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Identification and Characterization of a Mouse Oxysterol 7α-Hydroxylase cDNA*

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The synthesis of essential 7α-hydroxylated bile acids in the liver is mediated by two pathways that involve distinct 7α-hydroxylases. One pathway is initiated in the endoplasmic reticulum by cholesterol 7α-hydroxylase, a well studied cytochrome P450 enzyme. A second pathway is initiated by a less well defined oxysterol 7α-hydroxylase. Here, we show that a mouse hepatic oxysterol 7α-hydroxylase is encoded by Cyp7b1, a cytochrome P450 cDNA originally isolated from the hippocampus. Expression of a Cyp7b1 cDNA in cultured cells produces an enzyme with the same biochemical and pharmacological properties as those of the hepatic oxysterol 7α-hydroxylase. Cyp7b1 mRNA and protein are induced in the third week of life commensurate with an increase in hepatic oxysterol 7α-hydroxylase activity. In the adult mouse, dietary cholesterol or colestipol induce cholesterol 7α-hydroxylase mRNA levels but do not affect oxysterol 7α-hydroxylase enzyme activity, mRNA, or protein levels. Cholesterol 7α-hydroxylase mRNA is reduced to undetectable levels in response to bile acids, whereas expression of oxysterol 7α-hydroxylase is modestly decreased. The liver thus maintains the capacity to synthesize 7α-hydroxylated bile acids regardless of dietary composition, underscoring the central role of 7α-hydroxylated bile acids in lipid metabolism.

The formation of bile acids from cholesterol[1] serves two important physiological functions. First, bile acid biosynthesis provides a pathway of cholesterol catabolism by which excess sterol can be disposed. Second, the end products of this pathway serve as natural detergents in the gut that facilitate the solubilization and absorption of dietary sterols, triglycerides, and fat-soluble vitamins. Individual bile acids differ in the positions of the hydroxyl groups on the ring structure of cholesterol and in the length and degree of oxidation of the side chain. To date, the chemical structures of several hundred bile acids in dozens of different species have been elucidated (1). Included in this roster are bile acids that range in size from 19 to 27 carbons and that contain hydroxyl groups with either α or β stereochemistries at one or more of these carbon atoms.

Although the chemical structures of bile acids from different species are diverse, almost all vertebrates contain 7α-hydroxylated bile acids (1, 2), which suggests that compounds with this substituent play essential roles in cholesterol and lipid metabolism. Two pathways that lead to the synthesis of 7α-hydroxylated bile acids have been described. One is the neutral or microsomal pathway (3, 4) and involves an initial 7α-hydroxylation of cholesterol by a microsomal cytochrome P450 enzyme termed cholesterol 7α-hydroxylase (EC 1.14.13.17). This enzyme has been extensively studied at the biochemical, genetic, and molecular levels (reviewed in Refs. 5 and 6). The output from the microsomal pathway is tightly regulated by controlling the transcription of the cholesterol 7α-hydroxylase gene in the liver (7–10).

The second pathway is the mitochondrial or acidic pathway and utilizes a slightly different sequence of initial steps in the formation of 7α-hydroxylated bile acids (3, 4). Cholesterol is first converted in mitochondria to an oxysterol by hydroxylation of the side chain (11). This intermediate is then a substrate for a microsomal oxysterol 7α-hydroxylase that is distinct from cholesterol 7α-hydroxylase (12–16). Although oxysterol 7α-hydroxylase activity has been reported in several tissues of the pig, rat, hamster, and mouse, a cDNA encoding this enzyme has not yet been reported, nor has the regulation of the enzyme, and hence the output of the mitochondrial pathway, been extensively studied.

A genetic demonstration of the physiological importance of 7α-hydroxylated bile acids came from mice in which the contribution of the microsomal pathway was eliminated by targeted disruption of the cholesterol 7α-hydroxylase gene (17). Approximately 90% of animals homozygous for a null allele die in the first 3 weeks of life from fat and vitamin malabsorption. Death can be prevented by the addition of a 7α-hydroxylated bile acid and vitamins to the diet, whereas survival in the absence of these supplements is correlated with the induction of the oxysterol 7α-hydroxylase/mitochondrial pathway (16, 17). The activation of this pathway in mice normally occurs at about 3 weeks of age in response to yet undefined regulatory signals (16).

A candidate cDNA for the oxysterol 7α-hydroxylase was recently isolated by differential hybridization screening of a hippocampal cDNA library (18). This cDNA, termed *hct*-1 for hippocampal transcript 1, encoded a cytochrome P450 enzyme that shared 39% sequence identity with cholesterol 7α-hydroxylase. The high degree of sequence conservation placed *hct*-1 in the cytochrome P450 gene family 7 with the official designation of

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[1] The abbreviations and trivial names used are: cholesterol, 5-cholesten-3β-ol; 25-hydroxycholesterol, cholest-5-ene-3β,25-diol; 7α-hydroxylated 25-hydroxycholesterol, cholest-5-ene-3β,7α,25-triol.

Cyp7b1 (Ref. 19). Besides the hippocampus, the *hct*-1 mRNA was detected in several nonneuronal tissues, including the liver (18). Subsequent expression studies showed that the *hct*-1 enzyme is a steroid 7α-hydroxylase that acts on dehydroepiandrosterone and pregnenolone (20). These results, together with the expression of *hct*-1 in the brain, suggested that the enzyme was involved in the synthesis of neurosteroids (20).

In the current study, we provide evidence that *hct*-1 is also an oxysterol 7α-hydroxylase that participates in hepatic bile acid biosynthesis. The biochemical, pharmacological, and ontological properties of the *hct*-1 cDNA-encoded enzyme match those of an oxysterol 7α-hydroxylase activity present in liver microsomes. It is further shown that the oxysterol 7α-hydroxylase and the cholesterol 7α-hydroxylase enzymes, and hence their respective pathways, are differentially regulated in response to diet.

EXPERIMENTAL PROCEDURES

Materials—^{32}P-Labeled nucleotides and [^{3}H]cholest-5-ene-3β,25-diol (25-[^{3}H]hydroxycholesterol) were purchased from NEN Life Science Products. Thin layer chromatography plates (LK5DF silica gel 150 Å) were from Whatman (Clifton, NJ). Reagents used in cDNA cloning and sequencing were from New England Biolabs (Beverly, MA), Boehringer Mannheim, or Life Technologies, Inc. Common laboratory chemicals and stigmastanol were from Sigma. Nafimidone was obtained from D. C. Swinney at Roche Biosciences (Palo Alto, CA).

Animals and Diets—C57BL/6J mice (male, 3 months old) were obtained from the Jackson Laboratory (Bar Harbor, ME) and were housed individually in a humidity- and temperature-controlled room (22 °C) with alternating 12-h light/12-h dark cycles. Mice were fed a cereal-based rodent diet (7001, Harlan Teklad, Madison, WI), which contained ≥4% (w/w) fat, ≥24% (w/w) protein, and ≤5% (w/w) fiber. Where indicated, this diet was supplemented with 2% (w/w) cholesterol (ICN, Irvine, CA), 2% (w/w) colestid (ICN), 0.5% (w/w) cholic acid (ICN), or a combination of 2% cholesterol and 0.5% cholic acid. Mice (n = 4 or 5 per group) were fed individual diets for 10 days. Body weights for each animal were recorded before and after the feeding period. On the last day of the experiment, mice were fasted for 6 h, anesthetized with nembutal (3 mg/animal, delivered intraperitoneally), and bled from the inferior vena cava. Total plasma cholesterol was enzymatically determined (Cholesterol/HP kit, Boehringer Mannheim). Plasma cholesterol levels (mean ± S.E.) after 10 days on normal diet or diet mixed with 2% cholesterol, 2% colestid, 0.5% cholic acid, or 2% cholesterol plus 0.5% cholic acid were 75.8 ± 4.9, 69.8 ± 3.8, 65.7 ± 2.1, 58.0 ± 7.6, and 131.2 ± 21.5 mg dl^{-1}, respectively. None of the values measured in animals fed supplemented diets were significantly different from those of normal diet-fed mice (*i.e.* $p > 0.01$, Student's two-tailed test assuming equal variance).

The wet weights of the livers were determined, and the tissue from each mouse was divided into three aliquots. Two aliquots of 500 mg each were frozen in liquid nitrogen and stored at −70 °C until used for the preparation of mRNA and microsomal membranes, respectively. A third aliquot (~200 mg) was immediately saponified in 5 ml of ethanolic KOH (2 h, 65 °C). Free sterols were extracted with petroleum ether, an internal standard was added (1 mg stigmastanol), and the organic phase was analyzed by gas chromatography to determine total hepatic cholesterol content (21). Hepatic cholesterol levels (mean ± S.E.) on the normal, cholesterol, colestipol, cholic acid, and cholesterol plus cholic acid diets were 2.3 ± 0.05, 3.24 ± 0.19 ($p \leq 0.001$), 2.44 ± 0.09, 3.41 ± 0.18 ($p \leq 0.0003$), and 23.5 ± 3.0 ($p \leq 0.0001$) mg/g of tissue, respectively. Values measured in cholesterol-, cholic acid-, and cholic acid plus cholesterol-fed mice were significantly higher (*i.e.* $p \leq 0.01$, Student's two-tailed test assuming equal variance) than those determined in control mice.

Enzymatic Assays—Assays for oxysterol 7α-hydroxylase activity were carried out as described previously (16). The hepatic oxysterol 7α-hydroxylase utilizes both 25-hydroxycholesterol and 27-hydroxycholesterol as substrate (14). In the present study, commercially available 25-[^{3}H]hydroxycholesterol was used as a substrate.

Transfections—A plasmid vector capable of expressing the murine Cyp7bl protein was constructed via standard methods of genetic engineering. Briefly, an *hct*-1 cDNA encompassing nucleotides −20 to 1880 (Ref. 18), was ligated into the mammalian expression vector pCMV6 and transformed into *E. coli* DH5α cells. The desired recombinants were characterized by restriction enzyme mapping and DNA sequencing. The expression plasmid was purified and transfected into human embryonic kidney 293 cells using an MBS transfection kit (Stratagene Corp., La Jolla, CA). Twenty-four h after transfection, the indicated concentrations of 25-[^{3}H]hydroxycholesterol were added to the medium of transfected and mock-transfected cells. At various times after the addition, lipid metabolites were extracted from the medium using Folch reagent (chloroform/methanol, 2:1, v/v) and analyzed by thin layer chromatography (solvent system toluene/ethyl acetate, 2:3, v/v) and autoradiography.

Nafimidone Inhibition Experiments—Microsomal membranes from mouse liver were prepared after Dounce homogenization by sequential centrifugation as described below. Microsomal membranes from 293 cells were prepared by harvesting cells 24 h after transfection, washing once with ice cold phosphate-buffered saline, and swelling for 10 min in a hypotonic buffer containing 10 mM Hepes, pH 7.6, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 9000 trypsin inhibitory units/ml aprotinin, 10 μg/ml leupeptin, 5 μg/ml pepstatin, and 5 μM phenylmethylsulfonyl fluoride. Cells were disrupted by passing the suspension through a 22.5-gauge needle 15 times. The nuclei and cell debris were removed by a 5-min centrifugation at 1000 × g at 4 °C. The supernatant was transferred to an ultracentrifuge tube, and microsomal membranes were pelleted by centrifugation at 4 °C for 30 min at 130,000 × g. Microsomal membranes from mouse liver and transfected 293 cells were resuspended in Buffer A (50 mM Tris-HCl, pH 7.4, 20% (v/v) glycerol, 1 mM EDTA, and protease inhibitors as described above) at a final concentration of 20 mg of membrane protein/ml and assayed for oxysterol 7α-hydroxylase activity in the presence of the indicated concentrations of nafimidone.

For each incubation, the required amount of an ethanolic solution of inhibitor was dried down in a test tube and preincubated with 100 μg of microsomal protein, 2 mM dithiothreitol, 0.03% (v/v) Triton X-100 in buffer A for 10 min at 37 °C. 25-[^{3}H]Hydroxycholesterol was added to a final concentration of 0.2 μM, and the reaction (total volume 0.5 ml) was started by the addition of NADPH to a final concentration of 1.5 mM. Mouse liver microsomes were incubated for 10 min, and 293 cell membranes were incubated for 30 min, both at 37 °C. Thereafter, 6 ml of methylene chloride were added to each incubation to terminate the reaction and to extract 25-hydroxycholesterol metabolites. The organic phase was dried down under nitrogen, and lipids were redissolved in 40 μl of Folch reagent and analyzed by thin layer chromatography (solvent system toluene/ethyl acetate, 2:3, v/v) and autoradiography. Radiolabeled products were quantitated using a System 200 Imaging Scanner (Bioscan, Inc., Washington, D. C.).

Antibody Production—A 15-amino acid peptide (QDLLKRYYRHDDSEIG) derived from residues 266–281 of the cDNA-deduced sequence of the murine Cyp7b1 protein (18), was synthesized by Biosynthesis Inc. (Lewisville, TX), as a multiple-antigen peptide. The multiple-antigen peptide was used to immunize four New Zealand White rabbits (male, 3 months old). For the initial injection, 250 μg of multiple-antigen peptide were emulsified with complete Freund's adjuvant and administered intramuscularly. Over the next 3 months, four boosts were given using the same dose of multiple-antigen peptide but with incomplete Freund's adjuvant. Antibody titers in serum samples were tested in immunoblotting experiments using as antigen extracts from 293 cells transfected with a murine Cyp7bl cDNA expression vector.

Chemical Analysis of 25-Hydroxycholesterol Metabolites—Samples were first converted to trimethylsilyl ethers by treatment with 50 μl of pyridine/hexamethyldisilazane/trimethylchlorosilane (3:2:1, v/v/v) at 60 °C for 30 min. After evaporation of the solvent under argon, the residue was dissolved in hexane and transferred to an autosampler vial. Gas chromatography-mass spectrometry was performed on a Hewlett Packard 5890 Series II Plus gas chromatograph equipped with an HP-5MS capillary column (30 m × 0.25 mm, 0.25-μm phase thickness), connected to an HP 5972 mass selective detector and an HP 7673A automatic sample injector. The oven temperature program was 180 °C for 1 min, 20 °C/min to 250°, followed by 5 °C/min to 300 °C, where the temperature was kept for 8.0 min. Helium was the carrier gas. The gas chromatograph was operated in constant flow mode at a flow rate of 0.8 ml of helium/min. The injector was operated in splitless mode at 270 °C. The detector transfer line was maintained at 280 °C.

RESULTS

To determine if a Cyp7b1 cDNA encoded an oxysterol 7α-hydroxylase activity, a murine cDNA was inserted into an expression vector, and the resulting plasmid was transfected into cultured human embryonic kidney 293 cells. A substrate for the murine oxysterol 7α-hydroxylase, 25-[^{3}H]hydroxycho-

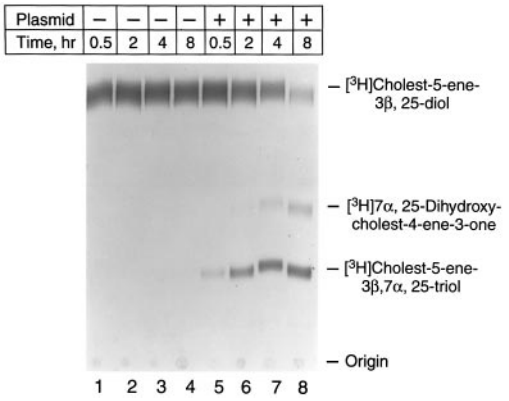

FIG. 1. **Cyp7b1 cDNA encodes an oxysterol 7α-hydroxylase activity.** Cultured human embryonic kidney 293 cells were transfected with either an expression plasmid containing no cDNA (−) or the same plasmid (+) containing a nearly full-length Cyp7b1 cDNA isolated from mouse (18). After a 24-h expression period, transfected cells were incubated for the indicated periods of time with 0.12 μM [^{3}H]cholest-5-ene-3β,25-diol (25-[^{3}H]hydroxycholesterol), and the formation of 7α-hydroxylated products was determined by thin layer chromatography as described under "Experimental Procedures." Nafimidone (50 μM), an inhibitor of an endogenous 293 cell oxysterol 7α-hydroxylase (see "Results"), was included in culture media throughout the experiment. The positions and identities of radiolabeled sterols are indicated on the *right*. [^{3}H]Cholest-5-ene-3β,7α,25-triol (^{3}H-labeled 7α-hydroxylated 25-hydroxycholesterol) is initially formed in cells transfected with the Cyp7b1 cDNA. This product is subsequently converted into ^{3}H-labeled 7α,25-dihydroxycholest-4-ene-3-one by an endogenous 3β-hydroxysteroid dehydrogenase activity of 293 cells.

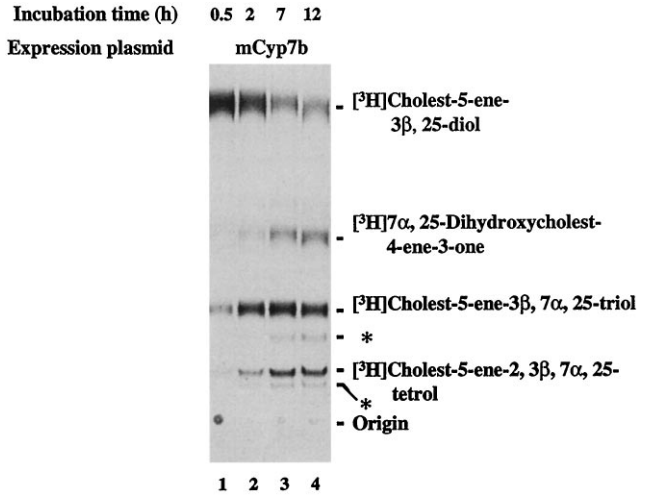

FIG. 2. **Formation of additional oxysterols in cells transfected with Cyp7b1 cDNA.** Dishes of cultured 293 cells were transfected with an expression plasmid containing the mouse Cyp7b1 cDNA. After a 24-h expression period, 25-[^{3}H]hydroxycholesterol (0.04 μM) was added to the culture media, and the incubations were continued for the indicated times. Thereafter, sterols were extracted from the cell media and analyzed by thin layer chromatography as described under "Experimental Procedures." The locations and identities of several of the sterol products are shown to the *right* of the *autoradiogram*. *Asterisks* mark minor products whose identities were not determined.

lesterol (16), was then added to the cell media at a final concentration of 0.12 μM. The conversion of this compound into [^{3}H]cholest-5-ene-3β,7α,25-triol (7α-hydroxylated 25-hydroxycholesterol) was monitored by thin layer chromatography. In initial experiments, the presence of an endogenous human oxysterol 7α-hydroxylase activity in 293 cells prevented an unambiguous demonstration of plasmid-encoded enzyme activity (data not shown). To overcome this problem, an inhibitor (nafimidone) of cytochrome P450 enzymes[2] was used to selectively block the human oxysterol 7α-hydroxylase in the 293 cells. The concentration of nafimidone required to inhibit 50% of endogenous oxysterol 7α-hydroxylase enzyme activity in intact 293 cells was approximately 3 μM, whereas concentrations above 500 μM were required to inhibit activity expressed from a transfected murine Cyp7b1 cDNA (data not shown).

In the presence of 50 μM nafimidone, mock-transfected cells did not metabolize 25-[^{3}H]hydroxycholesterol to more polar compounds during the 8-h course of the experiment (Fig. 1, *lanes 1–4*). Cells transfected with the murine Cyp7b1 expression vector began producing a product identified as 7α-hydroxylated 25-hydroxycholesterol within 30 min of incubation with substrate (*lane 5*). With longer incubation times, the amount of this product increased, and another metabolite identified as [^{3}H]7α,25-dihydroxy-cholest-4-ene-3-one (Fig. 1, *lanes 6–8*) appeared; this secondary metabolite arises due to an endogenous 3β-hydroxysteroid dehydrogenase enzyme activity present in the 293 cells. The chemical structures of these products were previously determined by thin layer chromatography and gas chromatography-electron impact mass spectrometry using authentic standards (16). We concluded from these experiments that the murine Cyp7b1 cDNA encodes an oxysterol 7α-hydroxylase active against 25-hydroxycholesterol.

When the transfection experiments with the murine Cyp7b1 cDNA were carried out in the absence of nafimidone, additional cDNA-specific, polar products arising from 25-[^{3}H]hydroxycholesterol were detected (Fig. 2). Within 2 h, a major product with a mobility slower than 7α-hydroxylated 25-hydroxycholesterol was detected (*lane 2*). With longer incubation times, the amount of this metabolite rose and several additional minor products appeared (*lanes 3* and *4*). Mock-transfected cells produced only low levels of 7α-hydroxylated 25-hydroxycholesterol (data not shown). To obtain sufficient mass to determine the chemical structure of the initial metabolite produced in transfected cells, the number of dishes in the experiment was increased by a factor of 30. After an 8-h incubation with 25-[^{3}H]hydroxycholesterol, the sterol products were separated by preparative thin layer chromatography, and the compound of interest was isolated from the silica gel plate by extraction with organic solvents.

Gas chromatography of the isolated compound revealed a single peak indicative of a high degree of purity (Fig. 3*A*). The mass spectrum of this compound is shown in Fig. 3*B*. A prominent peak at *m/z* 632 corresponded to the M-90 peak of a tetrahydroxylated cholesterol species. The *m/z* 131 ion was characteristic of metabolites with hydroxylation at carbon 25, while the ion at *m/z* 251 indicated the presence of one double bond and three hydroxyl groups on the steroid nucleus. Finally, the *m/z* 129 ion was characteristic of a fragment containing the $\Delta^{5,6}$-unsaturated bond and the 3β-hydroxyl group on carbon 3. The positions of three of the four hydroxyl groups in the metabolite could thus be assigned to carbons 3, 7, and 25 based on previous analyses of Cyp7b1-generated products and on the known structure of the starting substrate.

The location of the fourth hydroxyl group was determined by incubating the metabolite with 2,2-dimethoxypropane, which produces an acetonide derivative if vicinal (adjacent) hydroxyl groups are present on the treated compound. An acetonide was produced at high efficiency (data not shown), which was indicative of vicinal hydroxylation at either carbons 2 and 3 or carbons 3 and 4. Position 4 was ruled out by the absence of prominent ions at *m/z* 147 or 181 in the mass spectrum (Fig. 3*B*; Ref. 22), which left carbon 2 as the recipient of the additional hydroxyl group. The configuration of the hydroxyl group at this carbon was not determined. Taken together, these data

[2] D. C. Swinney, personal communication.

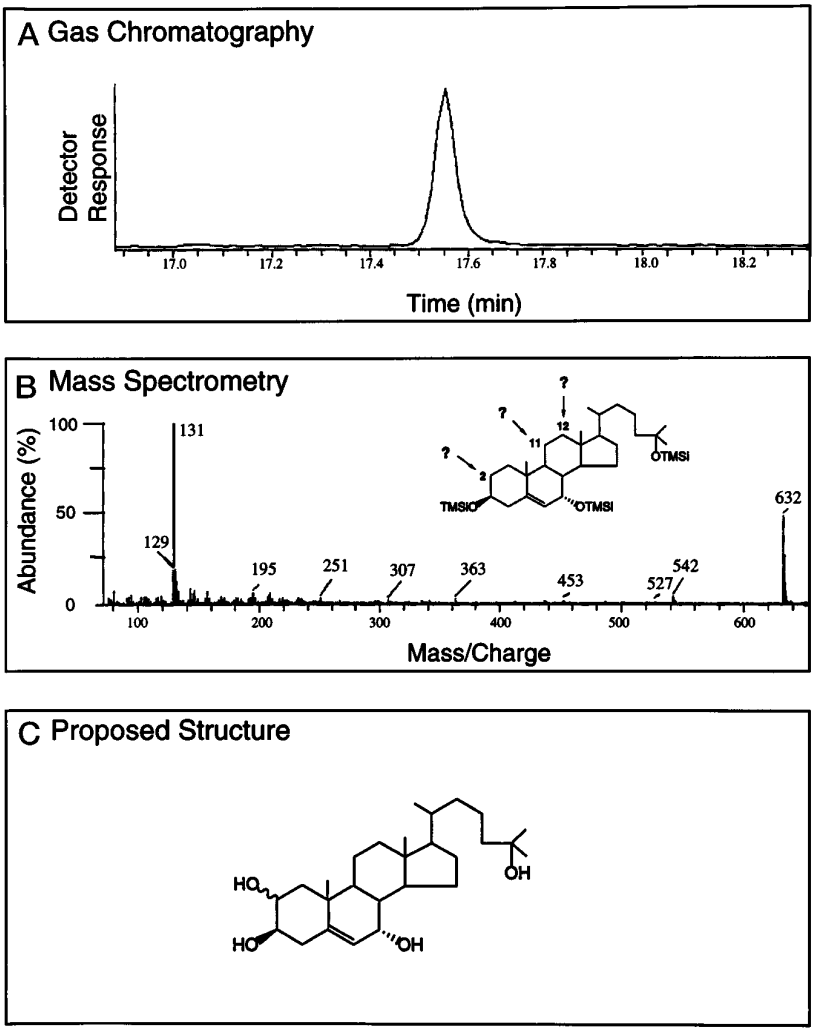

FIG. 3. **Chemical analyses of oxysterol formed in cells transfected with murine Cyp7b1 cDNA.** The most abundant side product arising in Cyp7b1-transfected cells (see Fig. 2) was isolated by preparative thin layer chromatography or high pressure liquid chromatography and subjected to gas chromatography and electron ionizing mass spectrometry to deduce a proposed structure for the compound. *A*, profile obtained after gas chromatography showing a single compound with an elution time of 17.55 min. *B*, mass spectrum obtained after bombardment of the compound eluting at 17.55 min from the gas chromatography column. *C*, proposed chemical structure after interpretation of the mass spectrum and additional derivatization experiments (see "Results" for further details).

indicated that the identity of the novel polar product arising from cells transfected with the Cyp7b1 cDNA was cholest-5-ene-2,3β,7α,25-tetrol (Fig. 3*C*). Cyp7b1 was thus both an oxysterol 7α-hydroxylase and a 2-hydroxylase.

The transfection studies were consistent with the notion that the enzyme encoded by the Cyp7b1 cDNA was an oxysterol 7α-hydroxylase of bile acid biosynthesis. To gain further support for this hypothesis, we exploited the ability of nafimidone to block oxysterol 7α-hydroxylase activity. The concentration of nafimidone required to inhibit 50% of enzyme activity in mouse microsomes (the IC_{50} value) was approximately ~14 μM (Fig. 4). To determine if the oxysterol 7α-hydroxylase enzyme activity encoded by the mouse Cyp7b1 cDNA showed the same inhibition profile, microsomes prepared from transfected 293 cells were assayed for nafimidone sensitivity. The inhibition curve generated with the transfected cell membranes was indistinguishable from that produced with liver microsomes (Fig. 4). The IC_{50} value for the recombinant enzyme was ~17 μM. The concentrations of nafimidone required to achieve similar inhibition in intact cells were far greater (see above), suggesting that nafimidone crosses the cell membrane poorly. The similarity in nafimidone inhibition profiles obtained with hepatic and transfected cell membranes supported the idea that the Cyp7b1 cDNA encoded a hepatic oxysterol 7α-hydroxylase enzyme activity in mice.

Oxysterol 7α-hydroxylase enzyme activity is absent or low in the livers of young mice and is induced around the time of weaning (16). If the murine Cyp7b1 gene encodes the oxysterol 7α-hydroxylase of bile acid biosynthesis, then the expression of Cyp7b1 mRNA and protein should closely follow the developmental induction of enzyme activity. To test this idea, livers were isolated from mice ranging in age from 5 to 55 days. Three to 10 animals per time point were used. Poly(A)$^+$-enriched mRNA and microsomal membranes were prepared from pooled samples. RNA blotting was performed using a radiolabeled Cyp7b1 cDNA probe. Oxysterol 7α-hydroxylase activity in the microsomal membranes was assayed with 25-[^{3}H]hydroxycholesterol as substrate, and immunoblotting was performed using a polyclonal antipeptide antibody.

As shown in Fig. 5, there was a very good correlation between the level of oxysterol enzyme activity (*upper panel*), and the levels of both the Cyp7b1 mRNA (*middle panel*) and protein (*lower panel*). The time course of induction closely paralleled our previous findings (16) that the hepatic oxysterol 7α-hydroxylase activity is induced around the third week of life (*i.e.* day 18, Fig. 5). Hybridization with a control probe (cyclophilin) showed equal amounts of RNA in each lane of the blot, and Ponceau S staining of the immunoblots showed equal amounts of protein in each lane (data not shown).

To compare and contrast the regulation of this enzyme and that of cholesterol 7α-hydroxylase, mice were placed on diets having well-characterized effects on the regulation of cholesterol 7α-hydroxylase. These included normal laboratory diet or diets supplemented with 2% colestipol, 2% cholesterol, 0.5% cholic acid, or 2% cholesterol plus 0.5% cholic acid. After 10 days, livers were isolated from the different groups, pooled, and

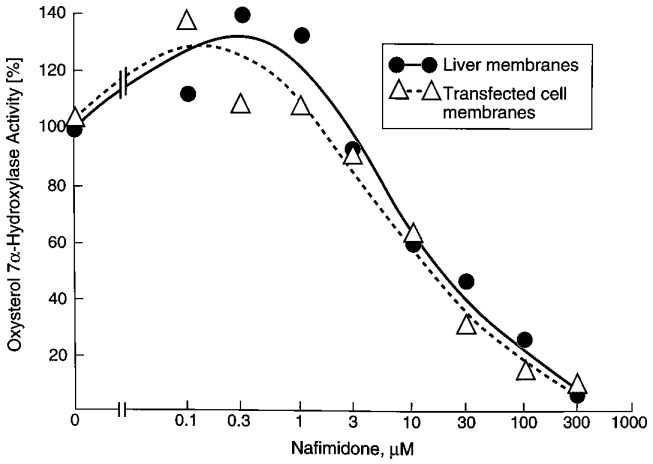

FIG. 4. **Nafimidone inhibition of oxysterol 7α-hydroxylase activity in microsomes and transfected cells.** Microsomal membranes were prepared from mouse liver or human embryonic kidney 293 cells transfected with an expression vector containing the murine Cyp7bl cDNA. Aliquots (100 μg of protein) were incubated with 0.2 μM 25-[^{3}H]hydroxycholesterol, 1.5 mM NADPH, and nafimidone as indicated. Incubation times were 10 min (liver membranes) or 30 min (transfected cell membranes), both at 37 °C. Reactions were terminated by extraction with organic solvents, sterols were separated by thin layer chromatography, and the amounts of substrate and product were determined by phosphor imaging. The reasons why low concentrations of inhibitor increased the level of enzyme activity in both preparations of microsomes were not determined, but they may be related to the inhibition of other cytochrome P450 enzymes in the membranes that deplete the effective concentrations of cofactor or that produce endogenous inhibitors of the reaction.

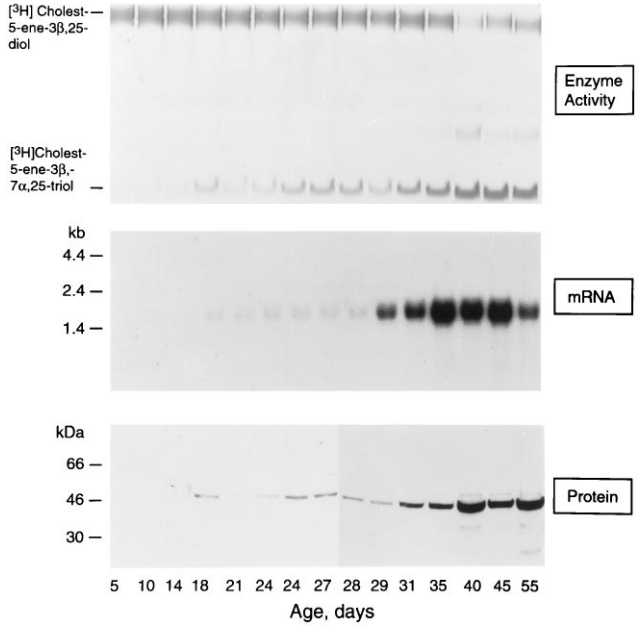

FIG. 5. **Induction of hepatic oxysterol 7α-hydroxylase in mice.** Microsomes and poly(A)$^+$-enriched mRNA were prepared from the livers of mice of the indicated ages (3–10 animals/time point) and assayed for oxysterol 7α-hydroxylase enzyme activity and Cyp7b1 mRNA and protein. *Upper panel*, aliquots of pooled liver microsomes (100 μg of protein) were incubated with 25-[^{3}H]hydroxycholesterol (0.072 μM) and NADPH (1.5 mM) for 15 min. Sterols were extracted and analyzed by thin layer chromatography. The identities and positions to which radiolabeled sterols migrated to are shown on the *left* of the autoradiogram. *Middle panel*, 15-μg aliquots of poly(A)$^+$-enriched mRNA were electrophoretically separated on a 1.5% (w/v) agarose gel, transferred to a nylon membrane, and probed with a radiolabeled fragment of the Cyp7b1 cDNA. The washed filter was subjected to autoradiography for a period of 18 h. The positions to which size standards migrated to are shown on the *left* of the autoradiogram. Subsequent hybridization with a cyclophilin cDNA probe showed that each lane contained similar amounts of RNA. *Bottom panel*, aliquots of liver microsomal proteins (30 μg) were separated on a 10% (w/v) polyacrylamide gel containing SDS and transferred to a PolyScreen membrane (NEN Life Science Products). The membrane was incubated with a polyclonal antibody directed against a 15-amino acid peptide whose sequence was derived from the Cyp7b1 protein predicted from the cDNA. Primary antibody was detected on the membrane by enhanced chemiluminescence. The exposure time was 5 min.

assayed for both cholesterol and oxysterol 7α-hydroxylase mRNA levels as well as oxysterol 7α-hydroxylase enzyme activity and protein levels.

As expected from previous work (23, 24), colestipol-containing diets increased steady state levels of cholesterol 7α-hydroxylase mRNA (2.8-fold), as did the addition of cholesterol (1.4-fold) (Fig. 6*A*). Also, as expected, diets containing cholic acid or cholesterol plus cholic acid led to a marked suppression of the cholesterol 7α-hydroxylase mRNA (Fig. 6*A*). In contrast, dietary supplementation with either colestipol or cholesterol had little or no effect on oxysterol 7α-hydroxylase mRNA levels (Fig. 6*A*) or on enzyme activity or protein levels (Fig. 6*B*), whereas the addition of cholic acid or cholesterol plus cholic acid decreased the amount of oxysterol 7α-hydroxylase mRNA activity and protein levels by 40–70% (Fig. 6, *A* and *B*). Under these experimental conditions, oxysterol 7α-hydroxylase mRNA, protein, and activity closely paralleled one another.

All of these findings were consistent with Cyp7bl encoding an oxysterol 7α-hydroxylase that participates in bile acid biosynthesis. To determine the extent to which this enzyme activity, and hence the mitochondrial pathway leading to 7α-hydroxylated bile acids, was present in other vertebrates, liver microsomal membranes isolated from 14 different species were assayed for their ability to convert 25-[^{3}H]hydroxycholesterol into [^{3}H]7α-hydroxylated 25-hydroxycholesterol (Fig. 7). Oxysterol 7α-hydroxylase activity was detected in all species tested, including humans (*lane 1*), ungulates (*lanes 2–8*), carnivores (*lanes 9* and *10*), lagomorphs (*lane 11*), rodents (*lanes 12–16*), and aves (*lanes 17* and *18*). Among these, the highest levels of enzyme activity were detected in mouse, hamster, rabbit, and pig livers. In the sheep, enzyme activity was present in both sexes and in young and adult animals (*lanes 6–8*). In each species, the initial 7α-hydroxylated oxysterol product was further converted into a spectrum of metabolites that presumably reflected downstream intermediates of bile acid biosynthesis (Fig. 7).

DISCUSSION

In the current paper, we present several lines of evidence to support the hypothesis that the murine oxysterol 7α-hydroxylase of bile acid biosynthesis is identical with Cyp7b1, a microsomal cytochrome P450 enzyme expressed in multiple tissues. First, transfection of an expression vector containing the mouse Cyp7b1 cDNA into cells produces oxysterol 7α-hydroxylase enzyme activity. Second, the nafimidone inhibition profile of the mouse oxysterol 7α-hydroxylase activity in microsomes is identical to that of the recombinant enzyme produced in transfected cells. Third, oxysterol 7α-hydroxylase enzyme activity and Cyp7b1 mRNA and protein show concordant age-dependent induction patterns in mouse liver. Fourth, expression of oxysterol 7α-hydroxylase enzyme activity and Cyp7b1 mRNA and protein are down-regulated by dietary bile acid. We further show that oxysterol 7α-hydroxylase enzyme activity is widely distributed among vertebrate species and that the regulation of this enzyme is different from that of cholesterol 7α-hydroxylase.

The Cyp7b1 cDNA utilized in this study was identified in a screen for genes that were preferentially expressed in the rodent hippocampus (18). DNA sequence analysis revealed that the encoded protein shared 39% sequence identity with choles-

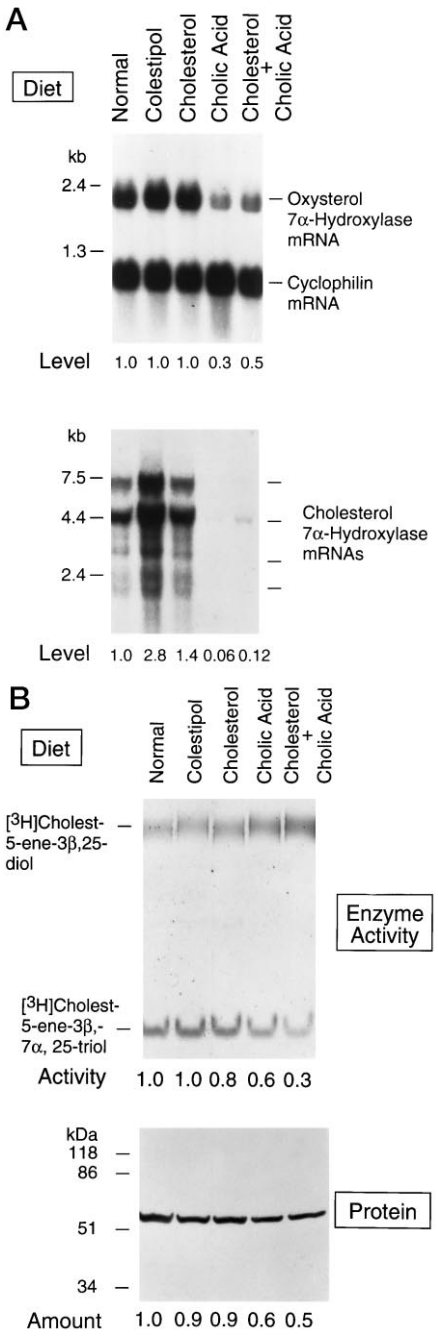

FIG. 6. **Effect of diet on hepatic oxysterol 7α-hydroxylase expression.** Livers were isolated from groups of mice maintained on diets supplemented with the indicated sterols or drug and assayed for the presence of oxysterol 7α-hydroxylase activity, mRNA and protein, and cholesterol 7α-hydroxylase mRNA. *A*, *upper panel*, oxysterol 7α-hydroxylase mRNA; *lower panel*, cholesterol 7α-hydroxylase mRNA. Poly(A)$^+$-enriched mRNA (15 μg) was first subjected to blot hybridization using radiolabeled fragments from the oxysterol 7α-hydroxylase and cyclophilin cDNAs. The washed filter was subjected to autoradiography for 18 h, stripped, and reprobed with a radiolabeled fragment from the cholesterol 7α-hydroxylase cDNA. The washed filter was subjected to autoradiography for 18 h. The positions of size standards are shown on the *left* of the *autoradiograms*. *Numbers below* the *autoradiograms* indicate the levels of 7α-hydroxylase mRNAs in each liver pool relative to those in normal diet-fed mice. *B*, *upper panel*, oxysterol 7α-hydroxylase enzyme activity. Aliquots of microsomal protein (100 μg) were incubated with 25-[^{3}H]hydroxycholesterol (0.072 μM) and 1.5 mM NADPH for 15 min at 37 °C. Radiolabeled sterols were separated by thin layer chromatography and visualized by autoradiography. The positions to which enzyme substrate and product migrated are shown to the *left* of the *autoradiogram*. Numbers *below* the autoradiogram represent the diet induced changes in enzyme activity relative to control mice. *Lower* panel, immunodetection of oxysterol 7α-hydroxylase protein. Liver microsomal proteins (100 μg) were separated on a 10% (w/v) polyacrylamide-SDS gel, transferred to a PolyScreen membrane (NEN Life Science Products), and blotted with an antipeptide antibody. Size standards are indicated on the *left*. *Numbers below* the *lumigram* indicate amounts of oxysterol 7α-hydroxylase protein in each liver pool relative to that in control mice.

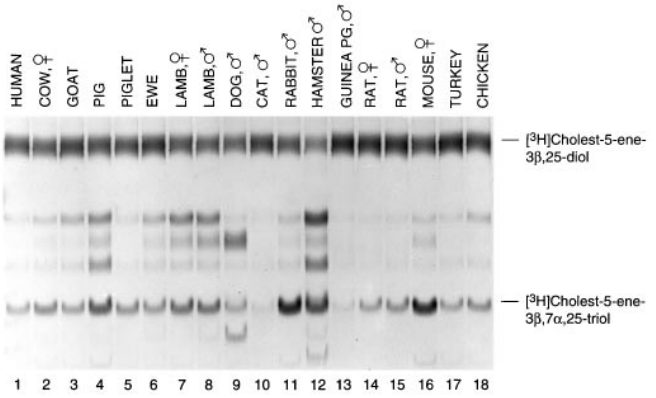

FIG. 7. **Hepatic expression of oxysterol 7α-hydroxylase in vertebrates.** Liver microsomes were isolated from the indicated species, and 200-μg aliquots of protein were combined with 0.12 μM 25-[^{3}H]hydroxycholesterol and 1.5 mM NADPH in a volume of 500 μl. After a 15-min incubation at 37 °C, reactions were stopped by the addition of organic solvent, and radiolabeled sterols were separated by thin layer chromatography on silica plates and visualized by autoradiography. The positions to which the substrate ([^{3}H]cholest-5-ene-3β,25-diol) and product ([^{3}H]cholest-5-ene-3β,7α,25-triol) migrated to are shown to the *right* of the *autoradiogram*.

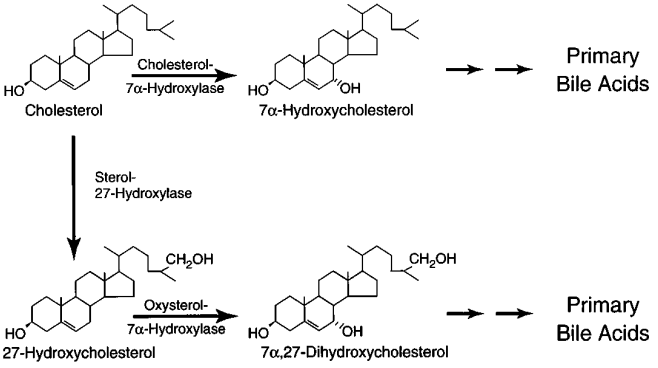

FIG. 8. **Two pathways of 7α-hydroxylated bile acid biosynthesis.** The substrates and products of three biosynthetic enzymes that participate in the initial steps of 7α-hydroxylated bile acid biosynthesis are shown. Subsequent steps leading to the formation of primary bile acids may be shared between the two pathways or distinct.

terol 7α-hydroxylase, which suggested that Cyp7b1 would also be capable of 7α-hydroxylating steroid substrates. This hypothesis has been confirmed with the findings that Cyp7b1 is capable of 7α-hydroxylating 25-hydroxycholesterol (Fig. 1) and the neurosteroids dehydroepiandrosterone and pregnenolone (20). In addition, a study published while this paper was in review reported that recombinant Cyp7b1 converted 27-hydroxycholesterol to cholest-5-ene-3β,7α,27-triol (25). Here, we show that the murine enzyme, when overexpressed in transfected cells, also is an oxysterol 2-hydroxylase (Figs. 2 and 3). It is presently not clear whether the latter activity is peculiar to the mouse enzyme, is substrate-specific, or represents only an *in vitro* detectable side activity. Nevertheless, taken together, these studies indicate that Cyp7b1 is a diverse enzyme capable of participating in the synthesis of neurosteroids in the brain and of multiple bile acids in the liver.

The biosynthesis of 7α-hydroxylated bile acids in the liver is

accomplished by two pathways that differ in their initial steps (Fig. 8). A microsomal pathway begins with the 7α-hydroxylation of cholesterol by the cytochrome P450 cholesterol 7α-hydroxylase (5), whereas a mitochondrial pathway begins with hydroxylation of the side chain of cholesterol by sterol 27-hydroxylase or other enzymes (11). The second step of the mitochondrial pathway involves 7α-hydroxylation of the oxysterol intermediate and is catalyzed by the oxysterol 7α-hydroxylase characterized here. The cDNA-derived sequence of this cytochrome P450 enzyme suggests that it is localized to the endoplasmic reticulum (18). This enzyme is expressed in the liver of many species (Fig. 7; Refs. 12–14 and 16) and in peripheral tissues like the rodent brain (18) and possibly ovary (15). The enzyme participates in the synthesis of 7α-hydroxylated bile acids in the liver of these species and in peripheral tissues; the oxysterol 7α-hydroxylase may work in concert with the similarly expressed sterol 27-hydroxylase enzyme (26, 27) to facilitate excretion of oxysterols (28).

The observation of redundancy in the enzymes that synthesize 7α-hydroxylated sterols suggests that bile acids with this substituent are pivotal in lipid metabolism. Most mice that are deficient in cholesterol 7α-hydroxylase die within the first 3 weeks of life, but those that survive this period thereafter have a normal life span (16). We previously proposed that another enzymatic pathway, specifically the oxysterol 7α-hydroxylase pathway, was able to at least partially compensate for the absence of cholesterol 7α-hydroxylase activity. This compensatory pathway is induced at approximately day 18 in the mouse, and expression of the oxysterol 7α-hydroxylase enzyme leads to the synthesis of 7α-hydroxylated bile acids (16). Our findings that oxysterol 7α-hydroxylase activity is present in many different vertebrates (Fig. 7), further support the physiological importance of 7α-hydroxylated bile acids.

The two hepatic pathways of 7α-hydroxylated bile acid biosynthesis are differentially regulated (Fig. 6). Cholesterol 7α-hydroxylase is induced by dietary cholesterol and by colestipol, which decreases the return of bile acids to the liver via the enterohepatic circulation. Dietary bile acid supplementation dramatically reduces cholesterol 7α-hydroxylase. In contrast, oxysterol 7α-hydroxylase remains unaffected by dietary cholesterol or colestipol and is only modestly down-regulated (~50%) by excess bile acids. Taken together, these data suggest that the liver is never without a capacity to synthesize 7α-hydroxylated bile acids, even when presented with an excess of the end product of the pathways.

What then is the crucial physiological role of 7α-hydroxylated bile acids? This stereochemistry may be important for some as yet undefined aspect of lipid uptake in the gut, or 7α-hydroxylated bile acids may be less cholestatic (toxic) to the liver than bile acids without a 7α-hydroxyl group. 7α-Hydroxylated bile acids, but not 7β-hydroxylated bile acids (29), mediate feedback regulation of cholesterol 7α-hydroxylase; thus, the 7α-configuration is of regulatory importance. We previously showed that mice deficient in cholesterol 7α-hydroxylase, and therefore dependent on the oxysterol 7α-hydroxylase/mitochondrial pathway for bile acid biosynthesis, contained abnormally low levels of vitamin E (16). Accordingly, chemical diversity arising from two pathways may be required to absorb the full complement of lipids and vitamins required for health. It is conceivable that as diets changed over time, the ability to synthesize a more diverse spectrum of bile acids was selected for, giving rise to the two pathways that are observed today in multiple vertebrates. In the future, we will search for additional pathways of bile acid biosynthesis and study the physiological roles of specific classes of bile acids in mice that are deficient in both cholesterol 7α-hydroxylase and the oxysterol 7α-hydroxylase.

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REFERENCES

1. Matschiner, J. T. (1971) in *The Bile Acids, Chemistry, Physiology, and Metabolism* (Nair, P. P., and Kritchevsky, D. eds) Vol. 1, pp. 11–46, Plenum Publishing Corp., New York
2. Haslewood, G. A. D. (1967) *J. Lipid. Res.* **8,** 535–550
3. Wachtel, N., Emerman, S., and Javitt, N. B. (1968) *J. Biol. Chem.* **243,** 5207–5212
4. Axelson, M., and Sjövall, J. (1990) *J. Steroid Biochem.* **36,** 631–640
5. Russell, D. W. and Setchell, K. D. R. (1992) *Biochemistry* **31,** 4737–4749
6. Hylemon, P. B., Stravitz, R. T., and Vlahcevic, Z. R. (1994) *Prog. Liver Dis.* **12,** 99–120
7. Jelinek, D. F., Andersson, S., Slaughter, C. A., and Russell, D. W. (1990) *J. Biol. Chem.* **265,** 8190–8197
8. Noshiro, M., Nishimoto, M., and Okuda, K. (1990) *J. Biol. Chem.* **265,** 10036–10041
9. Li, Y. C., Wang, D. P., and Chiang, J. Y. L. (1990) *J. Biol. Chem.* **265,** 12012–12019
10. Sundseth, S. S., and Waxman, D. J. (1990) *J. Biol. Chem.* **265,** 15090–15095
11. Björkhem, I. (1992) *J. Lipid Res.* **33,** 455–471
12. Axelson, M., Shoda, J., Sjövall, J., Toll, A., and Wikvall, K. (1992) *J. Biol. Chem.* **267,** 1701–1704
13. Martin, K. O., Budai, K., and Javitt, N. B. (1993) *J. Lipid Res.* **34,** 581–588
14. Toll, A., Wikvall, K., Sudjana-Sugiaman, E., Kondo, K., and Björkhem, I. (1994) *Eur. J. Biochem.* **224,** 309–316
15. Payne, D. W., Shackleton, C., Toms, H., Ben-Shlomo, I., Kol, S., deMoura, M., Strauss, J. F., and Adashi, E. Y. (1995) *J. Biol. Chem.* **270,** 18888–18896
16. Schwarz, M., Lund, E. G., Setchell, K. D. R., Kayden, H. J., Zerwekh, J. E., Björkhem, I., Herz, J., and Russell, D. W. (1996) *J. Biol. Chem.* **271,** 18024–18031
17. Ishibashi, S., Schwarz, M., Frykman, P. K., Herz, J., and Russell, D. W. (1996) *J. Biol. Chem.* **271,** 18017–18023
18. Stapleton, G., Steel, M., Richardson, M., Mason, J. O., Rose, K. A., Morris, R. G. M., and Lathe, R. (1995) *J. Biol. Chem.* **270,** 29739–29745
19. 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) *Pharmacogenetics* **6,** 1–42
20. Rose, K. A., Stapleton, G., Dott, K., Kieny, M. P., Best, R., Schwarz, M., Russell, D. W., Björkhem, I., Seckl, J., and Lathe, R. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 4925–4930
21. Turley, S. D., Herndon, M. W., and Dietschy, J. M. (1994) *J. Lipid Res.* **35,** 328–229
22. Dumaswala, R., Setchell, K. D., Zimmer-Nechemias, L., Iida, T., Goto, J., and Nambara, T. (1989) *J. Lipid Res.* **30,** 847–856
23. Rudling, M. (1992) *J. Lipid Res.* **33,** 493–501
24. Dueland, S., Drisko, J., Graf, L., Machleder, D., Lusis, A. J., and Davis, R. A. (1993) *J. Lipid Res.* **34,** 923–931
25. Martin, K. O., Reiss, A. B., Lathe, R., and Javitt, N. B. (1997) *J. Lipid Res.* **38,** 1053–1058
26. Skrede, S., Björkhem, I., Kvittingen, E. A., Buchmann, M. S., Lie, S. O., East, C., and Grundy, S. (1986) *J. Clin. Invest.* **78,** 729–735
27. Andersson, S., Davis, D. L., Dahlbäck, H., Jörnvall, H., and Russell, D. W. (1989) *J. Biol. Chem.* **264,** 8222–8229
28. Björkhem, I., Andersson, O., Diczfalusy, U., Sevastik, B., Xiu, R., Duan, C., and Lund, E. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 8592–8596
29. Shefer, S., Nguyen, L. B., Salen, G., Ness, G. C., Tint, G. S., Batta, A. K., Hauser, S., and Rani, I. (1991) *J. Biol. Chem.* **266,** 2693–2696