Coordinated control of bile acids and lipogenesis through FXR-dependent regulation of fatty acid synthase[1]

Karen E. Matsukuma,[*] Mary K. Bennett,[*] Jiansheng Huang,[†] Li Wang,[†] Gregorio Gil,[§] and Timothy F. Osborne[2,*]

Department of Molecular Biology and Biochemistry,[*] University of California, Irvine, CA; Departments of Medicine and Pharmacology,[†] University of Kansas Medical Center, Kansas City, KS; and Department of Biochemistry,[§] Medical College of Virginia, Virginia Commonwealth University, Richmond, VA

Abstract **We discovered a nuclear receptor element in the FAS promoter consisting of an inverted repeat spaced by one nucleotide (IR-1) and located 21 bases downstream of a direct repeat sequenced by 4 nucleotides (DR-4) oxysterol liver X receptor response element. An IR-1 is present in promoters of several genes of bile acid and lipid homeostasis and binds farnesoid X receptor/retinoid X receptor (FXR/RXR) heterodimers to mediate bile acid-dependent transcription. We show that FXR/RXRα specifically binds to the FAS IR-1 and that the FAS promoter is activated ~10-fold by the addition of a synthetic FXR agonist in transient transfection assays. We also demonstrate that endogenous FXR binds directly to the murine FAS promoter in the hepatic genome using a tissue-based chromatin immunoprecipitation procedure. Furthermore, we show that feeding wild-type mice a chow diet supplemented with the natural FXR agonist chenodeoxycholic acid results in a significant induction of FAS mRNA expression. Thus, we have identified a novel IR-1 in the FAS promoter and demonstrate that it mediates FXR/bile acid regulation of the FAS gene. These findings provide the first evidence for direct regulation of lipogenesis by bile acids and also provide a mechanistic rationale for previously unexplained observations regarding bile acid control of FAS expression.**—Matsukuma, K. E., M. K. Bennett, J. Huang, L. Wang, G. Gil, and T. F. Osborne. **Coordinated control of bile acids and lipogenesis through FXR-dependent regulation of fatty acid synthase.** ***J. Lipid Res.*** **2006.** 47: **2754–2761.**

Supplementary key words farnesoid X receptor • nuclear receptors • small heterodimer partner

In the intestine, bile acids serve to emulsify lipids from the diet, thereby facilitating efficient absorption of fatty acids, sterols, and fat-soluble vitamins. However, increased bile acid levels are cytotoxic (1, 2), so their levels must be tightly regulated. One of the key transcription factors responsible for the regulation of bile acid homeostasis is the nuclear receptor farnesoid X receptor (FXR) (3). Physiologic concentrations of select bile acids, such as chenodeoxycholic acid (CDCA) and cholic acid, are endogenous agonists for FXR (4–6). Activation of the FXR/retinoid X receptor (RXR) heterodimer by bile acids stimulates the synthesis of bile acid-regulatory proteins, including I-BABP (7), BSEP (8), and OATP1B3 (9), that modulate the flux of bile acids in the liver and intestine. Additionally, indirect regulation of the bile acid biosynthetic enzymes cytochrome P (CYP)7A1 and CYP8B1 is accomplished through FXR induction of the negative acting small heterodimer partner (SHP) protein in the liver, which binds to and represses the positive acting liver receptor homologue (LRH)-1 nuclear receptor in the promoters for these genes (10–12). Another twist in the mechanism for bile acid-dependent repression of CYP7A1 was recently identified that involves the activation of FGF15 expression in the small intestine by bile acids and FGFR4 expression in the liver (13). Here, intestinal FGF15 is proposed to act through ligand-dependent stimulation of FGFR4 signaling in the liver.

A second lipid-sensing nuclear receptor, liver X receptor (LXR), binds oxysterols and plays a key role in cholesterol homeostasis (14–18). As cholesterol is the direct precursor for bile acid biosynthesis, a complex interplay between the actions of LXR and FXR must exist to meet the metabolic needs of the organism. Serum cholesterol levels are in turn dependent on fatty acids as they are the organic substrate for cholesterol esterification, which is important for cholesterol storage. Thus, bile acids, cholesterol, and fatty acids are intimately tied to one another in the overall maintenance of lipid homeostasis.

Several studies have also established that bile acids and FXR play important roles in both triglyceride and glucose metabolism. The triglyceride-lowering effect of bile acids was first described >20 years ago when clinicians ob-

Manuscript received 26 July 2006 and in revised form 5 September 2006.
Published, JLR Papers in Press, September 6, 2006.
DOI 10.1194/jlr.M600342-JLR200

[1] This paper is dedicated to the memory of Professor Edward K. Wagner, who died during the preparation of the manuscript.

[2] To whom correspondence should be addressed.
e-mail: tim.osborne@uci.edu

served that bile acid therapy for the treatment of gallstone disease had the added effect of decreasing serum triglycerides (19–21). More recently, FXR induction of its target gene SHP was shown to correlate with decreased expression of the master lipogenic regulator sterol-regulatory element binding protein (SREBP)-1c (as well as many of its gene targets), thus providing at least a partial mechanistic explanation for the triglyceride-lowering effect of bile acids (22). However, SHP modulation of SREBP-1c levels is unlikely to be the sole mechanism regulating the lipogenic response to bile acids because the repression of many of SREBP-1c's downstream targets (including FAS) is transient despite ongoing expression of SHP (22).

Because FAS occupies a pivotal position for lipogenic flux, its activity is highly regulated. Significant regulation occurs at the transcriptional level, where major metabolic signals such as insulin and carbohydrates (23–26), thyroid hormone (27), fatty acids (28), and sterols (29) have all been shown to influence gene transcription. The promoter for the FAS gene contains a complex nuclear receptor response region at ~−700. This region contains a classic direct repeat sequenced by 4 nucleotides (DR-4) nuclear receptor site that mediates the FAS response to thyroid hormone as well as LXR activators (27, 30). In this study, we report the identification of an inverted repeat spaced by one nucleotide (IR-1) located 21 bases downstream of the FAS DR-4 and demonstrate that it mediates bile acid regulation of the FAS gene. We thus establish FAS as an FXR target gene and provide the first example of the direct regulation of lipogenesis by bile acids.

MATERIALS AND METHODS

Cell culture

HEK293T cells were obtained from the American Type Culture Collection, cultured in Dulbecco's Minimum Essential Medium (Irvine Scientific) containing penicillin/streptomycin (100 μg/ml), L-glutamine (10 mM), nonessential amino acids, (100 μM), 5 mM HEPES, pH 7.2, and 10% fetal bovine serum, and maintained at 37°C and 5% CO_2. One day before transfection, cells were plated at a density of 3.5×10^5 cells/well on a six-well plate in 3 ml of normal culture medium.

Transient DNA transfections

Twelve to 24 h after plating, cells were transfected by the standard calcium phosphate method as described (31). Cytomegalovirus X promoter (CMX)-rat FXRα (30 ng), CMX-human RXRα (100 ng), luciferase reporter (4 μg), CMV-β-galactosidase (2 μg), and salmon sperm DNA (to a final mass of 12 μg) were transfected per well as noted in the figure legends. Six to 8 h after transfection, cells were rinsed two times with sterile PBS. Medium was replaced with Defined Serum-Free Medium [Dulbecco's Minimum Essential Medium, 100 μg/ml penicillin/streptomycin, 2 mM L-glutamine, 100 μM nonessential amino acids, 5 mM HEPES, pH 7.2, insulin/transferrin/selenite (5 μg/ml; 5 μg/ml; 5 ng/ml; Sigma), 4% BSA (A-3803; Sigma), and 0.1 μg/ml 25-hydroxycholesterol] plus either DMSO (0.1%) as a vehicle control or the synthetic FXR agonist GW4064 (final concentration of 1 μM). (25-Hydroxycholesterol was added to suppress endogenous SREBP expression and thus minimize basal transcriptional activity.) Cells were allowed to incubate at 37°C and 5% CO_2 for an additional 16–20 h before harvesting. For each experiment, duplicate wells were plated for each condition. Transfection experiments were performed at least twice with similar results.

Plasmids

The FAS −700/+65 pGL2 luciferase reporter construct was subcloned by PCR from the rat FAS −1594/+65 pGL2 described previously (29). All mutant reporter constructs were generated using the Quikchange site-directed mutagenesis kit (Stratagene). The remaining reporter constructs were generated by PCR amplification and subcloning. pSynTATALuc is a reporter vector containing the minimal promoter region of the hamster HMG-CoA synthase promoter (−28/+39) and has been described previously (32). The mouse SREBP-1c (−937/+29) promoter reporter (33), the rat sterol 12-α hydroxylase promoter reporter (34), and the rat acetyl-CoA carboxylase promoter reporter were described previously (35). The following expression vectors were generously provided by other laboratories: CMX-human thyroid receptor (TR)β (Barry Forman, City of Hope), CMX-human RXRα (Ron Evans, Salk Institute), CMX-rat FXR (Bruce Blumberg, University of California, Irvine), and CMX-mouse LRH-1 (David Mangelsdorf, University of Texas Southwestern).

Enzyme assays

At the time of harvest, cells were rinsed once with PBS and then lysed in a reporter lysis buffer (25 mM Gly-Gly, 15 mM $MgSO_4$, 4 mM EGTA, and 0.25% Triton). Luciferase activity of the lysates was measured in an Analytical Luminescence Monolight 2010 luminometer using 5–20 μl of cell extract plus 100 μl of luciferase assay reagent (Promega), with data expressed in relative light units (RLUs). β-Galactosidase activity was measured by a standard colorimetric assay at 420 nm absorbance using 10–20 μl of cell lysate and 2-nitrophenyl β-galactopyranoside as the substrate. Luciferase activity for each sample was divided by the β-galactosidase activity to yield normalized RLUs. Fold activation was determined by dividing the normalized RLUs for a given sample by the normalized RLUs for the control sample (no activators plus vehicle). Each transfection was performed at least twice with similar results.

Electrophoretic mobility shift assay

In vitro transcribed and translated TRβ (human), LXRα (human), FXR (rat), and RXRα (human) proteins were generated using the T7 TnT Rabbit Reticulocyte Lysate (Promega). Five microliters of each translation was added to each binding mixture [containing 10 mM HEPES, pH 7.6, 50 mM NaCl, 2.5 mM $MgCl_2$, 0.1 μg/μl poly dI:dC, 0.05% Nonidet P-40 (v/v), and 10% glycerol] in a final volume of 20 μl. A double-stranded oligonucleotide DNA probe containing the wild-type sequence of IR-1 was 5′ end-labeled using T_4 polynucleotide kinase (USB) and added to the binding mixtures at 1 ng per reaction. Binding mixtures were incubated at 4°C for 1–2 h. Samples were then run on 5% polyacrylamide:bis-acrylamide (19:1) gels at room temperature for 1.5 h, fixed in a solution of 10% methanol/10% acetic acid, and dried onto 3MM chromatography paper at 80°C for 1 h. Dried gels were exposed to X-ray film at −20°C for 12–48 h.

Chromatin immunoprecipitation

B6/129 mice (6 week old males) were purchased from Taconic, allowed to adapt for 2 weeks to a 12 h light/12 h dark

cycle, and euthanized at the end of the dark cycle (8 AM). Livers from four mice were placed in 40 ml of ice-cold PBS containing a cocktail of protease inhibitors (1 μg/ml leupeptin, 1.4 μg/ml pepstatin, and 2 μg/ml PMSF) plus 1 mM EDTA and 1 mM EGTA. The tissue was disrupted in a Tekmar Tissumizer at the lowest setting. Formaldehyde was added from a 37% stock (v/v) to a final concentration of 1%, and samples were rotated on a shaker for 6 min followed by the addition of glycine to a final concentration of 0.125 M. Samples were returned to the shaker for an additional 5 min, and then cells were collected by centrifugation (2,000 rpm in a Sorvall RC3B at 4°C). The cell pellet was washed once with homogenization buffer A (10 mM HEPES, pH 7.6, 25 mM KCl, 1 mM EDTA, 1 mM EGTA, 2 M sucrose, 10% glycerol, and 0.15 mM spermine) plus protease inhibitors as described above. The final pellet was resuspended in buffer A and homogenized in a Dounce homogenizer with a B pestle to release nuclei. The solution was layered over buffer A and centrifuged in a Beckman ultracentrifuge (1 h at 26,000 rpm, 4°C), and the nuclear pellet was resuspended in nuclei lysis buffer (1% SDS, 50 mM Tris, pH 7.6, and 10 mM EDTA). Nuclei were disrupted using an Ultrasonic model W-220F sonicator for 5 × 10 s to shear chromatin. Chromatin size was checked by agarose electrophoresis to ensure that the average size was between 200 and 500 bp. Aliquots were used in immunoprecipitation experiments with an antibody to FXR (Santa Cruz H130X) and processed as described (36). Final DNA samples were analyzed by quantitative PCR in triplicate with a standard dilution curve of the input DNA performed in parallel. Oligonucleotide pairs for the FAS FXR binding region or exon 4 from the YY1 gene used in the quantitative PCR are as follows: FAS-700 (5′) ATCCTGGTCTCCAAGGTG; FAS-534 (3′) TAGGCAATAGGGTGATGGG; YY1 (5′) TCTGACGAGAGGATTGTGTGGAC; YY1 (3′) CTGAAGGGCTTTTCTCCAGTATG.

Animal feeding studies

C57/BL6 mice (8 week old males) were maintained in a 12 h light/12 h dark controlled environment with free access to water and standard laboratory rodent chow. At the start of the feeding experiment, each group of mice (n = 4) was fed either chow or chow mixed with 0.25% CDCA (w/w) for 6 days. Animals were euthanized and tissues harvested 6 h into the dark cycle. Tissues were homogenized and extracted for RNA using Trizol reagent (Invitrogen). For each liver sample, 8 μg of total RNA was separated on a 1% formaldehyde gel and transferred to a charged nylon membrane. FAS, L32, and SHP mRNA levels were detected by Northern blotting using α-^{32}P-labeled DNA probes. Densitometry was performed using Bio-Rad Quantity One software. FAS expression was normalized to L32 expression, and fold activation by CDCA feeding was determined by setting the normalized FAS mRNA expression of the control fed animals to 1. The feeding study was performed twice with similar results.

RESULTS

We previously defined a DR-4 element in the rat FAS promoter as an LXR responsive site (30). In the course of these studies, we discovered an additional nuclear receptor half-site located near the DR-4 that was also indirectly required for LXR signaling through binding the monomeric nuclear receptor LRH-1 (K. E. Matsukuma et al., unpublished data). A closer inspection of the sequence surrounding this additional half-site suggested that it comprised half of a potential IR-1 nuclear receptor element (**Fig. 1A**). LXR/RXR heterodimers do not characteristi-

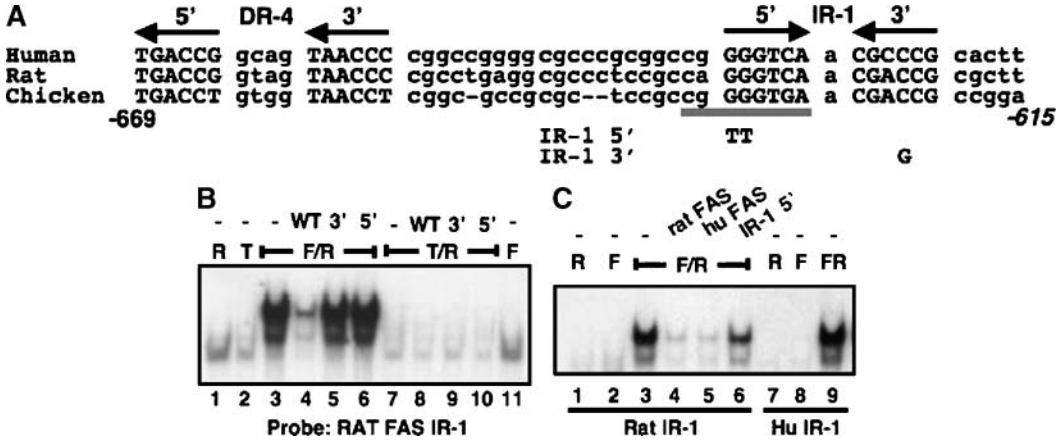

Fig. 1. A: Alignment of the FAS promoter sequences from human, rat, and chicken. Nuclear receptor half-sites are shown in uppercase letters and denoted by arrows. The positions of the direct repeat sequenced by 4 nucleotides (DR-4) and inverted repeat spaced by one nucleotide (IR-1) elements are noted, and the liver receptor homologue (LRH)-1 binding site is denoted by a gray underline. The bases mutated in key electrophoretic mobility shift assay and reporter plasmids (IR-1 5′ or 3′) are also noted. B: Electrophoretic mobility shift assay demonstrating that the farnesoid X receptor/retinoid X receptor (FXR/RXR) heterodimer binds FAS IR-1. In vitro translated RXRα (R), thyroid receptor (TR)β (T), or FXR (F) were incubated alone or in combination as indicated with a ^{32}P-labeled sequence containing the FAS IR-1 [wild type (WT)] and analyzed by electrophoretic mobility shift assay. Fifty-fold excess cold competitor DNAs corresponding to the wild-type or mutated sequences (IR-1 5′ or 3′) were added along with the probe as noted at the top of the gel. C: FXR/RXR binding to human (hu) FAS IR-1 by electrophoretic mobility shift assay. In vitro translated RXRα (R), FXR (F), or both (F/R) were incubated with a ^{32}P-labeled sequence containing to either rat or human FAS IR-1 as indicated. Unlabeled competitor DNAs (50-fold molar excess) corresponding to the rat or human FAS probes or a mutated competitor (IR-1 5′) were added along with the probe as noted at the top of the gel.

cally bind to this half-site configuration; however, it is a known high-affinity site for the FXR/RXR heterodimer (37). This site is conserved in the mouse promoter as well.

To determine whether FXR/RXR heterodimers could bind this putative IR-1, we tested the binding of in vitro translated FXR and RXRα on a ^{32}P-labeled probe containing the sequence of the rat FAS IR-1 (Fig. 1B). We also tested the binding of TRβ and LXRα (data not shown) to the IR-1. Individually, none of the nuclear receptors (including FXR) bound the DNA probe. In addition, when combined with RXRα, neither TRβ (lanes 7–11) nor LXRα (data not shown) interacted efficiently with the DNA. In contrast, incubation of FXR and RXRα proteins with the radiolabeled probe resulted in a strong shifted band (lane 3). The binding was effectively competed off by 50-fold excess cold wild-type competitor DNA (lane 4) but not by cold competitor DNA containing minimal mutations in either half of the putative IR-1 (lanes 5, 6). Thus, the rat FAS IR-1 specifically binds FXR/RXRα and not TRβ/RXRα or LXRα/RXRα.

Because the human FAS promoter diverges by one base from the rat promoter in the region of the putative IR-1 sequence, we investigated whether the site from the human FAS promoter could also bind the FXR/RXRα heterodimer (Fig. 1C). The FXR/RXRα heterodimer bound to the human FAS element (lane 9) at a level comparable to that seen with the rat sequence (lane 3). In addition, competition of FXR/RXRα binding to the rat FAS IR-1 element by 50-fold excess cold competitor DNA containing the human FAS sequence was equivalent to that seen with competitor DNA containing the rat FAS sequence (lanes 4, 5).

To determine whether FXR/RXRα could activate FAS through the IR-1 site, we transfected HEK293T cells with expression vectors for FXR and RXRα and measured the activation of a FAS promoter construct containing the IR-1 (**Fig. 2**). We chose HEK293T cells because they do not show significant endogenous LRH-1 activity (K. E. Matsukuma et al., unpublished data). This is important because the LRH-1 binding site overlaps the FXR binding site in the FAS promoter, thus complicating a straightforward study of promoter activation by FXR. Cell lines derived from hepatocytes would be expected to express significant amounts of endogenous LRH-1 and therefore would not be appropriate model systems. When the synthetic FXR agonist GW4064 was added along with 10, 30, or 100 ng of the FXR expression vector, FAS activation increased significantly in a dose-dependent manner, and addition of the RXRα expression vector amplified this effect. In contrast, a point mutation in the putative IR-1 (FAS IR-1 5′) extinguished the positive FXR response.

Because the DR-4 that is required for the LXR activation of FAS is in close proximity to the IR-1 and because half of the IR-1 is an LRH-1 binding site essential for signaling through LXR, we analyzed the potential role of the DR-4 in FXR signaling (**Fig. 3**). In contrast to LXR activation of FAS, mutations in the DR-4 had little impact on activation by FXR. However, point mutations in either of the half-sites of the IR-1 significantly reduced the FXR response. Thus, FXR-dependent activation of FAS is specifically mediated through the IR-1 element.

In other studies, we have shown that LXR activation of FAS is inhibited by SHP (K. E. Matsukuma et al., unpublished data); therefore, we investigated whether FXR activation of FAS was also affected by SHP expression in a cotransfection assay. Unlike LXR activation, however, FXR activation of FAS is independent of SHP expression (**Fig. 4**). It should be noted that in companion dishes from this same experiment, SHP addition did inhibit LXR activation of FAS (data not shown).

To determine whether other LRH-1 activated genes might also contain dual-function binding elements for LRH-1 and FXR/RXR, we tested the effect of FXR activation on SREBP-1c and CYP8B1, two other genes known to be activated by LRH-1 overexpression (22, 34) (**Fig. 5**). In addition, the effect of FXR activation was tested on the promoter for acetyl-CoA carboxylase, a lipogenic gene closely linked to FAS in the pathway of basic fatty acid biosynthesis (that has not been investigated for LRH-1 dependence). In this experiment, FAS was the lone promoter stimulated efficiently by FXR. Thus, FXR activation is neither a common feature of all LRH-1 gene targets nor a property shared by all lipogenic genes.

To determine whether bile acid-dependent FAS activation could be recapitulated in an animal model, we fed C57/BL6 wild-type mice either a normal chow diet or a normal chow diet supplemented with 0.25% CDCA, a bile acid and potent activator of FXR, for 6 days and compared

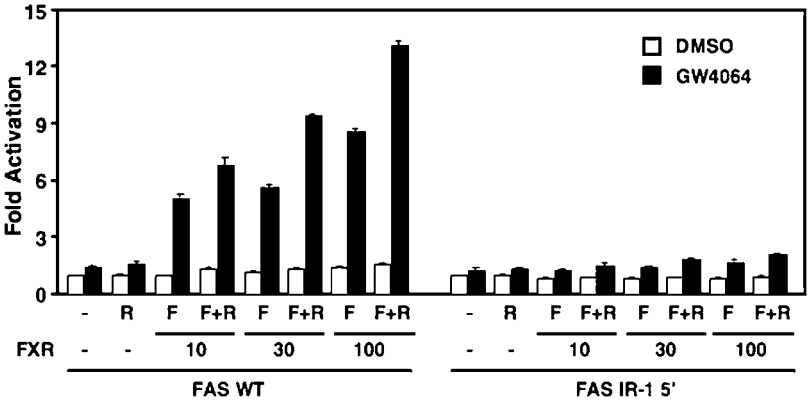

Fig. 2. FXR/RXR activates FAS through IR-1. HEK293T cells were transiently transfected with increasing amounts (10, 30, and 100 ng each) of cytomegalovirus X promoter (CMX)-RXRα (R) or CMX-FXR (F) and FAS −700/+65 wild type (WT) or FAS −700/+65 IR-1 5′ mutant reporter construct. Cells were treated with or without the synthetic FXR agonist GW4064 (1 μM) for 16 h and harvested for measurement of luciferase and β-galactosidase activity as described in Materials and Methods. Error bars represent standard deviation.

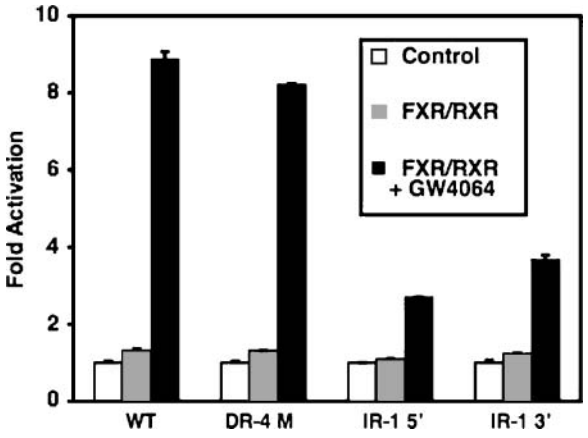

Fig. 3. Both IR-1 half-sites contribute to the FAS FXR response. HEK293T cells were transiently transfected with 100 ng of CMX-RXRα and 30 ng of CMX-FXR and one of four FAS reporter constructs as indicated. Cells were then treated with the synthetic FXR agonist GW4064 (1 μM) for 16 h and harvested for measurement of luciferase and β-galactosidase activity as described in Materials and Methods. Error bars represent standard deviation.

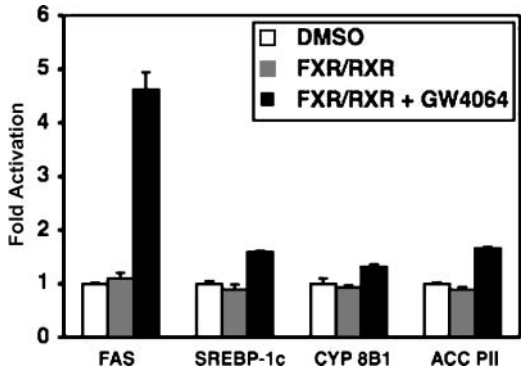

Fig. 5. FXR/RXR activation is specific to the FAS promoter. HEK293T cells were transiently transfected with 100 ng of CMX-RXRα, 100 ng of CMX-FXR, and reporter plasmids as indicated. Cells were then treated with the synthetic FXR agonist GW4064 (1 μM) or DMSO for 16 h and harvested for measurement of luciferase and β-galactosidase activities as described in Materials and Methods. ACC PII, promoter for acetyl-CoA carboxylase; SREBP, sterol-regulatory element binding protein. Error bars represent standard deviation.

hepatic FAS gene expression (**Fig. 6A, B**). FAS mRNA increased ~2-fold in livers of animals fed the 0.25% CDCA diet with respect to the control fed animals. In this same experiment, the expression of SHP, a known FXR target gene, increased by ~3-fold. Thus, bile acids induce FAS expression in vivo, and induction of FAS occurs at a level comparable to that of other FXR target genes. Unfortunately, feeding bile acids to FXR-deficient mice is highly toxic, and the animals become cachetic and begin to die after a few days of bile acid feeding (38). Thus, a knockout mouse model was not available for further verification of the role of FXR in FAS activation.

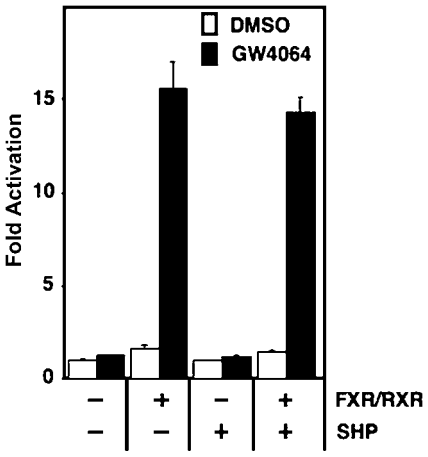

Fig. 4. LRH-1 interferes with FXR/RXR activation of FAS; SHP does not. HEK293T cells were transiently transfected with 100 ng of CMX-RXRα, 30 ng of CMX-FXR, 100 ng of CMX-SHP, or combinations as noted. The reporter plasmid was the FAS promoter construct with mutations in both halves of the DR-4 (DR-4M). Transfected cells were then treated with the synthetic FXR agonist GW4064 (1 μM) for 16 h and harvested for measurement of luciferase and β-galactosidase as described in Materials and Methods. Error bars represent standard deviation.

Because the bile acid feeding study by itself did not provide direct evidence for FXR regulation of FAS expression in animals, we performed chromatin immunoprecipitation studies on livers of wild-type mice expressing endogenous levels of FXR to determine whether FXR is directly associated with the endogenous FAS promoter. Chromatin was analyzed for FXR binding to the FAS promoter using an antibody to FXR and PCR primers specific for the relevant region of the FAS promoter or a genomic region from the mouse YY1 gene as a negative control (**Fig. 7**). The FAS promoter DNA was enriched >100-fold relative to an equivalent sample in which a control IgG fraction was used. In addition, FXR binding to the negative control YY1 locus was negligible (Fig. 7). Thus, endogenous FXR is specifically recruited to the native FAS promoter.

DISCUSSION

Although FXR was originally identified as a key regulator of cholesterol and bile acid homeostasis (38), it is now understood to also play an important role in fatty acid metabolism. The first evidence that bile acids might be involved in fatty acid metabolism derived from the incidental observation >20 years ago that administration of bile acids decreased serum triglycerides in patients with gallstone disease (19–21). An FXR-dependent mechanism for this effect was proposed by Watanabe et al. (22), who observed that SREBP-1c levels decreased ~50% but SHP levels increased by 300% after 1 day of treatment of wild-type or prediabetic (KK-A^y) mice with 0.5% cholic acid. In in vitro experiments, these authors demonstrated that LXR activation of the SREBP-1c promoter was significantly inhibited by the expression of SHP or the addition of bile acids. However, in this same study, levels of several key lipogenic targets of SREBP-1c did not correlate with SREBP-1c levels when the feeding study was extended to

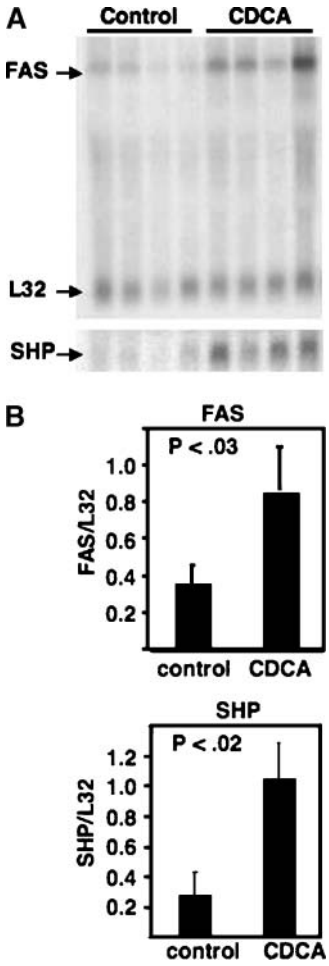

Fig. 6. Six day chenodeoxycholic acid (CDCA) feeding (0.25%) increases FAS mRNA in livers of wild-type mice. Wild-type mice were fed ad libitum either a normal chow diet (control) or a normal chow diet supplemented with 0.25% CDCA for 6 days. RNA was extracted from livers of mice and individually analyzed by Northern blotting. A: Autoradiogram of Northern blot probed for FAS and L32 or SHP. Each lane was loaded with 20 μg of RNA extracted from an individual animal in each group. B: Densitometric analysis of the data in A with the ratios of FAS/L32 and SHP/L32 represented in the graphs. Error bars represent standard deviation.

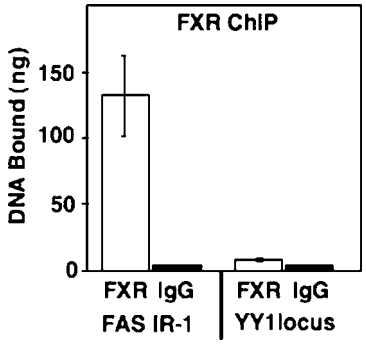

Fig. 7. FXR binds FAS promoter in hepatic chromatin. Endogenous hepatic FXR binding to the FAS promoter was analyzed by a chromatin immunoprecipitation (ChIP) experiment as described in Materials and Methods. The level of DNA precipitated by the FXR or control IgG antibody is presented for the FAS promoter or exon 4 from the YY1 gene as a control. The amount of DNA precipitated was calculated using a serial dilution curve of the input DNA from native chromatin that was quantified by optical density at 260 nm. Error bars represent standard deviation.

7 days. In particular, FAS mRNA decreased by ~50% after 1 day of cholic acid feeding but returned to control levels at 1 week, despite ongoing SREBP-1c repression and continued SHP elevation. Thus, only the transient inhibitory effect of bile acids is consistent with SHP inhibition of SREBP-1c-dependent lipogenesis, so additional mechanisms for the bile acid regulation of lipogenesis under more long-term bile acid-feeding conditions must exist. Therefore, we designed our feeding study to reflect this more "chronic" increase of bile acids.

In this study, we identified an IR-1 element in the FAS promoter that mediates the bile acid activation of the FAS gene. Although neither FAS nor other lipogenic gene targets have been identified as a result of FXR microarray experiments, analysis of these microarray data sets reveals that whereas some well-established FXR target genes (such as SHP) are activated by the synthetic ligand GW4064, they are not significantly activated by CDCA, or vice versa (39, 40). Other studies have reported opposing effects of a single FXR ligand on different known FXR target genes (41) and differential patterns for the recruitment of coactivators by the various endogenous bile acid agonists (42). These findings suggest that an exhaustive list of FXR gene targets may not be achieved simply by use of broad microarray analyses. Physiologically speaking, it seems likely that the discrepancies in FXR target gene activation observed in these experiments underlie complex regulatory mechanisms in which the specific ligand and the unique architecture of the nuclear receptor response regions in each promoter influence the final gene output response.

Demonstration of a direct activating role for FXR on the FAS promoter was unexpected given the well-established triglyceride-lowering effect of bile acids. However, an important role for fatty acid synthesis may be revealed when cholesterol levels are chronically increased. High cellular cholesterol levels increase oxysterols and therefore LXR signaling, leading to the upregulation of fatty acid synthase (30). Increased fatty acid production facilitates cholesterol storage by increasing the availability of the cosubstrate for cholesterol esterification. In the rodent, increased LXR activity resulting from the presence of oxysterols also results in the upregulation of CYP7A1 and the conversion of the excess cholesterol to bile acids (14, 15). If this process is allowed to continue, however, bile acids eventually accumulate to levels that activate the endogenous bile acid receptor FXR and result in the activation of FXR target genes such as SHP (10, 11). Because SHP negatively regulates both CYP7A1 (11) and FAS (22) (K. E. Matsukuma et al., unpublished data), activation of SHP would both limit the flow of cholesterol into the bile acid synthetic pathway and simultaneously decrease fatty acid synthesis, resulting in a shift in the balance of cholesterol and fatty acids to a state of cholesterol excess. This would limit the ability of the liver to

store excess cholesterol as cholesteryl ester and result in cholesterol toxicity (43). The direct mechanism for the bile acid activation of FAS presented in this study provides a plausible means by which FAS may circumvent SHP inhibition to maintain an adequate fatty acid pool for cholesterol esterification when both cholesterol and bile acid levels are chronically high.

Finally, it is interesting that the FXR/RXR binding site in the FAS promoter overlaps an LRH-1 binding site that is required for efficient LXR activation (K. E. Matsukuma et al., unpublished data). Although the significance of this binding site arrangement is not known, overlapping nuclear receptor binding sites have been found in many other promoters, including CYP7A1, CYP8B1, and apolipoprotein C-III (12, 44, 45). In the promoters of CYP7A1 and CYP8B1, binding sites for the nuclear receptors HNF-4α and FTF overlap. Using the technique of chromatin immunoprecipitation, investigators found that in the absence of bile acids, HNF4α preferentially bound to the overlapping site in the CYP7A1 promoter. In the presence of bile acids, however, only FTF bound this site. Given that each nuclear receptor has a unique transcriptional activation potential, the differential binding of these two nuclear receptors may function to ensure that the precise amount of gene product accumulates for optimal physiologic responses. In this way, overlapping binding sites may be an effective mechanism by which dynamic physiologic responses are achieved.

The authors thank Drs. Bruce Blumberg, Ronald Evans, Barry Forman, Peter Tontonoz, and David Mangelsdorf for plasmid reagents and Timothy Willson for the GW4064. The authors gratefully acknowledge support from the National Institutes of Health to T.F.O. (Grants HL-48044 and DK-71021), G.G. (Grant DK-38030), and L.W. (Grant P20 RR-016475 from the IdeA Network of Biomedical Research Excellence (INBRE) Program of the National Center for Research Resources). L.W. also was supported in part through a grant from Kansas Masonic Cancer Research. K.E.M. was supported by fellowships from the Achievement Rewards for College Scientists Foundation and the American Diabetes Association and is a member of the Medical Scientist Training Program in the University of California Irvine School of Medicine.

REFERENCES

1. Schmucker, D. L., M. Ohta, S. Kanai, Y. Sato, and K. Kitani. 1990. Hepatic injury induced by bile salts: correlation between biochemical and morphological events. *Hepatology.* **12:** 1216–1221.
2. Spivey, J. R., S. F. Bronk, and G. J. Gores. 1993. Glycochenodeoxycholate-induced lethal hepatocellular injury in rat hepatocytes. Role of ATP depletion and cytosolic free calcium. *J. Clin. Invest.* **92:** 17–24.
3. Forman, B. M., E. Goode, J. Chen, A. E. Oro, D. J. Bradley, T. Perlmann, D. J. Noonan, L. T. Burka, T. McMorris, W. W. Lamph, et al. 1995. Identification of a nuclear receptor that is activated by farnesol metabolites. *Cell.* **81:** 687–693.
4. Makishima, M., A. Y. Okamoto, J. J. Repa, H. Tu, R. M. Learned, A. Luk, M. V. Hull, K. D. Lustig, D. J. Mangelsdorf, and B. Shan. 1999. Identification of a nuclear receptor for bile acids. *Science.* **284:** 1362–1365.
5. Parks, D. J., S. G. Blanchard, R. K. Bledsoe, G. Chandra, T. G. Consler, S. A. Kliewer, J. B. Stimmel, T. M. Willson, A. M. Zavacki, D. D. Moore, et al. 1999. Bile acids: natural ligands for an orphan nuclear receptor. *Science.* **284:** 1365–1368.
6. Wang, H., J. Chen, K. Hollister, L. C. Sowers, and B. M. Forman. 1999. Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. *Mol. Cell.* **3:** 543–553.
7. Grober, J., I. Zaghini, H. Fujii, S. A. Jones, S. A. Kliewer, T. M. Willson, T. Ono, and P. Besnard. 1999. Identification of a bile acid-responsive element in the human ileal bile acid-binding protein gene. Involvement of the farnesoid X receptor/9-cis-retinoic acid receptor heterodimer. *J. Biol. Chem.* **274:** 29749–29754.
8. Ananthanarayanan, M., N. Balasubramanian, M. Makishima, D. J. Mangelsdorf, and F. J. Suchy. 2001. Human bile salt export pump promoter is transactivated by the farnesoid X receptor/bile acid receptor. *J. Biol. Chem.* **276:** 28857–28865.
9. Jung, D., M. Podvinec, U. A. Meyer, D. J. Mangelsdorf, M. Fried, P. J. Meier, and G. A. Kullak-Ublick. 2002. Human organic anion transporting polypeptide 8 promoter is transactivated by the farnesoid X receptor/bile acid receptor. *Gastroenterology.* **122:** 1954–1966.
10. Lu, T. T., M. Makishima, J. J. Repa, K. Schoonjans, T. A. Kerr, J. Auwerx, and D. J. Mangelsdorf. 2000. Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors. *Mol. Cell.* **6:** 507–515.
11. Goodwin, B., S. A. Jones, R. R. Price, M. A. Watson, D. D. McKee, L. B. Moore, C. Galardi, J. G. Wilson, M. C. Lewis, M. E. Roth, et al. 2000. A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis. *Mol. Cell.* **6:** 517–526.
12. del Castillo-Olivares, A., and G. Gil. 2001. Suppression of sterol 12alpha-hydroxylase transcription by the short heterodimer partner: insights into the repression mechanism. *Nucleic Acids Res.* **29:** 4035–4042.
13. Inagaki, T., M. Choi, A. Moschetta, L. Peng, C. L. Cummins, J. G. McDonald, G. Luo, S. A. Jones, B. Goodwin, J. A. Richardson, et al. 2005. Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis. *Cell Metab.* **2:** 217–225.
14. Lehmann, J. M., S. A. Kliewer, L. B. Moore, T. A. Smith-Oliver, B. B. Oliver, D. A. Winegar, D. E. Blanchard, T. A. Spencer, and T. M. Willson. 1997. Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. *J. Biol. Chem.* **272:** 3137–3140.
15. Peet, D. J., S. D. Turley, W. Ma, B. A. Janowski, J-M. A. Lobaccard, R. E. Hammer, and D. J. Mangelsdorf. 1998. Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXRα. *Cell.* **93:** 693–704.
16. Repa, J. J., S. D. Turley, J. A. Lobaccaro, J. Medina, L. Li, K. Lustig, B. Shan, R. A. Heyman, J. M. Dietschy, and D. J. Mangelsdorf. 2000. Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science.* **289:** 1524–1529.
17. Laffitte, B. A., J. J. Repa, S. B. Joseph, D. C. Wilpitz, H. R. Kast, D. J. Mangelsdorf, and P. Tontonoz. 2001. LXRs control lipid-inducible expression of the apolipoprotein E gene in macrophages and adipocytes. *Proc. Natl. Acad. Sci. USA.* **98:** 507–512.
18. Luo, Y., and A. R. Tall. 2000. Sterol upregulation of human CETP expression in vitro and in transgenic mice by an LXR element. *J. Clin. Invest.* **105:** 513–520.
19. Angelin, B., K. Einarsson, K. Hellstrom, and B. Leijd. 1978. Effects of cholestyramine and chenodeoxycholic acid on the metabolism of endogenous triglyceride in hyperlipoproteinemia. *J. Lipid Res.* **19:** 1017–1024.
20. Bateson, M. C., M. D. Evans, Jr., and I. A. Bouchier. 1978. Chenodeoxycholic acid therapy for hypertriglyceridaemia in men. *Br. J. Clin. Pharmacol.* **5:** 249–254.
21. Bell, G., B. Lewis, A. Petrie, and R. Dowling. 1973. Serum lipids in cholelithiasis: effect of chenodeoxycholic acid therapy. *BMJ.* **3:** 520–523.
22. Watanabe, M., S. M. Houten, L. Wang, A. Moschetta, D. J. Mangelsdorf, R. A. Heyman, D. D. Moore, and J. Auwerx. 2004. Bile acids lower triglyceride levels via a pathway involving FXR, SHP, and SREBP-1c. *J. Clin. Invest.* **113:** 1408–1418.
23. Moustaid, N., K. Sakamoto, S. Clarke, R. S. Beyer, and H. S. Sul. 1993. Regulation of fatty acid synthase gene transcription. *Biochem. J.* **292:** 767–772.
24. Moustaid, N., R. S. Beyer, and H. S. Sul. 1994. Identification of an insulin response element in the fatty acid synthase promoter. *J. Biol. Chem.* **269:** 5629–5634.
25. Wolf, S. S., G. Hofer, K. F. Beck, K. Roder, and M. Schweizer. 1994. Insulin-responsive regions of the rat fatty acid synthase gene promoter. *Biochem. Biophys. Res. Commun.* **203:** 943–950.

26. Ishii, S., K. Iizuka, B. C. Miller, and K. Uyeda. 2004. Carbohydrate response element binding protein directly promotes lipogenic enzyme gene transcription. *Proc. Natl. Acad. Sci. USA.* **101:** 15597–15602.
27. Xiong, S., S. S. Chirala, M. H. Hsu, and S. J. Wakil. 1998. Identification of thyroid hormone response elements in the human fatty acid synthase promoter. *Proc. Natl. Acad. Sci. USA.* **95:** 12260–12265.
28. Clarke, S. D., M. K. Armstrong, and D. B. Jump. 1990. Dietary polyunsaturated fats uniquely suppress rat liver fatty acid synthase and S14 mRNA content. *J. Nutr.* **120:** 225–231.
29. Bennett, M. K., J. M. Lopez, H. B. Sanchez, and T. F. Osborne. 1995. Sterol regulation of fatty acid synthase promoter. *J. Biol. Chem.* **270:** 25578–25583.
30. Joseph, S. B., B. A. Laffitte, P. H. Patel, M. A. Watson, K. E. Matsukuma, R. Walczak, J. L. Collins, T. F. Osborne, and P. Tontonoz. 2002. Direct and indirect mechanisms for regulation of fatty acid synthase gene expression by liver X receptors. *J. Biol. Chem.* **277:** 11019–11025.
31. Yieh, L., H. B. Sanchez, and T. F. Osborne. 1995. Domains of transcription factor Sp1 required for synergistic activation with sterol regulatory element binding protein 1 of low density lipoprotein receptor promoter. *Proc. Natl. Acad. Sci. USA.* **92:** 6102–6106.
32. Sanchez, H. B., L. Yieh, and T. F. Osborne. 1995. Cooperation by SREBP and Sp1 in sterol regulation of low density lipoprotein receptor gene. *J. Biol. Chem.* **270:** 1161–1169.
33. Zhang, C., D. J. Shin, and T. F. Osborne. 2005. A simple promoter containing two Sp1 sites controls the expression of sterol-regulatory-element-binding protein 1a (SREBP-1a). *Biochem. J.* **386:** 161–168.
34. del Castillo-Olivares, A., and G. Gil. 2000. Alpha 1-fetoprotein transcription factor is required for the expression of sterol 12alpha -hydroxylase, the specific enzyme for cholic acid synthesis. Potential role in the bile acid-mediated regulation of gene transcription. *J. Biol. Chem.* **275:** 17793–17799.
35. Lopez, J. M., M. K. Bennett, H. B. Sanchez, J. M. Rosenfeld, and T. F. Osborne. 1996. Sterol regulation of acetyl CoA carboxylase: a mechanism for coordinate control of cellular lipid. *Proc. Natl. Acad. Sci. USA.* **93:** 1049–1053.
36. Bennett, M., J. I. Toth, and T. F. Osborne. 2004. Selective association of sterol regulatory element binding protein isoforms with target promoters in vivo. *J. Biol. Chem.* **279:** 37360–37367.
37. Laffitte, B. A., H. R. Kast, C. M. Nguyen, A. M. Zavacki, D. D. Moore, and P. A. Edwards. 2000. Identification of the DNA binding specificity and potential target genes for the farnesoid X-activated receptor. *J. Biol. Chem.* **275:** 10638–10647.
38. Sinal, C. J., M. Tohkin, M. Miyata, J. M. Ward, G. Lambert, and F. J. Gonzalez. 2000. Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. *Cell.* **102:** 731–744.
39. Pircher, P. C., J. L. Kitto, M. L. Petrowski, R. K. Tangirala, E. D. Bischoff, I. G. Schulman, and S. K. Westin. 2003. Farnesoid X receptor regulates bile acid-amino acid conjugation. *J. Biol. Chem.* **278:** 27703–27711.
40. Downes, M., M. A. Verdecia, A. J. Roecker, R. Hughes, J. B. Hogenesch, H. R. Kast-Woelbern, M. E. Bowman, J. L. Ferrer, A. M. Anisfeld, P. A. Edwards, et al. 2003. A chemical, genetic, and structural analysis of the nuclear bile acid receptor FXR. *Mol. Cell.* **11:** 1079–1092.
41. Dussault, I., R. Beard, M. Lin, K. Hollister, J. Chen, J. H. Xiao, R. Chandraratna, and B. M. Forman. 2003. Identification of gene-selective modulators of the bile acid receptor FXR. *J. Biol. Chem.* **278:** 7027–7033.
42. Lew, J. L., A. Zhao, J. Yu, L. Huang, N. De Pedro, F. Pelaez, S. D. Wright, and J. Cui. 2004. The farnesoid X receptor controls gene expression in a ligand- and promoter-selective fashion. *J. Biol. Chem.* **279:** 8856–8861.
43. Warner, G. J., G. Stoudt, M. Bamberger, W. J. Johnson, and G. H. Rothblat. 1995. Cell toxicity induced by inhibition of acyl coenzyme A:cholesterol acyltransferase and accumulation of unesterified cholesterol. *J. Biol. Chem.* **270:** 5772–5778.
44. Stroup, D., and J. Y. L. Chiang. 2000. HNF4 and COUP-TFII interact to modulate transcription of the cholesterol 7{alpha}-hydroxylase gene (CYP7A1). *J. Lipid Res.* **41:** 1–11.
45. Claudel, T., Y. Inoue, O. Barbier, D. Duran-Sandoval, V. Kosykh, J. Fruchart, J. C. Fruchart, F. J. Gonzalez, and B. Staels. 2003. Farnesoid X receptor agonists suppress hepatic apolipoprotein CIII expression. *Gastroenterology.* **125:** 544–555.