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New Proteins in the Rat CYP2B Subfamily: Presence in Liver Microsomes of the Constitutive CYP2B3 Protein and the Phenobarbital-Inducible Protein Product of Alternatively Spliced CYP2B2 mRNA

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ABSTRACT. The rat *CYP2B* gene subfamily includes *CYP2B1*, *CYP2B2* and *CYP2B3*. Translation of an alternatively spliced hepatic CYP2B2 mRNA would generate a CYP2B2 variant, CYP2B2v, having eight additional amino acid residues inserted between CYP2B2 positions 274 and 275. The presence of CYP2B3 and CYP2B2v in rat liver has yet to be demonstrated. cDNA expression vectors were obtained for CYP2B1, CYP2B2, CYP2B3 and CYP2B2v. All four proteins react with an anti-CYP2B1 antibody and can be resolved by SDS–PAGE. A CYP2B3-specific polyclonal antibody raised against an undecapeptide (SPVDPNTIDMT) from near the C-terminus of CYP2B3 detected a constitutive protein on immunoblots of rat liver microsomes, thus demonstrating that the CYP2B3 mRNA is translated in the liver. Similarly, a CYP2B2v-specific polyclonal antibody was raised against a peptide containing the eight additional amino acid residues (VSPAWMRE) predicted to be present in the CYP2B2v protein. It detected a phenobarbital- and Aroclor 1254-inducible protein in rat liver microsomes. Microsomes of Ad293 cells expressing cDNAs for CYP2B2 and CYP2B2v were used to metabolize 7,12-dimethylbenz[*a*]anthracene (DMBA), and the metabolites produced were compared with those generated by microsomes of cells expressing CYP2B1 cDNA. CYP2B2v had activity similar to that of CYP2B2 for DMBA metabolism. Both CYP2B2 forms preferentially catalyzed 12-hydroxylation, whereas CYP2B1 preferred 7-hydroxylation and exhibited turnover that was strongly suppressed as previously reported. These results demonstrate the existence in rat liver of two new CYP2B proteins: CYP2B3, the major constitutive CYP2B form, and CYP2B2v, which represents a rare case of non-aberrant alternative splicing among xenobiotic-metabolizing P450s. BIOCHEM PHARMACOL 52;8:1311–1319, 1996.

KEY WORDS. phenobarbital; CYP2B; cytochrome P450; alternative splicing; antipeptide antibodies; rat liver

The monooxygenase system in hepatic microsomes contains multiple CYP¶ forms and is involved in the metabolism of many endogenous compounds as well as in the detoxification of xenobiotics [1, 2]. Toxicity and carcinogenicity of certain xenobiotics are enhanced by CYP-dependent metabolism [3]. Hence, the characterization of CYP forms may be critical for the prediction of individual susceptibility to adverse drug reactions or of the toxic and carcinogenic potential of certain drugs or chemicals [4, 5].

There are at least 8–11 genes in the rat *CYP2B* subfamily [6–8]. In rat liver, the closely related CYP2B1 and CYP2B2 proteins are the major PB-inducible CYP2B forms [9]. The original CYP2B1 and CYP2B2 sequences differ in 14 of 491 amino acids and are identical over the first 302 residues [10]. Despite their near identity of amino acid sequence, CYP2B1 and CYP2B2 have slightly different electrophoretic mobilities on SDS–polyacrylamide gels [11, 12]. After PB treatment of rats, liver CYP2B1 and CYP2B2 levels increase by >200-fold and ~27-fold, respectively, over those of untreated rats [13]. Rat CYP2B1 or CYP2B2 proteins or enzymatic activities have also been found in various extrahepatic tissues such as small intestine, adrenals, kidneys, and lungs, although generally at low levels compared with liver [13–15].

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¶ *Abbreviations:* anti-2B1, rabbit polyclonal anti-CYP2B1 antibody; anti-2B3, rabbit polyclonal anti-CYP2B3 antibody; anti-2B2v, rabbit polyclonal anti-CYP2B2v antibody; CYP, cytochrome P450; CYP2B2v, variant CYP2B2 protein with eight additional amino acid residues between CYP2B2 positions 274 and 275; $CYP2B2^M$ and $CYP2B2v^M$, polymorphic CYP2B2 forms having Met at position 473; PB, phenobarbital; MC, 3-methylchloanthrene; ARO, Aroclor 1254; βNF, β-naphthoflavone; PCN, pregnenolone 16α-carbonitrile; DMBA, 7,12-dimethylbenz[α]anthracene; MBS, *m*-maleimido benzoyl-*N*-hydroxysuccinimide ester; KLH, keyhole limpet hemocyanin; and DEX, dexamethasone.

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The rat *CYP2B3* gene has been isolated and characterized recently in one of our laboratories [16]. It is transcribed into a constitutive hepatic mRNA, the level of which does not respond to PB or ARO treatment [17, 18]. This mRNA encodes a protein the deduced amino acid sequence of which has 77% identity with the CYP2B1/CYP2B2 proteins [18]. We have also demonstrated that alternative splicing can give rise to a variant CYP2B2 mRNA containing an additional 24 bp at the boundary between exon 5 and exon 6. If translated, this mRNA would generate a variant CYP2B2 protein, CYP2B2v, having eight additional amino acid residues between CYP2B2 positions 274 and 275 [19]. Finally, amino acid substitution polymorphisms are known at various positions for both CYP2B1 and CYP2B2, including CYP2B2 position 473, which may be occupied by Lys or Met [7, 20].

We have reported the existence of a constitutive rat liver microsomal protein, referred to as band 5 protein, which is immunologically related to CYP2B1 and CYP2B2 and has a higher electrophoretic mobility on SDS–PAGE than CYP2B1. Using anti-2B1, a polyclonal antibody directed against CYP2B1 that also recognizes CYP2B2 and CYP2B3, we showed that cDNA-expressed CYP2B3 comigrates with band 5 protein [16]. There have been other reports of a similar protein in liver microsomes of untreated or inducer-treated rats [14, 21–23] and in cultured rat hepatocytes [24–27]. In addition, a PB-inducible protein with a lower electrophoretic mobility than CYP2B2 and immunologically closer to CYP2B2 than to CYP2B1 has been identified in rat liver microsomes [22]. A similar low mobility CYP2B protein has also been observed by others in liver microsomes of inducer-treated rats [21, 24]. This form, which we refer to as band 1 protein [16], constitutes a candidate for the CYP2B2v protein.

In the present study, we show, using polyclonal antibodies specifically recognizing CYP2B3 or CYP2B2v, that the band 1 and band 5 proteins indeed correspond to CYP2B2v and CYP2B3, respectively. We also demonstrated that CYP2B2v hydroxylates DMBA with a regioselectivity similar to that of CYP2B2 and that the Lys/Met polymorphism at CYP2B2 position 473 is without appreciable effect on the regioselectivity of DMBA hydroxylation by CYP2B2 forms.

MATERIALS AND METHODS

Materials

Ad293 cells, which are adenovirus-transformed human embryonic kidney cells [28], were provided by Dr. M. Seidman (Otsuka Pharmaceutical Co., Rockville, MD). They have been shown to permit synthesis of enzymatically active CYP1A2 from a pMT2-1A2 cDNA expression vector [29] and to have little or non endogenous activating activity for a variety of promutagens [29, 30]. The pMT2 expression vector [31], anti-2B1 [32], PCN, and ARO were provided by Dr. Randal Kaufman (Genetics Institute, Cambridge, MA), Dr. D. J. Waxman (Boston University, Boston, MA), G. D. Searle & Co. (Oakville, Ontario), and the Monsanto Chemicals Co. (St. Louis, MO), respectively. T7 DNA sequencing kits were from Pharmacia Canada (Baie d'Urfé, Québec); $NaCNBH_3$ and MC were from Aldrich (Milwaukee, WI); MBS, KLH, and SDS were from ICN Biomedicals Canada (St-Laurent, Québec). Freund's complete and incomplete adjuvant and βNF were from Sigma (St. Louis, MO). Horseradish peroxidase-labeled donkey anti-rabbit immunoglobulin and the enhanced chemiluminescence immunoblot detection system were from Amersham Canada (Oakville, Ontario). Oligopeptides and oligodeoxyribonucleotides were synthesized by Guy Drapeau and Michel Lambert (Centre de recherche en cancérologie de l'Université Laval, L'Hôtel-Dieu de Québec).

cDNA Constructs and Expression Vectors

To characterize further the two CYP2B2-like proteins, full-length cDNAs with (CYP2B2v) and without (CYP2B2) the additional 24 bp were constructed and subcloned in the eucaryotic expression vector pMT2. Plasmid pTZ2B2-1.4v was obtained by subcloning the 1.4-kb *Bam*HI-*Pst*I fragment of the PB22 CYP2B2v cDNA insert [19] into the polylinker of pTZ19R (Pharmacia Canada). Plasmid pTZ2B2-1.4 was obtained by exchanging the 1.1-kb *Msc*I-*Pst*I fragment of pTZ2B2-1.4v with the corresponding fragment of the PB13 CYP2B2 cDNA insert [19]. The *Bam*HI-*Pst*I fragment of pTZ2B2-1.4v and of pTZ2B2-1.4 were then subcloned into pBluescript KS (Stratagene, La Jolla, CA). A *Bam*HI-*Sal*I fragment of both resulting constructions was subcloned into the *Bam*HI-*Sal*I site of pTZ19R. A composite polylinker in a pTZ plasmid background with an *Eco*RI site on each side of the cDNA insert was thus obtained. The full-length cDNAs were finally obtained by subcloning a 0.5 kb 5′ *Bam*HI fragment of the CYP2B1 cDNA [33] into the composite vectors pTZc2B2-1.4 and pTZc2B2-1.4v; this gave pTZc-2B2 and pTZc-2B2v, the cDNA inserts of which were then subcloned into the unique *Eco*RI site of the pMT2 expression vector to obtain pMT2-2B2 and pMT2-2B2v. The coding portions of the cDNA inserts of both constructions were then fully sequenced. The CYP2B2 amino acid sequence encoded by pMT2-2B2 corresponded to the normal CYP2B2 sequence employed in the experiments of Christou *et al.* [34] (see also Aoyama *et al.* [35]), except for position 473 which in our case is occupied by Lys instead of Met [19]. The pMT2-2B2v expression vector encodes a protein identical to CYP2B2 except for 8 additional amino acids V^{275}SPAWMRE^{282} encoded by the 24 additional bp at the boundary between exon 5 and 6 in the CYP2B2v cDNA sequence [19]. An expression vector with the CYP2B2 cDNA insert in the inverted orientation, pMT2-2B2(i), was also constructed. The pMT2-2B1, pMT2-2B1(i), and pMT2-2B3 vectors have been described [16, 33]. The CYP2B1 amino acid sequence encoded by pMT2-2B1 has Val instead of Glu at position 282 [36]. This difference does not alter the testosterone hydroxylation activity of CYP2B1 [35].

Site-Directed Mutagenesis

A 374-bp *Bgl*II fragment of pMT-2B2v containing codon 473 was subcloned into the *Bam*HI site of the pSELECT phagemid vector (Promega, Madison, WI), and site-directed mutagenesis was carried out using the Promega Altered Sites *in vitro* Mutagenesis System. The oligonucleotide primer was 5′-TCACGCCCAtGGAGAGTGG-3′, where the lowercase letter denotes the changed nucleotide. Following mutagenesis, the 374-bp *Sau*3A modified fragment from pSELECT was exchanged with the corresponding 374-bp unmodified *Bgl*II fragment in pTZc-2B2 to produce pTZc-2B2^M. The 5′ 1.1-kb *Kpn*I fragment of pTZc-2B2v was exchanged with the corresponding 1.1-kb *Kpn*I fragment in pTZc-2B2^M to produce full-length CYP2B2v^M cDNA. Both full-length mutated cDNAs were subcloned into the unique *Eco*RI site of pMT2 to obtain pMT2-2B2^M and pMT2-2B2v^M. The presence of the ATG codon 473 in the mutated CYP2B2^M and CYP2B2v^M cDNAs was confirmed by sequencing. pMT-2B2^M encodes a CYP2B2 protein with a Met at amino acid position 473 which represents a polymorphic CYP2B2 cDNA obtained from a rat liver cDNA library [17] and should be identical to the normal CYP2B2 used in the experiments of Christou *et al.* [34]. The mutant pMT2-2B2v^M encodes a hypothetical CYP2B2v^M protein that would be produced if alternative splicing also occurs in the pre-mRNA from the *CYP2B2*^{M} allele [19].

Crude Extracts and Microsomes from Transfected Cells and Quantitation of DMBA Metabolites

Ad293 cells were grown and transfected, and crude cell extracts were prepared and assayed for protein content essentially as described previously [29, 37]. For microsome preparation, Ad293 cells were pooled from 30 to 40 150-mm diameter tissue-culture dishes at 48 to 72-hr post-transfection. Cells were washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 3.4 mM Na_2HPO_4, 1.5 mM KH_2PO_4 at pH 7.4) and dispersed in 40 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl. After centrifugation (300 *g*; 10 min), the cell pellet was frozen in dry ice. Microsomes were prepared [38], incubated with DMBA in reaction buffer, and metabolite profiles were obtained using reverse-phase high pressure liquid chromatography [39].

Treatment of Rats and Preparation of Rat Liver Microsomes

Male Sprague–Dawley rats (150–200 g) from Charles River Canada (St. Constant, Québec) were injected intraperitoneally, on 4 consecutive days, with βNF in corn oil (daily dose, 80 mg/kg), or with PB in PBS (daily dose, 75 mg/kg), or with 3-MC in corn oil (daily dose, 25 mg/kg), or, on 3 consecutive days, with DEX in PBS (daily dose, 10 mg/kg). PCN was emulsified in a 2% Tween 80 solution and administered intragastrically on 4 consecutive days (daily dose, 50 mg/kg). Food was removed at the time of the last treatment and killing was by decapitation 24 hr later. A single intraperitoneal injection of ARO, at 500 mg/kg, was administered in corn oil 6 days before decapitation; food was removed 24 hr before killing. Tissues (liver, kidneys, lungs, prostate, and small intestine) were homogenized in 300 mM potassium phosphate, pH 7.25, 150 mM KCl using a glass–teflon homogenizer, centrifuged twice at 10,000 *g* for 15 min, and the final supernatant centrifuged at 105,000 *g* for 90 min. The 105,000 *g* microsomal pellet was suspended in 50 mM Tris–HCl, pH 7.5, 250 mM sucrose using a Dounce homogenizer.

Generation of Anti-2B3 and Anti-2B2v Antibodies

The synthetic CYP2B3 peptide Ser-**Pro-Val-Asp**-Pro-**Asn-Thr**-Ile-Asp-**Met**-Thr-Cys ([461-471]CYP2B3 plus the C-terminal Cys introduced to facilitate coupling) was chosen because 6 of the 11 residues (those in boldface) are different from the corresponding residues in CYP2B1/CYP2B2. It was linked to KLH [40] using MBS as the coupling reagent [41] and used to generate a rabbit polyclonal anti-2B3 antibody [42] designated ZL426. Purified immunoglobulin G fractions were prepared from immune and preimmune sera by caprylic acid precipitation [43].

The peptide resin Cys-Arg-Met-Glu-Lys-**Val-Ser-Pro-Ala-Trp-Met-Arg-Glu**-NovaSyn KD was prepared. It contains the peptide [271-282]CYP2B2v plus an N-terminal Cys residue and includes the 8 CYP2B2v-specific residues (in boldface), which are absent from all known CYP2B proteins since they are encoded by the alternatively spliced CYP2B2v mRNA [19]. NovaSyn KD consist of a polydimethylacrylamide/Keiselguhr composite derivatized with ethylenediamine; the peptide was synthesized directly on the free amino group of the base resin [44]. The peptide resin in aqueous suspension was sonicated to a white paste and used to generate a rabbit polyclonal anti-2B2v antibody [42] designated BLO70.

Immunochemical Methods

SDS–polyacrylamide electrophoresis gels [45] were run at 35 mA/gel until the dye front had exited from the gel, and proteins were transferred subsequently to nitrocellulose [46] using a Hoefer (San Francisco, CA) Transphor apparatus. The blocking reagent was 5% dried milk in antibody dilution buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20). Immunodetection was carried out using an enhanced chemiluminescence detection kit with horseradish peroxidase-labeled donkey anti-rabbit immunoglobulin as a secondary antibody (1:10,000 dilution). Molecular mass markers were run in parallel on all gels to identify the expected area of migration of the CYP proteins in the 50-kDa range.

RESULTS

Electrophoretic Properties of CYP2B1, CYP2B2, CYP2B2v and CYP2B3

The identification and integrity of the cDNA-expressed CYP2B proteins were verified by SDS–PAGE and western

immunoblotting using anti-2B1, which recognizes all CYP2B forms tested. cDNA-expressed CYP2B2v, CYP2B2, CYP2B1 and CYP2B3 proteins could be resolved by western blot analysis (Fig. 1). Their characteristic pattern of migration in the 50-kDa region was, from slowest to fastest, CYP2B2v, CYP2B2, CYP2B1 and CYP2B3 (Fig. 1A, lanes 2–5; Fig. 1B, lanes 2–4). The electrophoretic mobilities of $CYP2B2^M$ and $CYP2B2v^M$, also recognized by anti-2B1, were identical to those of CYP2B2 and CYP2B2v, respectively (data not shown). Variation in transfection efficiencies accounts for differences in signal intensities observed in Fig. 1 for the different cDNA-expressed CYP2B proteins. Anti-2B1 did not recognize any protein in the 50-kDa region in crude extracts of Ad293 cells transfected with the control vectors pMT2-2B1(i) or pMT2-2B2(i) (Figs. 1–3).

Using 25 μg of liver microsomal protein isolated from untreated rats, up to five proteins (designated, in order of increasing electrophoretic mobility, band 2 protein to band 6 protein) have been detected on western immunoblots by anti-2B1 [16]. Bands 2–5 are evident in Fig. 1A (lane 1) and Fig. 2A (lane 7), but band 6 was not resolved here. Similar analysis of 1 μg of liver microsomal protein from PB-treated rats revealed the presence of up to three protein bands (bands 1, 2, and 3, in order of increasing electrophoretic mobility) [16]. When 25 μg of liver microsomal protein from a PB-treated rat was analyzed, up to five proteins were detected (bands 1–5 in Fig. 2A, lane 6, lower); although resolution was poor because of the intensity of bands 2 and 3, previously identified with CYP2B2 and

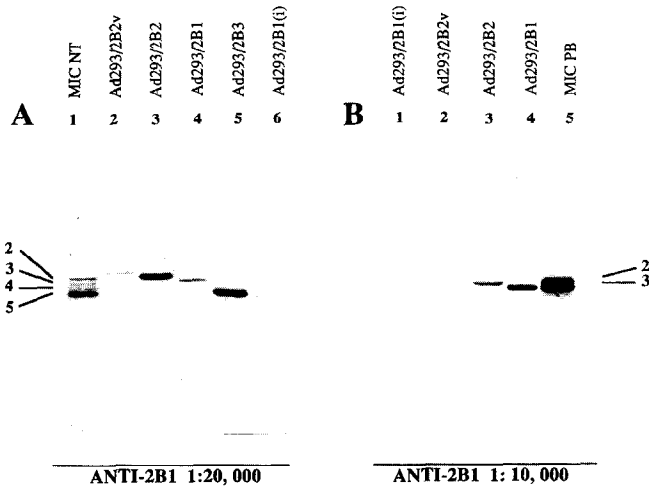

FIG. 1. Western blot analysis using the anti-2B1 antibody. (A) Liver microsomes of an untreated rat (MIC NT) or crude extracts of Ad293 cells transfected with pMT2-2B2v (Ad293/2B2v), pMT2-2B2 (Ad293/2B2), pMT2-2B1 (Ad293/2B1), pMT2-2B3 (Ad293/2B3), or pMT2-2B1(i) (Ad293/2B1(i)) were analyzed. All lanes had 25 μg of protein except lane 6 which had 50 μg of protein. (B) Crude extracts (50 μg of protein) of Ad293 cells transfected with pMT2-2B1(i), pMT2-2B2v, pMT2-2B2 and pMT2-2B1 or microsomal proteins (5 μg) of a PB-treated rat (MIC PB) were analyzed. Crude extracts of Ad293 cells for panels (A) and (B) were from different transfections.

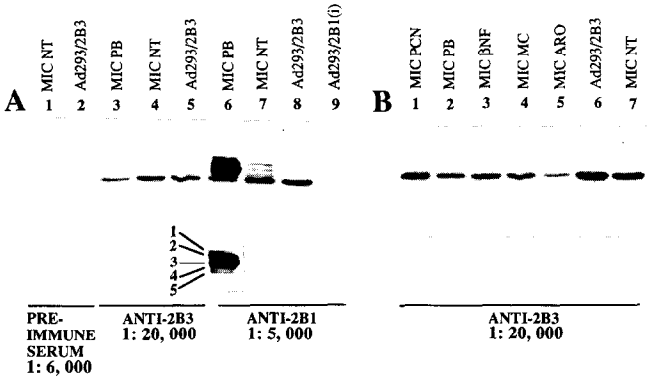

FIG. 2. Characterization of an anti-2B3 antibody and identification of CYP2B3 in rat liver microsomes. Western blot analysis was performed on crude extracts of Ad293 cells transfected with pMT2-2B3 (Ad293/2B3) or pMT2-2B1(i) (Ad293/2B1(i)) and with liver microsomes of an untreated rat (MIC NT) and of rats treated with PB (MIC PB), PCN (MIC PCN), βNF (MIC βNF), MC (MIC MC), or ARO (MIC ARO). Panels (A) and (B) represent results of two separate experiments. All lanes had 25 μg of protein except lane 9 in panel (A) which had 50 μg of protein. The upper and lower images in panel (A), lane 6, represent two different exposure times for the same material. The anti-2B3 serum samples used were obtained after the third boost.

CYP2B1, respectively [16], the proteins of bands 2–5 co-migrated with the corresponding proteins from an untreated rat (Fig. 2A, lanes 6 and 7).

As expected, cDNA-expressed CYP2B1 and CYP2B2 co-migrated with the corresponding proteins detected by anti-2B1 in microsomes of an untreated rat (Fig. 1A, compare lane 1, bands 2 and 3, with lanes 3 and 4) or of a PB-treated rat (Figure 1B, compare lane 5, bands 2 and 3, with lanes 3 and 4). In addition, and as shown previously [16], cDNA-expressed CYP2B3 co-migrated with a protein of electrophoretic mobility greater than that of CYP2B1 and was detected by anti-2B1 in liver microsomes of untreated rats (Fig. 1A, compare lane 1, band 5, with lane 5; Fig. 2A, compare lane 7, band 5, with lane 8). This protein was also detected by anti-2B1 in liver microsomes of a PB-treated rat (Fig. 2A, lane 6, band 5).

As noted above, cDNA-expressed CYP2B2v migrated more slowly than CYP2B2 (Fig. 1A and B, lanes 2 and 3). It co-migrated with band 1 protein, which is visible as a shadow above the CYP2B2 band in Fig. 1B, lane 5, and which is apparent in Fig. 2A, lane 6. CYP2B2v and band 1 protein have an electrophoretic mobility similar to that of the CYP2B2-related PB-inducible protein identified by Oesch *et al.* [22]. In agreement with previous work [16], band 1 protein was PB-inducible in liver microsomes (compare Fig. 1A, lane 1, and Fig. 2A, lane 7, with Fig. 1B, lane 5 and Fig. 2A, lane 6).

Detection by Anti-2B2v and Anti-2B3 of CYP2B2v and CYP2B3 in Rat Liver Microsomes

To test the hypothesis that CYP2B2v and CYP2B3 are represented, respectively, by band 1 and band 5 proteins

detected in rat liver microsomes by anti-2B1, specific polyclonal antibodies for each were generated using short specific peptide sequences as immunogens. The resulting antisera were tested by western blotting for their reactivity against crude extracts of Ad293 cells transfected individually with the pMT2-2B1, pMT2-2B2, pMT2-2B2v, or pMT2-2B3 vectors and against hepatic microsomes from PB-treated or untreated rats. Antibodies specific for CYP2B3 or for CYP2B2v were thus obtained.

The anti-2B3 antibody detected the constitutive band 5 protein in liver microsomes of PB-treated rats (Fig. 2A, lane 3) and untreated rats (Fig. 2A, lane 4) which co-migrated with cDNA-expressed CYP2B3 (Fig. 2A, lane 5). CYP2B3 was also detected in liver microsomes of five additional untreated rats (data not shown). No CYP2B-like proteins were detected with the pre-immune serum in such preparations (Fig. 2A, lanes 1 and 2, and data not shown). The anti-2B2v antibody detected the cDNA-expressed CYP2B2v protein (Fig. 3A, lane 2), but not the cDNA-expressed CYP2B2 protein (Fig. 3A, lane 1); both of these proteins were detected, as expected, by anti-2B1 (Fig. 3B, lanes 1 and 2). The anti-2B2v antibody also detected a protein in liver microsomes of PB-treated rats (Fig. 3A, lane 3, and Fig. 3C, lane 2), which co-migrated with the cDNA-expressed CYP2B2v protein (Fig. 3A, lane 2, and Fig. 3C, lane 3). Typically, this protein was undetectable with anti-2B2v in liver microsomes of untreated rats (Fig. 3A, lane 4, and Fig. 3D, lane 7), but it was detected in such microsomes after very long exposure times (Fig. 3C, lane 1). Hence, CYP2B2v, like CYP2B2 [14], is highly inducible by PB, although it is present at an appreciable constitutive level in rat liver microsomes.

Tissue Distribution of CYP2B2v and CYP2B3 in Rats Treated with Different Inducers

To investigate whether the CYP2B2v and CYP2B3 proteins are present in other rat tissues and to determine whether their levels are affected by known P450 inducers, microsomes of various tissues were prepared from untreated or inducer-treated rats and analyzed by western blotting. As before, the anti-2B3 antibody detected a protein in liver microsomes of an untreated rat (Fig. 2B, lane 7) that co-migrated with the cDNA-expressed CYP2B3 protein (Fig. 2B, lane 6). The level of CYP2B3 in the liver was not increased by treatment of rats with PCN, PB, βNF, MC, or ARO (Fig. 2B, lanes 1–5) or with DEX (data not shown). Indeed, there seemed to be a decrease in the amount of immunoreactive CYP2B3 in liver microsomes of rats treated with some inducers, notably ARO (Fig. 2B, compare lanes 5 and 7) and to a lesser extent PB (Fig. 2B, compare lanes 2 and 7; see also Fig. 2A, lanes 3 and 4). No protein in the 50-kDa range was detected by anti-2B3 in lung, kidney, prostate, or small intestine microsomes of untreated rats or of rats treated with PCN, PB, βNF, MC, or ARO (data not shown). Microsomes from lung, kidney, and prostate of untreated rats were used in western blot analysis

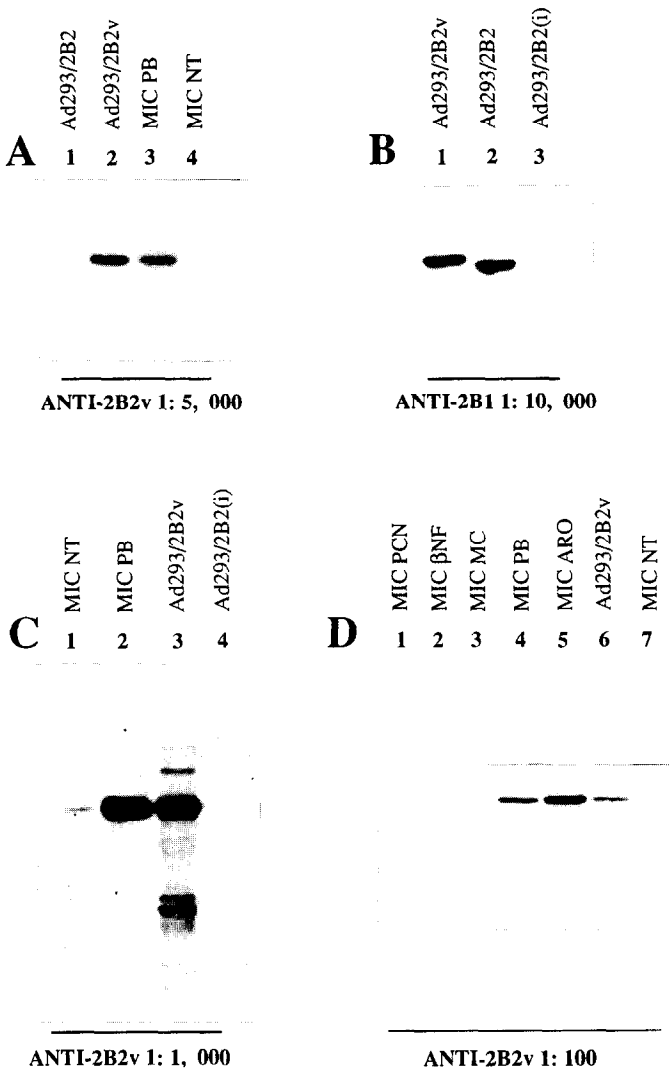

FIG. 3. Characterization of an anti-2B2v antibody and identification of CYP2B2v in rat liver microsomes. Western blot analysis was performed on crude extracts of Ad293 cells transfected with pMT2-2B2 (Ad293/2B2), pMT2-2B2v (Ad293/2B2v), or pMT2-2B2(i) (Ad293/2B2(i)) and with liver microsomes of an untreated rat (MIC NT) and of rats treated with PB (MIC PB), PCN (MIC PCN), βNF (MIC βNF), MC (MIC MC), or ARO (MIC ARO). All lanes had 25 μg of protein. The film for panel (C) was overexposed to permit detection of the basal level of immunoreactive protein in lane 1, which explains the heavy background in lane 3. The anti-2B2v serum samples were obtained after the sixth (panels A and C or the second panel D) boost. Samples for panels (A) and (B) were run on a single gel and the crude extracts of Ad293 cells for these two panels were from the same transfections.

with the polyclonal anti-2B1 antibody and, except for lungs where a high constitutive level of CYP2B1 was observed as previously described [13], no protein co-migrating with CYP2B1, CYP2B2, CYP2B2v, or CYP2B3 was detected in those preparations (data not shown).

As noted above, the anti-2B2v antibody detected, in rat liver microsomes, a PB-inducible protein that co-migrated with the cDNA-expressed CYP2B2v protein (Fig. 3, A and

C). The CYP2B2v protein was also induced in rat liver by ARO (Fig. 3D, compare lanes 5 and 7). CYP2B2v was not detected in liver microsomes of rats treated with DEX (data not shown), PCN, βNF, or MC (Fig. 3D, lanes 1–3). No protein in the 50-kDa range was detected by anti-2B2v in lung microsomes of an untreated rat or of rats treated with PCN, DEX, βNF, MC, ARO, or PB (data not shown).

DMBA Metabolism by Microsomes of Transfected Ad293 Cells

Activities and regioselectivity for the metabolism of DMBA by microsomes of Ad293 cells transfected with pMT2-2B1, pMT2-2B2, and pMT2-2B2v, as well as pMT2-2B2^M and pMT2-2B2v^M [and pMT2-2B1(i) as a control] were determined and compared (Table 1). Microsomes containing cDNA-expressed CYP2B1 and CYP2B2 formed 5,6-dihydrodiols and both 7- and 12-hydroxymethyl metabolites of DMBA in ratios similar to those previously obtained using a vaccinia virus expression system with HepG2 cells [34]. More specifically, CYP2B2 showed a strong preference for hydroxylation of DMBA at the 12-methyl position rather than the 7-methyl position whereas CYP2B1 showed a slight preference for hydroxylation at the 7-methyl position. CYP2B2v metabolized DMBA, and the metabolite profile was similar to that obtained with CYP2B2. Thus, the addition of 8 amino acids to the CYP2B2 protein did not modify appreciably methyl hydroxylation activity towards DMBA, suggesting that this addition does not disturb the overall structure of the protein. CYP2B2^M and CYP2B2v^M also metabolized DMBA, and the metabolite profile was similar to that of CYP2B2 and CYP2B2v. Thus, the presence of a Lys or Met at position 473 does not affect appreciably CYP2B2 activity towards DMBA. The cDNA-expressed CYP2B3 protein had no detectable activity towards DMBA.

DISCUSSION

Four proteins, designated band 2 protein to band 5 protein, were detected by anti-2B1 in liver microsomes of untreated and PB-treated rats, and an additional form, band 1 protein, was also detected in liver microsomes of PB-treated rats. Co-migration with cDNA-expressed CYP2B proteins, as well as previous results [16], associated band 1 protein with CYP2B2v, band 2 protein with CYP2B2, band 3 protein with CYP2B1, and band 5 protein with CYP2B3. The protein of band 4, and that of band 6, detected previously [16] but not resolved in the experiments reported herein, have yet to be identified. They may correspond to other members of the CYP2B subfamily.

Based on our results and on those of Edwards *et al.* [47], antipeptide antibodies provide a powerful method for the detection and identification of closely related P450s. The CYP2B2v- and CYP2B3-specific antibodies showed the presence in rat liver of the PB- and ARO-inducible CYP2B2v and the constitutive CYP2B3 protein, which appear to correspond to proteins detected previously by several groups [14, 21–27]. CYP2B2v represents a rare case of a functional xenobiotic-metabolizing rat liver P450 produced by alternative splicing [48]. The level of CYP2B3 in rat liver microsomes did not increase after treatment with any of the six known P450 inducers tested. Indeed, there was a decrease in the CYP2B3 protein level in microsomes of ARO- and PB-treated rats which may result from destabilization of CYP2B3 in the presence of these agents.

TABLE 1. Metabolism of DMBA by CYP2B proteins produced by cDNA expression vectors in Ad293 cells

	DMBA metabolites (pmol·mg^{-1}·hr^{-1})				
		Hydroxymethyl			
Source of microsomes	5,6-Dihydrodiols	7-OH	12-OH	Total	Corrected total*
CYP2B1 (3.75)†	43.2	29.4	25.1	97.7	26.1
CYP2B2 (3.69)	26.7	125.2	924.2	1076.0	291.6
CYP2B2v (.50)	2.2	30.3	85.1	117.7	235.4
CYP2B2v (1.40)	4.4	20.1	144.0	168.6	120.4
CYP2B2^M (2.34)	16.6	64.4	523.5	604.6	258.4
CYP2B2v^M (0.45)	2.4	18.4	58.8	79.6	176.9
CYP2B2v^M (0.30)	2.0	4.8	55.8	62.6	208.7
CYP2B2v^M (1.00)	8.8	33.9	295.7	338.4	338.4
CYP2B3‡	ND§	ND	ND	ND	
CYP2B1(i)	ND	ND	ND	ND	

Ad293 cell microsomes were incubated as described in Materials and Methods. Values for the individual enzymatic activities represent means from duplicate incubations; variation between duplicates was generally less than 10%. Results are shown for two different transfections for CYP2B2v, for three different transfections for CYP2B2v^M, and for one transfection for CYP2B1, CYP2B2, CYP2B2^M, CYP2B3, and the CYP2B1(i) control. Quantitation of CYP2B forms was achieved by reference to a standard curve generated by serial dilution of microsomes containing recombinant CYP2B1. Integration to obtain relative band intensities was carried out by laser densitometry.

* Total DMBA metabolites/relative band intensity.

† Values in parentheses are relative band intensities for CYP2B1, CYP2B2, or CYP2B2v forms for each set of microsomes with the band intensity of one of the CYP2B2v^M samples being arbitrarily set at 1.00.

‡ Relative band intensity was not determined for this sample, but the intensity of the immunoreactive CYP2B3 protein band was comparable to that of the most intense CYP2B2v^M sample.

§ ND, metabolite formed at levels of < 0.5 pmol·mg^{-1}·hr^{-1}.

Assays of microsomal DMBA metabolism were analyzed with a variation between duplicates of approximately 10%, although the variation in the ratio (total DMBA metabolites/relative band intensity of microsomal P450 protein) between microsomes prepared from separate cultures was as much as 2-fold (see Table 1). The source of this 2-fold variation is not clear, but it may represent variable heme incorporation. Within the limits of the observed experimental variation, the cDNA-expressed CYP2B2v protein hydroxylates DMBA in a manner similar to that of the cDNA-expressed CYP2B2 protein, although it is not excluded that the specificity for other substrates is changed by the presence of 8 additional amino acids in CYP2B2v. The results also show that Met at position 473 in CYP2B2^{M} and CYP2B2v^{M} does not alter appreciably enzymatic activity or regioselectivity for DMBA hydroxylation and that, with the expression system used, CYP2B3 does not have any detectable enzymatic activity towards DMBA. The latter result may mean that DMBA is not a substrate for CYP2B3, although it is not inconceivable that the pMT2-2B3 construct does not generate an enzymatically active protein, regardless of the expression system, or that CYP2B3 is not active in Ad293 cells. In any case, the biological functions of CYP2B2v and CYP2B3 remain to be established.

The cDNA-expressed CYP2B1 gave activities for DMBA metabolism that were about 10 times lower than those for the CYP2B2 forms. This contrasts with the typically higher activity of CYP2B1 for various substrates [49], but confirms a previous observation of Christou *et al.* [34] that metabolism of polycyclic hydrocarbons by CYP2B1 is selectively suppressed in the microsomal membrane relative to the reconstituted preparations of the purified CYP2B1 and CYP2B2 proteins. The approximate 10-fold activity difference between the cDNA-expressed CYP2B1 and CYP2B2 forms was essentially the same as that seen previously [34], indicating that the host cell (Ad293 vs HepG2) does not play a role in the suppression. Based on the 3-fold higher activity of purified CYP2B1 relative to CYP2B2 [34], this low DMBA metabolism indicates a selective 30-fold suppression of CYP2B1 activity in the microsomal environment. Clearly this suppression, which evidently results from one or more of the amino acid differences between the two forms, is not affected appreciably by the insert in CYP2B2v or by the presence of Met or Lys at CYP2B2 position 473.

Although conserving relatively little amino acid sequence similarity, vertebrate P450s and the procaryotic CYP101 have a similar distribution of major α-helix and β-sheet motifs as judged by computer assessment of the sequences [50, 51]. The crystal structure of CYP101 has been used as a basis for modeling the active site of CYP2B1 [52]. The predicted amino acid contacts with substrates have been used to analyze the data concerning effects of site-directed substitutions that affect these contacts, and the results have generally validated the model [52]. Data presented in this paper indicate that the 8-amino acid insert in CYP2B2v, which, based on the structural alignment with CYP101, probably occurs between helices H and I [19], does not greatly affect the substrate binding for DMBA. Notably, there is no change in the preference for 12-hydroxylation of DMBA, the distinguishing feature compared to CYP2B1. The substitution of Met for Lys at position 473 also appears not to affect DMBA orientation in the substrate pocket. Although at first glance this is more surprising since the loss of charge occurs in the putative β-sheet 5 close to the proposed substrate-contacting amino acids [52], He *et al.* [53] observed that substitutions at this position in CYP2B1 are without detectable effect on steroid hydroxylase activity.

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