Cloning, Expression, and Regulation of Lithocholic Acid 6β-Hydroxylase*

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We have isolated a hamster liver cDNA whose expression is induced upon feeding hamsters with a cholic acid-rich diet. It was identified as a cytochrome P450 family 3 protein, by sequence homology, and named CYP3A10. The activity of CYP3A10 was determined by transient expression of its cDNA in transfected COS cells and was found to hydroxylate lithocholic acid at position 6β. CYP3A10 RNA is 50-fold higher in males than in female hamsters. In males, it appears to be regulated by age with expression highest after puberty. Shortly after weaning (28 days), cholic acid feeding of male hamsters elevates the level of message over that of hamsters fed with normal laboratory chow. Females do not exhibit regulation by cholic acid. In hamster liver, murideoxycholic acid, the 6β-metabolite of lithocholic acid, is the major hydroxylated product of lithocholic acid. Lithocholic acid 6β-hydroxylase (6β-hydroxylase) activity is diminished in hamster female liver microsomes as would be expected due to the lack of CYP3A10 mRNA in females. Additionally, male liver microsomal 6β-hydroxylase activity was increased by cholic acid feeding, consistent with the cholic acid-mediated induction of its RNA. These results indicate that, in male hamsters, 6β-hydroxylation is the major pathway for detoxification of lithocholate and that, likely, CYP3A10 is responsible for that activity.

The cytochrome P450 (P450)* superfamily is a group of heme proteins that catalyze the oxidation of a variety of endogenous and exogenous substrates in concert with NADPH and NADPH-dependent cytochrome P450 reductase (P450 reductase) (Gonzalez, 1989; Nebert et al., 1991). Many P450s are involved in very different metabolic pathways such as steroid hormone metabolism (Gonzalez, 1989; Nebert et al., 1991), drug detoxification (Guengerich, 1987; Shaw et al., 1989; Shimada and Guengerich, 1985), and bile acid synthesis (Björkhem, 1985; Björkhem and Danielsson, 1974) and are regulated by poorly understood molecular mechanisms that induce or suppress expression or activity. Some P450s are subject to complex endocrine regulation that renders their expression tissue- and sex-specific and/or developmentally programmed (Gonzalez, 1989). The molecular details involved in any of these regulatory mechanisms have yet to be identified.

Among the systems of special interest is the involvement of P450s in cholesterol homeostasis, specifically in converting cholesterol to bile acids for their subsequent elimination from the body (see Fig. 1). Since mammals do not possess enzymes that degrade cholesterol absorbed from the diet, it must be eliminated via the bile acid metabolic pathway (Dietschy, 1984). It is known that 80–90% of intravenously injected cholesterol is catabolized to bile acids (Björkhem, 1985). Atherosclerosis, gallstone disease, and some lipid storage diseases may be affected by the rate of cholesterol elimination via bile acid biosynthesis and excretion (Björkhem, 1985).

Cholic acid and chenodeoxycholic acid are the primary bile acids synthesized by a number of hydroxylations of the steroid nucleus and side chain of the cholesterol molecule (see Fig. 1). These hydroxylations are catalyzed by different liver cytochrome P450s. Primary bile acids are then converted in the gut lumen by microbial enzymes to secondary bile acids such as lithocholic acid and deoxycholic acid by reduction of the 7α-hydroxyl group (Danielsson and Sjövall, 1975).

Lithocholic acid is a toxic bile acid that has been shown to cause cholestasis in experimental animals (Miya et al., 1975). Indeed, a clinical hallmark of cholestasis is the dramatic increase in serum bile acids, including lithocholic acid. The deleterious effects of this accumulation of lithocholic acid, as well as others, can be attenuated by two detoxification pathways found in the liver: oxidative reactions, mainly hydroxylations, and conjugation with sulfuric or glucuronic acids (Summerfield et al., 1976). Hydroxylation of bile acids makes them more hydrophilic and thus easily excreted in urine or feces. Lithocholic acid can be hydroxylated at position 6 (Fig. 1), either below the plane (α) or above the plane (β), by the enzymes lithocholic acid 6α-hydroxylase (6α-hydroxylase) and lithocholic acid 6β-hydroxylase (6β-hydroxylase) (Hoffman, 1988). In rats, the major hydroxylated product of lithocholic acid is the 6β-metabolite, murideoxycholic acid (Zmijak et al., 1989).
**Bile acid metabolic pathway.** A highly schematic depiction of the bile acid metabolic pathway is shown. Conversion of cholesterol to 7α-hydroxycholesterol by cholesterol 7α-monooxygenase (EC 1.14.13.17) is the initial and rate-limiting step in bile acid synthesis. 7α-Hydroxycholesterol undergoes subsequent enzymatic conversions, thus have been omitted, in its route to becoming the primary bile acids, cholic acid and chenodeoxycholic acid. Secondary bile acids, lithocholic acid and deoxycholic acid, are synthesized in the gut lumen from the primary bile acids by microbial enzymes and are efficiently reabsorbed into the enterohepatic circulation. In the liver these bile acids can be further hydroxylated for facilitated excretion. Shown are the enzymes and known metabolites involved in the 6-hydroxylation of secondary bile acids.

The perturbation of bile acid metabolism can be a consequence of biliary obstruction and alcoholism (Bremmelgaard et al., 1982), and liver disease and unknown genetic factors (Shoda et al., 1990), whereby the levels of 6-hydroxylated products of bile acids are dramatically elevated in serum and urine. The general evidence for this observation is in clinical cholestatics where the 6-hydroxylation of different bile acids leads to their 6α- and 6β-derivatives. These bile acids are found in substantial amounts in the urine and serum of patients with cholestasis and are not found in normal healthy individuals. It would seem logical that 6-hydroxylation can act as a bile acid-induced mechanism for excretion of excess bile acids due to biliary obstruction and liver dysfunction.

In order to better understand how the synthesis and catabolism of bile acids are regulated and, thus, develop a means for studying bile acid metabolism and the perturbation thereof, we are undertaking an effort to study the regulation of different enzymes involved in bile acid metabolism. We report here the isolation and sequence of a cDNA that encodes lithocholic acid 6β-hydroxylase. In accordance with the recommended nomenclature for P450s (Nebert et al., 1991), this gene has been named CYP3A10. Expression of this gene is 50-fold higher in males than in females. In young males, expression of CYP3A10 is induced by cholic acid feeding, in contrast to the bile acid-mediated suppression of the rate-limiting enzyme of bile acid synthesis, cholesterol 7α-monooxygenase (EC 1.14.13.17) (Jelinek and Russell, 1990; Li et al., 1990; Sundseth and Waxman, 1990).

**EXPERIMENTAL PROCEDURES**

**Materials**—32P-Labeled nucleotides were purchased from Du Pont-New England Nuclear and [14C]lithocholic acid from Amersham Corp. Thin layer chromatography plates were from Merck. Reagents used in cDNA cloning and sequencing were from New England BioLabs, Boehringer Mannheim, U. S. Biochemical Corp., or Gibco/BRL.

Subtractive hybridizations were done with reagents from Invitrogen, San Diego, CA. Authentic murideoxycholic acid was purchased from Steraloids, Wilton, NH and other bile acids from Sigma. Common laboratory chemicals were from Fisher, Sigma, or Bio-Rad. Golden Syrian hamsters were purchased from Charles River Laboratories, Wilmington, MA. DEAE-cellulose and LiCl-LiOH were from Pharmacia LKB Biotechnology Inc. Colestipol was purchased from The Upjohn Co.

**General Experimental Procedures—**Standard recombinant DNA procedures were carried out essentially as described by Sambrook et al. (1989). DNA probes were labeled by random hexanucleotide priming (Feinberg and Vogelstein, 1983) or by primer extension with M13 clones and the universal primer (Church and Gilbert, 1984). Nucleotide sequencing was done by the dideoxy chain termination method (Sanger et al., 1977) with M13 universal primers and the Klenow fragment of *Escherichia coli* polymerase I after subcloning into bacterial M13 vectors (Sambrook et al., 1989). Protein was quantified by the method of Bradford (Bradford, 1976). Total cytochrome P450s were quantified according to Anderson (1985).

**Preparation of RNA and Microsomes from Liver—**Golden Syrian hamsters were maintained on a 12-h light/12-h dark cycle, fed either regular laboratory chow or chow supplemented with 0.5% (w/w) cholic acid or 4% (w/w) colestipol for 5 days and were put to death at the middle of the dark cycle. Their livers were removed and used to prepare total cellular RNA by guanidine thiocyanate homogenization followed by centrifugation through a cesium chloride cushion (Chirgwin et al., 1979). Liver microsomes were prepared by differential centrifugation of the livers of 4-week-old hamsters fed with diets as for RNA preparation. The liver was homogenized in 5 volumes/g homogenization buffer (10 mM Tris acetate, pH 7.4, 1 mM EDTA, and 0.25 M sucrose) with 10 strokes of a Dounce homogenizer. The homogenate was centrifuged at 27,000 × g for 20 min at 4 °C. The supernatant was centrifuged at 100,000 × g for 1 h at 4 °C in a refrigerated ultracentrifuge. The resulting pellet was resuspended in one-fifth the original volume of homogenization buffer and quickly frozen for later use.

**Northern and Slot Blot Analyses—**Northern blot hybridizations were performed essentially as described (Sambrook et al., 1989). Briefly, 10-µg aliquots of RNA were denatured at 65 °C with glyoxal and sodium metabisulphite and electrophoresed overnight through an agarose gel buffered with 10 mM sodium phosphate, pH 7, and blotted onto a nylon membrane overnight. When slot blot analyses were done, 10 µg of RNA was denatured in an equal volume of 18% formaldehyde, 10 × SSC (1 × SSC, 150 mM NaCl, 15 mM sodium citrate) at 65 °C and spotted onto nylon. Blots were dried in a vacuum oven at 80 °C and prehybridized in a 50% formamide hybridization solution at 42 °C. 32P-Labeled probes were added to the solution at 2 × 106 cpm/ml and hybridized overnight. The blots were washed at 65 °C with 0.1 × SSC plus 0.1% sodium dodecyl sulfate and exposed to film at −70 °C. An autoradiographic slot blot analyzer was used to directly measure counts/min/band or slot.

**Preparation, Subtraction, and Screening of Libraries—**Poly(A)-RNA (10 µg) was selected from total RNA by two cycles of oligo (dT)-cellulose chromatography (Chirgwin et al., 1979) and used to prepare cDNA libraries from the livers of male hamsters fed colestipol (library A) or cholic acid (library B) as described above. After second strand synthesis, BstXI non-palindromic linkers were added by ligation, excess linkers removed, and cDNAs ranging in size from 1 to 8 kb size-selected by agarose gel electrophoresis. The selected cDNAs were eluted and ligated to pCDNAII, a plasmid vector that contains the M13 origin of replication to facilitate single strand synthesis (Duguid et al., 1988). The libraries were transformed into DH1α cells by electroporation generating 2 × 106 clones each before amplification.

Libraries were substracted according to the method of Duguid et al. (1988). Briefly, single-stranded DNA from the library B was biotinylated with photobiotin and UV irradiation and hybridized for 24 h with the single-stranded DNA from library A at 68 °C. After hybridization was complete, clones common to both libraries were precipitated by addition of streptavidin. The subtracted library was transformed into DH1α cells by electroporation (Potter et al., 1984) and used for screening.

The subtracted library was plated onto nitrocellulose, and replicas of each plate were made. Cells were lysed and the DNA denatured and immobilized on the filter. Filters were prehybridized overnight with 50% formamide, 1× SSC, 1% sodium dodecyl sulfate, and then hybridized at 50°C according to Sambrook et al. The set of replicas was probed with hexamer-primed, 32P-labeled cDNA library A subtracted with cholic acid-fed hamster liver mRNA. The other set was probed with cDNA library B subtracted with colestitol.
fed hamster liver mRNA and labeled as above with equal specific activity. After hybridization, filters were washed in 0.1 % SSC, 0.1 % sodium dodecyl sulfate at 65 °C and exposed to x-ray film. Plasmids were prepared from putative positives displaying different signal intensities. These were labeled by random hexanucleotide priming and an RNA blot was prepared with RNA from colesterol-fed and cholic acid-fed animals. Screening was completed when one clone, pFR29, displayed approximately 4-fold induction with RNA from cholic acid-fed animals versus RNA from normal or colesterol-fed animals.

Subcloning, Sequencing, and Analysis of FR29—pFR29-1 was digested at sites astride the BstXI cloning site of pDNAII with BamHI and XhoI and the 1.1-kb fragment inserted into M13 vectors. After Sanger dideoxy sequencing (Sanger et al., 1977), a 5'-end 200-bp fragment was isolated by digestion with BstXI and the 2.5-kb fragment inserted into the EcoRI site of pCMV (pFR29-5), an expression vector that contains the cytomegalovirus (CMV) promoter and facilitates expression of the cDNA in mammalian cells (Anderson et al., 1989). Complete sequencing of both strands of the full-length cDNA was accomplished by subcloning several fragments into M13 for Sanger dideoxy sequencing. DNA analyses were performed with Microgenie™ software from Beckman Instruments.

Transient Expression in COS Cells—COS cells were transfected by the DEAE-dextran method (Sambrook et al., 1989). The essentials or modifications are as follows. COS cells were seeded on day 0 at a density of 5 × 10^6 cells per 10-cm plate in Ham's F12 medium, 10% fetal calf serum, and 20 mM HEPES. The following day, cells were transfected for 30 min with 10 μg of pFR29-5, pCMV, and/or NADPH-cytochrome P450 reductase cDNA, inserted in the same pCMV vector in 500 μg/ml DEAE-dextran. Cells were then incubated at 37 °C for 3 h in media supplemented with 100 mM chloroquine followed by a 4-min shock with 20% (v/v) glycerol media. After 48 h of incubation at 37 °C, the cells were washed in ice-cold phosphate-buffered saline and harvested. Microsomes were prepared from the collected cells as for liver except that the first centrifugation was done at 2,000 × g.

Assay of Lithocholic Acid 6-Hydroxylase Activity—Lithocholic acid was prepared for substrate delivery by diluting 7 nmol of 14 Ci/mol [carboxy-^1^C]lithocholic acid in 20 μl of 1 mg of dilauroyl phosphatidylcholine/ml of tolune, dried down under Ne2, and resuspended in 250 mM potassium phosphate, pH 7. Incubations were done under linear conditions in 250 mM potassium phosphate, pH 7 at 37 °C, with 1 mM NADPH and microsomes from COS cells for 1 h or liver microsomes for 20 min. Reactions were stopped by dilution with 500 μl of ethanol, acified with 20 μl of 0.1 N HCl, and extracted with 5 ml of methyl ether (Danielsson, 1973). The ether phase was transferred, dried down under Ne2, and reextracted in ethanol for spotting on Silica Gel G TLC plates or for reversed phase high performance liquid chromatography. The plates were chromatographed with authentic standards in a sealed tank with isooctane:ethyl acetate:acetic acid (5:5:1) (Eneroth, 1963), dried, and exposed to x-ray film overnight. Radioactive spots were excised from the plate and counted in a liquid scintillation counter or else quantified by a Betagen Petascope analyzer to ensure accuracy and normalizing to the extent of a given sequencing experiment. Both strands of the entire cDNA were sequenced by the dideoxy chain termination method.

RESULTS

Using differential hybridization techniques (see "Experimental Procedures") we have isolated a hamster liver cDNA (initially called pFR29) whose expression is induced by cholic acid feeding. Fig. 2 is a schematic representation of the isolated cDNAs and the strategy used to determine its nucleotide sequence. The complete nucleotide sequence of pFR29-2 and the predicted amino acid sequence of the protein are shown in Fig. 3. pFR29 encodes a 503-amino acid protein with 76 nucleotides in the 5'-untranslated region and 430 nucleotides in the 3'-untranslated region. Its deduced amino acid sequence contains a putative heme-binding domain present in all mammalian P450s characterized to date (Gonzalez, 1989) as well as the residues that direct the specificity of P450s for steroids (Gotoh and Fujii-Kuriyama, 1989).

Computer analysis of the sequence revealed that the deduced amino acid sequence of pFR29 was highly homologous to P450 family 3 proteins. This homology approaches 70% with other members of the family previously isolated from human, rat, and rabbit. In Fig. 4, the alignment of the amino acid sequence of FR29 is shown with that of CYP3A1/bNL, the first of the P450 family 3 proteins whose cDNA was isolated (Gonzalez et al., 1985). Because of this homology, the isolated cDNA has been named CYP3A10 according to the recommended nomenclature system (Nebert et al., 1991).

RNA blot analysis was performed in order to determine the extent of regulation by cholic acid of the steady state level of CYP3A10 mRNA. The autoradiogram of a typical experiment is shown in Fig. 5. The size of the message, 2.1 kb, corresponds to that predicted from the sequence. Regulation of CYP3A10 was determined by measuring the counts/min/band with a Betascope analyzer to ensure accuracy and normalizing to the hybridizable actin mRNA expressed in the same manner. (Fig. 5B) Expression of CYP3A10 mRNA is much higher (50-fold) in males (lanes 1–8) than in females (lanes 9–16); in fact, only after exposure of the blot for 4 days (versus 2 h for the males) can CYP3A10 mRNA be detected in female RNA (data not shown). In young males (28 days old) CYP3A10 mRNA was induced approximately 3-fold by cholic acid feeding (lanes 7 and 8) in this particular experiment (see other experiments below). There was a slight induction (20%) by cholic acid in males at 7 weeks of age (lanes 5 and 6) and no regulation at other ages (lanes 1–4). In females, in addition to the much lower expression of CYP3A10, its RNA is not regulated by cholic acid feeding at any age.

Since family 3 proteins are highly homologous, at least in rat, we eliminated the possibility that our probes might be hybridizing to more than one RNA species under the conditions we normally do the RNA blot analyses (50% formamide hybridization solution and 0.1 × SSC, 65 °C washes) by performing a Southern analysis (Fig. 6) with hamster genomic DNA. When we probed one blot with the 5'-end EcoRI fragment (bp −71 to 166) of the CYP3A10 cDNA (panel A), two bands were visualized. This was expected since we have
observed an intervening sequence 73 bp (see Fig. 3) into the coding region of pFR29–2 that contains an EcoRI site.2 When a parallel blot was probed with the 3'-end fragment (bp 1718 to 2010) of the FR29 cDNA (panel B), we observed a single band in each lane. This probe was generated from the 3'-untranslated region of the cDNA where one would not expect there to be an intron. This strongly suggests that the conditions we used to do the RNA blot analyses were such that only the product of one gene was being detected however, considering the highly homologous nature of P450s in other species we cannot unequivocally rule out the possibility that we may be hybridizing to more than one RNA. Additionally, when the same analysis was done under lower stringency conditions (35% formamide hybridization solution and 2 × SSC, 55 °C washes), an innumerable amount of bands were visualized (data not shown), presumably due to hybridization with genes that are homologous to CYP3A10, including other family 3 members.

To determine the nature of the biological activity encoded by CYP3A10, we performed transient expression experiments in COS cells (Fig. 7). We placed CYP3A10 cDNA in the expression vector pCMV (Andersson et al., 1989) that contains the CMV promoter as well as the simian virus 40 (SV40) origin of replication. We transfected COS cells with either the

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Computer analysis of the amino acid sequences produced no activity that converts lithocholic acid with that of rat pen1, a well characterized family 3 protein. They share identity (indicated by a 0 in 69% of their residues, and only one gap in the sequence encoded by FR29 revealed that it shares a high degree of homology with that of rat pen1 and lithocholic acid 6-hydroxylase with that of rat pen1 and lithocholic acid 6-hydroxylase, considering both identical and conserved (indicated by a ½) amino acids increases to 81%. Planar vector pCMV, another construct that contains the CYP3A10 cDNA, or both CYP3A10 and P450 reductase cDNAs. About 48 h after transfection, cells were harvested and microsomes were prepared and assayed for lithocholic acid 6-hydroxylase activity (see "Experimental Procedures"). The reaction products were identified by com-

**Fig. 4. Amino acid alignment of CYP3A10/lithocholic acid 6β-hydroxylase with that of CYP3A1/pen1.** Computer analysis of the amino acid sequence encoded by FR29 revealed that it shares a high degree of homology with that of P450 family 3 proteins. They share identity (indicated by a 0 in 69% of their residues, and only one gap in the sequence encoded by FR29 revealed that it shares a high degree of homology with that of rat pen1 and lithocholic acid 6-hydroxylase with that of rat pen1 and lithocholic acid 6-hydroxylase, considering both identical and conserved (indicated by a ½) amino acids increases to 81%.

<table>
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<th>Female</th>
<th>Sex</th>
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<th>Control Diet</th>
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**Fig. 5. Regulation of lithocholic acid 6β-hydroxylase mRNA.** RNA was prepared from the indicated sources and analyzed by Northern analysis as described under "Experimental Procedures." λ/HindIII-digested DNA was used as a standard and visualized by ethidium bromide staining, and the relative mobilities of the fragments are indicated on the right. Panel A, single-stranded, 32P-labeled probes complementary to lithocholic acid 6β-hydroxylase mRNA were prepared as described under "Experimental Procedures" from the 5' EcoRI fragment (bp -71 to 186) of the cDNA and used to detect CYP3A10 RNA. Exposure to x-ray film was done for 1 h at -70 °C with intensifying screens. Panel B, a parallel blot containing the same RNAs was treated as above but hybridized with a single-stranded actin probe (Ma et al., 1986) and exposed for 4 h to film as above.

parental vector pCMV, another construct that contains the P450 reductase cDNA placed in pCMV, our construct containing the CYP3A10 cDNA, or both CYP3A10 and P450 reductase cDNAs. About 48 h after transfection, cells were harvested and microsomes were prepared and assayed for lithocholic acid 6-hydroxylase activity (see "Experimental Procedures"). The reaction products were identified by comparing their migration distances in a TLC system with those of authentic 6α-hydroxy-(hydroxyoxocholic acid) and 6β-hydroxylithocholic acid (murideoxyoxocholic acid). COS cells transfected with vector DNA (lane 1) or with P450 reductase cDNA alone (lane 2) produced no activity that converts lithocholic acid. Cells transfected with CYP3A10 cDNA (lane 3) converted 4% of the lithocholic acid used in the assay to a product that migrated with authentic murideoxyoxocholic acid. Cells transfected with both CYP3A10 and P450 reductase cDNAs (lane 4) converted about 15% of the added substrate to the same product. Two other products were visualized and have yet to be identified. Reversed-phase HPLC confirmed that the major radioactive product of the CYP3A10, reductase-transfected COS cell microsomes assay was murideoxyoxocholic acid with 5% of total counts/min loaded recovered as such (data not shown).

Fig. 7 also shows the lithocholic acid hydroxylase activity of liver microsomes from hamsters of either sex, fed with a control diet or the same diet supplemented with cholic acid (lanes 5–8). Cholic acid feeding, in male hamsters, induced 6β-hydroxylase activity by approximately 50–100% (lanes 5 and 6), consistent with the observed induction of CYP3A10 RNA (see Fig. 5). There was no observable induction of 6β-hydroxylase activity in females (lanes 7 and 8). Additionally, transfection with both CYP3A10 and P450 reductase cDNAs (lane 4) converted about 15% of the added substrate to the same product. Two other products were visualized and have yet to be identified. Reversed-phase HPLC confirmed that the major radioactive product of the CYP3A10, reductase-transfected COS cell microsomes assay was murideoxyoxocholic acid with 5% of total counts/min loaded recovered as such (data not shown).
TABLE 1: TRANSFECTED COS CELLS

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<tr>
<th>Vector</th>
<th>CYP3A10</th>
<th>F450 Reductase</th>
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TABLE 2: LIVER MICROSOMES

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<tr>
<td>Female</td>
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**Fig. 7.** Lithocholic acid 6β-hydroxylase activity in transfected COS cells and in hamster liver microsomes. Lithocholic acid 6β-hydroxylase activity was measured as described under “Experimental Procedures” with 100 μg of microsomal protein prepared from COS cells transfected with the indicated DNA or with 20 μg of hamster liver microsomal protein from the indicated sources. The zones corresponding to the migration distance of authentic standards are indicated on the left. The specific activity of 6β-hydroxylase was 0.001, 0.001, 0.014, 0.089, 0.339, 0.544, 0.076, and 0.090 nmol/min·mg of protein (lanes 1–8, respectively).

There was an induction of 6α-hydroxylase activity by cholic acid feeding.

A statistical analysis of the cholic acid-mediated induction of CYP3A10 RNA and lithocholic acid 6β- and 6α-hydroxylase activities was done, the results of which are shown in Fig. 8. In this experiment, hamsters (n = 15) were fed either standard laboratory chow or the same diet supplemented with 0.5% cholic acid for 5 days, and their livers were harvested for RNA and microsomes as described under “Experimental Procedures.” Slot blot analyses and 6β-hydroxylase assays were done with RNA and microsomes from the individual animals. We observed a 68% increase in CYP3A10 mRNA (solid bars) and a 110% increase in lithocholic acid 6β-hydroxylase activity (stippled bars), p < 0.025 and p < 0.0005, respectively. We also observed a 130% induction of lithocholic acid 6α-hydroxylase (data not shown). The induction in CYP3A10 RNA was less than the 3-fold observed in Fig. 5, which depicts the results of an experiment with a pool of RNA from three animals. We believe this is due to the high variability of CYP3A10 expression observed in individual animals which necessitated experimentation with a large number of animals and individual analysis to obtain statistically significant data.

**Discussion**

In the current paper, we report the isolation and nucleotide sequence of a full-length cDNA for the enzyme lithocholic acid 6β-hydroxylase. The cDNA has been identified as 6β-hydroxylase based on its ability to program the synthesis of active enzyme upon transfection of the cDNA when placed after the CMV promoter in COS cells (Fig. 7). Other circumstantial evidence supports this finding. First, by amino acid sequence homology, the isolated cDNA CYP3A10 belongs to family 3 of the P450 gene superfamily (Fig. 4), and all the members of that family characterized to date catalyze hydroxylation of different steroid substrates at position 6 (Gonzalez, 1989). Second, the major hydroxylation product of lithocholic acid in male hamsters is at position 6 (Fig. 7) as it is in both rats (Zimniak et al., 1989) and humans (Ritmelska et al., 1990). Third, our results in hamster agree with previous reports that cholic acid feeding to rats (Danielsson, 1973) induced lithocholic acid 6β-hydroxylase activity in rat liver microsomes. We have extended these observations by demonstrating a concomitant increase in message for CYP3A10 associated with increased lithocholic acid 6β-hydroxylase activity, indicating the induction is at the pretranslational level. It would not be unexpected for CYP3A10 to have other natural substrates since many P450s have broad substrate specificities, and absolute identification of the preferred or physiological substrate of any P450s may prove a formidable task. However, we have determined the K<sub>m</sub> for 6β-hydroxylase from hamster liver microsomes to be approximately 3 μM (data not shown), within the concentration range reported for lithocholic acid in rat liver (Kurtz et al., 1982). We, therefore, fully expect lithocholic acid to be a physiological substrate for CYP3A10.

Several new insights into bile acid metabolism are deduced from these studies. First, in this study, we unequivocally characterized lithocholic acid 6β-hydroxylase as a P450 family 3 protein, CYP3A10. This was done based on the degree of homology with other family 3 proteins (references found in Nebert et al. (1991)). Previous studies hypothesized that 6β-hydroxylation of lithocholic acid could be catalyzed by a P450 family 2 or 3 protein, based on immunological data (Zimniak et al., 1991). At the amino acid level, CYP3A10 is roughly 70% homologous with the other known members of this family. Due to its activity on lithocholic acid, we believe CYP3A10 is probably a new member of P450 family 3 rather than the hamster homolog of a previously characterized gene.
The best characterized members of the family, CYP3A11/pcn1 and CYP3A5/pcn3, prefer steroid hormone substrates and are not the rat lithocholic acid 6β-hydroxylase (Radinmska et al., 1990). Absolute identification of CYP3A10 as nonorthologous to other family 3 proteins characterized to date would necessitate the isolation of the rat lithocholic acid 6β-hydroxylase cDNA.

Second, our results suggest that different genes encode male and female 6β-hydroxylase isoenzymes. The level of CYP3A10/6β-hydroxylase mRNA is 50-fold higher in male versus female hamsters (Fig. 5), although the level of 6β-hydroxylase activity is only 5-fold lower in females (Fig. 7), which agrees with the 2-fold lower 6β-hydroxylase activity in females versus males described in rats (Zimniak et al., 1991). Interestingly, the level of chenodeoxycholic acid, the immediate precursor of lithocholic acid, is significantly higher in female rat liver homogenate (Kurtz et al., 1982; Youssef et al., 1972). This is probably due to the ability of male rat liver to readily convert chenodeoxycholic acid to a more excretable metabolite (Youssef et al., 1973). Sex differences in the enzymes involved in bile acid metabolism have been reported for conjugation (Barnes et al., 1979), oxidation (Björkhem, 1985), and bile acid-binding proteins in the liver (LeBlanc and Waxman, 1990), and it certainly would not be surprising if that were the case for GB-hydroxylase. The sex differences we have demonstrated in hamsters, 50-fold higher in females described in rats, 50-fold higher in livers of female hamsters, although the level of GB-hydroxylase isoenzymes. The level of GB-hydroxylase isoenzymes. The level of GB-hydroxylase isoenzymes. The level of lithocholic acid GB-hydroxylase activity in rat liver microsomes that indicates chenodeoxycholic acid feeding suppresses lithocholic acid 6β-dehydroxylation form of deoxycholic acid (the 7α-dehydroxylation form of cholic acid) may get converted to lithocholic acid by a 12α-dehydroxylation reaction. Experiments are in progress to study these possibilities.

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