0026-895X/91/050679-07$03.00/0

MOLECULAR PHARMACOLOGY, 40:679-685

Purification and Characterization of Human Liver Microsomal Cytochrome P-450 2A6

CHUL-HO YUN, TSUTOMU SHIMADA, and F. PETER GUENGERICH

Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146

Received May 3, 1991; Accepted August 6, 1991

SUMMARY

Cytochrome P-450 (P-450) 2A6 was purified by chromatography of human liver microsomes. The final preparation was electrophoretically homogeneous and contained 16 nmol of P-450/mg of protein. The amino-terminal amino acid sequence of the protein (first 13 residues) matched that of the reported cDNA exactly. The UV-visible spectrum indicated that the isolated hemoprotein was in the low-spin form. The protein was recognized by rabbit antibodies raised against rat P-450 2A1, and a rabbit antiserum against the P-450 2A6 preparation was also prepared. With these antibodies, it was estimated that P-450 2A6 accounted for a maximum of 1% of the total P-450 present in the human liver microsomes; the level varied >100-fold among the 20 samples examined. Purified P-450 2A6 catalyzed coumarin 7-hydroxylation and 7-ethoxycoumarin *O*-deethylation at rates similar to those measured in the human liver sample used to prepare P-450 2A6, and these two microsomal activities were strongly inhibited by the antibodies. The purified P-450 2A6 enzyme also catalyzed low levels of 4,4′-methylene-bis(2-chloroaniline) (MOCA) *N*-oxidation and activation of aflatoxin B_1, 6-aminochrysene, 2-amino-3-methylimidazo[4,5-*f*]quinoline, and 2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline to genotoxic products; the antibody inhibited the activity of purified P-450 2A6 towards aflatoxin B_1 and 6-aminochrysene but did not inhibit these reactions in human liver microsomes (MOCA *N*-oxidation was inhibited ~20%). Human P-450 2A6 did not catalyze testosterone 7α-hydroxylation, a characteristic activity of the related rat P-450 2A1 protein. These results emphasize the need to characterize individual P-450 enzymes in order to understand their functions in the context of more complex systems.

P-450s are the major enzymes involved in the biotransformation of drugs, steroids, carcinogens, pesticides, and several other classes of "xenobiotic" chemicals (1). At least 38 different P-450s have been identified in the rat to date (2), and it is highly likely that a similar number will be found in humans. The different P-450s vary in terms of regulation of expression, catalytic specificity, and sensitivity to inhibitors (3). Considerable effort has been expended towards the characterization of individual human P-450 enzymes in this and other laboratories (4).

In 1979, Ryan *et al.* (5) isolated an enzyme termed P-450a and showed that it is the principal catalyst of testosterone 7α-hydroxylation in rat liver microsomes. Our own laboratory later reported an independent purification of rat liver $P\text{-}450_{UT\text{-}F}$ (6). Other similar preparations apparently include "form 1" (7) and P-450 PB-3 (8). Gonzalez and associates (9) have cloned the P-450 2A1 cDNA and shown that the expressed product is this same enzyme. In the rat, there exists a highly related P-450 2A2 gene, and some properties of its product have been reported (10–12).

In retrospect, the P-450 2A6 sequence was the first one for which a portion of a human cDNA sequence was reported (13). This sequence was first thought to be more closely related to what is now recognized as the P-450 2B family (14), but Yamano *et al.* (15) isolated a full-length P-450 2A6 cDNA[1] and also found that the expressed protein is the major coumarin 7-hydroxylase in human liver. Miles *et al.* (16) reached a similar conclusion on the basis of some correlation and immunoinhibition studies. Recently, the P-450 2A6 sequence has been transfected into a stable cell line, and these cells have been shown to generate mutations in the presence of *N*-nitrosodi-

This work was supported in part by Grants CA 44353 and ES 00267 from the National Institutes of Health and by a Grant in Aid for Cancer Research from the Ministry of Education, Science, and Culture of Japan.

[1]In this work, the rat antigen $P\text{-}450_{UT\text{-}F}$ was used to prepare an antibody (6), which is used here. $P\text{-}450_{UT\text{-}F}$ is now termed P-450 2A1 (2), and the amino-terminal amino acid sequence analysis of an older preparation of rat $P\text{-}450_{UT\text{-}F}$ gave the expected results for the first 12 residues (not presented). Other rat preparations in the literature are mentioned in the introduction of the text. Human P-450 2A6 was previously termed P-450 2A3, before the last nomenclature revision (2). It should be emphasized that a highly related human cDNA has been identified and termed P-450 2A7 (2, 10). The human P-450 2A6 cDNA has been reported (9), as well as some catalytic properties of the cDNA-expressed enzyme (10).

ABBREVIATIONS: P-450, cytochrome P-450; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography; MOCA, 4,4′-methylene-bis(2-chloroaniline); B(*a*)P, benzo(*a*)pyrene; AFB_1, aflatoxin B_1; 6-AC, 6-aminochrysene; MeIQ, 2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline.

ethylamine or AFB_1, yielding the conclusion that P-450 2A6 may have an important role in the activation of both of these chemicals (17).

During the course of isolation of a P-450 (*S*)-mephenytoin 4′-hydroxylase from human liver microsomes (18), we purified a protein that proved to be P-450 2A6. We report its characterization here; some of the properties are of interest in evaluation of the overall role of this and other P-450 enzymes.

Materials and Methods

Chemicals. Coumarin and B(*a*)P were obtained from Sigma Chemical Co. (St. Louis, MO). 7-Ethoxycoumarin and 7-hydroxycoumarin were purchased from Aldrich Chemical Co. (Milwaukee, WI). 7*α*-Hydroxytestosterone was purchased from Steraloids, Inc. (Wilton, NH). [6,6′-^{3}H]MOCA (210 mCi/mmol) was kindly provided by Dr. F. F. Kadlubar, National Center for Toxicological Research (Jefferson, AR). Other chemicals were of the highest grade commercially available.

Purification of human P-450 2A6. Human liver samples were obtained from organ donors through Tennessee Donor Services (Nashville, TN) (denoted "HL," for human liver, with a code number) or the Liver Tissue Procurement and Distribution System, University of Minnesota (Minneapolis, MN). Microsomes were prepared as described elsewhere (19).

Twenty human liver microsomal preparations were screened for coumarin 7-hydroxylation activity. Sample HL 115 was selected for the purification of the coumarin 7-hydroxylase, because it had the highest catalytic activity. Microsomal proteins were solubilized with sodium cholate, loaded onto a 2.5- × 48-cm *n*-octylamino-Sepharose 4B column (20), and eluted with 100 mM potassium phosphate buffer (pH 7.4) containing 0.4% (w/v) sodium cholate, 1 mM EDTA, 20% (v/v) glycerol, and 0.06% (w/v) Emulgen 911 (eluate I); further elutions were performed with the same buffer containing 0.2% (w/v) Emulgen 911 (eluate II) or 0.5% (w/v) Emulgen 911 (eluate III). The fractions having high coumarin 7-hydroxylation activity (eluate I only) were pooled, dialyzed, and applied to a 2.5- × 10-cm column of hydroxylapatite (high resolution grade; Calbiochem, San Diego, CA). The hydroxylapatite column was eluted sequentially with 40, 90, 180, and 300 mM potassium phosphate buffers (pH 7.4) containing 0.2 mM dithiothreitol, 20% (v/v) glycerol, and 0.2% (w/v) Emulgen 911. Coumarin 7-hydroxylation activity was found primarily in the fractions eluted with 300 mM phosphate. These fractions were pooled, dialyzed extensively against 5 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 0.2 mM dithiothreitol, 20% (v/v) glycerol, and 0.2% (w/v) Emulgen 911, and applied to a 1.6- × 15-cm Whatman DE-52 DEAE-cellulose column (Whatman, Clifton, NJ), which had been equilibrated with the same buffer. The column was washed with an additional 100 ml of the same buffer, and then a 300-ml linear gradient of 0 to 200 mM NaCl in the same buffer was used to elute the column. Coumarin 7-hydroxylation activity was eluted in the void volume. Those fractions that were electrophoretically homogeneous and contained coumarin 7-hydroxylation activity were pooled, and Emulgen 911 was removed from the purified preparation by sequential adsorption of protein onto a 1.2- × 1.5-cm hydroxylapatite column, extensive washing with 5 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and 20% (v/v) glycerol, and elution with 0.5 M potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and 20% (v/v) glycerol. The final preparation was dialyzed against 50 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and 20% (v/v) glycerol and was stored at −20°.

Antibodies to P-450 2A6 were raised in rabbits and treated as described elsewhere (21). Anti-rat $P\text{-}450_{UT\text{-}F}$ was from previous work (6).[1] Human cytochrome b_5 was isolated previously (20).

The amino acid sequence was determined, using automated Edman degradation, in the Vanderbilt University facility by T. Porter, with the use of an Applied Biosystems 470A sequenator. P-450 2A6 (300 pmol) was dialyzed extensively against H_2O and precipitated with a mixture of acetone/H_2O (5:1, v/v). The sample was washed two more times with the same solvent mixture. Phenylthiohydantoins were analyzed on-line by HPLC. Yields of individual residues were estimated by integration of the chromatograms and comparison with external standards.

Assays. Protein was assayed using a bicinchoninic acid procedure, according to the manufacturer's directions (Pierce Chemical Co., Rockford, IL). Spectral determination of P-450 was done by Fe^{2+}-CO versus Fe^{2+} difference spectroscopy (22). SDS-polyacrylamide gel electrophoresis utilized the basic procedure of Laemmli (23). Protein staining was done with silver, according to the method of Wray *et al.* (24). Protein immunoblotting was done as described elsewhere, and the concentration of P-450 2A6 in human liver microsomal samples was estimated using a standard curve (25).

Coumarin 7-hydroxylation and 7-ethoxycoumarin *O*-deethylation assays were carried out as previously described (26), with slight modification; purified P-450 2A6 (25–100 pmol) was reconstituted immediately before use in the enzyme assays, in 50 mM Tris·HCl buffer (pH 7.4) containing 30 μM L-*α*-dilauroyl-*sn*-glycero-3-phosphocholine and a 2-fold molar excess of rabbit liver NADPH-P-450 reductase. In the case of microsomal assays, the microsomal protein (150 μg) was incubated in 0.5 ml of 50 mM Tris·HCl buffer (pH 7.4). After a 3-min preincubation in the presence of coumarin or 7-ethoxycoumarin (20 μM), the reactions were initiated by the addition of an NADPH-generating system, using open glass vials in a shaking water bath at 37°. The reactions were stopped by the addition of 60 μl of 15% (w/v) aqueous Cl_3CCO_2H and 1.0 ml of CH_2Cl_2. Aliquots (0.5 ml) of the organic extracts were then added to 3 ml of 30 mM sodium borate buffer (pH 9.0). After vortex mixing and centrifugation, the supernatants (which contained 7-hydroxycoumarin formed from coumarin and 7-ethoxycoumarin) were analyzed fluorometrically, with $\lambda_{excitation}$ of 358 nm and $\lambda_{emission}$ of 458 nm, and the readings were compared with those of 7-hydroxycoumarin standards.

Testosterone metabolites were analyzed by the procedure described by Wood *et al.* (27), with slight modification. Metabolite preparation was done on an octadecylsilyl (C18) HPLC column (Partisil 5, 4.6 × 250 mm; Phenonemex, Torrance, CA), using a linear gradient of an aqueous solution of 43% (v/v) CH_3OH and 1.1% (v/v) CH_3CN to 75% (v/v) CH_3OH and 1.9% (v/v) CH_3CN, over 25 min, at a flow rate of 2.0 ml/min. The eluent was monitored at 254 nm, and 7*α*-hydroxytestosterone was quantified by comparison with an external standard.

B(*a*)P 3-hydroxylation (28), (*S*)-mephenytoin 4′-hydroxylation (18), and MOCA *N*-oxidation (29) assays were carried out as described in earlier reports. The *umu* gene expression assay for DNA damage is described in detail elsewhere (30), except that the AFB_1 concentration was reduced to 1 μM.

Results

Purification of P-450 2A6. A summary of the purification of P-450 2A6 from human liver microsomes is presented in Table 1. The overall yield of P-450 2A6, based on total microsomal P-450, was 0.2%. Column chromatography was used to purify the P-450 responsible for the 7-hydroxylation of cou-

TABLE 1
Purification of P-450 2A6 from human liver microsomes

Step	Protein	P-450		Purification[a]	Yield[a]
		Total	Specific content		
	mg	*nmol*	*nmol/mg of protein*	*fold*	*%*
Microsomes[b]	3200	3200	1.0	1	100
Solubilized supernatant	3040	2950	1.0	1.0	92
Octylamino-Sepharose 4B	126	586	4.7	4.7	18
Hydroxylapatite	20	153	7.7	7.7	4.8
DE 52	0.32	5.0	16	16	0.2

[a] Based upon total microsomal P-450.
[b] Liver sample HL 115 was used.

marin, and fractions of the eluent were monitored by SDS-polyacrylamide gel electrophoresis, immunochemical detection with rabbit anti-rat P-450$_{UT-F}$, and coumarin 7-hydroxylation activity. *n*-Octylamino-Sepharose 4B column chromatography of cholate-solubilized microsomes yielded the majority of P-450 2A6 in fractions eluted with the buffer containing 0.06% (w/v) Emulgen 911. The fractions having the highest coumarin 7-hydroxylation activity were purified further by hydroxylapatite column chromatography. Most of the P-450 2A6 was found in the hemoprotein peak eluted with 300 mM potassium phosphate buffer after extensive washing with 40, 90, and 180 mM potassium phosphate buffers. Because fractions from this peak were not homogeneous, further purification was performed with Whatman DE 52 DEAE-cellulose column chromatography. P-450 2A6 was eluted in the void fraction. The purified P-450 2A6 thus obtained was homogeneous, as judged by SDS-polyacrylamide gel electrophoresis (Fig. 1A), and contained 16 nmol of P-450/mg of microsomal protein. The apparent fold purification of P-450 2A6 was ~16 (based on total microsomal P-450; subsequent measurements indicated that the true fold purification was ~1500). Immunochemical staining of purified P-450 2A6 and P-450$_{UT-F}$ preparations with anti-rat P-450$_{UT-F}$ preparations and anti-human P-450 2A6, respectively, is shown in Fig. 1, B and C.

Amino-terminal amino acid sequence. The purified protein was subjected to automated Edman degradation, for determination of the amino-terminal sequence (Table 2). The first 13 residues were found to be identical to those reported in the cDNA sequence of P-450 2A6 (15, 16).

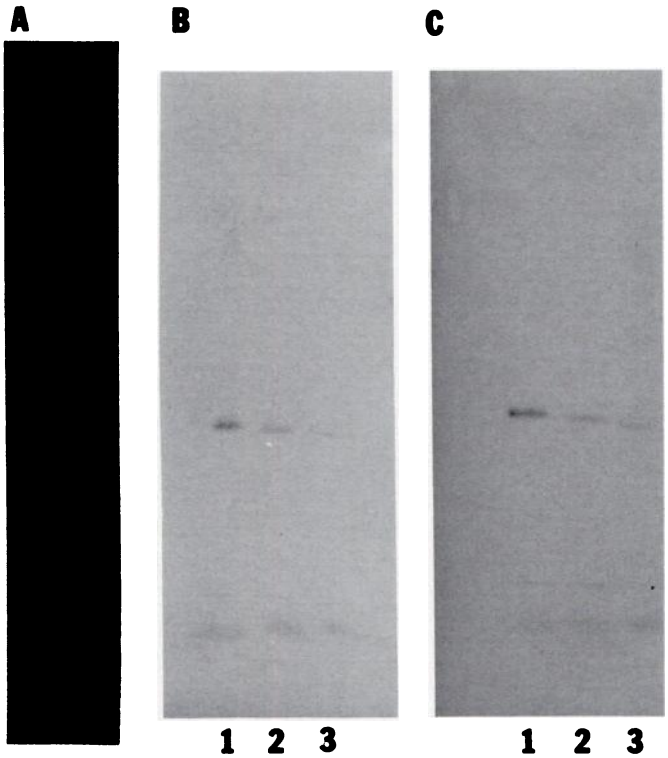

Fig. 1. SDS-polyacrylamide gel electrophoresis and immunoperoxidase staining of human microsomes and human and rat P-450 preparations. Purified P-450s and liver microsomes (sample HL 115) were applied to an electrophoresis gel. Purified human P-450 2A6 (0.10 μg, from sample HL 115) is shown with detection using silver staining (A). Purified P-450 and microsomes were separated by electrophoresis, transferred to nitrocellulose, and treated with anti-human P-450 2A6 (B) or anti-rat P-450$_{UT-F}$ (C), respectively. *Lane 1*, P-450 2A6 (0.13 μg); *lane 2*, human liver microsomes (30 μg); *lane 3*, P-450$_{UT-F}$ (0.03 μg). Three different gels were used in the electrophoresis (A, B, and C).

Spectral properties of P-450 2A6. P-450 2A6 was found to have an α band at 567–568 nm, a β band at 534–536 nm, and a Soret peak at 414 nm in the oxidized state; the wavelength maximum of the Fe^{2+}-CO complex was found to be at 450 ± 0.5 nm. The ferric protein appeared to be essentially completely low spin (results not shown).

Correlation of coumarin 7-hydroxylation activity in human liver microsomes with anti-rat P-450$_{UT-F}$ reactivity. Coumarin 7-hydroxylation rates ranged from 0.014 to 2.33 nmol of product formed/min/nmol of P-450 (Fig. 2) in microsomes prepared from 20 human liver samples, a difference of 167-fold. Immunoblotting experiments with the human liver microsomes under consideration yielded a single band in all lanes when staining was done with anti-rat P-450$_{UT-F}$ (Fig. 2). When the intensity of the individual bands was estimated, a high correlation was found with coumarin 7-hydroxylation activity ($r = 0.93$) (Fig. 2C).

Catalytic activities of purified human liver P-450 2A6 and comparison with human liver microsomes. Purified P-450 2A6 had coumarin 7-hydroxylation, 7-ethoxycoumarin *O*-deethylation, and MOCA *N*-oxidation activities (Table 3). No B(*a*)P 3-hydroxylation, (*S*)-mephenytoin 4′-hydroxylation, or testosterone 7α-hydroxylation activity was detected. Although testosterone 7α-hydroxylation is a characteristic reaction of the related rat protein P-450$_{UT-F}$ (2A1) (31), purified human P-450 2A6 showed no activity. The human liver microsomes prepared from sample HL 115 had relatively low testosterone 7α-hydroxylation activity; neither anti-rat P-450$_{UT-F}$ nor anti-human P-450 2A6 inhibited the testosterone 7α-hydroxylation activity in human liver microsomes.

Effect of cytochrome b_5 on P-450 2A6 activities. Human cytochrome b_5 was added to the reconstituted system in order to examine its effect on the catalytic activities of P-450 2A6. An inhibitory effect of cytochrome b_5 on the coumarin 7-hydroxylation and 7-ethoxycoumarin *O*-deethylation activities was observed, with the activities gradually decreasing to ~50% at a cytochrome b_5 to P-450 2A6 ratio of 6; at a ratio of 1, about 90% of the activity was observed (results not shown).

Immunoinhibition of P-450 2A6-linked catalytic activities of human liver microsomes by anti-rat P-450$_{UT-F}$ and anti-human P-450 2A6 antibodies. In or-

TABLE 2
Amino-terminal amino acid sequence of P-450 2A6

Residue	Amino acid	Yield[a]
		pmol
1	M	200
2	L	179
3	A	169
4	S	58
5	G	78
6	M	50
7	L	39
8	L	41
9	V	40
10	A	41
11	L	30
12	L	32
13	V	31

[a] Sequence analysis was done with 300 pmol of P-450 2A6, as described in Materials and Methods.

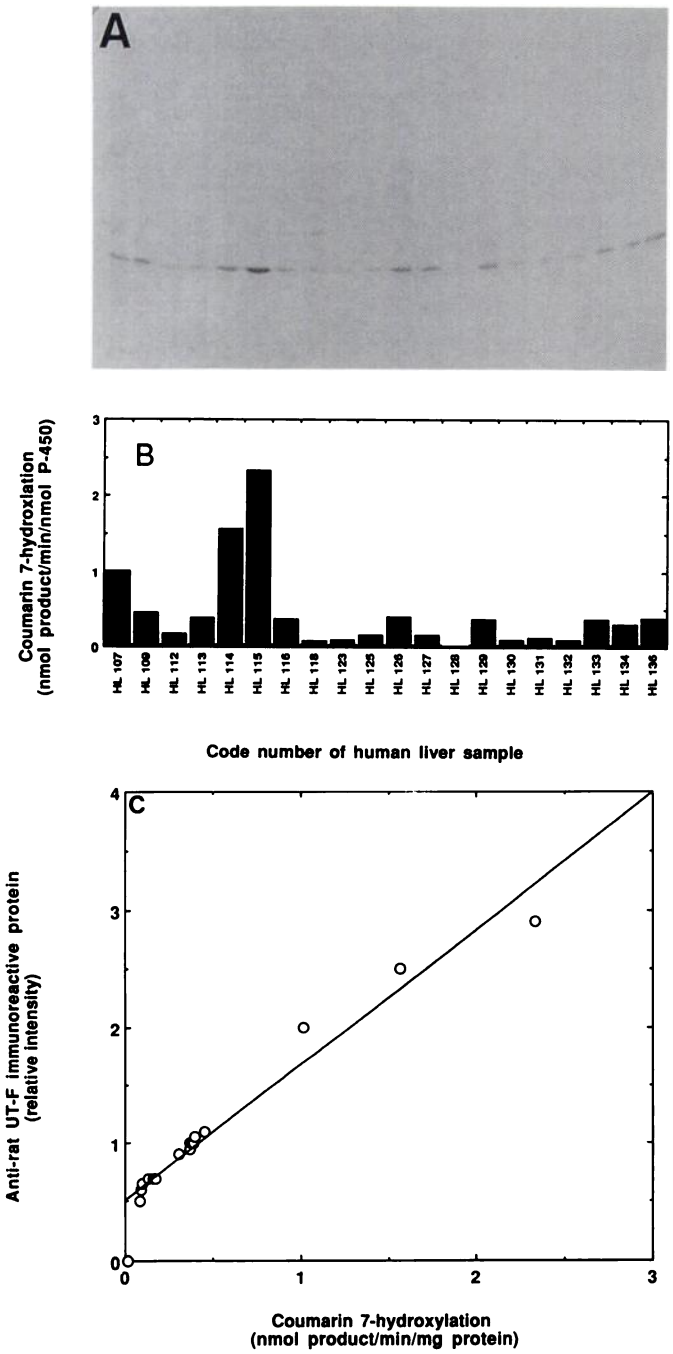

Fig. 2. Correlation of coumarin 7-hydroxylation activity in human liver microsomes with anti-rat P-450$_{UT-F}$ reactivity. Immunoblotting (A) was done and coumarin 7-hydroxylation activities were measured (B) in 20 human liver microsomal samples (immunoblotting analysis was done with 10 μg of protein/well). The apparent correlation coefficient (r) was 0.93 (C).

der to determine whether human hepatic coumarin 7-hydroxylation is primarily due to P-450 2A6, immunoinhibition studies with anti-rat P-450$_{UT-F}$ and anti-human P-450 2A6 were carried out. Both anti-rat P-450$_{UT-F}$ and anti-human P-450 2A6 completely inhibited the coumarin 7-hydroxylation activity in human liver sample HL 115 microsomes (Fig. 3). The reactivity of the homologous antibody anti-human P-450 2A6 was higher than that of anti-rat P-450$_{UT-F}$. These results strongly suggest that P-450 2A6 is responsible for most, if not all, of the coumarin 7-hydroxylation activity in human liver.

TABLE 3

Catalytic activities of purified human liver P-450 2A6 and comparison with human liver microsomes (sample HL115)

Reaction	Activity	
	HL 115	P-450 2A6
	nmol of product/min/nmol of P-450	
Coumarin 7-hydroxylation	2.33	2.09
7-Ethoxycoumarin *O*-deethylation	0.78	0.60
MOCA *N*-oxidation	1.87	0.36
B(*a*)P 3-hydroxylation	0.065	<0.01
(*S*)-Mephenytoin 4′-hydroxylation	0.20	<0.01
Testosterone 7α-hydroxylation	1.7	<0.1

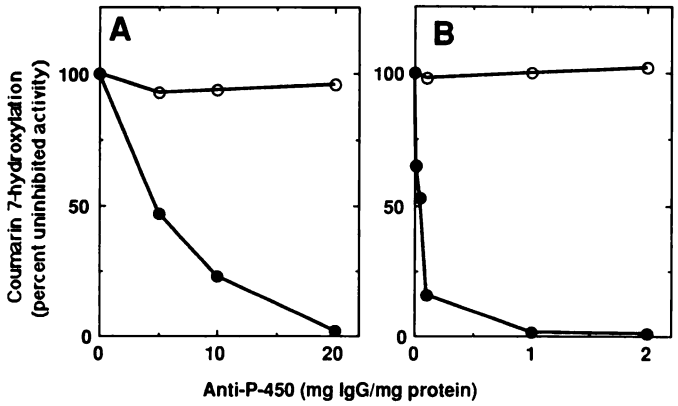

Fig. 3. Effect of anti-rat P-450$_{UT-F}$ (A) or anti-human P-450 2A6 (B) on coumarin 7-hydroxylation activity catalyzed by human liver microsomes. Microsomes (sample HL 115) were preincubated with preimmune IgG (○) or the anti-P-450 IgG preparation (●).

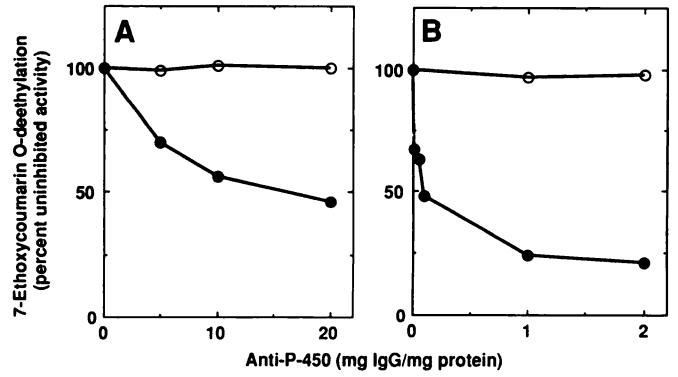

Fig. 4. Effect of anti-rat P-450$_{UT-F}$ (A) or anti-human P-450 2A6 (B) on 7-ethoxycoumarin *O*-deethylation activity catalyzed by liver microsomes. Microsomes were preincubated with preimmune IgG (○) or the anti-P-450 IgG preparation (●).

Human liver microsomal 7-ethoxycoumarin *O*-deethylation activity was only partially inhibited, on the other hand (Fig. 4). At the ratio of 20 mg of anti-rat P-450$_{UT-F}$/nmol of P-450 (where the coumarin 7-hydroxylation activity was completely inhibited), the 7-ethoxycoumain *O*-deethylation activity was inhibited by 54%. Anti-human P-450 2A6 inhibited ~80% of 7-ethoxycoumarin *O*-deethylation activity. These results indicate that P-450 2A6 appears to be a major P-450 catalyzing 7-ethoxycoumarin *O*-deethylation in human liver, although P-450 1A2 (32), P-450 2B6 (14), and P-450 2F1 (33) have all been reported to be capable of catalyzing 7-ethoxycoumarin *O*-deethylation in human liver.

Purified P-450 2A6 also catalyzes MOCA *N*-oxidation, albeit at a rate much lower than the microsomal activity. Anti-rat P-450$_{UT-F}$ and anti-human P-450 2A6 inhibited ~20% of MOCA *N*-oxidation in a human liver microsomal sample (Fig. 5). Thus, P-450 2A6 appears to be a minor catalyst of MOCA *N*-oxidation in human liver microsomes.

Estimation of P-450 2A6 levels in human liver microsomes. In order to estimate the concentration of P-450 2A6 in human liver microsomes, the immunoblotting intensities of purified P-450 2A6 and microsomal samples were compared, using anti-rat P-450$_{UT-F}$ (data not shown). The concentration of P-450 2A6 in sample HL 115 was ~11 pmol of P-450 2A6/mg of microsomal protein. The level of P-450 2A6 is much lower than those of the major P-450s (34); it accounts for only 1% of total P-450 in sample HL 115, which has the highest level of P-450 2A6 among the 20 human liver microsomal samples picked at random and a rate of coumarin 7-hydroxylation consonant with those reported by others (15, 16). P-450 2A6 was not detected, by immunoblotting analysis, at levels of >0.2 pmol/mg in the human lung, colon, breast, kidney, or placenta microsomes assayed.

Activation of AFB$_1$ and other procarcinogens by human liver microsomes and by purified P-450s. We first reported that P-450 3A4 is a principal enzyme involved in the metabolic activation of aflatoxins and 6-AC in human liver microsomes, as determined by the so called *umu* test using *Salmonella typhimurium* TA1535/pSK1002 (34, 35); several heterocyclic arylamines, including MeIQ and IQ, are transformed to reactive metabolites by human P-450 1A2 (P-450$_{PA}$), as revealed by this assay method. Recently, Ayoama *et al.* (36) reported that, in a cDNA-expressed system, P-450 2A6, as well as P-450 3A4, is involved in the metabolic activation of AFB$_1$. In order to determine whether P-450 2A6 has a major role in the activation of procarcinogens, we determined the activities using the *umu* test system. AFB$_1$ and 6-AC were converted to DNA-alkylating metabolites by purified P-450 2A6, P-450 3A4, and P-450 1A2; although the turnover numbers of the purified P-450 enzymes for AFB$_1$ were lower than those measured in human liver microsomes (sample HL 115), the rates of 6-AC activation in reconstituted P-450 systems were higher than those in liver microsomes (Table 4). In the activation of MeIQ and IQ, only P-450 1A2 had higher activity than that measured in human liver microsomes.

Immunoinhibition of P-450 2A6-catalyzed activation of AFB$_1$ and 6-AC by anti-P-450 antibodies. The results described above indicated the possible involvement of P-450 2A6 in the activation of AFB$_1$ and 6-AC in human liver microsomes. We also studied the effects of anti-P-450 IgG preparations on the activation of these two procarcinogens. Anti-rat P-450$_{UT-F}$ inhibited the activation of AFB$_1$ in a reconstituted monooxygenase system containing purified human P-450 2A6 (Fig. 6). However, when the effects of antibodies were determined in a liver microsomal system (human sample HL 115), anti-P-450 3A4 IgG strongly inhibited the activation of AFB$_1$ and 6-AC and anti-rat P-450$_{UT-F}$ did not (Fig. 7) (some effect of anti-human P-450 1A2 was seen). Also, we found that antibodies raised against the purified human P-450 2A6 enzyme had no effect on the activation of either AFB$_1$ or 6-AC in human liver microsomes (Fig. 8).

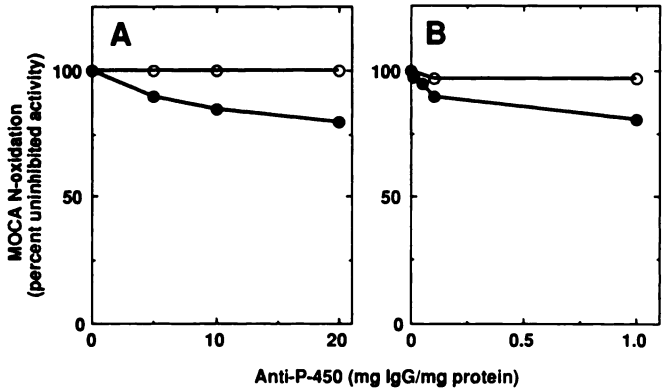

Fig. 5. Effect of anti-rat P-450$_{UT-F}$ (A) or anti-human P-450 2A6 (B) on MOCA *N*-oxidation activity catalyzed by liver microsomes. Microsomes were preincubated with preimmune IgG (○) or the anti-P-450 IgG preparation (●).

TABLE 4

Activation of procarcinogens by human liver microsomes and purified P-450s

Values are mean ± standard deviation of duplicate experiments.

	umu gene expression			
	Microsomes[a]	P-450 2A6	P-450 3A4[b]	P-450 1A2
	units/min/nmol of P-450			
AFB$_1$	1070 ± 37	64 ± 4	102 ± 18	129 ± 4
6-AC	329 ± 1	613 ± 21	562 ± 30	388 ± 81
MeIQ	1170 ± 135	194 ± 8	86 ± 23	1410 ± 32
IQ	935 ± 73	115 ± 11	96 ± 35	1680 ± 147

[a] Liver sample HL 115 was used.
[b] Cytochrome b_5 was added in the incubations.

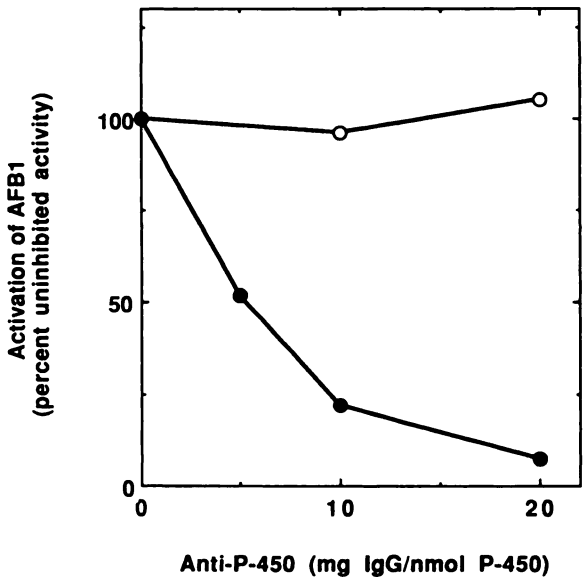

Fig. 6. Immunoinhibition of AFB$_1$-dependent *umu* gene expression by anti-rat P-450$_{UT-F}$ in a reconstituted system containing purified P-450 2A6. The effect of anti-rat P-450$_{UT-F}$ (●) or preimmune IgG (○) on the metabolic activation of AFB$_1$ is shown.

Discussion

Although human P-450 2A6 has been characterized through cDNA cloning and shown by this and other means to be a principal catalyst of coumarin 7-hydroxylation (15, 16), the enzyme had never been purified, nor was information available regarding the absolute level found in liver. We report the purification in this paper, along with spectral, immunochemi-

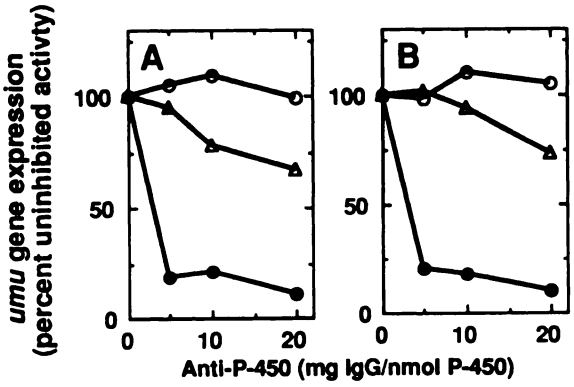

Fig. 7. Effect of anti-rat P-450$_{UT-F}$ (○), anti-human P-450 1A2 (△), or anti-human P-450 3A4 (●) on the activation of AFB_1 (A) or 6-AC (B) in human liver microsomes (sample HL 115).

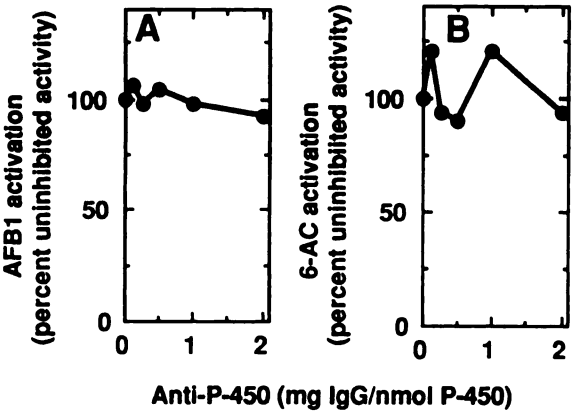

Fig. 8. Effect of anti-human P-450 2A6 on the activation of AFB_1 (A) and 6-AC (B) in human liver microsomes (sample HL 115).

cal, and catalytic properties. The protein is immunochemically related to rat P-450 2A1 (P-450$_{UT-F}$). It appears to be a major catalyst of coumarin 7-hydroxylation and 7-ethoxycoumarin *O*-deethylation activities in human liver microsomes but does not make a substantial contribution to the oxidation of the other substrates tested.

The anti-rat P-450$_{UT-F}$ preparation recognized a single human liver microsomal polypeptide band in SDS-polyacrylamide gel/immunoblotting analysis, and this was found to be P-450 2A6. No evidence of a second band was obtained, in contrast to reports of others (15, 16), although this difference could be a function of the details of electrophoresis in different laboratories (one sample, HL 118, did contain an additional high molecular mass band that is not accounted for). However, anti-human P-450 2A6 did recognize a faint additional band, of considerably higher molecular mass, when used at high titer. We have not yet determined the identity of this polypeptide and are uncertain whether it is an additional P-450 2A member. The more specific, heterologous, anti-rat P-450$_{UT-F}$ preparation was used in deriving estimates of P-450 2A6 levels.

The amount of P-450 2A6 present in human liver microsomes is rather low, and in the liver microsomes (sample HL 115) with the highest level (Fig. 2) the amount was only ~11 pmol/mg of protein, or 1% of the total P-450. With this information, the purification of the enzyme from liver microsomes was ~1500-fold and the yield was ~20% (Table 1). The low level of P-450 2A6 present in human liver microsomes explains why the enzyme appears to make only a minor contribution to certain activities, such as AFB_1 and 6-AC activation (Fig. 8). These results may be compared with those of Lai and Chiang (37); they demonstrated that, although purified hamster P-450 1A2 could catalyze the oxidation of AFB_1 to a genotoxic species, the addition of an antibody (prepared to the enzyme) to the microsomes actually enhanced the activation. It should be emphasized that all work with AFB_1 was done at the relatively low substrate concentration of 1 μM; although different results might be seen at substrate concentrations too low to utilize *in vitro*, the possibility seems unlikely. With the low level of P-450 2A6 present in the microsomes, it is not clear exactly why the rates of oxidation of coumarin and 7-ethoxycoumarin seen with the purified enzyme are not even higher (Table 3). The possibility must be considered that the reconstitution conditions are not completely optimal or that the enzyme has lost activity during purification [the apparent turnover number of P-450 2A6 expressed in vaccinia-infected HepG2 cells was ~12 min^{-1} for coumarin 7-hydroxylation and ~2 min^{-1} for 7-ethoxycoumarin *O*-deethylation (15)]. Nevertheless, the results of antibody inhibition clearly indicate that P-450 2A6 is a major catalyst of these activities in the microsomes. The conclusion regarding coumarin 7-hydroxylation is enhanced by the correlation analysis (Fig. 2). P-450 2A6 appears to make only a minor contribution towards the *N*-oxidation of MOCA, and efforts are in progress to characterize the major enzyme. P-450 2A6 does not appear to make a major contribution with any of the other potential substrates tested. Testosterone 7α-hydroxylation is a characteristic activity of rat P-450 2A1 (10, 38) and testosterone 15α-hydroxylation is characteristic of rat P-450 2A2 (10); both enzymes also form other minor products as well (10). Purified human P-450 2A6, although highly similar in its primary sequence, did not appear to oxidize testosterone at all.

At this point, it is not clear what the major physiological substrate for P-450 2A6 is, or even whether one exists. Indeed, the literature to date does not even indicate that any drugs or carcinogens are important substrates for this enzyme. Coumarin itself is an anticoagulant, mildly toxic, and possibly carcinogenic (39), so it may not be the ideal substrate for use in *in vivo* phenotyping of humans. At this time, there are also no known inhibitors of P-450 2A6 that can be used *in vivo*. Further studies will be required to determine whether human P-450 2A6 has important roles in the metabolism of xenobiotic or endogenous chemicals.

Acknowledgments

We thank Dr. F. F. Kadlubar for providing the arylamine substrates and T. Porter for amino acid sequence analysis.

Note Added in Proof

Very recently another report has appeared describing the isolation of a denatured human liver P-4502A6 preparation by immunoaffinity methods (Maurice, M., S. Emiliani, I. Daletheluche, J. Derancourt, and R. Lange. Isolation and characterization of a cytochrome-P-450 of the IIA subfamily from human liver microsomes. *Eur. J. Biochem.* **200**:511–517 (1991)). The conclusions support the results presented in References 15 and 16 and here. The immunoquantitation was not based on a primary standard bu the recovery of ~1% is consistent with our own estimates of the level of expression.

References

1. Guengerich, F. P. Enzymatic oxidation of xenobiotic chemicals. *Crit. Rev. Biochem. Mol. Biol.* **25**:97–153 (1990).
2. Nebert, D. W., D. R. Nelson, M. J. Coon, R. W. Estabrook, R. Feyereisen, Y. Fujii-Kuriyama, F. J. Gonzalez, F. P. Guengerich, I. C. Gunsalus, E. F. Johnson, J. C. Loper, R. Sato, M. R. Waterman, and D. J. Waxman. The P450 superfamily: update on new sequences, gene mapping, and recommended nomenclature. *DNA Cell Biol.* **10**:1–14 (1991).
3. Gonzalez, F. J. Molecular genetics of the P-450 superfamily. *Pharmacol. Ther.* **45**:1–38 (1990).

4. Guengerich, F. P. Characterization of human microsomal cytochrome P-450 enzymes. *Annu. Rev. Pharmacol. Toxicol.* **29**:241–264 (1989).
5. Ryan, D. E., P. E. Thomas, D. Korzeniowski, and W. Levin. Separation and characterization of highly purified forms of liver microsomal cytochrome P-450 from rats treated with polychlorinated biphenyls, phenobarbital, and 3-methylcholanthrene. *J. Biol. Chem.* **254**:1365–1374 (1979).
6. Guengerich, F. P., G. A. Dannan, S. T. Wright, M. V. Martin, and L. S. Kaminsky. Purification and characterization of liver microsomal cytochromes P-450: electrophoretic, spectral, catalytic, and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or β-naphthoflavone. *Biochemistry* **21**:6019–6030 (1982).
7. Lau, P. P., and H. W. Strobel. Multiple forms of cytochrome P-450 in liver microsomes from β-naphthoflavone-pretreated rats. *J. Biol. Chem.* **257**:5257–5262 (1982).
8. Waxman, D. J., A. Ko, and C. Walsh. Regioselectivity of androgen hydroxylations catalyzed by cytochrome P-450 isozymes purified from phenobarbital-induced rat liver. *J. Biol. Chem.* **258**:11937–11947 (1983).
9. Nagata, K., T. Matsunaga, J. Gillette, H. V. Gelboin, and F. J. Gonzalez. Rat testosterone 7α-hydroxylase: isolation, sequence, and expression of cDNA and its developmental regulation and induction by 3-methylcholanthrene. *J. Biol. Chem.* **262**:2787–2793 (1987).
10. Matsunaga, T., K. Nagata, E. J. Holsztynska, D. P. Lapenson, A. Smith, R. Kato, H. V. Gelboin, D. J. Waxman, and F. J. Gonzalez. Gene conversion and differential regulation in the rat P-450 IIA gene subfamily: purification, catalytic activity, cDNA and deduced amino acid sequence, and regulation of an adult male-specific hepatic testosterone 15α-hydroxylase. *J. Biol. Chem.* **263**:17995–18002 (1988).
11. Aoyama, T., K. Korzekwa, T. Matsunaga, K. Nagata, J. Gillette, H. V. Gelboin, and F. J. Gonzalez. cDNA-directed expression of rat P450s IIA1 and IIA2: catalytic activities toward steroids and xenobiotics and comparison with the enzymes purified from liver. *Drug Metab. Dispos.* **18**:378–382 (1990).
12. Hanioka, N., K. Korzekwa, and F. J. Gonzalez. Sequence requirements for cytochromes P450IIA1 and P450IIA2 catalytic activity: evidence for both specific and non-specific substrate binding interactions through use of chimeric cDNAs and cDNA expression. *Protein Eng.* **3**:571–575 (1990).
13. Phillips, I. R., E. A. Shephard, A. Ashworth, and B. R. Rabin. Isolation and sequence of a human cytochrome P-450 cDNA clone. *Proc. Natl. Acad. Sci. USA* **82**:983–987 (1985).
14. Yamano, S., P. T. Nhamburo, T. Aoyama, U. A. Meyer, T. Inaba, W. Kalow, H. V. Gelboin, O. W. McBride, and F. J. Gonzalez. cDNA cloning and sequence and cDNA-directed expression of human P450 IIB1: identification of a normal and two variant cDNAs derived from the *CYP2B* locus on chromosome 19 and differential expression of the IIB mRNAs in human liver. *Biochemistry* **28**:7340–7348 (1989).
15. Yamano, S., J. Tatsuno, and F. J. Gonzalez. The CYP2A3 gene product catalyzes coumarin 7-hydroxylation in human liver microsomes. *Biochemistry* **29**:1322–1329 (1990).
16. Miles, J. S., A. W. McLaren, L. M. Forrester, M. J. Glancey, M. A. Lang, and C. R. Wolf. Identification of the human liver cytochrome P-450 responsible for coumarin 7-hydroxylase activity. *Biochem. J.* **267**:365–371 (1990).
17. Crespi, C. L., B. W. Penman, J. A. Leakey, M. P. Arlotto, A. Stark, A. Parkinson, T. Turner, D. T. Steimel, K. Rudo, R. L. Davies, and R. Langenbach. Human cytochrome P450IIA3: cDNA sequence, role of the enzyme in the metabolic activation of promutagens, comparison to nitrosamine activation by human cytochrome P450IIE1. *Carcinogenesis (Lond.)* **11**:1293–1300 (1990).
18. Srivastava, P. K., C.-H. Yun, P. H. Beaune, C. Ged, and F. P. Guengerich. Separation of human liver tolbutamine hydroxylase and (*S*)-mephenytoin 4′-hydroxylase cytochrome P-450 enzymes. *Mol. Pharmacol.* **40**:69–79 (1991).
19. Wang, P. P., P. Beaune, L. S. Kaminsky, G. A. Dannan, F. F. Kadlubar, D. Larrey, and F. P. Guengerich. Purification and characterization of six cytochrome P-450 isozymes from human liver microsomes. *Biochemistry* **22**:5375–5383 (1983).
20. Shimada, T., K. S. Misono, and F. P. Guengerich. Human liver microsomal cytochrome P-450 mephenytoin 4-hydroxylase, a prototype of genetic polymorphism in oxidative drug metabolism: purification and characterization of two similar forms involved in the reaction. *J. Biol. Chem.* **261**:909–921 (1986).
21. Kaminsky, L. S., M. J. Fasco, and F. P. Guengerich. Production and application of antibodies to rat liver cytochrome P-450. *Methods Enzymol.* **74**:262–272 (1981).
22. Omura, T., and R. Sato. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239**:2370–2378 (1964).
23. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.)* **227**:680–685 (1970).
24. Wray, W., T. Boulikas, V. P. Wray, and R. Hancock. Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* **118**:197–203 (1981).
25. Guengerich, F. P., P. Wang, and N. K. Davidson. Estimation of isozymes of microsomal cytochrome P-450 in rats, rabbits, and humans using immunochemical staining coupled with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Biochemistry* **21**:1698–1706 (1982).
26. Greenlee, W. F., and A. Poland. An improved assay of 7-ethoxycoumarin *O*-deethylase activity: induction of hepatic enzyme activity in C57BL/6J and DBA/2J mice by phenobarbital, 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *J. Pharmacol. Exp. Ther.* **205**:596–605 (1978).
27. Wood, A. W., D. E. Ryan, P. E. Thomas, and W. Levin. Regio- and stereoselective metabolism of two C19 steroids by five highly purified and reconstituted rat hepatic cytochrome P-450 isozymes. *J. Biol. Chem.* **258**:8839–8847 (1983).
28. Guengerich, F. P. Analysis and characterization of enzymes, in *Principles and Methods of Toxicology* (A. W. Hayes, ed.). Raven Press, New York, 777–814 (1989).
29. Butler, M. A., F. P. Guengerich, and F. F. Kadlubar. Metabolic oxidation of the carcinogens 4-aminobiphenyl and 4,4′-methylene-bis(2-chloroaniline) by human hepatic microsomes and by purified rat hepatic cytochrome P-450 monooxygenases. *Cancer Res.* **49**:25–31 (1989).
30. Shimada, T., M. V. Martin, D. Pruess-Schwartz, L. J. Marnett, and F. P. Guengerich. Roles of individual human cytochrome P-450 enzymes in the bioactivation of benzo(*a*)pyrene, 7,8-dihydroxy-7,8-dihydrobenzo(*a*)pyrene, and other dihydrodiol derivatives of polycyclic aromatic hydrocarbons. *Cancer Res.* **49**:6304–6312 (1989).
31. Waxman, D. J., C. Attisano, F. P. Guengerich, and D. P. Lapenson. Human liver microsomal steroid metabolism: identification of the major microsomal steroid hormone 6β-hydroxylase cytochrome P-450 enzyme. *Arch. Biochem. Biophys.* **263**:424–436 (1988).
32. Yamano, S., T. Aoyama, O. W. McBride, J. P. Hardwick, H. V. Gelboin, and F. J. Gonzalez. Human NADPH-P450 oxidoreductase: complementary DNA cloning, sequence and vaccinia virus-mediated expression and localization of the *CYPOR* gene to chromosome 7. *Mol. Pharmacol.* **36**:83–88 (1989).
33. Nhamburo, P. T., S. Kimura, O. W. McBride, C. A. Kozak, H. V. Gelboin, and F. J. Gonzalez. The human *CYP2F* gene subfamily: identification of a cDNA encoding a new cytochrome P450, cDNA-directed expression, and chromosome mapping. *Biochemistry* **29**:5491–5499 (1990).
34. Shimada, T., M. Iwasaki, M. V. Martin, and F. P. Guengerich. Human liver microsomal cytochrome P-450 enzymes involved in the bioactivation of procarcinogens detected by *umu* gene response in *Salmonella typhimurium* TA1535/pSK1002. *Cancer Res.* **49**:3218–3228 (1989).
35. Shimada, T., and F. P. Guengerich. Evidence for cytochrome $P\text{-}450_{NF}$, the nifedipine oxidase, being the principal enzyme involved in the bioactivation of aflatoxins in human liver. *Proc. Natl. Acad. Sci. USA* **86**:462–465 (1989).
36. Aoyama, T., S. Yamano, P. S. Guzelian, H. V. Gelboin, and F. J. Gonzalez. 5 of 12 forms of vaccinia virus-expressed human hepatic cytochrome-P450 metabolically activate aflatoxin B_1. *Proc. Natl. Acad. Sci. USA* **87**:4790–4793 (1990).
37. Lai, T. S., and J. Y. L. Chiang. Aflatoxin B_1 metabolism by 3-methylcholanthrene-induced hamster hepatic cytochrome P-450s. *J. Biochem. Toxicol.* **5**:147–153 (1990).
38. Levin, W., P. E. Thomas, D. E. Ryan, and A. W. Wood. Isozyme specificity of testosterone 7α-hydroxylation in rat hepatic microsomes: is cytochrome P-450a the sole catalyst? *Arch. Biochem. Biophys.* **258**:630–635 (1987).
39. Egan, D., R. O'Kennedy, E. Moran, D. Cox, E. Prosser, and R. D. Thornes. The pharmacology, metabolism, analysis, and applications of coumarin and coumarin-related compounds. *Drug Metab. Rev.* **22**:503–529 (1990).

Send reprint requests to: F. Peter Guengerich, Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, TN 37232-0146.