Rat hepatoma L35 cells, a liver-differentiated cell line, display resistance to bile acid repression of cholesterol 7α-hydroxylase

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Abstract A stable hepatoma cell line (L35 cells) showing an activation of the cholesterol 7α-hydroxylase gene (CYP7) that had been silent in the parental hepatoma cell line (H35 cells) was used to examine the influence of bile acids on its gene expression and activity. L35 cells were found to concentrate taurocholate from the culture medium, without any significant effect on the expression of 7α-hydroxylase. At physiologic levels (up to 100 μM), CYP7 mRNA expression was not repressed by any bile acid. At supra-physiologic levels (1 mM), the more hydrophobic dihydroxy bile acids, taurodeoxycholate and taurochenodeoxycholate, decreased CYP7 mRNA without decreasing the relative abundance of beta-actin mRNA. Similar results were obtained by culturing cells with sodium dodecylsulfate (50 μM). The medium of L35 cells treated with either taurochenodeoxycholate (1 mM), taurodeoxycholate (1 mM), or sodium dodecylsulfate (50 μM) contained significantly greater activities of two cytosolic enzymes, lactate dehydrogenase and phosphoglucose isomerase, indicating a cytotoxic response. Activation of protein kinase C by phorbol esters decreased the expression of 7α-hydroxylase mRNA without evidence of cytotoxicity; therefore, the inability of L35 cells to show bile acid repression cannot be ascribed to a lack of an effect by this secondary messenger system. In addition, insulin decreased and dexamethasone increased 7α-hydroxylase mRNA without increasing the release of the cytoplasmic enzyme markers. The combined data suggest that L35 cells are resistant to repression of CYP7 gene expression by bile acids, but display physiologic expression to hormones and protein kinase C activation.—**Trawick, J. D., K. D. Lewis, S. Dueland, G. L. Moore, F. R. Simon, and R. A. Davis.** Rat hepatoma L35 cells, a liver-differentiated cell line, display resistance to bile acid repression of cholesterol 7α-hydroxylase. *J. Lipid Res.* 1996. **37:** 588–598.

Supplementary key words cell culture models • bile acids • cholesterol 7α-hydroxylase • gene expression • hepatoma cells

The first and rate-limiting step in bile acid synthesis is the conversion of cholesterol to 7α-hydroxycholesterol by the cytochrome P450 enzyme, cholesterol 7α-hydroxylase (EC 1.14.13.17). cDNAs encoding 7α-hydroxylase have been isolated and cloned from rats (1–3), mice (4), hamsters (5, 6), rabbits (7), and humans (8–10). The cholesterol 7α-hydroxylase gene (CYP7) is expressed only in the liver (1) and exhibits a complex regulation of expression dependent upon diurnal cycle, hormones, diet, and the enterohepatic circulation (11). In vivo, CYP7 expression is induced in response to glucocorticoid hormones such as dexamethasone (12, 13), thyroid hormone (14, 15), and dietary cholesterol (16, 17). Dexamethasone, either with (15) or without (12) thyroid hormone, appears to be essential for maintenance of CYP7 expression in rat hepatocytes and hepatoma cells, respectively. In contrast, CYP7 expression is reduced by insulin (18–20), and by adding bile acids to the diet (14, 21). Repression by feeding bile acids is thought to mimic the primary mechanism regulating bile acid synthesis (i.e., negative feedback).

In rats, biliary diversion and feeding of bile acid sequestering agents leads to a rapid increase in 7α-hydroxylase transcription, mRNA accumulation, and activity (22, 23). While bile acids are thought to act directly on the liver to initiate the negative feedback, there are several reports showing that infusing bile acids directly into the circulation of bile-diverted rats does not decrease bile acid synthesis (24) nor affect the activity and hepatic content of mRNA of 7α-hydroxylase (25). These

Abbreviations: DEX, dexamethasone; DMEM, Dulbecco's modified Eagle's medium; GLC, gas–liquid chromatography; PMA, phorbol 12-myristate, 13-acetate; SDS, sodium dodecylsulfate; SF, serum-free; TC, taurocholate; TCDC, taurochenodeoxycholate; TDC, taurodeoxycholate; TUDC, tauroursodeoxycholate.

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results obtained in vivo suggest that bile acids may require factors residing outside of the liver in order to initiate negative feedback regulation.

Attempts to recreate bile acid negative feedback regulation using cultured hepatocytes have yielded conflicting data. Earlier work using primary rat hepatocytes showed that bile acids had little, if any, inhibitory effect on bile acid synthesis or 7α-hydroxylase expression (17, 24, 26, 27). In contrast, using pig hepatocytes, suppression of 7α-hydroxylase was observed by adding taurocholate to the culture medium (28). These differences between rat and pig hepatocytes in bile acid repression were ascribed to the presence (pig) or absence (rat) of processes required for bile acid uptake. More recent studies show that when rat hepatocytes are cultured in the presence of both dexamethasone and thyroid hormone, taurocholate represses the expression of the CYP7 gene (15, 29). The human hepatoma cell line (HepG2 cells) also exhibits bile acid repression of CYP7 expression (30, 31). Using this model, a "bile acid repressible element" has been identified in the CYP7 promoter (32). The structure of the bile acid appears to be an important determinant of its ability to mediate regulation through this promoter element: more hydrophobic bile acids such as taurochenodeoxycholic acid and taurodeoxycholic acid are inhibitory, whereas the more hydrophilic bile acid taurocholate is not (32).

The rat hepatoma cell line (L35 cells) shows the unusual ability to express 7α-hydroxylase at levels similar to those of rat liver in vivo (12). When induced by dexamethasone, the level of expression of CYP7 is 100-fold greater than that observed in cultured rat hepatocytes and 10-fold greater than that expressed by HepG2 cells (both treated with dexamethasone) (12). These unique, highly differentiated cells provide a cell culture model that appears to reflect many of the properties of the in vivo liver parenchymal cell. Moreover, L35 cells provided us with a culture model to examine the regulation of CYP7 gene expression. While L35 cells displayed the ability to concentrate taurocholate from the culture medium, this bile acid did not repress CYP7. In order to observe a decrease in 7α-hydroxylase mRNA, hydrophobic bile acids were required at supra-physiologic levels (1 mM), which also resulted in cytotoxicity as demonstrated in the release of cytoplasmic enzyme activities to the culture medium. Although bile acids did not show physiologic regulation of 7α-hydroxylase in these cells, L35 cells responded to hormone regulation by dexamethasone (induction) and insulin (repression) of CYP7 expression, as well as repression by protein kinase C activation.

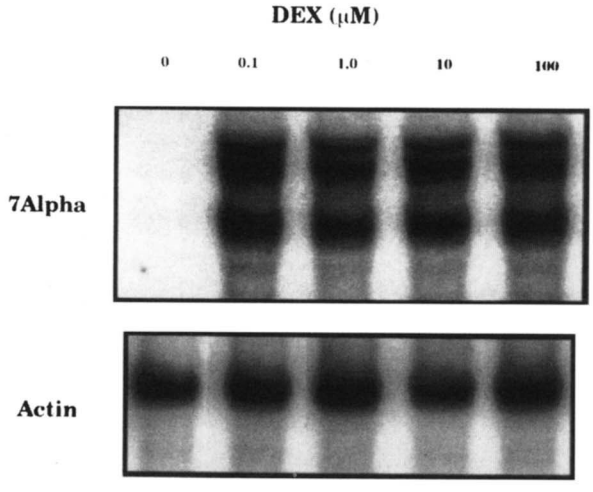

Fig. 1. Response of 7α-hydroxylase mRNA to dexamethasone. L35 cells were cultured as described in Materials and Methods. Duplicate plates were changed to serum-free DMEM and serum-free DMEM plus dexamethasone (DEX) at 0.1, 1.0, 10, and 100 μM. After 48 h the cells were harvested and polyA RNA was purified; a formaldehyde-agarose gel was run and northern blotted. Autoradiographs are from hybridizations successively probed for 7α-hydroxylase (7 alpha) and actin.

MATERIALS AND METHODS

Materials

All reagents, culture supplies, bacterial strains and supplies were obtained from Sigma, Gibco/BRL, and Boehringer Mannheim or from sources stated in the text.

Strains of cells and growth conditions

The cells used in these studies are L35 cells, a subclone of the original cell line previously described (12). Studies performed over 5 years showed that the original stock of L35 cells were heterogenous as defined by morphology, expression of 7α-hydroxylase and resistance to 25-hydroxycholesterol (data not shown). The L35 subclone, obtained by limiting dilution cloning and rescreening for resistance to 25-hydroxycholesterol cytotoxicity, has been cultured continuously for 2 years without a detectable change in phenotype. The cells were plated and cultured as described (12). At 50% confluence (2 to 3 days after plating), the medium was changed to serum-free DMEM and the indicated reagent was added, as described in legends. The sodium salts of taurocholate, taurodeoxycholate, tauroursodeoxycholate, or taurochenodeoxycholate (Sigma) were dissolved in serum-free DMEM with 10 mg fatty acid-free BSA/ml as a carrier and added at concentrations ranging between 0.01 mM to 1 mM.

Plasmid vectors containing cDNAs for hybridization probes were propagated in *E. coli* DH5-α. The cDNA encoding rat 7α-hydroxylase (1) was a generous gift from David Russell.

TABLE 1. L35 cells accumulate cholic acid from culture medium

	Cellular Cholic Acid
No taurocholate	164 ± 14
Plus taurocholate	379 ± 59

L35 cells were cultured in DMEM plus dexamethasone either with or without a supplement of 100 μM taurocholate. Triplicate samples of cells were harvested and extracts were prepared and subjected to GC–MS as described (17). Concentrations are given in μM assuming a cell volume estimated by gravimetric analysis of the average cell water mass. This analysis showed that the ratio of water:cell protein in L35 cells is 5.7:1. Using this ratio, the cellular concentration of bile acids/unit protein was converted into concentration/unit water volume.

RNA isolation and polyA RNA purification

Total RNA from L35 cells was isolated by a modification of the guanidinium isothiocyanate protocol (33). Medium from a 150-mm culture dish was removed by aspiration and 3 ml of guanidinium isothiocyanate lysis solution (33) was immediately added. The RNA was extracted with phenol and precipitated with isopropanol, pelleted, and reprecipitated with ethanol.

PolyA RNA was isolated (34) with 10–40 mg of oligo dT cellulose (type 3, Collaborative Biomedical Products) in a 1.5-ml microfuge tube. Previously, the oligo dT cellulose had been activated and washed as described (35). Binding was carried out in loading buffer (0.5 M LiCl, 0.01 M Tris-HCl, pH 7.5, 0.001 M Na-EDTA, 0.1% SDS) (35) for 1 h at room temperature with gentle rocking. Then the oligo dT cellulose was washed with loading buffer a total of three times and transferred to a spin column microtube (Invitrogen). The oligo dT cellulose–RNA mix was then washed four times with middle wash buffer (0.15 M LiCl, 0.01 M Tris-HCl, pH 7.5, 0.001 M Na-EDTA, 0.1% SDS) (35). The poly A RNA was eluted with H_2O and collected in a total of 0.4 ml.

Gel electrophoresis of RNA was performed as described (36). Capillary transfer in 10 × SSC was carried out onto Zetaprobe GT (Bio-Rad) nylon membranes. After 12–18 h of transfer, the blots were baked at 80°C under vacuum. Hybridization was performed according to the instructions for Zetaprobe GT, in 0.12 M $NaHPO_4$, 0.25 M NaCl, 7% (w/v) SDS, and 50% deionized formamide for 18 h at 44°C. Probes were labeled using 0.1 μg of cDNA with a nick translation kit (Boehringer Mannheim) and α-^{32}P dCTP (DuPont). After hybridization, northern blots were washed in a final wash of 0.1 × SSC, 0.1% SDS for 45 min at 44°C. Northern blots were autoradiographed using DuPont Reflection Autoradiography Film and an intensifying screen for between 4 h and 3 days. Alternatively, northern blots were exposed overnight using a Molecular Dynamics PhosphorImager.

The CYP7 probe used in hybridization was derived from the cloned rat cDNA (1) by digestion with EcoRI at the 5′ end and Ava II at 1640 bp to make a cDNA probe containing the entire coding region and only 80 bp of the 3′ noncoding region. This fragment eliminates the repetitive sequences found further downstream in the 3′ noncoding region. A beta-actin cDNA was used as a counterprobe as described previously (12).

Quantitation of bile acids

After incubation with taurocholate (described above), the culture medium was removed and the cells were washed with phosphate-buffered saline on ice. The cells were disrupted by sonication and bile acids were extracted and quantitated by GLC, as described (17).

Measurement of lactate dehydrogenase and phosphoglucose isomerase release into the culture medium

Lactate dehydrogenase activity was determined by measurement of change in absorbance at 340 nm in the presence of pyruvate and NADH, as described (37). Plates (100 mm) of L35 cells were cultured exactly as

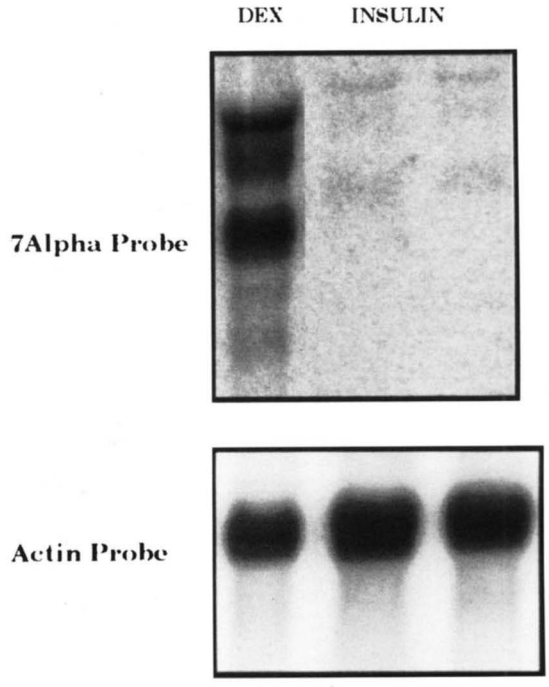

Fig. 2. Insulin represses expression of 7α-hydroxylase mRNA in L35 cells. L35 cells were cultured and 7α-hydroxylase mRNA was induced with 100 μM dexamethasone as described for Fig. 1. After 48 h, the medium was removed and replaced with fresh serum-free DMEM supplemented either with or without 0.1 μg porcine insulin/ml and incubated for 24 h. Shown are the positive control with no added insulin and duplicate lanes with insulin added.

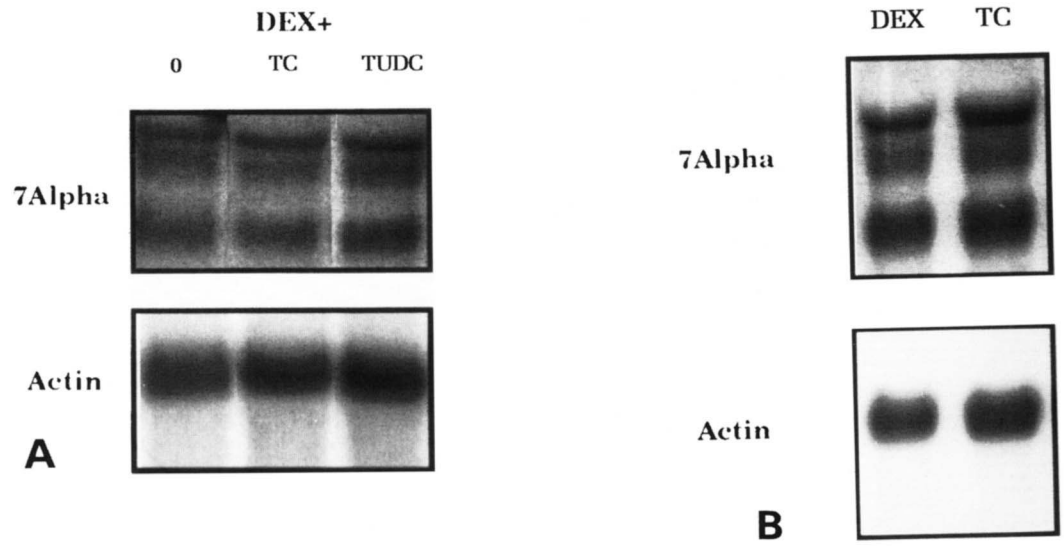

Fig. 3. Taurocholate neither blocks nor inhibits 7α-hydroxylase expression in L35 cells. A: L35 cells were cultured in serum-free DMEM plus dexamethasone (100 μM) for 48 h (+DEX) or with dexamethasone and 1.0 mM taurocholate for 48 h (+TC) or 1.0 mM tauroursodeoxycholate for 48 h (+TUDC). As above, poly A containing RNA was isolated, northern blotted onto nylon, and hybridized with probes for the coding region of CYP7 and for beta-actin. B: L35 cells were placed in serum-free DMEM plus dexamethasone for 48 h, then the medium was switched to serum-free DMEM without dexamethasone (DEX), or to serum-free DMEM without dexamethasone but with 1.0 mM taurocholate (TC), incubated an additional 24 h, then polyA RNA was harvested, northern blotted, and probed as described above.

described for the RNA experiments. In serum-free DMEM, the cells were incubated with dexamethasone (100 μM) and the indicated concentrations of bile acids for 48 h. Then the culture medium was harvested, any floating cells were removed by centrifugation, and the cells on the plate were harvested for protein determination using the Bio-Rad protein reagent. Preliminary studies showed that freezing the culture medium resulted in a marked loss of lactate dehydrogenase activity. As a result of these findings, lactate dehydrogenase activity was assayed using 0.1 ml of freshly harvested culture medium. The rate (zero order) of the reaction was calculated from the linear portions of the curves and corrected to the protein concentration.

The measurement of phosphoglucose isomerase was performed by assaying the change in absorbance by the reduction of NADP in the presence of fructose-6-phosphate and glucose dehydrogenase at 340 nm, as described (38). Conditions for cell culture, harvesting of culture medium, and assaying were as described above for the lactate dehydrogenase assay.

Statistical analysis

All values are reported as the mean ± SD. Student's *t* test was used to calculate statistical differences among groups; values of $P < 0.05$ (double-tailed) were considered to be significant.

RESULTS

Expression of 7α-hydroxylase is increased by dexamethasone and decreased by insulin: evidence for physiologic regulation

Expression of 7α-hydroxylase similar to the maximal level obtained in vivo in intact normal rats can be obtained by treating L35 cells with dexamethasone (12). We determined the concentration of dexamethasone necessary to maximally induce 7α-hydroxylase mRNA. Dexamethasone, at physiologic levels (0.1 μM), maximally induced the expression of 7α-hydroxylase mRNA (**Fig. 1**). Additionally, maximal induction of 7α-hydroxylase was displayed by levels of dexamethasone as low as 10 nM (data not shown). The multiple molecular weight forms of 7α-hydroxylase mRNA (i.e., 3.6, 2.9, 2.4, and 1.7 kilobases), characteristic of the rat (1), were all induced by dexamethasone. Interestingly, removing dexamethasone from the culture medium did not decrease the relative abundance of 7α-hydroxylase mRNA for up to 24 h (the longest period examined, **Fig. 2**). In contrast, changing the culture medium to serum-free DMEM containing insulin (0.1 μg/ml) decreased 7α-hydroxylase mRNA to undetectable levels (Fig. 2). The repression by insulin was dominant as shown by no expression of 7α-hydroxylase mRNA in cells incubated with dexamethasone and insulin together (data not shown). Thus, expression of 7α-hydroxylase by L35 cells

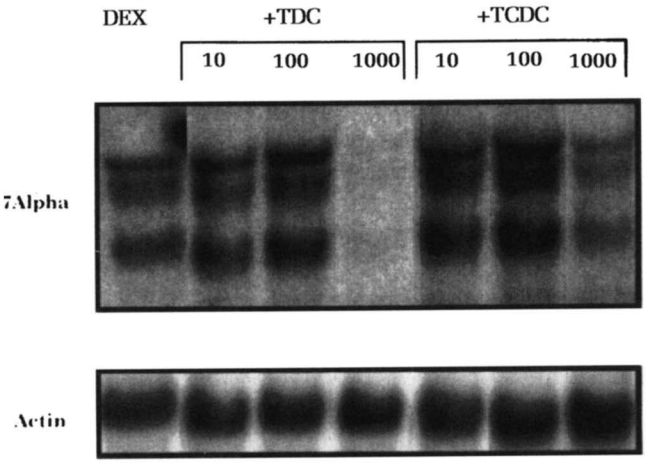

Fig. 4. In L35 cells, 7α-hydroxylase expression is inhibited by supra-physiologic concentrations of the hydrophobic bile acids, taurodeoxycholate and taurochenodeoxycholate. L35 cells were cultured as described above and shifted to serum-free DMEM with DEX and taurodeoxycholate (TDC) in the indicated concentrations from 10 to 1000 μM or taurochenodeoxycholate (TCDC) from 10 to 1000 μM. Duplicate samples of polyA RNA were isolated, northern blotted, and hybridized in succession with probes specific for CYP7 and beta-actin.

is responsive to these hormones at physiologic levels in a manner similar to rat livers in vivo (13, 20, 39).

L35 cells take up taurocholate from the culture medium and concentrate it intracellularly

To examine whether L35 cells could take up and concentrate taurocholate from the culture medium, L35 cells were incubated with taurocholate (100 μM) for 24 h, after which the cells were harvested and the intracellular concentration of cholic acid was quantitated by GLC. The intracellular concentration of cholic acid relative to the medium was increased by nearly 4-fold to 379 μM (**Table 1**). Based on these results, L35 cells phenotypically resemble hepatic parenchymal cells in their ability to concentrate bile acids from the medium.

At physiologic levels (up to 100 μM) taurine-conjugated bile acids do not repress 7α-hydroxylase mRNA

We examined the ability of bile acids to repress 7α-hydroxylase. L35 cells were incubated with either dexamethasone alone or with dexamethasone and taurocholate (1 mM) for 48 h. Taurocholate had no effect on the induction of 7α-hydroxylase mRNA levels by dexamethasone (**Fig. 3A**). When quantitated relative to actin message levels, no significant differences in 7α-hydroxylase message levels between the three treatments were observed. The more hydrophilic bile acid, tauroursodeoxycholate, also showed no effect on levels of 7α-hydroxylase mRNA.

We also performed a second type of an experiment in which L35 cells were incubated with dexamethasone, then it was removed and taurocholate was added (Fig. 3B). Removal of dexamethasone for 24 h had no affect on 7α-hydroxylase mRNA levels. Moreover, addition of taurocholate for 24 h also did not affect 7α-hydroxylase levels, relative to the actin control.

At supra-physiologic concentrations, hydrophobic dihydroxy bile acids both decrease 7α-hydroxylase mRNA and cause cytotoxicity

At higher concentrations (1 mM), the more hydrophobic bile acids taurochenodeoxycholate and taurodeoxycholate decreased 7α-hydroxylase mRNA levels (**Fig. 4**). The suppression caused by taurodeoxycholate ranged from 8- to 20-fold while the suppression due to taurochenodeoxycholate was 7-fold when corrected to actin mRNA levels. In contrast, even at this higher than physiologic level of bile acids, neither taurocholate nor tauroursodeoxcholate, caused any detectable change in 7α-hydroxylase mRNA (Fig. 3). Because beta-actin mRNA levels were unchanged by any of the incubation conditions, there was an apparent specific decrease in the relative level of 7α-hydroxylase mRNA levels in the presence of taurodeoxycholate and taurochenodeoxycholate.

In the presence of 1 mM concentrations of hydrophobic bile acids, cells appeared to have an abnormal morphology; they appeared rounded as opposed to a flattened appearance, normally observed under growth conditions (**Fig. 5**). This altered morphology was displayed by > 85% of the cells cultured in the presence of 1 mM of either taurochenodeoxycholate and taurodeoxycholate.

To examine whether this change in morphology was due to cytotoxic effects of the dihydroxy bile acids, the release of two cytosolic enzymes, lactate dehydrogenase and glucose-isomerase, into the culture medium was measured. At the (1 mM) concentration of hydrophobic dihydroxy bile acids (required to observe a decrease in 7α-hydroxylase mRNA) significantly increased activities of both lactate dehydrogenase and phosphoglucose isomerase in the culture media were observed (**Fig. 6**). In contrast, in all other culture conditions described above (the lower concentrations of bile acids, or no bile acids), there was no detectable release of lactate dehydrogenase or phosphoglucose isomerase activity into the medium (data not shown). The combined data suggest that both taurochenodeoxycholate and taurodeoxycholate at the supra-physiologic concentration of 1 mM caused cytotoxicity in L35 cells.

To further examine the relationship between decreases in 7α-hydroxylase mRNA and cytotoxicity, L35 cells were incubated with the detergent sodium dodecylsulfate (SDS) at a concentration of 50 μM. Cells responded to SDS in a manner similar to the effect of supra-physiologic concentrations of hydrophobic dihydroxy bile acids. SDS decreased the relative abundance of 7α-hydroxylase mRNA (with no detectable change in beta-actin mRNA) and increased the activity of both lactate dehydrogenase and phosphoglucose isomerase into the culture medium (**Fig. 7**). The combined data show that in L35 cells, bile acids and SDS repress 7α-hydroxylase mRNA in a manner associated with cytotoxicity.

L35 cells retain protein kinase C regulation of 7α-hydroxylase expression

In primary rat hepatocytes, 7α-hydroxylase gene expression is negatively regulated by inducers of the protein kinase C and this has been proposed to be the mechanism by which bile acids regulate 7α-hydroxylase (40). We examined the relationship between protein kinase C and CYP7 expression in L35 cells using PMA activation of this second messenger system. L35 cells were treated with dexamethasone for 48 h and then were incubated with PMA (250 nM) for 3 h. PMA markedly decreased 7α-hydroxylase mRNA about 2.6-fold (**Fig. 8**). At the same time, levels of beta-actin mRNA were increased 2.6-fold relative to the control. PMA induction of beta-actin mRNA has been reported in other types of hepatoma cells (41). The combined data show that L35 cells display a phenotype associated with resistance to bile acid repression of CYP7 expression. This resistance may be caused by an inability of bile acids to activate protein kinase C.

DISCUSSION

The liver consists of several different cell types, many having specific functions and patterns of gene expression. Within the lobules of the liver are localized regions where parenchymal cells exhibit differential gene expression (42, 43). Parenchymal cells localized to the periportal regions display a high expression of HMG-CoA reductase (44), whereas parenchymal cells in the pericentral regions display a high expression of 7α-hydroxylase (45). As parenchymal cells located in the periportal regions are mainly responsible for returning bile acids from the portal blood to the biliary system (46, 47) the decreased expression of 7α-hydroxylase in this region has been explained by negative feedback on 7α-hydroxylase. This differential localization of liver-specific functions may, in part, explain the difficulty in devising model cell systems that accurately reflect, in toto, the functional integrity of parenchymal cells of the liver in vivo.

In this report we further characterized L35 cells, a line of rat hepatoma cells that express 7α-hydroxylase in a regulated manner. To our knowledge these cells are the only cells that display in combination the following gene products typical of the adult differentiated liver: 7α-hydroxylase at in vivo levels, liver-specific glucokinase, the transcription factors DBP and CEBP, the beta sub-unit of Na^+,K^+-ATPase, the microsomal cytochrome P4502EI, the liver-specific form of phosphatidylethanolamine N-methyl transferase 2, LCAT, and plasma apolipoproteins. They form cell/cell contacts reminiscent of bile canaliculi (R. A. Davis, J. D. Trawick, and F. R. Simon, unpublished data). The combined data indicate that the L35 cell line displays a phenotype that more

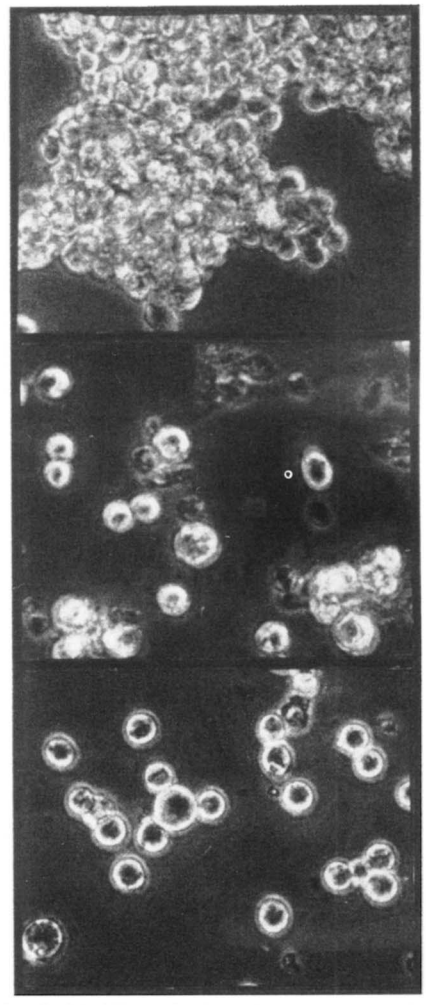

Fig. 5. Effect of the hydrophobic bile acids, taurodeoxycholate and taurochenodeoxycholate, on L35 cell morphology. After culturing L35 cells in serum-free DMEM supplemented with dexamethasone or in serum-free DMEM, dexamethasone, and 1.0 mM of the indicated bile acid for 48 h, photomicrographs of the culture dishes were made.

closely approximates that of the parenchymal cell in vivo than primary rat hepatocytes and HepG2 cells. This unique cell line allowed us to examine how this phenotype might affect bile acid repression of CYP7 expression, a response common to hormonally induced primary rat hepatocytes and HepG2 cells (29–31).

At physiologic concentrations (< 100 μM), none of the bile acids examined showed an effect (Fig. 4) on expression of 7α-hydroxylase mRNA. As L35 cells take up and concentrate taurocholate (Table 1), the absence of repression of 7α-hydroxylase cannot be attributed to the inability of the bile acids to enter L35 cells. Moreover, at concentrations higher than observed in rat portal blood (48), both taurochenodeoxycholate and taurodeoxycholate decreased 7α-hydroxylase mRNA, without affecting beta-actin mRNA (Fig. 4). An identical effect was observed using SDS (Fig. 7). While one might conclude from these data that taurochenodeoxycholate, taurodeoxycholate, and SDS may have elicited a specific decrease of 7α-hydroxylase, the additional finding showing a release of two cytoplasmic enzyme activities, lactate dehydrogenase and phosphoglucose isomerase (Figs. 6 and 7), suggests that this effect was caused by the detergent properties of these amphipaths. The additional finding showing an altered morphology of cells incubated with these detergents at concentrations necessary to decrease 7α-hydroxylase mRNA, but not actin mRNA, indicates that cellular integrity was impaired, i.e., cytotoxicity. Our finding that L35 cells cultured under these conditions display clear evidence of cytotoxicity without a decrease in actin mRNA suggests that a selective decrease in one mRNA compared to another may not necessarily be a valid indication of a "specific regulatory" response.

To establish that bioactive molecules (i.e., effectors) initiate physiologically relevant regulation of gene expression, they must conform to at least the following three criteria: *1)* changes in the concentration of the effector in the target tissue should correlate with physiologic changes in gene expression; *2)* they should show a structural specificity that correlates with potency of response; and *3)* in an appropriate experimental system that reflects the in vivo situation, they should, at physiologic concentrations, change the expression of the gene in a manner that accurately reflects what occurs in vivo.

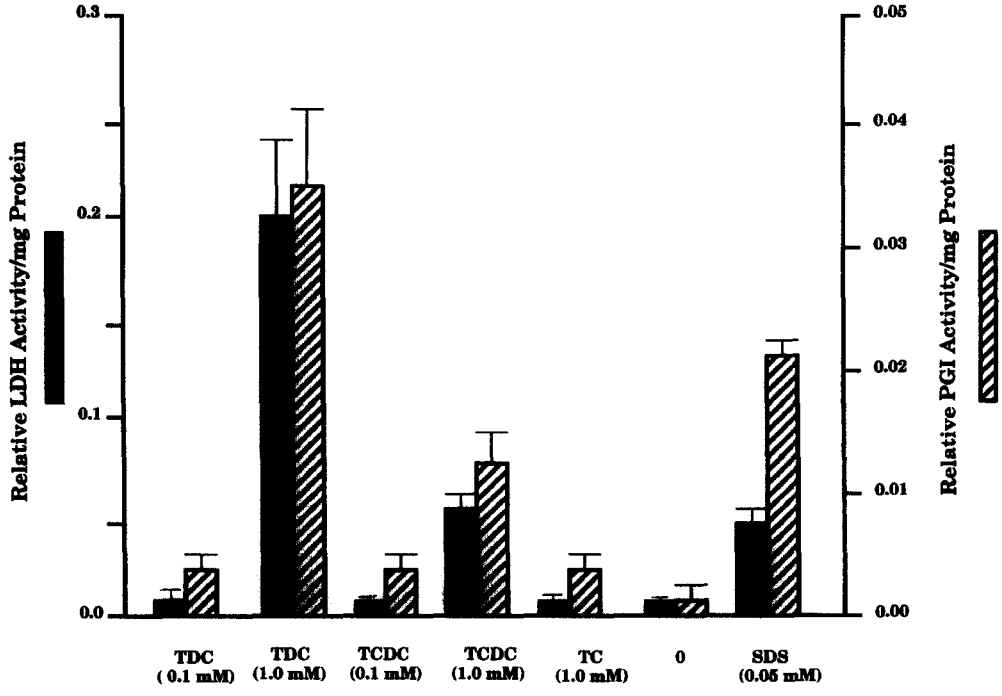

Fig. 6. Treatment of L35 cells with taurodeoxycholate and taurochenodeoxycholate at concentrations sufficient to inhibit CYP7 mRNA accumulation also lead to the release of the cytosolic enzymes lactate dehydrogenase and phosphoglucose isomerase into the culture medium. L35 cells were cultured, induced with dexamethasone, and treated with either taurodeoxycholate (TDC) or taurochenodeoxycholate (TCDC) at 0.1 mM to 1.0 mM, taurocholate (TC) at 1.0 mM, no added bile acids (0), or SDS at 0.05 mM as described for Fig. 4. Then the culture medium was sampled and assayed for activities corresponding to lactate dehydrogenase or phosphoglucose isomerase. Solid boxes, release of lactate dehydrogenase activity into the culture medium. Striped boxes, release of phosphoglucose isomerase into the culture medium. Both activities are expressed as relative rate of substrate conversion per mg of cell protein.

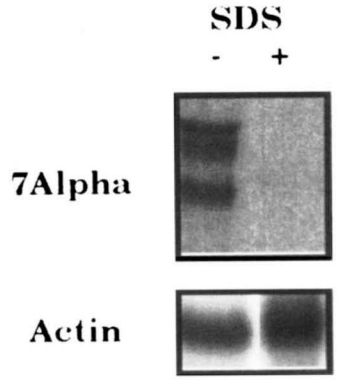

Fig. 7. The ionic detergent SDS can block 7α-hydroxylase expression in L35 cells. L35 cells were cultured and treated as in Figs. 4 and 5 except that sodium dodecylsulfate at a final concentration of 50 μM was added instead of a bile acid. The northern blot was probed with 7α-hydroxylase, stripped, and reprobed with actin.

In regard to bile acid negative feedback regulation of 7α-hydroxylase, there are many contradictory studies. With respect to the first criterion, removal of bile acids from the enterohepatic circulation by either biliary diversion or feeding bile acid sequestrants resulted in increased activity and mRNA for 7α-hydroxylase (22, 23, 49). While these two findings are consistent with the hepatic levels of bile acids being the effectors of 7α-hydroxylase negative feedback repression, there are a number of notable exceptions. These exceptions include the following: *1)* the intrahepatic concentrations of bile acids (50) do not vary inversely with the diurnal increase in activity caused by increased gene expression (51); *2)* it is well known that the activity (49) and mRNA (4) for 7α-hydroxylase show a diurnal maximum soon after food consumption, yet this is the same time when bile acids are no longer sequestered in the gallbladder but are actively being absorbed and delivered back to the liver via the enterohepatic circulation (51, 52); *3)* in bile duct-ligated rats, the concentration of bile acids in the liver is increased, as is the activity of 7α-hydroxylase (53, 54). With respect to the second criterion, the potency of bile acids in regard to repressing CYP7 expression correlates most strongly with hydrophobicity, rather than stereochemistry (55, 56). While a recent report argued that structure was important in bile acid action, hydrophobicity index correlated with 7α-hydroxylase repression at a highly significant level ($P < 0.0001$) (56). Therefore, one is faced with the dilemma of how bile acids having such extraordinary differences in structure might directly interact with a common receptor or regulatory subunit of an enzyme and initiate repression (2).

Recent studies may provide an explanation of this dilemma. Studies in primary rat hepatocytes (40) and HepG2 cells (57) show that bile acids repress 7α-hydroxylase and induce MHC class I antigens, respectively, by activation of protein kinase C. Both effects were greater with more hydrophobic bile acids. In non-hepatic cells, bile acid stimulation of protein kinase C is associated with the procoagulant activity of monocytes (58). Activation of protein kinase C by bile acids has been proposed to be caused by their effects on membrane lipids due to their detergent nature (57). The recent finding that taurocholate at a physiological concentration of 25 μM causes repression of CYP7 gene transcription by a protein kinase C-mediated event (40) may indicate that bile acids act indirectly on gene expression, rather than through a "classical" ligand-receptor model. As indicated by the similar response of both hepatic and extrahepatic cells toward bile acid activation of protein kinase C, this response neither requires the enterohepatic circulation nor the expression of the liver-specific sodium-dependent bile acid transporter (59).

Our findings showing that bile acids do not repress CYP7 expression in L35 cells without an associated cytotoxicity, even though these cells show expected responses to physiologic regulators of CYP7, further suggest that the simple model of bile acids acting directly on the liver transcription of CYP7 is unlikely to be valid. The inability of physiologic concentrations of taurocholate to repress CYP7 expression by L35 cells suggests that these cells, in contrast to rat hepatocytes (40), either have factors that prevent or lack factors necessary to activate protein kinase C by bile acids. These factors are not involved in dexamethasone induction and insulin repression of CYP7. Differences in the ability of bile acids to repress 7α-hydroxylase in vivo exists between species and strains. For example, feeding diets contain-

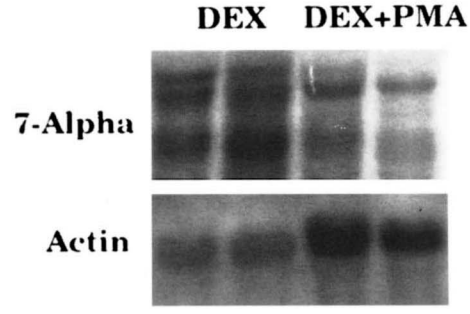

Fig. 8. Phorbol 12-myristate, 13-acetate (PMA), an inducer of PKC, negatively regulates 7α-hydroxylase mRNA in L35 cells. L35 cells were cultured and 7α-hydroxylase was induced with 100 μM dexamethasone as in Fig. 4. After 48 h, PMA was added to 250 nM and RNA was harvested after 3 h of PMA induction of PKC. Control cells induced with dexamethasone but not treated with PMA were harvested at the same time. The northern blot with duplicate samples probed in succession with 7α-hydroxylase and beta-actin is shown.

ing cholesterol and cholic acid to rats led to increased 7α-hydroxylase levels (60), whereas when fed to C57BL/6 mice there was a decrease (21). These data show that in vivo some animals are resistant to bile acid repression of 7α-hydroxylase, whereas some are susceptible. Our findings showing that L35 cells are resistant to taurocholate repression of 7α-hydroxylase whereas primary rat hepatocytes and HepG2 cells are susceptible may be reflecting these in vivo differences. Moreover, as activation of protein kinase C by PMA clearly decreases 7α-hydroxylase mRNA in L35 cells, it is likely that the differences in the ability of bile acids to activate protein kinase C may be responsible for the differences between cultured cell models and individual species and strains of mice in their response to bile acids. Species (61), individual (7), and strain-specific (21) differences in the mechanisms regulating CYP7 expression may contribute to the processes determining plasma lipoprotein levels and susceptibility to atherosclerosis.

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REFERENCES

1. 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.
2. Jelinek, D. F., and D. W. Russell. 1990. Structure of the rat gene encoding cholesterol 7α-hydroxylase. *Biochemistry.* **29:** 7781–7785.
3. Noshiro, M., M. Nishimoto, and K. Okuda. 1990. Rat liver cholesterol 7α-hydroxylase. Pretranslational regulation for circadian rhythm. *J. Biol. Chem.* **265:** 10036–10041.
4. Tzung, K-W., K. Ishimura-Oka, S. Kihara, K. Oka, and L. Chan. 1994. Structure of the mouse cholesterol 7α-hydroxylase gene. *Genomics.* **21:** 244.
5. 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.
6. Hayashi, K., H. Kurushima, M. Saeki, H. Amioka, J. Kurokawa, Y. Kuga, S. Nomura, Y. Ohkura, H. Ohtani, and M. Noshiro. 1993. Partial cloning of cDNA for hamster liver cholesterol 7α-hydroxylase. *Intern. Med.* **32:** 210–214.
7. Poorman, J. A., R. A. Buck, S. A. Smith, M. L. Overturf, and D. S. Loose-Mitchell. 1993. Bile acid excretion and cholesterol 7α-hydroxylase expression in hypercholesterolemia-resistant rabbits. *J. Lipid Res.* **34:** 1675–1685.
8. Cohen, J. C., J. J. Cali, D. F. Jelinek, M. Mehrabian, R. S. Sparkes, A. J. Lusis, D. W. Russell, and H. H. Hobbs. 1992. Cloning of the human cholesterol 7α-hydroxylase gene (CYP7) and localization to chromosome 8q11-q12. *Genomics.* **14:** 153–161.
9. Nishimoto, M., M. Noshiro, and K. Okuda. 1993. Structure of the gene encoding human liver cholesterol 7α-hydroxylase. *Biochim. Biophys. Acta.* **1172:** 147–150.
10. Noshiro, M., and K. Okuda. 1990. Molecular cloning and sequence analysis of cDNA encoding human cholesterol 7α-hydroxylase. *FEBS Lett.* **268:** 137–140.
11. Vlahcevic, Z. R., W. M. Pandak, D. M. Heuman, and P. B. Hylemon. 1992. Function and regulation of hydroxylases involved in the bile acid biosynthesis pathways. *Sem. Liver Dis.* **12:** 403–419.
12. Leighton, J. K., Dueland, M. S. Straka, J. Trawick, and R. A. Davis. 1991. Activation of the silent endogenous cholesterol-7α-hydroxylase gene in rat hepatoma cells: a new complementation group having resistance to 25-hydroxycholesterol. *Mol. Cell. Biol.* **11:** 2049–2056.
13. Princen, H. M. G., C. M. G. Huijsmans, F. Kuipers, R. J. Vonk, and H. J. M. Kempen. 1989. Dexamethasone regulates bile acid synthesis in monolayer cultures of rat hepatocytes by induction of cholesterol-7α-hydroxylase. *Biochem. J.* **262:** 341–348.
14. Myant, N. B., and K. A. Mitropoulos. 1977. Cholesterol 7α-hydroxylase. *J. Lipid Res.* **18:** 135–153.
15. Hylemon, P. B., Y. C. Li, J. Y. Chiang, E. J. Studer, E. C. Gurley, D. M. Heuman, 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.
16. Jones, M. P., W. M. Pandak, D. M. Heuman, J. Y. L. Chiang, P. B. Hylemon, and Z. R. Vlahcevic. 1993. Cholesterol 7α-hydroxylase: evidence for transcriptional regulation by cholesterol or metabolic products of cholesterol in the rat. *J. Lipid Res.* **34:** 885–892.
17. Davis, R. A., P. M. Hyde, J-C. W. Kuan, M. Malone-McNeal, and J. Archambault-Schexnayder. 1983. Bile acid secretion by cultured rat hepatocytes. Regulation by cholesterol availability. *J. Biol. Chem.* **258:** 3661–3667.
18. Omland, E., and O. Mathisen. 1991. Effect of insulin on hepatic bile secretion during normoglycaemia and hyperglycaemia. *Scand. J. Gastroenterol.* **26:** 645–651.
19. Subbiah, M. T., and R. L. Yunker. 1984. Cholesterol 7α-hydroxylase of rat liver: an insulin sensitive enzyme. *Biochem. Biophys. Res. Commun.* **124:** 896–902.
20. Twisk, J., M. F. M. Hoekman, E. M. Lehmann, P. Meijer, W. H. Mager, and H. M. G. Princen. 1995. Insulin suppresses bile acid synthesis in cultured rat hepatocytes by down-regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase gene transcription. *Hepatology.* **21:** 501–510.
21. Dueland, S., J. Drisko, L. Graf, D. Machleder, A. J. Lusis, and R. A. Davis. 1993. Effect of dietary cholesterol and taurocholate on cholesterol-7α-hydroxylase and hepatic LDL receptors in inbred mice. *J. Lipid Res.* **34:** 923–931.
22. Pandak, W. M., Z. R. Vlahcevic, D. M. Heuman, K. S. Redford, J. Chiang, and P. B. Hylemon. 1994. Effects of different bile salts on steady-state mRNA levels and transcriptional activity of cholesterol 7α-hydroxylase. *Hepatology.* **19:** 941–947.
23. Pandak, W. M., Y. C. Li, J. Y. 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.* **266:** 3416–3421.

24. Davis, R. A., C. A. Musso, M. Malone-McNeal, G. R. Lattier, P. M. Hyde, J. Archambault-Schexnayder, and M. S. Straka. 1988. Examination of bile acid negative feedback regulation in rats. *J. Lipid Res.* **29:** 202–211.
25. Pandak, W. M., D. M. Heuman, P. B. Hylemon, J. Y. L. Chiang, and Z. R. Vlahcevic. 1995. Failure of intravenous infusion of taurocholate to down-regulate cholesterol 7α-hydroxylase in rats with bile fistulas. *Gastroenterology.* **108:** 533–544.
26. Davis, R. A., W. E. Highsmith, M. Malone-McNeal, J. Archambault-Schexnayder, and J-C. W. Kuan. 1983. Inhibition by mevinolin, but not by bile acids. *J. Biol. Chem.* **258:** 4079–4082.
27. Kubaska, W. M., E. C. Gurley, P. B. Hylemon, P. S. Guzelian, and Z. R. Vlahcevic. 1985. Absence of negative feedback control of bile acid biosynthesis in cultured rat hepatocytes. *J. Biol. Chem.* **260:** 13459–13463.
28. Kwekkeboom, J., E. M. Van Vorthuizen, H. M. G. Princen, and H. J. M. Kempen. 1988. Feedback inhibition of bile acid synthesis in cultured pig hepatocytes. *Biochem. Biophys. Res. Commun.* **155:** 850–856.
29. Stravitz, R. T., P. B. Hylemon, D. M. Heuman, L. R. Hagey, C. D. Schteingart, H. T. 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.
30. Crestani, M., W. G. Karam, and J. Y. L. Chiang. 1994. Effects of bile acids and steroid/thyroid hormones on the expression of cholesterol 7α-hydroxylase mRNA and the CYP7 gene in HepG2 cells. *Biochem. Biophys. Res. Commun.* **198:** 546–553.
31. Taniguchi, T., J. Chen, and A. D. Cooper. 1994. Regulation of cholesterol 7α-hydroxylase gene expression in Hep-G2 cells. Effect of serum, bile salts, and coordinate and noncoordinate regulation with other sterol-responsive genes. *J. Biol. Chem.* **269:** 10071–10078.
32. Chiang, J. Y. L., and D. Stroup. 1994. Identification and characterization of a putative bile acid-responsive element in cholesterol 7α-hydroxylase gene promoter. *J. Biol. Chem.* **269:** 17502–17507.
33. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162:** 156–159.
34. Aviv, H., and P. Leder. 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci. USA.* **69:** 1408–1412.
35. Kingston, R. E. 1994. Poly A RNA purification. *In:* Current Protocols in Molecular Biology, F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, ed., J. Wily, 4.5.1–4.5.3.
36. Derman, E., K. Krauter, L. Walling, C. Weinberger, M. Ray, and J. E. Darnell. 1981. Transcriptional control in the production of liver-specific mRNAs. *Cell.* **23:** 731–739.
37. Stolzenbach, F. 1966. Lactic dehydrogenases. *Methods Enzymol.* **9:** 278–288.
38. Noltmann, E. A. 1966. Phosphoglucose isomerase. *Methods Enzymol.* **9:** 557–565.
39. Mitropolous, K. A., and S. Balasubramaniam. 1976. The role of glucocorticoids in the regulation of the diurnal rhythm of hepatic β-hydroxy-β-methylglutaryl-coenzyme A reductase and cholesterol 7α-hydroxylase. *Biochem. J.* **160:** 49–55.
40. Stravitz, R. T., Z. R. Vlahcevic, E. C. Gurley, and P. B. Hylemon. 1995. Expression of cholesterol 7α-hydroxylase transcription by bile acids is mediated through protein kinase C in primary cultures of rat hepatocytes. *J. Lipid Res.* **36:** 1359–1369.
41. Messina, J. L., and R. S. Weinstock. 1992. Regulation of beta-actin gene transcription by insulin and phorbol esters. *Exp. Cell Res.* **200:** 532–535.
42. Gumucio, J. J. 1989. Hepatocyte heterogeneity: the coming of age from the description of a biological curiosity to a partial understanding of its physiological meaning and regulation. *Hepatology.* **9:** 154–160.
43. Jungermann, K., and N. Katz. 1989. Functional specialization of different hepatocyte populations. *Physiol. Rev.* **69:** 708–764.
44. Singer, I. I., D. W. Kawka, D. M. Kazazis, A. W. Alberts, J. S. Chen, J. W. Huff, and G. C. Ness. 1984. Hydroxymethylglutaryl-coenzyme A reductase-containing hepatocytes are distributed periportally in normal and mevinolin-treated rat livers. *Proc. Natl. Acad. Sci. USA.* **81:** 5556–5560.
45. Ugele, B., J. M. Kempen, R. Gebhardt, P. Meijer, H-J. Burger, and H. M. G. Princen. 1991. Heterogeneity of rat liver parenchyma in cholesterol 7α-hydroxylase and bile acid synthesis. *Biochem. J.* **276:** 73–77.
46. Jones, A. L., G. T. Hradek, R. H. Renston, K. Y. Wong, G. Karlaganis, and G. Paumgartner. 1980. Autoradiographic evidence for hepatic acinar concentration gradient of bile acid derivatives. *Am. J. Physiol.* **238:** G233–G237.
47. Groothois, G., M. J. Hardonk, K. P. T. Keulemans, P. Miervenhuis, and D. K. F. Meijer. 1982. Autoradiographic and kinetic demonstration of acinar heterogeneity of taurocholate transport. *Am. J. Physiol.* **243:** G455–G462.
48. Botham, K. M., M. E. Lawson, G. J. Beckett, I. W. Percy-Robb, and G. S. Boyd. 1981. The effect of portal blood bile salt concentrations on bile salt synthesis in rat liver. *Biochim. Biophys. Acta.* **920:** 68–75.
49. Balasubramaniam, S., K. A. Mitropolous, and N. B. Myant. 1972. Rhythmic changes in the activity of cholesterol 7α-hydroxylase in the livers of fed and fasted rats. *In* Bile Acids in Human Diseases. II. Bile Acid Meeting, Freiburg. P. Back and W. Gerok, editors. FK Schattauer Verlag, Stuttgart. 97–102.
50. Bortz, W. M., and L. A. Steele. 1973. Synchronization of hepatic cholesterol synthesis, cholesterol and bile acid content, fatty acid synthesis and plasma free fatty acid levels in the fed and fasted rat. *Biochim. Biophys. Acta.* **306:** 85–94.
51. Duane, W. C., M. L. Gilberstadt, and D. M. Wiegand. 1979. Diurnal rhythm of bile acid production in the rat. *Am. J. Physiol.* **236:** R175–R181.
52. LaRusso, N. F., M. G. Korman, N. E. Hoffman, and A. F. Hofmann. 1974. Dynamics of the enterohepatic circulation of bile acids. Postprandial serum concentrations of conjugates of cholic acid in health, cholecystectomized patients, and patients with bile acid malabsorption. *N. Engl. J. Med.* **291:** 689–692.
53. Danielsson, H. 1973. Effect of biliary obstruction on formation and metabolism of bile acids in rat. *Steroids.* **22:** 567–580.

54. Dueland, S., J. Reichen, G. T. Everson, and R. A. Davis. 1991. Regulation of bile acid homeostasis in bile-obstructed rats. *Biochem. J.* **280:** 373–377.
55. Heuman, D. M., P. B. Hylemon, and Z. R. Vlahcevic. 1989. Regulation of bile acid synthesis. III. Correlation between biliary bile acid hydrophobicity index and the activities of enzymes regulating cholesterol and bile acid synthesis in the rat. *J. Lipid Res.* **30:** 1161–1171.
56. Twisk, J., M. F. M. Hoekman, L. M. Muller, T. Iida, T. Tamaru, A. Ijzerman, W. H. Mager, and H. M. G. Princen. 1995. Structural aspects of bile acids involved in the regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase. *Eur. J. Biochem.* **228:** 596–604.
57. Hirano, F., H. Tanaka, Y. Makino, K. Okamoto, M. Inaba, T. Miura, and I. Makino. 1995. Regulation of the major histocompatibility complex class I mRNA expression by bile acids in cultured human hepatoma cells. *Biochem. Biophys. Res. Commun.* **208:** 935–942.
58. Podevin, P., Y. Calmus, A. Robert, R. Poupon, and G. Cherqui. 1994. Inhibition of procoagulant activity of human monocytes by chenodeoxycholic acid: involvement of protein kinase C. *Hepatology.* **19:** 1164–1170.
59. Hagenbuch, B., B. Steiger, M. Foquet, H. Lubbert, and P. J. Meier. 1991. Functional expression cloning and characterization of the hepatocyte Na^+/bile acid cotransport system. *Proc. Natl. Acad. Sci. USA.* **88:** 10629–10633.
60. Spady, D. K., and J. A. Cuthbert. 1992. Regulation of hepatic sterol metabolism in the rat. Parallel regulation of activity and mRNA for 7α-hydroxylase but not 3-hydroxy-3-methylglutaryl-coenzyme A reductase or low density lipoprotein receptor. *J. Biol. Chem.* **267:** 5584–5591.
61. Horton, J. D., J. A. Cuthbert, and D. K. Spady. 1995. Regulation of hepatic 7α-hydroxylase expression and response to dietary cholesterol in the rat and hamster. *J. Biol. Chem.* **270:** 5381–5387.