MOLECULAR MECHANISM OF AVIAN AHR1-CYP1A5 SIGNALING PATHWAY: TCDD RESPONSES OF 5' FLANKING REGIONS OF CYP1A5 GENES FROM CHICKEN (*Gallus gallus*) AND CORMORANT (*Phalacrocorax carbo*)

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Abstract
To clarify the molecular mechanism of avian aryl hydrocarbon receptor (AHR)-cytochrome P4501A (CYP1A) signaling pathway, we cloned the 5' flanking regions of cormorant (*Phalacrocorax carbo*) CYP1A5 (*cc*CYP1A5) and chicken (*Gallus gallus*) CYP1A5 (*ck*CYP1A5) genes. Using the 5' flanking regions of CYP1A5 genes, we constructed an *in vitro* luciferase reporter gene assay system, where cormorant or chicken AHR1 was transiently expressed in COS-7 cells, and measured the luciferase activity in the cells treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Seven putative xenobiotic response elements (XREs) were identified within the 2.7kb upstream region of *cc*CYP1A5, and six XREs were within the 0.9kb of *ck*CYP1A5. Sequential deletion analyses of 5' flanking region of *cc*CYP1A5 gene by the reporter gene assay identified two major active regions: proximal 717bp from putative transcriptional start site and at a more distal area from -2127 to -1633bp. Each active region contained one XRE. Interestingly, the addition of exon 1 and intron 1 resulted in a loss of transactivation by TCDD, implying the presence of novel negative regulatory elements in this region. As for *ck*CYP1A5 gene, two functional XREs were found at -387 to -383bp and -262 to -258bp. EC_{50} value for the transcriptional activation by TCDD in cormorant AHR-CYP1A5 reporter construct was 10 fold higher than that in chicken AHR-CYP1A5 reporter construct, indicating that cormorant is more resistant to TCDD than chicken. Thus, the avian CYP1A5 gene is induced by TCDD via a conserved AHR signaling pathway, but there is a marked interspecies difference in sensitivity of CYP1A induction.

Introduction
CYP1 family has been the focus of intense scrutiny in view of toxicology, because this enzyme responds to dioxin-like compounds (DLCs) that are of concern for environmental health. It is known that the expressions of mammalian CYP1A1 and CYP1A2 are enhanced at the transcriptional level by the exposure to DLCs.[1] In addition, several studies have shown that expressions of chicken CYP1A4 and CYP1A5 genes, which may be the orthologues of mammalian CYP1A1 and CYP1A2 genes, respectively, are also inducible by the treatment of these compounds.[2] As for the transcriptional activation of mammalian CYP1As, ligands bind to aryl hydrocarbon receptor (AHR) in the cytosol, triggering its translocation to the nucleus.[3] In the nucleus, ligand-activated AHR dimerizes with aryl hydrocarbon receptor nuclear translocator (ARNT) and interacts with conserved xenobiotic response elements (XREs: 5'-GCGTG-3'), located in the 5'-flanking region of the CYP1As to activate the transcription. On the other hand, the novel induction mechanism was also suggested in rat CYP1A2 which does not contain consensus XREs in the regulatory region.[4]

Our previous studies have clarified that wild common cormorants (*Phalacrocorax carbo*) from Lake Biwa in Japan accumulate high levels of DLCs (360–50,000 pg/g lipid wt as TCDD toxic equivalents; TEQs) in the liver and there are the significant positive correlations between hepatic total TEQs and CYP1A4 or CYP1A5 mRNA expression levels, suggesting induction of both CYP1A isozymes by DLCs.[5] Recently, cDNAs of multiple AHRs (AHR1 and AHR2) and ARNTs (ARNT1, ARNT2 and their splice variants) from the cormorant have been isolated and characterized.[6, 7] These results imply that AHR/ARNT signaling pathway may be conserved in cormorant as well as mammalian model species, and the induction of cormorant CYP1A genes is a biomarker for assessing exposure to DLCs. However, there is little information on the molecular mechanism of CYP1A induction by DLCs in avian species. In addition, species difference in sensitivity is a major source of uncertainty in assessing the effects of DLCs in wildlife. A recent study reported that the structural properties of AHR contributes to the sensitivity in avian species.[6,8] However, it remains unclear whether the structural properties of 5' flanking region of the target genes contribute to the dioxin susceptibility.

To clarify the molecular mechanism of AHR-CYP1A signaling pathway in avian species, the present study initially cloned the promoter/enhancer region of cormorant and chicken CYP1A5 (*cc*CYP1A5 and *ck*CYP1A5, respectively) genes. Using these 5' flanking regions of CYP1A5 genes, we constructed an *in vitro* luciferase reporter gene assay system, where cormorant or chicken AHR1 was transiently expressed in COS-7 cells, and measured the luciferase activity in the cells treated with TCDD. Furthermore, the transactivation response to TCDD in cormorant AHR-CYP1A5 reporter construct was compared with that in chicken reporter construct.

Materials and Methods

The GenomeWalker kit (Clontech) was used for the isolation of the 5'-flanking region of the avian CYP1A5 genes. Briefly, the cormorant and chicken genomic DNAs were isolated from the liver samples using Wizard SV Genomic DNA Purification System (Promega) and digested individually with five different restriction enzymes, EcoR V, Dra I, Pvu II, Stu I and Hinc II. Digested DNA fragments were then adapter-ligated to produce five sets of DNA fragments with adapters at their ends. Each set of DNA fragment was amplified using an adapter-specific 5'-primer and a CYP1A5 gene-specific 3'-primer (5'-GCAGGGACTGGATGAGCAGGAAGACCA-3' for cormorant; 5'-AGCCGGGTGAGCACCAGGTGTGGGTCCTT -3' for chicken). The second PCR reaction was done using a nested adaptor primer and a nested gene specific primer (5'-GTGAGGAGGACCTCGGTGGCTGAAACA-3' for cormorant; 5'-CTGCACCATCACTTCCTCCGGCCCCATT -3' for chicken). The sequences of gene specific primers for cormorant were designed based on the sequence of the *cc*CYP1A5 cDNA previously reported[5] and those for chicken were based on the sequence of the *ck*CYP1A5 cDNA deposited in DDBJ database. *In silico* analysis for searching putative transcription factor binding sites in the upstream region of *cc*CYP1A5 and *ck*CYP1A5 was performed with Genomatix (www.genomatix.de) and TFSEARCH. Afircan green monkey kidney fibroblast cell (COS-7) was employed for transfection experiments. For the construction of cormorant ARNT1 expression vector (pcDNA-*cc*ARNT1), the pME18SFL3-*cc*ARNT1[7] was digested with EcoR I and Not I, and the fragment was ligated into pcDNA3.1/Zeo(+) (Invitrogen). Regarding constructs containing the cormorant AHR1 (pcDNA-*cc*AHR1) and chicken AHR1 (pcDNA-*ck*AHR1), the details have already been reported elsewhere.[6] Sequential deletion of the 5'-flanking region was accomplished by cloning of PCR fragments (0XRE; +3bp to -595bp, 1XRE; +3 to -717, 2XREs; +3 to -1023, 4XREs; +3 to -1632, 5XREs; +3 to -2127, 6XREs; +3 to -2308, 7XREs; +3 to -2683, 7XREs+e1+i1; +814 to -2683, -pi1; +240 to -2683 for cormorant, and 0XRE; +1 to -140, 1XRE; +1 to -249, 2XREs; +1 to -270, 3XREs; +1 to -657, 4XREs; +1 to -693, 5XREs; +1 to -738, 6XREs; +1 to -866 for chicken). The PCR products were initially cloned into a pGEM-T easy vector, and then excised by KpnI/XhoI for cormorant and Nhe I and Bgl I for chicken. The excised fragments were subcloned into the same restriction enzyme sites of the firefly luciferase reporter vector pGL4-Basic (Promega). Transient co-transfection was performed using the Lipofectamine 2000 (Invitrogene). Briefly, 60,000 cells were seeded onto 24-well plates prior to transfection. Five ng of AHR expression vector (pcDNA-*cc*AHR1 or pcDNA-*ck*AHR1), 50 ng of pcDNA-*cc*ARNT1, 20 ng of the respective pGL4-constructs and 3 ng of renilla luciferase control vector (Promega) were diluted into OPTI-MEM medium (Invitrogen Life Technologies) and mixed with Lipofectamine 2000. Cells were washed with OPTI-MEM and then incubated in a total 0.5 ml of transfection medium. Following 5h incubation, cells were washed with DMEM. Cells treated with dimethyl sulfoxide (DMSO) or TCDD dissolved in DMSO were incubated for an additional 18h. The Dual Luciferase Assay kit (Promega) was used to determine firefly (AhR agonist-dependent) and renilla (transfection control) luciferase activities in each well. Ligand-dependent transcriptional activity was determined by the relative firefly luciferase activity to renilla luciferase activity. The 50% effect concentration (EC_{50}) of TCDD was calculated using SigmaPlot, version 9.0.

Results and Discussion

Approximately 2.7kb of 5' flanking region of *cc*CYP1A5 gene was isolated and sequenced. The transcription start site (TSS) was determined by comparison of nucleotide sequences between 5' untranslated region reported by Kubota *et al*. (2006)[5] and genomic DNA of *cc*CYP1A5 isolated in this study. Exon 1/intron 1 junctions abide by the GT/AG splice donor/acceptor rule. Sequence analysis of the 2.7kb revealed the presence of potential DNA elements including Sp1, AP-1, Oct-1, GATAs, CdxA, HNF-1, HNF-4 and seven XREs; these XREs were assigned with "XRE1" to "XRE7" (Figure 1A). For *ck*CYP1A5 gene, we cloned 1.3kb including the 5' flanking region and the first exon (Figure 1B). The TSS of *ck*CYP1A5 was determined by 5'-RACE using a GeneRacer kit (Invitrogen). Multiple putative TSSs positioned at 254, 262 and 277 nucleotide upstream of the translational

initiation codon were found. According to the result from a previous report,[9] the most 3'-ward start site was designed as +1 of the *ck*CYP1A5 transcript. The upstream region of *ck*CYP1A5 gene contained a TATA box, Sp1, GATAs, CdxA, MZF1, Nkx-2 and six putative XREs ("XRE1" to "XRE6").

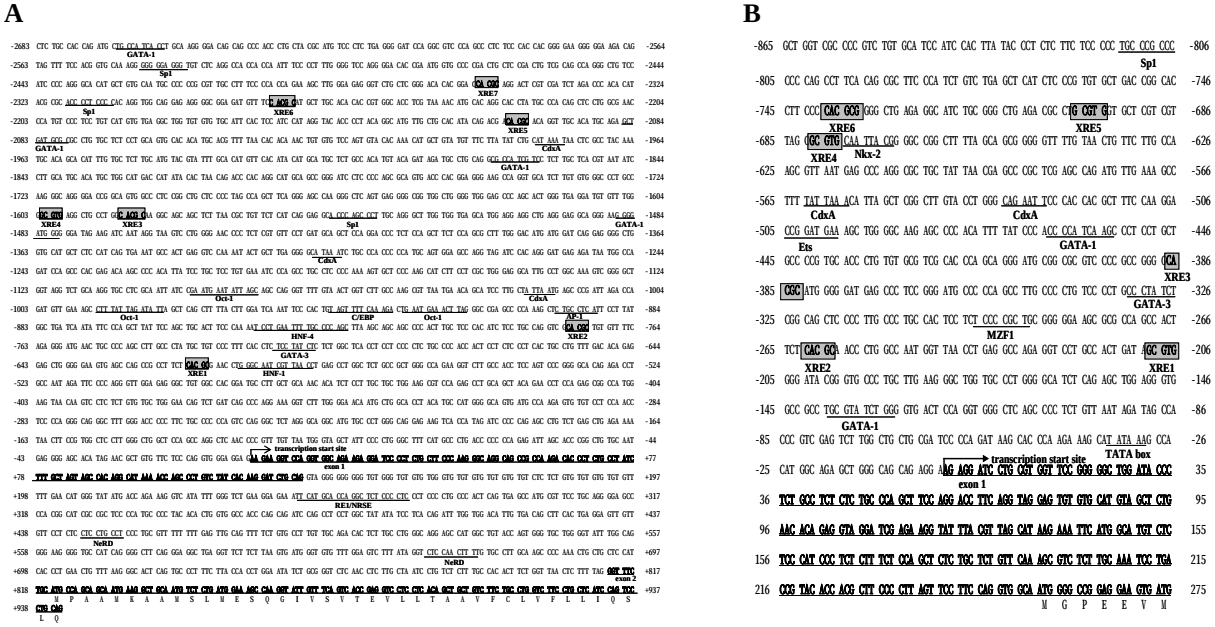

Figure 1. The nucleotide sequences of the upstream regions in cormorant (A) and chicken (B) CYP1A5 genes. Nucleotides representing the 5' flanking region from the putative transcription start site are numbered as (-). The putative XRE motifs are boxed. Other putative transcriptional factor binding sites are underlined. Nucleotides in exon are represented in bold type and underlined.

To identify the sequences responsible for the transactivation of the *cc*CYP1A5/*ck*CYP1A5 gene, a series of 5'-truncated CYP1A5 promoter/enhancer-luciferase reporter plasmids were constructed (Figure 2). As for *cc*CYP1A5 gene, the transactivation of 7XREs was significantly increased by the treatment of 14nM TCDD (Figure 2A), indicating that the promoter/enhancer is responsive to TCDD. Deletion of the sequence from -2127 to -1633, containing XRE5 or from -595 to -717, containing XRE1 markedly decreased the transcriptional activity in the presence of TCDD. With regard to *ck*CYP1A5 gene, the reporter vector which does not contain XRE2 or XRE3 showed a diminished activity by TCDD. This implies that the induction mechanism of avian CYP1A5 via a consensus XRE is conserved. Recent mounting evidences have shown that the downstream region of promoter, especially the untranslated sequences within intron, is critical for the transcriptional regulation of genes including dioxin-inducible genes.[10,11] The insertion of the sequence encoding exon 1 and intron 1 (7XREs+e1+i1) of *cc*CYP1A5 decreased the activity, implying the presence of negative regulatory elements in this region. The putative RE1/NRSF element[12] that is known to repress the transcription of CYP gene and NeRD element[13,14] were found in intron 1 of *cc*CYP1A5 gene (Figure 1A). To clarify the presence of negative regulatory elements in cormorant, the sequence from +240 to +813 containing the putative RE1/NRSE and NeRD elements was deleted, and the transactivation was measured (Figure 2A, -pi1). However, no reporter activity was recovered, implying the presence of unknown negative regulatory elements other than RE1/NRSE and NeRD in the region.

EC_{50} value for the transactivation by TCDD in cormorant AHR1-*cc*CYP1A5 reporter construct was 10 fold higher than that in chicken AHR-*ck*CYP1A5 reporter construct, indicating that cormorant is more resistant to TCDD than chicken (Figure 3). Thus, the avian CYP1A5 gene is induced by TCDD via a conserved AHR signaling pathway, but there is a marked interspecies difference in sensitivity of CYP1A induction. These results were consistent with our previous data showing the difference in AHR binding affinity with TCDD between cormorant and chicken.[6] On the other hand, the EC50 values for transactivation of cormorant AHR TCDD in *cc*AHR1 were not different regarding to the type of the reporter constructs; cormorant AHR-*cc*CYP1A5 vs

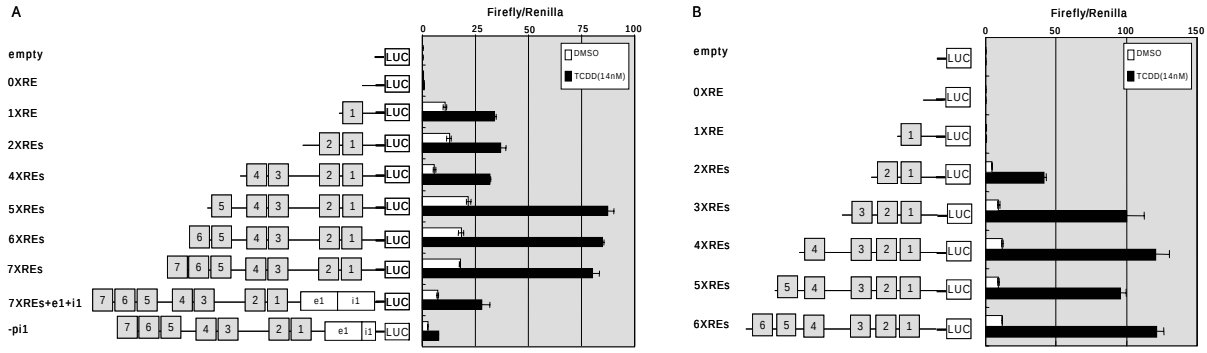

Figure 2. Deletion mapping and the transactivation of luciferase reporter gene construct of *cc*CYP1A5 (A) and *ck*CYP1A5 (B). Each reporter construct was transiently cotransfected with cormorant ARNT1 and cormorant AHR1 (A) or chicken AHR1 (B) into COS-7 cells, and then treated with 14nM TCDD.

cormorant AHR-*ck*CYP1A5. Similar results were obtained for chicken AHR. Therefore, it is likely that the structural difference of AHR is a major factor to the dioxin susceptibility and the regulatory region of CYP1A5 gene does not have a dramatic effect on the susceptibility between the two species.

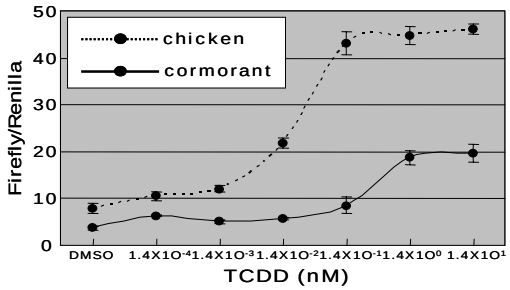

Figure 3. The comparison of transactivation by TCDD between cormorant AHR-*cc*CYP1A5 reporter construct and chicken AHR-*ck*CYP1A5 reporter construct.

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