Molecular Cloning and Expression of Two Novel Avian Cytochrome P450 1A Enzymes Induced by 2,3,7,8-Tetrachlorodibenzo-p-dioxin

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Transcriptional regulation by the aryl hydrocarbon receptor, for which the environmental toxin 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the most potent ligand, leads in mammalian liver to the induction of genes for two distinct cytochrome P450 (CYP)1A enzymes, CYP1A1 and -1A2. Fish seem to have only one CYP1A enzyme. CYP1A enzymes have been regarded as injurious largely because of their ability to activate chemical carcinogens. We report here the cloning and sequencing of cDNAs for two catalytically distinct TCDD-induced CYP enzymes in chick embryo liver. One mediates classic CYP1A1 activities. The other has some -1A2-like activities and is also responsible for TCDD-induced arachidonic acid epoxygenation, a much more conspicuous effect in liver of chicks than of mammalian species. Amino acid sequence analysis shows that although each chick enzyme can be classified in the CYP1A family, both are more like CYP1A1 than -1A2, and neither can be said to be directly orthologous to CYP1A1 or -1A2. Phylogenetic analysis shows that the two chick enzymes form a separate branch in the CYP1A family tree distinct from mammalian CYP1A1 and -1A2 and from fish CYP1A enzymes. The findings suggest that CYP1A progenitors split into two CYP enzymes with some parallel functions independently in two evolutionary lines, evidence for convergent evolution in the CYP1A family. Northern analysis shows that the chick enzymes have a different tissue distribution from CYP1A1 and -1A2. Polymerase chain reaction and in situ hybridization data show that both chick enzymes are expressed in response to TCDD even before organ morphogenesis. The findings further suggest that beyond their role in activating carcinogens, CYP1A enzymes have conferred evolutionary and developmental advantages, perhaps as defenses in maintaining homeostatic responses to toxic chemicals.

Activation of the aryl hydrocarbon (Ah) receptor by toxic polychlorinated aromatic hydrocarbons like TCDD leads to the transcriptional induction of genes in the CYP1 family and to a toxicity syndrome that includes immunologic, hormonal, and cardiac dysfunction and tumor promotion (1, 2). There are large unexplained species differences in sensitivity to TCDD toxicity that cannot be attributed to differences in Ah receptor concentration or binding affinity and seem to involve factors downstream of the Ah receptor (1, 2). Ligand activation of the Ah receptor in mammalian liver induces expression of CYP1A1 and -1A2 and the more recently discovered CYP1B1 (3). These enzymes exhibit differences in substrate specificity, regulation, and tissue distribution. Because single amino acid differences can change CYP catalytic activity (4), species differences in CYP1 structure and function may contribute to differences in sensitivity to TCDD toxicity.

The chicken and chick embryo close to hatching are among the more sensitive species to TCDD toxicity. They have long served as models for the study of Ah receptor-mediated toxicity and changes in heme and hemoprotein synthesis (2), but the chick TCDD-induced CYP enzymes have only been partially characterized. We have identified and purified two catalytically distinct TCDD-induced chick embryo liver CYP enzymes with nearly identical molecular weights (5). One, designated TCDDAAH, was selectively active in classic CYP1A1-mediated mixed function oxidations, aryl hydrocarbon hydroxylase (AHH) and 7-ethoxyresorufin deethylase. The other, TCDDAA, was active in epoxygenation of the endogenous fatty acid arachidonic acid (AA) and lacked AHH or 7-ethoxyresorufin deethylase activity. As TCDDAA was also preferentially active in some CYP1A2-selective activities, i.e. uroporphyrinogen decarboxylation (6) and estradiol-2-hydroxylation (7), and shared immunologic epitope(s) with rat CYP1A2 (5), it seemed that TCDDAA and TCDDAAH might be orthologous to mammalian CYP1A2 and -1A1, respectively.

MATERIALS AND METHODS

Treatment of Chick Embryos and Preparation of Poly(A)+ mRNA—White Leghorn chicken embryos (Burr Farm, Inc., Hampton, CT) were incubated in ovo at 37 °C at high humidity. They were treated at 16 days of development with 1 nmol of TCDD in 0.005 ml of dioxane or at 24 h of development with 0.5 nmol of TCDD in 0.005 ml of dioxane. Controls received solvent alone. Chemicals were injected through a hole in the shell into the fluids surrounding the embryo. For the older embryos, lysers or other organs as noted below were removed 16 h after treatment and immediately frozen in liquid nitrogen. mRNA was iso-

polymarkerase chain reaction; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; UTR, untranslated region; bp, base pair(s); kb, kilobase(s). Sinclair, P. R., Gorman, N., Walton, H. S., Sinclair, J. F., Bentivegna, C. S., Hamilton, J. W., Lee, C., and Rifkind, A. B. (1994) Proceedings of the 10th International Conference on Microsomes and Drug Oxidations, p. 533, University of Toronto, Toronto.

K. Hirada, C. A. Lee, and A. B. Rifkind, unpublished observations.
Chicken CYP1A Enzyme cDNAs

| TCDDAA | 150 bp |
|---------|--------|
| Pat | 5' UTR |
| Pat | 3' UTR |
| ORF | Pat |
| 2' UTR |
| Clones | 7 |
| antibody | 8 |
| nucleotide | 28 |
| RT2 | RT-PCR |

**RESULTS AND DISCUSSION**

Immunoscreening identified three partial clones as TCDDAA and two as TCDDAHH (Fig. 1). Rescreening of the library (6) with a 812-bp probe (full clone 8) and a 265-bp EcoRI/SphI fragment from clone 4 produced two 1.9-kb clones (clones 10 and 2B). Both contained the N-terminal amino acid sequence found for the purified TCDDAA protein (5). The full coding sequence of TCDDAHH was obtained by RT-PCR and 5'-RACE. It contained the N-terminal amino acids found for the purified protein (5) plus the initial methionine.

The TCDDAA cDNA (Fig. 2) contained a 5'-UTR of 147 bp, an open reading frame of 1584 bp coding for 528 amino acids, and a 3'-UTR of 107 bp. The TCDDAHH cDNA (Fig. 3) contained 71 bp of 5'-UTR sequence, 1590 bp coding for 530 amino acids, and a 3'-UTR of 425 bp. Both enzymes contained the characteristic heme binding motif (15) underlined in Figs. 2 and 3. Their distributions of basic, acidic, and hydrophobic amino acids were consistent with the distribution in other CYP enzymes (16) (64, 63, and 182, respectively, for TCDDAA and 60, 56, and 188 for TCDDAHH). The N-terminal amino acid sequences for both contained the typical hydrophobic signal sequence that anchors P450 in the endoplasmic reticulum membrane, followed by a halt-transfer signal containing several positively charged amino acids (15). The N-terminal sequences extending to the halt-transfer signals were longer for the chick than for the mammalian or fish CYP1 enzymes, suggesting that the chick enzymes have a longer segment inserted in the membrane or a part that is free in the lumen of the endoplasmic reticulum. The two cDNAs have dissimilar 3' and 5'-UTRs and 80% DNA sequence and 78% amino acid identities in their coding regions. A partial sequence reported by Murti et al. (16) resembles TCDDAA but lacks the canonical heme binding region and several other CYP1A consensus sites.

TCDDAA was found to have several potential secondary modification sites not present in TCDDAHH or the other CYP1 proteins listed in Table I. These include an N-linked glycosylation site (NPSI, amino acids 150–156) and 11 other potential sites (see FIG. 4). A C-terminal protease phosphorylation site (KQ5DPYR, amino acids 190–198), a protein kinase C phosphorylation site (SMK,
amino acids 523–525), three casein II phosphorylation sites (SVLE, amino acids 60–63; TAVE, amino acids 273–276; and SIQD, amino acids 491–494), and an amidation site (DGKK, amino acids 494–497). TCDDAHH had a protein kinase C phosphorylation site (SFK, amino acids 435–437) and a casein II phosphorylation site (SRTE, amino acids 450–453) not present in TCDDAA. These findings suggest that TCDDAA and to a lesser extent TCDDAHH have the potential to undergo several posttranslational modifications distinct from each other. Protein kinase C and casein II phosphorylation sites, amidation sites, and N-myristylation sites were also found that were common to both TCDDAA and TCDDAHH. Some but not all of those were also present in other CYP1 enzymes. Both chick enzymes, but not the mammalian or fish CYP1A enzymes, also contained a 10-amino acid sequence identified as a prokaryotic membrane lipoprotein lipid attachment site (IAASPTASSSC, amino acids 156–166).

Comparison of the amino acid sequences for the chick enzymes and other CYP enzymes (Fig. 4 and Table I) showed identities of both chick enzymes to CYP1A1 ranging from 58–63%, to CYP1A2 ranging from 53–61%, and to fish CYP1A enzymes ranging from 53–58%. Those values meet the criteria for inclusion in the CYP1A family (46% or greater similarity for amino acid sequences of different species to be included in the same family) (17). However, as shown in Table I, both chick enzymes were more similar to CYP1A1 than CYP1A2. Moreover, even though TCDDAA is enzymatically and immunologically more like -1A2 and TCDDAHH is enzymatically more like CYP1A1, the amino acid sequence of TCDDAA is more like that of CYP1A1 than is the sequence of TCDDAHH. Thus, neither TCDDAHH nor TCDDAA can be said to be directly orthologous to CYP1A1 or -1A2.

There is other evidence for differences between chick TCDDAA and TCDDAHH and mammalian CYP1A enzymes. 1) Whereas CYP1A2 and -1A1 iron are found in high and low spin states, respectively, both TCDDAA and TCDDAHH are low spin (18). 2) Furafylline, at concentrations selectively inhibitory for human and rat CYP1A2 (19), did not inhibit chick liver TCDD-

### TABLE I

Percent amino acid identities between TCDDAA and TCDDAHH and other CYP enzymes

| Percent identity | TCDDAA | TCDDAHH |
|------------------|--------|---------|
| Mammalian CYP1A1 | Human  | 61      | 59      |
| Mouse            |        | 63      | 59      |
| Hamster          |        | 60      | 58      |
| Rabbit           |        | 61      | 59      |
| Rat              |        | 62      | 60      |
| Guinea pig       |        | 61      | 58      |
| Mammalian CYP1A2 | Human  | 61      | 55      |
| Mouse            |        | 57      | 53      |
| Hamster          |        | 57      | 53      |
| Rabbit           |        | 58      | 53      |
| Rat              |        | 57      | 53      |
| Guinea pig       |        | 59      | 56      |
| Fish CYP1A       | Plaice | 55      | 53      |
| Scup             |        | 58      | 55      |
| Toadfish         |        | 55      | 55      |
| Trout            |        | 56      | 55      |
| Mammalian CYP1B  | Human  | 34      | 33      |
| Rat              |        | 35      | 34      |
| Mammalian CYP2   | Rat 2A1| 25      | 27      |
| Rat 2B1          |        | 28      | 29      |
| Rabbit 2C3       |        | 25      | 25      |
| Rat 2E1          |        | 25      | 26      |
| Chicken          | CYP2H1 | 25      | 27      |
| CYP2H2           |        | 25      | 27      |
| CYP17            |        | 22      | 23      |
induced arachidonic acid metabolism. Isosafrole, a selective CYP1A2 inducer (20), did not increase TCDD AA or TCDD AHH in chick embryo liver. CYP1A2 has been found only in liver (1), but TCDDAA is also present in kidney. The amino acid sequence of TCDDAHH is less like that of CYP1A1 than is the sequence of TCDDAA. TCDDAHH is expressed in myocardium, whereas fish CYP1A was found in endocardium and not in myocardium (22). For these reasons, and because one of the two trout enzymes has been designated “CYP1A3” (17), we suggest naming chicken TCDDAHH, CYP1A4 and TCDDAA, 1A5.

Phylogenetic analysis (Fig. 5) shows that neither chick enzyme clustered with the mammalian CYP1A1 or -1A2 sequences or with the fish 1A sequences. Rather, the two chick enzymes formed a separate branch between the fish and mammalian CYP1A sequences. Identification of the chick sequences, therefore, adds a new branch to the CYP1A phylogenetic tree (22) and demonstrates that the chick CYP1A enzymes are a separate lineage.

Data from Morrison et al. (22) that fish have only one CYP1A enzyme (with the possible exception of trout, which have two

\[\text{FIG. 4. Multiple sequence alignments for TCDDAA and TCDDAHH, human, rat, and rabbit CYP1A1 and -1A2, and two fish CYP1A enzymes. Amino acids common to six or more sequences are shaded; TCDDAA numbering is used as the reference.}\]

\[\text{FIG. 5. Phylogenetic tree for the CYP1A family enzymes. The scale represents the number of amino acid residue substitutions from the base of the tree to the furthest branch. The same relationships were found when parsimony analysis was done using Phylogenetic Analysis Using Parsimony (D. L. Swofford, Version 3.0 s).}\]
The amino acid sites that differ for CYP1A1 and -1A2 are principally contained in a sequence of about 127 amino acids (amino acids 197–323 in Fig. 4). That region contains 13 sites where fish and chick CYP1As are conserved with mammalian CYP1A1 but none with -1A2, suggesting that the ancestral CYP1A progenitors were more -1A1- than -1A2-like (see also Ref. 22). That is supported by evidence that fish CYP1A (21), mammalian CYP1A1 (25) and TCDDAHH, but not mammalian -1A2 or TCDDAA, are all expressed in vascular endothelial cells as well as liver. The 127-amino acid region also includes 11 sites selectively shared by TCDDAA and -1A2 but only three by TCDDAHH and -1A2. One or more of the former may contribute to the -1A2-like activity of TCDDAA. TCDDAA, however, lacks the CYP1A2-specific immunologic epitope (amino acids 291–298 in Fig. 4) (26). Thus, another region must account for the selective immunologic cross-reactivity of immunopurified anti-TCDDAA serum to TCDDAA and rat CYP1A2 (5).

On Northern analysis (Fig. 6A) mRNA from TCDD-treated but not control embryos contained single transcripts of approximately 1.9 kb for TCDDAA in liver and kidney but not heart and single transcripts of 2.3 kb for TCDDAHH in liver, kidney, and heart, consistent with the tissue-specific expression of the CYP enzyme proteins (5). The findings demonstrate definitively that TCDD induces two distinct chick CYP1A genes. They also provide additional evidence for differences between the chick enzymes and CYP1A1 genes. The findings demonstrate definitively that TCDD induces two distinct chick CYP1A genes. They also provide additional evidence for differences between the chick enzymes and CYP1A1 genes.

PCR analysis performed on whole 28–72-h-old embryos treated with TCDD at 24 h (middle panels of Fig. 6B) and for comparison on livers of 17-day-old TCDD-treated embryos (top panel) amplified bands of the same size (400 bp for TCDDAHH and 200 bp for TCDDAA) in both the younger embryos and the embryos close to hatching. From the top panel it is also seen that liver glyceraldehyde-3-phosphate dehydrogenase was increased by TCDD treatment in livers of the 17-day-old chick embryos, as has been reported in human keratinocytes exposed to TCDD (27). TCDDAHH (upper middle panel) was induced in 48-h-old embryos 24 h after TCDD treatment, and the induction was increased in the 72-h-old embryos. TCDDAA (lower middle panel) was expressed in the controls at the earliest time examined (28 h of development), suggesting that there is constitutive expression of this gene in the early embryo. The expression of TCDDAA was increased by TCDD in the 36-h-old embryos 12 h after treatment and further increased at 48–72 h of development. AHH induction has been reported in 5-day-old chick embryos (28). Our findings indicate that there is a functional Ah receptor in the developing chicken 3–4 days earlier than previously recognized.

**Fig. 6.** A, Northern analysis of TCDDAA, TCDDAHH, and actin mRNA in liver, heart, and kidney of 17-day-old chick embryos 16 h after treatment with 1 nmol of TCDD (+) or solvent alone (−) (0.005 ml of dioxane). Lanes 1 and 2, liver; Lanes 3 and 4, heart; Lanes 5 and 6, kidney (10 µg total RNA for each). B, RT-PCR analysis of TCDDAA and TCDDAHH expression. Amplification of TCDDAA (AA), TCDDAHH (AHH), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from livers of control (−) and TCDD-treated (+) embryos using gene specific primers. Top panel, livers of 17-day-old chick embryos. Lower three panels, whole embryos treated with TCDD at 24 h of development. The time (h) after TCDD treatment is shown at the top of each panel. TCDDAHH, upper middle panel; TCDDAA, lower middle panel; glyceraldehyde-3-phosphate dehydrogenase, lower panel. C, localization by in situ hybridization of TC-DDAHH and TCDDAA in 72-h embryos treated with TCDD at 24 h. Top left panel, control embryo probed with TCDDAHH antisense probe; top middle and bottom left panels, TCDD-treated embryo probed with TCDDAHH antisense probe; right panels, TCDD-treated embryo probed with TCDDAA antisense probe. g, gut endoderm; da, dorsal aorta; e, endocardium; md, mesonephric duct. Anterior, top; dorsal, left. Magnification: upper panels, ×25; lower panels, same areas at ×50.
In situ hybridization studies in 72-h-old embryos 48 h after TCDD treatment (Fig. 6C) showed that mRNAs for both enzymes are expressed in the areas of gut epithelium that later develop into liver. Sense probes did not hybridize (data not shown). Both antisense probes were negative for the solvent-treated controls and positive for the TCDD-treated embryos. TCDD_{AHH} but not TCDD_{AA} was expressed in the dorsal aorta and other vascular endothelial cells and in endocardium. TCDD_{AA} but not TCDD_{AHH} was expressed in the mesonephric duct, which gives rise to the kidney. The localization of TCDD_{AHH} in vascular endothelium further suggests that TCDD may have widespread effects via its expression in blood vessels.

Our evidence that mammalian and chick CYP1A enzymes branched into two functionally analogous pairs of enzymes independently suggests that the split in both cases may have responded to similar evolutionary pressures. That, together with the evidence that both chick enzymes are expressed very early in development, suggests that CYP1A enzymes have functions beyond their harmful actions as activators of carcinogens, perhaps providing a defense against environmental pollutants. For example, with respect to known activities of these enzymes, increased estradiol-2-hydroxylation may help to maintain calcium, fluid, and hormonal homeostasis (30). High activity of CYP1A1 for metabolism of growth-modulating retinoids has recently been shown (31).

The common view of CYP1A1 and -1A2 as injurious has not succeeded in explaining the role of these enzymes in TCDD toxicity. Thus, the carcinogenic effect of TCDD does not involve metabolic activation of TCDD by CYP1A enzymes (1, 2) and there is little CYP1A induction in the liver of TCDD-treated guinea pig, the species most sensitive to TCDD toxicity (2). Although CYP1A enzymes may have some potentially carcinogenic effects (1, 2, 32), our results suggest that investigation of their advantageous, possibly protective actions and of their effects on endogenous metabolism may prove productive.

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