Catalytic Activities of Human Liver Cytochrome P-450 IIIA4 Expressed in *Saccharomyces cerevisiae*†

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ABSTRACT: A human liver cytochrome P-450 (P-450) IIIA4 cDNA clone was inserted behind an alcohol dehydrogenase promoter in the plasmid vector pAAH5 and expressed in *Saccharomyces cerevisiae* (D12 and AH22 strains). A cytochrome P-450 with typical spectral properties was expressed at a level of ~8 × 10^5 molecules/cell in either strain of yeast. The expressed P-450 IIIA4 had the same apparent monomeric M_r as the corresponding protein in human liver microsomes (P-450_{NF}) and could be isolated from yeast microsomes. Catalytic activity of the yeast microsomes toward putative P-450 IIIA4 substrates was seen in the reactions supported by cumene hydroperoxide but was often lower and variable when supported by the physiological donor NADPH. The catalytic activity of purified P-450 IIIA4 was also poor in some systems reconstituted with rabbit liver NADPH-P-450 reductase and best when both the detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate and a lipid extract (from liver or yeast microsomes) or L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine were present. Under these conditions the expressed P-450 IIIA4 was an efficient catalyst for nifedipine oxidation, 6β-hydroxylation of testosterone and cortisol, 2-hydroxylation of 17β-estradiol and 17α-ethynylestradiol, N-oxygenation and 3-hydroxylation of quinidine, 16α-hydroxylation of dehydroepiandrosterone 3-sulfate, erythromycin N-demethylation, the 10-hydroxylation of (*R*)-warfarin, the formation of 9,10-dehydrowarfarin from (*S*)-warfarin, and the activation of aflatoxins B_1 and G_1, sterigmatocystin, 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene (both + and − diastereomers), 3,4-dihydroxy-3,4-dihydrobenz[*a*]anthracene, 3,4-dihydroxy-3,4-dihydro-7,12-dimethylbenz[*a*]anthracene, 9,10-dihydroxy-9,10-dihydrobenzo[*b*]fluoranthene, 6-aminochrysene, and tris(2,3-dibromopropyl) phosphate to products genotoxic in a *Salmonella typhimurium* TA1535/pSK1002 system where a chimeric *umuC' 'lacZ* plasmid is responsive to DNA alkylation. Reaction rates were stimulated by 7,8-benzoflavone and inhibited by rabbit anti-P-450 IIIA (anti-P-450_{NF}), troleandomycin, gestodene, and cimetidine. Evidence was obtained that rates of reduction of ferric P-450 IIIA4 in yeast microsomes and the reconstituted systems are slow and at least partially responsible for the lower rates of catalysis seen in these systems (relative to liver microsomes). The results of these studies with a defined protein clearly demonstrate the ability of P-450 IIIA4 to catalyze regio- and stereoselective oxidations with a diverse group of substrates, and this enzyme appears to be one of the most versatile catalysts in the P-450 family.

P-450[1] enzymes catalyze the oxidation of a great variety of endogenous and xenobiotic chemicals [for references and lead articles see Guengerich (1987a), Ortiz de Montellano (1986), and Wislocki et al. (1980)]. The current view is that the chemistry of the reaction of the activated oxygen atom with the substrate is relatively constant in such oxidation reactions (Guengerich & Macdonald, 1984), and the catalytic specificity of the different enzymes is the result of steric forces imposed by the individual proteins upon the substrates (or perhaps more accurately, the appropriate stabilization of diverse transition states). Knowledge of the forces that govern the catalytic specificity of cytochrome P-450 enzymes is imperative for an overall understanding of these proteins and, consequently, the many associated biochemical phenomena in pharmacology, toxicology, endocrinology, and clinical medicine. Many of the human P-450 enzymes have now been characterized, and their roles in the metabolism of individual drugs, steroids, and eicosanoids are of great interest with regard to human health [for reviews see Guengerich (1988b, 1989b)].

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[1] Abbreviations: P-450, microsomal cytochrome P-450;[2] HPLC, high-performance liquid chromatography; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DHEA-SO_4, dehydroepiandrosterone 3-sulfate; BA-3,4-diol, 3,4-dihydroxy-3,4-dihydrobenz[*a*]anthracene; BP-7,8-diol, 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene; DMBA-3,4-diol, 3,4-dihydroxy-3,4-dihydro-7,12-dimethylbenz[*a*]anthracene; BFA-9,10-diol, 9,10-dihydroxy-9,10-dihydrobenzo[*b*]fluoranthene; Tris-BP, tris(2,3-dibromopropyl) phosphate; DLPC, L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine; TAO, troleandomycin; DTT, dithiothreitol.

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In a few cases heterologous expression of single P-450 genes in yeast or mammalian cells, in combination with site-directed mutagenesis, has increased our understanding of the roles of individual residues in the catalytic specificity of individual proteins (Imai, 1988; Kronbach et al., 1989a; Lindberg & Negishi, 1989; Sakaki et al., 1985; Shimuzu et al., 1988). However, our knowledge is far from complete, particularly in cases where closely related genes exist and relationships between DNA sequence and protein catalytic specificity have not been rigorously established, such as with some members of the rabbit P-450 IIC (Imai, 1988) and P-450 IVA (Yokotani et al., 1989) and human P-450 IIC (Brian et al., 1989; Ged et al., 1988) families. Of particular interest is the human P-450 IIIA gene family—a protein in this group appears to be one of the first human P-450s to be purified (Kitada & Kamataki, 1979), and circumstantial evidence exists that the proteins encoded by genes in this family may have a great diversity of important catalytic functions (Guengerich, 1989b, 1990; Guengerich et al., 1986a; Kitada et al., 1987; Shimada & Guengerich, 1989; Shimada et al., 1989a,b; Watkins et al., 1985). However, efforts in this area have been hampered by the unusually low catalytic activities of P-450 IIIA proteins purified from rats (Elshourbagy & Guzelian, 1980; Guengerich et al., 1982; Halpert, 1988), rabbits (Schwab et al., 1988), and humans (Guengerich et al., 1986a; Kitada et al., 1987; Watkins et al., 1985). Much of the evidence that certain biotransformations are catalyzed by these human P-450 IIIA enzymes is based upon studies with liver microsomes, involving immunoinhibition with antibodies that undoubtedly recognize several of the P-450 IIIA proteins (Böcker & Guengerich, 1986; Guengerich, 1988a), and correlation experiments, in which two or more gene products that show coordinate regulation might not be distinguished (Beaune et al., 1986a). Many important steroids, drugs, and carcinogens are in the group of substrates attributed to the human P-450 IIIA enzymes (Guengerich, 1989b), and definition of the catalytic specificity of a major human P-450 IIIA protein is an important goal.

Several lines of evidence suggest that P-450 IIIA4 is a, if not the, major P-450 IIIA protein expressed in adult human liver (Bork et al., 1989). Gonzalez et al. (1988) and Aoyama et al. (1989) have expressed a cDNA in COS and HepG2 mammalian cell systems and shown that the protein (differing only in one residue from the P-450 IIIA4 protein in this study) can catalyze nifedipine oxidation (although the blank rate appeared unusually high), progesterone and testosterone 6β-hydroxylation, and three hydroxylations of the immunosuppressive agent cyclosporin. The latter set of reactions is of interest in that the cyclic peptide cyclosporin A appears to have the highest molecular weight of any known P-450 substrate (Combalbert et al., 1989). Nevertheless, many questions remain about the catalytic specificity of the enzyme. We constructed a plasmid in which the P-450 IIIA4 coding region was placed downstream of an alcohol dehydrogenase promoter and expressed the protein in the yeast *Saccharomyces cerevisiae*. The P-450 IIIA4 protein was partially purified and reconstituted with rabbit liver NADPH–P-450 reductase under conditions where catalytic activity was demonstrated, and the catalytic specificity of this system was demonstrated with 23 different substrates (including two inactivating substrates), with dramatic regio- and stereoselectivity observed in many cases.

Materials and Methods

Chemicals. Testosterone, cortisol, 17β-estradiol, dehydroepiandrosterone 3-sulfate (3β-hydroxy-5-androsten-17-one 3-sulfate), 3β,16α-dihydroxy-5-androsten-17-one, erythromycin, and cimetidine were purchased from Sigma Chemical Co. (St. Louis, MO). The 6β-hydroxy isomers of testosterone and cortisol were purchased from Steraloids (Wilton, NH). All radioactive compounds were purchased from Du Pont–New England Nuclear (Boston, MA), and radiopurity was evaluated by HPLC (>98% purity in all cases). The resolution of racemic warfarin into its enantiomers and the preparation of warfarin metabolites has been described elsewhere (Kaminsky et al., 1981). Gestodene (13-ethyl-17β-hydroxy-18,19-dinor-17α-pregna-4,15-dien-20-yn-3-one) was a gift of Dr. H. Kuhl (University of Frankfurt, FRG). TAO was provided by Dr. D. J. Waxman (Harvard University, Boston, MA). (*ring*-^{3}H)Phenacetin was synthesized as described elsewhere (Larrey et al., 1984). The sources of the procarcinogens are reported in Shimada and Guengerich (1989) and Shimada et al. (1989a,b). Either sources of other chemicals are listed previously or else materials of acceptable quality are routinely available from commercial sources (Guengerich, 1988a; Guengerich et al., 1986a,b).

Microorganisms and Media. Escherichia coli strain MC1060 was maintained on LB media [1% bactopeptone (w/v), 0.5% yeast extract (w/v) (Difco, Detroit, MI), and 1% NaCl (w/v)]. *E. coli* strain Y1088 and *Salmonella typhimurium* strain TA1535, containing the pSK1002 plasmid, were maintained on LB containing ampicillin (50p μg/mL, Sigma). For the *umuC* genotoxicity assays *S. typhimurium* TA1535/pSK1002 was grown in TGA media [1% bactotryptone (Difco) (w/v), 0.5% NaCl (w/v), and 2% glucose (w/v)]. *S. cerevisiae* strain AH22 [a, leu2-3, leu 2-112, his 4-519, can 1, (cir$^+$)] was a gift from Dr. Y. Yabusaki (Sumitomo Chemical Co., Takarazuka Hyogo, Japan). *S. cerevisiae* strain D12 [a, leu2, (cir$^+$)] was a gift from the Genex Corp. (Gaithersburg, MD). Both yeast strains (AH22 and D12) were maintained on YPD media [2% bactopeptone (w/v), 1% yeast extract (w/v), and 2% glucose (w/v)]. Transformed yeast were cultivated on minimal SD media [0.67% yeast nitrogen base without amino acids (w/v, Difco), 2% glucose (w/v), and 20 μg of histidine/mL (w/v)] or SD media supplemented with amino acids (minus leucine) (Sherman et al., 1986).

Expression Vector Construction. A 1.5-kb insert containing almost all of the coding region of NF 25 (Beaune et al., 1986b) was removed from λgt11 by digestion with enzymes *Nco*I and

[2] The P-450 proteins and genes are referred to as recommended by Nebert et al. (1989), with the exception that the designation P-450 IIIA6 is now used to describe the sequence reported by Komori et al. (1989a,b) (λHFL33, λHFL10, P-450HFLa) (Kitada & Kamataki, 1979; Kitada et al., 1985) instead of the rabbit protein (P-450 3c) previously known by this designation (D. W. Nebert, personal communication). P-450 IIIA6 probably corresponds to a protein termed HLp2 by Wrighton and Vandenbranden (1989). The other human P-450 IIIA proteins and sequences are known by several other nonsystematic names, including P-450 IIIA3: P-450HLp (Molowa et al., 1986; Watkins et al., 1985), hPCN2 (Aoyama et al., 1989); P-450 IIIA4: P-450$_{NF}$ (Beaune et al., 1986b; Bork et al., 1989; Guengerich et al., 1986a), hPCN1 and phPCN1 (Aoyama et al., 1989; Gonzalez et al., 1988), P-450 human-1 (Kawano et al., 1987), and possibly others (Combalbert et al., 1989; Shaw et al., 1989), P-450 IIIA5: hPCN3 (Aoyama et al., 1989), and HLp3 (Schuetz et al., 1989; Wrighton et al., 1989). The usefulness of the systematic nomenclature (Nebert et al., 1989) is apparent. In this paper both the protein expressed in human liver and protein expressed in the heterologous yeast expression system are termed "P-450 IIIA4", although the vector is given the trivial name "pAAH5/NF 25" on the basis of the original cDNA clone (Beaune et al., 1986b). The antibodies used here [see Guengerich et al., (1986a) for characterization] undoubtedly recognize all of the proteins in the P-450 IIIA4 family and are referred to as "anti-P-450 IIIA" even though raised against what is probably P-450 IIIA4.

*Nsi*I (New England Biolabs, Beverly, MA). The portion of the agarose electrophoresis gel containing the NF 25 insert was excised, and the DNA was purified by using GeneClean reagent (Bio 101, LaJolla, CA). The 5′ linker was designed to replace the nucleotides from the ATG initiation codon to the *Nco*I restriction site that were missing from the insert after removal from λgt11. Both linkers (5′ and 3′) also contained a *Hin*dIII site to place the insert in the pAAH5 vector (gift of Dr. B. Hall, University of Washington, Seattle). The 5′ sense strand oligomer and the 3′ 16-mer (anti-sense) were phosphorylated by using polynucleotide kinase (New England Biolabs), and then the other oligomers were added and linkers prepared by annealing as previously described (Brian et al., 1989).

The pAAH5 vector was linearized with *Hin*dIII (New England Biolabs) and treated with bacterial alkaline phosphatase (Bethesda Research Labs, Gaithersburg, MD) to prevent recircularization. Following ligation of pAAH5 with the NF 25 insert (containing the linkers), the ligation mixture was used to transform *E. coli* MC1060 cells, made competent by $CaCl_2$ treatment. Cells containing the expression vector were selected by plating on LB media containing ampicillin. Clones were screened for the NF 25 inset by using a ^{32}P-labeled oligomer specific for the NF 25 sequence [oligomer 305 (Bork et al., 1989)]. Vectors isolated from positive colonies were analyzed to find those containing the NF 25 insert in the correct orientation behind the ADH1 promoter using *Bam*HI and *Pst*I restriction analysis. AH22 yeast were transformed with a correct construct of pAAH5 containing the NF 25 insert (pAAH5/NF 25) or pAAH5 alone as described (Brian et al., 1989). The transformed yeasts are referred to as pAAH5 and pAAH5/NF 25 in the text. Yeast clones were screened for P-450 IIIA4 protein expression by spectral analysis and immunoblotting using rabbit polyclonal anti-P-450 IIIA, and the clone giving the highest level of expression was selected for further study.

Antibody and Enzyme Sources. Antibodies raised to purified human P-450 IIIA4 used in this study are described in Guengerich et al. (1986a). Rabbit NADPH–P-450 reductase was purified according to the method of Yasukochi and Masters (1976) as modified elsewhere (Guengerich et al., 1986a). Cytochrome b_5 was prepared from human liver sample HL 91 as described (Shimada et al., 1986).

Microsomal Preparations. Human liver microsomes were prepared from organ transplant donors as described (Wang et al., 1983). The convention "HL" denotes "human liver", and numbers associated with samples were simply assigned in chronological order. Immunoblot analysis of the microsomes used here with anti-P-450 IIIA has been published elsewhere (Guengerich, 1988a). Yeast microsomes were prepared by treating cells with yeast lytic enzyme (ICN Biochemicals, Cleveland, OH), sonicating, and fractionating cells by differential centrifugation (Brian et al., 1989). Microsomal pellets were resuspended in 10 mM Tris-HCl buffer (pH 7.5) containing 0.65 M sorbitol, 0.1 mM dithiothreitol, and 0.1 mM EDTA for assay of enzymatic activity.

P-450 IIIA4 Purification. P-450 IIIA4 ($P\text{-}450_{NF}$) was purified from human liver sample HL 110 microsomes according to Guengerich et al. (1986a). P-450 IIIA4 was partially purified from pAAH5/NF 25 transformed yeast by using a modification of this method. Yeast microsomes were solubilized with sodium cholate (3 g of sodium cholate/g of protein) and applied to an *n*-octylamino-Sepharose 4B column (1.5 × 25 cm) equilibrated with 0.1 M potassium phosphate buffer (pH 7.25) containing 1 mM DTT, 1 mM EDTA, 20% glycerol, 0.6% sodium cholate, and 0.25 mM phenylmethanesulfonyl fluoride. The column was washed with 0.1 M potassium phosphate buffer (pH 7.25) containing 1 mM DTT, 1 mM EDTA, 20% glycerol, and 0.4% sodium cholate, and then the P-450 IIIA4 was eluted with the same buffer containing 0.2% Emulgen 913. Fractions found to contain P-450 IIIA4 by A_{417} measurements were pooled and dialyzed against 5 mM potassium phosphate buffer (pH 7.25) containing 0.1 mM DTT and 20% glycerol. This pooled fraction was loaded onto a 1.6 × 2 cm hydroxylapatite column ("High Resolution", Calbiochem, San Diego, CA) equilibrated with the same buffer. The column was washed with this buffer and then eluted with 0.5 M potassium phosphate buffer (pH 7.25) containing 0.1 mM DTT and 20% glycerol. Fractions found to contain P-450 IIIA4 using A_{417} measurements were pooled. The presence of P-450 IIIA4 was confirmed by immunoblotting analysis; aliquots were saved for determination of enzymatic activity (stored at −80 °C).

Enzyme Assays. The general incubation and HPLC conditions described previously were used to assay the enzymatic oxidation of nifedipine to its pyridine metabolite (Guengerich et al., 1986a), the 2-hydroxylation of 17α-ethynylestradiol (Guengerich, 1988a), the 3-hydroxylation and N-oxygenation of quinidine (Guengerich et al., 1986b), and the oxidation of warfarin (Kaminsky et al., 1981).

Unless noted otherwise, measurements of enzyme activities had the following characteristics in common: incubation mixtures with microsomes isolated from human liver or pAAH5/NF 25 transformed yeast included 100 pmol of total P-450. Reconstituted mixtures including P-450 IIIA4 (purified from pAAH5/NF 25 transformed yeast or human liver microsomes) contained 100 pmol of P-450, either 15 nmol of DLPC or 25 μg of human microsomal lipid extract, 200 pmol of rabbit NADPH–P-450 reductase, and 165 nmol of CHAPS of 0.1 M potassium phosphate buffer (pH 7.4 or 7.7) in a final volume of 0.5 mL. The human lipid extract was prepared by extracting human liver microsomes (sample HL 39) with a mixture of N_2-sparged $CHCl_3/CH_3OH$ (2:1 v/v), drying the organic phase under N_2, and resuspending the lipid in water (purged with N_2) by sonication. These components were mixed in a small volume and incubated at 23 °C for 5 min, and then the other reaction components were added. The complete microsomal and reconstituted reaction mixtures were incubated at 23 °C for 5 min, and then reactions were initiated by addition of an NADPH-generating system (Guengerich et al., 1986a) or cumene hydroperoxide (to 0.1 mM). Reactions were incubated at 37 °C for 10 min, stopped by the addition of 2 volumes of CH_2Cl_2, mixed by using a vortex device, and centrifuged at 3000*g* for 10 min. Aliquots of the organic phases were dried under N_2, and samples were dissolved in CH_3OH and analyzed by HPLC.

Incubation mixtures for testosterone 6β-hydroxylation contained 50 μM testosterone. Product formation was measured by HPLC using a 4.5 × 250 mm Zorbax octyldecylsilyl (C_{18}) reverse-phase column (Mac-Mod, Chadds Ford, PA), eluted with a mixture of CH_3OH/H_2O (64:36) with a flow rate of 2 mL/min, using UV detection of the product at 254 nm. Retention times of 6β-hydroxytestosterone and testosterone were 1.8 and 6.0 min, respectively. A linear gradient of 5–100% CH_3OH showed that 6β-hydroxytestosterone accounted for more than 97% of all products formed in incubations with human liver microsomes; thus, isocratic conditions were used for all assays.

17β-Estradiol 2-hydroxylation was assayed (in 0.25 mL total volume) with 50 μM [6,7-^{3}H]-17β-estradiol (4 mCi/mmol). Product formation was determined by using the HPLC column described for determining testosterone hydroxylation (vide

supra), eluted with an isocratic solvent system consisting of 62% CH_3OH in aqueous 0.05% (w/v) ammonium phosphate buffer (pH 3.0), with a flow rate of 1 mL/min. Column output was directed through a UV monitor (254 nm) and then a Flo-One scintillation counter (Radiomatic, Tampa, FL), where it was mixed with Flo-scint II cocktail (Radiomatic) in a ratio of 1:3 (v/v). Typical retention times of 2-hydroxyestradiol and estradiol were 5.0 and 7.0 min, respectively.

Incubation mixtures for cortisol 6β-hydroxylation contained 0.2 mM [1,2-^{3}H]cortisol (1 mCi/mmol). HPLC conditions were the same as those described for 17β-estradiol, except that ammonium phosphate was omitted from the solvent. 6β-Hydroxycortisol and cortisol typically had retention times of 5.7 and 8.5 min, respectively.

Erythromycin N-demethylation was assayed in a total incubation volume of 0.15 mL containing 0.4 mM [*N-methyl*-^{14}C]erythromycin (1 mCi/mmol). The reaction proceeded for 15 min at 37 °C, and then the aqueous phase was extracted 3 times with 2 volumes of CH_2Cl_2 to remove unreacted erythromycin, as in an assay procedure used for benzphetamine N-demethylation (Guengerich, 1989a). The amount of H^{14}CHO formed was determined by scintillation counting of the aqueous phase.

DHEA-SO_4 16α-hydroxylation was measured by using the conditions of Kitada et al. (1987) except that 25 pmol of testosterone was added as internal standard to each vial prior to derivatization, which was carried out as described (Kitada et al., 1987). Gas chromatography–mass spectrometry was performed on a Hewlett–Packard 5890 gas chromatograph interfaced with a Finnigan INCOS 50 mass spectrometer, and the column was a capillary fused silica SPB1 (10 m, 0.25 mm i.d.). The initial gas chromatograph oven temperature was 190 °C: beginning 1.5 min after injection, the temperature was increased to 300 °C at a rate of 20 °C/min and maintained 1 min at 300 °C. Selected ion monitoring was carried out at m/z 389 (derivatized testosterone) and m/z 446 (derivatized 3β,16α-dihydroxy-5-androsten-17-one). DHEA-SO_4 16α-hydroxylation activities were calculated by reference to a standard curve generated from mass spectrometric analysis of different ratios of derivatized 3β,16α-dihydroxy-5-androsten-17-one (2.5–25 pmol) versus derivatized testosterone (25 pmol).

Procarcinogen activation was measured by using the *umuC* bioactivation assay (Nakamura et al., 1985; Oda et al., 1985; Shimada & Guengerich, 1989; Shimada et al., 1989a,b). Reaction mixtures contained 20 pmol of total P-450 in microsomes from liver sample HL 110 or pAAH5/NF 25 transformed yeast, or purified P-450 IIIA4 (from pAAH5/NF 25 transformed yeast) reconstituted with DLPC.

Phenacetin O-deethylation was assayed as described (Larrey et al., 1984) by using human liver microsomes (sample HL 110) or P-450 IIIA4 (purified from pAAH5/NF 25 transformed yeast) reconstituted with DLPC.

Inhibition Studies. Antibody inhibition of P-450 IIIA4 testosterone 6β-hydroxylase activity was examined in microsomes prepared from human liver microsomal sample HL 110 or pAAH5/NF 25 transformed yeast. Incubation mixtures contained 100 pmol of total P-450 protein in 0.5 mL final reaction volume. Microsomes were incubated with various amounts of immunoglobulin G prepared from polyclonal rabbit P-450 IIIA antiserum for 30 min at 23 °C, and then other components were added. Activity was determined as described above.

TAO and gestodene inhibition of nifedipine oxidation were determined with human liver microsomes (sample HL 110) or with purified P-450 IIIA4 (from pAAH5/NF 25 transformed yeast) reconstituted with NADPH–P-450 reductase, CHAPS, and human lipid extract. Reaction mixtures were prepared as described, using 100 pmol of P-450, with the exception of substrate and the NADPH-generating system. TAO or gestodene (at a final concentration of 20 μM) was added to some samples with or without 0.2 mM NADPH. Mixtures were incubated at 37 °C for 30 min, and then nifedipine and NADPH (0.2 mM) were added and nifedipine oxidation was assayed as described (Guengerich et al., 1986a).

P-450 Reduction Assays. The general methods used for manipulating the samples in anaerobic cuvettes are described elsewhere (Guengerich, 1983). Briefly, the samples were handled in cuvettes fabricated with graded Pyrex seals and matched joints, and alternate cycles of vacuum and scrubbed CO (>10) were used to render the environment anaerobic; a glucose oxidase–catalase system was included to remove residual oxygen. The P-450 IIIA4 in the yeast microsomes appered to be less stable than in the other samples, and the time for total equilibration was reduced from about 80 to 30 min, with the sample kept at 0–4 °C. In all cases mixing of NADPH was performed manually, and spectra of the samples were recorded in either a Hewlett-Packard 8542 or a Cary 210 spectrophotometer.

Other Assays. Sodium dodecyl sulfate electrophoresis and immunoblotting analysis with anti-P-450 IIIA were done as described elsewhere (Brian et al., 1989; Guengerich et al., 1982, 1986a; Laemmli, 1970).

P-450 concentrations in microsomes were estimated by Fe^{2+} versus Fe^{2+}–CO difference spectra, according to the method of Omura and Sato (1964). Spectra in whole yeast cells (approximately 1.5 × 10^9 cells/mL) were obtained as previously described (Brian et al., 1989) according to the procedure of Oeda et al. (1985), using a Cary 219 spectrophotometer in the automatic base-line correction mode.

Protein concentrations were estimated with the bicinchoninic acid (BCA) reagent system (Pierce Chemical Co., Rockford, IL), with bovine serum albumin as a standard.

Results

Plasmid Construction and Yeast Expression. S. cerevisiae strain AH22 was transformed with the pAAH5/NF 25 plasmid construct and transformants were screened by measuring P-450 spectra and by immunoblotting with rabbit polyclonal anti-P-450 IIIA in order to find colonies exhibiting high levels of P-450 IIIA4 expression. Microsomes prepared from yeast grown from this colony showed Fe^{2+} versus Fe^{2+}–CO difference spectra characteristic of P-450, with a large peak at 450 nm, whereas microsomes from yeast containing the plasmid pAAH5 alone showed only low amounts of endogenous yeast P-450 (Figure 1). Immunoblot analysis of microsomes prepared from yeast transformed with pAAH5 or pAAH5/NF 25 showed that only yeast containing the NF 25 clone produced a protein with the same M_r as human liver microsomal P-450 IIIA4 (Figure 2). From the Fe^{2+} versus Fe^{2+}–CO difference spectra of intact cells it was estimated that pAAH5/NF 25 transformed yeast contains approximately 8 × 10^5 molecules/cell.

Enzymatic Activity of P-450 IIIA4 Expressed in Yeast. Characterizing the catalytic specificity of the P-450 enzyme encoded by the NF 25 clone was first attempted with microsomes isolated from pAAH5/NF 25 transformed yeast using an NADPH-regenerating system, as done previously in this laboratory and others (Brian et al., 1989; Oeda et al., 1985; Shimuzu et al., 1988). It was found that nifedipine oxidation

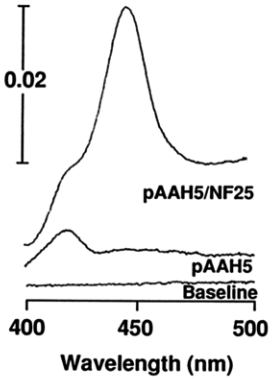

FIGURE 1: Fe^{2+} versus Fe^{2+}–CO difference spectra of microsomes from pAAH5 and pAAH5/NF 25 transformed yeast. Microsomes were prepared as described under Materials and Methods and diluted to a protein concentration of 3 mg/mL in 0.1 M potassium phosphate buffer (pH 7.7) containing 1 mM EDTA, 40% glycerol, 0.4% Emulgen 913, and 0.5% sodium cholate. The following were obtained: a (corrected) base line with microsomes in buffer only (Baseline); Fe^{2+}–CO versus Fe^{2+} spectrum of microsomes from pAAH5-containing yeast (pAAH5); Fe^{2+}–CO versus Fe^{2+} spectrum of microsomes from pAAH5/NF 25 transformed yeast (pAAH5/NF 25). Similar spectra were obtained, indicating P-450 IIIA4 was expressed at 8×10^5 molecules/cell.

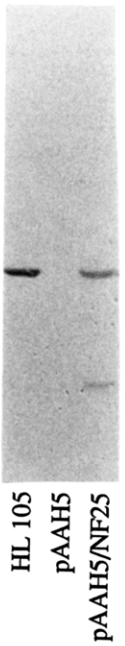

FIGURE 2: Immunochemical blotting of human liver and yeast microsomal proteins with anti-P-450 IIIA4. Electrophoresis of microsomal samples was done in 7.5% (w/v) sodium dodecyl sulfate–polyacrylamide gels (Laemmli, 1970), and nitrocellulose transfers were treated with rabbit polyclonal anti-P-450 IIIA. (Lane 1, HL 105 μS) HL 105 microsomes, 10 pmol of P-450; (lane 2, pAAH5) pAAH5-transformed yeast microsomes; (lane 3, pAAH5/NF 25) pAAH5/NF 25 transformed yeast microsomes, 2 pmol of P-450. Equivalent amounts of microsomal protein were used for the yeast samples.

and testosterone 6β-hydroxylation activities varied considerably among preparations of yeast, ranging from roughly equal to the average human liver microsomal values in some preparations to totally inactive in others (data not shown).

The reasons for the variable enzymatic activities were not apparent, even on closer examination. Spectral analysis of yeast microsomal samples showed that the variation was not due to differences among yeast preparations in the amount of P-450 IIIA4 expressed in the cells or isolated in the microsomes. Immunoblotting with anti-P-450 IIIA4 indicated

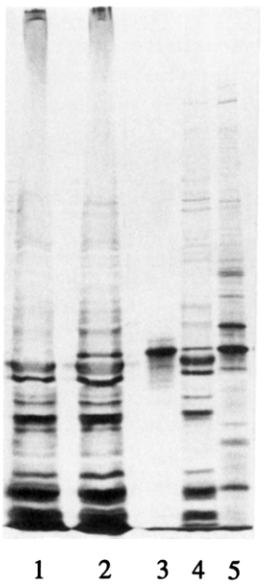

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of human liver and yeast microsomes and partially purified P-450 IIIA4 from yeast. Electrophoresis was done on microsomes from yeast transformed with pAAH5 alone (lane 1, 90 μg of protein), microsomes from pAAH5/NF 25 transformed yeast (lane 2, 90 μg of protein), P-45 IIIA4 purified from human liver microsomal sample HL 105 (lane 3, 0.3 μg of protein), sodium cholate solubilized microsomes from pAAH5/NF 25 transformed yeast (lane 4, 77 μg of protein), and partially purified P-450 IIIA4, pooled and concentrated after hydroxylapatite chromatography (lane 5, 1.1 μg of protein). Proteins were separated by electrophoresis on a 7.5% (w/v) polyacrylamide gel (Laemmli, 1970) and stained with silver (Wray et al., 1981).

that the expressed P-450 is the correct size relative to P-450 IIIA4 in human liver (Figure 2), and there is no evidence of glycosylation by the yeast, which might be expected to yield multiple bands of higher molecular weight [cf. Sanglard and Loper (1989)].[3]

The highest enzymatic activities with expressed P-450 IIIA4 were observed upon partial purification of the enzyme from pAAH5/NF 25 transformed yeast microsomes and reconstitution in the presence of phospholipid and detergent. Fractionation of detergent-solubilized microsomes prepared from pAAH5/NF 25 transformed yeast using *n*-octylamino-Sepharose and hydroxylapatite chromatography resulted in a 23-fold enrichment of P-450 IIIA4, with a 31% recovery (Figure 3). [Complete purification of the enzyme from liver results in poor catalytic activity (Guengerich et al., 1986a) and was not done.] The optimal amounts of reaction components for reconstitution of catalytic activity of the partially purified P-450 IIIA4 were determined by using nifedipine oxidation and testosterone 6β-hydroxylation activities as parameters of enzymatic function, and a wide variety of conditions, including those of Yamazoe et al. (1989), were considered (data not shown). Two sources of lipid were found to work best: DLPC or a $CHCl_3/CH_3OH$ extract of human liver microsomal lipids. The addition of CHAPS reproducibly increased activity, with 0.33 mM CHAPS being optimal (details not shown). The addition of cholate also enhanced the catalytic activity of phospholipid-reconstituted P-450 IIIA4 but not to so great an extent. The addition of cytochrome b_5 to the reconstitution mixture did not further increase either activity (toward nifedipine or testosterone) under these conditions and was not included in the assays (data not shown).

The reason for the low nifedipine oxidation and testosterone hydroxylation activities in pAAH5/NF 25 transformed yeast microsomes was probed further. Cumene hydroperoxide,

Table I: Drug Oxidation by P-450 IIIA4[a]

	nifedipine oxidation	erythromycin N-demethylation	quinidine 3-hydroxylation	quinidine N-oxygenation
microsomes, +NADPH				
HL 110	3.32	2.6	4.95	6.95
HL 107	1.92	0.6	1.45	2.35
HL 102	0.82	0.4	0.55	0.65
yeast (pAAH5/NF 25)	0.22	1.6	0.05	0.15
yeast (pAAH5)	≤0.02	<0.1	<0.005	<0.005
reconstituted system, +NADPH				
P-450 IIIA4, DLPC	0.7	0.7	0.35	0.35
P-450 IIIA4, human lipid extract	2.8	1.1	0.55	0.55
P-450 IIIA4	<0.1	<0.1	0.05	0.15
microsomes, +cumene hydroperoxide				
HL 110	1.4	0.1	1.95	2.05
HL 107	0.8	<0.1	0.35	0.15
HL 102	0.5	<0.1	0.15	0.15
yeast (pAAH5/NF 25)	0.4	1.7	1.35	0.15

[a] Activities expressed as nmol of product formed/(min·nmol of P-450). Mean values are presented from at least 3 determinations, and the variation is <20%. In each case the value measured in the absence of NADPH or cumene hydroperoxide has been subtracted.

rather than NADPH, can be used to oxidize P-450 in vitro, circumventing the need for interaction with NADPH–P-450 reductase in the normal catalytic cycle (Kadlubar et al., 1973; White & Coon, 1980). Microsomes from pAAH5/NF 25 transformed yeast catalyzed nifedipine oxidation and testosterone 6β-hydroxylation in the presence of cumene hydroperoxide (Tables I and II). Although this stability of the P-450 may appear to be surprising, it has also been documented in another instance (Zanger et al., 1988), and the cumene hydroperoxide supported reaction was sensitive to an inhibitory antibody (vide infra). Thus, P-450 IIIA4 itself appears to be structurally intact, and low activities measured in pAAH5/NF 25 transformed microsomes are not due to inhibition of P-450 IIIA4 by an endogenous compound in yeast that blocks substrate binding or oxidation per se.

Rates of P-450 reduction were examined in order to define why overall rates of catalysis were slow in some of the systems used. Most of the P-450 in human liver microsomal sample HL 110[4] was reduced within 10 s after the addition of NADPH (at 25 °C) (Figure 4). However, only a small fraction of the P-450 IIIA4 was rapidly reduced in either (a) a system reconstituted from purified yeast P-450 IIIA4, rabbit liver NADPH–P-450 reductase, human liver lipid extract, and CHAPS under optimal conditions found for nifedipine oxidation and testosterone 6β-hydroxylation or (b) yeast microsomes (Figure 4). In other experiments (results not presented), the presence of a $(FeO_2)^{2+}$ complex could not be detected in either yeast (P-450 IIIA4) or human liver microsomes (sample HL 110) during the steady state of the overall reaction using the protocol of Estabrook et al. (1971) [see also Guengerich et al. (1976)], with 50 μM testosterone present in the cuvettes. Thus, reduction of the $(FeO_2)^{2+}$ complex does not appear to be rate-limiting in either case.

Inhibitors. The effect of polyclonal antibodies raised to P-450 IIIA4 (isolated from human liver) and specific inhibitors of the IIIA family were determined in order to further characterize the P-450 IIIA4 expressed in yeast as a member of the IIIA family. Incubation of microsomes isolated from human liver or yeast transformed with pAAH5/NF 25 with polyclonal anti-P-450 IIIA resulted in a similar inhibition of testosterone 6β-hydroxylation activity (Figure 5). Specific inhibitors of the P-450 IIIA family that require oxidation to exert their effects include the macrolide antibiotic TAO (Watkins et al., 1985; Guengerich, 1988a, 1990) and the acetylenic steroid gestodene (Guengerich, 1990). Preincubation with TAO or gestodene in the presence of NADPH

[3] *S. cerevisiae* AH22 cells were transformed with the pAAH5/NF 25 plasmid on two other occasions in order to determine if the low activities were due to a problem with yeast transformation. Colonies arising from these transformations also had high levels of P-450 expression but low nifedipine oxidase activity. A different strain of *S. cerevisiae*, D12, was transformed with the pAAH5/NF 25 plasmid and produced high amounts of P-450 IIIA4 as judged by immunoblotting results, but microsomes prepared from these yeast had little or no nifedipine oxidation activity. The culture of yeast in different nutritional media can change the intermediary metabolism and might affect the activity of the heterologous P-450 IIIA4. However, yeast were grown in SD medium adjusted to pH 7 with potassium phosphate, SD medium plus amino acid supplements, and YPD medium—the amount of P-450 produced depended on the growth media, but there was no correlation between nifedipine oxidation activity measured in the microsomes and the media used for growth. Low enzymatic activity was also apparently not the result of preparing spheroplasts with yeast lytic enzyme or sonicating spheroplasts, since isolation of microsomes by vortex agitation of intact cells or spheroplasts with glass beads or by precipitation with large amounts of $CaCl_2$ (Cullin & Pompon, 1988) did not yield better activities. Microsomes from pAAH5/NF 25 transformed yeast that contained substantial nifedipine oxidation or testosterone 6β-hydroxylation activity immediately upon preparation rapidly lost catalytic activity upon storage at −20 or −80 °C, regardless of whether stored under N_2 or Ar or resuspended in Tris buffer containing either 0.65 M sorbitol or 20% glycerol. The catalytic activity of the microsomes was not stimulated by the addition of varying amounts of purified NADPH–P-450 reductase or cytochrome b_5, even when any of a variety of ionic or nonionic detergents were first added to disrupt the membrane structures. The amounts of endogenous yeast NADPH–P-450 reductase (assayed as NADPH–cytochrome *c* reductase) and cytochrome b_5 did not particularly vary between several preparations of microsomes from pAAH5 and pAAH5/NF 25 transformed yeast. [Yeast microsomes typically contained ~30 pmol of cytochrome b_5/mg of protein and reduced 40 nmol of cytochrome *c*/(min·mg of protein)—if the specific activity of the yeast reductase is assumed to be similar to those of the mammalian enzymes (Yasukochi & Masters, 1976), the amount of the flavoprotein would be ~10 pmol/mg of protein.] There was also no correlation between amounts of these proteins in pAAH5/NF 25 transformed yeast microsomes and the measured nifedipine oxidase or testosterone 6β-hydroxylase activities, nor did the concentration of endogenous P-450 or P-450 IIIA4 expressed per milligram of protein influence the catalytic activity (expressed on a P-450 basis). Further, mixing experiments provided no evidence for the presence of soluble inhibitors in yeast preparations that were low in catalytic activity.

[4] Human liver sample HL 110 has the highest levels of all of the catalytic activities ascribed to P-450 IIIA4 ever seen in this laboratory. Approximately 60% of the total P-450 (thus ~0.7 nmol of P-450/mg of protein) is thought to be P-450 IIIA4 (or a closely related protein) on the basis of (a) immunoblotting measurements and (b) the observation that 60% of the spectrally detectable P-450 is specifically destroyed during incubation with gestodene (Guengerich, 1990). mRNA blotting studies indicate that only very low levels of P-450 IIIA3 or P-450 IIIA5 can be present in this sample (Bork et al., 1989; Wrighton et al., 1990).

Table II: Steroid Hydroxylation by P-450 IIIA4[a]

	testosterone 6β-hydroxylation	cortisol 6β-hydroxylation	17β-estradiol 2-hydroxylation	17α-ethynylestradiol 2-hydroxylation	DHEA-SO_4 16α-hydroxylation[b]
microsomes, +NADPH					
HL 110	15.8	0.7	3.1	3.5	0.95
HL 107	9.3	0.5	1.6	1.9	0.31
HL 102	3.7	0.3	0.9	2.0	0.52
yeast (pAAH5/NF 25)	8.8	0.2	0.3	0.2	0.10
yeast (pAAH5)	0.1	<0.05	<0.05	<0.01	≤0.05
reconstituted system, +NADPH					
P-450 IIIA4, DLPC	2.2	0.5	2.4	3.0	0.11
P-450 IIIA4, human lipid extract	2.1	0.4	2.0	4.0	0.18
P-450 IIIA4	0.1	0.2	<0.1	0.2	0.03
microsomes, +cumene hydroperoxide					
HL 110	8.9	0.5	0.4	–[c]	0.39
HL 107	2.0	0.3	0.3	–[c]	0.21
HL 102	1.9	0.3	0.1	–[c]	0.45
yeast (pAAH5/NF 25)	4.4	0.2	0.2	–[c]	0.50

[a] Activities expressed as nmol of product formed/(min·nmol of P-450) unless otherwise noted. Mean values are presented from at least 3 determinations, and the variation is <20%. In each case the value measured in the absence of NADPH or cumene hydroperoxide has been subtracted. [b] Activities expressed as pmol of product formed/(min·nmol of P-450). [c] Blank values obtained with cumene hydroperoxide in the absence of P-450 were high, so catalytic activity could not be determined accurately.

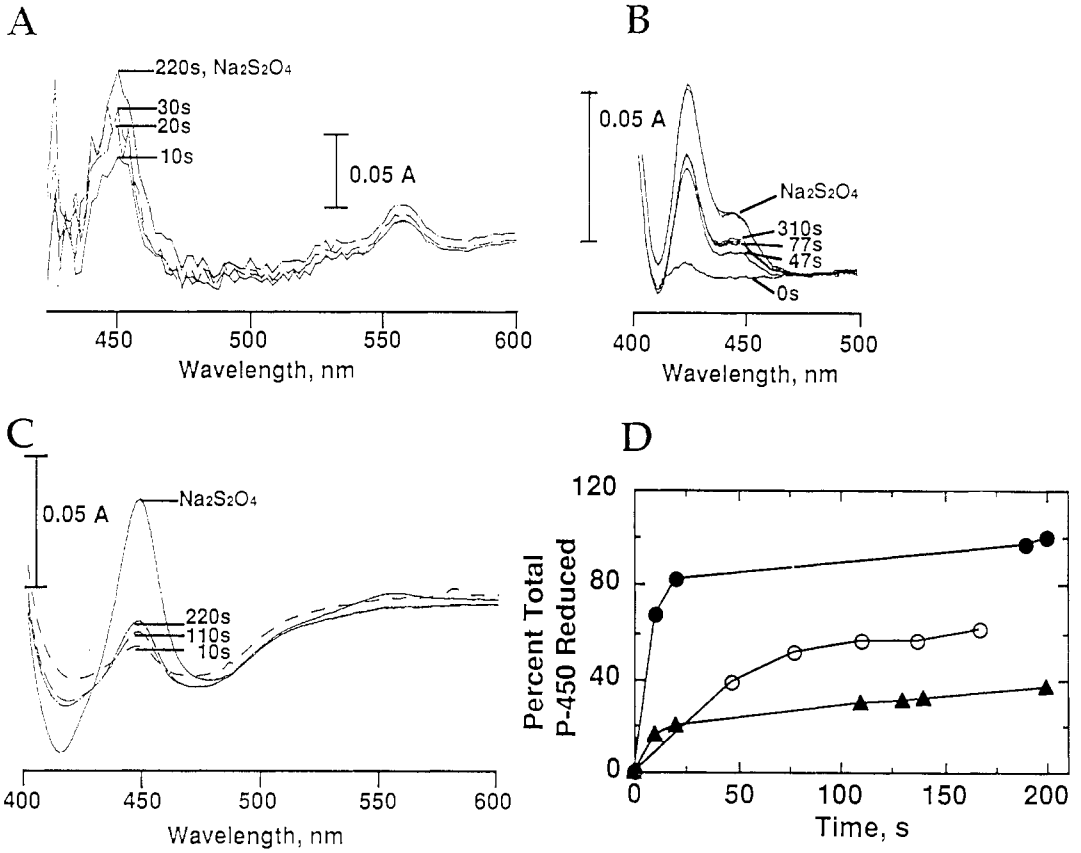

FIGURE 4: Reduction of P-450 by NADPH. Parts A–C show the spectra recorded at various times after the addition of 0.1 mM NADPH in the presence of a CO atmosphere. The final trace (after $Na_2S_2O_4$ addition) is also labeled . (A) Human liver microsomes (sample HL 110), 2.0 μM P-450. (B) pAAH5/NF 25 transformed yeast (P-450 IIIA4) microsomes, 0.42 μM P-450. (C) Mixture of 0.86 μM purified yeast P-450 IIIA4, 2.0 μM rabbit liver NADPH–P-450 reductase, 25 μg of human liver lipid extract/mL, and 0.33 mM CHAPS. All systems contained 0.1 M potassium phosphate, 10 units of (dialyzed) *Aspergillus niger* glucose oxidase/mL, 500 units of (dialyzed) bovine erythrocyte catalase/mL, 0.1 M glucose, and 50 μM testosterone (at 25 °C). In parts A and C spectra were recorded in a Hewlett-Packard 8542 diode-array spectrophotometer (every 10 s; only selected data are shown). In part C spectra were recorded in a Cary 210 spectrophotometer because of light scattering (the dead time was therefore greater); the peak at 424 nm was also seen in experiments devoid of CO and is probably due to cytochrome b_5, not cytochrome P-420. Part D shows the percentage of total P-450 (determined by $Na_2S_2O_4$ addition) reduced at each time point.

decrease nifedipine oxidation activity of the yeast-expressed P-450 IIIA4 in a fashion similar to that in human liver microsomes (Figure 6). In other experiments, cimetidine was found to inhibit the nifedipine oxidase activity of the yeast P-450 IIIA4 enzyme, without the need for NADPH-dependent biotransformation.[5]

Substrate Specificity: Drugs. The P-450 IIIA4 enzyme encoded by the NF 25 cDNA clone had nifedipine oxidase activity when measured in yeast microsomes or when partially

[5] R. G. Knodell, D. Browne, G. P. Gwodz, W. R. Brian, and F. P. Guengerich, submitted for publication.

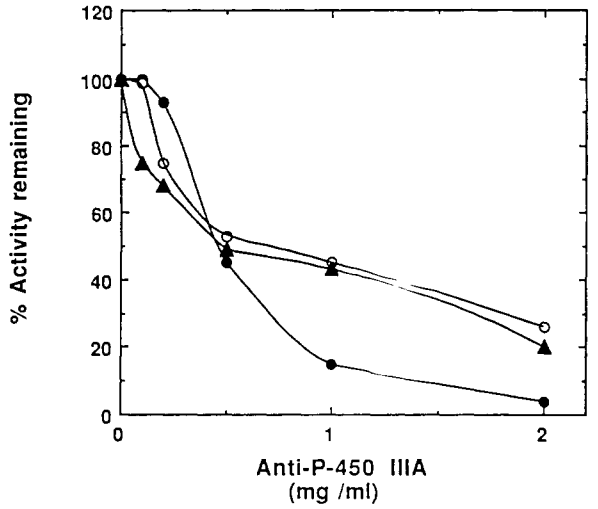

FIGURE 5: Inhibition of testosterone 6β-hydroxylation in microsomes from human liver and pAAH5/NF 25 transformed yeast by anti-P-450 IIIA4. Microsomes from human liver sample HL 110 and yeast transformed with pAAH5/NF 25, containing 0.1 nmol of P-450 in 0.5 mL final volume, were preincubated at 23 °C for 30 min with various amounts of rabbit anti-P-450 IIIA (immunoglobulin G). An NADPH-generating system (Guengerich et al., 1986a) or cumene hydroperoxide (0.1 mM) was used to start the reaction, and the assay was completed as described under Materials and Methods. Liver microsomes with NADPH (●); liver microsomes with cumene hydroperoxide (○); yeast microsomes with cumene hydroperoxide (▲). The (uninhibited) activities of human liver microsomes with NADPH or cumene hydroperoxide were 17 and 8.6 nmol of 6β-hydroxytestosterone formed/(min·nmol of P-450), respectively. Yeast microsomes had an (uninhibited) activity of 4 nmol of 6β-hydroxytestosterone formed/(min·nmol of P-450).

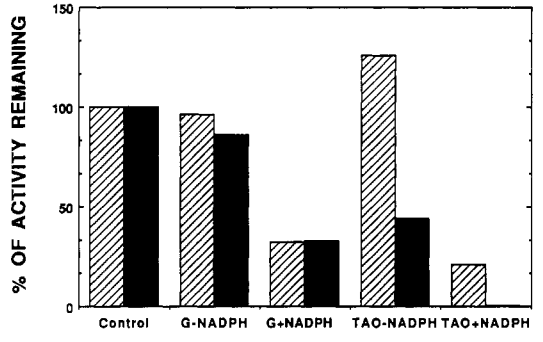

FIGURE 6: Inhibition of nifedipine oxidation by TAO and gestodene. Microsomes from human liver microsomal sample HL 110 or reconstituted yeast P-450 IIIA4 (partially purified enzyme) were used to examine inhibition of nifedipine oxidation by TAO and gestodene. Reaction mixtures, containing 0.1 nmol of P-450, were prepared as described under Materials and Methods without substrate or the NADPH-generating system. TAO or gestodene (G) was added when indicated (20 μM final concentration). All samples were preincubated 30 min at 37 °C with or without 0.2 mM NADPH present, as indicated. Nifedipine (200 μM) and NADPH were then added in all cases, and nifedipine oxidation was assayed. (Control) Reaction mixtures preincubated with NADPH only; (G − NADPH) preincubation with gestodene but not NADPH; (G + NADPH) preincubation with gestodene and NADPH; (TAO − NADPH) preincubation with TAO without NADPH; (TAO + NADPH) preincubation with TAO and NADPH. (Hatched bars) Microsomes from human liver microsomal sample HL 110; (filled bars) P-450 IIIA4.

purified and reconstituted (Table I). Reconstitution of partially purified P-450 with a human liver lipid extract produced a 3- to 4-fold increase in nifedipine oxidation activity compared to reconstitution with DLPC, with catalytic activity approaching the rate measured with the best liver microsomes (sample HL 110).[4]

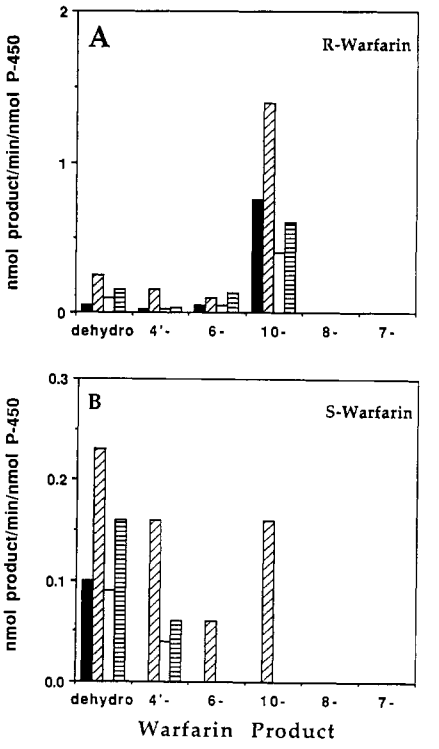

FIGURE 7: Rates of (*R*)- and (*S*)-warfarin oxidation by human liver microsomes or P-450 IIIA4 purified from pAAH5/NF 25 transformed yeast. Reaction mixtures (total volume 0.25 mL) contained 0.9 mM (*R*)- (part A) or (*S*)- (part B) warfarin and 0.25 nmol of total P-450, in human liver microsomal preparations HL 110 (diagonal hatched bars), HL 107 (open bars), or HL 102 (horizontal hatched bars) or yeast P-450 IIIA4 reconstituted with human lipid extract (filled bars). Metabolites measured are dehydrowarfarin (dehydro), 4′-hydroxywarfarin (4′-), 6-hydroxywarfarin (6-), 7-hydroxywarfarin (7-), 8-hydroxywarfarin (8-), and 10-hydroxywarfarin (10-).

Enzymes in the P-450 IIIA family appear to be involved in the metabolism of other drugs, including erythromycin (Watkins et al., 1985), quinidine (Guengerich et al., 1986b), and warfarin (Beaune et al., 1986a; Kaminsky et al., 1984). Microsomes and partially purified P-450 IIIA4 from pAAH5/NF 25 transformed yeast had erythromycin N-demethylation activity (Table I). NADPH-dependent catalytic activity was higher in microsomes than in the reconstituted system. P-450 IIIA4 had also been implicated in the 3-hydroxylation and N-oxygenation of quinidine [Guengerich et al,. 1986b), and the reconstituted P-450 IIIA4 prepared from the yeast catalyzed both of these activities, although rates determined with microsomes from pAAH5/NF 25 transformed yeast were below the limit of detection (Table I). Rates of product formation in the reconstituted enzyme mixture were low compared to those in human liver microsomes, with approximately equal amounts of the 3-alcohol and *N*-oxide formed (in the case of 3-hydroxylation, high activities were measured in the yeast microsomes in the presence of cumene hydroperoxide). Kaminsky et al. (1984) suggested that the P-450 IIIA family is involved in the metabolism of warfarin, catalyzing predominantly the 10-hydroxylation of (*R*)-warfarin. Reconstituted yeast P-450 IIIA4 demonstrated this activity and also catalyzed the 9,10-dehydrogenation of (*S*)-warfarin and the production of other minor metabolites (Figure 7). The human liver microsomes also catalyzed these warfarin oxidation reactions, as well as others.

Substrate Specificity: *Steroids*. Previous reports from this and other laboratories suggest that P-450 IIIA family proteins are the major steroid 6β-hydroxylase enzymes in human liver

Table III: Carcinogen Activation by P-450 IIIA4 and Stimulation by 7,8-Benzoflavone[a]

procarcinogen	substrate concentration (μM)	*umuC* gene expression [units/(min·nmol of P-450)][c] HL 110 microsomes	HL 110 microsomes + 7,8-benzoflavone	P-450 IIIA4[b]	yeast (pAAH5/NF 25) microsomes	yeast (pAAH5/NF 25) microsomes + 7,8-benzoflavone
aflatoxin B_1	10	256	914	57	6	122
aflatoxin G_1	10	189	540	14	7	191
sterigmatocystin	10	384	1042	149	39	124
(±)B(a)P-7,8-diol	10	190	764	6	41	134
(+)B(a)P-7,8-diol	10	156	539	6	19	40
(−)B(a)P-7,8-diol	20	225	931	12	34	99
6-aminochrysene	20	144	310	40	83	102
DMBA-3,4-diol	10	128	364	25		
BA-3,4-diol	20	71	209	5	21	79
BFA-9,10-diol	10	373	733	25	11	48
Tris-BP	20	33	53	<1	3	15

[a] Mean values are presented from duplicate determinations; variation was <15%. [b] Partially purified P-450 IIIA4 from pAAH5/NF 25 transformed yeast, reconstituted with DLPC. [c] Units are as defined by Miller (1972).

(Guengerich et al., 1986a; Komori et al., 1988; Waxman et al., 1988). Aoyama et al. (1989) recently reported that the hPCN1 clone, which encodes a protein with one amino acid different than the NF 25 encoded protein, had 6β-hydroxylation activity toward testosterone, progesterone, and androstenedione when expressed in HepG2 cells. Microsomes and partially purified P-450 IIIA4 from yeast transformed ith pAAH5/NF 25 catalyzed the 6β-hyroxylation of testosterone (Table II). As in the case of erythromycin N-demethylation activity, this activity was higher in the yeast microsomes than in the reconstituted system containing P-450 IIIA4. Of interest is the observation that several microsomal preparations with little or no detectable nifedipine oxidation activity had testosterone hydroxylation activities of ~4 nmol of 6β-hydroxytestosterone formed/(min·nmol of P-450). Other evidence suggests that a protein(s) in the P-450 IIIA family catalyzes cortisol 6β-hydroxylation (Ged et al., 1989). This hypothesis was confirmed by using partially purified P-450 IIIA from pAAH5/NF 25 transformed yeast (Table II). The reaction is of significance in that urinary 6β-hydroxycortisol levels can be readily measured in humans without the need for administration of exogenous cortisol (Ohnhaus & Park, 1979) and may reflect P-450 IIIA4 levels (Ged et al., 1989).

P-450 IIIA4 is thought to be the major human liver P-450 catalyzing 2- and 4-hydroxylation of 17β-estradiol (Guengerich et al., 1986a) and 2-hydroxylation of 17α-ethynylestradiol (Guengerich, 1988a). Partially purified P-450 IIIA4 from pAAH5/NF 25 transformed yeast catalyzed 2-hydroxylation of substrates at relatively high rates (Table II). However, 4-hydroxylation of 17β-estradiol was not detected at the level of sensitivity in these assays.

Kitada et al. (1987) reported that P-450 IIIA6 (P-450HFLa) catalyzed the 16α-hydroxylation of DHEA-SO_4. Partially purified P-450 IIIA4 from pAAH5/NF 25 transformed yeast also demonstrated this activity.

Substrate Specificity: Carcinogens. Recent evidence from this laboratory using a bioactivation assay with *S. typhimurium* TA1535/pSK1002 indicated that the P-450 IIIA family is responsible for the activation of many important procarcinogens (Shimada & Guengerich, 1989; Shimada et al., 1981a,b). P-450 IIIA4 purified from pAAH5/NF 25 transformed yeast was used to examine the role of the enzyme in the activation of these procarcinogens in further detail. The yeast P-450 IIIA4 had activity toward all of the procarcinogens tested (Table III). Activities were somewhat lower with the reconstituted P-450 IIIA4 relative to sample HL 110 human liver microsomes, but the general pattern of activation was consistent among them. The purified P-450 IIIA4 did not activate Tris-BP at a detectable level, although a microsomal preparation did have activity. As in the case of the purified human liver P-450 IIIA4, yeast P-450 IIIA4 was able to activate BA-3,4-diol, although correlation and immunoinhibition studies suggest that other microsomal P-450s must preferentially catalyze this reaction in human liver (Shimada et al., 1989b). No experiments were done with cumene hydroperoxide because of its own effects in the *umuC* gene activation assay.

Stimulation of Catalytic Activity. Certain flavones act as inhibitors of some P-450s but stimulate others (Huang et al., 1981). We have reported that stimulation of the P-450-mediated activation of aflatoxins and BP-7,8-diol to genotoxic products by flavones (Buening et al., 1981) can be attributed to the response of P-450 IIIA4 (Shimada & Guengerich, 1989; Shimada et al., 1989a,b). The P-450 IIIA4 protein was examined in yeast microsomes (Table III). Although the apparent rates of activation of the promutagens were lower than measured in the human liver microsomes (see also Tables I and II), the enzyme responded to the addition of 7,8-benzoflavone in every case. In other work, the response was shown to be concentration-dependent to 25 μM. It should be pointed out, however, that evidence exists that other P-450s may also be stimulated by flavones, although in human liver microsomes P-450 IIIA4 appears to be more responsive than most of the other enzymes (Huang et al., 1981; Shimada et al., 1989a).

Since the yeast-expressed P-450 IIIA4 had activity toward all of the drugs, steroids, and carcinogens chosen, another assay was considered to determine if the enzyme was acting specifically and not as a general oxidative catalyst, perhaps by release of partially reduced oxygen species. Partially purified P-450 IIIA4 was mixed with NADPH–P-450 reductase, DPLC, and CHAPS in the usual manner and assayed for phenacetin O-deethylation activity, which has been characterized as a property of a different human P-450, P-450 IA2 ($P\text{-}450_{PA}$) (Distlerath et al., 1985; Guengerich, 1989b). The yeast P-450 IIIA4 had no activity [<0.005 nmol of acetaminophen formed/(min·nmol of P-450)]—as a control, sample HL 124 human liver microsomes catalyzed phenacetin O-deethylation at a rate of 0.47 nmol of acetaminophen formed/(min·nmol of P-450).

Discussion

The use of yeast-based systems for the heterologous expression of eukaryotic P-450 proteins has a number of ad-

vantages. Among these are the lack of proteolytic activity (relative to prokaryotic expression systems), the capability to prepare relatively large amounts of enzyme, and the presence of endogenous NADPH–P-450 reductase and cytochrome b_5 and endoplasmic reticulum. In the case where the human P-450 IIC10 protein was expressed in yeast in our laboratory, the catalytic activity for tolbutamide methyl hydroxylation was even higher than in human liver microsomes, when expressed on a total P-450 basis (Brian et al, 1989). In the expression of human P-450 IIIA4 in yeast, we experienced a situation in which the catalytic activity measured in the yeast microsomes was rather variable when the system was supported by NADPH in the usual manner. This variation was somewhat unexpected and could not be attributed to variation in the levels of (expressed) P-450 IIIA4, endogenous yeast P-450, NADPH–P-450 reductase, or cytochrome b_5. Further, the rates of both nifedipine oxidation and testosterone 6β-hydroxylation supported by an active oxygen surrogate, cumene hydroperoxide, were relatively constant, at least in fresh yeast microsomal preparations. The activities in the yeast microsomes were often lower than measured in liver microsomes. When the expressed P-450 IIIA4 was partially purified, it exhibited reasonable activity when phospholipid and the detergent CHAPS were both present. These conditions, which were the most optimal of those examined to date, were utilized in the remainder of the assays related to catalytic specificity.

The reason for the lack of what might be expected to be the full catalytic activity was examined. Semiquantitative experiments on the kinetics of reduction indicated that, while the P-450 in human liver microsomes was reduced rapidly enough to support the observed rates of catalytic activity, only a small fraction of the P-450 in the yeast microsomes or even in the system reconstituted with purified P-450 IIIA4 and human liver phospholipids was reduced rapidly (Figure 4). Therefore, it is not possible to make a direct comparison of enzyme turnover numbers on the basis of the total amount of P-450 found in each system (i.e., quantitatively to compare other systems with the human liver microsomes in this case). If only the amounts of rapidly reducible P-450 are considered in the systems, then rates of catalysis in the reconstituted system become higher than found in the microsomes (Figure 4 and Tables I and II).

In many accounts of heterologous expression of P-450 enzymes, comparison is not made with tissue microsomes containing the particular enzyme. Such comparisons are important in establishing the true catalytic specificity of P-450 enzymes, particularly in complex multigene families. In principle, the rates measured in the expression system might be expected to exceed the apparent rates (on a nanomole of P-450 basis) in the liver microsomes, for the latter values are based upon composite concentrations of several proteins. Indeed, we found such comparison to be valid in our earlier studies on tolbutamide methyl hydroxylation by P-450 IIC10 (Brian et al., 1989). In the case of P-450 IIIA4 the comparison is not so readily made. It is of interest to note that Sakaki et al. (1989) found that bovine P-450 XVIIA1 showed only steroid 17α-hydroxylation activity and not $C_{17,20}$-lyase activity when expressed in a yeast system. Hardwick et al. (1987) have also reported that yeast microsomes containing rat P-450 IVA1 did not catalyze 12-hydroxylation of lauric acid unless exogenous NADPH–P-450 reductase and cytochrome b_5 were added. Even when a *Candida tropicalis* (yeast) alkane hydroxylase P-450 was expressed in *S. cerevisiae*, its turnover number was found to be less than in the homologous system (Sanglard & Loper, 1989). Mammalian expression systems have also produced unexpected results. For instance, the relative activities of P-450 XVIIA1 for 17-hydroxylation of progesterone and C_{17-20}-lyase action toward 17-hydroxyprogesterone seem to be variable in both yeast (Sakaki et al., 1989) and mammalian COS (Estabrook et al., 1988) expression systems. Recently Matsunaga et al. (1989) reported that the rate of bufuralol hydroxylation by COS cells expressing rat P-450 IID1 was very low (relative to microsomes), even in the presence of exogenous NADPH–P-450 reductase, and a somewhat higher level of activity was seen when the reaction was supported by cumene hydroperoxide instead of NADPH. Thus, it should be emphasized that heterologous expression systems cannot be trusted as a sole source of information concerning catalytic specificity and should be used in combination with other biochemical approaches.

Despite the difficulty in precisely quantifying the optimal rates of certain catalytic activities, the point should be emphasized that the expressed P-450 IIIA4 protein showed activity with all of the compounds examined which had previously been suspected as substrates (Guengerich et al., 1986a,b; Kitada et al., 1987; Komori et al, 1988; Shimada & Guengerich, 1989; Shimada et al., 1989a,b; Waxman et al., 1988; Watkins et al., 1985). The structures of these substrates are quite diverse, and the point should be emphasized that the regioselectivity and stereoselectivity of oxidation are quite dramatic with most of these compounds (Figure 7). The precise nature of the active site is of considerable interest, in that so many reactions are accommodated. The nature of the amino acid residues directly involved in catalysis is unknown at this time. It is of interest that at least some of the oxidation reactions are also catalyzed by other P-450 IIIA proteins (Aoyama et al., 1989; Kitada et al., 1987; Wrighton & Vandenbranden, 1989; Wrighton et al., 1989). Aoyama et al. (1989) compared some catalytic activities performed by the P-450 IIIA4 and P-450 IIIA5 proteins—both were active toward some substrates, but P-450 IIIA5 was always less active and, unlike P-450 IIIA4, only hydroxylated cyclosporin at one position and did not catalyze the 6β-hydroxylation of testosterone, androstenedione, or progesterone at rates >25% those measured with P-450 IIIA4. We have examined many of the catalytic activities of human liver P-450 IIIA5, provided by Dr. S. A. Wrighton (Eli Lilly Co., Indianapolis, IN), in the course of the work with the P-450 IIIA4 expressed in yeast—P-450 IIIA5 catalyzed nifedipine oxidation, testosterone 6β-hydroxylation, 17β-estradiol 2-hydroxylation, DHEA-SO_4 16α-hydroxylation, and cortisol 6β-hydroxylation at rates generally less than those of P-450 IIIA4 and was essentially inactive in 17α-ethynylestradiol 2-hydroxylation, erythromycin N-demethylation, and quinidine 3-hydroxylation and N-oxygenation (Wrighton et al., 1990). It is interesting to speculate how the small number of amino acid substitutions in P-450 IIIA5 (84% amino acid sequence identity with P-450 IIIA4) restricts the catalytic activity toward some, but not all, of the P-450 IIIA4 substrates. The substitutions do not appear to be particularly clustered. The diversity of catalytic function of P-450 IIIA4 is intriguing. In a sense, P-450 IIIA4 may be considered the most versatile of the P-450 enzymes. The phenobarbital-inducible rat P-450 IIB1 and rabbit P-450 IIB4 proteins accommodate and oxidize many small substrates (Guengerich, 1987b), although the hydroxylations that are catalyzed tend to be of a nature driven more by strictly chemical principles, and the range of large molecules that can serve as substrates is probably not so great as in the case of P-450 IIIA4.

The available evidence suggests that P-450 IIIA4 may be the major P-450 IIIA gene expressed in adult human liver. Hybridization of separated genomic DNA with nonoverlapping

cDNA probes indicates three common fragments, suggesting the presence of at least three genes or genelike sequences (Beaune et al., 1986b). The number of genes in the family is unknown, since no genomic clones have been characterized. The total amount of hybridizable DNA corresponds to 50–60 kb (Beaune et al., 1986b). At this time four or five different cDNAs in the human P-450 IIIA family have been reported (Aoyama et al., 1989; Beaune et al., 1986b; Bork et al., 1989; Komori et al., 1989a,b; Molowa et al., 1986; Schuetz et al., 1989). cDNA clones NF 25 and NF 10 were isolated from the same single-liver library, but NF 10 differs only in the absence of a nucleotide triplet. Analysis of mRNA with specific oligonucleotide probes indicates that NF 10, if it really does represent a distinct gene, is not expressed at an appreciable level (Bork et al., 1989). The cDNA sequences of P-450 IIIA3 and P-450 IIIA4 differ in 66 nucleotides, and all but one of the 16 differences in the protein-coding portion are found in the 5′ halves of the sequences (Molowa et al., 1986). The cDNA clone used in the original sequencing of P-450 IIIA3 was from the same library (individual human liver) as the NF 25 P-450 IIIA4 clone, so P-450 IIIA3 should not be an allelic variant (Beaune et al., 1986b; Molowa et al., 1986). mRNA analysis using selective oligonucleotide hybridization also indicates that P-450 IIIA3 is not expressed at a very high level in any of 12 human liver samples examined (Bork et al., 1989). The P-450 IIIA5 protein is electrophoretically distinguishable from P-450 IIIA4, and Wrighton et al. (1989, 1990) have reported that only about 20% of the adult population express this protein and, when so, at a level about 25% that of the P-450 IIIA4 protein. Komori et al. (1990) have also used specific oligonucleotide hybridization analysis to determine that human fetal liver expresses P-450 IIIA6 and not P-450 IIIA4 and that adult liver generally expresses P-450 IIIA4 but not P-450 IIIA6. Thus, at this time it would appear that P-450 IIIA4 may be the principal P-450 IIIA protein expressed in adult human liver, and the observation of catalytic activity toward all of the substrates previously assigned to P-450 IIIA proteins by the yeast-expressed P-450 IIIA4 protein expressed in this study is consistent with this view.[6]

Although the list of reactions catalyzed by the expressed P-450 IIIA4 is extensive (Tables I–III), it is not complete. The mechanism-based inactivation of P-450 IIIA4 by gestodene would suggest that the substituted acetylenic carbon is attacked by the activated oxygen species, since the heme is readily destroyed and protein alkylation is not extensive (Guengerich, 1990; Komives & Ortiz de Montellano, 1987). Renaud et al. (1990) demonstrated the role of P-450 IIIA4 in the oxidation of the amine nitrogen in troleandomycin. Aoyama et al. (1989) reported that HepG2 cell microsomes containing an expressed P-450 IIIA4 protein (differing from the NF 25 product only in a V392W substitution) also catalyzed 6β-hydroxylation of androstenedione and progesterone and the hydroxylation of cyclosporin at three different positions. In another report the same system was used to catalyze both 1′- and 4-hydroxylation of midazolam (Kronbach et al., 1989b). Other reactions are suggested by the literature. For instance, immunoinhibition experiments with human liver microsomes suggest that at least 16 different 1,4-dihydropyridines are oxidized by P-450 IIIA4 or a closely related enzyme (Böcker & Guengerich, 1986; Komori et al., 1988). P-450 IIIA4 or a closely related enzyme also appears to be involved in *d*-benzphetamine N-demethylation (Ged et al., 1989; Guengerich et al., 1986a). Work with microsomes indicates a potential role of P-450 IIIA 4 in the reduction of 1,6-dinitropyrene (probably reduction of one nitro group to an amine), a process that destroys the genotoxic activity of the compound toward bacteria (Shimada & Guengerich, 1990). Komori et al. (1988) also reported a rate of 16α-hydroxylation of progesterone that was considerbly lower than that of 6β-hydroxylation. Imaoka et al. (1990) have also obtained evidence that P-450 IIIA4 or a related protein is involved in the N-deethylation of the anaesthetic lidocaine. Finally, P-450 IIIA4 has also been implicated in the hydroxylation of lovastatin (Mevacor), a 3-hydroxymethylglutaryl-CoA reductase inhibitor used to lower cholesterol levels.[7]

The role of P-450 IIIA4 in the oxidation of the drugs, carcinogens, and steroids mentioned above should indicate that this enzyme can be important in matters related to human health. Levels of the enzyme vary considerably (>40-fold) among people (Bork et al., 1989; Guengerich, 1988a; Kleinbloesem et al., 1984; Schellens et al., 1988; Watkins et al., 1985), and both in vivo and in vitro evidence is available to support the view that the protein may be induced by barbiturates, antibiotics, and dexamethasone (Shaw et al., 1989; Watkins et al., 1985; Morel et al., 1990). The enzyme is involved in the bioactivation of aflatoxin B_1, and the hypothesis can be considered that elevated levels of the enzyme may predispose individuals to chemically induced cancer (Guengerich, 1988b; Shimada & Guengerich, 1989; Shimada et al., 1989a). Elevated levels of P-450 IIIA4 in women who ingest rifampicin or barbiturates can increase the rate of elimination of 17α-ethynylestradiol and render the contraceptive ineffective (Bolt et al., 1973; Guengerich, 1988a). On the other hand, mechanism-based inactivation of P-450 IIIA4 by gestodene can increase the levels of 17β-estradiol, 17α-ethynylestradiol, and cortisol (Guengerich, 1990; Jung-Hoffmann & Kuhl, 1989) and may lead to altered physiological effects. Thus, understanding the range of reactions catalyzed by P-450 IIIA4 is a challenging problem that has practical applications in medicine as well as basic implications about the extent of catalytic specificity in an enzyme.

During the course of this work, the original NF 25 cDNA clone was also expressed in a different yeast strain by another

[6] The possibility that other genes might exist in this family and that the corresponding proteins may contribute to the overall catalytic activity seen toward some of these substrates in microsomes cannot be dismissed. The similarity of the sequences of the genes in this family also makes analysis of expressed by selective hydridization difficult in that the analayses must be done between individual pairs of sequences. However, the suggestion of Schuetz et al. (1989) that the results of the oligonucleotide hybridization work of Bork et al. (1989) can be attributed to the binding of specific probes to P-450 IIIA5 instead of P-450 IIIA4 is unfounded, since Wrighton et al. (1989) showed that the level of expression of P-450 IIIA5 is usually considerably lower (than that of P-450 IIIA4) in the livers in which it is found—further, several of the samples used in this work (e.g., HL 110) were very high in erythromycin N-demethylation, 17α-ethynylestradiol 2-hydroxylation, and quinidine 3-hydroxylation and N-oxygenation activities (Guengerich, 1988a; Guengerich et al., 1986b), which were not catalyzed by the purified P-450 IIIA5 protein purified by Wrighton et al. (1989, 1990). The conclusion that several of the livers do not contain the P-450 IIIA5 protein has been extended by using a (cross-adsorbed) antibody that recognizes P-450 IIIA5 but not P-450 IIIA3 or P-450 IIIA4 (Wrighton et al., 1990). References (Schuetz et al., 1989) to individual figures of the work of Bork et al. (1989) are incorrect, and the conclusion that the P-450 IIIA3 sequence is expressed at a much lower level (<10%) than some other P-450 IIIA mRNA, if not P-450 IIIA4, is clearly supported by studies on 12 adult liver samples with two different oligonucleotide probes optimized for maximum mismatch (Bork et. al., 1989). A similar conclusion was reached by Kolars et al. (1990). However, it is unknown if these differences in mRNA levels also occur at the levels of the proteins themselves, for antibodies that discriminate between P-450 IIIA3 and P-450 IIIA4 have not been developed.

[7] A. Y. H. Lu, R. Wang, W. R. Brian, and F. P. Guengerich, to be submitted for publication.

group, and the studies are reported elsewhere (Renaud et al., 1990). The level of expression and the general conclusions regarding the substrates tested there (nifedipine, quinidine, and TAO) are similar to those reported here.

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Registry No. DHEA-SO_4, 651-48-9; B(a)P-7,8-diol, 13345-25-0; DMBA-3,4-diol, 72617-60-8; BA-3,4-diol, 60839-18-1; BFA-9,10-diol, 130221-15-7; Tris-BP, 126-72-7; TAO, 2751-09-9; P-450, 9035-51-2; monooxygenase, 9038-14-6; nifedipine, 21829-25-4; erythromycin, 114-07-8; quinidine, 56-54-2; testosterone, 58-22-0; cortisol, 50-23-7; 17β-estradiol, 50-28-2; 17α-ethynylestradiol, 57-63-6; aflatoxin B_1, 1162-65-8; aflatoxin G_1, 1165-39-5; sterigmatocystin, 10048-13-2; 6-aminochrysene, 2642-98-0; 7,8-benzoflavone, 604-59-1; gestodene, 60282-87-3; (*S*)-warfarin, 5543-57-7; (*R*)-warfarin, 5543-58-8; cimctidine, 51481-61-9.

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