The stimulatory effect of LXRα is blocked by SHP despite the presence of a LXRα binding site in the rabbit *CYP7A1* promoter

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Abstract The transcription of the cholesterol 7α-hydroxylase gene (*CYP7A1*) is greatly decreased in cholesterol-fed rabbits. To determine whether the molecular structure of the promoter is responsible for this downregulation, we cloned the rabbit *CYP7A1* promoter, identified the binding sites for α-fetoprotein transcription factor (FTF) and liver X receptor (LXRα), and studied the effects of FTF, LXRα, and SHP on its transcription. Adding LXRα/retinoid X receptor together with their ligands (L/R) to the promoter/reporter construct transfected into HepG2 cells greatly increased its activity. FTF did not increase promoter activity, nor did it enhance the stimulatory effect of L/R. Mutating the FTF binding site abolished the promoter baseline activity. Increasing amounts of SHP abolished the effect of L/R, and FTF enhanced the ability of SHP to decrease promoter activity below baseline levels. Thus, downregulation of *CYP7A1* in cholesterol-fed rabbits is attributable secondarily to the activation of farnesoid X receptor, which increases SHP expression to override the positive effects of LXRα. Although FTF is a competent factor for maintaining baseline activity, it does not further enhance and may suppress *CYP7A1* transcription.—Shang, Q., L. Pan, M. Saumoy, J. Y. L. Chiang, G. S. Tint, G. Salen, and G. Xu. **The stimulatory effect of LXRα is blocked by SHP despite the presence of a LXRα binding site in the rabbit *CYP7A1* promoter. *J. Lipid Res.* 2006.** 47: **997–1004.**

Supplementary key words farnesoid X receptor • liver X receptor • α-fetoprotein transcription factor • SHP • dietary cholesterol • cholesterol 7α-hydroxylase

All humans do not respond in a similar manner to the consumption of cholesterol-rich foods: some are sensitive so that high levels of dietary cholesterol lead to hypercholesterolemia, whereas others consuming similar diets are essentially unaffected (1). This phenomenon has also been observed in two useful animal models that may provide us with a means to understand how these differences arise. Rabbits develop increased levels of plasma cholesterol when fed a cholesterol-rich diet, but rats do not (2). Cholesterol-fed rats are able to upregulate the activity of cholesterol 7α-hydroxylase (CYP7A1) (3–5), the rate-controlling enzyme for classic bile acid synthesis (6), which diverts the excess dietary cholesterol into the bile acids and then to the feces. In contrast, NZW rabbits are very sensitive to dietary cholesterol. These rabbits accumulate large amounts of cholesterol in the plasma (7) and develop severe atherosclerosis, similar to that seen in humans. In contrast to what is observed in the rat, we found that CYP7A1 activity was downregulated in cholesterol-fed rabbits (8) and hypothesized that this specific response led to the accumulation of dietary cholesterol in plasma that, in the rat, would have been destined for bile acid synthesis and excretion. In these rabbits, the circulating bile acid pool expanded by nearly 2-fold (9) and hepatic oxysterols (oxidized cholesterol) increased significantly. That should have activated simultaneously the nuclear receptors farnesoid X receptor (FXR) and liver X receptor (LXRα), which have an inhibitory (10–12) and a stimulatory effect (13), respectively, on *CYP7A1* transcription. In our cholesterol-fed rabbits, inhibition by the activation of FXR overrode stimulation by activated LXRα so that *CYP7A1* expression was suppressed (14). Such regulatory mechanisms appear to be species-specific. Chiang, Kimmel, and Stroup (15) reported that the rat *CYP7A1* promoter bound to LXRα tightly, the hamster promoter bound LXRα loosely, and the human *CYP7A1* promoter had no LXR binding site at all. It is unclear whether the rabbit *CYP7A1* promoter fits this scheme. It is also possible

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that in rabbits, activated LXRα [i.e., the complex formed by the LXR/retinoid X receptor (RXR) heterodimer together with any of the oxysterols known to alter transcription] might repress *CYP7A1* expression, as has been reported in human hepatocytes (16). In addition, it remains unclear whether increased amounts of cholesterol would repress rabbit but induce rat *CYP7A1* transcription.

In this study, we chose to clone the rabbit *CYP7A1* promoter to investigate whether it possesses a LXR binding site and to measure its response to activated LXRα and FXR (via its target gene, SHP). Because α-fetoprotein transcription factor (FTF) is essential for the expression of human *CYP7A1* (17) and potentiates LXRα functionality (10, 18), we examined the rabbit promoter to determine whether there is also a functional FTF binding site and studied its possible role in the regulation of rabbit *CYP7A1*. In addition, the effect of cholesterol on rabbit and rat *CYP7A1* transcription was also evaluated.

MATERIALS AND METHODS

Cloning the rabbit CYP7A1 promoter

The rabbit CYP7A1 promoter sequence was isolated using the GenomeWalker Kit (BD Biosciences, Palo Alto, CA). The genomic DNA was extracted from the rabbit ileum. 5′ oligonucleotide primers (AP1 and AP2) were provided with the kit, whereas 3′ oligonucleotide primers (GSP1 and GSP2) were designed according to the reported DNA sequence of the rabbit *CYP7A1* gene (19): GSP1, 5′-TCCTTAGTCCCAGAATAAGCCAC-3′; GSP2, 5′-CCACAAACAACAGCACACTGATAG-3′. The obtained PCR DNA was recovered and ligated into a pCR4-TOPO vector (Invitrogen, Carlsbad, CA) and transformed into a DH5α-competent cell (Invitrogen). Based on the sequence of the positive clone, a new pair of primers containing the *Xho*I site was designed: 5′ primer, 5′-CCGCTCGAGTATCATCTCATTTTCTT C-3′; 3′ primer, 5′-CCGCTCGAGAACTCC TGACAG GGACAATC-3′. The *CYP7A1* gene promoter fragment was amplified with those primers from the positive clone mentioned above. The resultant promoter fragment was then ligated, expanding from −1,025 to +46. The resultant promoter sequence was then ligated to a pGL3.basic vector (Promega, Madison, WI) and transformed into the DH5α cell.

The transcription start site was determined on rabbit total RNA using the First Choice RLM-RACE Kit (Ambion, Austin, TX). 5′ primers were provided with the kit, and 3′ primers were designed based on the cDNA sequence of the rabbit *CYP7A1* gene: GsP1, 5′-CCATCTCTTGGGTCAATGCTTCTATG-3′; GsP2, 5′-CATTTAGTTTGCAGGTAAAAACATGAC-3′. PCR products (400 bp) were then sequenced directly to determine the transcription start site.

Point mutations were performed on the putative LXR and FTF binding sites using the Quikchange Site-Directed Mutagenesis Kit (Stratagene, Cedar Creek, TX). Corresponding mutated constructs pGL3-CYP7A1-LXRm and pGL3-CYP7A1-FTFm were isolated. All clones were proofread by sequencing.

Electrophoretic mobility shift assays

Double-stranded oligonucleotide probes were obtained by annealing equal moles of single-stranded complementary oligonucleotides. The probes corresponding to the LXRα and FTF binding sites, identified in the rabbit *CYP7A1* gene promoter, were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase from the Gel Shift Assay System (Promega). LXRα, RXR, and FTF proteins were synthesized from the expression plasmids of human LXRα, RXR, and FTF using the coupled TNT Transcription/Translation system (Promega). Gel-shift analysis was conducted with human LXRα, RXR, and FTF proteins and labeled probes, again using the Gel Shift Assay System (Promega). The assay was carried out on a 4% acrylamide gel using the following radiolabeled probes containing either wild-type or mutated LXRα or FTF binding sites (mutated nucleotides are shown in boldface italic type): LXRα wt (LXRE), 5′-GCTTTGGTCACTCAAGTTCAAGTT-3′; mutated LXRα (LXRm), 5′-GCTTTGGTCACTC***CTTA***TCAAGTT-3′; FTF wt (FTFE), 5′-CTGTGGACTTAGTTCAAGGCTAGTTAA-3′; mutated FTF (FTFm), 5′-CTGTGGACTTAGTTC***CTAT***CTAGTTAA-3′.

Cell culture

HepG2 and HEK 293 cells (American Type Culture Collection, Manassas, VA) were grown at 37°C in an atmosphere of 5% CO_2. The cells were cultured in Eagle's minimal essential medium (EMEM; Sigma, St. Louis, MO) supplemented with ampicillin (100 U/ml; Sigma) and 10% FBS for HepG2 cells and with 10% heat-inactivated horse serum for HEK 293 cells. Confluent cultures of the cells were grown in 60 mm culture dishes. Once the cell density reached 70–80%, the medium with HepG2 cells was replaced with EMEM supplemented with ampicillin (100 U/ml) and 10% charcoal/dextran-treated FBS (delipidated), whereas the medium with HEK 293 cells was replaced with the same supplemented EMEM mentioned above. An intact rabbit *CYP7A* promoter (−1,125/+125), a rabbit *CYP7A* promoter with a mutated LXRα binding site, and a rabbit *CYP7A1* promoter with a mutated FTF binding site were inserted into pGL3 vectors (Promega). An intact rat *CYP7A1* promoter (−778/+38) inserted into pGL2 vector (from Dr. John Chiang's laboratory) was used as the positive control in this experiment. A synthetic *Renilla* luciferase reporter, phRG-TK (Promega), was used as a luciferase internal standard. *CYP7A1* promoter (600 ng) and 50 ng of phRG-TK vector (internal standard) were cotransfected in each dish. The expression plasmids CMX-human LXRα, CMX-human RXR, pCDM8-human FTF, CMV-mouse SHP, and an empty CMV vector were added in varying amounts, and the total amount of DNA transfected in each dish was then adjusted to 4 μg. All plasmids were cotransfected using FuGENG6 reagent (Roche, Indianapolis, IN). In the experiments in which expression plasmids for LXRα/RXR were transfected, 25 μM LXRα agonist, 22(*R*)-hydroxycholesterol, and 1 μM RXR agonist, 9-*cis*-retinoic acid, were always added after an additional 2 h of incubation. Cells were then incubated for another 48 h, harvested, and lysed, and luciferase activity was assayed using the Luciferase Assay System (Promega). The amount of luciferase activity in transfectants was measured using a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA) and normalized to the amount of phRG-TK luciferase activity. Transfections were carried out in triplicate, and each experiment was repeated six times.

Statistical analysis

Data are shown as means ± SD and were compared statistically by ANOVA followed by the Bonferroni multiple comparisons test. GraphPad InStat V.3 (GraphPad Software, San Diego, CA) was used for all statistical evaluations.

RESULTS

Figure 1A depicts the cloned 1.1 kb 5′-flanking region of the rabbit *CYP7A1* promoter schematically. The putative

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A
-1125                           ATCATCTGCATTTTCTTCCTGACTC
-1100 TCATTATCTTCCTCCTCACCTTGGACTGATCTCCCCGAGTGAAGTGTCTG
-1050 TGGCCTGGGGTTGAATTCTGTTTCTTTCTTTGATCTTTCTTTCTCCCTAA
-1000 GTGATTTCATAGGACCTCTTGGCTTTAAATACCATCCAAATGCTGGTGAA
-950  TTGATATTTATATCTCCAACTCTAGCCTTTCTCCTTCACTTTAGACTCAC
-900  TCAATTGTCAACTTGACATTGACCCGATGGTTCCTAAAAGATCTCCCAAA
-850  CATCATATGTCCAAAATAGTATTCTGATGTATTGCCTTCCAAGACCTCTC
-800  TCCCTGCTCACAAGCAATTCTGTATGAATTGTTTCTTATCTAACTTTTTG
-750  TGGTTTTAATCTTAGGTAACTTTGTCGGATTCACTCTGCTCCAGTTAGTT
-700  GTACTAAATTTTCCAGTTAGTAGATGTACAAAGTTTATGTTTGAAATGAG
-650  GTGGGTTTCTTTAATAAATAGAAAAATTAATAAACAAATTTAAGGTATAA
-600  AAAAGTTTGATAATGTTTTTATGCTTAAAAGAAACTGTTTTATTTACTTT
-550  CTTGATACTAGGTGAGTAATATGTTATGATCTATGTAAATTGTCTATGAT
-500  TTTGGAAAAATACTGAGACTTGAAGCAAGAGAATCTAGTTCTCTTCCCTT
-450  TGAACTTAACCATTGGATCTCTCTGGCATTTAAATTAATTTCTTCTTCAA
-400  AATGTAATAAGATAAAAAAAGCAACAAGATCAAATCACTAGTTTGATGAA
-350  TAGTTTATACTTAGTTTTCTTTATGTGTCACATATTTACTACATATGTTC
-300  ATCTTGAACAGATTTGTTTTTATACACATACCTAACTTGTAATACTAGCT
-250  GCTGTCCCCAGGAATGAATGTTGAGTCAACATATGTTTGAGAGACCTTCA
-200  ACTTATCAAGTATTGCAGATTTCTGATTGTTTTGGAAGATCTTCTGATGC
-150  CTGTGGACTTAGTTCAAGGCTAGTTAATACCACTATCTTTTTTTTCCTAA
-100  TAGGATGAACAAATGGTTAATTGTTTGCTTTGGTCACTCAAGTTCAAGTT
-50   ATTGAATCAGGGTCCTACGTATATAAAAACTCTAGCTTCAGACTGTTCAG

B
                FTF                   LXR
Mouse  ...tagtTCAAGGCCAgata...gctcTGGTCAcccaAGTTCAagt...
Human  ...tagtTCAAGGCCAgtta...tgctTTGTCAaccaAGCTCAagt...
Rat    ...tagtTCAAGGCCGggta...gcttTGGTCActcaAGTTCAagt...
Rabbit ...tagtTCAAGGCTAgtta...gcttTGGTCActcaAGTTCAagt...
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Fig. 1. Nucleotide sequence of the rabbit cholesterol 7α-hydroxylase (CYP7A1) promoter. A: The cloned 1.1 kb (−1 to −1,125) of the 5′-flanking region of rabbit *CYP7A1*. Putative binding sites for the liver X receptor (LXRα) and α-fetoprotein transcription factor (FTF) transcription factors are located at −55/−70 and −129/−137, respectively, and are indicated in boldface. B: FTF and LXRα binding sites are conserved. The proposed rabbit FTF and LXRα binding sites are compared with the mouse, human, and rat sites. The proposed FTF binding site in the rabbit *CYP7A1* promoter was 89% homologous to the human and mouse sites and 78% homologous to the rat site. The proposed LXR binding site in the rabbit *CYP7A1* promoter was identical to that in the rat and was 94% and 75% homologous, respectively, to the mouse and human sites.

binding sites for the LXRα and FTF transcription factors, identified by Genomatix (Munich, Germany) and Accelrys (San Diego, CA), are indicated in boldface (Fig. 1A). The sequence of the proposed LXR binding site (TGGTCACT-CAAGTTCA) located at −55/−70 in the rabbit *CYP7A1* promoter is identical to the previously identified rat LXR binding site. The proposed FTF binding site (TCAAGGCTA) located at −129/−137 in the rabbit *CYP7A1* promoter was 89% and 78% match to human and rat promoters respectively (Fig. 1B). To confirm that

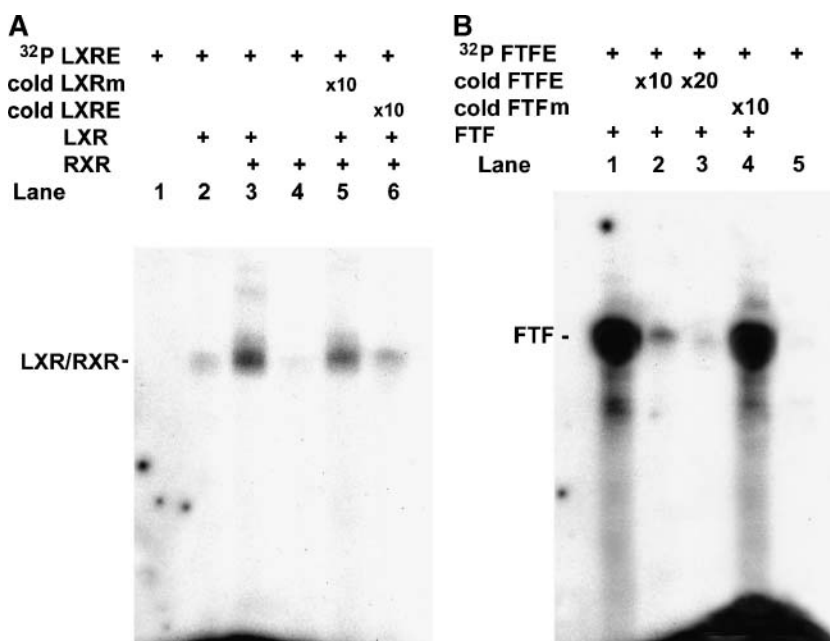

Fig. 2. Electrophoretic mobility shift assays: the putative LXR and FTF binding sites bind specifically to the relevant proteins. A: The ^{32}P-labeled LXR binding site (^{32}P LXRE) in the rabbit *CYP7A1* promoter (see Materials and Methods) binds to the human LXRα/retinoid X receptor (RXR) heterodimer (lane 3). The cold mutated LXR probe (cold LXRm) does not compete with LXRα/RXR binding (lane 5), whereas addition of a 10-fold increased amount of cold LXRα (cold LXRE) does compete (lane 6). B: The ^{32}P-labeled FTF binding site (^{32}P FTFE) in the rabbit *CYP7A1* promoter strongly binds to human FTF protein (lane 1). A 10-fold (lane 2) and a 20-fold (lane 3) amount of cold FTF probe (cold FTFE) blocks FTF binding, but the cold mutated FTF probe (cold FTFm; lane 4) does not.

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these sites are functional, we used electrophoretic mobility shift assays to determine whether LXRα and FTF can bind to their respective sites (**Fig. 2**). ^{32}P-labeled rabbit LXRα probe (LXRE) indeed bound to LXRα/RXR (Fig. 2A, lane 3), whereas excess cold LXRα probe (cold LXRE; lane 6) but not mutated LXR probe (LXRm; lane 5) competed with the labeled probe for binding. Similarly, in Fig. 2B, labeled FTF probe (^{32}P FTFE) bound FTF protein (lane 1). The binding of labeled FTFE was reduced markedly by cold FTF probe (lanes 2, 3) but not by cold mutated FTF probe (FTFm; lane 4).

To determine whether FTF and LXRα proteins actually regulate the activity of rabbit *CYP7A1*, we transfected the cloned rabbit *CYP7A1* promoter fused to a luciferase reporter gene into human HepG2 cells, which naturally express FTF. *CYP7A1* promoter activity is reported as normalized luciferase activity units. In this cell system, adding human FTF protein did not increase but rather reduced promoter activity (**Fig. 3A**), so that 400 ng of added FTF protein (the expression plasmid for human FTF), for example, suppressed promoter activity by 39% ($P < 0.001$) compared with baseline (7.4 ± 0.8 vs. 12.1 ± 0.9 units). However, adding >400 ng of FTF resulted in only marginally reduced activities. We also examined the effect of low doses of FTF protein (0.5–20 ng) on promoter activity in HepG2 cells, but none was observed (data not shown). In a second experiment, we studied the effect of FTF on rabbit *CYP7A1* transfected into HEK 293 cells, which do not naturally express FTF. Baseline *CYP7A1* promoter activity in HEK 293 cells was low (0.44 ± 0.05 units), only 1/27th of that in HepG2 cells (12.1 ± 0.9 units). The addition of 200 and 400 ng of FTF protein increased promoter activity by 50% (0.66 ± 0.15; $P < 0.05$) and 89% (0.83 ± 0.07; $P < 0.001$), respectively. Promoter activity did not increase with the further addition of FTF; instead, it tended to decrease, just as we had observed for higher doses of FTF in HepG2 cells (Fig. 3B).

The effect of LXRα/RXR on rabbit *CYP7A1* promoter activity in HepG2 cells is shown in Fig. 3C. When 200 ng of human LXRα/RXR protein (expression plasmids for human LXRα and RXR) plus 25 μM 22(*R*)-hydroxycholesterol and 1 μM 9-*cis*-retinoic acid were added, promoter activity increased 2.4-fold (24 ± 3 units; $P < 0.001$) compared with the baseline value of 9.9 ± 1.5 units. Addition of FTF up to 800 ng did not further enhance the stimulation of the *CYP7A1* promoter by LXRα/RXR. The results in HEK 293 cells (Fig. 3D) are similar except that both baseline and stimulated activities were considerably

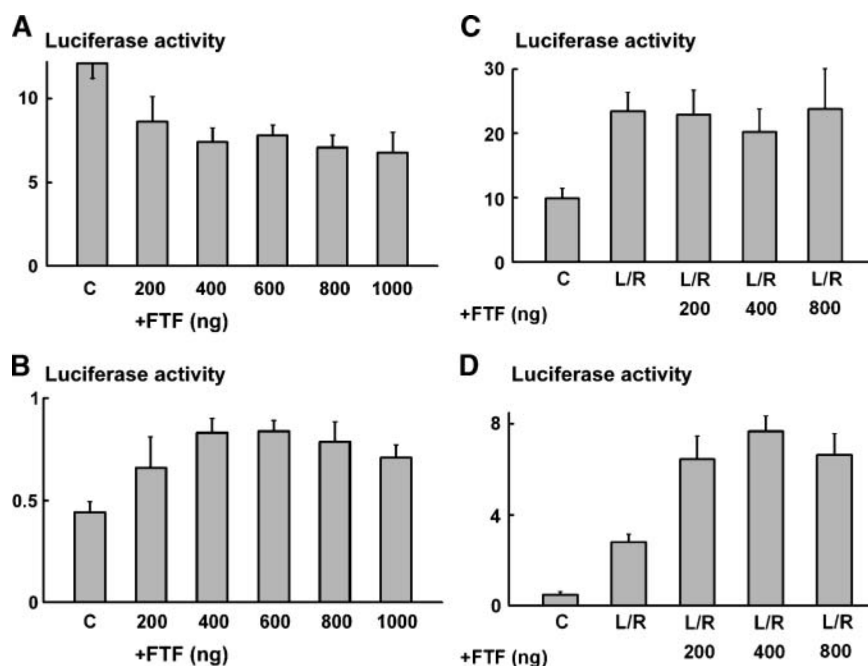

Fig. 3. Effect of FTF on the *CYP7A1* promoter in HepG2 and HEK 293 cells. *CYP7A1* promoter activity is reported as normalized luciferase activity units. Data are presented as means ± SD. For studies in HepG2 cells, n = 6; for studies in HEK 293 cells, n = 4. A: Human HepG2 cells were cotransfected with 600 ng of the cloned rabbit *CYP7A1* promoter fused to a luciferase reporter gene. C, control; +FTF, addition of 200 to 1,000 ng of expression plasmid for human FTF. B: HEK 293 cells were cotransfected with 600 ng of the cloned rabbit *CYP7A1* promoter fused to a luciferase reporter gene. +FTF, addition of 200 to 1,000 ng of expression plasmid for human FTF. C: HepG2 cells were cotransfected with 600 ng of the rabbit *CYP7A1* promoter fused to a luciferase reporter gene. +FTF, addition of 200 to 800 ng of expression plasmids for human FTF; L/R, addition of 200 ng of expression plasmids for human LXRα and RXR plus 25 μM 22(*R*)-hydroxycholesterol and 1 μM 9-*cis*-retinoic acid. D: HEK 293 cells were cotransfected with 600 ng of the rabbit *CYP7A1* promoter fused to a luciferase reporter gene. +FTF, addition of 200 to 800 ng of expression plasmid for human FTF; L/R, addition of 200 ng of expression plasmids for human LXRα and RXR with 25 μM 22(*R*)-hydroxycholesterol and 1 μM 9-*cis*-retinoic acid.

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lower than the activities observed in HepG2 cells, whereas adding 200 ng of the FTF appeared to restore the essential FTF that was missing from these cells, so that activity increased by >2-fold.

To further confirm the effects of FTF (human) and LXRα/RXR (human) proteins on the promoter, we produced two different rabbit *CYP7A1* promoters with mutated LXR (LXRm, TGGTCACTC**CTTA**TCA) and FTF (FTFm, TC**CTAT**CTA) binding sites, respectively. The mutated promoter (LXRm) transfected into HepG2 cells was essentially inactive, 0.13 ± 0.02 units compared with wild-type promoter activity of 10.7 ± 1.6 units, and no additional response could be elicited by adding LXRα/RXR or LXRα/RXR + FTF (**Fig. 4A**). The baseline luciferase activity of the promoter with a mutated FTF binding site (FTFm) in HepG2 cells was sharply (20-fold) less (0.52 ± 0.12 units; $P < 0.001$) than the activity observed with the wild-type promoter (9.1 ± 1.3 units). Additional activity could not be elicited by the addition of more FTF protein (0.72 ± 0.23 units). Although adding LXRα/RXR or LXRα/RXR + FTF increased the activity of FTFm (2.9 ± 0.5 or 5.0 ± 1.2 units, respectively), it was still significantly lower than the baseline value of the nonmutated promoter (Fig. 4B) and was only one-eighth to one-fifth as high as that in the wild-type promoter under the same conditions (Fig. 3C).

The effect of SHP on the rabbit *CYP7A1* promoter activity is shown in **Fig. 5A**. Adding 200 ng of mouse SHP protein (the expression plasmid for mouse SHP) decreased activity in HepG2 cells by 42% (from 9.2 ± 1.8 to 5.3 ± 1.0 units; $P < 0.001$). Addition of 400 ng of SHP reduced promoter activity by 56% compared with baseline (4.0 ± 0.7 units; $P < 0.001$), but a further increase of SHP had no effect (Fig. 5A).

Figure 5B illustrates that the stimulatory effect of human LXRα/RXR on the rabbit *CYP7A1* promoter in HepG2 cells was offset by mouse SHP. Adding 200 ng of

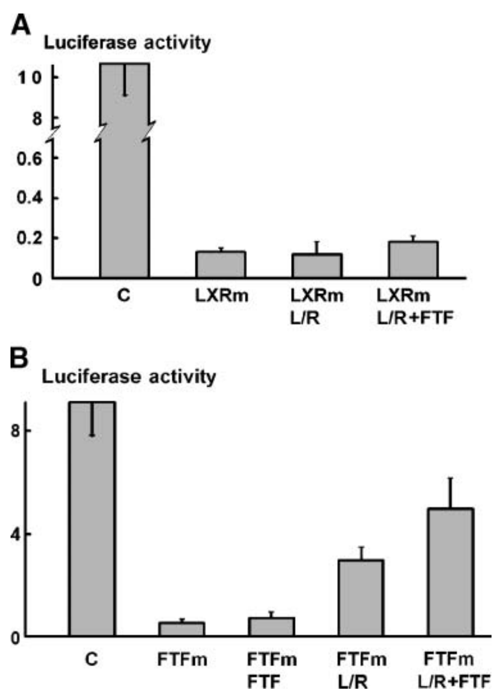

Fig. 4. *CYP7A1* promoter activity with a mutated LXR or FTF binding site. The experiments were carried out in HepG2 cells. *CYP7A1* promoter activity is reported as normalized luciferase activity units. Data are presented as means ± SD; n = 6. A: Effect of mutating the LXR binding site. C, transfected with 600 ng of intact rabbit *CYP7A1* promoter; FTF, 200 ng of expression plasmid for human FTF; L/R, 200 ng of expression plasmids for human LXRα and RXR plus 25 μM 22(*R*)-hydroxycholesterol and 1 μM 9-*cis*-retinoic acid; LXRm, transfected with 600 ng of rabbit *CYP7A1* promoter with a mutated LXR binding site. B: Effect of mutating the FTF binding site. C, transfected with 600 ng of intact rabbit *CYP7A1* promoter; FTF, 200 ng of expression plasmid for human FTF; FTFm, transfected with 600 ng of the rabbit *CYP7A1* promoter with a mutated FTF binding site; L/R, 200 ng of expression plasmids for human LXRα and RXR plus 25 μM 22(*R*)-hydroxycholesterol and 1 μM 9-*cis*-retinoic acid.

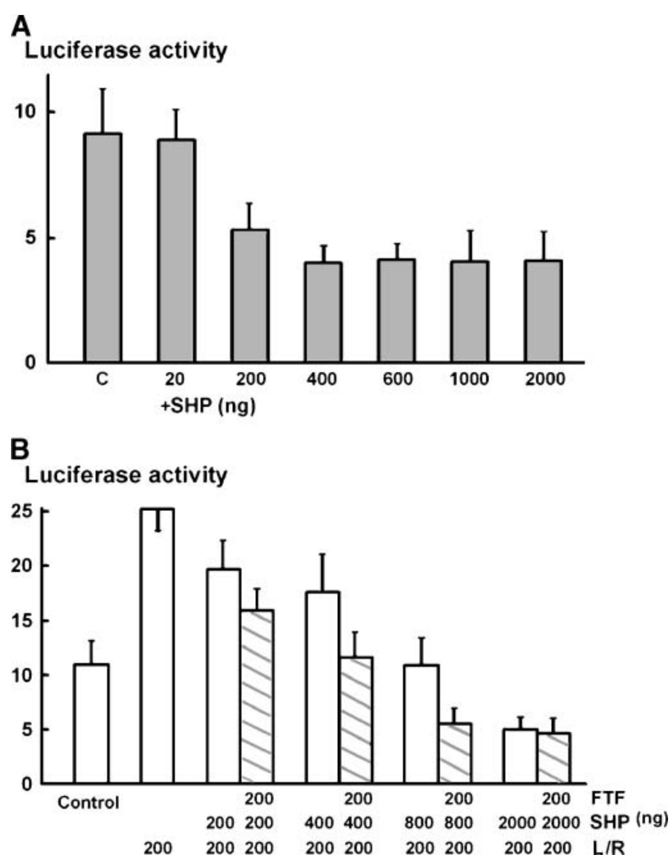

Fig. 5. Repressive effect of SHP on rabbit *CYP7A1* promoter activity. *CYP7A1* promoter activity is reported as normalized luciferase activity units. Data are presented as means ± SD; n = 6. A: Suppressive effect of SHP alone. C, controls; HepG2 cells were cotransfected with 600 ng of the *CYP7A1* promoter. Expression plasmid for mouse SHP was added from 20 to 2,000 ng, respectively, to cells transfected with the same amount of the promoter. B: FTF enhanced the inhibitory effect of SHP. In each experiment, HepG2 cells were cotransfected with 600 ng of the cloned *CYP7A1* promoter fused to a luciferase reporter gene. Open bars, no FTF; hatched bars, plus 200 ng of expression plasmid for human FTF. L/R, 200 ng of expression plasmids for human LXRα and RXR plus 25 μM 22(*R*)-hydroxycholesterol and 1 μM 9-*cis*-retinoic acid; SHP, addition of 200 to 2,000 ng of expression plasmid for mouse SHP.

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SHP with LXRα/RXR decreased the already increased promoter activity by 22% (19.7 ± 2.7 vs. 25.3 ± 2.1 units; $P < 0.01$), whereas adding 800 ng of SHP abolished the stimulatory effect of LXRα/RXR entirely. Interestingly, in the presence of 200 ng of FTF (human), the inhibitory effect of SHP was enhanced significantly. Adding 400 ng of SHP together with 200 ng of FTF decreased the previously increased activity by 54%, which was a significantly greater effect than that achieved by adding 400 ng of SHP alone (11.6 ± 2.3 vs. 17.6 ± 3.5 units; $P < 0.001$). Similarly, 800 ng of SHP with 200 ng of FTF decreased promoter activity by almost 50% more than adding 800 ng of SHP alone (5.6 ± 1.3 vs. 10.9 ± 2.4 units; $P < 0.01$). The further reduced promoter activity was significantly lower ($P < 0.01$) than baseline levels.

To further clarify whether the rabbit *CYP7A1* promoter responds to FTF and LXRα/RXR in opposite directions, another set of paired experiments was carried out in HepG2 cells transfected with rat and rabbit *CYP7A1* and then treated with the same amounts of human FTF, LXRα/RXR, and mouse SHP. **Figure 6A** (rabbit) and B (rat) demonstrate that LXRα/RXR increased activity in both the rabbit and rat *CYP7A1* promoter. The promoter activity was induced 2.4-fold in the rabbit and 13-fold in the rat after the addition of human LXRα/RXR. Adding FTF to LXRα/RXR did not further enhance the increased promoter activity either in the rabbit (20 ± 4 vs. 23 ± 2 units) or the rat (2.6 ± 0.5 vs. 2.4 ± 0.3 units). However, the addition of SHP to LXRα/RXR reduced the increased activity in both the rabbit (−26%; $P < 0.05$) and the rat (−33%; $P < 0.05$). When FTF was added together with SHP, the LXRα/RXR-induced activity was further repressed in both the rabbit (−53%; $P < 0.001$) and the rat (−38%) compared with those with only SHP + LXRα/RXR (L/R/F/S vs. L/R/S in Fig. 6A, B).

To investigate whether cholesterol can repress CYP7A1 directly, the rabbit and rat *CYP7A1* promoters were transfected into HepG2 cells. Increasing the cholesterol concentration in the medium from 10 to 50 μM did not reduce *CYP7A1* promoter activity in either one (**Fig. 7A, B**). Furthermore, the addition of 22(*R*)-hydroxycholesterol by itself did not alter promoter activity, whereas LXRα/RXR with their agonists 22(*R*)-hydroxycholesterol and 9-*cis*-retinoic acid did stimulate promoter activity in both the rabbit (2.5-fold; $P < 0.001$) and the rat (12-fold; $P < 0.001$).

DISCUSSION

In cholesterol-fed rabbits, unlike in rats, *CYP7A1* is not upregulated, and although LXR is activated simultaneously, the inhibitory effect of FXR is dominant (14). The major objective in this study was to determine whether

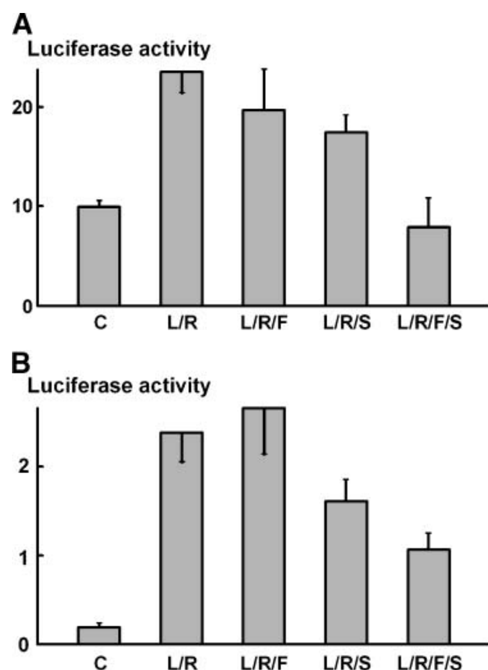

Fig. 6. Comparison of the response of the rabbit (A) and rat (B) *CYP7A1* promoter to LXRα/RXR, FTF, and SHP. *CYP7A1* promoter activity is reported as normalized luciferase activity units. Data are presented as means ± SD; n = 6 for rabbit and n = 4 for rat. In each experiment, 600 ng of the promoter was transfected. C, controls; F, 200 ng of expression plasmid for human FTF; L/R, 200 ng of expression plasmids for human LXRα and RXR plus 25 μM 22(*R*)-hydroxycholesterol and 1 μM 9-*cis*-retinoic acid; S, 200 ng of expression plasmid for mouse SHP.

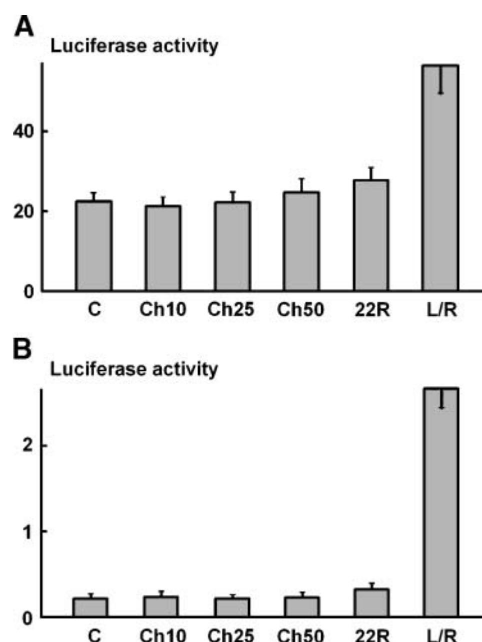

Fig. 7. Effect of cholesterol on the rabbit (A) and rat (B) *CYP7A1* promoter. *CYP7A1* promoter activity in HepG2 cells is reported as normalized luciferase activity units. Data are presented as means ± SD. In the rabbit, n = 6; in the rat, n = 4. In each experiment, 600 ng of the promoter was transfected. C, controls; Ch10, Ch25, and Ch50, cholesterol concentration in medium of 10, 25, and 50 μM, respectively; L/R, 200 ng of expression plasmids for human LXRα and RXR plus 25 μM 22(*R*)-hydroxycholesterol and 1 μM 9-*cis*-retinoic acid; 22R, 25 μM 22(*R*)-hydroxycholesterol.

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the molecular structure of the rabbit *CYP7A1* promoter is responsible for this difference.

Cholesterol feeding results in increased amounts of oxidized cholesterol (oxysterols), ligands for LXR. It has been reported that in mice (13) and rats, LXRα is a strong positive regulator of *CYP7A1* transcription. In this study, we asked whether the rabbit *CYP7A1* promoter has an LXR binding site at all, or, alternatively, whether there might be "weak" or imperfect binding that cannot respond positively to activated LXRα (the LXR/RXR/oxysterol/retinoic acid complex). Our data demonstrate that not only does the rabbit *CYP7A1* promoter contain a functional LXR binding site identical to that in the rat but that the LXRα/RXR complex stimulates rabbit promoter activity significantly (Figs. 3C, 6A). The putative binding site in the rabbit *CYP7A1* promoter that we identified is specific for LXR because when the site was mutated, the stimulatory effect of activated LXRα was absent (Fig. 4A). In fact, by mutating the LXR binding site, promoter activity was nearly abolished, being at most 1% of baseline activity. Adding FTF could not restore the activity of the mutated promoter. Thus, the mutation in the LXR binding site probably destroyed the "core promoter" that is essential for maintaining the activity of the promoter.

We demonstrated that there is also a functional FTF binding site in the promoter similar to that in the rat. FTF, however, does not enhance the stimulatory effect of LXRα/RXR on the rabbit *CYP7A1* promoter, as the addition of increasing quantities of FTF did not further increase promoter activity (Fig. 3C). However, the strong increase of the promoter activity by the LXRα/RXR complex in wild-type *CYP7A1* (24 ± 3 units) (Fig. 3C) was nearly abolished (2.9 ± 0.5 units) (Fig. 4B) when the FTF binding site in the rabbit *CYP7A1* promoter was mutated. This result agrees with Lu et al. (10) and Luo, Liang, and Tall (18) that FTF is a competent factor for the stimulation of *CYP7A1* expression by LXRα.

Nitta et al. (17) reported that human *CYP7A1* promoter binding factor, a homolog of FTF, represents a specific transcriptional inducer of human *CYP7A1* gene expression. We believe that FTF itself is also a competent factor for maintaining a baseline level of the rabbit *CYP7A1* promoter because *a*) mutation of the FTF binding site abolished baseline activity (Fig. 4B); *b*) the amount of FTF protein naturally synthesized by HepG2 cells was sufficient to maintain rabbit *CYP7A1* expression in culture, but more FTF did not increase its activity (Fig. 3A, C); *c*) in contrast, activity of the rabbit *CYP7A1* promoter after its transfection into HEK 293 cells, which do not synthesize FTF, was barely detectable before but increased markedly after the cells were supplied with FTF (Fig. 3B, D); and *d*) the activation pattern in the two cell lines was similar in that a baseline level of FTF (endogenous in HepG2 but exogenous in HEK 293) was needed to stimulate promoter activity, but additional FTF led to no further increase in activity (Fig. 3).

Although FTF is a competent factor for maintaining baseline promoter activity, an excess will not further stimulate but will, in fact, suppress it in HepG2 cells (Fig. 3A) and probably in HEK 293 cells as well (Fig. 3B). Furthermore, when even a moderate amount (200 ng) of FTF was added together with SHP and LXRα/RXR, FTF enhanced the inhibitory effect of SHP but not the stimulatory effect of activated LXRα (Fig. 5B). Thus, we propose that FTF may also have negative effects on the rabbit *CYP7A1* promoter under other conditions. At the least, FTF appears to assist SHP in offsetting the stimulatory effect of activated LXRα. These results agree with the hypothesis that FTF acts as a negative regulator by competing with HNF4 for binding to the overlapping site within the *CYP7A1* promoter (20, 21).

To ensure that there are no major functional differences between the rabbit and rat *CYP7A1* promoter, we carried out a set of paired experiments. As shown in Fig. 6, the responses of the two *CYP7A1* promoters are similar: both are stimulated by LXRα/RXR but not further enhanced by the addition of FTF, whereas SHP represses both promoters and FTF reinforces this inhibitory effect.

Another important question we have answered in this study is whether increased levels of cholesterol directly repress rabbit but not rat *CYP7A1* transcription. We demonstrate in Fig. 7 that in vitro cholesterol has no direct effect on either the rabbit or rat *CYP7A1* promoter. We also note that 22(*R*)-hydroxycholesterol alone does not increase rabbit and rat promoter activity but that 22(*R*)-hydroxycholesterol coupled with LXRα/RXR does strongly stimulate promoter activity in both species (Fig. 7). This induction of *CYP7A1* promoter activity is attributable to the activation of LXRα by its ligand oxysterols. Thus, cholesterol itself does not have an inhibitory effect on the rabbit and rat *CYP7A1* promoter. However, cholesterol's oxidized product together with LXRα/RXR will strongly stimulate promoter activity in the rabbit as well as in the rat. These results agree with our previous finding that *CYP7A1* was actually upregulated in rabbits fed 2% cholesterol for only 1 day (14). In these rabbits, the bile acid pool size had not yet expanded (it takes an average of 4 days) and FXR was not activated, so that SHP expression was not increased. Under these circumstances, *CYP7A1* is upregulated because LXRα is activated by the increased oxysterol concentration in the liver. Thus, downregulation of *CYP7A1* in long-term cholesterol-fed rabbits is not attributable to the direct effect of cholesterol.

We reported previously that in rabbits fed 2% cholesterol for 10 days, the circulating pool of FXR ligand (bile acid) expanded by 2-fold (9), FXR was activated, and the expression of its target gene *SHP* was increased by 4-fold (14). The results shown in Fig. 5B demonstrate that in the presence of FTF, when the increase of SHP is sufficient, *CYP7A1* promoter activity is suppressed to a level significantly lower than that at baseline, regardless of whether LXRα is activated simultaneously (LXRα/RXR together with their ligands). We conclude that in rabbits, cholesterol feeding downregulates *CYP7A1* because the FXR ligand (the pool of bile acids) is enlarged, activating FXR, which then induces the increased expression of *SHP*. Increased levels of SHP protein, with the assistance of FTF, enable the activation of FXR, which overrides the

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stimulatory effect of activated LXRα. We have also demonstrated conclusively that this is certainly not because the rabbit *CYP7A1* promoter lacks a functional LXRα binding site. In rats, the reason that dietary cholesterol upregulates *CYP7A1* is not just that the rat *CYP7A1* promoter has an LXR binding site that strongly responds to activated LXRα. More importantly, *SHP* expression is not increased because FXR is not activated in these animals, as the pool of circulating FXR ligands (bile acids) is not enlarged and the proportion of hydrophobic bile acids is reduced (22). These findings are leading us to focus on the mechanisms by which cholesterol feeding results in an expanded bile acid pool in rabbits but not in rats.

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