Supporting Text

Oligonucleotide Primers

Primers were synthesized by Life Technologies, Integrated DNA Technologies, or Midland Certified Reagent and were used as described. Primer sequences were:

B-21F: 5'-GAAGACAGACCTGAGTTTCAACGTCAGC-3';

B-25F: 5'-GCAGAGCTGTGTATGAGAGGAACAGGATACC-3';

B-31R: 5'-AGAAGGCTTGCCAAGCGATCCAACTCCGC-3';

B-52R: 5'-TGTAGCTGACGTTGAAACTCAGGTCTGTC-3';

Bkoz: 5'-GATGGTCGACGCCACCATGAACCCCAATGTCACC-3';

Bterm: 5'-GATGGAGCTCTAGATCCGAATCACATAAATCCAC-3';

NcoF: 5'-AGTTTCTCCATGGACAGAACAAGAAAGG-3';

NcoR: 5'-GTTCTGTCCATGGAGAAACTTTAATCG-3';

NarF: 5'-CAGATGAAGAAGGCGCCGAACATCTACG-3';

NarR: 5'-CGTAGATGTTCGGCGCCTTCTTCATCTG-3';

f258mt: 5'-GAAAGGGAAGGATGGTGCTGCTTTGTCTCCTCAGCTTGC-3';

f325mt: 5'-GAGAGGAACAGGATACCAGTTTATTCATGCAGCTGATATGC-3';

f381mt: 5'-GAAGACCGGATTACATCATTTCCACGCAAAGACCTCTTAC-3';

r258mt: 5'-GCAAGCTGAGGAGACAAAGCAGCACCATCCTTCCCTTTC-3';

r325mt: 5'-GCATATCAGCTGCATGAATAAACTGGTATCCTGTTCCTCTC-3';

r381mt: 5'-GTAAGAGGTCTTTGCGTGGAAATGATGTAATCCGGTCTTC-3'.

DNA Sequence Analysis

DNA sequences were assembled and translated by using MACVECTOR/ASSEMBLYLIGN sequence analysis software (Oxford Molecular Group, Madison, WI). Multiple sequence alignments were performed by using CLUSTALX (1). The aligned nucleotide sequences were used to construct phylogenetic trees using Maximum Parsimony and distance criteria in PAUP*4.0b8 (2).

Saturation Binding Analysis

Saturation binding was performed using two methods. The initial analyses used a modification of the hydroxylapatite (HAP) adsorption assay of Gasiewicz and Neal (3). Aryl hydrocarbon receptor (AHR) proteins were synthesized by *in vitro* transcription and translation as described above. For each AHR, four 50-μl TnT reactions were combined and then diluted 4-fold in MEEDG buffer (25 mM Mops, 1 mM EDTA, 5 mM EGTA, 0.02% NaN_3, 10% vol/vol glycerol, 1 mM DTT, pH 7.5). The diluted TnT-expressed AHRs were then split into 50-μl aliquots that were incubated in 10 × 75 mm glass tubes for 22 h at 4°C with each of eight concentrations of [^{3}H]2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in DMSO (0.1, 0.2, 0.5, 1, 2, 4, 8, and 10 nM, each in duplicate). A 5-μl aliquot was taken from each tube to determine the total concentration of [^{3}H]TCDD. After the incubation, 200 μl of resuspended HAP (50%, vol/vol in MEEDG) were added to each tube and the tubes were incubated on ice for 30 min. The HAP was then washed

either by repeated cycles of centrifugation, aspiration, and buffer addition (4) or by filtration. For the filtered HAP assay, the AHR-HAP mixture was transferred onto a Whatman GF/F filter in a Millipore 12-position vacuum apparatus, the tube was washed with 0.5 ml of MEEDG buffer, and the wash was added to the filter. After application of the vacuum, the filter was washed two additional times with 0.75 ml of MEEDG buffer. The filter was then transferred to a scintillation vial and the radioactivity (total bound [^{3}H]TCDD) was measured on a Beckman 5000 scintillation counter. The centrifuged HAP and filtered HAP assays produced similar measures of specific binding.

In other experiments, saturation binding was performed by using velocity sedimentation at several [^{3}H]TCDD concentrations. In these experiments, the TnT reactions for chicken AHR were diluted with unprogrammed lysate (UPL) before the [^{3}H]TCDD binding assay, to minimize any effect that the higher binding capacity of the chicken AHR might have on the measurement of binding affinities. For chicken AHR, two 50-μl TnT reactions were combined and diluted with 100 μl of UPL, followed by 600 μl of MEEDMG buffer. For tern AHR or UPL control, four TnT reactions were combined (200 μl total) and diluted with 600 μl of MEEDMG buffer. Each sample was split into eight aliquots and incubated with [^{3}H]TCDD (various concentrations) overnight at 4°C in glass tubes. The [^{3}H]TCDD concentration was verified by sampling each tube for total counts. Samples were analyzed on 10-30% sucrose gradients (5).

Saturation binding curves were plotted as “free” [^{3}H]TCDD (nM) vs. bound [^{3}H]TCDD. The amount of bound [^{3}H]TCDD was determined directly from measured radioactivity, either adsorbed to HAP or in the specific binding peaks of sucrose gradients (typically fractions 10-20). Free [^{3}H]TCDD was determined by subtracting the bound [^{3}H]TCDD concentration from the total [^{3}H]TCDD concentration for each tube. Nonspecific binding (binding of [^{3}H]TCDD to UPL, as measured either by HAP adsorption or in fractions 10-20 of sucrose gradients) was plotted as a function of the free [^{3}H]TCDD concentration and fit to a linear model; this relationship was used to calculate the nonspecific binding at each concentration of free [^{3}H]TCDD in the incubations with *in vitro* synthesized AHRs. Specific binding for each AHR was calculated as the difference between the actual total

binding and the calculated nonspecific binding. The equilibrium dissociation constants (K_d) for each AHR were obtained from the respective specific binding curves using the equation for the Langmuir binding isotherm: $B = \frac{B_{max}[L]}{[L] + K_d}$, where B is specifically bound [^{3}H]TCDD, B_{max} is maximum bound receptor, L is the concentration of free ligand, and K_d is the equilibrium dissociation constant. Nonlinear regression analysis was performed by using PRISM version 3 software for the Macintosh (GraphPad, San Diego) as described (6).

Discussion

Relationships among avian and mammalian AHRs

The chicken and tern AHRs reported here both belong to the AHR1 clade, indicating that these avian AHRs are orthologs of the human and mouse AHRs. Although AHR2 forms very recently have been identified in birds (7, 8) including chicken and tern (J. Lapseritis and M.E.H., unpublished data), their functional properties have not yet been determined. Therefore, we focused on characterizing the chicken and tern AHR1 forms and their possible role in the species difference in sensitivity to HAHs. The chicken and common tern AHRs share a surprising degree of sequence conservation: 92% amino acid identity overall, with most of the differences occurring in the C-terminal half of the proteins. Chickens (Galliformes; Gallianseres) and terns (Charadriiformes; Neoaves) belong to distinct avian lineages that diverged 82-90 million years ago (9). This is only slightly less than the estimated divergence time of 96 MYA between primates and rodents (10), yet mouse and human AHRs share only 72% amino acid identity overall. Thus, the degree of sequence conservation between these two avian AHRs appears to be greater than that seen among mammalian AHRs. Despite their closely related sequences, the chicken and tern AHRs differ substantially in their functional characteristics.

Structural and functional differences within LBD of chicken and tern AHRs

The LBDs of chicken and tern AHRs differ at three amino acid residues: Ala-257, Ile-324, and Ser-380 in the chicken AHR are Thr-258, Val-325, and Ala-381 in the tern AHR (Fig. 5). Tern AHR mutant T258A was functionally similar to the wild-type tern AHR, indicating that this residue is not responsible for the functional differences between tern and chicken AHRs. Similarly, this position is not at all conserved in vertebrate AHRs (Fig. 8*A*), suggesting that variability in this residue is well tolerated in other AHRs.

In contrast, the tern AHR mutants V325I and A381S exhibited [^{3}H]TCDD binding and transactivation abilities approaching those of the wild-type chicken AHR. The position corresponding to tern Val-325 is Ile in most other vertebrate AHRs (Fig. 8*A*) but it is Val in several other avian species that, like the tern, exhibit reduced sensitivity to HAHs (Fig. 8*B*). Moreover, this residue is Val in invertebrate AHRs (Fig. 8*A*), which do not bind [^{3}H]TCDD (11). Thus, the present results suggest that Ile/Val may be a key determinant of [^{3}H]TCDD binding in diverse animal species.

Tern Ala-381 is homologous to Ala-375 of the high-affinity mouse Ah^{b-1} protein and Val-375 and Val-380 of the lower affinity mouse Ah^d and human AHR proteins, respectively (Fig. 8*A*). Most other vertebrate AHRs have Ala at this position, while AHR homologs from *Drosophila melanogaster* and *Caenorhabditis elegans* possess Cys and Leu, respectively, at this site (Fig. 8*A*). Previous reports have demonstrated that differences in this residue are responsible for the different TCDD-binding affinities of mouse Ah^b and Ah^d variants (12-15), that mutating this residue in the rat AHR from Ala to Val (A379V) reduced the ability of low-affinity ligands to activate the AHR (16), and that changing the corresponding aa in the human AHR from Val to Ala (V381A) increased the binding of TCDD (13, 17). Our results obtained with chicken and tern AHRs demonstrate the more general importance of this residue in determining AHR ligand-binding function in vertebrate species, including nonmammalian vertebrates.

Potential value and limitations of AHR and AHR LBD analysis in relation to HAH sensitivity and risk assessment

The present results localizing the difference in TCDD-binding properties of chicken and tern AHRs to two residues suggest that sequencing and *in vitro* functional analysis of AHRs or AHR LBDs could be used in a predictive way in risk assessment, to supplement current approaches involving *in vivo* exposures (18, 19) or use of cultured hepatocytes (20, 21) to assess species differences in HAH sensitivity. Consistent with this, the AHR LBD sequences of other bird species that have reduced sensitivity to HAHs (or are congeneric with HAH-resistant species) are identical to that of the tern AHR. As compared to chickens, the herring gull (*Larus argentatus*), Pekin duck (*Anas platyrhynchos*), and double-crested cormorant (*Phalacrocorax auritus*) are less sensitive to various HAH effects by 50- to 1,000-fold, 250- to 1,000-fold, and 15- to 100-fold, respectively (18, 20-25). The AHR LBD sequences of herring gull, Pekin duck, and the congeneric cormorant *Phalacrocorax carbo* share Val-325 and Ala-381 with the tern AHR (Fig. 8*B*). Thus, the limited data on avian AHRs to date support the potential value of examining AHR LBD sequences.

Despite the apparent concordance between AHR LBD sequences and HAH sensitivity among species of birds, data from mammals suggests that the relationship between AHR structure and HAH sensitivity may be more complex, involving not only the LBD but also regions outside of the LBD as well as non-AHR factors. As noted earlier, molecular studies of AHRs in mouse strains and humans show that specific differences within the AHR LBD and the resulting differences in affinity for TCDD explain the apparent strain and species differences in sensitivity to HAHs (12, 13, 16, 17, 26). In contrast, differences in the LBD may not fully explain the dramatic difference in sensitivity of guinea pigs and hamsters to the toxicity of HAHs. Guinea pigs are ≈1,000-fold more sensitive than hamsters to the lethal effects of TCDD (27, 28), although these species exhibit similar sensitivity to CYP1A induction and other sublethal responses (29-31). Biochemical characterization of AHR proteins in these two species suggests that the guinea pig AHR has a somewhat higher ligand-binding affinity (32, 33) and is more easily activated to the nuclear, DNA-binding form (34, 35). Sequence comparisons show that while the guinea pig and hamster AHRs differ at 24/168 residues in the LBD, there are even more dramatic differences in the C-terminal half of the proteins (36, 37). The C-

terminal TAD of AHRs is known to contribute to species and allelic differences in AHR function (38, 39). Thus, differences outside the LBD are likely to be important in determining species sensitivity. Another example of this is provided by the Han/Wistar (*Kuopio*) rat strain, which is extremely resistant to the lethal effects of TCDD as compared to other rat strains such as Long-Evans, but retains sensitivity to biochemical and sublethal toxic effects (28, 40). AHRs from sensitive (Long-Evans) and resistant (Han/Wistar) rat strains exhibit similar ligand-binding affinities (41). Consistent with this, their AHRs are identical in the LBD but differ substantially in the TAD as a result of point mutation causing altered mRNA splicing (42). These and other studies (43) suggest that differences in the C-terminal half of these AHRs, rather than the LBD, contribute to the differential sensitivity among rat strains. Together, these data demonstrate that, although differences in the AHR LBD may underlie species-specific sensitivity in some taxa, including birds, differences in the LBD do not provide a universal explanation for differential sensitivity among species and individuals.

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1. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997) *Nucleic Acids Res.* **25,** 4876-4882.

2. Swofford, D. L. (1998) PAUP*: *Phylogenetic Analysis Using Parsimony (*and Other Methods)* (Sinauer, Sunderland, MA), Version 4.

3. Gasiewicz, T. A. & Neal, R. A. (1982) *Anal. Biochem.* **124,** 1-11.

4. Kim, E.-Y. & Hahn, M. E. (2002) *Aquat. Toxicol.* **58,** 57-73.

5. Karchner, S. I., Powell, W. H. & Hahn, M. E. (1999) *J. Biol. Chem.* **274,** 33814-33824.

6. Jensen, B. A. & Hahn, M. E. (2001) *Toxicol. Sci.* **64,** 41-56.

7. Hahn, M. E. (2002) *Chem.-Biol. Interact.* **141,** 131-160.

8. Yasui, T., Kim, E. Y., Iwata, H. & Tanabe, S. (2004) *Mar. Environ. Res.* **58,** 113-118.

9. Harrison, G. L., McLenachan, P. A., Phillips, M. J., Slack, K. E., Cooper, A. & Penny, D. (2004) *Mol. Biol. Evol.* **21,** 974-983.

10. Nei, M., Xu, P. & Glazko, G. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 2497-2502.

11. Butler, R. B., Kelley, M. L., Powell, W. H., Hahn, M. E. & Van Beneden, R. J. (2001) *Gene* **278,** 223-234.

12. Poland, A., Palen, D. & Glover, E. (1994) *Mol. Pharmacol.* **46,** 915-921.

13. Ema, M., Ohe, N., Suzuki, M., Mimura, J., Sogawa, K., Ikawa, S. & Fujii-Kuriyama, Y. (1994) *J. Biol. Chem.* **269,** 27337-27343.

14. Maier, A., Micka, J., Miller, K., Denko, T., Chang, C. Y., Nebert, D. W. & Puga, A. (1998) *Environ. Health Persp.* **106,** 421-426.

15. Murray, I. A., Reen, R. K., Leathery, N., Ramadoss, P., Bonati, L., Gonzalez, F. J., Peters, J. M. & Perdew, G. H. (2005) *Arch. Biochem. Biophys.* **442,** 59-71.

16. Backlund, M. & Ingelman-Sundberg, M. (2004) *Mol. Pharmacol.* **65,** 416-25.

17. Ramadoss, P. & Perdew, G. H. (2004) *Mol. Pharmacol.* **66,** 129-36.

18. Brunstrom, B. (1988) *Poultry Sci.* **67,** 52-57.

19. Hoffman, D. J., Melancon, M. J., Klein, P. N., Eisemann, J. D. & Spann, J. W. (1998) *Environ. Toxicol. Chem.* **17,** 747-757.

20. Kennedy, S. W., Lorenzen, A., Jones, S. P., Hahn, M. E. & Stegeman, J. J. (1996) *Toxicol. Appl. Pharmacol.* **141,** 214-230.

21. Sanderson, J. T., Kennedy, S. W. & Giesy, J. P. (1998) *Environ. Toxicol. Chem.* **17,** 2006-2018.

22. Brunstrom, B. & Halldin, K. (1998) *Comp. Biochem. Physiol. C* **121,** 213-219.

23. Sanderson, J. T. & Bellward, G. D. (1995) *Toxicol. Appl. Pharmacol.* **132,** 131-145.

24. Jin, X., Kennedy, S. W., Di Muccio, T. & Moon, T. W. (2001) *Toxicol. Appl. Pharmacol.* **172,** 241-248.

25. Powell, D. C., Aulerich, R. J., Meadows, J. C., Tillitt, D. E., Powell, J. F., Restum, J. C., Stromborg, K. L., Giesy, J. P. & Bursian, S. J. (1997) *Environ. Toxicol. Chem.* **16,** 1450-1455.

26. Moriguchi, T., Motohashi, H., Hosoya, T., Nakajima, O., Takahashi, S., Ohsako, S., Aoki, Y., Nishimura, N., Tohyama, C., Fujii-Kuriyama, Y. & Yamamoto, M. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 5652-5657.

27. Poland, A. & Knutson, J. C. (1982) *Annu. Rev. Pharmacol. Toxicol.* **22,** 517-554.

28. Pohjanvirta, R. & Tuomisto, J. (1994) *Pharmacol. Rev.* **46,** 483-549.

29. Gasiewicz, T. A., Rucci, G., Henry, E. C. & Baggs, R. B. (1986) *Biochem. Pharmacol.* **35,** 2737-2742.

30. Birnbaum, L. S. (1994) *Environ. Health Perspect.* **102,** 157-167.

31. Olson, J. R. & McGarrigle, B. P. (1992) *Chemosphere* **25,** 71-74.

32. Gasiewicz, T. A. & Rucci, G. (1984) *Mol. Pharmacol.* **26,** 90-98.

33. Denison, M. S. & Wilkinson, C. F. (1985) *Eur. J. Biochem.* **147,** 429-435.

34. Bank, P. A., Yao, E. F., Phelps, C. L., Harper, P. A. & Denison, M. S. (1992) *Eur. J. Pharmacol. - Environ. Toxicol. Pharmacol. Sect.* **228,** 85-94.

35. Henry, E. C., Rucci, G. & Gasiewicz, T. A. (1989) *Biochemistry* **28,** 6430-6440.

36. Korkalainen, M., Tuomisto, J. & Pohjanvirta, R. (2000) *Biochem. Biophys. Res. Commun.* **273,** 272-281.

37. Korkalainen, M., Tuomisto, J. & Pohjanvirta, R. (2001) *Biochem. Biophys. Res. Commun.* **285,** 1121-1129.

38. Ramadoss, P. & Perdew, G. H. (2005) *Biochemistry* **44,** 11148-59.

39. Wong, J. M., Okey, A. B. & Harper, P. A. (2001) *Biochem. Biophys. Res. Commun.* **288,** 990-996.

40. Simanainen, U., Tuomisto, J. T., Pohjanvirta, R., Syrjala, P., Tuomisto, J. & Viluksela, M. (2004) *Toxicol Appl Pharmacol* **196,** 11-9.

41. Pohjanvirta, R., Viluksela, M., Tuomisto, J. T., Unkila, M., Karasinska, J., Franc, M. A., Holowenko, M., Giannone, J. V., Harper, P. A., Tuomisto, J. & Okey, A. B. (1999) *Toxicol Appl Pharmacol* **155,** 82-95.

42. Pohjanvirta, R., Wong, J. M. Y., Li, W., Harper, P. A., Tuomisto, J. & Okey, A. B. (1998) *Mol. Pharmacol.* **54,** 86-93.

43. Tuomisto, J. T., Viluksela, M., Pohjanvirta, R. & Tuomisto, J. (1999) *Toxicol Appl Pharmacol* **155,** 71-81.