In nonhepatic cells, cholesterol 7α-hydroxylase induces the expression of genes regulating cholesterol biosynthesis, efflux, and homeostasis

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Abstract **CHO cells expressing the liver-specific gene product cholesterol-7α-hydroxylase showed a 6-fold increase in the biosynthesis of [^{14}C]cholesterol from [^{14}C]acetate, as well as increased enzymatic activities of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and squalene synthase. Cells expressing cholesterol-7α-hydroxylase contained less sterol response element-binding protein 1 (SREBP1) precursor, whereas the cellular content of mature SREBP1, as well as the mRNAs of cholesterol biosynthetic genes (HMG-CoA reductase and squalene synthase), were all increased ~3-fold. Cells expressing cholesterol-7α-hydroxylase displayed greater activities of luciferase reporters containing the SREBP-dependent promoter elements derived from HMG-CoA reductase and farnesyl diphosphate synthase, in spite of accumulating significantly more free and esterified cholesterol and 7α-hydroxycholesterol. While cells expressing cholesterol-7α-hydroxylase displayed increased SREBP-dependent transcription, sterol-mediated repression of SREBP-dependent transcription by LDL-cholesterol and exogenous oxysterols was similar in both cell types. Cells expressing cholesterol-7α-hydroxylase displayed greater rates of secretion of cholesterol as well as increased expression of the ABC1 cassette protein mRNA. Adding 25-hydroxycholesterol to the culture medium of both cell types increased the expression of ABC1 cassette protein mRNA. The combined data suggest that in nonhepatic CHO cells multiple regulatory processes sensitive to cellular sterols act independently to coordinately maintain cellular cholesterol homeostasis.**—Spitsen, G. M., S. Dueland, S. K. Krisans, C. J. Slattery, J. H. Miyake, and R. A. Davis. **In nonhepatic cells, cholesterol-7α-hydroxylase induces the expression of genes regulating cholesterol biosynthesis, efflux, and homeostasis.** ***J. Lipid Res.*** **2000.** 41: **1347–1355.**

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The unique ability of the liver to synthesize bile acids from cholesterol provides the major quantitative pathway through which cholesterol is removed from the body of mammals via the excretion of biliary bile acid–phospholipid–cholesterol micelles (1). Bile acid synthesis transforms the hydrophobic cholesterol molecule into an amphipathic molecule capable of forming mixed micelles with many lipid-insoluble substrates. This is achieved by adding hydroxyl groups, reducing the double bond, and oxidatively cleaving the side chain of cholesterol to form a carboxylic acid that usually forms an amide with either taurine or glycine (1). The initial step controlling bile acid synthesis is catalyzed by the liver-specific gene product cholesterol 7α-hydroxylase (C7αH: EC 1.14.13.17), a cytochrome *P*-450 enzyme located in the endoplasmic reticulum (2–4). Bile acids are also produced by an alternative pathway involving the 7α-hydroxylation of oxysterols (5). The amphipathic properties of bile acids are essential for the two major functions of bile acids: *i*) facilitating the digestion and absorption of fat-soluble nutrients (6) and *ii*) maintaining cholesterol homeostasis by facilitating the formation and excretion of biliary bile acid–phospholipid–cholesterol micelles (7). Distinct transporter molecules generally conforming to the ATP-binding cassette (ABC) family act in concert to transport bile acid–phospholipid–cholesterol micelles into the bile canaliculus (8–11).

Most mammalian cells do not express the bile acid synthetic pathway, yet cholesterol homeostasis is still tightly regulated by negative feedback control of synthesis and uptake (12). In response to the accumulation of cholesterol, the rate-limiting isoprenoid biosynthetic enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) re-

Abbreviations: ALLN, *N*-acetyl-leucyl-leucyl-norleucinal; CHO, Chinese hamster ovary; CMV, cytomegalovirus; FCS, fetal calf serum; LDL, low density lipoprotein; MEM, modified Eagle's medium; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SREBP1, sterol response element-binding protein 1; SSC, 1.5 M NaCl, 0.15 M sodium citrate, pH 7.0; TLC, thin-layer chromotography.

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ductase is rapidly inactivated via several pre- and posttranscriptional mechanisms (13, 14). Transcriptional regulation of several lipid biosynthetic enzymes and the low density lipoprotein (LDL) receptor occurs in response to the sterol response element-binding protein (SREBP) family of transcription factors (15). Substrate activation of acyl-CoA:cholesterol acyltransferase (ACAT) diverts free cholesterol from membrane bilayers to storage in the form of cholesteryl esters (16). The discovery of a sterol-activated ABC1 protein provided evidence that cholesterol homeostatic excretory pathways exist in nonhepatic cells (17).

To gain an understanding of the role of C7αH in regulating cellular cholesterol homeostasis, we have examined how the stable expression of C7αH altered the phenotype of Chinese hamster ovary (CHO) cells, which normally do not express this liver-specific gene product (18). Unexpectedly, in CHO cells, C7αH expression led to increased expression of the LDL receptor in spite of increases in the cellular content of both free and esterified cholesterol (18). Thus, as a result of C7αH expression the ability of cellular sterols to signal negative feedback regulation of the LDL receptor was attenuated (18).

In the present study we examined how the expression of C7αH in CHO cells influences the expression of genes controlling cellular cholesterol homeostasis. Specifically, we examined how C7αH expression affected the processing of SREBP, the expression of several SREBP-regulated genes, the transcription of SREBP-dependent reporters, and the expression of ABC1. Our results show that cellular and exogenous sterols display distinct differences in their ability to influence expression of several genes contributing to regulation of cellular cholesterol homeostasis.

MATERIALS AND METHODS

Cell culture and stable transfection

All tissue culture supplies, chemicals, and radioactive chemicals were obtained from suppliers, as described (18). Cells were maintained in modified Eagle's medium with 1.5% fetal bovine serum and 3.5% newborn calf serum (MEM Plus serum, Gemini Bioproducts, Woodland, CA) at 37°C in an atmosphere of 5% CO_2. The plasmid expressing rat C7αH (pCMV-7α) was generously supplied by D. Russell and was constructed with the expression vector pCMV2 and the cDNA for rat C7αH (3). JD15 cells, expressing this plasmid and cotransfected with the neomycin resistance plasmid pRSVneo, were obtained and cultured as described (18). K1-7α cells were obtained by transfecting CHO-K1 cells with an expression plasmid (pcDNA3-7α), which contained the coding sequence of rat C7αH ligated into pcDNA3 (Invitrogen, San Diego, CA), as described (19). CHO-K1 cells transfected with pcDNA3-7α were screened for resistance to G418 (400 μg/mL) and then single cell cloned. Cells stably expressing an SREBP-activated HMG-CoA reductase promoter/luciferase reporter [pRED*luc*, generously supplied by T. Osborne (20)], were obtained by transfecting CHO-K1 with pRED*luc* and pRSVneo and by transfecting JD15 cells with pRED*luc* and a plasmid expressing hygromycin B phosphotransferase (21). The resulting cell lines (DH1, without C7αH and DH2, with C7αH), expressing pRED*luc*, were maintained in serum containing MEM with G418 (400 μg/mL) and hygromycin B (500 μg/mL). Before use in experiments, G418 and hygromycin B were removed prior to plating and growth (to 80% confluence).

[^{14}C]Acetate labeling

Cells were incubated in serum-containing culture medium with [2-^{14}C]acetate (5 μCi; specific activity, 47 mCi/mol) for 2 h, extracted with chloroform–methanol, and separated on silica gel thin-layer chromatography (TLC) plates, and the radioactivity in sterols and sterol esters was quantitated as described (22).

Enzyme assays

One day prior to reaching 80% confluence, the cell medium was changed as described in the figure legends. Delipidated serum was obtained by the cabosil extraction procedure (23). After culturing (as described in the figure legends), cell extracts were assayed for HMG-CoA reductase (24) and squalene synthase (25). Each assay was performed in duplicate from three individual culture dishes of cells. Protein concentrations were determined by dye-binding assay (Bio-Rad, Hercules, CA). Values are reported as the mean ± SD for three individual culture dishes. For the cholesterol C7αH activity assay microsomes were isolated and assayed by the method described in Straka et al. (26).

Genomic DNA isolation and quantitation of the number of copies of pRED*luc* integrated into the genome

To estimate the relative copy numbers of the reporter plasmid, pRed*luc*, in the CHO cell lines stably transfected with it, genomic DNA was isolated from both DH1 and DH2 cell lines. Genomic DNA was isolated with a QIAamp kit (Qiagen, Chatsworth, CA). Approximately 10^7 cells were scraped from a 150-mm dish and washed with phosphate-buffered saline (PBS) and then resuspended in 0.2 mL of PBS. To this, proteinase K and 0.2 mL of Qiagen solution AL were added and the solution was mixed by vortexing. The lysate was incubated at 70°C for 10 min and then applied to the QIAamp column and subjected to centrifugation in a microcentrifuge at 8,000 rpm for 1 min. The column was washed twice with Qiagen buffer AW according to the manufacturer instructions. The genomic DNA was eluted with 10 mM Tris-HCl, pH 9.0, by centrifugation in a microcentrifuge at 8,000 rpm for 1 min. The optical absorbance of the DNA was read at 260 and 280 nm and found to have an OD_{260}/OD_{280} ratio of greater than 1.90. Genomic DNA (5.0 μg) from DH1 and DH2 cells was subjected to digestion with restriction endonucleases *Bam*HI and *Bgl*II and analyzed for luciferase plasmid relative to endogenous C7αH by Southern blotting (27). After washing, the image was read and quantitated on a Molecular Dynamics (Sunnyvale, CA) PhosphorImager. To control for equal loading of samples, the gel was stained with ethidium bromide prior to blotting. After hybridization with the luciferase probe, the blot was stripped and reprobed with a probe recognizing the endogenous C7αH gene. Quantitation demonstrated that the molar ratio of DH1 to DH2 DNA was 0.86, in accordance with both the original optical density measurements and with staining of the loaded DNA.

Transient transfections of promoter-luciferase reporters

Transfections were performed by lipofection under optimized conditions. Typically, CHO-K1 cells were transfected with 200 ng of the farnesyl diphosphate synthase (FPPS) promoter-luciferase reporter construct, a kind gift from P. Edwards (28), in the presence of 1 μL of LipofectAMINE (Life Technologies, Gaithersburg, MD) per well in a 12-well plate according to the manufacturer instructions. Transfection efficiencies were normalized by cotransfecting with pRL-TK (Promega, Madison, WI), a control

vector containing a sea pansy (*Renilla reniformis*) luciferase gene driven by a thymidine kinase (TK) promoter in the molar ratio of 1:10.

Quantitation of luciferase activity

After incubation of cells as described in the figure legends, the medium was removed, and the cells were washed twice with 1× PBS. The cells were harvested by scraping with a rubber policeman in 1× lysis buffer (enhanced luciferase kit; Analytical Luminescence Laboratory, San Diego, CA). The lysate was centrifuged at 5,000 rpm for 5 min at 4°C to pellet the cellular debris, and the supernatant was transferred to a new tube. Luciferase activity of each sample was measured in triplicate, using an Analytical Luminescence Laboratory Monolight 2010 luminometer and reagents supplied by the manufacturer. Protein concentrations were determined by the method of Lowry et al. (29). The production of light was linear with respect to time and protein throughout the assay. For the dual luciferase assay, after incubation of cells as described in the figure legends, the medium was removed, and the cells were washed twice with 1× PBS. The cells were lysed with 1× lysis buffer and the luciferase activity of each sample was measured in triplicate with an Analytical Luminescence Laboratory Monolight 2010 luminometer and reagents supplied by Promega.

Quantitation of cholesterol and cholesteryl esters

Cells were washed twice with PBS. PBS (1 mL) was added to each plate and then the cells were scraped into tubes. A portion of the cells was assayed for protein by the method of Lowry et al. (29). To another portion, β-sitosterol was added as an internal standard and the cells were extracted with chloroform–methanol 1:1 (v/v) and stored under N_2 until use. The solvent was evaporated under a stream of N_2 and the samples were then resuspended in isopropanol. Samples were injected into a Hewlett-Packard (Palo Alto, CA) gas chromatograph and cholesterol mass was quantitated as described (30).

Northern blot analysis

Poly(A)$^+$ RNA was isolated from cells by a modification of the guanidinium isothiocyanate method (31), as described (32). Two to 5 μg of the resulting mRNA was separated by 0.8% agarose-formaldehyde gel electrophoresis, transferred to a nitrocellulose membrane, UV cross-linked to nitrocellulose, and probed with nick-translated [^{32}P]cDNA probes prepared from gel-purified inserts (19) and washed with a stringent SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

SREBP analysis of nuclei and membrane fractions

Cells were harvested on ice in cold PBS containing a protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride [PMSF], aprotinin [100 μg/mL], and leupeptin [50 μg/mL]), using a rubber policeman. Nuclei and membrane fractions were obtained as described (33). Cells were centrifuged at 1,000 rpm for 10 min, and the pellets were resuspended in 10 volumes of cell homogenization buffer (10 mM HEPES-KOH at pH 7.6, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 1 mM EDTA, and the protease inhibitors described above). The cells were disrupted by passage through a 22-gauge needle (15 times) and then centrifuged at 1,000 rpm for 10 min at 4°C. The crude nuclear pellet was extracted with an equal volume of nuclear extraction buffer [20 mM HEPES-KOH at pH 7.6, 25% (v/v) glycerol, 0.5 M NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, and the protease inhibitor cocktail] and centrifuged at 12,000 rpm for 30 min at 4°C. The supernatant was used as nuclear extract for immunoblotting analysis. The microsomal membrane fraction was obtained by further centrifugation of the supernatant obtained from the nuclear pellet by ultracentrifugation at 45,000 rpm for 2 h at 4°C, using a TLA45 rotor (Beckman, Fullerton, CA).

Western blot analysis

Western blotting was performed as described (19). After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 1–15% gradient), the gels were electroblotted onto nitrocellulose membranes. The nonspecific binding sites of the membranes were blocked with 10% defatted dried milk, followed by addition of the appropriate primary antibody. The relative amount of primary antibody bound to the proteins on the nitrocellulose membrane was detected with the species-specific horseradish peroxidase-conjugated IgG. Blots were developed by chemoluminescence with an ECL detection kit (Amersham, Arlington Heights, IL). The antibodies used are described in the figure legends. All values are reported as means ± SD. Statistical differences were calculated by using Student's *t*-test, double tailed. Values of $P < 0.05$ were considered to be significant.

RESULTS

Characterization of CHO cells expressing C7αH (JD15 cells)

To obtain CHO cells that would stably express C7αH, CHO-K1 cells were cotransfected with plasmids expressing rat C7αH (pCMV-7a) and one that confers neomycin resistance (pRSVneo) (18). Cells were selected for resistance to both G418 (400 μg/mL) and 25-hydroxycholesterol, as described in detail (18). A representative clone (designated as JD15 cells) expressed C7αH mRNA and enzyme activity that was comparable to that of rat liver (18). JD15 cells were resistant to cytotoxic killing by 25-hydroxycholesterol, whereas CHO-K1 cells were sensitive (**Fig. 1**). To examine whether the resistance of JD15 cells to 25-hydroxycholesterol required C7αH, they were screened for

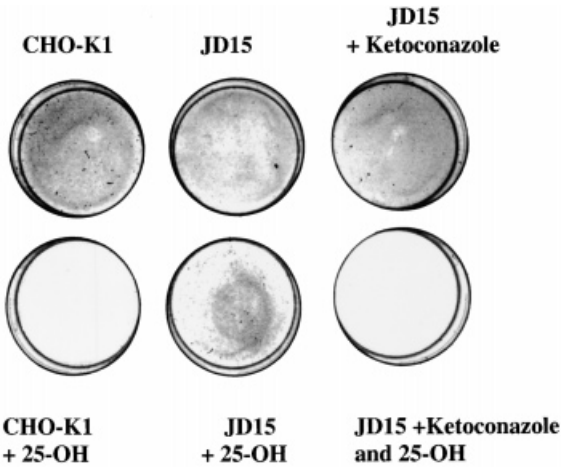

Fig. 1. Effect of ketoconazole on the resistance of CHO cells to 25-hydroxycholesterol (25-OH) cytotoxicity. Wild-type CHO-K1 cells and JD15 cells were cultured in MEM containing 0.5% FCS and 1% nutridoma, as described (18). 25-Hydroxycholesterol (0.25 μg/mL) and/or the cytochrome *P*-450 inhibitor ketoconazole (5 μM) were added to the cultured medium as indicated. Cells were maintained in the cultured medium for 3 days. Cells were washed and then stained, as described (27).

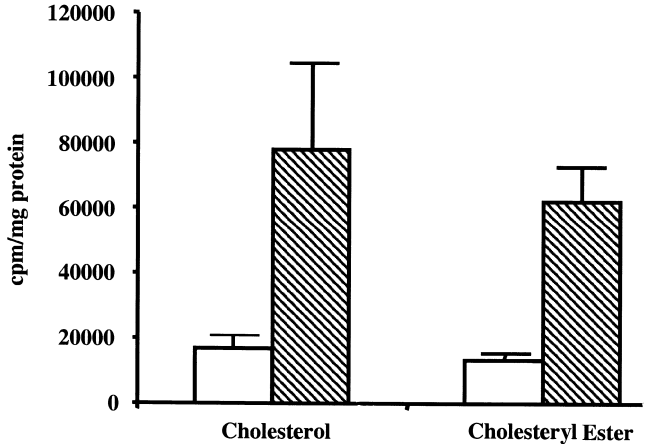

Fig. 2. [^{14}C]Cholesterol biosynthesis by CHO cells. Wild-type CHO-K1 cells and JD15 cells were plated and grown to 80% confluence. [2-^{14}C]Acetate (3.3 μCi/mL) was added to the medium and cells were incubated for 2 h. Cells were then harvested and lipids separated by TLC and the radioactivity was quantitated by β-scintillation analysis. Open columns represent wild-type CHO-K1 cells; hatched columns represent JD15 cells. Values represent the mean ± SD of three separate cell extracts in each group of cells. There were significant increases in both [^{14}C]cholesterol and cholesteryl esters in JD15 cells compared with CHO-K1 cells ($P < 0.001$).

25-hydroxycholesterol resistance in the presence of the cytochrome *P*-450 inhibitor ketoconazole (27). Whereas, by itself, ketoconazole had no apparent effect on the growth of JD15 cells, when 25-hydroxycholesterol was included in the medium, JD15 cells were rapidly killed (Fig. 1). These data show that the resistance of JD15 cells to 25-hydroxycholesterol cytotoxicity can be blocked by the cytochrome *P*-450 inhibitor ketoconazole. We have reported that JD15 cells expressed higher levels of LDL receptor mRNA than wild-type CHO-K1 cells did (32). Unexpectedly, JD15 cells contained more free and esterified cholesterol than CHO-K1 cells did (32). To examine the possibility that the increased cholesterol levels in JD15 cells were derived from de novo synthesis, we determined the relative rate of [^{14}C]cholesterol synthesis from [^{14}C]acetate (**Fig. 2**). When cultured in serum-containing medium, the relative rates of ^{14}C-labeled cholesterol and cholesteryl esters were 8-fold and 6-fold greater, respectively, compared with the rates exhibited by CHO-K1 cells (Fig. 2).

Expression of C7αH in CHO cells increases the activities of HMG-CoA reductase and squalene synthase

To examine how the activities of cholesterol biosynthetic enzymes contribute to the increase in cholesterol biosynthesis, we measured the activities of two rate-limiting enzymes that act at the branch points of the isoprenoid (HMG-CoA reductase) and the sterol (squalene synthase) biosynthetic pathways (1). The activities of both HMG-CoA reductase and squalene synthase were increased up to 5-fold in JD15 cells as compared with wild-type CHO-K1 cells (**Fig. 3**). In addition, while culturing conditions affected the activities of both enzymes similarly in both cell types, under all conditions the activities of HMG-CoA reductase and squalene synthase were significantly greater in JD15 cells (Fig. 3).

Expression of C7αH increases the transcription of luciferase driven by a promoter containing the HMG-CoA reductase sterol response element

JD15 cells and wild-type CHO cells were stably transfected with a luciferase reporter construct containing the sterol response element (SRE) of the HMG-CoA reductase promoter (pRed*luc*) (20). Southern blot analysis of the single-cell clones used for each cell type showed that relative to the endogenous C7αH gene, there were 12 times more copies of pRed*luc* in JD15 cells compared with CHO-K1 cells (data not shown). These single-cell clones (DH1 and DH2 cells, derived from CHO-K1 and JD15 cells, respectively) were examined for the effects of different culture conditions, which are known to affect SREBP-regulated gene expression, on the transcriptional activity of the HMG-CoA reductase SRE as reflected by the activity of the luciferase reporter. To relate changes in transcriptional activity between the two cell types, luciferase activity per unit of genome-integrated plasmid was calculated. The presence of C7αH (DH2 cells) increased the expression of luciferase under all culture conditions (**Fig. 4**). The greatest difference (10-fold) in luciferase expression by DH1 and DH2 cells occurred when the cells were cultured under conditions known to decrease the transcrip-

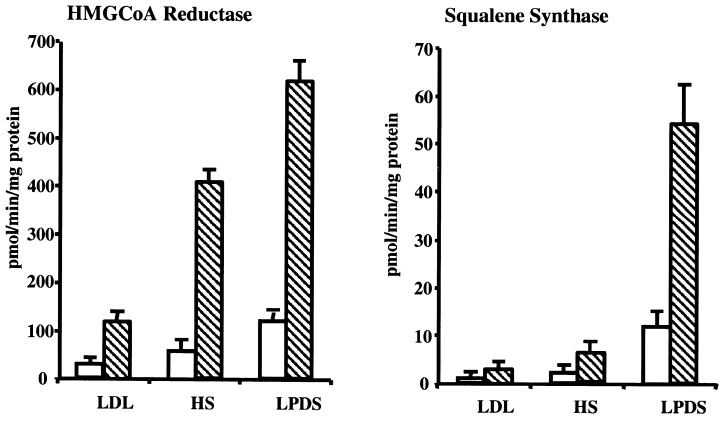

Fig. 3. HMG-CoA reductase and squalene synthase enzyme activities in CHO-K1 and JD15 cells. CHO-K1 (open columns) and JD15 (hatched columns) cells were grown to 80% confluence in MEM with 5% calf serum. The medium was changed for overnight incubation to MEM with the following additions: 5% lipoprotein-deficient serum and LDL at 50 μg/mL (LDL); 5% calf serum (HS); or 5% lipoprotein-deficient serum alone (LPDS). Results are expressed as the mean ± SD for triplicate dishes of cells. There were significant increases in the activities of both enzymes under all culturing conditions in JD15 cells compared with CHO-K1 cells ($P < 0.001$).

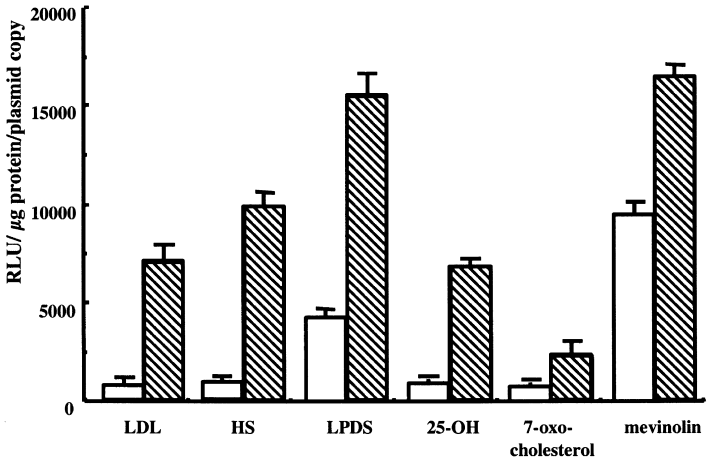

Fig. 4. Effect of culturing conditions on luciferase expression in CHO cells. DH1 (no expression of C7αH: open columns) and DH2 (expression of C7αH: hatched columns) cells were grown in MEM with 5% calf serum for 24 h and then changed for 24-h incubation to MEM with either 5% calf serum (HS) or 5% lipoprotein-deficient serum and one of the following additions: no addition (LPDS), LDL at 50 μg/mL (LDL), 25-hydroxycholesterol (1 μg/mL) + cholesterol at 10 μg/mL (25-OH), 7-oxo-cholesterol at 10 μg/mL (7-oxo-cholesterol), or mevinolin at 30 μg/mL (mevinolin). Results, in light units per μg of protein per plasmid copy, are expressed as the mean ± SD for triplicate dishes of cells. There were significant increases in the activity of luciferase under all culturing conditions in DH2 cells compared with DH-1 cells ($P < 0.001$).

tion of SREBP-regulated genes by blocking site 1 proteolytic cleavage of transmembrane SREBP (34): serum-containing medium, LDL-containing medium, and 25-hydroxycholesterol-containing medium (Fig. 4). Incubation of DH2 cells with 7-oxo-cholesterol, a competitive inhibitor of C7αH enzyme activity (35), repressed the expression of pRed*luc* to the greatest extent, reaching a level only 2.8-fold greater than that of DH1 cells.

Expression of C7αH in CHO cells increases the cellular content of both free and esterified cholesterol

Under all culturing conditions examined, the cellular free cholesterol levels for each cell type remained constant. However, compared with CHO-K1 cells, JD15 cells contained significantly greater levels of free cholesterol (**Fig. 5**). In contrast, the cholesteryl ester levels in both cell types changed appropriately in response to culturing conditions: lipoprotein-deficient serum (LPDS) < serum-containing medium < LDL-containing medium (Fig. 5). Under all culturing conditions examined, JD15 cells contained more cholesteryl esters than CHO-K1 cells did. These data suggest that while the expression of C7αH significantly increased cellular cholesterol pools, it did not interfere with the ability of the cells to change their cellular cholesterol content in response to culturing conditions.

Recapitulation of the JD15 phenotype in CHO-K1 cells transfected with pcDNA3-7α and screened for resistance to neomycin

The possibility existed that the sterol-resistant regulation of SREBP-regulated genes displayed by JD15 cells was caused by the screening for resistance to 25-hydroxycholesterol. To address this possibility we derived a new line of CHO-K1 cells expressing C7αH by using a different expression plasmid (pcDNA3), which contains both a CMV promoter and a neomycin resistance marker (19). CHO-K1 cells transfected with pcDNA3-7α were screened for resistance to G418 and then single cell cloned. One representative single-cell clone (K1-7α) maintained in cultured medium containing G418 (400 μg/mL) expressed C7αH mRNA, protein, and enzyme activity at levels that were similar to those of JD15 cells (32). K1-7α cells synthesized about 2-fold more [^{14}C]cholesterol and [^{14}C]cholesteryl esters from [^{14}C]acetate than did CHO-K1 cells (**Fig. 6A**). It is interesting to note that there was approximately 3-fold more [^{14}C]cholesterol and 2-fold more [^{14}C]cholesteryl esters secreted into the culture medium by K1-7α cells compared with CHO-K1 cells (Fig. 6B). These combined findings indicate that expressing C7αH in CHO cells increases cholesterol biosynthesis and excretion. To examine whether C7αH increased the genetic expression

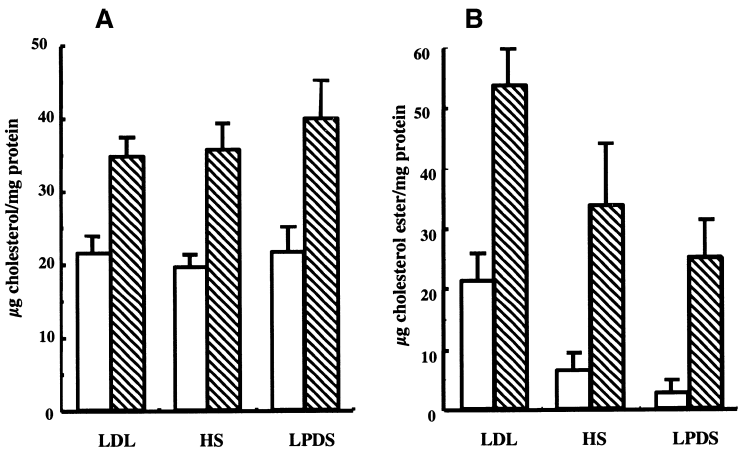

Fig. 5. Free (A) and esterified (B) cholesterol levels in K1 cells and JD15 cells. CHO-K1 (open columns) and JD15 (hatched columns) cells were grown to 80% confluence in MEM with 5% serum. The medium was changed for overnight incubation to MEM with the following additions: 5% lipoprotein-deficient serum and LDL at 50 μg/mL (LDL), 5% calf serum (HS); or 5% lipoprotein-deficient serum alone (LPDS). Values represent the mean ± SD of three separate plates of cells. There were significant increases in the cellular concentration of free and esterified cholesterol under all culturing conditions in JD15 cells compared with CHO-K1 cells ($P < 0.001$).

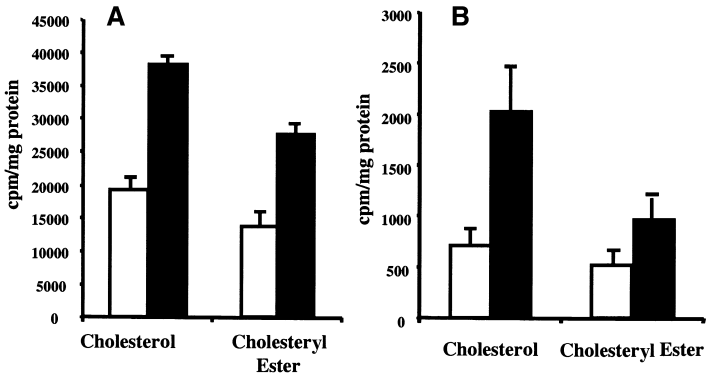

Fig. 6. Synthesis (A) and secretion (B) of [^{14}C]cholesterol and cholesteryl esters by CHO cells. Wild-type CHO-K1 cells and K1-7α cell were plated and grown to 80% confluence. [2^{14}C]Acetate (3.3 μCi/mL) was added to the medium and cells were incubated for 2 h. Cells and medium were then harvested and lipids separated by TLC and the radioactivity was quantitated by β-scintillation analysis. Open columns represent wild-type CHO-K1 cells; closed columns represent K1-7α cells. Values represent the mean ± SD of three separate cell extracts in each group of cells. There were significant increases in the synthesis of both free and esterified cholesterol in K1-7α cells compared with CHO-K1 cells ($P < 0.001$).

of SREBP-regulated cholesterol biosynthetic enzymes, we determined the relative content of mRNAs encoding HMG-CoA reductase and squalene synthase in CHO-K1 and K1-7α cells (**Fig. 7).** Compared with CHO-K1 cells, K1-7α cells displayed a ~3-fold increased expression of mRNAs encoding the rate-limiting enzymes HMG-CoA reductase and squalene synthase (Fig. 7). In addition, similar to what has been reported to occur in JD15 cells (32), K1-7α cells displayed ~3-fold greater levels of LDL receptor mRNA compared with CHO-K1 cells. The increased expression of HMG-CoA reductase and squalene synthase mRNAs displayed by K1-7α cells suggested the possibility that C7αH expression may have increased SREBP-regulated gene transcription. To examine this possibility, the cellular content of mature and precursor SREBP1 in both CHO-K1 and K1-7α cells was determined by Western blotting. Compared with CHO-K1 cells, K1-7α cells treated with *N*-acetyl-leucyl-leucyl-norleucinal (ALLN, a protease inhibitor) for 4 h prior to harvest contained ~3-fold more mature SREBP1, whereas the precursor form was decreased ~50% (**Fig. 8A**). Thus, there was a concordance in the relative increase in mature SREBP1 and mRNAs encoding HMG-CoA reductase and squalene synthase displayed by K1-7α cells. Adding 25-hydroxycholesterol to the culture medium of both cell types decreased the cellular content of mature SREBP1 to levels that were at the limits of detection in both cell types (Fig. 8B). It is important to point out that in the absence of ALLN, the amount of mature SREBP1 in both cell types was barely detectable, whereas the amount of precursor was similar to the amount detected in cells treated with ALLN (data not shown). ALLN blocks the rapid degradation of mature SREBPs, but not the precursor form (33). Thus, the finding of a 4-fold increase in mature SREBP1 in K1-7α cells treated with ALLN (Fig. 8A) suggests that C7αH expression increased the proteolytic production of mature SREBP1, which is regulated by the site 1 protease (33, 36, 37). In contrast to the differences in SREBP1, both cell types contained similar levels of SREBP2 (data not shown).

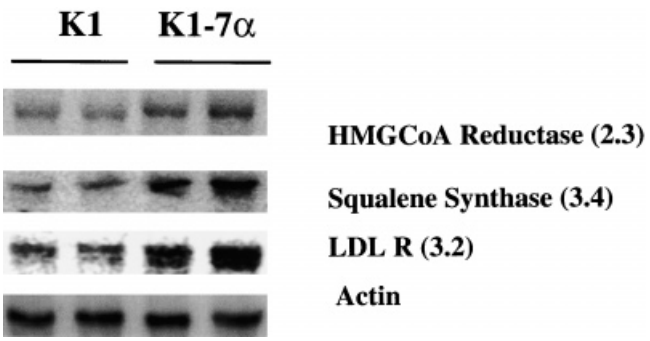

Fig. 7. The relative abundance of mRNAs encoding HMG-CoA reductase, squalene synthase, and the LDL receptor. Wild-type CHO-K1 cells and K1-7α cells were plated and grown to 80% confluence. Cells were harvested and poly(A) mRNA was obtained. The mRNA was Northern blotted and hybridized with ^{32}P-labeled cDNAs, as indicated. Lanes 1 and 2 are from two separate plates of CHO-K1 cells; lanes 3 and 4 are from two separate plates of K1-7α cells. The K1-7α/CHO-K1 ratios for each mRNA relative to β-actin are indicated.

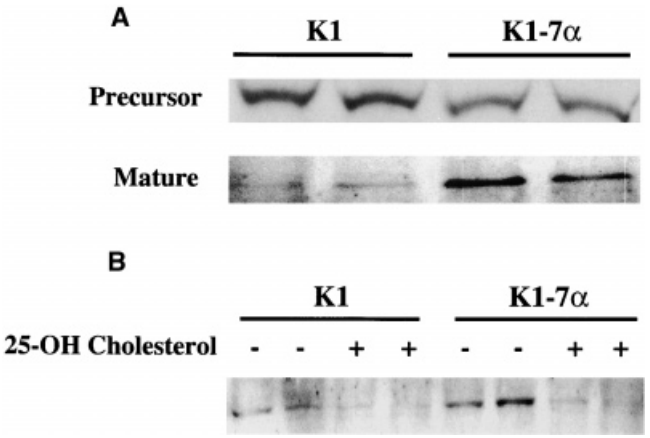

Fig. 8. Cellular content of SREBP1. (A) Wild-type CHO-K1 cells and K1-7α cells were cultured in MEM containing 5% FCS. Four hours before harvest, cells were treated with ALLN (50 μg/mL). Nuclei (mature) and membrane fractions (precursor) were isolated (33) and subjected to Western blot analysis with a monoclonal antibody against SREBP1 (19, 33). (B) Wild-type CHO-K1 cells and K1-7α cells were cultured in MEM containing 5% FCS with (+) and without (−) sterols (cholesterol [10 μg/mL] and 25-hydroxycholesterol [2 μg/mL]) for 16 h. Four hours before harvest, cells were treated with ALLN (50 μg/mL). Nuclei fractions containing mature SREBP were isolated and 50 μg of each sample was separated by SDS-PAGE and subjected to Western blot analysis with a monoclonal antibody against SREBP1 (19, 33). Immunoreactive bands correspond to the mature form of SREBP1.

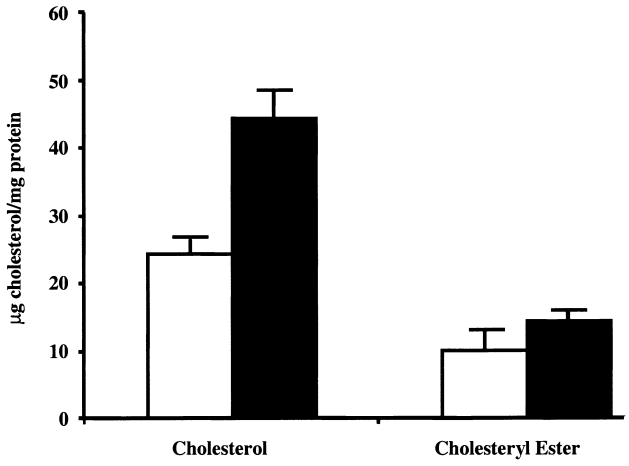

Fig. 9. Free and esterified cholesterol levels in CHO-K1 cells and K1-7α cells. Wild-type CHO-K1 cells (open columns) and K1-7α cells (closed columns) were cultured to 80% confluence in MEM containing 5% FCS. Cells were harvested and the amount of cholesterol and cholesteryl esters was determined by GLC. Values represent the mean ± SD of three separate plates of cells. There were significant increases in cellular concentrations of both free and esterified cholesterol in K1-7α cells compared with CHO-K1 cells ($P < 0.001$).

While the cellular content of mature SREBP1 was clearly increased in K1-7α cells when compared with wild-type CHO cells, similar to JD15 cells (Fig. 8), the cells contained significantly greater amounts of both free and esterified cholesterol than CHO cells (**Fig. 9**). These combined data show that K1-7α cells displayed a phenotype similar to that of JD15 cells: increased expression of SREPB-regulated genes in a manner that is paradoxically associated with increased cellular content of cholesterol and cholesteryl ester. The similarity of the phenotypes displayed by JD15 and K1-7α cells rules out the possibility that screening for resistance to 25-hydroxycholesterol, used as the metabolic selection, or the plasmids transfected into these cells were responsible for altering the wild-type phenotype.

K1-7α cells accumulate 7α-hydroxycholesterol while displaying increased SREBP-dependent gene transcription

The enzymatic activity of C7αH was determined with microsomes from each cell type (38). Whereas no C7αH activity was detectable in microsomes from CHO-K1 cells, microsomes from K1-7α cells displayed an activity of 17.1 pmol/min/mg protein, which is similar to the activity expressed by JD15 cells (i.e., 11.2 pmol/min/mg protein) (32). It is interesting to note that freshly isolated microsomes from K1-7α cells contained 103.8 pmol of 7α-hydroxycholesterol per mg protein, whereas none was detected in microsomes obtained from CHO-K1 cells. Because 7α-hydroxycholesterol decreases the production of mature SREBP1 (33) its accumulation in K1-7α cells is incompatible with the finding that these cells also contain more mature SREBP1 than CHO-K1 cells did. Thus, a unique characteristic of the phenotype of CHO cells expressing C7αH is the apparent inability of cellular cholesterol and 7α-hydroxycholesterol to repress SREBP-dependent gene expression by inhibiting the production of mature SREBP1.

To examine the ability of exogenous cholesterol and oxysterols to repress SREBP-dependent gene expression, cells were transiently transfected with a luciferase reporter containing the FPPS promoter sequences that can be activated by SREBP (28). K1-7α cells displayed about 2 to 3 times more luciferase activity than CHO-K1 cells did (**Fig. 10**). Moreover, while both cell types showed decreased luciferase activities when cultured in medium containing cholesterol or 25-hydroxycholesterol, the activity remained significantly greater in K1-7α cells. It is interesting to note that treating K1-7α cells with either 7-oxo-cholesterol or 7α-hydroxycholesterol decreased the expression of the luciferase reporter to levels that were similar to those of CHO-K1 cells. These findings show that K1-7α cells respond to the repression of SREBP-dependent transcription by exogenous cholesterol and oxysterols in a manner similar to wild-type CHO-K1 cells.

Increased expression of ABC1 in K1-7α cells

To examine the possibility that the ABC1 protein may have contributed to the 3-fold increase in secretion of free

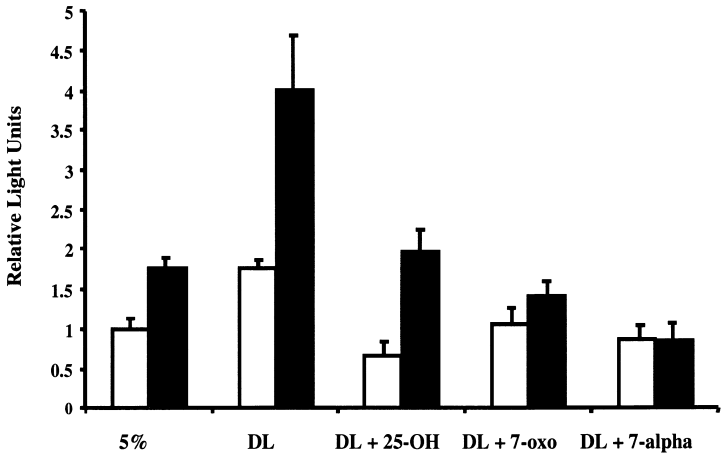

Fig. 10. Effect of culturing conditions on SREBP-dependent luciferase expression in K1 and K1-7α cells. K1 (open columns) and K1-7α (closed columns) were grown in MEM with 5% calf serum. After 16 h of incubation the cells were transfected as described in Materials and Methods. Twenty-four hours after transfection the wells were rinsed twice with 1× PBS and the medium was changed to either 5% calf serum (5%) or lipoprotein-deficient serum and one of the following conditions: no addition (DL), 25-OH cholesterol at 2 µg/mL + cholesterol at 10 µg/mL (DL + 25-OH), 7-oxo-cholesterol at 10 µg/mL (DL + 7-oxo), or 7α-hydroxycholesterol at 10 µg/mL (DL + 7-alpha). Twenty-four hours after incubation the cells were harvested and respective luciferase activity was quantitated. Results are presented as light units generated by firefly luciferase (FPPS-FL) per *Renilla* luciferase (TK-RL)

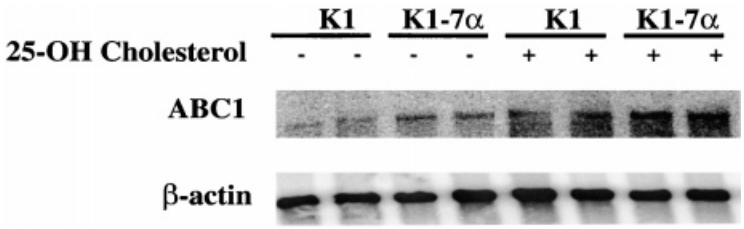

Fig. 11. Cellular content of ABC1 mRNA. Wild-type CHO-K1 and K1-7α were plated and grown to 80% confluence. Twenty-four hours prior to harvest, the medium was changed to 5% calf serum containing MEM with (+) and without (−) 25-OH cholesterol (2 μg/mL). Cells were harvested and poly(A) mRNA was obtained. The mRNA was Northern blotted and hybridized with ^{32}P-labeled ABC1 cDNA.

cholesterol displayed by K1-7α cells (Fig. 6), we determined the relative expression of its mRNA (**Fig. 11**). Compared with wild-type CHO-K1 cells, K1-7α cells contained ~two times more ABC1 mRNA relative to β-actin mRNA. Furthermore, when cultured in the presence of 25-hydroxycholesterol, the relative amount of ABC1 mRNA was increased ~3-fold in both cell types, suggesting that oxysterols induce ABC1 mRNA expression. Additional studies showed that adding 7α-hydroxycholesterol to the culture medium also increased ABC1 mRNA ~3-fold (data not shown). These findings are consistent with a study demonstrating that the expression of ABC1 mRNA by macrophages increases by loading the cells with cholesterol derived from acetyl-LDL (17).

DISCUSSION

Our results support three novel conclusions: *i*) in CHO cells, the expression of C7αH leads to the induction of genes controlling cholesterol biosynthesis (HMG-CoA reductase and squalene synthase), import (the LDL receptor), and excretion (ABC1 protein); *ii*) regulatory sterols coordinately regulate cellular cholesterol homeostasis by repressing the expression of genes controlling cholesterol biosynthesis and import (the LDL receptor) while increasing cholesterol excretion; and *iii*) regulatory sterols can exist in forms that diminish their ability to repress SREBP-dependent transcription, while maintaining their ability to induce ABC1 expression. Our findings support those showing that cellular uptake of acetyl-LDL cholesterol increased the expression of ABC1 in cultured macrophages (17).

Our finding that the expression of C7αH in CHO cells increases the cellular content of mature SREBP1 and the expression of mRNAs whose transcription is SREBP dependent, such as HMG-CoA reductase, squalene synthase, and the LDL receptor (18), is similar to what occurred in cultured rat hepatoma cells (19), with one potentially important distinction. In McArdle rat hepatoma cells, C7αH expression led to decreased cellular cholesterol levels (19), whereas in CHO cells it resulted in cholesterol accumulation (Figs. 5 and 9). We have found that unlike McArdle rat hepatoma cells and most other cultured cells, CHO cells do not express the sterol 27-hydroxylase and the oxysterol-7α-hydroxylase (data not shown). The limited ability of CHO cells to express sterol hydroxylases and thus metabolize cholesterol and oxysterols could account for the increased sterol content displayed by both JD15 and K1-7α cells.

Surprisingly, K1-7α cells accumulated more mature SREBP1 in spite of the increased cellular content of sterols (i.e., cholesterol and 7α-hydroxycholesterol), which act to reduce the proteolytic activation of SREBPs (15). These findings suggest that expression of C7αH either interfered with the site 1 protease reaction or the signaling by these regulatory sterols. However, because the expression of C7αH in CHO cells did not interfere with the regulation of SREBP-dependent transcription by adding exogenous sterols to the culture medium (Fig. 8B), it is unlikely that C7αH either interfered with the site 1 protease reaction or the signaling sterols in general. Our findings suggest that in CHO cells SREBP-regulated gene expression is less sensitive to endogenous de novo synthesized sterols compared with exogenous sterols added to the cultured medium.

In contrast to these differences in how endogenous and exogenous sterols affected SREBP-dependent gene expression, the sterol-induced ABC1 gene displays similar induction by both endogenous and exogenous sterols. Our findings clearly show that the expression of ABC1 mRNA is increased by the expression of C7αH and by adding 25-hydroxycholesterol to the culture medium (Fig. 11). On the basis of these findings, it is reasonable to propose that the increased expression of ABC1 by K1-7α cells is caused by the accumulation of cholesterol and/or 7α-hydroxycholesterol as a result of the C7αH enzyme reaction. Thus, while the accumulation in K1-7α cells of the regulatory sterols cholesterol and 7α-hydroxycholesterol did not repress SREBP-dependent gene expression, it did induce the expression of ABC1 mRNA. These findings are consistent with the proposal that distinct signals are separately responsible for sterol inhibition of SREBP site 1 proteolysis and sterol induction of ABC. Because sterol repression of SREBP site 1 proteolysis and induction of ABC1 may act in concert, albeit as independent processes, differences in signaling sterol repression and induction may provide greater versatility in homeostatic lipid regulation. Our experiments provide evidence demonstrating that the functional expression of C7αH influences gene expression regulated by both SREBP-dependent and independent mechanisms.

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REFERENCES

1. Edwards, P. A., and R. A. Davis. 1996. Isoprenoids, sterols and bile acids. *In* New Comprehensive Biochemistry. Vol. 31. D. E. Vance and J. Vance, editors. Elsevier, Amsterdam. 341–362.
2. Noshiro, M., and K. Okuda. 1990. Molecular cloning and sequence analysis of cDNA encoding human cholesterol 7 alpha-hydroxylase. *FEBS Lett.* **268:** 137–140.
3. Jelinek, D. F., S. Andersson, C. A. Slaughter, and D. W. Russell. 1990. Cloning and regulation of cholesterol 7-alpha-hydroxylase, the rate-limiting enzyme in bile acid biosynthesis. *J. Biol. Chem.* **265:** 8190–8197.
4. Li, Y. C., D. P. Wang, and J. Y. L. Chiang. 1990. Regulation of cholesterol 7 alpha-hydroxylase in the liver: cloning, sequencing and regulation of cholesterol 7 alpha-hydroxylase mRNA. *J. Biol. Chem.* **265:** 12012–12019.
5. Schwarz, M., E. G. Lund, and D. W. Russell. 1998. Two 7 alpha-hydroxylase enzymes in bile acid biosynthesis. *Curr. Opin. Lipidol.* **9:** 113–118.
6. Carey, M. C., and D. M. Small. 1972. Micelle formation by bile salts. Physical–chemical and thermodynamic considerations. *Arch. Intern. Med.* **130:** 506–527.
7. Hardison, W. G., and J. T. Apter. 1972. Micellar theory of biliary cholesterol excretion. *Am. J. Physiol.* **222:** 61–67.
8. Simon, F. R., E. M. Sutherland, and M. Gonzalez. 1982. Regulation of bile salt transport in rat liver. Evidence that increased maximum bile salt secretory capacity is due to increased cholic acid receptors. *J. Clin. Invest.* **70:** 401–411.
9. Elferink, R. P., G. N. Tytgat, and A. K. Groen. 1997. Hepatic canalicular membrane 1: the role of mdr2 P-glycoprotein in hepatobiliary lipid transport. *FASEB J.* **11:** 19–28.
10. Keppler, D., and I. M. Arias. 1997. Hepatic canalicular membrane. Introduction: transport across the hepatocyte canalicular membrane. *FASEB J.* **11:** 15–18.
11. Gerloff, T., B. Stieger, B. Hagenbuch, J. Madon, L. Landmann, J. Roth, A. F. Hofmann, and P. J. Meier. 1998. The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. *J. Biol. Chem.* **273:** 10046–10050.
12. Brown, M. S., and J. L. Goldstein. 1986. A receptor-mediated pathway for cholesterol homeostasis. *Science.* **232:** 34–47.
13. Goldstein, J. L., and M. S. Brown. 1990. Regulation of the mevalonate pathway. *Nature.* **343:** 425–430.
14. Edwards, P. A., and J. Ericsson. 1998. Signaling molecules derived from the cholesterol biosynthetic pathway: mechanisms of action and possible roles in human disease. *Curr. Opin. Lipidol.* **9:** 433–440. [Published erratum appears in *Curr. Opin. Lipidol.* 1999; **10:** 83 (following)].
15. Brown, M. S., and J. L. Goldstein. 1999. A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. *Proc. Natl. Acad. Sci. USA.* **96:** 11041–11048.
16. Chang, T. Y., C. C. Chang, and D. Cheng. 1997. Acyl-coenzyme A:cholesterol acyltransferase. *Annu. Rev. Biochem.* **66:** 613–638.
17. Langmann, T., J. Klucken, M. Reil, G. Liebisch, M. F. Luciani, G. Chimini, W. E. Kaminski, and G. Schmitz. 1999. Molecular cloning of the human ATP-binding cassette transporter 1 (hABC1): evidence for sterol-dependent regulation in macrophages. *Biochem. Biophys. Res. Commun.* **257:** 29–33.
18. Dueland, S., J. D. Trawick, M. S. Nenseter, A. A. MacPhee, and R. A. Davis. 1992. Expression of 7-alpha-hydroxylase in non-hepatic cells results in liver phenotypic resistance of the low density lipoprotein receptor to cholesterol repression. *J. Biol. Chem.* **267:** 22695–22698.
19. Wang, S-L., E. Du, T. D. Martin, and R. A. Davis. 1997. Coordinate regulation of lipogenesis and the assembly and secretion of apolipoprotein B-containing lipoproteins by sterol response element binding protein 1. *J. Biol. Chem.* **272:** 19351–19364.
20. Osborne, T. F., M. Bennett, and K. Rhee. 1992. Red 25, a protein that binds specifically to the sterol regulatory region in the promoter for 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *J. Biol. Chem.* **267:** 18973–18982.
21. Blochlinger, K., and H. Diggelmann. 1984. Hygromycin B phosphotransferase as a selectable marker for DNA transfer experiments with higher eucaryotic cells. *Cell. Mol. Biol.* **4:** 2929–2931.
22. Davis, R. A., P. M. Hyde, J. C. Kuan, M. M. Malone, and S. J. Archambault. 1983. Bile acid secretion by cultured rat hepatocytes. Regulation by cholesterol availability. *J. Biol. Chem.* **258:** 3661–3667.
23. Borensztajn, J., G. S. Getz, and T. J. Kotlar. 1988. Uptake of chylomicron remnants by the liver: further evidence for the modulating role of phospholipids. *J. Lipid Res.* **29:** 1087–1096.
24. Sinensky, M., R. Torget, and P. A. Edwards. 1981. Radioimmune precipitation of 3-hydroxy-3-methylglutaryl coenzyme A reductase from Chinese hamster fibroblasts. Effect of 25-hydroxycholesterol. *J. Biol. Chem.* **256:** 11774–11779.
25. Shechter, I., E. Klinger, M. L. Rucker, R. G. Engstrom, J. A. Spirito, M. A. Islam, B. R. Boettcher, and D. B. Weinstein. 1992. Solubilization, purification, and characterization of a truncated form of rat hepatic squalene synthetase. *J. Biol. Chem.* **267:** 8628–8635.
26. Straka, M. S., L. H. Junker, L. Zaccaro, D. I. Zogg, S. Dueland, G. T. Everson, and R. A. Davis. 1990. Substrate stimulation of 7alpha-hydroxylase, an enzyme located in the cholesterol-poor endoplasmic reticulum. *J. Biol. Chem.* **265:** 7145–7149.
27. Leighton, J. K., S. Dueland, M. S. Straka, J. Trawick, and R. A. Davis. 1991. Activation of the silent endogenous cholesterol-7-alpha-hydroxylase gene in rat hepatoma cells: a new complementation group having resistance to 25-hydroxycholesterol. *Mol. Cell. Biol.* **11:** 2049–2056.
28. Ericsson, J., S. M. Jackson, B. C. Lee, and P. A. Edwards. 1996. Sterol regulatory element binding protein binds to a *cis* element in the promoter of the farnesyl diphosphate synthase gene. *Proc. Natl. Acad. Sci. USA.* **93:** 945–950.
29. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
30. Davis, R. A., W. E. Highsmith, M. M. McNeal, J. A. Schexnayder, and J. C. Kuan. 1983. Bile acid synthesis by cultured hepatocytes. Inhibition by mevinolin, but not by bile acids. *J. Biol. Chem.* **258:** 4079–4082.
31. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thioguanate–phenol–chloroform extraction. *Anal. Biochem.* **162:** 156–159.
32. Trawick, J. D., K. D. Lewis, S. Dueland, G. L. Moore, F. R. Simon, and R. A. Davis. 1996. Expression of cholesterol 7 alpha-hydroxylase by differentiated rat hepatoma 135 cells: inability to distinguish bile acid repression from cytotoxicity. *J. Lipid Res.* **37:** 24169–24176.
33. Wang, X., R. Sato, M. S. Brown, X. Hua, and J. L. Goldstein. 1994. SREBP-1, a membrane-bound transcription factor released by a sterol-regulated proteolysis. *Cell.* **77:** 53–62.
34. Brown, M. S., and J. L. Goldstein. 1997. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell.* **89:** 331–340.
35. Breuer, O., E. Sudjana-Sugiaman, G. Eggertsen, J. Y. Chiang, and I. Bjorkhem. 1993. Cholesterol 7 alpha-hydroxylase is up-regulated by the competitive inhibitor 7-oxocholesterol in rat liver. *Eur. J. Biochem.* **215:** 705–710.
36. Sakai, J., A. Nohturfft, D. Cheng, Y. K. Ho, M. S. Brown, and J. L. Goldstein. 1997. Identification of complexes between the COOH-terminal domains of sterol regulatory element-binding proteins (SREBPs) and SREBP cleavage-activating protein. *J. Biol. Chem.* **272:** 20213–20221.
37. Sakai, J., R. B. Rawson, P. J. Espenshade, D. Cheng, A. C. Seegmiller, J. L. Goldstein, and M. S. Brown. 1998. Molecular identification of the sterol-regulated luminal protease that cleaves SREBPs and controls lipid composition of animal cells. *Mol. Cell.* **2:** 505–514.
38. Hylemon, P. B., E. J. Studer, W. M. Pandak, D. M. Heuman, Z. R. Vlahcevic, and J. Y. L. Chiang. 1989. Simultaneous measurement of cholesterol 7alpha-hydroxylase activity by reverse-phase high-performance liquid chromatography using both endogenous and exogenous [4-^{14}C]cholesterol as substrate. *Anal. Biochem.* **182:** 212–216.