Dioxins including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induce various toxic effects through the aryl hydrocarbon receptor (AhR) signaling pathway. Here, we investigated the structural and functional characteristics and molecular evolution of multiple AhRs in black-footed albatross (Phoebastria nigripes) and common cormorant (Phalacrocorax carbo). We report the complementary DNA sequences of two distinct AhRs, designated AhR1 and AhR2, from these species as well as the identification of an AhR2-like gene sequence from the chicken genome database. Phylogenetic analysis reveals that avian AhR1 and AhR2 are orthologous to mammalian AhR1 and fish AhR2, respectively, supporting the hypothesis that an ancestral AhR gene underwent a tandem duplication prior to the divergence of fish and tetrapod lineages. In vitro–expressed AhR1 and AhR2 isoforms from both albatross and cormorant exhibited specific binding to [3H]TCDD, as assessed by velocity sedimentation. An in vitro reporter gene transactivation assay revealed that both AhR1 and AhR2 are transcriptionally active, but AhR2 appears to have reduced transcriptional efficacy. Hepatic messenger RNA expression level of cormorant AhR1 was greater than that of AhR2. Together, these results suggest that AhR1 is the dominant form of avian AhRs, in contrast to fish, in which AhR2 is the major form. Comparative analysis of AhR diversity and gene synteny among chicken, zebrafish, and human suggests that additional, independent AhR duplications have occurred in the fish and tetrapod lineages following the initial tandem duplication on the ancestral chromosome. The identification and characterization of avian AhR1 and AhR2 provide new insight into the evolution of AhR structure and function in vertebrates.

Key Words: aryl hydrocarbon receptor 2 (AhR2); chromosomal duplication; specific binding; transcriptional activation; black-footed albatross; common cormorant.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and related planar halogenated aromatic hydrocarbons are widespread contaminants of the environment, causing an extensive range of adverse effects in various organisms. These effects are initiated by ligand binding to the aryl hydrocarbon receptor (AhR), a cytoplasmic transcription factor (Poland and Knutson, 1982; Schmidt and Bradfield, 1996).

The AhR and its homologs are known to be present in a variety of animal species ranging from Drosophila melanogaster and Caenorhabditis elegans to mammals and birds (reviewed in Hahn, 2002). Most animals contain only one AhR isoform, while fishes possess at least two types of AhR isoforms, designated AhR1 and AhR2. This has been shown in a variety of fish species, including cartilaginous fishes (Hahn et al., 1997) as well as bony fishes (Andreasen et al., 2002; Hahn et al., 1997; Karchner et al., 1999, 2005; Yamauchi et al., 2005). Considering the evolutionary history and presence of AhR1 and AhR2 in both bony and cartilaginous fishes, the existence of multiple AhR isoforms in other vertebrate species, particularly in more recently diverging animals such as mammals and birds, has been expected. However, a survey of AhR-related genes in sequenced mammalian genomes found no evidence for an AhR2 in mammals (Hahn et al. 2006).

Intra- and interspecies differences in sensitivity to TCDD and dioxin-like compounds have been reported with several lines of evidence in laboratory animals, and have also been anticipated in wildlife. The difference between the two mouse strains in the LD50 for TCDD sensitivity can be explained by a similar difference in the dioxin-binding affinities of their respective AhR proteins (Okey et al., 1989; Poland et al., 1994). This suggests that AhR can be a critical determinant for the sensitivity to dioxin and the mechanism of dioxin action.

Our previous studies revealed that black-footed albatross (Phoebastria nigripes) and common cormorant (Phalacrocorax carbo) accumulated high levels of dioxins, including polychlorinated dibenzo-p-dioxins, furans, and coplanar PCBs in the livers (Iwata et al., 2001; Kubota et al., 2005). The hepatic toxic equivalent (TEQ) levels detected in these species exceeded the EC50/Lowest observed effect level for CYP1A
induction in primary hepatocyte culture of various avian species (Kennedy et al., 1996). Further investigations demonstrated that there were significant positive correlations between hepatic TEQs and CYP1A expression levels or 7-ethoxyresorufin O-deethylase activities in the wild population of both species, indicating CYP1A induction via activation of the AhR signaling pathway (Iwata et al., 2001; Kubota et al., 2005, 2006). Considering the dramatic differences in sensitivity to CYP1A induction among avian species (Kennedy et al., 1996; Lorenzen et al., 1997), research focusing on the avian AhRs may contribute to a better understanding of the mechanisms responsible for this differential susceptibility. However, the structure and function of avian AhRs at the molecular level has been limited to the full-length sequences of single AhR isoforms (corresponding to mammalian AhR) in each of two species, chicken and common tern (Karchner et al., 2006; Walker et al., 2000).

In a recent short communication, we provided a preliminary report describing the identification of three full-length and one partial complementary DNA (cDNA) sequences of novel AhR isoforms from the livers of black-footed albatross and common cormorant; these novel AhRs were grouped in the same clade as fish AhR1 and AhR2 by phylogenetic analysis (Yasui et al., 2004). However, nothing is known about the structural and functional role of the novel avian AhR2 isoforms that we have identified.

Here the complete cDNA sequence for the cormorant AhR2 was determined and phylogenetic analyses were performed. Comparative analysis of AhR diversity and gene synteny among chicken, zebrafish, and human suggests that in addition to the tandem duplication leading to AhR1 and AhR2, additional, independent AhR duplications have occurred in the fish and tetrapod lineages. To begin to understand the functional and regulatory characteristics of these avian AhRs, the messenger RNA (mRNA) expression levels were measured in multiple tissues of cormorants. In addition, to investigate the basic function of these AhR isoforms, their ability to participate in TCDD specific binding and transactivation was also determined.

The present study revealed that both of the avian species contained two distinct AhR isoforms, which possess TCDD-binding affinity. All avian AhRs exhibited transcriptional activation in an in vitro reporter gene assay, but AhR2s were less effective at activating the transcription of the reporter gene. Comparison of hepatic mRNA expression levels of AhR isoforms revealed that AhR1 was expressed at higher levels than AhR2. These findings strongly suggest that AhR1 is the dominant isoform that is involved in TCDD-induced toxicity in avian species, whereas AhR2 appears to be the major form in some fish species.

MATERIALS AND METHODS

**Chemicals.** 2,3,7,8-Tetrachloro[1,6-3H]dibenzo-p-dioxin was obtained from Chemsyn Science Laboratories ([3H]TCDD, 35 Ci/mmol, > 99% radiochemical purity) (Lenexa, KS) and EaglePicher ([3H]TCDD, 27.5 Ci/mmol, > 97% radiochemical purity by high-performance liquid chromatography) (Lenexa).

[3H]Methionine and methylated [methyl-14C]ovalbumin were purchased from Amersham Biosciences and NEN Life Science Products Inc. (Boston, MA), respectively. Methylated [methyl-14C]catalase was synthesized by PerkinElmer (Boston, MA).

**Sample collection and RNA isolation.** Black-footed albatrosses (P. nigripes) were accidentally caught by gill nets from the North Pacific in 1995. Common cormorants (P. carbo) were captured from Lake Biwa, Japan in 2001, 2002, and 2003 under the license of Shiga Prefecture. After dissection, subsamples of liver and other tissues (kidney, heart, lung, spleen, muscle, pancreas, brain, gonad, and intestine) were immediately frozen in liquid nitrogen and stored at −80°C until total RNA isolation. For the cloning of AhRs, total RNA was isolated using RNAagent Total RNA Isolation System (Promega, Madison, WI), and poly(A)+ RNA was purified by oligo(dT) spin columns (mRNA Purification Kit; Amersham Biosciences, Piscataway, NJ). For the measurement of AhR mRNA expression level, total RNAs were isolated from tissues of common cormorants using Nucleic Acid Purification Kit (TOYOBO, Turun, Japan) and Nucleic Acid Purification System MagExtractor MFX-2100 (TOYOBO).

**cDNA cloning and sequencing.** The albatross and cormorant AhR isoforms were cloned using a reverse transcription-PCR (RT-PCR) method as described earlier (Kim and Hahn, 2002). One microgram of poly(A)+ RNA from liver was reverse-transcribed with random hexamers using the Gene-Amp RNA-PCR kit (Applied Biosystems, Foster City, CA) following the manufacturer’s instruction. Full-length AhR1 sequences of albatross and cormorant were obtained by RT-PCR, employing avian specific primers designed based on the chicken AhR mRNA sequence, as described in our previous study (Yasui et al., 2004).

The partial sequences of AhR2 for albatross and cormorant were cloned by RT-PCR. Primer sequences and temperature conditions of RT-PCR have already been described elsewhere (Yasui et al., 2004). For 5′- and 3′-rapid amplification of cDNA ends (RACE) of AhR2, adaptor-ligated, oligo(dT)-primed, double-stranded cDNAs were synthesized using a Marathon cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA). For 5′- and 3′-RACE, AhR2 gene-specific primers (BFA5′-2′ and BFA-3′-4′ for albatross, and BFA5′-4′ and BLC3′-5′ for cormorant) were coupled with adaptor primers in the PCR reactions and the products were cloned and sequenced. Primers used for AhR cloning are listed in Table 1. The conditions of touch-down PCR were as follows: 30 s at 94°C, 5 cycles of 5 s at 94°C and 4 min at 72°C, 5 cycles of 5 s at 94°C and 4 min at 70°C, 25 cycles of 5 s at 70°C, and 4 min at 68°C.

As for the full-length sequences of AhR2, specific primer pairs, BFA2-T3/BFA2-T4 for albatross and BLC2-1F/BLC2-1R for cormorant, were used. PCR was performed under the same temperature conditions as in the case of RACE method.

All PCR products were ligated into the pGEM-T Easy vector (Promega) and sequenced by ABI PrismTM 310 Genetic Analyzer (Applied Biosystems). AhR sequences were assembled and translated using MacVector 7.2 (Accelrys, San Diego, CA).

**Sequence alignment and phylogenetic analysis.** A multiple alignment of amino acid sequences for vertebrate AhR and AhR repressor (AhRR) sequences was performed using ClustalX (Thompson et al., 1997). The invertebrate AhR homologs AHR-1 (C. elegans) and spinesless (D. melanogaster) were included as outgroups. Regions of ambiguous alignment, including most of the C-terminal halves of the proteins, were omitted. The alignment used for phylogenetic inference included most of the basic helix loop helix (bHLH) and Per-Arnt-Sim homology (PAS) domains and contained 394 total characters, of which 265 were parsimony informative. Phylogenetic relationships among avian AhRs and AhRs from other vertebrate species were inferred from this alignment using maximum parsimony (MP) and distance (minimum evolution [ME]) as optimality criteria and by Bayesian analysis. MP and ME trees were constructed using
TABLE 1
The Sequences of Oligonucleotide Primers and Probes Used for Cloning of Avian AhR2 cDNAs (A) and for Quantification of AhR and CYP1A mRNAs by Real-Time RT-PCR (B)

<table>
<thead>
<tr>
<th></th>
<th>Primer sequence</th>
<th>Reverse primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Albatross AhR2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RACE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BFA5’-2</td>
<td>5’-gacgacagtacctgagagctgt-3’</td>
<td>5’-acccattctagcaacagacgca-3’</td>
</tr>
<tr>
<td>BFA3’-4</td>
<td>5’-caaggctacatgctgtaaggcgt-3’</td>
<td>5’-cttcgcccaggattgctgtaaggctgt-3’</td>
</tr>
<tr>
<td>Full-length</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BFA2-T3</td>
<td>5’-ccccgggtggagcttctacaggg-3’</td>
<td>5’-gcgcaggagttgctgtaaggctgacg-3’</td>
</tr>
<tr>
<td>BFA2-T4</td>
<td>5’-ccccgggtggagcttctacaggg-3’</td>
<td>5’-gcgcaggagttgctgtaaggctgacg-3’</td>
</tr>
</tbody>
</table>

| **Cormorant AhR2** |                          |                                 |
| RACE           |                          |                                 |
| BFA5’-4        | 5’-tctccggcgagagtgctgtaaggctgt-3’ | 5’-cttcgcccaggattgctgtaaggctgt-3’ |
| BLC3’-5        | 5’-cttcgcccaggattgctgtaaggctgt-3’ | 5’-tctccggcgagagtgctgtaaggctgt-3’ |
| Full-length    |                          |                                 |
| BFA2-T2        | 5’-gccgggatgtacgccggagaggaaga-3’ | 5’-tctccggcgagagtgctgtaaggctgt-3’ |
| BFA2-T4        | 5’-gccgggatgtacgccggagaggaaga-3’ | 5’-tctccggcgagagtgctgtaaggctgt-3’ |

(A)  
(B)  

- **Expression constructs.** The full-length cDNAs of albatross and cormorant AhR1 were cut out of pGEM-T Easy vector with restriction enzyme pairs NotI/XhoI, NotI/XhoI, and NotI/XhoI, respectively. Albatross and cormorant AhR2 were digested with EcoRI. These DNA fragments were inserted into their corresponding restriction enzyme sites in pcDNA3.1/zeo (+) vector (Invitrogen, Carlsbad, CA, USA). For the expression of avian AhRM, the pcDNA-ccArnt1 expression construct was prepared by the insertion of full-length cormorant AhR1 cDNA into pcDNA3.1/zeo (+) vector (Invitrogen). From cormorant liver, two distinct Amb cDNAs, Arnt1 and Arnt2, and some splicing variants were identified and cloned (Lee et al., in press). Cormorant Arnt1 that is the same type as chicken Arnt was employed in this study. As a reporter vector, the plasmid pGL3-cycCYP1A5 consisted of firefly luciferase reporter gene linked with cormorant CYP1A5 promoter, which contains six XREs in an approximately 2.6-kbp fragment (Lee et al., unpublished data).

- **In vitro protein synthesis.** Unlabeled or [35S]methionine-labeled proteins were synthesized by TnT Quick-Coupled Reticulocyte Lysate Systems (Promega) following the manufacturer’s instructions. Five micrograms of the [35S]methionine-labeled TnT reactions was separated on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. The gels were fixed, dried onto filter paper, and exposed to X-ray film overnight. For the velocity sedimentation analyses, the nonlabeled-TnT reactions were incubated at 4°C with [3H]TCDD (2nM). Nonspecific binding was determined by reactions containing an empty vector (unprogrammed lysate) as described previously (Karchner et al., 1999; Kim and Hahn, 2002).

- **Velocity sedimentation analyses.** Two different protocols conducted by two laboratories were used for the velocity sedimentation/binding analyses of albatross AhR1 and cormorant AhRs/albatross AhR2. Chicken AhR was used as a positive control to compare the binding condition in both analyses.

- **In vitro–expressed AhR proteins were analyzed by velocity sedimentation on sucrose gradients as described earlier (Karchner et al., 1999).** For albatross AhR1 and chicken AhR, 50 μl of TnT reactions was diluted 1:1 with MEEDMG buffer (25mM 4-Morpholinepropanesulfonic acid, pH 7.5, 20°C), containing 1mM dithiothreitol, 1mM ethylenediaminetetraacetic acid, 5mM ethyleneglycol-bis-(2-aminoethyl)ether)-N,N,N’,N’-tetraacetic acid, 0.02% NaN3, 20mM Na2MoO4, 10% (vol:vol) glycerol plus a mixture of protease inhibitors), and incubated in 10 × 75 mm glass tubes with [3H]TCDD in dimethyl sulfoxide (DMSO) (2nM final TCDD concentration) overnight at 4°C. The [3H]TCDD concentration was verified by sampling 5 μl from each tube for total counts. After incubation, 90 μl from each tube were applied to 10–30% sucrose gradients that were prepared in MEEDMG buffer. The gradients were spun for 130 min at 60,000 rpm (327,800 × g) at 4°C in a vertical tube rotor (VTI 65.2), after which 150-μl fractions were counted using a Beckman LS500TD scintillation counter. [14C]Ovalbumin (3.6S) and [14C]catalase (VTi 65.2), after which 150-μl fractions were added as internal sedimentation markers; they were eluted in fractions 4–5 and 15–16, respectively. Sedimentation coefficients of the AhR proteins were determined by the method of Martin and Ames (1961).

- **For cormorant AhRs and albatross AhR2, 750 μl of TnT reactions was diluted 1:1 with MEEDMG buffer (25mM 4-Morpholinepropanesulfonic acid, pH 7.5, 20°C), containing 1mM dithiothreitol, 1mM ethylenediaminetetraacetic acid, 5mM ethyleneglycol-bis-(2-aminoethyl)ether)-N,N,N’,N’-tetraacetic acid, 0.02% NaN3, 20mM Na2MoO4, 10% (vol:vol) glycerol plus a mixture of protease inhibitors), and incubated in 10 × 75 mm glass tubes with [3H]TCDD in dimethyl sulfoxide (DMSO) (2nM final TCDD concentration) overnight at 4°C. The [3H]TCDD concentration was verified by sampling 5 μl from each tube for total counts. After incubation, 90 μl from each tube were applied to 10–30% sucrose gradients that were prepared in MEEDMG buffer. The gradients were spun for 130 min at 60,000 rpm (327,800 × g) at 4°C in a vertical tube rotor (VTI 65.2), after which 150-μl fractions were counted using a Beckman LS500TD scintillation counter. [14C]Ovalbumin (3.6S) and [14C]catalase (VTi 65.2), after which 150-μl fractions were added as internal sedimentation markers; they were eluted in fractions 4–5 and 15–16, respectively. Sedimentation coefficients of the AhR proteins were determined by the method of Martin and Ames (1961).
Transfection and luciferase reporter assay. COS-7 cells were kindly provided by Prof. Chong Kyu-Hyuck (Sungkyunkwan University, Korea) and maintained in Dulbecco’s modified Eagle’s medium (Sigma, St Louis, MO) supplemented with fetal calf serum (10% final concentration) at 37°C under 5% CO₂. Cells were plated at 6 × 10⁴ cells/well in 24-well plates. Transfections were carried out in triplicate wells 20–24 h after plating. DNA and Lipofectamine 2000 transfection reagent (Invitrogen) were diluted in serum-free Opti-MEM (minimal essential medium) medium (Invitrogen). For each well, a total of approximately 300 ng of DNA was complexed with 1 μl of Lipofectamine 2000 reagent. The mixture was then added to cells in serum-free Opti-MEM medium. Cells were dosed 4–5 h after transfection with DMSO or TCDD (14nM final concentration), which were diluted by charcoal/dextran-treated MEM supplemented with 10% charcoal/dextran-treated fetal bovine serum (HyClone, Logan, UT). Renilla luciferase (phRL-TK, Promega) was used as the transfection control. The amounts of transfected expression vectors were 5 ng of each AhR, 50 ng of Arnt, 20 ng of pG3-l-cCYP1A5, and 3 ng of phRL-TK. The total amount of transfected DNA was kept constant at 300 ng by addition of pcDNA3.1(zeo+) vector with no insert. Cells were lysed 18–20 h after dosing and kept at −80°C for at least 1 h. Luminescence was measured using the Dual Luciferase Assay kit (Promega) in a TD-20/20 Luminometer (Promega). The final luminescence values were expressed as a ratio of the luciferase units to the Renilla luciferase units.

RESULTS

Cloning of AhR cDNAs

Two distinct AhR sequences (designated AhR type 1 and AhR type 2, see below) were identified from each of two avian species, common cormorant and black-footed albatross, by RT-PCR using hepatic RNA. As reported in our preliminary study (Yasui et al., 2004) and described in detail here, black-footed albatross and common cormorant AhR type 1 cDNAs had open reading frames (ORFs) of 861 and 860 amino acid residues, respectively, and shared high amino acid identity (96%) (Fig. 1).

Previously (Yasui et al., 2004), we reported an AhR type 2 cDNA sequence from albatross and a partial AhR type 2 cDNA sequence from cormorant, but no functional or expression analyses were performed. In the present study we used RACE to complete the cormorant sequences in preparation for detailed functional, regulatory, and phylogenetic studies. The full-length cDNA of black-footed albatross AhR type 2 encodes a 925 amino acid ORF with a predicted molecular mass of 101 kDa, larger than that of AhR type 1 (Fig. 2). Approximately 1.3 kb of 3'-untranslated region (UTR) sequence was obtained. The full-length cDNA of common cormorant AhR type 2 encodes a 995 amino acid residue ORF with a predicted molecular mass of 107 kDa (Fig. 2). As a result of 3'-RACE, approximately 2.0 kb of UTR sequence with a polyadenylation signal (ATTAAA) and a poly A tail were identified. Compared with albatross AhR type 2 cDNA, the cormorant AhR type 2 cDNA lacked one cytosine nucleotide in the C-terminal half, inducing a frame-shift (Fig. 2). Although the 3'-UTR sequence of black-footed albatross AhR type 2 was not complete, the UTR sequence obtained was similar to that of cormorant AhR type 2 (69% nucleotide identity).
Phylogenetic Analyses of Avian AhR1 and AhR2

To characterize the novel AhR sequences from cormorant and albatross and their relationship to previously identified AhRs from birds, mammals, and fishes, we conducted phylogenetic analysis of AhR amino acid sequences from various vertebrate species. We used distance (ME) and MP as optimality criteria, and also inferred the most probable tree using Bayesian analysis, as described in "Methods." Figure 3 shows the 50% majority rule tree from Bayesian analysis; Table 2 summarizes the results of analyses by ME, MP, and MrBayes for key clades. In all trees, the new avian AhR1 sequences formed a strongly supported monophyletic group with the previously identified chicken and tern AhR sequences (tern results not shown) and were part of a larger, strongly supported clade of tetrapod AhR1 sequences, which also included mammalian and amphibian AhRs (see "tetrapod AhR1" in Table 2). The relationship between fish and tetrapod AhR1 forms was not resolved by Bayesian analysis, although in the ME tree the vertebrate AhR1 clade was strongly supported (Table 2). Further analyses involving additional fish AhR1 sequences will be needed to resolve this relationship.

FIG. 1. Nucleotide sequences for black-footed albatross and common cormorant AhR1 cDNAs. The nucleotides are numbered on the right side. The identical nucleotides in albatross and cormorant AhR1s are shown as periods (.), and lack of nucleotide in either sequence is shown as a hyphen (-). Capital letters indicate the sequence of the ORF, and italic indicates the start and stop codon. The nucleotide sequences have been deposited in the DDBJ/EMBL/GenBank database under accession number AB106109 (albatross AhR1) and AB109545 (cormorant AhR1).
Because AhR2 genes were identified initially in fishes but have not been found in mammals (Hahn et al., 2006), we were especially interested in the relationship of the putative avian AhR2 isoforms to the previously identified AhR2 proteins from bony fishes, as well as to the AhR1 and AhRR clades within the AhR gene family. A vertebrate (bird + bony fish) AhR2 clade that is distinct from the AhR1 and AhRR clades was strongly supported in all analyses, with greatest support from MrBayes (100% posterior probability; Table 2). Together, these results show that avian AhR type 2 isoforms are orthologous to mammalian AhR (and possibly fish AhR1s) and that avian AhR type 2 isoforms are orthologous to fish AhR2s. The results of these phylogenetic analyses support our designation of the two putative AhRs as AhR1 and AhR2. Our results (Yasui et al., 2004 and the present study) provide the first complete cDNA sequences of AhR2s from vertebrate species other than fish.

Comparison of Amino Acid Sequences of Avian AhR1 and AhR2

Additional sequence analysis was performed using the aligned amino acid sequences (Fig. 4). The alignment of AhR amino acid sequences indicated that all AhR1 and AhR2 isoforms of albatross and cormorant contained bHLH and PAS domains, which are known to be essential for dimerization with Arnt, and for ligand and DNA binding (Fig. 4).

Cormorant AhR1 shared the greatest amino acid sequence identity with albatross AhR1 (96%) and chicken AhR (92%) (Table 3). Cormorant AhR2 showed a high amino acid identity with albatross AhR2 (77%) but exhibited only 32% identity with cormorant AhR1. The bHLH and PAS domains were highly conserved in albatross and cormorant AhR1s, sharing 100% amino acid identity with the chicken AhR in the bHLH domain, and 98–99% identity in the PAS domain. Albatross and cormorant AhR2 showed 78% and 76% identity with killifish AhR2 in the bHLH domain, and 63% and 64% in the PAS domain, respectively.

There were several isoform-specific amino acid sequences in the main functional domains or critical sites of avian AhR2. Some amino acids of avian AhR2 in regions containing nuclear localization/export signal motifs (Ikuta et al., 1998) and a tetratricopeptide repeat (TPR) site that is required for the assembly of XAP2 with hsp90 and AhR (Levine et al., 2000) were different from those of AhR1s (e.g., human AhR-bird AhR2; R16-K8, A78-V71). Site-directed mutagenesis of A78D at mouse AhR completely inhibited TCDD-dependent activation of a XRE-driven gene expression construct in transfected COS-1 and BP8 cells. In vitro DNA binding analysis demonstrated that loss of transactivation potential by the A78D mutation at TPR site resulted in impaired XRE binding (Fig. 4).

Because AhR2 genes were identified initially in fishes but have not been found in mammals (Hahn et al., 2006), we were especially interested in the relationship of the putative avian AhR2 isoforms to the previously identified AhR2 proteins from bony fishes, as well as to the AhR1 and AhRR clades within
TABLE 2
Phylogenetic Analyses of Vertebrate AhR Sequences: Comparison of Results for Selected Clades

<table>
<thead>
<tr>
<th>Clade</th>
<th>Optimality criterion or algorithm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bird AhR1</td>
<td>Bayes 100 MP 100 ME 100</td>
</tr>
<tr>
<td>Mammal AhR1</td>
<td>Bayes 100 MP 99 ME 100</td>
</tr>
<tr>
<td>Tetrapod AhR1</td>
<td>Bayes 100 MP 98 ME 100</td>
</tr>
<tr>
<td>Fish AhR1</td>
<td>Bayes 100 MP 100 ME 100</td>
</tr>
<tr>
<td>Vertebrate AhR1</td>
<td>Bayes &lt; 5 MP 41 ME 100</td>
</tr>
<tr>
<td>Bird AhR2</td>
<td>Bayes 100 MP 100 ME 100</td>
</tr>
<tr>
<td>Fish AhR2</td>
<td>Bayes 99 MP 58 ME 59</td>
</tr>
<tr>
<td>Vertebrate AhR2</td>
<td>Bayes 100 MP 84 ME 81</td>
</tr>
<tr>
<td>Vertebrate AhRR</td>
<td>Bayes 100 MP 100 ME 100</td>
</tr>
<tr>
<td>Vertebrate AhR + AhRR</td>
<td>Bayes 100 MP 96 ME 100</td>
</tr>
</tbody>
</table>

Note. Phylogenetic analyses were performed as described in “Materials and Methods.” Support for selected clades is indicated. For Bayesian analysis, numbers indicate posterior probabilities. For MP, and ME, numbers represent bootstrap values based on 100 resamplings of the data.

In vitro Translation of Avian AhRs and their Specific Binding of [3H]TCDD

Black-footed albatross and cormorant AhR proteins were synthesized by an in vitro transcription/translation system to

224–228 amino acids) that in the human AhR might be important for interaction with coactivators during transactivation (Ikuta et al., 2002), was not conserved in avian AhR2s (FRCLL, 221–225). However, a Leu to Ala mutation at a corresponding site in human AhR indicated that this motif affects neither the subcellular localization nor transcriptional activity (Ikuta et al., 2002). Considering that the killifish AhR2, which is capable of activating transcription, contains the same amino acid as bird AhR2s in these sites (FRCLL, 231–235), amino acid sequences at these sites may be AhR2 isoform-specific and avian AhR2 may also be functional. An “LxCxE” motif, which has been suggested to be important for interaction of the mammalian AhR with the retinoblastoma protein (Ge and Elferink, 1998), is conserved in albatross and cormorant AhR1s, whereas the sequence of avian AhR2s at this location is “MYCAE” (Fig. 4).

It has been reported that the differential dioxin sensitivity between chicken and tern is mainly due to the difference in two amino acids (chicken AhR: Ile324 and Ser380, tern AhR: Val325 and Ala381) in the ligand-binding domain (LBD) of their AhRs (Karchner et al., 2006). The amino acid in the latter position corresponds to the amino acid which is responsible for the altered ligand binding affinity of AhRs in two mouse strains (Ala375 in C57BL/6 and to Val375 in DBA/2 mouse) (Ema et al., 1994). While the corresponding amino acid residues in cormorant AhR1, AhR2 and albatross AhR2 were the same type as in the tern AhR (Val325 and Ala381), albatross AhR1 had residues (Ile325 and Ala381) that were intermediate between those of the chicken and tern AhRs (Fig. 4). The three-dimensional model of the AhR showed that the Ile332 in mouse AhR (Ile325 in avian AhR) is expected to have a structural role as part of the long central helix (helical connector) that forms part of the binding pocket (Pandini et al., 2007).

Other key amino acids, such as Cys327, Thr343, Phe345, and Leu347, which might be related to the size of ligand-binding pocket and the interaction with TCDD, were indicated in a three-dimensional model of the mouse AhR (Procopio et al., 2002). In avian AhR1 and AhR2, amino acids with properties similar to those of amino acids in the mouse AhR were found in these positions.

It has been suggested that the glutamine (Q)–rich region in the transcriptional activation domains (TAD) of mammalian AhRs (Fukunaga et al., 1995) and fish AhR1s (Karchner et al., 1999), may be involved in their transactivation function. Avian AhR1s possess a similar domain in the TAD, while there is no prominent Q-rich region in the TAD of avian AhR2s as well as fish AhR2s. Nevertheless, fish AhR2 can apparently activate the transcription of target genes (Andreasen et al., 2002; Karchner et al., 2002).

Tissue Expression Profiles of AhR1 and AhR2

To investigate the tissue expression profiles, AhR1 and AhR2 mRNA levels were determined in various tissues and organs of cormorants by one-step real-time RT-PCR. Levels of AhR1 mRNA expression were similar in most tissues except muscle and pancreas, in which mRNA levels were low. On the other hand, AhR2 mRNA was mainly expressed in the liver, and was detectable in gonad, brain, and intestine (Fig. 5A), suggesting that each AhR isoform may play a distinct role. In addition, mRNA expression levels of two distinct CYP1A isoforms corresponding to chicken CYP1A4 and 1A5 were also determined in the same tissues as those in which AhR mRNAs were measured (Kubota et al., 2006). The CYP1A4 mRNA expression levels were the highest in the liver, followed by kidney, heart, and muscle (Fig. 5A). The tissue distribution profile of CYP1A5 showed a similar tendency in liver and kidney, but was not detectable in most other tissues (Fig. 5A).

In order to understand the quantitative relationship of AhR1 and AhR2 mRNA expression levels, the absolute contents of both AhR transcripts were determined using two-step real-time RT-PCR in 21 liver samples of cormorants that previously had been examined for TEQ and CYP1A protein levels (Kubota et al., 2005). There was no significant difference (p > 0.05) in AhR mRNA levels between young and adult (data not shown). A significant sex difference (p < 0.05) was only obtained for AhR2 mRNA levels (data not shown), but not for AhR1. AhR1 mRNA content accounted for 80 ± 12% of total AhR (AhR1 + AhR2) (Fig. 5B), and showed no significant correlation with AhR2 (data not shown), implying that avian AhR1 and AhR2 may be regulated by different transcriptional mechanisms. Neither AhR1 nor AhR2 mRNA levels were correlated with levels of CYP1A protein or hepatic TEQs (data not shown).
determine whether these AhR cDNAs actually have potential to encode functional proteins. Gel electrophoresis of the $[^{35}S]$ methionine-labeled translation products of albatross and cormorant AhR1 cDNAs revealed single bands similar in size to chicken AhR (Fig. 6A). The avian AhR2 bands were slightly larger than those of avian AhR1s, consistent with their predicted sizes from their cDNA sequences (e.g., 101 kDa for albatross AhR2 vs. 97 kDa for albatross AhR1).

To examine the ligand-binding ability of albatross and cormorant AhRs, the in vitro–translated AhRs were incubated...
with [3H]TCDD and separated by velocity sedimentation on sucrose density gradients. Compared to the nonspecific binding detected using in vitro translation products of an empty vector, a peak of [3H]TCDD specific binding was observed for each AhR (Fig. 6). These results demonstrate that AhRs of these species encode proteins that are capable of specific binding with TCDD, as seen for the chicken AhR (Karchner et al., 2006). The predicted sedimentation coefficients of albatross AhR1, cormorant AhR1, and chicken AhR were 10.4, 10.5, and 10.0S, respectively. These avian AhR1 sedimentation coefficients were similar to those previously published for fish AhRs (Karchner et al., 1999). Both albatross and cormorant AhR2s also exhibited detectable peaks of [3H]TCDD-specific binding, in fractions eluting from the gradient slightly earlier than those seen for AhR1s; sedimentation coefficients were 9.1S and 8.6S for albatross AhR2 and cormorant AhR2, respectively (Fig. 6). Thus, both avian AhR forms, including the novel AhR2, are capable of specific binding of TCDD.

Luciferase Reporter Assay of Avian AhRs

To examine the transactivation abilities of these avian AhRs, a luciferase reporter gene vector was constructed using cormorant CYP1A5 promoter region containing six XREs. Each AhR isoform together with cormorant Arnt and the reporter vector was transiently expressed in COS-7 cells. Compared to the relative luciferase activity in “no AhR” transfected cells treated with 14nM TCDD, AhR1-transfected cells exposed to TCDD exhibited reporter gene activity that was 28-fold (albatross) or 11-fold (cormorant) greater (Fig. 7), while luciferase activity of cells transfected with chicken AhR and exposed to TCDD were 48-fold greater than in the TCDD-exposed, no-AhR controls. As compared to the luciferase activities of DMSO-treated cells for each AhR1, TCDD caused a 4.9-, 17.5-, and 41-fold induction for cormorant, albatross, and chicken, respectively.

In cells transfected with cormorant and albatross AhR2s, the luciferase activities after TCDD exposure were 1.5- and 3.6-fold greater than that of TCDD-exposed, no-AhR cells, respectively (Fig. 7). For cormorant and albatross AhR2s, the TCDD-induced luciferase activity was 5.1- and 8.0-fold greater than that of the respective DMSO controls. Together, these results show that both AhR1 and AhR2 are capable of supporting TCDD-inducible transactivation of reporter gene expression; however, the avian AhR2 forms appear to be less efficacious than the AhR1 forms.

DISCUSSION

Evolutionary History of Avian AhRs

The identification of both AhR1 and AhR2 forms in birds and their comparison with AhRs in fishes and mammals illuminates

### Table 3

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Note. (B) The boundary between the N- and C-terminal region is residue 403, 404, 404, 404, 399, 397, 397, 396, and 409 for chicken AhR, albatross AhR1, cormorant AhR1, human AhR, killifish AhR1, albatross AhR2, cormorant AhR2, Killifish AhR2, and zebrasfish AhR2, respectively (the end of LBD). N-terminal comparisons at lower left; C-terminal comparisons at upper right.
the evolutionary history of AhR genes in vertebrates. Fish, including zebrafish, have multiple AhR genes including both AhR1 and AhR2 forms. Initially, zebrafish AhR1 was reported as nonfunctional for ligand binding and transactivation of a dioxin-responsive reporter gene (Andreasen et al., 2002). Recently, a new functional AhR1 isoform was identified in zebrafish (Karchner et al., 2005). This novel AhR1, named AhR1b, is located adjacent to the AhR2 gene on chromosome 22 (zf-chr22), whereas the AhR1a, which was previously identified, is located on chromosome 16 (zf-chr16) (Karchner et al., 2005). This suggests that AhR1 and AhR2 derive from a tandem duplication on the ancestral chromosome, and that the duplicated AhR1 originates from the subsequent fish whole-genome duplication after the divergence of fish and tetrapod lineages (Karchner et al., 2005).

From the chicken genomic database (cited in National Center for Biotechnology Information [NCBI] Map Viewer: http://www.ncbi.nlm.nih.gov/mapview/) and cDNA sequences of cormorant and albatross AhR2s, a predicted AhR2-like sequence was identified in chicken (Fig. 8; see also Hahn et al., 2006). The chicken AhR2-like gene is located on chromosome 7 (chick-chr7), while the originally identified AhR (AhR1) is on chromosome 2 (chick-chr2).

To understand the evolution of avian AhRs, we compared gene syntenies on chick-chr2 and -chr7 with those on the human and zebrafish orthologous chromosomal segments, using Ensemble Genome Browser (http://www.ensembl.org/index.html) and NCBI Map Viewer. In this analysis, we disregarded genes that are not yet annotated. Chick-chr2 displays extensive shared synteny with human chromosome 7.
(hu-chr7) and zf-chr16 (Fig. 8A). In addition to AhR, two other orthologous genes (pon2 and tbx20) were found in common on all three chromosomes. In contrast, chick-chr2 shares no orthologous genes other than AhR1 with zf-chr22. These results suggest that chicken AhR1, zebrafish AhR1A, and human AhR are orthologous genes.

To determine whether chick-chr7 (where the chicken AhR2-like gene is located) shares conserved synteny with chromosomes containing chicken AhR or zebrafish AhR2 (chick-chr2 and zf-chr22), we searched for orthologous or paralogous genes located on these chromosomes. Adjacent to the AhR2-like gene on chick-chr7, we found a novel, predicted bHLH/
et al. appears to be a second AhR1-like gene (Fig. 8B; see also Hahn PAS gene that based on its inferred amino acid sequence the result of comparative analysis of the chicken genome revealed extensive shared syntenic (Fig. 8C), consistent with (Fig. 8B). In addition, comparison of chick-chr7 with hu-chr2 (14nM). The luciferase activity was quantified as the firefly luciferase activity relative to the transfection control Renilla luciferase. The results are expressed as the median value ± SEM, n = 3.

PAS gene that based on its inferred amino acid sequence appears to be a second AhR1-like gene (Fig. 8B; see also Hahn et al., 2006). This supports the idea that avian AhR1 and AhR2 arose from a tandem gene duplication, as did the fish AhR1 and AhR2. The comparison of chromosomal genes showed that only a few homologous genes are shared between chick-chr7 and zf-chr22, whereas large segments of homologous (paralogous) genes are shared between chick-chr7 and chick-chr2 (Fig. 8B). In addition, comparison of chick-chr7 with hu-chr2 revealed extensive shared synteny (Fig. 8C), consistent with the result of comparative analysis of the chicken genome (Hillier et al., 2004).

The synteny comparisons in Figure 8 suggest that the AhR-containing segments of chick-chr2 and human-chr7 are derived from the same chromosome in the most recent common ancestor of mammals and birds, that segments of chick-chr7 and human-chr2 are derived from the same ancestral chromosome, and that parts of chick-chr2 and chick-chr7 originated by duplication of a chromosome or chromosomal segment. To determine the timing of the latter duplication, we looked for evidence of interchromosomal segmental duplication between the regions of human-chr7 and human-chr2. As shown by McLysaght et al. (2002; see also http://wolfe.gen.tcd.ie.dup/), human-chr7 and -chr2 contain paralogous segments (paralogons), including in the region that contains the AhR. This shared synteny between human-chr7 and human-chr2 suggests that the duplication event that led to the two AhR1-like genes in chickens preceded the divergence of birds and mammals. This duplication might have occurred in the tetrapod lineage after its divergence from the fish lineage, as illustrated in Figure 9. Alternatively, it might have occurred prior to the fish/tetrapod divergence, for example in one of the two whole-genome duplications that are thought to have occurred early in vertebrate evolution (McLysaght et al., 2002). More detailed phylogenetic and genomic analyses involving additional species will be needed to distinguish between these possibilities.

Regardless of the precise timing of the duplications that led to multiple AhR genes in birds, the human–chicken comparisons described above suggest that a bird/mammal ancestor may have possessed four AhR genes, and that the AhR2 gene located on a chromosome ancestral to chick-chr2 and hu-chr7 was lost prior to the divergence of the bird and mammalian lineages. Although chick-chr7 exhibits shared synteny with hu-chr2 (Fig. 8C), no AhR-like genes have been found in the corresponding region of hu-chr2, suggesting the loss of both AhR genes after the divergence of the bird and mammalian lineages (Fig. 9). Considering that avian AhR2 appears to have reduced ligand binding and transactivation function as compared to fish AhR2, there may have been reduced selective pressure to retain a mammalian AhR2.

Interspecies and Isoform Comparisons of Avian AhR Function

The [3H]TCDD-binding assays demonstrated that both AhR isoforms in albatross and cormorant exhibit specific binding of TCDD (Fig. 6). Considering the two amino acid residues responsible for the differential binding affinity between chicken AhR (Ile324 and Ser380) and tern AhR (Val325 and Ala381) (Karchner et al., 2006), the binding affinity of avian AhR1 was predicted to follow the order: chicken AhR (I-S type) > albatross AhR1 (I-A) > cormorant AhR1 (V-A). In a preliminary comparison using the relative peak areas of specific binding to [3H]TCDD, the chicken AhR-binding area (1.0) was greater than those of albatross AhR1 (0.61) and cormorant AhR1 (0.14), when the specific binding of the same concentration of [3H]TCDD to chicken AhR is regarded as 1.0 (Fig. 6). The differences in relative peak areas suggest that the relative binding affinities or capacities (or a combination) for these AhRs is the same as the order predicted from the identity of amino acid residues. Saturation binding analysis will be needed to confirm these initial estimates of relative binding properties.

The ability of these AhRs to transactivate the luciferase reporter gene in the presence of TCDD mirrored the results of the TCDD-binding assays (Fig. 7). Overall, our observations of TCDD binding and transactivation properties are consistent with the predictions made using the results of the site-directed mutagenesis of the tern AhR (Karchner et al., 2006).

Like the AhR1 isoforms, both albatross AhR2 and cormorant AhR2 exhibited specific binding to [3H]TCDD (Fig. 6). Although the predicted molecular mass of AhR2 is higher than that of AhR1, the smaller AhR1 proteins sediment more rapidly than the AhR2 proteins (Fig. 6C). This implies either that AhR1 and AhR2 are bound to different chaperone proteins in reticulocyte lysate or that the AhR1 complex has a more spherical shape than the AhR2 complex.
FIG. 8. Mapping of chicken AhR1 (A) and AhR2-like (B and C) genes on chicken chromosome 2 (chick-chr2) and chromosome 7 (chick-chr7), respectively, and comparison of the gene syntenies with those on human and zebrafish chromosomes. The locations of genes displayed were identified from the NCBI map.
Compared to “no AhR”-transfected cells treated with TCDD, avian AhR2-transfected cells exposed to TCDD had only slightly increased luciferase expression: 3.7-fold for albatross and 1.5-fold for cormorant (Fig. 7). However, for cormorant and albatross AhR2s, the TCDD-induced luciferase activity was five- to eightfold greater than that of the respective DMSO controls. These results suggest that the albatross and cormorant AhR2 are responsive to TCDD, but may be less efficacious at activating target gene transcription than the AhR1s. Alternatively, avian AhR2 isoforms may be expressed at a lower level in COS-7 cells, or they may regulate the transcription of a distinct set of target genes as compared to AhR1s. One possible cause of the difference in transactivation may be the structural difference in transactivation regions between AhR1 and AhR2. Amino acid sequences in the C-terminal halves of AhR1 and AhR2 are highly divergent (11% amino acid identity in each species) suggesting that structural and functional differences between avian AhR1 and AhR2 in C-terminal regions may be involved in the differences in transactivation efficacy.

Some studies on chicken reported that CYP1A4/1A5 was detected in liver and kidney after TCDD-treatment, and only CYP1A4 was expressed in heart and the vascular endothelium (Mahajan and Rifkind, 1999). In the present study, cormorant CYP1A4/1A5 mRNAs showed tissue distribution profiles similar to those of chicken CYP1As (Fig. 5A). This suggests that liver, kidney, and heart may be target tissues for dioxin toxicity mediated by the AhR signaling pathway and/or

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**FIG. 9.** Hypothesis for duplication and loss of AhR genes in vertebrate evolutionary history. In an early vertebrate ancestor, AhR1 and AhR2 arose from a tandem gene duplication event. Following the divergence of fish and tetrapods, independent genome, chromosome, or chromosome segment duplications occurred in the two lineages. These events led to four copies of AhR genes in ancestral species of bird and mammal. One AhR gene (an AhR2) was lost prior to the divergence of bird and mammalian lineages. Two additional AhR genes (an AhR1 and an AhR2) were subsequently lost in the mammalian lineage. Note: The pattern shown for bony fish is that of zebrafish. Other fish species (e.g., *Takifugu rubripes*, *Oryzias latipes*, and *Tetraodon nigroviridis*) have retained both pairs of AhR genes (Hahn et al., 2006). For additional discussion and alternative hypotheses, see text.
CYP1A in avian species, and at least in kidney and heart, AhR1 may be responsible for CYP1A expression. In addition, the comparison of hepatic AhR expression levels revealed that the majority (80 ± 12%) of hepatic AhR transcripts were those of AhR1 (Fig. 5B).

Considering the function and expression levels of avian AhR1 and AhR2, our results indicate that AhR1 may be the form primarily responsible for CYP1A induction in avian species. The relative importance of avian AhR1 with regard to TCDD exposure implies that differences in sensitivity to TCDD among avian species may depend primarily on the function of AhR1. Based on that assumption, of the species examined here chicken is predicted to be the most sensitive, albatross intermediate in sensitivity, and cormorant the most resistant. Additional data, such as the relative expression of AhR1 and AhR2 protein in avian tissues, will be required for a more definitive statement about the relative roles of these two AhR paralogs.

In contrast to the postulated primary role of AhR1 in birds, AhR2 has been reported to be the predominant AhR isoform mediating the response to TCDD exposure in fish (Abnet et al., 1999; Andreasen et al., 2002; Karchner et al., 1999; Prasch et al., 2003; Yamauchi et al., in press). These reports imply that fish AhR2 can mediate CYP1A induction and other effects. In mammals, only one AhR isoform, an AhR1, has been identified (reviewed in Hahn et al., 2006). Together with these results regarding AhRs from a variety of taxa, the present study suggests that in fish and birds, different AhR isoforms play the predominant role in the response to TCDD. In view of the functional weakness and limited expression of avian AhR2 in comparison with fish AhR2, we hypothesize that the contribution of vertebrate AhR2 to overall functions declined during the evolution from fish to avian species, and mammalian species would be able to survive even though the AhR2 gene has been lost. Therefore, avian species may be an important model organism to bridge the evolutionary gap between mammal and fish AhRs, and to understand the molecular mechanisms underlying the functional transition of AhR isoforms during vertebrate evolution.

**FUNDING**

Grants-in-Aid for Scientific Research (A) (No. 17208030 and 16201014), (B) (No. 13480170), and (C) (No. 18510059); Grant-in-Aid for Exploratory Research from the Japan Society for the Promotion of Science (No. 17651030); Grant-in-Aid for Scientific Research on Priority Areas (A) (No. 13027101); “21st Century COE Program” from the Ministry of Education, Culture, Sports, Science and Technology, Japan; Hayashi Memorial Foundation for Female Natural Scientists and Feasibility Studies for Basic Research in ExTEND2005 (Enhanced Tack on Endocrine Disruption) from the Ministry of the Environment, Japan; NOAA National Sea Grant College Program, Department of Commerce grants (NA46RG0470, NA16RG2273); U.S. National Institutes of Health grant (R01ES006272) to M.E.H.; and Doctoral Fellowship for Researcher from the Japan Society for the Promotion of Science to T.Y. (no. 08490).

**ACKNOWLEDGMENTS**

The authors would like to thank Prof. Chung Kyu-Hyuck, Sunkyunkwan University, Korea, for providing COS-7 cell and Tomoko Suda, Ehime University, Japan, for technical assistance of in vitro reporter gene assay. We also thank Dr Jared Goldstone, Woods Hole Oceanographic Institution, for advice on the Bayesian analyses.

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