Cloning and Sequence Determination of a Complementary DNA Related to Human Liver Microsomal Cytochrome P-450 S-Mephenytoin 4-Hydroxylase†

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ABSTRACT: A cDNA sequence related to the human cytochrome P-450 responsible for S-mephenytoin 4-hydroxylation (P-450$_{MP}$) has been isolated from a human liver bacteriophage λgt11 library with antibodies specific for P-450$_{MP}$. The total length of the cDNA is 2.5 kilobases (kb), of which there is a 1.6-kb *Eco*RI fragment coding for all but five amino acids corresponding to the N-terminus of the protein and including a small noncoding region at the 3′ end. This 1.6-kb fragment has been sequenced and used as a probe to analyze human genomic DNA and liver RNA. The sequence shows extensive sequence similarity with that of rabbit liver cytochrome P-450 progesterone 21-hydroxylase [Tukey, R. H., Okino, S., Barnes, H., Griffin, K. J., & Johnson, E. F. (1985) *J. Biol. Chem. 260*, 13347–13354], and this cDNA, like the rabbit clone, appears to be part of a multigene family. At least two liver mRNA species, 2.2 kb and 3.5 kb, hybridize to the cDNA sequence. The cloning of this gene should aid in analyzing the molecular basis for the genetic polymorphism of S-mephenytoin 4-hydroxylation reported in humans.

Cytochrome P-450[1] enzymes are responsible for the monooxygenation of a wide variety of compounds, including endogenous substrates as well as xenobiotics. In humans as well as animals, there is ample evidence to support the existence of multiple forms of P-450, each with somewhat unique biochemical properties and substrate specificities. Recently, differences in P-450 have been implicated in part in the wide interindividual variation of xenobiotic metabolism in humans. In addition to differences due to induction of P-450s, humans also exhibit polymorphic variation in the activities of certain of the P-450 forms toward the oxidation of specific drugs [for review, see Küpfer and Preisig (1983)]. Although the precise bi chemical basis for these polymorphisms is not known, several have a genetic basis.

The enzyme responsible for 4-hydroxylation of the anticonvulsive drug mephenytoin exhibits genetic polymorphism (Küpfer & Preisig, 1984; Wedlund et al., 1984). About 5–10% of Caucasian populations show a slow metabolizer phenotype. We have previously purified and characterized the enzyme responsible for mephenytoin 4-hydroxylation, termed P-450$_{MP}$ (Shimada et al., 1986). The enzyme actually exists in at least two similar forms, with almost identical catalytic and immunological properties, and in vitro translation studies suggest that they are coded by two separate messages. We now report the cloning of the DNA coding sequence related to these forms as a means of studying the regulation of this enzyme and its relationship to the "poor metabolizer" phenotype.[2]

Experimental Procedures

Screening of Bacteriophage λgt11 Library. A human liver cDNA library constructed in λgt11 (a generous gift of Drs. George Ricca and William Drohan, Meloy Laboratories, Inc., Springfield, VA) was screened for fusion proteins according to the general method described by Young and Davis (1983) using nitrocellulose, 0.1-μm pore size (Millipore, Milford, MA), with antibody against P-450$_{MP}$ (Wang et al., 1983; Shimada et al., 1986). These antibodies had been shown previously to specifically recognize P-450$_{MP}$ and to inhibit mephenytoin 4-hydroxylase activity (Shimada et al., 1986). The antiserum was adsorbed with *Escherichia coli* lysate bound to Sepharose 4B (Pharmacia, Piscataway, NJ) to decrease the screening background. Clones that were positive with anti-P-450$_{MP}$ were isolated from contaminating phage by several cycles of plating and rescreening. The immunospecificity of each clone was verified by mixing and diluting purified phage with phage known not to cross-react with anti-P-450$_{MP}$ (λgt11 and human serum albumin in λgt11) to assure that the signal was not due to cross-reactivity of the antibody with *E. coli* proteins. Phage λ DNA was prepared (Maniatis et al., 1982) and analyzed on agarose gels after digestion with restriction endonucleases (New England Biolabs, Beverly, MA). The clone containing the largest cDNA insert (2.5 kb) (designated MP-8) was further characterized.

Subcloning into M13mp9. A 1.6-kb *Eco*RI fragment from λgt11 MP-8 containing the coding sequence for P-450$_{MP}$ was subcloned into M13mp9 (New England Biolabs, Beverly, MA) according to Messing (1983) and propagated in *E. coli* UT 481. An asymmetrically positioned *Sac*I site in the cDNA (Figure 1) was used to orient the insert in the M13 phage.

DNA Sequencing. DNA sequencing was carried out with a ^{32}P sequencing kit from New England Nuclear (Boston, MA) according to their protocol, with minor modifications. A higher dideoxynucleotide concentration was used to obtain less ambiguous short sequences, and reactions were carried out at 37

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[1] Abbreviations: P-450, cytochrome P-450; P-450$_{MP}$, cytochrome P-450 S-mephenytoin 4-hydroxylase; 1× SSC, saline sodium citrate buffer [15 mM sodium citrate buffer (pH 7.0) containing 150 mM NaCl]; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

[2] This work has been presented in abstract form (Umbenhauer et al., 1986).

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°C in the absence of NaCl. Radioactive DNA fragments were separated on a 6% polyacrylamide gel (36 × 92 cm) with a buffer gradient and autoradiographed directly. After a sequence of 300–350 bases was read, an appropriate synthetic primer (20-mer) was made (Biosearch, San Rafael, CA) and the sequencing continued from that primer (Figure 1). Overlapping and complementary sequences were read for verification, and each primer was used at least 4 times.

Preparation of Genomic DNA and Total RNA. Genomic DNA and total RNA were isolated simultaneously from the same preparations by the CsCl cushion method described by Chirgwin et al. (1979). The RNA was collected as a pellet on the bottom of the centrifuge tube, dissolved in water, and precipitated with 0.1 volume of 2.5 M sodium acetate and 2 volumes of ethanol. The DNA was collected as a viscous solution at the interface between the CsCl and the homogenate. This solution was extracted once with a mixture of phenol and $CHCl_3$ (1:1) and once with $CHCl_3$ and then precipitated with ethanol. The DNA was dissolved in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, treated with proteinase K (50 μg/mL) in 0.1% (w/v) sodium dodecyl sulfate, and reextracted with phenol/$CHCl_3$ and $CHCl_3$ as above. The purified DNA was then precipitated with ethanol.

Nick Translation of cDNA and Transfer Blot Analyses of Genomic DNA and RNA. The 1.6-kb *Eco*RI fragment was purified from agarose by a phenol-freeze extraction (Benson, 1984). A 1.5-kb *Eco*RI–*Pvu*II fragment containing only coding sequences was also purified in the same way. These fragments were then nick translated by using a ^{32}P-CTP nick translation kit from New England Nuclear to a specific activity of approximately 10^8 cpm/μg of DNA.

Total RNA was electrophoresed through 1% formaldehyde–agarose gels. The RNA was transferred by capillary action to Gene Screen Plus according to the instructions from New England Nuclear for "Northern" blotting (Alwine et al., 1977). Hybridizations at 42 °C were carried out in the presence of dextran sulfate and 50% formamide. Membranes hybridized with the nick-translated probe were washed twice for 30 min at 65 °C in 2× SSC (with 0.1% sodium dodecyl sulfate, w/v) followed by a room temperature wash in 0.1× SSC (30 min). An oligonucleotide (50-mer) complementary to the 5′ end of human serum albumin mRNA (Dugaliczyk et al., 1982) was synthesized and used as an internal standard for quantity of intact mRNA loaded. Membranes hybridized with the 50-mer were washed at 50 °C in 2× SSC instead of at 65 °C. Human genomic DNA samples were digested with endonucleases, electrophoresed through 0.8% agarose gels, and transferred by capillary action to Gene Screen Plus according to the protocol from the manufacturer and Southern (1975). Hybridizations were done as for RNA blots.

Results and Discussion

DNA Cloning and Sequencing. Initial screening of the bacteriophage λgt11 library with anti-P-450$_{MP}$ yielded 20 immunopositive clones that were isolated and purified (out of a total of 4 × 10^5 screened). The clone with the largest cDNA insert, 2.5 kb, was then further characterized (Figure 1). Since the cDNA was inserted into the λ phage by the endonuclease *Eco*RI, *Eco*RI sites should have been present at both ends of the cDNA. However, the *Eco*RI site at the 3′ end of the gene was lost, apparently in the initial construction of the library. An *Eco*RI site in the cDNA was found approximately 1.6 kb from the 5′ end. Knowing that the apparent monomeric M_r of the protein is 48 000–50 000 (Shimada et al., 1986), we reasoned that the 1.6-kb *Eco*RI fragment could code for the entire protein sequence and would be the most interesting part

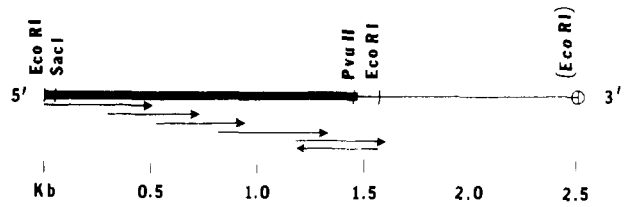

FIGURE 1: Partial restriction map of clone MP-8 isolated from the λgt11 cDNA library. The restriction sites relevant to subcloning into M13 and construction of nick-translated probes are shown. The solid bar indicates the region coding the protein. The arrows indicate the segments and direction of sequences read from the synthetic primers.

of the cDNA to study. This 1.6-kb fragment was therefore subcloned into M13mp9 in order to facilitate isolation of cDNA and sequencing of the gene.

The DNA sequencing was carried out according to the dideoxy termination method (Sanger et al., 1977) by first reading from a general primer for M13 and then by "walking" along the gene with synthetic primers designed from the determined sequence. The positions of the primers and the representative sequences read are shown by the arrows in Figure 1.

The sequence determined by this method is shown in Figure 2. The first 1458 nucleotides comprise an open reading frame ending with a TGA termination codon, followed by 110 nucleotides of 3′-noncoding region. The remaining 900 nucleotides of the cDNA that were not subcloned into M13 must therefore also be part of the 3′-noncoding region. The cDNA does not contain an ATG initiation codon or any 5′-untranslated sequences.

Comparison between Purified P-450$_{MP}$ and the cDNA Sequence. The length of the cDNA coding sequence (486 amino acids) is very similar to that expected for a 50 000-dalton protein (the calculated M_r of the apoprotein is 55 015). Although the cDNA is missing the coding sequence for the first five amino acids, the deduced protein sequence is identical with the determined sequence of the protein at all comparable unambiguous positions through the first 29 amino acids (Table I). Positions 10 and 13 in the protein analysis are ambiguous: traces of tyrosine and tryptophan were found at the respective positions; carboxymethylation of denatured and reduced P-450$_{MP}$ with 2-iodo[^{14}C]acetic acid and Edman degradation did not provide evidence for the presence of cysteine. Treatment of native or denatured P-450$_{MP}$ with carboxypeptidase A, B, or Y did not release a residue, even after 6 h. If the protein sequence shown in Figure 2 corresponds exactly to P-450$_{MP}$, the proline would have been expected to block release of the C-terminus.

The nucleotide sequence of the cDNA shares approximately 80% similarity with that of rabbit liver progesterone 21-hydroxylase (P-450 1) (Tukey et al., 1985) and with two other rabbit cDNA sequences for which proteins have not been identified, cytochrome P-450 I and cytochrome P-450 II (Leighton et al., 1984). All reported N-terminal human P-450 sequences are compared in Table I, and rabbit liver P-450 I is included. These related rabbit sequences were found with the aid of computer access to the National Biomedical Research Foundation data bank (Georgetown University, Washington, DC). This similarity exists at both the nucleotide level and amino acid level and suggests that we have maintained the correct reading frame throughout the entire sequence. At positions 810–818, there is a net addition of three codons in the human P-450$_{MP}$ sequence as compared to the rabbit progesterone 21-hydroxylase, but the similarity is maintained after that insertion. Much less structural similarity

Table I: Comparison of Determined N-Terminal Sequence of P-450$_{MP}$ with Deduced Amino Acid Sequence of cDNA Clone (MP-8) and Other P-450s[a]

	1				5				10				15				20				25				29
cDNA						V	L	V	L	C	L	S	C	L	L	L	L	S	L	W	R	Q	S	S	G	R	G	K	L
Human P-450 MP-1	M	D	S	L	V	V	L	V	L	X	L	S	X	L	L	L	L	S	L	W	R	Q	S	S	G				
Human P-450 MP-2	M	D	S	L	V	V	L	V	L	X	L	S	X	L	L	L	L	S	L	W	R	Q	S	S	G	X	G	X	L
Rabbit P-450 1	M	D	P	V	V	V	L	V	L	G	L	C	C	L	L	L	L	S	I	W	K	Q	N	S	G	R			
Human P-450$_{NF}$	M	A	L	I	P	D	L	A	M	E	T	W	L	L	L	A	V	S	L	V	L								
Human P$_1$-450	M	L	F	P	I	S	M	S	A		T	E	F	L	L	A	S	V	I	F	C								
Human P-450d	M	A	L	S	Q	S	V	P	F	S	A	T	E	L	L	L	A	S	A										

[a] Sequences of P-450$_{MP-1}$ and P-450$_{MP-2}$ are from Shimada et al. (1986) and were determined by automated Edman degradation. "X" indicates unidentified residue. Other sequences are deduced from DNA sequences [P-450$_{NF}$, Beaune et al. (1986) [identical with protein sequence of Watkins et al. (1985)]; P$_1$-450, Jaiswal et al. (1985)] or sequencing of a protein isolated by immunoaffinity chromatography (Wrighton et al., 1986), and recently also found by cDNA sequencing (Quattrochi et al., 1986).

exists with other P-450s in the data bank (≤50%), suggesting that while they are not closely related proteins, all P-450s share a significant degree of similarity.

Two of the most characteristic catalytic activities of rabbit P-450 I are progesterone 21-hydroxylation (Dieter et al., 1982) and 17β-estradiol 2-hydroxylation (Schwab & Johnson, 1985). Human liver microsomes apparently contain both of these activities, as measured in our laboratory. However, none of three preparations of purified P-450$_{MP-1}$ catalyzed either activity at detectable rates [>0.1 nmol of product formed min^{-1} (nmol of P-450)$^{-1}$], although all catalyzed the 4-hydroxylation of S-mephenytoin. In other experiments, we have attributed human liver 2- and 4-hydroxylation of 17β-estradiol to P-450$_{NF}$, which has little structural similarity to rabbit P-450 1 or P-450$_{MP}$ (Beaune et al., 1986).

The sequence at positions 1267–1329 (underlined in Figure 2) encodes a peptide for which there is a consensus sequence among all of the P-450s analyzed (Adesnik & Atchison, 1986; Beaune et al., 1986). This region contains a cysteine that is presumably the heme binding site, and the sequence deduced from our cDNA fits very well with the consensus sequence derived from other P-450 forms and with the general positioning of the binding site near the C-terminus of the protein.

When the overall amino acid composition of the coding region was compared to the published composition (Shimada et al., 1986), a substantial discrepancy was found. However, we were confident that the cDNA was closely related to the P-450$_{MP}$ since the N-terminal protein sequences agreed perfectly and the clone was isolated with antibodies raised against P-450$_{MP}$. When the amino acid analysis was repeated in our own laboratory on the same protein (isolated from liver sample HL 36), it became obvious that there was a large difference in the determination of several amino acids. The data in Table II show the comparison between the amino acid composition from the cDNA and the protein, expressed as the mean and standard error from three separate determinations on the protein done after publication of the earlier results, which were obtained in another laboratory. With the exception of a few amino acids, notably aspartic acid/asparagine, glycine, and alanine, the two compositions agree relatively well. This discrepancy could be due to the limit of accuracy in determining the composition of an entire protein, especially a membrane protein with limited solubility in aqueous solutions. On the other hand, we may have isolated a cDNA coding for a related protein but not the one identical with the P-450$_{MP}$

Table II: Amino Acid Composition of Purified P-450$_{MP}$ Protein and Derived cDNA Composition[a]

	P-450$_{MP}$	cDNA		P-450$_{MP}$	cDNA
Ala	31 ± 7	18	Met	12 ± 1	13
Arg	22 ± 5	21	Phe	26 ± 4	32
Asx	33 ± 5	47	Pro	31 ± 2	31
Glx	41 ± 6	46	Ser	31 ± 4	31
Gly	39 ± 1	27	Thr	23 ± 4	24
His	12 ± 2	12	Trp	3 ± 1	3
Ile	26 ± 4	34	Tyr	14 ± 3	11
Leu	51 ± 5	57	Val	30 ± 3	29
Lys	46 ± 12	35			

[a] A single purified P-450$_{MP-1}$ preparation (HL 36; Shimada et al., 1986) was analyzed for amino acid composition by the Picotag method (Waters Associates, Milford, MA). Results are expressed as the mean of three determinations ± standard error.

analyzed. If the P-450$_{MP}$ is indeed part of a multigene family where more than one mRNA is transcribed (vide infra), then there may exist a family of related proteins; two catalytically active forms sharing the same N-terminal sequence have already been identified (Shimada et al., 1986).

Analysis of Human Genomic DNA and RNA. Human genomic DNA was digested with *Eco*RI, *Taq*I, or *Pst*I and electrophoresed to separate the fragments. The DNA was then transferred to a nylon membrane (Gene Screen Plus) and hybridized to the 1.6-kb cDNA nick-translated fragment. The resulting autoradiogram (after the membrane was washed at 65 °C) is shown in Figure 3A. In every case, there are multiple restriction fragments that hybridize to the cDNA probe suggesting that, like the rabbit progesterone 21-hydroxylase, P-450$_{MP}$ is part of a complex multigene family. Digestion with the three enzymes did not show any differences between the two DNAs examined. When the membrane was washed at 45 °C instead of at 65 °C or hybridized with the 1.5-kb fragment instead of the 1.6-kb probe, an identical pattern of banding was seen (data not shown) suggesting that the bands that appear are closely related to the probe, and weakly related sequences that disappear under conditions of increased stringency do not seem to be present.

When total RNA was electrophoresed, transferred, and hybridized to either the 1.5- or 1.6-kb probe, two bands were seen, one at approximately 2.2 kb and a very light one at approximately 3.5 kb (Figure 3B). Both bands were present after washing at 45 or 65 °C, suggesting that they are both closely related to the nick-translated probe. When RNA was

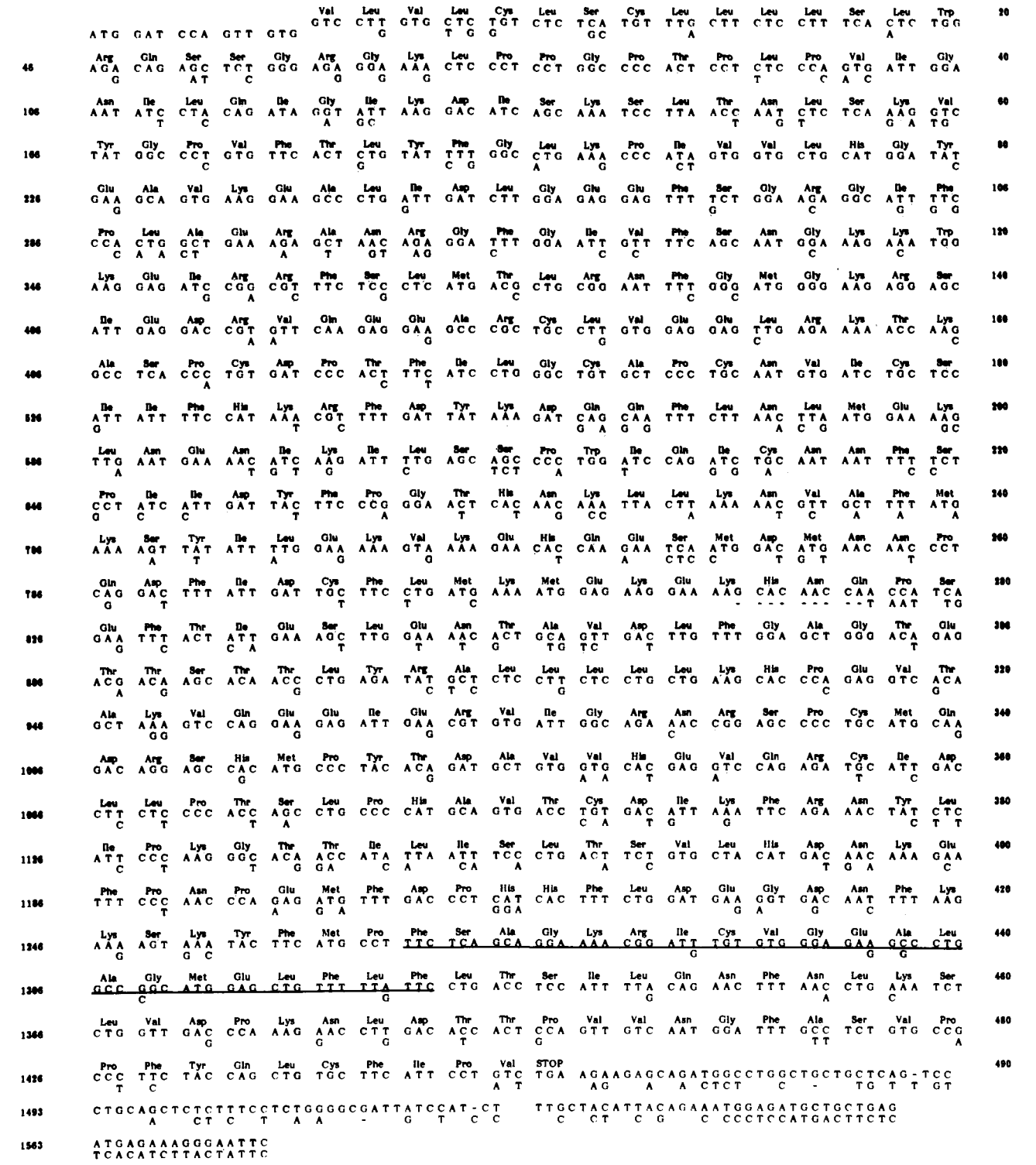

FIGURE 2: Nucleotide and deduced amino acid sequence of cDNA clone MP-8. For comparison, differences in the rabbit liver progesterone 21-hydroxylase cDNA (Tukey et al., 1985) are shown in the bottom line. Numbers on the left indicate nucleotide position starting with the first nucleotide of MP-8. Numbers on the right indicate amino acid position beginning with the methionine determined by automated Edman degradation. The heme binding peptide is underlined.

isolated from fibroblasts or from human fetal liver, no bands appeared on the autoradiogram with the 1.5-kb probe corresponding to the coding region.

We synthesized an oligonucleotide (51-mer, positions 1516–1567) complementary to the noncoding region (to which there is very little similarity to the rabbit sequence) in the hope of finding a more specific probe. Surprisingly, however, this oligonucleotide hybridized to an mRNA in fibroblasts that did not exist in human liver and that did not correspond to any of the bands which hybridized to the nick-translated probes (data not shown). Fibroblasts in culture would not be expected to contain P-450$_{MP}$, and fetal livers do not have any mephenytoin 4-hydroxylase activity or immunoreactive protein (Shimada et al., 1986; Cresteil et al., 1985).

The ratio of intensity of the two mRNA bands (seen in Figure 3B) was relatively constant (approximately 5:1) in 12 individual samples that were examined, although some variation (of the ratio) was seen. In these 12 samples, some correlation between the immunochemically determined levels of P-450$_{MP}$ and mRNA was seen ($r = 0.41$). The correlation

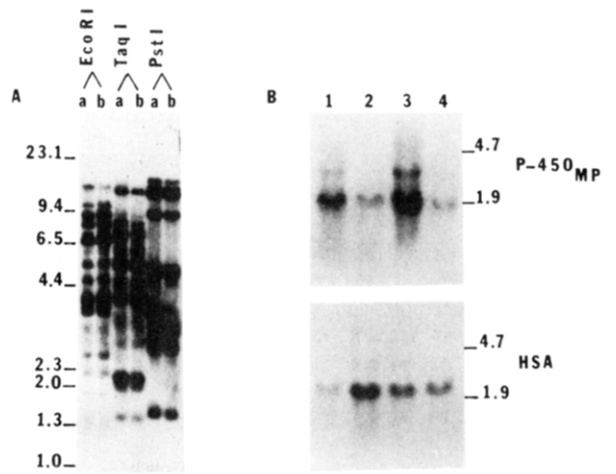

FIGURE 3: DNA and RNA blot analyses. (A) DNA samples isolated from two livers were digested with the restriction enzymes shown and analyzed by "Southern" blotting with the 1.6-kb nick-translated probe. (B) Total RNA samples from four livers were analyzed by "Northern" blot hybridized with the 1.5-kb nick-translated fragment of MP-8 (top) or an oligonucleotide complementary to human serum albumin (HSA) (bottom).

was only somewhat better when a synthetic oligonucleotide complementary to human serum albumin was used as an internal standard for normalization to correct for degradation ($r = 0.58$). The S-mephenytoin 4-hydroxylase activities of the 12 samples (which varied by more than an order of magnitude) did not correlate well with either mRNA levels or, as previously reported, the levels of P-450$_{MP}$ (Shimada et al., 1986). Thus, mRNA levels may not be the sole determinant of P-450$_{MP}$ levels; however, the possibility exists that minor changes in the nucleotide sequence may drastically alter the epitopes of the protein recognized by the antibody. Likewise, differences in nucleotide sequences that decrease hybridization with the nucleic acid probe may not greatly alter the portion of the protein that is recognized by the antibody. The number of different transcripts hidden under each band derived from this multigene family is yet not clear, even in a single individual. Preliminary comparison of restriction fragment analyses among several of the clones hybridizing to nick-translated MP-8 cDNA indicates that small sequence differences exist among the clones isolated from the library. It is important to note that this human library was constructed from mRNA isolated from a single human liver; therefore, it is very likely that several related transcripts exist in each individual liver.

CONCLUSIONS

In summary, we have used specific antibodies to isolate human cDNA clones related to P-450 S-mephenytoin 4-hydroxylase. One of these was sequenced and found to be only 14 nucleotides short of containing the entire protein coding sequence; the predicted N-terminal sequence matches that determined for both P-450$_{MP-1}$ and P-450$_{MP-2}$ at all unambiguous positions. Of all P-450 sequences in the literature, only that of rabbit P-450 1 is closely related. While rabbit P-450 1 distinctively catalyzes progesterone 21-hydroxylation and 17β-estradiol 2-hydroxylation, human P-450$_{MP}$ has neither activity. The size of the gene family related to P-450$_{MP}$ is large, and at least several genes are present, as suggested by genomic DNA blot analysis and the existence of multiple mRNAs in the cDNA library. The transcription of multiple mRNA species is indicated by blotting analysis, restriction analysis of λgt11 clones, and prior studies on the in vitro translation of P-450$_{MP-1}$ and P-450$_{MP-2}$ (Shimada et al., 1986).

While the cDNA sequences of several human liver P-450s have been reported (Jaiswal et al., 1985; Phillips et al., 1985; Quattrochi et al., 1986; Beaune et al., 1986), only in one case reported to date has the protein been isolated in functional form and characterized (Beaune et al., 1986). As in that case (Beaune et al., 1986), a multigene family appears to be present, and the precise relationship of the protein(s) and cDNA has not been possible. However, the amino acid composition and N-terminal sequence comparisons of our cDNA with the protein argue that the cDNA sequence is highly related to if not identical with P-450$_{MP-1}$ or P-450$_{MP-2}$. The availability of this DNA clone and sequence should prove to be a valuable step on the way to elucidating the molecular basis of the complex polymorphism in human liver S-mephenytoin 4-hydroxylase activity.

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Registry No. Cytochrome P-450, 9035-51-2; mephenytoin 4-hydroxylase, 96779-46-3.

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Pseudomonas cepacia 3-Hydroxybenzoate 6-Hydroxylase: Induction, Purification, and Characterization†

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ABSTRACT: A single strain of *Pseudomonas cepacia* cells was differentially induced to synthesize salicylate hydroxylase, 3-hydroxybenzoate 6-hydroxylase, or 4-hydroxybenzoate 3-hydroxylase. A procedure was developed for the purification of 3-hydroxybenzoate 6-hydroxylase to apparent homogeneity. The purified hydroxylase appears to be a monomer with a molecular weight of about 44 000 and exhibits optimal activity near pH 8. The hydroxylase contains one FAD per enzyme molecule and utilizes NADH and NADPH with similar efficiencies. The reaction stoichiometry for this enzyme has been determined. In comparison with other aromatic flavohydroxylases, this enzyme is unique in inserting a new hydroxyl group to the substrate at a position para to an existing one.

Microbial flavoprotein hydroxylases are a class of external monooxygenases. Most of such flavohydroxylases are induced in soil pseudomonads to catalyze the hydroxylation of various benzenoids including pollutants and toxicants. The hydroxylated products are readily subject to further catabolism to produce metabolites that can be used by microbes for growth. Through the initial action of hydroxylases, many benzenoid compounds can be degraded and detoxified by microbial actions (Stanier & Ornston, 1973).

Although enzyme induction is an important property shared by microbial flavohydroxylases, the nature of the induction mechanism(s) is not well understood. With respect to the inducer–induced enzyme relationship, our understanding is mostly limited to the observation that a single strain of microorganism can be induced by using a benzenoid compound as the sole carbon source for growth to synthesize a specific flavohydroxylase. This induced hydroxylase is capable of catalyzing the hydroxylation of the very same benzenoid used as the carbon source for growth [Massey and Hemmerich (1975) and references cited therein]. In one case, the differential induction of orcinol and resorcinol hydroxylases (using orcinol and resorcinol, respectively, for induction) in cells of *Pseudomonas putida* ORC has been established (Ohta & Ribbons, 1976). The question as to whether a single strain of microbe can be differentially induced to synthesie multiple species of flavohydroxylases deserves further investigations.

Using a strain of *Pseudomonas cepacia* cells identified in our laboratory, we have previously reported the induction and isolation of salicylate hydroxylase (Tu et al., 1981; Wang & Tu, 1984). In this work, we demonstrate that *m*-hydroxybenzoate hydroxylase and *p*-hydroxybenzoate hydroxylase can also be differentially induced in the same strain of cells. The newly induced *m*-hydroxybenzoate hydroxylase has been purified to apparent homogeneity and its general structural and catalytic properties have been characterized. On the basis of the position of substrate hydroxylation, this hydroxylase is designated 3-hydroxybenzoate 6-hydroxylase. The feature that the new hydroxyl group is inserted at a position para to the existing hydroxyl function is unique among known flavohydroxylases that utilize benzenoid substrates.

Experimental Procedures

Materials. Salicylic acid, *m*-hydroxybenzoic acid, and *p*-hydroxybenzoic acid, obtained from Aldrich, were recrystallized twice from hot water. For large scales of cell growth for enzyme purifications, these benzenoid acids were used directly as supplied. 2,5-Dihydroxybenzoic acid was a product of Eastman, and all other derivatized benzoic acids used were from Aldrich. NADH, dithiothreitol, FAD, venom phosphodiesterase I (type VII), deoxyribonuclease I (type III), and horse liver alcohol dehydrogenase were all purchased from Sigma. FAD was further purified by DEAE-cellulose chromatography (Massey & Swoboda, 1963). Hen egg white lysozyme was from Boehringer-Mannheim GmbH. DEAE-Sephadex A-50, Sephadex G-100, PBE 94 chromatofocusing exchanger, and polybuffer 74 were products of Pharmacia. Hydroxyapatite (Bio-Gel HTP) and horseradish peroxidase

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