Characterization of cDNAs, mRNAs, and Proteins Related to Human Liver Microsomal Cytochrome P-450 (*S*)-Mephenytoin 4′-Hydroxylase[†,‡]

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ABSTRACT: A cytochrome P-450 (P-450) multigene family codes for several related human liver enzymes, including the P-450 responsible for (*S*)-mephenytoin 4′-hydroxylation. This enzyme activity has previously been shown to be associated with a genetic polymorphism. Genomic (Southern) blot analysis using nonoverlapping 5′ and 3′ portions of a cDNA clone suggests that approximately seven related sequences are present in this gene family. In this study four cDNA clones, all nearly full-length, were isolated from a bacteriophage λgt11 library prepared from a single human liver. These clones can be grouped into two categories that are approximately 85% identical at the level of DNA sequence. The cDNA clones in one category (MP-4, MP-8) both match the N-terminal sequences of the P-450$_{MP-1}$ and P-450$_{MP-2}$ proteins, which had previously been shown to be catalytically active in (*S*)-mephenytoin 4′-hydroxylation. These two cDNAs, MP-4 and MP-8, differ in only two bases in the coding region but are quite distinct in their 3′ noncoding regions. Another protein (P-450$_{MP-3}$) was isolated on the basis of its immunochemical similarity to P-450$_{MP-1}$ but was found to be catalytically inactive; amino acid sequencing of tryptic peptides of P-450$_{MP-3}$ showed a correspondence to the second category of cDNA clones (MP-12, MP-20), which differ from each other in only four (nonsilent) base changes. Oligonucleotides specific for the two groups of cDNA clones were used as probes of human liver mRNAs—individual liver samples examined expressed both types of mRNAs but no correlation was observed between the abundance levels of any mRNA and catalytic activity. Further, oligonucleotide probes indicated that mRNAs corresponding to both the MP-4 and MP-8 clones were apparently present in individual liver samples. A monoclonal antibody was isolated that recognized P-450$_{MP-1}$ but not P-450$_{MP-2}$ or P-450$_{MP-3}$; the amount of protein detected by the antibody in different liver samples was not correlated with the mephenytoin 4′-hydroxylase activity. These results indicate that several closely related P-450 genes are all expressed in individual human livers. The MP-4/MP-8 gene products are proposed to be the ones most likely involved in mephenytoin 4′-hydroxylation, and much of the variation in catalytic activity among individuals is not a result of differences in levels of P-450$_{MP-1}$ or mRNA but may be due to base differences in the structural gene(s).

Mephenytoin, an anticonvulsive drug, shows a distinct genetic polymorphism in its oxidation in humans. This polymorphism has a variation in enzymatic 4′-hydroxylation of the *S* enantiomer as its basis (Wedlund et al., 1984; Küpfer & Preisig, 1984). While the incidence of the "poor metabolizer" phenotype is less than 5% in Caucasians, the incidence rises to above 20% in Japanese (Nakamura et al., 1985). The slow-oxidation phenotype is of further significance in that the oxidation of some other substituted hydantoins and barbiturates, such as hexobarbital, appears to cosegregate with this phenotype (Knodell et al., 1988; Hall et al., 1987).

In an effort to understand the biochemistry involved in this phenomenon, our laboratory purified two human liver cytochrome P-450[1] enzymes, P-450$_{MP-1}$ and P-450$_{MP-2}$, that were both catalytically active in (*S*)-mephenytoin 4′-hydroxylation (Shimada et al., 1986). The two proteins, which are both present in some individual livers, could be distinguished by their apparent monomeric M_rs and appear to be encoded for by different mRNAs, as judged by the results of in vitro translation experiments. Subsequently, antibodies raised against P-450$_{MP-1}$ were used to isolate human cDNA clones from a bacteriophage λgt11 library. One nearly full-length clone (MP-8) was sequenced (Umbenhauer et al., 1987b).[2]

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[1] Abbreviations: P-450, cytochrome P-450; PBS, phosphate-buffered saline [20 mM potassium phosphate buffer (pH 7.4) containing 0.15 M NaCl]; 1× SSC, saline–sodium citrate buffer [0.015 M sodium citrate buffer (pH 7.0) containing 150 mM NaCl]; 1× SSPE, saline–sodium phosphate–EDTA buffer [10 mM sodium phosphate buffer containing 180 mM NaCl and 1 mM EDTA (pH 7.7)]; NaDodSO$_4$, sodium dodecyl sulfate; Denhardt's reagent, mixture of 0.02% (w/v) Ficoll, 0.02% (w/v) poly(vinylpyrrolidone), and 0.02% (w/v) bovine serum albumin; Ig, immunoglobulin; IgG, immunoglobulin G; IgM, immunoglobulin M; IgK, immunoglobulin K; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; ELISA, enzyme-linked immunoadsorbent assay; T_m, melting temperature. In this report the terms P-450$_{MP-1}$, P-450$_{MP-2}$, and P-450$_{MP-3}$ refer to individual proteins and MP-4, MP-8, MP-12, and MP-20 refer to cDNAs. The term P-450$_{MP}$ is used in a generic sense to include all of the proteins related to P-450$_{MP-1}$, P-450$_{MP-2}$, and P-450$_{MP-3}$.

[2] The gene family is tentatively classified in the IIC8 and IIC9 categories of the scheme of Nebert et al. (1987).

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Ultimately, an understanding of the precise mechanisms of regulation of individual genes in this human multigene family and structure–activity relationships among the proteins will require detailed characterization of the genes and their products. In this article we report the isolation and sequencing of several cDNAs originating from a single liver, the partial sequencing of a protein corresponding to one cDNA, the results of studies on human liver mRNA and DNA samples using specific oligonucleotide probes and restriction fragment analysis, and the preparation of a monoclonal antibody that shows specificity for the P-450$_{MP-1}$ protein.[3]

Experimental Procedures

Screening of a Bacteriophage λgt11 Library. A human liver cDNA library constructed in bacteriophage λgt11 had been previously screened with antibodies directed against P-450$_{MP-1}$ (Wang et al., 1983; Shimada et al., 1986), and the longest clone, MP-8, was further characterized (Umbenhauer et al., 1987b). In the present work, two additional clones MP-4 (2.17 kb) and MP-12 (1.77 kb) were studied, which had been identified during the same screening in which MP-8 was isolated but differed from MP-8 and each other in their restriction patterns. Another cDNA clone was isolated by a new screening (10^5 plaques) of the same library using a ^{32}P-end-labeled 20-mer oligonucleotide probe specific for MP-12. The longest clone (1.8 kb) was further analyzed and designated MP-20. λgt11 clones were propagated in *Escherichia coli* Y1088 or Y1090, and λ DNA was isolated from phage lysates on DE-52 DEAE-cellulose columns (Whatman Separation Products, Clifton, NJ) according to Carlock (1986).

Subcloning in M13 Phage. All three λ DNAs (MP-4, MP-12, MP-20) were subcloned into M13 mp18 and mp19 phage in order to sequence inserts from both ends.

A 3.2-kb *Eco*RI–*Kpn*I fragment from λgt11 MP-4, which included a 1-kb *Eco*RI–*Kpn*I fragment of λgt11 DNA, was subcloned into M13 phage. λgt11 MP-12 and MP-20 both contain an internal *Eco*RI site and were subcloned by using *Kpn*I and *Sac*I cuts, adding 1 kb of λgt11 sequence to each end of the insert. Subcloning procedures were performed according to Messing (1983) using *E. coli* UT481 cells for the propagation of M13 subclones.

DNA Sequencing. DNA sequencing was carrid out using the dideoxy chain termination method (Sanger et al., 1977) with a ^{32}P-sequencing kit from New England Nuclear (Boston, MA) and slight modifications of their protocol, as previously described (Umbenhauer et al., 1987b). After a sequence of 300–350 bp was read, appropriate 20-mer oligonucleotides were synthesized as primers for further sequencing along the same template. Every reaction was run at least four times, and overlapping or complementary sequences were read as necessary.

Preparation of Total and Poly(A+) RNA. RNA was isolated either by the CsCl cushion method according to Chirgwin et al. (1979) or by a recently described single-step method using guanidium thiocyanate–phenol–$CHCl_3$ extraction (Chomczynski & Sacchi, 1987). Oligo(dT)-cellulose columns were used to enrich the preparation in poly(A+) RNA according to Aviv and Leder (1972) for use in studies with oligomeric probes.

Preparation of Genomic DNA. Genomic DNA was isolated from human liver by the CsCl cushion method described by Chirgwin et al. (1979). DNA was also isolated from white blood cell nuclei, by proteinase K digestion and successive phenol–$CHCl_3$ extractions according to Kunkel et al. (1977), from subjects previously phenotyped for mephenytoin 4′-hydroxylation. Twelve samples were obtained from Dr. G. R. Wilkinson, Department of Pharmacology, Vanderbilt University, Nashville, TN.

Preparation of the Probes for Transfer Blot Analysis. MP-8, MP-12, and MP-20 cDNA inserts were isolated from M13 phage DNA on agarose gels and purified by phenol freeze extraction according to Benson (1984). The MP-8 probe was a 1.5-kb *Eco*RI–*Pvu*II fragment containing only the coding sequence. A 5′-end *Eco*RI–*Sma*I fragment 665 bp long and a 3′-end *Sma*I–*Eco*RI fragment 911 bp long were also obtained. The MP-12 probe was isolated in two pieces covering the coding sequence: an *Eco*RI–*Eco*RI fragment 757 bp long (5′) and a *Pst*I–*Eco*RI fragment 572 bp long (3′). The MP-20 probe was also cut into two pieces with *Eco*RI: the 5′-end piece was 811 bp long and the 3′ end was 989 bp long. These fragments were nick-translated with a [^{32}P]dCTP nick-translation kit from New England Nuclear to give a specific activity of (3–5) × 10^6 cpm (μg of DNA)$^{-1}$. Oligonucleotides were synthesized by S. T. Wright, Center in Molecular Toxicology, Vanderbilt University, either on a Biosearch Sam I synthesizer using phosphodiester chemistry or on a Biosearch Cyclone synthesizer using β-phosphoramidite methods (Biosearch, San Rafael, CA), and were further purified on denaturing polyacrylamide gels. When used as probes, oligonucleotides were ^{32}P-end-labeled with T_4 polynucleotide kinase from New England Biolabs (Beverly, MA) and [γ-^{32}P]ATP (ICN Radiochemicals, Irvine, CA).

Nucleic Acid Transfer Blot Analysis. Human genomic DNA samples were digested with restriction endonucleases obtained from New England Biolabs. Typically, 10 μg of genomic DNA was digested over a 24-h period with three successive additions of each enzyme [5 units (μg of DNA)$^{-1}$]. The DNAs were separated by electrophoresis in 0.8% agarose gels and transferred by capillary action to Gene Screen Plus (New England Nuclear) according to the protocol provided by the supplier and the reference of Southern (1975). Hybridizations were performed using nick-translated MP-8 insert as the probe, and washings were done at 65 °C in the same buffer as for RNA analysis using the same conditions described by Umbenhauer et al. (1987b). Total or poly(A$^+$) RNA samples [20 μg of total RNA or 1–4 μg of poly(A$^+$) RNA] were separated by electrophoresis in 1% formaldehyde–agarose gels. Resolved RNA samples were transferred to Gene Screen Plus or nitrocellulose membranes by a "Northern" blotting technique (Alwine et al., 1977) according to the instructions of the suppliers, New England Nuclear and Schleicher and Schuell (Keene, NH), respectively. Gene Screen Plus membranes were used for hybridization with nick-translated probes. Hybridizations were carried out in the presence of 10% (w/v) dextran sulfate, 50% (w/v) formamide, and 1 M NaCl at 42 °C, and washings were done at 60 °C in 2× SSC buffer containing 0.5% $NaDodSO_4$ (twice for 30 min), with a last washing at room temperature in 0.1× SSC. Nitrocellulose was used when the membranes were probed with oligonucleotides. The hybridization mixture contained 6× SSPE, 0.1% $NaDodSO_4$, 1× Denhardt's reagent, 100 μg of calf thymus DNA mL^{-1}, and 100 μg of carrier tRNA mL^{-1}. Hybridizations were carried out 5–10 °C below the calculated T_m for the appropriate oligonucleotide probes, and after two 15-min washes at room temperature in 6× SSPE containing 0.1% $NaDodSO_4$, the filters were washed in the same buffer for 1 min at the hybridization temperature, estimated ac-

[3] Portions of this work have been presented in abstract form (Umbenhauer et al., 1987a; Guengerich et al., 1988).

cording to the manufacturer's recommendations and the method of Wallace and Miyada (1987).

Preparation of Peptides and Amino Acid Sequencing. A P-450 preparation was isolated from human liver sample HL 99 by a general procedure described elsewhere (Shimada et al., 1986); purification was monitored by NaDodSO$_4$–polyacrylamide gel electrophoresis/immunoblotting with rabbit anti-P450$_{MP-1}$. The procedure yielded a homogeneous preparation, as judged by NaDodSO$_4$–polyacrylamide gel electrophoresis, with the migration distance being equivalent to that of catalytically active P-450$_{MP-1}$ (Shimdada et al., 1986). The detergent (Emulgen 913) was removed by adsorption of the protein to a small column of hydroxyapatite (1.2 × 4 cm), extensive washing with 50 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v), and elution with 0.5 M potassium phosphate buffer containing 20% glycerol. A portion (~55 nmol P-450 in 22 mL) was dialyzed overnight versus H_2O. The protein was carboxymethylated under reducing conditions in the presence of guanidinium hydrochloride (Barra et al., 1984; Shimada et al., 1986). The sample was then dialyzed three times versus H_2O and taken to near dryness in a Speed-Vac concentrator device (Savant Instruments, Hicksville, NY). An aliquot (~10 nmol as judged by quantitative amino acid analysis) was dissolved in 200 μL of 0.1 M NH_4HCO_3 buffer with sonication. Trypsin that had been treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (TPCK) was purchased from Worthington Biochemicals (Freehold, NJ) and added at a weight ratio of 1:100; after 5 h another aliquot was added to give a final ratio of 1 part trypsin:50 parts P-450 (by weight). The digestion proceeded for 21 h at 37 °C with occasional monitoring by HPLC (vide infra). Prior to preparative separation of the peptides, the sample was acidified by the addition of CF_3CO_2H to 1% (v/v). Peptide resolution utilized HPLC with a 4.6 × 30 mm Pierce RP octylsilyl (C8) guard column (Pierce Chemical Co., Rockford, IL) attached to a 6.2 × 80 mm Du Pont Bioseries PEP RP/1 Zorbax octylsilyl (C8) column (Du Pont Instruments, Wilmington, DE). Solvent A consisted of 4% CH_3CN (v/v) and 0.1% CF_3CO_2H (v/v) (Pierce) in H_2O, and solvent B consisted of 80% CH_3CN and 0.1% CF_3CO_2H in H_2O. The flow rate was 1.0 mL min^{-1}. From 0 to 5 min after injection, the system was held at 100% A. Between 5 and 100 min, the solvent composition was increased to 50% B in a linear manner. From 100 to 120 min, the solvent composition was increased to 100% B, and this composition was held for another 10 min. The absorbance was monitored at 214 nm (0–0.5 unit full-scale). Fractions (total of 136 corresponding to ~50 apparent peaks) were collected by hand into polypropylene tubes. Portions of these aliquots were concentrated in a Speed-Vac device (vide supra) prior to further analysis.

Amino acid analysis and sequencing were done by T. Porter and Dr. L. G. Warnock in the Vanderbilt Protein Chemistry Facility. Samples were hydrolyzed for 24 h in 6 N HCl and analyzed by a Waters "Pico-tag" phenyl isothiocyanate derivatization procedure, with analysis using a Waters HPLC system described by the supplier (Waters Chromatography Division, Millipore Corp., Milford, MA). Quantitative amino acid analysis of peptides was done prior to sequencing to estimate peptide concentrations and to verify compositions subsequently derived from sequencing. Sequencing work was done with an Applied Biosystems 470A automated Edman sequenator connected in-line with an Applied Biosystems 120 analyzer (Applied Biosystems, Foster City, CA). Recoveries of residues were determined by integration of signals and comparison to external standards.

Monoclonal Antibody Preparation and Assay. An 8-week-old Balb/c female mouse (Charles River, Wilmington, MA; viral- and mycoplasma-free) was immunized with 50 μg of purified human P-450$_{MP}$ [a mixture of P-450$_{MP-1}$ and P-450$_{MP-2}$ isolated from liver sample HL 91; Shimada et al., (1986)] mixed with an equal volume of complete Freund's adjuvant. The same dose was injected 4 and 12 weeks later with incomplete Freund's adjuvant. After 10 days, a test bleed was positive as judged by ELISA and NaDodSO$_4$–polyacrylamide gel electrophoresis/immunoblotting, and the mouse was primed (ip) with 50 μg of antigen. Three days later the animal was sacrificed; the spleen was harvested for fusion as modified from Fazekas de St. Groth and Scheidegger (1980). Spleen cells (3 × 10^8) were pooled with mouse myeloma P3X63Ag8.653 (Kearney et al., 1979) at a ratio of 4 spleen cells:1 myeloma. The cells were centrifuged (800*g*, 5 min), the supernatant was decanted, and the pellet was loosened by tapping the tube. One milliliter of fusion solution was added over 1 min [35% (w/v) poly(ethylene glycol) 4000 (Merck, Darmstadt, Germany, EM grade), 50% RPMI 1640 (Moore et al., 1967), 15% $(CH_3)_2SO$]. The mixture was stirred for 30 s, and then RPMI 1640 (no serum) was added over 6 min at a rate of 1 mL min^{-1}. RPMI 1640 (10 mL) containing 20% serum (v/v) was added over 5 min. The cells were collected by centrifugation (800g, 5 min) and then distributed into six 24-well plates at a density of 2 × 10^6 cells/well in 1.5 mL of RPMI 1640 containing 20% serum (heat inactivated at 56 °C for 30 min), 200 mM L-glutamine, 100 mM sodium pyruvate, 1.14 mM oxalacetate, 0.2 unit of insulin mL^{-1}, 50 μM 2-mercaptoethanol, 100 units of penicillin mL^{-1}, 0.17 mM streptomycin, and Littlefield's HAT mixture (Littlefield, 1964).

Plates were incubated undisturbed for 72 h at 37 °C in the presence of 7% CO_2. The media was partially replaced every 3–5 days over a period of 2 weeks until the wells were $^1/_2$ to $^3/_4$ confluent. The supernatants were assayed by ELISA and NaDodSO$_4$–polyacrylamide gel electrophoresis/immunoblotting. Positive wells were recloned at limiting dilution in 96-well plates containing 2 × 10^5 thymocyte feeder cells/well. Positive hybridomas were recloned at least twice to ensure monoclonality as well as to stabilize the cell line. Wells containing more than one clone were excluded. Ascites fluid was generated by inoculating 2 × 10^6–2 × 10^7 hybridoma cells into pristane-primed Balb/c mice.

Hybridoma clones were initially screened by a noncompetitive ELISA (Engvall, 1980). Poly(vinyl chloride) plates (Dynatech, Alexandira, VA) were coated for 2 h at 37 °C with 50 μL of purified P-450$_{MP}$ (1 μg mL^{-1}) diluted in 0.1 M $NaHCO_3$ buffer (pH 9.6). The plates were washed twice with PBS and blocked for 30 min with PBS containing 20% calf serum (v/v). The plates were washed twice with PBS, and 50 μL of cell culture supernatant was added overnight at room temperature. The plates were washed five times with PBS containing 0.05% Tween 20 (w/v), and 50 μL of affinity-purified rabbit anti-mouse Ig–horseradish peroxidase conjugate (Accurate, Westbury, NY) was added at a 1:2000 dilution in PBS. The plates were incubated for 60 min at room temperature, washed six times with PBS containing 0.05% Tween 20, and developed with *o*-phenylenediamine (10 mM in 0.1 M citric acid buffer containing 0.2 M sodium phosphate, pH 5.5). A_{492} was measured in the plates with a reading device.

Positive clones were isotyped by double immunodiffusion analysis using Biometics (Kensington, MO) or Miles Scientific (Naperville, IL) mouse-isotyping reagents.

Hybridomas were screened by $NaDodSO_4$–polyacrylamide gel electrophoresis/immunoblotting with human microsomes prepared from liver sample HL 97 as the antigen source (Laemmli, 1970; Towbin et al., 1979; Guengerich et al., 1982b) with a Bio-Rad Mini-Protean II gel apparatus and Bio-Rad MiniTransblot cell (Bio Rad, Richmond, CA). Nitrocellulose sheets were blocked for 30 min in PBS containing 20% calf serum (v/v), washed twice with PBS, and incubated overnight at room temperature in hybridoma supernatant or ascites fluid diluted in PBS. Nitrocellulose sheets were washed in PBS with three changes of buffer over 30 min. Affinity-purified rabbit anti-mouse Ig–horseradish peroxidase conjugate (Accurate, Westbury, NY) was incubated for 60 min at room temperature at a 1:2000 dilution. Nitrocellulose sheets were washed as before and developed by using 4-chloro-1-naphthol as substrate (Guengerich et al., 1982a), and the reaction was stopped by rinsing the nitrocellulose sheet with PBS.

The screening of bacteriophage λgt11 clones containing $P\text{-}450_{MP}$ inserts was performed with monoclonal antibody 1C6B6B5, which recognized only $P\text{-}450_{MP\text{-}1}$, using a Vectastain ABC kit based on avidin–biotin interactions (Vector Laboratories, Burlingame, CA) (Young et al., 1985; French et al., 1986; Young & Davis, 1985). Tween-20 was eliminated from all incubations and washes because of interference with binding of the monoclonal antibody to $P\text{-}450_{MP}$-related proteins. To minimize nonspecific binding of antibody, the filters were blocked for 30 min with buffer containing 20% horse serum. The initial washes as well as the incubations with the monoclonal and the biotinylated secondary antibody contained 2% horse serum. Washes following the secondary antibody incubation were with buffer only because of serum inhibition of the biotin–avidin interaction.

Other Assays. Mephenytoin 4′-hydroxylase activity was measured either with a radiochromatographic procedure (Shimada et al., 1985) or by HPLC analysis (Shimada et al., 1986). Tolbutamide methyl hydroxylation (Knodell et al., 1987) and hexobarbital 3′-hydroxylation (Knodell et al., 1988) were measured by HPLC as described. $NaDodSO_4$–polyacrylamide gel electrophoresis/immunoblotting and quantitation of stained bands (to estimate amounts of antigens) were done essentially as previously described (Guengerich et al., 1982b). P-450 was measured as described by Omura & Sato (1964), and protein concentrations were measured with the Pierce bicinchoninic acid (BCA) reagent system (Pierce Chemical Co., Rockford, IL).

Results and Discussion

Studies with Human Genomic DNA. Previous studies had shown that the mephenytoin 4′-hydroxylase gene belongs to a complex P-450 multigene family (Umbenhauer et al., 1987b).[2] After digestion with three restriction enzymes (*Eco*RI, *Pst*I, *Taq*I) and transfer to Gene Screen Plus membranes, identical patterns of hybridization were seen with all DNA samples using nick-translated MP-8 insert as a probe. One way to estimate the number of genes in a multigene family is to probe genomic restriction patterns with long but nonoverlapping 5′ and 3′ probes corresponding to the coding region of a gene and determine the number of bands recognized in common by the two probes. When such an experiment was done (Figure 1), at least seven genes or genelike sequences were detected for the P-450IIC8/9 family.[2]

In this study, a large panel of restriction enzymes (a total of 22) was used to search for possible restriction fragment length polymorphisms among DNA samples from 12 subjects who had been phenotypes previously in vivo as poor or extensive metabolizers of mephenytoin. The enzymes *Sac*I, *Hin*dIII, and *Msp*I were found to yield polymorphic patterns. Unfortunately, no correlation could be drawn between the in vivo phenotype and polymorphic patterns observed with any of those restriction enzymes.[4]

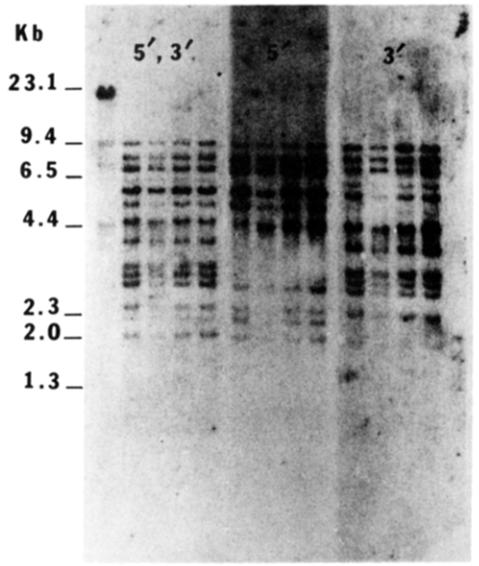


FIGURE 1: Genomic blot ("Southern") analysis of $P\text{-}450_{MP}$-related genes. DNA from four different human liver samples was digested with *Eco*RI and probed with the complete cDNA MP-8 insert (left), the 5′ 665-bp piece (middle), or the 3′ 911-bp piece (right).

cDNA Cloning and Sequencing. In an effort to better understand this complex multigene family, more cDNA clones from the single liver cDNA library were examined (Umbenhauer et al., 1987b). Restriction analyses showed that cDNA clone MP-4 could be distinguished from MP-8 and MP-12 had a different pattern (from MP-8), as shown by the restriction map in Figure 2.

The MP-4 clone contains a 2.17-kb insert in which 1152 bp are part of an open reading frame, identical with the MP-8 coding sequence except for two single base changes (Figure 3). In the 5′ portion of the cDNA, starting with a poly(T) region, a stretch of 625 bp was found to be repeatedly interrupted by stop codons. With computer analysis (National Biomedical Research Foundation data bank, Georgetown University, Washington, DC), this sequence was identified as the complementary strand of the cytochrome *b* gene (a mitochondrial gene) and is considered to be a cloning artifact in the library.[5] The 3′ noncoding region is 382 bp long and is identical with the MP-8 sequence only through the first 72 bp. A polyadenylation signal was present and was followed 20 bp downstream by a poly(A) tail.

The MP-12 clone has a 1.77-kb insert containing a 1388-bp open reading frame, which is 85% identical with the MP-8 sequence. Although the MP-8 and MP-12 sequences differ in regions scattered all along the open reading frame, some long stretches of complete identity were observed (50–60 bp) and the termination codon TGA is located at exactly the same position in MP-8 and MP-12. When compared to MP-8, 78 nucleotides are missing on the 5′ end of this clone and the 3′ untranslated region differs from both MP-4 and MP-8 along

[4] An *Msp*I polymorphism has been recently described with a cDNA probe for the P-450 I family (Nebert et al., 1987) on chromosome 15 (Spurr et al., 1987).

[5] This observation was highly unexpected and the reason for this library artifact remains unclear. The junction of the MP-4 and cytochrome *b* sequences does not contain an *Eco*RI site or any other known restriction endonuclease site.

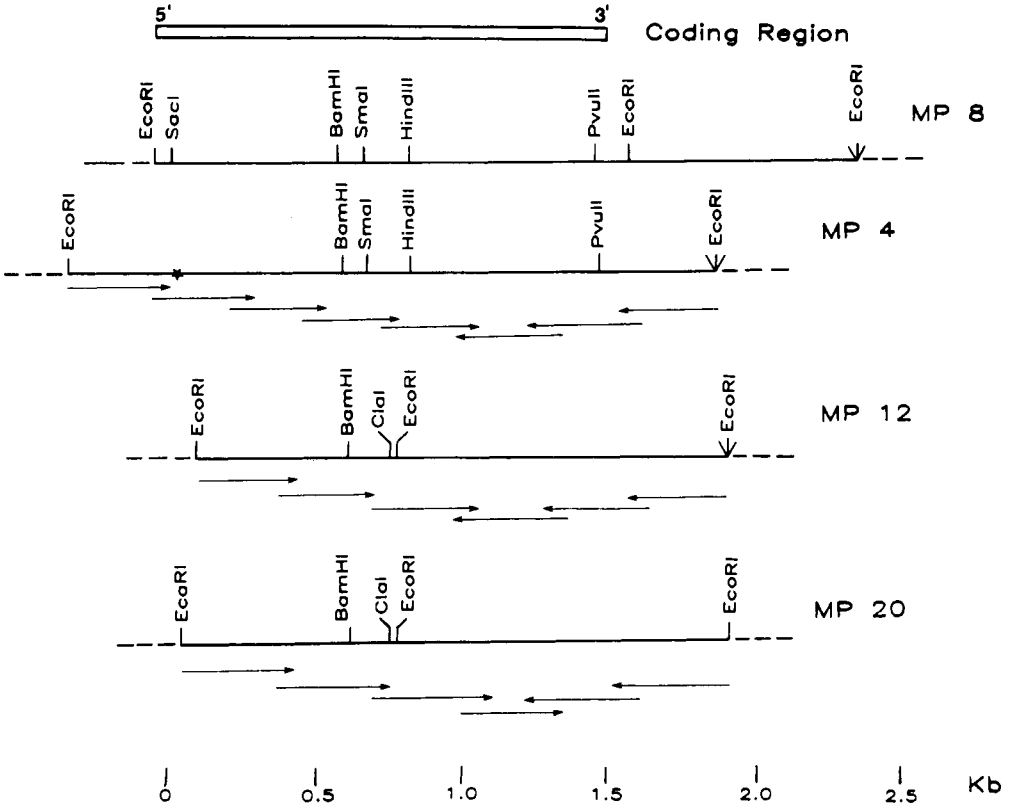


FIGURE 2: Restriction maps of P-450-related clones and sequencing strategy. Each clone was determined to be unique by restriction mapping and then by direct DNA sequencing. The coding sequence is represented at the top by the box. The arrows indicate the segments and direction of sequences read from the synthetic primers for MP-4, MP-12, and MP-20 cDNAs. * indicates the beginning of what is believed to be the actual coding sequence in MP-4. Ψ indicates a missing restriction site in λ DNA.

its 382 bp. A polyadenylation signal was also found, followed 17 bp later by a poly(A) tail.

The same human liver λgt11 cDNA library was screened in a search for a full-length clone corresponding to the MP-12 sequence. A 20-mer oligonucleotide was designed to be complementary to the sense strand of the cDNA and located in the sequence where MP-8 and MP-12 clones differ most (positions 290–309). From a total of 10^5 plaques screened, 5 positives were isolated. Four λ DNA clones had the 5′ *Eco*RI and the internal *Cla*I sites, which are specific for MP-12 (absent in MP-8). The four cDNA clones and inserts all had the same size and appeared slightly larger than the MP-12 insert.

The MP-20 clone (the first one that was subcloned in M13 phage) has a 1.8-kb insert of which 1441 bp are coding sequence and identical with the MP-12 sequence except for four single base changes, all of which code for amino acid substitutions. This clone is 48 bp longer than MP-12 at its 5′ end, and the deduced N-terminal amino acid sequence differs from the amino acid sequence predicted by the MP-8 nucleotide sequence. MP-20 was still missing the initiation codon and the first 30 nucleotides present in MP-8. MP-12 and MP-20 have the same 3′ untranslated region, but the polyA tail was missing in MP-20 and the cloned sequence ended 22 bp after the polyadenylation signal.[6]

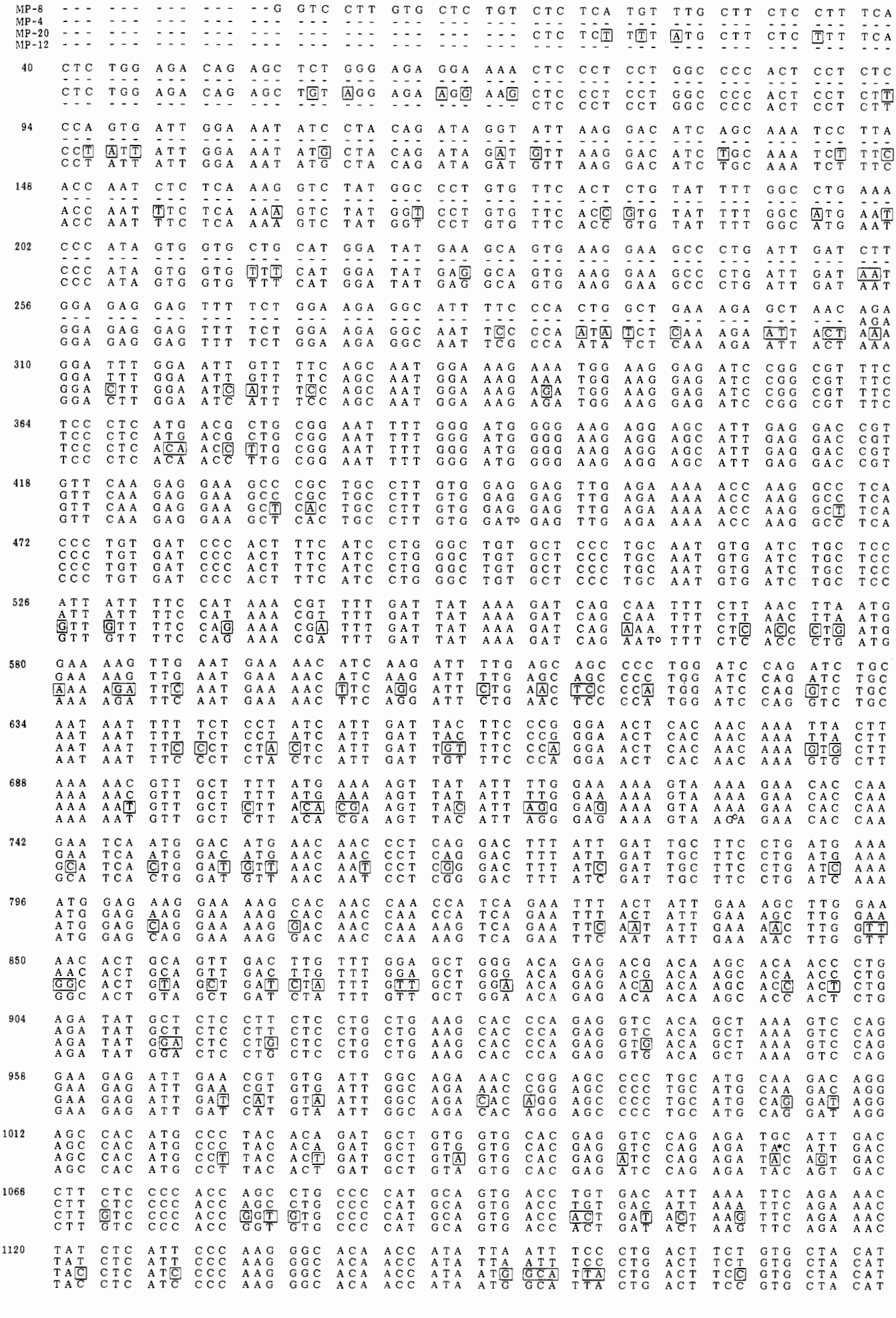
The cDNAs can be grouped into two closely related categories, the MP-4/MP-8 and the MP-12/MP-20 types. Computer analysis had previously shown (Umbenhauer et al., 1987b) that the MP-8 sequence shares 80% similarity with that of a rabbit liver progesterone 21-hydroxylase P-450 1 cDNA (Tukey et al., 1985). The same degree of identity was observed between MP-12/MP-20 and rabbit P-450 1 clones (Tukey et al., 1985). Yoshioka et al. (1987) have also characterized a rat cDNA clone highly similar (71%) to MP-8, and this cDNA appears to code for a constitutive male-specific P-450 protein (P-450M1).

The deduced amino acid sequences for MP-8 and MP-20 are given in Figure 4.[7] The extent of sequence similarity was estimated at 77%, with consensus sequences as long as 20–25 identical amino acids. Near the C-terminal region of the protein, the heme-binding consensus sequence observed for all P-450s analyzed (Black & Coon, 1986) was highly conserved between MP-8 and MP-20 (nucleotides 1267–1329).

Two other laboratories have recently purified human liver proteins whose N-terminal amino acid sequences match the MP-20 deduced amino acid sequence. Wrighton et al. (1987) isolated an MP-20-like protein that was immunochemically related to several rat P-450s. Lasker et al. (1987) were able to purify both MP-8- and MP-20-like proteins, as judged by the published partial N-terminal sequences. Interestingly, those results, consistent with our work, show that the proteins related to MP-8 and MP-20 have exactly the same amino acid sequence length; i.e., the initiation codons (ATG) absent in the MP-8 and MP-20 cDNAs are predicted to be located at the same position in both clones and also at the exact same

[6] Out of the three remaining clones derived from this new screening, two contained the 20 bp corresponding to the very 5′ end of MP-20, as shown by dot blot hybridization using λgt11 DNA and a specific 20-mer oligonucleotide as the probe. These were subcloned into M13 phage and the 5′ regions were sequenced; unfortunately all three were identical and no longer than MP-20. A new screening (2×10^5 plaques) failed to give rise to a MP-12-like clone containing the expected initiation codon or 5′ untranslated regions, suggesting that for some reason this sequence may be absent from the library.

[7] Yields of individual residues of the peptides are available upon request as supplementary material.


MP-8
MP-4
MP-20
MP-12

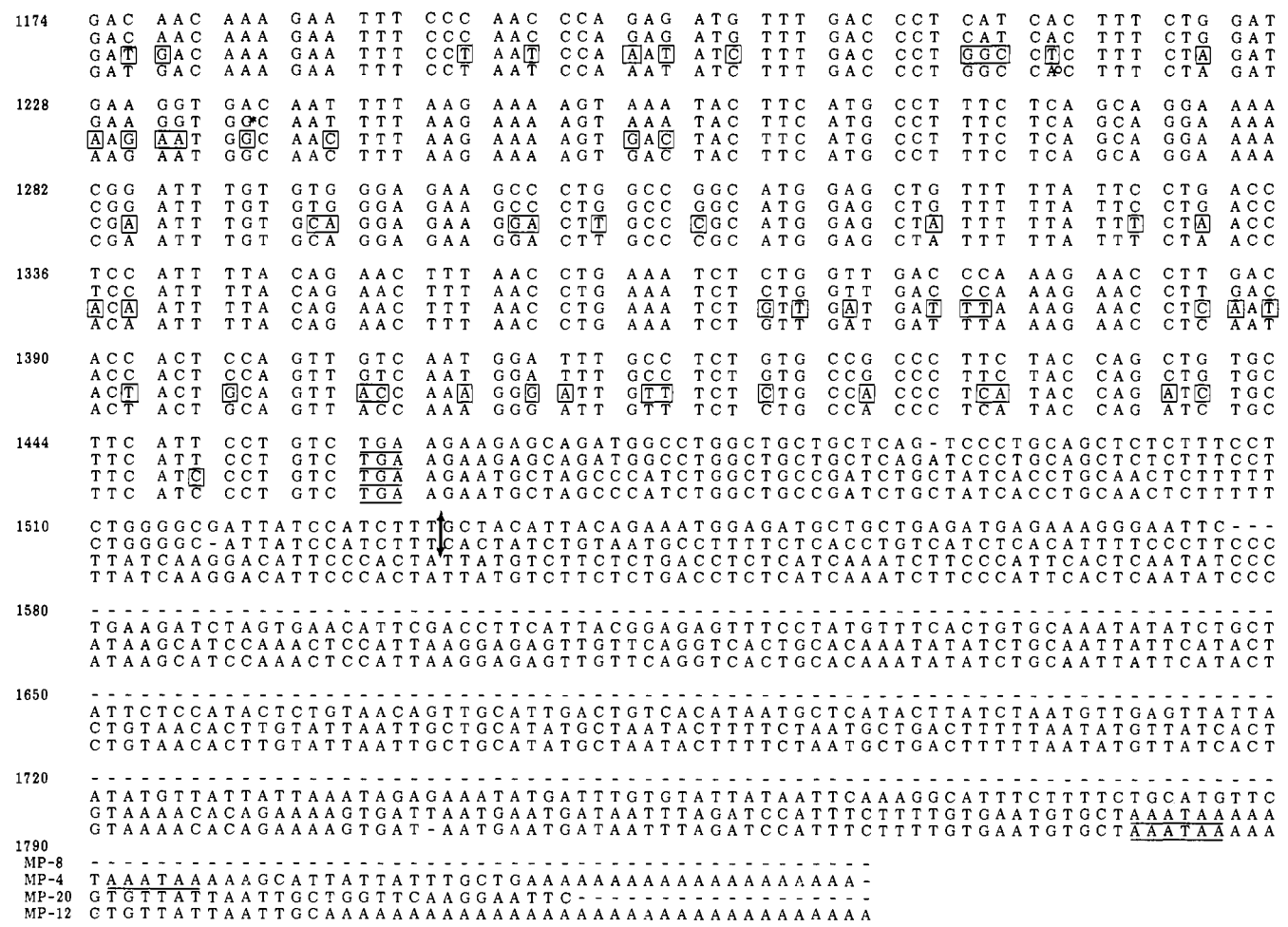


FIGURE 3: Nucleotide sequences of cDNA clones. All of the cDNA clones were derived from the liver of a single individual (Umbenhauer et al., 1987b) whose phenotype was not determined. MP-8 (line 1), MP-4 (line 2), MP-20 (line 3), and MP-12 (line 4) are shown. Numbers on the left indicate nucleotide position starting with the first nucleotide of MP-8. The dashed line (---) represents missing 5′ nucleotides, as judged by amino acid sequencing data. All sequences are aligned with MP-8 for comparison. Differences in the sequence of MP-4 and MP-8 are shown by asterisks (*), MP-20 dissimilarities with MP-8 are boxed, and differences between MP-12 and MP-20 are represented by small circles (O). The vertical arrow in MP-8 and MP-4 shows the origin of divergence between their 3′ untranslated regions. The termination codons (TGA) and the consensus polyadenylation signal (AATAA) are underlined. Clones MP-12 and MP-20 predict the same amino acid sequences differing in four residues, which differ from both MP-4 and MP-8, the predicted amino acid sequences of which also differ from each other. Yasumori et al. (1987) have very recently characterized a cDNA clone identical with our MP-4 cDNA except for the artifactual 5′ region. That clone, termed P-450 human-2, is nearly full-length (10 nucleotides missing on the 5′ end) and shows four single base changes when compared to our MP-8 coding sequence (two of these changes are conserved in MP-4). The untranslated 3′ noncoding region sequences of the MP-4 and P-450 human-2 (Yasumori et al., 1987) cDNAs are nearly identical. Kimura et al. (1987) also recently reported a clone (designated P450IIC2) that differs from our MP-4 in only two nucleotides of the protein coding region and is also nearly identical in the 3′ noncoding region. Okino et al. (1987) reported the isolation of a human cDNA clone (designated Hpl-1) nearly identical with MP-20, using a rabbit P-450 1 cDNA (Tukey et al., 1985) insert to screen a human liver cDNA library. Nucleotide sequences are in good agreement between MP-20 and Hpl-1 along the coding region except for four single base changes (two of those changes correspond to the MP-12 sequence) and also along the 3′ untranslated region (one base change). None of these changes give rise to amino acid replacements. Two cDNA clones were described, Hpl-1 and Hpl-2. Hpl-1 is the longest, encoding a putative 490 amino acid protein; the first 237 nucleotides are missing in Hpl-2, and Hpl-2 contains an additional 39-bp insert located upstream from the heme binding region and interrupted by two nonsense codons. These 39 bp are absent in our MP-12 and MP-20 clones, and no evidence was found for such clones in these screenings.

Table I: Catalytic Activities of Human Liver Microsomes and Purified P-450$_{MP}$ Preparations[a]

	nmol of product formed min^{-1} (nmol of P-450)$^{-1}$			
	microsomes[a] (n = 16)[b]	P-450$_{MP-1}$[a] (n = 3)[b]	HL 99 microsomes	HL 99 P-450$_{MP-3}$[c]
mephenytoin 4′-hydroxylation	0.15 (±0.03)	0.43 (±0.04)	0.068	0.0005 ($-b_5$)
hexobarbital 3′-hydroxylation	0.38 (±0.06)	4.8 (±0.6)	0.33	0.011 ($-b_5$) 0.009 ($+b_5$)
tolbutamide methyl hydroxylation	0.65 (±0.13)	4.3 (±0.6)	0.94	0.13 ($-b_5$) 0.10 ($+b_5$)

[a] Samples and data are from studies reported previously (Shimada et al., 1986; Knodell et al., 1987, 1988). [b] Samples derived from different individuals were used, and rates reported are means ±SD (n denotes the number of different samples used). [c] Rates were determined in the presence (+) and absence (−) of cytochrome b_5 [3-fold molar excess with respect to P-450$_{MP}$; Shimada et al. (1986)].

location described in the rabbit P-450 1 (Tukey et al., 1985) and rat P-450M$_1$ (Yoshioka et al., 1987) cDNAs.

Amino Acid Sequencing. During the course of this work, a preparation of what was first believed to be P-450$_{MP-1}$ was isolated from liver sample HL 99. The purification scheme was similar to that used for P-450$_{MP-1}$, and the procedure was monitored by NaDodSO$_4$–polyacrylamide gel electrophoresis/immunoblotting analysis with rabbit anti-P-450$_{MP-1}$.

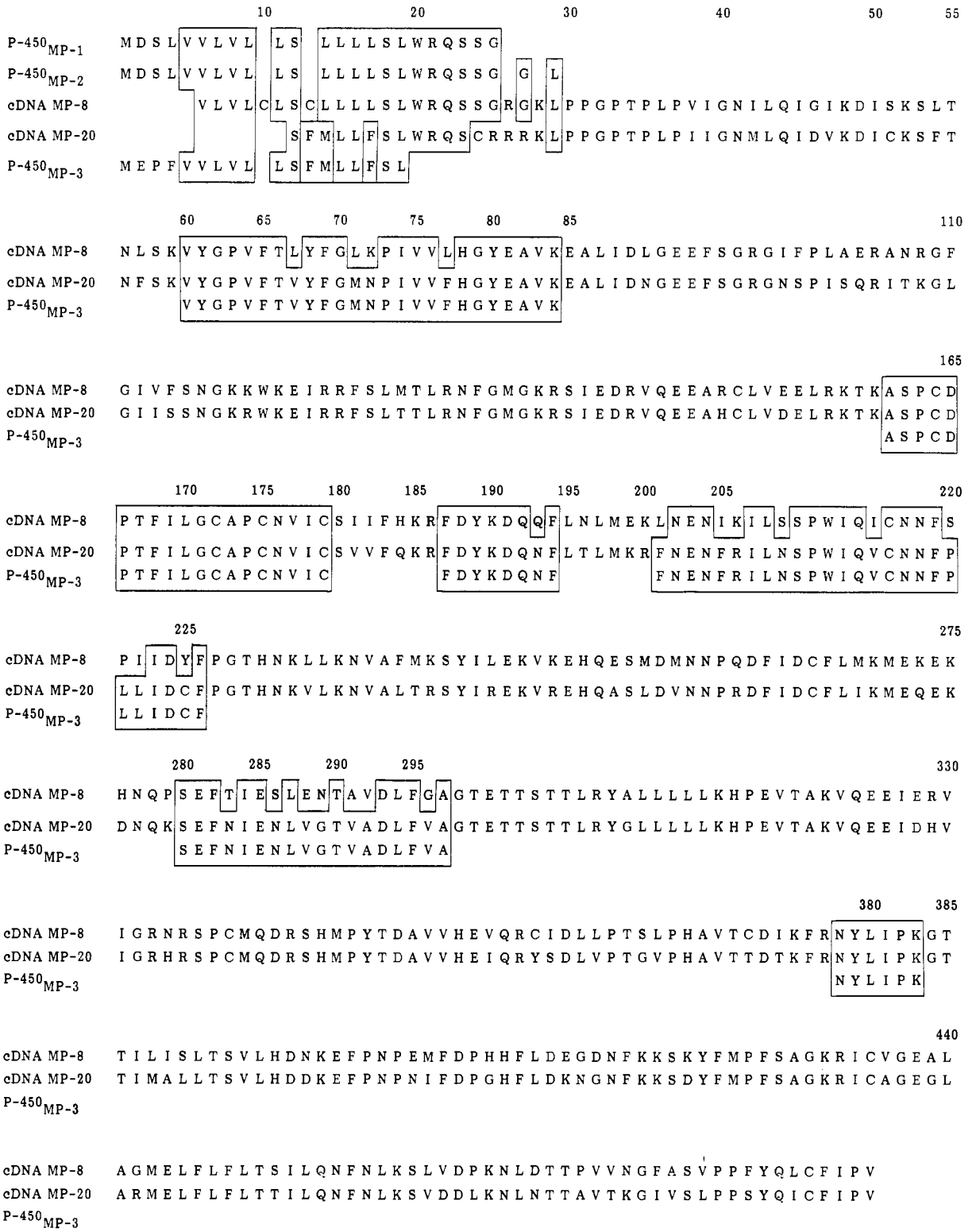


FIGURE 4: Amino acid sequences derived from proteins (P-450$_{MP-1}$, P-450$_{MP-2}$, P-450$_{MP-3}$) and predicted from cDNA clones (MP-8, MP-20). See Figure 3.[7]

Although the purified protein had an apparent monomeric M_r identical with that of P-450$_{MP-1}$ and displayed a typical P-450 ferrous–carbon monoxide near-UV spectrum after removal of detergent, it had relatively low catalytic activity toward mephenytoin, hexobarbital, and tolbutamide when compared to typical P-450$_{MP-1}$ preparations (Table I) and was termed P-450$_{MP-3}$.

Most of the sequences of the resolved tryptic peptides aligned with the protein structure predicted by cDNA clones MP-12 and MP-20 but not clones MP-4 and MP-8 (Figures 3, 4). However, some evidence was found for the presence of MP-8-specific amino acid residues in the N-terminal sequence, and a tetrapeptide (HNQP, data not shown) related to the MP-8-predicted sequence (possibly positions 276–279) was also found, although further residues could not be identified and the amount of this peptide present was difficult to establish.

Since previous N-terminal amino acid sequencing studies on catalytically active P-450$_{MP-1}$ and P-450$_{MP-2}$ preparations showed concordance with the MP-8 cDNA clone (Shimada et al., 1986; Umbenhauer et al., 1987b) and the P-450$_{MP-3}$

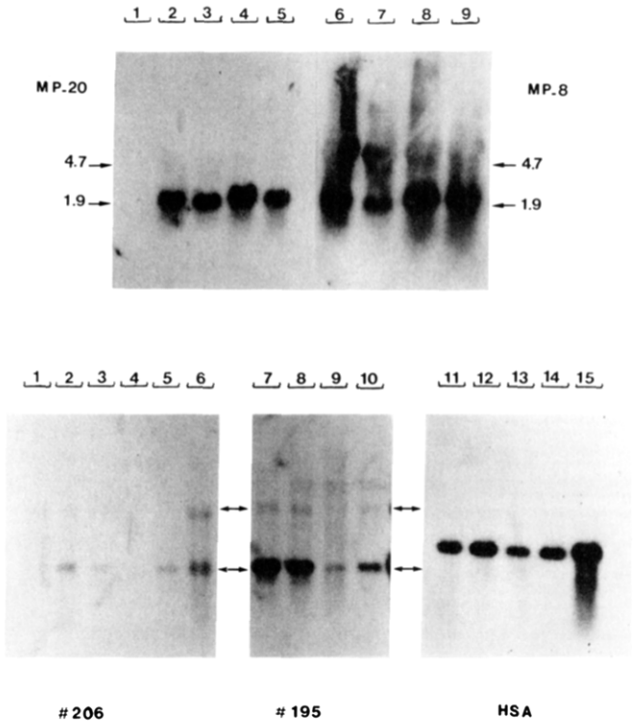


FIGURE 5: mRNA (Northern) blot analyses for MP-4/MP-8 and MP-12/MP-20 sequences. Upper panel: total RNA was probed with a nick-translated MP-8 insert (right) or MP-20 insert (left). As a negative control, total RNA from a lymphoblast cell line is shown in lane 1. Lanes 2–5 and 6–9 are duplicate blots of total RNA from human liver samples HL 96, HL 104, HL 105, and HL 110, respectively. Lower panel: poly(A+) RNA was probed with labeled oligomer 206 (left, 5′CTGTTAGCTCTTTCAGCCAG3′; T_m 60 °C), oligomer 195 (middle, 5′TTAGTAATTCTTTGAGATAT3′; T_m 48 °C), and an oligomer specific for human serum albumin mRNA (Umbenhauer et al., 1987b) (right). The oligomers match positions 290–309 (Figure 4). The same negative control (lymphoblast RNA) is in lane 1; lanes 2–5, 7–10, and 11–14 are triplicate blots of poly(A+) RNA from human liver samples HL 99, HL 101, HL 102, and HL 104, respectively. The rates of mephenytoin 4′-hydroxylation measured in microsomes prepared from these four liver samples were 59, 19, 40, and 12 pmol (product formed) min^{-1} (mg of protein)$^{-1}$, respectively. Lanes 6 and 15 represent total RNA from liver sample HL 96 and show nonspecific binding of 20-mer oligonucleotides to 18S and 28S RNA. Molecular weights of markers are given in kilobases (1.9, 4.7) and indicated with arrows (in both panels).

preparation, which was largely inactive toward the compounds tested here (Table I) and consisted primarily of a sequence related to the MP-12 and MP-20 clones, these latter sequences are apparently not related to mephenytoin 4′-hydroxylation. The low amount of activity may be due to the residual amount of P-450$_{MP-1}$. Proteins resembling P-450$_{MP-3}$ have been reported to show some *N*-demethylase activity toward *d*-benzphetamine (Wrighton et al., 1987) and aminopyrine (Lasker et al., 1987) (these activities were not examined in this work).[8]

RNA Studies. Blots of RNA probed with nick-translated MP-8 and either MP-12 or MP-20 showed the same pattern: a major band migrating at ~2.2 kb and a much lighter band at 3.5 kb. These results were not surprising, since MP-8 and MP-20 belong to the same gene family and have coding regions of a similar size. As shown previously (Umbenhauer et al., 1987b), cultured lymphoblasts provide a negative control with no mRNA corresponding to either MP-8 or MP-20 (Figure 5).

Experiments were designed with more specific probes in order to distinguish mRNA species related to individual cDNA clones. Oligomer 195 was specific for MP-20/MP-12 sequences; oligomer 206 was specific for MP-8/MP-4 sequences, and its sequence was located in the same region as oligomer 195 in the 5′ end portion of the cDNA. Specificity for the cloned cDNA sequences was checked (Figure 6) by using dot blots of MP-8 and MP-20 inserts (purified from M13 clones) under the same hybridization conditions as used for Northern blots (with hybridization and a short stringent wash performed 10 °C below the calculated T_m). As previously described (Umbenhauer et al., 1987b), a 51-mer oligonucleotide specific for human serum albumin was used as a standard for the amount of RNA actually transferred and subjected to hybridization. Interestingly, the liver samples examined had both messages (i.e., MP-8 *and* MP-20 sequences); among different liver samples some difference in levels of binding of the two probes was seen (e.g., compare HL 101 and 104 in Figure 5). Thus, expression of the different genes does not seem to be coordinately regulated. No correlation was found between rates of microsomal (*S*)-mephenytoin 4′-hydroxylase activity and any of the mRNA levels measured in the same samples (see legend to Figure 5). The complexity of this gene family and the apparent absence of catalytic activity of some of the proteins, such as P-450$_{MP-3}$ and perhaps others, might be responsible for this lack of correlation.

The two differences between the MP-4 and MP-8 cDNA clones (Figure 3) would result in the substitution of Tyr for Cys and of Gly for Asp in going from the MP-8 to the MP-4 deduced amino acid sequences, and these minor differences (in the C-terminal region) might or might not influence catalytic activity. Oligonucleotides (294, 295) that recognized the marked differences in the sequences of the 3′ noncoding regions of these genes were synthesized and used to examine the expression of mRNAs related to the two genes (Figure 7). [Oligonucleotide 295, for MP-4, actually matches cDNA MP-12/MP-20 better than MP-8; however, the number of mismatches–20/30–prevents hybridization at this degree of stringency (Figure 3).] The patterns of intensity among the four liver samples to which the probes hybridized were similar for oligomers 295 (3′ noncoding of MP-4) and 206 (coding region of MP-4/MP-8) (Figure 5). Thus, the MP-4 and MP-8 genes both exist and code for very closely related proteins and both genes are expressed in individuals. The relative levels of the mRNAs appeared to vary among individuals (Figure 7), and the genes are apparently not regulated in the same manner.

Specificity of Antibodies for Gene Products. The two human liver proteins P-450$_{MP-1}$ and P-450$_{MP-2}$ apparently exist together in some liver samples and can be separated by NaDodSO$_4$–polyacrylamide gel electrophoresis under appropriate conditions but cannot be distinguished by several of their physical properties. P-450$_{MP-2}$, the slower migrating of the two species, does not appear to give rise to P-450$_{MP-1}$ by proteolysis, as judged by the results of in vitro translation experiments (Shimada et al., 1986). In order to better characterize the proteins related to the gene family under consideration, specific monoclonal antibodies were sought. Hybridoma growth was observed in 74% of the wells resulting from the initial fusion. Fifteen of these wells were positive when initially screened by ELISA for reactivity with human P-450$_{MP-1}$ and P-450$_{MP-2}$. Three clones were selected on the

[8] Although the proteins designated P-450$_{MP-1}$ and P-450$_{MP-3}$ were not separated by NaDodSO$_4$–polyacrylamide gel electrophoresis in this work, Lasker et al. (1987) reported an apparent 5000-Da difference between P-450-C and P-450-B, two proteins whose N-terminal sequences are very similar, although not identical, to P-450$_{MP-1}$ and P-450$_{MP-3}$. The basis of these possible differences in properties is not clear; either the sequence differences or peculiarities associated with the electrophoretic systems could be involved.

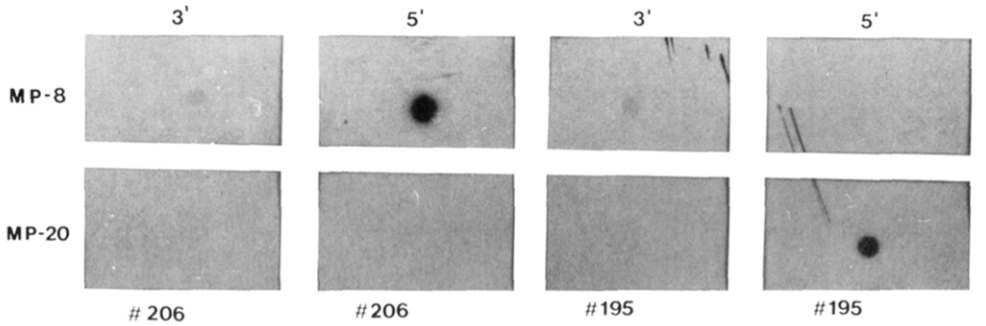


FIGURE 6: Oligomers 195 and 206 sequence specificity as demonstrated by dot blot analysis. Ten of the 20 bases were common to the two oligomers (see Figures 3 and 5). The 3′ and 5′ fragments (see text) of the MP-8 (upper panels) and MP-20 (lower panels) inserts were spotted on nitrocellulose in duplicate and probed with ^{32}P-end-labeled oligomers 206 (left side) and 195 (right side), which are specific for MP-8 cDNA and MP-20 cDNA, respectively.

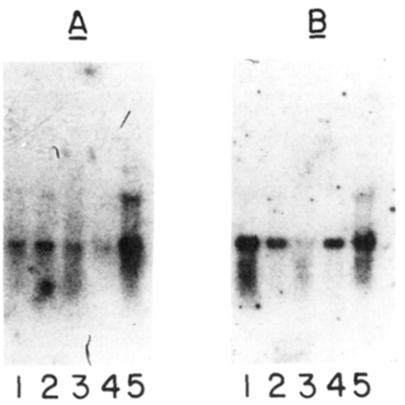


FIGURE 7: mRNA (Northern) blot analyses for MP-4 and MP-8 sequences. Poly(A+) RNA preparations from human liver samples HL 99, HL 101, HL 102, and HL 104 (lanes 1–4) and total RNA from human liver sample HL-96 (lane 5) were separated by electrophoresis and hybridized with ^{32}P-labeled probes for cDNA clones MP-4 (oligomer 295, part A) and MP-8 (oligomer 294, part B). Both oligomer probes were end-labeled at the same time and have approximately the same specific radioactivity. Oligomers 294 (MP-8, 5′TCTCAGCAGCATCTCCATTTCTGTAATGTA3′; T_m 75 °C) and 295 (MP-4, 5′ATGACAGGTGAGAAAAGGCATTACAGATAG3′; T_m 75 °C) correspond to positions 1534–1563. Only 3 of 30 bases match, and no cross-hybridization was detected with inserts under the conditions used in the mRNA work (data not shown).

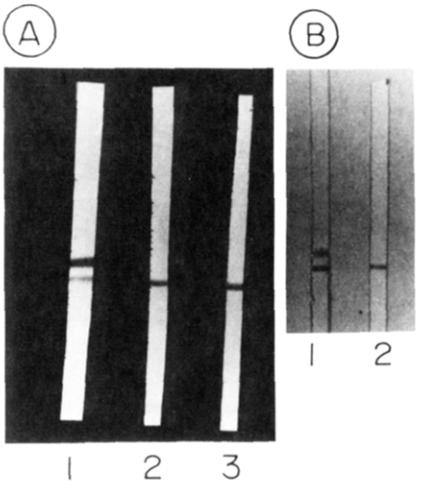


FIGURE 8: Immunoblotting analysis of human liver microsomal proteins. In each lane, a sample of human liver microsomes (HL 97, 15 μg of protein) was electrophoresed in an NaDodSO$_4$–polyacrylamide gel system (Laemmli, 1970), and the resolved proteins were electrophoretically transferred to a sheet of nitrocellulose and treated with the indicated antibody, followed by the visualization procedure described under Experimental Procedures. (A) Lane 1, mouse polyclonal anti-P-450$_{MP-1}$; lanes 2 and 3, two different clones of monoclonal antibody 1C6B6B5. (B) Lane 1, monoclonal antibody 7A2D12; lane 2, monoclonal antibody 1C6B6B5.

basis of NaDodSO$_4$–polyacrylamide gel electrophoresis/immunoblotting results for further evaluation: 7A2D12 (IgG$_1$, IgK) reacted with P-450$_{MP-1}$ and P-450$_{MP-2}$, 1C6B6B5 (IgM, IgK) reacted only with P-450$_{MP-1}$, and 4D4H9 (IgG$_1$, IgK) was negative in this assay (Figure 8).

P-450$_{MP-1}$ and P-450$_{MP-3}$, described above, both react with polyclonal anti-P-450$_{MP-1}$ are not separated in our electrophoretic systems. Since the cDNA clones under consideration here are nearly full-length (Figures 2, 3), the use of λgt11 β-galactosidase fusion products provided a definitive screen for antibodies related to the MP-4/MP-8 and the MP-12/MP-20 sequences. Monoclonal antibody 1C6B6B5 recognized the MP-4 and MP-8 fusion proteins but not proteins expressed by the MP-12 or MP-20 inserts or λgt11 itself (Figure 9).

Thus, antibody 1C6B6B5 recognizes P-450$_{MP-1}$ but not P-450$_{MP-3}$, which appears to be ~85% identical as judged by cDNA studies (Figures 3, 4), or P-450$_{MP-2}$, whose sequence beyond the immediate N-terminal region is unknown. The data reinforce the view that P-450$_{MP-2}$ is a protein distinct from P-450$_{MP-1}$, although its sequence remains undefined. (Antibody 1C6B6B5 did not inhibit mephenytoin 4-hydroxylase activity.) Because of the specificity of the 1C6B6B5 antibody, the relationship between reactivity (with this antibody) and mephenytoin 4-hydroxylase activity was reexamined in a group of 13 randomly selected human liver microsomal samples that

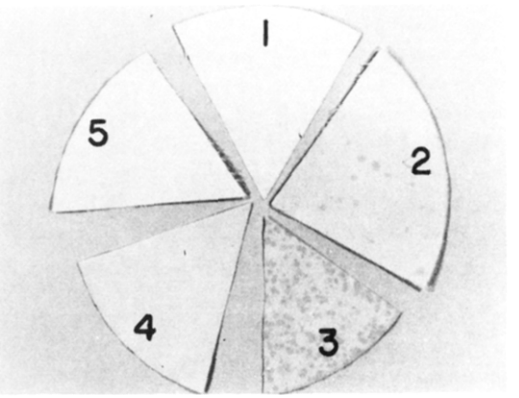


FIGURE 9: Staining of λgt11 fusion proteins with monoclonal antibody 1C6B6B5. λgt11 phage containing the inserts MP-4, MP-8, MP-12, and MP-20 infected *E. coli* Y 1090, and plaques were lifted onto nitrocellulose paper after 8 h as in the general screening procedure, except that 0.45 μm filters were used to reduce the background. Sections of the plaque lifts were developed with the monoclonal antibody as described under Experimental Procedures. Section: (1) λgt11 without insert; (2) MP-4; (3) MP-8; (4) MP-12; (5) MP-20.

varied 25-fold in rates of specific catalytic activity, by quantitation of NaDodSO$_4$–polyacrylamide gel electrophoresis/immunoblotting assays. No significant correlation was ob-

served ($r = 0.015$, $p > 0.10$), consonant with previously reported (Shimada et al., 1986) and subsequent studies with polyclonal anti-P-450$_{MP-1}$ and anti-P-450$_{MP-2}$.

Conclusions

It is important to understand details regarding the individual gene segments in this complex human P-450IIC family for a number of reasons. Both the work of Okino et al. (1987) and our own (Riddell et al., 1987) show the clustering of the genes on human chromosome 10. The observation that immunohistochemical localization of the proteins shows a diffuse pattern in liver (Ratanasavanh et al., 1986) must be considered with the caveat that individual products may be regionally distributed. The mechanisms of regulation of individual genes must also be considered. No immunochemically detected P-450$_{MP}$ protein or mRNA related to a full-length MP-8 cDNA probe can be detected in fetal liver (Cresteil et al., 1985; Shimada et al., 1986; Umbenhauer et al., 1987b), and it is known that levels of protein detected with rabbit anti-P-450$_{MP-1}$ decrease in primary hepatic tumors (El Mouelhi et al., 1987) and catalytic activities toward tolbutamide, hexobarbital, and mephenytoin decrease in liver disease and can be increased by rifampicin treatment (Zilly et al., 1975; Zhou et al., 1988). However, the question of how the expression of each gene product is regulated is still open.

Currently, protein preparations can be grouped into two categories. P-450$_{MP-1}$ and P-450$_{MP-2}$ from our own laboratory (Shimada et al., 1986), P-450 human-2 (Yasumori et al., 1987), and P-450-C of Lasker et al. (1987) fall into one group, which corresponds to our MP-8 and MP-4 clones [and the recently reported P-450 human-2 (Yasumori et al., 1987) and P-450IIC2 (Kimura et al., 1987) cDNA clones]. The reported N-terminal amino acid sequence of P-450-C differs from P-450$_{MP-1}$ and P-450$_{MP-2}$ in one residue (10), as deduced by cDNA clones [in the amino acid sequencing, we have been unable to identify residues 10 and 13 [Shimada et al. (1986) and unpublished subsequent work]]. The second group of P-450s is related to the MP-12 and MP-20 clones [and, recently reported, the clones of Okino et al. (1987) and the P-450IIC1 cDNA clone (Kimura et al., 1987)] and includes P-450 HLx (Wrighton et al., 1987), P-450-B (Lasker et al., 1987), and P-450$_{MP-3}$ proteins (this report). The N-terminal amino acid sequence of P-450-B differs from the others at two positions (13, 17). Evidence presented here is not conclusive but suggests that the MP-4/MP-8 sequence is most likely to be related to (*S*)-mephenytoin 4′-hydroxylase activity. The possibility exists that tolbutamide hydroxylation may be catalyzed by a distinct but related P-450 (Knodell et al., 1987).

In conclusion, there exist (at least) two categories of genes in this family whose products can both be expressed concurrently. Evidence was obtained for this view in livers of individuals at three levels: cDNA clones (Figures 2, 3), mRNA (Figures 5, 7), and protein (Figures 4, 8). Further, the MP-4 and MP-8 genes appear to code for nearly identical proteins and transcription of both genes also occurs in individuals. It is possible that both the MP-4 and MP-8 gene products may be active mephenytoin 4′-hydroxylases. Firm conclusions as to the mechanism underlying the polymorphism still cannot be drawn. The lack of correlation of activity with mRNA levels measured with the oligonucleotide probes suggests the lack of a major role for transcriptional regulation underlying the interindividual differences in catalytic activity, although the probes may still not be specific enough for analyzing this multigene family. The evidence for induction of catalytic activity by drugs has been alluded to above. Thus, in several regards the polymorphism would appear to differ from that of human P-450$_{DB}$, the debrisoquine 4-hydroxylase (Distlerath & Guengerich, 1984; Gonzalez et al., 1988). While no restriction fragment length polymorphisms have been found to be related to mephenytoin 4-hydroxylase activity, further analysis may be necessary.

Acknowledgments

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Supplementary Material Available

A table of the amino acid sequence of peptides derived from P-450$_{MP-3}$ isolated from liver sample HL 99 (1 page). Ordering information is given on any current masthead page.

Registry No. P-450, 9035-51-2; (*S*)-mephenytoin 4′-hydroxylase, 96779-46-3.

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