MOLECULAR PHARMACOLOGY, 22:779–786



# Quantitation of Hepatic Cytochrome $P_1$-450 mRNA with the Use of a Cloned DNA Probe

## Effects of Various P-450 Inducers in C57BL/6N and DBA/2N Mice

ROBERT H. TUKEY, MASAHIKO NEGISHI, AND DANIEL W. NEBERT

*Developmental Pharmacology Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20205*

Received March 29, 1982; Accepted June 18, 1982

SUMMARY

Clone 46 previously has been shown by both immunological and genetic criteria to represent a unique cloned cDNA associated with 3-methylcholanthrene-induced cytochrome $P_1$-450, a protein regulated by the *Ah* receptor. From the livers of control C57BL/6N and DBA/2N inbred mice and mice treated with each of 10 P-450 inducers, we concomitantly have examined microsomal aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase (EC 1.14.14.1) activity, immunoprecipitable $P_1$-450 microsomal protein, $P_1$-450 mRNA content, and *HindIII*- and *BamHI*-digested DNA fragments. $P_1$-450 mRNA was quantitated by means of $R_0t$ analysis with the clone 46 DNA. In C57BL/6N mice, $P_1$-450 mRNA is induced most effectively by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (> 200-fold over control values), followed in order by benzo[*a*]anthracene, 3-methylcholanthrene, benzo[*a*]pyrene, β-naphthoflavone, Aroclor 1254, and isosafrole. In both C57BL/6N and DBA/2N mice, isosafrole induces a form(s) of P-450 other than $P_1$-450. Phenobarbital, pregnenolone 16α-carbonitrile, and *trans*-stilbene oxide do not induce any measurable $P_1$-450 mRNA, yet they increase aryl hydrocarbon hydroxylase activity 2- to 3-fold in both C57BL/6N and DBA/2N mice. None of these 10 P-450 inducers causes any detectable change in the organization of chromosomal DNA, as evidenced by hydridization to clone 46 DNA. Quantities of aryl hydrocarbon hydoxylase activity, immunoprecipitable $P_1$-450 protein, and $P_1$-450 mRNA correlate well among these 10 groups of drug-treated mice, although some induced aryl hydrocarbon hydroxylase "activities" must be derived from induced forms of P-450 other than $P_1$-450. At lowest $R_0t$ values (maximally induced $P_1$-450 mRNA), inducible aryl hydrocarbon hydoxylase activity reaches a plateau, suggesting that translational processes may be involved in limiting the expression of the enzyme activity.

## INTRODUCTION

Cytochrome $P_1$-450 is defined (2) as that form of polycyclic-aromatic-inducible P-450 most closely associated with polycyclic-aromatic-inducible aryl hydrocarbon hydroxylase activity. This laboratory has defined cytochrome P-448 as that form of polycyclic-aromatic-inducible P-450 having a Soret peak shifted maximally to the blue when reduced and combined with CO. $P_1$-450 is distinct from P-448 not only in mice (3) but also in rabbits, rats, and fish (reviewed in ref. 4). The antibody to mouse $P_1$-450 inhibits rat $P_1$-450-catalyzed activity toward benzo[*a*]pyrene, and the mouse clone 46 cDNA cross-hybridizes to rat $P_1$-450 mRNA and the rat $P_1$-450 structural gene (5).

The *Ah* locus regulates the induction of $P_1$-450 and other proteins by polycyclic aromatic compounds such as 3-methylcholanthrene and TCDD[1] (reviewed in ref. 2). By taking advantage of the *Ah* receptor difference between "responsive" B6 and "nonresponsive" D2 mice, this laboratory has cloned a portion of the $P_1$-450 cDNA (6). Both immunological and genetic criteria confirm the fact that clone 46 represents indeed an 1100-base pair portion of $P_1$-450 cDNA (6, 7). By use of the clone 46 probe, it also was shown (7) that $P_1$-450 induction is under transcriptional control.

It is well known that treatment of mice, rats, and

This work was presented at the Meeting of the American Society for Pharmacology and Experimental Therapeutics, Calgary, Alberta, Canada, August 1981 (1).

[1] The abbreviations used are: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; B6, the inbred C57BL/6N mouse strain; D2, the inbred DBA/2N mouse strain; DBM, diazobenzyloxymethyl; $NaDodSO_4$, sodium dodecyl sulfate.

0026-895X/82/060779-08$02.00/0

rabbits with numerous P-450 inducers (8)—even phenobarbital (9)—enhances hepatic aryl hydrocarbon hydroxylase activity to some extent. In mice it is clear from recent studies (3, 10, 11) that more than just $P_1$-450 contributes to the aggregate polycyclic-aromatic-inducible aryl hydrocarbon hydroxylase activity. The use of a specific $P_1$-450 DNA probe therefore can be employed to determine definitively whether increases in the enzyme activity directly reflect $P_1$-450 mRNA induction. In this report we thus have examined simultaneously the effects on genomic DNA, specific $P_1$-450 mRNA content, relative concentrations of immunoprecipitable $P_1$-450 protein, and the catalytic hydroxylase activity in B6 and D2 mice treated with any one of 10 selected P-450 inducers.

Clone 46 was shown (6) to react specifically only with the $P_1$-450 gene. Clone 46 is therefore a specific probe for induced $P_1$-450 mRNA. This study could not have been carried out with any antibody, unless that antibody were monospecific for the $P_1$-450 protein. Any antibody—(including monoclonal antibodies) cross-reacting with any antigenic sites on other forms of P-450—would not be as specific as the clone 46 probe in detecting $P_1$-450 mRNA and only $P_1$-450 mRNA. Hence, the specific detection of the single $P_1$-450 mRNA species by clone 46 is the key to this study.

## EXPERIMENTAL PROCEDURES

*Materials and animals.* Benzo[*a*]anthracene and isosafrole were purchased from Eastman Laboratory and Specialty Chemicals (Rochester, N. Y.); benzo[*a*]pyrene from Sigma Chemical Company (St. Louis, Mo.); Aroclor 1254 from Analabs, Inc. (North Haven, Conn.); *trans*-stilbene oxide from Aldrich Chemical Company (Milwaukee, Wisc.); and $S_1$ nuclease from Miles Laboratories (Elkhart, Ind.). Pregnenolone 16$\alpha$-carbonitrile was a generous gift from G. D. Searle and Company (Chicago, Ill.). Sources of all other chemicals have been provided previously (3, 6, 7, 12, 13). *Ah*-responsive B6 and *Ah*-nonresponsive D2 mice were purchased as sexually immature weanlings of either sex from the Veterinary Resources Branch, National Institutes of Health (Bethesda, Md.).

*Treatment of mice.* All animals were treated i.p. each day with the following chemicals dissolved in corn oil (25 ml/kg): benzo[*a*]anthracene, 3-methylcholanthrene, benzo[*a*]pyrene, $\beta$-naphthoflavone, or Aroclor 1254, 125 mg/kg; isosafrole, 125 mg/kg; pregnenolone 16$\alpha$-carbonitrile, 150 mg/kg; and *trans*-stilbene oxide, 100 mg/kg. The mice were killed 24 hr after the fourth dose. Sodium phenobarbital in 0.85% NaCl (25 ml/kg) was administered i.p. on consecutive days at doses of 40, 60, 80, and 80 mg/kg, respectively, and the mice were killed 24 hr after the fourth dose. TCDD in *p*-dioxane (0.4 ml/kg) was given as a single i.p. dose of 25 $\mu$g/kg 48 hr before killing. In prior studies, it had been determined that all 10 inducers exerted their maximal effect (induction of total P-450 content) at these dosage regimens chosen. Livers were perfused with ice-cold 1.15% KCl, and 10 livers generally were combined. Aliquots of the tissue were then used for preparation of DNA, poly($A^+$)-enriched RNA, and microsomes from the same mice.

*Preparation of liver DNA.* The aliquots were frozen immediately in liquid nitrogen. DNA was then extracted from the frozen tissue with phenol/*m*-cresol (14).

*Isolation of total poly($A^+$)-enriched RNA.* The liver RNA was extracted by the guanidine HCl procedure (15), followed by oligo(dT)-cellulose chromatography twice (16) and precipitation in ethanol, as described in detail (7).

*Preparation and isolation of clone 46 cDNA.* Clone 46 has an internal *PstI* site (6). This clone was grown in *Escherichia coli* LE392 and purified (7). The plasmid was digested with *PstI* and the fragments were electrophoresed in 2% agarose containing 20 mM Tris-borate (pH 7.8). The 700- and 400-base pair fragments were cut out of the agarose gel and electroeluted for 4 hr in dialysis bags containing 2–3 ml of 10 mM Tris-HCl buffer (pH 7.0) plus 10 mM $Na_3BO_3$. The contents of the dialysis bag were applied to a DEAE-cellulose column (1.5 × 3 cm) which had been equilibrated with 20 mM Tris-HCl (pH 7.5) containing 0.1 M KCl. The contents of the column were washed with 10 ml of 20 mM Tris-HCl containing 0.3 M KCl, and the DNA was eluted in 5 ml of 20 mM Tris-HCl (pH 7.5) containing 0.6 M KCl. The DNA was recovered after precipitation in 2 volumes of 95% ethanol at −20°. These isolated DNA fragments from clone 46 were labeled with [$^{32}$P]CTP by means of nick translation (17).

*Blot hybridization of DNA and poly($A^+$)-enriched RNA.* Following digestion with *HindIII* or *BamHI*, genomic DNA fragments were electrophoresed in 1% agarose and transferred to DBM paper (18). The poly($A^+$)-enriched RNA was electrophoresed on 1% agarose gels containing 10 mM methylmercury(II) hydroxide (19) and then adsorbed to DBM paper (7). All hybridizations were performed with nick-translated [$^{32}$P]DNA purified from clone 46 (both the 700- and 400-base pair fragments).

*$R_0t$ analysis with [$^{32}$P]DNA from clone 46.* RNA excess hybridization experiments were performed (20), with certain modifications (21). Concentrations of poly($A^+$)-enriched RNA ranging from 10 ng/ml to 10 mg/ml were boiled for 3 min with the 700-base pair [$^{32}$P]DNA fragment of clone 46 (approximately 1000 cpm) in 40-$\mu$l sealed capillary tubes and then incubated at 68° for 10 min–36 hr. Following this hybridization, each sample was pipetted into a 1-ml solution containing 0.5 M NaCl, 2.5 mM $ZnCl_2$, 0.2 mM sodium acetate (pH 4.5), and 100 $\mu$g of salmon sperm DNA. The mixture was then divided in half: one-half was treated for 30 min at 37° with $S_1$ nuclease (200 units); the other half served as control. After $S_1$ nuclease treatment, 100% trichloroacetic acid (1:10 volume) was added, and the samples were chilled on ice for 15 min and then filtered over Whatman glass-fiber filters. Each filter was washed with 15 ml of 5% cold trichloroacetic acid, dried in air at room temperature, and counted in 10 ml of Aquasol in a Beckman 2000 liquid scintillation counter.

*Isolation of liver microsomes, NaDodSO$_4$-polyacrylamide gel electrophoresis, and quantitation of immunoprecipitable $P_1$-450 protein.* Preparation and −80° storage of the microsomes were performed as previously detailed (3). NaDodSO$_4$-polyacrylamide gel electrophoresis of the microsomes was performed at room temperature with the use of a Hoefer Scientific Model SE-500 apparatus with the discontinuous buffer system of Laemmli (22), with certain modifications (23). The protein bands in the gels were stained with Coomassie blue,

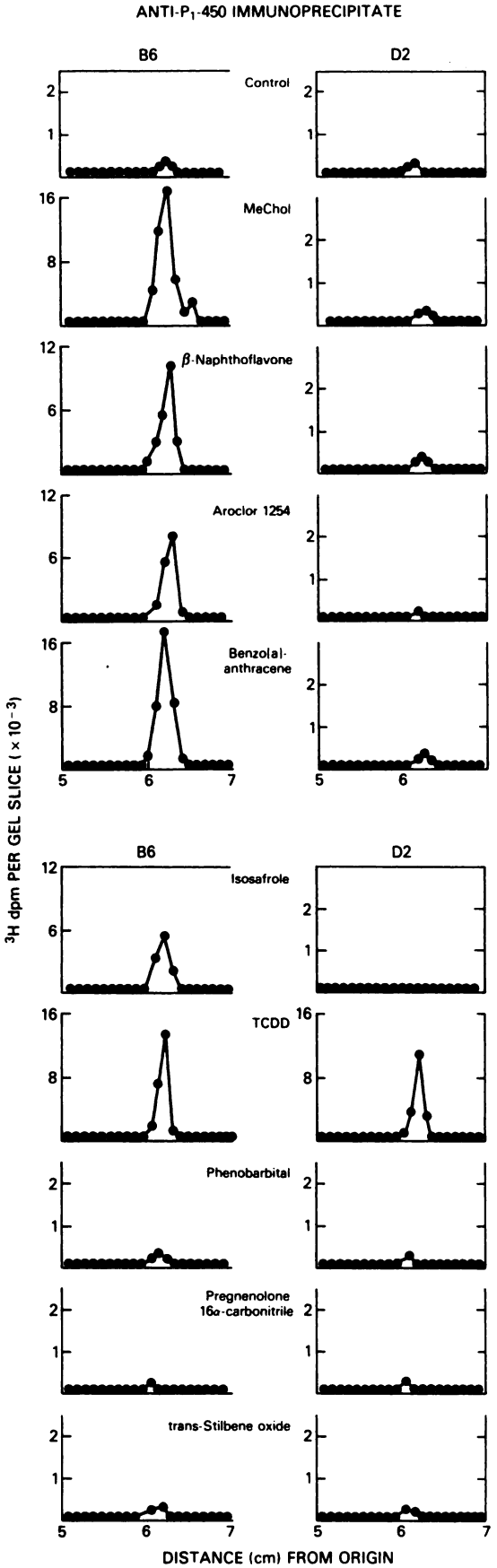


FIG. 1. *Electrophoresis of the radioactivity precipitated by anti-($P_1$-450) following the in vitro labeling of intact liver microsomes with NaB[$^3$H]$_4$ and then digestion with sodium cholate*

Results from control B6 and D2 mice and mice treated with each of nine P-450 inducers are illustrated. Experimental details have been described (3, 12).

and densitometric tracings were quantitated (24). Purification of mouse liver $P_1$-450 and the development of anti-($P_1$-450) has been described (3). The *in vitro* labeling of intact microsomes with NaB[$^3$H]$_4$ and the immunoprecipitation of the detergent-solubilized microsomes were carried out (3, 12).

*Enzyme assay.* Liver microsomal aryl hydrocarbon hydroxylase activity was determined (25). One *unit* of the hydroxylase activity is defined as that amount of enzyme catalyzing in 1 min at 37° the formation of hydroxylated products causing fluorescence equivalent to that of 1.0 pmole of the 3-hydroxybenzo[*a*]pyrene recrystallized standard. *Specific activity* denotes units per milligram of microsomal protein. Protein concentrations were measured by the method of Lowry *et al.* (26) with the use of bovine serum albumin as standard.

## RESULTS AND DISCUSSION

*Analysis of the inducible immunoprecipitable $P_1$-450 protein and aryl hydrocarbon hydroxylase activity.* Following treatment of B6 and D2 mice with each of the 10 P-450 inducers, liver microsomes from controls and treated mice were compared for immunoprecipitable $P_1$-450 (Fig. 1) and aryl hydrocarbon hydroxylase activity (Table 1). Differences of 5- to 28-fold in aryl hydrocarbon hydroxylase activity[2] between B6 and D2 were found for benzo[*a*]anthracene, 3-methylcholanthrene, benzo[*a*]pyrene, β-naphthoflavone, and Aroclor 1254 (Table 1). A large B6-D2 difference was measurable with immunoprecipitable radioactivity representing $P_1$-450 (Fig. 1). TCDD induced the $M_r$ ~ 55,000 immunoprecipitable $P_1$-450 protein (Fig. 1) and aryl hydrocarbon hydroxylase activity (Table 1) equally well in B6 and D2 mice.

Benzo[*a*]anthracene induced the total P-450 content in both B6 and D2 mice, yet the hydroxylase activity per nanomole of P-450 actually decreased almost 3-fold in D2 mice. These data are consistent with a second form of polycyclic-aromatic inducible P-450, one that has no catalytic activity toward benzo[*a*]pyrene.

Isosafrole induced aryl hydrocarbon hydroxylase activ-

[2] The assay for aryl hydrocarbon hydroxylase activity reflects principally the highly fluorescent 3-hydroxy- and 9-hydroxybenzo[*a*]pyrene, although all of the other benzo[*a*]pyrene phenols probably contribute (27). By high-pressure liquid chromatographic studies, this laboratory has found that induced $P_1$-450, governed by the $Ah^b$ allele and therefore the *Ah* receptor in mice, most specifically oxygenates the benzo[*a*]pyrene 7,8-bond and, as long as the epoxide hydrolase is not rate-limiting, the increases in 7,8-diol formation parallel very closely the formation of the benzo[*a*]pyrene phenols, especially 3-hydroxybenzo[a]pyrene (28). This is true, whether the inducer in mice is TCDD, 3-methylcholanthrene, benzo[*a*]anthracene, or β-naphthoflavone and whether the tissue is liver, intestine, lung, kidney, skin, marrow, or ovary (C. Legraverend, T. M. Guenthner, and D. W. Nebert, manuscript in preparation). Polycyclic-aromatic-inducible $P_1$-450 is thus viewed as catalyzing the most important step on the way to producing the ultimate carcinogen of numerous polycyclic hydrocarbons (reviewed in ref. 29): attack occurs on the angular benzo-ring adjacent to the "bay region" (30); there is first monooxygenation of the bond opposite the bay region; *trans*-dihydrodiol formation and then diol-epoxide formation follow readily.

TABLE 1

*Total P-450 content, aryl hydrocarbon hydroxylase, the $P_1$-450 protein, and $P_1$-450 mRNA following treatment of B6 and D2 mice with the 10 P-450 inducers*

Ten livers were pooled for each treatment group. Results of a second group of 10 livers each gave virtually identical results.

| Treatment | Total P-450 content | | Aryl hydrocarbon hydroxylase activity | | | | Densitometric units from $NaDodSO_4$-polyacrylamide gel electropherogram ($M_r$ ~ 55,000) | | Anti-($P_1$-450)-precipitable radioactivity | | Log equivalent $R_0t_{1/2}$ for $P_1$-450 mRNA | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | B6 | D2 | B6 | D2 | B6 | D2 | B6 | D2 | B6 | D2 | B6 | D2 |
| | *nmoles/mg protein* | | *pmoles/min/mg protein* | | *pmoles/min/nmoles P-450* | | | | | | | |
| Control | 0.34 | 0.30 | 200 | 180 | 590 | 610 | 25 | 30 | 750 | 800 | >3000 | 2,400 |
| TCDD | 1.90 | 1.80 | 3,400 | 3,100 | 1,790 | 1,770 | 70 | 65 | 54,700 | 36,000 | 14 | 28 |
| Benzo[*a*]anthracene | 1.05 | 0.70 | 4,310 | 150 | 4,100 | 220 | 60 | 20 | 36,200 | 880 | 16 | |
| 3-Methylcholanthrene | 1.80 | 0.75 | 3,690 | 370 | 2,040 | 490 | 90 | 30 | 38,700 | 1,050 | 18 | 480 |
| Benzo[*a*]pyrene | 1.40 | 0.70 | 4,430 | 310 | 3,210 | 440 | 55 | 25 | | | | |
| β-Naphthoflavone | 0.96 | 0.50 | 3,100 | 250 | 3,230 | 490 | 55 | 30 | 23,000 | 950 | 40 | |
| Aroclor 1254 | 1.30 | 1.00 | 2,200 | 480 | 1,750 | 470 | 50 | 30 | 21,000 | 1,010 | 45 | |
| Isosafrole | 1.60 | 1.40 | 680 | 360 | 430 | 260 | 60 | 70 | 12,800 | 800 | 170 | |
| Phenobarbital | 1.30 | 1.40 | 500 | 570 | 400 | 400 | 40 | 45 | 750 | 1,100 | | |
| Pregnenolone 16α-carbonitrile | 1.00 | 0.97 | 420 | 640 | 410 | 660 | 40 | 40 | 650 | 850 | | |
| *trans*-Stilbene oxide | 1.10 | 1.00 | 550 | 470 | 500 | 450 | 35 | 40 | 800 | 800 | | |

ity slightly in both strains of mice (Table 1). A 2-fold difference was found between B6 and D2. Isosafrole induced an $M_r$ ~ 55,000 electrophoretic band in both B6 and D2 mice (Table 1), yet immunoprecipitable $P_1$-450 was found in B6 but not D2 mice (Fig. 1). Although isosafrole greatly increased the total P-450 content in both B6 and D2 mice, the hydroxylase activity per nanomole of P-450 actually decreased in both B6 and D2 mice. These results further indicate that isosafrole induces another form of P-450 that has little or no specificity toward benzo[*a*]pyrene metabolism. This form of induced P-450 exists along with $P_1$-450 in isosafrole-treated B6 mice but exists without $P_1$-450 in isosafrole-treated D2 mice. Recently this isosafrole-induced P-450 was highly purified from D2 mice and named $P_2$-450 (31).

Phenobarbital, pregnenolone 16α-carbonitrile, and *trans*-stilbene oxide each induced aryl hydrocarbon hydroxylase activity 2- to 4-fold in both strains of mice without any B6-D2 differences (Table 1), and essentially no immunoprecipitable $P_1$-450 was detectable (Fig. 1). In both B6 and D2 microsomes, the increases in intensity of each major electrophoretic band were found at about $M_r$ 52,000, 53,000, and 55,000 for phenobarbital, pregnenolone 16α-carbonitrile, and *trans*-stilbene oxide, respectively (data not illustrated). These phenobarbital- and pregnenolone 16α-carbonitrile-induced increases in electrophoretic bands from mouse liver have been reported previously (32).

*Induction of $P_1$-450 mRNA.* Large increases in $P_1$-450 (23 S) mRNA (denoted by *arrows* in Fig. 2), as measured by hybridization to [$^{32}$P]DNA from the clone 46 probe, were observed in TCDD-treated D2 mice and in B6 mice after treatment with 3-methylcholanthrene, TCDD, β-naphthoflavone, Aroclor 1254, benzo[*a*]anthracene, benzo[*a*]pyrene, and isosafrole—in the same seven instances showing the greatest aryl hydrocarbon hydroxylase inducibility and immunoprecipitable $P_1$-450 (Table 1). An mRNA species of about 23 S corresponds to 3000–3500 base pairs in length. Control D2 exhibits some hybridizable $P_1$-450 mRNA not seen in control B6; this was noted before (7), although the significance of this finding is unclear. 3-Methylcholanthrene produced an increase in D2 $P_1$-450 mRNA (arrow at *lower left* in Fig. 2); such an increase most likely is reflected in the 2-fold rise in aryl hydrocarbon hydroxylase activity in 3-methylcholanthrene-treated D2 mice but was not apparent in the immunoprecipitable $P_1$-450 protein from 3-methylcholanthrene-treated D2 mice (Fig. 1).

Isosafrole treatment did not enhance the $P_1$-450 mRNA in D2 mice. This difference between isosafrole-treated and control D2 mice therefore affords a distinct means with which to exploit in the cloning of a gene for an isosafrole-induced form of P-450 other than $P_1$-450 (33).

Phenobarbital, pregnenolone 16α-carbonitrile, and *trans*-stilbene oxide produced no detectable rise in $P_1$-450 mRNA in B6 or D2 mice—even after long exposures of the DBM paper to the X-ray film (Fig. 2, *bottom*). These data further support the fact that forms of P-450 other than $P_1$-450 are involved in the induction mechanism by these drugs and that clone 46 cDNA is specific for $P_1$-450 and does not hybridize detectably with other P-450 mRNAs.

*$R_0t$ analysis.* Analysis of the hybridization kinetics by means of $R_0t$ curves (Fig. 3) indicates that TCDD, benzo[*a*]anthracene, and 3-methylcholanthrene are most effective in B6 mice at inducing maximal $P_1$-450 mRNA levels. The $P_1$-450 mRNA concentration in TCDD-treated D2 and in β-naphthoflavone- and Aroclor 1254-treated B6 was 3–6 times less, and in isosafrole-treated B6 at least 10 times less, than the maximal level found in TCDD-treated B6 mice. In D2 mice, 3-methylcholan-

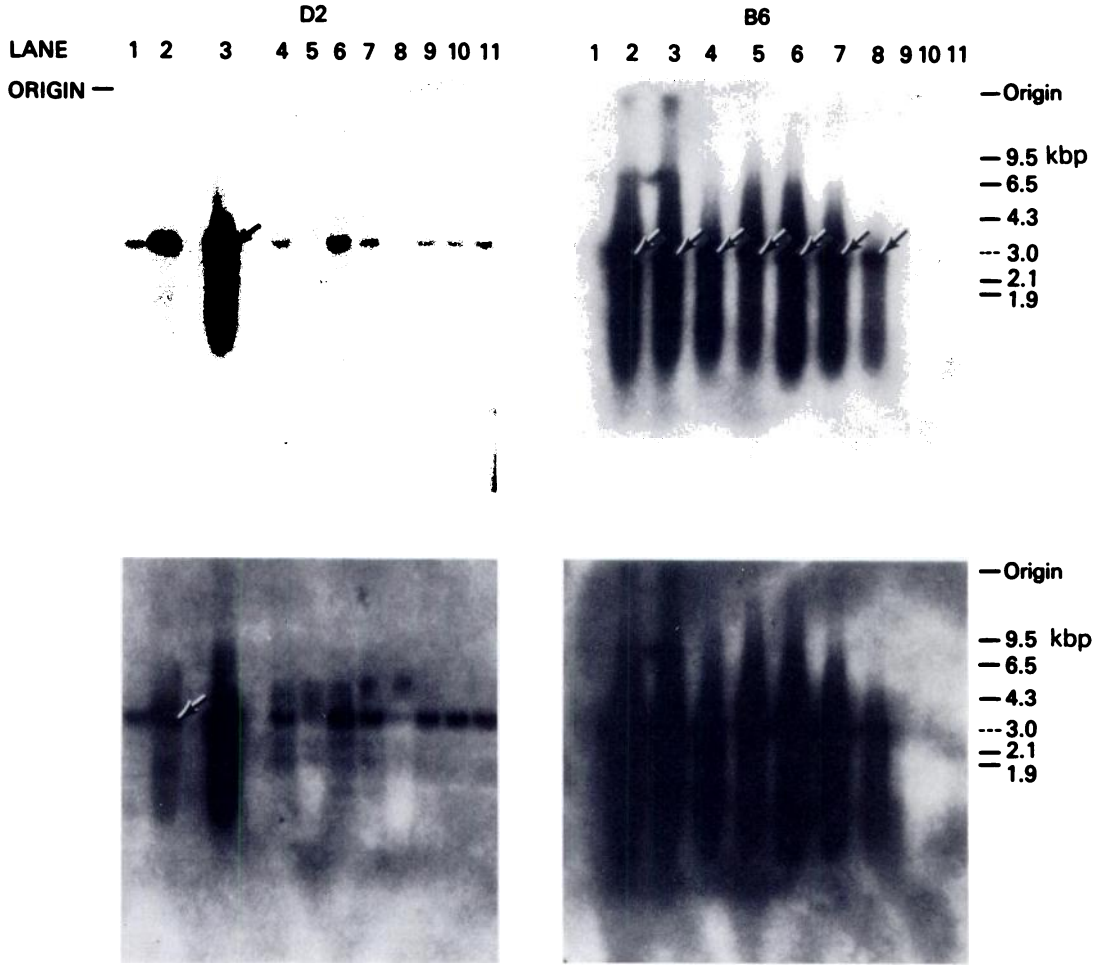


FIG. 2. *Northern blot of total poly(A⁺)-enriched B6 and D2 RNA probed with clone 46 [³²P]DNA*

Total hepatic poly(A⁺)-enriched RNA was prepared from control mice and from mice receiving each of 10 P-450 inducers. Lanes *1* through *11* represent control, 3-methylcholanthrene, TCDD, β-naphthoflavone, Aroclor 1254, benzo[*a*]anthracene, benzo[*a*]pyrene, isosafrole, phenobarbital, pregnenolone 16α-carbonitrile, and *trans*-stilbene oxide treatment, respectively. For the mRNA from D2 mice, each well contained 15 μg of RNA, except well 3, which contained 5 μg. All wells with B6 mRNA contained 5 μg of RNA. In this figure and in Fig. 4, electrophoretic migration is from *top* to *bottom*. Electrophoresis of the liver mRNA in 1% agarose gels under denaturing conditions, adsorption to DBM paper, and hybridization to nick-translated [³²P]DNA from clone 46 have been previously detailed (7). The same autoradiograms are shown after a 3-hr (*top*) and an 8-hr (*bottom*) exposure of the DBM paper of the film. Standards derived from *HindIII*-digested λ phage DNA provide approximations of size in kilobase pairs (*kbp*) at *right*.

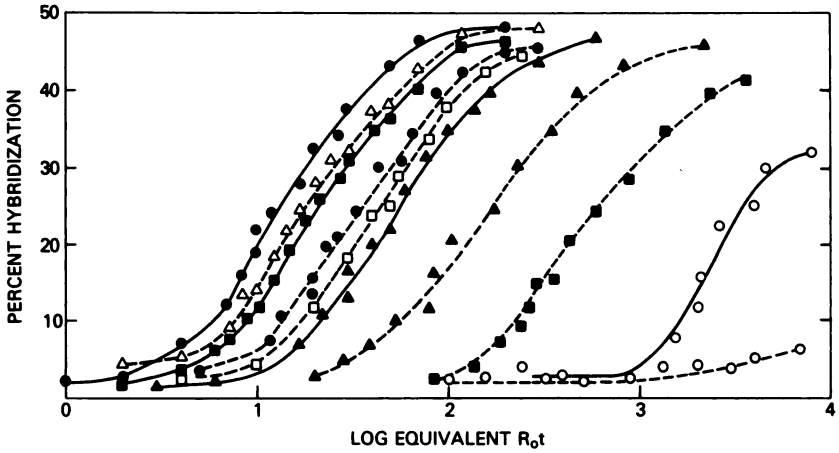


FIG. 3. *Isolation of poly(A⁺)-enriched liver RNA from control mice and mice treated with one of six P-450 inducers and hybridization of excess mRNA with clone 46 [³²P]DNA*

Details are provided under Experimental Procedures. The curves from *left* to *right* represent TCDD-treated B6, benzo[*a*]anthracene-treated B6, 3-methylcholanthrene-treated B6, TCDD-treated D2, β-naphthoflavone-treated B6, Aroclor 1254-treated B6, isosafrole-treated B6, 3-methylcholanthrene-treated D2, control D2, and control B6. Fifty per cent hybridization represents complete hybridization because the 700-base pair fragment of clone 46 DNA is double-stranded.

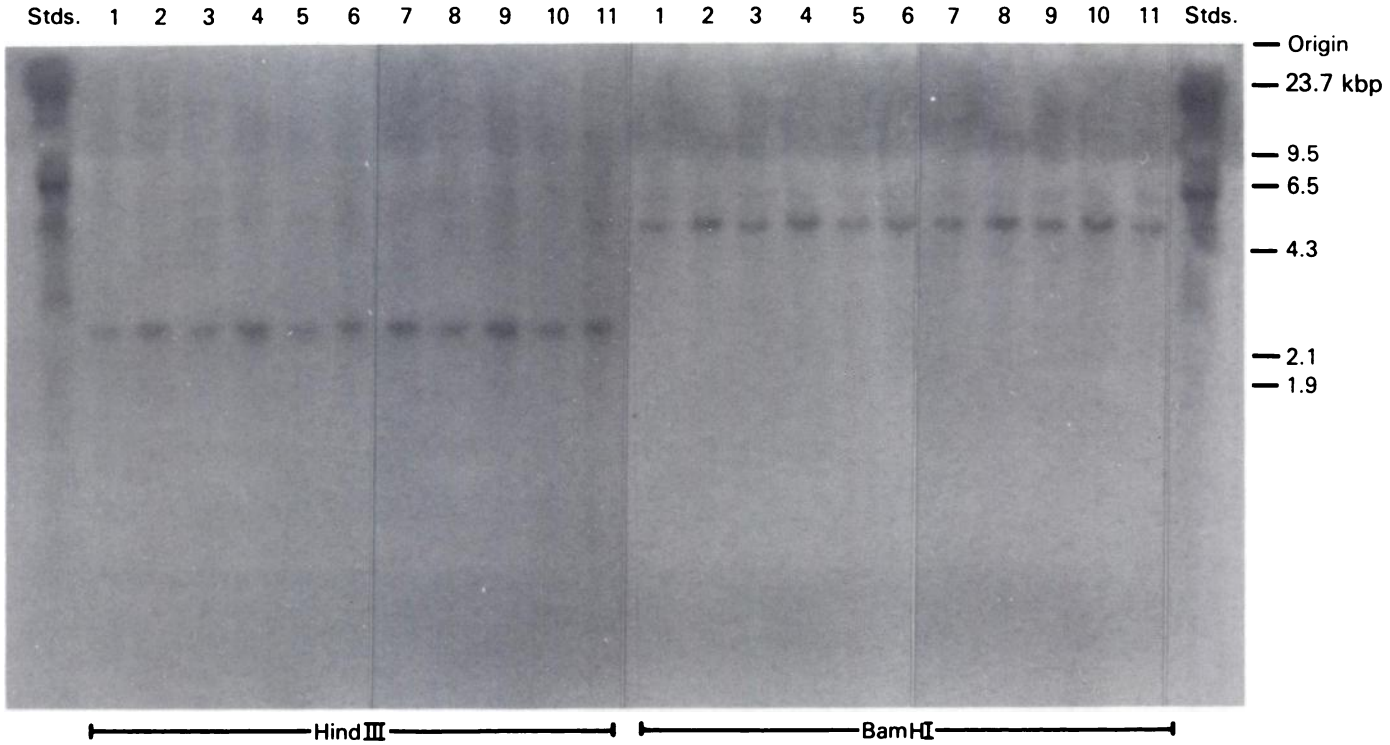


FIG. 4. *Northern blot of HindIII-digested (left) or BamHI-digested (right) genomic B6 DNA fragments with the clone 46 probe*

Total hepatic DNA was isolated from control mice and from mice receiving each of 10 P-450 inducers, as detailed under Experimental Procedures. Lanes *1* through *11* represent control followed by the same order of 10 inducers listed in Fig. 2. Each well contained 20 μg of DNA. Electrophoresis of the DNA fragments in 1% agarose gels, adsorption to DBM paper, and hybridization to nick-translated [$^{32}$P]DNA from clone 46 have been described previously (7). This autoradiogram represents a 48-hr exposure of the DBM paper to X-ray film and then magnification with the intensifier screen. Standards (*Stds.*) derived from *HindIII*-digested λ phage DNA are depicted at *right* in kilobase pairs (*kbp*).

threne induced approximately a 4-fold increase in $P_1$-450 mRNA, compared with control D2 values. The shapes of these curves for all eight drug-treated examples and for control D2 are within the experimental error of $R_0t$ analysis for demonstrating a single mRNA species (34). This induced $P_1$-450-specific mRNA represents less than 0.1% of the total mRNA in the liver cell. From the data in Table 1 and Figs. 1–3, therefore, we conclude that TCDD, benzo[*a*]anthracene, 3-methylcholanthrene, benzo[*a*]pyrene, β-naphthoflavone, Aroclor 1254, and isosafrole induce $P_1$-450 mRNA, which in turn is translated into the immunoprecipitable $P_1$-450 that is catalytically active in hydroxylating benzo[*a*]pyrene.

The data in Figs. 1 through 3 and Table 1 are consistent with the hypothesis that all of the above-mentioned seven chemicals interact at least to some degree with the *Ah* receptor, thereby evoking $P_1$-450 mRNA and protein induction and increases in aryl hydrocarbon hydroxylase activity. Phenobarbital, pregnenolone 16α-carbonitrile, and *trans*-stilbene oxide do not. This hypothesis is strengthened by previous *Ah* receptor binding studies with these 10 compounds (35–37). In terms of induction not associated with the *Ah* locus, whether some of these inducers such as isosafrole, Aroclor 1254, phenobarbital, pregnenolone 16α-carbonitrile, or *trans*-stilbene oxide effect their inductive process via some other receptor will require further study.

*DNA-DNA hybridization.* It is possible that various P-450 inducers might cause changes in the $P_1$-450 structural gene that are detectable by hybridization of mouse liver DNA with the clone 46 probe. Figure 4 shows that *HindIII*- and *BamHI*-digested fragments of B6 DNA which hybridize to clone 46 DNA resulted in restriction fragments of about 2.5 and 6.0 kilobase pairs, respectively. The same size of restriction fragment was seen in controls and in mice exposed to any of the 10 P-450 inducers. It is thus concluded that none of these inducers produces any detectable gene amplification or gross form of genomic rearrangement in this portion of the mouse $P_1$-450 structural gene.

*Comparison of mRNA and protein parameters.* In concomitantly studied groups of control mice plus mice treated with each of the 10 inducers (Fig. 5), the isosafrole-treated samples can be seen to stand out. When aryl hydrocarbon hydroxylase specific activity is plotted as a function of immunoprecipitated protein (Fig. 5, *left*), the isosafrole-induced B6 sample demonstrates induction of both the enzyme activity and the $P_1$-450 protein, whereas the isosafrole-induced D2 sample shows little induction of the hydroxylase activity and no induction of the $P_1$-450 protein. These data suggest that isosafrole uses the *Ah* receptor in inducing the $P_1$-450 protein (31, 38). When aryl hydrocarbon hydroxylase activity is plotted as a function of densitometric units from the $NaDodSO_4$-polyacrylamide gel electropherogram (Fig. 5, *right*), both B6 and D2 isosafrole-treated samples appear out of line. This graph supports the possibility that isosafrole induces in both B6 and D2 mice one or more forms of P-

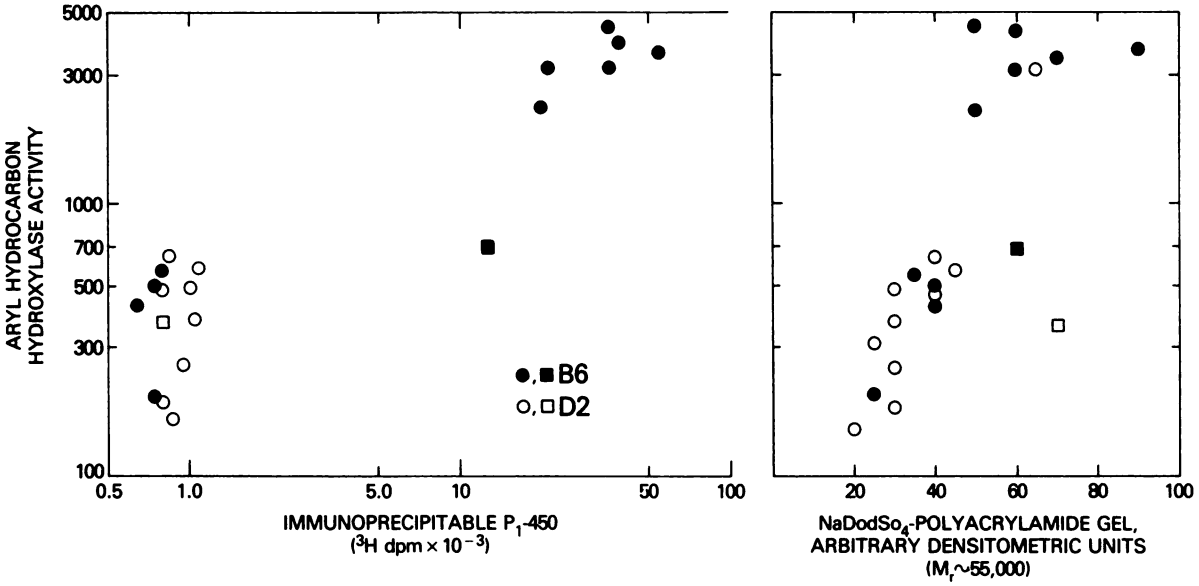


FIG. 5. *Aryl hydrocarbon hydroxylase activity as a function of anti-($P_1$-450)-precipitated radioactivity (left) and arbitrary densitometric units in the $M_r$ ~ 55,000 range following NaDodSO4-polyacrylamide gel electrophoresis (right)*
All values from Table 1 are included. *Squares* represent isosafrole-treated samples. Values of 1,000 dpm of immunoprecipitable $P_1$-450 (*left*) are regarded as negligible.

450 other than the one (i.e., $P_1$-450) responsible for polycyclic-aromatic-induced aryl hydrocarbon hydroxylase activity.

At specific hydroxylase activities between 200 and 700 units/mg of microsomal protein, following treatment with phenobarbital, pregnenolone 16α-carbonitrile, or *trans*-stilbene oxide, no distinct increase in immunoprecipitable $P_1$-450 was observed. Control aryl hydrocarbon hydroxylase activity is known not to reflect $P_1$-450 because immunoprecipitable $P_1$-450 protein is not detectable in control B6 mice (13). These 500 units of hydroxylase activity therefore represent form(s) of induced P-450 other than $P_1$-450.

When aryl hydrocarbon hydroxylase specific activity was plotted as a function of $P_1$-450 mRNA content quantitated by $R_0t$ analysis (Fig. 6), an excellent correlation was observed—except at the highest mRNA concentrations. The $P_1$-450 mRNA concentration in TCDD-, benzo[*a*]anthracene-, and 3-methylcholanthrene-treated B6 mice reached a plateau; in other words, excessive amounts of the mRNA produce no further increases in the already maximally induced hydroxylase activity. In B6 mice treated maximally with these three inducers, therefore, the rate of translation of available $P_1$-450 mRNA appears to become rate-limiting.

The data in Figs. 5 and 6 illustrate that $P_1$-450 mRNA, and ultimately the immunologically identifiable $P_1$-450 protein, can account for most, but not all, of the induced aryl hydrocarbon hydroxylase "activities" that are possible. Other inducers—such as TCDD, benzo[*a*]anthracene, Aroclor 1254, isosafrole, phenobarbital, pregnenolone 16α-carbonitrile, and *trans*-stilbene oxide—apparently induce forms of P-450 in addition to $P_1$-450. Although these induced forms of P-450 most likely are more specific toward other substrates, these forms also use benzo[*a*]pyrene as a substrate because a small

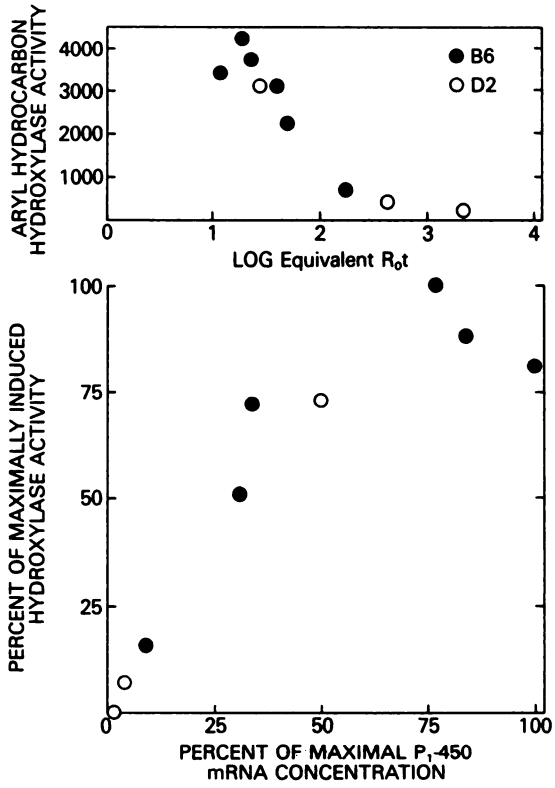


FIG. 6. *Aryl hydrocarbon hydroxylase activity as a function of the log equivalent $R_0t$ value (top) and the percentage of maximally inducible hydroxylase activity as a function of the percent of $P_1$-450 mRNA estimated by $R_0t$ analysis (bottom)*

The lowest log equivalent $R_0t$ value is regarded as the 100% level of (maximally inducible) $P_1$-450 mRNA. The nine successful experiments shown in Fig. 3 are illustrated; the control B6 sample could not be plotted. The highest three $P_1$-450 mRNA values represent TCDD-, benzo[*a*]anthracene-, and 3-methylcholanthrene-treated B6 mice.

amount of non-$P_1$-450-associated induced aryl hydrocarbon hydroxylase activity is detectable.

Other laboratories have begun the cloning of rat cDNA associated with phenobarbital-induced (39, 40) and 3-methylcholanthrene-induced (41) forms of P-450. If these cDNA probes are shown to be as specific for a single form of induced P-450 as the clone 46 probe, studies similar to those in this report should provide more precise information than what has been possible to obtain in the past by polyspecific antibodies (reviewed in ref. 4).

## ACKNOWLEDGMENT

The expert secretarial assistance of Ms. Ingrid E. Jordan is greatly appreciated.

## REFERENCES

1. Tukey, R. H., M. Negishi, and D. W. Nebert. A study of cytochrome $P_1$-450 mRNA enhancement by various inducers with the use of a cloned DNA probe. *Pharmacologist* **23**:175 (1981)
2. Nebert, D. W., M. Negishi, M. A. Lang, L. M. Hjelmeland, and H. J. Eisen. The *Ah* locus, a multigene family necessary for survival in a chemically adverse environment: Comparison with the immune system. *Adv. Genet.* **21**:1–52 (1982).
3. Negishi, M., and D. W. Nebert. Structural gene products of the *Ah* locus: genetic and immunochemical evidence for two forms of mouse liver cytochrome P-450 induced by 3-methylcholanthrene. *J. Biol. Chem.* **254**:11015–11023 (1979).
4. Nebert, D. W., and M. Negishi. Multiple forms of cytochrome P-450 and the importance of molecular biology and evolution. *Biochem. Pharmacol.* **31**:2311–2317 (1982).
5. Chen, Y.-T., M. A. Lang, N. M. Jensen, M. Negishi, R. H. Tukey, E. Sidransky, T. M. Guenthner, and D. W. Nebert. Similarities between mouse and rat liver microsomal cytochromes P-450 induced by 3-methylcholanthrene: evidence from catalytic, immunologic, and recombinant DNA studies. *Eur. J. Biochem.* **122**:361–368 (1982).
6. Negishi, M., D. C. Swan, L. W. Enquist, and D. W. Nebert. Isolation and characterization of a cloned DNA sequence associated with the murine *Ah* locus and a 3-methylcholanthrene-induced form of cytochrome P-450. *Proc. Natl. Acad. Sci. U. S. A.* **78**:800–804 (1981).
7. Tukey, R. H., D. W. Nebert, and M. Negishi. Structural gene product of the [*Ah*] complex: evidence for transcriptional control of cytochrome $P_1$-450 induction by use of a cloned DNA sequence. *J. Biol. Chem.* **256**:6969–6974 (1981).
8. Conney, A. H. Pharmacological implications of microsomal enzyme induction. *Pharmacol. Rev.* **19**:317–366 (1967).
9. Nebert, D. W., J. E. Gielen, and F. M. Goujon. Genetic expression of aryl hydrocarbon hydroxylase induction. III. Changes in the binding of *n*-octylamine to cytochrome P-450. *Mol. Pharmacol.* **8**:651–666 (1972).
10. Lang, M. A., and D. W. Nebert. Structural gene products of the *Ah* locus: evidence for many unique P-450-mediated monooxygenase activities reconstituted from 3-methylcholanthrene-treated C57BL/6N mouse liver microsomes. *J. Biol. Chem.* **256**:12058–12067 (1981).
11. Lang, M. A., J. E. Gielen, and D. W. Nebert. Genetic evidence for many unique liver microsomal P-450-mediated monooxygenase activities in heterogeneic stock mice. *J. Biol. Chem.* **256**:12068–12075 (1981).
12. Negishi, M., N. M. Jersen, G. S. Garcia, and D. W. Nebert. Structural gene products of the murine *Ah* locus: differences in ontogenesis, membrane location, and glucosamine incorporation between liver microsomal cytochromes $P_1$-450 and P-448 induced by polycyclic aromatic compounds. *Eur. J. Biochem.* **115**:585–594 (1981).
13. Negishi, M., and D. W. Nebert. Structural gene products of the *Ah* complex: increases in large mRNA from mouse liver associated with cytochrome $P_1$-450 induction by 3-methylcholanthrene. *J. Biol. Chem.* **256**:3085–3091 (1981).
14. Tiemeier, D., S. Tilghman, and P. Leder. Purification and cloning of a mouse ribosomal gene fragment in phage lambda. *Gene* **2**:173–191 (1977).
15. Cox, R. A. The use of guanidinium chloride in the isolation of nucleic acids. *Methods Enzymol.* **12**:120–129 (1968).
16. Aviv, H., and P. Leder. Purification of biologically active globin mRNA chromatography on oligo(dT)-cellulose. *Proc. Natl. Acad. Sci. U. S. A.* **69**:1408–1412 (1972).
17. Tsai, S. Y., D. R. Roop, M. Tsai, J. P. Stein, A. R. Means, and B. W. O'Malley. Effect of estrogen on gene expression in the chick oviduct: regulation of the ovomucoid gene. *Biochemistry* **17**:5773–5780 (1978).
18. Wahl, G. M., M. Stern, and G. R. Stark. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxmethyl paper and rapid hybridization by using dextran sulfate. *Proc. Natl. Acad. Sci. U. S. A.* **76**:3683–3687 (1979).
19. Bailey, J. M., and N. Davidson. Methylmercury as a reversible denaturing agent for agarose gel electrophoresis. *Anal. Biochem.* **70**:75–85 (1976).
20. Harris, S. E., R. J. Schwartz, M. J. Tsai, A. K. Roy, and B. W. O'Malley. Effect of estrogen on gene expression in the chick oviduct: *in vitro* transcription of the ovalbumin gene in chromatin. *J. Biol. Chem.* **251**:524–529 (1976).
21. Chan, L., A. Dugaiczyk, and A. R. Means. Molecular cloning of the gene sequences of a major apoprotein in very low density lipoproteins. *Biochemistry* **19**:5631–5637 (1980).
22. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**:680–685 (1970).
23. Tukey, R. H., and T. R. Tephly. Purification and properties of rabbit liver estrone and p-nitrophenol UDP-glucuronyltransferases. *Arch. Biochem. Biophys.* **209**:565–578 (1981).
24. Boobis, A. R., D. W. Nebert, and J. S. Felton. Comparison of $\beta$-naphthoflavone and 3-methylcholanthrene as inducers of hepatic cytochrome(s) P-448 and aryl hydrocarbon (benzo[a]pyrene) hydroxylase activity. *Mol. Pharmacol.* **13**:259–268 (1977).
25. Nebert, D. W. Genetic differences affecting microsomal electron transport: the *Ah* locus. *Methods Enzymol.* **LII**:226–240 (1978).
26. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
27. Holder, G., H. Yagi, W. Levin, A. Y. H. Lu, and D. M. Jerina. Metabolism of benzo[a]pyrene. III. An evaluation of the fluorescence assay. *Biochem. Biophys. Res. Commun.* **65**:1363–1370 (1975)
28. Legraverend, C., B. Mansour, D. W. Nebert, and J. M. Holland. Genetic differences in benzo[a]pyrene-initiated tumorigenesis in mouse skin. *Pharmacology* **20**:242–255 (1980).
29. Pelkonen, O., A. R. Boobis, and D. W. Nebert. Genetic differences in the binding of reactive carcinogenic metabolites to DNA, in *Carcinogenesis: Polynuclear Aromatic Hydrocarbons* (P. W. Jones and R. I. Freudenthal, eds.), Vol. 3. Raven Press, New York, 383–400 (1978).
30. Nordquist, M., D. R. Thakker, H. Yagi, R. E. Lehr, A. W. Wood, W. Levin, A. H. Conney, and D. M. Jerina. Evidence in support of the bay region theory as a basis for the carcinogenic activity of polycyclic aromatic hydrocarbons, in *Molecular Basis of Environmental Toxicity* (R. S. Bhatnagar, ed.). Ann Arbor Science Publishing, Inc., Ann Arbor, Mich., 329–357 (1980).
31. Ohyama, T., R. H. Tukey, D. W. Nebert, and M. Negishi. Genetic regulation of mouse liver microsomal $P_2$-450 induction by isosafrole (ISF) and 3-methylcholanthrene (MC), in *Cytochrome P-450: Biochemistry, Biophysics and Environmental Implications.* Elsevier/North Holland Biomedical Press, Amsterdam, in press (1982).
32. Haugen, D. A., M. J. Coon, and D. W. Nebert. Induction of multiple forms of mouse liver cytochrome P-450: evidence for genetically controlled *de novo* protein synthesis in response to treatment with $\beta$-naphthoflavone or phenobarbital. *J. Biol. Chem.* **251**:1817–1827 (1976).
33. Tukey, R. H., T. Ohyama, M. Negishi, and D. W. Nebert. Isolation and characterization of cloned cDNA encoding cytochrome $P_2$-450. *Pharmacologist* **24**:207 (1982).
34. Hood, L. E., J. H. Wilson, and W. B. Wood. *Molecular Biology of Eucaryotic Cells.* W. A. Benjamin Inc., Menlo Park, Calif., 192–201 (1975).
35. Poland, A. P., E. Glover, and A. S. Kende. Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin by hepatic cytosol: evidence that the binding species is the receptor for the induction of aryl hydrocarbon hydroxylase. *J. Biol. Chem.* **251**:4936–4946 (1976).
36. Okey, A. B., G. P. Bondy, M. E. Mason, G. F. Kahl, H. J. Eisen, T. M. Guenthner, and D. W. Nebert. Regulatory gene product of the *Ah* locus: characterization of the cytosolic inducer-receptor complex and evidence for its nuclear translocation. *J. Biol. Chem.* **254**:11636–11648 (1979).
37. Bigelow, S. W., and D. W. Nebert. The *Ah* regulatory gene product: survey of nineteen polycyclic aromatic compounds' and fifteen benzo[a]pyrene metabolites' capacity to bind to the cytosolic receptor. *Toxicol. Lett.* **10**:109–118 (1982).
38. Ikeda, T., M. Altieri, M. Nakamura, D. W. Nebert, and M. Negishi. Induction by 3-methylcholanthrene or isosafrole of two P-450 mRNA's which share sequence homology, in *Cytochrome P-450: Biochemistry, Biophysics and Environmental Implications.* Elsevier/North-Holland Biomedical Press, Amsterdam, in press (1982).
39. Fujii-Kuriyama, Y., T. Taniguchi, Y. Mizukami, M. Sakai, Y. Tashiro, and M. Muramatsu, Construction and identification of a hybrid plasmid containing DNA sequence complementary to phenobarbital-inducible cytochrome P-450 messenger RNA from rat liver. *J. Biochem.* **89**:1869–1879 (1981).
40. Adesnik, M., S. Bar-Nun, F. Maschio, M. Zunich, A. Lippman, and E. Bard. Mechanism of induction of cytochrome P-450 by phenobarbital. *J. Biol. Chem.* **256**:10340–10345 (1981).
41. Bresnick, E., J. Levy, R. N. Hines, W. Levin, and P. E. Thomas. The molecular cloning of cytochrome P-450c information. *Arch. Biochem. Biophys.* **212**:501–507 (1981).

**Send reprint requests to:** Dr. Daniel W. Nebert, Building 10, Room 13-N-266, National Institutes of Health, Bethesda, Md. 20205.