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cDNA Cloning and Sequence and cDNA-Directed Expression of Human P450 IIB1: Identification of a Normal and Two Variant cDNAs Derived from the *CYP2B* Locus on Chromosome 19 and Differential Expression of the IIB mRNAs in Human Liver†

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ABSTRACT: A cDNA designated hIIB1, representing the entire coding sequence of a P450 in the IIB gene family, was isolated from a human liver λgt11 library by using the rat IIB1 cDNA as a probe. The hIIB1 protein, deduced from the cDNA sequence, contained 491 amino acids, had a calculated molecular weight of 56 286, and displayed 76% amino acid similarity with the rat IIB1 protein. Expression of this cDNA, using the vaccinia virus system, yielded a P450 that had a reduced CO-binding spectrum with an absorption maximum of 452 nm. The expressed human enzyme was able to catalyze the deethylation of 7-ethoxycoumarin. Total RNA from 13 livers was probed for levels of hIIB mRNA. Two livers had high levels, four contained moderate levels, and eight contained very low, or no detectable, mRNA. These data suggest either that defective hIIB1 genes exist in humans or that the hIIB1 gene is regulated and variably induced in our liver specimens. To search for mutant mRNA transcripts, libraries were constructed from livers expressing low levels of hIIB1 mRNA. A cDNA, designated hIIB2, was isolated that was identical with the hIIB1 cDNA except for the presence of an unusual alteration of the DNA near its 5′ end corresponding to the putative exon 4. This alteration was caused by a deletion of 29 bp and an insertion of 44 bp of nonhomologous DNA. This sequence replacement occurs at the junction of the third and fourth exons as predicted from the structure of the rat IIB1 gene, suggesting that a faulty splice might have given rise to the variant hIIb2 transcript. Due to the presence of an in-frame termination codon in the inserted DNA, this variant transcript can only produce a prematurely terminated protein. A third cDNA, designated hIIB3, was identified in two separate libraries that displayed 95% nucleotide and 93% cDNA-deduced amino acid sequence similarities to hIIB1. This transcript was found to possess a C → T change that resulted in a termination codon. The IIB genes (*CYP2B* locus) were localized to human chromosome 19 using the somatic cell hybrid mapping strategy. High-frequency restriction fragment length polymorphisms were detected in both *Bam*HI and *Bgl*II digests.

Hepatic P450s are the principal enzymes required for the metabolism and ultimate removal of foreign chemicals from the body. The great majority of foreign substances, including drugs and toxins, when ingested, pass through the liver and are converted to other chemical forms by P450. Hydrophobic compounds are frequently hydroxylated, and the hydroxy group can then be further metabolized by conjugating enzymes, the net result being that lipophilic material is converted to a more hydrophilic compound that can be easily eliminated through the bile or urine. P450 reactions on carcinogenic compounds sometimes yield highly reactive intermediates that can damage DNA and initiate tumorgenesis. Individual differences in P450 levels could determine susceptibility or resistance to the detrimental effects of drugs or carcinogens. In fact, genetically inherited P450-mediated drug oxidation polymorphisms have been described, including the debrisoquine/sparteine polymorphism (Mahgoub et al., 1977; Ei-

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Table I: Characteristics of Liver Donors

liver	race	age (years)	sex	known drug intake
K10	caucasian	21	male	amitriptyline, cimetidine
K12	caucasian	23	female	cocaine
K14	caucasian	35	female	phenytoin, phenobarbital
K15	caucasian	39	male	dopamine, penicillin, levophed, furosamide
K16	caucasian	52	male	mannitol, dopamine
K18	caucasian	20	male	insulin, lasix
K19	caucasian	37	female	dopamine, heparin
K20	caucasian	50	female	diazepam, phenobarbital, nitropruside, dexamethasone
K21	caucasian	50	female	propranol, methyldopahydrochlorothiazide, amitriptyline, chlorodiazapoxide
M1	caucasian	21	male	unknown
M2	caucasian	23	male	cocaine, dopamine, mannitol, lasix, heparin
KDL12 (no information)				
KDL24	caucasian	54	male	thiopental

chelbaum et al., 1979) and the mephenytoin polymorphism (Kupfer & Preisig, 1984; Wilkinson et al., 1989). The enzymology of rat (Guengerich, 1987) and human (Distlerath & Guengerich, 1987) P450s has been reviewed.

Human P450s that metabolize debrisoquine (Distlerath et al., 1985; Gut et al., 1986) and mephenytoin (Shimata et al., 1986; Gut et al., 1986) have been purified. The debrisoquine/sparteine defect was determined to be due to mutant alleles of the debrisoquine 4-hydroxylase gene (Gonzalez et al., 1988), and at least two of these mutant genes can be detected by Southern analysis of lymphocyte DNA (Skoda et al., 1988). The nature of the mephenytoin polymorphism is still unclear even though cDNAs to the mephenytoin hydroxylase have been isolated (Umbenhauer et al., 1987).

The fact that some P450 genes are defective in the human population would intuitively suggest that some of these enzymes are not critical for survival or do not carry out reactions important to physiological homeostasis. Indeed, no adverse metabolic conditions have been described that seem to be associated with the lack of the debrisoquine- or mephenytoin-metabolizing P450s. These results indicate that some P450s may have evolved to metabolize only foreign chemicals, probably plant toxins, since human diets have become highly restricted and noxious plant materials can be avoided (Gonzalez, 1988). Under conditions of dietary changes, certain P450s may be dispensible, and hence their genes are beginning to accumulate mutations. With this premise, it is likely that other human P450 gene families may have members with a certain frequency of defective alleles, yet their frequency and substrate preferences may be such that they have not been detected by drug reaction anomalies. To this end, we have used rodent P450IIB cDNA probes to characterize human liver specimens for expression of the orthologous human P450 genes. Analysis of RNA from several livers has revealed that the human IIB locus displays tremendous variability in expression of hIIB-related mRNA. In the present report, we have characterized three distinct full-length cDNAs, one that is derived from a normal mRNA and two that are derived from variant mRNAs that cannot produce functional P450. These three cDNAs appear to be encoded by three different members of a multigene family.

Materials and Methods

Materials. Human liver specimens were obtained from the University of Toronto Liver Bank (K series), the Biocenter, University of Basel (KDL series), and the University of Miami Kidney Transplant Unit (M series). The characteristics of these livers are shown in Table I. Wild-type vaccinia virus strain WR, vector pSC11, and human TK$^-$ 143 cells were obtained from Dr. Bernard Moss at the National Institutes of Health and Hep G2 (ATCC HB 8065) and CV-1 (ATCC CCL70) cells from the American Type Culture Collection, Rockville, MD. cDNA synthesis kits were purchased from Bethesda Research Laboratories, Gaithersburg, MD. Phage DNA packaging extracts were obtained from Stratagene Cloning Systems, La Jolla, CA. Phosphorylated *Eco*RI linkers, CsTFA, and Sepharose CL-4B were from Pharmacia Inc., Milwaukee, WI.

Isolation and Analysis of RNA. Total liver RNA was extracted from frozen tissue by using the guanidine thiocyanate procedure of Chirgwin et al. (1979) except that the CsCl cushion was replaced with CsTFA. The RNA was extracted twice with a phenol–chloroform emulsion and then ethanol precipitated prior to use. RNA was electrophoresed on 1% agarose–2.2 M formaldehyde gels (Lehrach et al., 1977) and blotted to nylon membranes. The membranes were hybridized with nick-translated cDNA probes according to the conditions of Church and Gilbert (1984).

Cloning and Sequencing of Human IIB cDNAs. Poly(A) RNA was isolated from purified human liver RNAs using oligo(dT)–cellulose (Aviv & Leder, 1977). Double-stranded cDNA was synthesized by using the strand replacement method (Okayama & Berg, 1982) with murine sarcoma virus reverse transcriptase and DNA polymerase (Watson & Jackson, 1985). Double-stranded DNA was made blunt-ended using T4 DNA polymerase, methylated with *Eco*RI methylase, and ligated to *Eco*RI linkers. The DNA was then digested with *Eco*RI and size fractionated on Sepharose CL-4B. Fragments of greater than 1 kbp were collected and ligated to *Eco*RI-digested and phosphatase-treated λgt11 arms. The ligated DNA was packaged, and the resultant phage particles were plated on *Escherichia coli* Y1088. The libraries were screened by plaque hybridization using the rat IIB1 cDNA as a probe (Gonzalez & Kasper, 1982).

The largest cDNAs obtained from screening three libraries were subcloned into pUC9. The inserts were then sequenced by using the sonication shotgun cloning method (Deininger, 1983) in conjunction with *m*13 cloning and dideoxynucleotide sequencing (Sanger et al., 1977) except Sequenase (Tabor & Richardson, 1985) was substituted for DNA polymerase Klenow fragment. The sequence data were analyzed by using the Beckman Microgenie program.

Expression of the hIIB1 cDNA. The hIIB1[1] cDNA was excised from pUC9 using a partial *Eco*RI digest and made blunt ended with DNA polymerase Klenow fragment. The cDNA was then inserted into the *Sma*I site of the recombinant

[1] The nomenclature used in this report is according to Nebert et al. (1989). The hIIB1 and hIIB3 genes have been designated *CYP2B7* and *CYP2B8*, respectively.

plasmid pSC11 (Chakrabarti et al., 1985). Recombinant vaccinia virus expressing hIIB1 was produced by using the strategy outlined by Mackett et al. (1984) and Chakrabarti et al. (1985). Briefly, CV-1 cells were infected with vaccinia virus strain WR and then transfected with purified WR DNA and pSC11 containing the hIIB1 cDNA. After a period of 2 days, viral particles were harvested and used to infect TK^- cells that lack thymidine kinase. The virus obtained from the CV-1 infection consists of a mixture of wild-type virus and virus that had integrated pSC11 DNA. The integration event results from homologous thymidine kinase gene sequences in pSC11 that recombine with the functional thymidine kinase gene in the vaccinia virus. This event inactivates the thymidine kinase gene. In the presence of 5-bromodeoxyuridine, the wild-type vaccinia virus will not propagate on TK^- cells. The plaques containing recombinant vaccinia viruses are identified on the basis of their ability to metabolize X-Gal. Final verification of expression of hIIB1 protein was by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) and Western immunoblotting (Towbin et al., 1979). The recombinant virus was amplified on Hep G2 cells to produce IIB1 protein for enzyme activity analysis. Spectral measurements were performed as described by Omura and Sato (1966). Aryl hydrocarbon hydroxylase (Nebert & Gelboin, 1968) and ethoxycoumarin *O*-deethylase (Greenlee & Poland, 1978) activities were assayed as described.

Chromosome Localization of the CYP2B Gene Locus. The human *CYP2B* locus was localized on a human chromosome using the somatic cell hybrid strategy. Panels of human–mouse and human–hamster cells containing subsets of human chromosomes in a rodent chromosome background were prepared and analyzed in earlier reports (McBride et al., 1982a,b,c). These lines were characterized by karyotypic analysis of banded mitotic chromosomes and electrophoretic analysis of human biochemical markers. The IIB genes were localized by Southern blotting and hybridization of DNA using the hIIB1 probe as described (McBride et al., 1986).

Analysis of Gene Copy Number in CYP2B. To determine the gene copy number in the IIB locus, fragments, ranging from 140 to 320 bp and representing the first 4 putative exons within the hIIB genes, were sequentially hybridized to panels of lymphocyte DNAs from 10 different individuals that were digested separately with 12 restriction endonucleases. The number of restriction fragments that reacted with each probe was compared with the number of fragments that annealed with the 5′ coding sequence of hIIB1 containing exons 1–5.

Results

Isolation and Sequencing of Human hIIB cDNAs. Preliminary experiments were carried out to identify livers that expressed human mRNA corresponding to rat P450 IIB mRNAs. Northern blots on RNA from several liver specimens revealed livers that contained a high level of a 3.2-kb mRNA that reacted with the rat IIB1 cDNA probe. Other livers were found that had low levels of this mRNA (see below). Poly(A) mRNAs from low- and high-expressing livers were used to construct cDNA libraries. These libraries were screened by using the rat IIB1 cDNA, and three cDNAs containing the complete reading frames of proteins in the human IIB subfamily were isolated and sequenced. One cDNA, designated hIIB1, was isolated, and its deduced amino acid sequence of 491 amino acids had a calculated molecular weight of 56 286 and displayed 76% similarity to rat IIB1 (Figure 1). The ATG at nucleotide 7 probably represents the initiator Met codon since this residue aligns with the initiator Met in the rat IIB1 protein. Another cDNA, designated hIIB3, was isolated from a second cDNA library, M2, that contained only trace levels of the 3.2-kb mRNA. The hIIB3 cDNA-deduced amino acid sequence displayed 93% similarity with hIIB1 except this cDNA contained a C → T transition at residue 1148 resulting in the appearance of a TGA termination codon that replaced the Arg residue. These results indicate that hIIB3 is derived from a mutant gene transcript. This variant h11B3 transcript was also isolated from a separate human liver cDNA library derived from mRNA containing low levels of the 3.2-kb transcript. No hIIb3 cDNA was found in any of the three libraries that lacked the termination codon at position 1148.

The hIIB1 and hIIB3 cDNA nucleotide sequences were 3045 and 2907 bp, respectively, and displayed 95% similarity across their aligned regions. hIIB3 contained four deletions of 12, 126, 5, and 1 bp in the 3′-untranslated region relative to hIIB1 (Figure 1). Interestingly, the 126 bp deletion encompasses the second of two *Alu* sequences found in the 3′-untranslated region of hIIB1 (Figure 1). These repetitive units display 85% sequence similarity with the complement of the consensus *Alu* sequence (Deininger et al., 1982; Kariya et al., 1987; Britten et al., 1988). The orientation of both *Alu* sequences is opposite to that of the P450 IIB1 gene. The second *Alu* sequence contains a 116 bp truncation at its 5′ end, accompanied by a 33 bp insertion, and the 126 bp deletion in hIIB3 includes 5 bp of this inserted sequence. Both *Alu* sequences are members of a very old subfamily [class II of Britten et al. (1988)], and they were probably inserted into their present location greater than 43 million years ago. However, both sequences are flanked by nearly perfect direct target site repeats (Figure 1), indicating independent insertion events in close proximity. The presence of the same *Alu* sequences in the 3′ flank of both hIIB1 and hIIB3 genes indicates that both *Alu* insertions preceded reduplications of the IIB locus to form the human IIB1 and IIB3 genes. Interestingly, three inverse/complement *Alu* sequences were found in the 3′-flanking region of the human IA2 mRNA (Quattrochi et al., 1986; Jaiswal et al., 1987). The significance of this finding is unclear since the cellular role of *Alu* sequences has not been elucidated.

A third variant cDNA, designated hIIB2, was isolated from the M2 library. Three separate hIIB2 cDNAs were isolated from the M2 library suggesting that this transcript is not a cloning artifact. The hIIB2 cDNA was identical with the hIIB1 cDNA except for the presence of eight base changes in the 3′-untranslated region (Figure 1 legend) and a region of sequence between residues 490 and 519 (Figure 1) that appears to have been generated by deletion of 29 bp of sequence complementary to hIIB1 and insertion of 44 bp of noncomplementary sequence (Figure 2). Assuming that the human IIB genes share the same intron–exon boundaries as their rat counterparts (Suwa et al., 1985), this altered sequence would be located beginning at the 3′ junction of exon 3 and exon 4 (Figure 2). It appears that the unusual sequence in hIIB2 is either due to (i) an alternative splice of the hIIB1 gene, (ii) a mutant hIIB1 gene, or (iii) the presence of a recently duplicated and nonfunctional gene. In any case, the newly inserted sequence introduces a termination codon in the hIIB2 mRNA resulting in a truncated protein.

Expression of hIIB mRNAs in Human Liver. To examine the expression of IIB mRNA in human liver, Northern blotting was carried out by using a probe derived from the 5′-coding region of the hIIB1 cDNA that was devoid of *Alu* sequence. A 3.2-kb mRNA was detected in several of the 13 liver specimens derived from kidney transplant donors (Figure 3).

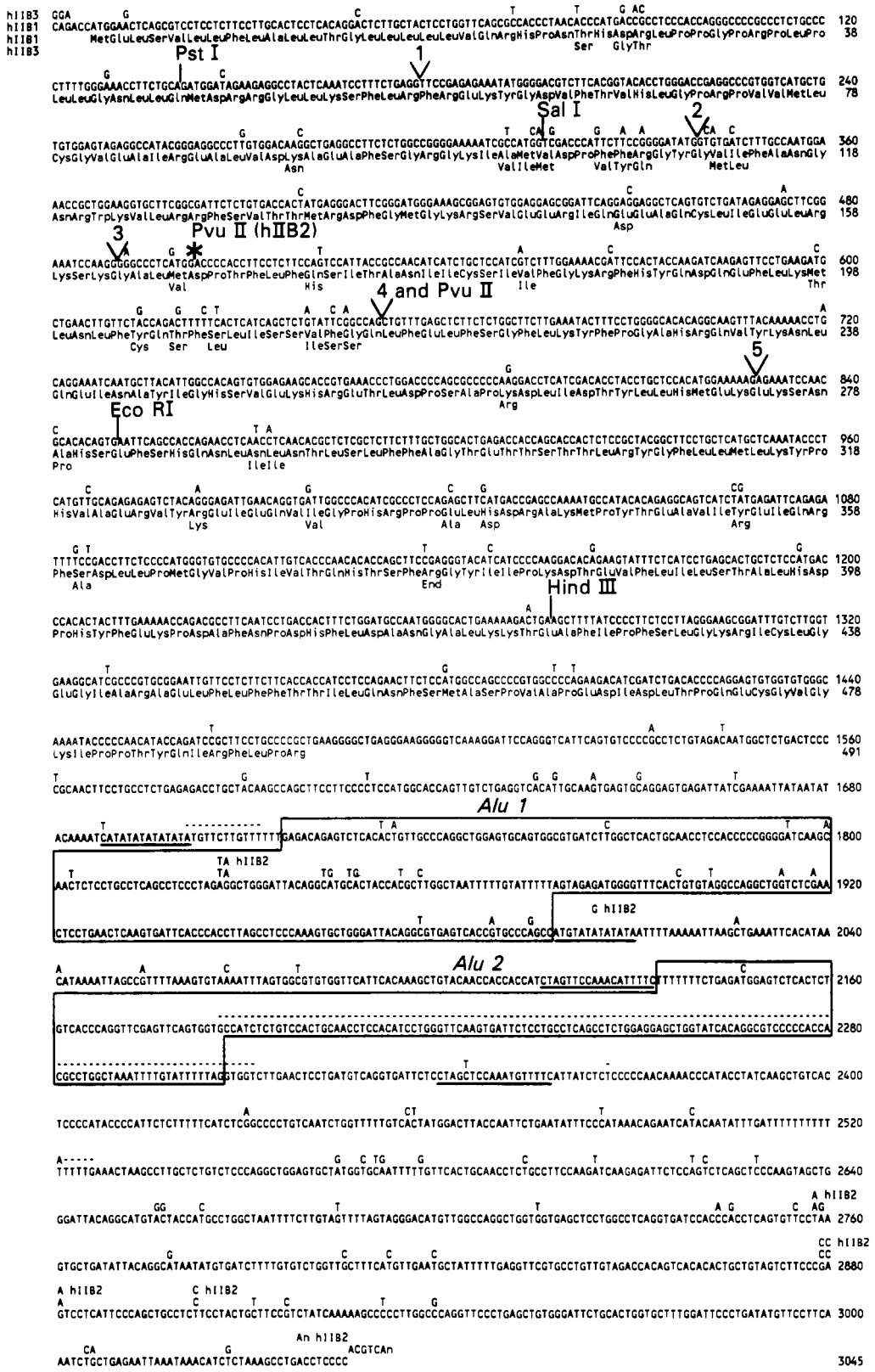

FIGURE 1: Sequence of the hIIB1 and hIIB3 cDNAs. The complete nucleotide and deduced amino acid sequence of hIIB1 is shown. Only those nucleotide and amino acid residues of hIIB3 that differ from hIIB1 are shown above and below the hIIB1 sequences, respectively. Dashes represent the absence of sequence residues in hIIB3. The arrows denote the putative positions of hIIB gene exons, deduced from the sequence of the rat IIB1 gene (Suwa et al., 1985). The restriction sites used to generate various probes from hIIB1 are displayed above their recognition sites. *Alu* sequences are enclosed by boxes, and the direct repeats flanking the *Alu* sequences are underlined. hIIB2 is identical with hIIB1 except for an insertion of sequence between residues 490 and 491 (Figure 2) and the following: A at 1826, G at 1827, A at 2004, T at 2758, G at 2879, A at 2880, G at 2881, and T at 2902 (these numbers correspond to the hIIB1 sequence).

We did not note the presence of two distinct mRNAs despite the fact that hIIB1 and hIIB3 cDNAs differ in length by about 100 bp. However, this small difference in size may not be large enough to resolve on our Northern blots.

The level of expression of the 3.2-kb mRNA was quite variable among the different liver specimens. Two livers (K14 and K19) had a high level of the 3.2-kb mRNA, whereas four livers (K10, K12, K20, and KDL 24) had levels about one-

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rIIB1 EXON 3  TGTTTGGTGGAGGAACTGCGGAAATCCCAGG gtgaa---INTRON 3---tccag GAGCCCCACTGGATCCCACCTTCCTCTTCCAGTGCATCACAGCCAAC EXON 4
              CysLeuValGluGluLeuArgLysSerGlnG                       lyAlaProLeuAspProThrPheLeuPheGlnCysIleThrAlaAsn

hIIB1 EXON 3  TGTCTGATAGAGGAGCTTCGGAAATCCAAGG---------INTRON 3---------GGGCCCTCATGGACCCCACCTTCCTCTTCCAGTCCATTACCGCCAAC EXON 4
              CysLeuIleGluGluLeuArgLysSerLysG                       lyAlaLeuMetAspProThrPheLeuPheGlnSerIleThrAlaAsn

hIIB2 EXON 3  TGTCTGATAGAGGAGCTTCGGAAATCCAAGG                                                  CAGTCCATTACCGCCAAC EXON 4
              CysLeuIleGluGluLeuArgLysSerLysA
                                            ACCACTCTCCCTCCAGCTGGGGCCAGTGCTGAGCCTGGTGTATA <-- INSERTED SEQUENCE
                                            spHisSerProSerSerTrpGlyGlnCysEnd
```

FIGURE 2: Sequence of the exon 3 and 4 regions of the hIIB1 and hIIB2 cDNAs and comparison to the rat IIB1 (rIIB1) gene (Suwa et al., 1985). The hIIB1 and hIIB2 sequences are shown across the defective region of hIIB2. The intron 4 of rIIB1 and the putative intron 4 of hIIB1 are shown. The GT, AG splice consensus sequences of the rat gene are underlined. The inserted sequence in hIIB2 begins at the exon 3 3′ junction and extends into exon 4. The first 29 bp of exon 4 in hIIB1 is missing in hIIB2.

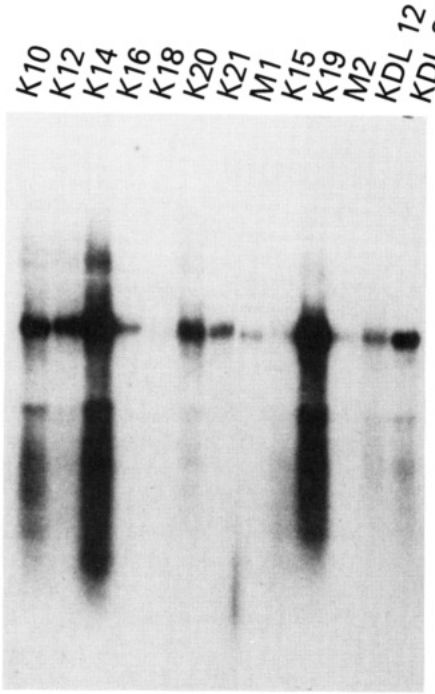

FIGURE 3: Northern blotting of RNA isolated from 13 human liver samples. The RNAs (10 μg/well) were electrophoresed, transferred to Nytran paper, and hybridized with the 5′-*Eco*RI fragment derived from hIIB1 (Figure 1). The filter was exposed to film for 48 h with the aid of a Dupont "Lightning Plus" intensifying screen.

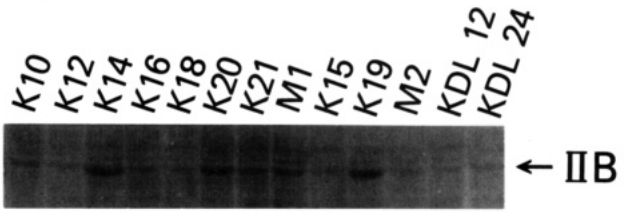

FIGURE 4: Western immunoblot of IIB protein expressed in human livers. Thirteen human liver microsomes samples (50 μg/well) were electrophoresed and transferred to nitrocellulose paper, and the IIB proteins were stained using rabbit anti-rat IIB1 and alkaline phosphatase conjugated goat anti-rabbit IgG.

fourth those of K14 and K19, and the remaining seven livers had low or undetectable 3.2-kb mRNA. The reason for this variability in expression between individual livers is unknown. However, it is possible that IIB mRNA expression is regulated by the presence of inducers since the rat IIB1 and IIB2 genes are inducible by phenobarbital (Hardwick et al., 1983; Omiecinski et al., 1985). Indeed, the K14 patient was an epileptic that had been receiving phenobarbital. The K19 patient, on the other hand, was not known to have received any drug suspected to be a phenobarbital-type inducer. Furthermore, the patients from which K20 and KDL 24 were derived were administered phenobarbital and thiopental, respectively, and yet these samples contained only moderate levels of the 3.2-kb mRNA (Figure 3). Finally, there does not appear to be any correlation between the age or sex of the donors and the level of the hIIB mRNA.

Summary of cDNA Cloning and Northern Blotting Results. By use of oligonucleotide probes specific to mRNAs corresponding to hIIB1, hIIB2 and hIIB3 cDNAs were used to screen these Northern blots. Due to low levels of mRNA, we were only able to detect hIIB1 in K14 and K19 livers.[2] We therefore do not know the frequency of expression of each mRNA in these livers. By direct cDNA cloning and sequencing, we found two hIIB1 cDNAs and one hIIB3 cDNA in the K19 library. On the basis of restriction enzyme mapping of five other cDNAs, they all appeared to correspond to hIIB1. The M2 library, constructed from a liver expressing trace levels of IIB mRNAs (Figure 3), yielded hIIB3 and hIIB2 cDNAs. Five other clones were restriction mapped and corresponded to either hIIB1 or hIIB2. This distinction could not be made because the cDNAs were not long enough to encompass the variable region between hIIB1 and hIIB2. The M2 library was not further analyzed due to the low level of hIIB cDNA clones.

At present, it is unclear what accounts for the low level of hIIB mRNAs in several of the livers we have studied. It is tempting to speculate that these livers contain only defective mRNAs incapable of producing P450. In general, mRNAs that are defective and contain premature termination codons are unstable in the cell (Daar & Maquat, 1988).

Expression of hIIB Proteins in Human Liver. To determine if the levels of 3.2-kb mRNA were correlated with the amount of hIIB protein in each liver sample, Western immunoblotting was performed using antibody against rat IIB1. The antibody reacted weakly with human microsomal IIB protein, yet this protein had a relative molecular weight of 52 000 and comigrated on SDS–polyacrylamide gels with rat IIB1. The most prominent immunoreactive band was found in livers K14 and K19 (Figure 4). These two livers had high levels of the 3.2-kb mRNA. Very faint proteins were detected in K18, K15, K16, M2, and KDL 12; these livers had trace levels of the hIIB mRNA. Less apparent correlation is found between the mRNA and protein levels detected in the other liver specimens, however.

Mapping of the CYP2B Locus. The chromosome location of the IIB genes was determined by analyzing somatic cell hybrids segregating human chromosomes. Human–rodent hybrid cell DNAs were digested with *Eco*RI and subjected to Southern blotting analysis. All human sequences hybridizing with the hIIB1 cDNA probe (3.1, 3.4, 3.8, 4.2, 4.7, and 9.0 kb) appeared to segregate concordantly (i.e., either all present or all absent) in the hybrids, but some bands could not be resolved from cross-hybridizing rodent bands (Figure

[2] Unpublished experiments.

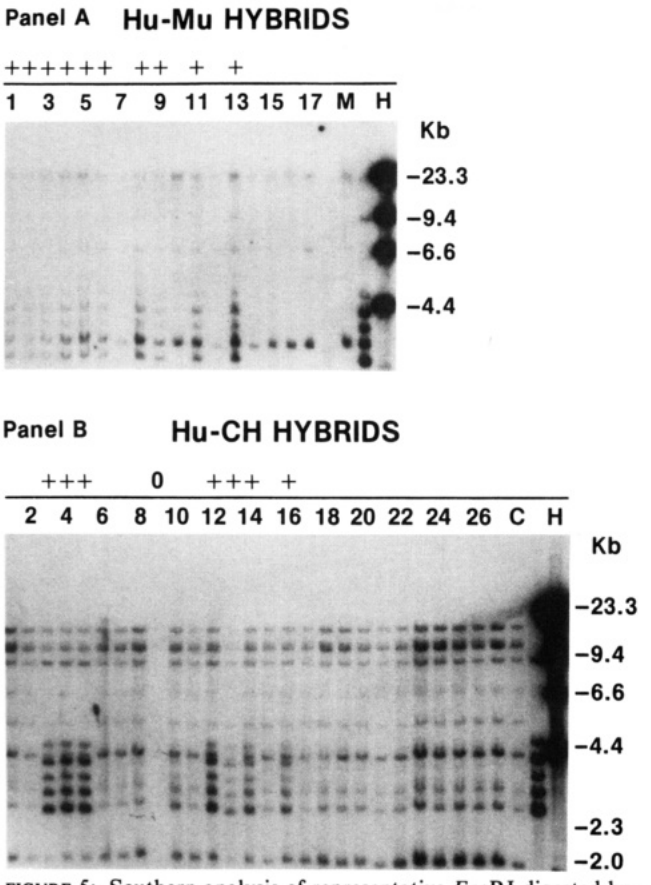

FIGURE 5: Southern analysis of representative *Eco*RI-digested human–hamster (lanes 1–27 bottom) and human–mouse (lanes 1–18 top) somatic cell hybrid DNAs with a 800 bp hIIB1 5′-cDNA probe containing exons 1–5. A different hybrid cell DNA (10 μg) is present in each lane; parental mouse (M), Chinese hamster (C), and human placental (H) DNAs are also shown. Positions and sizes of *Hin*dIII-digested λ phage markers are shown at the right of each figure. Hybrids containing human hIIB1 hybridizing sequences (3.1, 3.4, 3.8, 4.2, 4.7, and 9.0 kb) are indicated (+) above the lanes; no DNA is present in lane 9 (0) of the human–hamster digests. Cross-hybridizing 2.1-, 3.1-, 3.4-, 4.3-, 5.4-, 6.8-, 9.0-, 11.9-, and 17.5-kb hamster (top) and 3.4-, 6.6-, and 23-kb mouse (bottom) sequences were also detected. The panel of *Eco*RI-digested human–rodent hybrid cell DNAs was also hybridized with an 800 bp hIIB3 cDNA probe (results not shown), and these blots were washed at high stringency (i.e., 66 °C in 0.1 × SSC–0.2% SDS) due to minor contamination of this probe with *Alu* sequences. An identical pattern of hybridizing human bands was observed, but only the 2.1-kb cross-hybridizing hamster bands, and no mouse bands, were observed.

5). These results were confirmed by hybridization of the same blots with a 160 bp probe consisting of only exon 3 sequences, but the complexity of cross-hybridizing rodent sequences was not appreciably reduced. However, all human bands could be detected with an 800 bp hIIB1 5′-cDNA probe, and these bands all segregated concordantly under more stringent hybridization conditions (not shown) which failed to detect the overlapping rodent bands. The multigene hIIB family could be assigned (Table II) to human chromosome 19 (*CYP2B* locus), and it segregated discordantly (≥17%) with all other human chromosomes. The four apparent discordancies with chromosome 19 resulted from failure to detect the human IIB fragments in hybrid lines that retained chromosome 19 in a small fraction of the cell population.

Restriction Fragment Length Polymorphisms at the CYP2B Locus. Simple two-allele polymorphisms, due to loss or gain of a restriction site, were detected at the *CYP2B* locus with *Bam*HI and *Bgl*II (Figure 6). The sizes of allelic bands in *Bam*HI digests (Figure 6, bottom) were 19 kb (A1) and 8.9

Table II: Segregation of the Human P450 IIB Multigene Family with Chromosome 19[a]

human chromosome	gene/chromosome +/+	+/−	−/+	−/−	% discordancy
1	21	3	13	58	17
2	16	8	10	61	19
3	18	6	17	54	24
4	21	3	37	34	42
5	18	6	11	60	18
6	24	0	24	47	25
7	13	11	26	45	39
8	18	6	19	52	26
9	18	6	13	58	20
10	10	14	9	62	24
11	14	10	16	55	27
12	11	13	17	54	32
13	14	10	21	50	33
14	15	9	27	44	38
15	17	7	29	42	38
16	13	11	23	48	46
17	20	4	37	34	43
18	17	7	33	38	42
19	24	0	4	67	4[b]
20	21	3	19	52	23
21	22	2	40	31	44
22	14	10	14	57	25
X	14	10	34	37	46

[a] The hIIB genes were detected in an *Eco*RI digest of hybrid cell DNAs as 9.0, 4.8, 4.2, 3.8, 3.4, and 3.1 kbp fragments that annealed with an 840 bp probe derived from the 5′-coding region of the hIIB1 cDNA. This probe also cross-reacted with numerous rodent sequences. However, most of the human fragments could be resolved from the rodent fragments, allowing accurate chromosome assignment (see text). Detection of the IIB genes is correlated with the presence or absence of each human chromsome in the group of somatic cell hybrids. Discordancy represents the presence of the gene in the absence of the chromosome (−/−) or the absence of the gene despite the presence of the chromosome (−/+), and the sum of these numbers divided by the total hybrids examined (×100) represents percent discordancy. The human–hamster hybrids consisted of 27 primary clones and 14 subclones (14 positive of 41 total), and the human–mouse hybrids represented 14 primary hybrids and 40 subclones (10 positive of 54 total). Assignment of the P450 IIB gene family was confirmed by hybridization of the same hybrid cell DNA panel with a P450 IIB2 5′-cDNA probe; the hybridization pattern was simplified by washing at high stringency which prevented detection of cross-hybridizing rodent sequences in these digests. All human bands segregated concordantly (i.e., together) under these circumstances. [b] All discordancies resulted from failure to detect hIIB-hybridizing sequences in one human–hamster and three human–mouse hybrids retaining chromosome 19 in only a small fraction of the cell population.

kb (A2) whereas 21-kb (B1) and 16.6-kb (B2) allelic bands were found in *Bgl*II digests (Figure 6, top). Invariant bands were also detected in both digests, and these bands appear to represent nonallelic members of this hIIB multigene family at the same locus. The allele frequencies in 39 individuals were A1:A2 = 0.47:0.53 and B1:B2 = 0.81:0.19. The use of both RFLPs at this locus provides a highly informative three- or four-allele system, and the observed haplotype frequencies were A1B1 = 0.28–0.37, A1B2 = 0.11–0.20, A2B1 = 0.44–0.53, and A2B2 = 0–0.09. These polymorphisms will permit further localization of the *CYP2B* locus on chromosome 19 by genetic linkage analysis in families. Two probable RFLPs were detected in *Msp*I digests, but alleles could not be identified in the complex pattern of hybridizing bands. No RFLPs were detected in DNAs from 10 individuals using *Eco*RI, *Hin*dIII, *Taq*I, *Sac*I, *Xba*I, *Pvu*II, *Eco*RV, or *Kpn*I. A single 2.7-kb variant (frequency 0.01) band allelic with a 2.4-kb band was found in *Pst*I digests.

Determination of Gene Copy Number. The very complex pattern of human bands hybridizing with the hIIB1 5′-cDNA probe in *Eco*RI digests (Figure 5), as well as 11 other re-

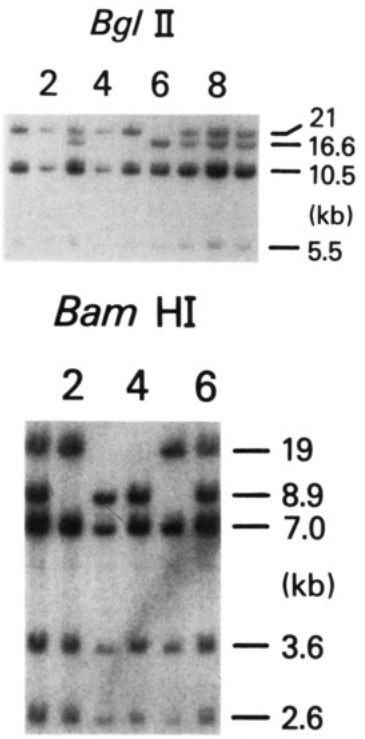

FIGURE 6: Restriction fragment length polymorphisms (RFLPs) detected in human genomic DNAs with human hIIB1 cDNA probes. *Bgl*II (top) and *Bam*HI (bottom) digests of DNAs isolated from peripheral leukocytes of unrelated individuals were fractionated by 0.7% agarose gel electrophoresis, transferred to nylon membranes, and hybridized with an 800 bp human hIIB1 5′-cDNA probe. In *Bam*HI digests, a 19-kb band (A1) (lanes 2 and 5) is allelic with an 8.9-kb band (lanes 3 and 4); lanes 1 and 6 depict heterozygotes. A 21-kb band (B1) (lanes 1, 2, 4, and 5) is allelic with a 16.6-kb band (B2) (lane 6) in *Bgl*II digests, and heterozyotes are shown in lanes 3, 7, 8, and 9. Invariant bands of 2.6, 3.6, and 7.0 kb or 5.5, 10, and 11 kb are present in *Bam*HI and *Bgl*II digests, respectively, and represent nonallelic members of the *CYP2B* family. Allele frequencies in 39 individuals were A1:A2 = 0.47:0.53 and B1:B2 = 0.81:0.19.

striction digests (not shown), suggested that multiple human hIIB genes exist at the *CYP2B* locus. To determine the gene copy number, Southern blots of DNA restriction digests from 10 individuals were probed with 4 small contiguous fragments of the hIIB1 cDNA corresponding to exons 1–4. The same blots were used sequentially for hybridization with each probe, thereby permitting precise alignment of bands hybridizing with each probe. It was anticipated that the number of identical bands hybridizing with any two probes representing contiguous cDNA sequences would reflect the minimum copy number of hIIB genes. The results, summarized in Table III, suggest the presence of four distinct hIIB genes at this locus.

Results of hybridization with the exon 1 probe are particularly informative since the number of different hybridizing bands detected in most restriction digests should reflect the number of genes. A single band hybridizing with the exon 1 probe can be anticipated for any member of this multigene family unless the relevant restriction site is present in exon 1. Moreover, different sized hybridizing fragments would probably be found with each gene since one member of each pair of restriction sites is located within the nonconserved flanking region. The detection of four nonallelic fragments hybridizing with the 140 bp exon 1 probe after digestion with nearly all restriction enzymes (Table III) strongly suggests the presence of four genes. Detection of identical bands with the exon 2 probe after digestion with enzymes such as *Bam*HI and *Bgl*II demonstrates that these multiple bands do not result from the presence of internal restriction sites within a smaller

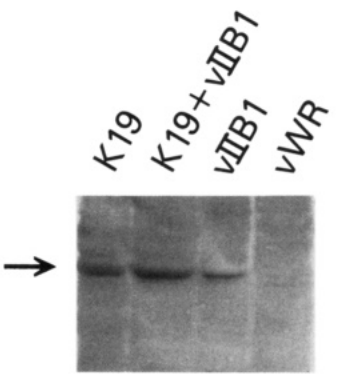

FIGURE 7: Analysis of cells infected with vhIIB1. TK⁻ cells were infected with wild-type vaccinia virus vWR or recombinant vaccinia virus vhIIB1 containing the hIIB1 cDNA. Cells were sonicated, and 50 μg of total cell protein and 50 μg of human microsomal protein were subjected to immunoblot analysis using rabbit antibody against rat IIB1 and phosphatase-conjugated goat antibody against rat IgG.

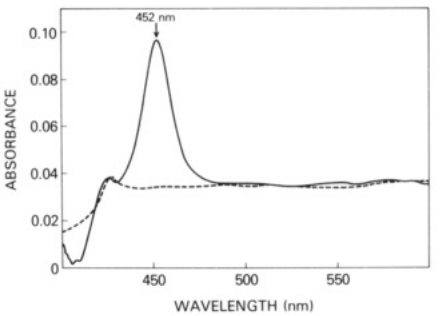

FIGURE 8: Spectral analysis of cells infected with vIIB1. Microsomes were isolated from TK⁻ cells infected with vWR and vIIB1. Reduced CO-binding absorption spectra were measured as described by Omura and Sato (1966). Microsomes were suspended in 100 mM potassium phosphate buffer (pH 7.25), 20% w/v glycerol, and 0.20% w/v Emulgen 911. Spectra were measured by using an Aminco DW 2000 spectrophotometer.

number of genes. The loss of some hybridizing bands and the appearance of new bands with different exonic probes, as observed with *Eco*RI or *Xba*I, indicate the presence of these specific internal restriction sites (i.e., probably intronic) within some, but not all, members of this multigene family. The failure to detect at least four hybridizing bands with each probe may reflect differing extents of sequence similarity of the individuals genes with different portions of the cDNA or the presence of fragments not adequately resolved from other hybridizing bands. In any case, the results are most readily explained by the existence of four genes at the *CYP2B* locus.

Expression of the hIIB1 cDNA. To determine if the hIIB1 cDNA can produce a normal P450, it was inserted into vaccinia virus using the pSC11 vaccinia virus expression system (Chakrabarti et al., 1985). The expressed enzyme reacted with antibody to rat IIB1 and comigrated with hIIB protein found in human K19 microsomes (Figure 7). Spectral analysis of hIIB1 P450 revealed a reduced CO-complexed pigment with an absorption maximum at 452 nm, typical of a P450 (Figure 8). The cDNA-expressed enzyme was able to catalyze the O-deethylation of 7-ethoxycoumarin, a reaction that is also carried out by rat IIB1 (Guengerich, 1987). Cells infected with hIIB1 recombinant virus produced the product coumarin at a rate of 40 ± 3.0 pmol min^{-1} (mg of cell lysate protein)$^{-1}$, whereas cells infected with wild-type vaccinia yielded only 4.9 ± 0.9 pmol min^{-1} mg^{-1}. hIIB1-expressing cells were also able to metabolize benzo[*a*]pyrene, albeit at a rate much lower than that for 7-ethoxycoumarin metabolism. The ability of hIIB1 to catalyze the oxidation of other drugs and chemicals needs to be examined further.

Table III: Analysis of Gene Copy Number at the Human *CYP2B* Locus on Chromosome[a] 19

enzyme	fragments (kbp)	exon 1	exon 2	exon 3	exon 4
*Bam*HI	19.0/8.9[b]	+	+	+	+
	7.0	+	+	+	+
	3.6	+	+	–	–
	2.6	+	+	–	–
*Bgl*II	21.0/16.6[b]	+	+	+	+
	10	+	+	+	+
	11.0	+	+	–	–
	5.5	+	+	–	–
	1.4, 1.1	–	–	+	–
*Eco*RI	9.0	+	+	–	–
	4.7	+	+	–	–
	4.2	–	+	+	+
	3.8	+	+[c]	+	+
	3.4	+	+[c]	+	+
	3.1	–	+	+	+
*Hin*dIII	9.4	+	+	+	+
	8.0	–	–	+[c]	–
	6.3	+	+	–	–
	4.7	+	+	+	+
	3.8	+[c]	–	–	–
	2.4	–	+	+	+
*Sac*I	10.5	+	+	–	–
	8.8	+	+	+	+
	8.2	–	+	+	+
	4.6	+	+	–	+[c]
*Xba*I	22.8	+	+[c]	–	–
	13.5	–	+	+	+
	9.2	–	+	+	+
	5.7	+	+	–	–
	3.4	+	+	–	+[c]
	2.7	+	+	–	–

[a] A panel of DNA from 10 individuals were separately digested with each of the restriction enzymes shown in the left column. The probes used (Figure 1) were an *Eco*RI–*Pst*I fragment of 149 bp corresponding to most of exon 1 (5′ end to base 140), a *Pst*I–*Sal*I fragment of 176 bp corresponding to exon 2 (bases 141–316), a *Sal*I to *Pvu*II fragment of 190 bp corresponding to exon 3 (bases 317–513) that was derived from hIIB2, and a *Pvu*II–*Pvu*II fragment of 138 bp corresponding to exon 4 (bases 514–651, Figure 1) derived from hIIB2. [b] Allelic bands. [c] Weakly hybridizing fragments.

Discussion

By direct cDNA cloning from human liver libraries, three distinct *CYP2B* gene products have been identified. The hIIB1 cDNA codes for an apparently normal enzyme that is very similar in sequence to rat IIB1. A second cDNA, hIIB2, is almost identical with hIIB1 except for the presence of an unusual sequence in its putative fourth exon region. This sequence appears to have been generated by both deletion of normal DNA and insertion of DNA foreign to the hIIB1 gene. The IIB2 variant cDNA was isolated from two separate libraries, thereby excluding the possibility that it is the result of a cloning artifact. This variant IIB2 RNA could have been generated from (i) a faulty splice of the third intron from a mutant hIIB1 gene, (ii) alternative splicing of a normal IIB1 gene, or (iii) a second gene that had recently duplicated and accumulated an insertion of a foreign sequence via a spurious recombination event or a transposition. The IIB2 variant transcript is very similar to that noted by Kimura et al. (1988) in which a variant transcript of the rat PB-1 (IIC6) gene was found that had an unusual replacement block of sequence that encompassed the putative eighth exon of the IIC6 gene. These investigators proposed that either an alternative splicing of a cryptic exon or a recombinant gene generated by gene conversion could have given rise to this unusual transcript. A variant transcript in the IID subfamily of mouse was also found that lacked coding sequence corresponding to exon 6 (Wong et al., 1987). It is unclear whether this transcript originated from an alternative splice or from a mutant gene. Others identified a variant human IIC8 transcript that had a 39 b insertion that is between the putative seventh and eight exons of the IIC8 gene (Okino et al., 1987). It was proposed that this transcript was generated through alternative pre-mRNA processing in which another splice acceptor site is used. The presence of a mutant IIC8 allele, however, could not be excluded. These unusual variant transcripts may be common to genes in the P450 superfamily.

The hIIB3 cDNA we characterized was apparently derived from a mutant gene. This cDNA was isolated from two libraries made from separate individual livers. We have not been able to isolate the normal cDNA counterpart of hIIB3. The hIIB3 gene is capable of producing only a truncated protein that lacks the critical heme-binding cysteine residue. Therefore, this protein would not be enzymatically active. Southern blotting data indicate that a fourth gene also exists in the human IIB subfamily.

Others have recently cloned cDNAs in the human IIB gene subfamily (Miles et al., 1988). One of these cDNAs, λMP1, clearly was a truncated variant of hIIB1 and had the carboxy-terminal half of the protein sequence beginning at amino acid 275 (Figure 1) and the complete 3′-untranslated region. This cDNA also contained a portion of the putative fifth intron of the gene and a deletion of sequence within the eighth exon. The second cDNA, λMP2, had less amino acid coding sequence, yet was otherwise normal (Miles et al., 1988). We found only five nucleotide differences between hIIB1 and λMP1/λMP2.

The *CYP2B* locus was mapped to human chromosome 19. This confirms results recently published by Miles et al. (1988) and Santisteban et al. (1988), who sublocalized these genes to the region 19q12-q13.2. *Bam*HI and *Msp*I RFLPs were also found to exist around the *CYP2B* locus (Miles et al., 1988). We confirmed the *Bam*HI RFLP, but we could not interpret the complex *Msp*I RFLPs detected with our probes. In addition, we detected a high-frequency two-allele RFLP using *Bgl*II. These RFLPs should prove very useful for studies to further sublocalize this locus using family linkage analysis.

The hIIB1 cDNA is capable of producing an enzyme with a normal heme environment as evidenced by the typical reduced CO-complexed Soret absorption maximum at 452 nm. The expressed P450 also catalyzes the O-deethylation of 7-ethoxycoumarin, similar to its rat counterpart. Further experiments screening additional substrates will be necessary to establish the specificity of hIIB1.

Finally, by use of a bank of human liver specimens, we have identified a subfamily of P450s with substantial variability in levels of expression among different individuals. At least a portion of this individual variability in expression is due to the presence of mutant genes, including hIIB3. It is also possible that some of the observed variability was due to the presence of inducing agents, since the rat IIB1 and IIB2 genes are known to be highly inducible. This question, however, is very difficult to answer in humans unless an in vivo assay is developed for the human IIB gene products.

Registry No. DNA (human liver clone hIIB1 cytochrome P450 isoform IIB mRNA complementary), 121787-66-4; cytochrome P450 IIB (human liver clone hIIB1 isoform IIB1 protein moiety reduced), 121787-63-1; DNA (human liver clone hIIB3 cytochrome P450 isoform IIB mRNA complementary), 121787-68-6; cytochrome P450 IIB (human liver clone hIIb3 isoform IIB3 protein moiety reduced), 121787-65-3; cytochrome P450, 9035-51-2; DNA (human liver clone hIIB2 cytochrome P450 isoform IIB2 moiety reduced), 121787-67-5; cytochrome P450 (human liver clone hIIB2 isoform IIB2 protein), 121787-64-2.

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