0026-895X/94/050915-07$03.00/0

MOLECULAR PHARMACOLOGY, 46:915-921, 1994.

Analysis of the Four Alleles of the Murine Aryl Hydrocarbon Receptor

ALAN POLAND, DAVID PALEN, and ED GLOVER

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706

Received March 28, 1994; Accepted August 25, 1994

SUMMARY

The cDNAs for the four murine aryl hydrocarbon (Ah) receptor alleles were cloned and sequenced, and the deduced amino acid sequences were compared. The Ah^{b-1} allele encodes a protein of 805 amino acids, the Ah^{d} and Ah^{b-2} alleles encode proteins of 848 amino acids, and the Ah^{b-3} allele encodes a protein of 883 amino acids. The alleles differ by eight point mutations in the common open reading frame (the initial 805 amino acids) and by additional sequences at the carboxyl end. The amino halves of the proteins, containing a spliced leader sequence, a basic helix-loop-helix motif, and two 50-amino acid repeats (PAAS), have identical sequences except for a single amino acid change in the second PAAS box. The Ah^{d} allele, which has a lower ligand binding affinity, differs from the Ah^{b-2} receptor by only two amino acids. Mutagenesis experiments with these cloned cDNAs, using *in vitro* transcription and translation and 2-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin binding, indicate that the low ligand binding affinity of the Ah^{d} allele is attributable to a valine at residue 375; changing this amino acid to an alanine, as in the Ah^{b-2} protein, enhances the affinity 4-fold. For *in vitro* translated Ah^{b-1} and Ah^{b-2} alleles the K_d values were ~6–10 pM and for Ah^{d} the K_d value was ~37 pM. Using 5′ truncation and mutations to produce 3′ translation truncation sites, we mapped the ligand binding region for the Ah^{b-1} allele.

The *Ah* locus was defined as a difference in responsiveness of inbred strains of mice to polycyclic aromatic hydrocarbons, originally in their response to 7,12-dimethylbenzanthracene-induced skin inflammation (1) and then in the induction of cytochrome P450IA1-mediated monooxygenase activity in liver and other tissues by systemic administration of 3-MC (2). C57BL/6 mice challenged with 3-MC show an induction of hepatic cytochrome P450IA1 and associated AHH activity, whereas DBA/2 mice fail to respond. In genetic crosses and backcrosses between these mice, the trait of aromatic hydrocarbon responsiveness is inherited as an autosomal dominant trait and the alleles are designated responsive (Ah^{b}, for B6) and nonresponsive (Ah^{d}, for DBA/2). Administration of TCDD, a more potent inducer than 3-MC, induces cytochrome P450IA1 and AHH activity (3) in all inbred strains of mice, those responsive and those nonresponsive to polycyclic aromatic hydrocarbons; however, a larger dose of TCDD is required in the aromatic hydrocarbon-nonresponsive strains (4).

Further studies established that the *Ah* locus determined a soluble protein that specifically binds [^{3}H]TCDD with high affinity and that has rank-ordered binding affinities for various ligands that match their potencies to induce AHH activity (5). Thus, the *Ah* locus encodes the Ah receptor, a protein that binds to polycyclic aromatic hydrocarbons and TCDD (and other isosteric halogenated aromatic hydrocarbons) and mediates their pleiotropic gene expression. The Ah^{d} allele expresses a protein with diminished binding affinity for ligands, resulting in virtual insensitivity to weak agonists such as 3-MC and diminished sensitivity to more potent agonists such as TCDD (4).

With the aid of [^{125}I]Br_2DpD to photoaffinity label murine liver cytosol, it was possible to identify four different allelic forms of the Ah receptor, namely Ah^{d} (low affinity, apparent molecular mass of ~104 kDa, stabilized by sodium molybdate) and three high affinity alleles, Ah^{b-1} (~95 kDa), Ah^{b-2} (~104 kDa), and Ah^{b-3} (~105 kDa) (6, 7). The Ah^{d} allele is found in AKR, DBA/2, and 129 strains, the Ah^{b-1} allele is carried by C57, C58, and MA/My strains, the Ah^{b-2} allele is carried by BALB/cBy, A, and C3H strains, and the Ah^{b-3} allele is found in *Mus caroli*, *Mus spretus*, and MOLF/Ei.

Subsequent work has established many of the molecular details of activation of the receptor. The unliganded receptor

This work was supported in part by National Institute of Environmental Health Sciences Grant ES01884 and National Cancer Institute Core Grant CA07175.

ABBREVIATIONS: Ah, aryl hydrocarbon; PAAS, periodicity; Ah, Arnt, and single minded; 3-MC, 3-methylcholanthrene; AHH, aryl hydrocarbon hydroxylase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; Arnt, Ah receptor nuclear translocator; ORF, open reading frame; PCR, polymerase chain reaction; hsp90, 90-kDa heat shock protein; [^{125}I]Br_2DpD, 2-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin; TCDBF, 2,3,7,8-tetrachlorodibenzofuran; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

exists as a large complex (280 kDa, associated with hsp90), similar to some of the steroid hormone receptors (8, 9). Upon ligand binding, the Ah receptor dissociates from hsp90 and becomes phosphorylated, and the activated receptor-ligand complex specifically binds to an enhancer region, a dioxin-responsive element, with a consensus sequence of T(T/A)GCGTG (10, 11). It was subsequently shown that the activated receptor binding to this enhancer is a heterodimer composed of the liganded Ah receptor and a second protein, Arnt (12, 13). Cloning of the human Arnt cDNA (12, 13) and the murine Ah^{b-1} (14, 15) and human Ah (16) receptors revealed that both Arnt and the Ah receptor are basic helix-loop-helix proteins that share a second motif, i.e., two imperfect 50-amino acid repeats (PAAS boxes) spaced 100 amino acid apart. A database search showed homology of these cDNAs with two other *Drosophila* genes, *sim* and *per* (12, 14). In summary, the Ah receptor is a novel transcription activation factor sharing some similarities with steroid hormone receptors in ligand binding and dissociation from hsp90 but differing in that the Ah receptor and Arnt are basic helix-loop-helix proteins and not zinc-finger proteins. In this report we clone the cDNA for the alleles of the Ah receptor, examine the mutation in the Ah^d allele that is responsible for the low affinity ligand binding, and further characterize the ligand binding domain of the Ah receptor.

Materials and Methods

Cloning and sequencing of the cDNA for the *Ah* alleles. Poly(A)$^+$ RNA was prepared from the livers of C57BL/6 congenic mice homozygous for each of the four *Ah* alleles (6, 7). We synthesized primers (with 5′ restriction enzyme sequences) based on the known sequence of the Ah^{b-1} allele ORF (14, 15), and cDNA prepared by PCR was subcloned and sequenced (Fig. 1). We subcloned and sequenced first the 5′ and 3′ halves of the ORFs (nucleotides 1–1263 and 1241–2418) and then the entire ORF, ending at the Ah^{b-1} stop site (nucleotide 2418). Lacking success in one-sided PCR extension (rapid amplification of cDNA ends), we identified a region in the published Ah^{b-1} cDNA, 3′ to the translation stop site, that was largely preserved in the human *Ah* cDNA (15, 16). Thus, the ultimate primers for PCR of the entire ORF of each allele were primer 17 [GGCTAAGCTTATGAGCAGCGGCGCCATCA, which contains a 5′ *Hind*III site (underlined) and sequence corresponding to nucleotides 1–21] and antisense strand primer 47X [CGATCTCGAGAAAAGCACCAAACCATTTA, corresponding to nucleotides 3137–3119 with a *Xho*I restriction site (underlined)]. The cDNAs were cut with restriction enzymes, subcloned into Bluescript SK$^-$ (Stratagene, La Jolla, CA), and sequenced by the dideoxynucleotide method, using [α-^{35}S]ATP and primers based on the Ah^{b-1} allele (16). For each allele, cDNAs from two independent reverse transcription/PCRs were sequenced on both strands, to avoid reverse transcription/polymerase errors. Plasmids were purified using Qiagen adsorption (Qiagen, Chatsworth, CA) (17).

Cloning into phagemid and mutagenesis. The cDNA of the Ah receptor allele, generated by PCR with a 5′ primer containing a *Hind*III site and a Kozak consensus sequence (18) and a 3′ primer containing an *Xba*I site, was inserted into the multiple cloning site of the phagemid p-ALTER-1 (Promega, Madison, WI) (19). This plasmid also contains adjacent T7 and SP6 RNA polymerase promoters, a β-lactamase coding region (producing ampicillin resistance), and a phage f1 region so that single-stranded DNA can be formed and mutagenized. Single-stranded DNA was generated, purified, and hybridized to the specific mutation primer and a second primer to convert the frame-shifted β-lactamase gene back to ampicillin resistance; the DNA was prepared with T4 polymerase and T4 ligase and then introduced into competent cells (BMH71-18 *mutS* and then *Escherichia coli* JM109), all according to the manufacturer's instructions (20). The mutagenic primer for conversion of $Ah^{b\text{-}2}{}_{348}$ (phenylalanine) to Ah_{d348} (leucine) was TGTTTTGCAAGAAGCGGGAA, and for the reverse conversion of $Ah^{d}{}_{348}$ (leucine) to $Ah^{b\text{-}2}{}_{348}$ (phenylalanine), guanine was changed to adenine (at the underlined position); for conversion of $Ah^{b\text{-}2}{}_{375}$ (alanine) to $Ah^{d}{}_{375}$ (valine) the primer was TCTCTGAGTGACGATGATGT, and for the reverse conversion of $Ah^{d}{}_{375}$ (valine) to $Ah^{b\text{-}2}{}_{375}$ (alanine) adenine was changed to guanine (see Table 3).

For 3′ truncations in the Ah^{b-1} allele, a codon in the ORF was mutagenized to a translation stop site, at the amino acid positions indicated in Fig. 4. These constructs were checked by sequencing over the mutagenized region and by comparison of the apparent molecular mass of [^{35}S]methionine-labeled translation product with that predicted (21, 22).

For 5′ truncations, the cDNA was prepared from the cloned Ah^{b-1} allele with primers with suitable restriction sites (5′, *Hind*III; 3′, *Xba*I), 5′ Kozak consensus sequence (18), sequence complementary to the desired start site of the ORF cDNA, and standard 3′ primer sequence at the translation termination site. For the combined 3′ and 5′ truncations in Fig. 4, cDNA was prepared by PCR with 5′ primers as described above and a 3′ primer containing complementary sequence, a translation truncation site at codon 401, and an *Xba*I restriction sequence. The most complex construction, shown in Fig. 5, consisted of the two PAAS boxes (spaced by seven amino acids) from two constructs made by PCR, cut with restriction enzymes, and then ligated, i.e., 1) *Hind*III restriction sequence/Kozak consensus sequence/ORF amino acid sequence 130–188/*Xho*I restriction sequence and 2) *Xho*I restriction sequence/ORF amino acid sequence 288–805/*Xba*I. These constructs were checked by sequencing over 5′ ends, determination of the approximate size of the cDNA by agarose gel electrophoresis, and determination, on sodium dodecyl sulfate-polyacrylamide gels, of the size of the [^{35}S]methionine-labeled translation product (21), which was visualized by fluorography (22) using Amplify (Amersham, Arlington Heights, IL).

***In vitro* transcription and translation.** All cDNAs were cloned into p-ALTER-1 and expressed using a coupled transcription/translation system (T_NT coupled reticulocyte lysate system or T_NT coupled

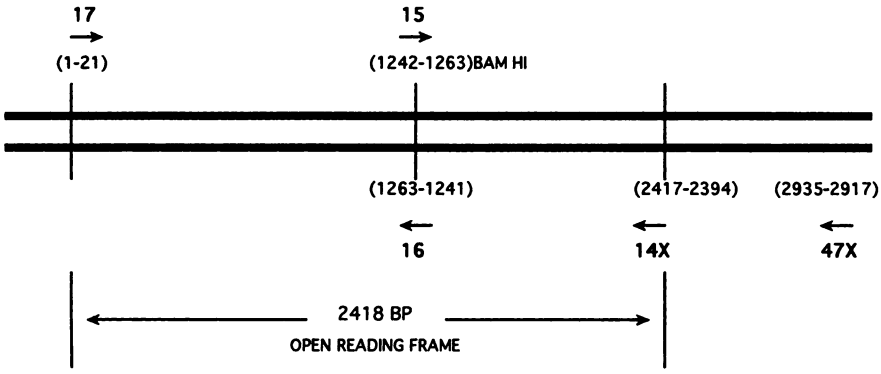


Fig. 1. Diagram of Ah^{b-1} cDNA. Primers used to amplify alleles by PCR were primers 17, 15, 16, 14X, and 47X. The nucleotides are numbered from the translation start site of the ORF (*numbers in parentheses*).

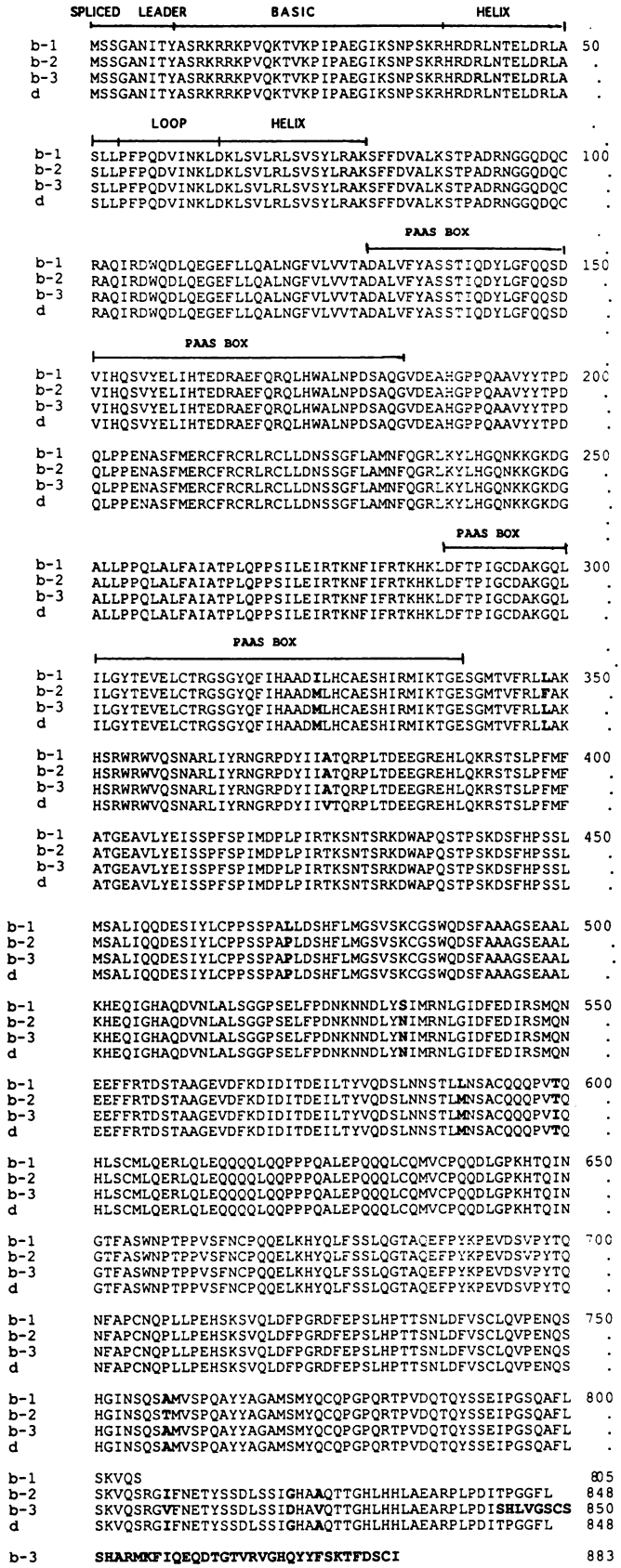


Fig. 2. ORF of the four murine alleles of the Ah receptor. The amino acid sequences of the four murine alleles were deduced by cDNA sequencing. Each cDNA was generated by two independent reverse transcription/PCRs, and each strand was sequenced at least twice for verification. Any amino acid that differs between alleles is emphasized by *bold type*. Major motifs are indicated by *overlining* (spliced leader, basic helix-loop-helix region, and PAAS boxes).

wheat germ extract system; Promega) with 1 μg of phagemid DNA/50-μl reaction for 1 hr at 30°, according to the manufacturer's instructions (20). All transcripts were transcribed with SP6 RNA polymerase, using circular DNA without capping. Where indicated, the translation products were radiolabeled with [^{35}S]methionine (Amersham no. S1015, 1000 Ci/mmol, translation grade, ~40 μCi/50-μl reaction mixture).

Radioligand binding. [^{125}I]Br_2DpD (2176 Ci/mmol) was synthesized and purified as described previously (23). Murine liver cytosol from C57BL/6 congenic mice was prepared by homogenization in MDENG buffer [20 mM MOPS, pH 7.5 (20°), 1 mM dithiothreitol, 1 mM EDTA, 0.02% sodium azide, 10% glycerol] plus 1 mM EGTA, with or without 20 mM sodium molybdate, at a final protein concentration of about 7 mg of protein/ml and was stored at −80° until use. Liver cytosol was diluted to 100 μg of protein/ml in MDENG buffer (without molybdate or EGTA).

The translation products were diluted (2.5 μl into 1 ml of MDENG buffer; final concentration, ~100 μg of reticulocyte lysate protein/ml) and incubated with varying concentrations of [^{125}I]Br_2DpD, with or without a 200-fold molar excess of unlabeled TCDBF. For saturation plots, the liver cytosol and/or translation products were incubated overnight at 4°, the free ligand was absorbed with charcoal/gelatin (final concentrations, 1%/0.1%), and the supernatant was quantified by γ scintillation counting as reported previously (24). In some cases, as noted, the incubation was at 20° for varying periods of time.

Results

Poly(A)$^+$ RNA was prepared from the livers of C57BL/6 congenic mice homozygous for each of the *Ah* alleles and, using primers based on the cloned sequence from Hepa 1 cells (Ah^{b-1} alleles), cDNA was prepared, subcloned, and sequenced. In Fig. 2 are shown the deduced amino acid sequences for the ORF of each of the four murine *Ah* alleles. The deduced sequence for the Ah^{b-1} allele is identical to that isolated from Hepa 1 cells (14) and contains 805 amino acids; the Ah^{b-2} and Ah^d alleles each encode 848 amino acids and the Ah^{b-3} allele encodes 883 amino acids. In the ORF common to all of the alleles, i.e., the first 805 amino acids, there are only eight point mutations that result in amino acid differences between the alleles. The first nine amino acids, a spliced leader sequence for the Ah^{b-1} allele, and the basic helix-loop-helix sequences are identical for all of the alleles. The next >600 base pairs, encoding two 50-amino acid imperfect repeats (PAAS boxes) spaced by 100 amino acids, are identical in all four alleles except for a single point mutation in the second PAAS box. The translation termination codon, TGA in the Ah^{b-1} allele, has been mutated to CGA (arginine) in the other alleles, extending the ORF.

Because three other groups have reported sequencing of one or more of the murine Ah receptor alleles (14, 15, 25), it is useful to note any inconsistencies. Burbach *et al.* (14) reported sequence for the Ah^{b-1} allele in Hepa 1 cells, Ema *et al.* (15) for the Ah^{b-1} allele from C57BL/6 mice, and Chang *et al.* (25) for the Ah^{b-1} and Ah^d alleles from C57BL/6 and DBA/2 mice, respectively. We note the following disparities. 1) Amino acid 74 was reported by all authors for all alleles as serine, except in Hepa 1 cells, where it is threonine. 2) Amino acids 132, 133, 171, and 172 were found by us for all four alleles and by Burbach *et al.* (14) for Ah^{b-1} to be leucine, valine, glutamine, and leucine, respectively. In contrast, Ema *et al.* (15) found for Ah^{b-1} and Chang *et al.* (25) found for Ah^{b-1} and Ah^d that these residues

were phenylalanine, leucine, histidine, and valine, respectively. 3) Chang *et al.* (25) found amino acid 324 in Ah^d to be isoleucine, and we find it to be methionine. All reports agree that in Ah^{b-1} residue 324 is methionine. The *Ah* locus was first defined as a strain difference in responsiveness to polycyclic aromatic hydrocarbons (Ah^b, responsive; Ah^d, nonresponsive), and the difference was shown to be due to a difference in ligand binding affinity for the Ah^b versus Ah^d receptors. Because the Ah^{b-2} and Ah^d alleles encode proteins of the same size, it is instructive to compare their sequences (Fig. 2). They differ at only three amino acids, as follows: Ah^{b-2}, amino acid 348, phenylalanine; amino acid 375, alanine; and amino acid 754, threonine; Ah^d, amino acid 348, leucine; amino acid 375, valine; amino acid 754, isoleucine.

The cDNAs for the Ah^{b-1}, Ah^{b-2}, and Ah^d alleles were subcloned into the p-ALTER-1 phagemid (which can produce single-stranded DNA for mutagenesis and has two bacteriophage promoters for *in vitro* transcription). The cloned cDNAs were expressed in a combined transcription/translation system, using SP6 polymerase and rabbit reticulocyte lysate or wheat germ extract. Using 1 μg of plasmid DNA, the quantity of Ah receptor protein produced in the coupled reticulocyte or wheat germ system was roughly the same for each allele, as determined by [^{35}S]methionine incorporation (Fig. 3) and by immunochemical staining of Western blots (data not shown). The translated products were evaluated for ligand binding. Specific binding of [^{125}I]Br_2DpD was observed only with products formed in the reticulocyte lysate system and not in the wheat germ system (Table 1). Similar results have been observed for the translation of steroid hormone receptors. The hsp90, which is thought to be essential for conformational stability and ligand binding of the steroid hormone receptors and the Ah receptor, is present in reticulocyte lysate, but an immuno-cross-reacting species

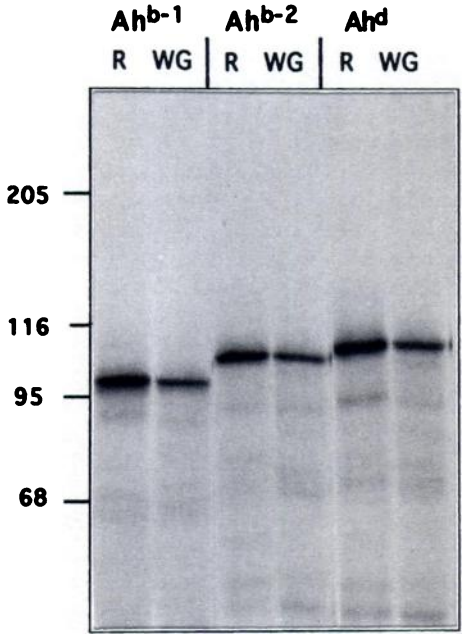


Fig. 3. Autoradiograph of translated products of Ah^{b-1}, Ah^{b-2}, and Ah^d cDNAs. One microgram of plasmid cDNA containing each of the alleles was transcribed and then translated in a combined system (T_NT; Promega) with SP6 polymerase plus rabbit reticulocyte lysate (*R*) or wheat germ (*WG*) extract, with [^{35}S]methionine. An aliquot (2.5 μl) of each reaction mixture was resolved on a 7.5% polyacrylamide gel, and the bands were visualized by autoradiography (see Materials and Methods).

TABLE 1

Radioligand binding to *in vitro* translated Ah^{b-1}, Ah^{b-2}, and Ah^d alleles of the murine Ah receptor

The p-ALTER-1 plasmids containing the Ah^{b-1}, Ah^{b-2}, and Ah^d cDNAs were transcribed and translated in a combined system (T_NT, with SP6 polymerase) that included rabbit reticulocyte lysate or wheat germ extract. A 2.5-μl aliquot of the reaction mixture was diluted with 1 ml of ice-cold MDENG buffer and incubated with 2.5×10^4 dpm of [^{125}I]Br_2 DpD, with or without a 200-fold molar excess of TCDBF, for 18 hr at 4°, unbound ligand was removed by charcoal/gelatin adsorption, and the specifically bound ligand was quantified.

Allele	Specific binding	
	Rabbit reticulocyte lysate	Wheat germ extract
	dpm/ml	
Ah^{b-1}	8161	0
Ah^{b-2}	8267	0
Ah^d	2170	0
Control (no cDNA added)	273	0

TABLE 2

Saturation binding to Ah^{b-1}, Ah^{b-2}, and Ah^d receptors in mouse liver cytosol and expressed in an *in vitro* translation system

Hepatic cytosol was prepared at ~7 mg/ml in MDENG buffer with +20 mM sodium molybdate and stored; for use, it was diluted to 100 μg/ml in MDENG buffer. One microgram of plasmid cDNA containing each of the *Ah* cDNAs was transcribed and translated in a 50-μl reaction, and the translation product was diluted (1.25 μl/ml of MDENG buffer). Binding was performed at various ligand concentrations, with or without a 200-fold molar excess of TCDBF, for 18 hr at 4°. The radioligand concentrations were 10, 20, 40, 60, 80, 100, and 200×10^3 dpm/ml and 400×10^3 dpm/ml for Ah^d.

	Number of points	Correlation coefficient	K_D	B_{max}
			pM	*fmol/mg*
Liver cytosol				
Ah^{b-1}	8	0.967	34	134
Ah^{b-2}	7	0.927	26	89
	8	0.913	35	109
Ah^d	7	0.847	91	40
	8	0.910	146	49
	8	0.892	146	51
	6	0.916	94	31
	8	0.892	146	51
				fmol/1.25 μl
Translation product				
Ah^{b-1}	7	0.857	10	11
	7	0.912	9	10
Ah^{b-2}	7	0.874	8	5
	7	0.928	6	5
Ah^d	7	0.838	37	3
	7	0.948	37	4

was not detected in wheat germ extract (26, 27). At a single ligand concentration, [^{125}I]Br_2DpD specific binding to the translated Ah^{b-1} and Ah^{b-2} receptors was approximately 4 times greater than binding to the low affinity Ah^d receptor.

Ligand binding to the murine Ah receptors was examined in full saturation curves (Table 2). For the *in vitro* translated Ah^{b-1} and Ah^{b-2} receptors, the equilibrium dissociation constants (K_D) ranged from 6 to 10 pM, whereas for the Ah^d allele the K_D was 37 pM, roughly 4–5 times larger. We also examined the specific ligand binding to hepatic cytosol from C57BL/6 congenic mice homozygous for the Ah^{b-1}, Ah^{b-2}, and Ah^d alleles. As noted previously, the apparent K_D is a function of the Ah receptor concentration and total protein concentration, i.e., at higher total protein concentrations there is increasing misclassification of nonspecific binding and an increase in the apparent K_D value (24). Thus, the Ah^{b-1} and Ah^{b-2} receptors at 100 μg of

cytosolic protein/ml had K_D values of ~20–35 pM and the Ah^d receptor had a K_D value of ~90–140 pM (mean, 125 pM), which is roughly 4–5-fold higher.

The difference in sensitivity to polycyclic aromatic hydrocarbons that originally defined the *Ah* locus appears to reside solely in the Ah receptor, because we see a difference in specific ligand binding to liver cytosol from C57BL/6 congenic mice (Ah^b versus Ah^d) and to the *in vitro* translated products of the *Ah*$^{b-2}$ versus *Ah*^{d} alleles. As noted above, the *Ah*$^{b-2}$ and *Ah*^{d} alleles encode receptors that differ at only three amino acids. We mutated each of the alleles, at one or both of the amino acid positions in the ligand binding domain at which they differ, to encode the amino acid of the alternate allele. As shown in Table 3, the translated product of the wild-type *Ah*$^{b-2}$ allele had about 3.5 times the specific binding of the *Ah*^{d} product (at one ligand concentration). Mutation of the *Ah*$^{b-2}$ allele to change amino acid 348 from phenylalanine to leucine yielded a protein that retained ligand binding affinity. In contrast, mutation of the *Ah*$^{b-2}$ allele to change amino acid 375 from alanine to valine greatly reduced specific binding, as did the double mutation. For the *Ah*^{d} allele, mutation of amino acid 348 from leucine to phenylalanine did not enhance binding, but conversion of amino acid 375 from valine to alanine, or the double mutation, resulted in greater binding. In summary, the data indicate that the lower affinity binding in the *Ah*^{d} allele is attributable to the point mutation changing Ala_{375} to Val_{375}, and hence this site is critical to the ligand binding domain.

One can further define the ligand binding domain of the Ah receptor by using receptor constructs that delete part of the protein. Dolwick *et al.* (28) previously characterized the ligand binding domain of the *Ah*$^{b-1}$ allele. We examined three series of truncated receptor constructs, containing 1) carboxyl-end truncations produced by mutating the ORF codons to translation termination codons, 2) a series of amino-terminal deletions produced largely by PCR, usually with a 5′ primer containing a *Hind*III restriction site and a consensus translation start sequence connected to sequence complementary to progressively more 3′ sequence in the ORF, or 3) amino-terminal deletions combined with carboxyl translation termination at amino acid 402. The constructs were examined by sequencing over point mutations, determination of the size of the cDNA, and determination on gels of the sizes of the [^{35}S]methionine-labeled translation products, compared with their calculated sizes.

In Fig. 4 are seen the results of ligand binding by a series of carboxyl-terminally truncated forms of the Ah^{b-1} receptor. Specific binding equivalent to that seen with the full length construct (1–805 amino acids) was unaffected by successive truncations down to 403 amino acids, but further shortening to 377 amino acids eliminated binding. Thus, the carboxyl-terminal side of the ligand binding domain is sharply defined.

In a second series we examined the effect of amino-terminal truncations (Fig. 5). Deletions from the amino-terminal side produced a gradual loss of ligand binding. 1) Removal of amino acids 2–78, the basic helix-loop-helix region, resulted in a ~30% decrease in binding; 2) further truncation from amino acid 79 to amino acid 129 produced loss of ~30% of the original binding; 3) further shortening by removal of amino acids 130–181, the first of the two 50-amino acid repeats (or PAAS boxes), resulted in an additional loss of binding of ~10%; and 4) removal of amino acids 182–287 produced complete loss of specific binding. In a third series of truncation experiments (Fig. 5, *bottom*), the same amino-terminal truncations were tested with carboxyl termination at amino acid 402. In this series, approximately the same graded loss of ligand binding as observed with amino-terminal deletions was seen. The reason for this graded loss of binding with amino-terminal deletions is unknown but may represent reduced binding to hsp90 and hence reduced stabilization of the Ah receptor. Our findings corroborate those of Dolwick *et al.* (28).

Discussion

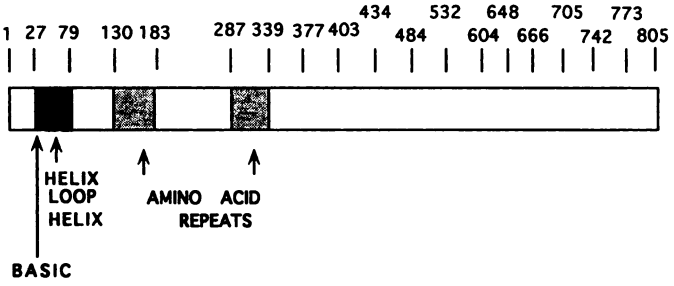
The murine *Ah* locus was first defined as a strain difference in response to polycyclic aromatic hydrocarbons and was later

TABLE 3

Effect of point mutations on the specific radioligand binding of the transcribed/translated products of the cDNAs of the *Ah*$^{b-2}$ and *Ah*^{d} alleles

The cDNAs for *Ah*$^{b-1}$ and *Ah*^{d} alleles, cloned into p-ALTER-1, were mutated at two specific sites (see Materials and Methods). Equivalent amounts of plasmid DNA containing each of the constructs were transcribed and translated in the combined reticulocyte system, and specific ligand binding was determined with an aliquot of the reaction mixture (1.25 μl diluted to 1 ml with MDENG) by incubation at 4° overnight with 4 × 10^4 dpm/ml [^{125}I]Br_2DpD, with or without a 200-fold molar excess of TCDBF (see Materials and Methods).

	Specific binding
	dpm/ml
Ah^{b-2} wild-type (F_{348}, A_{375})	7,048
Ah^d wild-type (L_{348}, V_{375})	2,143
Ah^{b-2} $F_{348} \rightarrow L_{348}$	10,445
Ah^{b-2} $A_{375} \rightarrow V_{375}$	555
Ah^{b-2} $F_{348} \rightarrow L_{348}$, $A_{375} \rightarrow V_{375}$	1,737
Ah^d $L_{348} \rightarrow F_{348}$	760
Ah^d $V_{375} \rightarrow A_{375}$	12,477
Ah^d $L_{348} \rightarrow F_{348}$, $V_{375} \rightarrow A_{375}$	6,931



AA	SPECIFIC BINDING (dpms)	AA	SPECIFIC BINDING (dpms)
1-805	5800	1-805	8481
1-773	5843	1-604	8131
1-742	7209	1-532	9994
1-705	7737	1-484	7583
1-666	6638	1-434	8656
1-648	5901	1-403	7914
		1-377	86

Fig. 4. Effect of carboxyl-terminal truncations in the *Ah*$^{b-1}$ allele on ligand binding. The diagram is representative of the Ah^{b-1} receptor protein, depicting the amino-terminal basic helix-loop-helix region and two 50-amino acid repeats (PAAS boxes) spaced 100 amino acids apart. The *Ah*$^{b-1}$ cDNA cloned into p-ALTER-1 was mutagenized by converting codons in the ORF to translation stop sites. The cDNA was transcribed and translated in a combined system; the mixture was diluted in MDENG buffer and specific binding of radioligand was determined. *AA*, amino acids.

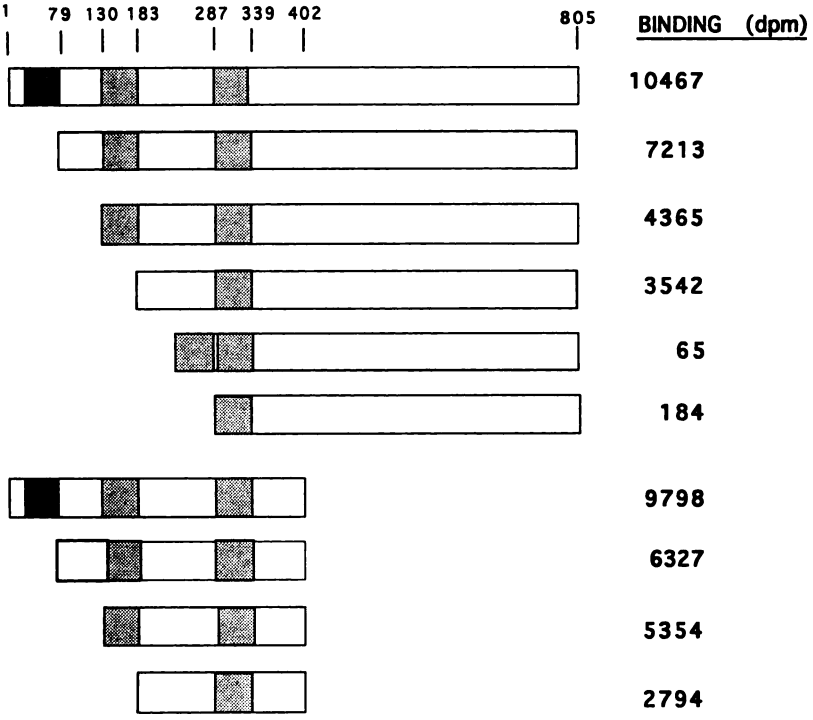


Fig. 5. Effect of amino-terminal truncations of the Ah^{b-1} receptor on ligand binding. The Ah^{b-1} constructs were synthesized, transcribed, and translated and radioligand binding was determined as described in Materials and Methods.

shown to encode the Ah receptor, which binds these planar aromatic ligands to mediate their pleiotropic effects. In this report we present the amino acid sequences for all four murine alleles, deduced from their cloned and sequenced cDNA.

All of the Ah^b alleles (Ah^{b-1}, Ah^{b-2}, and Ah^{b-3}) display high affinity ligand binding. These alleles differ by a few point mutations in the common ORF and by additional sequence at their carboxyl ends; Ah^{b-1} contains 805 amino acids, Ah^{b-2} contains 848 amino acids, and Ah^{b-3} contains 883 amino acids. The amino-terminal half of the protein contains the DNA binding domain (the basic region), the Arnt binding domain (helix-loop-helix), and the ligand binding domain. In truncation experiments the carboxyl side of the ligand binding domain was sharply defined at amino acid 402, and amino-terminal truncations produced a more graded loss of binding. These results are in accord with those of Dolwick *et al.* (28). The function of the carboxyl half of the Ah receptor remains to be determined, but the glutamine-rich region may be involved in transcription activation.

The Ah^d allele, which encodes the receptor with the lower ligand binding affinity, is most appropriately compared with the Ah^{b-2} allele, because the two alleles express proteins of the same size that differ in only two amino acids. In this report, it is shown that the lower ligand affinity of the Ah^d receptor is attributable to valine at position 375 and that mutation of this residue to alanine, as found in the Ah^{b-2} receptor, enhances ligand affinity (Table 3). For *in vitro* translated products, we found that [^{125}I]Br_2DpD binding shows a K_D of ~7 pM for the Ah^{b-2} allele and a K_D of ~37 pM for the Ah^d allele, an approximately 5-fold difference. [The *in vitro* translated Ah^{b-1} allele has a K_D of ~9 pM, close to its estimated true K_D (24).] The apparent K_D for the Ah receptor is a function of the receptor concentration, relative to total protein concentration, and approaches a limiting value at infinite dilution that is equivalent to the kinetic K_D ($K_D = k_{-1}/k_{+1}$). As also shown in Table 3, when liver cytosol is the source of receptor the apparent K_D values are ~30 pM for Ah^{b-2} and ~125 pM for Ah^d, also a 4–5-fold difference, but with higher absolute values attributable to the receptor/protein concentration.

It is common to contrast C57BL/6 and DBA/2 mice, because the *Ah* locus was first defined as a difference in response to polycyclic aromatic hydrocarbons between these two strains. Because these strains differ in many genes and traits, other than the *Ah* locus, that can influence drug action and pharmacokinetics, it is preferable to compare congenic strains, i.e., mice with the same genetic background differing only in the trait of interest, e.g., C57BL/6·(Ah^{b-1}) versus C57BL/6·(Ah^d). However, there is another, perhaps less widely appreciated, difference between these two strains; C57BL/6 carries the Ah^{b-1} allele, so Ah^{b-1} is contrasted with Ah^d. The Ah^{b-1} receptor is more thermostable (7) than the Ah^{b-2} receptor and is not as easily activated *in vitro*. The addition of sodium molybdate to buffer stabilizes the Ah^{b-2} and Ah^d receptors much more than the Ah^{b-1} receptor (7, 29). These observations all suggest that the Ah^{b-1} receptor is more stable and binds to hsp90 more avidly than do the Ah^{b-2} and Ah^d variants. With these caveats in mind, it is useful to examine pertinent observations from the literature.

For the induction of hepatic AHH activity by TCDD, the ED_{50} values were found to be ~1.7×10^{-9} mol/kg for C57BL/6 mice and ~1.6×10^{-8} mol/kg for DBA/2 mice, a 9-fold difference (4). In general, an approximately 10-fold difference was found between Ah^{b-1} or Ah^{b-2} and Ah^d strains (4). Using [^{3}H] TCDD, stabilization of the Ah^d receptor with sodium molybdate, and velocity sedimentation assays, Okey *et al.* (29) reported that in liver cytosol from C57BL/6 mice the K_D (Ah^{b-1}) was $1.8 \pm 0.2 \times 10^{-9}$ M and in that from DBA/2 mice the K_D was $16 \pm 2.5 \times 10^{-9}$ M, a 9-fold difference.

Whereas Ah^d mice show a nearly absolute insensitivity to polycyclic aromatic hydrocarbons *in vivo*, primary fetal cell

cultures from Ah^d mice respond to polycyclic aromatic hydrocarbons *in vitro* (30, 31). Niwa *et al.* (30) reported that the EC_{50} for TCDD induction of AHH activity in fetal cell cultures derived from C57BL/6 mice was 0.12×10^{-9} M, compared with 1.9×10^{-9} M for cells derived from DBA/2 mice, a 16-fold difference. A similar ~10-fold difference was seen with 3-MC as an inducer.

In a more recent report, Harper *et al.* (32) compared AHH induction in fetal cell cultures derived from C57BL/6 and DBA/2 mice and found that the EC_{50} values for TCDD and 1,2-benzanthracene were virtually identical in cultures from the two strains; furthermore, the binding affinities for [^{3}H]TCDD were similar (2–3-fold differences in K_d values between strains). It is difficult to explain these data showing the loss in fetal cell cultures of the differences seen in adult mice. Our results in the current study indicate that the lower ligand binding affinity and reduced sensitivity of the Ah^d receptor are intrinsic in the primary structure of the receptor. It is possible that 1) post-translational modification is present in rabbit reticulocyte lysate but not in fetal tissue or 2) there are higher receptor concentrations in fetal tissue versus adult tissue, enhancing mass action; this might explain the results of Harper *et al.* (32).

In summary, in this report we present the amino acid structures of the four murine variants and identify the point mutation in the *Ah^d* allele that accounts for lower affinity binding. Further analysis of the Ah receptor domains should provide a deeper understanding of structure-function relationships.

References

1. Schmid, A., I. Elmer, and G. S. Tarnowski. Genetic determination of differential inflammatory reactivity and subcutaneous tumor susceptibility of AKR/J and C57BL/6J mice to 7,12-dimethylbenz[*a*]anthracene. *Cancer Res.* **29**:1585–1589 (1969).
2. Nebert, D. W., F. M. Goujon, and J. E. Gielen. Aryl hydrocarbon hydroxylase induction by polycyclic hydrocarbons: simple autosomal dominant trait in the mouse. *Nature New Biol.* **236**:107–110 (1972).
3. Poland, A., and E. Glover. Comparison of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, a potent inducer of aryl hydrocarbon hydroxylase, with 3-methylcholanthrene. *Mol. Pharmacol.* **10**:349–359 (1974).
4. Poland, A., and E. Glover. Genetic expression of aryl hydrocarbon hydroxylase by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin: evidence for a receptor mutation in genetically non-responsive mice. *Mol. Pharmacol.* **11**:389–398 (1975).
5. Poland, A., E. Glover, and A. S. Kende. Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin by hepatic cytosol. *J. Biol. Chem.* **251**:4936–4946 (1976).
6. Poland, A., E. Glover, and B. A. Taylor. The murine *Ah* locus: a new allele and mapping to chromosome 12. *Mol. Pharmacol.* **32**:471–478 (1987).
7. Poland, A., and E. Glover. Characterization and strain distribution pattern of the murine Ah receptor specified by the *Ah^d* and *Ah^{b-3}* alleles. *Mol. Pharmacol.* **38**:306–312 (1990).
8. Wilhelmsson, A., S. Cuthill, M. Denis, A.-C. Wikstrom, and J.-Å. Gustafsson. The specific DNA-binding activity of the dioxin receptor is modulated by the 90 kd heat shock protein. *EMBO J.* **9**:69–76 (1991).
9. Perdew, G. H. Comparison of the nuclear and cytosolic forms of the Ah receptor from Hepa1c1c7 cells: charge heterogeneity and ATP binding properties. *Arch. Biochem. Biophys.* **291**:284–290 (1991).
10. Denison, M. S., J. M. Fisher, and J. P. Whitlock, Jr. The DNA recognition site for the dioxin-Ah receptor complex. *J. Biol. Chem.* **263**:17221–17224 (1988).
11. Lusska, A., E. Shen, and J. P. Whitlock, Jr. Protein-DNA interactions at the dioxin-responsive enhancer. *J. Biol. Chem.* **265**:6575–6580 (1993).
12. Hoffmann, E. C., H. Reyes, F.-F. Chu, F. Sander, L. H. Conley, B. A. Brooks, and O. Hankinson. Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science (Washington D. C.)* **252**:952–958 (1991).
13. Reyes, H., S. Reisz-Porszasz, and O. Hankinson. Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA-binding form of the Ah receptor. *Science (Washington D. C.)* **256**:1193–1195 (1992).
14. Burbach, K. M., A. Poland, and C. A. Bradfield. Cloning of the Ah receptor cDNA reveals a distinctive ligand-activated transcription factor. *Proc. Natl. Acad. Sci. USA* **89**:8185–8189 (1992).
15. Ema, M., K. Sogawa, N. Watanabe, Y. Chujoh, N. Matsushita, O. Gotoh, Y. Funae, and Y. Fujii-Kuriyama. cDNA cloning and structure of mouse putative Ah receptor. *Biochem. Biophys. Res. Commun.* **184**:246–253 (1992).
16. Dolwick, K. M., J. V. Schmidt, L. A. Carver, H. I. Swanson, and C. A. Bradfield. Cloning and expression of the human Ah receptor cDNA. *Mol. Pharmacol.* **44**:911–917 (1993).
17. Sanger, F., S. Nicklen, and A. R. Coulson. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467 (1977).
18. Kozak, M. An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* **15**:8125–8132 (1987).
19. Promega Corp. Altered Sites *in vitro* mutagenesis system. Manufacturer's instructions. Promega Corp., Madison, WI.
20. Promega Corp. T_NT coupled reticulocyte lysate system and T_NT coupled wheat germ extract system. Manufacturer's instructions. Promega Corp., Madison, WI.
21. Laemmli, U. K. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**:680–685 (1970).
22. Laskey, R. A., and A. D. Mills. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* **56**:335–341 (1975).
23. Poland, A., E. Glover, F. H. Ebetino, and A. S. Kende. Photoaffinity labeling of the Ah receptor. *J. Biol. Chem.* **261**:6352–6365 (1986).
24. Bradfield, C. A., A. S. Kende, and A. Poland. Kinetic and equilibrium studies of aryl hydrocarbon receptor-ligand binding: use of [^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin. *Mol. Pharmacol.* **34**:229–237 (1988).
25. Chang, C.-Y., D. R. Smith, V. S. Prasad, C. L. Sidman, D. W. Nebert, and A. Puga. Ten nucleotide differences, five of which cause amino acid changes, are associated with the Ah receptor locus polymorphism of C57BL/6 and DBA/2 mice. *Pharmacogenetics* **3**:312–321.
26. Dalman, F. C., E. H. Bresnick, P. D. Patel, G. H. Perdew, S. J. Watson, Jr., and W. B. Pratt. Direct evidence that the glucocorticoid receptor binds to hsp90 at or near the termination of receptor translation *in vitro*. *J. Biol. Chem.* **25**:19815–19821 (1989).
27. Dennis, M., and J.-Å. Gustafsson. Translation of glucocorticoid receptor *in vitro* yields a nonactivated protein. *J. Biol. Chem.* **264**:6005–6008 (1989).
28. Dolwick, K., H. I. Swanson, and C. A. Bradfield. *In vitro* analysis of Ah receptor domains involved in ligand-activated DNA recognition. *Proc. Natl. Acad. Sci. USA* **90**:8566–8570 (1993).
29. Okey, A. B., L. M. Vella, and P. A. Harper. Retention and characterization of a low affinity form of Ah receptor in livers of mice "nonresponsive" to induction of cytochrome $P_1$450 by 3-methylcholanthrene. *Mol. Pharmacol.* **35**:823–830 (1989).
30. Niwa, A., K. Kumaki, and D. W. Nebert. Induction of aryl hydrocarbon hydroxylase activity in various cell cultures by 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin. *Mol. Pharmacol.* **11**:399–408 (1975).
31. Hitchins, V. M., B. M. Laine, and J. J. Hutton. Genetic regulation of aryl hydrocarbon hydroxylase in primary cultures of fetal cells established from inbred strains of mice. *Cancer Res.* **40**:427–431 (1980).
32. Harper, P. A., C. L. Golas, and A. B. Okey. Ah receptor in mice genetically "nonresponsive" for cytochrome P450IA1 induction: cytosolic Ah receptor, transformation to the nuclear binding state, and induction of aryl hydrocarbon hydroxylase by halogenated and nonhalogenated aromatic hydrocarbons in embryonic tissues and cells. *Mol. Pharmacol.* **40**:818–826 (1991).

Send reprint requests to: Alan Poland, McArdle Laboratory for Cancer Research, University of Wisconsin, 1400 University Ave., Madison, WI 53706.