cDNA Cloning of Mouse and Human Cholesterol 25-Hydroxylases, Polytopic Membrane Proteins That Synthesize a Potent Oxysterol Regulator of Lipid Metabolism*

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Oxysterols regulate the expression of genes involved in cholesterol and lipid metabolism and serve as intermediates in cholesterol catabolism. Among the most potent of regulatory oxysterols is 25-hydroxycholesterol, whose biosynthetic enzyme has not yet been isolated. Here, we report the cloning of cholesterol 25-hydroxylase cDNAs from the mouse and human. The encoded enzymes are polytopic membrane proteins of 298 and 272 amino acids, respectively, which contain clusters of histidine residues that are essential for catalytic activity. Unlike some other sterol hydroxylases, cholesterol 25-hydroxylase is not a cytochrome P450, but rather it is a member of a small family of enzymes that utilize diiron cofactors to catalyze the hydroxylation of hydrophobic substrates. The cholesterol 25-hydroxylase gene lacks introns, and in the human it is located on chromosome 10q23. The murine gene is expressed at low levels in multiple tissues. Expression of cholesterol 25-hydroxylase in transfected cells reduces the biosynthesis of cholesterol from acetate and suppresses the cleavage of sterol regulatory element binding protein-1 and -2. The data suggest that cholesterol 25-hydroxylase has the capacity to play an important role in regulating lipid metabolism by synthesizing a co-repressor that blocks sterol regulatory element binding protein processing and ultimately leads to inhibition of gene transcription.

Oxysterols are formed by the hydroxylation of the side chain of cholesterol.1 This modification renders the sterol more hydrophilic and confers two important biological properties. First, the increased hydrophilicity enhances the ability of the oxysterol to cross membranes and thereby facilitates its movement between intracellular compartments, cells, and tissues. Second, oxysterols delivered in ethanol to cultured cells are potent regulators of the expression of genes involved in sterol and fatty acid metabolism (1, 2).

The enhanced solubility of oxysterols is exploited by the body to maintain cholesterol homeostasis. In several tissues and cell types, including the brain, kidney, endothelium, and macrophages, cholesterol is converted into oxysterols that subsequently traverse the plasma membrane and are transported to the liver (3–5). In the liver, they are converted into bile acids by a newly described biosynthetic pathway (6). These bile acids are essential for normal lipid and fat-soluble vitamin metabolism (7).

Oxysterols are both positive and negative regulators of gene expression. As positive effectors, they bind to and activate the nuclear receptor LXR (8), which in turn increases transcription of the cholesterol 7α-hydroxylase gene (9). This activation stimulates the conversion of cholesterol into bile acids (10). Mutation of the LXR gene in mice causes a loss of 7α-hydroxylase gene induction and a build up of cholesterol in the liver (11). As negative regulators, oxysterols suppress the cleavage of two transcription factors known as sterol regulatory element binding proteins-1 and -2 (SREBP-1 and -2) (12). These proteins are synthesized as inactive precursors in the membrane compartment of the cell. When intracellular cholesterol levels decline, SREBPs are cleaved to release amino-terminal fragments that migrate to the nucleus and activate the transcription of a network of genes involved in cholesterol synthesis and supply (12). This activation in turn restores intracellular cholesterol levels.

Several oxysterols occur naturally, including 25-hydroxycholesterol (cholest-5-ene-3β,25-diol), 24-hydroxycholesterol (cholest-5-ene-3β,24-diol), and 27-hydroxycholesterol (cholest-5-ene-3β,27-diol) (13). Of these three oxysterols, 25-hydroxycholesterol is the most potent regulator of gene transcription when assayed in vitro (1, 2, 9, 11). Despite this potency, it has proven difficult to document the biosynthesis of this oxysterol and thus its role as an in vivo regulator of lipid metabolism has remained questionable.

The objectives of the current study were to obtain in vivo evidence for 25-hydroxylation of cholesterol and to identify the gene responsible for this putative activity. We here report the isolation of cDNAs encoding murine and human cholesterol 25-hydroxylases, and we demonstrate that expression of these cDNAs in cultured cells down-regulates cholesterol synthesis and SREBP processing.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™ via EBI Data Bank with accession number(s) AF059213, AF059214, AF059211, and AF059212.

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The abbreviations and trivial names used are: cholesterol, 5-cholesten-3β-ol; SREBP, sterol regulatory element binding protein; CHOP, Chinese hamster ovary poloma; 25-hydroxycholesterol, cholest-5-ene-3β,25-diol; 24-hydroxycholesterol, cholest-5-ene-3β,24-diol; 27-hydroxycholesterol, cholest-5-ene-3β,27-diol; cholesterol, 5α-cholestan-3β-ol; epicholesterol, 5-cholest-3a-ol; coprostanol, 5β-cholestan-3β-ol; desmosterol, 5,24-cholestadien-3β-ol; sitosterol, 5,22-cholesta-4β,6α-ol; 25-oxo-27-norcholesterol, 27-nor-25-oxo-5-cholesten-3β-ol; DMEM, Dulbecco’s modified Eagle’s medium; bp, base pair; kb, kilobase pair;
**EXPERIMENTAL PROCEDURES**

**Expression Cloning**—Total RNA was prepared from 400 mg of an SREBP-1a transgenic mouse liver (14) using RNA-Stat 60 (Tel-Test, Inc. Friendswood, TX). Poly(A)+ RNA was prepared from total RNA by two cycles of chromatography on oligo(dT) (mRNA Purification Kit, Amersham Pharmacia Biotech). A size-fractionated, directional cDNA library with SacII and NotI cohesive ends at the 5′ and 3′ termini, respectively, was constructed from 4 μg of poly(A)+ RNA using a Superscript Plasmid Kit (Life Technologies, Inc.). Size-fractionated cDNA (>1.0 kb, 10 ng) was ligated with 50 ng of CMV6 expression vector (a derivative of pCMV4 (15) containing a NotI site in the polylinker) using a protocol and reagents supplied with the Superscript kit. Prior to ligation, the pCMV6 vector (1.2 μg) was digested for 2 h with 10 units of NotI and SacII, respectively, in 30 μl of 1× SacII restriction buffer (New England Biolabs, Beverly, MA). The transformed plasmid DNA was purified by phenol/chloroform (1:1, v/v) extraction, electrophoresed on a 0.8% agarose gel, and recovered from the gel using a QIAquick Gel Extraction Kit (Qiagen GmbH, Germany).

Plasmid DNA was purified from the ligation reaction by precipitation with ammonium acetate/ethanol and resuspended in 4 μl of water, of which 1 μl was used to transform 40 μl of Electromax Escherichia coli DH10B cells (Life Technologies, Inc.). The transformed bacteria were diluted into 1000 ml of LB medium containing ampicillin. Aliquots of cells were plated on LB ampicillin plates for calculation of the total number of recombinants. The remainder was divided into 400 pools of 2.5 ml each that were grown to saturation overnight at 37 °C. The total number of independent recombinants in the cDNA library was 1.5 × 107, and each pool contained an average of 3800 recombinants. DNA was isolated from individual pools using a Wizard Miniprep Kit (Promega Inc, Madison, WI). The yield of plasmid DNA from each pool was approximately 25 μg.

Human embryonic kidney 293 cells (ATCC CRL 1573) were plated on day 0 at a density of 7 × 104 cells/ml in Medium A (Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate). On day 1, individual dishes were transfected with a mixture of plasmid DNAs that included pool DNA (5 μg), pCMV-STAR (2 μg), pCAGI (2 μg), and pVA-1 (1 μl). The expression plasmid pCMV-STAR contains a full-length cDNA encoding the mouse steroidalogenic acute regulatory protein (STAR, Ref. 16) and was a kind gift of Dr. Douglas Stocco, Texas Tech University Medical School, Lubbock, TX. The expression plasmid pCAGI contains a full-length cDNA encoding the mouse oxyysterol 7α-hydroxylase enzyme (17, 18). The original b21 cDNA was a kind gift of Dr. Richard Lathe, University of Edinburgh, Edinburgh, Scotland. The plasmid pVAI contains the adenovirus type 5 VAI gene (19). A positive control, in which cells were transfected with 1 ng of a murine sterol 27-hydroxylation enzyme plasmid diluted into 5 μg of pCMV6 vector alone, was included in every experiment. Aliquots (40 μl) of the transformation mixture were added to 20,000 transformed bacteria in 5 ml of LB medium containing ampicillin and divided into 20 pools, each containing ~490 recombinants for secondary screening. DNA was prepared from individual pools, and 293 cells were transfected as above, except that 6-7 plates were used in place of 60-mm dishes, and amounts of reagents were scaled down accordingly. Tertiary screening was similarly performed except that pools of 200 recombinants were transfected. Quaternary screening was carried out with pools of 10 cDNA isolates derived from a matrix array of individual cDNAs to identify a single cholesterol 25-hydroxylase cDNA.

**Measurement of Cholesterol 25-Hydroxylase Activity in Whole Cells**—The transfection medium containing the cationic lipid was aspirated and replaced with 3 ml of Medium B (DMEM containing 10% newborn calf serum) supplemented with 5 μl of 4×-Cholesterol (56.6 μCi/mmol; 0.040 μCi/μl; NEN Life Science Products). Cells were incubated for a further 6 h at 37 °C in an atmosphere of 8.8% CO2.

Media from the transfected cells were collected and extracted with 8 ml of chloroform/methanol (2:1, v/v). The organic phase from each sample was taken to dryness under a stream of nitrogen, and residues were dissolved in 40-μl aliquots of chloroform/methanol (2:1, v/v) and applied to 20 × 20 cm precoated LK5DF silica gel TLC plates (Whatman, Hillsboro, OR) with preadsorbent layers. The plates were developed in ethyl acetate/toluene (4:6, v/v) and exposed to a Fuji BAS-MP phosphorimager plate overnight. Phosphorimager analysis was then performed on a Fuji BAS-2500 system.

**Isolation of Human Cholesterol 25-Hydroxylase cDNA**—A 372-base pair expressed sequence tag (EST, GenBank accession number W101328) with high sequence identity to a portion of the murine cholesterol 25-hydroxylase cDNA was identified by BLAST search. A bacterial stab transformed with a plasmid containing the EST sequence cloned into the pT37 vector was obtained from Research Genetics, Inc., Huntsville, AL. The plasmid DNA was purified by the polymerase chain reaction using oligonucleotide primers of the following sequence: 5′-CTGGGACACCTTGAGGCGCTC-3′ (forward primer) and 5′-GCCCAATGCACGGGCTC-3′ (reverse primer). The thermocycler program consisted of 55 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The amplified cDNA fragment was cloned into pGEM-T Easy (Promega Corp., Madison, WI). The insert was excised from the plasmid with EcoRI and used for the preparation of a radiolabeled probe by random octamer priming (Megaprime Labeling Kit, Amersham Pharmacia Biotech). The probe was used to screen 200,000 plaques of a human lung cDNA library in bacteriophage λgt10 (catalog number HL3004a, CLONTECH, Palo Alto, CA) using standard hybridization procedures (20). One positive probe was inserted whose cDNA insert was subcloned into the EcoRI sites of pBluescript SK+ (Stratagene Corp., La Jolla, CA) and pCMV6, yielding plasmids pBS-h25 and pCMV-h25, respectively.

**Gene Mapping**—Cholesterol 25-hydroxylase gene sequences were derived from a murine genomic library prepared from 128SVeV DNA (a kind gift of Dr. Alan Bradley, Baylor College of Medicine, Houston, TX) and a human genomic library (catalog number 946204, Stratagene), both in bacteriophage λ FIX II, by screening with full-length cDNA probes corresponding to the human and murine cholesterol 25-hydroxylase cDNAs, respectively, using standard protocols (20). Approximately 600 murine and 400,000 human recombinants were screened, and one positive clone from each library was identified and purified to homogeneity. The corresponding genomic DNA inserts were excised from the bacteriophage vectors and ligated into the NotI site of pBluescript SK+, yielding plasmids pBS-mg25 and pBS-h25, respectively.

The chromosomal location of the human cholesterol 25-hydroxylase gene was determined by fluorescence in situ hybridization (FISH) and by polymerase chain reaction amplification of somatic cell and radiation hybrid panel DNAs. FISH mapping was performed by See DNA Biotech, Huntsville, AL. Most hybrid panel DNAs were labeled by nick translation with biotinylated dATP for use as a FISH probe. Of 100 mitotic figures analyzed, 91 showed hybridization signals on paired sister chromatids corresponding to chromosome 10. Comparison of the signal positions with bands generated by staining with 4,6-diamidino-2-phenylindole indicated that hybridization occurred at band q23. Radiation and somatic cell hybrid panel DNA was used to map a radiation hybrid mapping panel of DNAs in the Somatic Cell Hybrid Mapping Panel 2 (Coriell Institute of Medical Research, Camden, NJ) and the Stanford G-3 radiation hybrid panel (Research Genetics, Huntsville, AL). The primer pair used for amplification was 5′-CTGGGACACCTTGAGGCGCTC-3′ (forward primer) and 5′-GCCCAATGCACGGGCTCAG-3′ (reverse primer), which, respectively, correspond to nucleotides 79–98 and 333–314 of the human gene sequence (Fig. 6A). The thermocycler program consisted of 35 cycles of 94 °C for 15 s and 68 °C for 30 s on a Perkin-Elmer GeneAmp 9600 machine. Only somatic cell hybrid DNAs containing human chromosome 10 produced a positive amplification signal. Analysis of the radiation hybrid data through the Stanford Genome Center server2 indicated linkage of the cholesterol 25-hydroxylase gene to the SHGC-15188 marker (LOD score = 9.4, cR_1000 = 45.76) on chromosome 10 in the vicinity of band q23.

**DNA Sequencing and RNA Blotting**—DNA sequencing was performed on an ABI Prism 377 sequencer using thermocycler sequencing protocols and fluorescent dye terminators. Contiguous DNA sequences were assembled using MacVector software (IBI-Kodak Corp., New Haven, CT). Cloned restriction-enzyme DNA was used to probe cosmid libraries, Restriction-site DNA was amplified by polymerase chain reaction using oligonucleotide primers corresponding to the following sequence: 5′-GATCTCTGCTAGGCGCTC-3′ (forward primer) and 5′-GCCCAATGCACGGGCGCTCAG-3′ (reverse primer). The thermocycler program consisted of 55 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The amplified cDNA fragment was cloned into pGEM-T Easy (Promega Corp., Madison, WI).

DNA blotting, a murine multiple tissue RNA blot (CLONTECH, catalog number 7762-1) was hybridized overnight in 50% formamide-
hybridization buffer at 42 °C with a full-length murine 25-hydroxylase cDNA probe using standard procedures (20). The probe was radiolabeled by random nonamer priming with [32P]dCTP. The blot was washed stringently at 65 °C, in 0.1× SSC containing 0.1% (v/v) SDS before exposure for 5 days to Kodak X-OMAT AR film at -80 °C using an intensifying screen.

**Antibodies**—An antipeptide antibody against the sequence RRYKIHPDFFSPSVKQ, representing amino acids 69–83 of the murine cholesterol 25-hydroxylase (Fig. 3A), was raised in rabbits. This sequence was synthesized as a multiple antigen peptide by Bio-Synthesis, Inc. (Lewisville, TX). For the initial immunization, 100 μg of peptide was administered intramuscularly as a dispersion in Freund's complete adjuvant to two New Zealand White male rabbits, 3 months of age. Boosts of 100 μg of antigen in Freund's incomplete adjuvant were given on average every 5 weeks, and bleeds were drawn 7 days after each boost. One of the two resulting antisera, U104, was used here after affinity purification on peptide antigen columns (21).

**Epitope Tagging**—To construct an epitope-tagged version of the murine cholesterol 25-hydroxylase enzyme, a cDNA fragment spanning the coding region and having BspDI and XhoI restriction sites at the 5′ and 3′ ends, was amplified by the polymerase chain reaction using the oligonucleotide primers 5′-AAATCATGCCGTGCACACGATTCGGA-3′ (forward primer) and 5′-AAATCTAGAATACTTGAACATGCAACAATCTCAGTTTAACTGC-3′ (reverse primer). The template was the plasmid pCMV-m25, and the thermocycler program consisted of 35 cycles of 94 °C at 30 s, 57 °C at 15 s, and 72 °C at 60 s. The amplified cDNA fragment was purified on a Centricon-100 column, and ligated into a modified pcDNA3 plasmid. The recovery was done in a 1:1 solution of DMEM/Ham's F12 medium. The cells were incubated for 1 h with 2% (w/v) 2-hydroxypropyl-beta-cyclodextrin, and the cell pellet was resuspended in 1 ml of Ham's F12 medium containing 5% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate). On day 0, cells were plated at a density of 7 × 10⁴ cells/60-mm dish. On day 1, transfections were carried out using 4 μg/dish of the indicated plasmid DNA and Lipofectamine reagent (Life Technologies, Inc.) according to the manufacturer's instructions and with modifications as described (26). After transfection, fresh media supplemented with 50 μg/ml neomycin, 50 μg/ml compactin, and 0.2% ethanol containing either no sterol or a mixture of sterols (final concentrations of 1 μg/ml 25-hydroxycholesterol and 10 μg/ml cholesterol) were added. The cells were returned for 20 h to a 37 °C incubator, and the samples were then harvested into a reaction mix containing 100 μl of pfx-8 lipid was used as a transfection reagent as described above and incubated for 48 h in Medium D supplemented with 10 μg/ml cholesterol. Thereafter, media were extracted with chloroform/methanol (2:1, v/v; 5 ml/well), and the organic phase was separated and taken to dryness under a stream of nitrogen. Extracts from 6 wells were combined for subsequent procedures. The samples were purified on Isolute silica columns (International Sorbent Technology, Mid Glamorgan, UK), and hydroxyl groups were converted to trimethylsilyl ethers as described previously (15).

**Gas chromatography-mass spectrometry**—Gas chromatography-mass spectrometry was performed on a Varian 3400 gas chromatograph equipped with an HP-5MS capillary column (30 m × 0.25 mm, 0.25-μm phase thickness) connected to a Finnigan SQ700 mass spectrometer. The gas chromatography temperature program was 180 °C for 1 min, followed by a temperature gradient of 1 °C/min to 300 °C, and held at 300 °C for 1 min. Nitrogen was used as the carrier gas at an injector valve pressure of 6 square inches. Injector and transfer line temperatures were set to 280 °C, and the injector was operated in the splitless mode. The mass detector was operated in the electron ionization mode with electron energy set to 70 eV, and the quadrupole was scanned between m/z 100 and 500 at a rate of 1 scan/1.5 s.

**Measurement of Cholesterol 25-Hydroxylase Activity in Cell Lysates**—To examine the sensitivity of N-linked carbohydrates on cholesterol 25-hydroxylase to endoglycosidase digestion, COS M6 cells were initially plated at a density of 5 × 10⁵ cells/60-mm dish in Medium A on Day 0 of the experiment. On Day 1, one dish each was transfected with 4.5 μg of pcMV-m25, pcDNA3-NH₂-myc-h₂₅, pcDNA3-h₂₅, or pcDNA3-NH₂-myc-m₂₅, and the cells were transfected with pCMV-m25 or with vector alone as described above and incubated for 48 h in Medium D supplemented with 10 μg/ml cholesterol. Thereafter, media were extracted with chloroform/methanol (2:1, v/v; 5 ml/well), and the organic phase was separated and taken to dryness under a stream of nitrogen. Extracts from 6 wells were combined for subsequent procedures. The samples were purified on Isolute silica columns (International Sorbent Technology, Mid Glamorgan, UK), and hydroxyl groups were converted to trimethylsilyl ethers as described previously (15).

**Measurement of N-Linked Carbohydrates**—To examine the sensitivity of N-linked carbohydrates on cholesterol 25-hydroxylase to endoglycosidase digestion, COS M6 cells were initially plated at a density of 5 × 10⁵ cells/60-mm dish in Medium A on Day 0 of the experiment. On Day 1, one dish each was transfected with 4.5 μg of pcMV-m25, pcDNA3-NH₂-myc-m₂₅, pcMV-m25-COOH-myc, pcCMV-h₂₅, or pcDNA3-NH₂-myc-m₂₅, and the cells were transfected with pCMV-m25 or with vector alone as described above and incubated for 48 h in Medium D supplemented with 10 μg/ml cholesterol. Thereafter, media were extracted with chloroform/methanol (2:1, v/v; 5 ml/well), and the organic phase was separated and taken to dryness under a stream of nitrogen. Extracts from 6 wells were combined for subsequent procedures. The samples were purified on Isolute silica columns (International Sorbent Technology, Mid Glamorgan, UK), and hydroxyl groups were converted to trimethylsilyl ethers as described previously (15).

**Measurement of Cholesterol 25-Hydroxylase Activity**—On Day 0, a derivative of Chinese hamster ovarian cells expressing the polyoma virus middle T antigen (31) (CHO cells) were plated at a density of 750,000 cells/100-mm dish in Medium C (1/1 (v/v) DMEM/ Ham's F12 medium containing 5% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate). On day 1, the cells were transfected with 1.5 μg of pVA1-1 and 13.5 μg of pcMV6, pcCMV-m25-HH2420Q4, or pcCMV-m25 per dish for vector, mutant, and wild type 25-hydroxylase, respectively (121 and 67 μg/mg protein, respectively) as a transfection reagent as described above. On day 2, cells were incubated for 1 h with 2% (v/v) 2-hydroxypropyl-beta-cyclodextrin dissolved in 1:1 solution of DMEM/Ham's F12 medium. The cells were washed once with ice-cold PBS and then harvested in the same buffer using a rubber policeman. After centrifugation at 1000 × g for 5 min, the buffer was aspirated, and the cell pellet was resuspended in 1 ml of 50 mM potassium phosphate buffer, pH 7.4, containing protease inhibitors (Boehringer Mannheim Complete Mini, EDTA-free, at the concentration recommended by the supplier). A cell lysate was prepared using a Polytron set at 10,000 rpm, with three bursts of 3 s each with 30-s intervals between bursts. Incubations were performed at 37 °C with 100 μg/ml of lysolecithin in 50 mM potassium phosphate buffer, pH 7.4, containing 5 mM NADPH. [4-14C]Cholesterol was added in 4 μl of 45% (v/v) 2-hydroxypropylcyclohexedrine in water to a final concentration of 5 μM. The total volume of the incubation was adjusted to 200 μl. After 2 h, reactions were extracted with chloroform/methanol (2:1, v/v) and analyzed by thin layer chromatography.

**Cytotoxicity**—For indirect immunocytochemistry, COS M6 cells were plated at a density of 4 × 10⁴ cells per well on glass coverslips
placed in 6-well dishes containing Medium B. On Day 1, cells were transfected with either a vector alone control (pCMV6) or with pCMV-m25-COOH-myc-m25 or pCDNA3-NH2-myc-m25. Three μg of plasmid DNA and 12 μl of pfx-8 lipid mixture were used per well. After transfection, cells were cultured in Medium B. Indirect immunochemistry was then performed with the indicated antibody and lectin probes as follows. Cells were fixed for 30 min with 3% (v/v) paraformaldehyde in Hanks’ balanced salt solution, pH 7.4. Following fixation, the coverslips were briefly rinsed with PBS (0.1 M phosphate buffer, pH 7.4, 0.15 M NaCl), and free aldehyde groups were quenched by incubation in PBS containing 50 mM NH₄Cl for 30 min. Permeabilization was accomplished by incubation in 0.1% (v/v) Triton X-100 in H₂O for 7 min on ice, followed by rinsing in PBS containing 1% (w/v) bovine serum albumin (blocking buffer) for 30 min at room temperature. Coverslips were incubated with rabbit anti-c-Myc IgG (Upstate Biotechnology Inc., 10 μg/ml in blocking buffer) for 2 h at room temperature. Finally, coverslips were incubated with fluorescein isothiocyanate goat anti-rabbit IgG (Zymed Laboratories Inc.; 20 μg/ml in blocking buffer) for 1 h at room temperature. For Golgi compartment staining, rhodamine-labeled wheat germ agglutinin was added during the second antibody incubation at a concentration of 1.25 μg/ml. Coverslips were washed three times with PBS containing 0.1% bovine serum albumin after each antibody or lectin incubation. Cells were photographed using a Zeiss Photomicroscope.

**Inhibitor Studies**—Transfection of CHOP cells was carried out as described above with the following exceptions. Cells were plated on Day 0 at a density of 150,000 cells/well in 6-well plates containing Medium C. On Day 1, cells were transfected with 2.7 μg of the indicated cholesterol 25-hydroxylase expression plasmid and 0.3 μg of pVA-1, using 12 μl of pfx-8 as a transfection lipid. After 4 h, the lipid/DNA mixture was removed, and 1.5 ml of Medium D was added. In certain experiments, this medium was aspirated and replaced on Day 2 with 1 ml per well of a 20 mg/ml solution of 2-hydroxypropyl-β-cyclodextrin (Sigma) in DMEM/Ham’s F12 (1:1) medium and returned to the incubator. The cyclodextrin-containing medium was replaced after 1–1.5 h with 1.5 ml of Medium D, and substrate and inhibitors were then added, each in a volume of 4.5 μl of ethanol. The concentration of [4-14C]cholesterol substrate (specific activity = 26.5 mCi/mmol) was 3 μM. Inhibitors were added to final concentrations of 3, 10, or 30 μM as indicated in Fig. 9. Cells and/or cells plus medium were harvested at the indicated times, extracted, and the conversion of substrate into [4-14C]25-hydroxycholesterol product was determined by thin layer chromatography as described above. The inhibitors used were cholesterol (5-cholastene-3α-ol), cholesatol (5a-cholest-3α-ol), epicholesterol (5-cholostanol-3α-ol), co- prostanol (5β-cholest-3β-ol), desmosterol (5, 24-cholestadien-3α-ol), β-sitosterol (5β-cholest-24β-ethyl-3α-ol), 25-hydroxycorticosterol (chole- last-5-ene-3β-ol), 25-diol, and 27-nor-25-oxocorticosterone (27-nor-25-oxo-5-cholastene-3β-ol). All steroids were purchased from Steraloids Inc. (Wilton, NH), except cholesterol, which was from Sigma.

**Stable Cell Lines**—Ecr-CHO cells (Invitrogen), a cell line stably expressing the subunits of the Droso phila ecdds receptor, RKR and VgECR (31), were plated on Day 0 at a density of 500,000 cells/100-mm dish in Medium C supplemented with 250 μg/ml Geneticin and 200 μg/ml zeocin. On Day 1, cells were transfected with 5 μg of pIND-m25 using 15 μl of Fugene 6 (Boehringer Mannheim) according to the instructions of the manufacturer. The plasmid pIND-m25 was constructed by insertion of a full-length mouse 25-hydroxylase cDNA fragment from plasmid pCMV-m25 into the pIND vector (Invitrogen). On Day 2, cells were split 1:15 and plated in fresh Medium C supplemented with 250 μg/ml Geneticin and 700 μg/ml zeocin. Cells were refed this medium every 2nd day, and on Day 10, groups of five geneticin-resistant colonies were replated in individual wells. Following expansion, cells were tested for cholesterol 25-hydroxylase expression by addition of ponasterone (Invitrogen) to a final concentration of 5 μM (16 h), followed by immunoblot analysis of total cell protein. Antibody U-104 (affinity purified) was used to detect expression of the enzyme as described above. One positive group of cells was selected and subcloned through one additional round to ensure clonality. One of the resulting cell strains, designated TR3102a, which manifests high level expression of cholesterol 25-hydroxylase upon ponasterone induction, and another, designated TR3102g, with no detectable inducible enzyme expression, were selected and maintained as lines. For routine induction experiments, 10 μM ponasterone was used for the indicated periods.

**Cholesterol Biosynthesis**—TR3102a and TR3102g cells were plated on Day 0 at a density of 250,000 cells/60-mm dish in Medium C containing Zeocin and geneticin as above. On Day 1, the medium was changed to Medium F (Medium D containing 250 μg/ml Zeocin, 700 μg/ml Geneticin, and 10 μM ponasterone). On Day 2, 20 μl of an aqueous solution containing 15 μCi of [1,2-3H]acetate (American Radiolabeled Chemicals), adjusted with cold acetate to a final mass of 1 μmol, was added to each dish. The additions were made in a staggered fashion so that all cells were harvested at the same time, corresponding to incubation times of 2, 4, and 6 h, respectively. The total time of induction with ponasterone was 27 h, including the acetate labeling period. Non-saponifiable lipids were isolated and analyzed by thin layer chromatography as described (32) except that 5 μCi of [26,27-3H]25-hydroxycholesterol (NEN Life Science Products) was used as a standard. Quantification of acetate incorporation into cholesterol was via phosphorimage analysis.

**RESULTS**

The livers of transgenic mice overexpressing the transcription factor SREBP-1a accumulate large quantities of cholesterol and triglycerides, owing to the overproduction of lipid synthesizing enzymes (14). An analysis of stool lipids by mass spectrometry revealed that these animals also excrete high levels of several oxysterols, including 25-hydroxycholesterol. To isolate cDNAs that encode putative oxysterol synthesizing enzymes from the livers of these transgenic mice, an expression cloning strategy in cultured mammalian cells was conceived and optimized. The basic premise of the screen was to transfect cells with pools of hepatic cDNAs cloned into an expression vector, add [14C]cholesterol to the medium, and then measure the conversion of this substrate into oxysterols by thin layer chromatography assay. Initially, we used a previously isolated sterol 27-hydroxylase cDNA, whose encoded enzyme converts cholesterol into the sterol 27-hydroxycholesterol (15), to optimize assay parameters.

Chromatography studies with oxysterol standards revealed that the separation between cholesterol and some oxysterols was poor on silica gel plates. Furthermore, in control transfection studies with the sterol 27-hydroxylase expression vector, the strong phosphorimage signal from the substrate often obscured a weaker product signal. To overcome these problems, a cDNA encoding a murine oxysterol 7α-hydroxylase (18) was cotransfected into the cells. This addition should result in the...
conversion of oxysterol products to their 7α-hydroxylated forms. The oxysterol 7α-hydroxylase also possesses a minor 2-hydroxylase activity against 7α-hydroxylated sterols (18); thus the formation of 2,7α-hydroxylated oxysterols was expected. Both of these classes of hydroxylated oxysterols were readily separated from cholesterol by thin layer chromatography assay. An autoradiogram derived from the silica gel plate is shown with the positions of cholesterol, 25-hydroxycholesterol, and sterol esters marked on the left. The percent conversion of substrate into product determined by quantification of the phosphorimage analysis is indicated below each lane. B, chemical analyses of cholesterol 25-hydroxylase products. Sterols from the media of cells transfected for 4.0 h with plasmid vector alone or vector containing a murine cholesterol 25-hydroxylase cDNA were extracted with organic solvent, derivatized, and subjected to gas chromatography-mass spectrometry. A cDNA-dependent product eluting at 23.56 min from the gas chromatograph (left panel) had an ionization spectrum virtually identical to that of authentic 25-hydroxycholesterol (right panels).

The pool containing the 25-hydroxylase cDNA was progressively subdivided and expressed to isolate a single cDNA. As the purity of the cDNA increased, the level of product generated in the transfected cells also increased to the point that cotransfection of the oxysterol 7α-hydroxylase cDNA was dispensable. Additional experiments revealed that the cDNA-encoded enzyme was not stimulated by inclusion of the steroidogenic acute activator cDNA, suggesting that it was not a mitochondrial protein. Transfection of the pure cDNA into CHOP cells produced abundant 25-hydroxylase enzyme activity that increased with time of incubation (Fig. 2A). The activity was stimulated approximately 10-fold by treatment of transfected cells with 2-hydroxypropyl-β-cyclodextrin (Fig. 2A). This compound presumably removes endogenous cholesterol from the membranes of the transfected cells that otherwise competes with the exogenously added radiolabeled cholesterol substrate (34). The chemical structure of the oxysterol produced by the isolated cDNA was determined by gas chromatography-electrospray mass spectrometry (Fig. 2B). The media from cells transfected with the putative cholesterol 25-hydroxylase cDNA contained a prominent sterol eluting at 23.56 min from the gas chromatography column. This sterol was not present in the media of mock-transfected cells (Fig. 2B, left panels). The mass spectrum of the cDNA-generated product was virtually identical to that of an authentic 25-hydroxycholesterol standard (Fig. 2B, right panels).

A search of the DNA data bases revealed a human EST with
Fig. 3. Structure of murine and human cholesterol 25-hydroxylases. A, line up of the cDNA-deduced protein sequences of the murine and human enzymes. Individual amino acids are shown in single letter code. Sequence identities are indicated by the black boxes. Amino acids are numbered on the right. The GenBank accessions numbers for the murine and human cDNA sequences are AF059213 and AF059214, respectively. B, hydropathy analyses comparing the primary sequences of the murine cholesterol 25-hydroxylase (upper panel) and the human cholesterol 25-hydroxylase (lower panel). Predicted hydrophobic regions of the protein sequences fall below the midlines, whereas hydrophilic sequences rise above the midlines. MacVector software was used to generate the data. The window size in the scan was 12 amino acids.

The subcellular localization of cholesterol 25-hydroxylase was assayed in two ways. First, the presence and structure of asparagine-linked carbohydrates were analyzed by endoglycosidase digestion (Fig. 5A). Expression of murine or human cDNAs in COS cells produced two forms of the enzyme that differed in mass by approximately 3 kDa as judged by immunoblotting (Fig. 5A, left panel) but eliminated enzyme activity in transfected cells (Fig. 5A, right panel). Similar results were obtained in a second experiment in which cholesterol 25-hydroxylase enzyme activity was measured in cell lysates rather than in intact cells (Fig. 4B).

The second approach to examine the subcellular location of cholesterol 25-hydroxylase were important for enzyme activity, a pair of histidine codons at positions 242 and 243 in the murine protein were changed to glutamine codons by site-directed mutagenesis of the wild type cDNA. The resulting mutant cDNA was transfected into CHOP cells and assayed for expression of the protein and for cholesterol 25-hydroxylase enzyme activity (Fig. 5A). Mutation of the two histidine residues had no effect on steady state expression levels as judged by immunoblotting (Fig. 5A, left panel) but eliminated enzyme activity in transfected cells (Fig. 5A, right panel).
Cholesterol 25-Hydroxylase cDNA

Fig. 4. Structure-function analysis in murine cholesterol 25-hydroxylase. A, expression of wild type and mutant cholesterol 25-hydroxylase cDNAs in intact cells. Plasmids containing no cDNA insert (lanes 1 and 4, labeled Vector), a mutant 25-hydroxylase cDNA in which histidine codons at positions 242 and 243 were changed to glutamine codons (lanes 2 and 5, labeled Mutant), or a wild type 25-hydroxylase cDNA (lanes 3 and 6, labeled Wild Type) were introduced into CHOP cells. 24 h later, the cells were assayed for expression of 25-hydroxylase protein by immunoblotting of cell lysates (left panel) and for enzyme activity in intact cells (right panel) as described under “Experimental Procedures.” B, cholesterol 25-hydroxylase enzyme activity in cell lysates. CHOP cells were transiently transfected with the indicated plasmids as described in A. 24 h later, cell lysates were prepared and assayed for 25-hydroxylase protein (left panel) and enzyme activity (right panel) as described under “Experimental Procedures.” In both A and B, mutation of the two histidine codons did not affect expression of the protein but did inactivate the enzyme. A closely spaced doublet corresponding to differentially glycosylated forms of the 25-hydroxylase protein (see Fig. 5A) is detected in A, but not B, because the polycyclonamide gel used here is electrophoresed for an extended period prior to immunoblotting.

We next isolated the murine and human cholesterol 25-hydroxylase genes. DNA sequence analysis of the isolated genomic DNAs and comparison to the respective cDNA sequences revealed that both genes lacked introns (Fig. 6A). This structure was confirmed by Southern blotting analyses of murine and human DNA and by direct amplification of the genes from genomic DNA (data not shown). Transfection into 293 cells of a plasmid containing the genomic DNA insert from bacteriophage λ clone that encompassed ~10 kb of 5′-flanking DNA and ~3 kb of 3′-flanking DNA of the murine 25-hydroxylase gene resulted in the expression of enzyme activity, which suggested that the isolated gene was not a pseudogene and that requisite regulatory sequences were located close to the coding region (data not shown). The human gene was localized to chromosome 10q23.3 by somatic and radiation hybrid DNA panel mapping and fluorescence in situ hybridization (Fig. 6B).

The tissue distribution of the murine cholesterol 25-hydroxylase mRNA was assessed by blot hybridization (Fig. 7). Low levels of a 1.5-kb mRNA were present in the heart, lung, and kidney. The mRNA was not detected in the livers of control mice (Fig. 7); however, it was present in RNA from the liver of the SREBP-1a transgenic mouse used to prepare the original cDNA expression library (data not shown). RNA blotting experiments using commercially available filters revealed only very low levels of human cholesterol 25-hydroxylase mRNA in 16 different tissues (data not shown).

The potent regulatory effects of 25-hydroxycholesterol were first observed in assays that measured the suppressive effects of oxysterols on cholesterol synthesis (1, 2). To determine if the 25-hydroxycholesterol synthesized by the 25-hydroxylase enzyme could suppress cholesterol synthesis, a line of CHO cells containing an ecdysone-inducible 25-hydroxylase cDNA was isolated as described under “Experimental Procedures.” These cells, and a control cell line that did not contain the 25-hydroxylase cDNA, were induced with the ecdysone analog ponasterone, and the incorporation of [14C]acetate into cholesterol was measured as a function of time. As shown in Fig. 8A, induction with ecdysone led to a marked reduction of cholesterol synthesis in cells containing the 25-hydroxylase cDNA but had no effect on this parameter in the control cells.

The experiments shown in Fig. 8B were carried out to determine if expression of cholesterol 25-hydroxylase in transfected cells affected the processing of SREBP transcription factors. Cultured CHO-7 cells were transiently transfected with either vector alone or an expression vector containing the murine 25-hydroxylase cDNA. After 24 h, fractions enriched in membrane or nuclear proteins were prepared from the transfected cells. Equal amounts of protein from each subcellular compartment were separated by gel electrophoresis, and the levels of SREBP-1a and SREBP-1c were determined by immunoblotting. Mock-transfected cells grown in the absence of sterols to induce SREBP-1 cleavage contained intact, uncleaved SREBP-1 in the membrane fraction and cleaved SREBP-1 in the nuclear fraction (Fig. 8B, lane 1). Mock-transfected cells grown in the presence of sterols (cholesterol plus 25-hydroxycholesterol) contained a majority of the immunodetectable SREBP-1 in the membrane fraction (lane 2). Cells transfected with the 25-hydroxylase cDNA and grown in the absence of sterols contained a majority of SREBP-1 in the membrane fraction even though no exogenous sterols were added (lane 3), presumably because the 25-hydroxycholesterol produced by the expressed 25-hydroxylase suppressed cleavage of the transcription factor. Similar results were obtained when the processing of SREBP-2 was followed by subcellular fractionation and immunoblotting (Fig. 8B, right panels, lanes 4–6).
Fig. 5. Analyses of asparagine-linked carbohydrates and subcellular localization of cholesterol 25-hydroxylase. A, simian COS cells were transfected with the indicated cholesterol 25-hydroxylase cDNA, and membrane proteins were treated as described under “Experimental Procedures” with endoglycosidase H (Endo H), which cleaves high mannose and some hybrid asparagine-linked carbohydrates or peptide-N-glycosidase F (PNGase F), which cleaves high mannose, hybrid, and complex asparagine-linked carbohydrates. Treated proteins were resolved by SDS-polyacrylamide gel electrophoresis, and the expressed cholesterol 25-hydroxylase was detected by immunoblotting with an antibody (U-104) that recognizes both the murine and human proteins. B, simian COS cells were transfected with a plasmid vector encoding a carboxyl terminus, epitope-tagged (c-Myc) form of the murine cholesterol 25-hydroxylase. After transient expression, cells were fixed, permeabilized, and incubated with a murine monoclonal antibody directed against the c-Myc epitope (green fluorescence, upper panel) and a wheat germ agglutinin conjugate (orange fluorescence, lower panel) that binds glycoproteins in the Golgi compartment. A goat anti-rabbit IgG serum conjugated with fluorescein was used to detect the primary antibody. The lectin probe was conjugated with rhodamine. The epitope-tagged cholesterol 25-hydroxylase is detected in both the endoplasmic reticulum and Golgi compartment of expressing cells (upper panel).
When desmosterol and [14C]cholesterol were present in equimolar amounts (3 μM), enzyme activity was decreased by 30%, whereas coprostanol did not inhibit the enzyme at this concentration. The observed inhibition of 25-hydroxylase activ-

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**FIG. 6.** Comparison of murine and human cholesterol 25-hydroxylase gene sequences and chromosomal location of the human gene. A, line up of the human and murine 25-hydroxylase gene sequences. The MegAlign subroutine of the DNA Star sequence analysis program was used to align the two DNA sequences for maximum identity. The black boxes indicate identical nucleotides. Sequences are numbered on the left. Position 1 was arbitrarily assigned to the first nucleotides of the illustrated sequences. The sequences specifying the initiation codons (ATG) of both genes begin at nucleotide 11 in the 1st lines of the DNA. The sequence specifying the termination codon of the human gene occurs at nucleotide 827 on the 10th line. The mouse termination codon occurs at nucleotide 905 on the 11th line. The GenBank accessions numbers for the murine and human gene sequences are AF059211 and AF059212, respectively. B, idiogram of human chromosome 10 showing location of cholesterol 25-hydroxylase gene. The position of the gene was determined by polymerase chain reaction analyses of radiation hybrid panel DNAs and by fluorescence in situ hybridization analyses using a genomic DNA probe. Both methods positioned the gene on chromosome 10q23.2.

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desmosterol > cholestanol > 25-hydroxycholesterol > epicholesterol > sitosterol ≃ coprostanol = 25-oxo-27-nor-cholesterol (Fig. 9). When desmosterol and [14C]cholesterol were present in equimolar amounts (3 μM), enzyme activity was decreased by 30%, whereas coprostanol did not inhibit the enzyme at this concentration. The observed inhibition of 25-hydroxylase activ-
ity could be due to individual sterols acting as either true inhibitors of the enzyme (i.e., not as substrates) or as competitors of the cholesterol substrate. In the case of desmosterol (5,24-cholestadien-3β,ol), which cannot be 25-hydroxylated due to the Δ24 bond, this sterol may be acting as a true inhibitor.

**DISCUSSION**

We report the isolation of cDNAs encoding enzymes that convert cholesterol to 25-hydroxycholesterol. An expression cloning assay was initially developed in 293 cells, and screening of >10⁶ independent clones produced a murine cDNA specifying cholesterol 25-hydroxylase. A cDNA encoding the human homologue was subsequently isolated by hybridization screening. The encoded murine and human cholesterol 25-hydroxylases are hydrophobic enzymes that synthesize 25-hydroxycholesterol, share 78% sequence identity, and are predicted to span the membrane multiple times. In transfected cells, the enzyme is localized to the endoplasmic reticulum and the Golgi compartment. The cholesterol 25-hydroxylase gene is expressed at low levels in several murine tissues as judged by RNA blotting. The expression of a cholesterol 25-hydroxylase cDNA in transfected cells suppresses the incorporation of acetate into cholesterol and the proteolytic activation of SREBPs. Taken together, the data suggest that cholesterol 25-hydroxylase may play an important regulatory role in cholesterol metabolism by synthesizing 25-hydroxycholesterol, a known sterol inhibitor of SREBP-dependent gene transcription.

Unlike other enzymes that catalyze hydroxylation reactions on sterol and steroid substrates, the murine and human cholesterol 25-hydroxylases are not cytochrome P450s (Fig. 3A). Rather, they belong to a growing family of enzymes that utilize oxygen and a diiron cofactor to catalyze hydroxylation reactions on different substrates. The diiron cofactor can be either Fe—O–Fe or Fe—OH–Fe and is bound to the enzyme through interactions with clustered histidine or glutamate residues (37, 41). Sequence comparisons reveal three classes of enzymes in this family, one of which is composed of membrane-bound steraryl-CoA desaturases, alkane hydroxylase, and xylene monooxygenase (37). These proteins contain multiple membrane-spanning domains and an unique arrangement of histidine clusters that are postulated to bind the diiron cofactor and to have catalytic function. The murine and human cholesterol 25-hydroxylases contain both of these shared structural features (Fig. 3), and mutation of two of the conserved histidine residues in the murine enzyme eliminates activity (Fig. 4). Similar landmarks are also present in the C-4 sterol methyl oxidase isolated from Saccharomyces cerevisiae and man (38, 39), a rat protein of unknown function termed neurep 1 (42), and in several cDNA sequences present in the data bases (e.g., GenBank™ accession number U40841 from Caenorhabditis elegans). The cholesterol 25-hydroxylases are 27% identical in sequence to the C-4 sterol methyl oxidases, and they share 25–30% sequence identity with the neurep 1 and C. elegans proteins. These four proteins may thus have arisen from a common ancestor involved in sterol metabolism.

Cells transfected with the murine cholesterol 25-hydroxylase cDNA produce authentic 25-hydroxycholesterol (Fig. 2B), and the oxysterol synthesized in cells transfected with the human cDNA comigrates with the mouse product in thin layer chromatography plates (Fig. 2A). No cDNA-dependent metabolism of 25-hydroxycholesterol, dehydroepiandrosterone, pregnenolone, 5α-androstane-3α, 17β-diol, 5α-androstane-3β, 17β-diol,
cholesterol 25-hydroxylase expression vector using a lipofection procedure. On Day 1, we transiently transfected CHOP cells with a murine cholesterol synthesis (1, 2) and the transcription of genes that encode 25-hydroxylase cDNA. These data suggest that the major enzyme involved in the synthesis of a co-repressor of gene transcription is 25-hydroxycholesterol. We thank Daphne Davis, Kevin Anderson, Jeff Cormier, Mark Daris, Tammy Dinh, and Michele Laremore for excellent technical assistance; Hitoshi Shimano and Jay Horton for SREBP-1a transgenic mice; and Joe Goldstein and Mike Brown for critical reading of the manuscript.

Cellular cholesterol homeostasis is maintained by feedback repression of genes involved in cholesterol synthesis and supply (12). Conclusive evidence obtained in transfected cells (data not shown). 24-Hydroxycholesterol was converted to 24,25-dihydroxycholesterol in cells expressing a 25-hydroxylase cDNA. These data suggest that the major enzymatic activity encoded by the isolated cDNAs involves 25-hydroxylation of cholesterol and 24-hydroxycholesterol. However, because we have been unable to estimate kinetic constants for the cholesterol 25-hydroxylase enzymes due to the presence of endogenous cholesterol in the transfected cells and their lysates, and because an exhaustive list of potential substrates has not yet been tested, the possibility remains that the observed cholesterol 25-hydroxylase activity is a side reaction detectable only in transfected cells overexpressing these enzymes. Additional biochemical and genetic experiments will be performed to exclude formally this possibility.

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cDNA Cloning of Mouse and Human Cholesterol 25-Hydroxylases, Polytopic Membrane Proteins That Synthesize a Potent Oxysterol Regulator of Lipid Metabolism

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