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Polymerase Chain Reaction-Directed Identification, Cloning, and Quantification of Human CYP2C18 mRNA

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SUMMARY

Sequencing of genomic polymerase chain reaction (PCR) products synthesized using primers generated from the CYP2C8 and CYP2C9 cDNAs revealed the presence of a new *CYP2C* gene in the human genome. Primers specific to exons of this new gene were used to perform PCR on human liver cDNA libraries and cDNA synthesized from human liver mRNA to generate a cDNA containing a complete cytochrome P450 amino acid reading frame. This cytochrome P450 cDNA, designated CYP2C18, displayed 85% and 87% nucleotide and 77% and 81% amino acid sequence similarities, respectively, with cDNAs and proteins corresponding to CYP2C8 and CYP2C9. cDNA-directed synthesis of CYP2C18 revealed a protein with relative M_r 49,000 on sodium dodecyl sulfate-polyacrylamide gels, which is considerably less than that calculated from the deduced amino acid composition, M_r 55,747. A preferred substrate for this enzyme has not been uncovered. Levels of CYP2C8, CYP2C9, and CYP2C18 mRNAs were examined in 17 human liver specimens using a PCR-based assay. CYP2C18 mRNA was found in all livers examined, albeit at mean levels 7–8-fold lower than those of mRNAs encoding CYP2C8 and CYP2C9. Marked interindividual differences in levels of expression of all three CYP2C mRNAs were also found.

P450s represent a superfamily of enzymes that contain a covalently associated heme and use O_2 and NADPH to oxidize numerous substrates (1). Ten families of P450 have been identified in mammals. P450s in the CYP1, CYP2, and CYP3 families comprise the major foreign compound-metabolizing enzymes, a single form of which is capable of oxidizing numerous structurally diverse substrates, thus accounting for the large capacity of the P450 system to deal with drugs and to activate promutagens and procarcinogens (2, 3).

Remarkable species differences exist in the regulation and catalytic activities of P450s (4, 5), indicating that rodents may not be accurate models for human drug metabolism, toxicity, and carcinogenecity studies. Recent efforts, therefore, have focused on directly examining the catalytic activities of human P450s. These studies have been accomplished by direct purification and analysis of human P450 forms, immunoquantification-based correlations, and cDNA-directed expression (5). Indeed, numerous human P450 cDNAs have been isolated using antibody and cDNA probes generated from rodent enzymes.

P450 cDNAs have been isolated that represent enzymes that have never been purified from human tissue sources, such as CYP2A6, CYP2F1, CYP3A5, CYP4B1, and others (5). Because P450 subfamilies can have multiple closely related members, it is difficult to determine whether all P450s or cDNAs within a subfamily have been isolated. In practice, cDNAs encoding P450s produced from abundant mRNAs are preferentially isolated. For example, the cDNA of CYP3A4, which is one of the major P450 forms in human liver (6), was the first to be isolated (7, 8).

In the CYP2C subfamily, numerous individual genes exist in rats and rabbits but only limited cDNAs have been isolated from human sources. In an earlier study, we screened three separate human liver cDNA libraries but could only identify those encoding CYP2C8 and CYP2C9 (9). However, Southern blotting analysis suggested the presence of numerous *CYP2C* genes in humans (10). New strategies, therefore, were developed to identify and clone cDNAs representing low abundance P450 mRNAs.

In the present report, we describe the identification, isolation, and sequence of a cDNA encoding a new member of the human *CYP2C* subfamily, designated *CYP2C18*. This gene was identified by PCR analysis of genomic DNA using primers generated from the *CYP2C9* sequence. Primers specific to *CYP2C18* exons were synthesized and used to perform PCR on human liver λgt 11 libraries and on reverse-transcribed liver mRNA, to amplify and clone a cDNA containing the full coding sequence of the CYP2C18 mRNA. This cDNA and its sequence were then used for cDNA expression and to develop a PCR-based mRNA quantification procedure to determine the relative levels of expression of CYP2C8, CYP2C9, and CYP2C18 in human livers.

ABBREVIATIONS: P450, cytochrome P450; PCR, polymerase chain reaction; bp, base pairs; SDS, sodium dodecyl sulfate; Taq, *Thermoactinomyces aquaticus*.

Experimental Procedures

Materials. Oligonucleotides were synthesized on an Applied Biosystem 380B DNA synthesizer and purified by Centricon-10 filtration (Amicon Corp.). Oligonucleotides used for the sequencing and PCR reaction are summarized in Table 1. All genomic DNA and RNA samples were isolated from the peripheral blood or liver tissue obtained from kidney donors. A cDNA library, in the vector λgt 11, was described in an earlier study (11, 12).

DNA manipulations. DNA sequencing was carried out according to a modified dideoxy nucleotide chain-termination method (13) after subcloning of individual fragments into the vector pUC18. To subclone PCR products into a plasmid vector, DNA polymerase Klenow fragment was used to generate blunt-ended DNA, as described (14). The PCR products that were subcloned and sequenced are shown in Fig. 1.

Synthesis and analysis of PCR products. All PCR reactions were performed in a volume of 100 μl containing 20 mM Tris·HCl (pH 8.3), 1.5 mM $MgCl_2$, 25 mM KCl, 0.05% (w/v) Tween 20, 100 μg/ml gelatin, 2% (w/v) dimethylsulfoxide, and 200 μM each dATP, dCTP, dGTP, and dTTP. Genomic DNA (0.5 μg), 12.5 μl of cDNA library solution (10^9 plaque-formation units/ml), or cDNA transcribed from 5 μg of total liver RNA were used directly as a template for PCR. Reactions were carried out for 35 cycles at an annealing temperature of 55° for 90 sec, a polymerization temperature of 72° for 1 min/1 kilobase of target DNA fragment, and a heat-denaturation temperature of 93° for 1 min, using a Perkin-Elmer Cetus DNA thermal cycler. An aliquot (5 μl) from each of the completed PCR reactions was subjected to electrophoresis in a 1.5% agarose gel. At the completion of electrophoresis, the gel was stained in ethidium bromide (1.0 μg/ml) for 15 min, destained in water for 10 min, and photographed by UV transillumination.

Transient expression of CYP2C18. To study the function of *CYP2C18* gene product, we used the transient COS-1 cell expression system. Clones C and E (Fig. 1) were fused at the *PstI* restriction site, and the fragment was then subcloned into the plasmid pUC18 and designated pUCF-7. This plasmid was digested with *AseI* and *NdeI* to release the 1.7-kilobase CYP2C18 cDNA-containing fragment. The insert DNA was treated with DNA polymerase Klenow fragment and ligated with the expression vector pCMV4 (15), which was previously digested with *SmaI* and treated with bacterial alkaline phosphatase. The construct was designated pCMF-60. This plasmid DNA was transfected into COS-1 cells using DEAE-dextran and a dimethylsulfoxide shock. Transfected cells were incubated for 60 hr at 37°, and cells were harvested for analysis. Protein electrophoresis (16) and Western immunoblotting (17) were carried out as described, using rabbit antibodies against rat P450s CYP2C7 and CYP2C11 and alkaline phosphatase-conjugated goat anti-rabbit IgG.

Competitive PCR for quantitation of mRNA. Quantitation of mRNA was performed as described (18), with minor modifications. Total RNA (5 μg) was transcribed into cDNA using oligo(dT) and Avian Myoblastosis Virus reverse transcriptase. After cDNA synthesis, half of the products were used as a PCR template. To amplify a portion of the CYP2C8 mRNA, primers HF 53R and HF 54 were used to produce a 452-bp fragment. Primers HF 51R and HF 52 were used to amplify a 356-bp fragment derived from the CYP2C9 mRNA. The CYP2C8 and CYP2C9 primers were based on the previously published sequences (19) and were designed to specifically amplify their respective cDNAs and to produce fragments of different size from the tubulin PCR product we used as an internal control. Primers HF 46 and HF 50R (shown in Fig. 3) were used to amplify a 282-bp fragment of the CYP2C18 cDNA. For an internal control, HF 55 and HF 56R, which amplify a portion of the 570-bp 3′ noncoding region of α-tubulin mRNA (19), were applied. The internal standard primers were used in the same reaction tube as the test primers. PCR reactions were carried out at an annealing temperature of 55° for 90 sec, a polymerization temperature of 72° for 1 min, and a heat denaturation temperature of 93° for 1 min. A tracer of [^{32}P]dCTP (3000 Ci/mmol; New England Nuclear, Beverly, MA) was added to each reaction. When the cycle number reached 10, an aliquot (5 μl) was withdrawn from each reaction tube and subjected to electrophoresis. We repeated this procedure every 5 cycles until reaching 35 cycles. Aliquots of the PCR reaction products were withdrawn at various cycles and were subjected to electrophoresis in a mixture of 3% NuSieve agarose and 1% standard agarose. The gel was dried and subjected to autoradiography, and the densities of amplified CYP2C DNA fragments were compared with those of the α-tubulin fragments by scanning of the film using a Beckman DU-8 spectrophotometer. We only scanned those reaction cycle aliquots that were in the linear range of the amplification reactions. This was generally between cycles 15 and 25. Scans were performed at different levels of exposure of the autoradiographic film, to ensure linearity of the signal with exposure time.

Results

Cloning and sequencing of the CYP2C18 cDNA. To analyze the complexity of the CYP2C subfamily, we used a PCR strategy. This strategy was based on the known sequences

TABLE 1

Oligonucleotides used for this study

The oligonucleotides are 5′ to 3′. The restriction endonuclease recognition sites used for subcloning of PCR fragments are underlined.

Primers	Sequence	Restriction site to be generated
HF 2R	GCCTTGGTTTTTCTCAACTC	
HF 7	GTGGTGCTGCATGGATATGAAG	
HF 19	AAGTTTTCCAGTGGCTGAAAAAGTTAACAAAGGAC	
HF 20R	GTCCTTTGTTAACTTTTTCAGCCACTGGAAAACTT	
HF 25R	CTTAAGCTTCAGACAGGAATGAAGCA	*Hin*dIII
HF 27	TATTAATAAGAAGAGAAGGCTTCA	*Ase*I
HF 28	GATTATTGGAAATATCCTGCAGTTAGA	
HF 29R	CAAGTGTTACAGAGTATG	
HF 30	GATATTGACATCACCCCCA	
HF 41R	TCTAACTGCAGGATATTTCCAATAATC	
HF 46	GGGCATGACCATAATAACATCC	
HF 50R	ACCAAATGCATTGGCAATGGGG	
HF 51R	CAAATCCATTGACAACTGGAGTGG	
HF 52	ACATTGACCTTCTCCCCACCAGCC	
HF 53R	AGAAACAATCCCTTTGGTAACTGC	
HF 54	GATCATGTAATTGGCAGACACA	
HF 55	TGACAGAATTCCAGACCAACCTGG	
HF 56R	TCAACAGAATCCACACCAACCTCC	

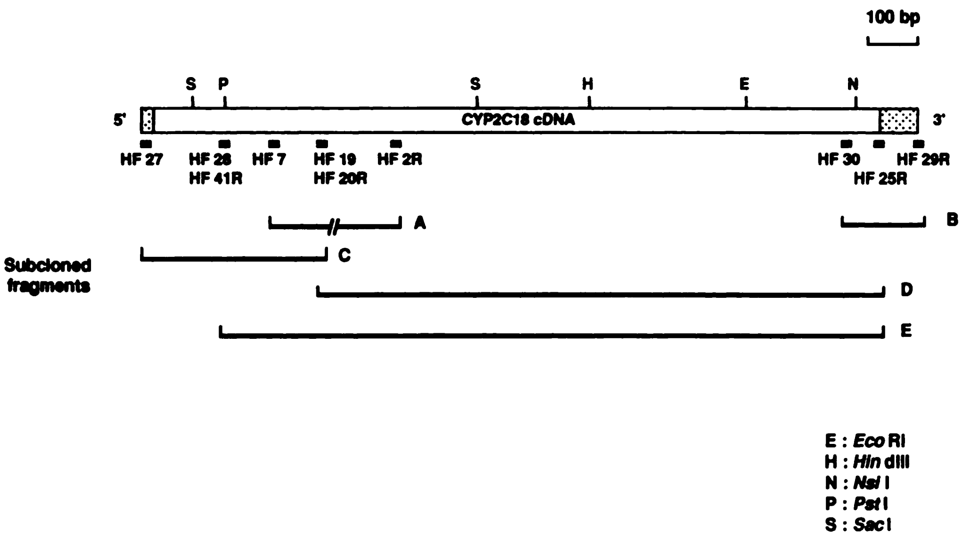

Fig. 1. Diagram of the CYP2C18 cDNA, positions of primers used in this study, and the subcloned PCR fragments.

of CYP2C cDNAs and the predicted exon and intron structure of rodent *CYP2C* genes. Primers HF 7 and HF 2R were synthesized from exons 2 and 3, respectively, representing regions of absolute nucleotide conservation between the CYP2C8 and CYP2C9 cDNAs (9). Because these exons flank the smallest intron of the rat *CYP2C11* and *CYP2C12* genes (20, 21), we predicted that the human *CYP2C* genes would also have a small intron. PCR products were cloned and sequenced, and these data confirmed that introns 2 of *CYP2C8* and *CYP2C9* were 168 and 171 bp, respectively (Fig. 2). PCR products also confirmed the presence of another *CYP2C* gene, designated *CYP2C18*, having intron and exon sequences that were markedly different from those of *CYP2C8* and *CYP2C9*. We also uncovered what appeared to be a pseudogene that lacked the 3′ splice recognition site "AG" in intron 2.[1]

The exon sequence of *CYP2C18* allowed the synthesis of specific primers that could distinguish between CYP2C8 and CYP2C9. These were used in the identification of CYP2C18 cDNA in liver λgt 11 cDNA libraries and CYP2C18 mRNA in liver RNA preparations. Fragments that were cloned and sequenced, along with the primers used for their amplification, are displayed in Fig. 1. Primers HF 7 and HF 2R were used to produce the clone shown in Fig. 2, which allowed synthesis of CYP2C18-specific primers, designated HF 20R and HF 19, that were used to produce clones C and D, respectively. The primers used to synthesize from the 5′ and 3′ end of clones C and D, respectively (HF 27 and HF 25R, respectively), were designed to react with both CYP2C8 and CYP2C9. We reasoned that these primers would also amplify CYP2C18 cDNA. Thus, primers specific to CYP2C18 would only amplify CYP2C18 cDNA and the opposite primers would amplify all three cDNAs but would only produce CYP2C18 by virtue of the specificity of the A-derived primers. Clone B was produced by the same strategy, using a CYP2C18-specific primer derived from clone D sequence (HF 30) and another CYP2C-common primer (HF 29R). Clone E was amplified using CYP2C18-specific primers designed from clone C (HF 28) and clone D (HF 25R) sequences. Clones C and E were then joined to produce a DNA fragment containing the full amino acid coding sequence of CYP2C18.

The complete sequence of CYP2C18 is shown in Fig. 3. Due to the PCR cloning strategy, we do not have the sequence of

[1] H. Furuya and F. J. Gonzalez, unpublished results.

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                                                                                             HF 7
                                                                                    GTGGTGCTGCATGGATATGAAG

C9    CAGTGAAGGAAGCCCTGATTGATCTTGGAGAGGAGTTTTCTGGAAGAGGCATTTTCCCACTGGCTGAAAGAGCTAACAGAGGATTTGgtaggtgtgcaag
C8                           AA                          A  C    A AT  C     AT  CT A    C          ca  ta
C18             G             A                        AAG   T   G         A  T     A    C      aa      t

C9    t-gcctgtttcag----catctgt-----ct------tg------gggatggggaggatggaaaacagagacttacagagctcctcggcagagcttggc
C8     -tt    g    ---- t tg  -----aa------ct------   g  a  g               c c  a a    t   -a         a
C18    at g   g at tgta   g   atgta  gggcag  gctata                     --  g   ga a g     g  a    a    a

C9    ccatccacatggctgcccagtgt--cagcttcctctttcttgcctgggatctccctcctagtttc-gtttctct-tcctgttagGAATTGTTTTCAGCAA
C8     t   tg          a     tg    actt    c    g   t  at --    c      t ccc  t - tt a        CA   C
C18    tg     g        g     --     ct  tg c    tt   at        g   c   -tg  t   g t   c       CC

C9    TGGAAAGAAATGGAAGGAGATCCGGCGTTTCTCCCTCATGACGCTGCGGAATTTTGGGATGGGGAAGAGGAGCATTGAGGACTGTGTTCAAGAGGAAGCC
C8            G                             CA  CT                                      C                T
C18           G                       G         T                                C      C

                              HF 2R
C9    CGCTGCCTTGTGGAGGAGTTGAGAAAAACCAAGGC
C8     A
C18
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Fig. 2. Sequences of PCR products of exons 2 and 3 and intron 2. The complete sequence of CYP2C9 is displayed. Only those nucleotides in CYP2C8 and CYP2C18 differing from those of CYP2C9 are shown. —, Gaps in the sequence introduced by the sequence alignment program.

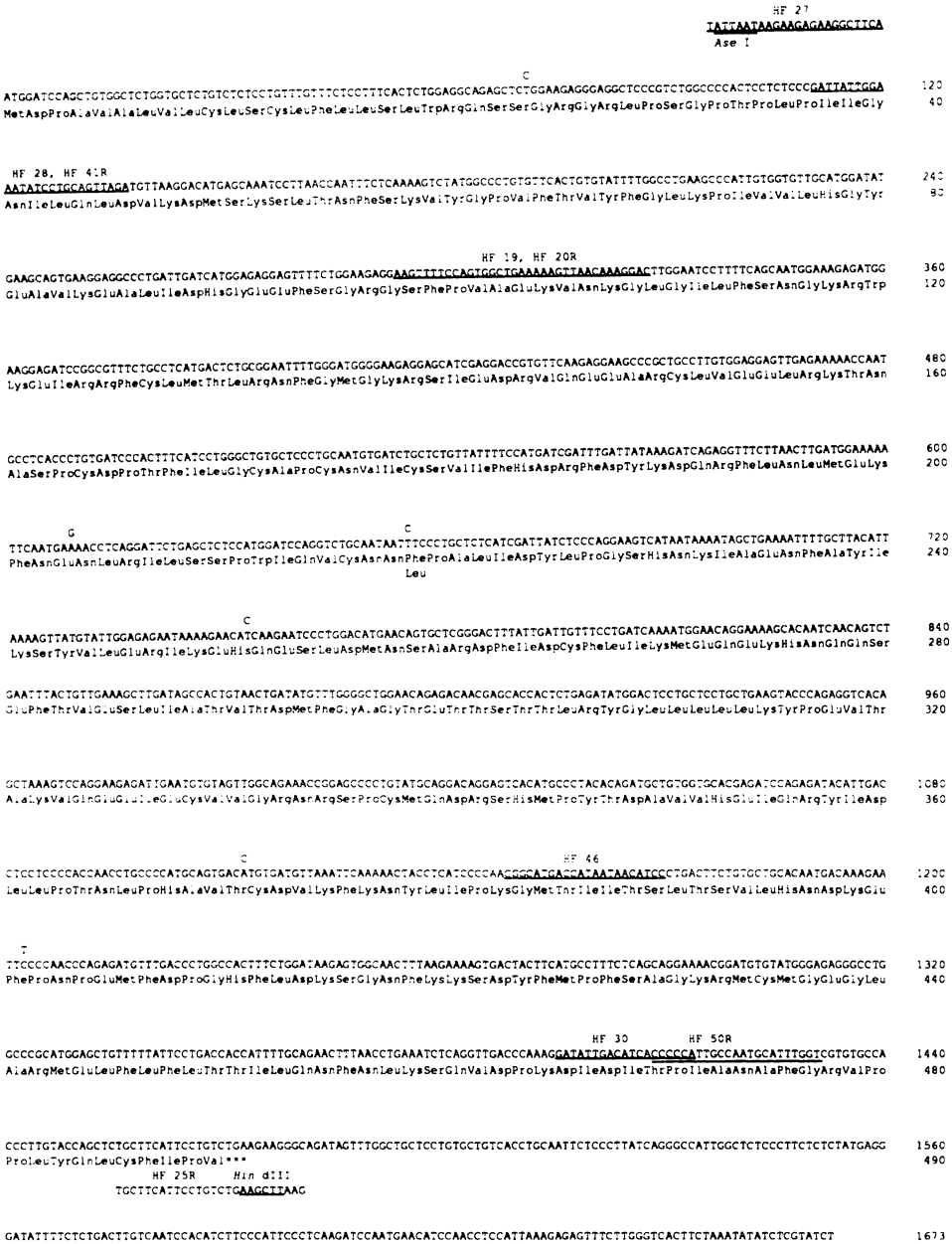

Fig. 3. Nucleotide and deduced amino acid sequences of CYP2C18. Primers used for PCR and pertinent restriction sites are *underlined*. Those nucleotides and amino acids found in an allelic variant of CYP2C18 are displayed *above* and *below* the CYP2C18 sequences, respectively.

the complete 5′ untranslated region of the CYP2C18 mRNA or the terminal 3′ untranslated region. The CYP2C18 protein contains 490 amino acids, of calculated M_r 55,747. The available nucleotide sequences of CYP2C18 are 85% and 87% similar to those of CYP2C8 and CYP2C9, respectively. The amino acid sequence of CYP2C18 displays 77% and 81% similarities with the CYP2C8 and CYP2C9 proteins, respectively. An allelic variant of CYP2C18 was uncovered having six nucleotide and one amino acid substitutions (Fig. 3). The latter is a conservative substitution, $Phe_{219} \rightarrow Leu$.

cDNA-directed expression of CYP2C18. The CYP2C18 cDNA was inserted into the vector pCMV4 (15). This vector contains the powerful cytomegalovirus promoter and SV40 origin of replication for expression of foreign proteins in COS cells. Transfection of COS-1 cells with the CYP2C18 construct resulted in production of a protein of about 49,000 Da, estimated based on SDS-polyacrylamide gel electrophoresis, that was of greater mobility than CYP2C8 and CYP2C9 (Fig. 4). This protein reacts with rabbit antibodies prepared against rat P450s CYP2C7 (Fig. 4A) and CYP2C11 (Fig. 4B). These antibodies were chosen because they are known to cross-react with human CYP2C8 and CYP2C9 and, therefore, were assumed to react with the related CYP2C18 protein. Expressed CYP2C18 migrates slightly faster than the faintly labeled, greatest mobility, immunoreactive protein detected in human liver microsomes, which we believe is CYP2C18. However, upon mixing of COS cell extracts with human liver microsomes, the expressed CYP2C18 protein could not be resolved from the faintly labeled human immunoreactive protein. The differences in electrophoretic mobilities between the microsomal and COS

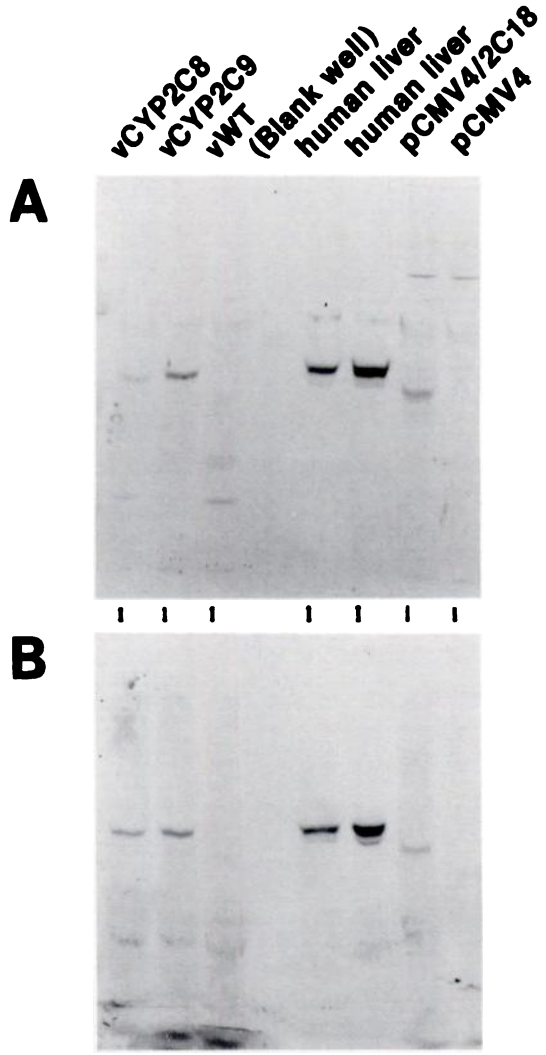

Fig. 4. Western immunoblot analysis of expressed CYP2C8, CYP2C9, and CYP2C18 using antibodies produced against CYP2C7 (A) and CYP2C11 (B). Total cell lysate protein (50 μg) of expressed material and microsomal protein from two human livers (10 μg) were analyzed. vCYP2C8 and vCYP2C9 are extracts from vaccinia virus-infected Hep G2 cells. vWT is an extract from wild-type vaccinia virus-infected Hep G2 cells. pCMVA/2C18 and pCMV4 are COS cells lysates from transfected cells.

cell-expressed proteins might be due to the differing compositions of mixtures applied to the gels.

To test the catalytic activity of expressed CYP2C18, several compounds were examined that are known substrates for other P450s in the various CYP2 subfamilies in humans and rodents. CYP2C18 was unable to deethylate 7-ethoxycoumarin or 7-ethoxyresorufin, hydroxylate benzo(*a*)pyrene at the 3-position, or hydroxylate any position of testosterone. Expressed CYP2C18 also did not display (*R*)- or (*S*)-mephenytoin 4-hydroxylase activity. Unfortunately, we have not found a favored substrate for this P450.

Analysis of expression of CYP2C8, CYP2C9, and CYP2C18 mRNAs in human liver. To estimate relative levels of CYP2C mRNAs in human liver specimens, we used PCR of cDNA synthesized using oligo(dT) from total liver RNA. Primers were used that could specifically amplify small regions of each CYP2C mRNA. PCR of α-tubulin mRNA (cDNA) was used as an internal standard in the same PCR reaction mixes, so that the levels of each CYP2C mRNA relative to tubulin mRNA could be estimated. To ensure that tubulin mRNA was expressed at similar levels in different livers, we performed PCR on each RNA specimen as a function of input RNA. Expression of α-tubulin mRNA was within 30% between different livers.

Large interindividual variations in levels of each mRNA were detected, which varied over 10-fold between livers (Fig. 5). The mean levels of CYP2C18 (0.22 ± 0.11), relative to α-tubulin, were about 7–8-fold lower than those of CYP2C8 (1.9 ± 1.6) and CYP2C9 (1.6 ± 0.97). These data confirm that CYP2C18 mRNA is expressed at a low level in liver and offer a reason why we could not identify this cDNA by screening three λgt 11 libraries using the CYP2C9 cDNA. The highest expression for CYP2C8 was found in K14 and was 6-fold greater than that for α-tubulin. Interestingly, in KDL-4, CYP2C18 was the most abundantly expressed mRNA; CYP2C18 mRNA was expressed at <0.2% of the tubulin mRNA level (Fig. 5). These data demonstrate the utility of PCR in determining relative mRNA levels of individual members of a closely related P450 subfamily using only small amounts of total mRNA. Therefore, small wedge or even needle biopsy samples could be used to estimate mRNA levels from as little as 1 μg of total RNA.

Discussion

Direct cloning of human P450 cDNAs from cDNA libraries has generally been productive using anti-rodent and -human P450 antibodies and rodent P450 cDNA probes. We believed, however, that these procedures only result in the cloning of the most abundantly expressed P450 mRNAs. For example, on the basis of complexity of Southern blotting patterns (10) and the large number of P450s identified in rabbits and rats (1), one would expect that more than two or three CYP2C P450 genes exist and would be expressed in humans. Large sequence similarities among P450s within a single gene family render them difficult to selectively extract from a liver cDNA library. The high degree of variation between individuals in their expression of P450s necessitates the screening of libraries prepared from different liver samples (11, 12). It is also difficult, in many cases, to distinguish closely related P450 cDNAs from allelic variants. For example, CYP2C9 and CYP2C10 are extremely similar across their amino acid coding regions but markedly differ at the 3′ noncoding region (3). It should be noted that only a single CYP2C10-type cDNA has been isolated. It remains a possibility that the CYP2C10 cDNA is a result of an artificial ligation during construction of the λgt 11 library that was used in this report.

Expression of CYP2C18 cDNA using COS cells yielded a protein of about 49,000 Da, as assessed by SDS-polyacrylamide gel electrophoresis. This is a lower molecular mass than that calculated from the cDNA-deduced protein sequence (55,747 Da). This discrepancy has been noted for other P450s. For example, CYP2A6 has a calculated M_r of 56,547, whereas its cDNA-expressed counterpart migrates with relative M_r 49,000 on SDS-polyacrylamide gels (12). The reason for this trend of underestimation of true P450 molecular weight by electrophoresis is unknown.

Despite the successful production of CYP2C18 protein, we were unable to demonstrate catalytic activities toward several P450 substrates. Even ethoxycoumarin, a substrate for several other human P450s, was not metabolized by CYP2C18.

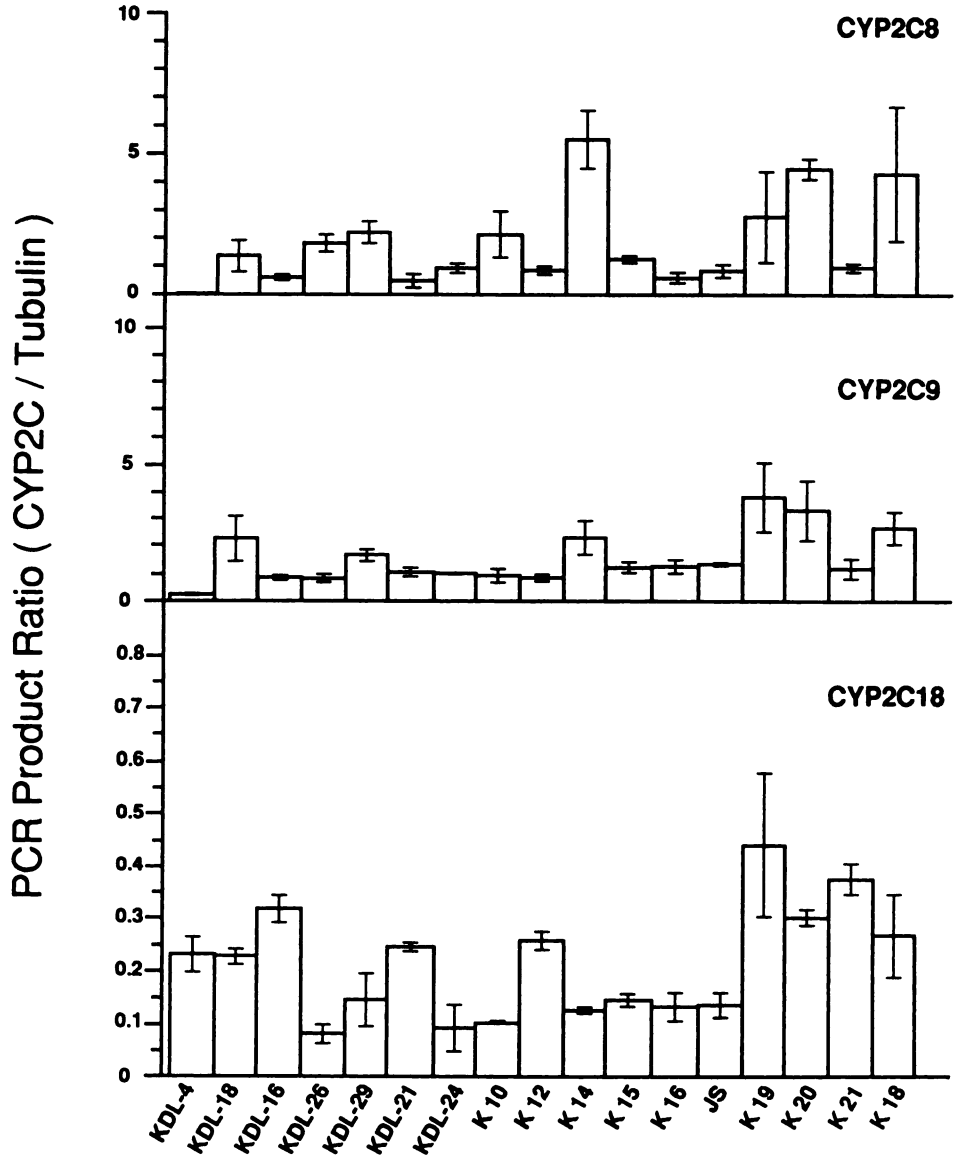

Fig. 5. PCR quantitation of CYP2C8, CYP2C9, and CYP2C18 mRNAs. cDNA was synthesized from total RNA and divided into three samples. These were then subjected to PCR using mixtures of each CYP2C primer and tubulin primer. The products were electrophoresed and the CYP2C/tubulin fragment ratio was determined by autoradiography and scanning densitometry. The values are expressed as mean ± standard deviation.

TABLE 2

Amino acid polymorphisms between CYP2C18 cDNA clones

The roman numerals designate DNA base differences (polymorphisms) and amino acid substitutions in CYP2C18 cDNAs. Numbering corresponds to the position of the cDNA in this report (Fig. 3). Haplotypes P and Q are reported in this paper (Fig. 3). Haplotype P was cloned from three individuals, three from a liver designated Pa72 and one each from livers KDL21 and KDL35. Haplotype Q was cloned from Pa72. Haplotypes R and S were reported by Romkes *et al.* (22). Haplotype R is reported as clone 6b and S as clone 29c.

Haplotype	No. of clones	Polymorphism			
		I	II	III	IV
P	5	^{5}AT ^{2}Asp	^{655}T ^{219}Phe	^{1154}T ^{385}Met	^{1413}CATTGCCAATGCATTTGGTCG ^{472}IleAlaAsnAlaPheGlyArg
Q	1	^{5}AT ^{2}Asp	^{655}C ^{219}Leu	^{1154}T ^{385}Met	^{1413}CATTGCCAATGCATTTGGTCG ^{472}IleAlaAsnAlaPheGlyArg
R	2	^{5}TA ^{2}Val	^{655}T ^{219}Phe	^{1154}T ^{385}Met	^{1413}ATTGCCAATGCATTTGGTCGG ^{472}LeuProMetHisLeuValGly
S	1	^{5}TA ^{2}Val	^{655}T ^{219}Phe	^{1154}C ^{385}Thr	^{1413}ATTGCCAATGCATTTGGTCGG ^{472}LeuProMetHisLeuValGly

The present study also demonstrates the utility of PCR to identify P450 mRNA expressed at low abundance. PCR can be used to clone a cDNA directly and to quantify relative levels of mRNAs. During the course of these studies, we cloned and sequenced numerous PCR products that had been subjected to amplification of 25 to 35 cycles with no evidence of artifactual PCR-generated mutations. Thus, this procedure may be of general utility for cloning cDNAs.

Quantification of mRNAs by PCR was also highly reproducible, with variations of 21 ± 14% (mean of all standard devia-

tions ± standard deviation) in CYP2C/tubulin mRNA PCR product ratios between experiments with a given sample of liver mRNA. This procedure can be used with as little as 1 μg of total RNA. It should be recognized, however, that the interindividual levels of a specific mRNA are dependent on the assumption that there exists a constant level of expression of the internal standard mRNA between liver specimens. We found one liver RNA specimen, of a total of 18, that had levels of tubulin mRNA that were about one half of those found in 17 other livers. This liver was excluded from our analysis. We feel, however, that α-tubulin mRNA, encoding a structural, ubiquitously expressed protein that is not significantly influenced by hormones, would be the best internal standard.

Analysis of the mRNA quantification data revealed a marked interindividual variation in levels of the CYP2C mRNAs. There appears to be a correlation between levels of expression of CYP2C8 and CYP2C9 mRNAs in the different liver specimens. For example, KDL-4 has very low levels of these mRNAs, compared with other livers, whereas CYP2C18 mRNA in this liver is present at a level comparable to that in other livers. Levels of CYP2C8 and CYP2C9 mRNAs in K14, K18, K19, and K20 are generally higher than those in most other livers, suggesting that CYP2C8 and CYP2C9 are co-regulated. It is interesting that livers K14 and K19 also possess high levels of mRNAs encoding CYP2A and CYP2B P450s (11, 12). Although the K14 liver was indeed derived from a patient receiving phenobarbital, an inducer of P450s in rodents, the patient donating the K19 liver was, to our knowledge, treated only with dopamine and heparin, which are not known to induce P450s.

While this manuscript was being reviewed, a report appeared describing two apparent CYP2C18 alleles that were isolated from a human liver cDNA library (22). Comparison of their sequences with ours revealed several differences, which can be classified into four haplotypes, arbitrarily designated P, Q, R, and S (Table 2).[2] We recognize that these differences may represent simple sequencing reading errors or Taq polymerase nucleotide misincorporation errors. However, we cloned and sequenced six independently amplified cDNAs from three individuals; five correspond to haplotype P and one corresponded to haplotype Q, which was identical to P except for a C at position 655 (Fig. 3) instead of a T, resulting in a Leu in place of a Phe.[2] The finding of the same sequence in five clones of independently amplified PCR products would rule out the possibility of Taq polymerase nucleotide misincorporation. Furthermore, we determined these sequences on both strands of the cDNAs and, on this basis, believe that there are no sequencing errors. We have never seen the sequences corresponding to polymorphisms I, III, and IV in our cDNAs. Polymorphism I is a TA in haplotypes R and S instead of an AT in our P and Q haplotypes. Polymorphism IV is especially interesting, because it results from a C deletion (residue 1413 in Fig. 3) and a G insertion (after residue 1432 in Fig. 3), compared with our sequence. It is also noteworthy that three of the seven amino acid residues encoded by the DNA between residues 1413 and 1432 of haplotypes P and Q are conserved in CYP2C8 or CYP2C9, whereas none of the amino acid residues in polymorphism IV in haplotypes R and S are conserved. These data would suggest that sequence misreading could account for polymorphisms I and IV in haplotypes R and S. In any case, the true nature of these polymorphisms awaits a PCR analysis of genomic DNAs. If, indeed, they do represent different alleles, it will be interesting to determine whether these amino acid changes affect CYP2C18 catalytic activities.

[2] Due to the few base changes observed, we are tentatively designating these cDNAs as allelic variants, although we cannot, at present, rule out the possibility that they are different gene products.

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