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Molecular Basis for Feedback Regulation of Bile Acid Synthesis by Nuclear Receptors

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Summary

The catabolism of cholesterol into bile acids is regulated by oxysterols and bile acids, which induce or repress transcription of the pathway's rate-limiting enzyme cholesterol 7α-hydroxylase (CYP7A1). The nuclear receptor LXRα binds oxysterols and mediates feed-forward induction. Here, we show that repression is coordinately regulated by a triumvirate of nuclear receptors, including the bile acid receptor, FXR; the promoter-specific activator, LRH-1; and the promoter-specific repressor, SHP. Feedback repression of *CYP7A1* is accomplished by the binding of bile acids to FXR, which leads to transcription of *SHP*. Elevated SHP protein then inactivates LRH-1 by forming a heterodimeric complex that leads to promoter-specific repression of both *CYP7A1* and *SHP*. These results reveal an elaborate autoregulatory cascade mediated by nuclear receptors for the maintenance of hepatic cholesterol catabolism.

Introduction

Cholesterol homeostasis is maintained by coordinate regulation of three primary pathways in the liver (Russell and Setchell, 1992; Princen et al., 1997). Two of these pathways involve maintaining cholesterol supply by regulation of de novo synthesis from acetate and uptake of plasma cholesterol via the LDL receptor, SR-B1, and LRP. The third pathway involves the elimination of cholesterol through the synthesis of bile acids. In this pathway, bile acids are direct end-products of cholesterol catabolism that are excreted via the bile and intestine into the feces. Additionally, bile acids serve to stimulate the excretion of excess hepatic cholesterol into the bile. Although bile acids are a crucial component of cholesterol excretion, they are also essential for solubilization and absorption of dietary cholesterol and fat-soluble vitamins. As a consequence of their importance in governing cholesterol homeostasis, the level of bile acids in the enterohepatic system is tightly controlled. This regulation is achieved through transcription of cholesterol 7α-hydroxylase (CYP7A1), the rate-limiting enzyme in the classic pathway of bile acid biosynthesis (Russell and Setchell, 1992). In certain animal species, high levels of dietary cholesterol stimulate CYP7A1 transcription, resulting in an increase in bile acid synthesis and cholesterol excretion. When the bile acid pool size grows too large, bile acids feed back and repress the transcription of CYP7A1, hence down-regulating their own synthesis (Hofman, 1994; Princen et al., 1997).

While the physiological responses of CYP7A1 to cholesterol and bile acids are well known, the molecular mechanism by which these compounds regulate CYP7A1 has only recently begun to be understood. A major advance toward understanding this regulation has come with the discovery of two classes of nuclear receptors, the liver X receptors (LXRs) and the farnesoid X receptor (FXR). The LXRs are comprised of two members, LXRα (NR1H3) and LXRβ (NR1H2), that have distinct but overlapping patterns of expression and are enriched in enterohepatic tissues (Repa and Mangelsdorf, 2000). LXRs function as RXR heterodimers, and their ligands are a select group of oxysterols that are derived from tissue-specific metabolism of cholesterol in the liver, brain, and gonads (Janowski et al., 1996; Lehmann et al., 1997). In the liver, genetic ablation studies have unequivocally demonstrated the role of LXRα as the key sensor of dietary cholesterol that up-regulates CYP7A1 mRNA and subsequently eliminates excess cholesterol via bile acid synthesis and excretion (Peet et al., 1998b). Consistent with these in vivo studies, the murine *Cyp7a1* promoter has been shown to contain a potent LXR response element (LXRE) that directly binds RXR/LXRα heterodimers and activates transcription in a ligand-dependent manner (Lehmann et al., 1997; Peet et al., 1998a).

In the opposing regulatory pathway, FXR (NR1H4) functions as the nuclear receptor for bile acids (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999). FXR activity also requires RXR heterodimerization (Forman et al., 1995), and when bound by bile acids, this complex effectively inhibits bile acid synthesis by repressing *CYP7A1* gene transcription (Makishima et al., 1999). In contrast to the mechanism by which *CYP7A1* is transactivated by LXRα, the mechanism of *CYP7A1* repression by FXR has remained unknown. Although the *CYP7A1* promoter contains no FXR/RXR binding sites, two bile acid–responsive regions (termed BARE-I and BARE-II) have been implicated in the repression response (Stroup et al., 1997). Interestingly, BARE-I corresponds to the LXRE (Lehmann et al., 1997), and BARE-II has been shown to be the binding site for liver receptor homolog-1 (LRH-1, also called CPF; NR5A2), a monomeric orphan receptor that functions as a tissue-specific transcription factor (Becker-André et al., 1993; Galarneau et al., 1996; Nitta et al., 1999). LRH-1 is required for hepatic expression of CYP7A1 (Nitta et al., 1999) and CYP8B1, a downstream enzyme of bile acid synthesis (del Castillo-Olivares and Gil, 2000). LRH-1 is also required for the expression of short heterodimer partner (SHP; NR0B2), an orphan nuclear receptor with potent repressor activity that binds to and inhibits the function of other nuclear receptors (Seol et al., 1996; Johansson et al., 1999; Lee et al., 1999).

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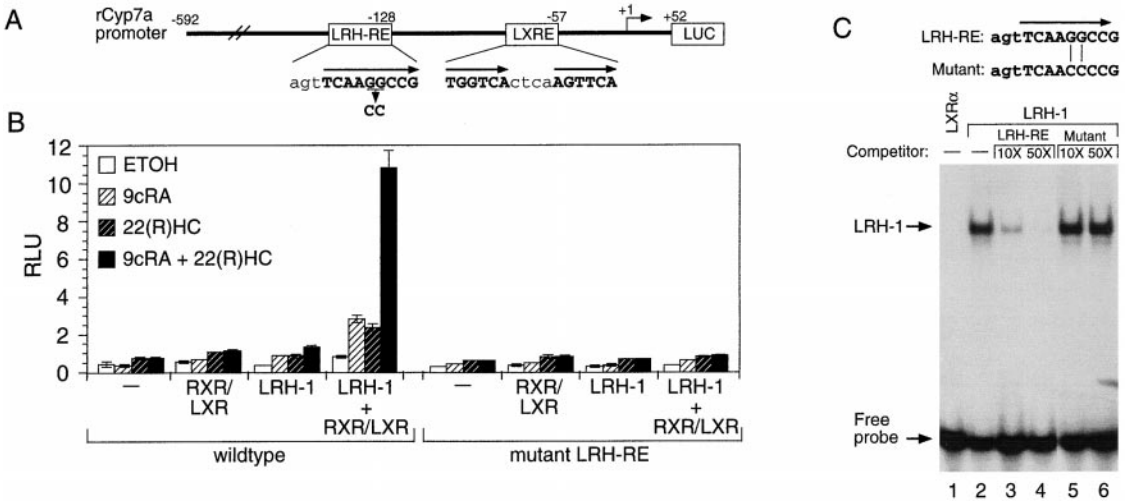

Figure 1. Transactivation of Rat *CYP7A1* by LXRα Requires LRH-1

(A) Schematic of the rat *CYP7A1* promoter and luciferase reporter construct. The LRH-1 response element (LRH-RE) at position –128 is conserved in rodent and human promoters.

(B) LRH-1 is required for *CYP7A1* transactivation by RXR/LXR. HEK293 cells were cotransfected with equivalent amounts (15 ng) of either control plasmid or expression plasmids for mRXRα, mLXRα, and/or mLRH-1 as indicated in combination with a p650-rCYP7A1-luciferase reporter (50 ng). Cells were then incubated with ethanol (ETOH), 1 μM 9cRA, 10 μM 22(R)HC, or both ligands and assayed for luciferase activity. Transcriptional activity was compared between wild-type and a mutant reporter in which the LRH-RE was mutated from GG to CC as shown in (A). RLU, relative light units.

(C) Mutation of the *CYP7A1* LRH-RE abolishes LRH-1 binding. Competition electrophoretic mobility-shift assays (EMSA) were performed as described in the Experimental Procedures. The ^{32}P-labeled LRH-RE oligonucleotide probe specifically binds to LRH-1 (lane 2) but not LXRα (lane 1). LRH-1 binding is specifically competed with unlabeled LRH-RE (lane 3, 10-fold molar excess; lane 4, 50-fold molar excess), but not a mutant LRH-RE (lane 5, 10-fold molar excess; lane 6, 50-fold molar excess).

In this paper, we show that the *SHP* gene is a direct target of FXR and that its expression is dramatically upregulated in the liver by bile acids. The increased level of SHP results in the complete repression of the *CYP7A1* promoter and eventually the *SHP* promoter itself. Finally, we show that the mechanism of SHP repression on both *CYP7A1* and *SHP* promoters is due to the direct interaction of SHP with the competence factor LRH-1. These findings demonstrate the existence of an autoregulatory cascade involving at least five different nuclear receptors that govern bile acid metabolism. The data further illustrate the importance of nuclear receptors as master regulators of cholesterol homeostasis.

Results

Transactivation of the *CYP7A1* Promoter by LXRα Requires LRH-1

Previous work has demonstrated that expression of the murine *CYP7A1* gene is regulated by LXRα in vivo (Peet et al., 1998b). However, despite the widespread expression of LXRα, the LXR-dependent regulation of CYP7A1 by oxysterols is liver specific. Analysis of the 5′-flanking regions of the mouse, rat, hamster, and human *CYP7A1* genes (Stroup et al., 1997; Nitta et al., 1999) reveals a highly conserved element upstream of the LXR response element (LXRE) that resembles the binding sites for the orphan nuclear receptors LRH-1 and steroidogenic factor-1 (SF-1) (Figure 1A). Recent studies document the binding of LRH-1 to this sequence and implicate LRH-1 in the tissue-specific expression of human *CYP7A1* (Nitta et al., 1999).

SF-1 is a well characterized competence factor, directing the expression of genes involved in steroid hormone metabolism in the gonads and adrenal (Parker, 1998). To test whether LRH-1 could serve as the hepatic counterpart to SF-1 and permit LXR-induced expression of *CYP7A1*, plasmids encoding LRH-1, LXRα, and RXRα were cotransfected into a kidney cell line (HEK293) together with a rat *CYP7A1*-luciferase reporter construct containing the proximal 650 bp of the promoter (Figure 1A). These cells were tested for ligand-dependent activation of the RXR/LXR heterodimer following incubation with 22(*R*)-hydroxycholesterol and 9-*cis* retinoic acid. As shown in Figure 1B, in the presence of either the RXR/LXR heterodimer or LRH-1 alone, there was no significant ligand-dependent activation of the rat *CYP7A1* promoter. However, cotransfection of all three receptors led to a marked induction of the promoter by LXR or RXR ligands and a synergistic induction when both ligands are present. Additional studies showed that ectopic expression of *CYP7A1* could be accomplished in a variety of cell lines provided that LRH-1 was added in *trans* along with LXRα and RXRα (data not shown).

To further demonstrate the requirements of LRH-1 and LXRα for *CYP7A1* regulation, the binding site for LRH-1 in the rat *CYP7A1* promoter was mutated at the position underlined in Figure 1A and the mutant construct was tested for its ability to be activated by LXRα. This mutation eliminated LRH-1 binding in vitro (Figure 1C) and completely abolished *CYP7A1* promoter activity (Figure 1B). Mutation of the LXRE also eliminated promoter activity (data not shown). Together, this series of experiments identify LRH-1 as a competence factor that permits LXRα transactivation of *CYP7A1*.

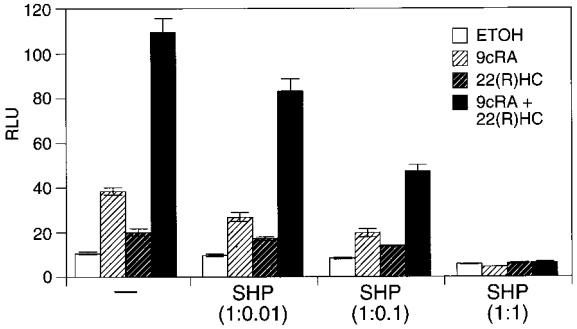

Figure 2. SHP Potently Represses LXR-Dependent Transactivation of *CYP7A1*

HEK293 cells were cotransfected with 15 ng of expression plasmids for mLRH-1, mLXRα, and mRXRα and increasing concentrations of mSHP expression plasmid (0 ng, 0.15 ng, 1.5 ng, or 15 ng) in combination with the p650-rCYP7A1-LUC reporter. The ratio of LRH-1/RXR/LXR to SHP plasmid used in the transfections are shown. Cells were then incubated with ethanol (ETOH), 0.1 μM 9cRA, 10 μM 22(R)HC, or both ligands and assayed for luciferase activity as in Figure 1.

SHP Is a Potent Repressor of *CYP7A1* Transcription

The experiments above as well as those of others (Nitta et al., 1999) suggest a role for LRH-1 as a competence factor in enterohepatic tissues that is remarkably similar to that shown previously for SF-1 in steroidogenic tissues (Parker, 1998). In considering the similarity between LRH-1 and SF-1 signaling pathways, we noted that the liver is one of the primary tissues that expresses SHP, an atypical orphan nuclear receptor that is most closely related to the orphan receptor DAX1 (Seol et al., 1996). DAX1 is selectively expressed in steroidogenic tissues and is an antagonist of SF-1 (Ito et al., 1997; Crawford et al., 1998; Nachtigal et al., 1998). SHP has a dimerization domain but no DNA-binding domain and can dimerize with and repress a subset of nuclear receptors (Seol et al., 1996). Based on these observations, we tested the hypothesis that SHP may regulate LRH-1 transactivation of the *CYP7A1* promoter. Utilizing the HEK293 cell transfection assay described in Figure 1, we first determined the effect of SHP on the transactivation potential of the rat *CYP7A1*-luciferase reporter gene. As shown in Figure 2, cotransfection of increasing concentrations of SHP expression plasmid resulted in a dose-dependent decrease in the ligand-induced transactivation of the *CYP7A1* promoter. The inhibitory effect of SHP appeared to be quite potent; *CYP7A1* promoter activity was completely abolished at a 1:1 molar ratio of SHP to the LRH-1, LXRα, and RXRα expression plasmids.

LRH-1 Is a Target of SHP Repression

The similarity of SHP to DAX1, which has been shown to interact with and repress SF-1, suggested that SHP may also interact with and repress LRH-1. To test this hypothesis, mammalian two-hybrid analysis and in vitro GST-pulldown assays were utilized to monitor LRH-1/SHP interaction (Figure 3). HEK293 cells were transfected with a Gal4 reporter gene and expression plasmids for either the Gal4 DNA-binding domain alone (Gal4-DBD) or the Gal4 DNA-binding domain fused to the LRH-1 ligand binding domain (Gal4-LRH-1). Significantly, cotransfection of SHP elicited a potent (>10-fold) repression of transcription mediated by Gal4-LRH-1 but did not affect the activity of the Gal4-DBD control (Figure 3A). This result suggests that SHP does not inhibit LRH-1 DNA binding, but rather binds to LRH-1 on DNA and actively represses transcription. This conclusion is supported by gel-shift experiments that demonstrate that SHP fails to inhibit LRH-1 from binding to its response element in vitro (data not shown). We also tested a VP16-SHP construct that contains the transactivation domain of the Herpes simplex virus VP16 protein

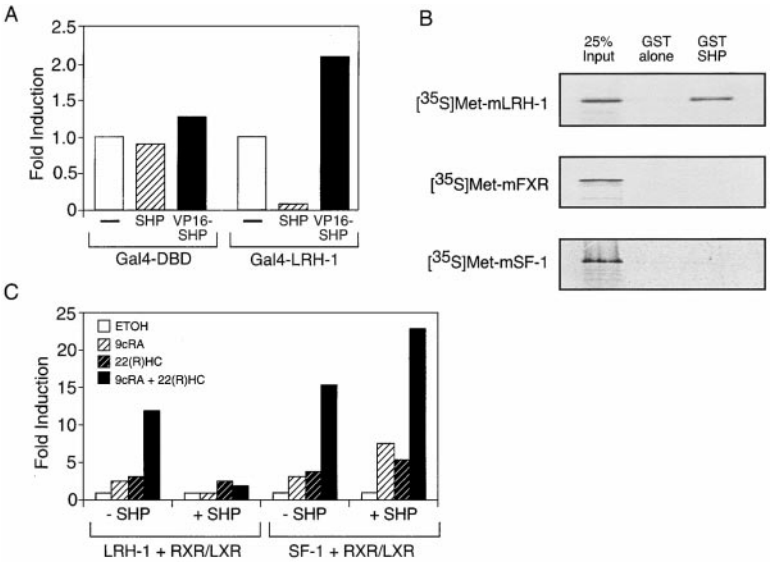

Figure 3. SHP Selectively Represses LRH-1 Activity

(A) SHP interacts with LRH-1 in vivo. Mammalian two-hybrid analysis in HEK293 cells was utilized to demonstrate interaction of LRH-1 with SHP and VP16-SHP. Cells were cotransfected with expression plasmids (15 ng) for Gal4-DBD (control) or Gal4-LRH-1 and empty vector (pCMX) or expression plasmids (15 ng) for SHP or VP16-SHP in combination with a TK-MH100x4-LUC (50 ng) reporter. Luciferase activity of the reporter is expressed as fold induction relative to conditions in the absence of any SHP expression plasmid.

(B) SHP directly interacts with LRH-1 in vitro. GST-pulldown assays were performed to demonstrate a direct interaction between full-length LRH-1 and SHP. GST-SHP specifically interacts with [35S]Met-mLRH-1 (top), but not [35S]Met-mFXR (middle) or [35S]Met-mSF-1 (bottom). Binding reactions were carried out with equivalent amounts of GST or GST-SHP as determined by Coomassie blue staining. Bound protein was analyzed by SDS–PAGE and visualized by autoradiography.

(C) SF-1 serves as a competence factor for *CYP7A1* activity, but does not mediate SHP-dependent repression. SF-1 or LRH-1 (15 ng) was cotransfected with LXRα and RXRα (15 ng each) in combination with the p650-rCyp7A1-LUC reporter (50 ng). For each condition, control empty vector (–SHP), or an expression plasmid for SHP (15 ng) was also transfected. Transcriptional activity induced by ethanol (ETOH), 0.1 μM 9cRA, 10 μM 22(R)HC, or both ligands together was measured as fold induction compared to ETOH control.

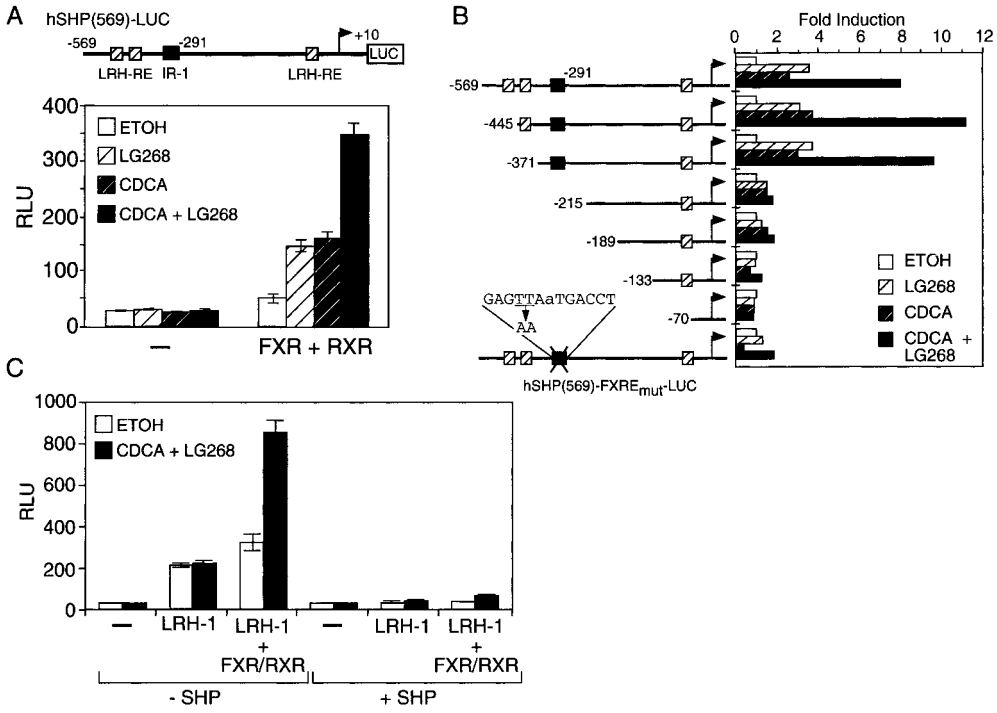

Figure 4. The *SHP* Promoter Is Activated by FXR and Bile Acids

(A) FXR/RXR heterodimer mediates bile acid/retinoid activation of the *SHP* promoter. HEK293 cells were transfected with expression plasmids with no receptor (–) or FXR and RXRα (15 ng each) in combination with the hSHP(569)-LUC reporter (50 ng). Cells were then incubated with ethanol (ETOH), 50 μM CDCA, 100 nM LG268, or both ligands together and measured for luciferase activity. Shown at the top of the figure is the hSHP(569)-LUC vector with the positions of the LRH-REs indicated as hatched boxes and the IR-1 indicated as a closed box.

(B) Identification of an FXRE in the *SHP* promoter. Serial deletions of the human *SHP* promoter were made as indicated and assayed for transcriptional induction by FXR and RXR ligands. The FXRE was localized between –371 and –215 upstream of the transcriptional start site. Point mutations were generated in the 5' half-site of the IR-1 located at position –291 and assayed for transcriptional activity. Transfection and luciferase assays were performed as described above.

(C) SHP feedback represses its own transcription. HEK293 cells were cotransfected with control vector (–) or expression plasmids (15 ng) for LRH-1 and/or hFXR/hRXR and the hSHP(569)-LUC reporter plasmid (50 ng). Transcriptional activity was determined in the presence or absence of a cotransfected SHP expression plasmid (15 ng) as described above.

fused to the amino terminus of SHP. When the Gal4-LRH-1 construct was cotransfected with VP16-SHP, the repression of SHP was counterbalanced by the VP16 activation domain, resulting in increased transcriptional activity. Neither SHP or VP16-SHP had any affect on the Gal4-DBD control. These results demonstrate the ability of LRH-1 and SHP to interact on DNA in cells.

To show that the interaction between LRH-1 and SHP is through direct binding, in vitro GST-pulldown assays were performed using purified GST-SHP fusion protein and in vitro translated, [^{35}S]-labeled LRH-1, FXR, or SF-1. As shown in Figure 3B, SHP efficiently interacted with LRH-1, but not with FXR or SF-1 in this assay. To demonstrate the specificity of the LRH-1/SHP interaction, the response of the *CYP7A1* promoter to LRH-1 and SF-1 was compared. Cotransfection of either LRH-1 or SF-1 with RXRα/LXRα resulted in ligand-dependent activation (Figure 3C), indicating that both LRH-1 and SF-1 can confer competence to the *CYP7A1* promoter. Importantly, however, when SHP was also expressed, repression of *CYP7A1* was seen in the presence of LRH-1 but not SF-1. This result clearly demonstrates that LRH-1 (and not the RXR/LXR heterodimer) is an essential target of SHP repression. Although it cannot be ruled out that other targets of SHP action may exist, we note that SHP also failed to interact with FXR and LXRα in the presence or absence of their ligands (data not shown). Taken together, these data provide strong evidence that LRH-1 is a primary target of SHP's ability to repress the *CYP7A1* promoter. Furthermore, these results show that the interaction of SHP with other factors that act on this promoter, such as RXR and LXR, is not sufficient for SHP-mediated repression.

SHP Gene Expression Is Regulated by Bile Acids and FXR

The ability of SHP to repress *CYP7A1* transcription suggested that the mechanism by which bile acids and FXR antagonize *CYP7A1* transcription may be through their regulation of SHP expression. Examination of the proximal 600 bp of the human and murine *SHP* promoter sequences revealed the presence of a conserved inverted repeat spaced by one nucleotide (IR-1) that could potentially serve as an FXR/RXR binding site (Figure 4A). This IR-1 element is similar to the FXR response element (FXRE) that we have previously identified in the ileal bile acid–binding protein gene (Makishima et al., 1999). Strikingly, the *SHP* promoter also contains several nuclear receptor binding motifs that have recently been

shown to function as LRH-1 response elements (Lee et al., 1999). To test whether the *SHP* gene promoter is also responsive to FXR, HEK293 cells were cotransfected with expression plasmids for FXR, RXRα, and a luciferase reporter construct containing a 569 bp fragment spanning the 5'-flanking region of human *SHP* (hSHP(569)-LUC; see Figure 4A). These cells were then tested for ligand-dependent activation by the FXR ligand, chenodeoxycholic acid (CDCA) and/or the RXR-selective ligand, LG268. As shown in Figure 4A, the FXR/RXR heterodimer strongly induced *SHP* promoter activity in the presence of both ligands, thereby confirming *SHP* as an FXR target gene.

To characterize the FXRE in the *SHP* promoter, serial deletions of the hSHP(569)-LUC reporter were generated and tested for activity in the cotransfection assay (Figure 4B). This analysis localized the response element between base pair positions –215 and –371 of the promoter, which contains the IR-1 element (position –291). Mutating the IR-1 completely eliminated FXR and RXR ligand-responsiveness of the *SHP* promoter (Figure 3B), confirming this site as the FXRE. As mentioned above, this IR-1 element is conserved in the murine *Shp* promoter, and in similar experiments, we have demonstrated that it also functions as a potent FXRE (data not shown). From these data, we conclude that the *SHP* promoter is a direct target of FXR transactivation by bile acids.

SHP Represses Its Own Transcription through LRH-1

Since the *SHP* promoter contains several nuclear receptor half-sites that have been characterized as LRH-1 binding sites, the potential for SHP to repress its own transcription was investigated. In a manner similar to that observed on the *CYP7A1* promoter, SHP potently repressed both the basal activity mediated by LRH-1 alone and the ligand-induced activity of the FXR/RXR heterodimer on its own promoter (Figure 4C). Thus, in addition to repressing bile acid synthesis by inhibiting *CYP7A1* transcription, SHP also feeds back and represses its own transcription.

Bile Acid–Mediated Repression of *CYP7A1* Gene Expression Correlates with Induction of *SHP* Gene Expression In Vivo

The data presented above suggest that bile acids repress CYP7A1 synthesis by inducing the expression of SHP. To confirm these results in vivo, we performed a series of experiments in wild-type and *Cyp7a1* knockout mice (Ishibashi et al., 1996) treated with compounds that are known to activate LXR (cholesterol), FXR (cholic acid), and/or RXR (LG268) in vivo (Peet et al., 1998b; Mukherjee et al., 1997; Makishima et al., 1999). In the first experiment, Northern analysis was performed on poly(A)$^+$ RNA isolated from livers of wild-type mice fed 0.2% cholesterol supplemented with vehicle, LG268, and/or cholic acid for 10 days. As shown in Figure 5A, treatment with LG268 and/or cholic acid resulted in a marked repression of CYP7A1 mRNA levels and a diametric induction of SHP mRNA. Importantly, the levels of other nuclear receptors involved in this pathway (LXRα and LRH-1) were not altered under these dietary conditions.

In the second set of experiments, mouse SHP and

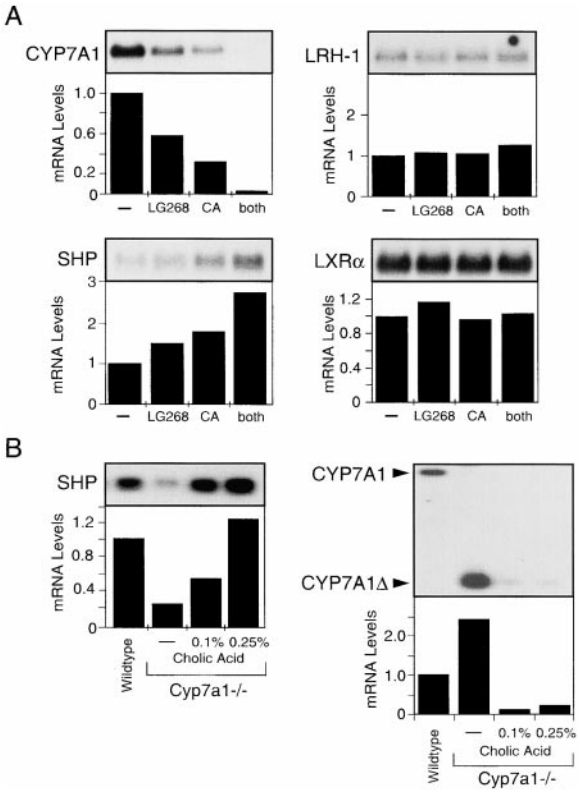

Figure 5. *Shp* and *Cyp7a1* Expression Are Diametrically Regulated by FXR/RXR Ligands In Vivo

(A) Induction of *Shp* correlates with repression of *Cyp7a1* expression in mice. Northern analysis of adult male A129 mice fed 0.2% cholesterol in combination with vehicle (0.9% carboxymethyl cellulose, 9.95% PEG-400, 0.05% Tween 80), cholic acid (0.1%), and/or LG268 (3 mg/kg body weight). 5 μg of liver poly(A)$^+$ mRNA was pooled from six mice per diet. The top of each panel shows Northern blot autoradiograms of CYP7A1, SHP, LRH-1 and LXRα mRNA expression. The bottom of each panel shows respective relative expression levels corrected by a β-actin internal standard.

(B) Bile acid–dependent regulation of *Shp* and *Cyp7a1* transcription in *Cyp7a1*$^{-/-}$ mice. Left: Expression of SHP mRNA is severely diminished in *Cyp7a1*$^{-/-}$ mice but is restored by feeding FXR ligands. Right: Transcription of the truncated CYP7A1 mRNA (CYP7A1Δ) in *Cyp7a1*$^{-/-}$ mice is still appropriately repressed by bile acids. Wild-type and *Cyp7a1*$^{-/-}$ mice were fed diets supplemented with 0.2% cholesterol only or supplemented with cholic acid (0.1% and 0.25% as indicated). The top of each panel shows Northern blot autoradiograms, and the bottom of each panel shows respective relative expression levels corrected by a β-actin internal standard.

CYP7A1 expression were measured by Northern analysis in wild-type and *Cyp7a1*$^{-/-}$ mice fed 0.2% cholesterol supplemented with increasing concentrations of cholic acid for 10 days (Figure 5B). Since *Cyp7a1*$^{-/-}$ mice have a significantly lower bile acid pool size due to a reduction in bile acid synthesis (Schwarz et al., 1998), it was of interest to determine if basal levels of SHP would be lower due to the deficiency in bile acid synthesis. As expected, in wild-type mice fed a 0.2% cholesterol diet there was a robust expression of SHP mRNA (Figure 5B, left panel) that is consistent with the known elevation of *Cyp7a1* gene expression and bile acid synthesis that results from LXRα activation (Peet et al., 1998b). In marked contrast, *Cyp7a1*$^{-/-}$ mice, which synthesize dramatically reduced levels of bile acids, also express

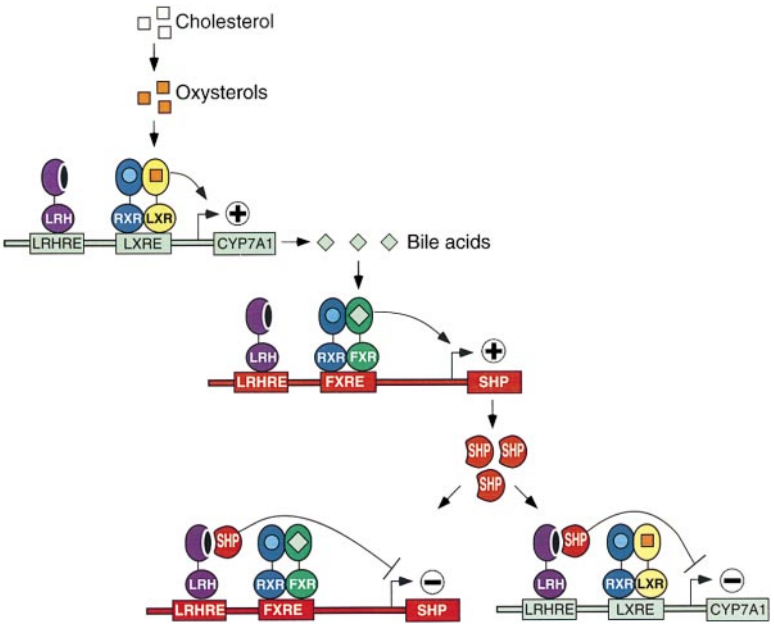

Figure 6. Model Describing Feedback Regulation of Bile Acid Homeostasis by Nuclear Receptors

Elevated cholesterol results in the formation of oxysterols that bind LXRα and activates *CYP7A1* transcription. Up-regulation of CYP7A1 results in increased bile acid synthesis and subsequent excretion of cholesterol. As bile acid levels increase, FXR is activated, which in turn induces the transcription of *SHP*. Elevated levels of SHP protein result in repression of both the *CYP7A1* and *SHP* promoters by SHP forming an inhibitory heterodimeric complex with LRH-1. In this way, bile acids effectively down-regulate their own synthesis.

significantly lower levels of SHP mRNA, even in the presence of 0.2% cholesterol (Figure 5B, left panel). As would be predicted by replenishing the bile acid pool size, feeding increasing concentrations of cholic acid to these animals leads to a complete dose-dependent restoration of SHP expression. To demonstrate that the FXR-mediated repression pathway is still intact in *Cyp7a1*$^{-/-}$ mice, we monitored the activity of the CYP7A1 promoter in these animals in response to cholic acid. The *Cyp7a1*$^{-/-}$ mice still contain a functional CYP7A1 promoter that produces a truncated mRNA that can be detected by Northern analysis (CYP7A1Δ, Figure 5B, right panel). As expected, the transcription of the truncated message responded normally and was elevated in the absence of SHP (Figure 5B, right panel, lane 2) and repressed upon elevation of SHP by cholic acid feeding (lanes 3 and 4). These data provide direct in vivo evidence that the FXR repression pathway was not altered by the knockout of *Cyp7a1*. Taken together, these experiments unequivocally demonstrate the bile acid dependence of *SHP* expression in vivo and strongly support the role of SHP as the mediator of the feedback repression of bile acid synthesis.

Discussion

A New Model for the Bile Acid Sensor

Bile acids play an integral role in lipid homeostasis. They are essential for the solubilization and absorption of lipids and fat-soluble vitamins in the intestine, and they provide the major pathway by which cholesterol is excreted from the body. In addition, like other important regulatory lipids, the metabolism and transport of bile acids is tightly governed. This regulation is achieved in part by the body's ability to accurately sense the concentration of bile acids in their target tissues. Given the structural and functional similarity of bile acids to oxysterols, which bind to the LXR subfamily of nuclear receptors (Janowski et al., 1999), it is perhaps not surprising that the key sensor governing bile acid homeostasis has recently been shown to be FXR, a nuclear receptor specifically expressed in enterohepatic and renal tissues (reviewed in Russell, 1999). However, in spite of the fact that the identification and study of these receptors has provided a rather complete picture of the feedforward pathway that activates bile acid synthesis (Repa and Mangelsdorf, 2000), the mechanism governing the feedback repression pathway has not been elucidated. In this paper, we provide evidence for a mechanism of feedback repression that it is intricately linked to the coordinate regulation of several liver-enriched nuclear receptors, including FXR, LRH-1, and SHP.

A model summarizing this mechanism is illustrated in Figure 6. In response to elevated levels of cholesterol, LXRα acts as a cholesterol sensor and up-regulates the expression of *CYP7A1* (Peet et al., 1998b). Transactivation of the *CYP7A1* promoter requires the concerted binding of RXR/LXRα and the competence factor LRH-1 (this work and Nitta et al., 1999). CYP7A1 enzymatic activity leads to the production of bile acids that bind to and activate FXR (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999). As demonstrated here, a key target of FXR transactivation that is likely to keep bile acid levels in check is SHP, an orphan nuclear receptor that functions as a potent transcriptional repressor of LRH-1. This finding is supported by similar observations in which a synthetic FXR agonist was shown to up-regulate *SHP* expression and repress *CYP7A1* transcription (Goodwin et al., 2000 [this issue of *Mol. Cell*]). Although the precise mechanism by which SHP represses has not been clearly defined, our work suggests that SHP does not prevent LRH-1 from binding DNA. Rather, our data are consistent with recent reports suggesting that SHP recruits co-repressor activity to its DNA-bound target (Lee et al., 2000). The increased expression of *SHP* effectively inhibits bile acid synthesis by repressing *CYP7A1* gene expression. In addition, we have shown that SHP autoregulates its synthesis by feedback-repressing transcription of the *SHP* promoter. Thus, the ability of FXR to induce SHP, which in turn represses its own synthesis, provides an elegant mechanism to explain how the bile acid sensor is both turned

on and off. In keeping with this model, the expression of *SHP* is markedly reduced in bile acid–deficient *Cyp7a1*$^{-/-}$ mice, and this expression is completely restored by giving back exogenous bile acids. This regulatory scheme is further supported by the finding that *Fxr*$^{-/-}$ mice have severely reduced levels of SHP and lack the ability to feedback repress *CYP7A1* (Sinal et al., 2000).

An important advance in understanding the mechanism of feedback repression by bile acids has come with the observation that LRH-1 is the major target of SHP's repressor activity. The interaction with LRH-1 allows SHP to specifically repress target genes that normally rely on LRH-1 for expression. This outcome leads to the prediction that in addition to *CYP7A1* and *SHP*, other LRH-1 dependent genes will also be repressed by SHP. One attractive candidate for such regulation is the gene encoding sterol 12α-hydroxylase (CYP8B1), an enzyme that is downstream of CYP7A1 and is required for synthesis of the primary bile acid, cholic acid. Like *CYP7A1*, the *CYP8B1* gene has recently been shown to require LRH-1 for liver-specific expression (del Castillo-Olivares and Gil, 2000), and it is known to be repressed by bile acids (Vlahcevic, et al., 2000). The regulation of this enzymatic cascade by LRH-1 and SHP in the enterohepatic system bears a striking resemblance to that found in the adrenal/gonadal axis. In these tissues, key *CYP* genes encoding steroidogenic biosynthetic enzymes are regulated by the nuclear orphan receptors SF-1 and DAX1 (Hammer and Ingraham, 1999), which are paralogs of LRH-1 and SHP.

Therapeutic Potential

The ability of SHP to selectively interact and repress LRH-1 activity provides an explanation for why FXR-mediated repression is dominant over the LXRα-mediated activation of the *CYP7A1* promoter. Because LRH-1 is essential for *CYP7A1* transcription, FXR-induced SHP is able to repress *CYP7A1* expression even in the presence of ligand-activated RXR/LXR heterodimer. Although other mechanisms have been proposed for how FXR may mediate *CYP7A1* repression (e.g., via FXR interference with the RXR/LXR heterodimer; Wang et al., 1999), such mechanisms cannot explain the repression that is observed when the *CYP7A1* promoter is activated by other factors or in species that lack a potent RXR/LXR binding site in the *CYP7A1* promoter (e.g., humans). The finding that SHP repression is mediated through LRH-1 and possibly other nuclear receptors provides a plausible explanation for why bile acids can repress *CYP7A1*, irrespective of the activator. This observation further explains why RXR-selective agonists (i.e., rexinoids), which can activate both RXR/LXR and RXR/FXR heterodimers, repress rather than activate *CYP7A1* expression in vivo (Figure 5A). This finding may have important therapeutic implications, since it implies that any agent that activates or inhibits *SHP* expression in vivo will dramatically alter bile acid homeostasis. Thus, the identification of SHP ligands should allow the pharmacological manipulation of the bile acid sensor to affect increased cholesterol excretion in the treatment of hypercholesterolemia or decreased bile acid synthesis for treatment of cholestasis.

Experimental Procedures

Plasmids

Expression vectors for pCMX (Umesono et al., 1991), pCMX-mLXRα (Peet et al., 1998b), pCMX-mRXRα (Mangelsdorf et al., 1990), pCMV5-SF-1 (Lala et al., 1997), pCMX-hFXR or -mFXR (Makishima et al., 1999) were created as described. Vectors including mLRH-1 and mSHP were constructed using cDNAs obtained from RT–PCR of mouse liver poly(A)$^+$ RNA. Primers for full-length mLRH-1 (nucleotides 159–1841, GenBank Accession Number M81385) were: 5′-CGAATTCCAAAGGACTGCCAATAATTTCGC-3′ and 5′-GTTGGATCCTAGAGCAAGCTTCCAGGGG-3′. PCR-amplified products were digested with EcoRI and BamHI and cloned into the pCMX-PL2 vector (Willy et al., 1995). For full-length mSHP (nucleotides 33–815, GenBank Accession Number L76567), primers were: 5′-AAGGATCCGCTGGGAAGAAACAGGAACAAG-3′ and 5′-CTAGCTAGCTGGAGGCACCAGACTCCATTC-3′. SHP PCR products were digested with BamHI and NheI and cloned into pCMX-PL1 and pCMX-VP16 (Willy et al., 1995). For construction of GAL4-LRH-1, the ligand binding domain of LRH-1 (nucleotides 675–1841) was PCR amplified, digested with EcoRI and BamHI, and cloned into the pCMX-GAL4 vector.

The p650-rCYP7A1 promoter-luciferase reporter was constructed by inserting 650 bp of the proximal rat *CYP7A1* promoter into the SalI and BglII site of TK-luciferase vector (Willy et al., 1995). Mutations in the rat *CYP7A1* promoter LRH-1 and LXR response elements were generated by Stratagene QuikChange Site-Directed Mutagenesis kit.

The human *SHP* promoter was obtained by PCR amplification using the published sequence (GenBank Accession Number AF044316), and 569 bp of the proximal promoter was cloned into the luciferase reporter pGL3 (Promega) to generate hSHP(569)-LUC. Human *SHP* promoter deletion constructs were generated by PCR amplification. These PCR fragments were subsequently ligated into the HindIII and BglII sites of TK-LUC. The reporter plasmid hSHP(569)-FXREmut-LUC was generated as indicated in Figure 4B utilizing the Stratagene QuikChange Site-Directed Mutagenesis kit. TK-MH100x4-LUC was utilized for mammalian two-hybrid analysis as described (Willy et al., 1995). All plasmids were sequenced prior to use to verify DNA sequence fidelity.

Cell Culture and Cotransfection Assays

HEK293 cells were maintained at 37°C, 5% CO_2 in DMEM containing 10% fetal bovine serum (FBS). Transfections were performed in media containing 10% dextran-charcoal–stripped FBS by the calcium phosphate coprecipitation assay as previously described (Makishima et al., 1999). Eight hours after transfection, ligands were added at 1000-fold dilutions in media containing 10% dextran-charcoal–stripped FBS. Ligands were handled under gold light and stored in ethanol at –20°C. Cells were harvested after 18 hr for luciferase and β-galactosidase activity using a Torcon Instruments, Inc. AML-34 luminometer and a Dynatech model MR5000 spectrophotometer, respectively. For most experiments, DNA cotransfection experiments involved utilization of 50 ng of reporter plasmid, 20 ng of pCMX-β-galactosidase, 15 ng of each receptor expression plasmid, and pGEM carrier DNA to give 150 ng of DNA total per well of a 96-well plate. Empty pCMX vector was utilized for no receptor controls and to maintain equivalent amounts of pCMX vector plasmids for each transfection condition. Luciferase data were normalized to an internal β-galactosidase control and represent the mean (± standard deviation) of triplicate assays.

Electrophoretic Mobility-Shift Assays

Electrophoretic mobility-shift assays were performed as described (Willy et al., 1995). Briefly, receptor proteins were in vitro translated with the TNT Quick Coupled Transcription/Translation System (Promega). Double-stranded oligonucleotides with HindIII overhangs were generated for the LRH-response element (LRH-RE) and the mutant as shown in Figure 1C. Binding reactions were performed in a total volume of 20 μl consisting of 75 mM KCl, 20 mM HEPES at pH 7.4, 2 mM DTT, 7.5% glycerol, 0.1% NP-40, 2 μg of poly[d(I-C)] (Pharmacia), 40 pmol of a nonspecific single-stranded oligonucleotide (to remove nonspecific binding), and 2 μl of LRH-1 lysate or LXRα (control) lysate. Reactions containing competing oligonucleotides were incubated for 30 min on ice, followed by the addition of 40 fmol of ^{32}P-labeled LRH-RE probe (labeled by end-filling) and further incubation at room temperature for 20 min. Samples were

then analyzed on 5% polyacrylamide gels run in 0.5 × TBE buffer and were visualized by autoradiography.

GST-Pulldown Assays

Mouse SHP cDNA was cloned into the GST-fusion vector pGEX5X-2 (Pharmacia). GST-SHP fusion protein was expressed in BL21 DE3 cells (Promega) and affinity purified onto glutathione-Sepharose 4B beads according to the manufacturer (Pharmacia). ^{35}S-labeled mLRH-1, mFXR, and mSF-1 were generated by using the TNT Quick Coupled Transcription/Translation System (Promega). For GST-pulldown assays, approximately 1 μg of GST-SHP and GST alone (as determined by Coomassie staining) was bound and equilibrated in binding buffer (20 mM Tris-HCl at pH 7.9, 150 mM NaCl, 1 mM EDTA, 4 mM $MgCl_2$, 0.2% NP-40, 10% glycerol). Bound GST proteins were then incubated with labeled mLRH-1, mFXR, or mSF-1 for 30 min at room temperature. After binding, beads were washed five times with binding buffer, resuspended in SDS–PAGE sample buffer, and loaded onto a 10% SDS–polyacrylamide gel. After electrophoresis, bound proteins were visualized by autoradiography.

Northern Analysis

Murine liver poly(A)$^+$ RNA was isolated and probed as previously described (Peet et al., 1998b). RNA was taken from male wild-type or *Cyp7a1*$^{-/-}$ mice (Ishibashi et al., 1996) maintained on a 12 hr light/dark cycle and fed ad libitum a cereal-based mouse/rat diet (No. 7001, Harlan-Teklad) supplemented with 0.2% cholesterol. As indicated in Figure 5, diets were further supplemented with vehicle (0.9% carboxymethyl cellulose, 9.95% PEG-400, 0.05% Tween 80), cholic acid (0.1% or 0.25%), and/or LG268 (3 mpk) for 10 days. Hybridization probes for Northern blots included PCR-amplified cDNA for CYP7A1 (Ishibashi et al., 1996), SHP, LRH-1, and LXRα. Relative expression levels were quantified utilizing a phosphorimager (Molecular Dynamics) and standardized against β-actin controls.

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