Regulation of Mouse Cytochrome P_3-450 by the *Ah* Receptor. Studies with a P_3-450 cDNA Clone†

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ABSTRACT: The *Ah* locus in the C57BL/6N mouse regulates at least two cytochrome P-450 gene products, termed in the mouse P_1-450 and P_3-450; these two enzymes are so named because each is responsible for the highest turnover number for the substrates benzo[*a*]pyrene and acetanilide, respectively. A cDNA library was prepared in pBR322 from sucrose gradient fractionated total liver poly(A^+)-enriched RNA (~20 S) from 2,3,7,8-tetrachlorodibenzo-*p*-dioxin- (TCDD) treated C57BL/6N (Ah^b/Ah^b) mice. Differential colony hybridization screening, with [^{32}P]cDNA probes derived from total liver mRNA of both TCDD-treated and control C57BL/6N mice, yielded $pP_3$450-21 (1710 base pair) and $pP_1$450-57 (1770 base pair) cDNA clones. $pP_1$450-57 was found to have 690 base pairs 5′-ward of the original P_1-450 cDNA cloned in this laboratory. Restriction maps of $pP_3$450-21 and $pP_1$450-57 are markedly different and clearly are derived from separate genes. By means of hybridization–translation–arrest experiments, anti-(P_3-450) precipitates the translation product ($M_r \cong$ 55 000) of mRNA specifically hybridizing to $pP_3$450-21. It is also shown that hybridization–translation–arrest experiments using polyclonal antibodies are not specific for proof of a P-450 cDNA clone. $pP_3$450-21 was used to probe liver mRNA from Ah^b/Ah^b, Ah^b/Ah^d, and Ah^d/Ah^d mice treated with 3-methylcholanthrene, β-naphthoflavone, aroclor 1254, isosafrole, low TCDD, or high TCDD. These genetic data rigorously demonstrate control of the P_3-450 (20S) mRNA induction process by the *Ah* receptor. $pP_3$450-21 fragments hybridized to TCDD-induced C57BL/6N mRNA and to a portion of the cloned 5′ end of the P_1-450 gene from a mouse MOPC 41 plasmacytoma library. Fragments of four genomic clones having at least parts of the P_1-450 gene were probed with $pP_3$450-21 and $pP_1$450-57. It is concluded that the P_1-450 (23S) mRNA and the P_3-450 (20S) mRNA exhibit a highly homologous region of at least several hundred base pairs in the 5′ portion.

The murine *Ah* locus is a particularly interesting model system for studying gene expression [reviewed in Eisen et al. (1983)]. The *Ah* locus encodes a cytosolic receptor that regulates the induction of certain drug-metabolizing enzymes by polycyclic aromatic compounds such as 3-methylcholanthrene and TCDD.[1] These enzymes in B6 mice include P_1-450 (Negishi & Nebert, 1979; Kimura et al., 1984b) P_3-450 (Negishi & Nebert, 1979; Kimura et al., 1984a,b), and UDP-glucuronosyltransferase (Owens, 1977). The induction process includes highly specific binding of certain foreign chemicals to the *Ah* receptor in the cytosol (Poland et al., 1976; Okey et al., 1979), translocation of the activated inducer–receptor complex into the nucleus (Okey et al., 1980), and the increased rate of transcription of the P_1-450 and P_3-450 genes (Gonzalez et al., 1984b). Maximal increases in an intranuclear high molecular weight precursor mRNA in 3-methylcholanthrene-treated B6 mice precede by several hours the maximal increases in cytosolic P_1-450 (23S) mRNA (Tukey et al., 1981). The appearance of the inducer–receptor complex in the nucleus is rigorously associated with P_1-450 mRNA induction (Tukey et al., 1982a). The 3′ end of the adult B6 P_1-450 gene appears to be less methylated than that of the adult D2 P_1-450 gene, although hypomethylation is not correlated with developmental changes or inducer treatment (Chen et al., 1982). Translation of P_1-450 mRNA occurs principally on membrane-bound polysomes (Chen & Negishi, 1982).

Many of these recent findings would not have been possible without the successful cloning of P_1-450 cDNA (Negishi et al., 1981b). It will be advantageous to compare the P_1-450 gene with other genes controlled by the *Ah* locus. In this report we describe the cloning, isolation, and characterization of mouse P_3-450 cDNA.

Experimental Procedures

Treatment of Mice. Weanling (either sex) B6 (Ah^b/Ah^b) and D2 (Ah^d/Ah^d) inbred mice were purchased from the Veterinary Resources Branch, National Institutes of Health (Bethesda, MD) and were used experimentally before sexual

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[1] Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; B6, the C57BL/6N inbred mouse strain; D2, the DBA/2N inbred mouse strain; isosafrole, 4-propenyl-1,2-(methylenedioxy)benzene; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; bp, base pair(s); kbp, kilobase pair(s). The term "P-450" is used to designate any or all forms of microsomal cytochrome P-450 (multisubstrate monooxygenases). Mouse "P_1-450" and "P_3-450" are defined as those forms of 3-methylcholanthrene- or TCDD-induced P-450 in B6 liver with the highest turnover numbers for induced aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase (EC 1.14.14.1) and acetanilide 4-hydroxylase activity, respectively (Negishi & Nebert, 1979). Mouse "P_2-450" represents that form of isosafrole-induced P-450 in D2 liver with the highest turnover number for isosafrole metabolite formation (Ohyama et al., 1984). We now believe that D2 P_2-450 corresponds to B6 P_3-450, i.e., that a polymorphism exists between these two inbred mouse strains. P_3-450 in several reports (Tukey et al., 1982c; Ikeda et al., 1983) was erroneously named P_2-450. The size of all three membrane-bound proteins is the same ($M_r \cong$ 55 000), as judged by $NaDodSO_4$-polyacrylamide gel electrophoresis. Soret peaks of the reduced cytochrome·CO complex for P_1-450, P_2-450, and P_3-450 are 449, 448, and 448 nm, respectively. Because we have now characterized two proteins with a spectral maximum of 448 nm, we have changed the name of "mouse P-448" characterized previously (Negishi & Nebert, 1979) to "P_3-450". Concentrations of the constitutive P_3-450 are at least 5 times higher than those of constitutive P_1-450, and concentrations of the 3-methylcholanthrene- or TCDD-induced forms of P_3-450 are at least 5 times greater than those of P_1-450 (Negishi et al., 1981a).

maturity. Heterozygotes (Ah^b/Ah^d) having the high-affinity receptor and homozygotes (Ah^d/Ah^d) having the poor-affinity *Ah* receptor (Eisen et al., 1983) were generated from the $B6D2F_1 \times D2$ backcross in our own mouse colony. Unless otherwise indicated, mice were treated intraperitoneally each day with the following chemicals (125 mg/kg) dissolved in corn oil (25 mL/kg): 3-methylcholanthrene, β-naphthoflavone, isosafrole, or aroclor 1254 (Tukey et al., 1982b). The animals were killed 24 h after the fourth dose. Unless otherwise indicated, TCDD in *p*-dioxane (0.4 mL/kg) was given as a single intraperitoneal dose of 25 μg/kg 48 h before killing. In earlier studies, it had been determined that all these inducers exert their maximal effect (induction of total P-450 content) at these dosage regimens. Controls received the corn oil (25 mL/kg) alone on the same time schedule. Livers were perfused with ice-cold 1.15% KCl; generally, ten livers were combined. Aliquots of the tissue were then used for preparation of poly(A^+)-enriched RNA and microsomes.

Isolation of Poly(A^+)-Enriched RNA for Cloning. Total RNA was extracted from the livers of 50 TCDD-treated B6 mice by the guanidine hydrochloride method (Cox, 1968), followed by oligo(dT)–cellulose chromatography twice (Aviv & Leder, 1972) and ethanol precipitation. Poly(A^+)-enriched RNA was size fractionated on a 5–20% linear sucrose density gradient containing 10 mM sodium acetate (pH 6.0), 130 mM NaCl, 1 mM ethylenediaminetetraacetic acid, and 0.2% $NaDodSO_4$. Gradient fractions corresponding to ~20S RNA were diluted with water and precipitated twice with 2 volumes of ethanol at −20 °C.

Preparation of Double-Stranded cDNA. First-strand synthesis was performed via reverse transcription (Miller & McCarthy, 1979). The reaction mixture (total volume = 0.1 mL) included 50 μg of poly(A^+)-enriched RNA (~20 S), 1 mM dATP, 1 mM dGTP, 1 mM dCTP, 1 mM dTTP, 10 μCi of [^{32}P]dCTP (Amersham Searle, Arlington Heights, IL), 50 mM KCl, oligo[$dT(pT)_{12-18}$] (Collaborative Research, Lexington, MA), and 4 mM $Na_4P_2O_7$. Following incubation of the mixture on ice for 10 min, 200 units of reverse transcriptase (Life Sciences Inc., St. Petersburg, FL) was added, and the incubation was carried out for 1 h at 45 °C. The mixture was then made 0.2% $NaDodSO_4$ and 0.3 M NaOH and incubated an additional 6 h at room temperature. After neutralization with glacial acetic acid, extraction with phenol, and chromatography on Sephadex G-50, the cDNA was precipitated with ethanol.

Double-stranded cDNA was generated in a reaction mixture (total volume = 0.2 mL) containing 10 μg of cDNA, 50 mM Tris-HCl (pH 8.3), 10 mM $MgCl_2$, 1 mM β-mercaptoethanol, 0.1 mM dATP, 0.1 mM dGTP, 0.1 mM dCTP, 0.1 mM dTTP, and 50 units of reverse transcriptase (Monahan et al., 1976). The mixture was incubated for 2 h at 45 °C and the reaction terminated by the addition of 20 μL of 10% $NaDodSO_4$. The DNA was extracted with phenol and precipitated twice with ethanol. cDNA (2 μg) was incubated with 100 units of S_1 nuclease (Miles Laboratory, Elkhart, IN) for 1 h at 37 °C in a 0.1-mL reaction mixture containing 30 mM sodium acetate (pH 4.5), 0.3 M NaCl, and 4 mM $ZnCl_2$ (Leong et al., 1972). The cDNA was then extracted with phenol and chromatographed (Sepharose 4B column, 1.2 × 30 cm) in 20 mM Tris-HCl (pH 7.5) and 1 mM ethylenediaminetetraacetic acid. Fractions containing the largest double-stranded cDNA fragments were pooled, and the cDNA was precipitated with ethanol.

Poly(dC) tailing of the 3′ ends of S_1 nuclease digested double-stranded cDNA was performed with 10 units of terminal deoxynucleotidyltransferase (Roychoudhury et al., 1976) in 0.1 mL of 100 mM potassium cacodylate (pH 7.0), 4 mM β-mercaptoethanol, 1 mM $CoCl_2$, and 100 μM dCTP. After a 5-min incubation at 37 °C, the DNA was extracted with phenol, chromatographed on a Sephadex G-50 column, and precipitated with ethanol. Approximately 200 ng of poly(dC)-tailed double-stranded DNA was annealed with 50 ng of poly(dG)-tailed pBR322 (New England Nuclear, Boston, MA) in 20 μL of 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid, and 20 mM Tris-HCl (pH 7.5) (Cook et al., 1980). The resultant chimeric plasmids were then used to transform *E. coli* strain LE392 in the presence of $CaCl_2$ (Tiemeier et al., 1977). Transformants were selected for tetracycline resistance and ampicillin sensitivity.

Selection of cDNA-Containing Plasmids Associated with the Ah Locus. Fifteen hundred colonies identified by differential antibiotic sensitivity were selected for further characterization. Colonies were screened by the in situ colony hybridization assay (Grunstein & Hogness, 1975) and a differential screening protocol in which duplicate filters were hybridized with different cDNA preparations: one [^{32}P]cDNA preparation from total liver mRNA of TCDD-treated B6 mice and the other from total liver mRNA of control B6 mice.

Other Procedures. Hybridization–arrest experiments (Negishi et al., 1981b) performed with filter-bound plasmid DNA and precipitation of specific mRNA translation products by anti-(P_3-450), preparation of B6 P_3-450 antigen and the development of anti-(P_3-450) in goat (Negishi & Nebert, 1979), isolation of the mouse DNA insert by restriction endonuclease digestion and electroelution following agarose gel electrophoresis (Tukey et al., 1981), DNA–DNA hybridization (Southern, 1975), and RNA–DNA hybridizations (Alwine et al., 1977) were performed by procedures described in the references cited.

Results

Identification of cDNA Clones Associated with the Ah Locus. Because TCDD-inducible P-450 is regulated by the *Ah* receptor, we reasoned that differential hybridization (i.e., TCDD-treated vs. control) would give us a promising high rate of success for finding another P-450 gene governed by the *Ah* receptor. By means of anti-(P_3-450)-precipitated translation products from different sizes of mRNA by procedures previously detailed (Negishi & Nebert, 1981), it was determined that the mRNA size for the major polycyclic aromatic inducible form of mouse P-450 was ~20 S. This size of gradient-fractionated poly(A^+)-enriched RNA from TCDD-treated B6 mice was thus used to prepare cDNA clones, as described under Experimental Procedures. Approximately 1500 ampicillin-sensitive tetracycline-resistant transformants were screened with [^{32}P]cDNA probes that had been derived from total liver mRNA of either control or TCDD-treated B6 mice. Ten recombinants hybridized much more strongly with the TCDD-treated probe than with the control probe. The ten promising inserts were removed from the pBR322 and sized on agarose gel electrophoresis. By detailed patterns of restriction endonuclease digestion, six clones appeared to belong to one distinct class and the other four clones belonged to a second distinct class. Clone p21 represented the longest (1710 bp) of the former class, and p57 represented the longest (1770 bp) of the latter class.

Clone 46, a 1080-bp P_1-450 cDNA clone, has been previously characterized in this laboratory (Negishi et al., 1981b; Tukey et al., 1981). Clone 46 hybridized intensely to all four of the "p57-like" clones but not to any of the six "p21-like" clones. By restriction enzyme analysis (Figure 1), pP_1450-57

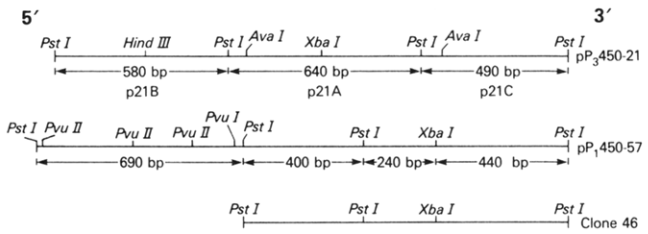

FIGURE 1: Restriction maps of three cDNA clones: pP_3450-21, pP_1450-57, and the original clone 46. For comparative purposes, the 3′-ward artificial *Pst*I site of all three clones is aligned.

was found to contain 690 bp more 5′-ward than clone 46. Complete cDNA sequencing studies (Kimura et al., 1984b) have confirmed that pP_1450-57 represents about two-thirds of the entire P_1-450 cDNA. Figure 1 shows that the restriction map of pP_3450-21 is very different from that of P_1-450 cDNA.

Correlation of Anti-(P_3-450) with pP_3450-21. pP_3450-21 was examined by hybrid translation–selection experiments with anti-(P_3-450) (Figure 2). The plasmid was linearized, denatured, and then bound to nitrocellulose filters. Total liver poly(A^+)-enriched RNA from TCDD-treated B6 mice was hybridized for 24 h with the pP_3450-21-bound filter. The nonhybridizable and hybridized mRNA were then each reisolated and translated in vitro in the presence of [^{35}S]-methionine. Figure 2 demonstrates that the specifically hybridized mRNA directs the synthesis of anti-(P_3-450)-precipitable protein ($M_r \cong 55000$). The data in Figure 2 confirm by hybridization–arrest that anti-(P_3-450), the antibody that most effectively blocks acetanilide 4-hydroxylase activity (Negishi & Nebert, 1979), also precipitates the protein translated from the mRNA that hybridizes most specifically to pP_3450-21.

Of interest, anti-(P_1-450) and anti-(P_2-450) also precipitate the protein translated from the mRNA hybridizing to pP_3450-21, and anti-(P_3-450) precipitates the protein translated from the mRNA hybridizing to pP_1450-57 (data not illustrated). There are several possible explanations for these results. (a) P_1-450 mRNA, P_2-450 mRNA, and P_3-450 mRNA may exhibit sufficient homology to hybridize to the filter-bound cDNA under the conditions described in Figure 2. (b) The P_1-450, P_2-450, and P_3-450 proteins may possess common antigenic determinants. Anti-(P_1-450) and anti-(P_3-450) are not claimed to be monospecific antibodies (Negishi & Nebert, 1979), and therefore anti-(P_1-450) may precipitate P_3-450 and anti-(P_3-450) may precipitate P_1-450. It remains of interest, however, that anti-(P_1-450) blocks aryl hydrocarbon hydroxylase but not acetanilide 4-hydroxylase activity and anti-(P_3-450) inhibits acetanilide 4-hydroxylase but not aryl hydrocarbon hydroxylase activity (Negishi & Nebert, 1979). Moreover, microsomes labeled wth NaB^3H_4, which are then detergent solubilized and immunoprecipitated by anti-(P_1-450) or anti-(P_3-450), yield distinctly different patterns (Negishi et al., 1981a). In summary, hybridization–arrest experiments with polyclonal antibodies to P-450 will never be conclusive if there is sufficient mRNA homology or if common antigenic determinants exist on the two or more P-450 proteins under study.

Induction of pP_3450-21-Hybridizable mRNA by Polycyclic Aromatic Compounds. Five foreign chemicals that mediate P_1-450 mRNA induction via the *Ah* receptor exhibit an approximate order of potency of TCDD > 3-methylcholanthrene > β-naphthoflavone > aroclor 1254 > isosafrole (Tukey et al., 1982b). We therefore examined by Northern analysis total liver poly(A^+)-enriched RNA from B6 and D2 mice that had been pretreated with each of these inducers (data not illustrated). The pP_3450-21 probe exhibited increases in intensity of 20S mRNA in excellent agreement with the potency of these five inducers and with the magnitude of acetanilide 4-hydroxylase induction in liver microsomes from these same two strains (Atlas & Nebert, 1976): increases were noted in B6 mice treated with each of the five chemicals and in D2 mice only after TCDD (50 μg/kg) treatment. Unexpectedly, the pP_3450-21 probe also hybridized to 23S mRNA (but with an intensity considerably less than that for 20S mRNA). Furthermore, the pP_1450-57 probe hybridized to the 20S messenger, in addition to the more striking hybridization to the 23S band [in excellent agreement with the potency of these five inducers and with the magnitude of aryl hydrocarbon hydroxylase induction (Tukey et al., 1982b)]. The most likely explanation is that pP_3450-21 represents a P-450 cDNA associated with the *Ah* receptor and that pP_3450-21 exhibits sequence homology with P_1-450 (23S) mRNA sufficient to show up on Northern blots.

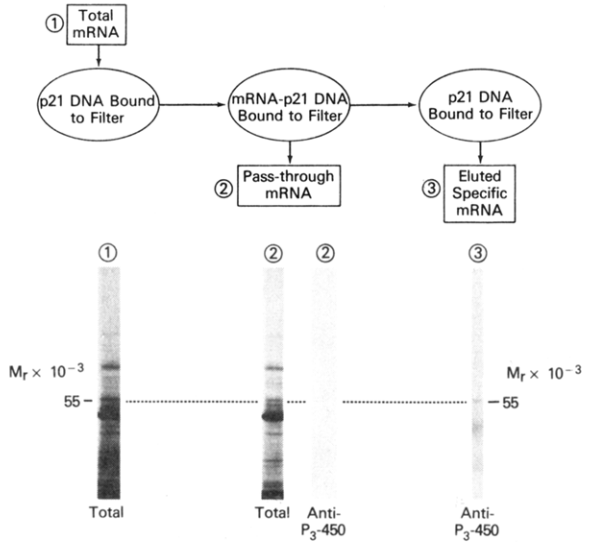

FIGURE 2: Cell-free translation of poly(A^+)-enriched RNA before and after hybridization to pP_3450-21 fixed to a diazobenzyloxymethyl filter. Total liver poly(A^+)-enriched RNA freshly prepared from TCDD-treated B6 mice was exposed to the filter under hybridization conditions. (1) Total translation products derived from TCDD-treated B6 mouse liver mRNA; (2) total (left) and anti-(P_3-450)-precipitable (right) translation products from the mRNA that failed to hybridize to the filter-bound pP_3450-21; (3) anti-(P_3-450)-precipitable translation product of mRNA that hybridized specifically to the filter-bound pP_3450-21 but subsequently had been released with water at 100 °C. Autoradiograms were developed from $NaDodSO_4$–polyacrylamide gels containing the [^{35}S]methionine-incorporated translated proteins. Eight times more RNA was used for the immunoprecipitation lanes than for the total translation lanes. In this figure and in subsequent figures, migration is from top to bottom. The major band at $M_r \cong 71000$ in lane 1 and lane 2, left, represents preproalbumin (Brown & Papaconstantinou, 1979), and the band at $M_r \cong 55000$ in lane 1 and lane 2, left, represents (at least in part) TCDD-induced P_1-450 and P_3-450. The immunoprecipitated material at $M_r \cong 55000$ in lane 3 represents P_3-450.

Rigorous Correlation of pP_3450-21 with the Ah Locus. It is well-known (Poland et al., 1974; Eisen et al., 1983) that low TCDD doses induce aryl hydrocarbon hydroxylase activity (P_1-450) in B6 (Ah^b/Ah^b) mice and B6D2F_1 hybrids (Ah^b/Ah^d) but not in D2 (Ah^d/Ah^d) mice, due to the receptor defect in Ah^d/Ah^d mice. High doses of TCDD overcome this receptor defect and thus induce P_1-450 equally well in Ah^b/Ah^b, Ah^b/Ah^d, and Ah^d/Ah^d individuals. Presumably this same differential response works for P_3-450 as well. The pP_3450-21 and clone 46 probes are compared in Figure 3. P_3-450 mRNA (at left) is induced by the low dose of TCDD in B6 and the

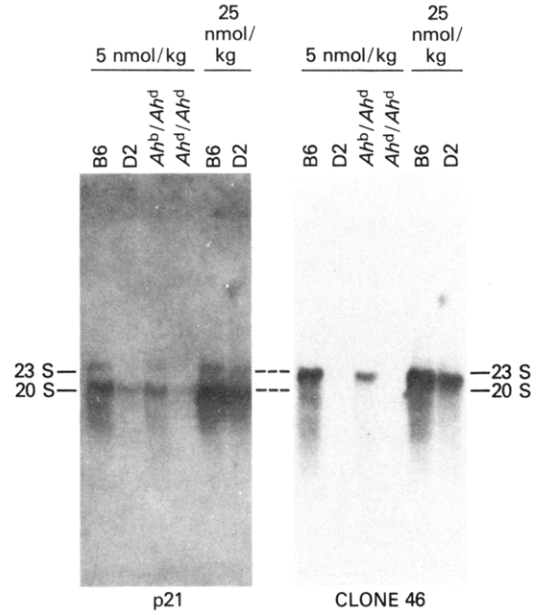

FIGURE 3: Northern blot of total liver poly(A^+)-enriched RNA probed with pP_3450-21 (left) and clone 46 (right), as a function of TCDD pretreatment. Ah^b/Ah^d and Ah^d/Ah^d offspring from the $B6D2F_1 \times$ D2 backcross had been *Ah* phenotyped 10 days before this experiment by means of the zoxazolamine paralysis test (Robinson & Nebert, 1974). Five male mice (age 4–6 weeks) per group were killed 18 h after a single dose of intraperitoneal TCDD. Each well contains 5 μg of RNA. Other experimental details are described under Experimental Procedures and in Tukey et al. (1981, 1982a).

Ah^b/Ah^d heterozygote offspring, but not in D2 and the Ah^d/Ah^d homozygote offspring; P_3-450 mRNA is induced about equally well by the high TCDD dose in both B6 and D2 inbred mice. The pattern is the same for P_1-450 (23S) mRNA probed by clone 46 (at right). The pP_3450-21 probe therefore (i) demonstrates a strict association with the *Ah* receptor, (ii) hybridizes predominantly to 20S mRNA, but hybridizes slightly to P_1-450 (23S) mRNA, and (iii) hybridizes to considerable quantities of constitutive P_3-450 mRNA (which are at least 5 times greater than constitutive levels of P_1-450 mRNA).

Clone 46 does not hybridize to 20S mRNA (Figure 3). Hence, the 690-bp region of pP_1450-57 not present in clone 46 (Figure 1) must contain the homologous segment that is able to hybridize to P_3-450 (20S) mRNA.

Hybridization of pP_3450-21 Fragments to TCDD-Induced mRNA. Digestion of pP_3450-21 with *Pst*I (Figure 1) yields three fragments named according to size: p21A, 640 bp; p21B, 580 bp; p21C, 490 bp. pP_3450-21 and the three fragments were each used as probes for Northern blots of control and TCDD-treated B6 mRNA (Figure 4). Although p21A, p21B, and p21C all hybridized equally well to the 20S mRNA, p21B showed homology with P_1-450 (23S) mRNA, p21A showed very little homology with P_1-450 (23S) mRNA, and p21C showed no homology with P_1-450 (23S) mRNA. Of interest, the orientation of these fragments (Figure 1) is 5′-p21B-p21A-p21C-3′, suggesting that a major portion of the 5′-most fragment, p21B, and probably the first 50 or 100 bp of the 5′ end of p21A display a highly homologous region with P_1-450 mRNA.

Regions of the P_1-450 Gene from Mouse MOPC 41 Plasmacytoma That Are Homologous with the pP_3450-21 and pP_1450-57 cDNA Clones. The entire cDNA clone and fragments of pP_3450-21 and pP_1450-57 were studied via Southern blots with various fragments of the P_1-450 genomic clones from a mouse MOPC 41 plasmacytoma library (Nakamura et al., 1983). One such experiment is illustrated in Figure 5. The probe used in Figure 5 is pAhP-2.9, a 2.9-kbp *Eco*RI fragment that includes the first two exons and the first two introns of the P_1-450 tumor gene (Figure 6). When pP_3450-21 was cut with *Pst*I only, the 580-bp piece (i.e., p21B) hybridizes intensely to pAhP-2.9, while p21A and p21C do not. When pP_3450-21 was cut with *Pst*I plus *Hin*dIII, this procedure results in p21B being divided (Figure 1) into a 5′-ward 300-bp fragment and a 3′-ward 270-bp fragment. It is concluded from Figure 5 that the most 5′-ward region of the entire pP_3450-21 cDNA clone of 1710 bp exhibits highly homologous sequences to pAhP-2.9.

λ3NT12, λ3NT13, λ3NT14, and λAhP-1 (Figure 6) represent the four clones by which colleagues in this laboratory

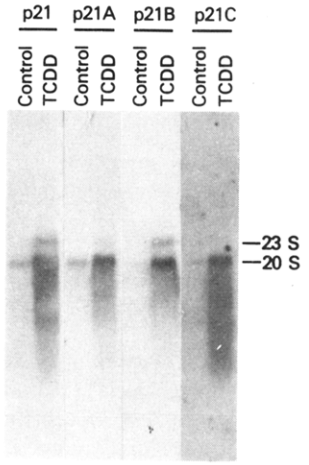

FIGURE 4: Northern analysis of total liver poly(A^+)-enriched RNA from control and TCDD-treated B6 mice; the probes are pP_3450-21, p21A, p21B, and p21C. The latter three probes represent *Pst*I-digested fragments of pP_3450-21 (Figure 1). Each well contains 5 μg of RNA. Further details are described under Experimental Procedures and in Tukey et al. (1981). The X-ray film was exposed to the filter paper for 24 h.

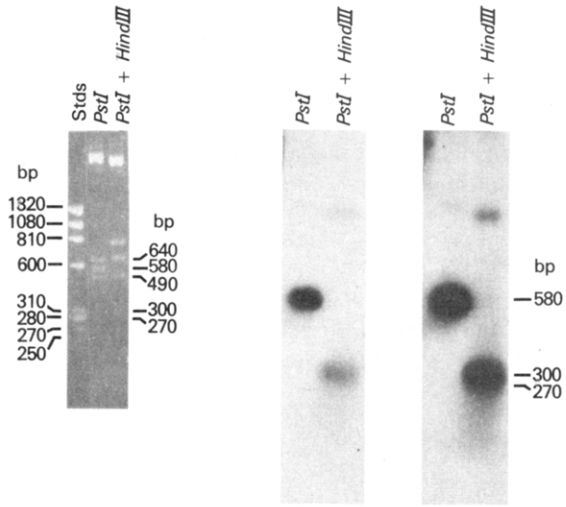

FIGURE 5: Southern blot of *Pst*I-digested and *Pst*I plus *Hin*dIII digested pP_3450-21 fragments probed with pAhP-2.9. Following digestion of pP_3450-21 DNA with one or both restriction enzymes and electrophoresis on 1% agarose gels, the fragments were fixed on nitrocellulose paper and probed with nick-translated pAhP-2.9 [^{32}P]DNA, which is an *Eco*RI-digested fragment of λAhP-1 that includes the 5′ end of the P_1-450 tumor gene (Ikeda et al., 1983; Nakamura et al., 1983). Each well contains 1 μg of DNA. Left, photograph of the restricted DNA after staining with ethidium bromide. Exposure of X-ray film to the filter paper was carried out for 6 h (center) and for 24 h (right). Standards (Stds) at left represent *Hae*III-digested λ phage DNA.

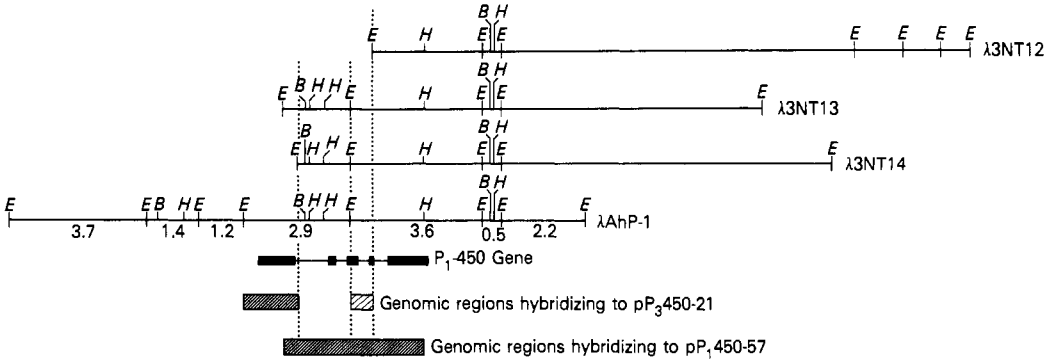

FIGURE 6: Positional alignment of four genomic clones from a mouse MOPC 41 plasmacytoma library (Nakamura et al., 1983), the approximate location of the P_1-450 gene, and regions of the gene that hybridize strongly (densely hatched rectangles) or weakly (lightly hatched rectangles) to the cDNA clones $pP_3$450-21 and $pP_1$450-57. E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII. The number below each *Eco*RI fragment of λAhP-1 denotes the size in kilobase pairs. The approximate sizes and locations of the five exons (solid rectangles) and four introns (lines between solid rectangles) of the P_1-450 tumor gene are illustrated. Vertical dotted lines to aid the reader are arbitrarily dropped from the 5′ end of λ3NT14, the first biologic *Eco*RI site of λ3NT13 and λ3NT14, and the 5′ end of λ3NT12. See text for discussion of regions of $pP_3$450-21 homology. It should be emphasized that the genomic regions hybridizing to these cDNA clones are illustrated as spanning the P_1-450 introns and 5′ flanking region, although no intron or 5′ flanking region should correspond to the cDNA.

have walked up the chromosome until the entire mouse P_1-450 tumor gene was isolated and characterized (Ikeda et al., 1983; Nakamura et al., 1983). By means of the appropriate single or double digestions with *Eco*RI, *Bam*HI, or *Hin*dIII of all four genomic clones, electrophoresis on 1% agarose gels, transfers of the fragments to nitrocellulose paper, and probing with nick-translated $pP_3$450-21 or $pP_1$450-57, regions of genomic DNA that hybridize intensely to these cDNA clones have been pinpointed (Figure 6). For example, $pP_3$450-21 hybridized intensely to λAhP-1 and λ3NT13, but not to λ3NT14 or λ3NT12. $pP_3$450-21 hybridized weakly to the 3.6-kbp *Eco*RI fragment of λAhP-1, λ3NT14, and λ3NT13, but not at all to the 3.0-kbp *Eco*RI piece of λ3NT12. $pP_1$450-57 hybridized strongly to the 2.9-kbp *Eco*RI fragment of λAhP-1, the 1.4-kbp *Eco*RI fragment of λ3NT14, and the 1.8-kbp *Eco*RI fragment of λ3NT13. $pP_1$450-57 hybridized intensely to the 2.0-kbp *Eco*RI–*Hin*dIII fragment of λAhP-1, λ3NT14, and λ3NT13 and to the 1.4-kbp *Eco*RI–*Hin*dIII fragment of λ3NT12. No other hybridizations were noted.

Discussion

$pP_3$450-21 is a cDNA clone whose mRNA induction, like that of P_1-450, is regulated by the *Ah* receptor. The P_1-450 (23S) mRNA and the P_3-450 (20S) mRNA appear to exhibit a high degree of homology in a region of at least several hundred base pairs in the 5′ portion of the P_1-450 gene. From restriction map analysis of these two cDNA clones, clearly P_1-450 and P_3-450 represent separate genes.

Until recently there has been no precedent for genes under similar regulatory control to have homology in their 5′ regions: the ovalbumin multigene family induced by progesterone (Lawson et al., 1982); the interferon gene family induced by viral infections (DeGrado et al., 1982; Miyata & Hayashida, 1982); vitellogenin genes induced by estrogen (Wahli et al., 1979, 1982); metallothionein genes induced by heavy metals and steroids (Schmidt & Hamer, 1983). Rat cytochrome P-450 related genes (Mizukami et al., 1983; Kumar et al., 1983), however, exhibit extensive homology over at least 755 bp in the 5′ region, suggesting the possibility of constant and hypervariable regions to explain P-450 diversity via gene conversion or unequal crossing-over [reviewed in Nebert & Negishi (1982)]. Because these genes were derived from similar rats but in different countries, however, it remains to be determined whether the same P-450e gene (vs. two closely related P-450e-like genes from the same subfamily) has been characterized.

Our laboratory has recently completed the nucleotide sequence on full-length cDNA clones corresponding to $pP_3$450-21 (Kimura et al., 1984a) and $pP_1$450-57 (Kimura et al., 1984b). Sufficient 5′ homology exists for us to be certain that mouse P_1-450 and P_3-450 correspond to rat P-450c and P-450d, respectively. A 5′ segment of 456 bp having 97% homology between P_1-450 and P_3-450 cDNA (Kimura et al., 1984b) has confirmed, at the nucleotide sequencing level, the data shown in this report via Northern and Southern hybridization analyses.

Added in Proof

The P_1-450 and P_3-450 genes from normal B6 liver both have seven exons (Gonzalez et al., 1984a); the larger exons 2 and 7 of the normal P_1-450 gene correspond to exons 1 and 5 illustrated in the tumor gene (Figure 6). One of the four small normal gene's exons (3, 4, 5, or 6) was overlooked in the original R-loop electron micrographs (Nakamura et al., 1983), and an interesting rearrangement appears to have occurred 5′-ward of exon 2 (illustrated as exon 1 in the P_1-450 tumor gene, Figure 6) (S. Kimura, F. J. Gonzalez, and D. W. Nebert, unpublished results).

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Registry No. Cytochrome P-450, 9035-51-2; cytochrome P_1-450, 9035-50-1.

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