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Isolation and sequence determination of a cDNA clone related to human cytochrome P-450 nifedipine oxidase

(gene cloning/polymorphism/dihydropyridines/calcium blockers)

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ABSTRACT **Human liver cytochrome P-450$_{NF}$ is the form of cytochrome P-450 responsible for the oxidation of the calcium-channel blocker nifedipine, which has been reported to show polymorphism in clinical studies. By screening a bacteriophage λgt11 expression cDNA library, we isolated two clones: NF95 with an insert length of 0.8 kilobases which gave a stable fusion protein and NF25 with an insert length of 2.2 kilobases. The two clones were both sequenced and shown to be identical in their overlapping section. The sequence of NF25 is 77% similar to that reported for a rat cytochrome "P-450$_{PCN}$" cDNA (PCN = pregnenolone-16α-carbonitrile). The similarity decreases to 45–53% when the sequence is compared to human cytochromes P-450 belonging to other families [i.e., "pH P-450(1)," "P$_1$-450," "P$_3$-450," and "P-450$_{MP}$." The deduced amino acid sequence is 73% similar to that of rat cytochrome P-450$_{PCN}$, and the first 21 amino acids are identical to those reported for human liver cytochrome "P-450p." Sections of these clones were nick-translated and used as probes for analyses of human mRNA and genomic DNA. The number and size of bands indicate that P-450$_{NF}$ belongs to a multigene family, the so-called pregnenolone-16α-carbonitrile-inducible family.**

Cytochrome P-450 (P-450) plays an important role in the oxidation of drugs and carcinogens as well as endogenous substrates. Interindividual variation in oxidative metabolism can be attributed, at least in part, to the composition of individual P-450 forms, and these differences are partly due to genetic factors. Since 1977, several genetic polymorphisms of drug oxidation have been demonstrated (1–3), and in some cases the involved form of P-450 has been identified in humans (4, 5). Recently Kleinbloesem *et al.* (6) reported that oxidation of nifedipine, a vasodilator and calcium-channel blocker, was distributed in a polymorphic manner—17% of the Dutch population studied were phenotypically poor metabolizers. Recently we identified and purified the human liver P-450 form that is responsible for this oxidation of nifedipine (7). This protein, P-450$_{NF}$, was shown to be related or identical to P-450s previously isolated from human liver in this laboratory (8). To better understand the mechanism underlying this polymorphism, we used polyclonal and monoclonal antibodies to screen a human liver bacteriophage λgt11 cDNA expression library. The selected clones were analyzed, sequenced, and used to prepare nick-translated probes for the analysis of mRNA and genomic DNA.

MATERIALS AND METHODS

Enzymes and Antibodies. Human livers were obtained through the Nashville Regional Organ Procurement Agency, and protocols were approved by the Vanderbilt Committee for the Protection of Human Subjects. Livers were perfused immediately after circulatory arrest, chilled on ice, and brought to the laboratory. The livers were cut in small pieces, frozen in liquid nitrogen, and stored at −70°C (8).

P-450$_{NF}$ was purified as described, and polyclonal and monoclonal antibodies were produced (7, 9). These antibodies recognized a single band migrating with purified P-450$_{NF}$ when human liver microsomes were electrophoresed and immunoblotted (7, 9). Before use in screening the λgt11 library, antibodies were adsorbed twice overnight at 4°C with *Escherichia coli* (BNN97) (10) lysate bound to CNBr-activated Sepharose 4B (Pharmacia, Piscataway, NJ) in order to eliminate reaction with *E. coli* proteins; after immunoadsorption, the antibody reactivity toward P-450$_{NF}$ and toward *E. coli* was assayed.

Library Screening. The human liver cDNA phage λgt11 expression library was a gift of G. A. Ricca and W. Drohan, Meloy Laboratories (Springfield, VA). It was screened with both polyclonal and monoclonal antibodies basically as described by Young and Davis (10); the nitrocellulose filters were developed with an immunochemical technique (11) using 4-chloro-1-naphthol in place of 3,3′-diaminobenzidine (12). Antisera or ascites fluids were used at dilutions of about 1:50. Positive plaques were checked and plaque-purified by at least two additional rounds of dilution and screening. High-titer phage stocks were purified through CsCl step and equilibrium gradients (13). λgt11 DNAs were prepared for restriction mapping and subcloning in phage M13 (14). Lysogens were prepared by infection of Y1089 *E. coli* with λgt11 phage clones, and colonies were selected for growth at 30°C and not 42°C. Fusion proteins were produced by initially growing cells in exponential phase at 30°C, heat-shocking them at 42°C for 15 min, and then growing them at 37°C in the presence of 10 mM isopropyl-β-D-thiogalactose until the A_{600} stabilized (between 0.5 and 2 hr). Cells were then collected by centrifugation at $10^4 \times g$ for 10 min, and the pellet was solubilized for electrophoresis, with subsequent visualization of bands by silver staining or immunoblotting.

Subcloning and Sequencing. λgt11 DNA containing inserts was digested with *Eco*RI, *Hin*dIII, *Sac* I, or *Hin*dIII/*Sac* I and was subcloned in M13 phage as described (15), except that the UT481 strain was used instead of JM101 or JM103. *E. coli* UT481 was constructed by and obtained from C. Lark (Salt Lake City, UT). M13 plaques containing inserts were plaque-purified, and single- and double-stranded DNAs were prepared as described (15); the orientation was checked by asymmetric restriction digestion (i.e., *Pst* I or *Bam*HI).

Single-stranded M13 DNA was used as a template for DNA sequencing by the dideoxy termination method (16). DNA sequencing kits were obtained from New England Nuclear

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Abbreviations: P-450, liver microsomal cytochrome P-450; PCN, pregnenolone-16α-carbonitrile; kb, kilobase(s).
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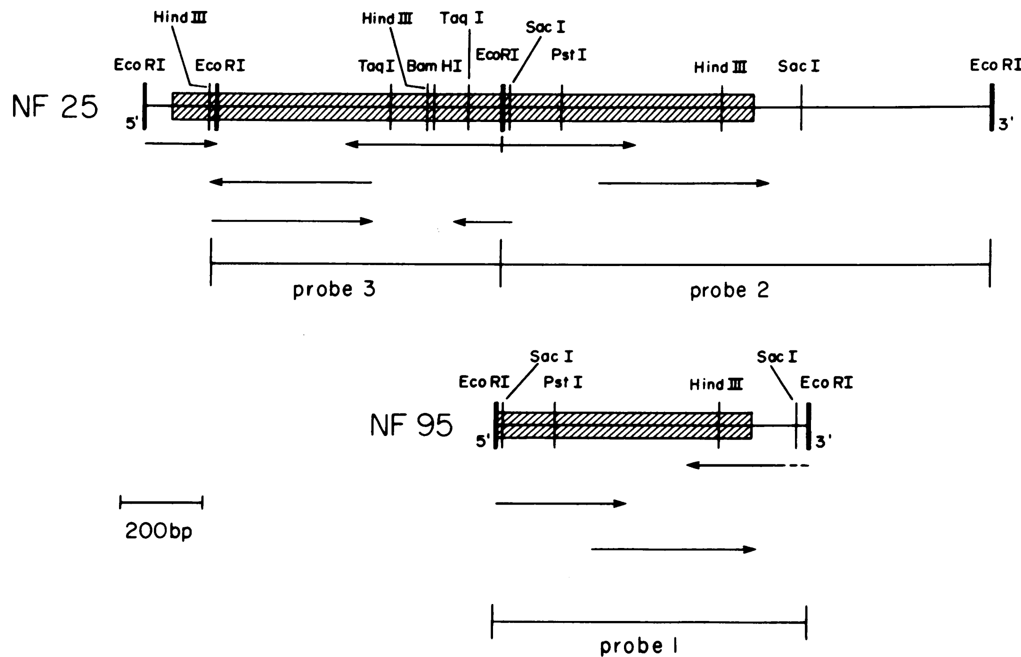

FIG. 1. Restriction map of clones NF95 and NF25 and sequencing strategy. The hatched region is the coding sequence. Arrows indicate the direction and extent of sequence determined for cDNA fragments subcloned in M13mp19 or M13-mp18. Recognition sites for restriction nucleases are indicated. Except for *Taq* I, these were all determined experimentally and confirmed by computer analysis of the sequence.

and used according to the supplier's instructions except that the incubation temperature was 37°C and no NaCl was included in the buffer. Additional sequencing primers were 18-mers or 20-mers synthesized with a BioSearch Sam-One Series II DNA Synthesizer; oligonucleotides were purified as described by Lloyd *et al.* (17). Sequences were compared with the aid of computer access to the protein sequence data base of the National Biomedical Research Foundation.†

Blotting Analysis of RNA and DNA. Genomic DNA and total RNA were isolated from the same preparation by using the CsCl cushion method described by Chirgwin *et al.* (18). The RNA was collected as a pellet on the bottom of the centrifuge tube, dissolved in water, and precipitated with 0.1 vol of sodium acetate and 2 vol 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 phenol/$CHCl_3$, 1:1 (vol/vol) and once with $CHCl_3$ and then was precipitated with ethanol. The DNA was dissolved in 10 mM Tris·HCl buffer, pH 8.0/1 mM EDTA, treated with proteinase K (50 μg/ml) in 0.1% sodium dodecyl sulfate, and reextracted with phenol/$CHCl_3$ and $CHCl_3$ as above. The purified DNA was then precipitated with ethanol. For Southern blots, 20 μg of genomic DNA was cut with various restriction enzymes and electrophoresed through a 0.8% agarose gel. The gel was processed by the Wahl *et al.* (19) modification of the Southern (20) procedure. The DNA was transferred to GeneScreen*Plus* (New England Nuclear) and processed as suggested by the supplier. For RNA blot analyses, 15 μg of RNA was submitted to agarose/formaldehyde electrophoresis as described (21). RNA was then transferred to GeneScreen*Plus* (New England Nuclear) and processed as suggested by the supplier. DNA and RNA blots were probed with nick-translated DNA inserts ($\approx 10^8$ dpm/μg). DNA fragments were purified from agarose gels basically as described (22). Hybridization and washings were performed as described by the supplier. Filters were then autoradiographed with Kodak XAR film (Kodak) with two screens from 3 to 7 days at −70°C.

†National Biomedical Research Foundation (1986) *Protein Sequence Data Base of the Protein Identification Resource* (Washington, DC), Release No. 8.0.

RESULTS AND DISCUSSION

Isolation and Characterization of cDNA Clones. A human liver cDNA expression library in bacteriophage λgt11 was screened with polyclonal and monoclonal antibodies raised against P-450_{NF}. After the screening of a total of 3×10^5 plaques, 15 clones were positively identified by both antibodies. These clones were characterized by insert length and by the presence of fusion proteins in lysogen preparations. Two clones were selected: NF25, the insert of which was long

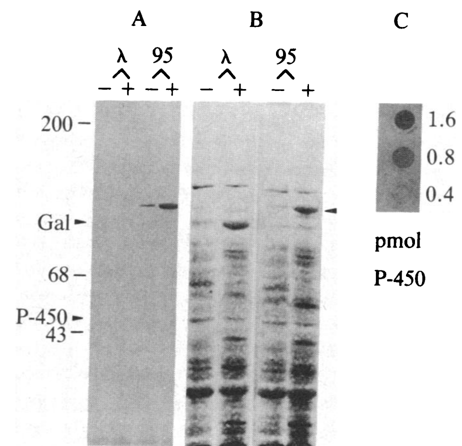

FIG. 2. (*A* and *B*) Electrophoresis and immunoblotting of λgt11 lysogen proteins: immunoblotting with anti-P-450_{NF} (*A*) and silver-staining of the gel (*B*). Lanes: λ, *E. coli* lysogen infected with λgt11 bacteriophage without insert; 95, *E. coli* lysogen infected with λgt11 clone 95; −, cells grown at 30°C pelleted and electrophoresed; +, cells grown at 30°C, heat-shocked 15 min at 42°C, and treated with isopropyl-β-D-thiogalactose. Molecular mass is shown in KDa. P-450 and Gal indicate the migration positions of P-450_{NF} and β-galactosidase, respectively. The arrowhead at the right indicates the migration of the fusion protein. Clone NF25 gave results similar to those in lanes λ. Development of immunoblots with anti-β-galactosidase visualized β-galactosidase in lane λ+ and the fusion protein with a higher molecular mass in lane 95+. (*C*) Various amounts of purified P-450_{NF} (from sample HL 93) (7) were spotted on nitrocellulose with a "dot" apparatus, incubated with a 1:50 dilution of rabbit antibody raised against β-galactosidase/clone NF 95 fusion protein (see text), and developed as described (11, 12).

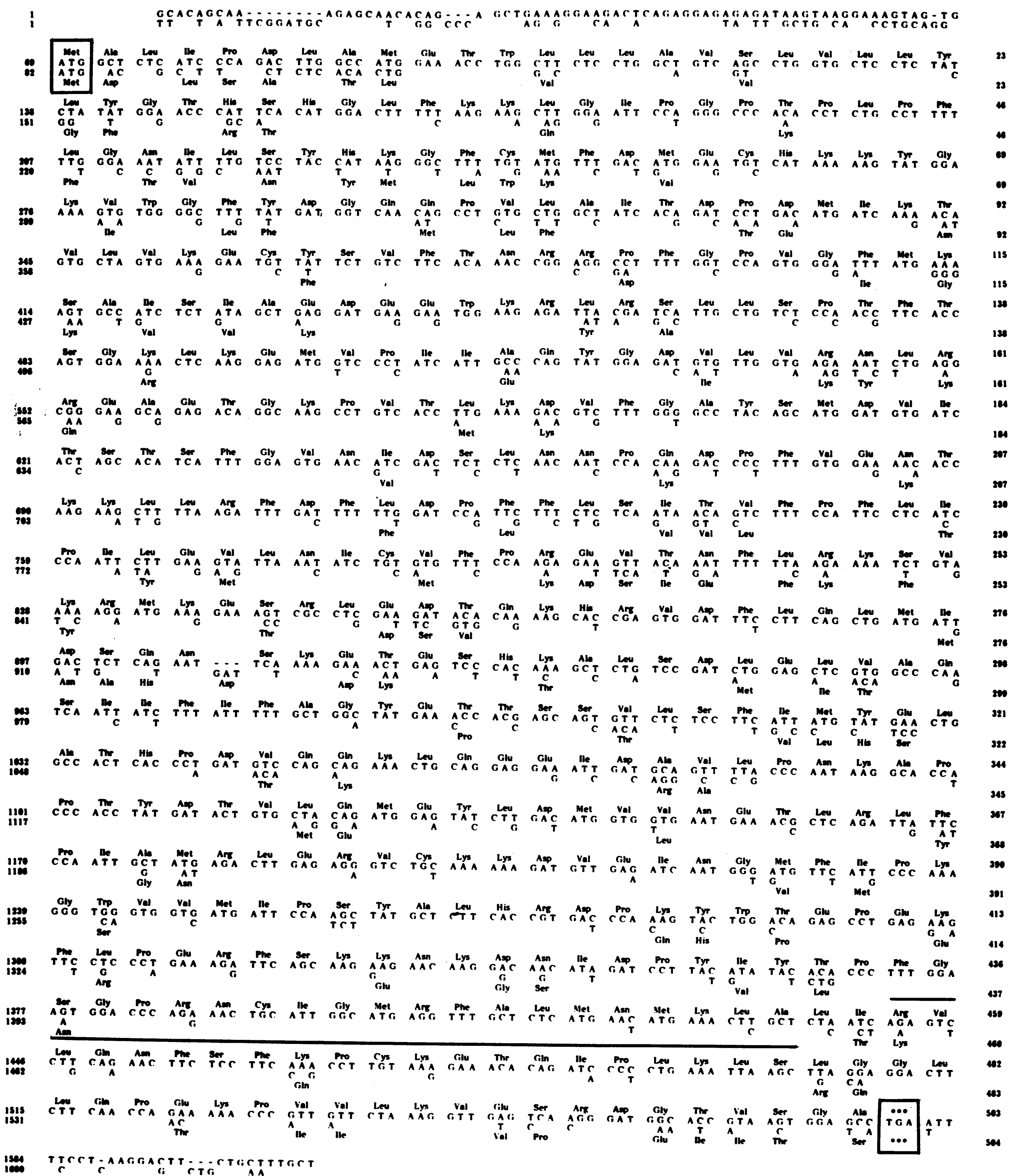

FIG. 3. Nucleotide and deduced amino acid sequences of NF25 (upper line) and comparison with rat P-450_{PCN} sequence (24) (lower line). Only the differences from NF25 are indicated in the rat P-450_{PCN} sequence. Initiation and termination codons are boxed. The cysteine-containing peptide thought to bind heme (25) is underlined. Dashes indicate the three-nucleotide gap introduced to increase the base and amino acid match.

enough to code for the entire protein (Fig. 1), and NF95, which gave a very strong antibody response and produced a fusion protein recognized by both anti-β-galactosidase and monoclonal and polyclonal anti-P-450_{NF} antibodies (Figs. 1 and 2). The increase in molecular weight of the fusion protein (compare β-galactosidase) correlated well when compared to the length of the insert (Fig. 2 *A* and *B*). Antibodies were raised against the fusion protein from NF95 by transferring the fusion protein to nitrocellulose paper, solubilizing the nitrocellulose in dimethylsulfoxide, and injecting this material into rabbits (23). These antibodies recognized P-450_{NF} (Fig. 2*C*). NF25 contained two internal *Eco*RI sites, which

were used for subcloning into M13, while NF95 did not contain any internal *Eco*RI sites. Three probes were prepared by nick-translation of restriction fragments of insert DNA (probes 1, 2, and 3; see Fig. 1) obtained after subcloning in M13. These were used to confirm the alignment of the subinserts of NF25 by Southern blotting of λgt11 DNA that had been cut with *Eco*RI and electrophoresed through a 1% agarose gel. Probe 1 recognized the same subinsert as probe 2 (data not shown). All other λgt11 clones selected with antibodies were recognized by probe 1.

Sequence Analysis of NF95 and NF25. NF25 and NF95, in phage M13, were sequenced by the dideoxy termination method (16) using the strategy indicated in Fig. 1. Each region represented by an arrow was sequenced between three and eight times. The sequence is shown in Fig. 3 along with the deduced amino acid sequence. The NF25 insert is 2.2 kilobases (kb) long and the sequenced portion represents 1606 nucleotides, including 68 nucleotides in the 5′ untranslated region, 29 nucleotides in the 3′ noncoding region, and an open reading frame of 1509 nucleotides, which corresponds to a protein of 503 residues. The termination codon is TGA, the most common termination codon published in known human and mouse sequences (26). The position −3 upstream from the initiation codon is a purine, as for all known eukaryotic sequences (27).

The deduced amino acid sequence was compared to those of two P-450s, rat P-450_{PCN} (24) (PCN = pregnenolone-16α-carbonitrile) and human P-450p (28); the latter is known to be highly similar or identical to P-450_{NF} by comparison of the procedures of purification (8, 28) and by immunochemical cross-reactivity (7, 28). The first 21 amino acids for NF25 (deduced sequence) and P-450p (28) are identical (Fig. 4). P-450_{PCN} (24) and NF25 share 77% nucleotide similarity, and only a one-codon gap has to be introduced (P-450_{PCN} has 504 amino acids instead of 503 for NF25). The NF25 nucleotide sequence shares 53%, 45%, 45%, and 47% similarity, respectively, with human pH P-450(1) (31), P_1-450 (26), P_3-450 (32), and P-450_{MP} cDNA coding sequences (D.R.U., R.S.L., and F.P.G., unpublished results) (5), respectively, as analyzed with these programs, indicating that P-450_{NF} belongs to a family different from the phenobarbital- and 3-methylcholanthrene-inducible families—the rat and rabbit PCN-inducible P-450 gene family (25). A more extensive comparison of the sequences of the N-terminal region and C-terminal cysteine-containing peptide, a highly conserved region, is shown in Fig. 4. All P-450s of the PCN-inducible family (human P-450_{NF}, rat P-450p, rat P-450_{PCN}, and rabbit P-450 LM3c) have similar N-terminal sequences, but they are not similar to human P_1-450 and P-450_{MP}. The heme ligand cysteine-containing peptide is more conserved within the gene families than within species: the nucleotide sequence similarity in this region can reach 96% within a family (P-450_{NF} versus P-450_{PCN}, Fig. 4). The cDNA-deduced amino acid composition was compared with the one obtained with purified P-450_{NF} (7); the difference index of 7.5 shows that they fit well (33). The amino acids that show the greatest deviation (glycine and alanine) are those that are known to generally yield discrepancies between amino acid analysis and cDNA-predicted sequences (34). Comparison of rat P-450_{PCN} and P-450_{NF} compositions gave a difference index of 3.7, confirming the strong relationship between the two proteins. The deduced molecular weight of the P-450_{NF} protein is slightly higher than the one estimated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis, as is the case for other hydrophobic P-450s, which may bind more sodium dodecyl sulfate than soluble proteins (34). A complete restriction map was obtained by computer analysis of the sequence and fitted perfectly with the experimental map (*Bam*HI, *Cla* I, *Eco*RI, *Hin*dIII, *Kpn* I, *Pst* I, *Pvu* I, *Sac* I, and *Sal* I). NF95 was also sequenced and, in the overlapping region with NF25, no differences were seen, indicating that both clones were probably derived from the same mRNA.

mRNA and Genomic DNA Analysis. Genomic DNA was cut with three different restriction enzymes, and the fragments were analyzed by Southern blotting (Fig. 5). Several bands were obtained with the three different cuts and the two nonoverlapping probes used (probes 2 and 3), even when relatively high-stringency hybridization conditions were used, indicating that P-450_{NF} very likely belongs to a multigene family. The size (50–60 kb with the two probes) and the numbers of bands practically exclude the existence of a single gene. Liver samples HL 32 and HL 34 showed some small differences in Southern blotting patterns (Fig. 5*A*); comparison with three other liver DNAs has not shown any specific pattern for poor or extensive metabolizers to date.

The multiplicity of P-450_{NF} genes was confirmed by Northern blot analysis (Fig. 5*B*), which shows that probe 1 recognizes three or four bands, indicating that there are several transcripts, the sizes of which (≈2–4 kb) are all compatible with the size of the protein and the lengths of other P-450 mRNAs (25). Clearly the major size class is about 2.0 kb, although the number of distinct transcripts within this

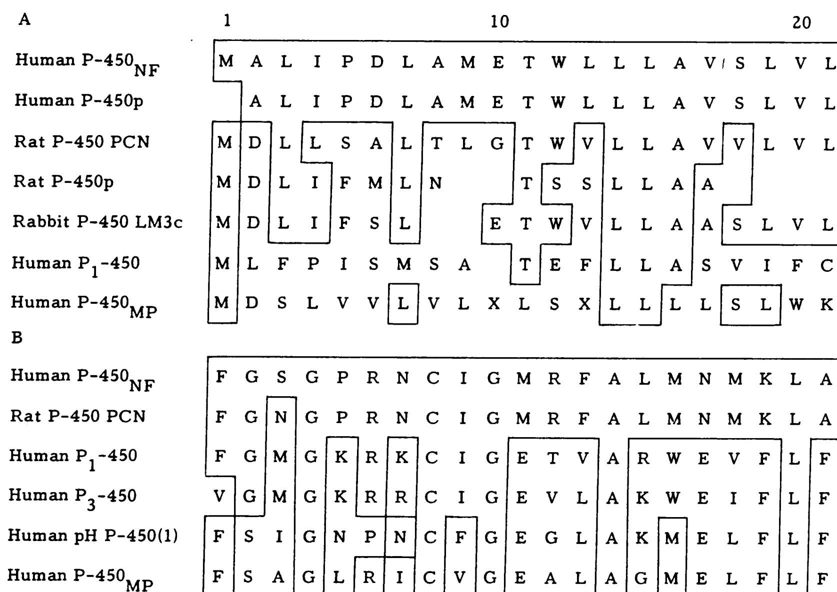

FIG. 4. Comparison of deduced amino acid sequences in two regions: N-terminal region (*A*) and the heme-binding cysteine-containing peptide (*B*) (25). Important similarities between the sequences are boxed. Amino acid sequences were obtained from the following sources in parentheses: rat P-450_{PCN} (24), rat P-450p (29), rabbit P-450 LM3c (30), human P-450p (28), pH P-450(1) (31), human P_1-450 (26), human P_3-450 (32), human P-450_{MP} (D.R.U., R.S.L., and F.P.G., unpublished results).

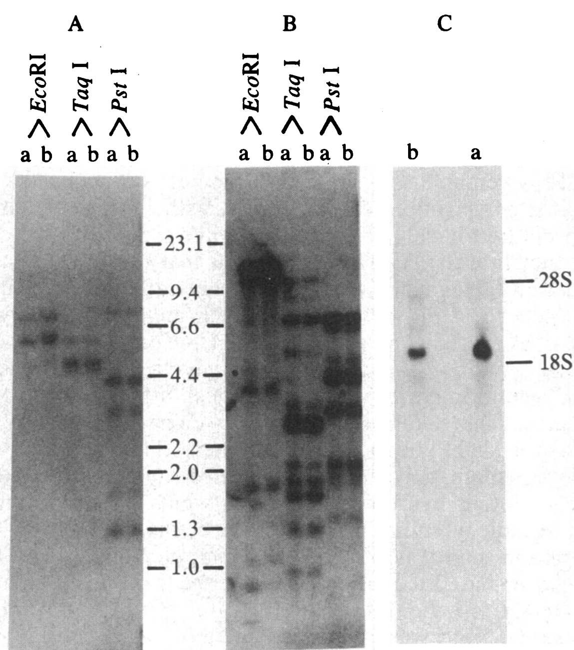

FIG. 5. DNA and RNA analysis by blotting. Microsomes prepared from human liver samples HL 32 (lanes a) and HL 34 (lanes b) oxidized nifedipine at rates of 0.88 and 0.65 nmol of product formed per min/nmol of P-450, respectively. (*A* and *B*) Southern DNA blots. DNA was loaded (20 μg) and hybridized with probe 3 (*A*) and probe 2 (*B*). (*C*) RNA blots. RNA was loaded (15 μg) and hybridized with probe 1. Probes 1 and 2 gave similar results. Restriction enzymes used are indicated at the tops of the lanes. Ribosomal RNA markers are indicated at 18S and 28S. Sizes are shown in kb.

zone is unknown, and we do not know conclusively which mRNA size-class codes for the protein. Our previous work suggested that catalytic activity is related to the amount of immunochemically detectable P-450$_{NF}$ (7), and these results have been confirmed. Analysis of mRNA levels in nine liver samples (with probe 2) showed a 20-fold variation [after normalization of mRNA hybridization with an oligomer (50-mer) complementary to the 5′ end of human serum albumin mRNA (35)]. However, the mRNA levels could not be correlated to either levels of nifedipine oxidase activity or immunochemically determined P-450$_{NF}$ in microsomes prepared from this set of samples.

CONCLUSIONS

While cDNA clones have been previously obtained for human P-450s (26, 31, 32), in no case has a sequence been obtained that is related to a P-450 protein that has been isolated and characterized in terms of physical properties and catalytic activity. We obtained a sequence for a cDNA related to a major human liver P-450 that appears to be involved in an oxidation polymorphism where a significant population is affected (6). The protein has been suggested to be inducible in humans (28), and other work in our laboratory indicates that P-450$_{NF}$ is involved in a number of reactions, including 6β-hydroxylation of testosterone and androstenedione, estradiol 2- and 4-hydroxylation, aldrin epoxidation (7), benzphetamine N-demethylation (7, 8), quinidine 3- and N-oxidation (36), and the oxidation of nifedipine and 17 other dihydropyridine analogs to pyridine compounds (37). Several related sequences are found in this gene family, and we cannot state with certainty that the coding sequence deduced from the cDNA is identical to that of P-450$_{NF}$. However, the cloning and sequence determination should provide a basis for further studies on the basis of regulation of activities related to this P-450 in humans.

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