

# A Regulatory Cascade of the Nuclear Receptors FXR, SHP-1, and LRH-1 Represses Bile Acid Biosynthesis

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## Summary

Bile acids repress the transcription of cytochrome P450 7A1 (*CYP7A1*), which catalyzes the rate-limiting step in bile acid biosynthesis. Although bile acids activate the farnesoid X receptor (FXR), the mechanism underlying bile acid-mediated repression of *CYP7A1* remained unclear. We have used a potent, nonsteroidal FXR ligand to show that FXR induces expression of small heterodimer partner 1 (SHP-1), an atypical member of the nuclear receptor family that lacks a DNA-binding domain. SHP-1 represses expression of *CYP7A1* by inhibiting the activity of liver receptor homolog 1 (LRH-1), an orphan nuclear receptor that is known to regulate *CYP7A1* expression positively. This bile acid-activated regulatory cascade provides a molecular basis for the coordinate suppression of *CYP7A1* and other genes involved in bile acid biosynthesis.

## Introduction

Cholesterol is essential for a number of cellular functions, including membrane biogenesis and steroid hormone and bile acid biosynthesis. However, in excess, cholesterol can contribute to disease processes such as atherosclerosis and gallstone formation. Therefore, cholesterol biosynthesis and catabolism must be coordinately regulated. The metabolism of cholesterol to bile acids represents a major pathway for its elimination from the body, accounting for approximately half of daily excretion. Cytochrome P450 7A (*CYP7A1*) is a liver-specific enzyme that catalyzes the first and rate-limiting step in one of the two pathways for bile acid biosynthesis (Chiang, 1998; Russell and Setchell, 1992). The gene encoding *CYP7A1* is regulated by a variety of small, lipophilic molecules, including steroid and thyroid hormones, cholesterol, and bile acids. Notably, *CYP7A1* expression is stimulated by cholesterol feeding and repressed by bile acids. Thus, *CYP7A1* is under both feedforward and feedback regulation.

*CYP7A1* expression is regulated by several members

of the nuclear receptor superfamily of ligand-activated transcription factors (Chiang, 1998; Gustafsson, 1999; Russell, 1999). Recently, two nuclear receptors, the liver X receptor  $\alpha$  (LXR $\alpha$ ; NR1H3) (Apfel et al., 1994; Willy et al., 1995) and farnesoid X receptor (FXR; NR1H4) (Forman et al., 1995; Seol et al., 1995), were implicated in the feedforward and feedback regulation of *CYP7A1*, respectively (Peet et al., 1998; Russell, 1999). Both LXR $\alpha$  and FXR are abundantly expressed in the liver and bind to their cognate hormone response elements as heterodimers with the 9-*cis* retinoic acid receptor RXR (Mangelsdorf and Evans, 1995). LXR $\alpha$  is activated by the cholesterol derivative 24,25(S)-epoxycholesterol and binds to a response element in the *CYP7A1* promoter (Lehmann et al., 1997). Mice lacking LXR $\alpha$  do not induce *CYP7A1* expression in response to cholesterol feeding (Peet et al., 1998). Moreover, these animals accumulate massive amounts of cholesterol in their livers when fed a high cholesterol diet. These data establish LXR $\alpha$  as the cholesterol sensor responsible for feedforward regulation of *CYP7A1* expression.

Bile acids stimulate the expression of genes involved in bile acid transport, such as the intestinal bile acid-binding protein (*I-BABP*), and repress *CYP7A1* and other genes encoding enzymes involved in bile acid biosynthesis, such as *CYP8B1*, which converts chenodeoxycholic acid (CDCA) to cholic acid, and *CYP27*, which catalyzes the first step in the alternative, "acidic" pathway for bile acid synthesis (Russell and Setchell, 1992; Javitt, 1994; Russell, 1999). Recently, FXR was shown to be a bile acid receptor (Wang et al., 1996; Makishima et al., 1999; Parks et al., 1999). Several different bile acids, including CDCA and its glycine and taurine conjugates, bind and activate FXR at physiologic concentrations. Moreover, FXR response elements (FXREs) were identified in both the mouse and human *I-BABP* promoters (Grober et al., 1999; Makishima et al., 1999), which provided strong evidence that FXR mediates the positive effects of bile acids on *I-BABP* expression. Notably, the rank order of bile acids that activate FXR correlates with that for repression of *CYP7A1* in a hepatocyte-derived cell line (Makishima et al., 1999). These data suggested that FXR also has a role in the negative effects of bile acids on gene expression. However, since the region of the *CYP7A1* promoter that is necessary for bile acid-mediated repression lacks a strong FXR-binding site (Chiang and Stroup, 1994; Chiang et al., 2000), it seemed unlikely that this repression was a direct effect of FXR. Thus, the molecular mechanism for bile acid-mediated repression of *CYP7A1* remained in question.

In this report, we have used a potent, nonsteroidal FXR ligand to demonstrate that FXR regulates the hepatic expression of small heterodimer partner 1 (SHP-1; NR0B2), an atypical, orphan member of the nuclear receptor family that lacks a DNA-binding domain (Seol et al., 1996). SHP-1 has been shown to bind to other nuclear receptors and to repress their transcriptional activities (Seol et al., 1996; Masuda et al., 1997; Johansson et al., 1999; Lee et al., 2000). We show that SHP-1 represses the *CYP7A1* promoter through interaction with liver receptor homolog 1 (LRH-1; NR5A2), an orphan nuclear receptor that binds as a monomer to a response

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element in the *CYP7A1* promoter and activates transcription (Becker-Andre et al., 1993; Galarneau et al., 1996; Nitta et al., 1999). LRH-1 is a mammalian homolog of the *Drosophila* fushi tarazu F1 gene product, which regulates *Drosophila* metamorphosis (Lavorgna et al., 1991; Broadus et al., 1999). Our findings define a novel regulatory cascade of three orphan nuclear receptors that provides a molecular basis for the coordinate repression of gene expression by bile acids.

## Results

### Identification of GW4064 as a Potent, Selective FXR Activator

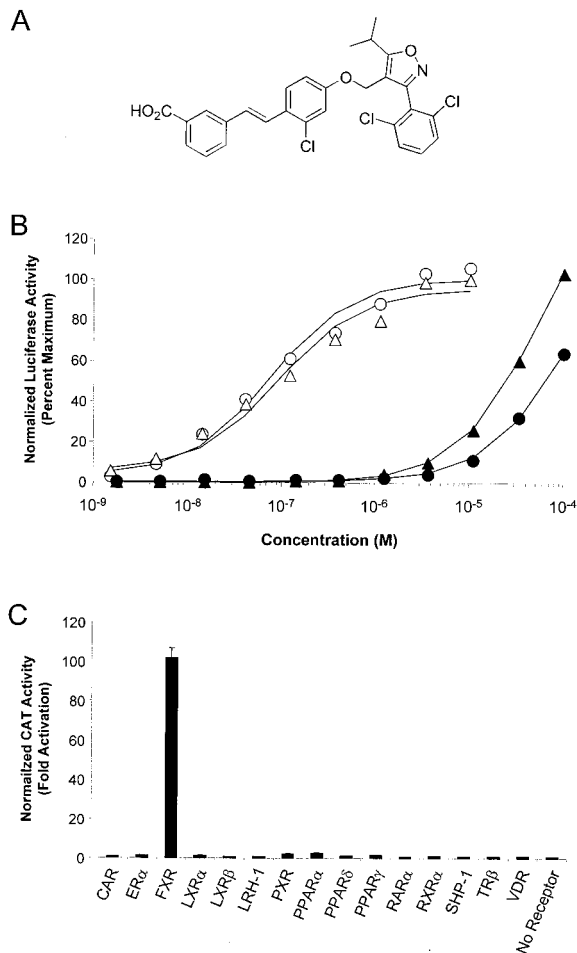
FXR was recently shown to be a receptor for CDCA as well as other bile acids (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999). However, these compounds bind to FXR with only micromolar affinities and at these concentrations also interact with other proteins, including bile acid-binding proteins and transporters. We sought to identify a potent, selective FXR ligand for use as a chemical tool in elucidating the genes regulated by FXR. Combinatorial libraries of compounds were screened using a ligand-sensing fluorescence resonance energy transfer assay that detects interactions between FXR and a peptide derived from the steroid receptor coactivator 1 (SRC-1) as previously described (Parks et al., 1999). Among the compounds that promoted an interaction between FXR and SRC-1 was the isoxazole GW4064 (Figure 1A), which bound to FXR with a half-maximal effective concentration ( $EC_{50}$ ) of 15 nM (Maloney et al., 2000). GW4064 activated mouse and human FXR with  $EC_{50}$  values of 80 and 90 nM, respectively, in CV-1 cells transfected with FXR expression vectors and a reporter plasmid containing two copies of an established FXR response element (FXRE) derived from the *Drosophila* heat shock protein 27 (hsp27) promoter (Forman et al., 1995) (Figure 1B). Thus, GW4064 is ~1000-fold more potent than CDCA in activating FXR in CV-1 cells (Figure 1B).

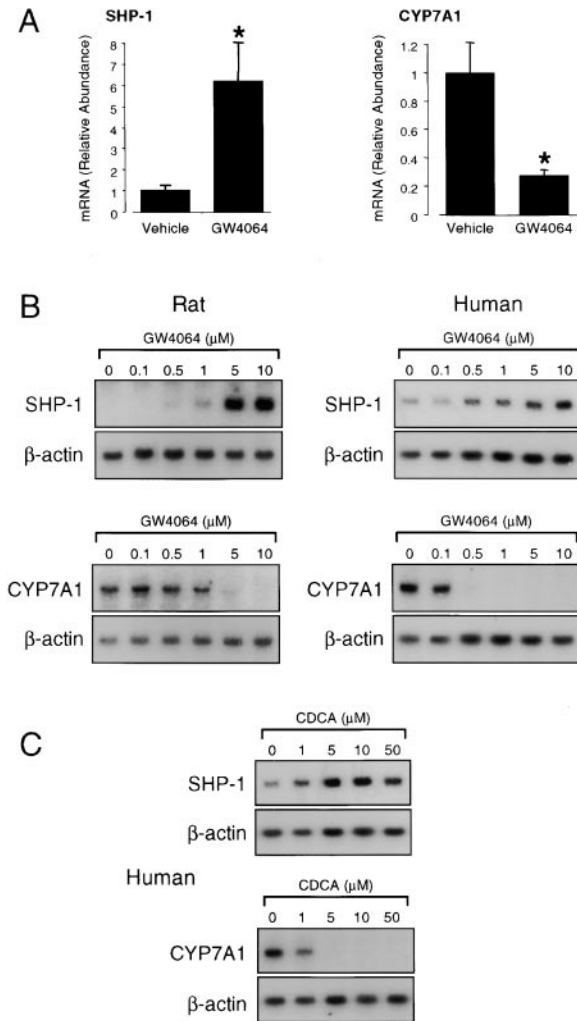
GW4064 was tested for selectivity against a panel of nuclear receptors. CV-1 cells were transfected with expression plasmids for various nuclear receptor–GAL4 chimeras and the reporter plasmid ( $UAS$ )<sub>5</sub>-tk-CAT as previously described (Parks et al., 1999). GW4064 activated only the FXR–GAL4 chimera (Figure 1C). Thus, GW4064 is a highly selective activator of FXR.

### FXR Regulates *SHP-1* Expression in the Liver

GW4064 was exploited as a chemical tool to identify the genes regulated by FXR in the liver. Male Fisher rats were treated for 7 days with GW4064 or vehicle alone (methyl cellulose). Following treatment, RNA was prepared from the livers of GW4064- and vehicle-treated animals, and genes that were either induced or repressed by GW4064 treatment were determined using CuraGen GeneCalling™ differential gene expression technology (Shimkets et al., 1999). A comprehensive list of the liver genes regulated by GW4064 will be published elsewhere. Interestingly, the gene that was most strongly induced by GW4064 treatment was that encoding the orphan nuclear receptor *SHP-1*. Northern analysis showed that *SHP-1* expression was increased ~6-fold in the livers of GW4064-treated rats relative to vehicle-treated animals (Figure 2A).

Bile acids are known to repress the expression of





**Figure 2. FXR Ligands Induce *SHF-1* and Repress *CYP7A1* Expression**

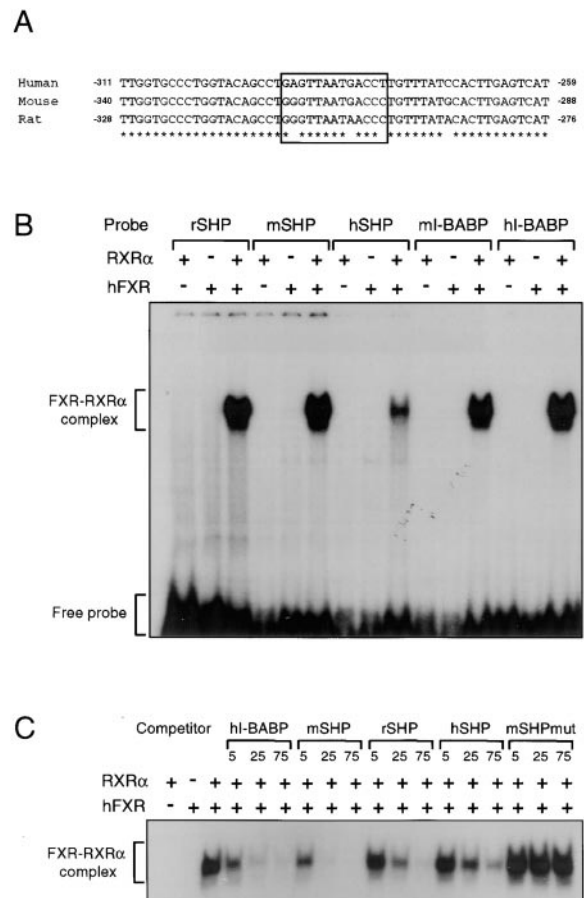
(A) Total RNA was prepared from the livers of male Fisher rats treated for 7 days with GW4064 or vehicle alone. Northern analysis was performed using probes for rat *SHP-1* and *CYP7A1*. Data represent the mean ( $n = 3$ )  $\pm$  standard error of the means. The asterisk denotes a statistically significant difference between vehicle- and GW4064-treated animals;  $P < 0.05$ .

(B) Total RNA was prepared from primary rat or human hepatocytes treated for 48 hr with the indicated concentrations of GW4064 or vehicle alone. Northern analysis was performed using probes for rat or human *SHP-1*, *CYP7A1*, or  $\beta$ -actin.

(C) Total RNA was prepared from primary human hepatocytes treated for 48 hr with the indicated concentrations of CDCA. Northern analysis was performed using probes for human *SHP-1*, *CYP7A1*, or  $\beta$ -actin.

effects of naturally occurring FXR ligands, namely bile acids, on *CYP7A1* expression. This observation provides compelling evidence that FXR mediates feedback repression of *CYP7A1* by bile acids.

To substantiate the *in vivo* data and extend them to human hepatocytes, we examined whether *SHP-1* and *CYP7A1* expression were regulated by FXR in primary cultures of rat and human hepatocytes. Hepatocytes were treated with increasing concentrations of GW4064, and the levels of *SHP-1* and *CYP7A1* expression were



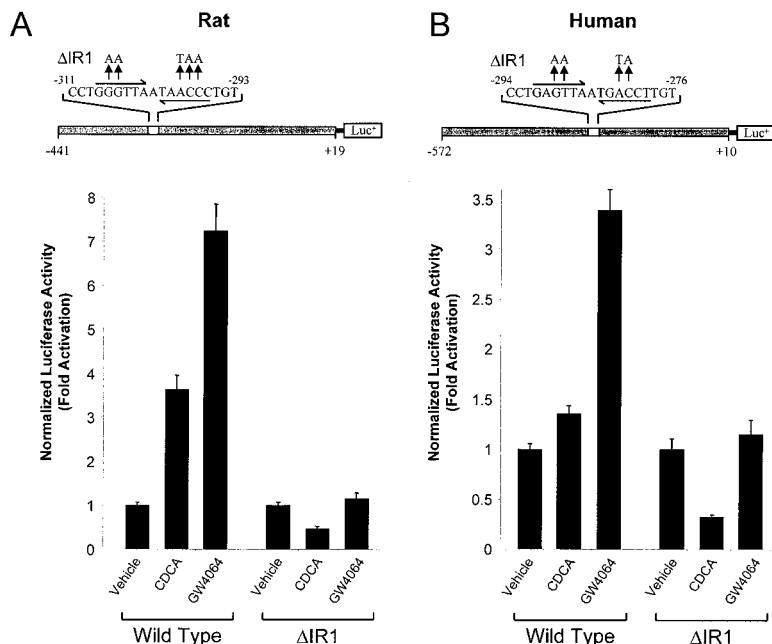
**Figure 3. Identification of FXR Binding Sites in the Human, Rat, and Mouse *SHP-1* Promoters**

(A) Alignment of the proximal regions of the human, rat, and mouse *SHP-1* promoters. The conserved IR1 FXR binding site is boxed. Conserved nucleotides are indicated by asterisks.

(B) Electrophoretic mobility-shift assays were performed with in vitro synthesized human FXR and/or human RXR $\alpha$  as indicated and [ $^{32}$ P]-labeled oligonucleotides containing the IR1 motif from the rat, mouse, or human *SHP-1* promoters or the mouse or human *I-BABP* promoters. The positions of the shifted FXR/RXR $\alpha$  complex and free probes are indicated.

(C) Electrophoretic mobility-shift assays were performed with in vitro synthesized human FXR and/or human RXR $\alpha$ , a [ $^{32}$ P]-labeled oligonucleotide containing the human *I-BABP* FXRE, and either a 5-, 25-, or 75-fold excess of unlabeled oligonucleotides containing the IR1 motifs from the human *I-BABP* promoter, the mouse, rat, or human *SHP-1* promoters, or a mutated derivative of the mouse *SHP-1* IR1 motif (mSHPmut). The position of the shifted FXR/RXR $\alpha$  complex is indicated.

examined by Northern blot analysis. GW4064 treatment markedly increased *SHP-1* expression and decreased *CYP7A1* expression in hepatocytes from both species in a dose-dependent fashion (Figure 2B). Similar results were obtained in human hepatocytes treated with the natural FXR ligand CDCA (Figure 2C). As expected, CDCA was less potent than GW4064 in its effects on gene expression (compare Figures 2B and 2C). These data strongly suggest that FXR regulates *SHP-1* and *CYP7A1* expression in both human and rodent hepatocytes. Notably, there was a striking reciprocal relationship between the regulation of *SHP-1* and *CYP7A1*



**Figure 4. FXR Activates the Rat and Human *SHP-1* Promoters**

HepG2 cells were transfected with the human FXR expression plasmid and luciferase reporter plasmids containing the proximal promoters of the rat ([A], nucleotides -441 to +19) or human ([B], nucleotides -572 to +10) *SHP-1* genes or the corresponding reporter plasmids in which the IR1 elements had been mutated (ΔIR1). Following transfection, cells were treated for 48 hr with GW4064 (1  $\mu$ M) or CDCA (100  $\mu$ M). Data represent the mean  $\pm$  S.D. of six individual transfections.

expression: GW4064 and CDCA repressed *CYP7A1* expression at the same concentrations that were required to induce *SHP-1* expression (Figures 2B and 2C). Since *SHP-1* is known to heterodimerize with several other members of the nuclear receptor superfamily and to repress their transcriptional activity (Seol et al., 1996; Masuda et al., 1997; Johansson et al., 1999), these data raised the intriguing possibility that FXR-mediated induction of *SHP-1* might underlie the repression of *CYP7A1* expression (see below).

#### FXR Binds and Activates *SHP-1* Promoters

We next sought to determine whether *SHP-1* expression is directly regulated by FXR. FXR preferentially binds as a heterodimer with RXR to FXREs composed of two nuclear receptor half-sites of consensus AG(G/T)TCA organized as an inverted repeat and separated by a single nucleotide (IR1) (Forman et al., 1995). IR1-type FXREs have been identified in the human and mouse *I-BABP* promoters (Grober et al., 1999; Makishima et al., 1999). The mouse, rat, and human *SHP-1* promoters were examined for IR1 motifs. A highly conserved IR1-like element was identified ~300 nucleotides upstream of the transcription initiation site in the *SHP-1* promoter of all three species (Figure 3A). Electrophoretic mobility-shift analyses demonstrated that the FXR/RXR complex binds efficiently to the IR1 element from the *SHP-1* promoter of each species (Figure 3B). In agreement with earlier observations (Grober et al., 1999), the FXR/RXR heterodimer also bound to the mouse and human *I-BABP* FXREs (Figure 3B). Competition binding analyses showed that these interactions were specific: no competition was seen with a mutated derivative of the IR1 motif derived from the mouse *SHP-1* promoter (Figure 3C).

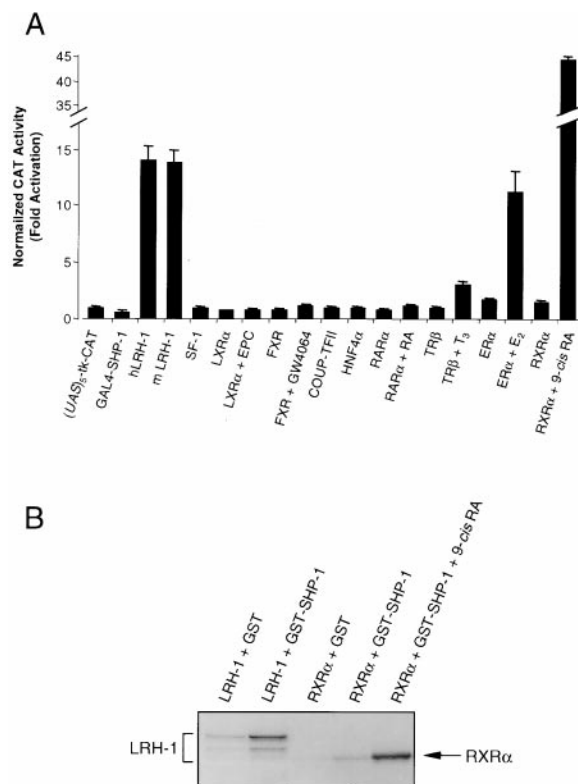
The presence of an FXR/RXR binding site suggested that the *SHP-1* gene is directly regulated by FXR. To test this hypothesis, HepG2 cells were transfected with an FXR expression plasmid and reporter plasmids expressing luciferase under the control of either the rat or

human *SHP-1* promoters. GW4064 treatment of cells transfected with the FXR expression plasmid and either promoter construct resulted in a marked induction of reporter activity (Figures 4A and 4B). Based on Northern blot analysis of *SHP-1* expression (Figure 2B), the magnitude of the response from the rat (7-fold) and human (3-fold) *SHP-1* promoters was somewhat lower than expected and it is possible that other promoter or enhancer elements contribute to the regulation of *SHP-1* expression. Alternately, additional factors present in well differentiated cultures of rat hepatocytes but not HepG2 cells may be required for maximal FXR responsiveness. In the absence of exogenously expressed FXR, the rat and human *SHP-1* promoters exhibited a modest (~1.5-fold) induction on exposure to GW4064, which is most likely due to endogenous FXR in HepG2 cells (data not shown). FXR responsiveness was eliminated when mutations were introduced into the IR1 motifs in either the rat or human *SHP-1* promoters (Figures 4A and 4B). These data provide strong evidence that *SHP-1* expression is regulated directly by the FXR/RXR heterodimer in multiple species.

#### *SHP-1* Interacts with Orphan Nuclear Receptor LRH-1

The finding that *SHP-1* expression is regulated by FXR together with the reciprocal relationship between *SHP-1* and *CYP7A1* regulation (Figure 2) suggested that *SHP-1* might play a pivotal role in bile acid-mediated repression of *CYP7A1* expression. Regulation of the *CYP7A1* promoter is complex and involves numerous transcription factors, including nuclear receptors with known ligands such as the thyroid hormone receptor (TR), retinoic acid receptor (RAR), RXR and LXR $\alpha$ , and the orphan receptors COUP-TFII, HNF4 $\alpha$ , and LRH-1 (Lehmann et al., 1997; Stroup et al., 1997; Chiang, 1998; Peet et al., 1998; Nitta et al., 1999; Russell, 1999; Stroup and Chiang, 2000). *SHP-1* has previously been shown to bind to and repress the transcriptional activities of TR, RAR, and RXR in the presence of their ligands and HNF4 $\alpha$  in the





**Figure 5.** SHP-1 Interacts with the Orphan Nuclear Receptor LRH-1 (A) Mammalian two-hybrid experiments were performed in CV-1 cells cotransfected with expression plasmids for the GAL4-human SHP-1 chimera and various VP16-nuclear receptor ligand-binding domain chimeras. Transfection assays containing the LXR $\alpha$ -, FXR-, RAR $\alpha$ -, TR $\beta$ -, ER $\alpha$ -, and RXR $\alpha$ -GAL4 chimeras were performed in the absence or presence of the indicated ligands [respectively: EPC, 24(S),25-epoxycholesterol (10  $\mu$ M), GW4064 (1  $\mu$ M); RA, all-*trans* retinoic acid (0.1  $\mu$ M); T<sub>3</sub>, triiodothyronine (0.1  $\mu$ M); E<sub>2</sub>, estradiol (0.1  $\mu$ M); 9-*cis* RA, 9-*cis* retinoic acid (0.1  $\mu$ M)]. Data are expressed as fold activation over cells transfected with the (UAS)<sub>5</sub>-tk-CAT reporter alone and represent the mean of assays ( $n = 8$ )  $\pm$  S.D. (B) GST pull-down assays were performed with [<sup>35</sup>S]-labeled LRH-1 or RXR $\alpha$  in the presence of GST or GST-SHP-1 as indicated. 9-*cis* retinoic acid (9-*cis* RA) was added to the binding reaction to a final concentration of 10  $\mu$ M.

absence of any exogenous ligand (Seol et al., 1996; Masuda et al., 1997). Using a mammalian two-hybrid approach, we examined whether SHP-1 interacts with these and other nuclear receptors that have been implicated in the regulation of *CYP7A1*. CV-1 cells were transfected with an expression plasmid for a GAL4-SHP-1 chimera, the (UAS)<sub>5</sub>-tk-CAT reporter, and expression plasmids for chimeras between the strong transcriptional activation domain of VP16 and the isolated ligand-binding domains of a panel of nuclear receptors (Figure 5A). When transfected alone, the GAL4-SHP-1 chimera caused a minor reduction ( $\sim$ 0.3-fold) in reporter activity (Figure 5A). However, reporter activity was strongly induced when GAL4-SHP-1 was coexpressed with VP16-RXR $\alpha$  ( $\sim$ 44-fold) or VP16-estrogen receptor  $\alpha$  (ER $\alpha$ ,  $\sim$ 11-fold) in the presence of 9-*cis* retinoic acid and estradiol, respectively (Figure 5A). These interactions were strongly dependent on the presence of ligand. Little or no interaction was detected between SHP-1 and LXR $\alpha$ ,

FXR, COUP-TFII, HNF4 $\alpha$ , RAR $\alpha$ , or TR $\beta$  in our mammalian two-hybrid assay (Figure 5A). The lack of a stronger interaction between SHP-1 and either TR $\beta$ , RAR $\alpha$ , or HNF4 $\alpha$  was surprising in light of the previous results of others (Seol et al., 1996; Masuda et al., 1997) and may reflect differences in the assay systems used. Notably, strong reporter activity was detected when GAL4-SHP-1 was expressed with VP16-human LRH-1 or VP16-mouse LRH-1 ( $\sim$ 14-fold activation for both human and mouse). This activity was completely dependent on the presence of GAL4-SHP-1 (data not shown). These data demonstrate that SHP-1 can interact with LRH-1 in cells. Interestingly, little or no interaction was detected between SHP-1 and steroidogenic factor 1 (SF-1) (Figure 5A), a closely related orphan receptor that shares  $\sim$ 60% amino acid identity with LRH-1 in the ligand-binding domain (Tsukiyama et al., 1992; Honda et al., 1993; Ikeda et al., 1993).

Using a glutathione S-transferase (GST) pull-down assay, we examined whether SHP-1 binds directly to LRH-1. SHP-1 was expressed in *E. coli* as a fusion protein with GST, and [<sup>35</sup>S]-labeled LRH-1 was synthesized in vitro. Glutathione-Sepharose beads efficiently coprecipitated [<sup>35</sup>S]-labeled LRH-1 in the presence of GST-SHP-1 but not in its absence (Figure 5B). In parallel incubations, GST-SHP-1 interacted strongly with [<sup>35</sup>S]-labeled human RXR $\alpha$  in the presence of 9-*cis* retinoic acid (Figure 5B). These data are in close agreement with those derived from mammalian two-hybrid experiments (Figure 5A). Thus, SHP-1 interacts directly with LRH-1.

### SHP-1 Represses Expression of *CYP7A1*

Does SHP-1 have a role in the repression of *CYP7A1* expression by FXR ligands? We addressed this question by performing cotransfection experiments with a rat *CYP7A1* luciferase reporter plasmid (pGL3-rCYP7A1 [−1573/+36]) containing nucleotides −1573 to +36 of the rat *CYP7A1* promoter, which includes a conserved LRH-1 binding site (Nitta et al., 1999). In the absence of exogenously expressed LRH-1, the activity of the pGL3-rCYP7A1(−1573/+36) reporter was low when transiently transfected into HepG2 cells (data not shown). Cotransfection of increasing amounts of an LRH-1 expression plasmid resulted in a dose-dependent increase in reporter activity (Figure 6). This LRH-1-dependent reporter activity was completely blocked by the cotransfection of SHP-1 expression plasmid (Figure 6). These data suggest that interactions between SHP-1 and LRH-1 represent a basis for bile acid-mediated repression of *CYP7A1* expression.

### Discussion

The recent discovery that FXR is a bile acid receptor provided a great deal of insight into the molecular mechanisms underlying bile acid signaling. In particular, these studies uncovered the mechanism whereby bile acids stimulate the transcription of genes, such as *I-BABP*, involved in bile acid transport. High-affinity binding sites for the FXR/RXR heterodimer have been identified in both the human and mouse *I-BABP* promoters (Grober et al., 1999; Makishima et al., 1999). By contrast, the mechanism underlying bile acid-mediated repression of *CYP7A1* expression remained a puzzle, since an FXRE had not been identified in the bile acid response elements of this gene (Chiang and Stroup, 1994; Chiang et

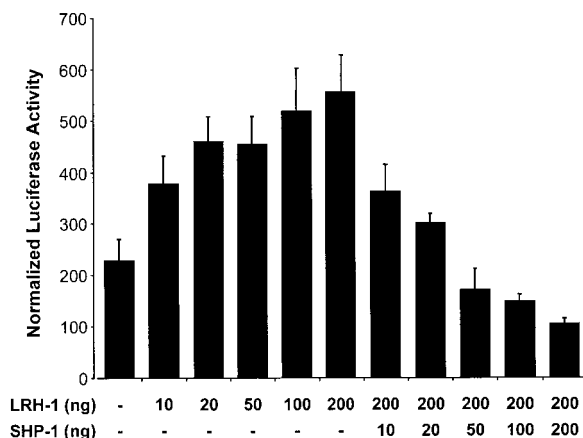


Figure 6. SHP-1 Represses LRH-1-Dependent Activation of the Rat *CYP7A1* Promoter

HepG2 cells were transfected with the rat *CYP7A1* reporter plasmid, pGL3-rCYP7A1(-1573/+36), and the indicated amounts of LRH-1 and/or SHP-1 expression plasmids. Data represent the mean of assays performed in triplicate  $\pm$  S.D.

al., 2000). We now present evidence that FXR does not repress *CYP7A1* expression directly, but rather through induction of the gene encoding the orphan nuclear receptor SHP-1, which, in turn, represses *CYP7A1* expression. Similar findings have been reported by Lu et al. (2000 [this issue of *Mol. Cell*]). Consistent with this model, it was recently shown that *SHP-1* expression is markedly lower and not inducible by cholic acid in the livers of mice lacking FXR (Sinal et al., 2000). Taken together, these data provide a molecular explanation for the coordinate suppression of gene expression by bile acids.

#### SHP-1 Represses *CYP7A1* Expression

We encountered the orphan nuclear receptor SHP-1 as part of a comprehensive, unbiased effort to identify FXR target genes in the liver. *SHP-1* expression was strongly induced in the livers of rats treated with the potent, nonsteroidal FXR ligand GW4064. *SHP-1* expression was also markedly induced by GW4064 in primary cultures of human and rat hepatocytes, whereas *CYP7A1* expression was suppressed under the same conditions. The reciprocal relationship between *SHP-1* and *CYP7A1* regulation, together with the established inhibitory effects of SHP-1 on nuclear receptor activity, suggested that SHP-1 might repress *CYP7A1* expression. Indeed, expression of SHP-1 repressed the activity of the rat *CYP7A1* promoter in HepG2 cells.

SHP-1 is unusual in that it lacks the highly conserved

DNA-binding domain typically found in members of the nuclear receptor family. SHP-1 was originally cloned in yeast two-hybrid experiments using the orphan nuclear receptors CAR or PPAR $\alpha$  as bait, but it interacts with a number of additional nuclear receptors, including ER $\alpha$  and ER $\beta$ , RAR, RXR, and TR (Seol et al., 1996; Masuda et al., 1997; Seol et al., 1998; Johansson et al., 1999). In each case, SHP-1 represses the ligand-induced transcriptional activity of these receptors. How does SHP-1 repress transcription of the *CYP7A1* promoter? Our data indicate that SHP-1 exerts much of its effect through interaction with the orphan nuclear receptor LRH-1. SHP-1 interacted strongly with LRH-1 in both a mammalian two-hybrid assay and an in vitro pull-down assay. Moreover, SHP-1 efficiently repressed LRH-1-dependent activation of the rat *CYP7A1* promoter. LRH-1 was recently shown to activate the human *CYP7A1* promoter by binding to an extended nuclear receptor half-site sequence that is conserved in the mouse, rat, and hamster *CYP7A1* promoters (Nitta et al., 1999). Earlier studies had defined DNA response elements in the *CYP7A1* and *CYP8B1* gene promoters that conferred repression in response to bile acids (Chiang and Stroup, 1994; Chiang et al., 2000; del Castillo-Olivares and Gil, 2000). Notably, each of these negative bile acid response elements contains an LRH-1 binding site. Consistent with these data, *CYP8B1* expression was repressed 3-fold in Fisher rats treated with GW4064 (S. A. J., unpublished data). Thus, interactions between SHP-1 and LRH-1 are likely to be important for the coordinate repression of a number of genes by bile acids. Among the genes that may be regulated by the interaction between SHP-1 and LRH-1 is *SHP-1* itself. An LRH-1-responsive region of the murine *SHP-1* gene has been identified (Lee et al., 1999). Thus, SHP-1 is likely to regulate its own expression. This feedback regulation may provide a mechanism for attenuating the bile acid-mediated repression of genes by SHP-1. A model for bile acid-mediated repression of gene expression via increased SHP-1 levels is shown in Figure 7.

Two recent reports showed that SHP-1 represses the transcriptional activation of ER $\alpha$  and ER $\beta$ , RXR, and the orphan receptor HNF4 $\alpha$  by competing with coactivator binding to these receptors (Johansson et al., 1999; Lee et al., 2000). In addition, SHP-1 contains a strong transcriptional repressor domain in its C terminus (Lee et al., 2000). Furthermore, SHP-1 has been shown to inhibit DNA binding of RAR-RXR heterodimers (Seol et al., 1996). Taken together, these studies suggest that SHP-1 inhibits the transcriptional activity of nuclear receptors through multiple mechanisms. To date, we have been unable to demonstrate inhibition of LRH-1 binding to its response element in the *CYP7A1* promoter by SHP-1 (data not shown). Thus, the mechanism by which SHP-1

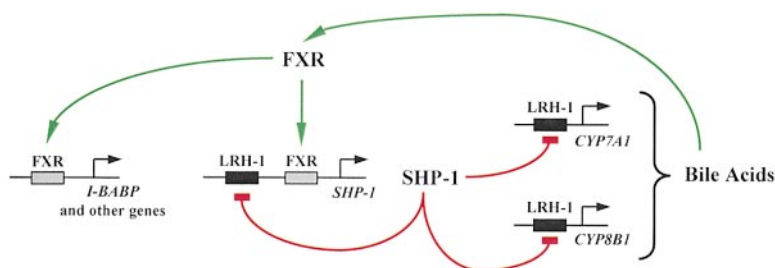


Figure 7. Model for the Feedforward and Feedback Regulatory Effects of Bile Acids on Gene Expression

Activation of FXR by bile acids results in the induction of *I-BABP* and *SHP-1* expression. SHP-1, in turn, interacts with LRH-1 and represses expression of *CYP7A1* and *CYP8B1*. SHP-1 may also repress expression of its own gene.

inhibits LRH-1-mediated transactivation of the *CYP7A1* promoter remains unresolved.

In addition to the interactions between SHP-1 and LRH-1, other mechanisms may play a role in bile acid-mediated repression of *CYP7A1* expression. First, SHP-1 binds to and represses the transcriptional activity of other nuclear receptors that regulate *CYP7A1*, including RXR and TR (Seol et al., 1996; Masuda et al., 1997). These interactions may also contribute to bile acid-mediated repression of *CYP7A1* expression. Second, ligand-bound FXR was reported to repress LXR $\alpha$  activity on an LXR $\alpha$  response element (Wang et al., 1999), although the mechanism for this *trans*-repression is not clear. Since LXR $\alpha$  stimulates rodent *CYP7A1* expression in response to oxysterols, repression of LXR $\alpha$  activity may contribute to the overall repression of *CYP7A1*. Thus, SHP-1/LRH-1 interactions may be one of several mechanisms whereby bile acids repress expression of *CYP7A1* and other genes.

#### Parallels between SHP-1/LRH-1 and Other Nuclear Receptor Pairs

Intriguing parallels exist between the SHP-1/LRH-1 interaction and another pair of nuclear receptors. LRH-1 is most closely related to the orphan receptor SF-1, which regulates the expression of enzymes required for steroid hormone biosynthesis (Parker, 1998; Hammer and Ingraham, 1999). SF-1 and LRH-1 are ~85% identical in the amino acid sequences of their DNA-binding domains, and both bind as monomers to the same extended nuclear receptor half-site sequence. Notably, the transcriptional activity of SF-1 is repressed by binding to DAX-1 (dosage-sensitive sex-reversal adrenal hypoplasia congenital region on the X chromosome, region 1; NR0B1), an orphan nuclear receptor most closely related to SHP-1 that also lacks the DNA-binding domain characteristic of nuclear receptors (Zanaria et al., 1994; Hammer and Ingraham, 1999). Thus, both SF-1 and LRH-1 are negatively regulated in a *trans*-dominant fashion by heterodimerization with orphan receptors lacking DNA-binding domains. Since SHP-1 expression is stimulated by bile acids, it will be interesting to determine whether DAX-1 expression is also regulated by hormones.

A second nuclear receptor pair with similarities to SHP-1/LRH-1 occurs in *Drosophila*. Hormonal activation of the ecdysone receptor (EcR) during the third larval instar phase of *Drosophila* metamorphosis results in an increase in the expression of two orphan nuclear receptors, DHR3, which has a functional DNA-binding domain, and E75B, which does not. E75B binds to DHR3 and represses its transcriptional activity (Thummel, 1997; White et al., 1997). This interaction is critical for determining the temporal progression of metamorphosis. The EcR/E75/DHR3 and FXR/SHP-1/LRH-1 regulatory cascades are remarkably similar in that hormone-mediated activation of a nuclear receptor (either FXR or EcR) induces expression of a second nuclear receptor, which, in turn, binds to and represses the activity of a third nuclear receptor. The similarities in these genetic hierarchies across evolution suggest that repression via heterodimerization may represent an important paradigm for the modulation of orphan receptor activity.

#### Conclusions

The mechanism whereby FXR represses expression of *CYP7A1* and other genes has until now remained an

enigma. Through the use of a potent, nonsteroidal FXR ligand, we have identified *SHP-1* as an FXR target gene in the liver of humans and rodents. Furthermore, we have demonstrated that SHP-1 can interact with LRH-1 and efficiently repress expression of *CYP7A1*. Thus, bile acid-induced repression of *CYP7A1* is mediated by a novel regulatory cascade of three nuclear receptors. Since both the *CYP7A1* and *CYP8B1* gene promoters contain LRH-1 binding sites, the SHP-1/LRH-1 partnership is likely to have broad implications in bile acid signaling. Both SHP-1 and LRH-1 are orphan receptors, which raises the possibility that bile acid biosynthesis will be regulated by additional, unidentified hormones. Regardless of whether SHP-1 and LRH-1 have natural ligands, pharmacologic modulation of their interaction represents an exciting new opportunity for the discovery of drugs that regulate cholesterol homeostasis.

#### Experimental Procedures

##### Materials

The synthesis of GW4064 will be described elsewhere (Maloney et al., 2000). CDCA, dexamethasone, estradiol, all-*trans* retinoic acid, 9-*cis* retinoic acid, and charcoal-stripped, delipidated calf serum were acquired from the Sigma Chemical Co. (St. Louis, MO). 24(S),25-epoxycholesterol was synthesized in-house. DNA-modifying enzymes, polymerases, and restriction endonucleases were provided by Roche Molecular Biochemicals (Indianapolis, IN). Charcoal/dextran-treated fetal bovine serum (FBS) was purchased from Hyclone Laboratories Inc. (Logan, UT). The human hepatocellular carcinoma cell line HepG2 was obtained from the American Type Culture Collection (ATCC number HB-8065, Manassas, VA). Matrigel was provided by Becton Dickinson Labware (Bedford, MA). All other tissue culture reagents were obtained from Life Technologies Inc. (Gaithersburg, MD).

##### Animals

Male Fisher rats were obtained from Charles River Laboratories Inc. (Raleigh, NC) and maintained on a 12 hr light/12 hr dark cycle. Animals were allowed food and chow ad libitum. GW4064 (30 mg/kg) was administered by gavage twice a day for 7 days and the animals sacrificed by cervical dislocation 4 hr after the final treatment. Livers were excised and snap-frozen in liquid nitrogen. Differential gene expression analysis was performed by CuraGen Corp. (New Haven, CT).

##### Plasmid Constructs

Expression plasmids for the human nuclear receptor-GAL4 chimeras were prepared by inserting amplified cDNAs encoding the ligand-binding domains into a modified pSG5 expression vector (Stratagene, La Jolla, CA) containing the GAL4 DNA-binding domain (amino acids 1–147) and the Simian virus 40 (SV40) large T antigen nuclear localization signal (APKKKKRVG). The (UAS)<sub>5</sub>-tk-CAT and (hsp27EcR)<sub>2</sub>-tk-LUC reporter constructs have been previously described (Forman et al., 1995; Parks et al., 1999). p $\beta$ -actin-SPAP, an expression vector containing the human secreted placental alkaline phosphatase (SPAP) cDNA under the control of  $\beta$ -actin promoter, was used as an internal control in all transfections. The expression plasmids for human and mouse FXR (pSG5-hFXR and pSG5-mFXR, respectively) and human SRC-1 are described elsewhere (Kliwer et al., 1998; Parks et al., 1999). The full-length coding regions for human LRH-1 (GenBank Accession Number AB019246) and human SHP-1 (GenBank Accession Number L76571) were amplified by PCR and cloned into pSG5, creating pSG5-hLRH-1 and pSG5-hSHP-1, respectively. A consensus Kozak sequence was created during amplification. The rat (bases –441 to +19, GenBank Accession Number D86745) (Masuda et al., 1997) and human (bases –572 to +10, GenBank Accession Number AF044316) (Lee et al., 1998) *SHP-1* promoters were amplified by PCR using the following primer pairs: Rat, 5'-gggtgtgagatctCCCTGGCTGGCTCCTGGCTCTGT-3' (sense) and 5'-gggtgtgagatctCCTGTTCTTCTGGCTCTGT



GGC-3' (antisense); and human, 5'-gggtgtgcgagatctTCCTAGACT GGACAGTGGGCAAAG-3' (sense) and 5'-gggtgtgcgagatctCTTCC AGCTCTCTGGCTCTGTGT-3' (antisense). The resultant fragments were inserted into the *Bgl*II site of pGL3-Basic, a promoter-less luciferase reporter vector (Promega, Madison, WI). Site-directed mutagenesis of putative FXREs in the rat and human *SHP-1* promoters was performed using the Transformer mutagenesis system (CLONTECH Laboratories, Palo Alto, CA) with the  $\Delta$ ratLR1 (bases -321 to -287, 5'-CCTGGTACAGCCTGGaaTAATAtaaCTGTTTATAC-3') and  $\Delta$ humanLR1 (bases -304 to -270, 5'-CCTGGTACAGCCTGA aaTAATGtaCTGTTTATCC-3') primers. Mutated constructs were verified to be free of nonspecific base changes by sequencing. pGL3-rCYP7A1(-1573/+36) contains bases -1573 to +36 of the rat *CYP7A1* promoter (GenBank Accession Number Z14108) inserted into the *Nhe*I site of pGL3-Basic. VP16-nuclear receptor chimeras contain the 80 aa Herpes virus VP16 transactivation domain linked to the ligand-binding domain of the following nuclear receptors in a modified pSG5 expression vector: human COUP-TFII, ER $\alpha$ , LRH-1, LXR $\alpha$ , RAR $\alpha$ , and TR $\beta$ ; mouse FXR, LRH-1, RXR $\alpha$ , and SF-1; and rat HNF4 $\alpha$ .

#### Transient Transfection Assays

Transient transfection of CV-1 cells was performed exactly as described elsewhere (Jones et al., 2000). Typically, transfection mixes contained 2–5 ng of receptor expression vector, 20 ng of reporter construct, and 8 ng of p $\beta$ -actin-SPAP. The amount of DNA used in each transfection was adjusted to 80 ng with carrier plasmid (pBluescript, Stratagene). Mammalian two-hybrid experiments utilized transfection mixes containing 20 ng of VP16 nuclear receptor ligand-binding domain expression vector, 5 ng of pSG5-GAL4-SHP-1, 15 ng of (UAS) $_3$ -tk-CAT, and 8 ng of p $\beta$ -actin-SPAP. Cells were maintained for 24 hr in the presence of drug (added as a 1000 $\times$  stock in dimethyl sulfoxide) in DMEM/F-12 nutrient mixture containing 10% charcoal-stripped, delipidated calf serum. An aliquot of medium was assayed for SPAP activity, and the cells were lysed prior to determination of luciferase expression. Luciferase activities were normalized to SPAP. HepG2 cells were maintained in DMEM/F-12 supplemented with 10% heat-inactivated FBS (Life Technologies Inc.). Plasmid DNA was transfected into HepG2 cells using the FuGENE6 transfection reagent according to the manufacturer's instructions (Roche Molecular Biochemicals). Thus, 24-well culture plates (15 mm diameter) were inoculated with  $7 \times 10^5$  cells 24 hr prior to transfection. Cells were transfected overnight in serum-free DMEM/F-12 with 100 ng of reporter construct, 32 ng of p $\beta$ -actin-SPAP, and 0–400 ng of receptor expression vectors (adjusted to 400 ng with carrier plasmid). Following transfection, the medium was aspirated and the cells were cultured for a further 48 hr in DMEM/F-12 supplemented with 10% heat-inactivated FBS. SPAP and luciferase values were determined as described above.

#### Primary Culture of Human and Rat Hepatocytes and Northern Blot Analysis

Primary human hepatocytes were obtained from Dr. Steve Strom (University of Pittsburgh). Rat hepatocytes were isolated as described elsewhere (LeCluyse et al., 1996). Cells ( $1.5 \times 10^6$ ) were cultured on Matrigel-coated 6-well plates in serum-free Williams' E medium supplemented with 100 nM dexamethasone, 100 U/ml penicillin G, 100  $\mu$ g/ml streptomycin, and insulin-transferrin-selenium (ITS-G, Life Technologies Inc.). Twenty-four hours after isolation, hepatocytes were treated with either GW4064 (0.1–10  $\mu$ M) or CDCA (1–100  $\mu$ M), which were added to the culture medium as 1000 $\times$  stocks in dimethyl sulfoxide. Control cultures received vehicle alone. Cells were cultured for a further 48 hr prior to harvest, and total RNA was isolated using a commercially available reagent (Trizol, Life Technologies Inc.) according to the manufacturer's instructions. Total RNA (10  $\mu$ g) was resolved on a 1% agarose/2.2 M formaldehyde denaturing gel and transferred to a nylon membrane (Hybond N+, Amersham Pharmacia Biotech Inc., Piscataway, NJ). Blots were hybridized with  $^{32}$ P-labeled cDNAs corresponding to human *SHP-1* (GenBank Accession Number L76571), human *CYP7A1* (bases 99–1564, GenBank Accession Number M93133), mouse *SHP-1* (bases 30–783, GenBank Accession Number L76567), or rat *CYP7A1* (bases 235–460, GenBank Accession Number J05460).

Subsequently, blots were stripped and reprobed with a radiolabeled  $\beta$ -actin cDNA (CLONTECH Laboratories).

#### Electrophoretic Mobility-Shift Assays

Electrophoretic mobility-shift assays (EMSA) were performed essentially as described elsewhere (Lehmann et al., 1997). hFXR and hRXR $\alpha$  were synthesized from pSG5-hFXR and pSG5-hRXR $\alpha$  expression vectors, respectively, using the TNT T7 Coupled Reticulocyte System (Promega). Unprogrammed lysate was prepared using the pSG5 expression vector (Stratagene). Binding reactions contained 10 mM HEPES (pH 7.8), 60 mM KCl, 0.2% Nonidet P-40, 6% glycerol, 2 mM dithiothreitol (DTT), 2  $\mu$ g of poly(dI-dC)•poly(dI-dC), and 1  $\mu$ l each of synthesized hFXR or hRXR $\alpha$ . Control incubations received unprogrammed lysate alone. Reactions were preincubated on ice for 10 min prior to the addition of [ $^{32}$ P]-labeled double-stranded oligonucleotide probe (0.2 pmol). Competitor oligonucleotides were added to the preincubation at 5-, 25-, and 75-fold molar excess. Samples were held on ice for a further 20 min, and the protein–DNA complexes resolved on a pre-electrophoresed 5% polyacrylamide gel in 0.5 $\times$  TBE (45 mM Tris-borate, 1 mM EDTA) at room temperature. Gels were dried and autoradiographed at -70°C for 1–2 hr. The following double-stranded oligonucleotides were used as probes and competitors in EMSA: rSHP, 5'-gatcCCTGGTAAATAACCCTGT-3'; mSHP, 5'-gatcCCTGGGTTAATGACCC TGT-3'; hSHP, 5'-gatcCCTGAGTTAATGACCTTGT-3'; ml-BABP, 5'-gatcTTAAGGTGAATAACCTTGG-3'; hl-BABP, 5'-gatcCCAGGTGAATAACCTCGG-3' (Grober et al., 1999); and mSHPmut 5'-gatcCC TGGaaTAATGttCCTGT-3'.

#### GST Pull-Down Assays

GST-SHP-1 fusion protein was expressed in BL21(DE3)plysS cells and bacterial extracts prepared by one cycle of freeze-thaw of the cells in protein lysis buffer containing 50 mM Tris (pH 8.0), 250 mM KCl, 1% Triton X-100, 10 mM DTT and 1 $\times$  Complete Protease Inhibitor (Roche Molecular Biochemical) followed by centrifugation at 40,000  $\times$  g for 30 min. Glycerol was added to the resultant supernatant to a final concentration of 10%. Lysates were stored at -80°C until use. [ $^{35}$ S]-labeled human LRH-1 or human RXR $\alpha$  was generated using TNT T7 Coupled Reticulocyte System (Promega) in the presence of Pro-Mix (Amersham Pharmacia Biotech Inc.). Coprecipitation reactions included 25  $\mu$ l of lysate containing GST-SHP-1 fusion protein or control GST; 25  $\mu$ l of incubation buffer (50 mM KCl, 40 mM HEPES [pH 7.5], 5 mM  $\beta$ -mercaptoethanol, 0.1% Tween 20 and 1% nonfat dry milk); and 5  $\mu$ l of [ $^{35}$ S]-labeled LRH-1 or RXR $\alpha$ . The mixtures were incubated for 25 min with gentle rocking at 4°C prior to the addition of 20  $\mu$ l of glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech Inc.) that had been extensively washed in protein lysis buffer. Reactions were incubated at 4°C with gentle rocking for a further 20 min. The beads were pelleted at 3000 rpm in a microfuge and washed four times with protein incubation buffer. Following the final wash, the beads were resuspended in 25  $\mu$ l of 2 $\times$  SDS-PAGE sample buffer containing 50 mM DTT. Samples were heated to 100°C for 5 min and resolved on a 10% acrylamide gel. Autoradiography was performed overnight.

#### Statistical Analyses

Unless otherwise stated, data are expressed as mean  $\pm$  standard deviation (S.D.). The significance of differences in *SHP-1* and *CYP7A1* expression between vehicle- and GW4064-treated animals were analyzed using an unpaired Student's *t*-test.

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