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Structure of the Rat Gene Encoding Cholesterol 7α-Hydroxylase[†,‡]

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ABSTRACT: Cholesterol 7α-hydroxylase (7α-hydroxylase) is a microsomal cytochrome P-450 that catalyzes the first and rate-limiting step in bile acid biosynthesis, the major catabolic pathway in cholesterol homeostasis. The gene encoding the rat 7α-hydroxylase has been isolated and characterized. Southern blotting experiments demonstrated that the gene is present in a single copy in the rat genome. DNA sequence analysis showed that the 7α-hydroxylase gene is unique among the characterized cytochrome P-450s in that it contains only six exons. Nuclease S1 and primer-extension mapping experiments positioned the 5′-ends of the 7α-hydroxylase mRNA approximately 20–25 nucleotides downstream of a consensus TATAAA sequence. RNA blotting experiments demonstrated the presence of multiple 7α-hydroxylase mRNAs that differ in the lengths of their 3′-untranslated regions.

The metabolic pathways of cholesterol supply and catabolism are tightly regulated to prevent a shortage or oversupply of this essential but toxic molecule. Cholesterol supply is accomplished through biosynthesis from acetate precursors (Brown & Goldstein, 1980) or via receptor-mediated endocytosis of cholesterol-carrying lipoprotein particles (Brown & Goldstein, 1986). Cholesterol catabolism occurs by conversion in the liver to bile acids followed by their excretion from the body via the bile and intestine (Carey, 1982). The regulatory cross-talk between these three pathways is now under intense study as a consequence of the recent cloning of cDNAs encoding key rate-limiting enzymes in each of the pathways. These enzymes are 3-hydroxy-3-methylglutaryl-coenzyme A reductase in cholesterol biosynthesis (Chin et al., 1984), the low-density lipoprotein receptor in endocytosis (Yamamoto et al., 1984), and cholesterol 7α-hydroxylase (7α-hydroxylase)[1] in cholesterol catabolism (Noshiro et al., 1989; Jelinek et al., 1990).

The genes encoding these enzymes are subject to regulation at the level of transcription (Goldstein & Brown, 1990; Jelinek et al., 1990). The synthesis of enzymes in the cholesterol biosynthetic and uptake pathways is suppressed by the consumption of diets rich in cholesterol (Goldstein & Brown, 1990) and induced by drug-supplemented diets that decrease cellular cholesterol levels (Goldstein & Brown, 1990). Classical promoter studies have identified a conserved octanucleotide sequence, termed a sterol regulatory element, that is necessary and sufficient for the observed feedback regulation (Goldstein & Brown, 1990). In the case of 7α-hydroxylase, the levels of mRNA in rat liver are suppressed by dietary bile acids and induced by dietary cholesterol (Jelinek et al., 1990). The molecular mechanisms that underlie the end-product suppression and substrate induction of 7α-hydroxylase are at present unknown.

Genetic defects in the enzymes of cholesterol supply have severe consequences. In the most well-studied example, mutations in the low-density lipoprotein receptor underlie the prevalent genetic disease familial hypercholesterolemia (Hobbs et al., 1990), which is characterized by premature atherosclerosis. Little is presently known about the genetics of cholesterol catabolism, as cDNAs encoding enzymes in this pathway are only now becoming available for study (Andersson et al., 1989; Noshiro et al., 1989; Jelinek et al., 1990). However, there is compelling evidence to suggest that abnormalities in the pathway can cause a variety of syndromes ranging from sterol storage disorders (Björkem & Skrede, 1989) to cholesterol gallstones (Marks et al., 1976).

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[1] Abbreviations: 7α-hydroxylase, cholesterol 7α-monooxygenase (EC 1.14.13.17); kb, kilobase.

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To gain preliminary insight into the regulation of cholesterol catabolism and the genetics of this pathway, we here report the isolation and characterization of the rat 7α-hydroxylase gene.

Experimental Procedures

Genomic Cloning. Rat genomic libraries obtained from Clontech Laboratories, Inc., or from Stratagene were screened with ^{32}P-labeled probes derived from the coding region of the 7α-hydroxylase cDNA (Jelinek et al., 1990) by standard methods (Sambrook et al., 1989). Hybridization-positive plaques were purified, and the size of the genomic DNA insert was determined by restriction endonuclease digestion of plate lysate DNA (Xu, 1986). Genomic clones were characterized by Southern blot analysis (Lehrman et al., 1985) using ^{32}P-labeled oligonucleotides corresponding to different regions of the 7α-hydroxylase cDNA. The *Eco*RI fragments of each genomic clone were further subcloned into the plasmid vector pBluescript (Stratagene) for DNA sequence analysis.

Nucleic Acid Sequencing and Blotting Analysis. DNA sequence analysis of the exon–intron junctions and the 5′-flanking sequence was carried out on alkaline-denatured plasmid DNA templates, or after subcloning into bacteriophage M13 vectors (Pharmacia) and sequencing by automated methods on an Applied Biosystems Model 370A DNA sequencer (Smith et al., 1986). The polymerase chain reaction (Saiki et al., 1988) was used to estimate the size of the introns. Southern blotting of rat genomic DNA was carried out as previously described (Lehrman et al., 1985). The coding region specific probe was prepared by random hexanucleotide labeling of a cDNA fragment corresponding to nucleotides 1–1463 of the published sequence (Jelinek et al., 1990). A 3′-untranslated region specific probe was synthesized (Church & Gilbert, 1984) from two bacteriophage M13 clones containing cDNA inserts spanning nucleotides 1760–2015 and 2938–3150 of the 7α-hydroxylase cDNA. Hybridization and washing of filters were carried out at high stringency as described (Lehrman et al., 1985).

RNA blotting was carried out as described previously (Lehrman et al., 1987) on poly(A+) RNA isolated from the livers of female Sprague-Dawley rats (150 g) (Sasco). Prior to sacrifice in the middle of the dark period, animals were fed ad libitum normal rat chow supplemented with 2% (w/w) cholestyramine (Colestid; Upjohn) for 7 days. Radiolabeled probes used in RNA blotting experiments were prepared as described above.

Primer Extension and Nuclease S1 Protection. For primer-extension analysis, a ^{32}P-labeled oligonucleotide complementary to nucleotides 31–51 of Figure 2 was annealed at 68 °C to 20 μg of rat liver poly(A+) RNA and extended with reverse transcriptase (GIBCO) as described (Südhof et al., 1987). For nuclease S1 protection, a ^{32}P-labeled single-stranded DNA probe complementary to nucleotides −592 to +52 of Figure 2 was prepared from a bacteriophage M13 template as described above with [^{32}P]dCTP and [^{32}P]dATP (Du Pont, New England Nuclear). Rat liver poly(A+) RNA (20 μg) was hybridized with the probe at 22 °C for 16 h, digested with 200 units of nuclease S1 (Sigma), and analyzed on a 7 M urea–6% (w/v) polyacrylamide gel.

Results

Structure of 7α-Hydroxylase Gene. To isolate the gene encoding the rat 7α-hydroxylase, two commercially available genomic DNA bacteriophage λ libraries were screened with probes derived from the cDNA. A total of three hybridization-positive clones were isolated after screening of approximately 2 × 10^6 bacteriophages. The genomic DNA inserts of these clones were subjected to restriction mapping, Southern blotting, plasmid subcloning, and DNA sequence analysis to determine the structure of the 7α-hydroxylase gene. All inserts represented one region of the rat genome and overlapped to varying extents.

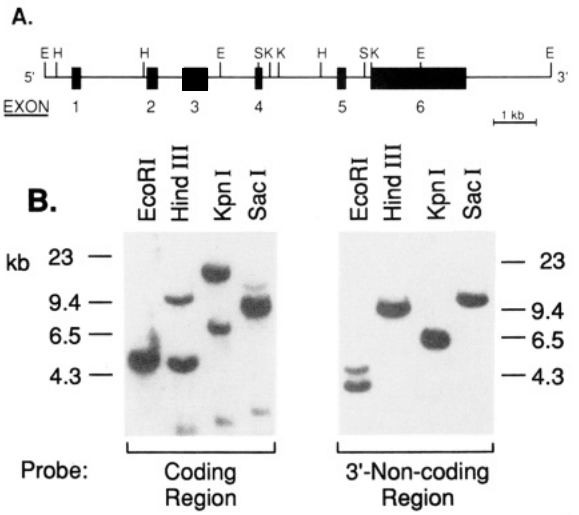

FIGURE 1: Structure of the rat 7α-hydroxylase gene. (A) The exon–intron organization of the gene is shown drawn to scale. Exons are indicated by the numbered boxes and introns by the connecting lines. The size of the last exon of the gene is based on that present in the most abundant (3.6 kb) mRNA (see text for details). The positions of all cleavage sites for the restrictions enzymes *Eco*RI (E), *Hin*dIII (H), *Sac*I (S), and *Kpn*I (K) are indicated along the gene schematic. (B) Southern blot analysis of rat genomic DNA. Twenty micrograms of rat liver DNA was digested with the indicated restriction enzyme and subjected to Southern blotting analysis as described under Experimental Procedures with probes derived from the coding region of the 7α-hydroxylase cDNA (left panel) or from the 3′-noncoding region of the cDNA (right panel). The washed filters were exposed to X-ray film for 48 h with two intensifying screens. The positions to which standards of known size migrated in an adjacent well of the agarose gel are shown on the left and right of the panels.

As indicated in Figure 1A, the 7α-hydroxylase gene spans approximately 10 kilobases (kb) and is divided into six exons separated by five introns. The exact locations of the introns were determined by DNA sequence analysis, while their sizes were deduced by amplification via the polymerase chain reaction. The DNA sequences of the intron–exon junctions are shown in Table I and obey the GT···AG rule of eukaryotic genes (Mount, 1982).

To confirm the deduced structure of the cloned 7α-hydroxylase gene and to determine if there were multiple copies of the gene, Southern blotting experiments were carried out with rat genomic DNA. As indicated in Figure 1B, the sizes and number of hybridizing fragments detected with the indicated probes in four different restriction enzyme digests agree very well with those predicted from the gene map of Figure 1A. At the hybridization stringency employed in these experiments (Figure 1B) and in others using additional enzymes (data not shown), only those DNA fragments predicted from the structure of the cloned gene were detected.

DNA Sequence of 5′-Flanking Region. The DNA sequence of 738 nucleotides corresponding to the 5′-end of the 7α-hydroxylase gene is shown in Figure 2. A consensus TATAAA sequence is located 90 nucleotides upstream of the initiator methionine codon, and the more distal DNA contains several recognition sequences for known transcription factors (Figure 2).

Determination of Transcription Start Sites. The 5′-ends of 7α-hydroxylase mRNAs transcribed from the gene were

Table I: Exon–Intron Organization of Rat Cholesterol 7α-Hydroxylase Gene

Exon Number	Exon Size (bp)[a]	Sequence at exon-intron junction[b] 5′ Splice donor	3′ Splice acceptor	Amino acid interrupted[c]
1	141,144[d]	AGG AGA AGgtatgg ...~1.6kb... 80	gtctctgtcttccagG AAA GCT 81	Arg(27)
2	241	TCT GCG AAGgtaatt ...~0.7kb... 321	tctattttctaccagGCA TTT GGA 322	Lys(107)
3	587	ATG ATC AGgtaact ...~1.1kb... 908	tggctctttccacagG AGT CCT 909	Arg(303)
4	131	GTA CTA Ggtgtgc ...~1.8kb... 1039	ttttaaaatatctag AC AGC ATC 1040	Asp(347)
5	176	GAC CCT TTGgtaagt ...~0.6kb... 1215	gtattcttattgcagACT TTC AAA 1216	Leu(405)
6	>770			

[a] bp, base pairs. [b] Capital letters and numbers refer to exon sequences, with the A of the ATG initiation codon being assigned number 1. Small letters refer to intron sequences. [c] Amino acids are numbered according to Jelinek et al. (1990). [d] The two sizes for exon 1 reflect the two different start sites detected by primer-extension mapping.

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-592          GAATTCTTCAAGCTGTGGCTTCCTGGTAGATGAGTCCTGGGAGGTTTTCTATTTCGATGATGGTAGATAGGTAACCTGTCATATACCACATGAAATACCTGT   -501

-500  GGCTTTGTAAACACACCGAGCAGTCAAGCAGGAGAATAGTTCCATACAGTTCGCGTCCCTTAGGATTGGTTTCGGGATACTTCTGGAGGTTCATTTAAATAATTTTCCCC   -401

-400  GAAGTACATTATGGGCAGCCAGTGTTGTGATGGGAAGCTTCTGCCTGTTTTGCTTTGCGTCGTGCTCCACACCTTTGACAGATGTGCTCATCTGTTTACTTCTTTTTCTA   -301

-300  CACACAGAGCACAGCATTAGCTGCTGTCCCGGCTTTGGATGTTATGTCAGCACATGAGGGACAGACCTTCAGCTTATCGAGTATTGCAGCTCTCTGTTTGTTCTGGAGCC   -201

-200  TCTTCTGAGACTATGGACTTAGTTCAAGGCCGGGTAATGCTATTTTTTTCTTCTTTTTTCTAGTAGGAGGACAAATAGTGTTTGCTTTGGTCACTCAAGTTCAAGTTATT   -101

-100  GGATCATGGTCCTGTGCACATATAAAGTCTAGTCAGACCCACTGTTTCGGGACAGCCTTGCTTTGCTAGGCAAAGAGTCTCCCCTTTGGAAATTTTCCTGCTTTTGCAAA   -1

          Met Met Thr Ile Ser Leu Ile Trp Gly Ile Ala Val Leu Val Ser Cys Cys Ile Trp Phe Ile Val Gly Ile Arg Arg   Intron 1
    +1    ATG ATG ACT ATT TCT TTG ATT TGG GGA ATT GCC GTG TTG GTG AGC TGT TGC ATA TGG TTT ATT GTT GGA ATA AGG AGA AGgtatgg...
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FIGURE 2: DNA sequence of the 5′-flanking region of the 7α-hydroxylase gene. The nucleotide sequence of the 5′-flanking region of the 7α-hydroxylase gene is shown. The sequence is numbered on the left and right with positive numbers assigned to the coding region and negative numbers assigned to the 5′-flanking nucleotides. Amino acids encoded by the first exon and the position of the first intron of the gene are shown on the bottom line. The two asterisks above the sequence indicate sites of transcription initiation as determined by primer-extension mapping, while the single asterisk below the sequence indicates an approximate site of transcription initiation determined by nuclease S1 mapping (see Figure 3 and text for details). A consensus TATAAA sequence is overlined as are nucleotides present in the recognition sequence for liver transcription factor LF-B1 (Frain et al., 1989; Baumhueter et al., 1990) (GTTATT) and cytochrome P-450 BTE transcription factor (Yanagida et al., 1990) (AGTAGGAGG).

localized by both primer extension mapping and nuclease S1 digestion experiments. As indicated in Figure 3, when a uniformly labeled single-stranded DNA probe derived from the 5′-flanking region of the gene (nucleotides −592 to +52, Figure 2) was annealed to liver mRNA and then digested with nuclease S1, a single protected fragment of about 110 nucleotides was detected (lane 1). This protected fragment was not detected when mRNA was omitted from the hybridization reaction (lane 2). Comparison of the length of this fragment to the DNA sequence of the 5′-flanking region allowed the approximate positioning of the 5′-end of the 7α-hydroxylase mRNA at nucleotide −65 of Figure 2.

Similar results were obtained when an oligonucleotide complementary to nucleotides 31–51 of Figure 2 and radiolabeled at its 5′-end was annealed to liver mRNA and extended with reverse transcriptase. Two closely spaced extension products were detected only when mRNA was present in the reaction (lanes 3 and 4, Figure 3). Although the two products are not well resolved on this gel, electrophoresis of the reaction mixture on sequencing gels adjacent to a DNA sequence ladder derived with the same oligonucleotide allowed both the resolution of the two equally abundant products (data not shown) and an assignment to specific nucleotides in the 5′-flanking DNA sequence of the gene (asterisks, Figure 2). This positioning agreed well with the results of the nuclease S1 experiments.

Origin of Multiple 7α-Hydroxylase mRNAs. Previous RNA blotting experiments revealed the presence of at least four 7α-hydroxylase mRNAs of 4.7, 3.6, 2.4, and ~1.9 kb in rat liver (Jelinek et al., 1990). To determine if these mRNAs were the result of differential polyadenylation at the 3′-end of the gene, we carried out the RNA blotting experi-

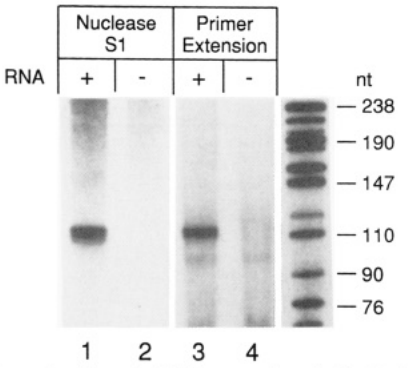

FIGURE 3: Determination of mRNA start sites in the 7α-hydroxylase gene. In lane 1, liver poly(A+) mRNA (20 μg) was annealed with a 643-nucleotide probe derived from the 5′-flanking sequence shown in Figure 2 and then digested with 200 units of nuclease S1. The protected fragments were analyzed by electrophoresis on a denaturing polyacrylamide gel followed by autoradiography for 48 h. In lane 2, the poly(A+) mRNA was omitted from the annealing reaction. Primer-extension analysis (lanes 3 and 4) was carried out as described under Experimental Procedures with a ^{32}P-labeled 20-nucleotide primer complementary to the 7α-hydroxylase mRNA. Primer-extension products were analyzed on the same gel as the nuclease S1 products. The experiment in lane 3 included 20 μg of poly(A+) mRNA while that shown in lane 4 did not. Autoradiography was carried out for 8 h. Radiolabeled standards shown on the right were derived from an *Msp*I digest of the plasmid pBR322.

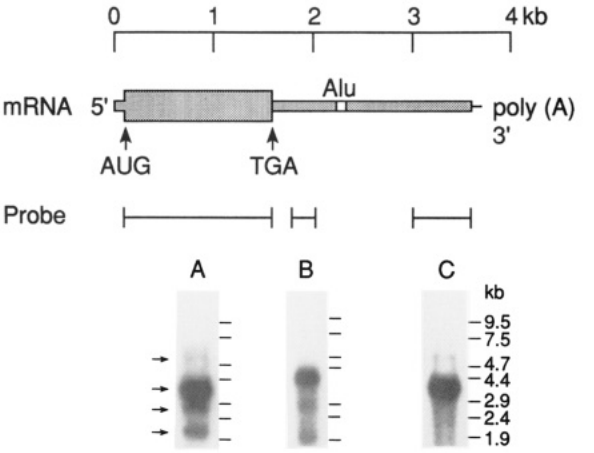

FIGURE 4: Origin of multiple mRNAs transcribed from the 7α-hydroxylase gene. A schematic of the most abundant 3.6-kb 7α-hydroxylase mRNA is shown at the top, with coding regions indicated by a thick stippling and noncoding regions indicated by thin stippling. An Alu middle repetitive sequence present in the 3′-noncoding region of the mRNA is indicated by a white box. For the RNA blotting experiments shown below the mRNA schematic, 10 μg of liver poly(A+) mRNA was size fractionated on a 1.5% (w/v) agarose gel, transferred to a nylon membrane, and hybridized with the indicated probes derived from the 7α-hydroxylase cDNA. The four mRNAs detected with the coding-region probe (probe A) are indicated by arrows on the left of the first panel. Standards are indicated on the right of each of the panels. Autoradiography was carried out for 4 h (probe A), 8 h (probe B), and 24 h (probe C). The hybridization of probe B to the largest mRNA is difficult to visualize. However, longer exposures of the same filter indicated hybridization to this species (data not shown).

ments shown in Figure 4. Polyadenylated mRNA from liver was size fractionated on an agarose gel, transferred to a nylon membrane, and then hybridized with radiolabeled probes derived from three different regions of the 7α-hydroxylase cDNA.

A probe derived from the coding region of the cDNA detected all four mRNAs (probe A, Figure 4). Similarly, a probe derived from the proximal portion of the 3′-untranslated region of the cDNA detected these same four mRNAs (probe B). However, a probe derived from the distal portion of the 3′-untranslated region of the mRNA detected only the major 3.6-kb mRNA and the less abundant 4.7-kb mRNA (probe C, Figure 4). These results suggest that all four mRNAs are derived from a single gene and that they differ in the lengths of their 3′-untranslated regions. They further suggest that the 3′-ends of the two shorter mRNAs of 2.4 and 1.9 kb are located between the 5′-ends of probes B and C (Figure 4). The 3′-untranslated region of the most abundant 7α-hydroxylase mRNA of 3.6 kb does not contain any classical polyadenylation signals with the sequence AATAAA (Jelinek et al., 1990); thus, we cannot at present assign a precise 3′-end to the multiple mRNAs.

Discussion

This paper describes the isolation and characterization of the rat 7α-hydroxylase gene. The gene contains six exons and five introns and spans approximately 10 kb in the rat genome. Transcription of mRNAs from the gene begins approximately 20–25 nucleotides downstream of a canonical TATAAA sequence and terminates at one of at least four positions in the last exon of the gene. All of the available evidence from these and other molecular cloning studies (Noshiro et al., 1989; Jelinek et al., 1990) indicates the presence of only a single gene encoding 7α-hydroxylase in the rat genome.

Cholesterol 7α-hydroxylase is a microsomal member of the cytochrome P-450 superfamily (Gonzalez, 1989). The 7α-hydroxylase gene with its six exons is so far unique among eukaryotic cytochrome P-450s for which gene structure information has been reported. The most recent survey revealed that cytochrome P-450 genes usually contain between 7 and 13 exons (Gonzalez, 1989). An inverse correlation was noted between the apparent evolutionary age of a given cytochrome P-450 and the number of introns in the gene, with older genes such as members of the P-450I family having the fewest exons (Gonzalez, 1989). The presence of only six exons in the 7α-hydroxylase gene fits this correlation as the capacity to synthesize 7α-hydroxylated bile acids extends throughout the vertebrate subphylum (Haselwood, 1967).

The position of introns in the genes of membrane proteins often correlates with functional domains in the protein (Südhof et al., 1985). In the case of 7α-hydroxylase, certain predicted domains are encoded by discrete exons, including the amino-terminal hydrophobic membrane anchoring sequence (exon 1; Nelson & Strobel, 1988) and the cysteine to which the heme cofactor is bound (exon 6; Gonzalez, 1989). The roles of other exonic sequences in the 7α-hydroxylase gene cannot be predicted in the absence of functional studies. However, it is interesting to note that exon 3 is unusually large (587 nucleotides, Table I) and encodes a very large segment (196 residues) in the center of the protein.

The expression of 7α-hydroxylase in the liver is negatively regulated by bile acids and induced by cholesterol. This regulation occurs at the level of expression of the mRNA (Jelinek et al., 1990) and is well correlated with 7α-hydroxylase enzyme activity and mass (Chiang et al., 1990; Jelinek et al., 1990; Nguyen et al., 1990). The DNA sequences in the 7α-hydroxylase gene that respond to bile acids and cholesterol are presently unknown; however, the elucidation of the DNA sequence of the 5′-flanking region of the gene (Figure 2) is a first step toward their identification. This region of the gene includes the sequence GTTANT (Figure 2), which is the most highly conserved segment of a cis-acting element found in all genes that respond to the liver-preferential transcription factor LF-B1 (Frain et al., 1989; Baumhueter et al., 1990). The presence of this sequence may underlie the liver-specific dis-

tribution of the 7α-hydroxylase mRNA (Jelinek et al., 1990). The 7α-hydroxylase 5′-flanking region shown in Figure 2 does not contain a sequence that is identical with an 8-nucleotide ($CACC^{CC}_{GT}AC$) sterol regulatory element that is common to many feedback-regulated genes in the cholesterol supply pathways (Goldstein & Brown, 1990). In three well-studied genes, this element is present within 350 base pairs of the mRNA start sites and leads to a reduction in transcription in the presence of sterols (Goldstein & Brown, 1990). The absence of this sequence in the proximal 5′-flanking region of the 7α-hydroxylase gene is further evidence that this gene may be regulated by mechanisms different from those of the cholesterol supply genes.

The multiple mRNAs detected in blot hybridization experiments appear to arise as a consequence of differential polyadenylation in the 3′-end of the 7α-hydroxylase gene (Figure 4) and not as a consequence of different 5′ start sites (Figure 3) or of multiple genes (Figure 1). At least six overlapping 7α-hydroxylase cDNAs have so far been isolated (Noshiro et al., 1989; Jelinek et al., 1990), and on the basis of DNA sequence analysis, all of these correspond to the gene isolated here. The presence of only one gene encoding a given cytochrome P-450 is frequently observed for enzymes that act on so-called endogenous substrates, such as steroids or cholesterol, as does 7α-hydroxylase (Gonzalez, 1989). This observation is in contrast to the common occurrence of multiple genes encoding cytochrome P-450 enzymes that act on exogenous substrates such as drugs and carcinogens (Gonzalez, 1989). There are several reports in the literature that suggest the presence of multiple 7α-hydroxylase enzymes (Chiang et al., 1990; Nguyen et al., 1990). The work described here indicates that if these isozymes do exist, they must arise as a consequence of posttranslational modification and not from multiple 7α-hydroxylase genes.

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Registry No. Cholesterol 7α-hydroxylase, 9037-53-0.

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