INDUCIBLE MONOOXYGENASE ACTIVITIES AND 3-METHYLCHOLANThRENE-INITIATED TUMORIGENESIS IN MOUSE RECOMBINANT INBRED SUBLINES

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ABSTRACT

The induction of a certain group of hepatic monooxygenase activities by polycyclic aromatic compounds is regulated by the same locus or gene cluster controlling the formation of cytochrome P-450 (P-448) in mice. Certain inbred strains of mice are "responsive" (Ahb) to such induction, whereas others are "nonresponsive" (Ahd). A pair of closely related sublines that differ with respect to the Ah locus (for aromatic hydrocarbon responsiveness) were used to identify or confirm the pleiotropic effects of this gene. The lines were derived by sibling-mating without selection from (C57L/J x AKR/J)F2 mice; the two sublines were separated at the F12 generation. Ten microsomal monooxygenase activities and one cytosol enzyme activity known to be associated with the Ah locus were similarly associated with cytochrome P-450 formation in these recombinant inbred sublines as well. Nine additional hepatic monooxygenase activities studied were found not to be associated with the Ah locus; certain of these activities were increased slightly, following treatment of nonresponsive as well as responsive mice with polycyclic aromatic compounds. The Ahb-containing subline was highly susceptible to 3-methylcholanthrene-induced subcutaneous sarcomas, whereas the Ahd-containing subline was relatively resistant. These results emphasize the potential importance of this particular enzyme for the study of coordinated regulation in mammals.

He mixed function oxidases, or monooxygenases, are a group of membrane-bound microsomal enzymes involved in the metabolism of certain drugs, polycyclic aromatic hydrocarbons and other lipophilic xenobiotics, as well as certain classes of hydrophobic endogenous compounds such as steroids and fatty acids. These transformations involve the insertion of one atom of molecular oxygen into the hydrophobic substrate, resulting in the formation of ring- or N-hydroxylated metabolites or in the oxidative O- or N-dealkylation of the parent compound. These enzymatic activities, which are dependent on NADPH and molecular oxygen, are associated with an electron-transport chain whose terminal

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oxidase is a CO-inhibitable hemoprotein, cytochrome P-450—so named because of its characteristic Soret maximum at about 450 nm for the difference spectrum of the reduced hemoprotein-CO complex (Omura and Sat0 1964).

Treatment of various mammalian species with polycyclic aromatic hydrocarbons such as MC* or BP results in increases in certain monooxygenase activities but not in others (Kato and Takayanagi 1966; Conney 1967; Sher 1971; Nebert, Considine and Owens 1973). The increase in enzymatic activities following aromatic hydrocarbon treatment is associated with an increase in the microsomal content of cytochrome P-450 as well as a qualitative change in its CO-difference spectrum, i.e. a shift of the Soret maximum from 450 to 448 nm (Alvares et al. 1967). This newly found cytochrome "P-450" (or P-448) possesses other distinct spectral properties (Sladek and Mannering 1966; Jefcoate, Gaylor and Calabrese 1969; Nebert, Gieilen and Goujon 1972) and is correlated with genetically mediated changes in spin state of the heme iron (Nebert and Kon 1973; Nebert, Robinson and Kon 1973). One associated monooxygenase activity, aryl hydrocarbon (BP) hydroxylase, or AHH, has been studied extensively. Increases of this activity in fetal rat liver primary cells cultures following aromatic hydrocarbon treatment require both RNA and protein synthesis (Nebert and Gieilen 1971). It has furthermore recently been shown that hepatic cytochrome P-450 "formation" accompanying AHH induction in mice in vivo is due to an increase in one of several preexisting P-450 apoproteins and requires de novo protein synthesis (Haugen, Coon and Nebert 1976).

The capacity of mice to respond to induction of AHH and cytochrome P-450 by MC is associated with the Ah locus (for aromatic hydrocarbon responsiveness), certain inbred strains being "responsive" (Ahb) to such treatment and others "nonresponsive" (Ah0) (Gieilen, Goujon and Nebert 1972). In this regard, the noncarcinogenic aromatic compound BNF acts as an equivalent inducer, displaying the same genetic specificity in the liver and other tissues (Nebert, Gieilen and Goujon 1972). The results of appropriate intercrosses and backcrosses from twelve inbred strains suggest that the regulation of responsiveness involves several alleles at two (or more) non-linked genetic loci (Robinson, Considine and Nebert 1974; Nebert et al. 1975). The difference, however, between two prototype inbred strains, the responsive C57BL/6N and the non-responsive DBA/2N, is almost completely explained by a mutation at a single locus, the allele Ahb for responsiveness being dominant (Nebert and Gieilen 1972; Niwa et al. 1975). The inducibility of at least ten additional hepatic monooxygenase activities by MC or BNF is regulated at or near this same Ah locus: p-nitroanisole O-demethylase, 7-ethoxycoumarin O-deethylase, 3-methyl-4-methylaminoazobenzene N-demethylase (Nebert, Considine and Owens 1973), zoxazolamine 6-hydroxylase (Robinson and Nebert 1974), phenacetin O-deethylase (Poppers, Levin and Conney 1975), 2-acetylaminofluorene N-hydroxylase (Thorgerisson, Felton and Nebert 1975), biphenyl 2-hydroxylase.

* The abbreviations used are: MC, 3-methylcholanthrene; BP, benzo[a]pyrene; BNF, β-naphthoflavone (7,8-benzo-flavone); DMBA, 7,12-dimethylbenz[a]anthracene.
biphenyl 4-hydroxylase, naphthalene 1,2-dihydrodiol formation, and acetanilide 4-hydroxylase (Atlas, Daly and Nebert 1975).

The usefulness of recombinant inbred lines in the genetic analysis of the regulation of enzyme induction has previously been described in detail (Bailey 1971; Swank and Bailey 1973). In addition to testing the closeness of genetic linkage, analysis of such lines also permits studies which are not possible in a single F2 individual, such as the time course of enzyme induction or (as in this paper) the simultaneous measurement of a large number of enzymatic activities. The purpose of this report is to confirm that the regulation of cytochrome P-450 induction and MC-initiated tumorigenesis in mice is linked to the expression of the above-mentioned monooxygenase activities by using two sublines of a recombinant inbred strain. Evidence is also presented that other monooxygenase activities, certain of which are increased by MC or BNF treatment, are not regulated at this same locus.

**MATERIALS AND METHODS**

**Materials:** BNF was purchased from Aldrich Chemical Co. The sources of chemicals used in the various enzyme assays were as cited in the respective references (see below). The C57BL/6N, DBA/2N, NZW/BLN, AKR/N, C3H/HeN and BALB/cAnN inbred mouse strains were obtained from the National Institutes of Health Veterinary Resources Branch.

A pair of closely related sublines (AKXL-38 and AKXL-38A) was derived by brother-sister inbreeding among the progeny of a pair of (C57L/J) × (AKR/J) F1 mice; the sublines were separated at the F12 generation and were further inbred for an additional ten generations. The two sublines, although genetically very similar with respect to the entire genotype, were found to differ at the Ah locus: AKXL-38 being nonresponsive like AKR/J and AKXL-38A being responsive like C57L/J. Because of their overall genetic similarity, this pair of sublines was chosen as material for studies of the pleiotropic effects of the Ah locus. Mice of these two sublines were produced at The Jackson Laboratory and shipped to the National Institute of Child Health and Human Development at 3 to 4 weeks of age. Following one week for stabilization in their new animal room, the mice were then treated with inducer and sacrificed.

**Treatment of mice and preparation of microsomes:** The environment of the animal room was rigidly controlled, as described previously (Robinson, Considine and Nebert 1974). All mice used in this study were 4 to 6 weeks of age. BNF (80 mg per kg) was administered intraperitoneally in corn oil 48 hours prior to sacrifice. Control animals received vehicle alone. Hepatic microsomes were prepared as previously described (GieLEN, GouJON and NeBERT 1972). Enzyme assays and spectrophotometry: Microsomal cytochrome P-450 content was determined by modifications (NeBERT, GieLEN and GouJON 1972) of the method of Omura and Sato (1964). The following enzymatic activities were measured by previously described methods: aryl hydrocarbon hydroxylase (GieLEN, GouJON and NeBERT 1972), 7-ethoxycoumarin O-deethylase, p-nitroanisole O-demethylase, 3-methyl-4-methyl-aminoazobenzene N-demethylase (NeBERT, Considine and Owens 1973), zoxazolamine 6-hydroxylase (Robinson and NeBERT 1974), 2-acetylaminofluorene N-hydroxylase (ThorGrensson, Felton and NeBERT 1975), aniline hydroxylase (Chhabra, Gram and Fouts 1972), benzenesulfonanilide hydroxylase (Daly 1970) as modified by Oesch et al. (1973), benzphetamine N-demethylase (Lu et al. 1972b), chlorcyclizine N-demethylase (Jacobson et al. 1972), ethylmorphine N-demethylase (Lü et al. 1972b), pentobarbital hydroxylase (Kuntzman et al. 1967), and testosterone 7α-, 16α- and 6β-hydroxylases (Kuntzman et al. 1968). Biphenyl 2- and 4-hydroxylases were measured by modification (Atlas and NeBERT 1976) of the method of Creaven, Parke and Williams (1965). Assays for acetanilide 4-hydroxylase and naphthalene 1,2-dihydrodiol formation were modifications (Atlas and NeBERT 1976) of the methods of Daly (1970) and Oesch and Daly (1972) respectively.
Reduced NAD(P):menadione oxidoreductase was measured by modification (K. KUMAKI, N. M. JENSON, J. G. M. SHIRE and D. W. NEBERT, manuscript submitted for publication) of the method of LIND and ERNST (1974). All enzymes were assayed at 37°, except that the testosterone hydroxylases and reduced NAD(P):menadione oxidoreductase were assayed at 25°.

Induction of tumors with MC: Eight- to ten-week-old male and female mice of the sublines AKXL-38 and AKXL-38A were injected subcutaneously with 150 µg of MC dissolved in 0.1 ml of trioctanoin. Subcutaneous sarcomas were recorded over the next 8-month period.

RESULTS

Treatment of responsive C57BL/6N mice with BNF resulted in a 2-fold increase in hepatic cytochrome P-450 content associated with a 2-nm Soret Shift, whereas neither the increase nor the shift occurred in similarly treated nonresponsive DBA/2N mice (Table 1). A similar distinction was found between the responsive AKXL-38A and the nonresponsive AKXL-38 recombinant inbred sublines (Table 1), which had previously been identified by their responsiveness to DMBA-induced skin ulcers (THOMAS, HUTTON and TAYLOR 1973).

Ten microsomal monooxygenase activities which were induced by BNF 2- to 6-fold in C57BL/6N but not in DBA/2N mice were similarly induced in the responsive (AKXL-38A) but not in the nonresponsive (AKXL-38) recombinant inbred subline (Table 2). This occurred without exception and was true as well for reduced NAD(P):menadione oxidoreductase, a cytosol enzyme whose regulation has tentatively been linked to that of cytochrome P₄₅₀ and associated monooxygenase activities (K. KUMAKI, N. M. JENSON, J. G. M. SHIRE and D. W. NEBERT, manuscript submitted for publication). Not shown in Table 2 are the values for benzenesulfonanilide hydroxylase activity, which is not associated with cytochrome P₄₅₀ induction in inbred mice. Values for the responsive AKXL-38A subline (control 1.56 nmol per min per mg, BNF 1.80) were not substantially different from those in the nonresponsive AKXL-38 subline (control 1.55, BNF 1.41).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype at &quot;Ah locus&quot;</th>
<th>Cytochrome P-450 content (nmol per mg microsomal protein)</th>
<th>Soret maximum (nm)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control  BNF Control  BNF</td>
<td>Control  BNF</td>
</tr>
<tr>
<td>DBA/2N</td>
<td>Nonresponsive</td>
<td>0.80 ± 0.13  0.82 ± 0.15</td>
<td>450  450</td>
</tr>
<tr>
<td>C57BL/6N</td>
<td>Responsive</td>
<td>0.76 ± 0.11  1.51 ± 0.21</td>
<td>450  448</td>
</tr>
<tr>
<td>AKXL-38</td>
<td>Nonresponsive</td>
<td>0.73 ± 0.09  0.72 ± 0.07</td>
<td>450  450</td>
</tr>
<tr>
<td>AKXL-38A</td>
<td>Responsive</td>
<td>0.68 ± 0.11  1.42 ± 0.18</td>
<td>450  448</td>
</tr>
</tbody>
</table>

* Each value represents the mean of three determinations, each of which was performed in duplicate. Samples for each determination consisted of pooled livers from three control or three BNF-treated animals of each strain. Values are expressed as the means ± standard deviations. Microsomal pellets from the recombinant inbred (AKXL) sublines were stored anaerobically at −20° for one day prior to analysis, while microsomes from the inbred strains were used fresh.

† Soret maximum of the reduced hemoprotein-CO complex by difference spectroscopy.
**TABLE 2**

*Effect of BNF treatment on ten microsomal monooxygenase activities and one cytosol enzyme activity in DBA/2N and C57BL/6N inbred mice and AKXL recombinant inbred sublines*.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Enzymatic activity</th>
<th>DBA/2N</th>
<th>C57BL/6N</th>
<th>AKXL-38</th>
<th>AKXL-38A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Control</td>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BNF</td>
<td>BNF</td>
<td>BNF</td>
<td>BNF</td>
</tr>
<tr>
<td>Aryl hydrocarbon hydroxylase†</td>
<td>610</td>
<td>590</td>
<td>3670</td>
<td>290</td>
<td>320</td>
</tr>
<tr>
<td>7-Ethoxycoumarin O-deethylase‡</td>
<td>1.8</td>
<td>2.1</td>
<td>2.2</td>
<td>13</td>
<td>0.45</td>
</tr>
<tr>
<td>p-Nitroanisole O-demethylase§</td>
<td>4.5</td>
<td>4.6</td>
<td>4.6</td>
<td>16</td>
<td>3.3</td>
</tr>
<tr>
<td>3-Methyl-4-methylaminoazobenzene N-demethylase§</td>
<td>6.6</td>
<td>6.8</td>
<td>7.4</td>
<td>7.2</td>
<td>6.9</td>
</tr>
<tr>
<td>Zoxazolamine 6-hydroxylase†</td>
<td>10</td>
<td>13</td>
<td>11</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>2-Acetylaminofluorene N-hydroxylase†</td>
<td>200</td>
<td>220</td>
<td>240</td>
<td>1270</td>
<td>180</td>
</tr>
<tr>
<td>Biphenyl 2-hydroxylase†</td>
<td>1270</td>
<td>1250</td>
<td>1180</td>
<td>7800</td>
<td>1230</td>
</tr>
<tr>
<td>Biphenyl 4-hydroxylase†</td>
<td>3160</td>
<td>3120</td>
<td>3020</td>
<td>7690</td>
<td>2300</td>
</tr>
<tr>
<td>Acetanilide 4-hydroxylase†</td>
<td>100</td>
<td>120</td>
<td>91</td>
<td>720</td>
<td>100</td>
</tr>
<tr>
<td>Naphthalene trans-1,2-dihydrodiol formation‡</td>
<td>510</td>
<td>570</td>
<td>580</td>
<td>1470</td>
<td>610</td>
</tr>
<tr>
<td>Reduced NAD(P):menadione oxidoreductase</td>
<td>80</td>
<td>100</td>
<td>120</td>
<td>360</td>
<td>90</td>
</tr>
</tbody>
</table>

*The same microsomal samples described under Table 1 were used to assay the monooxygenase activities; reduced NAD(P):menadione oxidoreductase activities were estimated in the cytosol fraction of the same preparations and are expressed as nmol cytochrome c reduced per min per mg cytosol protein. The coefficients of variance (i.e., standard deviation divided by the mean) for three similar experiments ranged between 0.08 and 0.19 for each group.*

† Expressed as pmol product formed per min per mg microsomal protein.
‡ Expressed as nmol product formed per min per mg microsomal protein.
§ Expressed as nmol HCHO formed per min per mg microsomal protein.

Although none of the cytochrome P-450-associated activities is appreciably induced by BNF or MC in DBA/2N mice, there are other monooxygenase activities which are increased to a small extent in nonresponsive as well as responsive strains (NEBERT, CONSIDINE and OWENS 1973). As seen in Table 3, of nine additional monooxygenase activities studied, aniline hydroxylase and two N-demethylase activities are increased 50% to 100% following BNF treatment of three aromatic hydrocarbon nonresponsive strains (DBA/2N, NZW/BLN, and AKR/N); the small increases seen for ethylmorphine N-demethylase are probably not significant. Of these nine enzyme activities in three aromatic hydrocarbon-responsive strains (C57BL/6N, C3H/HeN, and BALB/cAnN), only aniline hydroxylase is increased to the same degree following BNF treatment.

That testosterone 7α-hydroxylase is not induced by BNF in the mouse differs from previous studies (KUNTZMAN et al. 1968) in the rat. Our result emphasizes the fact that species differences in the induction of monooxygenase activities often occur (ATLAS et al. 1975).

Table 4 shows the results of testing the two sublines for susceptibility to subcutaneous tumor formation by MC. The responsive AKXL-38A mice were highly susceptible, 11 of 15 mice (73%) developing tumors during the 8-month period.
<table>
<thead>
<tr>
<th>Enzymatic activity</th>
<th>Nonresponsive strain</th>
<th>Responsive strain</th>
<th>Nonresponsive strain</th>
<th>Responsive strain</th>
<th>Nonresponsive strain</th>
<th>Responsive strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenacetin N-demethylase</td>
<td>1.3</td>
<td>1.4</td>
<td>1.9</td>
<td>3.4</td>
<td>3.5</td>
<td>3.8</td>
</tr>
<tr>
<td>Benzphetamine N-demethylase</td>
<td>1.0</td>
<td>1.1</td>
<td>1.2</td>
<td>1.3</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Chloropheniramine N-demethylase</td>
<td>1.4</td>
<td>1.5</td>
<td>1.6</td>
<td>1.7</td>
<td>1.8</td>
<td>1.9</td>
</tr>
<tr>
<td>Ethylmorphine N-demethylase</td>
<td>0.8</td>
<td>0.9</td>
<td>1.0</td>
<td>1.1</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>Testosterone 17β-hydroxylase</td>
<td>0.6</td>
<td>0.7</td>
<td>0.8</td>
<td>0.9</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Testosterone 6β-hydroxylase</td>
<td>0.2</td>
<td>0.3</td>
<td>0.4</td>
<td>0.5</td>
<td>0.6</td>
<td>0.7</td>
</tr>
</tbody>
</table>

* Each value represents the mean of duplicate determinations on each of six animals. Specific hydroxylase activities are expressed as nmol HCHO formed per min per mg microsomal protein. The coefficients of variance (i.e., standard deviation divided by the mean) range between 0.08 and 0.14 for each group.
TABLE 4

Tumorigenesis in AKXL sublines 38 and 38A by 150 μg of subcutaneous MC

<table>
<thead>
<tr>
<th>Mouse sublines</th>
<th>Phenotype at Aκ locus</th>
<th>Animals with tumors per number of animals</th>
<th>Average tumor latency (Weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKXL-38</td>
<td>nonresponsive</td>
<td>Male 1/7, Female 0/4, Total 1/11 9</td>
<td>22</td>
</tr>
<tr>
<td>AKXL-38A</td>
<td>responsive</td>
<td>Male 5/8, Female 6/7, Total 11/15 73</td>
<td>23</td>
</tr>
</tbody>
</table>

* Tumors were rhabdomyosarcomas and fibrosarcomas which developed at the site of injection. A few leukemias occurred in both sublines.

Only one of 11 (9%) of the nonresponsive AKXL-38 mice developed a subcutaneous tumor. We found no significant difference in the mean latency period between the two sublines, and there was no difference in tumor susceptibility between males and females.

DISCUSSION

We have shown that the induction of cytochrome P450 by polycyclic aromatic compounds is associated, in recombinant inbred sublines as well as in inbred strains of mice, with increases in ten microsomal monooxygenase activities and one cytosol activity. In studies of more than 500 offspring from intercrosses and backcrosses from C57BL/6N and DBA/2N animals, responsiveness to aryl hydrocarbon hydroxylase induction and cytochrome P450 by MC or BNF segregates almost exclusively as a single dominant allele. The linkage of each enzymatic activity listed in Table 2 to this single locus or gene cluster has been established by comparing aryl hydrocarbon hydroxylase activity with another particular activity in MC-treated individuals of these same crosses. In all of these studies (see beginning of paper for references), not a single recombinant F2 or backcross animal has been found, suggesting that either a single gene or gene cluster is responsible for the pleiotropic effect of all these induced enzyme activities and cytochrome(s) P450. Even though only two recombinant inbred sublines (equivalent to two F2 individual mice) were used in the present study to arrive at this same conclusion, the chances are statistically significantly small that this conclusion is incorrect.*

The derivation of multiple AKXL recombinant inbred strains from AKR/J and C57L/J has been recently described in detail (Taylor and Meier 1976). At the F12 generation, two sublines of many of the lines were established. Although no selection was practiced in developing the sublines, the lines were monitored at

* If two dominant phenotypes A and B are studied in a cross of two inbred strains of genotypes AABB and aabb, where the A and B loci are not linked, then the probability is 10/16 or .625 that an F2 individual will be phenotypically identical to one of the parents. If only two F2 individuals are studied the probability is (.625)² or .39 that they will both be phenotypically like one of the parents; thus there is a 39% chance that one would incorrectly conclude that the A and B loci are linked in fact they are not. If this argument is extended to 10 dominant phenotypes in the cross AABB...J1 x aabb...ij2, the probability is .056 that an F2 individual will be like one parent or the other, and the chance is only (.056)² or 0.31%, that one would conclude that a single or small number of tightly linked genes is responsible when in fact ten segregating loci are involved.
several of the generations with respect to the skin ulceration response to topically administered DMBA (Thomas, Hutton and Taylor 1973), a response genetically controlled by the \( \text{Ah} \) locus. The \( F_1 \) parents of the AKXL-38 line were evidently both heterozygous at the \( \text{Ah} \) locus, because their progeny displayed a 3:1 ratio of responsive to nonresponsive. Following additional generations of brother-sister mating, the AKXL-38 subline was then found to be uniformly resistant to the DMBA-induced inflammatory response, whereas the "auxiliary" subline (designated AKXL-38A) was found to be uniformly susceptible. Although these two sublines have been found to be identical at 22 additional loci (Taylor and Meier 1976), AKXL-38 and AKXL-38A were found to be fixed at the hexose-6-phosphate dehydrogenase-1 locus \( (\text{Gpd-I}) \) for the AKR/J and C57L/J alleles, respectively. Because the unmapped \( \text{Ah} \) locus is not linked to \( \text{Gpd-I} \) (Thomas, Kouri and Hutton 1972), the concordant segregation of these two loci in the two sublines is therefore probably coincidental.

The probability that sublines of a recombinant inbred strain separated at the \( F_{12} \) generation will differ at a particular autosomal locus (and hence the expected proportion of loci at which two such lines will differ) was computed using the method of transition matrices. The probability of occurrence of the three nonfixed mating types at the \( F_{11} \) generation of the breeding pair was computed: heterozygote \( \times \) heterozygote \( (Aa \times Aa) \), \( P_1 = 0.04804 \); heterozygote \( \times \) homozygote \( (Aa \times aa \text{ or } Aa \times AA) \), \( P_2 = 0.06289 \); and unlike homozygotes \( (AA \times aa) \), \( P_3 = 0.00600 \). Given these parameters, one can show that the probability of the two sublines being different at any one locus is \( 1/2 P_1 + 3/8 P_2 + 1/2 P_3 \), or very nearly 0.05. This value was the basis for our choice of the \( F_{12} \) generation for establishment of the sublines. Natural selection which favors heterozygotes would of course have the effect of delaying gene fixation and thus increasing \( P_1, P_2, \) and \( P_3 \). In the present study, on the basis of the observed difference between the AKXL-38 (AKR/J-like and AKXL-38A (C57L/J-like) sublines with respect to the DMBA response and the eleven inducible enzyme activities, we could hypothesize that if all of the responses were pleiotropic effects of the same locus (\( \text{Ah} \)), then AKXL-38 would resemble AKR with respect to that response and AKXL-38A would resemble C57L. If one or more of the responses is controlled by any other unlinked locus, then the \( a \text{ priori} \) probability that AKXL-38 and AKXL-38A would inherit the AKR and C57L alleles of the unlinked locus, respectively, is 0.025. Thus we can say with a 97.5\% confidence that these responses are not controlled by unlinked genes. The possibility that these responses are controlled by a cluster of closely linked loci is not excluded. In the future as genetic markers are mapped in the vicinity of the \( \text{Ah} \) locus, it may be possible to place strict bounds on the size of the genetic region controlling the responses. Thomas, Hutton and Taylor (1973) estimated that if the \( \text{Ah} \) locus is distinct from the locus that controls the DMBA inflammatory response, it is unlikely that the two loci are more than 6.0 centimorgans apart.

Our observation that the AKXL-38A subline was much more susceptible toward MC-initiated sarcomas than AKXL-38 (Table 4) further supports the hypothesis (Nebert, Benedict and Kouri 1974) that the cytochrome \( P_1-450 \)
monooxygenase system is important in chemical carcinogenesis. Presumably there are genetic differences in the metabolism of MC by the increased amount of cytochrome P-450 in the responsive mouse, compared with that in the non-responsive mouse, thereby leading to a higher steady-state level of the proximal or ultimate carcinogen of MC in the target tissues of the responsive mouse. More indirect mechanisms can also be imagined. Our results stress the importance of careful selection of experimental animals for carcinogenesis experiments and strongly support the early work of Koury and coworkers (KOURI, RATRIE and WHITMIRE 1973; KOURI, SALERNO and WHITMIRE 1973).

The fact that the induction of 10 monooxygenase activities and one cytosol activity is controlled by a single regulatory system provides, however, no information on the number of structural genes (or induction-specific proteins) involved (cf. NEBERT et al. 1975 for discussion of this subject). The precise nature of the enzyme-active sites involved in monooxygenase activities,—i.e. their relationship to cytochrome P-450, and whether a multitude of sites exist or whether a limited number of "pliable" sites can accept a wide variety of structurally dissimilar substrates—is not completely understood. Electrophoresis of solubilized hepatic microsomes from rats (ÁLVARES and SIEKEVITZ 1973; WELTON and AUST 1974), rabbits (HAUGEN, VAN DER HOEVEN and COON 1975) and mice (HAUGEN, COON and NEBERT 1976) reveal three or more polypeptide bands in the 45,000- to 60,000-dalton range corresponding to cytochrome P-450 apoproteins. Partially purified preparations of P-450 cytochromes (isolated by techniques which prevent the dissociation of the heme component), representing one or two electrophoretic bands, have been used in "reconstituted systems", containing NADPH-cytochrome P-450 reductase and a lipid fraction (presumably needed to recreate a hydrophobic environment), which possess monooxygenase activities toward a number of substrates (Lu and COON 1968; Lu, STROBEL and COON 1970; Lu and LEVIN review 1974). Furthermore, different classes of microsomal inducers (of which MC, BP and BNF represent one class and phenobarbital another) cause increases in differing groups of monooxygenase activities and in different electrophoretic bands; by using the reconstituted system, it appears that the substrate specificity resides in the various cytochromes themselves (Lu et al. 1971; Lu et al. 1972a; NEBERT et al. 1973; HAUGEN, VAN DER HOEVEN and COON 1975).

The polycyclic aromatic compounds primarily induce one (HAUGEN, COON and NEBERT 1976) or perhaps two (J. S. FELTON and D. W. NEBERT 1976) electrophoretic bands in mice. Although it is not known whether each of these bands can be resolved into several distinct apoproteins of similar molecular weight or, even if not, whether a single cytochrome possesses one or several different catalytic sites, the possibility remains that all the monooxygenase activities listed in Table 2 occur at a single hydrophobic, but pliable, enzyme-active site in mice. If inferences can be drawn from studies on other mammalian species, however, that possibility may be unlikely. In the adult rabbit—in which MC or BNF appears to induce a single electrophoretic species (HAUGEN, VAN DER HOEVEN and COON 1975)—only two of these enzymatic activities, 2-acetylamino-
fluorene N-hydroxylase and acetanilide 4-hydroxylase, are inducible by MC, one activity, 7-ethoxycoumarin O-deethylase, is markedly decreased by MC treatment, and the remainder of the enzyme activities are unchanged (Atlas et al. 1975). These data suggest that at least three proteins are responsible for these activities in the rabbit.

The concept of a single regulatory system governing the MC inducibility of cytochrome P₄₅₀ and associated enzymatic activities by MC in mice has been strengthened by the recent finding that nonresponsive strains are able to respond to the more potent inducer 2,3,7,8-tetrachlorodibenzo-p-dioxin (Poland et al. 1974). The fact that nonresponsive strains are less sensitive to 2,3,7,8-tetrachlorodibenzo-p-dioxin than responsive strains suggests that failure of nonresponsive strains to respond to MC or BNF may be due to a defective "receptor" (Poland and Glover 1975). Thus the small increases in other monoxygenase activities observed after BNF treatment of nonresponsive strains (Table 3), which are not associated with cytochrome P₄₅₀ induction, represent a different phenomenon: perhaps some sort of activation not requiring protein synthesis, or else induction by a different mechanism. With regard to the latter possibility, a small increase in one of the P₄₅₀ apoproteins has been observed following MC treatment of nonresponsive strains (J. S. Felton and D. W. Nebert, unpublished data).

Although the ultimate resolution of these issues may require as-yet-undeveloped techniques, genetic analysis of the regulation of cytochrome P₄₅₀-associated monoxygenase activities has provided early clues to the nature of the molecular systems involved in biotransformations of drugs and chemical carcinogens. Analysis of inbred strains of mice has, furthermore, provided evidence that these enzyme systems are involved in the mediation of chemical carcinogenesis (Nebert, Benedict and Kouri 1974), mutagenesis (Felton and Nebert 1975), drug toxicity (Thorgersson, Felton and Nebert 1975; Robinson et al. 1975), and teratogenesis (Nebert, Thorgersson and Lambert 1976). By further studying these genetic models in congenic strains of mice, we hope to eliminate the possibility that other (unknown) gene differences between responsive and nonresponsive strains which cosegregate with the Ah locus may influence these phenomena.

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LITERATURE CITED

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Atlas, S. A. and D. W. Nebert, 1975 Genetic association of inducible naphthalene trans-1,2-dihydriodiol formation, acetanilide 4-hydroxylase, biphenyl 4-hydroxylase, and biphenyl 2-hydroxylase activities with inducible aryl hydrocarbon hydroxylase in mice. Arch. Biochem. Biophys. (In press.)


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