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Structure, Evolution, and Liver-Specific Expression of Sterol 12α-Hydroxylase P450 (CYP8B)[1]

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The rat CYP8B cDNA encoding sterol 12α-hydroxylase was cloned and sequenced. The amino acid sequence of the heme-binding region of CYP8B was close to those of CYP7A (cholesterol 7α-hydroxylase) and CYP7B (oxysterol 7α-hydroxylase). Molecular phylogenetic analysis suggests that CYP8B and the CYP7 family derive from a common ancestor. The P450s of the *CYP7* and *CYP8* families, except for *CYP8A* (prostacyclin synthase), catalyze the oxygenation of sterols from an alpha surface in the middle of the steroid skeleton. These facts suggest that *CYP8B* is a P450 closely linked to those of the *CYP7* family. CYP8B was expressed specifically in liver. Hepatic CYP8B mRNA level and the 12α-hydroxylase activity were altered by cholestyramine feeding, starvation, streptozotocin-induced diabetes mellitus, and administration of clofibrate, dexamethasone or thyroxin, indicating the pretranslational regulation of *CYP8B* expression. The enhanced CYP8B mRNA expression in streptozotocin-induced diabetic rats was significantly decreased by insulin within 3 h of its administration. These facts demonstrate a regulatory role of insulin in *CYP8B* expression as a suppressor.

Key words: bile acid, cytochrome P450, diabetes mellitus, molecular evolution, sterol 12-hydroxylase.

Sterol 12α-hydroxylase (CYP8B) is an important P450 enzyme catalyzing the monooxygenation at the 12α-position of 7α-hydroxy-4-cholesten-3-one (HCO) in the conversion of cholesterol to cholic acid, a major constituent of the bile acids of most mammals (Scheme 1). This enzyme is considered to be responsible for the regulation of the cholic acid/chenodeoxycholic acid ratio in the bile (*2*). This ratio seems to be of importance for gallstone formation, because there is less stone formation when the cholic acid/chenodeoxycholic acid ratio is low. Moreover, the suppression of this enzyme activity with competitive inhibitors has been proposed to increase the endogenous formation of chenodeoxycholic acid to dissolve gallstones (*3*).

The amino acid sequence of rabbit CYP8B has been deduced from the cDNA sequence (*4*). The classification name of this P450 as CYP8B[3] rests on the fact that its amino acid sequence showed 39% similarity to that of human prostacyclin synthase (CYP8A). However, CYP8B, sterol 12α-hydroxylase, is considered to be related functionally to the P450s of the *CYP7* family, such as cholesterol 7α-hydroxylase (CYP7A) and oxysterol 7α-hydroxylase (CYP7B). Therefore, the evolutionary relationship between CYP8B and CYP7 is of interest in relation to the structural and functional diversification of P450 enzymes.

As mentioned above, CYP8B is the key enzyme for cholic acid formation, and the regulation of the ratio of cholic acid to chenodeoxycholic acid may be physiologically important. Therefore, the mechanisms by which the activity of this enzyme is regulated are worth studying. A homogenous preparation of CYP8B was obtained from rabbit liver, and its enzymatic properties were studied (*5*). Preliminary examination also showed that the sterol 12α-hydroxylase activity of rat liver was increased several-fold by cholestyramine feeding, starvation, or markedly by treatment with streptozotocin (*5*). However, information on the regulatory mechanism of the level of hepatic CYP8B, especially the pretranslational events, is still limited. Most bile acids are produced in liver. However, chenodeoxycholic acid might also be produced in extra-hepatic tissues, because CYP7B and CYP27, both of which participate in the alternative pathway for chenodeoxycholic acid synthesis, are found in

[1] The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number of AB009686.

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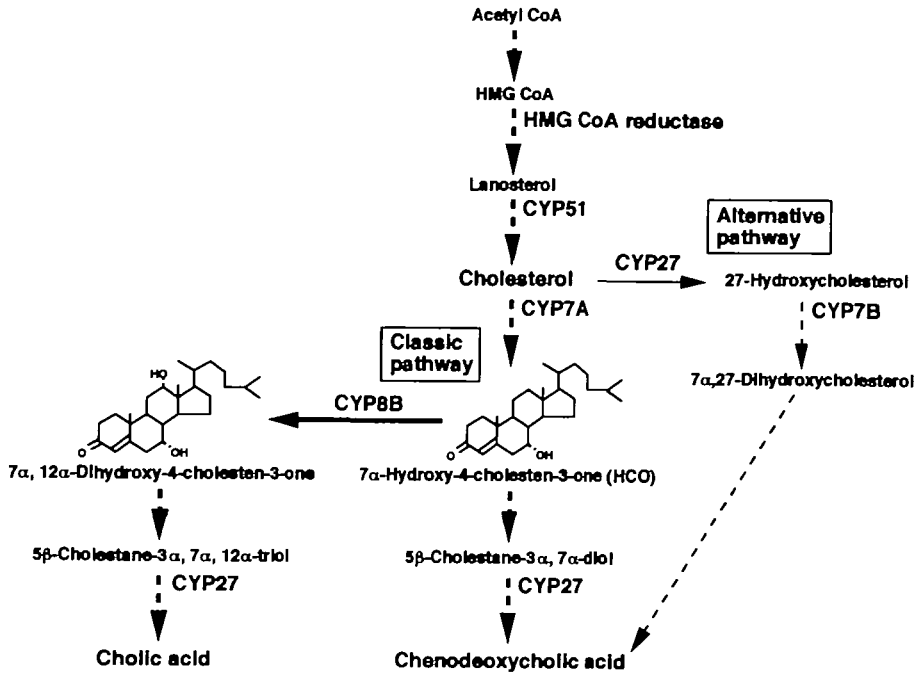
[3] According to the recommendations of the P450 nomenclature committee (Ref. *1* and URL: http://drnelson.ucmem.edu/nelson homepage.html), each member of the P450 monooxygenase superfamily is defined by the "CYP" code: sterol 12α-hydroxylase as CYP8B, prostacyclin synthase as CYP8A, cholesterol 7α-hydroxylase as CYP7A, oxysterol 7α-hydroxylase as CYP7B, lanosterol 14-demethylase as CYP51, and sterol 27-hydroxylase as CYP27.

Abbreviations: HCO, 7α-hydroxy-4-cholesten-3-one; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; bp, base pairs; kbp, kilobase pairs; RT-PCR, reverse transcription-polymerase chain reaction; RACE, rapid amplification of cDNA ends; HMG CoA red, hydroxymethyl glutaryl coenzyme A reductase.



Scheme 1

several extra-hepatic tissues (*6-10*). The question arises, therefore, whether cholic acid can be formed in tissues other than liver. This question can be answered by analysis of the tissue specificity of CYP8B expression, because CYP8B is an essential enzyme for cholic acid formation. To approach this question, we cloned and sequenced rat CYP8B cDNA and analyzed evolutionary relationship between *CYP8B* and the *CYP7* family. Using CYP8B cDNA, we also examined the tissue distribution of CYP8B mRNA and the effects of various treatments on the expression levels of CYP8B mRNA in rat liver by means of Northern blotting and RT-PCR.

MATERIALS AND METHODS

Materials—Alpha-[^{32}P]dCTP (111 TBq/mmol) was obtained from Du Pont-New England Nuclear. The Oligo-labelling kit and oligo (dT)-cellulose were products of Pharmacia Biotechnology (Uppsala, Sweden).

Cloning of Full-Length cDNA for Rat Sterol 12α-Hydroxylase and Nucleotide Sequencing—cDNA was synthesized from 1 μg of poly(A)$^+$ RNA of rat liver using the Marathon cDNA amplification kit (Clontech Laboratories, Palo Alto, CA) according to the instruction manual. Double-stranded cDNAs were ligated to the 44-mer adapter containing two overlapping primer sites for AP1 (5′-CCATCCTAATACGACTCACTATAGGGC-3′) and AP2 (5′-ACTCACTATAGGGCTCGAGCGGC-3′) supplied by the manufacturer. The polymerase chain reaction (PCR) was performed with the cDNA using the forward primer P1 (5′-GGCTACCTGAGTTTGTTCGG-3′) and the reverse primer P4 (5′-AAGAACCTCCCCGGGCAGAT-3′), which were synthesized based on the sequence of rabbit sterol 12α-hydroxylase cDNA (*4*). The 794-bp fragment amplified by PCR was cloned into the T-tailed vector pGEM-T Easy (Promega, Madison, WI), and the nucleotide sequence was determined. Full-length cDNA for rat sterol 12α-hydroxylase was isolated by 5′- and 3′-rapid amplification of cDNA ends (RACE) using the cDNA, a series of PCR primers synthesized based on the first isolated 794-bp PCR product, and the counter primers AP1 and AP2. The final specific primers used were P5 (5′-TTCCCCAGGTTTGTCTACTC-3′) and P8 (5′-TGGGAGGCCCAGAGCATCAT-3′). PCR was carried out under the following conditions: denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, and primer extension at 65°C for 3 min in a total of 30 cycles. The RACE products were subjected to Southern hybridization with the 794-bp PCR product as a probe. The hybridizable DNA fragments separated on the agarose gel were subcloned into pGEM-T Easy and subjected to nucleotide sequencing. DNA sequencing was performed by the modified method of Sanger *et al.* (*11*) using an ABI Prism 310 Autosequencer (Perkin-Elmer, Norwalk, CT).

Rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA was generated by RT-PCR using a forward primer (5′-GTCAAGGCTGAGAATGGGAA-3′) and a reverse primer (5′-GCTTCACCACCTTCTTGATG-3′) synthesized based on the sequence of rat GAPDH (EMBL/GenBank/DDBJ data base Accession No. M17701). These PCR products were subcloned into the pGEM T-vector (Promega, Madison, WI) and identified by sequencing. Rat cDNA probes for CYP7A (*12*) was prepared as described.

Construction of a Phylogenetic Tree—CYP7A, 7B, 8A, 8B, and 51 protein sequences were multiply aligned by the doubly nested randomized iterative refinement (DNR) method (*13*). For phylogenetic analysis, the columns containing 10% or more blank characters (deletions) were removed. Thus, the N-terminal *ca.* 60 sites containing the membrane anchor sequence specific to eukaryotic microsomal P450s were omitted from the present analysis. The resulting alignment consisting of 25 sequences and 429 columns was analyzed by the neighbor-joining (NJ) (*14*) method using the NEIGHBOR program distributed in PHYLIP-3.5c (*15*). The bootstrap test was performed with

100 repetitions of resampling and otherwise the default settings.

Northern Blot Analysis—Poly(A)$^+$ RNA preparations (1-2 μg/lane) were electrophoresed on 1% agarose gels containing 2.2 M formaldehyde as described by Thomas (*16*) and transferred to Nytran membranes (Schleicher & Schuell GmbH, Dassel). The membranes were hybridized with the ^{32}P-labeled probes of the 794-bp rat 12α-hydroxylase cDNA or 613-bp GAPDH cDNA in hybridization solution containing 6×SSC, 5×Denhardt's, 10 mM EDTA, 1% SDS, and 0.5 mg/ml sonicated salmon sperm DNA at 68°C. The membranes were washed with 0.1×SSC containing 0.5% SDS at 50°C and exposed to Kodak BMX films at −80°C. The radioactivities of the hybridized areas were quantified using a Bio-imaging Analyzer System BAS2000 (Fuji Photo Film, Tokyo).

RT-PCR for Tissue Distribution Study—The first-strand cDNA was synthesized from 50 ng of poly(A)$^+$ RNA prepared from various tissues using the Superscript pre-amplification system (Life Technologies, Rockville, MD). PCR for CYP8B was carried out under the conditions described above using the pair of primers P3 (5′-ATGATG-CTCTGGGCCTCCCA-3′) and P10 (5′-TGAGATGTCTAG-GAGGCAAG-3′). The PCR products were electrophoresed and subjected to hybridization with the ^{32}P-labeled CYP8B cDNA probe to detect the specific product.

Treatments of Rats and Preparation of Poly(A)$^+$ RNA and Liver Microsomes—Two-month-old Wistar rats were maintained under natural lighting conditions and fed *ad libitum* normal laboratory rat chow. For cholestyramine treatment, rats were fed the chow containing 3% (w/w) cholestyramine for 1 week. Other treatments were essentially the same as described previously (*5*). Clofibrate (400 mg/kg body weight) and thyroxin (150 μg/kg body weight) were administered daily for 4 days by intraperitoneal injection. Dexamethasone (100 mg/kg body weight) was administered once by intraperitoneal injection. In these treatments, rats were sacrificed one day after the last injection. Streptozotocin (65 mg/kg body weight) was administered once by intraperitoneal injection and rats were sacrificed 6 weeks after the injection. In the experiment in Fig. 6, the streptozotocin-induced diabetic rats were treated once with bovine insulin (8 units/kg body weight) administered subcutaneously. Since CYP7A is known to exhibit a marked circadian rhythm, all animals were sacrificed within 30 min at 10:00 to avoid experimental deviation due to the time of sample preparation. Livers from three to five rats for each treatment and untreated control were pooled and minced. The minced livers were divided into two parts for preparing poly(A)$^+$ RNA and microsomes. RNA was extracted from the minced liver by the guanidine HCl method (*17*), and poly(A)$^+$ RNA was enriched by oligo (dT)-cellulose column chromatography (*18*). Microsomes were prepared from the remaining portion of the minced liver by the previous method (*19*) and suspended in 50 mM potassium phosphate buffer (pH 7.5) containing 0.25 M sucrose, 1 mM EDTA, and 1 μg/ml leupeptin. Poly(A)$^+$ RNA of sixteen tissues used in the experiment shown in Fig. 4 were prepared by the above method.

Enzyme Assay—Microsomal HCO 12α-hydroxylase (CYP8B) and cholesterol 7α-hydroxylase (CYP7A) activities were assayed by the previous methods (*20*).

RESULTS AND DISCUSSION

Structural and Evolutionary Characterization of Rat CYP8B—Full-length cDNA for rat CYP8B was isolated by RT-PCR and 5′- and 3′-RACE as described in "MATERIALS AND METHODS." The entire nucleotide sequence of rat CYP8B cDNA was determined by sequencing of at least three independent clones for the 900-bp 5′- and the 1.3-kilobase 3′-RACE products. The resultant nucleotide sequence (DDBJ/EMBL/GenBank: AB009686) of rat CYP-8B cDNA contained a 1,497-bp open reading frame encoding a protein consisting of 499 amino acids (M_r = 57.5 kDa). The total length of 1,965 bases excluding a poly(A) tract agrees well with the mRNA size (2.2 kb) determined by Northern blot analysis (Figs. 4A and 5A).

Figure 1 shows the amino acid sequence of rat CYP8A deduced from the nucleotide sequence of the cDNA. This sequence was typical one of P450, and the heme binding region (CR-6 of Fig. 1) containing a cysteine serving of the characteristic axial ligand of P450 heme iron (arrowhead of Fig. 1) (*21*) and the putative substrate-recognition sites (SRS-0 through SRS-6 of Fig. 1) (*22*) were identified. As described by Yoshida *et al.* (*23, 24*), CYP8B is involved in an evolutionary cluster consisting of CYPs 7, 8, and 51. Functionally, CYP8B, which is a sterol 12α-hydroxylase, is closer to CYP7A (cholesterol 7α-hydroxylase) and CYP7B (oxysterol 7α-hydroxylase) than to another member of the *CYP8* family, CYP8A, which is a functionally unique P450 participating in the final step of prostacyclin synthesis. To obtain further information on the evolutionary situation of CYP8B, the primary structure of CYP8B was compared in detail with those of other members of *CYP7* and *8* families.

The amino acid sequences of CYP7, CYP8, and CYP51, which is the nearest neighbor of CYP7/8, were obtained from public databases and aligned by the improved multiple amino acid sequence alignment method (*13*). Then, intra- and inter-subfamily amino acid sequence conservation of *CYP7* and *CYP8* families was analyzed with respect

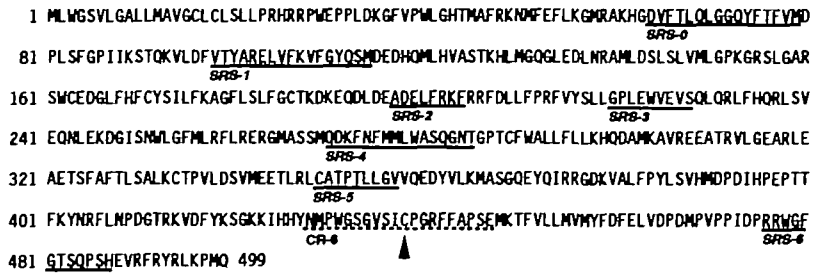


Fig. 1. **Amino acid sequence of rat CYP8B.** The amino acid sequence was deduced from the nucleotide sequence of rat CYP8B cDNA (DDBJ/EMBL/GenBank: AB009686). Underlines (SRS-0 through 6) indicate the regions corresponding to the putative substrate recognition sites (*22*). A heme-binding region designated as CR-6 (*23*) is broken-underlined. The arrowhead in CR-6 denotes the cysteine providing the thiolate heme ligand.

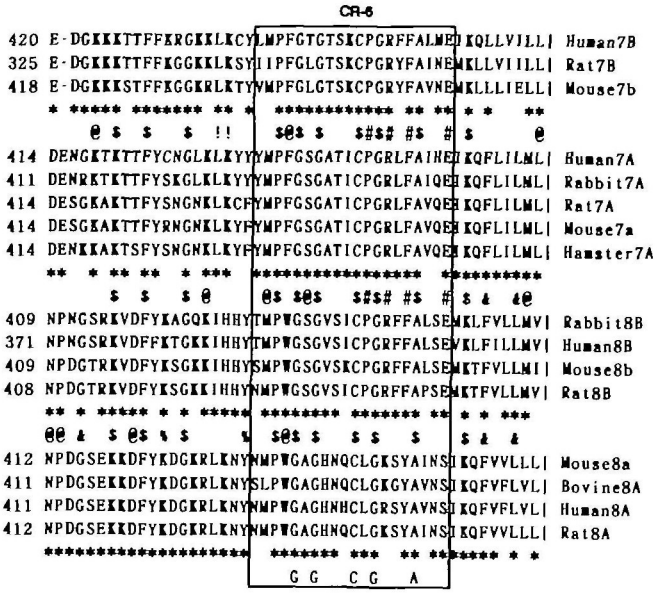


Fig. 2. **Amino acid conservation observed in the heme-binding region.** The amino acid sequences covering the heme-binding region (CR-6 in Fig. 1) were extracted from the multiple amino acid sequence alignment data obtained with the full-length sequences of CYP7A, 7B, 8A, and 8B. $, Amino acids common to all members of CYP7 and 8 families. #, Amino acids common to CYP8B and CYP7 family. &, Amino acids common to CYP8A, 8B, and 7A. !, Amino acids common to CYP7A, 7B, and 8A. %, Amino acids common to CYP8A, 8B, and 7B. @, Amino acids common to contiguous subfamilies. *, Amino acids conserved within each subfamily. Amino acid residues shown in the bottom line of the alignment indicate the amino acids common to CYP7A, 7B, 8A, 8B, and mammalian CYP51.

to SRSs (the putative substrate-recognition sites) and CR-6 (the heme-binding region). The amino acid sequences of SRSs were conserved within each subfamily, indicating their functional importance. However, the extents of sequence similarity among CYP8B, CYP7A, and CYP8A were comparable (data not shown), and no evidence indicating a specific evolutionary linkage among them was obtained. In contrast, interesting differences were observed in the similarity among the amino acid sequences of CR-6 (Fig. 2). Out of 20 CR-6 amino acids, 12 were conserved between CYP8B and CYP7A, and 10 of them were common to CYP8B, CYP7A, and CYP7B. However, only 7 identical amino acids observed between CYP8B and 8A, and 5 of them were common to all known members of the CYP7/8/51 cluster. Since structural alteration of CR-6 is restricted within the same subfamilies, as shown in Fig. 2, the high similarity of the CR-6 of CYP8B to those of CYP7A and CYP7B may be a remnant of the evolutionary relationship of CYP8B to CYP7, as expected from its function. The phylogenetic tree (Fig. 3) indicates that the evolutionary distance between the CYP7/8 divergence and the diverging point of mammalian CYP8B was shorter than that between CYP7/8 divergence and the diverging point of mammalian CYP8A. This suggests that CYP8B is closer than CYP8A to the common ancestral P450 of the *CYP7/8* families. Since most P450s of the *CYP7* and *CYP8* families catalyze hydroxylation of sterols, the ancestral P450 might be a sterol hydroxylase, and CYP8B has retained this feature of the ancestor. *CYP8A* might have diverged from an ancient *CYP8B* and subsequently acquired the novel function of a

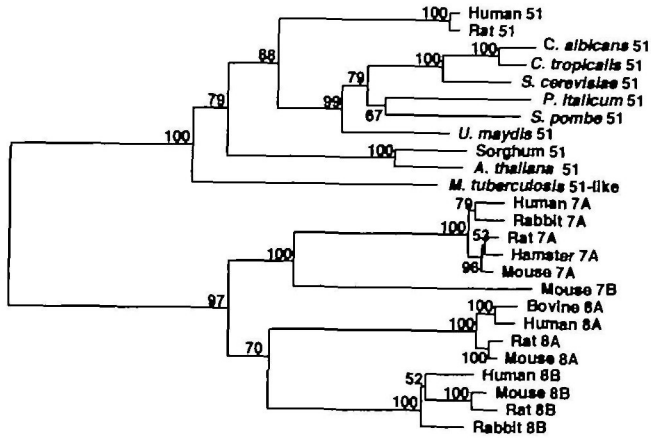


Fig. 3. **Neighbor-joining tree of *CYP8B* and related *CYP* families.** The phylogenetic tree of *CYP7, 8,* and *51* families was constructed by the neighbor-joining method based on the multiple amino acid sequence alignment data of *CYP7, 8,* and *51* families. This tree is the most probable one and the bootstrap probability values of each branch topology are shown. The accession numbers of the amino acid sequences employed for constructing this tree are M93133 (human CYP7A1), L10754 (rabbit CYP7A1), L04690 (hamster CYP7A1), J05430 (rat CYP7A1), L23754 (mouse Cyp7a1), U36993 (mouse Cyp7b1), U36992 (rat CYP7B1), D38145 (human CYP8A1), D30718 (bovine CYP8A1), U53855 (rat CYP8A), Y08269 (rabbit CYP8B1), D55681 (rat CYP51), D55653 (human CYP51), M21489 (*Saccharomyces cerevisiae* CYP51), X13296 (*Candida albicans* CYP51), M23673 (*C. tropicalis* CYP51), Z54096 (*Schizosaccharomyces pombe* CYP51), Z49750 (*Penicillium italicum* CYP51), Z48164 (*Ustilago maydis* CYP51), and Y09291 (*Triticum aestivum* CYP51).

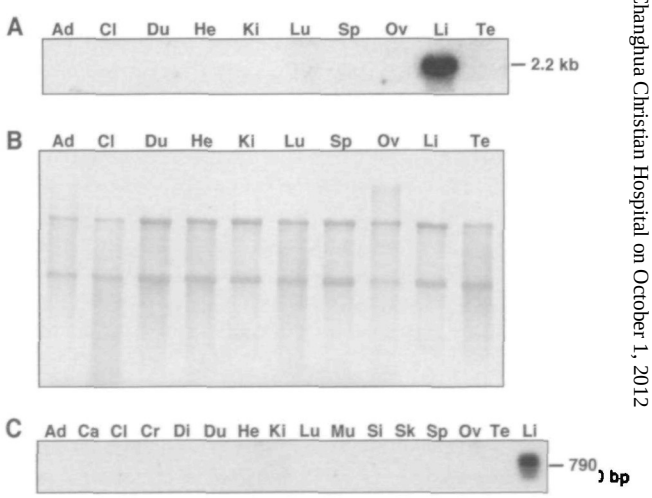


Fig. 4. **Tissue distribution of CYP8B mRNA in rat.** A: Northern hybridization of poly(A)$^+$ RNA obtained from various rat tissues with rat CYP8B cDNA. Poly(A)$^+$ RNA prepared from the indicated ten tissues were subjected to Northern blotting analysis as described in "MATERIALS AND METHODS." B: Methylene-blue staining of the blotted paper used in the Northern hybridization (A). C: Hybridization of the RT-PCR products obtained from poly(A)$^+$ RNA of various rat tissues with rat CYP8B cDNA. RT-PCR was performed as described in "MATERIALS AND METHODS" using P3 and P10 primers. Tissues used were adrenal gland (Ad), cartilage (Ca), cerebrum (Cr), cerebellum (Cl), diencephalon (Di), duodenum (Du), heart (He), kidney (Ki), lung (Lu), muscle (Mu), ovary (Ov), small intestine (Si), skin (Sk), spleen (Sp), testis (Te), and liver (Li). The sizes of CYP8B mRNA and the PCR product are indicated.

prostacyclin synthase through extensive alteration. The differentiation of the ancestral sterol hydroxylase P450 into CYP7A, 7B, and 8B might have formed the cholesterol catabolic pathway shown in Scheme 1, and the pathway to form cholic acid was established after the appearance of CYP8B. The P450s of the CYP7/8/51 cluster other than CYP8A participate in the oxygenation of a carbon atom in the middle of the steroid skeleton (C-7, C-12, and 14-methyl carbons) from the alpha-side. These facts strongly suggest that these P450s diverged from a common ancestral enzyme that catalyzed monooxygenation of the steroid ring from the alpha-side, and CYP8B, as well as CYP7 and CYP51, has retained this fundamental property.

The phylogenetic tree (Fig. 3) also shows that structural divergence of CYP8B among mammals was more extensive than those of CYP7A and CYP51; and the amino acid sequence identity between rat and human orthologues, which was calculated from the multiple amino acid sequence alignment data, decreased in the order of CYP51 (93.4%) > CYP7A (82.1%) > CYP8B (75.1%). This order parallels that of the array of these enzymes in the metabolic pathway shown in Scheme 1. CYP51 catalyzes an essential step of eukaryotic sterol synthesis and is considered to be one of the housekeeping enzymes of mammals, and CYP7A is the initial and key enzyme of cholesterol catabolism. In contrast, CYP8B participates in a downstream and diverged peripheral metabolic reaction of bile acid synthesis. These facts suggest that structural restriction of CYP8B may not be as strong as those of CYP51 and CYP7A, which play key metabolic roles.

Liver Specific Expression of CYP8B—Expression level of CYP8B mRNA was examined using CYP8B cDNA and poly(A)$^+$ RNA preparations from 16 tissues: adrenal gland, cartilage, cerebrum, cerebellum, diencephalon, duodenum, heart, kidney, lung, muscle, ovary, small intestine, skin, spleen, testis, and liver. Northern hybridization shows the presence of a 2.2-kb mRNA only in liver (Fig. 4A), when equal amount of RNA from each tissue was loaded on the gel (Fig. 4B). The possibility that CYP8B mRNA was expressed at low level in the tissues other than liver was examined by RT-PCR analysis with the ^{32}P-labeled cDNA probe of CYP8B. However, the positive RT-PCR product was obtained only from the hepatic poly(A)$^+$ RNA preparation (Fig. 4C), although the same poly(A)$^+$ RNA preparations of all tissues gave significant amounts of the RT-PCR product for GAPDH primers (data not shown). These observations clearly indicate that CYP8B is expressed specifically in liver, as in the case of CYP7A (Noshiro *et al.*, unpublished). Since CYP8B is indispensable for cholic acid formation, this finding strongly indicates the liver-specific formation of cholic acid. On the other hand, chenodeoxycholic acid is formed through two independent pathways (Scheme 1), and the enzymes participating in the alternative pathway are present not only in liver but also in some

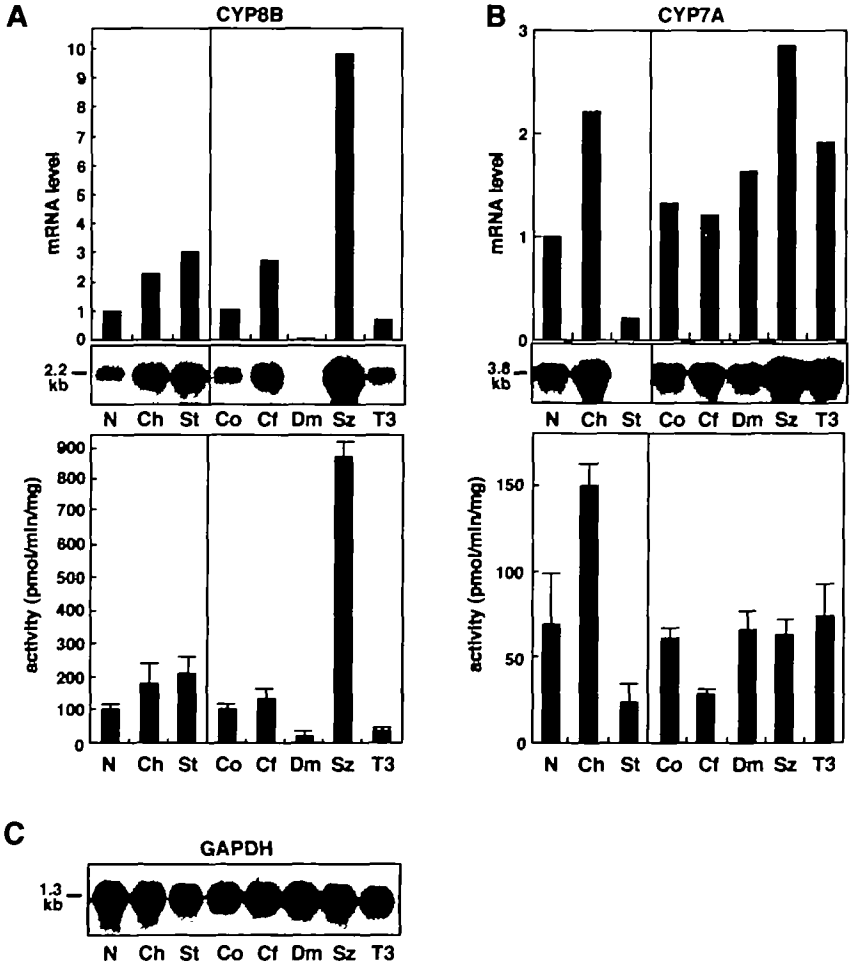


Fig. 5. **Effects of various treatments on the mRNA levels and activities of CYP8B (A) and CYP7A (B) in rat livers.** Rats were treated under various conditions as described in "MATERIALS AND METHODS." Treatments for estimating the effects of dietary conditions, *i.e.*, normal laboratory rat chow (N), cholestyramine-containing chow (Ch), and starvation (St), and of various modulator compounds, *i.e.*, corn oil as control (Co), clofibrate (Cf), dexamethasone (Dm), streptozotocin (Sz), and thyroxin (T3), were conducted separately. All animals were sacrificed at 10 a.m., and microsomes and poly(A)$^+$ RNA were prepared from the same liver mince as described in "MATERIALS AND METHODS." Top and middle panels show the mRNA levels of CYP8B (A) and CYP7A (B) estimated by Northern hybridization, and bottom panels show the specific activity of microsomal HCO 12α-hydroxylase (CYP8B) (A) and cholesterol 7α-hydroxylase (CYP7A) (B). The level of mRNA was expressed relative to the normal specimen (N of the left panel). The result of Northern hybridization for GAPDH mRNA is shown in C as the control. The sizes of CYP8B and CYP7A mRNA are indicated.

extra-hepatic tissues (*9, 25, 26*), which suggests the possible formation of chenodeoxycholic acid in these tissues. Recently, Andersson *et al.* (*27*) reported that rabbit CYP8B could metabolize 3α,7α-dihydroxy-5β-cholestanoic acid, which can be formed through the alternative pathway, to cholic acid. This fact suggests the possible formation of cholic acid without the contribution of CYP7A. Even so, cholic acid must be formed only in liver, because of the liver-specific expression of CYP8B.

Effects of Various Treatments on the Expression Levels of CYP8B in Rat Liver—Feeding of cholestyramine, a bile acid sequester, increased both mRNA and enzyme activity levels of CYP8B in the rat livers as shown in the left panel of Fig. 5A, as in the case of CYP7A (Fig. 5B, left panel and Ref. *12*). The induction of *CYP7A* by cholestyramine is considered to be due to the reduction of bile acids returning to the liver *via* enterohepatic circulation, because such bile acids suppress the *CYP7A* gene (*28-32*). Bile acids may regulate *CYP8B* gene expression by a similar mechanism to that for *CYP7A*. However, *CYP8B* expression was increased by starvation (Fig. 5A, left panel), whereas *CYP7A* expression was decreased under the same conditions (Fig. 5B, left panel and Refs. *4, 5,* and *12*). Starvation accumulates bile acids in liver and is considered to suppress *CYP7A* expression by the same mechanism as described above. Consequently, an additional potent inducing mechanism, which can overwhelm the repressive effect of bile acids, may operate in *CYP8B* expression under starvation.

The right panel of Fig. 5A presents the effects of several other modulators on *CYP8B* expression in rat liver. The most striking effect was observed in streptozotocin-treated diabetic rats. The enzyme activity and mRNA level of CYP8B in these rats were nearly 10 times those in the control rats. Streptozotocin treatment caused no effect on CYP7A activity (Fig. 5B, right panel), although it caused elevation of CYP7A mRNA level. Marked increase of CYP8B activity and unchanged CYP7A activity in the streptozotocin-treated rats agree with the *in vivo* observations that synthesis and pool size of cholic acid were increased in diabetic rats (*33*). Clofibrate tended to increase the expression of *CYP8B*, whereas dexamethasone and thyroxin strongly decreased the expression of *CYP8B* (Fig. 5A, left panel). Clofibrate and thyroxin caused opposite effects on the *CYP7A* expression, whereas dexamethasone had no effect (Fig. 5B, right panel).

The results summarized in Fig. 5 indicate that the regulation of *CYP8B* expression is independent of *CYP7A*. Independent regulation of *CYP8B* and *CYP7A* expression suggests that the regulation of *CYP8B* expression might effectively alter the biliary cholic acid/chenodeoxycholic acid ratio, because CYP7A is the rate-limiting enzyme of the bile acids synthesis (Scheme 1), and CYP8B activity was twice as high as CYP7A activity in control rats (see Fig. 5).

Down-Regulation of CYP8B by Insulin—Marked elevation of *CYP8B* expression by streptozotocin treatment suggested that *CYP8B* is down-regulated by insulin. Then, the effect of insulin administration to the streptozotocin-treated rats was examined. As shown in Fig. 6, the enhanced level of CYP8B mRNA in streptozotocin-treated rats (lane Sz) was significantly decreased by insulin within 3 h (lane Sz+In). This fact strongly supports the assumption that insulin acts as a direct suppressor of *CYP8B*. The starvation-induced elevation of *CYP8B* expression discussed in the preceding section (Fig. 5A, left panel) may be due to the reduction of insulin level by starvation.

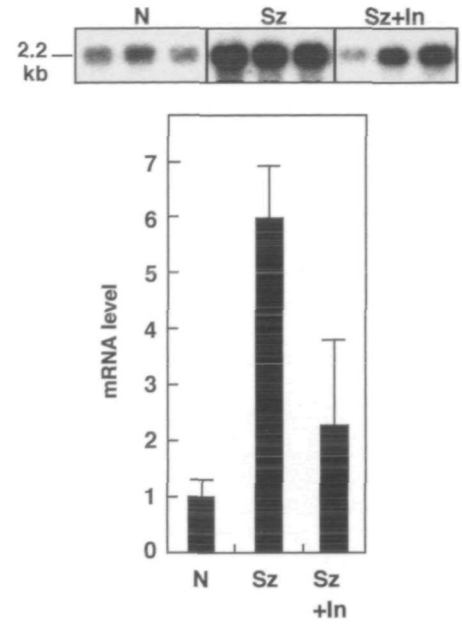


Fig. 6. **Effects of insulin-injection on CYP8B mRNA level in streptozotocin-induced diabetic rats.** Three rats were used for each group and the individual samples were assayed. Diabetes induced by intraperitoneal administration of streptozotocin (65 mg/kg body weight), and bovine insulin (8 units/kg body weight) was injected subcutaneously one week later. Poly(A)$^+$ RNA was prepared as described in "MATERIALS AND METHODS" and used for the determination of CYP8B mRNA level by Northern blotting. The individual CYP8B mRNA levels are shown in the upper panel. The level of mRNA is expressed relative to the normal specimen in the lower panel. N, normal; Sz, streptozotocin; Sz+In, streptozotocin plus insulin.

Insulin acts as a suppressor of *CYP7A* and *CYP27* in hepatocyte primary culture (*34*), whereas it acts as an inducer of HMG CoA reductase (*35*). In our preliminary observations, streptozotocin-induced diabetes mellitus decreased the mRNA of enzymes for cholesterol synthesis, such as HMG CoA reductase and CYP51, whereas it increased the mRNA of enzymes for cholesterol catabolism, such as CYP27 (data not shown), in addition to CYP8B and CYP7A (Fig. 5). These facts suggest an essential role of insulin in cholesterol metabolism. Further studies on the roles of insulin in the regulation of *CYP8B* and other sterol-metabolizing enzymes are in progress in our laboratories.

Note Added in Proof: After the acceptance of this paper, gene structures of human and mouse *CYP8B* were reported by M. Gåfvels *et al.* (*Genomics* **56**, 184-196, 1999, GenBank/EMBL/DDBJ No. AF090319 and AF090320 for mouse and human, respectively). In the 5′-flanking regions of both sequence data, we found similar core motifs [T(G/A)TTTTG] to an insulin response sequence (IRS), which are observed in 5′ upstream of the genes suppressively regulated by insulin such as glucose 6-phosphatase and phosphoenolpyruvate carboxykinase. This fact supports that *CYP8B* gene is also negatively regulated by insulin possibly at transcriptional level.

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