant SSGTSMPLSCSQQQPQ---QYVFPGMHATTSEFSYKSEANSSPYACRQEFMPYKQPTGMM
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Albatross PQLSNFAQMDFPVAGFDRSTYSASSNLEDFLSCLQQVPENHECGINSESVMLTPQTCYAG
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Chicken AVSMYQCTQEAQPNCMDQMQYDPMMANQQTLLNKFQNGFNGGNVNEAYPSQLDVISNSQT
Albatross GTHLQPLHHPTEPRSFSDLASSGFM
Cormorant ATHLQPLHHPTEPRSFSDLASSGFM
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Fig. 1

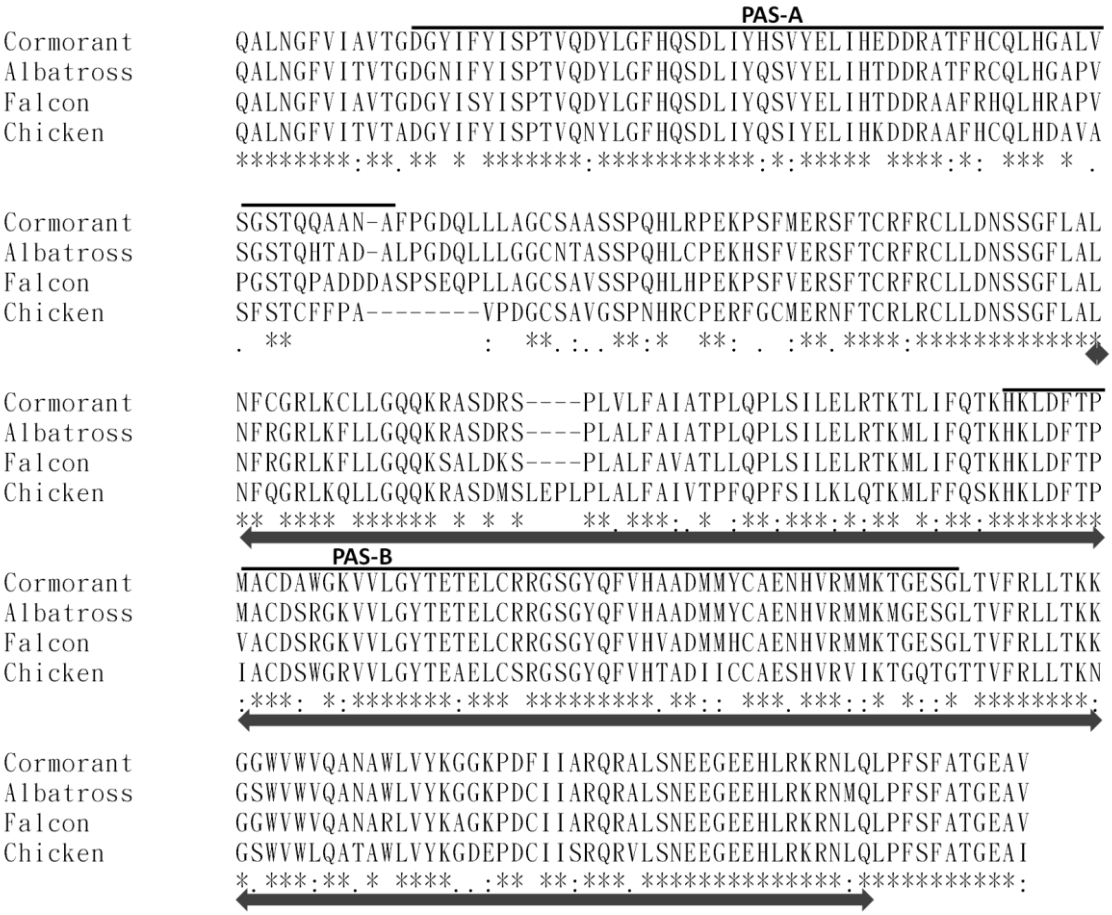

PAS-A
Cormorant QALNGFVIAVTGDGYIFYISPTVQDYLGFHQSDLIYHSVYELIHEDDRATFHCQLHGALV
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Falcon QALNGFVIAVTGDGYISYISPTVQDYLGFHQSDLIYQSVYELIHTDDRAAFRHQLHRAPV
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Cormorant SGSTQQAAN-AFPGDQLLLAGCSAASSPQHLRPEKPSFMERSFTCRFRCLLDNSSGFLAL
Albatross SGSTQHTAD-ALPGDQLLLGGCNTASSPQHLCPEKHSFVERSFTCRFRCLLDNSSGFLAL
Falcon PGSTQPADDDASPSEQPLLAGCSAVSSPQHLHPEKPSFVERSFTCRFRCLLDNSSGFLAL
Chicken SFSTCFFPA--------VPDGCSAVGSPNHRCPERFGCMERNFTCRLRCLLDNSSGFLAL
Cormorant NFCGRLKCLLGQQKRASDRS----PLVLFAIATPLQPLSILELRTKTLIFQTKHKLDFTP
Albatross NFRGRLKFLLGQQKRASDRS----PLALFAIATPLQPLSILELRTKMLIFQTKHKLDFTP
Falcon NFRGRLKFLLGQQKSALDKS----PLALFAVATLLQPLSILELRTKMLIFQTKHKLDFTP
Chicken NFQGRLKQLLGQQKRASDMSLEPLPLALFAIVTPFQPFSILKLQTKMLFFQSKHKLDFTP
PAS-B
Cormorant MACDAWGKVVLGYTETELCRRGSGYQFVHAADMMYCAENHVRMMKTGESGLTVFRLLTKK
Albatross MACDSRGKVVLGYTETELCRRGSGYQFVHAADMMYCAENHVRMMKMGESGLTVFRLLTKK
Falcon VACDSRGKVVLGYTETELCRRGSGYQFVHVADMMHCAENHVRMMKTGESGLTVFRLLTKK
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Falcon GGWVWVQANARLVYKAGKPDCIIARQRALSNEEGEEHLRKRNLQLPFSFATGEAV
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Fig. 2

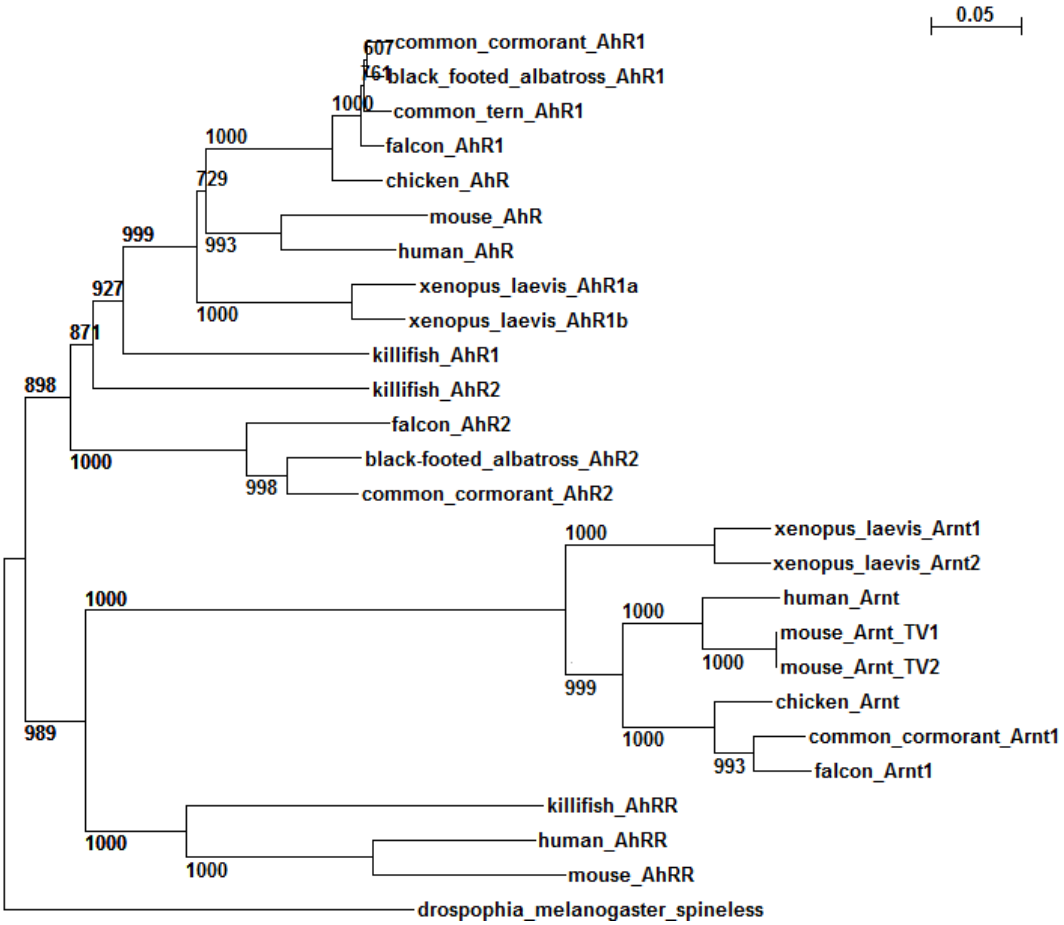

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Fig. 3

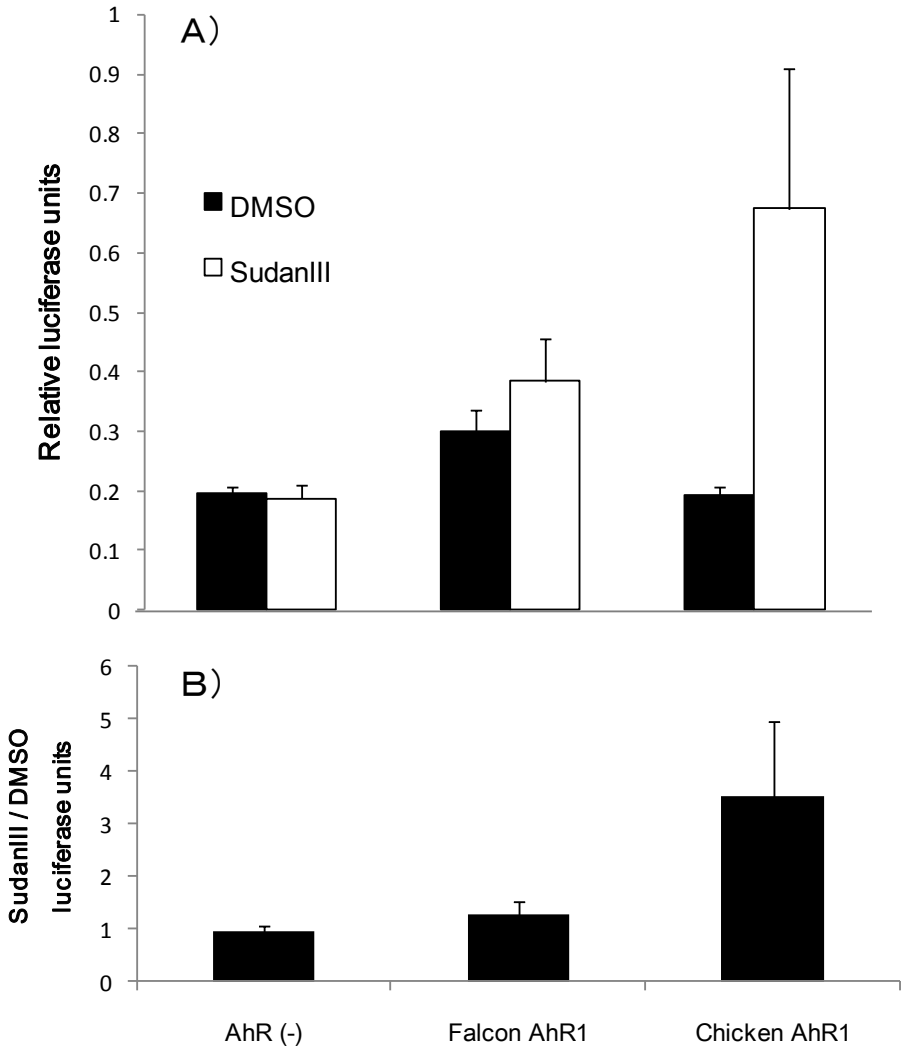

A)
Relative luciferase units
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
DMSO
SudanIII
B)
SudanIII / DMSO
luciferase units
6
5
4
3
2
1
0
AhR (-)
Falcon AhR1
Chicken AhR1

Fig. 4