Different Hepatocytes Express the Cholesterol 7α-Hydroxylase Gene During Its Circadian Modulation *In Vivo*

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Cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile salt synthesis from cholesterol is a P450 enzyme (CYP7A). Its expression and activity are regulated by bile salts, cholesterol, hormones and a circadian modulator. Here we define the hepatocytes contributing to the expression of the rat CYP7A gene during its *in vivo* circadian variation. The diurnal expression of the CYP7A messenger RNA (mRNA) was studied by *in situ* hybridization and correlated with the diurnal rate of CYP7A gene transcription and mRNA expression. At 10 AM, the time of lowest mRNA expression and gene transcription rate, only four to five hepatocytes, located close to the hepatic venules ("perivenular"), contained the CYP7A mRNA. At 10 PM, the time of highest mRNA expression and fastest *in vitro* transcription rate, approximately one half of the hepatocytes (still in a "perivenular" location) contained the cholesterol 7α-hydroxylase mRNA. In addition, the measured half-life of the CYP7A mRNA was shorter at 10 AM than at 10 PM suggesting that posttranscriptional mechanisms also contributed to the observed circadian differences. Therefore, the basal transcription rate of the CYP7A gene is maintained by four to five "perivenular" hepatocytes. During the circadian variation, the rate of gene transcription increases in these "perivenular" hepatocytes, but in addition, there is recruitment of other more proximal hepatocytes to transcribe the gene. It is proposed here that the response of specific hepatocytes to the various modulators of CYP7A gene expression is dependent on the relative position of these hepatocytes within the liver cell plate. (HEPATOLOGY 1995;21:1658-1667.)

Abbreviations: CYP7A, cytochrome P450 superfamily; mRNA, messenger RNA; HMGCoA, hepatic hydroxymethyl glutaryl coenzyme A; GLUT-1; erythroid/brain glucose transporter; ip, intraperitoneal; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, edetic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered solution; cDNA, complementary DNA; GLUT 2, liver glucose transporter; CTP, counts per minute; GTP, glutamyl transpeptidase; SDS, sodium dodecyl sulfate; C7αh, cholesterol 7α-hydroxylase.

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Cholesterol 7α-hydroxylase is a member of the cytochrome P450 superfamily (CYP7A), and it catalyzes the 7α-hydroxylation of cholesterol, the first and rate-limiting step in bile salt synthesis.[1] In liver, hepatocytes or parenchymal liver cells are responsible for the expression of this gene. The expression of the cholesterol 7α-hydroxylase gene is modulated by the concentration of bile salts[2] and cholesterol[3] as well as by hormones.[4,5] Bile salts exert a negative feedback regulation, and cholesterol feeding increases cholesterol 7α-hydroxylase messenger RNA (mRNA) and activity.[2,6] Both glucocorticoids and thyroid hormone increase cholesterol 7α-hydroxylase gene expression.[4,5] In addition, an interesting characteristic of cholesterol 7α-hydroxylase gene expression is that it follows a diurnal variation.[7] In the rat, cholesterol 7α-hydroxylase activity, protein mass, and mRNA levels exhibit a parallel circadian variation with peak expression at 10 PM and the lowest level of expression at 10 AM.[8] These data suggest that the diurnal variation is regulated at a pretranslational level. Another peculiarity of cholesterol 7α-hydroxylase expression is that the activity of the enzyme does not seem to be distributed uniformly among hepatocytes; rather it is predominantly located in a subgroup of hepatocytes.[9]

In this study, our objective was to define the pattern of cholesterol 7α-hydroxylase gene expression in the liver cell plate during its diurnal variation. Nuclear run-off assays were used to characterize the diurnal pattern of expression of the cholesterol 7α-hydroxylase gene at the transcriptional level. The hepatocytes which contribute to the diurnal expression of the gene have been identified by *in situ* hybridization and finally, the mechanisms that regulate the diurnal variation in expression of this gene in the liver cell plate have been partially defined. The results indicate that the cholesterol 7α-hydroxylase gene seems to be transcribed, under basal conditions, by few hepatocytes located very close to the hepatic venules. However, at times of maximal diurnal expression, other hepatocytes located closer to portal venules are recruited for gene transcription. Therefore, the circadian modulator(s) may increase transcription of the cholesterol 7α-hydroxylase gene by acting on different hepatocytes at various times of the day. In addition to the circadian differences in transcriptional activity of hepatocytes, we suggest that variations in the half-life of the choles-

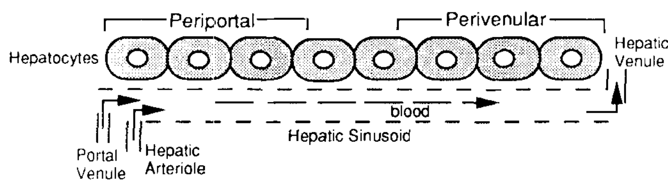


FIG. 1. The liver cell plate

terol 7α-hydroxylase mRNA also contribute to the circadian pattern of expression of this gene in the liver cell plate.

The liver cell plate, the basic structural element of liver tissue, consists of 15 to 25 hepatocytes, organized as a 1-cell-thick cord, which extends from a terminal portal venule to a hepatic venule (Fig. 1). Each cell plate is perfused unidirectionally, from portal to hepatic venule, with a dual blood supply originating in the portal venules and hepatic arterioles.[10] The cell plate represents the location where liver function is accomplished. During the last 20 years, it has become apparent that liver function is the result of the integration of multiple physiological processes that occur at different levels in the cell plate.[11] This compartmentation of function within the cell plate is caused, in part, by variations in hepatocyte phenotype, which, in turn, depend on the position these cells occupy in the cell plate. Thus, different genes are expressed by hepatocytes located closer to either the portal or the hepatic venules, as illustrated by Fig. 1. The position-dependent differential expression of genes in the cell plate results in the acquisition of diverse functional capabilities by hepatocytes. For instance, the hepatic hydroxymethyl glutaryl coenzyme A (HMGCoA) reductase gene is expressed in a few hepatocytes which surround the portal tracts.[12] In contrast, glutamine synthetase is expressed at the other end of the cell plate by few hepatocytes which surround the hepatic venules.[13] Similarly, the erythroid/brain glucose transporter or GLUT-1 gene is expressed on the cell membrane by one or two hepatocytes surrounding the hepatic venules.[14] Studies performed at one time interval have shown that rat liver cholesterol 7α-hydroxylase activity (determined in hepatocyte subpopulations) and bile salt synthesis predominate in hepatocytes located closer to hepatic venules or "perivenular" hepatocytes.[9] The explanation for this localization was not apparent. Therefore, the study of cholesterol 7α-hydroxylase gene expression in liver implies not only the definition of the mechanisms involved in the pretranslational modulation of the gene but also the characterization of the molecular mechanisms involved in the compartmentation of gene expression in selected hepatocytes in the liver cell plate.

Within this context, there are three elements that make the study of cholesterol 7α-hydroxylase gene regulation particularly interesting. First, bile salts have been identified as a major regulator of this gene.[15] There is evidence that bile salts inhibit the transcriptional activity of the cholesterol 7α-hydroxylase gene.[16,17] However, neither the role that bile salts play in regulating the physiological transcriptional activity of the gene *in vivo* nor the location of hepatocytes in the cell plate where this modulation occurs has been defined. Similarly, the role that hormones and other potential regulators play in the expression of the cholesterol 7α-hydroxylase gene *in vivo* has to be clarified. Second, the specific hepatocytes contributing to the expression of the gene during its diurnal variation and the extracellular signal(s) that trigger the circadian rhythm of expression have to be identified. Third, the role that the different modulators play *in vivo* and the possible interactions among modulators need to be elucidated.

In this study, we have defined that the diurnal expression of the cholesterol 7α-hydroxylase gene *in vivo* is a dynamic phenomenon involving, most likely, changes in the transcriptional activity of different hepatocytes. In addition, we propose that the hepatocytes responding to the circadian modulators of the CYP7A gene are located in a different position in the cell plate than are hepatocytes subjected to the main inhibitory action of bile salts.

MATERIALS AND METHODS

Animal Model. All experiments were performed using nonfasted, male Fischer 344 rats weighing 150 to 200 g exposed to a 12-hour light:dark cycle (lights on from 6 AM to 6 PM). Except when otherwise noted, livers were harvested at 10 AM. Anesthesia was induced by chloral hydrate 5% (1 cc/100 g body weight), administered by intraperitoneal (ip) injection. The care and use of the animals described in this protocol were in accordance with the provisions of the United States Department of Agriculture Animal Welfare Act, the United States Public Health Service *Guide for the Care and Use of Laboratory Animals,* and the United States Interagency Research Committee Principles for Animal Utilization.

Nuclei Isolation. Nuclei isolation and nuclear protein extraction were performed according to Hattori.[18] Livers were harvested, homogenized, and centrifuged in a sucrose gradient at 24,000 rpm for 60 minutes (Beckman SW28 rotor, Fullerton, CA). All work was performed on ice. For nuclear run-off assays, nuclei were counted on a hemocytometer and 2 to 3 × 10^8 nuclei were resuspended in 300 μL of nuclei storage buffer (25% glycerol, 50 mmol/L HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.6, 3 mmol/L $MgCl_2$, 0.1 mmol/L edetic acid [EDTA], 1.0 mmol/L dithiothreitol [DTT], and 0.1 mmol/L phenylmethylsulfonyl fluoride [PMSF]). The nuclei were frozen in liquid nitrogen and stored at −80°C for <1 week.

Preparation of Membranes for Nuclear Run-Off Assays. Slot blots were prepared with Nytran membranes (Schleicher and Schuell, Keene, NH) per the manufacturer's protocol. Five μg of each plasmid was loaded in duplicate, and the DNA was irreversibly bound to the Nytran membrane with ultraviolet light.

The plasmids were as follows: (1) the cholesterol 7α-hydroxylase plasmid, phosphate-buffered solution (PBS)-Sac7, contained 2.1 kb of the complementary DNA (cDNA) for rat cholesterol 7α-hydroxylase subcloned into PBS and was a generous gift of Dr D. Russell, Southwestern Medical Center, Dallas, TX,[19] (2) the liver glucose transporter (GLUT-2) contained 900 bp of the GLUT-2 cDNA subcloned into pGEM-4

and was generously provided by Dr M. Birnbaum, Harvard University, Boston, MA, and (3) the rRNA plasmid, pBC2, contained 8.4 kb subcloned into pBR322 and was generously provided by Dr K. Zaret, Brown University, Providence, RI.[20] The parent plasmids, pBS, pBR322, and pGEM-4Z, were also immobilized on Nytran for each experiment to serve as controls.

Nuclear Run-Off Transcription Assay. The nuclear run-off assay was performed according to Celano[21] with modifications. The assays were performed with approximately 2×10^8 nuclei in a total volume of 500 μL transcription buffer (35% glycerol, 30 mmol/L HEPES, pH 7.6, 5 mmol/L $MgCl_2$, 80 mmol/L KCl, 0.1 mmol/L EDTA, 0.5 mmol/L DTT, 1 mmol/L CTP, 1 mmol/L adenosine triphosphate [ATP], 1 mmol/L glutamyl transpeptidase, 40 μL [α-^{32}P]UTP [Amersham, Arlington Heights, IL], 3,000 Ci/mmole). As a control for nonspecific binding of RNA polymerase II independent transcripts, one set of nuclei per experiment was treated with 2 μg of α-amanitin and allowed to incubate on ice for 3 minutes before the addition of the nucleotides. The nuclei were incubated for 25 minutes at 27°C. The nuclei were then treated with 10 μL RNase-free DNase I (10 mg/mL) and 25 μL $CaCl_2$ (20 mmol/L) for 5 minutes at 27°C. Proteins were degraded with 5 μL proteinase K (10 mg/mL), and 62.5 μL 10 × SET (5% sodium dodecyl sulfate [SDS], 50 mmol/L EDTA, 100 mmol/L tris Cl, pH 7.4), and then transfer RNA was added. The sample was incubated at 37°C for 30 minutes. Nuclear RNA was isolated with guanidinium thiocyanate solution, extracted with phenol:chloroform, and ethanol precipitated. The radioactively labeled, elongated RNA transcripts (1×10^7 cpm/mL hybridization fluid) were hybridized to a previously prepared Nytran membrane (Schleicher and Schuell, Keene, NH) at 60°C for 16 to 18 hours. Membranes were washed for 3 hours with 6 wash changes containing decreasing concentrations of SSC (0.15 mol/L NaCl, 0.015 mol/L Na citrate). The final wash solution was 0.1 × SSC, 0.1% SDS. Membranes were quantitated with an Ambis Radiodetection system (San Diego, CA) and then autoradiography was performed at −70°C with an intensifying screen. The film (Hyperfilm, Amersham) was developed at 24, 48, and 96 hours. The data were analyzed using a Student's *t*-test.

Northern Blots. The plasmid containing 2.1 kilobases of the cholesterol 7α-hydroxylase cDNA, pBS-Sac7, was digested with *Eco*RI, and the cholesterol 7α-hydroxylase insert was isolated in a low melting point agarose gel (Sea Plaque, FMC Bioproducts, Rockland, ME). As a control, the glyceraldehyde-3-phosphate dehydrogenase cDNA subcloned into PBS, PBS-GAPDH/rat, (generously provided by Dr Jeffrey Ross, McArdle Laboratory for Cancer Research, Madison, WI)[22] was digested with *Hin*dIII and *Xba*I, and the insert was similarly isolated. The probes were prepared in gel using the random primer method (Multiprime Labelling Kit, Amersham, Arlington Heights, IL) and [α-^{32}P]dCTP (Amersham, 3,000 Ci/mmole) according to the manufacturer's directions. The specific activity of all of the probes was greater than 1×10^9 cpm/μg DNA.

Total RNA from each liver was extracted using TRIzol (Bethesda Research Laboratories, Bethesda, MD) according to the manufacturer's directions. RNA integrity was assessed on agarose-formaldehyde gels, and poly (A)+ RNA was isolated using the PolyATract IV system (Promega, Madison, WI). Three micrograms of poly (A)+ RNA were separated electrophoretically on a 1% formaldehyde-agarose gel. The RNA was transferred to Nytran Maximum Strength membranes (Schleicher and Schuell, Keene, NH) using the capillary blotting method according to the manufacturer's directions and then immobilized on the membranes using ultraviolet light crosslinking. The membranes were prehybridized in 5 × SSC, 5% SDS, 5× Denhardt's (50× contains 10 g of each of the following per liter: Ficoll, Type 400, polyvinylpyrrolidone, and bovine serum albumin, fraction 5), and 100 μg/mL salmon sperm DNA at 60°C for at least 2 hours. The membranes were hybridized for 16 to 20 hours using 1×10^6 counts per minute of probe/mL hybridization fluid, and then washed at 60°C in solutions containing 0.1% SDS and decreasing concentrations of SSC. The final wash was 0.1 × SSC, 0.1% SDS. Membranes were quantitated with an Ambis Radiodetection system, and then autoradiography was performed at −70°C with an intensifying screen. The film (Hyperfilm, Amersham, Arlington Heights, IL) was developed at 24, 48, and 96 hours.

In situ *Hybridization.* The plasmid containing cholesterol 7α-hydroxylase, PBS-Sac7, was linearized with *Sfu*I or with *Hin*fI. The Riboprobe Gemini II kit (Promega) was used according to the manufacturer's directions to generate ^{35}S-UTP labeled (Amersham) antisense and sense RNA probes of 175 bp and 143 bp in length, respectively. Livers were harvested, immediately placed on ice, cut into 0.5-cm^3 pieces, immersed in O.C.T. compound and then quick frozen in dry ice/ethanol. Eight-micrometer (Miles, Elkhart, IN) frozen sections were thawed and then fixed in 4% paraformaldehyde for 2 minutes, followed by 70% ethanol for 10 minutes at room temperature. The tissue was progressively rehydrated in decreasing concentrations of ethanol and then treated with acetic anhydride and 0.1 mol/L triethanolamine, pH 8.0 for 10 minutes. As controls, some of the slides were pretreated with 20 μg/mL RNase A for 30 minutes at 37°C. The tissue was dehydrated in increasing concentrations of ethanol. Then 25 μL of hybridization mixture (50% formamide, 100 mmol/L DTT [dithiothreitol], 1× TE [10 mmol/L tris, 1 mmol/L EDTA, pH 7.5], 0.6 mol/L NaCl, 1 mg/mL yeast transfer RNA, 100 μg/mL salmon sperm DNA, 2% dextran, and 1×10^5 cpm/μL riboprobe) was applied to the slide, covered, and maintained in a moist environment for 16 to 20 hours at 55°C. The slides were soaked in 2 × SSC, 50% formamide for 10 minutes to remove the cover slip, and then incubated at 37°C for 50 minutes in 20 μg/mL RNase A. The slides were washed in decreasing concentrations of SSC, 10 mmol/L 2-mercaptoethanol at 37°C such that the final dilution of SSC was 0.1×. The slides were allowed to air dry overnight, and autoradiography using Kodak (Science Park, New Haven, CT) NTB2 emulsion was performed. The relative density of silver grains along the liver cell plate was quantitated using an image analysis system. The data were analyzed using one-way ANOVA. Multiple comparisons between groups were achieved by application of the Tukey's Studentized Range Test.

mRNA Half-Life Experiments. For the mRNA half-life experiments, half of the animals were exposed to a reverse light cycle (lights on from 6 PM to 6 AM) for at least 2 months. The adequacy of the light cycle reversal was evaluated at 4 weeks. The livers from two reversed animals were harvested at 10 AM and 10 PM, and Northern analysis was performed using poly (A)+ RNA extracted from these livers. The results of the Northern analysis indicated that the diurnal rhythm of cholesterol 7α-hydroxylase had indeed reversed. The half-life studies were performed by administering α-amanitin (50 μg/100 g body weight, Sigma, St. Louis, MO) and actinomycin D (150 μg/100 g body weight, Sigma) by intraperitoneal injection at 10 AM to animals under normal and reversed lighting conditions. These agents were chosen in combination to ensure that transcriptional activity was quickly inhibited and that the inhibition was prolonged.[23] Run-off experiments

were performed at 0.5 and 7 hours after injection to evaluate whether transcription was adequately inhibited. At both time points, the transcription rate of cholesterol 7α-hydroxylase was inhibited by at least 90% of control value. To assess mRNA decay, livers were harvested from 0.5 to 5 hours after injection. A total of 16 livers from the rats under the conditions of reversed lighting (10-PM equivalent) and 15 livers from control (10 AM) rats were analyzed. The half-life curves were determined by regression analysis using the computer program Systat. Statistical differences between the slopes of the 10 AM and 10 PM time curves were determined using the t-test with 27 degrees of freedom.

RESULTS

Diurnal Variation in the Location of Cholesterol 7 α-Hydroxylase mRNA in the Liver Cell Plate. To assess the contribution of different hepatocytes in the liver cell plate to the diurnal variation in cholesterol 7α-hydroxylase mRNA, *in situ* hybridization was performed using livers harvested at 10 AM, 2 PM, and 10 PM. Following hybridization and autoradiography, the distribution of grains (corresponding to cholesterol 7α-hydroxylase mRNA) was semiquantitated using an image analysis system. As illustrated in the schematic diagram at the top of Fig. 2B, only liver cell plates that measured from 350 μm to 420 μm were analyzed. Each liver cell plate, from portal to hepatic venule, was divided into 10 boxes of equal area. Therefore, each box represented approximately two hepatocytes. The optical density of the area in the boxes was measured. At least 10 liver cell plates were analyzed from each of three different animals.

The distribution of cholesterol 7α-hydroxylase mRNA in the liver cell plate at 10 PM, the time of maximal expression, is shown in Fig. 2A. The autoradiographic grains were distributed heterogeneously with the majority of grains detected over the one half to two thirds of the hepatocytes located closer to the terminal hepatic venule. Few grains were noted over periportal hepatocytes. Pretreatment with RNase A eliminated this pattern of hybridization. Likewise, the sense RNA probe, which served as an additional control, hybridized at low levels in a uniform pattern. Using an image analysis system, the density of the grains was semiquantitated, and expressed as a gradient relative to the measurement of the box located closest to the portal venule (i.e., the first box in the schematic diagram at the top of Fig. 2B was assigned a value of 1). In Fig. 2B, each point on the graph represents 1 of the 10 boxes created when the liver cell plates were analyzed. At 10 PM, there was approximately a sevenfold difference between the first periportal and last perivenular measurement. Approximately 10 perivenular hepatocytes contained most of the grains. In comparison, at 10 AM, in Fig. 2C and 2D, the overall number of grains was less subtle and the gradient was more subtle than at 10 PM. The gradient was approximately 2.5-fold. Most of the grains were distributed at this time in the five hepatocytes closest to the hepatic venule. At 2 PM, the grains were distributed in an even more restricted number of hepatocytes, corresponding to the two to three hepatocytes contiguous to the hepatic venule, as shown in Fig. 2E and 2F. Therefore, at each time interval, the perivenous hepatocytes had the highest content of cholesterol 7α-hydroxylase mRNA. However, the number of perivenous hepatocytes containing high levels of mRNA decreased during the day.

Diurnal Variation in mRNA: Northern Analysis. Initially, we evaluated the magnitude of the diurnal variation of cholesterol 7α-hydroxylase mRNA in the Fischer 344 strain of rats under the food, light, and other environmental conditions routinely followed at our institution. Cholesterol 7α-hydroxylase has been studied in several rat strains.[8,19,24,25] These studies have shown that there is a marked variability in the magnitude of the diurnal variation of mRNA between strains of rats. The Fischer 344 strain was chosen for our studies because these rats are genetically more homogeneous and have been shown to show less interanimal variability in the expression of other cytochromes P450.[26] Northern analysis using the Fischer 344 rats confirmed the previously published data obtained in this strain.[24] Figure 3A is a representative Northern blot. As previously described, on Northern blots, cholesterol 7α-hydroxylase mRNA appears as two major bands (3.6 and 2.4 kb) and two minor bands (4.7 and 1.9 kb).[19] It has been suggested from RNA blotting experiments that the smaller bands are degradation products of the 4.7 kb species.[27] Quantitation of the 3.6 kb band, the most abundant species, using an Ambis radiodetector, revealed that in this strain of rats, the mRNA varied approximately 2.4-fold over the course of the day, with peak levels in the evening (Fig. 3B).

Diurnal Variation in Transcription Rate: Nuclear Run-Off Assays. To assess the mechanisms responsible for the diurnal variation in cholesterol 7α-hydroxylase mRNA, we determined transcription rates at 10 AM and 10 PM. Nuclear run-off assays were performed using nuclei isolated from total liver at each time interval. In Fig. 4A, the cholesterol 7α-hydroxylase cDNA (C7αh) is in the top row at each of the time points. The liver glucose transporter, GLUT-2, was used as a control as its relative rate of transcription does not vary throughout the day. Hybridization to the ribosomal RNA cDNA was used to normalize the data. The parent plasmids were also included on the blots to assess for background hybridization. In addition, at least two sets of nuclei at each of the time points were treated with α-amanitin before elongating the nascent transcripts to assess for nonspecific RNA polymerase II-independent transcript hybridization. Pretreatment with α-amanitin decreased hybridization by greater than 90% in all cases (data not shown).

As illustrated in Fig. 4B, at 10 PM, the time of maximal expression, the transcription rate of the cholesterol 7α-hydroxylase gene was also maximal. The transcriptional activity at 10 PM was approximately 1.6-fold higher than the transcription rate at 10 AM, the time of lowest mRNA accumulation. The difference in transcription rate between 10 AM and 10 PM was statistically significant (P = .019). These results are a mean

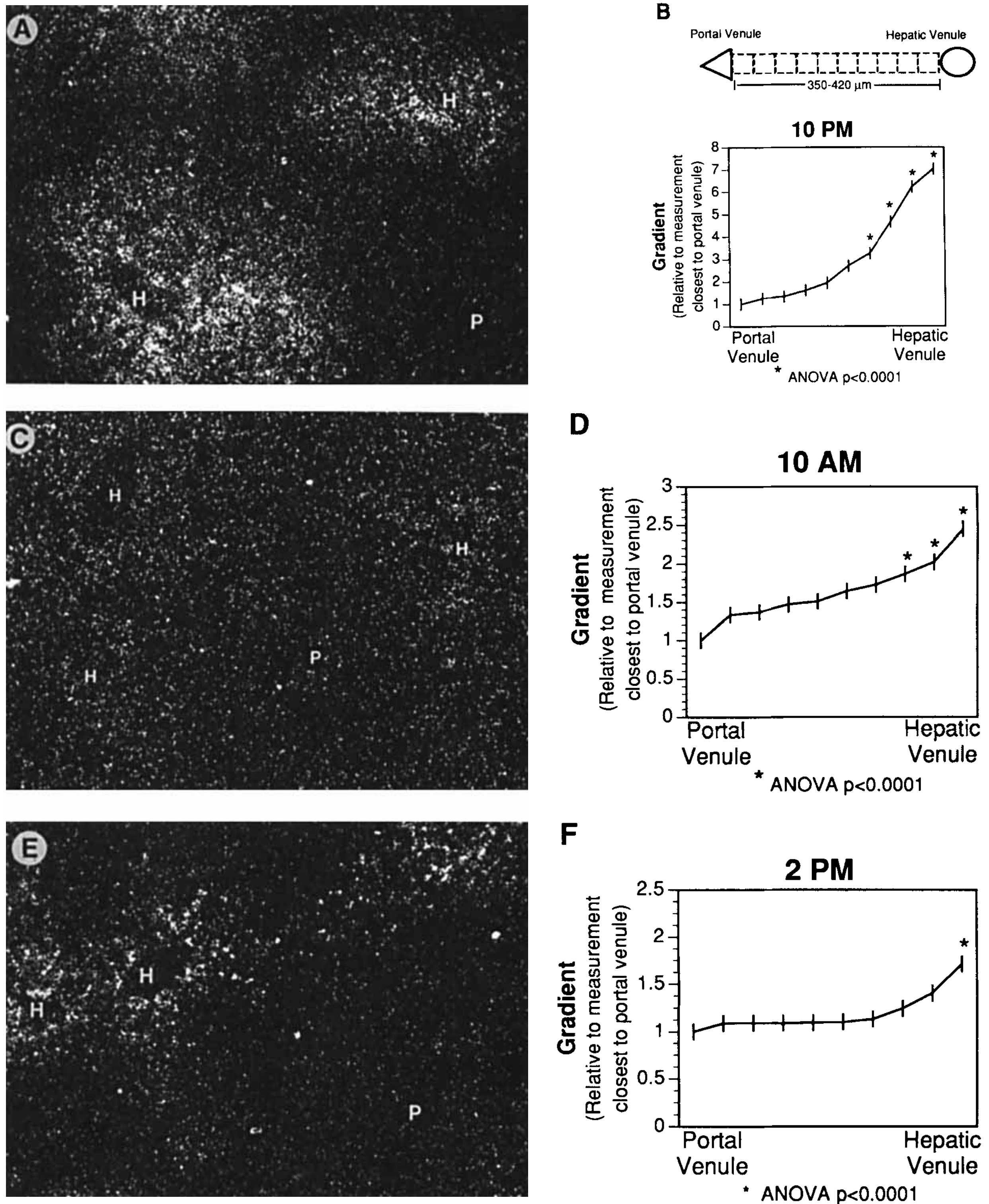


FIG. 2. *In situ* hybridization: Relative distribution of cholesterol 7α-hydroxylase mRNA in the liver cell plate during the diurnal cycle. Preparation of tissue sections, and hybridization and washing conditions are described in the Materials and Methods section. A, C, and E represent dark-field photographs of hybridizations from livers harvested at 10 PM, 10 AM, and 2 PM, respectively. B, D, and F show semiquantitation of the relative density of grains along the liver cell plate at 10 PM, 10 AM, and 2 PM, respectively. At least 10 liver cell plates from each of three different rats were analyzed. The results of each measurement were expressed relative to the first periportal measurement at each time point (the first portal measurement was arbitrarily assigned a value of 1). Statistical analysis = ANOVA; Tukey's Studentized Range Test. H, terminal hepatic venule; P, terminal portal venule.

of a minimum of three and a maximum of four different experiments (different nuclear preparations) per time point.

The *in situ* hybridization experiments showed the distribution of cholesterol 7α-hydroxylase mRNA in different hepatocytes. This distribution may reflect differences in the transcriptional activity among hepatocytes, in the half-life of mRNA or both. Therefore, an attempt was made to have a more direct measurement of the transcriptional activity of hepatocyte populations isolated from the proximal (periportal) or distal (perivenular) side of the liver cell plate. Periportal and perivenous hepatocytes were isolated by the digitonin-collagenase method of Lindros with modifications recently

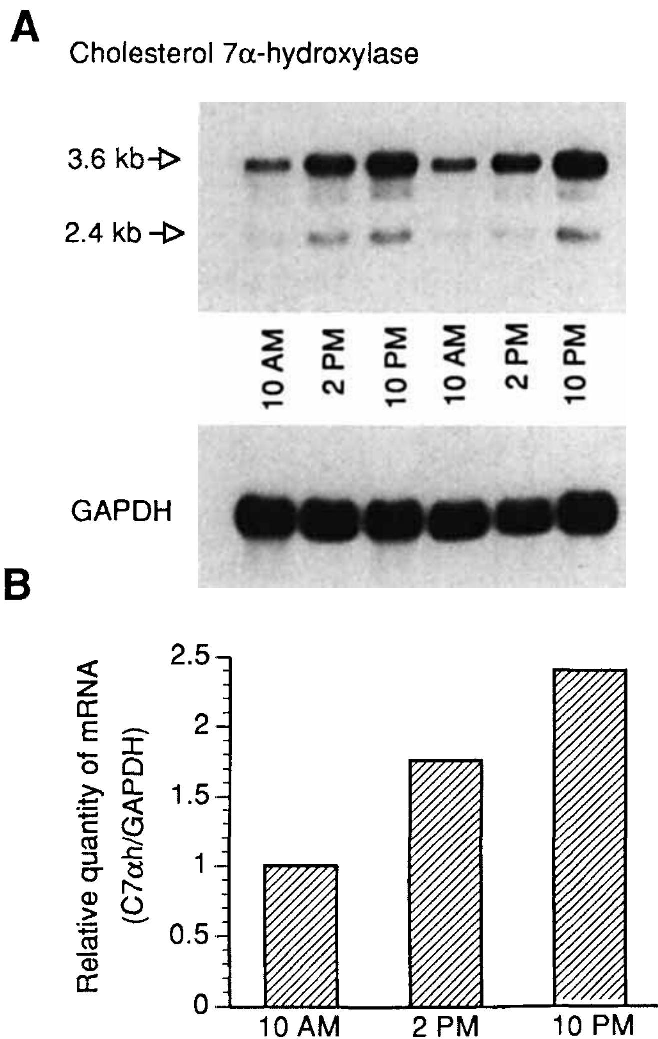
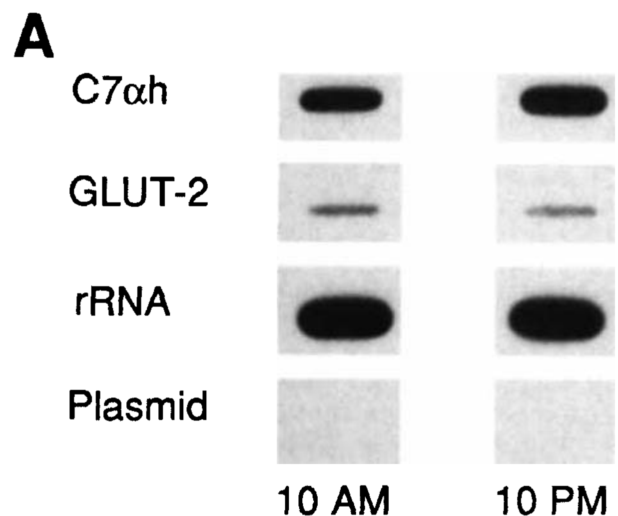


FIG. 3. Northern analysis of cholesterol 7α-hydroxylase mRNA in liver during the diurnal cycle. (A) Livers were harvested at the times indicated and poly (A)+ RNA extracted. Three micrograms of poly (A)+ RNA was separated electrophoretically, transferred to Nytran membranes, and hybridized as described in the Materials and Methods. After autoradiography was performed, the membranes were stripped and reprobed with a ^{32}P-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA to evaluate RNA loading. The RNA lanes are indicated in the figure. This is one representative sample of 4 individual experiments in each group. The autoradiogram was exposed for 48 hours and 12 hours for cholesterol 7α-hydroxylase and GAPDH, respectively. (B) β emissions from the most abundant band, identified by the 3.6 kb arrow, were quantitated using an Ambis radiodetector and expressed relative to the 10 AM measurement. The cholesterol 7α-hydroxylase data were normalized using relative GAPDH hybridization in each lane.



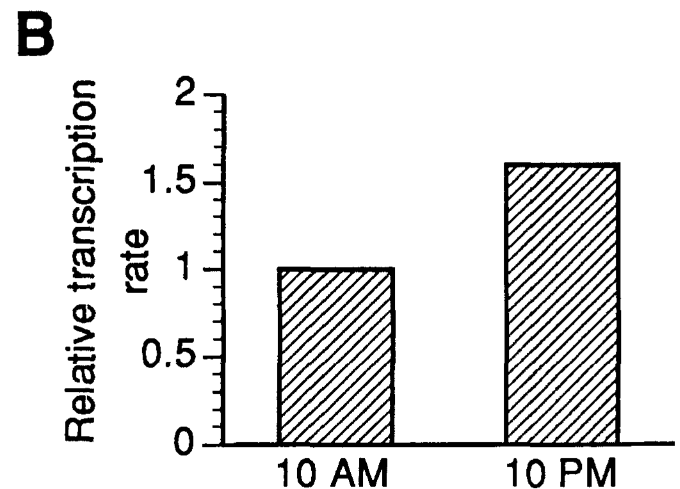


FIG. 4. Relative transcriptional activity of the cholesterol 7α-hydroxylase gene during the diurnal cycle. (A) Nuclear run-off assays were performed using isolated nuclei from livers harvested at 10 AM and 10 PM. RNA transcripts generated in each transcription assay were hybridized against blots containing duplicate 5-μg samples of each of the plasmids as described in the Materials and Methods section. Both the GLUT-2 and the rRNA cDNA served as controls because the relative transcription rates of each of these genes do not vary appreciably throughout the day. The plasmid was used to control for nonspecific/background hybridization. This is one representative sample of three to four individual experiments in each group. The autoradiogram was exposed for 48 hours. The transcription rates at 10 AM and 10 PM were significantly different as assessed by the *t*-test (P = .019). C7αh, cholesterol 7α-hydroxylase; GLUT-2, the liver-type glucose transporter; rRNA, ribosomal RNA cDNA; plasmid, pBluescript. (B) The transcription signals for cholesterol 7α-hydroxylase were quantitated using an Ambis radiodetector and expressed relative to the 10 AM measurement.

described.[28,29] The procedure of hepatocyte subpopulation isolation takes approximately 3 hours. Subsequently, nuclei were isolated.[18] Nuclear run-off assays performed with these processed nuclei showed that the relative transcription rates of all the genes tested, including several acute phase reaction genes, had decreased by at least 90%. It was concluded that these results were unacceptable for analysis and that the direct approach to the assessment of transcriptional activity in subpopulations of hepatocytes was not feasible.

Diurnal Variation in mRNA Half-Life. To assess whether posttranscriptional mechanisms contributed to the observed circadian changes, the decay rate of cholesterol 7α-hydroxylase mRNA was determined at 10 AM and 10 PM. In preparation for the experiments, several rats were placed in a room with reversed lighting (6 PM to 6 AM was light). After 1 month, Northern analysis was performed on poly (A) + RNA extracted from the livers of rats harvested at 10 AM and 10 PM and confirmed that the time of maximal cholesterol 7α-hydroxylase mRNA content had reversed, i.e., was at 10 AM (data not shown). After this was confirmed, rats were further assessed to determine whether the administration of α-amanitin and actinomycin D inhibited transcription. Nuclei were isolated 30 minutes and 7 hours after injection, and nuclear run-off assays were performed. The relative transcription rates were decreased by greater than 90% at each of these time points (data not shown). Then the mRNA half-life was determined beginning at 10 AM for rats maintained under both normal and reversed lighting. The half-life of cholesterol 7α-hydroxylase mRNA at 10 AM under physiological conditions was 102 minutes (Fig. 5). In contrast, the half-life at 10 AM under reversed lighting (equivalent of 10 PM) was 66 minutes. The slopes of the curves representing the rate of mRNA decay were significantly different (P = .0097). These data support the possibility that the diurnal variation in cholesterol 7α-hydroxylase is under posttranscriptional as well as transcriptional regulation.

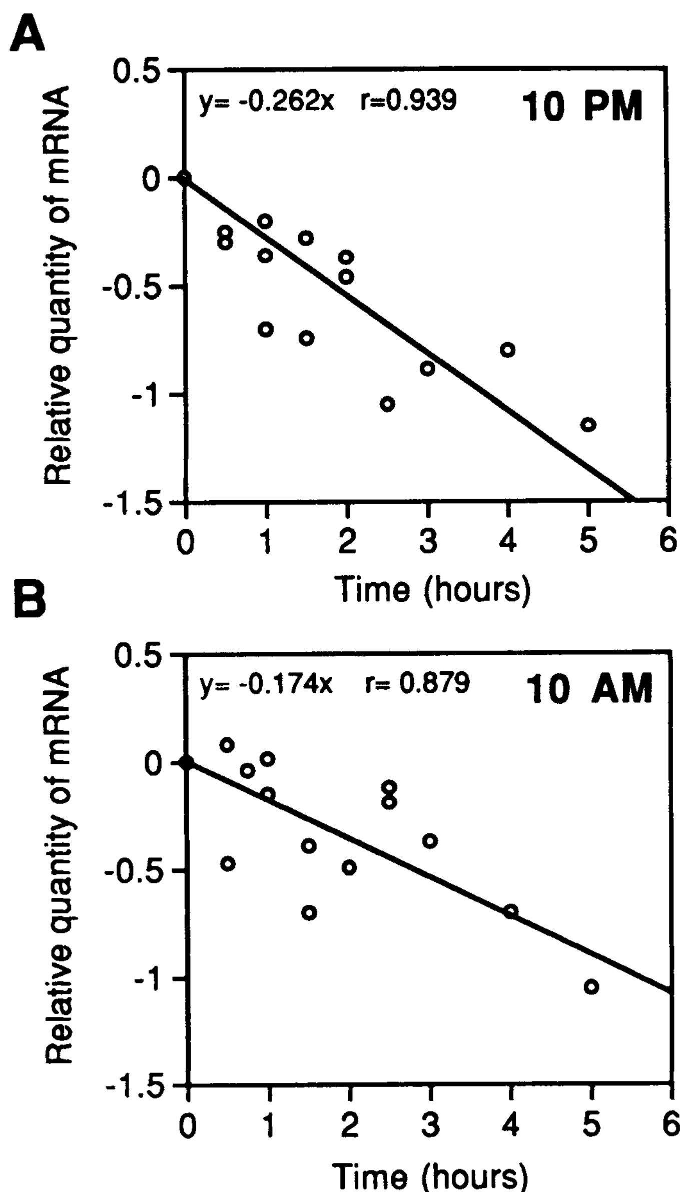


FIG. 5. The rate of cholesterol 7α-hydroxylase mRNA decay at 10 AM and 10 PM. As described in the Materials and Methods section, half of the rats were placed under reversed lighting conditions such that the time of highest cholesterol 7α-hydroxylase mRNA levels was at 10 AM (verified by Northern analysis). Transcription was inhibited in all animals at 10 AM with a combination of α-amanitin (50 μg/100 g body weight) and actinomycin D (150 μg/100 g body weight), and livers were harvested at various time intervals thereafter. The relative quantity of cholesterol 7α-hydroxylase mRNA was assessed by Northern analysis and quantitated using an Ambis radiodetector. The half-life of cholesterol 7α-hydroxylase mRNA was 66 minutes and 102 minutes at 10 PM and 10 AM, respectively. (A) 10 PM refers to the rats placed under reversed lighting conditions, i.e., 10 AM experiment time was equivalent to 10 PM under normal lighting conditions; (B) 10 AM refers to the rats tested under normal lighting conditions. Curves were generated by regression analysis using the computer program, Systat. The slopes of the 10 AM and 10 PM curves were significantly different as assessed by the t-test (P = .0097).

DISCUSSION

It is known that bile salts, hormones, and the diurnal cycle modulate the expression of the cholesterol 7α-hydroxylase gene.[2,4,5,8,15,19] However, the relative importance of each modulator in determining the physiological expression of the gene as well as the interactions among modulators *in vivo* are unknown. It has been proposed that bile salts may represent long-term modulators that set the level of expression of the gene. Food and hormones have been proposed to be short-term regulators, whereas the diurnal variation serves as a medium-term modulator.[24] In this work, we have studied the diurnal variation in cholesterol 7α-hydroxylase gene expression. We propose that to understand the relative role of these observed modulators on cholesterol 7α-hydroxylase gene expression *in vivo,* studies have to take into account that the effects of regulators may be exerted on different hepatocytes.

We have shown by *in situ* hybridization that the number of hepatocytes expressing detectable quantities of cholesterol 7α-hydroxylase mRNA in the liver cell plate varies throughout the day. In these experiments, at 10 AM, the time of the lowest cholesterol 7α-hydroxylase gene transcription rate, the corresponding mRNA was located in four to five hepatocytes that surround hepatic venules. At 10 PM, the time of the fastest transcription rate, the location of the cholesterol 7α-hydroxylase mRNA had extended more proximally (from the hepatic venule toward the portal venule) to encompass approximately 8 to 10 of the 15 to 20 hepatocytes comprising the cell plate. The initial five to six "periportal" hepatocytes were not apparently affected by the diurnal stimulus. At 2 PM, the number of hepatocytes containing the cholesterol 7α-hydroxylase mRNA was restricted to three to four "perivenular" hepatocytes. From these data, it would appear that the basal expression of the cholesterol 7α-hydroxylase gene in the cell plate is maintained, at least in part, by transcription of the gene in the three to four "perivenular" hepatocytes.

The rate of gene transcription as assessed by nuclear run-off assays also varies throughout the day. These findings suggest that different hepatocytes are recruited to transcribe the gene at various times of the day. This recruitment may imply that the more proximal hepatocytes transcribe the gene either only at the time of recruitment or that at these times, transcription becomes faster and easier to detect. Regardless, the data suggest that the circadian variation in transcription rate of the cholesterol 7α-hydroxylase gene can be explained both by variations in the number of hepatocytes transcribing the gene as well as by an increased rate of gene transcription in the hepatocytes engaged in basal transcription. This is supported by the increase in grain density observed at 10 PM in the three to four perivenular hepatocytes.

Northern blot analysis showed that the level of cholesterol 7α-hydroxylase mRNA at 10 PM was 2.4-fold higher than the level at 10 AM. In an attempt to further define the mechanisms involved in the regulation of the circadian variation in cholesterol 7α-hydroxylase expression, the half-life of cholesterol 7α-hydroxylase mRNA was measured. Previously, the half-life of the cholesterol 7α-hydroxylase enzyme activity was estimated to be 2 to 4 hours.[30-32] In these experiments, we measured the half-life of the mRNA after inhibition of cholesterol 7α-hydroxylase gene transcription by a combination of α-amanitin (to ensure rapid inhibition of transcription, and thus allow calculations of initial changes) and actinomycin D.[23] The *in vivo* mRNA half-life measurements showed that at 10 PM, the mRNA half-life was 1.1 hours, approximately 35% shorter than at 10 AM. Correlation of these results with data obtained by *in situ* hybridization showed that cholesterol 7α-hydroxylase mRNA was located, at 10 PM, in approximately two thirds of all hepatocytes (approximately 10 hepatocytes), whereas at 10 AM, grains were more restricted in location and distributed in approximately three to four perivenular hepatocytes. One possibility that explains the differences in mRNA half-life is that all cholesterol 7α-hydroxylase mRNA present at 10 PM had a shorter half-life than at 10 AM, regardless of its location. Another possibility is the existence in different hepatocytes of populations of cholesterol 7α-hydroxylase mRNA with different half-lives. The half-life of the mRNAs recruited at the time of maximal transcription and present in hepatocytes more proximal in location may be shorter than the half-life of the mRNA existing in hepatocytes located in a more restricted perivenular location. At present, we cannot distinguish between the two proposed alternatives. Regardless, the circadian variation in cholesterol 7α-hydroxylase expression in different hepatocytes in the cell plate seems to be modulated by both transcriptional and posttranscriptional events.

Ideally, for a definitive answer, the transcription rates of the cholesterol 7α-hydroxylase gene and mRNA half-lives should be measured in hepatocyte populations isolated from periportal and perivenular locations. However, at present, this experiment is not feasible. The isolation of hepatocyte subpopulations takes 3 hours.[29] The longest half-life of cholesterol 7α-hydroxylase mRNA measured here was 1.7 hours. Therefore, at least two, if not three, half-lives would have passed by the time the hepatocyte subpopulations could be examined. Also, the assessment of transcription rates using hepatocyte subpopulations has serious limitations. In these experiments, the transcription rate of several genes was measured using nuclei isolated from hepatocyte subpopulations. The transcriptional activity was found to be uniformly low.

Because the diurnal rhythm is synchronized by the photoperiod,[33] it is plausible that the circadian changes in cholesterol 7α-hydroxylase gene expression may be initially precipitated by neuroendocrine factors. Reversal of the photoperiod for several days results in a 12-hour shift in levels of expression, i.e., the highest levels of cholesterol 7α-hydroxylase mRNA, protein, and activity occur during the daytime dark period.[8] One possible modulator is cortisol. The pattern of the diurnal variation of cortisol is similar to that of cholesterol 7α-hydroxylase, except that the peak and nadir levels of cortisol precede the corresponding levels of cholesterol 7α-hydroxylase by approximately 3 to 4 hours.[34,35] In contrast, the absence of glucocorticoids, as attained by bilateral adrenalectomy in rats, has been shown to either abolish the diurnal variation of cholesterol 7α-hydroxylase activity or to decrease the amplitude of the peaks.[1,36,37] In addition, the circadian changes in the transcriptional activity of the cholesterol 7α-hydroxylase gene parallel the changes in the protein content of the transcription factor, DBP.[38] Recently, *in vitro* structural and functional experiments revealed that DBP bound to the cholesterol 7α-hydroxylase promoter and increased transcription of the gene.[39]

Our objective in these experiments was to define the pattern of expression of the cholesterol 7α-hydroxylase gene in the cell plate during its circadian variation. The

approximate distribution of cholesterol 7α-hydroxylase enzyme activity in the cell plate has already been described.[9] The cholesterol 7α-hydroxylase enzyme activity was severalfold higher in short-term cultures of perivenous hepatocytes compared with periportal cells.[9] In addition, as described, the mRNA and activity have similar half-lives. Given these data, and because cholesterol 7α-hydroxylase mRNA levels, protein mass, and enzyme activity follow a parallel circadian variation,[8] it is most likely that the protein is expressed during the day in a distribution similar to that of the mRNA. The lack of a reliable antibody has precluded us from defining the distribution of the protein during the diurnal cycle.

What may be the relationship between the circadian variation in gene expression and the inhibitory regulation of the cholesterol 7α-hydroxylase gene exerted by bile salts? Several studies have suggested that bile salts play a major role in the regulation of cholesterol 7α-hydroxylase activity.[15] Increasing concentrations of bile salts in hepatocytes inhibit the enzyme (negative feed-back regulation) while decreasing concentrations by bile salt depletion stimulate it.[40-42] In addition, recently, several studies have shown that bile salts inhibit the transcriptional activity of the cholesterol 7α-hydroxylase gene.[16,17,43] However, the role that bile salts play on the regulation of gene transcription *in vivo* has not been defined. Studies using autoradiography have shown that bile salts are taken up by hepatocytes in a heterogeneous fashion.[44,45] Thus, when the bile salt concentration in the portal blood is low, the content of bile salts is highest in "periportal" hepatocytes. Increasing the concentration of bile salts in portal blood results in recruitment of more distal hepatocytes for bile salt transport. From these data, it would be expected that the inhibitory action of bile salts on cholesterol 7α-hydroxylase gene transcription would be highest at the periportal side of the liver cell plate, and lowest at the perivenular side. Therefore, if bile salts were the only regulators of gene expression, "perivenular" hepatocytes should transcribe the cholesterol 7α-hydroxylase gene at the fastest rate.

Bile salt concentration in blood also undergoes a circadian variation.[46] However, the circadian variation in bile salt concentration cannot explain the circadian variation in cholesterol 7α-hydroxylase gene expression. Thus, in the rat, the serum level of bile salts, the concentration of bile salts in portal vein blood, and presumably, the level of bile salts in hepatocytes are lowest at approximately 10 AM.[46] Given the inhibitory effect of bile salts on the gene, if bile salts were responsible for the diurnal modulation of gene expression it would be expected that, at 10 AM, many or most hepatocytes in the cell plate should be involved in gene expression. This was not the case. At 10 AM, both the transcription rate and the number of hepatocytes expressing the gene were at the lowest levels. Moreover, hepatocytes were recruited to transcribe from a perivenular toward a periportal location at a time (10 PM) when the bile salt and cholesterol content of portal blood were the highest.[46] Therefore, neither the level of cholesterol 7α-hydroxylase gene transcription nor the location of the hepatocytes expressing the gene during its *in vivo* circadian variation can be explained by the circadian changes in bile salt concentration.

Of interest, the circadian modulator(s) stimulates cholesterol 7α-hydroxylase gene transcription predominantly in perivenular hepatocytes, which in the cell plate, are under the lowest inhibitory effect of bile salts. Conversely, at the time of maximal circadian stimulation of gene expression, 10 PM, the four to six more proximal periportal hepatocytes are not recruited for transcription. These "periportal" cells are exposed to the highest concentration of bile salts in sinusoidal blood. Therefore, we propose that the pattern of cholesterol 7α-hydroxylase gene expression *in vivo* may be determined, in part, by the inhibitory effect of bile salts acting on periportal hepatocytes and the stimulatory action of "the circadian modulator(s)" acting on perivenular hepatocytes.

In summary, it is proposed that the understanding of the relative role that modulators play in cholesterol 7α-hydroxylase gene expression *in vivo* requires, as shown here, the identification of the hepatocytes on which these modulators exert their *in vivo* action. These experiments provide the first evidence that the *in vivo* expression of genes in selected hepatocytes can be regulated by the diurnal cycle.

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