Cloning and Regulation of Cholesterol 7α-Hydroxylase, the Rate-limiting Enzyme in Bile Acid Biosynthesis*

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The rate-limiting step in bile acid biosynthesis is catalyzed by the microsomal cytochrome P-450 cholesterol 7α-hydroxylase (7α-hydroxylase). The expression of this enzyme is subject to feedback regulation by sterols and is thought to be coordinately regulated with enzymes in the cholesterol supply pathways, including the low density lipoprotein receptor and 3-hydroxy-3-methylglutaryl-coenzyme A reductase and synthase. Here we report the purification of rat 7α-hydroxylase and the determination of a partial amino acid sequence. Oligonucleotides derived from peptide sequence were used to clone a full-length cDNA encoding 7α-hydroxylase. DNA sequence analysis of the cDNA revealed a 7α-hydroxylase protein of 503 amino acids with a predicted molecular weight of 56,890 which represents a novel family of cytochrome P-450 enzymes. Transfection of a 7α-hydroxylase CDNA into simian COS cells resulted in the synthesis of a functional enzyme whose activity was stimulated in vitro by the addition of rat microsomal cytochrome P-450 reductase protein. RNA blot hybridization experiments indicated that the mRNA for 7α-hydroxylase is found only in the liver. The levels of this mRNA increased when bile acids were depleted by dietary cholestyramine and decreased when bile acids were consumed. Dietary cholesterol led to an increase in 7α-hydroxylase mRNA levels. The enzymatic activity of 7α-hydroxylase paralleled the observed changes in mRNA levels. These results suggest that bile acids and sterols are able to alter the transcription of the 7α-hydroxylase gene and that this control explains the previously observed feedback regulation of bile acid synthesis.

Cholesterol homeostasis in mammals represents a delicate balance between pathways of supply and catabolism. In the liver, the chief organ of cholesterol metabolism (1), supply is accomplished by a receptor-mediated pathway (2) and by the de novo synthesis of cholesterol from acetate precursors (3). A single pathway of catabolism involves the conversion of cholesterol into hydrophilic bile acids that are subsequently excreted from the body via the bile and intestine (4). Although the regulatory mechanisms underlying the expression of key enzymes in the cholesterol supply pathways are beginning to be understood (5–8), little is currently known about similar molecular mechanisms in cholesterol catabolism or about the coordinate regulation of the three pathways.

Bile acids play two opposing roles in the maintenance of cholesterol homeostasis. In one role, they are the end products of cholesterol catabolism, and their biosynthesis and excretion serve to decrease the levels of cholesterol in the liver. In a second role, their presence in the intestine facilitates the solubilization of dietary fats and cholesterol and the subsequent uptake of these essential nutrients (4). In this manner, bile acids increase whole body cholesterol levels. These observations suggest that the production of bile acids in the liver must be tightly regulated in order to balance these two antagonistic functions.

The synthesis of bile acids is carried out by at least 10 enzymes in the liver which hydroxylate the four-ring structure of cholesterol and shorten and oxidize the side chain (9). In the human and rat, the major products thus formed are the primary bile acids, cholic acid and chenodeoxycholic acid (Fig. 1). The rate-limiting step in this pathway involves the introduction of a hydroxyl moiety at the 7 position of cholesterol (10) and is catalyzed by cholesterol 7α-hydroxylase (7α-hydroxylase,1) Fig. 1), a microsomal enzyme that is a member of the cytochrome P-450 family. Once formed, 7α-hydroxycholesterol is rapidly converted into a primary bile acid, as few of the subsequent enzymatic steps appear to be regulated (9). The activity of 7α-hydroxylase is subject to end product repression by bile acids in the enterohepatic circulation (10). Thus, in animals maintained on a diet containing bile acids, the level of 7α-hydroxylase activity is reduced (10). Conversely, animals fed drugs such as cholestryamine, which enhance the excretion of bile acids (11), increase their hepatic levels of this enzyme and hence the production of bile acids (10). Because of the complexity of the enzyme assay and the lack of an independent measure of 7α-hydroxylase protein, the changes in enzyme activity have not yet been shown unequivocally to result from changes in the amount of enzyme as opposed to changes in its activity. Moreover, nothing is known about the regulation of the 7α-hydroxylase mRNA.

Recently, a preliminary report describing the purification and cloning of the rat 7α-hydroxylase was published by Okuda and colleagues (12). In the current studies, we confirm and extend this work by reporting the purification of rat 7α-hydroxylase and the subsequent determination of a partial protein sequence. These biochemical tools were then used to isolate cDNA clones that in turn allowed the determination of the structure of the protein and the expression of the

1 The abbreviations used are: 7α-hydroxylase, cholesterol 7α-monooxygenase (EC 1.14.13.1); DT T, dithiothreitol; SDS, sodium dodecyl sulfate; k b, kilobase(s); HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; HPLC, high performance liquid chromatography.

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Different enzymes (9). Cholesterol 7a-hydroxylase converts cholesterol into 7a-hydroxycholesterol and constitutes the rate-limiting step in the pathway.

Enzyme Assay—Cholesterol 7a-hydroxylase activity was assayed in the presence of 5-10 µg of [14C]cholesterol (6.6 Ci/mmol) in a 0.5-ml volume containing 50 mM Tris-acetate (pH 7.5), 20% glycerol, 1 mM EDTA, 2 mM dithiothreitol (DTT), 0.06% Triton X-100, 2 mM NADPH, and 1000 units of purified NADPH-cytochrome P-450 reductase. Reactions were carried out for 5-20 min at 37°C followed by extraction with either methylene chloride (purified fractions) or Pronto-Sol (reformulated methanol). The chlortohol. 2:1, COS cell extract phase was dried under an N2 stream at 50°C, resuspended in 100 µl of aceton, and subjected to thin layer chromatography in ethyl acetate/toluene (3:2, v/v). Autoradiography was used to identify areas on the plates corresponding to the various metabolites. These areas were scraped, and radioactivity was determined by liquid scintillation counting. The identities of the products were determined by comparison with the Rf values of known standards.

Preparation of Liver Microsomes—Freshly isolated rat livers were washed in ice-cold sucrose buffer (250 mM sucrose, 1 mM EDTA, 10 mM Tris-Cl, pH 7.4) and immediately homogenized in a Potter-Elvehjem Teflon glass homogenizer with 4 volumes of the same buffer/g of liver. All operations were carried out at 0-4°C. The homogenate was filtered through Miracloth (Calbiochem) and centrifuged for 20 min at 7,000 x g. The supernatant was filtered again through Miracloth and centrifuged 70 min at 106,000 x g. The microsomal pellet was renatured in 100 mM potassium pyrophosphate, pH 7.4, and centrifuged at 106,000 x g for 70 min. This pellet, referred to as washed liver microsomes, was resuspended in a minimal volume of sucrose buffer, divided into multiple aliquots, quick-frozen in liquid N2, and stored at -70°C.

Purification of Cholesterol 7a-Hydroxylase—Aliquots of washed microsomes were thawed and resuspended in 100 mM Tris-Cl (pH 7.5), 0.1 mM EDTA, and 20% glycerol as a protein concentration of 1 mg/ml. Solubilization of membrane proteins was accomplished by the slow addition of cholate to a final concentration of 1.8%. After stirring for 30-60 min at 4°C, the solubilized proteins were adjusted to 8% (v/v) polyethylene glycol by the addition of a solution of 50% polyethylene glycol and precipitated proteins were separated by centrifugation (20 min, 16,000 x g) and discarded. The polyethylene glycol concentration of the supernatant was then raised to 17%, and the resultant pellet containing 7a-hydroxylase activity was resolubilized in 100 mM potassium phosphate (pH 7.4), 20% glycerol, 0.1 mM EDTA, and 0.7% cholate acid. The final protein concentration of this solution was adjusted to 7 mg/ml. Material from approximately 75 rats (4,000 mg of protein) was applied to a 2.5 x 40 cm aminohexyl-Sepharose 4B columns previously equilibrated with 100 mM potassium phosphate (pH 7.4), 20% glycerol, 0.1 mM EDTA, and 0.5% cholate acid. The columns were then washed with the same buffer until the absorbance at 280 nm was less than 0.05. Elution of 7a-hydroxylase was carried out with a buffer containing 100 mM potassium phosphate, pH 7.4, 50% glycerol, 0.1 mM EDTA, 0.1 mM DTT, 0.4% cholate acid, and 0.06% Lubrol PX. Fractions of 20-ml were collected until the absorbance at 280 nm was less than 0.2 and the absorbance at 416 nm was less than 0.05. Fractions were assayed for 7a-hydroxylase activity as described above and pooled accordingly.

The active pool was adjusted such that the final buffer components were 20 mM potassium phosphate (pH 7.4), 20% glycerol, 0.1 mM EDTA, 0.2% cholate acid, and 0.2% Lubrol PX. A 1/15 volume of packed hydrolyxatique, pre-equilibrated in the same buffer, was added to the pool, and the resulting slurry was allowed to stir for 1 h at 4°C. A 1/30 volume of packed pre-equilibrated Whatman CF1 cellulose powder was then added, and the mixture was transferred to a glass column (2.5 x 40 cm). After packing, the column was equilibrated with the starting buffer containing 50 mM potassium phosphate (pH 7.4) in place of 20 mM potassium phosphate until the absorbance at 280 nm was less than 0.1. 7a-Hydroxylase was eluted by washing with buffer containing 180 mM potassium phosphate (pH 7.4). Active fractions were centrifuged at 20,000 x g for 20 min, and the supernatant was collected. The polypeptide was dissociated by the addition of 1 M NaCl and dialyzed against buffer containing 50 mM potassium phosphate (pH 7.4) in place of 20 mM potassium phosphate until the absorbance at 280 nm was less than 0.05. 7a-Hydroxylase activity was assayed in the presence of 5-10 µg of [14C]cholesterol (6.6 CI/mmol) in a 0.5-ml volume containing 50 mM Tris-acetate (pH 7.5), 20% glycerol, 1 mM EDTA, 2 mM dithiothreitol (DTT), 0.06% Triton X-100, 2 mM NADPH, and 1000 units of purified NADPH-cytochrome P-450 reductase.
Microsomes were prepared from 75 rats maintained for 7 days on a 2% cholestyramine diet and subjected to the indicated purification steps as detailed under "Experimental Procedures."

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TABLE II
Sequence of 7α-hydroxylase tryptic peptides

The M, 60,300 protein was isolated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and digested in situ with trypsin. The resulting peptides were resolved by HPLC and subjected to sequence analysis as described under "Experimental Procedures." A blank in the sequence of a given peptide indicates an amino acid residue for which an unambiguous identification could not be made.

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<th>Peptide</th>
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7α-Hydroxylase activity was assayed in the presence of 5–10 µg of [14C]cholesterol and 1000 units of cytochrome P-450 reductase.

b PEG, polyethylene glycol.
c AH-Sepharose, aminohexyl-Sepharose 4B.

RESULTS

Purification of 7α-Hydroxylase—To increase the level of enzyme in the liver, rats were maintained for 1 week on a normal chow diet supplemented with 2% cholestyramine and then killed in the middle of the dark period of a 12-h light/dark cycle. Cholestyramine increases 7α-hydroxylase activity by binding bile acids in the intestine and preventing their reutilization via the enterohepatic circulation (11). In compensation, the liver boosts the production of bile acids by increasing the synthesis of 7α-hydroxylase. Similarly, the production of this enzyme is maximized during the nocturnal feeding phase of the rat (10).

Washed microsomes were prepared and used as starting material for the isolation of the enzyme (Table I). The purification protocol was a modification of that described by Andersson et al. (24). Two key steps were employed in the purification: the first was chromatography on aminohexyl-Sepharose 4B, a mixed hydrophobic/ion exchange resin on which 7α-hydroxylase could be separated from the bulk of the material for the isolation of the enzyme (Table I). The purification was carried out as described previously (21).

Construction of 7α-Hydroxylase Expression Vector—A plasmid vector capable of expressing 7α-hydroxylase in mammalian cells was constructed via standard methods of genetic engineering. A DNA fragment corresponding to nucleotides 1–2172 of Fig. 5 was ligated into the EcoRI site of the pcMV2 eukaryotic expression vector (22). The desired recombinant plasmid (p7α-OHase) was characterized by Southern blotting and restriction mapping and banding twice in CsCl density gradients prior to transfection analysis.

Transfection of COS-M6 Cells—Stock cultures were maintained, grown, and transfected as described previously (23). To assay transfected cells for 7α-hydroxylase activity, microsomes were prepared as follows. Sixty h post-transfection, cells were washed with ice-cold phosphate-buffered saline, harvested with a rubber policeman, and homogenized in 0.5 M sucrose, 10 mM Tris-Cl (pH 7.5), 1.0 mM EDTA, 1.0 mM DTT, and 0.4% Lubrol PX. DEAE-Sepharose fractions (0.5 mg of protein/ml of resin) were then passed through the column at a flow rate of 0.5 ml/min. 7α-Hydroxylase-containing fractions in the flow-through were identified by thin layer chromatography assay, pooled, and stored at -70 °C. The desired recombinant plasmid (p7α-OHase) was characterized by Southern blotting and restriction mapping and bands twice in CsCl density gradients prior to transfection analysis.

DNA sequence analysis of both strands of the cDNA was carried out as described by Sanger et al. (19) or Smith et al. (20). RNA blotting was carried out as described previously (21).

2 S. Andersson, unpublished observations.
weights in the 40,000-50,000 range (Fig. 2, lane 6).

Despite further chromatography attempts on more than a dozen different resins, we were unsuccessful in obtaining a more highly enriched preparation of 7α-hydroxylase. In an attempt to identify which of the four major bands in the most highly purified material corresponded to 7α-hydroxylase, advantage was taken of the regulated expression of the enzyme. To this end, 7α-hydroxylase was simultaneously purified from microsomes derived from animals maintained on an induction diet (2% cholestyramine) and from microsomes from a similar number of animals maintained on a suppression diet (0.2% chenodeoxycholic acid). As shown in Fig. 3, a starting differential of 10-fold in 7α-hydroxylase activity was maintained throughout five steps of the purification scheme. When an equal mass of protein derived at purification step 5 (Table I) from the induced and suppressed microsomes was electrophoresed on SDS-polyacrylamide gels, only one protein was seen to vary in accordance with the difference in 7α-hydroxylase activity between the two preparations (data not shown). The regulated protein (apparent Mr, 50,300) corresponded to the largest of the four polypeptides visualized in Fig. 2, lane 6. These results suggested that this protein corresponded to 7α-hydroxylase.

In an attempt to identify 7α-hydroxylase further, we next took advantage of the observation that a majority of cytochrome P-450 enzymes do not have blocked amino termini and thus yield protein sequence data when subjected to Edman degradation. In addition, despite being members of a superfamily of proteins with hydrophobic amino termini, the sequences of cytochrome P-450s in this region are sufficiently different to distinguish the various members (25). To this end, material from step 6 of the purification (Table I) obtained from animals on an induction diet was electrophoresed, transferred to Immobilon membranes, and the four proteins were subjected individually to sequence analysis on an Applied Biosystems model 470A sequenator. The results obtained are shown in Fig. 4. The largest protein with an Mr of 50,300 corresponded to the cholestyramine-regulated protein and had a hydrophobic amino-terminal sequence that was characteristic of, but different from, all other cytochrome P-450 sequences reported to date (25). The amino-terminal sequences of the Mr, 46,600 and 45,400 proteins revealed them to be identical to the rat cytochrome P-450g (27) and cytochrome P-450a (28) enzymes, respectively. Cytochrome P-450g has been shown to catalyze the hydroxylation of testosterone at carbon atoms 6, 15, and 17, and an unknown additional position (29), whereas cytochrome P-450a catalyzes hydroxylation at the 7 position of testosterone (28). Consistent with these observations, step 6 material (Table I) also demonstrated these catalytic activities (data not shown). The Mr, 42,100 protein was somewhat small to be a eukaryotic cytochrome P-450, and consistent with this notion, the amino-terminal sequence of the protein contained a number of glycine residues (Fig. 4) that are generally underrepresented in the amino-terminal sequences of cytochrome P-450s (25).

The results of the regulation experiment shown in Fig. 3 and the unique hydrophobic amino-terminal sequence determined for the Mr, 50,300 polypeptide of Fig. 4 strongly suggested that this protein was 7α-hydroxylase. To aid in the isolation of cDNA clones, the sequences of seven internal tryptic peptides from this protein were subsequently determined (Table II).

cDNA Cloning of 7α-Hydroxylase—To isolate cDNA clones corresponding to the rat 7α-hydroxylase mRNA, size-fractionated cDNA libraries constructed from liver mRNA isolated from cholestyramine-fed animals were screened with

**Fig. 2.** SDS-polyacrylamide gel electrophoresis of 7α-hydroxylase protein at various stages of purification. 20 μg of rat microsomal 6–17% polyethylene glycol precipitate (lane 2), 2.5 μg of aminohexyl-Sepharose fraction (lane 3), 5.0 μg of hydroxylapatite fraction (lane 4), 1.5 μg of DEAE-Sepharose fraction (lane 5), and 1.0 μg of postmonoclonal antibody 2B4 fraction (lane 6) were electrophoresed on a 7% SDS-polyacrylamide gel and subsequently visualized by silver staining. The molecular weights of protein standards (lanes 1 and 7) are indicated on the left of the stained gel. The 7α-hydroxylase protein is indicated by an arrow on the right.

**Fig. 3.** Dietary regulation of 7α-hydroxylase enzyme activity. 7α-Hydroxylase was simultaneously purified from groups of 100 animals maintained for 2 weeks on rat chow supplemented with 2% cholestyramine (induced, I) or 0.2% chenodeoxycholic acid (suppressed, S) as described under “Experimental Procedures.” An equal amount of protein derived from the two groups at different purification steps was assayed for 7α-hydroxylase activity by thin layer chromatography. The purification steps correspond to those of Table I. Approximately 500 μg of protein from step 2 (lanes 2 and 3), 7 μg from step 3 (lanes 4 and 5), 6 μg from step 4 (lanes 6 and 7), and 1 μg from step 5 (lanes 8 and 9) were assayed for 5 min at 37°C in a reaction containing 25 μM [14C]cholesterol and 1000 units of cytochrome P-450 reductase. Lanes 1 contained only the starting isotope and was not subjected to solvent evaporation. The chromatogram was exposed to Kodak XAR-5 film for a period of 44 h at -70°C. The identities of cytochrome P-450s were determined by comparison with authentic standards. The 7-keto and 7α-hydroxylated forms of cholesterol represent spontaneous oxidation products derived from cholesterol during solvent evaporation in the workup of the various reactions.

**Fig. 4.** Protein sequencing of 7α-hydroxylase. A Coomassie Blue-stained gel of 20 μg of step 6 purified material (Table I) is shown together with the amino-terminal sequences determined from each of the four major proteins remaining at this stage of the purification. The calculated molecular weight of each protein is shown on the left. The identities of cytochromes P-450a and cytochrome P-450g were determined by comparison of their deduced amino-terminal sequences with those in the National Biomedical Research Foundation protein database and by enzyme assay (see “Results”).
multiple oligonucleotide probes derived from the peptide sequence of the M, 50,000 protein. After screening \( 8 \times 10^4 \) clones, a cDNA was isolated which hybridized with three oligonucleotide probes. The amino acid sequence deduced from the DNA sequence of this clone revealed an open translation reading frame of 135 residues which included peptides 1, 2, and 4 (Table II) that were used to design the hybridizing oligonucleotides. Comparison with other cytochrome P-450 sequences indicated that this cDNA encoded the carboxyl-terminal end of a member of this family of proteins. cDNAs spanning the complete \( \Delta 7 \)-hydroxylase mRNA were subsequently identified using hybridization probes derived from the 5'-end of the initial cDNA clone.

**Structure of \( \Delta 7 \)-Hydroxylase**—The nucleotide sequence and the predicted amino acid sequence of \( \Delta 7 \)-hydroxylase derived from five overlapping cDNA clones are shown in Fig. 5. The deduced amino acid sequence begins with the unusual occurrence of 2 methionine residues and proceeds for a total of 503 residues. As indicated by the underlines in Fig. 5, the 2 methionines were present in the mature \( \Delta 7 \)-hydroxylase protein as well as in the cDNA sequence. The first 6 amino acids of the \( \Delta 7 \)-hydroxylase cDNA including the two methionines (Met-Met-Thr-Ile-Ser-Leu) bear little resemblance to a previously published amino-terminal sequence (Met-Phe-Glu-Val/Ile-Ser-Leu) for \( \Delta 7 \)-hydroxylase derived from an apparently impure sample (50). The remainder of the protein sequence contains many of the hallmarks that identify a microsomal cytochrome P-450, including an overall hydrophobic nature and a conserved cysteine residue at position 444. The mRNA for \( \Delta 7 \)-hydroxylase has an unusually long 5'-untranslated region of over 2 kb (Fig. 5). Included within this region is a single copy of the Alu family of middle repetitive DNAs (nucleotides 2195-2313, Fig. 5).

**Expression of \( \Delta 7 \)-Hydroxylase cDNA in COS Cells**—To confirm that the sequence shown in Fig. 4 encoded a functional \( \Delta 7 \)-hydroxylase enzyme, we expressed the cDNA in simian COS-M6 cells. A DNA fragment corresponding to nucleotides 1-2172 of Fig. 5 was ligated into the pCMV2 eukaryotic expression vector and transfected into COS cells using a DEAE-dextran protocol. Enzyme activity could not be detected in transfected whole cells. However, as shown in Table III, \( \Delta 7 \)-hydroxylase activity was readily measured in microsomes prepared from COS cells transfected with the \( \Delta 7 \)-hydroxylase cDNA. This activity was stimulated approximately 18-fold by the addition of purified rat microsomal cytochrome P-450 reductase (experiment 2, Table III). No \( \Delta 7 \)-hydroxylase activity could be detected in mock-transfected COS cells. In experiments not shown, polyclonal antibodies raised against the purified \( \Delta 7 \)-hydroxylase cross-reacted with an M, 50,000 protein that was present in COS cells transfected with the \( \Delta 7 \)-hydroxylase cDNA but absent from the mock-transfected cells.

**Expression and Regulation of Cholesterol \( \Delta 7 \)-Hydroxylase**—To determine the tissue distribution of \( \Delta 7 \)-hydroxylase in the rat, polyadenylated RNA was prepared from eight organs and subjected to blot hybridization with \( \Delta 7 \)-hydroxylase cDNA. As indicated in Fig. 6, only the liver expressed \( \Delta 7 \)-hydroxylase mRNA. In this tissue, three species of mRNA were detected, including a prominent 3.6-kb mRNA and two less abundant mRNAs of 2.4 and 1.7 kb (Fig. 5A). With longer periods of autoradiography, an additional band of 4.7 kb was also detected (see below). As a control, the mRNA encoding a cis-trans-prolyl isomerase (31) was present in most lanes. Certain tissues did not express prolyl isomerase, and in these tissues we used \( \beta \)-actin mRNA as a control (32; data not shown).

We next examined the dietary regulation of hepatic \( \Delta 7 \)-hydroxylase mRNA expression. Rats were fed normal chow or chow supplemented with cholestyramine, bile acids, or cholesterol for periods of 7-10 days. Polyadenylated RNA was prepared from the pooled livers of multiple animals and subjected to blot analysis with probes corresponding to three different mRNAs. One probe was derived from the coding region of \( \Delta 7 \)-hydroxylase (Fig. 5). A second probe was derived from the cDNA for HMG-CoA synthase, an enzyme in the cholesterol biosynthetic pathway which is subject to negative feedback regulation by dietary cholesterol (33, 34). The third probe was complementary to the cis-trans-prolyl isomerase mRNA.

As indicated in Fig. 7A, the presence of chenodeoxycholic acid (first lane) or cholic acid (last lane) in the diet suppressed mRNA levels for \( \Delta 7 \)-hydroxylase relative to animals maintained on a normal chow diet (fourth lane). Conversely, cholestyramine (second lane) or cholesterol (third lane) in the diet induced \( \Delta 7 \)-hydroxylase mRNA levels. The mRNA for HMG-CoA synthase (Fig. 7B) demonstrated a similar pattern of suppression in the presence of dietary bile acids (first and last lanes) and of induction by cholestyramine (second lane). However, in contrast to the results obtained with \( \Delta 7 \)-hydroxylase, cholesterol suppressed hepatic levels of HMG-CoA synthase mRNA (third lane). Hybridization with the prolyl isomerase probe (Fig. 7B, bottom) indicated that near equal amounts of RNA were present in all lanes.

**DISCUSSION**

The current paper describes the purification, sequence, cDNA cloning, expression, and regulation at the protein and mRNA levels of rat hepatic \( \Delta 7 \)-hydroxylase. The structure and expression of this enzyme agree well with that described by Noshiro et al. (12).

The protein sequence of \( \Delta 7 \)-hydroxylase as deduced by peptide sequencing and from the cDNA (Fig. 5) indicates that this enzyme is a member of the cytochrome P-450 superfamily. Some 35% of the amino acids in the protein have hydrophobic side chains, and the ubiquitously conserved cysteine that is thought to be a protein ligand for the heme group is located at residue 444. Comparison of the sequence with those of other cytochrome P-450 enzymes (35) indicates that the \( \Delta 7 \)-hydroxylase represents a novel family of cytochrome P-450s (family VII) with a gene nomenclature of CYP7a.

The \( \Delta 7 \)-hydroxylase protein migrated on SDS-polyacrylamide gels as a single band with an M, of 50,300, well separated from the cytochrome P-450a and cytochrome P-450g species (Fig. 2). This migration is somewhat anomalous as \( \Delta 7 \)-hydroxylase is only 9 and 13 amino acids larger than the cytochrome P-450a (28) and cytochrome P-450g (27) proteins, respectively. Although well resolved on an SDS-polyacrylamide gel, the enzymes co-chromatographed on a large number of different resins. At the primary structure level, cytochrome P-450a and cytochrome P-450g are 46% identical in sequence and are classified into two subfamilies (IIA and IIC, respectively) (35). The sequence of \( \Delta 7 \)-hydroxylase is only 22% identical to these two proteins and as mentioned above, is classified into a different family (family VII). Despite these differences, however, the chromatographic behavior of these proteins indicates that they must share similar hydrophobic and hydrophilic surface properties.

At least four mRNAs for \( \Delta 7 \)-hydroxylase are present in the rat liver (Figs. 6 and 7). It is not known whether the multiple

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3 D. W. Nebert, personal communication.
mRNAs are the products of the same gene or of different members of a closely related gene family. However, all of the cDNA clones isolated in this study were identical in sequence to that reported in Fig. 5, and the four mRNAs demonstrated the same pattern of regulation (Fig. 7). The most abundant mRNA in the rat, including several encoding cytochrome P-450 enzymes, is circled at position 444. An Alu sequence in the 3'-untranslated region is overlined.

**Fig. 5.** Nucleotide sequence of the rat 7α-hydroxylase cDNA and predicted protein sequence. A dot is placed under every 10th nucleotide, and cysteine residues found in all cytochrome P-450 enzymes are numbered above the sequence, and amino acid residues determined from the amino terminus and from seven tryptic peptides is underlined. The protein sequence determined from the rat 7α-hydroxylase cDNA and predicted protein sequence matched that determined by Edman degradation (Fig. 4 and Table II) with the exception of the 14th residue of tryptic peptide 6, which was valine in the peptide and glycine (residue 323) in the predicted protein sequence.

![DNA sequence](http://www.jbc.org/)

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<th>Protein Sequence</th>
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<td><code>TCAGAAATTTTAATTGTTTAGATGAlGlAT~GAGTAACAC~TTClGTlATATACT~TCTGTAGl~CT~TTTGTTCTTAGAAC~GTTTGATGACTCTC~TTG~TGTATCC</code></td>
<td>Amino acids: Alanine, Leucine, Tryptophan, Alanine, Aspartic Acid, Arginine, Tyrosine, Leucine, Asparagine, Tryptophan, Serine, Tryptophan, Gln, Met, Ile, Arg, Serine, Proline, Glutamic Acid, Alanine, Lysine</td>
</tr>
</tbody>
</table>

The Alu family is a repetitive sequence of the rat Alu family (Fig. 7) and has an unusually long 3'-untranslated region of 2002 nucleotides. Contained within this region of the mRNA is a single copy of a repetitive sequence of the rat Alu family (Fig. 5). The Alu family in rodents is composed of DNA repeats that are approximately 130 base pairs in length and that are homologous to the right arm of the bipartite human Alu sequence (56). Although the function of these sequences is at present not known, they are found in a number of mature mRNAs in the rat, including several encoding cytochrome P-450s (26). Interestingly, multiple copies of an Alu repeat are also located in the 3'-untranslated region of the human low density lipoprotein receptor mRNA (37). The regulatory significance if any of this observation remains to be determined.

The RNA blotting results of Figs. 6 and 7 demonstrate that 7α-hydroxylase expression is limited to the liver and subject to feedback regulation by product as well as induction by...
TABLE III

Expression of 7α-hydroxylase in transfected COS-M6 cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Plasmid</th>
<th>Microsomal protein</th>
<th>Cytochrome P-450 reductase</th>
<th>Conversion*</th>
<th>Specific activity</th>
<th>% pmol/min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vector alone</td>
<td>250</td>
<td>+</td>
<td>&lt;0.1</td>
<td>&lt;5.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7α-Hydroxylase cDNA</td>
<td>250</td>
<td>+</td>
<td>&lt;0.1</td>
<td>&lt;2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>+</td>
<td>2.5</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.9</td>
<td>127</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Vector alone</td>
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<td>+</td>
<td>&lt;0.1</td>
<td>&lt;5.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7α-Hydroxylase cDNA</td>
<td>250</td>
<td>+</td>
<td>3.7</td>
<td>192</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>+</td>
<td>&lt;0.1</td>
<td>&lt;2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.4</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>+</td>
<td>7.2</td>
<td>187</td>
<td></td>
</tr>
</tbody>
</table>

* % conversion of [14C]cholesterol (25 μM) into 7α-hydroxycholesterol in 20 min.

FIG. 6. Tissue distribution of 7α-hydroxylase mRNA. Top, RNA was isolated from the indicated tissue or region of the brain by a guanidinium/CsCl procedure (17). Approximately 5 μg of polyadenylated RNA from each source was denatured with glyoxal and size fractionated by electrophoresis in a 1.5% agarose gel. Following transfer to a nylon membrane, hybridization was carried out with 32P-labeled single-stranded probes (21) derived from the coding region of the 7α-hydroxylase cDNA. The filter was washed as described previously (21) and subjected to autoradiography with intensifying screens at -70°C for 96 h. Bottom, the blot from panel A was stripped of radioactivity and reprobed with a cDNA corresponding to the cis-trans-prolyl isomerase mRNA. For the tissues that had low levels of this mRNA (adrenal, duodenum, pancreatic islets), previous studies with this same filter have shown that β-actin mRNA was present in these lanes (32).

substrate. These features of 7α-hydroxylase expression underscore the unique and important regulatory role of this microsomal cytochrome P-450 in the bile acid biosynthetic pathway. By way of comparison, the expression of sterol 26-hydroxylase, a mitochondrial cytochrome P-450 that catalyzes oxidations at the 26 position of sterol intermediates in this pathway, is neither liver specific nor subject to feedback regulation in this organ (22).

The mechanism by which dietary bile acids decrease the expression of 7α-hydroxylase mRNA remains to be determined. Given the precedence established for the regulation of other genes involved in cholesterol homeostasis (5-8), the observations reported here suggest the existence of DNA sequences in the promoter of the 7α-hydroxylase gene which respond to bile acids. The proteins that interact with these sequences would presumably sense the levels of bile acids in the enterohepatic circulation and regulate the expression of the gene accordingly. However, the addition of bile acids to the medium of cultured rat hepatocytes did not result in a decrease in bile acid production (38, 39). Similarly, the infusion of bile acids into bile-diverted animals did not down-regulate bile acid biosynthesis (40). Taken together, these results suggest that either an intact enterohepatic circulation is required for the regulation observed here or that bile acids act indirectly to influence 7α-hydroxylase expression (40), perhaps by influencing the total flux of cholesterol across the liver (41).

That the expression of 7α-hydroxylase mRNA can respond to cholesterol is demonstrated by the increase in the levels of this mRNA upon cholesterol feeding (Fig. 7). This response predicts the existence of regulatory elements in the promoter of the gene which respond in a positive fashion to cholesterol. Such elements may or may not be different from the sterol regulatory elements identified in genes such as HMG-CoA synthase and others in the cholesterol supply pathways which respond negatively to cholesterol (Fig. 7 and Refs. 5-7). Similarly, the 7α-hydroxylase promoter may share sequences with certain apolipoprotein genes whose expression is enhanced by dietary cholesterol (42). Clearly, the elucidation of the mechanisms that underlie the regulated expression of 7α-hydroxylase will be a rich area of future study.

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