Regulation of cholesterol 7α-hydroxylase expression by sterols in primary rat hepatocyte cultures

Kinchel C. Doerner,* Emily C. Gurley,* Z. Reno Vlahcevic,† and Phillip B. Hylemon[1,*]

Departments of Medicine and Microbiology and Immunology,* Medical College of Virginia-Virginia Commonwealth, Richmond, VA 23298, and McGuire Veterans Administration Medical Center,† Richmond, VA 23298

Abstract The importance of cholesterol and "oxysterols" in the regulation of cholesterol 7α-hydroxylase is not clear. Previous in vivo studies suggest that cholesterol may up-regulate cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile acid biosynthesis, but these studies are open to question as they were carried out in whole animals. Therefore, we used primary rat hepatocytes, cultured in serum-free medium, to determine the effects of cholesterol on the regulation of cholesterol 7α-hydroxylase. Squalestatin, a specific squalene synthase inhibitor, was used to block sterol but not isoprenoid biosynthesis in this system. Squalestatin (1 μM) decreased cholesterol 7α-hydroxylase specific activity to undetectable levels and decreased steady-state mRNA and transcriptional activity to 13% and 47% of controls, respectively. Mevalonolactone (2 mM) failed to restore cholesterol 7α-hydroxylase specific activity or steady-state mRNA levels in squalestatin-treated cells. Addition of cholesterol, delivered in β-cyclodextrin, to squalestatin-treated cells restored cholesterol 7α-hydroxylase specific activity and steady-state mRNA to control levels in a concentration (25 μM to 200 μM) -dependent manner. In contrast, the individual addition of selected "oxysterols" (5-cholesten-3β,7α-diol; 5α-cholestan-3β,6α-diol; cholestan-3β, 5α,6β-triol; 5-(25R)-cholesten-3β,26-diol, all at 50 μM) failed to restore cholesterol 7α-hydroxylase mRNA levels in squalestatin-treated cells. These experiments provide evidence that cholesterol rather than "oxysterols" regulate cholesterol 7α-hydroxylase gene expression. Squalestatin (1 μM) treatment increased HMG-CoA reductase specific activity by 229% of controls. Addition of cholesterol (200 μM), but not mevalonolactone (2 mM), to squalestatin-treated cells decreased HMG-CoA reductase specific activity to 19% of control. The primary rat hepatocyte culture system in conjunction with a specific squalene synthetase inhibitor should be a useful model for elucidating the mechanism of regulation of cholesterol 7α-hydroxylase gene expression by sterols.—**Doerner, K. C., E. C. Gurley, Z. R. Vlahcevic, and P. B. Hylemon.** Regulation of cholesterol 7α-hydroxylase expression by sterols in primary rat hepatocyte cultures. *J. Lipid Res.* 1995. **36:** 1168–1177.

Supplementary key words 3-hydroxy-3-methylglutaryl coenzyme A reductase • bile acid biosynthesis • squalestatin 1 • oxysterol • β-cyclodextrin • squalene synthase • gene expression

Hepatic cholesterol 7α-hydroxylase (EC 1.14.13.17) is the initial and rate-limiting enzyme in the "neutral" bile acid biosynthetic pathway (1). This enzyme is a P450 monooxygenase located in the smooth endoplasmic reticulum of the hepatocyte. Expression of cholesterol 7α-hydroxylase is regulated by the enterohepatic circulation of bile acids (2, 3). Heuman, Hylemon, and Vlahcevic (4) found that the relative hydrophobicity of the bile acid pool in the enterohepatic circulation is responsible for down-regulation of cholesterol 7α-hydroxylase. Pandak et al. (5) found that taurocholate decreased cholesterol 7α-hydroxylase specific activity, protein mass, steady-state mRNA levels, and gene transcriptional activities in the chronic bile fistula rat. Furthermore, Stravitz et al. (6) and Twisk, Lehmann, and Princen (7) have shown that bile acids also down-regulate cholesterol 7α-hydroxylase in primary rat hepatocytes. Also using primary rat hepatocytes, Hylemon et al. (8) demonstrated that dexamethasone and thyroxine synergistically increased cholesterol 7α-hydroxylase steady-state mRNA levels and transcriptional activity.

Cholesterol serves as the metabolic precursor of bile acids and is hypothesized to be an important regulator of hepatic cholesterol 7α-hydroxylase expression in the rat (5, 9). The rate of cholesterol biosynthesis is regulated by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (10). Using the chronic bile fistula rat, Pandak et al. (11) found that lovastatin infusion abrogates bile acid biosynthesis; however, this effect could be overcome by a

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary.

[1]To whom correspondence should be addressed.

bolus dose of mevalonate. Using the same model, Pandak et al. (12) have shown that AY9944, an inhibitor of 7-dehydrocholesterol reductase, also decreased the rate of bile acid biosynthesis; however, a bolus dose of mevalonate in combination with AY9944 did not return bile acid synthesis rates to control levels. Sundseth and Waxman (13) reported that mevalonate-treated rats increase cholesterol 7α-hydroxylase specific activity and mRNA levels. Ness, Zhao, and Keller (14) have recently shown in intact rats that injection of a squalene synthetase inhibitor (zaragozic acid) caused a rapid decrease in cholesterol 7α-hydroxylase steady-state mRNA levels. Using a genetically modified cholesterol 7α-hydroxylase promoter placed upstream of a chloramphenicol acetyltransferase reporter gene and transfected into a SV40-transformed mouse hepatocyte cell line, Ramirez et al. (15) showed an increased expression in response to low density lipoproteins. These data suggest that cholesterol exerts an up-regulatory effect on transcriptional activity that increases cholesterol 7α-hydroxylase specific activity and bile acid biosynthesis. Using the whole animal as a model for studies of cholesterol 7α-hydroxylase regulation by cholesterol has limitations. Björkhem, Eggertsen, and Andersson (16) report that feeding 2% cholesterol to rats results in bile acid malabsorption that could then cause a derepression of the cholesterol 7α-hydroxylase gene due to lack of bile acids returning to the liver. Therefore, an increase in cholesterol 7α-hydroxylase in cholesterol-fed rats may not be directly due to dietary cholesterol.

In the present study, we have used the primary rat hepatocyte model to investigate the effects of cholesterol and "oxysterols" on cholesterol 7α-hydroxylase expression. This system holds advantages over whole animal studies in that culture conditions and factors affecting cholesterol 7α-hydroxylase expression can be precisely defined. We have used serum-free medium for culturing hepatocytes and used squalestatin, an inhibitor of squalene synthase, to specifically block only the sterol arm of the cholesterol biosynthetic pathway (17). Using this model, we show that cholesterol or a cholesterol derivative is required for maintaining cholesterol 7α-hydroxylase gene transcription.

METHODS

Chemicals and other reagents

William's Medium E and the nick-translation kit were purchased from GIBCO-BRL, Gaithersburg, MD. Tri-Reagent was purchased from Molecular Research Center, Inc., Cincinnati, OH and Poly ATtract mRNA isolation system II was obtained from Promega. Radionuclides were purchased from DuPont-New England Nuclear. These included mevalonolactone, RS-[5-^{3}H(N)- (33 Ci/mmol); hydroxy-3-methylglutaryl coenzyme A, DL-3-[glutaryl-3-^{14}C], (59.9 mCi/mmol); deoxycytidine 5'-triphosphate, tetra(triethylammonium) salt, [α-^{32}P]-, (3000 Ci/mmol). Budget-Solve liquid scintillation cocktail was obtained from Research Products International, Mount Prospect, IL. Squalestatin 1, tripotassium salt was kindly provided by Glaxo Research Group, Greenford, Middlesex, United Kingdom, UB6 OHE. Squalestatin 1 will be referred to as squalestatin. β-Cyclodextrin (Molecusol-HPB) was purchased from Pharmatec, Inc., Alachua, FL. Dexamethasone, L-thyroxine, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), mevalonolactone, and cholesterol were purchased from Sigma Chemical Co., St. Louis, MO. "Oxysterols", including 5-cholesten-3β,7α-diol and 5α-cholestan-3β,6α-diol, were obtained from Steraloids. Cholestan-3β,5α,6β-triol was purchased from Sigma and 5-(25R)-cholesten-3β,26-diol was purchased from Research Plus, Inc. (Bayonne, NJ). AG2-X8 (200–400 mesh, chloride-form) resin was purchased from Bio-Rad Laboratories, Hercules, CA. All other chemicals were of the highest purity commercially available.

Isolation and culture of primary rat hepatocytes

Hepatocytes were isolated from male Sprague-Dawley rats (250–300 g) using the collagenase-perfusion technique of Bissell and Guzelian (18). Prior to plating, cells were judged to be >90% viable using trypan blue exclusion. Parenchymal cells (3.4×10^6) were plated onto 60-mm plastic petri dishes previously coated with rat tail collagen. Cells were incubated in 3 ml of Williams' Medium E supplemented with insulin (0.25 units/ml) and penicillin (100 units/ml) in a 5% CO_2 atmosphere at 37°C. Cells were routinely harvested at 72 h of culture. Unless otherwise indicated culture medium contained 0.1 μM dexamethasone and 1.0 μM L-thyroxine. Culture medium was changed daily. Squalestatin was added to the cells at 1 μM final concentration at 48 h of culture unless otherwise indicated. Mevalonolactone was added at 2 mM or 10 mM final concentration at 48 h of culture. Cholesterol dissolved in β-cyclodextrin was added at 200 μM final concentration at 48 h of culture.

Microsome preparation

Microsomes were prepared as follows. Medium was aspirated from the petri plates and the cells were washed once with phosphate-buffered saline (PBS). Microsomes to be assayed for cholesterol 7α-hydroxylase and active HMG-CoA reductase were prepared in buffer containing 100 mM potassium phosphate, pH 7.2, 100 mM sucrose, 50 mM KCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 200 mM NaF, 3 mM dithiothreitol, 100 μM leupeptin, 5 mM ethylenebis(oxyethylenenitrilo)-tetraacetic acid (EGTA), and 1 mM phenylmethylsulfonyl fluoride. Microsomes to be assayed for total active HMG-CoA reductase activity were prepared in buffer containing 200 mM NaCl in place of 200 mM NaF. Cells were scraped into the appropriate buffer using approximately 1 ml per

five plates. The cell suspension was placed on ice and sonicated for 1 min. The sample was then centrifuged at 12,000 *g*, for 10 min (4°C). The pellet was discarded and the supernatant fluid was subjected to centrifugation 110,000 *g* for 1.5 h (4°C). The resulting pellet was suspended in the appropriate volume of buffer (0.5–1.0 ml) using a hand-driven homogenizer and assayed immediately or stored at −20°C until assayed for HMG-CoA reductase activity or stored at −70°C until assayed for cholesterol 7α-hydroxylase activity.

Enzyme assays

Cholesterol 7α-hydroxylase activity was measured as previously described (19). Briefly, microsomes were incubated by shaking at 37°C with endogenous cholesterol used as the substrate. Upon termination of the reaction, cholesterol oxidase was added to convert 5-cholesten-3β,7α-diol to 4-cholesten-3-oxo, 7α-ol. To correct for recovery of product, 1 μg of 5-cholesten-7β-ol was added with cholesterol oxidase. Samples were extracted and the steroids were separated using a C-18 reverse-phase HPLC column. The steroid product was quantified by measuring absorption at 240 nm.

HMG-CoA reductase activity was determined using a modification of the method of Beg and Stonik (20). Final concentrations of the reaction components were as follows: 100 μg bovine serum albumin, 150 mM potassium phosphate, pH 6.9, 200 mM KCl, 6 mM sodium EDTA, 0.4 mM sodium azide, and 1 mM dithiothreitol. Microsome samples were pre-incubated at 37°C for 20 min prior to the addition of substrates to initiate the reaction. Final substrate concentrations were 2.3 mM NADPH and 340 μM HMG-CoA, which contained approximately 20,000 disintegrations per min of [3-^{14}C]3-hydroxy-3-methylglutaryl-CoA. The reaction was allowed to proceed for 5–20 min at 37°C in 100 μl and terminated with 50 μl of 10 N propionic acid. To correct for the amount of product recovered, approximately 20,000 dpm of [^{3}H]mevalonate in 15 μl was added to each assay. The average recovery of product using the ion-exchange assay for HMG-CoA reductase was approximately 93%. The samples were centrifuged (16,000 *g*; 4°C) to pellet any insoluble material and 100 μl of the supernatant fluid was applied to a AG 2-X8 resin column. Columns were prepared by equilibrating AG 2-X8 resin in 1 M propionic acid at room temperature with no less than four changes of 1 M propionic acid. Resin was pipetted to a height of 5 cm in glass transfer pipets plugged with glass wool. To remove excess propionic acid, the columns were washed with 2 ml of double-distilled water then positioned over liquid scintillation vials. The sample was applied to the column and washed with 2 ml of double-distilled water. Greater than 99% of the product formed required the addition of NADPH to the reaction mixture. Scintillation cocktail was added to the vials and the amounts of ^{3}H and ^{14}C were determined by liquid scintillation spectrometry.

RNA isolation and quantification

Total RNA was prepared from cultured hepatocytes using Tri Reagent protocol. Total RNA for dot-blot analysis was harvested from approximately 5 plates per experimental condition. One ml of Tri Reagent was added per plate and then RNA was prepared according to protocol. The total RNA was quantified by absorbency at 260 nm. The RNA was precipitated in one-tenth volume 3 M sodium acetate, pH 5.0, and two volumes of ethanol and stored at −20°C.

For HMG-CoA reductase mRNA analysis, it was necessary to prepare polyadenylated-RNA. Total RNA was prepared using the Tri Reagent protocol. Polyadenylated-RNA was isolated from 1 mg of total RNA using the poly-ATtract mRNA isolation system IV from Promega.

Dot-blot hybridization

Determination of steady-state mRNA levels of cholesterol 7α-hydroxylase and cyclophilin was performed as previously described (8).

Northern blot hybridization

Polyadenylated-RNA was analyzed by Northern blot analysis as previously described (21). The blot was probed with ^{32}P-labeled HMG-CoA reductase cDNA probe and also with ^{32}P-labeled cyclophilin cDNA.

Gene transcriptional activity (nuclear run-ons)

Nuclei isolation and gene transcriptional activity of cholesterol 7α-hydroxylase were performed as previously described (8).

Preparation and radiolabeling of cDNA probes

For the cholesterol 7α-hydroxylase cDNA probe, we isolated a 0.7 kb-AccI/EcoRI fragment of pBSK7α6 (22). The 0.43 Kb-EcoRI fragment of rat HMG-CoA reductase was used (23) and the BamHI fragment from p1B15 was used as the cyclophilin cDNA probe (24). cDNA probes were radiolabeled with [α-^{32}P]-dCTP using a commercially available nick-translation system. The radiolabeled cDNA was isolated using Nensorb 20 Nucleic Acid Purification Cartridges (NEN Research Products) as described by the manufacturer and stored at −20°C. The amount of radionuclide incorporated into the cDNA was determined by liquid scintillation spectrometry.

Protein determination

Protein was determined using the Bradford method (25) with Bio-Rad Protein Reagent (Bio-Rad Laboratories) or the Lowry et al. method (26). Bovine serum albumin was used as the protein standard.

Gas-liquid chromatography

The purity of cholesterol used was determined by gas-liquid chromatography as previously described (27).

Statistics

Data are reported as mean ± standard deviation. Where indicated, data were subjected to *t*-test analysis and determined to be significantly different if $P < 0.05$.

RESULTS

Effects of squalestatin on regulation of cholesterol 7α-hydroxylase

The effects of squalestatin on the regulation of cholesterol 7α-hydroxylase specific activity in primary rat hepatocyte cultures are shown in **Table 1.** β-Cyclodextrin was used as the solvent for cholesterol and other "oxysterols." The individual addition of mevalonate (2 mM) and cholesterol (200 μM) to culture medium resulted in a small increase in cholesterol 7α-hydroxylase specific activity (P = ns). In contrast, squalestatin (1 μM) addition for 24 h prior to harvest decreased cholesterol 7α-hydroxylase specific activity to an undetectable level. The addition of mevalonate (2 mM) to squalestatin-treated cells failed to restore any detectable cholesterol 7α-hydroxylase activity. In contrast, when cholesterol (200 μM) was added to squalestatin-treated cells, cholesterol 7α-hydroxylase specific activity returned to control levels. The cholesterol used in these experiments was found to be >99% pure by gas-liquid chromatography analysis, suggesting that auto-oxidation products of cholesterol were not being added to hepatocyte cultures in significant amounts. In control experiments, we examined the direct effects of squalestatin on cholesterol 7α-hydroxylase specific activity. Hepatic microsomes prepared from male Sprague-Dawley rats fed a 5% (w/w) cholestyramine-enriched diet were assayed for cholesterol 7α-hydroxylase activity in the presence and absence of squalestatin (150 μM). The inclusion of squalestatin did not detectably inhibit cholesterol 7α-hydroxylase activity (data not shown).

The effects of squalestatin concentration on cholesterol 7α-hydroxylase steady-state mRNA levels were also investigated (**Fig. 1**). Addition of squalestatin decreased cholesterol 7α-hydroxylase mRNA in a concentration-dependent fashion. The data show that at 50 nM squalestatin, cholesterol 7α-hydroxylase steady-state mRNA levels were reduced to less than 30% of control by 24 h. Maximal suppression of cholesterol 7α-hydroxylase mRNA levels of 14% of control was achieved by addition of 300 nM squalestatin.

The squalestatin-induced decrease of cholesterol 7α-hydroxylase mRNA levels was also time-dependent. The data in **Fig. 2** show that cholesterol 7α-hydroxylase mRNA levels rapidly decreased upon addition of squalestatin (1 μM). Cholesterol 7α-hydroxylase steady-state mRNA levels decreased to 50% of control in 2.7 h and to 14% of control by 24 h. We have determined that the half-life of cholesterol 7α-hydroxylase mRNA is approximately 1.5 h in primary rat hepatocyte cultures under our culture conditions (data not shown).

Next, we tested the effect of exogenous cholesterol addition on cholesterol 7α-hydroxylase mRNA levels in squalestatin-treated hepatocytes (**Fig. 3**). Cholesterol was added at increasing concentrations simultaneously with squalestatin (1 μM) for 24 h before RNA was extracted. This treatment caused a sigmoidal dose-response recovery of cholesterol 7α-hydroxylase mRNA levels. Approxi-

TABLE 1. Effects of squalestatin on cholesterol 7α-hydroxylase and HMG-CoA reductase specific activities in primary rat hepatocytes

Additions[a]	Specific Activity	
	Cholesterol 7α-Hydroxylase	HMG-CoA Reductase
	pmol/h/mg protein	*nmol/min/mg protein*
Control (hormones)	946 ± 295 (3)	1.08 ± 0.46 (3)
Squalestatin (1 μM)	<20 (3)[b]	2.47 ± 0.57 (5)[b]
Mevalonate (2 mM)	1110[c]	0.61 ± 0.23 (3)
Cholesterol (200 μM)	1664 ± 407 (3)	0.32 ± 0.15 (3)
Squalestatin (1 μM) + mevalonate (2 mM)	<20 (3)[b]	2.80 ± 0.44 (3)[b]
Squalestatin (1 μM) + cholesterol (200 μM)	1836 ± 589 (3)	0.46 ± 0.25 (4)

The specific activities of cholesterol 7α-hydroxylase and HMG-CoA reductase were determined as described in Methods. Data are shown as mean ± standard deviation and number in parentheses is the number of independent experiments (cell preparations) assayed in duplicate.

[a] Culture medium contained 0.1 μM dexamethasone, 1.0 μM L-thyroxine, and 2.5% (vol/vol) β-cyclodextrin. Squalestatin (1.0 μM), mevalonate 2 mM (as lactone), or cholesterol 200 μM was added to the cultures at 48 h of incubation and microsomes were prepared at 72 h.

[b] Indicates different from control (hormones) ($P < 0.05$).

[c] Average of two independent experiments performed in duplicate.

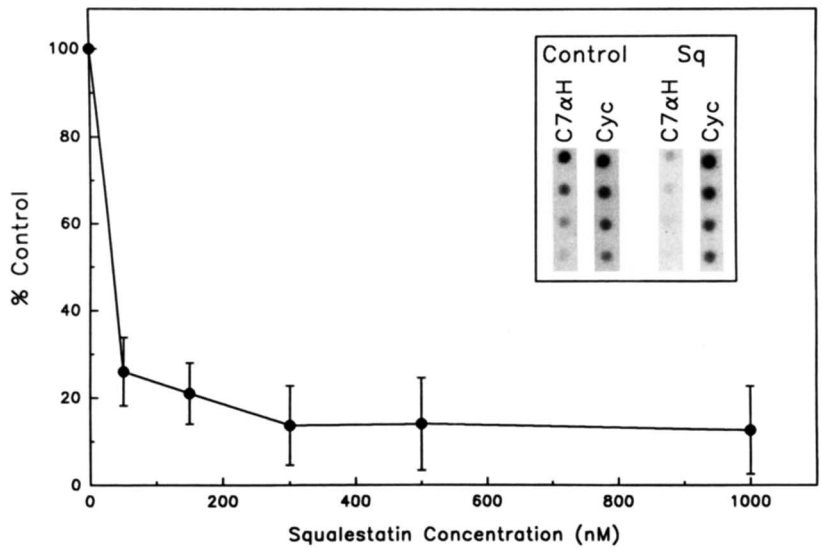

Fig. 1. Effects of squalestatin concentration on cholesterol 7α-hydroxylase mRNA levels. Squalestatin was added to the cultures at the concentrations indicated from 48 to 72 h at which time RNA was isolated. The amount of cholesterol 7α-hydroxylase mRNA was measured by dot-blot hybridization as described in Methods. Control cells were incubated in the absence of squalestatin. All mRNA measurements were normalized to cyclophilin mRNA levels and error bars represent the mean ± standard deviation. Inset: Dot blots of control and 1 μM squalestatin (Sq)-treated cells showing cholesterol 7α-hydroxylase (C7αH) and cyclophilin (cyc). Total RNA blotted was 10 μg, 5 μg, 2.5 μg, and 1.25 μg from top to bottom, respectively.

mately 200 μM cholesterol was required to restore cholesterol 7α-hydroxylase mRNA to control levels.

We also tested the effect of mevalonate in the presence and absence of squalestatin on cholesterol 7α-hydroxylase steady-state mRNA levels (**Fig. 4**). Addition of mevalonate (2 mM) alone slightly increased cholesterol 7α-hydroxylase steady-state mRNA levels and, as expected, squalestatin (1 μM) decreased cholesterol 7α-hydroxylase mRNA levels. The addition of mevalonate (2 mM) with squalestatin failed to restore cholesterol 7α-hydroxylase mRNA levels. This is contrary to the results obtained after addition of cholesterol.

The squalestatin-induced decrease in cholesterol 7α-hydroxylase steady-state mRNA levels could be due to either an increase in cholesterol 7α-hydroxylase mRNA degradation or a decrease in cholesterol 7α-hydroxylase gene transcription, or both. We investigated the latter possibility by performing nuclear "run-on" assays on nuclei isolated from primary rat hepatocytes that had been cultured in the presence and absence of squalestatin. Inclusion of squalestatin (1 μM) to the culture medium for 24 h significantly decreased cholesterol 7α-hydroxylase gene transcriptional activity to 47% of control levels ($P < 0.05$) (**Fig. 5**).

Effects of "oxysterols" on the regulation of cholesterol 7α-hydroxylase

Because cholesterol is known to be oxidized to various metabolites in the hepatocyte, i.e., 7α-hydroxycholesterol, 27-hydroxycholesterol, we next tested the effect of various

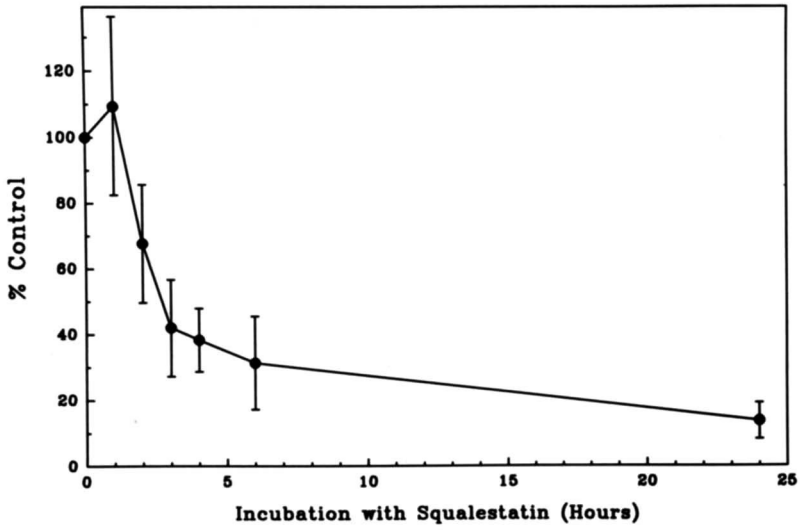

Fig. 2. Time course of squalestatin inhibition on cholesterol 7α-hydroxylase mRNA levels. Squalestatin additions (1 μM) were made in a staggered fashion to allow the harvesting of all time points at 72 h of culture. Hepatocytes were cultured with 1.0 μM L-thyroxine and 0.1 μM dexamethasone. mRNA measurements were normalized to cyclophilin mRNA levels and error bars represent the mean ± standard deviation.

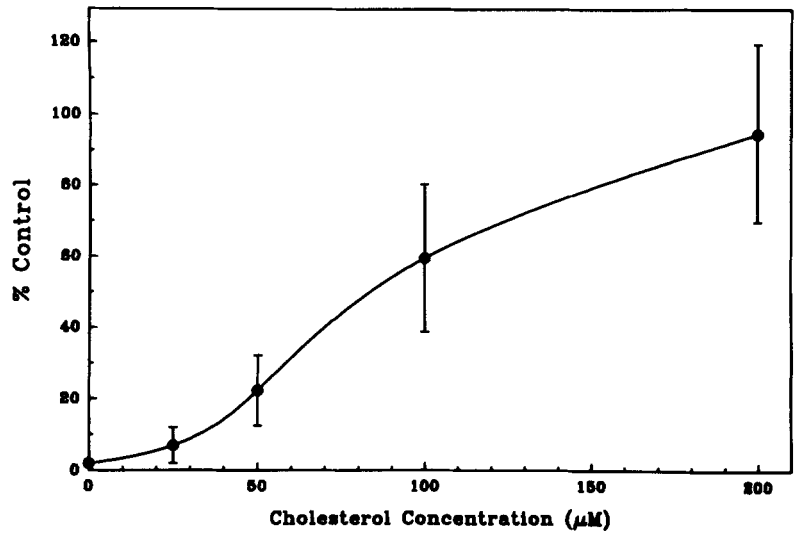

Fig. 3. Effects of cholesterol on inhibition of cholesterol 7α-hydroxylase mRNA by squalestatin. Squalestatin (1 μM) was added to culture medium at 48 h along with varying concentrations of cholesterol. All hepatocytes were cultured with 1.0 μM L-thyroxine and 0.1 μM dexamethasone. Cholesterol was solubilized using β-cyclodextrin. Vehicle alone (2.5% vol/vol) was added to control cultures. All mRNA measurements were normalized to cyclophilin mRNA levels and error bars represent the mean ± standard deviation.

"oxysterols" on the regulation of cholesterol 7α-hydroxylase steady-state mRNA levels. The data presented in **Table 2** show that 5α-cholestan-3β,6α-diol significantly increased ($P < 0.05$) cholesterol 7α-hydroxylase steady-state mRNA levels when added alone to primary rat hepatocyte cultures. However, in squalestatin-treated cells, cholesterol was the only sterol tested that could maintain control levels of cholesterol 7α-hydroxylase steady-state mRNA levels (**Fig. 6**). The effects of cholesterol were not altered by the addition of ketoconazole (Fig. 6).

Effects of hormones and squalestatin on HMG-CoA reductase specific activity and mRNA levels

In cultured rat hepatocytes dexamethasone and thyroxine synergistically increase cholesterol 7α-hydroxylase transcriptional activity (8). The effect of these hormones on HMG-CoA reductase has not been studied in primary hepatocyte cultures; we therefore explored the effects of L-thyroxine and dexamethasone on the regulation of HMG-CoA reductase. HMG-CoA reductase specific activity and steady-state mRNA levels increased 3.9-fold and 4.3-fold after the addition of 0.1 μM dexamethasone with 1.0 μM L-thyroxine to the culture medium, respectively (**Fig. 7**, and unpublished data). Addition of mevalonate (2 mM) or cholesterol (200 μM) reduced HMG-CoA reductase specific activity to 56% and 30% of control, respectively (Table 1). Treatment with squalestatin (1 μM) significantly increased HMG-CoA reductase specific activity to 229% of control ($P < 0.05$). The inclusion of cholesterol with squalestatin reduced HMG-CoA reductase specific activity to 19% ($P < 0.05$). Surprisingly, there was no effect on HMG-CoA reductase specific activity when mevalonate was added to squalestatin-treated cells (Table 1).

We next explored the effects of squalestatin on the phosphorylation-state of HMG-CoA reductase by using the method of Beg, Stonik, and Brewer (20, 28). Microsomes harvested in the absence of NaF (i.e., in the presence of NaCl) are indicative of the total amount of active HMG-CoA reductase in the cells and those harvested

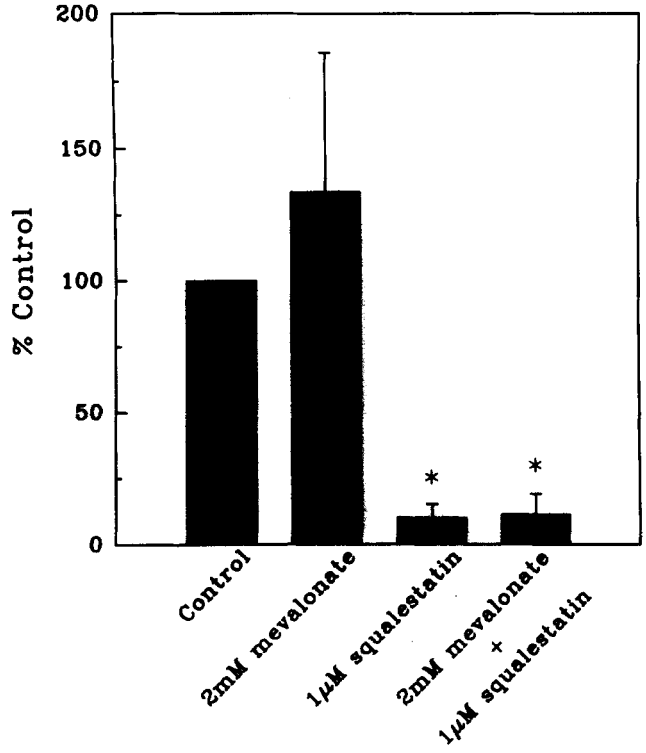

Fig. 4. Effects of mevalonate on inhibition of cholesterol 7α-hydroxylase mRNA levels by squalestatin. Squalestatin and mevalonate were added to the cultures at 48 h and RNA was extracted at 72 h. Hepatocytes were cultured with 1.0 μM L-thyroxine and 0.1 μM dexamethasone. mRNA measurements were normalized to cyclophilin mRNA levels and error bars represent the mean ± standard deviation. *Values are significantly different than the control ($P < 0.05$).

TABLE 2. Effects of selected "oxysterols" on cholesterol 7α-hydroxylase steady-state mRNA levels in primary rat hepatocyte cultures

Additions (50 μM)[a]	% Control (No Addition)
Control (3) no addition	100
Cholesterol (3)	142 ± 52
5-Cholesten-3β,7α-diol (3)	102 ± 13
5α-Cholestan-3β,6α-diol (3)	156 ± 23[b]
Cholestan-3β,5α,6β-triol (3)	121 ± 32
5-(25R)-Cholesten-3β,26-diol (3)	110 ± 21

[a] Culture medium contained 0.1 μM dexamethasone, 1.0 μM L-thyroxine, and 2.5% (vol/vol) β-cyclodextrin. "Oxysterols" solubilized in β-cyclodextrin were individually added to cultures at 48 h, total RNA was isolated at 72 h, and cholesterol 7α-hydroxylase steady-state mRNA levels were determined as described in Methods. Data are shown as mean ± standard deviation and the number in parentheses indicates the number of independent experiments (cell preparations) assayed in duplicate.
[b] Significantly different from control ($P < 0.05$).

in the presence of NaF are indicative of the percentage of the total HMG-CoA reductase that was active at the time of harvest. Results indicate the ratio of active to inactive HMG-CoA reductase was not altered by squalestatin (data not shown). Furthermore, previous work has shown that squalestatin does not directly inhibit HMG-CoA reductase activity (17).

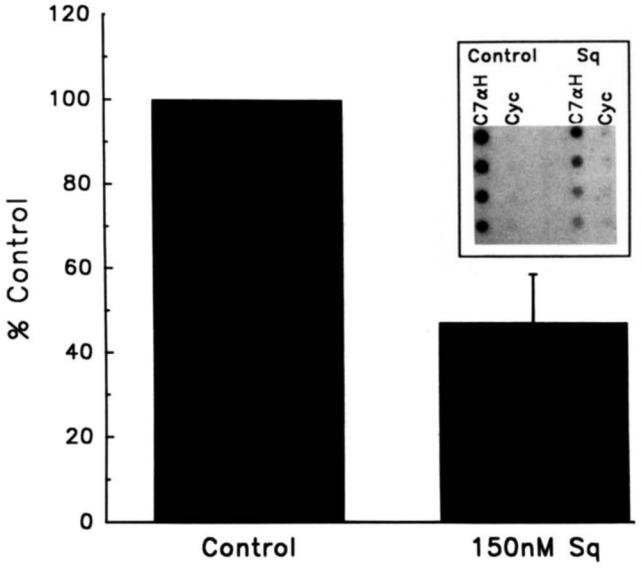

Fig. 5. Down-regulation of cholesterol 7α-hydroxylase transcriptional activity by squalestatin (Sq). Cells were incubated in the absence (control) or presence of 150 nM squalestatin from 48 to 72 h of culture, at which time nuclei were isolated. Transcriptional activity levels for cholesterol 7α-hydroxylase and cyclophilin were determined as described in Methods. C7αH transcriptional levels were normalized against cyc. The error bar represents mean ± standard deviation. Inset: Dot blot showing results of ^{32}P-labeled mRNA transcripts hybridized to various amounts of either C7αH or cyc cDNA. cDNA amounts are 2.5 μg, 1.25 μg, or 0.625 μg from top to bottom, respectively. Cells were cultured in the absence (control) or the presence of 1 μM squalestatin for 3 h and the nuclei were harvested at 12 h of culture.

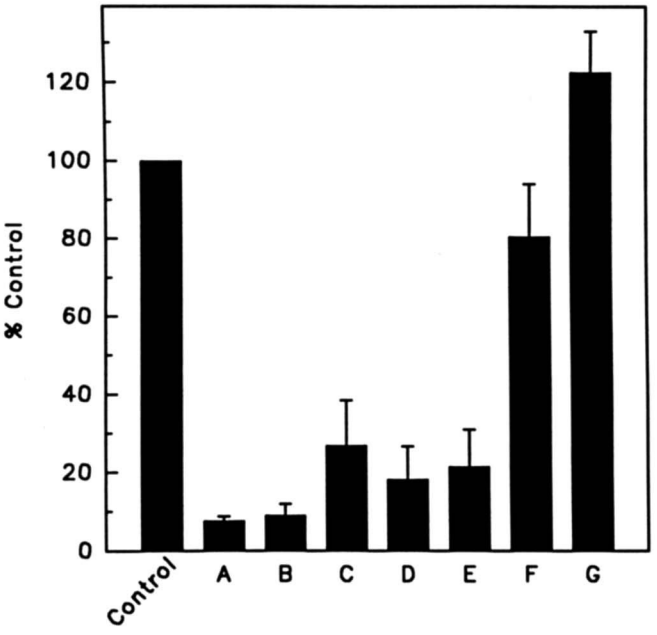

Fig. 6. Effects of cholesterol and selected "oxysterols" on cholesterol 7α-hydroxylase steady-state mRNA levels in squalestatin-treated hepatocytes. Primary rat hepatocyte cultures were prepared and incubated for 48 h in serum-free medium as described in Methods. Control cells were incubated in the absence of added cholesterol and "oxysterols". Squalestatin (Sq) alone (150 nM) or Sq (150 nM) plus individually added "oxysterols" (50 μM), in β-cyclodextrin 2.5% vol/vol, were added at 48 h, total RNA was isolated at 72 h, and the concentration of cholesterol 7α-hydroxylase steady-state mRNA was determined and normalized against cyclophilin mRNA. Error bars represent standard deviation. A) Sq alone; B) Sq + 5-cholesten-3β,7α-diol; C) Sq + 5α-cholestan-3β,6α-diol; D) Sq + cholestan-3β,5α,6β-triol; E) Sq + 5-(25R)-cholesten-3β,26-diol; F) Sq + cholesterol; G) Sq + cholesterol + ketoconazole.

DISCUSSION

The importance of cholesterol and oxysterols in the regulation of cholesterol 7α-hydroxylase gene expression has not been fully elucidated. Oxygenated metabolites of cholesterol have been shown to be powerful down-regulators of HMG-CoA reductase and LDL receptor gene expression (29–32). In order to resolve this issue, we have used the primary rat hepatocyte model and a highly specific inhibitor of cholesterol biosynthesis. In primary hepatocytes incubated in serum-free media, the need for cholesterol is satisfied solely by de novo biosynthesis. In primary rat hepatocytes, squalestatin is a potent (IC_{50} 12 nM) and specific inhibitor of squalene synthase, the first committed step in the sterol arm of the cholesterol biosynthetic pathway (17). Treatment with squalestatin deprives cells of endogenously synthesized sterols but does not impede the biosynthesis of isoprenoid compounds needed for ubiquinone biosynthesis, protein-isoprenylation, and other important metabolic processes (17, 33).

When squalestatin was added to the culture medium, cholesterol 7α-hydroxylase specific activity, steady-state

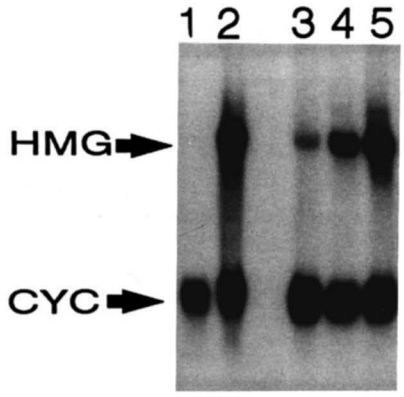

Fig. 7. Effect of hormones and squalestatin on HMG-CoA reductase steady-state mRNA levels in primary hepatocyte cultures. This Northern blot compares Poly(A)$^+$ mRNA levels in normal (lane 1), cholestyramine (lane 2)-fed rats, primary hepatocytes cultured in Williams' E medium alone (lane 3), with 1.0 μM L-thyroxine and 0.1 μM dexamethasone (lane 4), or with 1.0 μM L-thyroxine and 0.1 μM dexamethasone and 1 μM squalestatin (lane 5). Squalestatin was added at 48 h and Poly(A)$^+$-RNA was isolated at 72 h. Cyclophilin (cyc) was used as a RNA loading control. Details of the conditions for Northern blotting are described in the Methods section.

mRNA and transcriptional activity were all markedly decreased (Table 1; Figs. 1, 2, and 5). The addition of mevalonate (2 mM) to squalestatin-treated cells failed to restore cholesterol 7α-hydroxylase steady-state mRNA or specific activity (Table 1; Fig. 4) showing that non-sterol products derived from mevalonate do not up-regulate cholesterol 7α-hydroxylase in the hepatocyte. Ness and co-workers (14) have shown that inhibition of squalene synthesis decreases cholesterol 7α-hydroxylase mRNA in the intact rat. In contrast, the addition of cholesterol to squalestatin-treated cells increased cholesterol 7α-hydroxylase specific activity and mRNA levels to control levels (Table 1; Fig. 3). These observations show that cholesterol or a cholesterol metabolite may be required as an up-regulator of cholesterol 7α-hydroxylase.

Certain "oxysterols" stimulated cholesterol 7α-hydroxylase mRNA steady-state levels when added alone (Table 2), but none maintained cholesterol 7α-hydroxylase as well as cholesterol in squalestatin-treated cells (Fig. 6). The addition of ketoconazole (50 μM), a cytochrome P450 inhibitor, did not block the ability of cholesterol to maintain cholesterol 7α-hydroxylase in squalestatin-treated cells (Fig. 6). Gupta, Sexton, and Rudney (32) using rat intestinal epithelial cells showed that ketoconazole totally abolishes the inhibition of HMG-CoA reductase by LDL cholesterol. Our data suggest, but do not prove, that a cholesterol "regulatory pool" may control the levels of cholesterol 7α-hydroxylase gene expression. However, we cannot yet eliminate the possibility that a ketoconazole-insensitive cholesterol metabolite acts as an up-regulator of cholesterol 7α-hydroxylase.

The cDNA and genomic clones encoding cholesterol 7α-hydroxylase have been isolated and analyzed (9, 34–38). Analysis of the putative promotor region of the cholesterol 7α-hydroxylase gene (CYP7) shows a variety of nucleotide sequences known to bind various regulatory proteins (34–38). These *trans*-activating transcription factors and *cis*-acting regulatory elements involved in the regulation of the cholesterol 7α-hydroxylase gene have only begun to be identified by experimentation. However, the 5'-flanking region of the CYP7 gene does not contain any apparent sterol responsive elements (SREs) found in the promoters of other genes that respond to cholesterol levels such as HMG-CoA reductase, LDL-receptor, and HMG-CoA synthase (31). It should be stressed that the SREs described for genes involved in cholesterol biosynthesis and uptake are all down-regulated by sterols, whereas cholesterol up-regulates CYP7 gene expression. Therefore, different SRE motifs and specific transcription factors might be involved in the up-regulation of CYP7 by cholesterol.

Under most physiological circumstances cholesterol 7α-hydroxylase and HMG-CoA reductase specific activities fluctuate in tandem, as if they respond to similar regulatory signals. Hylemon et al. (8) has shown that dexamethasone and thyroxine maximally up-regulate cholesterol 7α-hydroxylase in primary hepatocyte cultures, raising the possibility that these hormones could also up-regulate HMG-CoA reductase. Results presented here show that a combination of these two hormones increased HMG-CoA reductase specific activity and mRNA levels (Fig. 7). Simonet and Ness (39) showed that feeding hypophysectomized rats thyroid powder increased HMG-CoA reductase transcriptional activity (5-fold) and also stabilized HMG-CoA reductase mRNA (up to 6-fold).

In contrast to cholesterol 7α-hydroxylase, HMG-CoA reductase specific activity decreased in response to cholesterol (40, 41). HMG-CoA reductase has been extensively studied in immortalized cell lines; however, its regulation in primary hepatocyte cultures has not been thoroughly investigated. The addition of squalestatin to the culture medium increased HMG-CoA reductase specific activity in primary hepatocyte cultures. Cholesterol, but not mevalonate (2 mM), down-regulated HMG-CoA reductase specific activity in squalestatin-treated hepatocytes (Table 1). Our data are consistent with those of Ness et al. (14), who have shown a marked increase in HMG-CoA reductase specific activity in the liver of intact rats using zaragozic acid, a squalene synthetase inhibitor. Panini et al. (42) have shown that Chinese hamster ovary (CHO) cells treated with a oxidosqualene cyclase inhibitor markedly increased HMG-CoA reductase specific activity. Mevalonate down-regulated HMG-CoA reductase specific activity in CHO cells. These

authors suggested that non-sterol mevalonate-derived products are the major regulators of HMG-CoA reductase activity in growing CHO cells. Our data suggest that cholesterol is a major regulator of HMG-CoA reductase activity in primary rat hepatocyte cultures. Our observations, however, do not eliminate a role for mevalonate-derived products in the regulation of HMG-CoA reductase, but suggest that cholesterol or a sterol metabolite is required for down-regulation in this system. Liscum et al. (43) treated rats with mevinolin and cholestyramine which increased HMG-CoA reductase specific activity, protein mass, and mRNA. When cholesterol was supplied in the diet of these animals, all parameters declined, suggesting that cholesterol is an important regulator of hepatic HMG-CoA reductase in the intact rat. Finally, Sudjana-Sugiaman, Eggertsen, and Björkhem (44) recently showed that in certain strains of rats, phenobarbital induced cholesterol 7α-hydroxylase activity with a resulting increase in HMG-CoA reductase specific activity in liver microsomes but not in other tissues. These authors postulated that cholesterol 7α-hydroxylase may up-regulate HMG-CoA reductase activity by metabolizing a regulatory pool of free cholesterol. Our data are consistent with this hypothesis.

In summary, we show that cholesterol or a metabolite is required for maintaining cholesterol 7α-hydroxylase in the primary rat hepatocyte. The primary rat hepatocyte system should be a useful model for studies of the regulation of cholesterol 7α-hydroxylase gene expression by sterols. In this system, it is possible to turn the cholesterol 7α-hydroxylase gene off (>85%) by inhibiting cholesterol biosynthesis and back on (100% of control) by adding free cholesterol to the culture medium.

This work was funded in part by the National Institutes of Health grant number PO1-DK38030-08, a grant from the Burroughs Wellcome Company, Inc., and a National Research Service Award (Doerner) F32DK08879 from the National Institutes of Health.

Manuscript received 20 June 1994 and in revised form 20 January 1995.

REFERENCES

1. Axelson, M., and J. Sjövall. 1990. Potential bile acid precursors in plasma—possible indicators of biosynthetic pathways to cholic and chenodeoxycholic acids in man. *J. Steroid Biochem.* **36:** 631–640.
2. Bergstrom, S., and H. Danielsson. 1958. On the regulation of bile acid formation in the liver. *Acta Physiol. Scand.* **43:** 1–7.
3. Shefer, S., S. Hauser, I. Bekersky, and E. H. Mosbach. 1969. Feedback regulation of bile acid biosynthesis in the rat. *J. Lipid Res.* **10:** 646–655.
4. Heuman, D. M., P. B. Hylemon, and Z. R. Vlahcevic. 1989. Regulation of bile acid synthesis. III. Correlation between biliary bile salt hydrophobicity index and the activities of enzymes regulating cholesterol and bile acid synthesis in the rat. *J. Lipid Res.* **30:** 1161–1171.
5. Pandak, W. M., Y. C. Li, J. Y. L. Chiang, E. J. Studer, E. C. Gurley, D. M. Heuman, Z. R. Vlahcevic, and P. B. Hylemon. 1991. Regulation of cholesterol 7α-hydroxylase mRNA and transcriptional activity by taurocholate and cholesterol in the chronic biliary diverted rat. *J. Biol. Chem.* **66:** 3416–3421.
6. Stravitz, R. T., P. B. Hylemon, D. M. Heuman, L. R. Hagey, C. D. Schteingart, H-T. Ton-Nu, A. F. Hofmann, and Z. R. Vlahcevic. 1993. Transcriptional regulation of cholesterol 7α-hydroxylase mRNA by conjugated bile acids in primary cultures of rat hepatocytes. *J. Biol. Chem.* **268:** 13987–13993.
7. Twisk, J., E. M. Lehmann, and H. M. G. Princen. 1993. Differential feedback regulation of cholesterol 7α-hydroxylase mRNA and transcriptional activity by rat bile acids in primary monolayer cultures of rat hepatocytes. *Biochem. J.* **290:** 685–691.
8. Hylemon, P. B., E. C. Gurley, R. T. Stravitz, J. L. Litz, W. M. Pandak, J. Y. L. Chiang, and Z. R. Vlahcevic. 1992. Hormonal regulation of cholesterol 7α-hydroxylase mRNA levels and transcriptional activity in primary rat hepatocyte cultures. *J. Biol. Chem.* **267:** 16866–16871.
9. Jelinek, D. F., S. Andersson, C. A. Slaughter, and D. W. Russell. 1990. Cloning and regulation of cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile acid biosynthesis. *J. Biol. Chem.* **265:** 8190–8197.
10. Brown, M. S., and J. L. Goldstein. 1980. Multivalent feedback regulation of HMG-CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. *J. Lipid Res.* **21:** 505–517.
11. Pandak, W. M., D. M. Heuman, P. B. Hylemon, and Z. R. Vlahcevic. 1990. Regulation of bile acid synthesis. IV. Interrelationship between cholesterol and bile acid biosynthesis pathways. *J. Lipid Res.* **31:** 79–90.
12. Pandak, W. M., Z. R. Vlahcevic, D. M. Heuman, and P. B. Hylemon. 1990. Regulation of bile acid synthesis. V. Inhibition of conversion of 7-dehydrocholesterol to cholesterol is associated with down-regulation of cholesterol 7α-hydroxylase activity and inhibition of bile acid synthesis. *J. Lipid Res.* **31:** 2149–2158.
13. Sundseth, S. S., and D. J. Waxman. 1990. Hepatic P-450 cholesterol 7α-hydroxylase. Regulation in vivo at the protein and mRNA level in response to mevalonate, diurnal rhythm, and bile acid feedback. *J. Biol. Chem.* **265:** 15090–15095.
14. Ness, G. C., Z. Zhao, and R. K. Keller. 1994. Effect of squalene synthase inhibition on the expression of hepatic cholesterol biosynthetic enzymes, LDL receptor, and cholesterol 7α-hydroxylase. *Arch. Biochem. Biophys.* **311:** 277–285.
15. Ramirez, M. I., D. Karaoglu, D. Haro, C. Barillas, R. Bashirzadeh, and G. Gil. 1994. Cholesterol and bile acids regulated cholesterol 7α-hydroxylase expression at the transcriptional level in culture and in transgenic mice. *Mol. Cell. Biol.* **14:** 2809–2821.
16. Björkhem, I., G. Eggertsen, and U. Andersson. 1991. On the mechanism of stimulation of cholesterol 7α-hydroxylase by dietary cholesterol. *Biochim. Biophys. Acta.* **1085:** 329–335.
17. Baxter, A., B. J. Fitzgerald, J. L. Hutson, A. D. McCarthy, J. M. Motteram, B. C. Ross, M. Sapra, M. A. Snowden, N. S. Watson, R. J. Williams, and C. Wright. 1992. Squalestatin 1, a potent inhibitor of squalene synthase, which lowers serum cholesterol in vivo. *J. Biol. Chem.* **267:** 11705–11708.
18. Bissell, D. M., and P. S. Guzelian. 1980. Phenotypic stability of adult rat hepatocytes in primary monolayer cultures.

Ann. NY Acad. Sci. **349:** 85–98.
19. 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 7α-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.
20. Beg, Z. H., and J. A. Stonik. 1982. Reversible inactivation of 3-hydroxy-3-methylglutaryl coenzyme A reductase: reductase kinase and mevalonate kinase are separate enzymes. *Biochem. Biophys. Res. Commun.* **108:** 559–566.
21. Duckworth, P. F., Z. R. Vlahcevic, E. J. Studer, E. C. Gurley, D. M. Heuman, Z. H. Beg, and P. B. Hylemon. 1991. Regulation of 3-hydroxy-3-methylglutaryl coenzyme a reductase activity, protein mass and mRNA levels by hydrophobic bile acids in the rat. *J. Biol. Chem.* **266:** 9413–9418.
22. Li, Y. C., D. P. Wang, and J. Y. L. Chiang. 1990. Regulation of cholesterol 7α-hydroxylase in the liver. Cloning, sequencing, and regulation of cholesterol 7α-hydroxylase mRNA. *J. Biol. Chem.* **265:** 12012–12019.
23. Day, R., R. L. Gebhard, H. L. Schwartz, K. A. Strait, W. C. Duane, B. G. Stone, and J. H. Oppenheimer. 1989. Time course of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and messenger ribonucleic acid, biliary lipid secretion, and hepatic cholesterol content in methimazole-treated hypothyroid and hypophysectomized rats after triiodothyronine administration: possible linkage of cholesterol synthesis to biliary secretion. *Endocrinology,* **125:** 459–468.
24. Danielsson, P. E., S. Forss-Petter, M. A. Brow, L. Calavetta, J. Douglass, R. J. Milner, and J. G. Sutcliffe. 1988. p1B15: a cDNA clone of the rat mRNA encoding cyclophilin. *DNA.* **4:** 261–267.
25. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
26. 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.
27. Hylemon, P. B., E. C. Gurley, W. M. Kubaska, T. R. Whitehead, P. S. Guzelian, and Z. R. Vlahcevic. 1985. Suitability of primary monolayer cultures of adult rat hepatocytes for studies of cholesterol and bile acid metabolism. *J. Biol. Chem.* **260:** 1015–1019.
28. Beg, Z. H., J. A. Stonik, and H. B. Brewer. 1984. In vivo modulation of rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase, reductase kinase and reductase kinase kinase by mevalonolactone. *Proc. Natl. Acad. Sci. USA.* **81:** 7293–7297.
29. Kandutsch, A. A., H. W. Chen, and H. J. Heiniger. 1978. Biological activity of some oxygenated sterols. *Science.* **201:** 498–501.
30. Takagi, K., J. G. Alvarez, M. F. Favata, J. M. Trzaskos, and J. F. Strauss III. 1989. Control of low density lipoprotein receptor gene promoter activity. Ketoconazole inhibits serum lipoprotein, but not oxysterol suppression of gene transcription. *J. Biol. Chem.* **264:** 12352–12357.
31. Goldstein, J. L., and M. S. Brown. 1990. Regulation of the mevalonate pathway. *Nature.* **343:** 425–430.
32. Gupta, A., R. C. Sexton, and H. Rudney. 1986. Modulation of regulatory oxysterol formation and low-density lipoprotein suppression of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and activity by ketoconazole. A role for cytochrome P-450 in the regulation of HMG-CoA reductase in rat intestinal epithelial cells. *J. Biol. Chem.* **261:** 8348–8356.
33. Dawson, M. J., J. E. Farthing, P. S. Marshall, R. F. Middleton, M. J. O'Neill, A. Shuttleworth, C. Stylli, R. M. Tait, P. M. Taylor, H. G. Wildman, A. D. Buss, D. Langley, and M. V. Hayes. 1992. The squalestatins, novel inhibitors of squalene synthase produced by a species of Phoma. I. Taxonomy, fermentation, isolation, physico-chemical properties and biological activity. *J. Antibiot.* **45:** 639–647.
34. Noshiro, M., M. Nishimoto, K. Morohashi, and K. Okuda. 1989. Molecular cloning of cDNA for cholesterol 7α-hydroxylase from rat liver microsomes. Nucleotide sequence and expression. *FEBS Lett.* **257:** 97–100.
35. Nishimoto, M., O. Gotoh, K. Okuda, and M. Noshiro. 1991. Structural analysis of the gene encoding rat cholesterol 7α-hydroxylase, the key enzyme for bile acid biosynthesis. *J. Biol. Chem.* **266:** 6467–6471.
36. Crestani, M., G. Galli, and J. Y. L. Chiang. 1993. Genomic cloning, sequencing, and analysis of the hamster cholesterol 7α-hydroxylase gene (CYP7). *Arch. Biochem. Biophys.* **306:** 451–460.
37. Molowa, D. T., W. S. Chen, G. M. Cimis, and C. P. Tan. 1992. Transcriptional regulation of the human cholesterol 7α-hydroxylase gene. *Biochemistry.* **31:** 2539–2544.
38. Lavery, D. J., and U. Schibler. 1993. Circadian transcription of the cholesterol 7α-hydroxylase gene may involve the liver-enriched bZIP protein DBP. *Genes and Dev.* **7:** 1871–1884.
39. Simonet, W. S., and G. C. Ness. 1988. Transcriptional and posttranscriptional regulation of rat hepatic 3-hydroxy-3-methylglutaryl-coenzyme A reductase by thyroid hormones. *J. Biol. Chem.* **263:** 12448–12453.
40. Shefer, S., L. B. Nguyen, G. Salen, G. C. Ness, I. R. Chowdhary, S. Lerner, A. K. Batta, and G. S. Tint. 1992. Differing effects of cholesterol and taurocholate on steady-state hepatic HMG-CoA reductase and cholesterol 7α-hydroxylase activities and mRNA levels in the rat. *J. Lipid Res.* **33:** 1193–1200.
41. Ness, G. C., R. K. Keller, and L. C. Pendleton. 1991. Feedback regulation of hepatic 3-hydroxy-3-methylglutaryl-CoA reductase activity by dietary cholesterol is not due to altered mRNA levels. *J. Biol. Chem.* **266:** 14854–14857.
42. Panini, S. R., R. Schnitzer-Polokoff, T. A. Spencer, and M. Sinensky. 1989. Sterol-independent regulation of 3-hydroxy-3-methylglutaryl-CoA reductase by mevalonate in Chinese hamster ovary cells: magnitude and specificity. *J. Biol. Chem.* **264:** 11044–11052.
43. Liscum, L., K. L. Luskey, D. J. Chin, Y. K. Ho, J. L. Goldstein, and M. S. Brown. 1983. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase and its mRNA in rat liver as studied with a monoclonal antibody and a cDNA probe. *J. Biol. Chem.* **258:** 8450–8455.
44. Sudjana-Sugiaman, E., G. Eggertsen, and I. Björkhem. 1994. Stimulation of HMG-CoA reductase as a consequence of phenobarbital-induced primary stimulation of cholesterol 7α-hydroxylase in rat liver. *J. Lipid Res.* **35:** 319–327.