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Primary Structures of Cytochrome P-450 Isozyme 5 from Rabbit and Rat and Regulation of Species-Dependent Expression and Induction in Lung and Liver: Identification of Cytochrome P-450 Gene Subfamily IVB

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SUMMARY

The primary structure of rabbit cytochrome P-450 isozyme 5 has been derived from the nucleotide sequence of cloned cDNA. Identical sequences were obtained for cDNAs constructed with mRNA from four different sources, lung and liver of untreated rabbits and liver from rabbits treated once or four times with phenobarbital. Isozyme 5 shows significant sequence identity only with rabbit P-450p2 (54%) and rat P-450LAω (53%), which places it in a previously unrecognized cytochrome P-450 gene subfamily (IVB). A cDNA library was also constructed from rat pulmonary mRNA and screened with cDNA encoding rabbit isozyme 5. The amino acid sequence derived from a positive clone was compared with that of rabbit isozyme 5 and found to be 87% identical, significantly greater than observed between other similar forms of cytochrome P-450 from rabbit and rat. Alignment of the primary structures of rabbit isozyme 5 (506 residues), rat isozyme 5 (511 residues), rabbit P-450p2, and rat P-450LAω shows 43% structural identity and a common 16-residue peptide near position 300 that is unique to these forms of cytochrome P-450. Analysis of mRNA from lung and liver of rabbit, rat, guinea pig, and hamster indicates that species and tissue differences in the expression and induction of isozyme 5 are likely regulated at the level of transcription. These differences fall into one of the following three groups: first, expression in lung and liver and induction in liver by phenobarbital (rabbit); second, expression in lung and liver but no hepatic induction (hamster); and third, expression in lung and little or no expression in liver regardless of treatment (rat and guinea pig).

The cytochrome P-450 monooxygenase system catalyzes the oxidative metabolism of a wide variety of exogenous and endogenous chemicals. This versatility stems from the existence of multiple forms of cytochrome P-450, each of which has a broad, but still somewhat unique, substrate specificity. Reactions of exogenous substrates catalyzed by cytochrome P-450 result in one of two general outcomes. First, the modification renders the chemical more suitable for excretion; second, the metabolite formed is reactive and binds covalently with some cellular constituent. The latter possibility is thought to be important in carcinogenesis, mutagenesis, and other toxic manifestations associated with numerous xenobiotics.

One of the many classes of compounds from which potentially harmful products can be formed is aromatic amines, a group of chemicals whose cytochrome P-450-mediated metabolism in rabbits has been thoroughly investigated (1-5). Of the forms of cytochrome P-450 known to be present in rabbit tissues, only two, isozymes 4 and 5, appear to be capable of metabolizing aromatic amines to reactive products (2, 3). Both of these isozymes are expressed in rabbit liver, but only isozyme 5 has been detected in lung and bladder, two tissues where the microsomal *N*-hydroxylation of 2-aminofluorene appears to be catalyzed entirely by this form of P-450 (3-5).

On the basis of structural, immunochemical, and functional properties, preparations of isozyme 5 from rabbit liver and lung are very similar, if not identical. However, at least one aspect of the regulation of isozyme 5 in these tissues differs; treatment of rabbits with PB induces the synthesis of isozyme 5 in liver but not in lung (6). In other species, however, forms of cytochrome P-450 related to rabbit isozyme 5 do not show the same pattern of expression (7). Whereas lungs of rats, mice, monkeys, guinea pigs, and hamsters contain cytochrome P-450 that is related immunochemically and functionally to isozyme 5, the same form of P-450 is expressed in liver only in the case of the hamster (7). In contrast to the rabbit, the concentration of this cytochrome P-450 in hamster liver is not increased following treatment with PB (7).

In the present study, we have investigated three questions regarding the structure and regulation of isozyme 5. First, are the hepatic and extrahepatic forms of the isozyme identical?

ABBREVIATIONS: PB, phenobarbital; kb, kilobase.

Second, what is the structural relationship between rabbit isozyme 5 and the isozyme from rat that exhibits similar immunochemical and functional properties? Third, do differences in expression of isozyme 5-like forms of cytochrome P-450 among various tissues and species appear to be regulated at the level of transcription?

Materials and Methods

Animals. Adult male New Zealand White rabbits, Sprague-Dawley rats (Dutchland Farms, Denver, PA), Hartley guinea pigs, and Syrian hamsters (Charles River Breeding Laboratories, Wilmington, MA) were used. Animals treated with PB received 40 mg/kg (rabbits) or 80 mg/kg (rats, guinea pigs, and hamsters) administered intraperitoneally daily for 4 days. Several rabbits were also killed 12 or 24 hr following a single treatment with PB (60 mg/kg). Food was taken from all animals 24 hr before they were killed.

Isolation of mRNA. The method of Deeley *et al.* (8) was used for the isolation of mRNA from the liver of a rabbit treated once with PB (60 mg/kg) for 12 hr. All other mRNA preparations were obtained by a modification of the methods of Chirgwin *et al.* (9) and Glisin *et al.* (10), as described by Gasser *et al.* (11). Polyadenylated mRNA was isolated by chromatography on oligo(dT)-cellulose (12). Messenger RNA for the construction of cDNA libraries was isolated from the liver of an untreated rabbit (Lv-C library), the liver of a rabbit treated once with PB for 12 hr (Lv-1 library), the liver of a rabbit treated daily for 4 days with PB (Lv-4 library), the lungs of an untreated rabbit (Lg library), and the lungs of an untreated rat (rat-Lg library).

Cloning of cDNA derived from pulmonary and hepatic mRNA. Double-stranded cDNA was synthesized from mRNA by sequential incubation with avian myeloblastosis virus reverse transcriptase and DNA polymerase I (13, 14), attached to synthetic *Eco*R1 linkers, chromatographed on Sepharose CL4B to remove molecules <1500 bases in length, and ligated into λgt11 (15) or λZAP (Stratagene, La Jolla, CA). About 6×10^6 recombinant clones were obtained per μg of cDNA. Recombinant DNA encoding isozyme 5 was isolated initially from the Lv-1 library by detection of the fused β-galactosidase-antigen product with antibody to isozyme 5 (16). Positive clones were purified to homogeneity and one insert (Lv-1:1, 930b) was subcloned into plasmid Bluescript (Stratagene) and used to isolate a related cDNA from the Lg library (Lg-1, 1764b). Additional clones suitable for sequencing were then isolated from the Lv-C library (Lv-C:1, 1762b), the Lv-4 library (Lv-4:1, 2039b), and the Lg library (Lg:2, 1240b) by plaque hybridization (17) with nick-translated Lg-1. The same methods, but with less stringent hybridization conditions, were also used to isolated a clone from the rat lung library (rat-Lg:1, 1930b). Fragments of these six clones (Lv-C:1, Lv-1:1, Lv-4:1, Lg:1, Lg:2, and rat-Lg:1), formed by sonication (18) or restriction (19), were subcloned into M13 and sequenced by the dideoxy-chain termination method (20, 21). Computer analysis of sequence data was carried out with the Sequence Analysis software Package from the University of Wisconsin Computer Group (22), and gap alignments were constructed with the algorithm developed by Wilbur and Lipman (23).

Analysis of mRNA and DNA. Samples of mRNA were analyzed by hybridization with Lv-4:1, following electrophoresis on agarose gels that contained methylmercury (24) and were transferred to a nylon (Nytran) membrane (Schleicher and Schuell, Keene, NH). In all cases, hybridization was carried out at 42° as described by Gasser *et al.* (11). Restriction fragments of hepatic genomic DNA, isolated by standard procedures (25, 26), were separated by electrophoresis, transferred to nylon membrane (Nytran), and analyzed by the method of Southern (27) with Lv-4:1 as the probe. Autoradiograms were scanned with a UV densitometer (Joyce Loebl, Gateshead, England).

Materials. All reagents not specifically noted above were obtained at the highest purity possible from commercial sources.

Results

Isolation and sequencing of cDNAs encoding for isozyme 5. Recombinant cDNA encoding for isozyme 5 was isolated from the Lv-1 library by detection of the fused β-galactosidase-antigen product with polyclonal antibodies to isozyme 5. Several clones were purified and one λgt11 insert (Lv-1:1, 930 bases) was subcloned and used as a probe in the isolation of several clones from the Lg library. The largest pulmonary clone (Lg:1, 1764b) was then used in the isolation of a number of hepatic and pulmonary clones (Lg:2, 1240 bases; Lv-4:1, 2034 bases; Lv-C:1, 1762 bases; and Rt-Lg:1, 1930 bases) that, along with Lv:1-1 and Lg:1, were subcloned and sequenced by the strategies shown in Fig. 1. The sequences of the rabbit cDNAs were identical at all corresponding positions. The longest of these sequences (Lv-4:1), which includes a 3′ *Eco*R1 fragment not retained by the other rabbit cDNAs, and the sequence of the rat lung cDNA are shown in Table 1. Alignment of these sequences requires that three gaps, two equal to three bases each and one equal to nine bases, be placed in the rabbit sequence near the 5′ end (Table 1). Aligned in this manner, the coding regions of the sequences (1518 bases for rabbit, 1533 bases for rat) show remarkable similarity (85%). In contrast, the similarity of the 3′-flanking sequences, which are shown unaligned, is less than 50%.

Primary structure of rabbit and rat forms of cytochrome P-450 isozyme 5. The amino acid sequences derived for forms of isozyme 5 from rabbit (506 residues) and rat (511 residues) are shown in Table 2. The first 21 amino acids of the rabbit sequence are the same as those determined from the purified protein (28). The sequences, as aligned, are identical

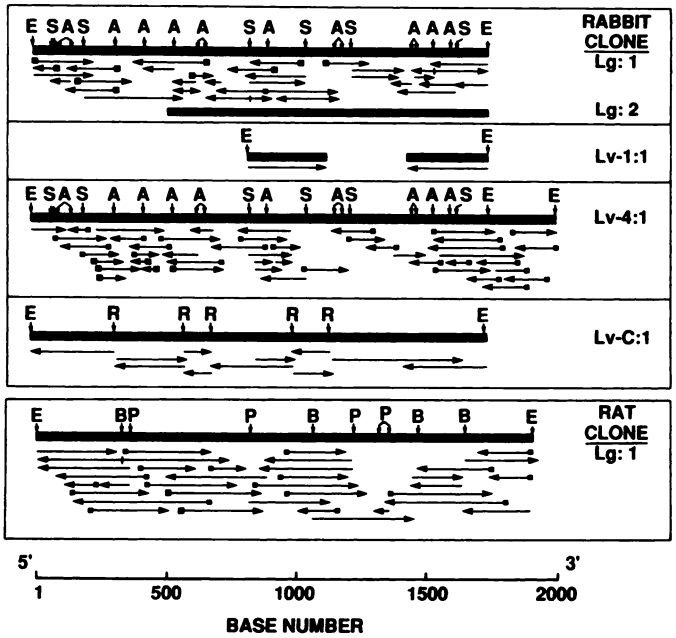

Fig. 1. Strategy used for sequencing cDNAs encoding for isozyme 5 isolated from hepatic and pulmonary libraries of rabbit and rat. At the *top* of each section is a graphical representation of the cDNA that was sequenced. The fragments from which the sequences were obtained are shown below each cDNA, with the direction of sequencing indicated by an *arrow*. Fragments were obtained by restriction (——) or by sonication (■—). The restriction enzymes used were *Alu*I (*A*), *Bam*HI (*B*), *Eco*RI (*E*), *Pst*I (*P*), *Rsa*I (*R*), and *Sau*3AI (*S*). A second rabbit pulmonary clone (Lg:2) is also shown.

TABLE 1

Nucleotide sequences of cDNAs encoding for cytochrome P-450 isozyme 5 (IVB) from rabbit and rat

Alignment of the same base in each sequence is designated by a vertical bar (|); alignment of different bases is designated by a colon (:). Insertion of a base in order to achieve the best alignment is noted by a dash (-). The 3′-flanking regions of the sequences show a great deal of divergence and are reported without any alignment. The initiation (*TAG*) and stop (*TAG*) codons are shown in italics.

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RABBIT 0001 GGGGCGGCTGCCGCTCCAGCCACG*ATG*CTC---GGCTTCCTCTCC---CGCCTG---------GGCCTGTGGGCTTCCGGACTGATCTTGATCCTAGGCT 0085
RAT 0001                    CC*ATG*GTCCTCAATTTCCTCTCCCCGAGCCTTTCCCGGCTCGGCTTGTGGGCTTCTGTAGTGATCCTGATGGTAATCG 0078

RABBIT 0086 TCCTCAAGCTCCTCCGCCTGCTGCTTCGAAGGCAGAGGTTGGCCCGGGCCATGGACAGCTTCCCAGGGCCACCCACTCACTGGCTGTTTGGGCACGCCCT 0185
RAT 0079 TTCTCAAGCTCTTCAGCCTGCTGCTTCGGAGGCAGAAGCTGGCCAGGGCTATGGACAGTTTCCCAGGGCCCCCCACACACTGGCTTTTTGGTCATGCCCT 0178

RABBIT 0186 CGAGATCCAGAAGACGGGGAGCCTGGACAAGGTGGTGACCTGGACCCAGCAGTTCCCCTACGCCCACCCTCTCTGGGTTGGACAGTTCATTGGCTTCCTG 0285
RAT 0179 TGAGATCCAGAAGTTAGGGAGCCTGGACAAGGTGGTATCCTGGGCCCAACAGTTCCCCCATGCCCACCCACTTTGGTTTGGACAATTCGTTGGTTTCCTG 0278

RABBIT 0286 AACATCTACGAGCCCGACTACGCCAAAGCTGTGTACAGCCGTGGGGACCCTAAAGCCCCGGATGTGTATGACTTCTTCCTCCAGTGGATTGGCAAAGGCC 0385
RAT 0279 AACATCTATGAGCCCGACTATGCGAAAGCTGTATACAGCCGCGGGGATCCGAAGGCTGCAGATGTGTATGACTTCTTCCTCCAGTGGATCGGGAAAGGCC 0378

RABBIT 0386 TGCTGGTTCTGGATGGGCCCAAGTGGTTCCAGCACCGCAAGCTGCTCACGCCTGGCTTCCATTACGACGTGCTGAAGCCCTACGTGGCCATCTTTGCCGA 0485
RAT 0379 TACTAGTTCTGGATGGGCCAAAATGGTTCCAGCACCGCAAGCTGCTCACACCTGGCTTCCATTATGATGTGCTGAAGCCCTATGTGGCCATATTTGCTGA 0478

RABBIT 0486 CTCCACACGCATCATGCTGGAAAAATGGGAGAAAAAGGCCTGTGAGGGTAAGAGCTTCGACATCTTCTCTGACGTGGGCCACATGGCGCTCGACACGCTC 0585
RAT 0479 GTCCACACGTATGATGCTGGACAAGTGGGAGAAAAAGGCTAGTGAAAATAAGAGCTTTGACATCTTCTGTGACGTAGGCCACATGGCCCTGGACACCCTC 0578

RABBIT 0586 ATGAAGTGTACGTTTGGCAAAGGAGACAGTGGCCTGAATCACAGGGACAGCAGCTACTACGTGGCAGTCAGCGAGCTCACGCTGCTGATGCAGCAACGCA 0685
RAT 0579 ATGAAGTGCACCTTCGGCAAAGGAGACAGCGGCCTAGGCCACAGAGACAACAGCTACTACCTGGCGGTCAGTGACCTCACACTGCTGATGCAGCAGCGCA 0678

RABBIT 0686 TCGACTCCTTCCAGTACCACAACGACTTCATCTACTGGCTCACTCCGCACGGCCGCCGCTTCCTGCGGGCCTGCAGGGCGGCCCACGACCACACCGACAG 0785
RAT 0679 TCGACTCCTTCCAGTACCATAATGACTTCATTTACTGGCTCACTCCACATGGCCGCCGCTTCCTGCGGGCCTGCAAGATAGCCCATGACCATACAGATGA 0778

RABBIT 0786 GGTCATCAGACAGCGGAAGGCAGCCCTGCAGGATGAGAAGGAGCGGGAGAAGATCCAGAACCGGAGACATCTGGACTTCCTGGACATTCTCTTGGATGTC 0885
RAT 0779 GGTCATCAGGCAGCGGAAGGCAGCACTGCAGGATGAGAAAGAGCGGGAAAAAGATTCAGCAGCGGAGGCACCTGGACTTCCTGGACATTCTCCTGGGTGTC 0878

RABBIT 0886 CGCGGTGAAAGTGGAGTCCAGCTGTCGGACACAGACCTCCGCGCTGAAGTGGACACGTTCATGTTCGAAGGTCATGACACCACCACCAGCGGCATCTCCT 0985
RAT 0879 CGGGATGAAAGTGGGATCAAGCTGTCAGATGCAGAGCTCCGGGCTGAAGTGGACACATTCATGTTTGAAGGCCATGACACCACCACTAGTGGTATCTCTT 0978

RABBIT 0986 GGTTCCTCTACTGCATGGCCTTGTACCCTGAGCACCAGCAGCGCTGTAGGGAGGAGGTCCGTGAGATCCTGGGAGACCAGGACTCCTTCCAGTGGGAGGA 1085
RAT 0979 GGTTTCTCTACTGCATGGCCCTTTACCCTGAGCACCAGCAGCTATGTAGGGAGGAGGTCCGTGGGATCCTAGGAGACCAAGACTCCTTCCAATGGGATGA 1078

RABBIT 1086 CTTGGCCAAGATGACCTACCTGACCATGTGCATGAAGGAGTGCTTCCGCCTCTACCCGCCCGTGCCCCAGGTGTACCGCCAGCTCAGCAAGCCCGTCAGC 1185
RAT 1079 TCTGGCCAAGATGACCTACCTGACCATGTGCATGAAGGAGTGCTTCCGCCTCTACCCACCTGTACCCCAAGTATACCGCCAGCTCAACAAGCCAGTCACC 1178

RABBIT 1186 TTTGTGGACGGCCGCTCCCTGCCTGCAGGCAGCCTGATCTCCCTGCATATCTACGCCCTCCATAGGAACAGCGACGTGTGGCCTGACCCTGAGGTCTTTG 1285
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RABBIT 1286 ACCCCCTGCGCTTTTCCCCGGAGAACTCGTCTGGACGCCACCCCTATGCCTTCATTCCCTTCTCTGCCGGGCCCAGGAACTGCATCGGGCAGCAGTTCGC 1385
RAT 1279 ACCCACTGCGCTTTTCTCCTGAGAATGCTGCAGGACGGCATCCCTTTGCCTTCATGCCTTTTTCTGCAGGGCCCAGGAATTGCATCGGGCAGCAGTTTGC 1378

RABBIT 1386 CATGAACGAGATGAAGGTGGTCACAGCCCTGTGCCTGCTCCGCTTCGAGTTCTCCGTGGACCCCCTGCGGCTGCCCATCAAGCTGCCCCAGCTGGTCCTG 1485
RAT 1379 CATGAACGAGATGAAGGTGGTCACAGCCCTTTGTTTACTGCGCTTTGAGTTCTCCCTGGATCCCTCAAAGATGCCCATTAAGGTCCCCCAGCTGATCTTG 1478

RABBIT 1486 CGCTCCAAGAATGGCATCCACCTCTACTTGAAGCCTCTGGGCCCCAAGGCTGAGAAG*TAG*CTCTGCTGAGAGCGGGGTCCCCGGCCGCCCAGGCTGCGGC 1585
RAT 1479 CGCTCCAAAAATGGTATCCACCTCTACCTGAAGCCGCTGGCTTCTAGGTCTGGAAAG*TAG*GTCTTCAGAGAACTAGGCCTGGGCAGCACAAGCCGTGACT 1578

RABBIT 1586 CTCTCCTGAGCGTCGCTGTCTCGTTGGGGGTTCCCTGCCTTCGGGATCTTGTAGCCTGGGAGGGGAGTAGGCACAGACACAGTCACCTCCAGGCCAGTGT 1685
RAT 1579 CTTCCTGGGATGTTGCCCTCCTAGATAGAATGTGGAGTCATCATGGATCCCTGCCTGTGGGAGGCTTTGGAGGCAAAAGCATGGACACTGACAGTCTCCT 1678

RABBIT 1686 CATGGAAACGCATGTGTCGACAGGTGCCTGCTGTGCATGCAAACATTTGGTGAGCACCTACTTGCTTCAGAATTCTCTCTTACTTTCCATAATGGACAGT 1785
RAT 1679 GGAAGACATTCTCTTGTAAACATGTGTGTGGAATGATTCAACGCCCAGGCTCCAGAACTTATTTCTTCACTCCACAACATTTGCTGAACGTTTTGCTGCC 1778

RABBIT 1786 CTTTCTAAAATGTACCAGAAACTTACAGTCCAGCCTCTGTGTCTTGGTGTGCGCACAGTGGAGCTCTGCCTCAGGATTTAAGGTCAGGAGCAGGGCCCGC 1885
RAT 1779 TTGAGAAACTCCATTGATTTCACATAGCTACATTTATTTAACCATCTTGGTGTGTATTTGGAATACGATGGACATGAGGAAAATTGCTCAAACTAAAACT 1878

RABBIT 1886 AGGACTGGGGACAGCTTGGGGGCCACCCTGCACTTGATCGGCTTTATCTGTGTGTGGGTGTGGGTGTTTGGAACACAACAGACATAAGGAAGATTTGCTT 1985
RAT 1879 GTACAAAATAAATAATACTTTAAAAAGCAAACTCCCACCACCAAAAAAAAAA 1930

RABBIT 1986 AGGCCCAGACTGTACACGATGCTGAATAAACAGAACTCAC 2026
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TABLE 2

Primary structures of the rabbit and rat forms of cytochrome P-450 derived from cDNA sequences

The complete amino acid sequence for the rabbit isozyme is given. The first 21 residues (underlined) are those reported for the protein by Parandoosh *et al.* (28). Residues are shown for the sequence of the rat isozyme at positions that differ from the rabbit sequence, and positions of agreement are noted by an equal sign (=). Stop codons are designated by *.

Rabbit	001	ML-GFLS-RL ---GLWASGL ILILGFLKLL RLLLRRQRLA RAMDSFPGPP	045
Rat	001	=VLN===PS= SRL=====VV ==MVIV===F S======K== ==========	050
Rabbit	046	THWLFGHALE IGKTGSLDKV VTWTQQFPYA HPLWVGQFIG FLNIYEPDYA	
Rat	051	========== ===L====== =S=A====H= ====F===V= ==========	100
Rabbit	096	KAVYSRGDPK APDVYDFFLQ WIGKGLLVLD GPKWFQHRKL LTPGFHYDVL	145
Rat	101	========== =A======== ========== ========== ==========	150
Rabbit	146	KPYVAIFADS TRIMLEKWEK KACEGKSFDI FSDVGHMALD TLMKCTFGKG	195
Rat	151	========E= ==M==D==== ==S=N===== =C======== ==========	200
Rabbit	196	DSGLNHRDSS YYVAVSELTL LMQQRIDSFQ YHNDFIYWLT PHGRRFLRAC	245
Rat	201	====G===N= ==L===D=== ========== ========== ==========	250
Rabbit	246	RAAHDHTDRV IRQRKAALQD EKEREKIQNR RHLDFLDILL DVRGESGVQL	295
Rat	251	KI======E= ========== ====K===Q= ========== G==D===IK=	300
Rabbit	296	SDTDLRAEVD TFMFEGHDTT TSGISWFLYC MALYPEHQQR CREEVREILG	345
Rat	301	==AE====== ========== ========== =========L ======G===	350
Rabbit	346	DQDSFQWEDL AKMTYLTMCM KECFRLYPPV PQVYRQLSKP VSFVDGRSLP	395
Rat	351	=======D== ========== ========== =======N== =T========	400
Rabbit	396	AGSLISLHIY ALHRNSDVWP DPEVFDPLRF SPENSSGRHP YAFIPFSAGP	445
Rat	401	========== ======T=== ========== ====AA==== F==M======	450
Rabbit	446	RNCIGQQFAM NEMKVVTALC LLRFEFSVDP LRLPIKLPQL VLRSKNGIHL	495
Rat	451	========== ========== =======L== SKM===V=== I=========	500
Rabbit	496	YLKPLGPKAE K*	506
Rat	501	=====ASRSG =*	511

TABLE 3

Percent identities of the primary structures of several isozymes of cytochrome P-450 from rabbit and rat

The species, trivial name, and gene family (38) are designated in the table. Data for rat P-450c, b, j, PCN1, and Laω and rabbit P-450, 6, 2, 3a, 3c, and p2 are from Refs. 29–37 and 11. Identities of less than 30% are denoted by *.

	Identity						
	Rat						Rabbit, 5 (IVB)
	c (IA)	b (IIB)	j (IIE)	PCN1 (IIIA)	LAω (IVA)	5 (IVB)	
			%				
Rabbit 6 (IA)	73	*	*	*	*	*	*
Rabbit 2 (IIB)	*	76	47	*	*	*	*
Rabbit 3a (IIE)	*	51	82	*	*	*	*
Rabbit 3c (IIIA)	*	*	*	70	*	*	*
Rabbit p2 (IVA)	*	*	*	*	74	52	
Rabbit 5 (IVB)	*	*	*	*	53	87	54
Rat 5 (IVB)	*	*	*	*	51		87

at 455 out of 511 positions (87%), with the terminal regions being the only areas of notable divergence. Seventeen differences are found in the first 38 positions (55% identity) and 11 in the final 34 (68% identity); 91% of the remaining 439 positions are the same, including the 21 residues (rabbit 441–461, rat 446–466) of a cysteine-containing peptide that is a characteristic feature of all cytochrome P-450 isozymes. Comparison of sequences of P-450 isozymes from rabbit and rat (29–37) shows that the degree of identity between the isozyme 5 sequences is greater than between any other set of corresponding sequences from these two species (Table 3). Further comparisons (33, 37) place isozyme 5 in the same gene family (IV) as rabbit P-450p2 (54% identity) and rat P-450 LAω (51% identity), but in a previously unrecognized subfamily (IVB), according to a recently devised classification scheme (38). Sequence alignment of the four isozymes in gene family IV (43% shared identity), two from rabbit and two from rat, is shown graphically in Fig. 2. More identity is observed with the C-terminal portions of the four sequences than with the N-terminal portions (55% versus 33%), with the first 100 N-terminal positions being particularly divergent (24% identity). The largest area of absolute identity among the sequences is a 16-residue peptide near position 300 (Table 4).

Hybridization of rabbit pulmonary and hepatic mRNA with cDNA for isozyme 5. The extent of hybridization of cDNA for isozyme 5 with mRNA from rabbit lung and liver, as determined by Northern blot analysis, is shown in Fig. 3. A single band (~2.2 kb) was observed with mRNA from either tissue and the amount detected in samples from lung was about 2.5 times greater than that in samples from liver. No hybridization was observed with samples from which the polyadenylated RNA had been removed (Fig. 3). Treatment of rabbits with PB greatly increased the amount of hepatic mRNA for isozyme 5 (Fig. 4) but had no effect on the pulmonary mRNA (data not shown). Little difference was seen between the increases observed 12 or 24 hr after a single treatment and 24 hr after four daily treatments with PB, all of which were between 10- and 15-fold (Fig. 4).

Hybridization of pulmonary and hepatic mRNA from rats, guinea pigs, and hamsters with cDNA for rabbit isozyme 5. The extent of the hybridization of cDNA for isozyme 5 with mRNA from rat lung was much less than with mRNA from rabbit lung but much greater than with mRNA from the liver of a rat treated with PB (Fig. 5). This differential between mRNA from lung and liver was even more pronounced with samples from the guinea pig (Fig. 6). In fact, the level of mRNA recognized by cDNA for rabbit isozyme 5 was below detection in hepatic samples from six different guinea pigs, three untreated and three treated with PB. In contrast, samples from livers and lungs of untreated and PB-treated hamsters contained similar amounts of mRNA detected by the cDNA for isozyme 5 (Fig. 7). (The difference between the intensities of

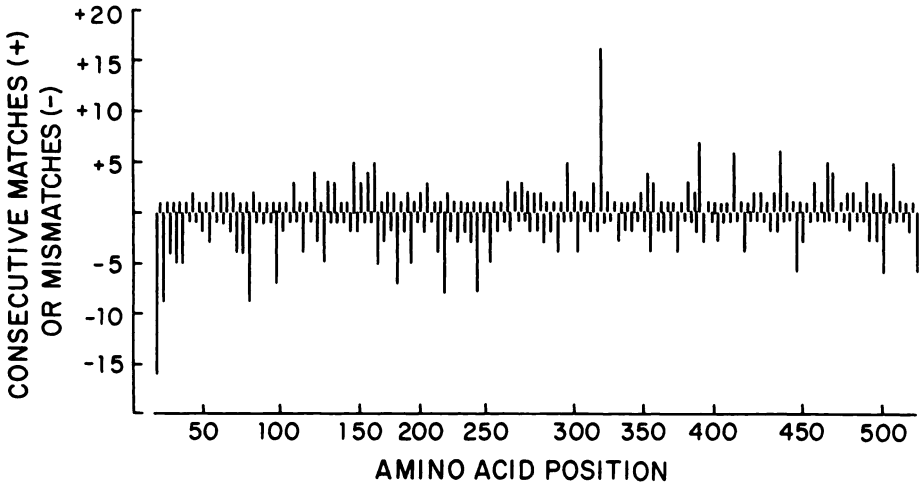

Fig. 2. A comparison of the amino acid sequences derived for four members of cytochrome P-450 gene family IV. The amino acid sequences derived for rabbit and rat forms of cytochrome P-450 isozyme 5, rabbit cytochrome P-450p2 (37), and rat P-450 Laω (33) were compared by gap alignment (23). Positions for which it was determined that all four sequences contain the same amino acid were designated as "matches"; all other positions were designated "mismatches." The numbers of consecutive matches (+) or mismatches (−) are shown.

TABLE 4

Peptide common to isozymes of cytochrome P-450 gene family IV

Species	Trivial Name	Gene Family	No.	Amino Acid	No.
Rabbit	5	IVB	292	VQ LSD TD LRAEVDTFMFEGHDTT T SG I S WF	322
Rat	5	IVB	298	IK LSD AE LRAEVDTFMFEGHDTT T SG I S SI	327
Rabbit	p2[a]	IVA	300	SS LSD QD LRAEVDTFMFEGHDTT A SG I S SI	329
Rat	LAω[b]	IVA	303	DS LSD KD LRAEVDTFMFEGHDTT A SG V S WI	332

[a] Sequence data from Matsubara *et al.* (37).
[b] Sequence data from Hardwick *et al.* (33).

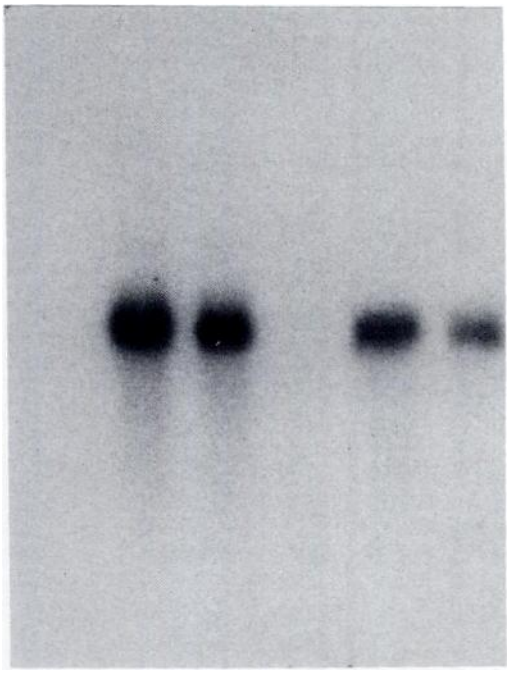

Fig. 3. Hybridization of cDNA encoding for isozyme 5 with mRNA from rabbit liver and lung. Pulmonary mRNA (10 μg, *lane 2*; 5 μg, *lane 3*) and hepatic mRNA (10 μg, *lane 5*; 5 μg, *lane 6*) were electrophoresed in an agarose gel, transferred to a nylon support, and hybridized with Lg:1, labeled by nick-translation with ^{32}P. *Lane 1* contains 10 μg of pulmonary RNA from which all polyadenylated RNA has been removed, and *lane 4* contains an equivalant hepatic sample. The autoradiogram was developed after 16 hr of exposure.

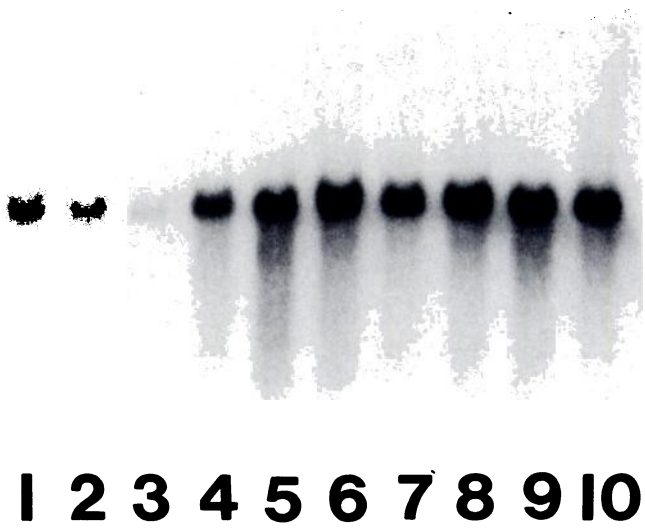

Fig. 4. Hybridization of cDNA encoding for isozyme 5 with mRNA from livers of untreated rabbits and rabbits treated with PB. Hepatic mRNA (5 μg) from individual untreated rabbits (*lanes 1*, *2*, and *3*) and from rabbits treated with PB (once for 12 hr, *lanes 4* and *5*; once for 24 hr, *lane 6*; and daily for 4 days, *lanes 7*, *8*, *9*, and *10*) was analyzed as described in the legend to Fig. 3. The autoradiogram was developed after 8 hr of exposure.

the samples from untreated and treated liver shown in Fig. 7 is well within the variation observed for either sample type.) The approximate sizes of the mRNAs recognized by cDNA for isozyme 5 (2.2 kb, rabbit; 2.35 kb, rat; and 2.4 kb, hamster and guinea pig) can be compared in Fig. 8.

Analysis of rabbit genomic DNA with cDNA for isozyme 5. Analysis of rabbit genomic DNA by hybridization (Southern blot) with cDNA for isozyme 5 indicated the presence of a minimum of two genes for isozyme 5 (Fig. 9). In

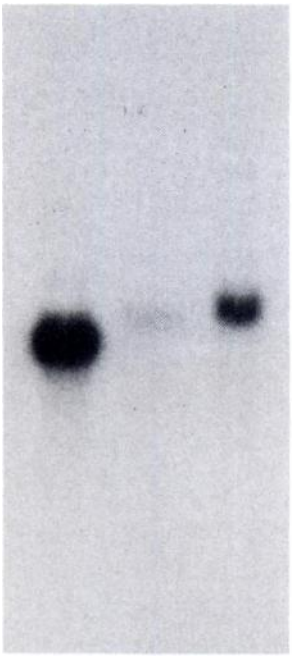

Fig. 5. Hybridization of cDNA encoding for rabbit isozyme 5 with mRNA from rat lung and liver. Samples of mRNA (12 μg) from liver (*lane 2*) and lung (*lane 3*) of a rat treated daily for 4 days with PB were analyzed as described in the legend to Fig. 3. Rabbit pulmonary mRNA (5 μg) was included for comparison (*lane 1*). The autoradiogram was developed after 24 hr of exposure.

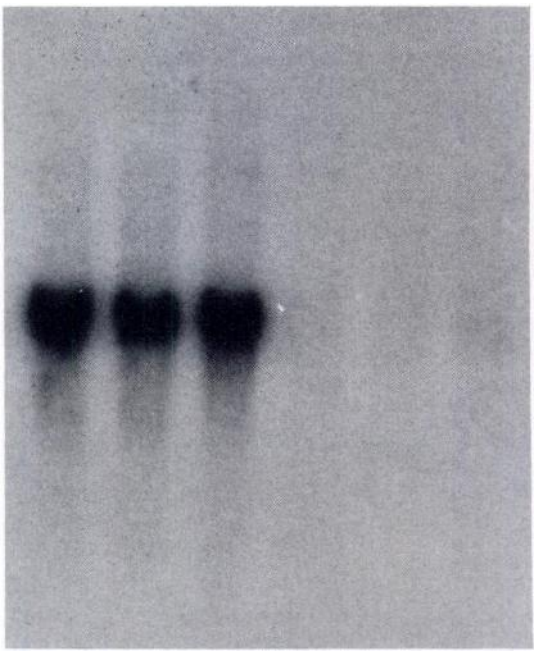

Fig. 6. Hybridization of cDNA encoding for rabbit isozyme 5 with mRNA from guinea pig lung and liver. Samples of mRNA (10 μg) from lungs (*lanes 1*, *2*, and *3*) and livers (*lanes 5* and *6*) of individual guinea pigs treated with PB daily for 4 days and from the liver of an untreated guinea pig (*lane 4*) were analyzed as described in the legend to Fig. 3. The mRNA samples in *lanes 1* and *5* and in *lanes 2* and *6* were obtained from the same animals. The autoradiogram was developed after 48 hr of exposure. Results similar to those shown for lung and for untreated liver were obtained with pulmonary and hepatic samples from three additional untreated animals.

addition, three distinct restriction patterns (two shown) were obtained by incubation of DNA samples from different individuals with *Kpn*I or *BstE*II. The evidence for polymorphism (multiple allelism) associated with one or more genes was not obtained with either *Nco*I or *Hind*III, both of which produced only one restriction pattern.

Discussion

A cDNA clone selected by screening with an antibody to cytochrome P-450 isozyme 5 was used to isolate additional clones from rabbit pulmonary and hepatic libraries. A direct relationship between these clones and isozyme 5 is established by the perfect match between the sequence of the first 21 N-terminal amino acids determined from the purified protein (28) and that derived from the nucleotide order. The partial structural identity (about 50%) of isozyme 5 with rabbit P-450p2 (37) and rat P-450LAω (33), members of cytochrome P-450 gene subfamily IVA (38), requires the designation of a new gene subfamily, IVB, for isozyme 5 if the newly proposed nomenclature is followed (38).

Identical nucleotide sequences of cDNAs isolated from pulmonary and hepatic libraries are consistent with the expression of a single form of isozyme 5 in rabbit lung and liver and in liver following treatment with PB. On the other hand, association of isozyme 5 with at least two genes and with multiple alleles suggests the possibility that there may be more than one form of the enzyme. In the case of isozyme 2, which, like isozyme 5, is a major form of P-450 in lung (39) and is induced in liver by PB (40), multiple forms of mRNA (11, 41) and protein (41, 42) have been characterized. Expression of both isozymes in lung and liver (lung > liver) and in liver following treatment with PB (about 10-fold induction) appears to be regulated by levels of mRNA (Ref. 11 and present work).

Based on structural information derived from a nucleotide sequence, a IVB form of P-450, remarkably similar to rabbit isozyme 5, is also expressed in rat. In fact, the conservation of primary structure seen with the IVB forms of P-450 is 5 to 15% greater than between other pairs of P-450 isozymes from rat and rabbit that have been assigned to the same gene subfamilies (37). The finding that the sequence derived for a human pulmonary IVB P-450[1] is 85% identical to the sequence of rabbit isozyme 5 provides additional evidence of extensive conservation of structure within the P-450 IVB subfamily. Alignment of the rabbit, rat, and human sequences shows that identical amino acids are present in over 81% of the positions. Some specific structural conservation is also evident when the sequences of the four isozymes in P-450 gene family IV (the two forms of isozyme 5, P-450LAω, and P-450p2) are aligned. All four have the same amino acids in 43% of the positions, and the same 16-amino acid peptide near position 300. This peptide is also present in a variant of rabbit P-450p2[2] and in the human

[1] Frank Gonzalez, National Institutes of Health, personal communication.
[2] Eric F. Johnson, Scripps Clinic and Research Foundation, personal communication.

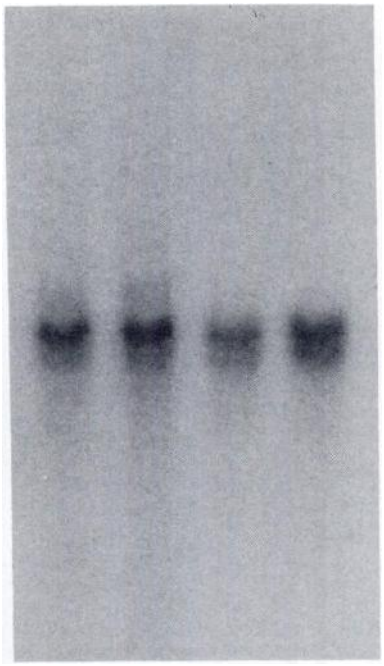

Fig. 7. Hybridization of cDNA encoding for rabbit isozyme 5 with mRNA from hamster lung and liver. Samples of mRNA (10 μg) from lungs (*lane 1*, pool of three) and liver (*lane 3*, individual) of untreated hamsters and from lungs (*lane 2*, pool of three) and liver (*lane 4*, individual) of hamsters treated daily for 4 days with PB were analyzed as described in the legend to Fig. 3. The autoradiogram was developed after 48 hr of exposure.

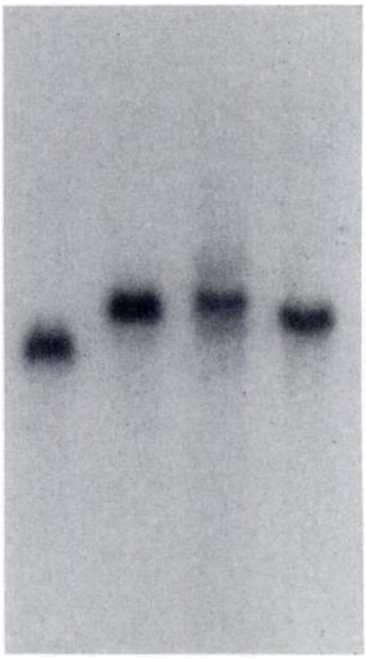

Fig. 8. Hybridization of cDNA encoding for rabbit isozyme 5 with pulmonary mRNA from rabbit, rat, guinea pig, and hamster. Samples of mRNA from lungs of an untreated rabbit (*lane 1*, 2 μg), guinea pig (*lane 2*, 5 μg), hamster (*lane 3*, 10 μg), and rat (*lane 4*, 10 μg) were analyzed as described in the legend to Fig. 3. The autoradiogram was developed after 48 hr of exposure.

IVB P-450[1] but is not present, even to the extent of 50% identity, in any other form of cytochrome P-450 for which the primary structure is available.

In addition to the structural resemblance between the forms of cytochrome P-450 IVB (isozyme 5) from rabbit and rat, we have demonstrated previously that both isozymes catalyze the *N*-hydroxylation of aromatic amines (7). In contrast to these similarities, some aspects of the expression of isozyme 5 in rabbit and rat are quite different. First, although it is evident from immunochemical determinations (7, 28) and analysis of mRNA content that the pulmonary concentration of isozyme 5 in rabbit is somewhat higher (2 to 3 times) than the hepatic concentration, this tissue difference is much greater in the rat. With the antibody to isozyme 5, we are able to detect a protein and to inhibit the *N*-hydroxylation of 2-aminofluorene in rat pulmonary microsomal preparations but not in preparations from rat liver (7). Northern analysis of samples from rats indicates that a small amount of isozyme 5 mRNA may be present in rat liver but that the level is at least 10-fold less than that found in pulmonary samples. This is consistent with the concentration of protein detected with antibodies to isozyme 5 being 5 times the limit of detection in rat pulmonary microsomes and below the limit of detection in hepatic microsomes (7). Also, assessments of protein content (7), antibody-inhibitable activity (7), and mRNA levels provide no evidence that PB has any effect on the concentration of isozyme 5 in rat liver. In contrast, treatment of rabbits with PB increases the hepatic concentration of isozyme 5 by up to 12-fold (6, 7, 17). As is the case for the induction of isozyme 2 by PB (11), the increase in isozyme 5 content is associated with increased mRNA levels.

Although the "PB-inducible" cytochrome P-450 genes in rabbit are generally thought to belong to subfamilies IIB and IIC (37), the induction of isozyme 5 by PB is not a trivial exception. In livers from rabbits treated with PB, over 10% of the total P-450 can be isozyme 5, and isozyme 5-catalyzed metabolism of aromatic amines to mutagenic products can account for 85% of the hepatic activity (6). This inductive effect in rabbits clearly represents a major species difference when compared with the lack of effect of PB on the hepatic expression of isozyme 5 in rats.

Final proof in the form of sequence data is not available, but it seems reasonable to conclude, on the basis of existing evidence, that species other than rabbit and rat also express forms of cytochrome P-450 belonging to subfamily IVB (7). Proteins recognized by antibodies to rabbit isozyme 5 are detected in lungs of guinea pigs, hamsters, mice, and monkeys, but in livers of hamsters only, results that are consistent with the finding that antibodies to isozyme 5 inhibit the pulmonary *N*-oxidation of aromatic amines in all four of these species but inhibit the hepatic activity only in the case of the hamster (7). We have now shown that the differences between guinea pig and hamster in the pulmonary and hepatic expression of isozyme 5 agree with differences in the contents of pulmonary and hepatic mRNA that hybridize with cDNA for rabbit isozyme 5. With respect to the hepatic expression of isozyme 5, the hamster represents a third variant, expression in livers from untreated animals but no response to treatment with PB.

In conclusion, the designation of a new subfamily (IVB) is

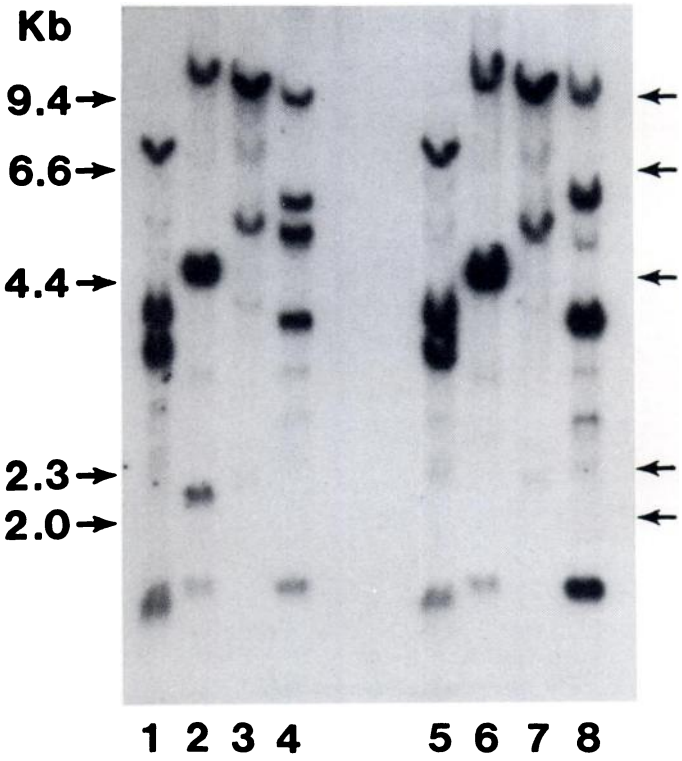

Fig. 9. Hybridization of cDNA encoding for rabbit isozyme 5 with rabbit hepatic genomic DNA. Samples (10 μg) of hepatic DNA from two rabbits (*lanes 1–4* and *lanes 5–8*) were digested for 4 hr with *NcoI* (*lanes 1* and *5*), *BstEII* (*lane 2* and *6*), *HindIII* (*lanes 3* and *7*), and *KpnI* (*lanes 4* and *8*). The digested samples were electrophoresed on 0.5% agarose gels for 16 hr at 50 V, transferred to a nylon support, and hybridized with clone Lg:1. The autoradiograms shown were developed after 3 days of exposure.

required in order to classify rabbit cytochrome P-450 isozyme 5 according to the recently proposed nomenclature (38). Direct structural evidence places rat isozyme 5 in the same subfamily, and results obtained from analysis of mRNA indicate that forms of isozyme 5 are also expressed in guinea pigs and hamsters. In addition, cytochrome P-450 related immunochemically and functionally to isozyme 5 has been detected in samples from mice and monkeys (7). Pulmonary expression of isozyme 5 and lack of induction in lung by PB have been observed with all species examined. In contrast to the uniformity of these findings, expression and induction of isozyme 5 in liver shows a great deal of species variability. In all cases, however, the amount of isozyme 5 present is consistent with the level of mRNA detected.

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