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Activation of the Silent Endogenous Cholesterol-7-Alpha-Hydroxylase Gene in Rat Hepatoma Cells: a New Complementation Group Having Resistance to 25-Hydroxycholesterol

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The oxysterol 25-hydroxycholesterol acts both as a regulatory sterol determining the expression of genes governed by sterol regulatory elements and as a substrate for 7-alpha-hydroxylase, the first and rate-limiting enzyme in the bile acid synthetic pathway. Most wild-type nonhepatic cells are killed by the cytotoxic action of 25-hydroxycholesterol. In contrast, liver cells, which express 7-alpha-hydroxylase activity, are resistant to killing by 25-hydroxycholesterol. We examined the possibility that selection for resistance to 25-hydroxycholesterol might lead to the derivation of a cell line expressing 7-alpha-hydroxylase. A rat hepatoma cell line (7-alpha-hydroxylase minus) was transfected with human DNA and screened for resistance to 25-hydroxycholesterol. Although parental hepatoma cells were all killed within a week, a 25-hydroxycholesterol-resistant cell line (L35 cells) which showed stable expression of 7-alpha-hydroxylase activity and mRNA was obtained. These cells exhibited normal inhibition of cholesterol biosynthesis by 25-hydroxycholesterol. Blocking 7-alpha-hydroxylase activity with ketoconazole also blocked the resistance of L35 cells to 25-hydroxycholesterol. Isolation of microsomes from these cells showed levels of 7-alpha-hydroxylase activity (22.9 pmol/min/mg of protein) that were comparable to the activity (33.2 pmol/min/mg) of microsomes isolated from the livers of rats killed during the high point of the diurnal cycle. Parental cells had no detectable activity. These data show a new complementation group for 25-hydroxycholesterol resistance: expression of 7-alpha-hydroxylase. Dexamethasone increased both the activity and the cellular content of mRNA coding for 7-alpha-hydroxylase. Since dactinomycin blocked the ability of dexamethasone to induce mRNA, active transcription is required. Southern analysis of genomic DNA showed that L35 cells contain the rat (endogenous) gene but not the human gene. Furthermore, the RNA expressed by L35 cells is similar in size to rat RNA and is distinct from the human form of 7-alpha-hydroxylase. The combined data indicate that L35 cells are resistant to 25-hydroxycholesterol because they express 7-alpha-hydroxylase. The mechanism responsible involves activation of the endogenous (silent) gene of the parental rat hepatoma cell.

The conversion of cholesterol to bile acids and their subsequent excretion via the biliary system is the major pathway through which cholesterol is removed from the body and cholesterol homeostasis is maintained (34). Bile acid synthesis is regulated by the activity of hepatic 7-alpha-hydroxylase (24) and the availability of substrate to the enzyme, which is located in the cholesterol-poor endoplasmic reticulum (36). Recent studies by Jelinek et al. (19) show that dietary cholesterol increases expression of 7-alpha-hydroxylase (EC 1.14.13.17) in rats. There are several substrates and products for the 7-alpha-hydroxylase reaction. In addition to cholesterol, both 25- and 26-hydroxycholesterol can be efficiently hydroxylated in the 7 position and converted to bile acids (2, 38).

The initial goal of this research was to use DNA-mediated gene transfer to obtain a rat cell line that would express high levels of the human gene for 7-alpha-hydroxylase in a stable manner. This cell line could then be used to isolate the human gene for 7-alpha-hydroxylase (by identification of the transfected [human] gene with the species-specific Alu repetitive sequence). For this technique to be successful, we needed an effective screen to isolate cells that expressed 7-alpha-hydroxylase. On the basis of the following rationale, we developed a metabolic selection for cells expressing 7-alpha-hydroxylase. Low concentrations (0.25 μg/ml) of 25-hydroxycholesterol kill wild-type nonhepatic cells (6, 7, 23, 33). In marked contrast, cultured rat hepatocytes are relatively insensitive to 25-hydroxycholesterol (50 μg/ml) (14). Since it has been shown that humans can metabolize 25-hydroxycholesterol to bile acids (38), we hypothesized that cells that express 7-alpha-hydroxylase activity will be resistant to 25-hydroxycholesterol-induced cytotoxicity.

Using this screening procedure, we obtained a stable line of rat hepatoma cells that are resistant to 25-hydroxycholesterol and express 7-alpha-hydroxylase at levels similar to those expressed by rats in vivo. However, unexpectedly, the 7-alpha-hydroxylase expressed by these cells is of endogenous (rat), not exogenous (human), origins. Using these cells, we show that expression of 7-alpha-hydroxylase is highly regulatable via changes in transcription.

MATERIALS AND METHODS

Materials. All reagents, culture supplies, and additives were obtained from suppliers, as described elsewhere (12, 36). Cultured cells (HepG2 and H35) were obtained from frozen stocks (27). Reagents for gas chromatography (GC)-mass spectroscopy analysis (7-alpha-hydroxylase) were obtained and used as described elsewhere (36). The cDNAs for 7-alpha-hydroxylase (a gift from Diane Jelinek and David

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Russell) (19) and β-actin (16) have been described elsewhere in detail.

Cell culture. Rat hepatoma cell line H35 (lacking 7-alpha-hydroxylase activity) (28) was adapted to growth in Dulbecco's modified Eagle's medium (DMEM) containing 0.5% fetal calf serum (FCS), 0.1 μg of insulin per ml, and 1% Nutridoma (Boehringer Mannheim Biochemicals, Indianapolis) (low-serum medium). The adapted cells (UC1) were used for transfection experiments.

Transfection and screening of UC1 cells. Human genomic DNA was isolated by guanidinium isothiocynate (4 M) extraction of HepG2 cells on culture plates. The cell lysate was collected and layered over a 4-ml pad of 5.7 M CsCl. The DNA was separated from other components by centrifugation at 40,000 rpm in an SW41 rotor for 6 h at 18°C. The viscous DNA was collected, digested with proteinase K and RNase A, and extracted with phenol-chloroform before dialysis. Human genomic DNA (size, about 50 kb; 20 μg) obtained from HepG2 cells was complexed with lipofectin (Bethesda Research Laboratories) and added to UC1 cells (5×10^5 cells per 60-mm dish) by using a procedure described by the manufacturer. After 18 h, the medium was changed to DMEM containing 0.5% FCS, 1% Nutridoma, and 25-hydroxycholesterol (0.25 μg/ml). After 14 days, cells remaining viable on the dish were replated and rescreened. After two rounds of screening, the cells were grown out in several dishes and characterized (see below).

7-Alpha-hydroxylase activity and mRNA from cultured cells. HepG2, UC1, and L35 cells were grown in DMEM containing 0.5% FCS, 0.1 mg of insulin per ml, and 1% Nutridoma. The cells were trypsinized, plated at 1.2×10^6 to 1.8×10^6/ml, and cultured for 3 days in DMEM with 8% FCS and insulin but with no Nutridoma. The medium was then changed to serum-free DMEM without any additions, and the cells were incubated for 48 h. In some of the experiments, dexamethasone dissolved in ethanol was added (controls received ethanol only). The medium was drawn off, and the cells were washed in phosphate-buffered saline (PBS) and either harvested with a rubber policeman or treated with buffer for RNA isolation (see below). The cells were centrifuged at 1,000 rpm ($100 \times g$) at 4°C for 5 min; suspended in 10 ml of a buffer containing 40 mM Tris-HCl, 1 mM EDTA, 5 mM dithiothreitol, 50 mM KCl, 50 mM KF, and 300 mM sucrose (pH 7.4); and subjected to two cycles of N_2 cavitation on ice at 500 lb/in^2 for 20 min as described previously (4). The homogenate was then centrifuged at $20,000 \times g$ for 20 min at 4°C. The supernatant was centrifuged in a Beckman TL-100 centrifuge at about $350,000 \times g$ (100,000 rpm) at 4°C for 15 min. The pellet was suspended in the same buffer without sucrose and frozen at −70°C until use.

The activity of 7-alpha-hydroxylase was determined by using a method described elsewhere in detail (36). The amount of 7-alpha-hydroxycholesterol produced was quantitated by isotope dilution GC-mass spectroscopy using [7-beta-^{2}H]7-alpha-hydroxycholesterol as internal standard.

Formation of [^{14}C]7-alpha-hydroxycholesterol in cultured cells. Cells grown as described above were incubated with [^{14}C]cholesterol (1 μCi/3 ml dissolved in 15 μl of dimethyl sulfoxide) for 24 h. The medium was collected, and the cells were washed two times in PBS, scraped, and centrifuged at 1,000 rpm ($100 \times g$) for 5 min at 4°C. The cells were suspended in 1 ml of distilled water. Medium and cells were extracted with chloroform-methanol (2:1) and reextracted with chloroform as previously described (36). The samples were chromatographed in a thin-layer chromatography system containing benzene-ethylacetate (1:4) with cholesterol, 7-alpha-hydroxycholesterol, and 7-beta-hydroxycholesterol as standards. The silica gel containing cholesterol, 7-alpha-hydroxycholesterol, 7-beta-hydroxycholesterol, and more-polar materials (identified by the migration of the standards) was scraped into counting vials, and the radioactivity was assayed by β-scintillation counting.

Isolation of RNA and Northern (RNA) blots. Total RNA was isolated by the acid guanidinium isothiocynate phenol-chloroform method of Chomczynski and Sacchi (9). L35 cells were plated at 1.8×10^6 cells per 60-mm dish and treated as indicated in the figures and tables. The cells were washed in PBS and lysed by the addition of 0.5 ml of 4 M guanidinium isothiocynate to the culture plate, and the lysate was transferred to a sterile 1.5-ml microfuge tube before sodium acetate and phenol-chloroform were added. After centrifugation, the RNA was precipitated by the addition of an equal volume of isopropanol, dissolved in guanidinium isothiocynate, reprecipitated with isopropanol, dissolved in water, precipitated with 2 volumes of ethanol and 0.2 M sodium chloride, and finally dissolved in 100 μl of diethylpyrocarbonate-treated water. RNA concentrations were determined by A_{260}. Poly(A) RNA was obtained by oligo(dT)-cellulose column chromatography as described elsewhere (1).

RNA was electrophoresed on a 1% agarose–2.2 M formaldehyde gel for 2 h at 45 V. The RNA was stained with ethidium bromide to visualize the rRNA and then transferred overnight to a Biodyne nylon membrane (ICN) in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), baked for 2 h in vacuo, and prehybridized for 4 h at 42°C in 50% formamide–5× SSPE (1× SSPE is 0.15 M NaCl, 0.01 M $NaPO_4$, 0.001 M EDTA 5× Denhardt's solution–1% sodium dodecyl sulfate (SDS). Hybridization of the RNA to cDNA probes was for 20 h in the solution described above. Probes were radiolabeled by nick translation (BRL) to a specific activity of 10^8 cpm/μg of DNA. Approximately 10^7 cpm was added per hybridization. Filters were washed at room temperature four times in 2× SSC–0.5% SDS for 5 min (each wash) and twice at 50°C in 0.1× SSC–0.5% SDS for 15 min (each wash). Filters were air dried before being exposed to film.

Isolation of DNA and Southern blots. High-molecular-weight genomic DNA was isolated from cultured cells and rat liver for use in Southern blots (35). Cells were trypsinized, scraped, pelleted by centrifugation, rinsed once in cold PBS, and incubated in digestion buffer (100 mM NaCl, 10 mM Tris [pH 8], 25 mM EDTA [pH 8], 0.5% SDS, 0.1 mg of proteinase K per ml). DNA was isolated from rat liver frozen in liquid nitrogen by grinding 1 g of liver tissue with a mortar and pestle and suspending it in 12 ml of digestion buffer. Cells or tissue was incubated for 18 h at 50°C in the digestion buffer. DNA was then extracted with an equal volume of phenol-chloroform-isoamyl alcohol (24:24:1), and the phases were separated by centrifugation for 10 min at $10,000 \times g$. The aqueous phase was removed to a new tube, and the DNA was precipitated by the addition of 0.5 volume of 7.5 M ammonium acetate and 2 volumes of cold ethanol. The DNA was spooled with a glass rod and dialyzed for 24 h with TE (0.01 M Tris-Cl, pH 7.6, 0.001 M EDTA).

For Southern blots, 10 μg of DNA was digested overnight with the indicated restriction enzymes (see Fig. 6) in the buffers supplied by the manufacturers. The DNA was separated on a 0.8% agarose gel using TEA buffer (0.04 M Tris-acetate, 0.001 M EDTA) at 30 V for 18 h. The DNA was stained with ethidium bromide to visualize the molecular weight markers. The DNA was denatured with 0.5 M NaOH

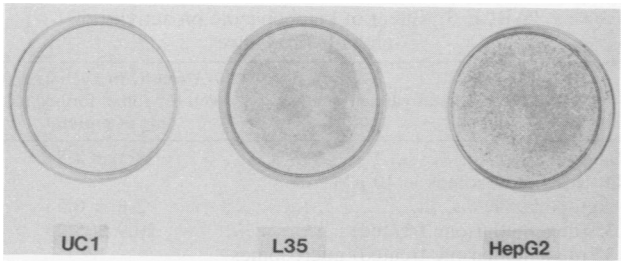

FIG. 1. Ability of 25-hydroxycholesterol to kill various hepatoma cells. The indicated hepatoma cells (UC1, L35, and HepG2) were cultured for 3 days in the presence of 25-hydroxycholesterol (0.25 μg/ml). Cells were then washed three times with PBS and stained.

for 30 min with gentle shaking and neutralized by 0.5 M Tris (pH 7.5)–1.5 M NaCl. The DNA was transferred to a Biodyne nylon membrane by capillary action in 20× SSC overnight, baked for 2 h in vacuo, and prehybridized, hybridized, and washed as for the Northern blots.

RESULTS

DNA-mediated gene transfer and screening for 25-hydroxycholesterol resistance. Previous studies by others have shown that rat H35 cells lack 7-alpha-hydroxylase activity but have the subsequent steps in the bile acid synthetic pathway (i.e., while H35 cells do not synthesize bile acids from cholesterol, incubation with 7-alpha-hydroxycholesterol produces bile acids [28]). In order to screen for resistance to 25-hydroxycholesterol, we needed a cell line that would replicate in medium containing low levels of serum. H35 cells were adapted to grow in medium that contained such levels. The low-serum-adapted cells (designated UC1) were able to replicate in culture medium containing 0.5% FCS and 1% Nutridoma. These cells were used for transformation and selection for 25-hydroxycholesterol resistance. Preliminary experiments showed that when cultured in DMEM containing 0.5% FCS, essentially all UC1 cells were killed by 25-hydroxycholesterol (0.25 μg/ml) (Fig. 1). In marked contrast, when HepG2 cells were cultured in the same medium containing 25-hydroxycholesterol, there was no significant killing (Fig. 1). Since HepG2 cells convert cholesterol to bile acids and express low levels of functional 7-alpha-hydroxylase (see below), we hypothesized that one mechanism responsible for resistance to 25-hydroxycholesterol (0.25 μg/ml), might be its metabolism (inactivation) by 7-alpha-hydroxylase.

One stable cell line derived from UC1 cells (designated L35) was resistant to 25-hydroxycholesterol (Fig. 1) and was found to express relatively high activity of 7-alpha-hydroxylase (see below). This cell line has been cultured in the absence of 25-hydroxycholesterol for 2 years with no detectable reversion to 25-hydroxycholesterol sensitivity (data not shown), indicating a stable phenotype.

25-Hydroxycholesterol inhibits cholesterol biosynthesis in L35 cells. A common mechanism for resistance to 25-hydroxycholesterol is loss of the down-regulation of cholesterol biosynthesis by 25-hydroxycholesterol (6, 7, 23, 33). We examined the ability of both low-density lipoproteins (LDL) and 25-hydroxycholesterol to inhibit the formation of [^{14}C]cholesterol from [^{14}C]acetate in 25-hydroxycholesterol-resistant L35 cells (Table 1). Both LDL and 25-hydroxycholesterol significantly inhibited the incorporation of [^{14}C]acetate into cholesterol. Furthermore, both LDL and 25-hydroxycholesterol more than doubled the percentage of [^{14}C]cholesterol that was esterified (Table 1), indicating that L35 cells exhibit feedback regulation of cholesterol synthesis and stimulation of cholesterol esterification (14). Similar results were obtained when parental UC1 cells were used (data not shown). The combined data show that loss of regulation of cholesterol synthesis cannot account for the resistance of L35 cells to 25-hydroxycholesterol.

TABLE 1. Effect of LDL and 25-hydroxycholesterol on [^{14}C]acetate incorporation by L35 cells[a]

Treatment	[^{14}C]acetate incorporation (cpm/mg of cell protein [%])	
	Cholesterol	Cholesterol Esters
None (control)	23,119 ± 2,191	3,828 ± 343 (14.2)
LDL	10,445 ± 787	6,080 ± 204 (36.8)
25-Hydroxycholesterol	7,015 ± 955	3,035 ± 166 (30.2)

[a] Cells (1.5 × 10^6/60-mm dish) were incubated with either LDL (100 μg/ml) or 25-hydroxycholesterol (5 μg/ml). After 18 h, cells were pulsed for 1.5 h with medium containing [^{14}C]acetate (1 μCi/ml). The cells were harvested, and lipids were extracted and separated by thin-layer chromatography. Bands corresponding to cholesterol and cholesterol esters were scraped and counted. Values shown are means ± standard deviations from three experiments.

L35 Cells express 7-alpha-hydroxylase. To determine if 25-hydroxycholesterol-resistant L35 cells express 7-alpha-hydroxylase, cells were harvested and disrupted by nitrogen cavitation, and microsomes were isolated by ultracentrifugation. The activity of 7-alpha-hydroxylase was determined by using a GC-mass spectrometer isotope dilution quantitation of 7-alpha-hydroxycholesterol produced in vitro by individual microsomes. This method quantitates the amount of product formed (i.e., the absolute activity of 7-alpha-hydroxylase can be compared among the different cell types) (36). Microsomes were isolated from rat liver, isolated rat hepatocytes, and UC1, HepG2, and L35 cells, and the 7-alpha-hydroxylase activity was determined.

When incubated with serum-free DMEM containing dexamethasone, microsomes prepared from L35 cells displayed 7-alpha-hydroxylase activity comparable to that of liver microsomes isolated from rats during the highest point of the diurnal variation (Table 2). In marked contrast, L35 cells

TABLE 2. Activity of 7-alpha-hydroxylase by isolated microsomal membranes[a]

Type of cell	7-Alpha-hydroxylase activity (pmol formed/min/mg of microsomal protein)	n^b
Rat liver	33.2 ± 3.7	3
HepG2 (dexamethasone treated)	3.3 ± 4.4	6
UC1 (no dexamethasone)	<0.2	2
UC1 (dexamethasone treated)	<0.2	4
L35 (dexamethasone treated)	22.9 ± 7.3	3
L35 (no dexamethasone)	<0.2	2
Isolated rat hepatocytes	0.34 ± 0.77	6

[a] Rat liver microsomes were isolated at mid-dark cycle. Rat hepatocytes were harvested 0 to 42 h after perfusion and isolation of the cells. HepG2, UC1, and L35 cells were grown as described in Materials and Methods. The formation of 7-alpha-hydroxylase was quantitated by GC-mass spectrometry. [^{2}H]7-alpha-hydroxycholesterol (1 μg) was added, and the cells were extracted.

[b] n, Number of experiments.

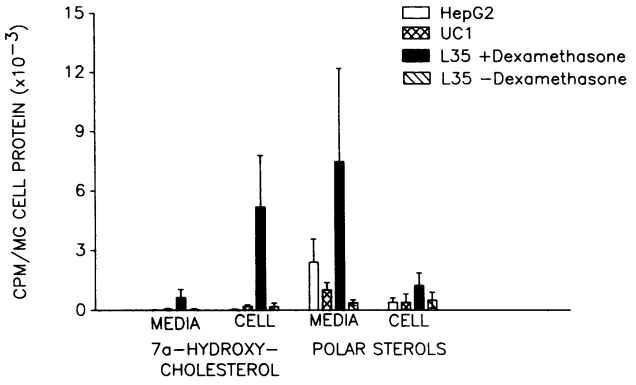

FIG. 2. Quantitation of metabolism of [^{14}C]cholesterol by different cells. Cultures of the indicated cells (1.5×10^6 cells per 60-mm dish) were incubated with [^{14}C]cholesterol (1 μCi/3 ml of medium) as described in Materials and Methods and the footnotes to Table 2. After 24 h, the cells and medium were harvested, extracted, and separated by thin-layer chromatography into 7-alpha-hydroxycholesterol and more-polar metabolites (hydroxy derivatives and bile acids) as described in Materials and Methods. Radioactivity migrated as shown. The means ± standard deviations of three to six individual experiments are shown.

cultured without dexamethasone showed no significant 7-alpha-hydroxylase activity. Parental UC1 cells cultured in the presence and the absence of dexamethasone displayed no detectable 7-alpha-hydroxylase activity. Compared with the activity of 7-alpha-hydroxylase in microsomes isolated from L35 cells cultured with dexamethasone, the activities in microsomes from HepG2 and rat hepatocytes were low (Table 2). We conclude that, compared with cultured rat hepatocytes and HepG2 cells, L35 cells express very high levels of 7-alpha-hydroxylase. Moreover, since both cultured rat hepatocytes (12, 18) and HepG2 cells (15) synthesize bile acids from cholesterol and are resistant to 25-hydroxycholesterol, it is likely that low levels of 7-alpha-hydroxylase activity are sufficient for these properties.

To prove that the compound isolated from microsomes from L35 cells is in fact 7-alpha-hydroxycholesterol, a sample was identified by using mass spectroscopy fragmentation patterns. The results show that the putative 7-alpha-hydroxycholesterol isolated from L35 cells displayed a GC retention time and a mass spectroscopy fragmentation pattern identical to those observed when an authentic sample of 7-alpha-hydroxycholesterol was used. Both the authentic sample of 7-alpha-hydroxycholesterol and the unknown isolated from L35 cells displayed a molecular ion at 546 (3- and 7-trimethyl silyl derivatives of OH) and a 456 peak (loss of one 0-trimethyl silyl). These data show that L35 cells produce 7-alpha-hydroxycholesterol.

We also examined the metabolism of exogenous [^{14}C]cholesterol. L35 cells incubated with dexamethasone and [^{14}C]cholesterol accumulated about 15 times more ^{14}C-labeled 7-alpha-hydroxycholesterol than did HepG2 cells or UC1 cells (Fig. 2). Without dexamethasone, L35 cells expressed the same inability to accumulate ^{14}C-labeled 7-alpha-hydroxycholesterol as did parental UC1 cells (Fig. 2). Dexamethasone had no effect on the ability of UC1 cells to take up [^{14}C]cholesterol (data not shown). In contrast to the exclusive ability of L35 cells to accumulate ^{14}C-labeled 7-alpha-hydroxycholesterol, all cells examined did have the capacity to produce polar metabolites from the ^{14}C-labeled cholesterol (Fig. 2). There were striking differences in the

TABLE 3. Effect of ketoconazole on activity of 7-alpha-hydroxylase[a]

Type of cell and treatment	7-Alpha-hydroxycholesterol activity (pmol formed/min/mg of protein)
Rat liver	28.1 ± 4.8
Rat liver microsomes + 10 μM ketoconazole	2.6 ± 0.5
L35 (dexamethasone treated)	20.9 ± 5.2
L35 (dexamethasone-treated) microsomes + 10 μM ketoconazole	0.0 ± 2.8

[a] L35 cells were incubated with dexamethasone (15 nM), the microsomes were isolated and incubated, and 7-alpha-hydroxycholesterol activity was determined as described in Table 2, footnote *a*. Ketoconazole (10 μM) was added to the microsomes in 2 μl of ethanol, and a similar amount of ethanol was added to the control incubations. Data are given as means ± standard deviations for triplicate determinations.

abilities of the radioactive metabolites to be secreted. Essentially all of the ^{14}C-labeled 7-alpha-hydroxycholesterol was isolated in the cells, and almost none was secreted into the culture medium. In contrast, the majority of the more-polar metabolites were isolated in the medium (Fig. 2). These data suggest that while all of the cell types examined have the ability to metabolize cholesterol to polar compounds, only L35 cells accumulate intracellular 7-alpha-hydroxycholesterol in significant amounts.

Ketoconazole inhibits 7-alpha-hydroxylase and blocks the resistance of L35 cells to 25-hydroxycholesterol. Ketoconazole has been shown to inhibit rat liver 7-alpha-hydroxylase (29). We investigated whether ketoconazole would similarly inhibit 7-alpha-hydroxylase expressed by L35 cells and, if so, whether this inhibition would cause L35 cells to revert to 25-hydroxycholesterol sensitivity. Adding ketoconazole directly to microsomes isolated from rat liver and from L35 cells caused a complete inhibition of 7-alpha-hydroxylase

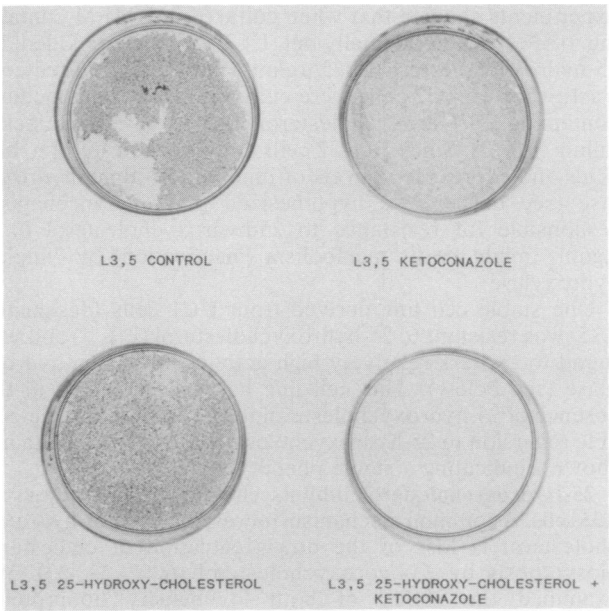

FIG. 3. Effect of ketoconazole on the sensitivity of L35 cells to 25-hydroxycholesterol. L35 cells were incubated for 3 days with and without 25-hydroxycholesterol (0.25 μg/ml) and 5 μM ketoconazole. Cells were then washed and stained.

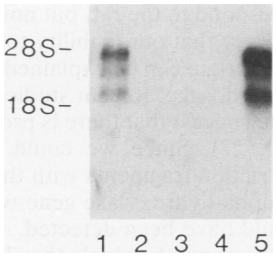

FIG. 4. Northern blot of poly(A) RNA isolated from cells and liver. The cells were cultured as described in Table 2 and Materials and Methods. Poly(A) RNA was isolated by oligo(dT)-cellulose chromatography. Equal amounts of RNA (2 μg) were applied to each well of an agarose gel and subjected to electrophoresis. The gel was blotted onto nitrocellulose and then incubated with ^{32}P-labeled cDNA probe for rat 7-alpha-hydroxylase. The washed nitrocellulose was autoradiographed for 2 days. Lane 1, L35 cells grown in medium containing dexamethasone (10 μM); lane 2, L35 cells grown without dexamethasone; lane 3, UC1 cells grown with dexamethasone (10 μM); lane 4, UC1 cells grown without dexamethasone; lane 5, RNA from the liver of a rat fed cholestyramine.

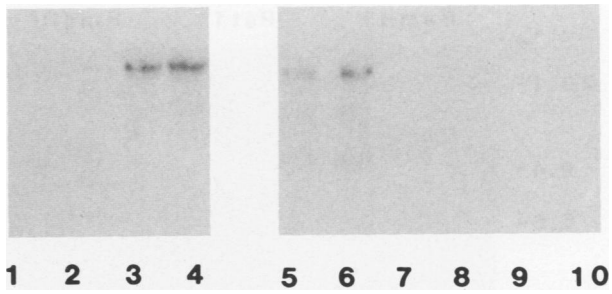

FIG. 5. Effect of dexamethasone and dactinomycin on 7-alpha-hydroxylase mRNA. L35 cells were incubated in serum-free DMEM with and without dexamethasone (10 μM) or dactinomycin (5 μg/ml). The RNA (total isolated) and equal amounts of RNA (10 μg) were electrophoresed, blotted, and hybridized with a ^{32}P-labeled cDNA probe for rat 7-alpha-hydroxylase. Lanes 1 and 2, grown without dexamethasone; lanes 3 and 4, incubated for 18 h with medium containing dexamethasone (10 μM); lane 5 and 6, incubated for 3 h with dexamethasone (10 μM); lanes 7 and 8, incubated with medium containing dactinomycin (5 μg/ml for 3 h); lanes 9 and 10, incubated with medium containing dexamethasone (10 μM) and dactinomycin (5 μg/ml) for 3 h.

(Table 3). Moreover, adding ketoconazole to the culture medium caused L35 cells to be rapidly killed by 25-hydroxycholesterol (Fig. 3). In contrast, under the same culture conditions, L35 cells were unaffected by either ketoconazole or 25-hydroxycholesterol alone. These data are consistent with the hypothesis that expression of 7-alpha-hydroxylase is responsible for the resistance of L35 cells to 25-hydroxycholesterol.

Dexamethasone induces expression of 7-alpha-hydroxylase. Using a cDNA which codes for rat liver 7-alpha-hydroxylase (19), we examined the expression of mRNA by L35 cells. Expression of mRNA for 7-alpha-hydroxylase (Fig. 4) paralleled the expression of activity (Table 2). L35 cells incubated with dexamethasone express three major molecular weight forms of mRNA that hybridize with the ^{32}P-labeled cDNA probe (Fig. 4, lane 1). The different forms of mRNA expressed by L35 cells are identical to the forms expressed by rat liver (lane 5). The multiple forms of rat liver 7-alpha-hydroxylase mRNA have been previously described in detail and are probably due to different polyadenylation sites on the primary transcript (19, 20). In marked contrast to the abundance of 7-alpha-hydroxylase mRNA expressed by L35 cells cultured in the presence of dexamethasone, almost no 7-alpha-hydroxylase mRNA was detected in L35 cultured without dexamethasone (lane 2) or in parental UC1 cells cultured either in the presence or absence of dexamethasone (lane 3 and 4). To ensure that similar amounts of mRNA were loaded in each lane, the blot was washed and reprobed with a beta-actin probe (16). All lanes showed similar amounts of beta-actin (data not shown), indicating that differences in hybridization with the 7-alpha-hydroxylase cDNA reflect relative abundance.

Dexamethasone regulates abundance of mRNA coding for 7-alpha-hydroxylase in L35 cells. We investigated whether transcription is required for dexamethasone induction of 7-alpha-hydroxylase mRNA. In this experiment, total RNA was extracted, and equal amounts were separated by agarose electrophoresis blotted onto nylon membranes and probed with ^{32}P-labeled cDNAs coding for beta-actin and rat 7-alpha-hydroxylase. In all lanes the amount of beta-actin RNA detected was the same (data not shown). L35 cells cultured without dexamethasone showed almost no detectable mRNA for 7-alpha-hydroxylase (Fig. 5, lanes 1 and 2). In marked contrast, after L35 cells were incubated with dexamethasone for 18 h, there was a marked elevation in 7-alpha-hydroxylase mRNA levels. The effect of dexamethasone could be easily observed after only 3 h (lanes 5 and 6), suggesting a rapidly mediated effect. Moreover, dactinomycin completely blocked the ability of dexamethasone to increase 7-alpha-hydroxylase mRNA levels (lanes 9 and 10). These data suggest that dexamethasone increases 7-alpha-hydroxylase mRNA levels via a mechanism requiring transcription.

Endogenous 7-alpha-hydroxylase gene is expressed by L35 cells. We used Southern restriction analysis of genomic DNA obtained from rats, humans, and L35 cells to investigate whether transfection with human DNA resulted in stable integration of the human 7-alpha-hydroxylase gene in L35 cells (Fig. 6). Clearly, the restriction pattern obtained from rats (Fig. 6, lane 1) was the same as that produced by L35 cells (lane 2) and parental UC1 cells (lane 3) but was distinctly different from that obtained with human DNA (lane 4). These data show that (i) there are no major alterations in the 7-alpha-hydroxylase gene contained in either L35 or UC1 cells and therefore its expression by L35 cells or its silence by UC1 cells cannot be explained by such changes, (ii) the human 7-alpha-hydroxylase gene can be differentiated from the rat gene, and (iii) the only 7-alpha-hydroxylase gene detected in L35 cells is of rat (not human) origin.

Recent studies show that the major form of 7-alpha-hydroxylase mRNA expressed by humans is smaller (i.e., migrates faster) than the major form expressed by rats (Fig. 4) (25). We examined the origin of the 7-alpha-hydroxylase expressed by L35 cells by comparing its mRNA to that of rat liver and human liver (Fig. 7). Clearly, the mRNA species produced by rat liver (lane 1) and L35 cells (lane 2) are identical to each other and distinct from the mRNA obtained from human liver (lane 3), which migrates faster, as described elsewhere (26). These data clearly show that the 7-alpha-hydroxylase mRNA expressed by L35 cells is of endogenous (rat) origin.

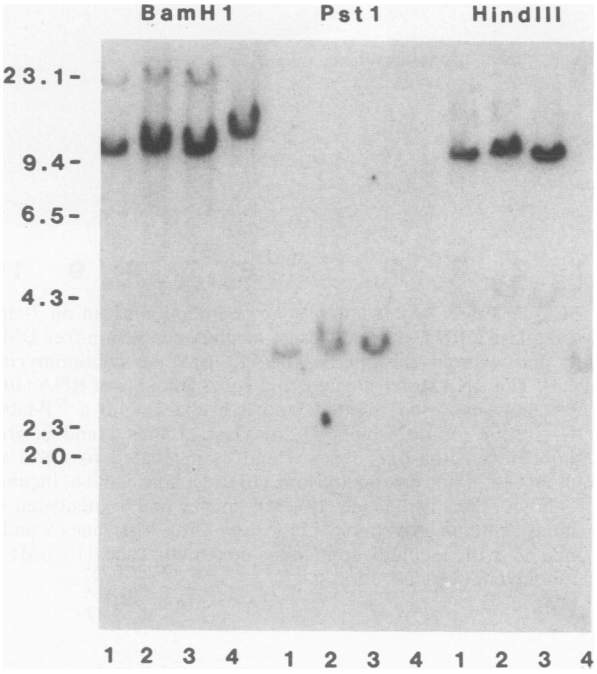

FIG. 6. Southern restriction analysis of genomic DNA. Genomic DNAs (10 μg) obtained from rat liver (lane 1), L35 cells (lane 2), UC1 cells (lane 3), and human HepG2 cells (lane 4) were digested with the indicated restriction enzymes and subjected to Southern blot analysis using ^{32}P-labeled cDNA probe for 7-alpha-hydroxylase. Molecular markers (in kilobase pairs) are shown at left.

DISCUSSION

Several lines of evidence support the conclusion that L35 cells express the rat, not the human, form of 7-alpha-hydroxylase. First, the restriction fragments produced by genomic DNA obtained from L35 cells are identical to those produced from both parental cell (UC1) and rat genomic DNA, and they are distinctly different from those produced from human genomic DNA (Fig. 6). Had the human gene been integrated into the genome of L35 cells, additional restriction fragments corresponding to the human gene would have been detected in the DNA obtained from L35 cells. This clearly did not happen. Second, human and rat 7-alpha-hydroxylase mRNAs are clearly distinguishable by electrophoretic migration (Fig. 7; 26). The mRNAs present in L35 cells correspond to the rat, but not the human, forms. Finally, it is unlikely that our inability to detect either DNA or RNA of human origin can be explained by species-specific differences in gene dosage. Recent studies of the rat 7-alpha-hydroxylase gene suggest that there is probably a single gene per genome (20, 22). Since we could clearly detect the human gene restriction fragments with the rat cDNA probe, if the human 7-alpha-hydroxylase gene was transfected into L35 cells, it would have been detected.

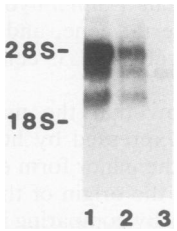

FIG. 7. Northern blot analysis of RNA obtained from rat liver, L35 cells, and human liver. Poly(A) RNA was subjected to electrophoresis in agarose, blotted onto nitrocellulose, and probed with a ^{32}P-labeled cDNA probe for rat 7-alpha-hydroxylase. Lane 1, 2.5 μg of poly(A) rat liver RNA obtained from a cholestyramine-treated rat; lane 2, 2.5 μg of poly(A) RNA obtained from L35 cells; lane 3, 5 μg of poly(A) RNA obtained from the liver of a human transplant patient.

The mechanism through which the 7-alpha-hydroxylase gene is expressed in L35 cells may be the same as the one responsible for its inactivation in UC1 cells. The restriction pattern recognized by the 7-alpha-hydroxylase cDNA probe and obtained from nonexpressing parental cells (UC1) is virtually identical to that produced by genomic DNA obtained from 7-alpha-hydroxylase-expressing L35 cells (Fig. 6). These data suggest that major rearrangements of the 7-alpha-hydroxylase gene cannot account for either the inability of UC1 cells or the ability of L35 cells to express 7-alpha-hydroxylase. Furthermore, since the intensities of the restriction fragments of L35 cells were identical to those produced with UC1 cells and rat genomic DNA, differences in gene dosage (i.e., gene amplification) cannot account for the differences in expression of 7-alpha-hydroxylase. Two mechanisms that may account for the activation of the endogenous 7-alpha-hydroxylase gene are an alteration in genomic DNA near or within the 7-alpha-hydroxylase gene (e.g., insertion, deletion of DNA, or changes in methylation) or addition or deletion of factors that act *trans* to the gene. The mechanism responsible for conferring expression of 7-alpha-hydroxylase by L35 cells allows regulatory but not constitutive expression. Additional data suggest that DNA transfection played a role in obtaining cells with 25-hydroxycholesterol resistance. In seven separate transfection experiments with genomic DNA, at least one 25-hydroxycholesterol-resistant colony was obtained in each experiment. In contrast, in three additional experiments without DNA transfection, not a single colony of 25-hydroxycholesterol-resistant cells was obtained. Further analysis of genomic DNA from L35 cells with a human repetitive DNA probe (13) displayed more hybridization than did parental UC1 cells (data not shown), suggesting that a portion of the human DNA was inserted into the rat hepatoma cell genome. Whether this insertion is directly (*cis* activation) or indirectly (*trans* activation) responsible for expression of the endogenous 7-alpha-hydroxylase gene remains to be determined. However, since the Southern restriction pattern was the same for inactive (UC1) and active (L35) cells, either the human DNA did not insert in or near the 7-alpha-hydroxylase gene or this insertion was too small to be detected by restriction fragment sizes.

Dexamethasone addition increased both 7-alpha-hydroxylase activity (Table 2) and mRNA levels (Fig. 4). This induction was rapid (within 3 h; Fig. 5) and was completely blocked by dactinomycin, suggesting that initiation of transcription is required. Previous studies show that in cultured rat hepatocytes, dexamethasone increased the activity of 7-alpha-hydroxylase (30). These findings showing that L35 cells are sensitive to dexamethasone induction of 7-alpha-hydroxylase suggest that these cells have regained the regulation exhibited by differentiated liver cells. Since the parental rat hepatoma cell line shows dexamethasone induction of several transcriptionally regulated gene products (5), it is unlikely that acquiring sensitivity to dexamethasone is the result of activation of the corticosteroid receptor (3). Dexamethasone increases the expression of many cy-

tochrome P450s via changes in transcription (31) and mRNA stabilization (31, 32). The exact mechanism responsible for dexamethasone induction of 7-alpha-hydroxylase remains to be determined, but it appears to involve transcription. The findings obtained with L35 cells (this study) and cultured rat hepatocytes (30) showing that dexamethasone induces the expression of 7-alpha-hydroxylase activity agree with the results of some (24), but not all (8, 22), in vivo studies.

To our knowledge, this is the first demonstration that microsomes obtained from a stable line of cultured cells express 7-alpha-hydroxylase at levels comparable to levels expressed by rat liver. Moreover, expression of 7-alpha-hydroxylase by L35 cells is regulatable in a physiologic manner. While both cultured rat hepatocytes (12, 18) and HepG2 cells (15) synthesize bile acids, isolation of their microsomes and measurement of 7-alpha-hydroxylase showed activities that are barely detectable and less than 10% of that expressed by microsomes obtained from fresh liver (Table 2; 18, 29, 30).

Our results show that we have derived a stable line of hepatoma cells which are resistant to the cytotoxicity of 25-hydroxycholesterol and express 7-alpha-hydroxylase. Several lines of evidence suggest that expression of 7-alpha-hydroxylase is responsible for 25-hydroxycholesterol resistance. First, L35 cells were clearly sensitive to the down-regulation of cholesterol biosynthesis by 25-hydroxycholesterol (Table 1), showing that the major mechanism through which CHO cells are resistant to 25-hydroxycholesterol (defective regulation of 3-hydroxy-3-methylglutaryl–coenzyme A reductase [6, 7, 23, 33]) cannot account for the 25-hydroxycholesterol resistance expressed by L35 cells. Second, L35 cells, which express 7-alpha-hydroxylase, are clearly resistant to 25-hydroxycholesterol, whereas parental UC1 cells, which do not express 7-alpha-hydroxylase, are rapidly killed by 25-hydroxycholesterol (Fig. 1). Finally, blocking 7-alpha-hydroxylase activity in L35 cells with ketoconazole (Table 3) completely restored the ability of 25-hydroxycholesterol to kill these cells (Fig. 3). On the other hand, L35 cells were obtained by screening for 25-hydroxycholesterol resistance in medium containing low levels (0.5%) of serum, 1% Nutridoma, and no dexamethasone. When cultured in this medium (without 25-hydroxycholesterol), L35 cells express low or undetectable 7-alpha-hydroxylase activity and mRNA. Since both rat hepatocytes and HepG2 cells are resistant to 25-hydroxycholesterol and since both express low (almost undetectable) levels of 7-alpha-hydroxylase (Table 2), this low expression may be sufficient to render cells resistant to 25-hydroxycholesterol. Additional studies show that expression of 7-alpha-hydroxylase by L35 cells is affected by several factors in addition to dexamethasone. We have found that 25-hydroxycholesterol induces the mRNA for 7-alpha-hydroxylase (21a). Additional studies show that serum and LDLs increase the expression of 7-alpha-hydroxylase mRNA and activity (data not shown). The mechanistic details of these findings remain to be elucidated.

The fact that screening cells for resistance to 25-hydroxycholesterol resulted in the isolation of hepatoma cells having an expressible endogenous 7-alpha-hydroxylase gene emphasizes the importance of this enzyme in the metabolism of 25-hydroxycholesterol. Previous in vivo studies have shown that oxysterols (25-hydroxycholesterol [38] and 26-hydroxycholesterol [2]) are efficiently converted to bile acids. Since bile acids do not act as oxysterol regulators, metabolism of oxysterols by 7-alpha-hydroxylase may play an important role in mediating their regulatory activity. While all the cells examined have the ability to metabolize exogenous [^{14}C]cholesterol to more-polar compounds, ^{14}C-labeled 7-alpha-hydroxycholesterol accumulated only in L35 cells treated with dexamethasone (Fig. 2). These data are consistent with the proposal that all cells can produce oxysterol regulators, whereas only the liver expresses 7-alpha-hydroxylase. Liver-specific expression of 7-alpha-hydroxylase has been demonstrated in the rat (19). Tissue-specific expression of 7-alpha-hydroxylase suggests that the hepatic metabolism of 25- and 26-hydroxycholesterol to bile acids may determine the amount of regulatory sterol available for regulating the expression of genes governed by sterol-responsive elements (27, 37). Oxysterol metabolism and inactivation by 7-alpha-hydroxylase may explain the liver's insensitivity toward LDL down-regulation of the LDL receptor (17) and the parallel induction of the LDL receptor (21) and 3-hydroxy-3-methylglutaryl–coenzyme A reductase (10, 11) when 7-alpha-hydroxylase activity is increased.

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