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Characterization and Strain Distribution Pattern of the Murine Ah Receptor Specified by the Ah^d and Ah^{b-3} Alleles

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SUMMARY

Two allelic forms of the Ah receptor have been previously identified by covalent labeling of the hepatic cytosol fractions of inbred strains of mice with the photoaffinity ligand 2-azido-3-[^{125}I] iodo-7,8-dibromodibenzo-*p*-dioxin and resolution of the labeled protein by denaturing gel electrophoresis: 1) a M_r 95,000 protein encoded by the Ah^{b-1} allele carried by the C57 and C58 family of mice, and 2) a M_r 104,000 protein encoded by the Ah^{b-2} allele present in other common inbred strains that are responsive to aromatic hydrocarbons (e.g., C3H/He, BALB/cBy, and A). In this report, ^{125}I-photoaffinity labeling is used to characterize two further murine variants and the strains that carry them: 1) the low affinity Ah receptor (Ah^d allele) in strains that are nonresponsive to aromatic hydrocarbons and 2) a newly identified, high affinity variant (Ah^{b-3}) found in several strains recently derived from feral mice. The low affinity Ah receptor has been recently characterized by reversible ligand binding by Okey *et al.* [*Mol. Pharmacol.* 35:823–830 (1989)], through the inclusion of sodium molybdate in the buffers during tissue preparation and ligand incubation to stabilize the receptor. Examination of the Ah receptor in hepatic cytosol from 18 strains of mice carrying the Ah^d allele, by preparation in molybdate and photoaffinity labeling, revealed that all strains express a M_r 104,000 protein. Tissue preparation in 20 mM sodium molybdate and subsequent dilution of the molybdate to ~0.5 mM during ligand incubation was found to enhance photoaffinity labeling of the high and low affinity allelic forms of the Ah receptor. A new variant of the receptor (Ah^{b-3}) expressing a M_r 105,000 protein was detected in *Mus molossinus*, *hortulanus*, *pahari*, *spretus*, and *caroli* but was absent from the strains of *Mus musculus* or *domesticus* that were examined. Allelic variants were also distinguishable by thermolability, i.e., the half-life of specific ligand binding capacity upon incubation at 35°. For the Ah^{b-1} allele (M_r 95,000) the $t_{½}$ (thermostability) is 20–30 min, for the Ah^{b-2} allele (M_r 104,000) the $t_{½}$ is 3–6 min, and for the Ah^{b-3} allele (M_r 105,000) the thermolability is intermediate.

The Ah receptor is a soluble protein detectable in a wide variety of tissues from vertebrate species that displays high affinity specific binding to certain planar aromatic compounds (i.e., polycyclic aromatic hydrocarbons, typified by 3-methylcholanthrene, and halogenated aromatic hydrocarbons, typified by TCDD) and initiates the coordinate expression of a number of genes (1–3). The canonical and best understood response mediated by the receptor is the induction of cytochrome P_1-450 (P4501A1) and associated monooxygenase activity (most often measured as AHH activity) (3). Several lines of evidence suggest that the ligand-receptor complex acts as a transcriptional activator by binding to specific "dioxin-responsive elements" with enhancer activity that are upstream from the first exon of the gene for cytochrome P_1-450 (4, 5). The Ah receptor is presumed to be a member of the erb-A superfamily (6) (which includes the steroid hormone, vitamin D, retinoic acid, and thyroid hormone receptors) because of its similarities to the steroid hormone receptors, i.e., 1) the unactivated receptor is associated with the M_r 90,000 heat shock protein (7, 8), 2) upon binding of a small molecule ligand, the receptor undergoes a temperature-dependent conformational change with enhanced affinity for DNA (i.e., activation), and 3) sodium molybdate partially stabilizes the Ah receptor, enhances ligand binding, and prevents activation (9).

The Ah receptor appears to differ from the steroid hormone receptors in two notable aspects, 1) to date, no endogenous or physiologic ligand has been identified and 2) there is remarkable variation in the structure of the Ah receptor among species and among strains within a species (10, 11).

The *Ah* locus, believed to be the structural gene for the Ah receptor, was first defined 20 years ago as a functional polymorphism (12–14), the difference among inbred strains of mice in response to polycyclic aromatic hydrocarbons. Certain inbred strains of mice (e.g., C57BL/6) when challenged with 3-methylcholanthrene respond with the induction of cytochrome P_1-

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ABBREVIATIONS: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDBF, 2,3,7,8-tetrachlorodibenzofuran; AHH, aryl hydrocarbon hydroxylase; [^{125}I] N_3Br_2DpD, 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin; [^{125}I]Br_2DpD, 2-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

450 (CYP1A1) and AHH activity, whereas other strains (e.g., DBA/2) fail to respond. In genetic crosses between C57BL/6 and DBA/2 mice, the trait of aromatic hydrocarbon responsiveness segregates in a simple autosomal dominant mode. TCDD, a far more potent agonist, was found to induce AHH activity in all strains of mice, both responsive (carrying the Ah^b allele) and nonresponsive (carrying the Ah^d allele) to polycyclic aromatic hydrocarbons (15).

These observations led to the hypothesis that 1) the *Ah* locus encodes a receptor that specifically binds agonists and initiates enhanced expression of cytochrome P_1-450 and 2) mice carrying the Ah^d allele express a receptor with reduced affinity for these compounds and, hence, display reduced sensitivity to induction. The advent of high specific activity [^{3}H]TCDD provided direct support for these conjectures, with the demonstration in hepatic cytosol from C57BL/6 mice of high affinity saturable binding sites for which other agonists showed an affinity that corresponded to their *in vivo* potency to induce AHH activity and little or no demonstrable specific binding of radioligand in the liver cytosol from DBA/2 mice (16).

Characterization of the Ah receptor has been approached with two methodologies, 1) most commonly, reversible binding of radioligand and examination of the physicochemical properties of the ligand-receptor complex under nondenaturing conditions (17–19) and 2) covalent labeling with the photoaffinity ligand [^{125}I]N_3Br_2DpD and analysis by denaturing gel electrophoresis and autoradiography (20). Among inbred strains of mice, three allelic variants of the Ah receptor have been described to date. For strains responsive to polycyclic aromatic hydrocarbons (i.e., Ah^b), two forms have been detected by photoaffinity labeling; the C57 and C58 family and MA/My mice express a M_r 95,000 ligand-binding protein of the Ah receptor (expressed by Ah^{b-1} allele), whereas the other responsive strains (e.g., A, BALB/c, C3H/He) express a M_r 104,000 protein (Ah^{b-2} allele) (10). The low affinity Ah receptor from mice that are nonresponsive to polycyclic aromatic hydrocarbons (Ah^d allele) has been undetectable in liver cytosol by reversible or covalent radioligand binding. However, following *in vivo* administration of [^{3}H]TCDD to Ah^d mice, the activated radioligand-Ah receptor complex extracted from the nucleus has been identified in a sucrose gradient (21). Recently, Okey *et al.* (22) were able to demonstrate [^{3}H]TCDD binding to the low affinity Ah receptor in hepatic cytosol from mice with the Ah^d allele and to characterize the radioligand-receptor complex by velocity sedimentation. This long sought-after goal was achieved by stabilization of the receptor with 20 mM sodium molybdate, present from tissue preparation through receptor binding. Sodium molybdate is well known to stabilize several steroid hormone receptors (23–25) but previously has been reported to have minimal effects on the Ah receptor from rat or mouse.

In this report we screen a number of mouse strains, including those more recently established from feral mice, in search of allelic variants of the Ah receptor. On the basis of apparent molecular weights of photoaffinity-labeled peptides and thermolability, we now report four alleles at the *Ah* locus in strains of *Mus*, Ah^{b-1} (M_r 95,000), Ah^{b-2} (M_r 104,000), Ah^d (low affinity, M_r 104,000), and the newly detected Ah^{b-3} (M_r 105,000).

Materials and Methods

Mice. Classical inbred strains of mice and some strains derived from more recently captured feral mice (as indicated in Table 1) were obtained from The Jackson Laboratory (Bar Harbor, ME). Other strains derived from recently captured feral mice (see Table 1) were obtained from the Hazelton Laboratory (Rockville Pike, MD), where they were maintained under contract for Dr. Michael Potter, National Cancer Institute. These latter mice were considered to be carriers of viral disease, and the single animal obtained from each strain was killed immediately upon receipt. More information on the geographic origin, capture, and husbandry of these more recently created strains can be obtained from The Jackson Laboratory, the Hazelton Laboratory, or standard references on mice (26–30).

Three strains of C57BL/6J mice congenic at the *Ah* locus were maintained, 1) C57BL/6J, carrying the Ah^{b-1} allele; 2) B6·C-H-38^c/By, constructed by Dr. Donald Bailey (31) to define a minor histocompatibility locus in BALB/cBy mice and carrying the Ah^{b-2} allele; and 3) B6J·D2N (Ah^d), expressing the Ah^d allele, originally constructed by Dr. Dan Nebert, National Institute of Child Health and Human Development, were bred, and phenotyped by our laboratory as previously described (37).

All mice were maintained in plastic cages with hardwood chip bedding, with a 12-hr light/dark diurnal cycle, at constant temperature and humidity. Water and food (Wayne Mouse Rodent Blox; Wayne Pet Food Division, Continental Grain Co., Chicago, IL) were available *ad libitum*. For the feral strains, the diet was supplemented with a birdseed mix.

Radioligands. [^{125}I]N_3Br_2DpD (20) and [^{125}I]Br_2DpD (32) were synthesized and purified to near homogeneity, as previously described, and had an initial specific activity of 2176 Ci/mmol.

Buffers. MEN (25 mM MOPS, 1 mM EDTA, 0.02% NaN_3, pH 7.5); MDEN (the same plus 1 mM dithiothreitol, and MDENG (the latter plus 10% glycerol) were used.

Preparation of hepatic cytosol. The mice were killed by cervical dislocation and the liver was perfused with MEN plus 150 mM NaCl, excised, weighed, and homogenized with 9 volumes of MDENG plus 5 mM EGTA, with or without 20 mM sodium molybdate. The homogenate was centrifuged at 10,000 × *g* for 20 min; the supernatant fraction was carefully aspirated to avoid the upper layer of lipid and centrifuged at 100,000 × *g* for 1 hr. The supernatant fraction (~10 mg of protein/ml) was stored at −70° until use.

Photoaffinity labeling. For mice carrying alleles that specify high affinity Ah receptors (i.e., Ah^{b-1}, Ah^{b-2}, and Ah^{b-3} alleles), the standard incubation conditions consisted of hepatic cytosol at 1 mg of protein/ml in MENG buffer and 1 nM [^{125}I]N_3Br_2DpD, with or without 200-fold unlabeled TCDBF, incubated for 30 min at 20°. The mixture was cooled to 0° for 5 min and a suspension of charcoal/dextran (final concentration, 0.5%/0.05%, w/v) was added, incubated for 5 min at 0°, and removed by centrifugation. The supernatant fraction was immediately irradiated with UV light and precipitated with acetone, as previously described (20). For mice expressing the low affinity receptor, the standard incubation conditions were modified as follows: 1) preparation of liver cytosol in buffer containing 20 mM sodium molybdate, 2) cytosolic protein concentration of 200 μg/ml plus 2 nM ^{125}I-photoaffinity ligand with or without 200-fold excess TCDBF in MENG buffer (Na_2MoO_4 thus diluted to ~0.4 mM), and 3) incubation at 0° for 2 hr and then addition of charcoal/dextran (final concentration, 1.0%/0.1%) and incubation for 30 min at 0° before removal. Photoaffinity labeling of the low affinity receptor has been carried out under a variety of conditions, and preparation of the cytosol in buffer containing sodium molybdate appears to be the most significant variable. UV irradiation and precipitation were the same as for high affinity receptors.

Gel electrophoresis and autoradiography. The precipitated photoaffinity-labeled samples were dissolved in electrophoresis sample buffer and subjected to denaturing electrophoresis on discontinuous polyacrylamide slab gels, by the method of Laemmli (33) (4% stacking gel; 7.5% separating gel; acrylamide/bisacrylamide, 30:0.8; 1.5-mm thick; 15 mA current/gel). The gels were fixed and dried, and an autoradiograph was developed as previously described (20). The molec-

TABLE 1
Strains and substrains of feral mice

Strain	Other names or designations	Origin	Generation	Source
MOLF/Ei	Inbred *Mus molossinus*	Japan	>F20	J[a]
PERA/Ei	Inbred Peru-Atteck	Peru	>F20	J
CAST/Ei	Inbred *Mus casteneus*		F43	J
Mus caroli		Thailand		J
Mus domesticus				J
Mus spretus		Spain		J
SK/Cam Ei		Stockholm, Sweden	>F20	J
SF/Cam Ei		San Francisco	>F20	J
Peromyscus maniculatus	Deer mouse			
Mus caroli	CARO	Thailand	F20	H[b]
Mus pahari	PARA I	Thailand	F11	H
Mus pahari	PARA II	Thailand	F16	H
Mus spretus	SPRET I	Spain	F16	H
Mus spretus	SPRET 2	Spain	F17	H
Mus hortulanus	HORT-H	Austria	F11	H
Mus cookii	COOK	Thailand	F15	H
Mus musculus	SKIVE	Denmark	F11	H
Mus musculus	Sanner's Farm	Maryland	F4	H
Mus musculus	CZECH I	Czechoslovakia	F19	H
Mus musculus	CZECH II	Czechoslovakia	F3	H
Mus domesticus brevirostris	BREV	Morocco	F2	H
Mus domesticus praetextus	PRAE	Morocco	F4	H

[a] J, The Jackson Laboratory (Bar Harbor, ME).
[b] H, Hazelton Laboratories (Rockville, MD). Maintained for the National Cancer Institute at the request of Dr. Michael Potter.

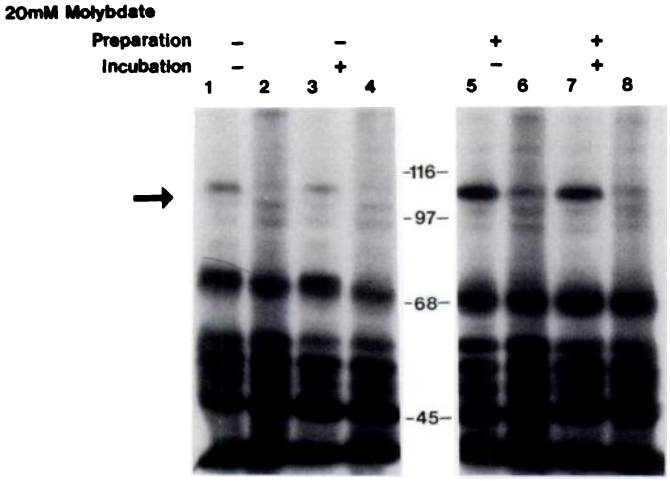

Fig. 1. Effect of sodium molybdate on photoaffinity labeling of the Ah receptor expressed by the *Ah*^{d} allele. The hepatic cytosol fraction from B6J·D2N (*Ah*^{d}) mice was prepared in MENG buffer, to which 20 mM sodium molybdate was or was not added. The cytosol, diluted to 150 μg of protein/ml, was incubated with 2 nM [^{125}I]N_3Br_2DpD, with or without a 200-fold excess of TCDBF, in MDENG buffer with either no sodium molybdate, dilution of the initial molybdate to ~0.5 mM, or 20 mM MoO_4^{2-} (see Materials and Methods). In the autoradiograph, *lanes 1*, *3*, *5*, and *7*, incubation with the photoaffinity ligand alone and *lanes 2*, *4*, *6*, and *8*, incubations with the photoaffinity label plus excess TCDBF.

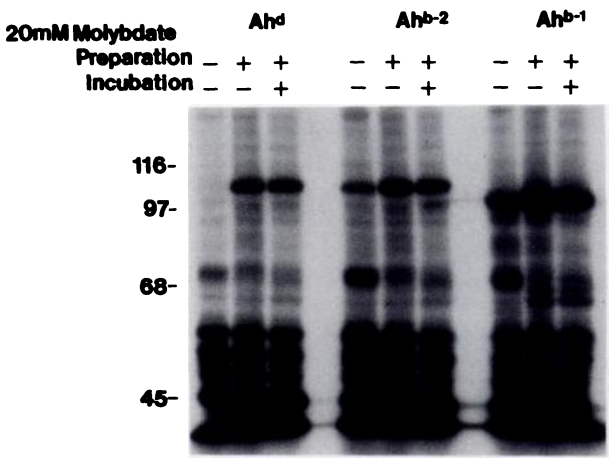

Fig. 2. Effect of sodium molybdate during tissue preparation and ligand incubation on photoaffinity labeling of the Ah receptor in hepatic cytosol of congenic B6 mice expressing *Ah*$^{b-1}$, *Ah*$^{b-2}$, or *Ah*^{d} alleles. The hepatic cytosolic fraction of B6 (*Ah*$^{b-1}$), B6·C-H-38^c/By (*Ah*$^{b-2}$), or B6J·D2N (*Ah*^{d}) mice was prepared in MENG buffer, with or without 20 mM sodium molybdate. The cytosols were then diluted to 200 μg/ml with the same buffer, with or without 20 mM molybdate so the final concentration of MoO_4^{2-} was 0 mM, ~0.4 mM, or 20 mM, and incubated with 2 nM ^{125}I-photoaffinity ligand for 2 hr at 0°. After removal of excess ligand, the sample was irradiated, subjected to gel electrophoresis, and visualized by autoradiography (see Materials and Methods). The radioactivity in the specifically labeled bands was quantified by γ-scintillation spectrometry. B6 (*Ah*^{d}): *lane 1*, 387 dpm; *lane 2*, 2396 dpm; *lane 3*, 2113 dpm; B6·C-H-38^c: *lane 4*, 1513 dpm; *lane 5*, 3116 dpm; *lane 6*, 2381; B6: *lane 7*, 3850 dpm; *lane 8*, 7684 dpm; *lane 9*, 5737 dpm.

ular weight standards were: myosin, M_r 205,000; β-galactosidase, M_r 116,000; phosphorylase b, M_r 97,500; bovine serum albumin, M_r 68,000; and ovalbumin, M_r 45,000.

Heat denaturation. Hepatic cytosol, prepared and stored as described above (~10 mg of protein/ml in MDENG buffer), was thawed on ice, diluted 1:3 with the same buffer prewarmed to 35°, and incubated in a 35° water bath. At the indicated time points (0, 10, 20, and 30 min for mice carrying the *Ah*$^{b-1}$ allele; 0, 3, 6, 9, 12, and 15 min for mice carrying the *Ah*$^{b-2}$ and *Ah*$^{b-3}$ allele), a 0.5-ml aliquot was removed from the water bath, added to 9 volumes of ice-cold MDENG buffer (final concentration, approximately 250 μg of cytosol protein/ml), and incubated overnight (~18 hr) at 4° with 50 pM [^{125}I]Br_2DpD, with or without a 400-fold molar excess of TCDBF. A charcoal/gelatin solution (final concentration, 1.0/0.1%, w/v) was added and incubated for 10 min at 0°, and the charcoal-adsorbed ligand was removed by centrifugation. Total bound ([^{125}I]Br_2DpD alone), nonspecifically bound ([^{125}I]Br_2DpD plus TCDBF), and specifically bound (the difference between total and nonspecifically bound) ligand were determined. Specifically bound ligand at each time point for heat inactivation was expressed as a fraction of initial specific binding (t = 0), and, from the mean least squares

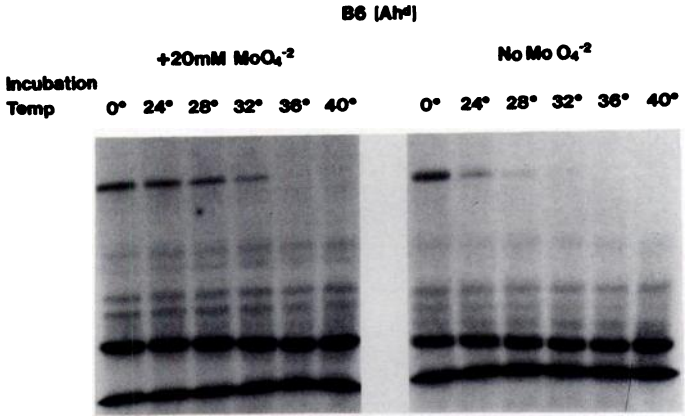

Fig. 3. Effect of sodium molybdate on the thermostability of the receptor encoded by the Ah^d allele. The hepatic cytosol fraction from B6J·D2N (Ah^d) mice was prepared in MENG buffer, in the absence or presence of 20 mM sodium molybdate. Aliquots of the cytosol fraction, diluted to 200 μg of protein/ml in the same buffer (with or without 20 mM sodium molybdate), were incubated for 15 min at the indicated temperatures, immediately cooled to 0°, labeled with the ^{125}I-photoaffinity ligand, and analyzed as described in Materials and Methods.

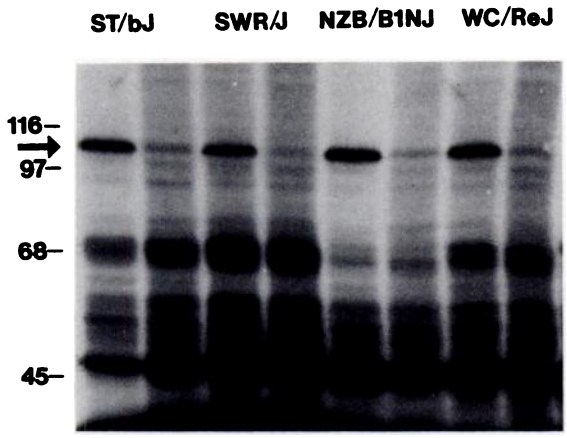

Fig. 4. ^{125}I-photoaffinity labeling of the Ah receptor in strains of mice carrying the Ah^d allele. Hepatic cytosols from strains of mice carrying Ah^d alleles were prepared in MENG buffer plus 20 mM Na_2MoO_4, diluted to 100 μg of protein/ml in the same buffer, incubated with 2 nM ^{125}I-photoaffinity label with or without a 200-fold excess of TCDBF, labeled, and resolved by gel electrophoresis. *Lanes 1, 3, 5,* and *7*, ^{125}I-photoaffinity ligand; *lanes 2, 4, 6,* and *8*, ^{125}I-photoaffinity ligand plus TCDBF.

regression line of fractional specific binding versus time, the heat inactivation rate and half-life of heat inactivation were determined ($t_{½}$ = ln $k_{inact.}$/2). Thermostability was assumed to be a first-order decay.

The phenotype $t_{½}$ (thermostability) is a complex variable (i.e., a function of total, nonspecific, and specific binding and the linear regression line of specific binding versus time) and is subject to modest day-to-day variation. Daily experiments always included controls of mice with the three known phenotypes (Ah^{b-1}, Ah^{b-2}, and Ah^{b-3}).

Results

Okey *et al.* (22) have recently characterized the low affinity Ah receptor from inbred strains of mice that are "nonresponsive" to aromatic hydrocarbons (i.e., homozygous for the Ah^d allele). Stabilization of the Ah receptor by the presence of sodium molybdate during tissue preparation and ligand binding enabled detection of the [^{3}H]TCDD-receptor complex by velocity sedimentation. We have examined the effect of molybdate on photoaffinity labeling of this low affinity variant of the Ah receptor. As seen in Fig. 1, in hepatic cytosol from B6J·D2N (Ah^d) congenic mice that was prepared and photoaffinity labeled in the absence of molybdate, there is one faint band of M_r ~104,000 that is selectively labeled, i.e., blocked with coincubation of excess TCDBF (Fig. 1, *lane 1* versus *lane 2*). Addition of 20 mM sodium molybdate during incubation does not enhance labeling (Fig. 1, *lane 3*). In contrast, if 20 mM Na_2MoO_4 is present during sample preparation, the intensity of labeling of the M_r 104,000 band is enhanced, regardless of whether the molybdate concentration is maintained (Fig. 1 *lane 7*) or diluted to 0.5 mM (Fig. 1, *lane 5*) during labeling.

In Fig. 2 is shown a comparison of the effects of molybdate on the photoaffinity labeling of hepatic cytosol from three congenic strains of C57BL/6J mice that are homozygous for the Ah^d, Ah^{b-1}, and Ah^{b-2} alleles. There are several noteworthy points in this autoradiograph. 1) In hepatic cytosol from B6J·D2N (Ah^d) mice, containing the low affinity receptor, that was prepared and labeled in the absence of molybdate, the M_r 104,000 protein in barely detectable. 2) Preparation of cytosol in the presence of 20 mM molybdate and reduction of the molybdate concentration (~0.5 mM) during labeling results in better photoaffinity labeling than preparation and labeling incubation in a high concentration of molybdate, and this is true for all three Ah receptor variants. 3) Preparation of the cytosol in the presence of molybdate reduces the labeled M_r 70,000 proteolytic fragment in all three variants, presumably due to enhanced stability of receptor conformation and diminished susceptibility to proteolysis by calpain II (34).

The enhanced stability of the receptor caused by molybdate can be demonstrated in another way. Hepatic cytosol from B6·D2N (Ah^d) mice, prepared in the presence or absence of 20 mM molybdate, was preincubated at varying temperatures for 15 min before incubation and labeling with the photoaffinity ligand (Fig. 3). Receptor prepared in the presence of molybdate is stabilized to heat inactivation and subsequently a greater fraction is labeled, compared with receptor heated in the absence of molybdate.

Strains with the Ah^d allele. Hepatic cytosol from a number of strains of mice classified as nonresponsive to aromatic hydrocarbons was prepared in the presence of molybdate, photoaffinity labeled, and analyzed by slab gel electrophoresis and autoradiography. As seen in Fig. 4, each of the hepatic cytosols showed a specifically labeled band (i.e., the labeling of which was blocked by coincubation with excess unlabeled TCDBF). On a long slab gel, all strains with the Ah^d allele had labeled proteins with an apparent molecular weight of 104,000 that comigrated with the photoaffinity-labeled receptor from mice carrying the Ah^{b-2} allele. A list of the 18 strains tested is found in Table 2.

Screen for other variants of the Ah receptor. Using the single phenotype of photoaffinity labeling and apparent molecular weight of the labeled protein species determined by denaturing gel electrophoresis, one can distinguish three variants of the Ah receptor among common inbred strains of mice, 1) the M_r 95,000 high affinity form determined by the Ah^{b-1} allele, 2) the M_r 104,000 high affinity receptor encoded by the Ah^{b-2} allele, and 3) the M_r 104,000 low affinity form stabilized by molybdate and specified by the Ah^d allele. This simple phenotype was used to screen for other variants of the receptor in mouse strains developed from more recently captured feral mice. Among certain strains, *Mus spretus*, *Mus caroli*, and

TABLE 2

Characterization of allelic forms of the Ah receptor and strains carrying them

Ah^d strains[a]	Ah^{b-1} strains[b]	Ah^{b-2} strains[c]	Ah^{b-3} strains[d]
1) Low affinity ligand binding	1) High affinity binding	1) High affinity binding	1) High affinity binding
2) Stabilized by sodium molybdate	2) M_r 95,000 protein	2) M_r 104,000 protein	2) M_r 105,000 protein
3) M_r 104,000 protein	3) $t_{½}$ (heat inactivation at 35°), 20–30 min	3) $t_{½}$ (heat inactivation at 35°), 3–6 min	3) $t_{½}$ (heat inactivation at 35°), ~6–15 min
AKR/J	C57BL/6J	PERA/Ei (J)	MOLF/Ei
BDP/J	C57BL/6ByJ	SK/CamEi (J)	*Mus caroli* (J) + (H)
DBA/2J	C57L/J	SF/Cam Ei (J)	*Mus spretus* (J) + (H)
ILn/J	C57BR/cdJ	*Mus musculus Skive (H)*	*Mus parahi* (H)
LG/J	C58/J	Sanner's Form (H)	*Mus hortulanus* (H)
LP/J	C57BL/KsJ	CZECH I + II (H)	*Mus cookii* (H)
NZB/BINJ	MA/MyJ	*Mus domesticus brevirostis* (H)	
RBF/DnJ		*Mus domesticus praetextus* (H)	
RF/J		A/J	
		BALB/cByJ	
SJL/J		C3H/HeJ	
ST/bJ		CBA/J	
SWR/J		CE/J	
129/J		BUB/BnJ	
WC/ReJ		HRS/J	
YBR/J		P/J	
CAST/Ei		SEA/GnJ	
Mus domesticus (J)		SEC/1ReJ	
B6J·D2N (Ah^d)		B6·C-H-38[c]/By	

[a] All Ah^d strains had stabilization of Ah receptor by preparation in buffer containing 20 mM Na molybdate.
[b] Strains tested positive for heat stability. For more complete list of strains with M_r 95,000 peptide, see Ref. 11.
[c] Strains tested positive for M_r 104,000 protein and heat stability. For more complete list of strains with Ah^{b-2} allele, see Ref. 11.
[d] Strains tested positive for M_r 105,000 protein and intermediate heat stability.

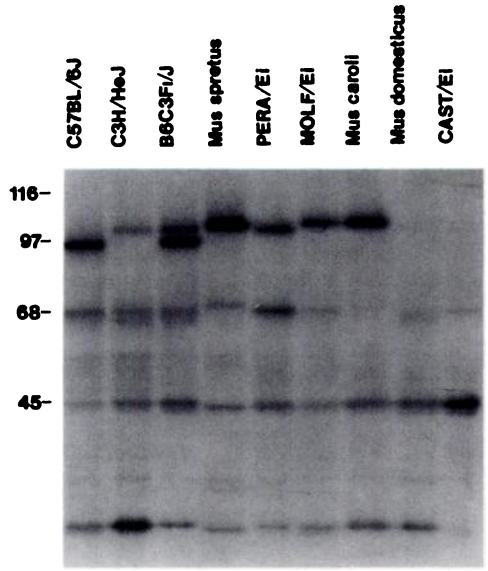

Fig. 5. Allelic variant of the Ah receptor expressing M_r 105,000 protein in newer strains of mice. Hepatic cytosol was prepared and labeled in MENG buffer (without molybdate) and processed, as described in Materials and Methods, on a long 10% polyacrylamide gel. Note the absence of labeling of the Ah receptor in *Mus domesticus* and CAST/Ei, which carry the Ah^d allele.

MOLF/Ei (inbred *Mus molossinus*), the labeled peptide has an apparent molecular weight of 105,000, just slightly larger than that found in C3H/HeJ or PERA/Ei (M_r 104,000), as seen in Fig. 5. This small difference in apparent M_r is very reproducible. The deer mouse, *Peromyscus maniculatus*, not closely related to *Mus* species, has a photoaffinity-labeled receptor with an apparent molecular weight of 130,000 (data not shown). In Table 2, we have summarized the results of these experiments. The *Mus musculus* or *domesticus* strains all expressed the M_r 104,000 peptide, whereas *Mus molossinus* (MOLF), *caroli*, *cookii*, *hortulanus*, *pahari*, and *spretus* all expressed the M_r 105,000 protein.

Thermolability. Relative thermolability is the most sensitive indirect method of detecting changes in primary protein structure (35). We screened mouse strains for variants of the Ah receptor using this phenotype. The hepatic cytosol fraction was incubated at 35° for various times and then incubated overnight with radioligand at 4° for determination of specific binding. In Fig. 6 is a plot of fractional specific binding (specific binding at a given time as a fraction of initial specific binding) versus time of incubation at 35°. For mice carrying the Ah^{b-1} allele (C57 and C58 family; M_r 95,000 protein), the Ah receptor is relatively heat stable, with a half-life of 20–30 min; in contrast, in strains carrying the Ah^{b-2} allele (M_r 104,000 protein), the $t_{½}$ of the heat stability is 4 to 7 min. For mouse strains that express the M_r 105,000 Ah receptor (Ah^{b-3}), the receptor is slightly more stable than that expressed by Ah^{b-2} mice, with a $t_{½}$ of 6 to 12 min. [The $t_{½}$ of heat stability for the Ah receptor in aliquots of the same cytosol varied modestly from day to day. Thus, it is more predictive to characterize an unknown sample by running known standards (e.g., A^{b-2} and Ah^{b-3}) than by depending on the absolute value of $t_{½}$.]

On the basis of the two measures studied, apparent molecular weight and heat stability of the receptor, we can distinguish four alleles fo the *Ah* locus in strains of *Mus*, 1) the Ah^{b-1} allele, which encodes a M_r 95,000, high affinity, and heat-stable receptor; 2) the Ah^{b-2} allele, which expresses a M_r 104,000, high

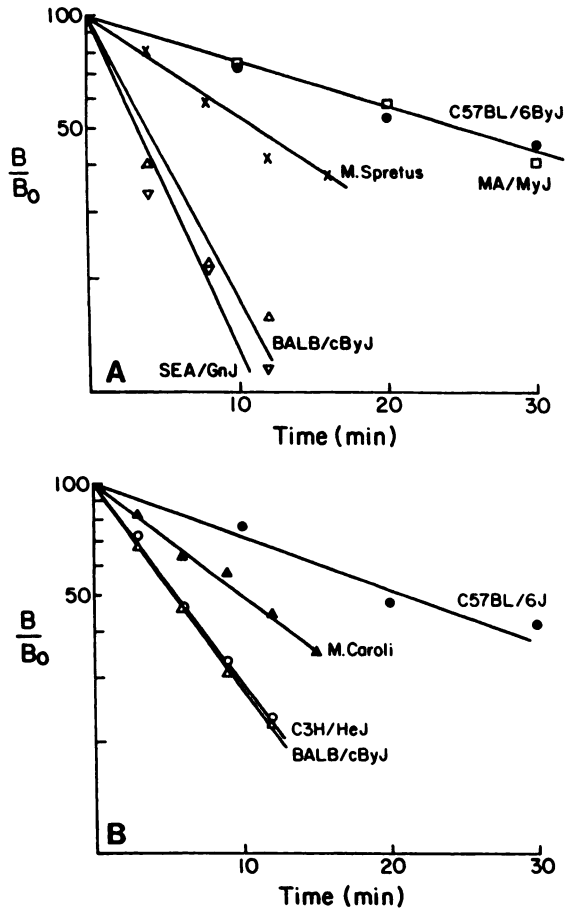

Fig. 6. Relative heat stability of Ah receptor variants. Hepatic cytosol was prepared from various inbred strains. Aliquots were incubated for various time periods (0, 3, 6, 9, 12, and 15 min or 0, 10, 20, and 30 min) at 35° and immediately cooled to 0°, and the specific binding of the reversible ligand [^{125}I]Br_2DpD was determined by overnight binding at 0°. The results are plotted as a function of specific binding (B) as a fraction of initial specific binding (B_0) versus the time of incubation at 35°. A and B are separate experiments. For C57BL/6ByJ mice, initial total binding = 66,800 dpm, initial nonspecific binding = 14,300 dpm, and initial specific binding = 52,500 dpm (100%), which was typical for Ah^{b-1} mice. For BALB/cByJ mice, initial total binding = 37,600 dpm, initial nonspecific binding = 10,600 dpm, and initial specific binding = 27,000 dpm, which was typical for Ah^{b-2} mice. For *Mus spretus*, initial total binding = 42,200 dpm, initial nonspecific binding = 8,200 dpm, and initial specific binding = 33,900 dpm.

affinity, heat-labile receptor; 3) the Ah^{b-3} allele, which expresses a M_r 105,000, high affinity receptor of heat lability intermediate between those of Ah^{b-1} and Ah^{b-2} products; and 4) the Ah^d allele, which determines a M_r 104,000, low affinity receptor that is stabilized by molybdate.

Discussion

In this report we have extended our survey of strains of mice, using improved methodologies, and detected four variants of the Ah receptor. Okey *et al.* (22) observed that inclusion of sodium molybdate in the buffer throughout the tissue preparation and incubation for binding stabilized the low affinity Ah receptor (encoded by the Ah^d allele) and permitted demonstration of reversible ligand binding. In the present study, inclusion of molybdate in the homogenization buffer permitted photoaffinity labeling of this low affinity receptor and the demonstration that all murine strains carrying the Ah^d allele express a M_r 104,000 ligand-binding peptide. We further found that inclusion of 20 mM sodium molybdate in the homogenization buffer and its dilution during the incubation with ligand enhanced labeling of the Ah receptor encoded by Ah^{b-1} and Ah^{b-2} alleles and reduced the presence of the labeled M_r 70,000 proteolytic product. We have previously demonstrated among the common inbred strains that express a high affinity receptor (i.e., Ah^b, responsive to aromatic hydrocarbons) that one can distinguish two allelic forms, the M_r 95,000 ligand-binding protein (Ah^{b-1} allele) in the C57 and C58 family and MA/My mice and the M_r 104,000 ligand-binding protein (Ah^{b-2} allele) found in the other inbred strains (11). As shown in this report, these two Ah receptor variants can be easily distinguished by their relative thermolability. We have identified a new variant of the Ah receptor among new murine strains produced by inbreeding recently isolated feral mice. This Ah receptor variant has an apparent molecular weight of 105,000 and a thermostability slightly greater than that of the M_r 104,000 receptor. It should be noted that the gene encoding this new variant (which we have designated the Ah^{b-3} allele) has not been shown formally, as yet, to be allelic with Ah^d, Ah^{b-1}, and Ah^{b-2}. Interestingly, the M_r 105,000 variant is found only in strains of *Mus* other than *Mus musculus* and *Mus domesticus* (the European house mouse). *Peromyscus maniculatus*, the deer mouse, was found to have an Ah receptor with an apparent molecular weight of approximately 130,000, but this species is too distant from *Mus* to warrant further consideration.

The four groups of variants were identified by using crude phenotypic markers, apparent molecular weight, high or low ligand affinity, and relative thermostability; with further investigation, more diversity may be found. The Ah^{b-2} allele is widely found in feral isolates of *Mus musculus* and *Mus domesticus*. The low affinity Ah^d allele is found in *Mus domesticus* (JAX strain) and other *Mus* strains, e.g., CAST/Ei (inbred *Mus castaneus*).

The Ah^{b-1} allele found in the C57 and C58 families and MA/My has not been traced to its origin in feral mice, but these stocks have origins from European and Japanese house mice. It is likely that the allele came from *Mus molossinus*. The only member of this strain tested (MOLF/Ei, inbred *Mus molossinus*) carried the Ah^{b-3} allele (M_r ~105,000).

The Ah receptor is a presumed member of the erb-A superfamily. In this light, the structural variation in the Ah receptor between vertebrate species and among strains within a species is remarkable, because most of the other members of the erb-A family (e.g., estrogen receptor) show great conservation in evolution. This variation in structure may reflect a lack of selection pressure to conserve the Ah receptor in vertebrates.

Do these allelic variants of the Ah receptor have any functional significance? Because the *Ah* locus was originally identified as a functional polymorphism, a difference in responsiveness to polycyclic aromatic hydrocarbons, one can detect a functional difference between Ah^{b-1} (and Ah^{b-2}) and the Ah^d alleles. We have some evidence of *in vivo* differences between mice carrying the Ah^{b-1} versus Ah^{b-2} allele; as yet, the Ah^{b-3} variant has not been examined. It is easiest to examine functional differences in these allelic variants if they are all on a common genetic background, i.e., congenic mice differing only in the allele at the *Ah* locus.

It is now technically possible to screen for variation in the Ah receptor in humans and address the earlier proposition

made by Kellermann *et al.* (36) that such variation might determine susceptibility to lung cancer from smoking.

References

1. Poland, A., and J. C. Knutson. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. *Annu. Rev. Pharmacol. Toxicol.* **22**:517–554 (1982).
2. Poland, A., and R. D. Kimbrough, eds. *Biological Mechanisms of Dioxin Action*, Banbury Report 18. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984).
3. Whitlock, J. P., Jr. The regulation of cytochrome P-450 gene expression. *Annu. Rev. Pharmacol. Toxicol.* **26**:333–369 (1986).
4. Denison, M. S., J. M. Fisher, and J. Whitlock. The DNA recognition site for the dioxin-Ah receptor complex. *J. Biol. Chem.* **263**:17221–17224 (1988).
5. Fujisawa-Sehara, A., K. Sogawa, M. Yamane, and Y. Fujii-Kuriyama. Characterization of xenobiotic responsive elements upstream from drug-metabolizing cytochrome P-450c: a similarity to glucocoticoid regulatory elements. *Nucleic Acids Res.* **15**:4179–4189 (1987).
6. Evans, R. M. The steroid and thyroid hormone receptor superfamily. *Science (Washington, D. C.)* **240**:889–895 (1988).
7. Perdew, G. Association of the Ah receptor with 90 kDa heat shock protein. *J. Biol. Chem.* **263**:13802–13805 (1988).
8. Denis, M., S. Cuthill, A.-C. Wikström, L. Poellinger, and J. Å. Gustafsson. Association of the dioxin receptor with the M_r 90,000 heat shock protein: a structural kinship with the glucocorticoid receptor. *Biochem. Biophys. Res. Commun.* **155**:801–807 (1988).
9. Denison, M. S., L. M. Vella, and A. B. Okey. Hepatic Ah receptor for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin: partial stabilization by molybdate. *J. Biol. Chem.* **261**:10189–10195 (1986).
10. Poland, A., and E. Glover. Variation in the molecular mass of the Ah receptor among vertebrate species and strains of rats. *Biochem. Biophys. Res. Commun.* **146**:1439–1449 (1987).
11. 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).
12. 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).
13. Nebert, D. W., J. E. Goujon, and J. E. Gielen. Aryl hydrocarbon hydroxylase induction by polycyclic hydrocarbons: simple autosomal dominant trait in the mouse. *Nature New Biol.* **235**:107–110 (1972).
14. Thomas, P. E., R. E. Kouri, and J. J. Hutton. The genetics of aryl hydrocarbon hydroxylase induction in mice: a single gene difference between C57BL/6J and DBA/2J. *Biochem. Genet.* **6**:157–168 (1972).
15. 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).
16. 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).
17. Poellinger, L., J. Lund, M. Gillner, L.-A. Hansson, and J.-Å. Gustafsson. Physicochemical characterization of specific and nonspecific polyaromatic hydrocarbon binders in rat and mouse liver cytosol. *J. Biol. Chem.* **258**:13535–13542 (1983).
18. Wilhelmsson, A., A.-C. Wikström, and L. Poellinger. Polyanionic properties of the receptor for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *J. Biol. Chem.* **261**:13456–13463 (1986).
19. Gasiewicz, T. A., and G. Rucci. Examination and rapid analysis of hepatic cytosolic receptors for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin using gel-permeation high performance liquid chromatography. *Biochim. Biophys. Acta* **798**:37–45 (1984).
20. Poland, A., E. Glover, F. H. Ebetino, and A. S. Kende. Photoaffinity labeling of the Ah receptor. *J. Biol. Chem.* **261**:6352–6365 (1986).
21. Mason, M. E., and A. B. Okey. Cytosolic and nuclear binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to the Ah receptor in extrahepatic tissues of rats and mice. *Eur. J. Biochem.* **123**:209–215 (1982).
22. Okey, A. B., L. M. Vella, and P. A. Harper. Detection and characterization of a low affinity form of cytosolic Ah receptor in livers of mice nonresponsive to induction of cytochrome P_1-450 by 3-methylcholanthrene. *Mol. Pharmacol.* **35**:823–830 (1989).
23. Nishigori, H., and D. Toft. Inhibition of progesterone receptor activation by sodium molybdate. *Biochemistry* **19**:77–83 (1980).
24. Nelson, C. J., J. J. Sando, W. M. Vogel, and W. B. Pratt. Glucocorticoid receptor inactivation under cell-free conditions. *J. Biol. Chem.* **252**:7568–7578 (1977).
25. Dahmer, M., P. R. Housley, and W. B. Pratt. Effects of molybdate and endogenous inhibitors on steroid-receptor inactivation, transformation, and translocation. Annu. Rev. Physiol. **46**:67–81 (1984).
26. Marshall, J. T. Taxonomy, in *The Mouse in Biomedical Research* (H. L. Foster, J. D. Small, and J. G. Fox, eds.), Vol. 1. Academic Press, New York (1981).
27. Sage, R. D. Wild mice, in *The Mouse in Biomedical Research* (H. L. Foster, J. D. Small, and J. G. Fox, eds.), Vol. 1. Academic Press, New York, 39–90 (1981).
28. Marshall, J. T., and R. D. Sage, Taxonomy of the house mouse in *Biology of the House Mouse* (R. J. Berry, ed.). Academic Press, New York, 15–25 (1981).
29. Potter, M., Comments on the relationship of inbred strains to the genus *Mus*, *in Origins of Inbred Mice* (H. C. Morse III, ed.). Academic Press, 497–509, New York, (1978).
30. Marshall, J. T. Brief review of the European house mouse, in *Origins of Inbred Mice* (H. C. Morse III, ed.). Academic Press, New York, 511–517 (1978).
31. Bailey, D. W. Genetics of histocompatibility in mice. I. New loci and congenic lines. *Immunogenetics* **2**:249–256 (1975).
32. Bradfield, C., A. S. Kende, and A. Poland. Kinetic and equilibrium studies of Ah receptor-ligand binding: use of [^{125}I]-2-iodo-7,8-dibromodibenzo-*p*-dioxin. *Mol. Pharmacol.* **34**:229–237 (1988).
33. Laemmli, U. K. Cleavage of structural proteins during assembly of the head of bacteriophage T_4. *Nature (Lond.)* **227**:680–685 (1970).
34. Poland, A., and E. Glover. Ca^{+2}-dependent proteolysis of the Ah receptor. *Arch. Biochem. Biophys.* **261**:103–111 (1988).
35. Paigen, K. The genetics of enzyme realization, in *Enzyme Synthesis and Degradation in Mammalian Systems* (M. Rechcigl, ed.). University Park Press, Baltimore, 1–46 (1971).
36. Kellermann, G., C. R. Shaw, and M. Luyten-Kellerman. Aryl hydrocarbon hydroxylase inducibility and bronchogenic carcinoma. *N. Engl. J. Med.* **289**:934–937 (1973).
37. Poland, A., P. Teitelbaum, E. Glover, and A. Kende. Stimulation of in vivo hepatic uptake and in vitro hepatic binding of [^{125}I]-2-Iodo-3,7,8-trichlorodibenzo-*p*-dioxin by the administration of agonists for the Ah receptor. *Mol Pharmacol* **36**:121–127 (1989).

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