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Cloning, Tissue Distribution, and Functional Expression of Two Novel Rabbit Cytochrome P450 Isozymes, CYP2D23 and CYP2D24[1]

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We cloned two novel cytochrome P450 cDNAs (CYP2D23 and CYP2D24) from a rabbit liver cDNA library. The open-reading frames of these cDNAs encode proteins that are each composed of 500 amino acids. The amino acid sequence identity of CYP2D23 with CYP2D24 is 91.6%, and the homology of these two isozymes with other known mammalian CYPs in the CYP2D subfamily range from 64.9 to 79.8%. Using RT-PCR, we determined the distribution of these two isozymes in 9 major organs, including brain tissue sections. CYP2D23 mRNA was abundantly expressed in the liver and small intestine, but only slightly in the brain sections, whereas CYP2D24 mRNA was expressed in the liver, small intestine, and stomach. CYP2D23 and CYP2D24 were heterogeneously expressed in 293T cells. CYP2D24 effectively catalyzed the oxidation of bufuralol and bunitrolol, the archetypal substrates of the CYP2D subfamily, while CYP2D23 exhibited catalytic activity only toward bufuralol. The results of this first study on rabbit CYP2D isozymes indicate that CYP2D23 and CYP2D24 are functionally expressed in rabbits, and have different organ distributions and metabolic properties.

Key words: CYP2D, cytochrome P450, degenerate primer, drug metabolism, molecular cloning.

Cytochrome P450 (CYP) comprises a superfamily of monooxygenases (*1*) that catalyze the metabolic activation, as well as the detoxification, of a broad spectrum of structurally unrelated compounds, including drugs, chemical carcinogens, environmental pollutants, and endogenous substrates, such as steroids, fatty acids, and prostanoids (*2*). To understand drug metabolism at the molecular level in various mammalian species, several properties of CYPs need to be clarified, including substrate specificity, primary structure, distribution of gene expression, and organization of subfamily members.

The isozymes of the CYP2D subfamily play a role in the metabolism of a wide range of drugs, particularly cardiovascular agents and psychotropic agents (*3, 4*). From 1 to 10% of individuals in certain ethnic groups lack CYP2D6 activity and are referred to as poor metabolizers (*5, 6*). In recent years, polymorphism in CYP2D6 has been studied intensively as to both its effects on drug metabolism and its possible role in the susceptibility to certain diseases, such as lung cancer (*7, 8*) and Parkinson's disease (*9, 10*). Isozymes of the CYP2D subfamily of a number of mammals, including rats, dogs, and humans, have been characterized and the primary structures of the cDNAs of these proteins have been sequenced (*11-13*).

Our preliminary experiments on rabbit liver microsomal drug oxidation indicated that rabbits have catalytic activities of bufuralol 1′-hydroxylation and bunitrolol 4-hydroxylation, typical reactions of the CYP2D subfamily (*14-16*), suggesting that there may be expressed CYP2D isozymes in rabbits. Because rabbits are often used in teratogenic tests and toxicological studies on new drugs and chemicals, it is important to determine the characteristics of the drug metabolizing ability of rabbit liver, which determines the fate of drugs and toxic substances. The CYP isozymes in rabbit liver has been extensively studied (*1*). Nevertheless, the expression of the CYP2D subfamily in rabbit liver has not been previously reported.

In this study, we present the isolation and characterization of cytochrome P450s belonging to the CYP2D subfamily. We successfully cloned two novel CYP2D isozymes, CYP2D23 and CYP2D24, from the liver of a male rabbit. We determined the distribution of these isozymes in organs and also established their catalytic activities.

MATERIALS AND METHODS

Animals and RNA Isolation—The tissues were frozen in liquid nitrogen immediately after sampling and stored at −80°C until use. A fresh liver sample from a male adult

[1] The cytochrome P450 isozymes whose cDNAs are described in this article were named CYP2D23 and CYP2D24 by the P450 nomenclature committee. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequenced databases under accession Nos. AB008784 (CYP2D23) and AB008785 (CYP2D24). This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan to S.F. (No. 07558236).

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New Zealand white rabbit (3.2 kg, 16 weeks old, purchased from SLC, Shizuoka) was used for the preparation of total RNA by the single-step method (*17*) using ISOGEN (NIPPON GENE, Toyama). Poly(A)$^+$ RNA was selected with Oligotex-dT30 (Takara, Tokyo).

Oligonucleotides

Primer A: 5′-ARGCCAARGGGAAYCCYRAGA-3′ (851-871 sense)
Primer B: 5′-GCCCTGGGCRTCCAGGAAGTG-3′ (1264-1284 antisense)
Primer C: 5′-CGGWRGGGCTTCTBCCAGAC-3′ (1231-1250 antisense)
Primer D: 5′-GCACCTCAATGTCCCGCGATGTCTGG-TG-3′ (1135-1162 antisense)
Primer E: 5′-CATCGGTGCTGAAGGACGAGGCC-3′ (1208-1230 sense)
Primer F: 5′-GGCCAGCTGCAGGCTGAACACGT-3′ (209-231 antisense)
Primer G: 5′-CGTGAAGCAGGAGGCCTTCATGC-3′ (1290-1312 sense)
Primer H: 5′-GCCATGGGGCTCCTGTCGGGAGA-3′ (−3-20 sense)
Primer I: 5′-CTAGCGGGCCACAGCACACAGCT-3′ (1481-1503 antisense)
Primer J: 5′-ACTTATGGCGAGGACAC-3′ (286-302 sense)
Primer K: 5′-ATGGGGATCACGTTCAG-3′ (679-695 antisense)
Primer L: 5′-ACCACGGTGCACGCCATCAC-3′ (594-613 G3PDH sense)
Primer M: 5′-TCCACCACCCTGTTGCTGTA-3′ (1026-1045 G3PDH antisense)

The nucleotide positions are those in Fig. 1. Degenerate primers were designed based on conserved regions of 10 mammalian CYP2D sequences (Primers A, B, and C).

cDNA Amplification and Isolation—cDNA samples were obtained using 200 ng of mRNA as the template for reverse transcription with SuperScript II reverse transcriptase and the oligo (dT) 12-18 primer (GIBCO BRL, Gaithersburg, MD). A cDNA fragment was amplified using degenerate primers targeted to nucleotide sequence 851-1284 (Primers A and B), in a reaction mixture comprising: 1.0 μl of cDNA, 0.5 μM of each primer, 2.5 units of *Taq* polymerase (Promega, Madison, WI), 30 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 200 μM dNTPs. The reaction conditions were denaturation for 1 min at 94°C, annealing for 1 min at 58°C, and extention for 1 min at 72°C, for 30 cycles. The polymerase chain reaction (PCR) product was amplified for use as a template for nested PCR using degenerate primers targeted to nucleotide sequence 851-1250 (Primers A and C). A single product of 400 base pairs (bp) was detected following analytical gel electrophoresis, and this was cloned into the pCR2.1 vector (Invitrogen, San Diego, CA) and sequenced.

To clone full-length novel CYP2D cDNAs, a Marathon cDNA amplification kit (Clontech, Palo Alto, CA) was used according to the manufacturer's instructions. Briefly, the 5′- and 3′-cDNA ends were amplified by PCR using a possible rabbit CYP2D cDNA sequence specific primer and the Marathon cDNA adapter sequence specific primer, AP1. Each rapid amplification of cDNA ends (RACE) was carried out twice (first RACE, Primer D or E; second RACE, Primer F or G) until fragments containing both start and stop codons were obtained (*18*). After determination of the 5′- and 3′-sequences of the RACE products, full-length cDNAs were amplified by PCR using a 5′- and 3′-cDNA terminal-specific primer pair (Primers H and I). The RACE reactions carried out comprised 25 cycles of denaturation for 30 s at 94°C, annealing and extension for 2 min at 68°C. The KlenTaq Polymerase used was purchased from Clontech.

Sequencing—The nucleotide sequence was determined with an ABI PRISM Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA) and an automated DNA sequencer (ABI PRISM 310 Genetic Analyzer). Four independent clones each for 5′-RACE, 3′-RACE, and six independent clones for each full-length cDNA were sequenced to check for PCR errors in the RACE and PCR products. Both sense and antisense fragments were sequenced for each clone to ascertain the matching of these sequences.

RT-PCR Analyses—To investigate the existence of CYP2D23 and CYP2D24 mRNAs in various tissues, reverse transcriptase (RT)-PCR was carried out, the first strand cDNA templates being transcribed with an oligo dT primer derived from total RNAs from various rabbit tissues. The cDNA was amplified using primer J as the sense primer and primer K as the antisense primer. The amplified fragments were digested with *Bss*HII to separate CYP2D23 (409 bp) and CYP2D24 (321 bp). A fragment (452 bp) of rabbit G3PDH (glyceraldehyde-3-phosphate dehydrogenase) was amplified using primer L as the sense primer and primer M as the antisense primer (*19*). The authenticity of these PCR products was confirmed by subcloning them into a plasmid pCR2.1 vector, followed by cycle sequencing.

Heterogeneous Expression of CYP2D23 and CYP2D24, and Drug-Metabolizing Activities—The cDNAs (2 μg) were transfected into 293T cells (human embryonic kidney cells) in 35 mm culture dishes using expression vector pCAGGS (*20*) with lipofectamine reagent (GIBCO BRL, Gaithersburg, MD). The cells were maintained for 18 h in Dulbecco's Modified Eagle's medium (high glucose) supplemented with 10% fetal bovine serum at 37°C in 5% CO_2 in air. 293T cells transfected after 48 h with pCAGGS were used for the assaying of drug metabolizing activities. To start the reactions, the medium was changed to another, containing 200 μM bufuralol or 500 μM bunitrolol, but no fetal bovine serum. After 1-h (bufuralol) or 3-h (bunitrolol) incubation, the supernatant was recovered. The metabolites were extracted and subjected to high-performance liquid chromatography essentially according to the published method (*21-23*).

Other Methods—Protein concentrations were determined by the method of Lowry *et al.* (*24*). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (*25*). Proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane and then immunostained with diaminobenzidine as a substrate. Anti-CYP2D6 peptide antibodies were prepared as previously described (*23, 26*). Rat CYP2D2 protein was generated with a baculovirus expression system using cDNA generously supplied by Dr. Gonzalez (*27*) as previously described (*16*).

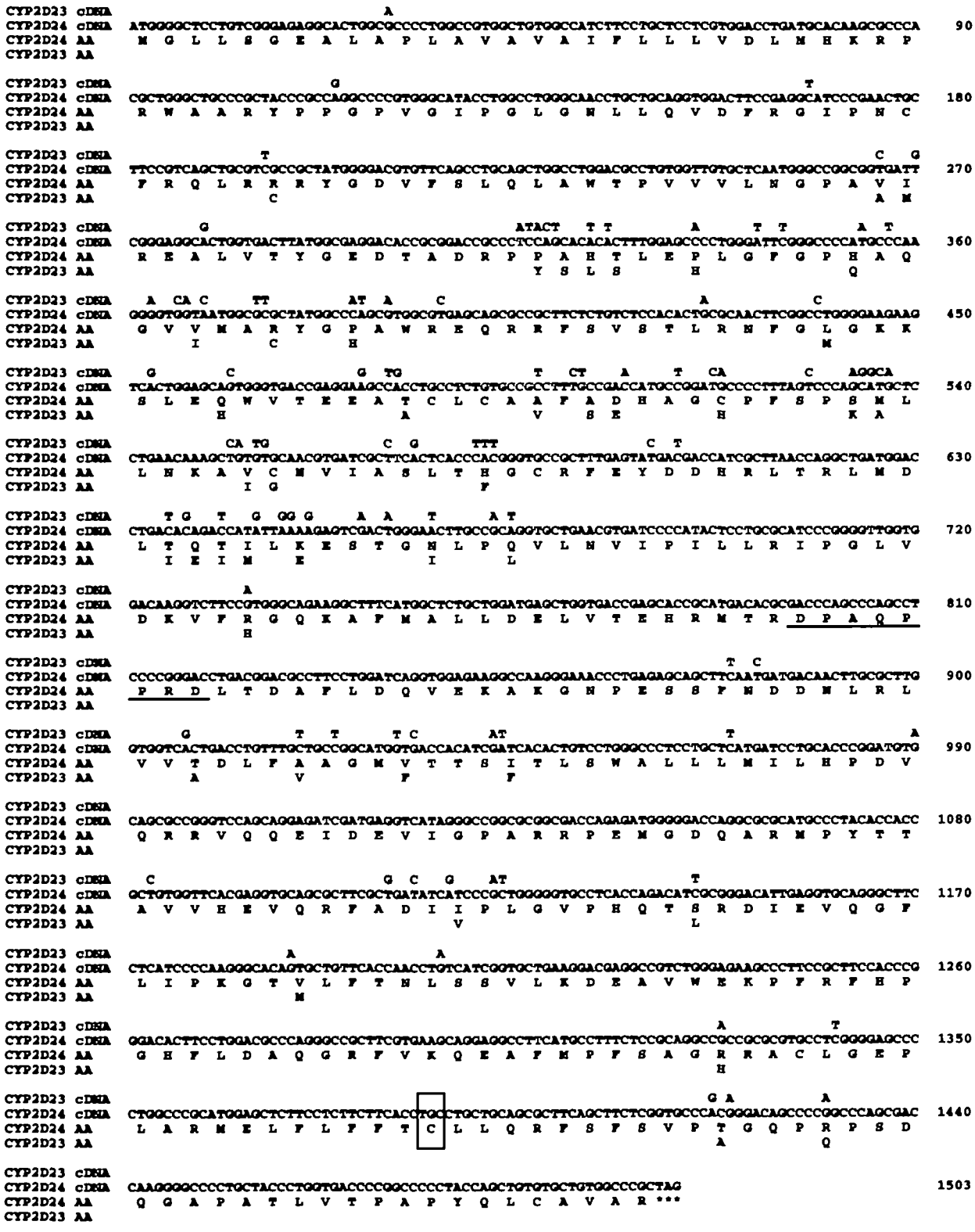

Fig. 1. **Comparison of the nucleotide and amino acid (AA) sequences of CYP2D23 and CYP2D24.** The sequences of CYP2D23 that are identical to those of CYP2D24 are not shown. The numbers on the right are the nucleotide numbers. The heme-binding cysteine is enclosed in a box. The termination codon is indicated by asterisks. The underline shows the LKM-1 region, which is the target of the anti-CYP2D6 peptide antibody (*16, 26*). This region is conserved within several CYP2D isozymes.

RESULTS AND DISCUSSION

The CYP2D subfamily in mammals has received considerable attention over the past ten years because of its clinical significance in the metabolism of a number of therapeutic drugs (*3, 4*). Although the rabbit is one of the most commonly used experimental animal species, the expression of the CYP2D subfamily has not been reported.

We isolated two full-length cDNAs from a rabbit liver cDNA library, each coding for a novel CYP2D, using the 5′-,3′-RACE method with gene specific-primers derived from the products of degenerate PCR, based on 10 mammalian CYP2D isozymes. Using these methods, we attempted to isolate several forms closely related to CYP2D isozymes. Several clones were obtained and sequenced. The form that appeared most often among the number of clones obtained was designated as CYP2D23, and the other as CYP2D24. Six independent clones for each of the full-length cDNAs were sequenced to check for PCR errors in the PCR products. In addition, we isolated a possible variant form of CYP2D24, which has only one amino acid substitution. Further studies on this variant form are in progress.

The nucleotide and amino acid sequences of CYP2D23 and CYP2D24 are shown in Fig. 1. The open-reading frames of CYP2D23 and CYP2D24 encode proteins that are each composed of 500 amino acids, which contain the highly conserved heme binding cysteine at position 446 and the conserved region in all the CYP family around the invariant cysteine residue. The overall amino acid sequence of CYP2D23 shows 64.9-77.5% identity with those of other known mammalian CYPs in the CYP2D subfamily. On the other hand, the overall amino acid sequence of CYP2D24 shows 65.3-79.8% identity with those of other known mammalian CYPs in the CYP2D subfamily (Table I). These results suggest that CYP2D23 and CYP2D24, novel members of the CYP2D subfamily, are the products of a multigene. It was reported that CYP2D isozymes in humans (*13*), rats (*11*), and mice (*28*) are members of a multigene family.

CYP2D23 mRNA was detected in the liver, lung, small intestine, stomach, and 6 out of 8 brain sections with remarkably high expression in the liver and small intestine (Fig. 2). The expression of CYP2D24 mRNA was lower in the liver and small intestine in comparison with CYP2D23. It should be noted that the stomach was the only organ in which the level of expression of CYP2D24 was higher than that of CYP2D23.

Immunoblotting analysis using anti-CYP2D6 peptide antibodies (*26*) specific to human 2D6 proteins was performed on a lysate of 293T cells transfected with expression vector pCAGGS containing CYP2D23 or CYP2D24

TABLE I. **Amino acid identities of the CYP2D family.** The coding regions of the CYP2D family were compared with that of CYP2D23 or CYP2D24. The results are expressed as the percentage of identity with CYP2D23 or CYP2D24. Family-subfamily names are as described by Nelson *et al.* (*1*).

Family/subfamily	Species	Amino acid identity (%)	
		CYP2D23	CYP2D24
CYP2D1	Rat	69.0	69.2
CYP2D2	Rat	68.6	69.6
CYP2D3	Rat	68.6	70.4
CYP2D4	Rat	73.2	75.6
CYP2D5	Rat	68.2	68.6
CYP2D6	Human	75.7	78.3
Cyp2d9	Mouse	67.8	68.2
Cyp2d10	Mouse	66.6	67.8
Cyp2d11	Mouse	64.9	65.3
CYP2D14	Cow	75.0	76.4
CYP2D15	Dog	70.6	72.4
CYP2D16	Guinea pig	71.6	72.2
CYP2D17	Monkey	77.5	79.8
CYP2D18	Rat	72.8	74.8
CYP2D19	Marmoset	76.5	79.7
CYP2D23	Rabbit	100	91.6
CYP2D24	Rabbit	91.6	100

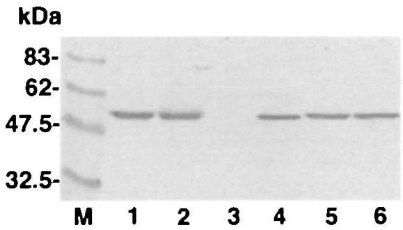

Fig. 3. **Immunoblotting analysis of 293T cell lysates transfected with expression vector pCAGGS containing CYP2D23 or CYP2D24 cDNA.** Cell lysates from cells transfected with pCAGGS containing CYP2D23 or CYP2D24 and liver microsomes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. The membrane was treated with anti-CYP2D6 peptide antibodies. Each lane contained 10 μg of protein: lane 1, rat liver microsomes; lane 2, rabbit liver microsomes; lane 3, lysate of cells transfected with the vector only; lane 4, CYP2D23; lane 5, CYP2D24; lane 6, rat CYP2D2. Lane M, marker proteins.

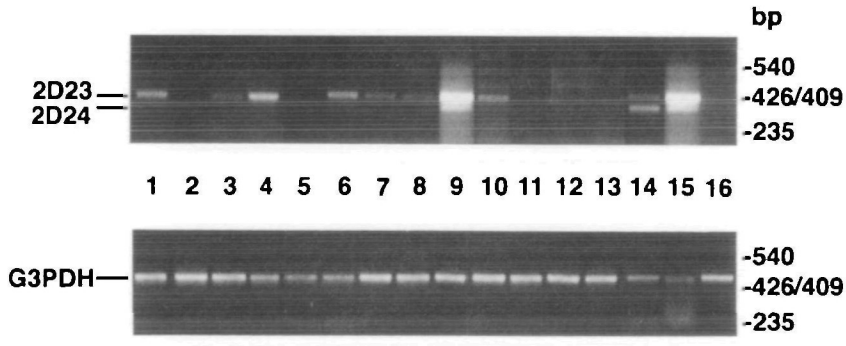

Fig. 2. **RT-PCR analyses of CYP2D23 and CYP2D24 mRNAs from various rabbit tissues.** Total RNAs from rabbit tissues were transcribed, PCR amplified, digested with *Bss*HII, and then separated by electrophoresis. The products from CYP2D23 mRNA (409 bp) and CYP2D24 mRNA (321 bp) are shown on the left. The tissues analyzed were: 1, hippocampus; 2, cerebellum; 3, cerebral cortex; 4, olfactory bulb; 5, pituitary grand; 6, medulla oblongata; 7, hypothalamus; 8, corpus striatum; 9, liver; 10, lung; 11, heart; 12, kidney; 13, adrenal grand; 14, stomach; 15, small intestine; and 16, testis. The base pair lengths of the pUC18/*Dde*I markers are indicated on the right.

TABLE II. **Bufuralol 1′-hydroxylation and bunitrolol 4-hydroxylation by rabbit CYP2D23 and CYP2D24 expressed in 293T cells.**

	2D23	2D24
Bufuralol 1′-hydroxylation	80.9±2.2	272±34
Bunitrolol 4-hydroxylation	N.D.	44.5±24

The results are means±SD (three independent determinations). Each value represents pmol/h·mg protein. N.D., not detectable (values lower than 1.0 pmol/h·mg protein).

cDNA. The peptide sequence (Fig. 1) occurs in CYP2D23 and CYP2D24 as well as human CYP2D6 and expressed rat CYP2D2 (*16*). Cells transfected with CYP2D23 or CYP2D24 contained an immunoreactive protein exhibiting similar mobility to CYP2D23 and CYP2D24 expressed in rabbit liver (Fig. 3). No CYP2D protein was detected in cells transfected with pCAGGS only.

Table II shows the bufuralol and bunitrolol metabolizing activities of heterogeneously expressed CYP2D23 and CYP2D24. CYP2D24 effectively catalyzed the oxidation of bufuralol and bunitrolol, the archetypal substrates of the CYP2D subfamily, while CYP2D23 exhibited catalytic activity only toward bufuralol. Although CYP2D24 was expressed at very low levels in the liver and small intestine, the activity of CYP2D24 was higher than that of CYP2D23 as to both bufuralol 1′-hydroxylation and bunitrolol 4-hydroxylation. CYP2D23 mRNA was detected in 6 out of 8 brain sections. This suggests that CYP2D23 should play some important roles in the metabolism of endogenous substrates. However, its catalytic activity as to testosterone metabolism is quite low (values lower than 50 pmol/h·mg protein). Further studies on its endogenous role are in progress.

Some species such as rats and mice have several CYP2D isozymes which differ in function and the organ distribution patterns (*11, 28*). Among rat CYP2Ds, CYP2D1 and CYP2D2 exhibit debrisoquine 4-hydroxylase activity, but only CYP2D2 exhibits bunitrolol 4-hydroxylase activity (*16*). CYP2D1 and CYP2D2 are distributed mainly in the liver, *i.e.* not in the brain. On the other hand, rat CYP2D4 and CYP2D18 are distributed mainly in the brain (*29, 30*). These findings demonstrate a significant aspect of drug metabolism. It is necessary to investigate the organization of subfamily members and their substrate specificity as well as their organ distributions to fully understand the metabolism of xenobiotics in the body.

In summary, we identified two novel rabbit CYP2Ds, CYP2D23 and CYP2D24, derived from rabbit liver cDNAs. We determined their amino acid sequences from the primary sequences of their cDNAs (Fig. 1). The amino acid sequence homologies of CYP2D23 and CYP2D24 with human CYP2D6 are 75.7 and 78.3%, respectively. CYP2D23 is expressed in several organs including the brain. Although the expression of CYP2D24 in the liver, small intestine, and stomach is not high, CYP2D24 shows high drug metabolizing activity. These data may provide some insight for the studies on drug metabolism and/or teratogenicity in which rabbits are used as experimental animals.

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REFERENCES

1. Nelson, D.R., Koymans, L., Kamataki, T., Stegeman, J.J., Feyereisen, R., Waxman, D.J., Waterman, M.R., Gotoh, O., Coon, M.J., Estabrook, R.W., Gunsalus, I.C., and Nebert, D.W. (1996) P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* **6**, 1-42
2. Guengerich, F.P. (1991) Reactions and significance of cytochrome P-450 enzymes. *J. Biol. Chem.* **266**, 10019-10022
3. Cholerton, S., Daly, A.K., and Idle, J.R. (1992) The role of individual human cytochromes P450 in drug metabolism and clinical response. *Trends Pharmacol. Sci.* **13**, 434-439
4. Gonzalez, F.J. (1996) The CYP2D subfamily in *Cytochromes P450: Metabolic and Toxicological Aspects* (Ioannides, C., ed.) pp. 183-210, CRC Press, Boca Raton, FL
5. Kalow, W. (1991) Interethnic variation of drug metabolism. *Trends Pharmacol. Sci.* **12**, 102-107
6. Daly, A.K., Brockmoller, J., Broly, F., Eichelbaum, M., Evans, W.E., Gonzalez, F.J., Huang, J.D., Idle, J.R., Ingelman-Sundberg, M., Ishizaki, T., Jacqz-Aigrain, E., Meyer, U.A., Nebert, D.W., Steen, V.M., Wolf, C.R., and Zanger, U.M. (1996) Nomenclature for human CYP2D6 alleles. *Pharmacogenetics* **6**, 193-201
7. Ayesh, R., Idle, J.R., Ritchie, J.C., Crothers, M.J., and Hetzel, M.R. (1984) Metabolic oxidation phenotypes as markers for susceptibility to lung cancer. *Nature* **312**, 169-170
8. Caporaso, N., Landi, M.T., and Vineis, P. (1991) Relevance of metabolic polymorphisms to human carcinogenesis: evaluation of epidemiologic evidence. *Pharmacogenetics* **1**, 4-19
9. Barbeau, A., Cloutier, T., Roy, M., Plasse, L., Paris, S., and Poirier, J. (1985) Ecogenetics of Parkinson's disease: 4-hydroxylation of debrisoquine. *Lancet* **2**, 1213-1216
10. Smith, C.A., Gough, A.C., Leigh, P.N., Summers, B.A., Harding, A.E., Maraganore, D.M., Sturman, S.G., Schapira, A.H., Williams, A.C., Spurr, N.K., and Wolf, C.R. (1992) Debrisoquine hydroxylase gene polymorphism and susceptibility to Parkinson's disease. *Lancet* **339**, 1375-1377
11. Matsunaga, E., Zanger, U.M., Hardwick, J.P., Gelboin, H.V., Meyer, U.A., and Gonzalez, F.J. (1989) The CYP2D gene subfamily: analysis of the molecular basis of the debrisoquine 4-hydroxylase deficiency in DA rats. *Biochemistry* **28**, 7349-7355
12. Sakamoto, K., Kirita, S., Baba, T., Nakamura, Y., Yamazoe, Y., Kato, R., Takanaka, A., and Matsubara, T. (1995) A new cytochrome P450 form belonging to the CYP2D in dog liver microsomes: purification, cDNA cloning, and enzyme characterization. *Arch. Biochem. Biophys.* **319**, 372-382
13. Gonzalez, F.J., Skoda, R.C., Kimura, S., Umeno, M., Zanger, U.M., Nebert, D.W., Gelboin, H.V., Hardwick, J.P., and Meyer, U.A. (1988) Characterization of the common genetic defect in humans deficient in debrisoquine metabolism. *Nature* **331**, 442-446
14. Gut, J., Catin, T., Dayer, P., Kronbach, T., Zanger, U., and Meyer, U.A. (1986) Debrisoquine/sparteine-type polymorphism of drug oxidation: purification and characterization of two functionally different human liver cytochrome P-450 isozymes involved in impaired hydroxylation of the prototype substrate bufuralol. *J. Biol. Chem.* **261**, 11734-11743
15. Suzuki, T., Narimatsu, S., Fujita, S., Masubuchi, Y., Umeda, S., Imaoka, S., and Funae, Y. (1992) Purification and characterization of a cytochrome P-450 isozyme catalyzing bunitrolol 4-hydroxylation in liver microsomes of male rats. *Drug Metab. Dispos.* **20**, 367-373
16. Yamamoto, Y., Tasaki, T., Nakamura, A., Iwata, H., Kazusaka, A., Gonzalez, F.J., and Fujita, S. (1998) Molecular basis of the Dark Agouti rat drug oxidation polymorphism: importance of CYP2D1 and CYP2D2. *Pharmacogenetics* **8**, 73-82
17. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159
18. Frohman, M.A., Dush, M.K., and Martin, G.R. (1988) Rapid

production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA* **85**, 8998-9002
19. Applequist, S.E., Keyna, U., Calvin, M.R., Beck-Engeser, G.B., Raman, C., and Jack, H.M. (1995) Sequence of the rabbit glyceraldehyde-3-phosphate dehydrogenase-encoding cDNA. *Gene* **163**, 325-326
20. Niwa, H., Yamamura, K., and Miyazaki, J. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193-199
21. Kronbach, T., Mathys, D., Gut, J., Catin, T., and Meyer, U.A. (1987) High-performance liquid chromatographic assays for bufuralol 1′-hydroxylase, debrisoquine 4-hydroxylase, and dextromethorphan O-demethylase in microsomes and purified cytochrome P-450 isozymes of human liver. *Anal. Biochem.* **162**, 24-32
22. Ono, S., Tsutsui, M., Gonzalez, F.J., Satoh, T., Masubuchi, Y., Horie, T., Suzuki, T., and Narimatsu, S. (1995) Oxidative metabolism of bunitrolol by complementary DNA-expressed human cytochrome P450 isozymes in a human hepatoma cell line (Hep G2) using recombinant vaccinia virus. *Pharmacogenetics* **5**, 97-102
23. Yamamoto, Y., Nakamura, A., Tasaki, T., Umeda, S., Sugimoto, C., Imaoka, S., Funae, Y., Masuda, M., Kazusaka, A., and Fujita, S. (1996) Impaired expression in DA rats of catalytically active P450BTL, immunochemically distinguishable from P4502D1: immunological evidence with specific anti-peptide antibodies. *Biomed. Res.* **17**, 331-337
24. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265-275
25. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
26. Nakamura, A., Yamamoto, Y., Tasaki, T., Sugimoto, C., Masuda, M., Kazusaka, A., and Fujita, S. (1995) Anti-peptide antibodies to the P4502D subfamily in rat, dog and man. *Xenobiotica* **25**, 1103-1109
27. Gonzalez, F.J., Matsunaga, T., Nagata, K., Meyer, U.A., Nebert, D.W., Pastewka, J., Kozak, C.A., Gillette, J., Gelboin, H.V., and Hardwick, J.P. (1987) Debrisoquine 4-hydroxylase: characterization of a new P450 gene subfamily, regulation, chromosomal mapping, and molecular analysis of the DA rat polymorphism. *DNA* **6**, 149-161
28. Wong, G., Itakura, T., Kawajiri, K., Skow, L., and Negishi, M. (1989) Gene family of male-specific testosterone 16 alpha-hydroxylase (C-P-450(16 alpha)) in mice. Organization, differential regulation, and chromosome localization. *J. Biol. Chem.* **264**, 2920-2927
29. Wyss, A., Gustafsson, J.A., and Warner, M. (1995) Cytochromes P450 of the 2D subfamily in rat brain. *Mol. Pharmacol.* **47**, 1148-1155
30. Kawashima, H., Sequeira, D.J., Nelson, D.R., and Strobel, H.W. (1996) Genomic cloning and protein expression of a novel rat brain cytochrome P-450 CYP2D18 catalyzing imipramine N-demethylation. *J. Biol. Chem.* **271**, 28176-28180