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Gene Structure of *CYP2C8* and Extrahepatic Distribution of the Human CYP2Cs

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Abstract: Extrahepatic tissue distribution of the mRNAs for the four human CYP2Cs (2C8, 2C9, 2C18, and 2C19) was examined in kidney, testes, adrenal gland, prostate, brain, uterus, mammary gland, ovary, lung, and duodenum. CYP2C mRNAs were detected by RT-PCR using specific primers for each individual CYP2C. CYP2C8 mRNA was detected in the kidney, adrenal gland, brain, uterus, mammary gland, ovary, and duodenum. CYP2C9 mRNA was detected in the kidney, testes, adrenal gland, prostate, ovary, and duodenum. CYP2C18 mRNA was found only in the brain, uterus, mammary gland, kidney, and duodenum and CYP2C19 mRNA was found only in the duodenum. Immunoblot analysis of small intestinal microsomes detected both 2C9 and 2C19 proteins. In addition, genomic clones for *CYP2C8* were sequenced, and long-distance PCR was performed to determine the complete gene structure. *CYP2C8* spanned a 31 kb region. Comparative analysis of the 2.4 kb upstream region of *CYP2C8* with *CYP2C9* revealed two previously unidentified transcription factors sites, C/EBP and HPF-1, and the latter might be involved in hepatic expression. Although *CYP2C8* has been shown to be phenobarbital inducible, neither a barbiturate-responsive regulatory sequence (a Barbie box) nor a phenobarbital-responsive enhancer module (PBREM) was found within the upstream region analyzed. © 1999 John Wiley & Sons, Inc. J Biochem Toxicol 13: 289–295, 1999

KEYWORDS: *CYP2C8* Gene Structure, Human CYP2C mRNA Expression, Extrahepatic Tissues.

INTRODUCTION

Cytochrome P450s are found in abundance in the liver and, to a lesser extent, in extrahepatic tissues. Within the CYP2C subfamily of P450s, cDNAs for four well characterized members, 2C8, 2C9, 2C18, and 2C19, have been isolated and characterized from human liver [1]. The gene structure and upstream regulatory regions for both *CYP2C9* and *CYP2C18* have been identified and characterized [2]. To date, only the 5′flanking region, exon 1, and the first 840 bp of intron 1 of *CYP2C8* have been characterized [3]. Polymorphisms for *CYP2C9*, *CYP2C18*, and *CYP2C19* have been reported [4–6], but to date no polymorphisms have been associated with *CYP2C8*.

Different 2C isoforms have been detected in rat, mouse, and porcine liver and extrahepatic tissues [7–10]. Three of these enzymes (2C8, 2C9, and 2C19) have also been identified in human liver [11]. Other investigators have detected some CYP2Cs or their mRNAs in human extrahepatic tissues. Zeldin *et al.* [12] were able to detect 2C8 and 2C9 (2C10) mRNA in kidney. Hukkanen *et al.* [13] detected CYP2C mRNA transcripts in bronchoalveolar macrophages using universal CYP2C primers in RT-PCR analysis. Nakajima *et al.* [14] reported CYP2C8 protein expressed in lung microsomes, and Macé *et al.* [15] detected both CYP2C8 and CYP2C18 mRNA transcripts in both bronchial mucosa and peripheral lung tissues. CYP2C8 and CYP1A1 mRNA were found to be the most frequently expressed CYP mRNAs in adult brain by McFadyen *et al.* [16]. CYP2C mRNA forms were detected in breast tissue by Huang *et al.* [17] with universal CYP2C primers in RT-PCR, but no individual forms were identified. Zaphiropoulos [18] found CYP2C18 mRNA to be abundantly expressed in the adult epidermis. Although no CYP2C mRNA was detected in full term placenta [19], CYP2C mRNA expression using universal CYP2C primers in RT-PCR was identified in first trimester placenta [20].

In this study, the complete gene structure of *CYP2C8* was determined by sequencing *CYP2C8* genomic clones and by long-distance PCR. In addition, the CYP2C mRNA distribution in human extrahepatic tissues was investigated. To identify the individual is-

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The 2C8 sequences reported in this article have been submitted to GenEMBI/Genbank under accession numbers AP13860 through AP136845.

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TABLE 1. Nucleotide Sequences of PCR Primers

CYP Isoform		*Nucleotide Sequences*	*Predicted Size of Amplified Fragment (bp)*
2C8	Sense	AGA TCA GAA TTT TCT CAC CC	158
	Antisense	AAC TTC GTG TAA GAG CAA CA	
2C9	Sense	AGG AAA AGC ACA ACC AAC CA	104
	Antisense	TCT CAG GGT TGT GCT TGT C	
2C18	Sense	ACC TCA GGA TTC TGA GCT CT	142
	Antisense	TGT TCT TTT ATT CTC TCC AAT AC	
2C19	Sense	ATT GAA TGA AAA CAT CAG GAT TG	182
	Antisense	GAG GGT TGT TGA TGT CCA TC	

oforms, specific primers for the four isoforms were designed and used in RT-PCR analyses. Immunochemical analysis for specific CYP2Cs was also performed on small intestinal and lung microsomes to identify which proteins were present.

MATERIALS AND METHODS

Human cDNA Samples

The following tissues were purchased from the International Institute for the Advancement of Medicine (Exton, PA): adrenal gland, testes, and prostate. Kidney and ovary were purchased from National Disease Research Interchange (Philadelphia, PA). Total RNA from these tissues was isolated using TRIzol™ LS Reagent (Life Technologies Inc., Gaithersburg, MD). Total RNAs from brain, uterus, mammary gland, and lung were purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA). Total RNA from duodenum was kindly donated by Dr. Paul Watkins (University of Michigan, Ann Arbor, MI) and was prepared by the methods of Chomczynski and Sacchi [21]. cDNA synthesis was performed in a total volume of 100 μL containing 1 μL RNA, 0.3 mM dNTPs, 1 μg Oligo $(dT)_{12–18}$ Primer (Life Technologies Inc., Gaithersburg, MD), 1× MMLV-Reverse Transcriptase Buffer (Stratagene, La Jolla, CA), 50 units of MMLV-Reverse Transcriptase, and DEPC-treated water. The RNA and other components were heated at 94°C for 2 minutes prior to the addition of reverse transcriptase. After the addition of reverse transcriptase, the mixture was incubated at 37°C for 90 minutes. The reaction was inactivated by heating at 94°C for 2 minutes.

Amplification of cDNA

The cDNA fragments were amplified using specific CYP2C primers for 2C8, 2C9, 2C18, and 2C19. Primers are shown in Table 1. Amplification was conducted in 1× PCR buffer (67 mM Tris-HCL, pH 8.8, 17 mM $(NH_4)_2SO4$, 10 mM β-mercaptoethanol, 7 μM EDTA, 0.2 mg/mL bovine serum albumin) containing 50 μM dNTPs, 0.25 μM concentrations of PCR primers, 2.5 units of AmpliTaq DNA polymerase (Applied Biosystems Inc., Foster City, CA), and 2.0 mM $MgCl_2$. Primers were synthesized using an Applied Biosystems Synthesizer (Applied Biosystems Inc., Foster City, CA). PCR amplification consisted of an initial denaturation step at 94°C for 5 minutes followed by 38 cycles of denaturation at 94(C for 20 seconds, annealing at 55°C for 10 seconds, and an extension at 72°C for 10 seconds. A final extension step at 72°C for 5 minutes was also performed in a Perkin Elmer 9600 GeneAmp PCR system (Perkin Elmer Cetus, Norwalk, CT). The PCR products were checked on a 3% Seakem ME agarose gel (FMC BioProducts, Rockland, ME).

Immunoblot Analysis of Small Intestinal and Lung Microsomes

Human small intestinal microsomes were kindly donated by Dr. Paul Watkins (University of Michigan, Ann Arbor, MI) and were prepared according to methods of Fitzsimmons and Collins [22]. Lung tissue was obtained from National Disease Research Interchange (Philadelphia, PA). Lung microsomes were prepared by standard methods. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (7.6% acrylamide) was performed as described by Laemmli [23]. The proteins were transferred to nitrocellulose sheets by the method of Towbin *et al.* [24], and immunoblots probed with a rabbit antibody to *CYP2C19*, which cross reacts with all four CYP2Cs. Immunoblots were developed using the ECL (enhanced chemiluminescence) Western blotting kit from Amersham (Arlington Heights, IL). Recombinant CYP2C proteins were expressed in *Escherichia coli* and partially purified as previously described [25].

Direct Sequencing and Long-Distance PCR of CYP2C8

A genomic library was constructed in the Lambda Dash II vector (Stratagene, La Jolla, CA) using liver DNA from an individual phenotyped in vitro as an extensive metabolizer of *S*-mephenytoin (2). The li-

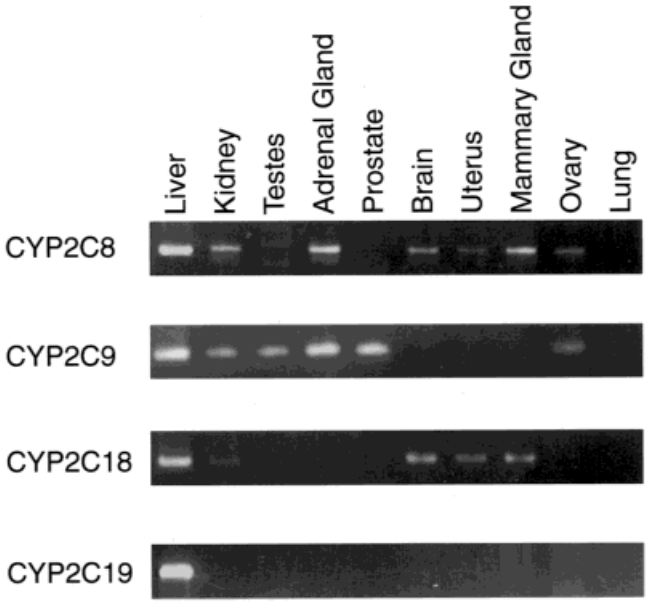

FIGURE 1. Expression of individual human CYP2C mRNA in various tissues. Expression was examined by RT-PCR using specific CYP2C primers, and amplified products were separated by electrophoresis using 3% agarose gels.

brary was screened by *in vitro* plaque hybridization using radiolabeled DNA fragments amplified by PCR from CYP2C19 cDNA. One isolated clone was found to contain exons 1–5 of *CYP2C8*. A PAC clone containing the entire gene structure of *CYP2C8* was obtained from Genome Systems, Inc. (St. Louis, MO). Direct sequencing of the intron–exon junctions and 2.4 kb upstream region of *CYP2C8* was accomplished using the cycle sequencing reaction with the Dye Terminator Cycle Sequencing Kit containing AmpliTaq DNA polymerase, FS from Applied Biosystems Inc., (Foster City, CA). Optimum conditions for sequencing the PAC clone consisted of 2.5 μg of DNA and 5 pmol of primer, and the reaction mix was cycled at an initial denaturation step of 94°C for 5 minutes followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 5 seconds, and an extension at 60°C for 4 minutes. The products were cleaned by ethanol precipitation and sequenced on the Applied Biosystems 373 DNA Stretch Sequencer (Applied Biosystems Inc., Foster City, CA). Long-distance PCR was accomplished using the GeneAmp(r) XL PCR Kit and protocol (Applied Biosystems Inc., Foster City, CA). The PCR products were checked on a 0.5%Seakem ME agarose gel (FMC BioProducts, Rockland, ME).

RESULTS AND DISCUSSION

The liver has long been considered to be the primary organ for metabolism of both endogenous and xenobiotic compounds. A vast number of xenobiotic-metabolizing enzymes such as cytochrome P450s, alcohol and aldehyde dehydrogenases, acetyltransferases, and glutathione *S*-transferases are found expressed in great abundance within the liver itself [26]. However, certain human drug metabolizing enzymes are found in extrahepatic tissues. For instance, CYP1A1 is found primarily in the lung [27], and CYP2J2 is expressed highly in the heart, small intestine, and colon [28,29]. The extrahepatic distribution of the CYP2Cs among tissues has not been systematically characterized. RT-PCR technology was utilized in this study for detecting the different isoforms of the human CYP2Cs present in extrahepatic tissues.

Among the panel of extrahepatic tissues examined in the present study, CYP2C8 mRNA was detected in the kidney, adrenal gland, brain, uterus, mammary gland, and ovary (Figure 1). Expression was highest in the adrenal gland, mammary gland, and kidney, followed by the brain, ovary, and uterus. In our study, detection of 2C8 mRNA was variable in lung even though other investigators were able to detect 2C8 mRNA or protein expression in lung [13–15]. The only tissues in which 2C8 mRNA was not detected were testes and prostate. In contrast, CYP2C9 mRNA was detected only in the kidney, testes, adrenal gland, prostate, and ovary (Figure 1), although Shimada *et al.* [30] were able to detect 2C9 protein in lung in only one of several pulmonary microsomal samples they examined. The intensity of the PCR product was the strongest with adrenal gland and prostate, and the intensity was weak for the ovary. Expression of CYP2C18 mRNA differed in that it was detected only in the brain, uterus, and mammary gland with a trace seen in the kidney. Although CYP2C19 mRNA was not observed in any of the above extrahepatic tissues, all four CYP2C mRNAs were detected in three separate duodenum cDNAs (Figure 2). The levels of mRNA expression in the duodenum among the 2Cs indicated 2C18 expression to be the greatest followed by 2C9, 2C19, and 2C8.

One interesting finding of this study was the differential levels of expression of 2C8 and 2C9 mRNA in various tissues. Figure 3 shows the differential level of expression for 2C8 and 2C9 mRNA in kidney, adrenal gland, testes, and prostate. Amounts of cDNA used included undiluted as well as serial dilutions of 1:2, 1:4, 1:10, 1:20, and 1:50. CYP2C8 mRNA was a predominant CYP2C RNA in kidney and was detected at all dilutions. However, 2C9 mRNA expression in kidney was only detected at high mRNA concentrations indicating higher levels of expression of 2C8 relative to 2C9 in kidney. Both 2C8 and 2C9 mRNAs were found in the adrenal gland but 2C9 appears to be expressed in somewhat higher quantities. In contrast, both testes and prostate displayed high levels of expression for 2C9 mRNA but expression of 2C8 mRNA was not detected in these tissues.

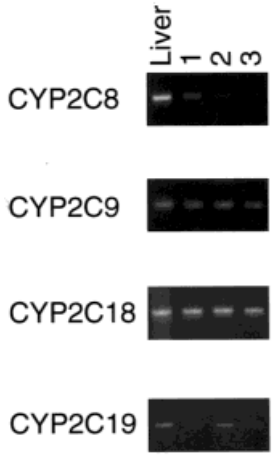

FIGURE 2. Expression of the individual human CYP2C mRNAs in duodenum. Liver cDNA was used as a positive control. Samples 1–3 are amplified cDNA products from three separate individuals. Expression was examined by RT-PCR using specific CYP2C primers and amplified products were separated by electrophoresis using 3% agarose gels.

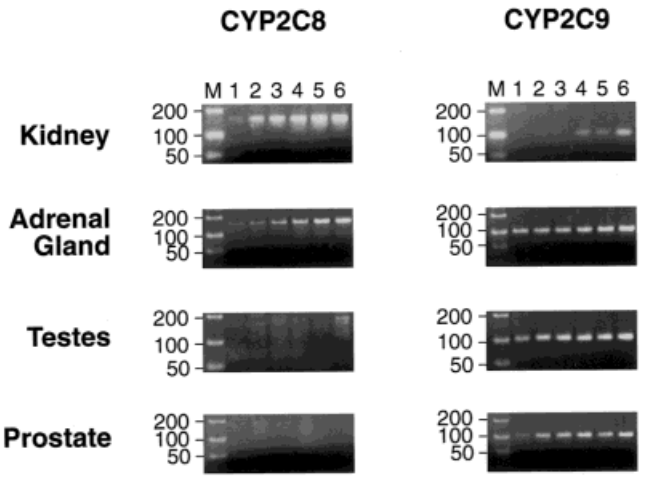

FIGURE 3. RT-PCR analyses of CYP2C8 and CYP2C9 cDNAs showing different levels of expression in kidney, adrenal gland, testes, and prostate. Lanes 1–6 represent the following dilutions of cDNAs: lane 1 is 1:50, lane 2 is 1:20, lane 3 is 1:10, lane 4 is 1:4, lane 5 is 1:2, and lane 6 is undiluted. The marker is GelMarker™.

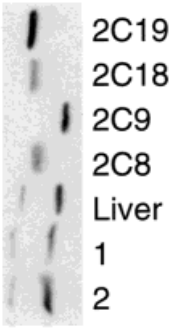

FIGURE 4. Immunoblot analysis of the CYP2C proteins from two human small intestines. 2C19, 2C18, 2C9, and 2C8 represent recombinant purified proteins and amounts used were 0.25 pmol, 5 pmol, 1 pmol, and 5 pmol, respectively. For a positive control, 8 μg of human liver microsomal protein was used. Samples 1 and 2 contained 80 μg of intestinal protein. Polyclonal antibodies for CYP2C19 cross reacted with all CYP2C proteins.

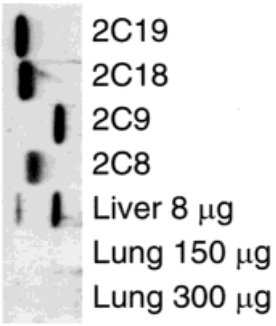

FIGURE 5. Immunoblot analysis of CYP2C proteins in human lung. 2C19, 2C18, 2C9, and 2C8 represent recombinant purified proteins and amounts used were 0.25 pmol, 5 pmol, 1 pmol, and 5 pmol. For a control, 8 μg of human liver microsomal protein was used. Expression of the 2Cs in human lung microsomes was examined at two different amounts (150 and 300 μg of protein).

Interestingly, immunoblot analysis of human small intestinal microsomes revealed the presence of both 2C9 and 2C19 proteins (Figure 4). Kolars *et al.* [31] detected abundant amounts of 3A4 protein in the small intestine as well. This is of particular interest in terms of omeprazole metabolism in which both 2C19 and 3A4 are the key CYP enzymes involved in omeprazole elimination [32]. Yamazaki *et al.* [33] determined that the extent to which either 2C19 or 3A4 in liver microsomes contributed to 5-oxidation of omeprazole depended on the amount of each protein present. Neither 2C8 nor 2C18 protein expression was detected in the small intestinal microsomes, although the mRNAs for both are expressed; particularly 2C18 mRNA levels had appeared high. Although, Nakijima *et al.* [14] reported very faint CYP2C8 protein expression in lung microsomes, we were not able to detect expression of any CYP2C protein in the lung (Figure 5) indicating that if present, the levels must be low. CYP2C18 and CYP2C8 are also recognized weakly by our antibody.

A map of the complete gene organization for *CYP2C8* is shown in Figure 6. *CYP2C8* spanned approximately a 31 kb region, making it the smallest gene among the known human CYP2C genes, with *2C9* and *2C18* spanning more than 39 and 55 kb, respectively

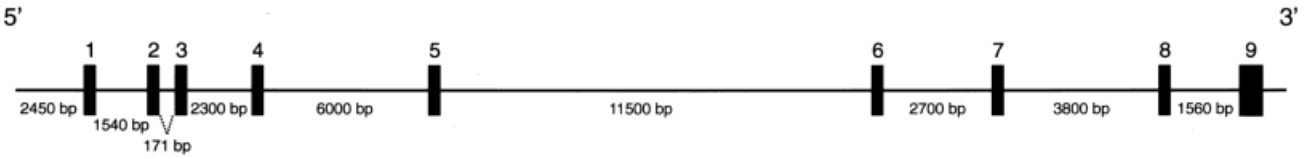

FIGURE 6. Representation of the structure of the *CYP2C8* gene. Long-distance PCR was utilized to determine the intron sizes of *CYP2C8* using specific 2C8 exon primers and the GeneAmp® XL PCR kit and protocol as described in the methods. The PCR products were analyzed on a 0.5% agarose gel.

TABLE 2. Location of Intron–Exon Boundaries in *CYP2C8*

Intron	*Donor*	*Intron Size*	*Acceptor*
1	TTTCACCAAATgtaagtctgc	1540	cctttcccagTTCTCAAAG
2	AAAGGAACTTGgtaggtgcac	171	tttttattagGAATCATTTC
3	AAAACCAAGGgtgggtgact	2300	aaatctttagCTTCACCCTG
4	ATGGATCCAGgtaaggccaa	6000	tctcttttagGTCTGCAATA
5	AATGGAGCAGgtaagatatt	11500	tcacttctagGAAAAGGACA
6	GAGGTCACAGgtaggaccac	2700	gtcttgtcagCTAAAGTCCA
7	CATCCCCAAGgtaagcttgt	3800	ttacttccagGGCACAACCA
8	TTCTCAGCAGgtaatagaaa	1560	ttatcttcagGAAAACGAAT

[2]. The intron–exon junctions are shown in Table 2. Intron sequences of *CYP2C8* will enable further studies of possible polymorphisms for *CYP2C8*.

Comparison of the 2.4 kb upstream region as well as the first 500 bases of the 5′-region of intron 1 of *CYP2C8* with the previously published sequence of Ged and Beaune [3] revealed a few minor differences. A possible explanation for these differences is that the automated sequencing technology now available is capable of producing more accurate sequencing data.

Comparative analysis of the upstream region of *CYP2C8* with that of *CYP2C9* revealed that *CYP2C8* contains two previously unidentified transcription factor sites. The first site was a C/EBP site at positions −1003 through −995, and the second was an HPF-1 site at positions −148 through −143. HPF-1 sites have been implicated in the liver specific regulation of the rabbit CYP2Cs [34]. In a previous study in our laboratory, the HPF-1 site was also identified as the predominant *cis*-acting element involved in the positive regulation of the *CYP2C9* promoter [35]. Morel *et al.* [36] found that mRNA content, which hybridized to a 2C8 cDNA, was increased in human hepatocytes after exposure to either phenobarbital or rifampicin, suggesting that *CYP2C8* may be a phenobarbital inducible gene. Chang *et al.* [37] also found that 2C8 protein was increased in primary hepatocytes following phenobarbital exposure. However, a barbiturate-responsive regulatory sequence (Barbie box) [38] identified in *CYP2C9* [2] was not identified in the 2.4 kb upstream region of *CYP2C8* in the present study. A Barbie box has been suggested to be important in phenobarbital inducibility of certain bacterial P450s [39], although its importance in positive regulation of mammalian P450s is more questionable [40]. Negishi and coworkers [41] identified a −51 bp upstream element as important in the phenobarbital inducibility of the mouse *Cyp2B10* gene. This is also found in the 1.7 kb upstream region of the human *CYP2B6* gene [42]. Within this region a 16 bp NR1 site has been suggested by Negishi (personal communication) to be the key element in mouse, rat, and human phenobarbital-responsive enhancer module (PBREM). We saw no evidence of this 16 bp site within the 2.4 kb upstream of *CYP2C8* sequenced in the present study. However, it is possible that similar elements could exist further upstream.

In conclusion, relative CYP2C expression in extrahepatic tissues was determined with RT-PCR technology. Different CYP2C isoforms were found to be expressed in a variety of extrahepatic tissues at different levels. Both CYP2C9 and CYP2C19 proteins were detected in the small intestine, but no CYP2C proteins were detected in lung. However, the role of each isoform in the metabolism of both endogenous and xenobiotic compounds in extrahepatic tissues has yet to be resolved. The gene structure for *CYP2C8* has also been elucidated. Currently there are no polymorphisms known to be associated with *CYP2C8*. However, the knowledge of the structure and sequence of this gene will allow for further studies of any possible genetic polymorphisms in this enzyme.

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