Immunochemical evidence for multiple steroid-inducible hepatic cytochromes *P*-450 in the rat

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It has been established that there are glucocorticoid-inducible hepatic cytochromes *P*-450 in the rat (*P*-450p), the rabbit (LM3c) and man (HLP) which share extensive structural, functional and regulatory features. We prepared immunochemical probes to *P*-450p and identified a unique monoclonal antibody, 1G8, that recognizes purified *P*-450p, but neither purified LM3c nor HLP, on immunoblot analysis. The *N*-terminal amino acid sequence of purified samples of *P*-450p was identical with that previously reported for *P*-450PCN1 [Gonzalez, Nebert, Hardwick & Kasper (1985) *J. Biol. Chem.* **260**, 7435–7441]. Immunoblot analyses of liver microsomes from untreated male rats revealed two 1G8-reactive proteins, whereas liver microsomes from untreated females contained none. Another monoclonal antibody, 13-7-10, reacted specifically with LM3c and HLP, but *not* with *P*-450p. A single 13-7-10-reactive microsomal protein was detected in untreated male and female rats, the latter protein exhibiting a greater apparent M_r . 1G8-reactive proteins were induced to the greatest extent by triacetyloleandomycin, followed by dexamethasone, chlordan, pregnenolone-16 α -carbonitrile and 2,4,2',4'-tetrachlorobiphenyl. In contrast, 13-7-10-reactive proteins were most strongly induced by dexamethasone, only moderately by triacetyloleandomycin and pregnenolone-16 α -carbonitrile, weakly by chlordan and not at all by 2,4,2',4'-tetrachlorobiphenyl. We conclude that the *P*-450p family in rat liver consists of three or more proteins that are structurally related and yet appear to be under distinct regulatory control.

INTRODUCTION

The liver cytochromes *P*-450 are a superfamily of haemoproteins located in the endoplasmic reticulum of the hepatocyte that play a critical role in the biotransformation of a diverse array of endogenous substances, such as steroid hormones, as well as xenobiotics, such as drugs and carcinogens (Lu & West, 1979; Adesnik & Atchison, 1985). Families of cytochrome *P*-450 proteins have been identified on the basis of similarities in their biochemical, immunochemical and genetic characteristics, including their responses to prototypic inducers. For example, *P*-450b and *P*-450e are immunochemically and structurally similar haemoproteins that are co-induced in the liver of rats treated with phenobarbital or phenobarbital-like inducers (Kumar *et al.*, 1983; Adesnik & Atchison, 1985). Likewise, *P*-450c and *P*-450d are products of homologous genes whose expression is stimulated by administration of 3-methylcholanthrene and other polycyclic aromatic hydrocarbons (Adesnik & Atchison, 1985; Guengerich *et al.*, 1982).

Several years ago we identified a unique form of cytochrome *P*-450 (Elshourbagy & Guzelian, 1980), now termed '*P*-450p', that is inducible by the synthetic steroid pregnenolone-16 α -carbonitrile (PCN) and by steroids of the glucocorticoid class such as dexamethasone (DEX) (Heuman *et al.*, 1982; Schuetz *et al.*, 1984). Cytochrome *P*-450p is structurally and functionally distinct

from the 'phenobarbital-inducible' and 'methylcholanthrene-inducible' forms of cytochrome *P*-450, and yet *P*-450p is inducible by phenobarbital and 'phenobarbital-like' inducers (Guengerich *et al.*, 1982; Heuman *et al.*, 1982; Schuetz *et al.*, 1986). Macrolide antibiotics represent a third group of compounds that induce *P*-450p (Wrighton *et al.*, 1985a). Cytochromes immunochemically and functionally related to *P*-450p have been found in all species thus far examined (Wrighton *et al.*, 1985b), including *P*-450 LM3c in the rabbit and *P*-450 HLP in man (Watkins *et al.*, 1985). Cytochrome *P*-450p is conserved among mammalian species, is regulated by an endogenous inducer (corticosterone) (Schuetz & Guzelian, 1984), and thus may be involved in important biotransformation reactions.

It is now evident that *P*-450p is a representative of a multigene cytochrome *P*-450 family. Cloned cDNAs to *P*-450p hybridize to at least two liver RNA species in PCN-treated rats (Wrighton *et al.*, 1985b) and to a sufficient amount of the rat genome to account for as many as five *P*-450p-related genes (Molowa *et al.*, 1986; Hardwick *et al.*, 1983). Until recently, the liver cytochromes isolated by others from PCN-treated rats [*P*-450PB/PCN-E (Guengerich *et al.*, 1982) and *P*-4502a (Waxman, 1986)] appeared to be the same as *P*-450p. Gonzalez and co-workers identified and isolated a PCN-inducible protein and corresponding cDNA (*P*-450PCN1) (Gonzalez *et al.*, 1985) apparently identical

Abbreviations used: TAO, triacetyloleandomycin; DEX, dexamethasone; CD, chlordan; PCN, pregnenolone-16 α -carbonitrile; TNC, *trans*-nonachlor; SKF, SKF-525A; SLN, spironolactone; MTP, metyrapone; PCB, 2,4,2',4'-tetrachlorobiphenyl; ISF, isosafrole; PB, phenobarbital; TSO, *trans*-stilbene oxide; ARO, arochlor 1254; PTZ, phenothiazine; PBB, 2,4,5,2',4',5'-hexabromobiphenyl; CLO, clofibrate; DPH, diphenylhydantoin; CPZ, chlorpromazine; ISO, propan-2-ol ('isopropanol'); PBS, phosphate-buffered saline (137 mM-NaCl/3 mM-KCl/8 mM-Na₂HPO₄/1 mM-KH₂PO₄, pH 7.4); e.l.i.s.a., enzyme-linked immunosorbent assay.

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with *P*-450p, and, in addition, have recently found a homologous cDNA (*P*-450PCN2) that differs from *P*-450PCN1 in its deduced *N*-terminal amino acid sequence (Gonzalez *et al.*, 1986). However, no laboratory has isolated more than one *P*-450p-related protein from a single species. Some apparently homogeneous preparations may, in fact, be mixtures of closely related polypeptides that cross-react with polyclonal antibodies.

In the present study, we prepared monoclonal antibodies that recognize selectively individual PCN-inducible hepatic cytochromes *P*-450. On the basis of analyses of rat liver microsomes (microsomal fractions) with these probes, we conclude that rat liver contains multiple immunochemically similar *P*-450p-related proteins that display separate regulatory characteristics.

EXPERIMENTAL

Materials

The polychlorinated and polybrominated biphenyl isomers were generously given by Dr. Stephen Safe and Dr. Stephen Aust respectively. CD and TNC were gifts from Velsicol (Chicago, IL, U.S.). ARO was obtained from Monsanto Co. (St. Louis, MO, U.S.A.) and SKF was provided by Smith, Kline and French (Philadelphia, PA, U.S.A.). TAO was a gift of Pfizer Laboratories (Brooklyn, NY, U.S.A.), and PCN was provided by the Upjohn Co. (Kalamazoo, MI, U.S.A.). PB was purchased from Amend Drug and Chemical Co. (Irvington, NJ, U.S.A.); ISF from Fluka (New York, NY, U.S.A.); h.p.l.c.-grade ISO from Burdick and Jackson (Muskegon, MI, U.S.A.); SLN, TSO, CPZ, PTZ, DPH, MTP, DEX, CLO and corn oil from Sigma (St. Louis, MO, U.S.A.); peroxidase-conjugated anti-mouse IgG, rabbit anti-goat IgG and goat peroxidase-anti-peroxidase from Miles Scientific (Elkhart, IN, U.S.A.); nitrocellulose paper from Bio-Rad (Richmond, CA, U.S.A.); and diaminobenzidine tetrahydrochloride from Pfaltz and Bauer (Stamford, CT, U.S.A.). All other chemicals used were of reagent grade or better.

Animals and treatments

Sprague-Dawley rats (100–150 g; Flow Laboratories, Dublin, VA, U.S.A.) were housed in pairs in wire-bottomed cages and given unlimited access to standard laboratory chow and water, unless otherwise indicated. All chemicals were dissolved in corn oil, except CPZ, PB and ISO (which were dissolved or diluted in water), MTP (which was dissolved in 0.9% NaCl) and TAO (which was added to powdered chow). Single intraperitoneal injections of PCB (500 μ mol/kg) and PBB (50 μ mol/kg) were given 4 and 7 days respectively before microsome preparation. A single intraperitoneal injection was also given for ARO (300 mg/kg) 4 days before microsome preparation. Daily intraperitoneal injections were given for SLN (100 mg/kg), TAO (300 mg/kg), ISF (150 mg/kg), CPZ (40 mg/kg), PTZ (100 mg/kg), PCN (300 mg/kg), DPH (50 mg/kg), TNC (46 mg/kg), and CD (42 mg/kg), each for 4 days, and SKF (80 mg/kg) and CLO (400 mg/kg), each for 3 days. DEX (300 mg/kg) and ISO (15 mmol/kg) were given by oral gavage daily for 4 days and for 1 day respectively; PB (0.1%) was administered in drinking water for 6 days; TAO (1% w/w) was fed in powdered chow for 10 days; and MTP (500 mg/kg) was given as a single intraperitoneal injection 1 day before microsome preparation.

Each animal was fasted overnight and killed by decapitation. The liver was perfused retrogradely with cold PBS, pH 7.4, excised and homogenized, and microsomes were isolated by differential centrifugation as previously described (Elshourbagy & Guzelian, 1980). The concentration of total CO-binding cytochrome *P*-450 in the microsomal preparations and in the purified proteins was determined by difference spectroscopy as described by Omura & Sato (1964). Microsomal protein concentrations were determined colorimetrically (Schacterle & Pollack, 1973).

Rat cytochrome *P*-450p (Wrighton *et al.*, 1985a), rabbit cytochrome LM3c (Koop *et al.*, 1981), and human cytochrome HLP (Watkins *et al.*, 1985) were purified by the indicated published procedures.

Preparation of antibodies

Five virus-free female Balb/c mice (15–18 g; Charles River Laboratories, Raleigh, NC, U.S.A.) were each injected intraperitoneally with 50 μ g of purified *P*-450p in 50 mM-potassium phosphate/50 mM EDTA/20% glycerol buffer, pH 7.4, combined with an equal volume of Freund's complete adjuvant in a total volume of 0.2 ml, followed 21 days later by a 20 μ g boost. Six days after the boost, blood samples from the mice were pooled and serum was prepared and assayed for the presence of antibody by e.l.i.s.a. A final 20 μ g intravenous boost was given 3 days before fusion. Spleen cells from one immunized mouse were fused with P3-X63-Ag8 multiple myeloma cells and the resulting hybridomas were expanded *in vitro*. Limiting dilutions were carried out to isolate individual clones. These were tested for antibody production by e.l.i.s.a., allowed to expand, and then injected intraperitoneally into female Balb/c mice (5×10^6 cells/mouse) which had been primed with pristane (0.5 ml intraperitoneally) 2 weeks previously. The resulting ascitic fluid was collected 9–14 days later, centrifuged to remove cells, and then stored at -20°C . The second monoclonal antibody used in these studies, termed 13-7-10, was prepared by Dr. Kremers as described elsewhere (Beaune *et al.*, 1985).

A polyclonal anti-*P*-450p IgG raised in a goat was prepared as previously described (Elshourbagy *et al.*, 1981). In some immunoblot experiments absorbed anti-*P*-450p was used. Briefly, liver microsomes prepared from a 3-methylcholanthrene-treated male rat were solubilized with cholate and bound to CNBr-activated Sepharose 4B (15 mg of microsomal protein/ml of Sepharose 4B) (Cuatrecasas, 1970; March *et al.*, 1974). The IgG fraction prepared from serum of a goat immunized with *P*-450p was incubated overnight with the immobilized microsomes (100 mg of IgG/ml of Sepharose 4B). The unbound IgG was collected and subjected to a second immunoabsorption step (25 mg of IgG/ml of Sepharose 4B) with liver microsomes (prepared from an untreated female rat) bound to the Sepharose matrix.

Immunoblot analysis

Quantitative analyses of immunoblots were performed as described previously (Schuetz *et al.*, 1984). Briefly, microsomal proteins were electrophoretically separated in 10% SDS/polyacrylamide slab gels. After the initial 2 h of electrophoresis, a different amount of purified protein standard was added to each well. Electrophoresis

was then continued for an additional 1–2 h. The resolved proteins were electrophoretically transferred to nitrocellulose paper, and non-specific protein-binding sites were blocked by an overnight incubation of the nitrocellulose paper in PBS containing 3% (w/v) bovine serum albumin and 10% (v/v) calf serum. After a 1 h exposure to the monoclonal antibody, the blots were washed with cold PBS, incubated with peroxidase-conjugated anti-mouse IgG for 30 min, and washed again with PBS. The immunoreactive proteins were revealed with diaminobenzidine in 0.006% H_2O_2 . The blots were scanned with a Zeiss densitometer to determine the integrated absorbance of each protein band. The amount of immunoreactive protein present in each microsomal sample was calculated from the linear portion of a standard curve derived from the densities of the purified proteins on the same blot. Qualitative immunoblots developed with goat anti-*P*-450p IgG required a 30 min exposure to rabbit anti-goat IgG and a 30 min exposure to goat peroxidase anti-peroxidase with intervening PBS washes.

RESULTS AND DISCUSSION

Monoclonal antibody 1G8 was selected because this unique probe reacted with purified rat *P*-450p on immunoblots, but did not react with the purified homologous proteins, rabbit cytochrome LM3c (Wrighton *et al.*, 1985a) or human cytochrome HLp (Watkins *et al.*, 1985) (Fig. 1). Nevertheless, these three homologous proteins are immunochemically related in being recognized by polyclonal antibodies raised against either *P*-450p (Fig. 2, top) or LM3c (Watkins *et al.*, 1985). Immunoblot analyses of liver microsomes from untreated male rats developed with 1G8 revealed two narrowly separated proteins (Fig. 1). The more slowly migrating of the two 1G8-reactive proteins in untreated males had the same mobility as did purified *P*-450p (Fig. 3). In contrast, no proteins were detected in liver microsomes isolated from untreated female rats when immunoblotting was performed under conditions of complete transfer (Schuetz *et al.*, 1984) (Fig. 1). When

Fig. 1. Immunoblots of purified cytochromes *P*-450 and rat liver microsomes

Protein samples were electrophoretically resolved in polyacrylamide slab gels, transferred to nitrocellulose paper, and then developed with either monoclonal 1G8 (top) or 13-7-10 (bottom) as described in the Experimental section. Shown are 1 μ g each of purified rat *P*-450p, rabbit LM3c and human HLp; 50 μ g of microsomal protein from untreated rats, male (M) and female (F); and 10 μ g of microsomal protein from a DEX-treated female rat.

Fig. 2. Specificity of anti-*P*-450p antibody before and after immunoabsorption

Purified cytochromes *P*-450 and rat liver microsomes were analysed by immunoblots, as described in the Experimental section, developed with either polyclonal anti-*P*-450p serum (top) or anti-*P*-450p antibody after 2 $\times$ immunoabsorption (bottom). Shown are 0.5 μ g each of purified rabbit LM3c, human HLp and rat *P*-450p, and 50 μ g of microsomal protein from untreated male (M) and female (F) rats.

Fig. 3. Relative electrophoretic mobility of 1G8-reactive microsomal proteins with purified *P*-450p

Immunoblot of purified rat *P*-450p (0.5 μ g) and of microsomal proteins from an untreated male rat (M) (50 μ g) and from a DEX-treated female rat (1 μ g) developed with monoclonal antibody 1G8.

these conditions were exceeded, trace amounts of 1G8-reactive protein were observed, indicative of very-low-level expression of *P*-450p in livers of untreated female rats (results not shown).

Next, we characterized a second monoclonal antibody, 13-7-10, which reacts specifically with HLp, a glucocorticoid-inducible human liver cytochrome *P*-450 (Molowa *et al.*, 1986). The reactivity of this monoclonal antibody was the opposite of that of 1G8 in that 13-7-10 specifically recognized purified rabbit LM3c and human

HLp, but did not recognize purified *P*-450p (Fig. 1). Immunoblots of rat liver microsomes developed with the 13-7-10 antibody revealed a reactive protein in both untreated males and untreated females (Fig. 1). A consistent finding was that the 13-7-10-reactive protein in females migrated more slowly than did the 13-7-10-reactive protein in males (Fig. 1). Thus two distinct 13-7-10-reactive proteins can be distinguished on the basis of their mobilities and sex specificities. One or both of these proteins may be inducible, because DEX treatment increased the amount of total 13-7-10-reactive microsomal protein in female rats (Fig. 1).

We cannot exclude the possibility that the microsomal proteins recognized by these monoclonal antibodies are only coincidentally related to *P*-450p. However, polyclonal antiserum directed against *P*-450p reacted not only with all three purified homologous proteins (*P*-450p, LM3c and HLp), but also with a pair of closely migrating, but separable, proteins in rat liver microsomes from an untreated male rat and a second pair in microsomes from an untreated female rat (Fig. 2). When the antiserum was absorbed first against immobilized rat liver microsomes prepared from 3-methylcholanthrene-treated males and then against those from untreated females, the resulting antibody still reacted strongly with purified *P*-450p, but only weakly with LM3c and HLp, and not at all with microsomes from untreated female rats (Fig. 2). Thus it appears that LM3c, HLp, and the 13-7-10-reactive protein present in untreated female rats share one or more epitopes that are recognized by neither the 1G8 monoclonal antibody nor the absorbed anti-*P*-450p IgG. Finally, when portions of the same immunoblot were developed with the anti-*P*-450p antiserum or with one of the two monoclonal antibodies, the pair of 1G8-reactive proteins in untreated male rats co-migrated exactly with the proteins identified by the polyclonal antiserum (Fig. 4). Likewise, the proteins identified by the 13-7-10 monoclonal antibody (one in untreated males and one in untreated females) exhibited electrophoretic mobilities identical with those recognized by the polyclonal antiserum (Fig. 4). Therefore the proteins reacting with the unabsorbed polyclonal antibody and the monoclonal antibodies are probably the same. We have no evidence to suggest that the faster-migrating 'female' protein, which was recognized only by the unabsorbed polyclonal antibody, is related to the *P*-450p family. Therefore we conclude that there are at least three related proteins expressed in the livers of untreated rats: *P*-450p, and the male- and female-specific 13-7-10-reactive proteins. It is possible that the faster migrating of the two 1G8-reactive proteins in untreated males and the male-specific 13-7-10-reactive protein are the same, since both proteins exhibit similar electrophoretic mobilities.

We have attempted to isolate and purify the proteins recognized by the monoclonal antibodies in order to identify definitively these cytochromes. To date we have been able to isolate only *P*-450p in homogeneous form. We purified several samples of *P*-450p from rats treated with TAO or PCN, using previously published techniques (Elshourbagy & Guzelian, 1980; Wrighton *et al.*, 1985a). Repeated analyses of the *N*-terminal amino acid sequence of these protein samples yielded sequences that, through the first 27 amino acids, were the same as that reported by Gonzalez *et al.* (1986) for *P*-450PCN1 (Table 1). The present sequence for *P*-450p obtained with the

Fig. 4. Analyses of rat liver microsomes by immunoblots developed with various antibodies

Shown are microsomal protein samples (50 µg) from untreated male (M) and female (F) rats and microsomal protein samples (5 µg) from a DEX-treated female rat. Before development, the immunoblot was cut into strips bisecting the lanes containing microsomal protein samples from untreated rats. Each strip was then developed with the indicated antibody, as described in the Experimental section. The dye front is shown to demonstrate proper re-alignment of the nitrocellulose paper.

use of a microsequencing device is different from that which we previously reported (Wrighton *et al.*, 1985a). The reason for this discrepancy is unknown, but probably is the result of technical error in the previous automated sequencing process. The remaining immunoreactive proteins have not yet been purified to homogeneity.

In the next series of experiments, we examined the effects of treatment of female rats with inducers of *P*-450p on the amounts of 1G8- and 13-7-10-reactive proteins as determined by quantitative immunoblot analyses of liver microsomes. Because it was not possible to sufficiently separate and individually quantify the pairs of proteins in induced rats, we have expressed our results as the total 1G8- or total 13-7-10-reactive proteins. Our studies utilized only one dose of each compound at a level reported to be maximal for induction of CO-binding haemoprotein. TAO induced the 1G8-reactive proteins to the greatest extent (> 300-fold increase over the minimum detectable value) (Table 2). This is consistent with the results of previous experiments in which *P*-450p was quantified by a polyclonal anti-*P*-450p IgG preparation (Wrighton *et al.*, 1985b). Other strong inducers of 1G8-reactive proteins were DEX (90-fold), CD (85-fold), PCN (79-fold), TNC (63-fold), SKF (41-fold), SLN (39-fold), MTP (32-fold) and PCB (20-fold) (Table 2). In contrast, TAO only moderately induced the 13-7-10-reactive proteins (21-fold increase) compared with DEX (49-fold increase) (Table 2). Most striking, however, was the relative lack of induction of 13-7-10-reactive proteins by CD and TNC, both of which were strong inducers of the 1G8-reactive proteins (Table

Table 1. N-Terminal amino acid sequences of PCN-inducible cytochromes P-450

P-450p was purified from TAO- or PCN-treated rats by published procedures (Elshourbagy & Guzelian, 1980; Wrighton *et al.*, 1985a). Sequence analyses were performed on 1.0 nmol aliquots of the purified proteins with an Applied Biosystems 470 automatic sequencer with an in-line amino-acid-phenylthiohydantoin-derivative analyser. The results for P-450p were obtained both on freshly purified protein samples and also on stored samples of P-450p that had previously been sequenced and the results reported (Wrighton *et al.*, 1985a). The sequences for P-450PCN1 and P-450PCN2 are those deduced from the nucleotide sequences of cDNA clones reported by Gonzalez *et al.* (1986). Amino acids in P-450PCN2 that are different from P-450p and P-450PCN1 are underlined.

Isoenzyme	Residue no. . . .	Residues identified																										
		1				5				10				15				20				25						
<i>P</i> -450p		M	D	L	L	S	A	L	T	L	E	T	W	V	L	L	A	V	V	L	V	L	L	Y	G	F	G	T
<i>P</i> -450PCN1		M	D	L	L	S	A	L	T	L	E	T	W	V	L	L	A	V	V	L	V	L	L	Y	G	F	G	T
<i>P</i> -450PCN2		M	D	L	L	S	A	L	T	L	E	T	W	V	L	L	A	V	<u>I</u>	L	V	L	L	Y	<u>R</u>	<u>L</u>	G	T

Table 2. Quantification with monoclonals 1G8 and 13-7-10 of immunoreactive cytochromes P-450 in rats treated with inducers

Female rats were treated and liver microsomes prepared and analysed by quantitative immunoblotting as described in the Experimental section. Results are expressed as densitometric units/ μ g of microsomal protein and the corresponding fold increase over the lowest detectable value. [Detection of 1G8- and 13-7-10-reactive proteins in microsomes isolated from untreated female rats requires analysis of samples that exceed the conditions established for quantitative electrophoretic transfer. The above values for fold induction are calculated as increases over the lowest detectable value (0.007 unit/ μ g) and thus underestimate the magnitude of induction.] Values are means $\pm$ S.D. for three individual rats.

Inducer	1G8-reactive	Fold increase	13-7-10-Reactive	Fold increase
Control	< 0.007	—	< 0.007	—
TAO	2.230 $\pm$ 0.562	319	0.147 $\pm$ 0.040	21
DEX	0.631 $\pm$ 0.121	90	0.341 $\pm$ 0.063	49
CD	0.594 $\pm$ 0.129	85	0.034 $\pm$ 0.012	5
PCN	0.555 $\pm$ 0.049	79	0.147 $\pm$ 0.029	21
TNC	0.441 $\pm$ 0.065	63	0.028 $\pm$ 0.004	4.0
SKF	0.284 $\pm$ 0.090	41	0.043 $\pm$ 0.006	6.1
SLN	0.270 $\pm$ 0.071	39	0.044 $\pm$ 0.012	6.3
MTP	0.223 $\pm$ 0.034	32	0.033 $\pm$ 0.013	4.7
PCB	0.141 $\pm$ 0.040	20	< 0.007	—
ISF	0.110 $\pm$ 0.039	16	0.033 $\pm$ 0.022	4.7
PB	0.096 $\pm$ 0.021	14	0.049 $\pm$ 0.018	7.0
TSO	0.081 $\pm$ 0.017	12	0.012 $\pm$ 0.005	1.7
ARO	0.078 $\pm$ 0.022	11	0.009 $\pm$ 0.022	1.3
PTZ	0.050 $\pm$ 0.019	7	< 0.007	—
PBB	0.040 $\pm$ 0.004	5.7	0.027 $\pm$ 0.017	3.0
CLO	0.039 $\pm$ 0.008	5.6	0.009 $\pm$ 0.002	1.3
DPH	0.022 $\pm$ 0.008	3.1	0.009 $\pm$ 0.002	1.3
CPZ	0.019 $\pm$ 0.006	2.7	< 0.007	—
ISO	0.012 $\pm$ 0.005	1.7	< 0.007	—

2). Similarly, SKF, SLN and MTP were strong inducers of the 1G8-reactive proteins, but relatively poor inducers of the 13-7-10-reactive proteins (Table 2). In addition, PCB only induced 1G8-reactive proteins (Table 2). Weaker inducers of the 1G8-reactive proteins included ISF, PB, TSO and ARO (Table 2). Each of these compounds was a superior inducer of the 1G8-reactive proteins as compared with the 13-7-10-reactive proteins (Table 2). PTZ, CPZ and ISO exclusively induced the 1G8-reactive proteins, whereas PBB, albeit a weak inducer, produced similar increases in the 1G8- and 13-7-10-reactive proteins (Table 2). The responses of the 1G8- and 13-7-10-reactive proteins to DPH and CLO were only marginal increases (Table 2).

This laboratory previously demonstrated that there is a striking, qualitative difference in the regulatory control of P-450p-related proteins between species (Wrighton *et al.*, 1985b). For example, rifampicin, the most efficacious inducer of P-450 LM3c in the rabbit, does not induce P450p in the rat at all. Conversely, PCN, an inducer of rat P-450p, is not an inducer of P-450 LM3c in the rabbit. In the present study, using quantitative analyses, we found a striking difference in the profile of induction among apparently related proteins within a single species. For example, the organochlorine pesticides CD and TNC, which strongly induced the 1G8-reactive proteins were weak inducers of the 13-7-10-reactive proteins (Table 2). Indeed, PCB appeared to exclusively

induce 1G8-reactive proteins (Table 2). Although preferential induction of cytochrome *P*-450 forms within a family has been demonstrated (Ryan *et al.*, 1980; Thomas *et al.*, 1983), we are unaware of previous examples of induction of one (or more) isoenzymes within an inducible family to the exclusion of other family members. Thus it may be concluded that, although the 13-7-10-reactive proteins are related to *P*-450p, they are subject to quantitatively and qualitatively distinct control.

Gonzalez *et al.* (1986) have reported differential regulation of two mRNAs encoding members of the PCN family (Gonzalez *et al.*, 1986). They found that *P*-450PCN1 mRNA was undetectable in both untreated male and female rats, but was readily inducible by PCN and DEX. The homologous *P*-450PCN2 mRNA was present in untreated male and female rats, was inducible by PB, but was refractory to induction by PCN and DEX. The reported expression of *P*-450PCN2 mRNA does not correspond to that of the immunoreactive proteins we have described, because none of the 1G8- nor 13-7-10-reactive proteins are present in both males and females, and both groups of proteins are readily inducible by PCN or DEX (Table 2). The resolution of these discrepancies awaits more complete characterization of the multiple mRNA and protein species.

The immunoquantification results provide a basis for ruling out the possibility that the 13-7-10-reactive proteins might be previously characterized rat liver cytochromes. For example, cytochromes *P*-450b and *P*-450e are elevated 20–30-fold in phenobarbital-treated rats (Waxman, 1986), which is inconsistent with the weak response of the 13-7-10-reactive proteins to phenobarbital (Table 2). Likewise, it is unlikely that 13-7-10 recognizes cytochromes *P*-450c and *P*-450d, which are elevated 30-fold by ARO and 20-fold by ISF treatments respectively (Waxman, 1986) (see Table 2). Cytochrome *P*-450 PB-C (*P*-450 PB-1) (Guengerich *et al.*, 1982; Waxman, 1986), which is induced by neither PCN nor ARO (Guengerich *et al.*, 1982) can also be ruled out. Finally, rat liver cytochromes *P*-450a, *P*-450f, *P*-450g, *P*-450h, *P*-450i (Ryan *et al.*, 1982, 1984a,b) and *P*-450 UT-H (Larrey *et al.*, 1984) are proteins that are not significantly induced and actually may decrease after treatment with PB, PCN, ISO and ARO (Guengerich *et al.*, 1982).

Vlasuk *et al.* (1982) used two-dimensional gel electrophoresis to tentatively identify as *P*-450p a polypeptide that was barely detectable in untreated rats, was induced by PCN, ISO, SKF, ARO and TSO, but was *not* induced by CD. We recently identified a protein that had mobility on two-dimensional gel electrophoresis identical with that of *P*-450p, was recognized by polyclonal anti-*P*-450p IgG, and was induced by CD (Schuetz *et al.*, 1986). Thus the polypeptide identified by Vlasuk and co-workers (Vlasuk *et al.*, 1982) may represent a distinct member of the PCN family (Schuetz *et al.*, 1986). The exact relationship between the latter protein and the *P*-450p-related proteins we have identified in the present report remains unclear, since our results indicate that SKF, ISO and CD elevate the 13-7-10-reactive proteins to approximately the same extent (about 5-fold above the minimum detectable amount), and ARO and TSO were only marginally efficacious inducers of 13-7-10-reactive proteins (Table 2).

We found that 13-7-10-reactive proteins were only weakly induced by TAO (Table 2). This might reflect differences in substrate specificity of the 13-7-10-reactive proteins, because the dramatic induction of 1G8-reactive *P*-450p in TAO-treated rats is associated with conversion of the drug by *P*-450p into a metabolite that binds *P*-450p specifically (Wrighton *et al.*, 1985a). In contrast, we have yet to find an agent that elevates 13-7-10-reactive proteins more than the 1G8-reactive proteins. The most effective in this regard is PBB, which elevated these proteins to a similar extent. Although we made no attempt to determine dose-response relationships for the inducers, structural differences among the polyhalogenated biphenyls may account for the apparent preferential or selective induction of the two types of immunoreactive proteins (Table 2). Indeed, we have recently demonstrated that induction of *P*-450p and *P*-450b/e by polychlorinated biphenyl isomers involves different structure-activity relationships (Schuetz *et al.*, 1986).

In summary, on the basis of immunoblot analyses of rat liver microsomes developed with the antibody preparations described, we have tentatively identified at least two heretofore-unrecognized proteins distinct from, but related to, *P*-450p. Final proof that these proteins are products of separate genes will require their purification, structural elucidation and assessment of their immunoreactivity. Nevertheless, specific monoclonal antibodies provide strong, albeit indirect, evidence to add to the growing recognition that there is a family of 'PCN-inducible cytochromes *P*-450' expressed in rat liver. These proteins appear to be disco-ordinately regulated. They are under complex control by factors associated with gender and are influenced to different extents by many structurally dissimilar exogenous compounds. The monoclonal antibodies described will be most helpful in eventually achieving a complete structural description of this unique family of proteins and the mechanisms of their regulation.

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REFERENCES

- Adesnik, M. & Atchison, M. (1985) CRC Crit. Rev. Biochem. **19**, 247–305
- Beaune, P., Kremers, P., Letawe-Goujon, F. & Gielen, J. E. (1985) Biochem. Pharmacol. **34**, 3547–3552
- Cuatrecasas, P. (1970) J. Biol. Chem. **245**, 3059–3065
- Elshourbagy, N. A. & Guzelian, P. S. (1980) J. Biol. Chem. **255**, 1279–1285
- Elshourbagy, N. A., Barwick, J. L. & Guzelian, P. S. (1981) J. Biol. Chem. **256**, 6060–6068
- Gonzalez, F. J., Nebert, D. W., Hardwick, J. P. & Kasper, C. B. (1985) J. Biol. Chem. **260**, 7435–7441
- Gonzalez, F. J., Sang, B. & Hardwick, J. P. (1986) Mol. Cell. Biol. **6**, 2969–2976
- Guengerich, F. P., Dannan, G. A., Wright, S. T., Martin, M. V. & Kaminsky, L. S. (1982) Biochemistry **21**, 6019–6030

- Hardwick, J. P., Gonzalez, F. J. & Kasper, C. B. (1983) *J. Biol. Chem.* **258**, 10182–10186
- Heuman, D. M., Gallagher, E. J., Barwick, J. L., Elshourbagy, N. A. & Guzelian, P. S. (1982) *Mol. Pharmacol.* **21**, 753–760
- Koop, D. R., Persson, A. V. & Coon, M. J. (1981) *J. Biol. Chem.* **258**, 10704–10711
- Kumar, A., Raphael, C. & Adesnik, M. (1983) *J. Biol. Chem.* **258**, 11280–11284
- Larrey, D., Distlerath, L. M., Dannan, G. A., Wilkinson, G. R. & Guengerich, F. P. (1984) *Biochemistry* **23**, 2787–2795
- Lu, A. Y. H. & West, S. B. (1979) *Pharmacol. Rev.* **31**, 277–295
- March, S. C., Parikh, I. & Cuatrecasas, P. (1974) *Anal. Biochem.* **60**, 149–152
- Molowa, D. T., Schuetz, E. G., Wrighton, S. A., Watkins, P. B., Kremers, P., Mendez-Picon, G., Parker, G. A. & Guzelian, P. S. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5311–5315
- Omura, T. & Sato, R. (1964) *J. Biol. Chem.* **239**, 2370–2378
- Ryan, D. E., Thomas, P. E. & Levin, W. (1980) *J. Biol. Chem.* **255**, 7941–7955
- Ryan, D. E., Thomas, P. E., Reik, L. M. & Levin, W. (1982) *Xenobiotica* **12**, 727–744
- Ryan, D. E., Dixon, R., Evans, R. H., Ramanathan, L., Thomas, P. E., Wood, A. W. & Levin, W. (1984a) *Arch. Biochem. Biophys.* **233**, 636–642
- Ryan, D. E., Iida, S., Wood, A. W., Thomas, P. E., Lieber, C. S. & Levin, W. (1984b) *J. Biol. Chem.* **259**, 1239–1250
- Schacterle, G. R. & Pollack, R. L. (1973) *Anal. Biochem.* **57**, 654–655
- Schuetz, E. G. & Guzelian, P. S. (1984) *J. Biol. Chem.* **259**, 2007–2012
- Schuetz, E. G., Wrighton, S. A., Barwick, J. L. & Guzelian, P. S. (1984) *J. Biol. Chem.* **259**, 1999–2006
- Schuetz, E. G., Wrighton, S. A., Safe, S. H. & Guzelian, P. S. (1986) *Biochemistry* **25**, 1124–1133
- Thomas, P. E., Reik, L. M., Ryan, D. E. & Levin, W. (1983) *J. Biol. Chem.* **258**, 4590–4598
- Vlasuk, G. P., Ryan, D. E., Thomas, P. E., Levin, W. & Walz, F. G., Jr. (1982) *Biochemistry* **24**, 6288–6292
- Watkins, P. B., Wrighton, S. A., Maurel, P., Schuetz, E. G., Mendez-Picon, G., Parker, G. A. & Guzelian, P. S. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6310–6314
- Waxman, D. J. (1986) *Cytochrome P-450: Structure, Mechanism and Biochemistry* (Ortiz de Montellano, P. R., ed.), Plenum Press, New York
- Wrighton, S. A., Maurel, P., Schuetz, E. G., Watkins, P. B., Young, B. & Guzelian, P. S. (1985a) *Biochemistry* **24**, 2171–2178
- Wrighton, S. A., Schuetz, E. G., Watkins, P. B., Maurel, P., Barwick, J., Bailey, B. S., Hartle, H. T., Young, B. & Guzelian, P. S. (1985b) *Mol. Pharmacol.* **28**, 312–321

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