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Null Phenotype for Cytochrome P450 2B2 in the Rat Results from a Deletion of Its Structural Gene

CURTIS J. OMIECINSKI, RICHARD RAMSDEN, ARFAAN RAMPERSAUD,[1] and FREDERICK G. WALZ, JR.

Department of Environmental Health, School of Public Health and Community Medicine, University of Washington, Seattle, Washington 98195 (C.J.O., R.R.), and Department of Chemistry, Kent State University, Kent, Ohio 44242 (A.R., F.G.W.)

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SUMMARY

The absence of phenobarbital (PB)-inducible cytochrome P450 2B2 (CYP2B2) in hepatic microsomes from Marshall 520 (M520) and Wistar Munich (WM) inbred strains of rat was previously reported [*Biochem. Genet.* **25**:527–534 (1987)], and it was subsequently shown for M520 rats that corresponding CYP2B2 mRNA was not detected in hepatic extracts from either control or PB-treated animals [*DNA* **8**:29–37 (1989)]. In the present study, solution hybridization was used to quantify PB-induced CYP2B2 and CYP2B1 mRNAs in livers from M520, WM, and outbred Sprague-Dawley rats, as well as additional inbred strains that express all known electrophoretic phenotypes for both of these closely related isozymes. Amounts of these mRNAs were also measured for F_1 and F_2 progenies of crosses involving M520 rats. The results indicated that the extent of PB induction of both isozymes appears to be independent of the electrophoretic phenotype. It was also shown that the null phenotype for CYP2B2 observed in M520 rats results from a mutation of a single autosomal gene and is inherited codominantly, regarding protein and mRNA phenotypes. Analyses of restriction digests and specific polymerase chain reaction products of hepatic DNAs revealed that the basis for the null phenotype of CYP2B2 in M520 and WM rats was a deletion of the *CYP2B2* gene.

In different mammalian tissues, the oxidative metabolism of xenobiotics and endogenous compounds is catalyzed primarily by the CYP450 system associated with the endoplasmic reticulum. The ability of this system to catalyze monooxygenase reactions with a bewildering variety of substrates results from the presence of different CYP450 isozymes, which usually exhibit distinct but overlapping substrate specificities. In the laboratory rat (*Rattus norvegicus*), the primary structures of almost 40 CYP450 isozymes have been categorized into nine families (members having ≥40% sequence identity) and 16 subfamilies (members sharing ≥59% sequence identity) (1, 2). Unique isozyme patterns are programmed for a given tissue and a large subset of CYP450 isozymes are expressed in the liver, where most are subject to additional inductive (or repressive) controls by hormones and xenobiotics (1).

The *CYP2B* subfamily in the rat has been studied extensively and consists of approximately 10 genes, based on hybridization studies with cDNA probes and limited genomic sequencing (3, 4). Complete primary structures are known for CYP2B1 and CYP2B2, which share ~97% sequence identity (5, 6). For rats, low levels of CYP2B2 but not CYP2B1 are expressed constitutively in the liver (7–9), whereas both of these isozymes are induced dramatically after PB administration (10, 11). In a recent study, cDNA sequencing revealed an alternatively spliced version of PB-induced CYP2B2 that predicted the existence of a protein containing an insert of eight additional amino acid residues (12), which may correspond to a PB-induced polypeptide that has a higher apparent molecular mass than other forms of CYP2B2 and is immunochemically more similar to CYP2B2 than to CYP2B1 (7). The complete primary structure of putative CYP2B3 was deduced from its cDNA sequence and was shown to be 77% identical to that of CYP2B1 (13). Polyadenylated mRNA coding for CYP2B3 was constitutively expressed in the liver, but its level was nonresponsive to PB treatment. In addition, the partial sequence of a cloned genomic fragment coding for the first 58 amino acids of putative CYP2B8 (originally designated the product of gene IV) has been reported and shown to be 62% similar to the corresponding region of CYP2B1 (4). Hepatic mRNA representing CYP2B8 has been shown to exist at low levels and was modestly induced (~6-fold) by PB (4). Unlike *CYP2B1* and *CYP2B2*, hemoprotein products of *CYP2B3* and *CYP2B8* genes have not yet been

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[1] Present address: Department of Biochemistry, Rutgers University, Piscataway, NJ 08854.

ABBREVIATIONS: CYP450, cytochrome P450; IF/SDS electrophoresis, two-dimensional electrophoresis with isoelectric focusing in the first dimension and sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the second dimension; PCR, polymerase chain reaction; SD, Sprague-Dawley/N; LEW, LEW/SSN; M520, Marshall 520/N; SHR, SHR/N; SHRsp, SHRsp/A3N; COP, Copenhagen 2331/NHsd; WF, Wistar Furth/Hsd; WM, Wistar Munich/Hsd; PB, phenobarbital; Kb, kilobase(s); bp, base pair(s).

definitively characterized and, therefore, the biological significance of their expression is not well understood.

The molecular complexity of the *CYP2B* subfamily in rat liver is augmented by the existence of allelic variants for CYP2B1 and CYP2B2, which have been detected either by electrophoretic analysis (14, 15) or from the results of protein/DNA sequencing (6, 12, 16–24). The demonstration that a structural polymorphism for CYP2B2 resulted in the loss of its ability to catalyze testosterone 16β-hydroxylation, when expressed in Hep G2 cells, suggests that such allelic variation may be physiologically significant (24). On the other hand, the *in vitro* catalytic properties of two purified electrophoretic variants of CYP2B1 were found to be the same when tested with testosterone and six other substrates, using reconstituted systems (25).

IF/SDS electrophoresis of hepatic microsomes from PB-treated rats, representing a variety of inbred strains, was used to study systematically allelic polymorphisms among members of the *CYP2B* and *CYP2C* subfamilies (15). Strains were shown to be homozygous for any one of four electrophoretic variants of CYP2B1 [designated CYP2B1-(e)*m* (m = 3, 4, 6, or 7)] and for either of two electrophoretic variants of CYP2B2 [designated CYP2B2-(e)*n* (n = 5 or 8)].[2] On the other hand, assuming no sequencing errors, at least three alleles, representing differences in protein sequences, have been shown to characterize *CYP2B1* (see Refs. 6 and 16–18) and at least five unique sequences represent *CYP2B2* variants (see Refs. 6, 12, and 19–24). The structural basis for the electrophoretic variants CYP2B1-(e)3 in F344 rats is now known, because the complete cDNA sequence for *CYP2B1* in this inbred strain has been reported (18). Because all of the remaining sequences were determined for protein/DNA derived from outbred rats and are incomplete in many cases, it is not possible to make additional identifications of sequences for a given electrophoretic variant. Nevertheless, the sequence information to date predicts differences in electrostatic charge that are consistent with the observed isoelectric focusing behavior of variants of both CYP2B1 and CYP2B2. On the other hand, this limited sequence information also suggests the possibility that some variants might have the same electrostatic charge.

In addition to these electrophoretic variants, a null allele (i.e., *CYP2B2-(r)0*) was found to characterize inbred M520 and WM rats (15). It was speculated that this variant might represent a structural gene deletion as a result of an unequal crossover event (15), because extremely homologous *CYP2B1* and *CYP2B2* genes are closely linked at the *CYP2B* locus (previously designated the *P450-b,e* locus) on rat chromosome 1 (26). Consistent with the latter view was the subsequent finding that CYP2B2 mRNA was not detected in hepatic RNA extracts after electrophoresis and blotting or in hepatocytes using *in situ* hybridization, for either control or PB-treated M520 rats (27). However, it is also possible that this null phenotype for *CYP2B2* reflects homozygosity at the *R* ("responsive") locus, because it was recently reported that the *RR* genotype, but not the *rr* genotype, results in normal PB-induced levels of mRNA for CYP2B1 but no detectable level of mRNA for CYP2B2 after PB treatment (28). In any event, the functional significance of the *CYP2B2-(r)0* allele in M520 rats was demonstrated by its dramatic effect on the metabolism of the carcinogen dimethylbenz[*a*]anthracene (8).

In the present study, we examined the molecular basis for the *CYP2B2-(r)0* allele first by elucidating its codominant inheritance as a single autosomal gene and then by demonstrating that its null phenotype is the result of a deletion encompassing the structural gene for *CYP2B2*. Furthermore, preliminary studies indicate no significant differences in PB induction among all known electrophoretic phenotypes of CYP2B1 and CYP2B2.

Experimental Procedures

Materials. [γ-^{32}P]ATP (approximately 4500 Ci/mmol) and [α-^{32}P]dCTP (>3000 Ci/mmol) were purchased from ICN. Restriction endonucleases were purchased from New England Biolabs or United States Biochemicals. Other materials were obtained from sources described previously (4, 11).

Animals. Outbred SD rats were obtained from Tyler Laboratories (Bellevue, WA). Inbred LEW, M520, SHR, and SHRsp rats were obtained as breeding couples from the National Institutes of Health Small Animal Section (Bethesda, MD). Harlan Sprague-Dawley, Inc. (Indianapolis, IN) was the source for inbred COP, WF, and WM rats. Animals were maintained and bred under conditions described previously (26, 27). PB treatment consisted of one intraperitoneal injection of 75 mg/kg, 16 hr before killing by decapitation. All animals utilized in these studies were 84–86-day old males, except for the F_2 progenies, which were 14 days of age and included females.

Microsomes and two-dimensional gel electrophoresis. The preparation of hepatic microsomes and IF/SDS electrophoresis of microsomal polypeptides were performed as previously described (15).

Nucleic acid isolations and analyses. Hepatic RNA and DNA were prepared from individual animals, as described previously (29, 30). DNA/RNA electrophoresis, blotting, and solution hybridization assays were performed according to published protocols (11, 27). Transfers of polynucleotides from electrophoretograms were performed using capillary blotting onto GeneScreen Plus nylon membranes (DuPont). Restriction endonucleases were utilized according to supplier's instructions, except that digestions were conducted overnight with a 4-fold excess of enzyme.

Hybridization probes. For hybridizations with RNA after electrophoresis (Northern blot) or slot-blotting, gene-specific 18-mer probes (i.e., 5′-GGTTGGTAGCCGGTGTGA-3′ for CYP2B1 mRNA and 5′-GGATGGTGGCCTGTGAGA-3′ for CYP2B2 mRNA) were used, as previously described (11, 31). For hybridizations with DNA after electrophoresis (Southern blot), probes were prepared from isolated Charon 4A library phage corresponding to either the 5′ or the 3′ region of the *CYP2B1* gene (4). The 5′ probe consisted of an *Acc*I-*Xba*I fragment spanning downstream position +10 to upstream position −345, relative to the transcription initiation site at +1 (4, 16). The 3′ probe was a 650-bp *Xba*I-*Xho*I fragment derived from the immediate 3′-flanking region of the *CYP2B1* gene (4). The probes were made radioactive with ^{32}P by a random hexamer labeling procedure (32).

PCR experiments. Specific analyses of genes for either CYP2B1 or CYP2B2 were accomplished using PCR genomic amplification procedures. For analysis of the 5′ region of the respective genes, nondiscriminatory primers for both genes were synthesized, i.e., a 20-mer forward (sense) primer, 5′-ATCACATGTACCCAGGACAC-3′, and a 25-mer reverse (antisense) primer, 5′-GGGACGAGGTCCTGGTGGGAAGTTG-3′. These PCR primers permitted amplification of a 640-bp fragment of both genes, which spanned positions −476 to +164 of each (4, 16). The products of this reaction were electrophoresed, blotted, and hybridized to oligomeric probes for nested sequences within the amplified fragments that distinguished either *CYP2B1* or *CYP2B2*

[2] The allelic designations of genes are updated according to the new nomenclature (2), with *CYP2B1-(e)m*, *CYP2B2-(e)n*, and *CYP2B2-(r)0* replacing *P-450-b(e)*^{m}, *P-450-e(e)*^{n}, and *P-450-(e)*^{0}, respectively. Suffixes indicating electrophoretic (e) or regulatory (r) variants were described previously (15).

genes. These 20-mer probes were 5′-CTGAATCCGCCCCTCCACTC-3′ for *CYP2B1* and 5′-CTGAATCTGCCCCTACACTC-3′ for *CYP2B2*. The PCR reaction conditions were as follows: after an initial 3-min denaturation at 93°, the reactions were cycled 30 times through 1-min denaturation at 93°, 2-min annealing at 54°, and 2.5-min extension at 72°, with a final extension step for 10 min at 72°. The reaction mixture (50-μl final volume) was 50 mM KCl, 25 mM Tris·HCl, pH 8.4 (at 25°), 2 mM $MgCl_2$, 0.3 mM deoxynucleotide triphosphates, containing 1 μg of genomic DNA, 1.5 units of *Thermus aquaticus* polymerase, and 10 pmol each of the respective primers. After electrophoretic separation on a 1% agarose gel, the PCR products were blotted and hybridized using conditions described previously (31), except that two additional wash steps with a 3 M tetramethylammonium chloride solution (33) were used, with the first at 37° and the second at 59°.

A second series of PCR reactions were performed to analyze downstream regions of the *CYP2B1* and *CYP2B2* genes spanning exons 6 and 7. Specific primers for either *CYP2B1* [5′-GCTCAAGTACCCCC-ATGTCG-3′ (forward) and 5′-ATCAGTGTATGGCATTTTACTGC GG-3′ (reverse)] or *CYP2B2* [5′-CTTTGCTGGCACTGAGACCG-3′ (forward) and 5′-ATCAGTGTATGGCATTTTGGTACGA-3′ (reverse)] were used in this case. Specific 18-mer oligomeric probes for discriminating nested sequences within these amplified fragments of *CYP2B1* or *CYP2B2* were the same as those used for RNA analysis described above (31).

Results

Genes for CYP2B1 and CYP2B2 are closely linked on rat chromosome 1, and alleles representing all known electrophoretic variants of these isozymes are expressed in a codominant fashion, when detected as polypeptides in IF/SDS electrophoretograms (14, 15, 26). However, the inheritance of the null allele, *CYP2B2-(r)0*, has not been studied, and the possibility that regulatory differences are linked to a given electrophoretic allele has not been investigated. In the present study, we used specifically designed oligomeric probes that can distinguish CYP2B1 and CYP2B2 mRNAs (11), to monitor their induction in representative strains of inbred rats expressing all known electrophoretically detected allelic forms of these isozymes (15).[3] In addition, F_1 and F_2 progenies were prepared and analyzed, to elucidate the inheritance of the *CYP2B2-(r)0* allele.

Analyses of CYP2B1 and CYP2B2 hepatic mRNAs in different rat strains. Northern blots of electrophoresed hepatic RNA from individual rats, representing eight different strains and four F_1 hybrids, were probed for either CYP2B1 or CYP2B2 mRNA sequences, using the appropriate antisense oligomers, and the results are shown in Fig. 1. Except for the

[3] The 18-mer segment of mRNAs coding for amino acid residues 334 through 339 of CYP2B1 and CYP2B2 involves four nucleotide differences (11); at this time, none of the sequenced alleles for either of these isozymes has revealed a difference in this segment, which suggests that the original antisense probes (11) can be used to detect mRNAs for the different electrophoretic variants tested in the present study. However, a single nucleotide difference in the probed sequence was suggested to characterize a variant of *CYP2B2* in a particular colony of SD rats (34).

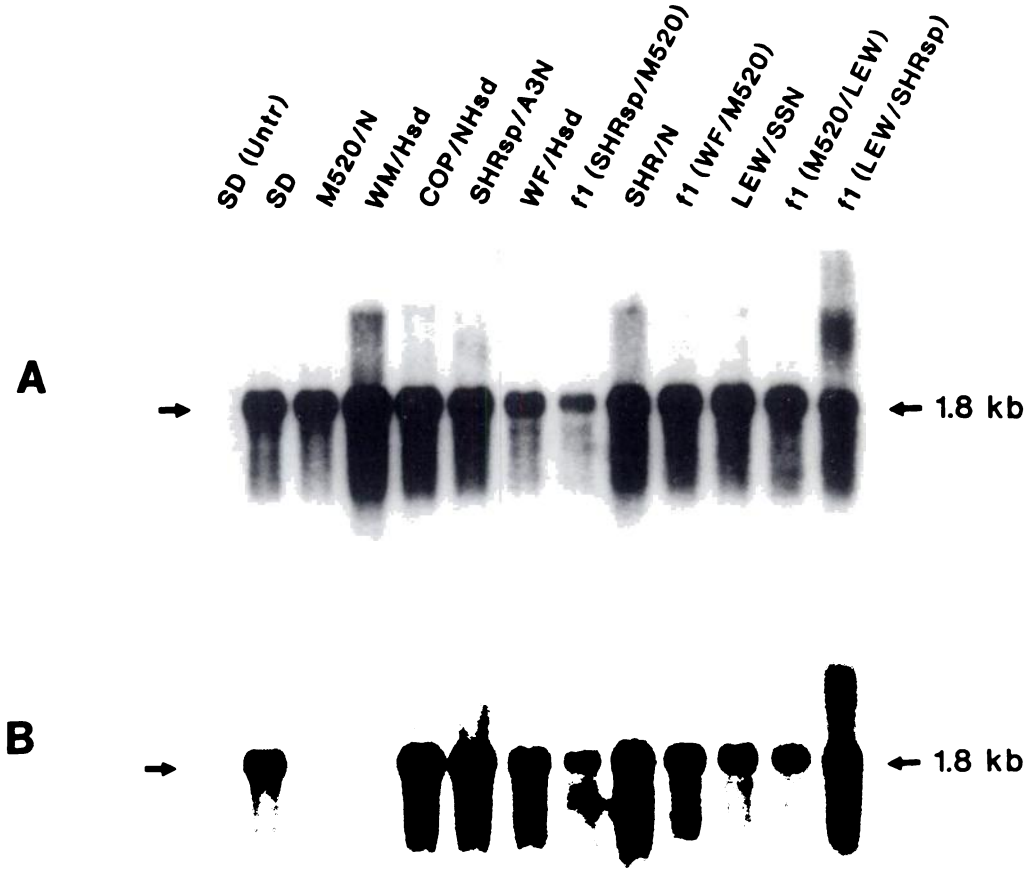

Fig. 1. Northern blots of hepatic RNA probed for CYP2B1 and CYP2B2 mRNAs in individual rats, representing various strains and F_1 progenies. Ten micrograms of total RNA were applied to each lane. Experimental conditions are described in Experimental Procedures. A, Hybridization with the probe for CYP2B1 mRNA; B, hybridization with the probe for CYP2B2 mRNA after removal of the probe that generated the result in A.

TABLE 1

Levels of hepatic mRNA for CYP2B1 and CYP2B2 measured by solution hybridization for various rat strains and F_1 progenies

Experiments are described in Experimental Procedures.

Rat strain	*CYP2B1/CYP2B2* genotype[a]	mRNA level[b]		mRNA ratio, CYP2B1/CYP2B2
		CYP2B1	CYP2B2	
	m/n	10^{-2} fmol/μg		
SD (untreated)[c]		ND[d]	0.04	
SD		3.7	1.2	3.1
M520	4/0	2.5	ND	
WM	4/0	6.0	ND	
COP	3/5	5.6	1.2	4.7
SHR	6/5	3.5	1.1	3.2
SHRsp	4/5	3.5	0.9	3.9
F_1 (SHRsp × M520)[e]	4/5+0	2.3	0.5	4.6
LEW	3/5	4.2	1.1	3.8
F_1 (M520 × LEW)	3+4/5+0	4.0	0.5	8.0
WF	7/8	2.5	0.8	3.1
F_1 (WF × M520)	4+7/8+0	3.7	0.6	6.2
F_1 (LEW × SHRsp)	3+4/5	2.3	0.7	3.3

[a] Genotypes were deduced from phenotypes determined using IF/SDS electrophoresis, and designations of alleles use the nomenclature described in the text and footnote 2, where *m* and *n* are taken from *CYP2B1-(e)m* and *CYP2B2-(e)n*, respectively (15). Electrophoretic phenotypes were not determined for the SD rats.

[b] Levels are expressed as 10^{-2} fmol/μg of total hepatic RNA and are the average of two experiments performed with duplicate samples; variation between experiments was <15%.

[c] All other rats were treated with a single intraperitoneal injection of PB, at 75 mg/kg, 16 hr before killing.

[d] ND, not detectable within the sensitivity limits of this assay (0.02×10^{-2} fmol/μg).

[e] For F_1 crosses: (sire × dam).

untreated SD rat, a prominent band at 1.8 kb was observed for all PB-treated animals when mRNA for CYP2B1 was probed, as shown in Fig. 1A. In contrast, the apparent absence of hepatic mRNA for CYP2B2 in PB-treated M520 and WM rats is dramatically illustrated by the results in Fig. 1B, where a 1.8-kb band for CYP2B2 mRNA was not visualized for these strains even after prolonged autoradiographic exposure (data not shown).

Considering the results for F_1 progenies, it is clear that the *CYP2B2-(r)0* is not dominant, because mRNA for CYP2B2 is present in all samples from PB-treated F_1 progenies of crosses involving M520 rats with inbred rats expressing this mRNA (see Fig. 1B). This conclusion was corroborated by the observation of polypeptides representing CYP2B2 protein in IF/SDS electrophoretograms of hepatic microsomes from the same rats (data not shown).

Inheritance pattern of the *CYP2B2-(r)0* allele. Because the *CYP2B2-(r)0* allele was never found in the colony of outbred SD rats used in the present study, it is most likely that all alleles for *CYP2B2* are capable of normal expression in this population. In any event, F_1 progenies of (M520 × SD) crosses were prepared, and these were mated to generate F_2 progenies. At 2 weeks of age, these F_2 progenies were treated with PB, after which hepatic RNA was prepared. CYP2B1 and CYP2B2 mRNAs for two F_2 progenies were analyzed by a slot-blot procedure, using the specific probes described above (11). Autoradiograms were subjected to densitometry, and the results are shown in Fig. 2.

As expected, all of the F_2 animals expressed mRNA for CYP2B1; however, it appears that PB induction of this isozyme is lower for female (rats 8–12) versus male (rats 1–7) animals at 2 weeks of age. On the other hand, this sex difference does not seem to characterize the induction of mRNA for CYP2B2, which can reasonably be categorized as follows: 1) no expression (four rats; rats 2, 3, 6, and 10), 2) low level (four rats; rats 5, 8, 11, and 12), 3) intermediate level (two rats; rats 7 and 9), and 4) high level (two rats; rats 1 and 4). Considering the error involved in these experiments, it is likely that the intermediate category is not real and that animals 7 and 9 might represent either high or low levels of CYP2B2 mRNA. The data presented in Fig. 2 are consistent with the most probable results expected for segregation of a single codominantly expressed gene, which are as follows: three animals with no expression, six animals with 50% of maximal levels, and three animals with maximal levels.

It also is clear from the data in Fig. 2 that untreated adult M520 rats express detectable levels of CYP2B1 mRNA, whereas untreated adult SD rats express detectable levels of mRNA only for CYP2B2, which is consistent with previous reports where either mRNA (27) or protein (8) was analyzed. The results further indicate that, as a fraction of total hepatic RNA, PB induction of mRNAs for both isozymes is greater in adult versus 2-week-old animals. The basis for this apparent developmental difference is currently unknown.

Quantitation of hepatic CYP2B1 and CYP2B2 mRNA levels in different rat strains, using solution hybridization. To provide more accurate quantitative information, solution hybridization (11) was also used to measure hepatic CYP2B1 and CYP2B2 mRNA levels in the various animals listed in Fig. 1. Table 1 lists the *CYP2B1* and *CYP2B2* genotypes for these animals, along with the solution hybridization data. The results indicate no apparent correlation between a given electrophoretic allele and the PB-induced level of its corresponding mRNA. For example, four rats were homozygous for *CYP2B1-(e)4* and the level of its mRNA ranged from 2.3×10^{-2} to 6.0×10^{-2} fmol/μg, but this range included all levels for CYP2B1 mRNA reported in Table 1. It is likely that some variability for these mRNA levels reflects experimental variation, perhaps as a result of using a single dose of PB. On the other hand, the CYP2B1/CYP2B2 mRNA ratio was considerably less variable than the level of either mRNA. For example, the mean ± standard deviation of this ratio was 3.6 ± 0.6 for all animals (other than F_1 progenies of crosses involving an M520 parent), whereas it was 6.3 ± 1.7 for F_1 progenies involving an M520 parent. The latter finding is consistent with codominant expression of *CYP2B2-(r)0*, where half the normal level of CYP2B2 mRNA would be expected in these F_1 rats. An additional test for codominance of this null allele of *CYP2B2* is to compare the levels of mRNA for both isozymes in PB-treated SHRsp, LEW, and WF rats with those in PB-treated F_1 progenies resulting from crosses of these strains with M520 rats (see Table 1). In this regard, the mean values ± standard deviations for CYP2B1 mRNA levels in these three inbred strains and in their F_1 hybrids with M520 rats were $3.4 \pm 0.9 \times 10^{-2}$ fmol/μg and $3.3 \pm 0.9 \times 10^{-2}$ fmol/μg, respectively. On the other hand, CYP2B2 mRNA levels in these strains and the same F_1 hybrids were different, being $0.93 \pm 0.15 \times 10^{-2}$ fmol/μg and $0.53 \pm 0.05 \times 10^{-2}$ fmol/μg, respectively. It also can be concluded that the gene represented by *CYP2B2-(r)0* is autosomal, because the same F_1 phenotype for PB-induced CYP2B2 mRNA was observed regardless of the sex of the M520 parents (see Table 1).

Analysis of hepatic DNA for *CYP2B1* and *CYP2B2* genes in M520 and WM rats. To investigate the molecular

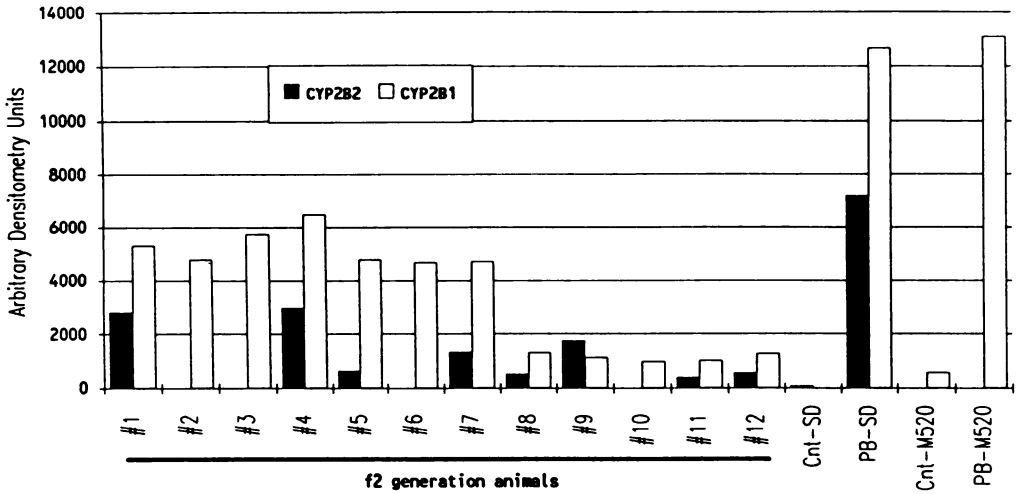

Fig. 2. Levels of hepatic CYP2B1 and CYP2B2 mRNA in PB-treated F_2 [F_1 (M520 × SD) × F_1 (M520 × SD)] rats. F_2 rats were 2 weeks old; control (*Cnt*) and PB-treated adult male M520 and SD rats were also analyzed. Ten micrograms of total hepatic RNA were applied to a slot-blot apparatus, and the resulting membrane was hybridized consecutively to oligomeric probes for CYP2B1 mRNA, CYP2B2 mRNA, and 18 S rRNA. The autoradiographs generated were densitometrically scanned, and the results were normalized with respect to the 18 S rRNA content.

basis for the null allele of *CYP2B2* in M520 and WM rats, restriction digests of hepatic DNA were analyzed using gene-specific probes. Fig. 3 depicts relevant structural features of the *CYP2B2* gene, including regions for which the hybridization probes were targeted.

The results of the Southern blot analyses presented in Fig. 4 compare genomic restriction patterns for M520 and SD rats, using *Bam*HI, *Hind*III, and *Xba*I as endonucleases. The blot in Fig. 4A illustrates the data obtained with a probe representing positions −345 to +10 at the 5′ end of the *CYP2B1* gene (*Xba*I-*Acc*I fragment). Because of extensive sequence identity, the 5′ and 3′ probes derived from the *CYP2B1* gene hybridized equally well with the *CYP2B2* gene under the conditions used (4, 16). For each of the three restriction endonucleases, one prominent DNA fragment appears to be absent from the digest of M520 DNA, compared with SD DNA. The absent bands are indicated by *arrows* in Fig. 4A and, based on DNA size standards run concurrently, correspond to 7000 bp (*Bam*HI), 4400 bp (*Hind*III), and 7800 bp (*Xba*I). These fragment sizes are consistent with those predicted for the *CYP2B2* gene (see Fig. 3).

Similarly, in Fig. 4B, data obtained with the 650-bp 3′ probe, representing a *Xba*I-*Xho*I fragment of the *CYP2B1* gene, also indicate the absence of specific fragments from digests of M520 DNA, compared with SD DNA. In this case, fragments with sizes of 10,600 bp (*Bam*HI), 6100 bp (*Hind*III), and 7900 bp (*Xba*I) that were observed with SD DNA were not detected with M520 DNA (compare the regions indicated by the *arrows* in Fig. 4B). Taken together, the Southern blot data suggest the absence of sequences corresponding to both the 5′ and 3′ regions of the *CYP2B2* gene in M520 rats.

In additional experiments, similar Southern blots were probed with a fragment encompassing positions −1530 to −1730 of the *CYP2B2* gene; again, single bands were absent in all three digests of M520 DNA, compared with the SD DNA (data not shown). Hepatic DNA from WM rats was also analyzed in the same kind of Southern blot experiments as those described in Fig. 4, and the results were identical to those observed with the DNA samples from M520 rats (data not shown).

Gene-specific PCR amplification of *CYP2B1* and *CYP2B2* DNA in M520 and WM rats. To eliminate possible confusion resulting from cross-hybridization of *CYP2B1/2* genomic probes with other members of the *CYP2B* subfamily, a PCR-based amplification strategy was developed that allowed for the specific detection of *CYP2B1* and *CYP2B2* genes by amplification of two regions of these closely related genes, as shown in Fig. 3. In addition to M520 and SD rats, single WM and SHR rats were also used in these PCR experiments.

Internal regions of the *CYP2B1* and *CYP2B2* genes were analyzed by PCR, using forward and reverse primer sets residing in exon 6 and exon 7, respectively. In these experiments, each of the individual primers and probe oligomers were designed to hybridize specifically with either the *CYP2B1* or

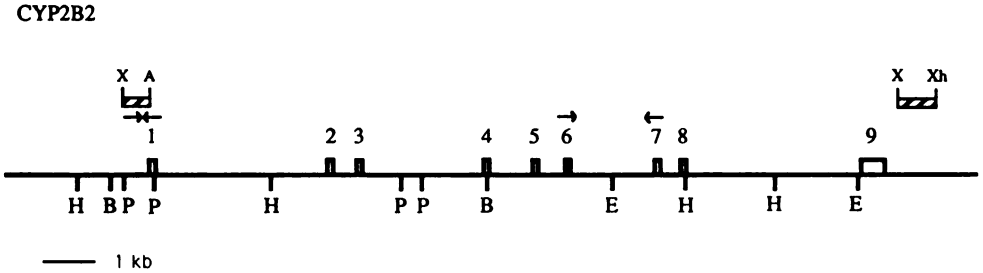

Fig. 3. Abbreviated structure of the *CYP2B2* gene. The nine exons are numbered and selected restriction sites (*A, Acc*I; *B, Bam*HI; *H, Hind*III; *P, Pst*I; *X, Xba*I; *Xh, Xho*I) are indicated (4, 16, 21). *Hatched boxes*, relative positions of the hybridization probes utilized for Southern blot experiments; *forward and reverse arrows*, regions of the gene subjected to PCR amplification.

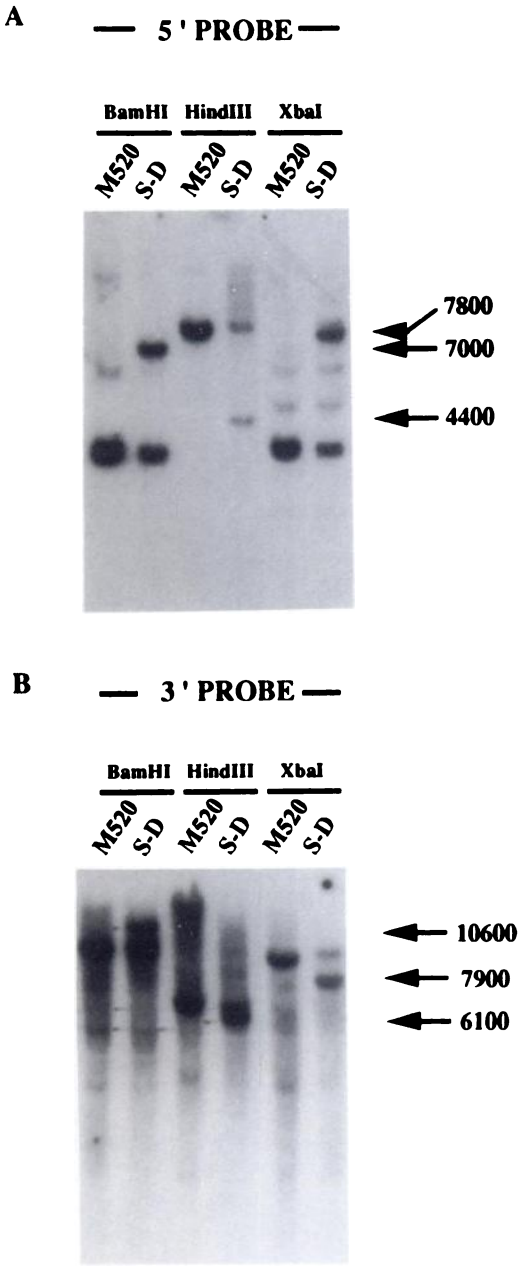

Fig. 4. Autoradiographs of Southern blots of genomic DNA from M520 and SD rats. Hepatic DNA was isolated from individual rats and was subjected to digestion with *Bam*HI, *Hind*III, or *Xba*I restriction endonucleases, and Southern blots were performed, as described in Experimental Procedures. A, Analysis with the 5′ probe; B, analysis with the 3′ probe. See Fig. 3 for the location of these genomic probes with respect to the *CYP2B2* gene.

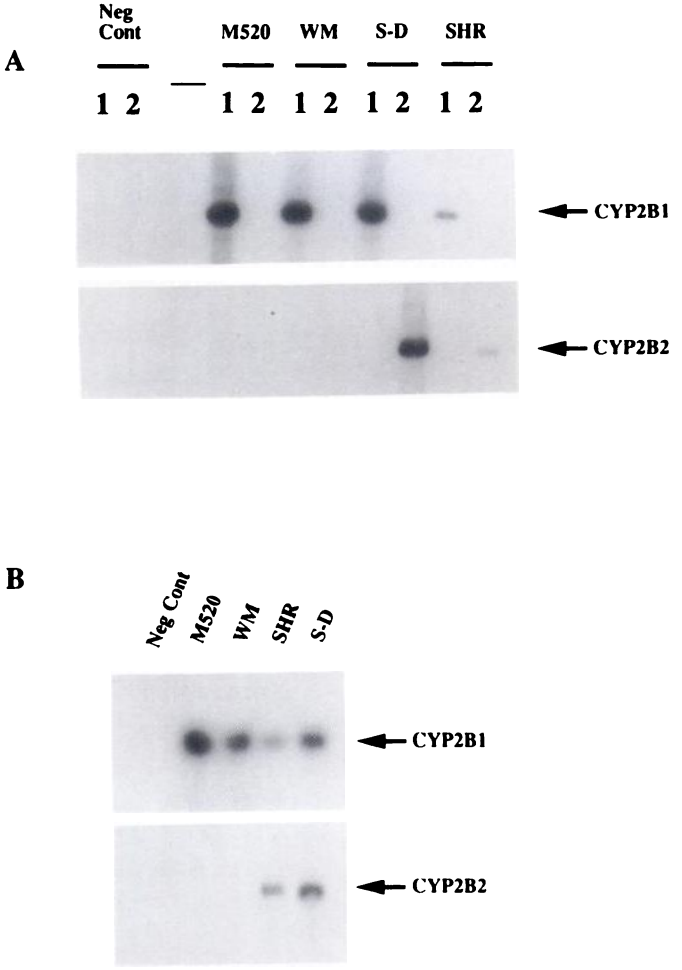

Fig. 5. PCR amplification of genomic DNA from M520, WM, SD, and SHR rats. A, PCR reactions amplified internal regions of either the *CYP2B1* gene (*lanes 1*) or the *CYP2B2* gene (*lanes 2*), using specific primer sets. The resulting products contained sequences in exons 6 and 7 of each gene that could be distinguished by probes for either *CYP2B1* (*upper*) or *CYP2B2* (*lower*). B, A single primer set that annealed to identical sequences of both genes was used, but the amplified products of *CYP2B1* and *CYP2B2* contained different nested sequences that could be discriminated using specific probes for either *CYP2B1* (*upper*) or *CYP2B2* (*lower*). The negative controls in A and B contained all of the common PCR reaction mixture components except template DNA. The DNA from SHR rats was obtained from a frozen liver and was partially degraded, which explains the lower intensity signals, compared with the other strains.

CYP2B2 genes (11, 31). The two sets of amplified genomic DNAs for the four rat strains were electrophoresed, blotted, and then hybridized to gene-specific probes nested within the amplified regions. The results of these experiments are presented in Fig. 5A, which clearly indicates the absence of amplified *CYP2B2* gene products with hepatic DNA from M520 or WM rats, despite clear evidence of such an amplification for the SD and SHR rats. In contrast, specific PCR-amplified products of *CYP2B1* DNA were readily evident in all four of the tested strains.

To assess the 5′ region of either the *CYP2B1* or the *CYP2B2* genes, a single forward and reverse primer set for PCR was chosen that shared identical sequences in the two genes, so that comparable annealing and subsequent amplification of both genes would occur simultaneously. However, the amplified products of the two genes would be different, having characteristic nested sequences that could be individually detected. In these experiments, the region amplified was between nucleotide positions −451 and +138 for the *CYP2B1* gene and between positions −473 and +138 for the *CYP2B2* gene. The PCR data in Fig. 5B clearly show the presence of *CYP2B1* genomic sequence in all four rat strains; however, the presence of this portion of the *CYP2B2* gene was only evidenced by amplification of genomic DNA from SD and SHR rats, because the corresponding lanes for amplified DNA from M520 and WM rats were blank.

Discussion

The results of genetic crosses in the present study indicated that *CYP2B2-(r)0* represents a single autosomal gene that is inherited in a codominant fashion. These features suggested that the locus for this allele is the *CYP2B2* gene itself, which was proven by the demonstration of its deletion using restriction fragment analyses and two different PCR-based strategies. Assuming a single deletion event, its minimal extent would be approximately 16 kb, including the complete *CYP2B2* structural region (14 kb) together with at least 1.5 kb of 5′ and 1.0 kb of 3′ flanking sequences. However, the deletion end-points for *CYP2B2-(r)0* are unknown at this time. Obviously, the absence of the *CYP2B2* gene explains why hepatic mRNA and protein for CYP2B2 were not found in untreated or PB-treated M520 rats (8, 27). In this regard, the *R* allele cannot be identified with *CYP2B2-(r)0*, because rats having the *RR* genotype are characterized by constitutive expression of CYP2B2 mRNA, which is completely suppressed after PB treatment (28).

A different phenotype for defective PB-induced expression of hepatic CYP2B2 was claimed to characterize a colony of Qdj:SD rats (34), whereby low levels of CYP2B2 (mRNA and protein) were expressed after PB treatment. However, because this study used outbred rats and homozygosity for this putative phenotype was not demonstrated in individual animals, it is possible that a high frequency of either *CYP2B2-(r)0* or *R* alleles (28) in the population of rats studied may be responsible for the reported results (34). Because the same null allele for *CYP2B2* is found in M520 and WM rats, which do not share a common pedigree, it is reasonable to expect that *CYP2B2-(r)0* would be found in other colonies of outbred rats.

It would have been interesting if a regulatory mutation had been evidenced in the present study, regarding PB induction of CYP2B1 and/or CYP2B2 mRNAs among the inbred strains tested, because studies of such a mutation could have elucidated putative *cis*-acting PB control elements in either or both of the corresponding genes. Unfortunately, such information was not obtained and, within the experimental error, it appears that PB-induced mRNA levels were essentially the same for all electrophoretic variants of these isozymes.

The deletion characterizing *CYP2B2-(r)0* might be associated with regulation of the *CYP2B1* gene, because untreated M520 rats express CYP2B1 mRNA, whereas untreated rats of other strains have been shown to express CYP2B2 mRNA (8, 27) (see Fig. 2). One could speculate that a large deletion of the *CYP2B2* gene could directly affect expression of *CYP2B1*, because genes for these isozymes are closely linked (26). However, because only one haplotype [i.e., *CYP2B1-(e)4:CYP2B2-(r)0*] has been investigated to date, it is also possible that the constitutive expression of CYP2B1 in M520 rats is independently affiliated with the *CYP2B1-(e)4* allele. A more indirect causation of significant constitutive expression of CYP2B2 in M520 rats might involve hormonal differences among inbred strains, because growth hormone and thyroid hormone are known to suppress expression of this isozyme (35). It was also suggested that CYP2B1 was expressed at higher levels in control and PB-treated M520 rats, compared with comparable rats of other strains, to compensate for the absence of CYP2B2 (8). Future genetic studies are planned to elucidate the molecular control of constitutive expression of CYP2B1 in M520 rats, using several inbred strains of rat that are representative of the five different haplotypes for *CYP2B1/CYP2B2* that are currently known (15).

While this work was in progress, an allele for a poor metabolizer phenotype in humans was found to involve a large deletion that included the entire *CYP2D6* gene (36). As is the case for *CYP2B2-(r)0*, the deleted gene was situated in a tightly linked cluster with closely related genes [i.e., *CYP2D8P* and *CYP2D7* (37)]. These similar chromosomal environments for the two deletions reinforce the suggestion that these deletions might have resulted from unequal crossover events (15, 37). Because many hepatic CYP450s are capable of catalyzing the synthesis of harmful electrophilic compounds, as well as detoxifying reactions, it is possible that the existence of functionally null alleles for a variety of isozymes in the gene pool of a given mammalian species is widespread and that this condition was selected over the course of evolution.

The CYP2B2-deficient M520 and WM strains characterized herein should prove valuable in further drug metabolism studies aimed at elucidating the respective roles of the structurally homologous CYP2B1 and CYP2B2 isozymes.

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Send reprint requests to: Dr. Curt Omiecinski, Department of Environmental Health, SC-34, University of Washington, Seattle, WA 98195.