Synchronous circadian rhythms of mRNA levels and activities of cholesterol 7α-hydroxylase in the rabbit and rat[1]

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Abstract Cholesterol 7α-hydroxylase, the key enzyme in a series of metabolic changes for the production of bile acids from cholesterol, shows circadian rhythms. The mechanism of the circadian rhythms is the subject of controversy; moreover, few pieces of information are presently available. Because the rabbit is a good animal model for the study of human cholesterol–bile acid metabolism, in the present study we isolated a complete cDNA encoding rabbit cholesterol 7α-hydroxylase (3022 base pairs (bp) long and 1503 bp open-reading frame encoding 501 amino acid residues). Using this isolate rabbit cDNA and the rat cDNA as probes, the circadian rhythms of this enzyme were studied in normal male rabbits and rats. The animals were maintained on a 12-h light–dark cycle. Three subjects each were then killed every 3 h for 1 day. The livers were harvested, and the mRNA levels and the activities of the enzyme were measured. Both mRNA levels and activities of the enzyme showed remarkable circadian rhythms, with higher values during the dark phase and lower values reaching minimum during the light phase. The enzyme activities correlated well with the mRNA levels. There was no shift in the cycles of the enzyme activities and the mRNA levels. These findings suggest that pre-translational regulation is the most likely mechanism for the circadian rhythms of cholesterol 7α-hydroxylase. Because the rhythms of mRNA levels and activities of this enzyme are synchronous, the turnover of the enzyme should be as fast as that of the mRNA, and we can hereafter measure the mRNA levels instead of the enzyme.—**Kai, M-H., T-A. Eto, K-H. Kondo, Y. Setoguchi, S. Higashi, Y. Maeda, and T. Setoguchi.** Synchronous circadian rhythms of mRNA levels and activities of cholesterol 7α-hydroxylase in the rabbit and rat. *J. Lipid Res.* 1995. **36:** 367–374.

Supplementary key words cDNA cloning • transcriptional activity • pre-translational regulation

Cholesterol 7α-hydroxylase (EC 1.14.13.17), which is localized in the endoplasmic reticulum of only hepatic parenchymal cells, is the rate-limiting enzyme in the bile acid biosynthetic pathway from cholesterol. This metabolic pathway plays a critical role in cholesterol homeostasis in mammalian systems. Changes in the rate of cholesterol metabolism and bile acid synthesis may be important contributing factors to various pathophysiological states including atherosclerosis, hyperlipoproteinemia, hyperlipidemia, coronary heart disease, and gallstones (1).

Although the precise mechanism should be amended in the light of molecular biology, the activity of this enzyme has been thought to be regulated in three ways: long-term, mid-term, and short-term regulation (2). The bile acid biosynthesis controlled by a negative feedback mechanism shows long-term regulation. This feedback depends on the flux and composition of bile acids that undergo enterohepatic circulation (3).

Enzyme activities that show a circadian rhythm, with enzyme activities high at night and low during the day in the rat (4, 5), are defined as mid-term regulation. This rhythm is maintained in the animals in which the long-term regulation was disturbed as in cholestyramine-fed and bile-fistula rats, indicating that it is independent of the long-term regulation (2).

Finally, cholesterol 7α-hydroxylase is responsive to short- to mid-term modulation by various hormones, drugs, and cholesterol precursors (6, 7), which can modulate cholesterol 7α-hydroxylase activities in a matter of hours.

Although the circadian rhythms of cholesterol 7α-hydroxylase activities have been previously documented in the rat (4, 5, 8–11), no information, except for a report on the mouse (9), is presently available on the existence of similar phenomena in other animal species. The rabbit seems to be a good animal model for the investigation of the regulation of this enzyme in humans because *1)* the

Abbreviations: (k)bp, (kilo)base pairs; C7 αH, cholesterol 7α-hydroxylase; DMSO, dimethyl sulfoxide; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger RNA; SDS, sodium dodecyl sulfate. The nucleotide sequence reported in this paper has been submitted to the GenBank™/EMBL Data Bank with the accession number L10754.

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species has bile acids similar to humans; *2)* bile acids (which are C-24 steroids, the principal final metabolites of cholesterol after it is split off its side chain) can be hydroxylated at the 6 and 7 position in the rat and mouse, but not in humans and the rabbit; and *3)* humans, mice, and the rabbit have a gallbladder, but the rat does not. Therefore, we carried out molecular cloning of cDNA for rabbit liver cholesterol 7α-hydroxylase to investigate the regulatory mechanism of the transcriptional activity of this enzyme.

The mechanism of circadian rhythm in the rat has been the subject of controversy. Noshiro, Nishimoto, and Okuda (4) and Sundseth and Waxman (5) suggested that the enzyme activities correlated well with the mRNA levels in the rat, indicating that pre-translational regulation was a mechanism for the circadian rhythm. Recently, Lavery and Schibler (12) offered additional evidence of the pre-translational regulation, stating that the rat albumin D-element-binding protein was a regulator of the circadian rhythm of rat cholesterol 7α-hydroxylase at the transcriptional level. Li, Wang, and Chang (10), however, suggested that the enzyme activities and protein levels were regulated post-translationally, because of discrepancies between mRNA levels and both enzyme activities and protein levels. Therefore, further examination has been required to resolve the issue.

In this study, we describe the structure of the isolated complete cDNA corresponding to the rabbit liver cholesterol 7α-hydroxylase. In addition, we report on the circadian rhythms of both mRNA levels and activities of this enzyme in normal male rabbits and rats after measuring them at eight time points per day. We also discuss whether the circadian rhythms of the activities reflect the mRNA levels.

MATERIALS AND METHODS

Materials

Oligo (dT) cellulose was purchased from Pharmacia (Uppsala, Sweden). The DNA ligation kit and restriction enzymes were from Takara Shuzo Co., Ltd. (Tokyo, Japan). Nitrocellulose filters came from Schleicher & Schuell (Dassel, Germany), and the λZAP-cDNA synthesis kit and Gigapack II packaging extract were from Stratagene (La Jolla, CA). Multiprime DNA labeling systems and [α-^{32}P]dCTP were obtained from Amersham (Buckinghamshire, U.K.). Fluorescent primers and a cycle sequencing kit from Applied Biosystems (Foster City, CA) and nylon membranes from Bio-Rad (Hercules, CA) were used. Cholesterol oxidase (from *Streptomyces violascence*) was supplied from Toyo Jozo Co., Ltd. (Tokyo, Japan). All other reagents used were of the highest quality commercially available.

The rat cholesterol 7α-hydroxylase cDNA used as a probe for library screening and determination of the mRNA of rat cholesterol 7α-hydroxylase was supplied by Dr. Kyu-Ichiro Okuda (Hiroshima University).

Animals

Male New Zealand White rabbits (1.0–1.5 kg initial weight and 5–7 weeks old) and male Wistar rats (6 weeks old) were obtained from Kuroda Laboratory Center (Kumamoto, Japan). To prepare the RNA for the rabbit liver cDNA library, a male New Zealand White rabbit was fed appropriate food containing 3% (w/w) cholestyramine for 1 week in order to induce cholesterol 7α-hydroxylase (4). To study the circadian rhythms, male New Zealand White rabbits and male Wistar rats were housed in an appropriate room where lighting was exclusively artificial, automatically turned on at 8 AM and off at 8 PM. They were fed ad libitum normal food (ORC 4 from Oriental Yeast Co., Ltd., Tokyo, Japan for rabbits and CE-2 from Clea Japan, Inc., Tokyo, Japan for rats), and acclimated to this food and a light–dark cycle for 1 week before the experiment. Three rabbits and three rats were killed every 3 h over a complete 24-h day under general anesthesia using ether. Livers were harvested after the animals were killed. The livers were excised to prepare RNA and microsomes immediately.

cDNA cloning and nucleotide sequencing

Total RNA was prepared from 0.5 g of cholestyramine-fed male rabbit liver by the guanidium thiocyanate–phenol–chloroform extraction method (13), and this RNA was applied to an oligo(dT) cellulose column (14) to isolate poly(A^+) RNA. The poly(A^+) RNA was used to construct a λZAP cDNA library (14, 15). The DNAs ligated to the λZAP were packaged, and the resultant phage particles were plated on XL1-Blue. The λZAP cDNA library was screened using a random hexanucleotide ^{32}P-labeled probe (16) corresponding to the rat cholesterol 7α-hydroxylase cDNA. Hybridization was performed with the radioactive probe for 12 h in 20% (v/v) formamide, 0.1% (w/v) each of Ficoll (Type 400, Pharmacia), polyvinylpyrrolidone, and bovine serum albumin, 0.9 M NaCl, 0.06 M NaH_2PO_4, 6 mM EDTA, 0.1% (w/v) sodium dodecyl sulfate (SDS), and 100 μg/ml denatured salmon sperm DNA at a temperature of 37°C. Washing was performed once in a solution containing 300 mM NaCl, 30 mM sodium citrate, 0.5% (w/v) SDS at 50°C for 30 min, and twice in a solution containing 75 mM NaCl, 7.5 mM sodium citrate, 0.1% (w/v) SDS at 50°C for 30 min. Forty nine positive clones were identified of which one had the longest cDNA insert of 3.0 kbp. For DNA sequence determination, deletion mutants of the clone were prepared by using the Exo/mung bean nuclease deletion system (17), and the cDNA fragments generated by different restriction endonucleases were subcloned into a pBluescript II vector. DNA sequencing was carried out on both strands by the dideoxy nucleotide

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chain termination method using a Taq Dye Primer Cycle Sequencing Kit and ABI 373A DNA Sequencer (Applied Biosystems) (18).

Northern blot hybridization

Twenty μg of total RNA for each sample was denatured in 6 M glyoxal, DMSO (dimethyl sulfoxide) and 0.1 M sodium phosphate (pH 7.0) at 50°C, and electrophoresed in a 1% agarose gel containing 10 mM sodium phosphate (pH 7.0) (14). RNAs were transferred to nylon membranes (Zeta-Probe Blotting Membrane, Bio-Rad) by blotting (VacuGene™ XL Vacuum blotting System, Pharmacia) with a transfer buffer of 3 M NaCl, 300 mM sodium citrate, pH 7.0. To fix the RNAs to the membranes, they were exposed to 120 mJ of ultraviolet irradiation (UV Stratalinker™ 1800, Stratagene), and then boiled with a solution of 15 mM NaCl, 1.5 mM sodium citrate, pH 7.0, for 20 min. The membranes were prehybridized in a solution containing 20% (v/v) formamide, 0.1% (w/v) each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin, 0.9 M NaCl, 0.06 M NaH_2PO_4, 6 mM EDTA, 0.1% (w/v) SDS, and 100 μg/ml denatured salmon sperm DNA. They were then hybridized overnight at 37°C in the same solution containing the radioactive probe. The membranes were washed and exposed to X-ray films for 2 days at −70°C.

Quantitation of cholesterol 7α-hydroxylase mRNA level

The relative amounts of cholesterol 7α-hydroxylase mRNA were determined by the Northern blot hybridization method. Total RNAs, prepared from the livers of rabbits and rats, were hybridized with the radioactive probes of cholesterol 7α-hydroxylase cDNA, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA as an internal control. The exposed X-ray films were developed and scanned using a laser densitometer (LKB 2202 ultroscan laser densitometer, Pharmacia), and the intensities of the bands were quantitated.

Assay for cholesterol 7α-hydroxylase activity

Cholesterol 7α-hydroxylase activity was assayed according to the method of Ogishima and Okuda (19) using internal microsomal cholesterol as the substrate (20). Protein concentration was determined by the method of Lowry et al. (21) using bovine serum albumin as the standard.

Statistical analysis

Statistical comparisons were made using Student's *t*-test for unpaired data. The differences in comparisons were judged significant at the $P < 0.05$ level. The data expressed in the figure are the means and standard errors of the means.

RESULTS

Nucleotide and amino acid sequences of rabbit cholesterol 7α-hydroxylase

The rabbit liver cDNA library constructed in the λZAP vector was screened and 49 positive clones out of approximately 3×10^5 clones were isolated by the plaque hybridization method. The longest clone (p7α-147), approximately 3.0 kbp, was subcloned into pBluescript II vector by using the in vivo excision method (15). The restriction map for p7α-147 and its sequencing strategy is shown in **Fig. 1.** The complete nucleotide sequence and its predicted amino acid sequence of rabbit cholesterol 7α-hydroxylase derived from p7α-147 cDNA clone is shown in **Fig. 2.** The overall length of the cDNA was 3022 bp including the poly(A^+) tail. This was coincident with the mRNA size (3.0 kbp) estimated by the Northern blot hybridization analysis (data not shown). It contains a 1503 bp open-reading frame encoding 501 amino acid residues (M_r = 58,097) as well as 84 bp of a 5'-untranslated region and 1432 bp of a 3'-untranslated region. A consensus sequence for the heme-binding domain of cytochrome P450 was observed at the residues 434–454. This sequence is underlined in Fig. 2.

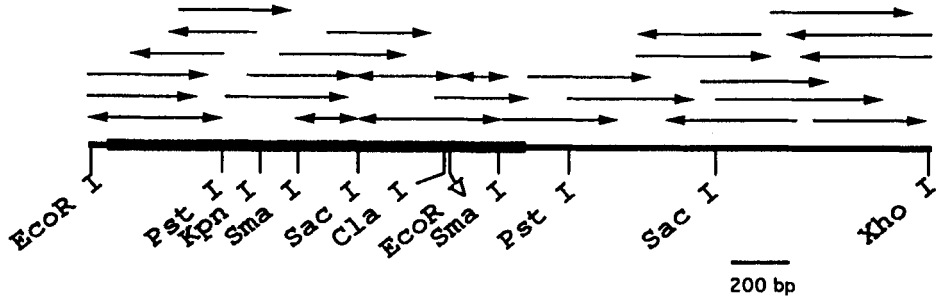

Fig. 1. Restriction map and sequencing strategy of p7α-147. Arrows indicate the direction and extent of sequencing. EcoRI and XhoI sites at both ends are multiple cloning sites of a pBluescript vector.

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Quantitation of cholesterol 7α-hydroxylase mRNA level

The quantitation of the densitometry of cholesterol 7α-hydroxylase mRNA was evaluated as follows. We performed a Northern blot hybridization analysis of the total RNA of a rabbit liver treated with cholestyramine to obtain the standard curve of the relative amounts of cholesterol 7α-hydroxylase mRNA. Relative absorbances of the hybridized areas in X-ray films were quantitated in a range of 2–40 μg of the total RNAs in a typical analysis. This produced a linear response ($y = 0.165x + 0.044$, $r^2 = 0.969$) to the amounts of total RNAs in a range of 0.5 to 8.0 of the relative absorbance units.

Circadian rhythms of cholesterol 7α-hydroxylase

In order to appreciate the circadian rhythms of

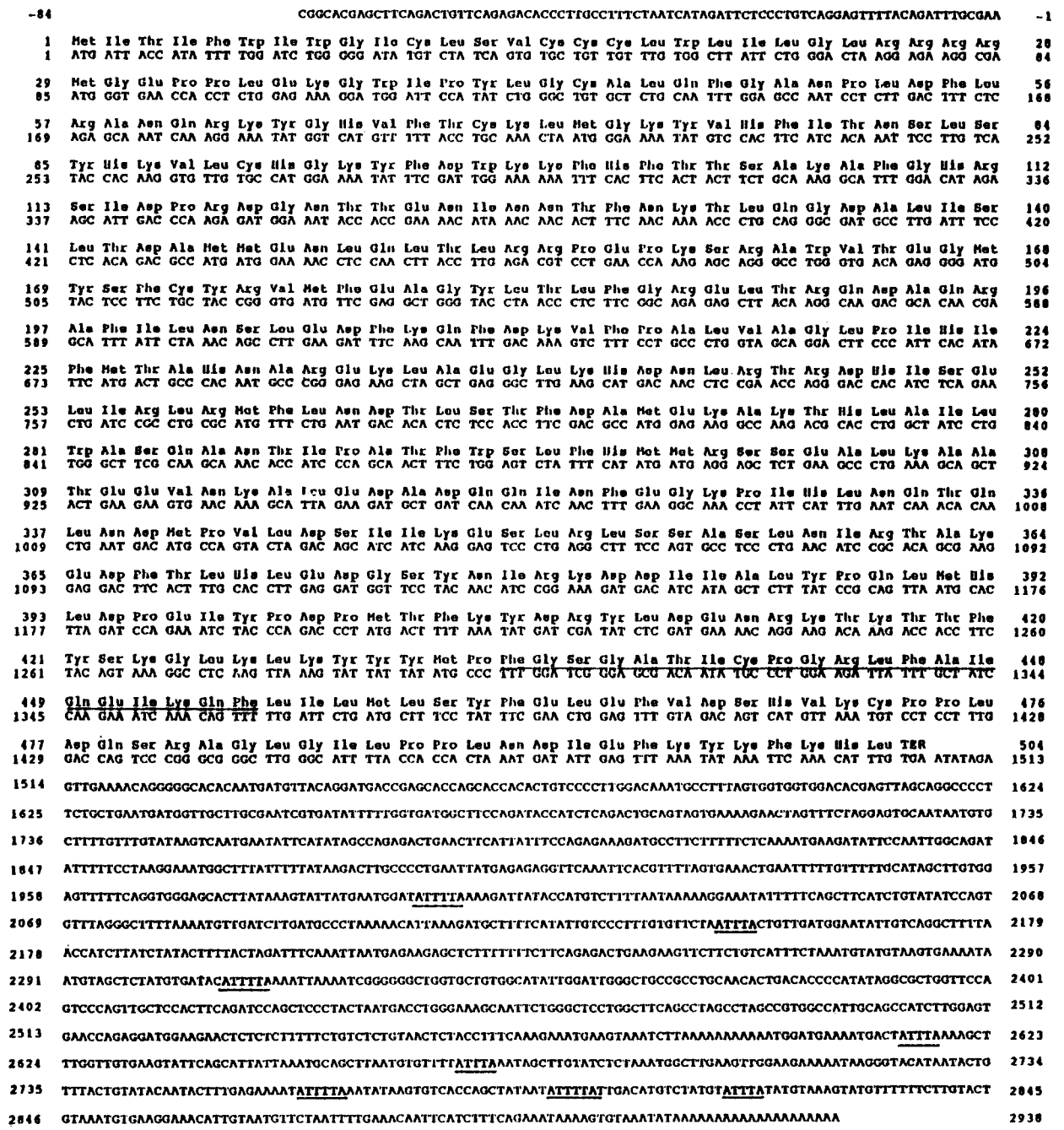

Fig. 2. Nucleotide sequence of the cDNA corresponding to the mRNA for rabbit cholesterol 7α-hydroxylase and the predicted amino acid sequence of the protein. Position 1 corresponds to the first nucleotide of the ATG triplet coded for the initiator methionine. The nucleotides on the 5' side of position 1 are indicated by negative numbers. The consensus sequence for the heme-binding domain of cytochrome P450 is underlined at residues 434 to 454. A motif, ATTTA, and nucleotides similar to it in the 3'-untranslated region are also underlined.

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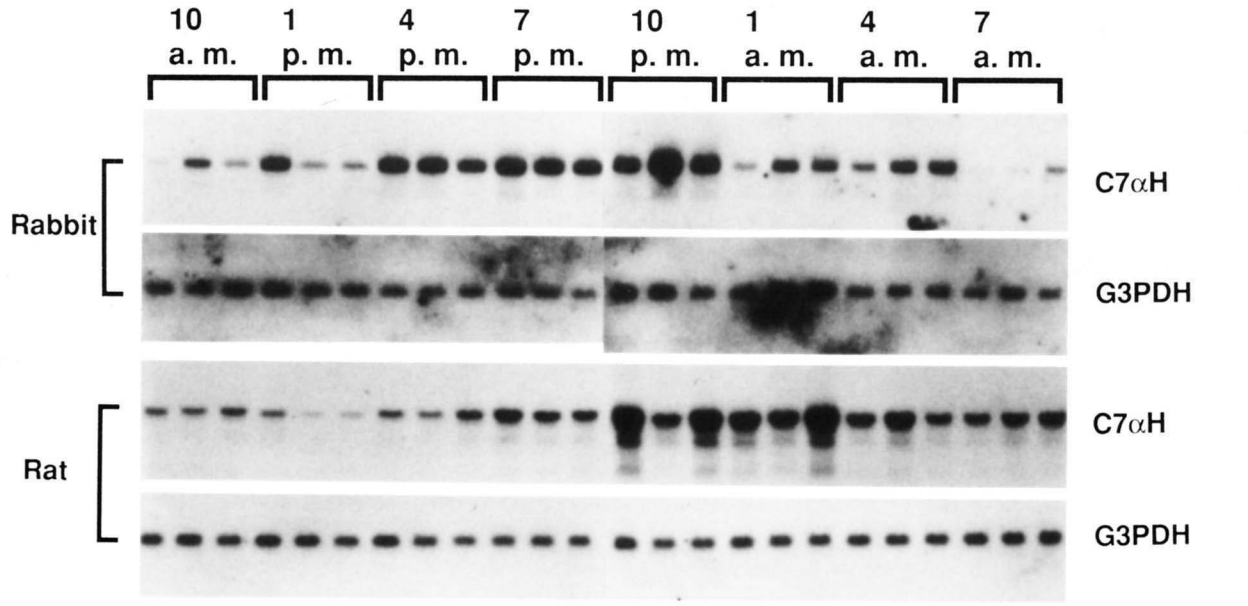

Fig. 3. Circadian rhythms of the mRNA levels of cholesterol 7α-hydroxylase (C7αH) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) for rabbits and rats. Northern blot hybridization analyses with probes of C7αH cDNA and G3PDH cDNA are shown. Twenty μg of total RNAs isolated from the rabbit and rat livers prepared at various times of the day was denatured and electrophoresed and then transferred to nylon membranes. The membranes were then hybridized with each radioactive probe. X-ray films were exposed to the radioactivities for 2 days at −70°C.

cholesterol 7α-hydroxylase in the rabbit and rat, their activities and mRNA levels were measured immediately after killing in both species of animals. They were killed every 3 h over a complete 24-h day. The Northern blot hybridization analyses of the rabbits and rats with the probes of cholesterol 7α-hydroxylase cDNA and G3PDH cDNA as an internal control were performed (**Fig. 3**). Although dispersion was found in each group, the intensities of the mRNA bands of cholesterol 7α-hydroxylase in both species varied according to the time of preparation. On the other hand, the mRNA bands of G3PDH showed about the same intensities all through a day for both species.

Cholesterol 7α-hydroxylase activities and mRNA levels of the rabbits and rats were quantitated (**Fig. 4**). The circadian rhythms of cholesterol 7α-hydroxylase activities and mRNA levels were clearly detectable in the rabbit as well as in the rat. In each species, both enzyme activities and mRNA levels exhibited the minimum level during the day time and the maximum level at night. In the rabbit, the mRNA levels were very low during the morning hours, minimum at 7 AM. At about 4 PM, the mRNA levels began to rise, reached maximum at 10 PM, and declined sharply during the night. In the rat, the mRNA levels were minimum at 1 PM, and at about 7 PM began to rise, reached maximum at 10 PM and declined continuously to 1 PM. In both species, the enzyme activities correlated well with the mRNA levels, although not proportionately. Statistically significant differences ($P < 0.05$, some: $P < 0.01$) were observed in both species between the minimum level and some of the other inter-

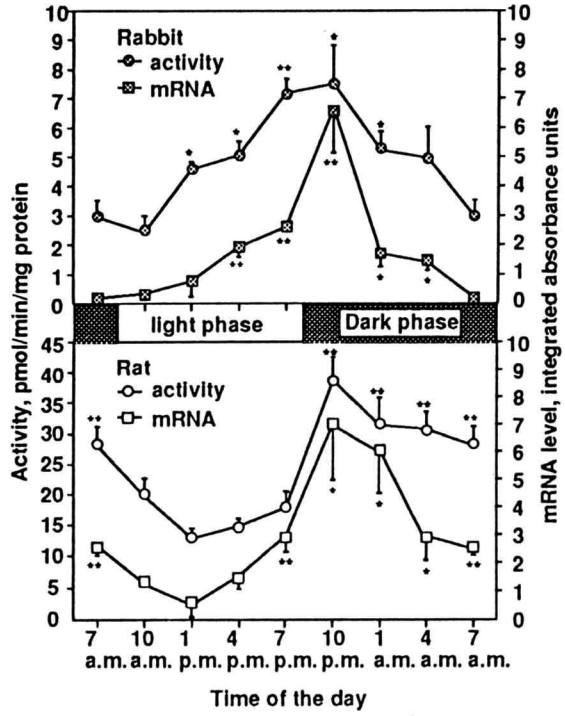

Fig. 4. Quantitation of mRNA and activities of cholesterol 7α-hydroxylase in rabbits and rats at various times of the day. The spots in the X-ray films were quantitated and, on the other hand, the enzyme activities were also quantitated. The results are given as means ± standard errors (vertical bar) of means of three subjects. An asterisk indicates significance (*, $P < 0.05$; **, $P < 0.01$) of the difference compared to the minimum levels of circadian rhythms.

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mediate levels in both the enzyme activities and mRNA levels.

In the rabbit, the ratios of the maximum level to the minimum level of the enzyme activities and mRNA levels were 3-fold and 35-fold, respectively. In the rat, 3-fold and 11-fold ratios were observed, respectively. The enzyme activities in the rat were always higher than those of the rabbit all through a day; even the minimum level in the rat was higher than the maximum level in the rabbit.

DISCUSSION

The amino acid sequence of rabbit cholesterol 7α-hydroxylase had 81% and 82% similarity to those of the rat and human enzyme (4, 22), respectively. The nucleotide sequence of the coding region of rabbit cDNA showed 82% and 86% similarity to those of the rat and human cDNA, respectively. The 3'-untranslated region of the rabbit cDNA, however, was shorter than that of the rat cDNA and longer than that of the human cDNA (the rabbit ca. 1400 bp, the rat ca. 2000, human ca. 1300) and showed a lower similarity to them (53% and 54%, respectively). A consensus amino acid sequence for the heme-binding domain of cytochrome P450 was observed at the residues 434–454. This was highly conserved in all three species; the sequence for the heme-binding region in the rabbit showed 95% similarity to the sequences of both the rat and human enzymes (4, 22). In this region, residue Ile-448 in the rabbit was substituted by Val-451 in the rat, and residue Gln-449 in the rabbit was substituted by His-452 in the human (**Fig. 5**).

It has been reported (4, 22) that the 3'-untranslated region of the rat and human cDNA of cholesterol 7α-hydroxylase is rich in AT nucleotides and often contains ATTTA motifs, 5'-AAT-3' or 5'-TAA-3' trinucleotides in a single-stranded region of the secondary structure. These have been reported to be reduced to rapid degradation of mRNA (23). Similar unique structures of about ten were also observed in the 3'-untranslated region of the rabbit cDNA. This suggests that the rabbit cholesterol 7α-hydroxylase mRNA degrades rapidly as well.

The circadian rhythms of cholesterol 7α-hydroxylase activities and mRNA levels previously described in the rat (4, 5) were also clearly detectable in the rabbit, with maximums around midnight and minimums during the daytime. These rhythms seem reasonable for the function of the enzyme in nocturnal animals, such as the rat and rabbit.

As for the mechanism causing circadian rhythms, two opposing suggestions have been presented. Li et al. (10) reported that the enzyme activities and the protein levels were regulated post-translationally. In their study, the cholesterol 7α-hydroxylase activities and protein levels were higher during the dark phase, and decreased and reached minimums at the middle of the light phase; however, no significant changes in the mRNA levels in the dark phase were observed. They have suggested that the enzyme activities and the protein levels may be regulated post-translationally.

On the other hand, Noshiro et al. (4) and Sundseth and Waxman (5) demonstrated that pre-translational regulation is most likely a mechanism in the circadian rhythms of cholesterol 7α-hydroxylase, because of the good correlation of the levels of mRNA to the enzyme activities and the protein levels. In addition, recently, Lavery and Schibler (12) reported evidence that the rat albumin D-element-binding protein was a regulator of the circadian rhythm of rat cholesterol 7α-hydroxylase, and the primary level of this circadian expression was at the transcriptional level.

In our study, statistically significant changes in the cholesterol 7α-hydroxylase mRNA levels in the dark phase and a good correlation of the levels of mRNA to the enzyme activities were observed in the rabbit as well as in the rat. Our results, taken together with those of Noshiro et al. (4), Sundseth and Waxman (5), and Lavery and Schibler (12) support the concept that the circadian rhythm of cholesterol 7α-hydroxylase is pre-translationally regulated.

The maximums and minimums of the enzyme activities correlate well with those of the mRNA levels. In the rabbit, the ratios of the maximum level to the minimum level of the enzyme activities and mRNA levels were 3-fold and 35-fold, respectively. Whereas, in the rat, 3-fold and 11-fold ratios were observed, respectively. In both species, the amplitudes of mRNA levels were far larger than those of enzyme activities. The ratios of the enzyme activities to the mRNA levels were the lowest at the maximums, the highest at the minimums of the en-

species	No.	Amino acid sequence
Rabbit	434	Phe Gly Ser Gly Ala Thr Ile Cys Pro Gly Arg Leu Phe Ala Ile Gln Glu Ile Lys Gln Phe
Rat	437	Phe Gly Ser Gly Ala Thr Ile Cys Pro Gly Arg Leu Phe Ala Val Gln Glu Ile Lys Gln Phe
Human	437	Phe Gly Ser Gly Ala Thr Ile Cys Pro Gly Arg Leu Phe Ala Ile His Glu Ile Lys Gln Phe

Fig. 5. Amino acid sequences of the heme binding region of cholesterol 7α-hydroxylase in the rabbit, rat, and human. The number (No.) is the first amino acid residue of respective sequence depicted in the figure. The amino acid sequences of rat and human cholesterol 7α-hydroxylase are taken from references 4 and 22, respectively. The differences between the rabbit and rat sequences and between the rabbit and human sequences are underlined in the figure.

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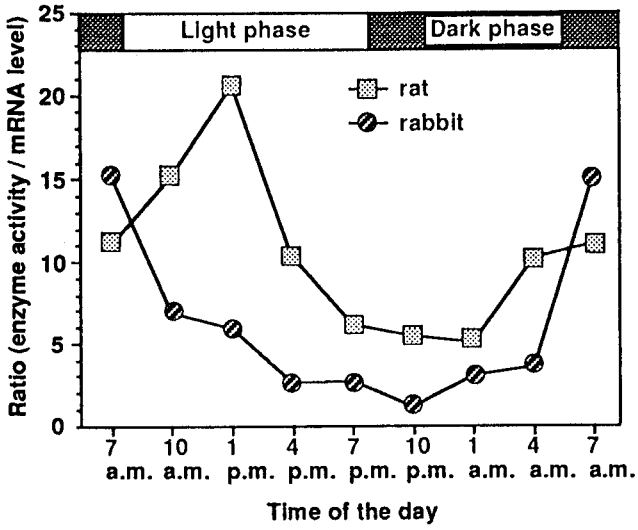

Fig. 6. The ratios of mean enzyme activities to mean mRNA levels in three rabbits and three rats at each time in Fig. 4.

zyme activities and mRNA levels (**Fig. 6**). These ratios almost equally increase or decrease after or before the maximums, respectively, like a mirror image of each other. This may show that there is no shift between two circadian rhythms of mRNA and enzyme activities of cholesterol 7α-hydroxylase.

Gielen et al. (11) evaluated the half-life of the enzyme protein in the rat as short as 2 h by calculation of the decline of normal rhythm. From the structural feature of rapid degradation in 3'-untranslated region, the mRNA of the enzyme should be rapidly degraded, as mentioned above. Although there might be a slight delay in the enzyme activities in the rat, it will not be as large a delay as reported by Gielen et al. (11). No such delay was observed in the rabbit either.

Noshiro et al. (4) reported the analysis of one rat for each 6-h span, and presented a figure showing that changes in the mRNA levels and the enzyme activities were both synchronous and parallel. Two hours of the half-life of the enzyme protein as shown above (Gielen et al. (11)) does not easily convince us that the two values move synchronously as shown in the figure of Noshiro et al. (4). In addition, one point of analysis in a 12-h interval using one subject cannot demonstrate that the two curves of the protein and mRNA peak at the same point in time. The present study was therefore designed to check the two peaks of the curves although the conclusion was the same after we studied the mRNA levels and the enzyme activities at three points in a 12-h interval, using three subjects at each point.

Van Cantfort and Gielen (9) reported that the ratio of the maximum level to the minimum level of the enzyme activities was much smaller in the mouse compared to the rat. They stated that the mouse, compared to the rat, synthesized bile acids at a more constant rate. They attributed this phenomenon to the fact that the mouse, unlike the rat, possesses a gallbladder. In our study in the rabbit, the ratio of the maximum level to the minimum level of the enzyme activities was 3-fold. Although the rabbit possesses a gallbladder, this was almost equal to that of the rat. This suggests that the amplitudes (the ratios) of circadian rhythms may not be affected by the existence or absence of the gallbladder, although the enzyme activities in the rat were always higher than those of the rabbit throughout the day; even the minimum level in the rat was higher than the maximum level in the rabbit.

Using complete cDNAs encoding cholesterol 7α-hydroxylase of the rabbit and rat as probes, circadian rhythms of the enzyme (activities) and its mRNA levels were carefully studied, every 3 h for a 24-h day. Enzyme activities showed rhythms synchronous with those of mRNA, which support the concept that the circadian rhythms of this enzyme are pre-translationally regulated. Instead of measuring the enzyme, from now on, we can measure the relative mRNA level to study the metabolism of cholesterol-bile acids, although there will be some factors that directly influence the enzyme (24). Because cholesterol 7α-hydroxylase is not stable, we must measure the enzyme activities soon after we prepare the liver fraction. The mRNA, on the other hand, is far more stable. It can be safely stored before measurement. We also emphasize the point that the marked circadian rhythms cannot be overlooked in the study of the metabolism of the enzyme concerned.

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REFERENCES

1. Danielsson, H., and J. Sjövall. 1975. Bile acid metabolism. *Annu. Rev. Biochem.* **44:** 37–47.
2. Myant, N. B., and K. A. Mitropoulos. 1977. Cholesterol 7α-hydroxylase. *J. Lipid Res.* **18:** 135–153.
3. Reihnér, E., I. Björkhem, B. Angelin, S. Ewerth, and K. Einarsson. 1989. Bile acid synthesis in humans: regulation of hepatic microsomal cholesterol 7α-hydroxylase activity. *Gastroenterology.* **97:** 1498–1505.
4. Noshiro, M., M. Nishimoto, and K. Okuda. 1990. Rat liver cholesterol 7α-hydroxylase. Pretranslational regulation for circadian rhythm. *J. Biol. Chem.* **265:** 10036–10041.
5. Sundseth, S. S., and D. J. Waxman. 1990. Hepatic P-450 cholesterol 7α-hydroxylase. Regulation in vivo at the protein and mRNA level in response to mevalonate, diurnal rhythm, and bile acid feedback. *J. Biol. Chem.* **265:** 15090–15095.
6. Gielen, J. E., J. Van Cantfort, and P. Kremers. 1976. Genetic and hormonal regulation of steroid hydroxylases and drug metabolizing enzymes in rat liver. *Arch. Toxicol.* **36:** 255–266.
7. Mitropoulos, K., A., S. Balasubramaniam, S. Venkatesan, and B. E. Reeves. 1978. On the mechanism for the regula-

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tion of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, of cholesterol 7α-hydroxylase and of acyl-coenzyme A:cholesterol acyltransferase by free cholesterol. *Biochim. Biophys. Acta.* **530:** 99–111.
8. Danielsson, H. 1972. Relationship between diurnal variations in biosynthesis of cholesterol and bile acids. *Steroids.* **20:** 63–72.
9. Van Cantfort, J., and J. E. Gielen. 1979. Comparison of rat and mouse circadian rhythm of cholesterol 7α-hydroxylase activity. *J. Steroid Biochem.* **10:** 647–651.
10. Li, Y. C., D. P. Wang, and Y. L. Chang. 1990. Regulation of cholesterol 7α-hydroxylase in the liver. Cloning, sequencing, and regulation of cholesterol 7α-hydroxylase mRNA. *J. Biol. Chem.* **265:** 12012–12019.
11. Gielen, J., J. Van Cantfort, B. Robaye, and J. Renson. 1975. Rat-liver cholesterol 7α-hydroxylase. 3. New results about its circadian rhythm. *Eur. J. Biochem.* **55:** 41–48.
12. Lavery, D. J., and U. Schibler. 1993. Circadian transcription of the cholesterol 7α-hydroxylase gene may involve the liver-enriched bZIP protein DBP. *Genes Dev.* **7:** 1871–1884.
13. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* **162:** 156–159.
14. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning. A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
15. Short, J. M., J. M. Fernandez, J. A. Sorge, and W. D. Huse. 1988. λ ZAP: a bacteriophage λ expression vector with in vivo excision properties. *Nucleic Acids Res.* **16:** 7583–7600.
16. Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132:** 6–13.
17. Henikoff, S. 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene.* **28:** 351–359.
18. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* **74:** 5463–5467.
19. Ogishima, T., and K. Okuda. 1986. An improved method for assay of cholesterol 7α-hydroxylase activity. *Anal. Biochem.* **158:** 228–232.
20. Noshiro, M., H. Ishida, S. Hayashi, and K. Okuda. 1985. Assays for cholesterol 7α-hydroxylase and 12α-hydroxylase using high performance liquid chromatography. *Steroids.* **45:** 539–550.
21. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
22. Noshiro, M., and K. Okuda. 1990. Molecular cloning and sequence analysis of cDNA encoding human cholesterol 7α-hydroxylase. *FEBS Lett.* **268:** 137–140.
23. Shaw, G., and R. Kamen. 1986. A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell.* **46:** 659–667.
24. Shefer, S., L. B. Nguyen, G. Salen, G. C. Ness, G. S. Tint, A. K. Batta, S. Hauser, and I. Rani. 1991. Regulation of cholesterol 7α-hydroxylase by hepatic 7α-hydroxylated bile acid flux and newly synthesized cholesterol supply. *J. Biol. Chem.* **266:** 2693–2696.

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