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The Role of α_1-Fetoprotein Transcription Factor/LRH-1 in Bile Acid Biosynthesis

A KNOWN NUCLEAR RECEPTOR ACTIVATOR THAT CAN ACT AS A SUPPRESSOR OF BILE ACID BIOSYNTHESIS*

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Two key regulatory enzymes in the bile acid biosynthesis pathway are cholesterol 7α-hydroxylase/CYP7A1 (7α-hydroxylase) and sterol 12α-hydroxylase/CYP8B1 (12α-hydroxylase). It has been shown previously that hepatocyte nuclear factor-4α (HNF-4) and the α_1-fetoprotein transcription factor (FTF) are activators of 7α- and 12α-hydroxylase transcription and that the small heterodimer partner (SHP) suppresses bile acid biosynthesis by heterodimerizing with FTF. However, the role of FTF in bile acid biosynthesis has been studied only in tissue culture systems. In heterozygous FTF knockout mice, 7α- and 12α-hydroxylase genes were expressed at 5–7-fold higher levels than in wild-type mice, an apparent direct contradiction to previous *in vitro* observations. This higher expression of the 7α- and 12α-hydroxylase genes resulted in a 33% higher bile acid pool in their gallbladders, bile more enriched in cholic acid, and a 13% decrease in plasma cholesterol levels. Adenovirus-mediated FTF overexpression in wild-type mice resulted in 10-fold lower expression of the 7α- and 12α-hydroxylase genes and up to 8-fold higher SHP expression, highlighting the dual role that FTF plays in different promoters. Shorter overexpression times still resulted in lower 7α- and 12α-hydroxylase expression, but unchanged SHP expression, suggesting that two different mechanisms are involved in the FTF-mediated suppression of 7α- and 12α-hydroxylase expression. This FTF-mediated suppression of the expression of two bile acid biosynthesis genes resulted in a 3-fold lower rate of bile acid synthesis in a rat bile fistula animal model. Based on these observations and on protein binding studies performed *in vitro* and by chromatin immunoprecipitation, we hypothesize that FTF has two synergetic effects that contribute to its role in bile acid biosynthesis: 1) it has the ability to activate the expression of SHP, which in turn heterodimerizes and suppresses FTF transactivation activity; and 2) it occupies the FTF/HNF-4 recognition site within the 7α- and 12α-hydroxylase promoters, which can otherwise be occupied by a factor (HNF-4) that cannot be suppressed by SHP.

In mammals, cholesterol is required for several important functions such as cell growth and differentiation, maintenance of cell membrane integrity and as a precursor of steroid hormones. Another important biological function of cholesterol is its role as a bile acid precursor. Bile acid synthesis is responsible for the catabolism of >50% of body cholesterol in mammals. The catalytic process of cholesterol to bile acids occurs exclusively in the liver (1). It is well known that both genetic defects and environmental factors lead to disarrangements in cholesterol metabolism, which, in turn, produce many different degenerative conditions such as hypercholesterolemia and cholesterol gallstone disease (2, 3).

The conversion of cholesterol to cholic acid and chenodeoxycholic acid (the primary bile acids in humans and rodents) is done through four routes (1). Two of the routes that are well studied are the "classic" or "neutral" pathway and the "alternative" or "acidic" pathway. In the neutral pathway, cholesterol 7α-hydroxylase (referred to throughout as 7α-hydroxylase) hydroxylates cholesterol to 7α-hydroxycholesterol. This is the rate-limiting and controlling step. In the alternative pathway, cholesterol is hydroxylated to produce 27-hydroxycholesterol, a reaction catalyzed by the enzyme sterol 27-hydroxylase/CYP27A1. This oxysterol then undergoes 7α-hydroxylation by the enzyme oxysterol 7α-hydroxylase (1). 7α-Hydroxycholesterol is ultimately converted into cholic acid by the action of sterol 12α-hydroxylase/CYP8B1 (referred to throughout as 12α-hydroxylase), which is the specific step in cholic acid synthesis. If 12α-hydroxylase is not present or active, then the 7α-hydroxycholesterol is transformed to chenodeoxycholic acid. Chenodeoxycholic acid has higher hydrophobicity compared with cholic acid. Thus, 12α-hydroxylase determines the hydrophobicity of the bile acid pool and the absorption of dietary sterols, one of the primary functions of bile acids (1). The ratio of cholic acid to chenodeoxycholic acid has been postulated to play a role in cholesterol gallstone formation (3) and affects bile cholesterol and phospholipid secretion, thus altering intestinal cholesterol absorption and receptor-mediated lipoprotein uptake by the hepatocyte (4).

Bile acids negatively regulate the transcription of the 7α-hydroxylase gene, which controls output from the classic pathway. Recent studies have delineated many of the factors involved in this regulation. LRH-1 (liver receptor homolog-1), also known as CYP7A promoter-binding factor (5), NR5A2 (6), and the α_1-fetoprotein transcription factor (FTF)[1] (Genome

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[1] The abbreviations used are: FTF, α_1-fetoprotein transcription factor; SHP, small heterodimer partner; JNK, c-Jun N-terminal kinase; HNF-4, hepatocyte nuclear factor-4α; ChIP, chromatin immunoprecipitation; AFP, α_1-fetoprotein; HMG, 3-hydroxy-3-methylglutaryl; Q-RT, quantitative reverse transcription; Ad, adenovirus; CMV, cytomegalovirus; DR-1, direct repeat-1.

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Database Nomenclature Committee) (7), has been proposed to be required for the transcription of the 7α-hydroxylase (5, 8) and 12α-hydroxylase (9, 10) genes. We refer to this nuclear receptor as FTF. Bile acids activate the transcription of the small heterodimer partner (SHP) via binding of the hormone receptor farnesoid X receptor to its binding site in the SHP promoter. In turn, it has been proposed that SHP dimerizes with FTF and diminishes its activity on the 7α-hydroxylase promoter by mechanisms not yet well understood (11, 12). An alternative mechanism has recently been proposed for this down-regulation of 7α-hydroxylase transcription that involves the c-Jun N-terminal kinase (JNK) (13). Via this mechanism, the JNK pathway is activated by bile acids, which in turn activate c-Jun, resulting in higher SHP transcription. Another SHP-independent mechanism has also been proposed for the down-regulation of 7α-hydroxylase transcription in which bile acids suppress 7α-hydroxylase transcription by inhibiting the transactivation potential of hepatocyte nuclear factor-4α (HNF-4), which binds to and activates the 7α-hydroxylase promoter (15), through a mitogen-activated protein kinase pathway (14). Finally, a fourth mechanism has been proposed to be involved in the bile acid-mediated regulation of 7α-hydroxylase transcription, which also implicates HNF-4 (46). Via this mechanism, bile acids impair the recruitment of peroxisome proliferator-activated receptor-γ coactivator-1α and cAMP-responsive element-binding protein-binding protein by HNF-4. Chromatin immunoprecipitation (ChIP) assays revealed that bile acid-induced dissociation of coactivators from HNF-4 decreases the recruitment of RNA polymerase II on the core promoter and downstream in the 3′-untranslated regions of these two genes, reflecting the reduction of gene transcription (14). All of these studies have been carried out in cells in culture and *in vitro*.

Our laboratory has performed several studies directed toward understand the molecular mechanisms involved in the transcription and bile acid-mediated regulation of the 12α-hydroxylase gene. All of these studies have also been carried out in cells in culture and *in vitro*. We have shown that FTF is required for 12α-hydroxylase promoter activity (9). FTF binds to two sites within the rat 12α-hydroxylase promoter, and both sites are required for both promoter activity and bile acid-mediated regulation. We have also shown that SHP is involved in the down-regulation of the 12α-hydroxylase promoter (10). Overexpression of SHP in HepG2 cells suppresses 12α-hydroxylase promoter activity. We have also shown that, despite the interaction between HNF-4 and SHP and the apparent requirement for HNF-4 for 12α-hydroxylase promoter activity, bile acids suppress only FTF-activated 12α-hydroxylase promoters.

Although FTF was first functionally characterized as a key transcription factor for the transcription of the α_1-fetoprotein (AFP) gene (hence its name), in the last few years, it has become apparent that it might play a major role in bile acid metabolism and more generally in cholesterol and lipid metabolism. In addition to the role that FTF seems to play in the transcription and regulation of the 7α- and 12α-hydroxylase genes, it has been proposed recently that it might also be involved in intestinal bile acid transport through its involvement in the transcription and regulation of the apical sodium-dependent bile acid transporter (16). Also, human enterocyte MRP3 (multidrug resistance protein-3) has been reported to work as a bile acid transporter in human enterocytes, and its expression is induced by bile acids through a fundamentally different mechanism than the mechanism involved in the bile acid-mediated regulation of 7α- and 12α-hydroxylase genes (17). FTF also participates in self-regulation of its transcription (18), and it has been proposed to be involved also in the transcription and regulation of SHP (12, 19), potentially playing an additional role in the regulation of the transcription of the 7α- and 12α-hydroxylase genes. In addition, FTF is required for reverse cholesterol transport because it induces the expression of the human cholesteryl ester transfer protein gene (20) and the scavenger receptor class B type I gene (21). However, as is the case for the transcriptional regulation of the 7α- and 12α-hydroxylase genes, all the evidence for these roles of FTF comes from experiments in tissue culture cells.

As indicated above, the involvement of FTF in bile acid synthesis has been studied only in tissue culture. Animal studies should be performed to demonstrate whether FTF is indeed involved in the transcription of key genes implicated in bile acid biosynthesis and regulation. We have used three complementary animal models to study the *in vivo* role of FTF in bile acid biosynthesis. First, a knockout mouse model in which the FTF gene has been deleted was used. Second, *in vivo* overexpression of FTF in the mouse was accomplished using an adenovirus system. Third, we used a bile fistula animal model to quantify bile acid synthesis *in vivo* in rats overexpressing FTF. All three models show that FTF acts as a suppressor of 7α- and 12α-hydroxylase gene expression. These studies, combined with *in vitro* gel retardation assays and ChIP experiments, led us to hypothesize that FTF has two synergetic effects that contribute to its role in bile acid biosynthesis: 1) it has the ability to activate the expression of SHP, which in turn heterodimerizes and suppresses FTF transactivation activity; and 2) it occupies the FTF/HNF-4 recognition site within the 7α- and 12α-hydroxylase promoters, which can otherwise be occupied by a nuclear receptor (HNF-4) that cannot be suppressed by SHP.

EXPERIMENTAL PROCEDURES

Materials—Reagents used in DNA cloning and sequencing were from New England Biolabs Inc. and Invitrogen. Common laboratory chemicals were from Fisher, Sigma, or Bio-Rad. Oligonucleotides were prepared in the Medical College of Virginia DNA Synthesis Facility by the phosphoramidite method on an automated DNA synthesizer. Plasmids for *in vitro* synthesis of FTF and HNF-4 have been described previously (10). Anti-FTF antibodies were prepared against amino acids 179–197 of the mouse FTF sequence and were affinity-purified before use. Anti-HNF-4 antibodies were obtained from Santa Cruz Biotechnology.

Western Blot Analysis—Liver nuclear extracts were prepared as described previously (22). 10 μg of nuclear protein was fractionated on an SDS-polyacrylamide gel, transferred to nitrocellulose, incubated with the affinity-purified anti-FTF antibody (8 μg/ml), and processed with a Western Lightning Chemiluminescence Reagent Plus kit (PerkinElmer Life Sciences) according to the manufacturer's recommendations.

Animals—All mice were ~8 weeks of age at the time of the experiments. Mice were maintained in a 12-h light/12-h dark controlled environment with free access to water and standard laboratory rodent food. Harvesting of the indicated tissues was done at the middle of the dark period. $FTF^{+/-}$ mice were a generous gift from Dr. L. Belánger (Laval University, Quebec) and have been described previously (23). $FTF^{+/-}$ mice were received at the Virginia Commonwealth Animal Facility after at least 10 generations of inbreeding. A colony was generated and used as described under "Results." Heterozygous mice were detected by PCR analysis using oligonucleotides from the neomycin gene used in the generation of knock-in mice. FTF-null mice are embryonic lethal at embryonic day 7.5.

For the bile acid synthesis rate quantification, age- and weight-matched adult male Sprague-Dawley rats weighing between 275 and 325 g were housed under controlled lighting conditions on a natural light/dark cycle (4 a.m. to 4 p.m.). For the overexpression experiments, male C57BL/6 mice were purchased from Charles River Laboratories and maintained for 2 weeks prior to the experiments. They were injected with the indicated number of viral particles through the tail vein and harvested after either 3 or 5 days as indicated.

Adenovirus Preparation and Propagation—The adenovirus construct used in this study was obtained through the Massey Cancer Center Shared Resource Facility of the Virginia Commonwealth University. pZeroTG/CMV-hFTF needed to create that construct was prepared as

follows. A 1.6-kb fragment from pCI-hFTF (10) cut with SacII and ScaI was cloned into pCMX, which had been linearized with BamHI and blunt-ended, which resulted in pCMX-hFTF. An EcoRV/NheI fragment generated from pCMX-hFTF was cloned into the EcoRV site of pZeroTG/CMV. The resulting pZeroTG/CMV-hFTF plasmid was cotransformed with ClaI-linearized pTG/CMV (containing the entire Ad5*dl*324 genome) into *Escherichia coli*. Recombinant plasmids were transfected into human embryonic kidney 293 cells (American Type Culture Collection, Manassas, VA). Adenovirus DNA from the resulting plaques was further screened by analytical digestions for the presence of the insert. To purify the recombinant virus, the crude supernatant was carefully layered over a two-step CsCl gradient as described (24). For the mouse experiments, a control virus was used that contained the LacZ sequence. For the rat experiments, the control virus used was prepared with empty pZeroTG/CMV.

RNA Isolation and Quantification—Total liver RNA was isolated by the guanidine thiocyanate lysis/cesium chloride centrifugation method (25) or using the SV total RNA isolation kit (Promega). In some experiments, 7α-hydroxylase, 12α-hydroxylase, 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, and cyclophilin mRNAs were quantified by RNase protection assay as described (26) according to the manufacturer's protocol (Ambion Inc., Austin, TX). In other experiments, 7α-hydroxylase, 12α-hydroxylase, mouse FTF, human FTF, SHP, HNF-4, and AFP mRNAs were quantified by quantitative reverse transcription (Q-RT)-PCR using the primers and probes shown in Table I and the Brilliant QRT-PCR Core Reagent kit (Stratagene) using either TaqMan probes (7α- and 12α-hydroxylase) or SYBR Green (SHP, mouse HNF-4, human FTF, and mouse FTF) with either a DNA Engine Opticon2 (MJ Research, Inc.) or an iCycler iQ system (Bio-Rad). All values were normalized to the levels of actin mRNA, quantified also by Q-RT-PCR either in separate SYBR Green reactions or in duplex reactions when TaqMan probes were used.

Quantification of Bile Acid Synthesis Rates—Adult male Sprague-Dawley rats weighing between 275 and 325 g were housed under controlled lighting conditions on a natural light/dark cycle (4 a.m. to 4 p.m.). Groups of age- and weight-matched animals were used in all experiments. Under brief methoxyflurane anesthesia, bile fistulas and intravenous cannulas were placed as described previously (4). All animals received a continuous infusion of glucose/electrolyte replacement solution at 1.1 ml/h during the course of the experiment. Following surgery, the rats were housed in individual metabolic cages with free access to water and chow. Dietary intake, activity, and bile flow were carefully monitored. 1.5×10^{11} particles of the adenovirus (Ad) containing the human FTF cDNA in front of the cytomegalovirus (CMV) promoter (Ad-FTF) or the control virus were infused through the jugular immediately after surgery. Diverted bile was collected in 4-h increments throughout the course of the experiment. At the end of 72 h, animals were briefly anesthetized and decapitated, and blood was collected for determination of serum alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase. Under these chronic biliary diverted conditions, bile acid secretion is equivalent to bile acid synthesis.

Bile Acid Quantification and Bile Cholesterol and Phospholipids—Mouse gallbladders were collected on a piece of filter paper. Bile acids were extracted by the Folch method (27). Bile collected from the biliary diverted rats was also extracted by the Folch method. Conjugated bile acids were quantified from both types of samples by reverse-phase high pressure liquid chromatography as described previously (28). The chloroform phase was stored for later analysis of cholesterol and phospholipids using a quantitative enzymatic assay from commercially available kits (Wako Bioproducts), with minor modifications, using 1 ml of the chloroform phase, which was evaporated under nitrogen before adding the color reagent.

Electrophoretic Mobility Shift Analysis—Electrophoretic mobility shift assays were performed as described (10) using 8 fmol of the indicated rat 12α-hydroxylase and AFP ^{32}P-labeled DNA probes. *In vitro* proteins were prepared using the TnT T7 coupled reticulocyte lysate system (Promega).

ChIP—We used a modification of the method described by Weinmann and Farnham (29). Briefly, cross-linking of proteins to DNA was accomplished by adding formaldehyde to 400 mg of chopped liver at a final concentration of 1% in 5 ml of phosphate-buffered saline, followed by a 15-min incubation at room temperature. Glycine was added to a final concentration of 125 mM to stop the cross-linking reaction. Tissue was collected by centrifugation, washed twice with cold phosphate-buffered saline, and disaggregated in 4 ml of phosphate-buffered saline with a Dounce homogenizer. Cells were resuspended in cell lysis buffer and incubated for 10 min in ice. Nuclei were collected by centrifugation and resuspended in 7 ml of hypotonic nuclear lysis buffer. After 15 min on ice, glass beads (0.7 g, 212–300 μm) were added and sonicated five times for 15 s at setting 2.5 in a Sonic Dismembrator 550 (Misonix Inc.) to an average length of 500–1000 bp. The chromatin solution was adjusted to a protein concentration of 4.0 mg/ml. Chromatin aliquots corresponding to 100 μg of protein were precleared with 20 μl of preblocked staphylococcus A in a 300-μl final volume of radioimmune precipitation assay (RIPA) buffer supplemented with 1% Nonidet P-40 for 1 h at 4 °C. Precleared chromatin was incubated with 1 μg of nonspecific antibody (sc-8954, Santa Cruz Biotechnology), anti-HNF-4 antibody H-171 (sc-8987, Santa Cruz Biotechnology), or no antibody at 4 °C for 12 h. Immunoprecipitation, washing, and elution of immune complexes were carried out as described (29). After cross-link reversions and RNase A and proteinase K digestions, samples were resuspended in 30 μl of sterile 10 mM Tris-HCl (pH 8) and 1 mM EDTA, and 2 μl was used in each PCR with the primers shown in Table I. Total input chromatin samples (supernatants from no-antibody immunoprecipitations) were resuspended in 100 μl of sterile H_2O and then diluted 1:10 before PCR.

RESULTS

Increased 7α- and 12α-Hydroxylase Expression and Gallbladder Bile Acid in FTF$^{+/-}$ Mice—We obtained heterozygous FTF-null mice (FTF$^{+/-}$) from Dr. L. Belánger. These mice were generated by homologous recombination in embryonic stem cells as reported previously (23). Homozygous FTF-null mice are nonviable. These FTF$^{+/-}$ mice exhibited one-third of the normal amount of FTF protein and mRNA compared with wild-type mice (Fig. 1), probably a consequence of the FTF gene being self-regulated (18), as shown by Western blotting and Q-RT-PCR analysis.

Tissue culture studies suggest that FTF is a positive transcription factor for both 7α- and 12α-hydroxylase transcription (5, 8–12). Thus, we expected that the expression of the 7α- and 12α-hydroxylase genes would be lower in FTF$^{+/-}$ mice than in wild-type mice. Total RNAs from male wild-type and FTF$^{+/-}$ mice were prepared from pooled livers at equal quantities, and 7α- and 12α-hydroxylase mRNAs were quantified. Surprisingly, the expression of both genes was dramatically increased in FTF$^{+/-}$ mice (Fig. 2). 7α-Hydroxylase expression was 5–7-fold higher in FTF$^{+/-}$ mice, and 12α-hydroxylase RNA was 3–5-fold higher. As a control, we quantified cyclophilin mRNA, which was expressed at the same levels in both sets of mice. HMG-CoA reductase mRNA was also quantified in the same experiment, and its expression was also induced by at least 5-fold in FTF$^{+/-}$ mice.

Because 7α-hydroxylase is the key regulatory enzyme of the classic pathway of bile acid biosynthesis and 12α-hydroxylase is the enzyme responsible for cholic acid synthesis, studies were carried out to determine whether changes in 7α- and 12α-hydroxylase mRNAs in FTF$^{+/-}$ mice affect bile acid biosynthesis. This analysis is crucial for bile acid biosynthesis because of the existence of at least two biosynthesis pathways (1). Altered 7α-hydroxylase expression might not necessarily lead to altered bile acid synthesis because 7α-hydroxylase is involved only in the neutral pathway, and the acidic pathway could potentially compensate for the lack of synthesis through the neutral pathway. Similarly, because 12α-hydroxylase expression is increased in the FTF$^{+/-}$ mouse and 12α-hydroxylase is the specific enzyme for cholic acid synthesis, we expected that cholic acid synthesis would be more affected than chenodeoxycholic acid synthesis as a consequence of lower FTF expression. To this end, as a first approach to quantify bile acid synthesis in wild-type and FTF$^{+/-}$ mice, gallbladder bile acids were extracted from wild-type and FTF$^{+/-}$ mice, followed by quantification of the total bile acid pool and the relative amounts of cholic acid and muricholic acid, a major bile acid in the mouse to which most of the rodent chenodeoxycholic acid gets converted. Fig. 3 shows that the total bile acid pool and the

TABLE I

Oligonucleotides used in the Q-RT-PCR quantification of RNAs and in the ChIP experiments

For the Q-RT-PCR oligonucleotides (m, mouse; h, human), the nucleotide numbers correspond to the GenBank™/EBI Data Bank accession numbers. An F in the oligonucleotide name means forward; an R, reversed; and a T, TaqMan probe. For the ChIP, nucleotides are numbered with respect to the transcriptional initiation site, which has been set as +1.

Oligo	Gene	GenBank™ no.	Nucleotides	Sequence
m7α-F	m7α-hydroxylase	NM_007824	914–935	CAT CTC AAG CAA ACA CCA TTC C
m7α-R			984–1006	GAA AGC AGC CTC TGA AGA AGT GA
m7α-T			947–976	CAG GGC TCC TGA TCA TTT GAA ATA AGC TCC
m12α-F	m12α-hydroxylase	AF090319	2220–2239	GAA CTC AAC CAG GCC ATG CT
m12α-R			2280–2298	AGT CTG GGT GCC AGC TCC T
m12α-T			2244–2272	TTA GGC CCT AGC ATC ACC AAG GAT AGG CT
mFTF-F	mFTF	NM_030676	1404–1424	CAT GGG AAG GAA GGG ACA ATC
mFTF-R			1474–1494	GGT TGT TGA ACG CGA CTT CTG
hFTF-F	hFTF	NM_003822	1241–1263	TTT ACC GAC AAG TGG TAC ATG GA
hFTF-R			1309–1330	CGG CTT GTG ATG CTA TTA TGG A
mSHP-F	mSHP	NM_011850	549–569	CTG AAG GGC ACG ATC CTC TTC
mSHP-R			643–663	ACC AGG GCT CCA AGA CTT CAC
mAFP-F	mAFP	NM_007423	126–147	GAT AGC TTC CAC GTT AGA TTC C
mAFP-R			201–220	TGG CTT CCG GAA CAA ACT GG
mβ-actin-F	mβ-actin	NM_007393	574–594	TCT ACG AGG GCT ATG CTC TCC
mβ-actin-R			699–721	TCT TTG ATG TCA CGC ACG ATT TC
mβ-actin-T			605–626	CCT GCG TCT GGA CCT GGC TGG C
mApoC-III ChIP-F	mApoC-III	NT_039473	−187 to −168	CGT GAA AAG CAT GGG CAA TC
mApoC-III ChIP-R			−2 to +18	AGG GAT AAA ACT GAG CAG GC
m12α ChIP-F	m12α-hydroxylase	NT_039482	−172 to −153	CCA AAC TCT GCT GTG TCA TG
m12α ChIP-R			+6 to +25	TAG GCT CCA GCA GGC TGA GC

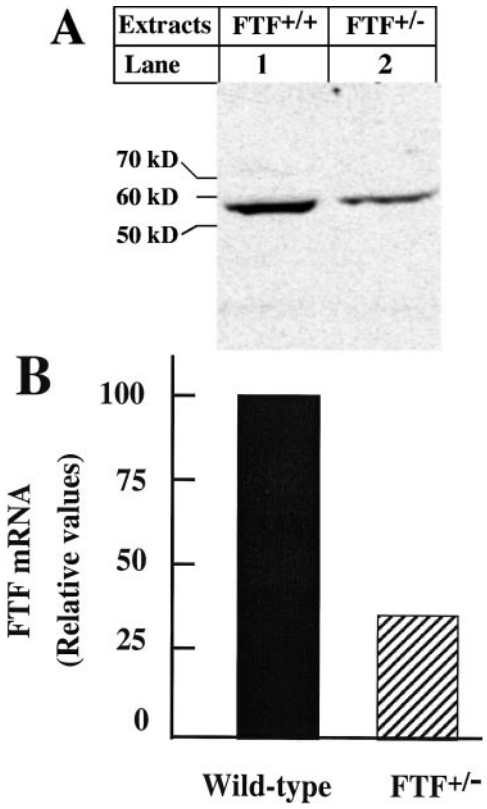

FIG. 1. **Differential FTF protein and mRNA levels in wild-type and FTF$^{+/-}$ mice.** *A*, Western blotting was performed with the indicated nuclear extracts. The antibody used was an affinity-purified anti-peptide antibody (amino acids 179–197 of the mouse FTF sequence). *B*, the relative amounts of FTF mRNA in the two groups of mice are indicated.

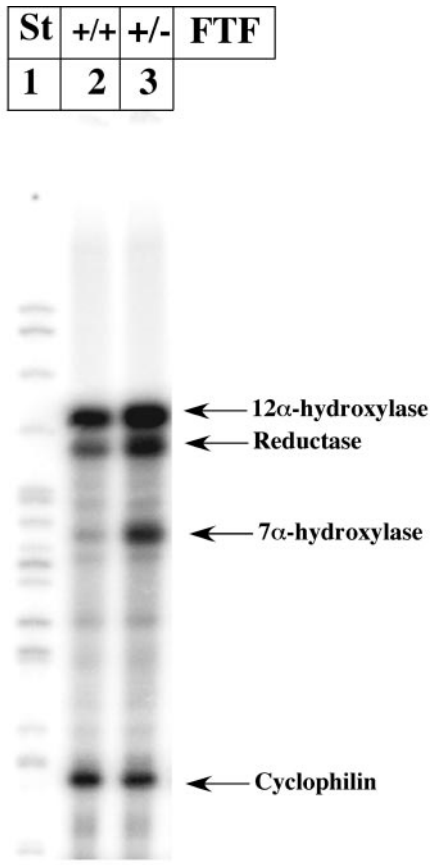

FIG. 2. **Differential expression of bile acid biosynthesis genes in wild-type and FTF$^{+/-}$ mice.** Total RNA was isolated from equal aliquot amounts of liver from the indicated male mice (*lanes 2* and *3*). A total of six mice were used in each group. An RNase protection assay was performed to detect 12α- and 7α-hydroxylase and HMG-CoA reductase mRNAs. Cyclophilin mRNA was quantified as a control. ^{32}P-Labeled, HaeIII-cut ΦX174 DNAs were used as size markers (*lane 1*). *St*, standards.

cholic and muricholic acid levels were ~1.5-fold higher in FTF$^{+/-}$ mice than in wild-type mice. Also, the ratio of cholic acid to muricholic acid was higher in the gallbladders of FTF$^{+/-}$ mice. These changes were also reflected in a 13% lower plasma cholesterol level in FTF$^{+/-}$ mice compared with wild-type mice ($p < 0.01$) (data not shown), emphasizing the role of bile acid synthesis in overall cholesterol homeostasis. Female mice gave the same results in terms of both 7α- and 12α-hydroxylase as well as gallbladder bile acids and plasma cholesterol (data not shown).

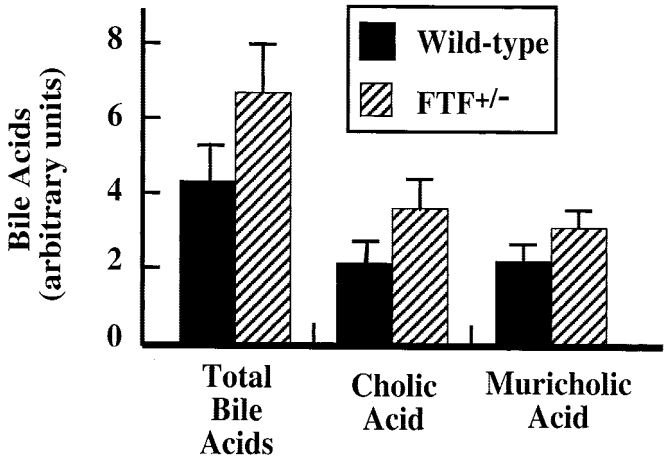

FIG. 3. **Total and specific bile acid levels in gallbladders from wild-type and FTF$^{+/-}$ mice.** Values represent the means ± S.D. of 10 mice/group ($p < 0.05$).

Overexpression of FTF in Mice Leads to a Decrease in 7α- and 12α-Hydroxylase mRNAs—Given the unexpected results, we turned to another animal model, *i.e.* mice overexpressing FTF. We used an adenovirus carrying the FTF cDNA driven by the CMV promoter. Male C57BL/6 mice were injected with 3×10^{10} viral particles of either a control adenovirus containing the bacterial galactosidase coding sequence in front of the CMV promoter (Ad-Gal) or an adenovirus containing the human FTF cDNA in front of the CMV promoter (Ad-FTF). We used human FTF to be able to distinguish between the exogenous and endogenous mRNAs. 5 days later, these two sets of mice, together with control mice that had been injected with saline solution, were killed, and their livers were harvested. The body weight of these animals did not change as a result of the viral injections, and all mice had similar weights of between 25 and 28 g. Liver RNA was isolated and used to quantify 7α- and 12α-hydroxylase mRNAs by TaqMan Q-RT-PCR. Actin RNA was quantified and used to normalize RNA values. As expected, given the results obtained with FTF$^{+/-}$ mice, 7α- and 12α-hydroxylase mRNAs were suppressed (~10-fold) in the group of mice injected with Ad-FTF, with a minor effect caused by the control virus (Fig. 4, *A* and *B*). The expression of two other genes thought to be regulated by FTF was also quantified. SHP has been shown to be induced upon FTF overexpression in tissue culture cells (12), and both the human SHP promoter (12) and the mouse SHP promoter have functional FTF sites. The FTF sites in these promoters are structurally different from the sites in the 7α- and 12α-hydroxylase promoters in the sense that they do not have HNF-4 sites overlapping the FTF recognition sites. Contrary to the effect of FTF overexpression on 7α- and 12α-hydroxylases, SHP expression was induced by ~8-fold (Fig. 4*C*) and could explain the suppression of 7α- and 12α-hydroxylase expression. Although the expression of AFP in the non-infected mice was low, it was still detectable and was also induced by 5-fold upon FTF overexpression (Fig. 4*D*). As expected, infection with Ad-FTF produced a dramatic increase (~50-fold) in FTF mRNA (Fig. 4*E*).

The adenovirus infection described above produced a limited liver toxicity based on the plasma levels of liver marker enzymes such as alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase, although similar for both the control and FTF viruses. In an attempt to avoid this toxicity, we performed a similar overexpression experiment, but we harvested the mice 3 days after infection instead of 5 days. We also used three different amounts of virus: 1, 2, and 3×10^{10} particles. Under these conditions, liver marker enzymes were unaffected by the virus infection. Both 7α- and 12α-hydroxylase expression was suppressed in a virus concentration-dependent manner, reaching 3-fold suppression at the highest concentration (Fig. 5, *A* and *B*). However, expression of both SHP and AFP was unaffected during these shorter infection times (Fig. 5, *C* and *D*), suggesting that FTF has two effects on gene expression. Expression of HNF-4 was not altered under any condition used (data not shown).

Overexpression of FTF in Rats Leads to a Decrease in Bile Acid Biosynthesis in Vivo—Because 7α-hydroxylase is required only for the neutral bile acid biosynthesis pathway, a decrease in its expression does not necessarily mean lower bile acid synthesis because the acidic pathway could potentially compensate for the lower activity of the neutral pathway. Thus, a better reflection of bile acid synthesis rates is to quantify bile acid synthesis in the entire animal by performing a bile fistulation and collecting bile after the circulating bile has been emptied (30). Under these conditions, bile acid secretion equals synthesis (28). We performed such an experiment in rats injected with 1.5×10^{11} particles of Ad-FTF, and bile was collected at different times. Both non-injected rats and rats injected with a control virus were used as controls. Only data on control virus-injected rats are shown, as no significant difference was observed with the non-injected group. Bile was collected every hour for 72 h; the volume was determined by weight; and bile acids were quantified. Fig. 6 shows the result of such an experiment. During the first 24 h, bile acid secretion fell, and the amount of bile acids collected diminished until the system was drained, reaching a low point at 24 h. This low point represents basal bile acid synthesis. Then, bile acid secretion increased again as a result of de-repression of the bile acid biosynthesis pathways, which is a consequence of the lack of return of bile acids through the enterohepatic circulation, eliminated by the bile fistula. This increase in bile acid release in the bile fistula animals is actually considered proof that, at those time points, bile acid secretion equals bile acid synthesis (28, 31). Most important, this increase in bile acid synthesis was lower in the Ad-FTF-injected animals than in the control virus-injected rats (compare the control virus- and Ad-FTF-injected rats at the 68- and 72-h times points). There was an ~2-fold increase in the control virus-injected rats, but only a 1.4-fold increase in the Ad-FTF injected rats, which strongly suggests that FTF indeed suppresses bile acid synthesis. Bile cholesterol and phospholipid levels were also quantified, and they changed similarly to bile acid levels (data not shown). Plasma liver enzyme levels did not increase as a result of the adenovirus infection.

Competition between HNF-4 and FTF for an Overlapping Site in the 12α-Hydroxylase Promoter—Binding of HNF-4 to the direct repeat-1 (DR-1) site in the 12α-hydroxylase (10) and 7α-hydroxylase (32) promoters has been reported *in vitro*. The DR-1 site in the 12α-hydroxylase promoter completely overlaps the FTF sites (9, 10) such that binding of both proteins to the same DNA molecule cannot occur simultaneously. In the 7α-hydroxylase promoter, the two sites overlap partially (5, 32). This suggests that more HNF-4 could bind to the 12α-hydroxylase promoter FTF/DR-1 site in FTF$^{+/-}$ mice given the lower levels of FTF expression. As a first approach to study this, we investigated whether HNF-4 could prevent FTF from binding to the rat 12α-hydroxylase promoter FTF/DR-1 site using a gel retardation assay with proteins made *in vitro*. As a control, we used the rat AFP FTF site, which does not bind HNF-4. Fig. 7*A* shows the results from such an experiment. As we have seen before (10), the rat 12α-hydroxylase promoter FTF/DR-1 site bound both FTF and HNF-4 (*lanes 1* and *2*), whereas the AFP promoter FTF site bound only FTF (*lanes 7* and *8*). When the

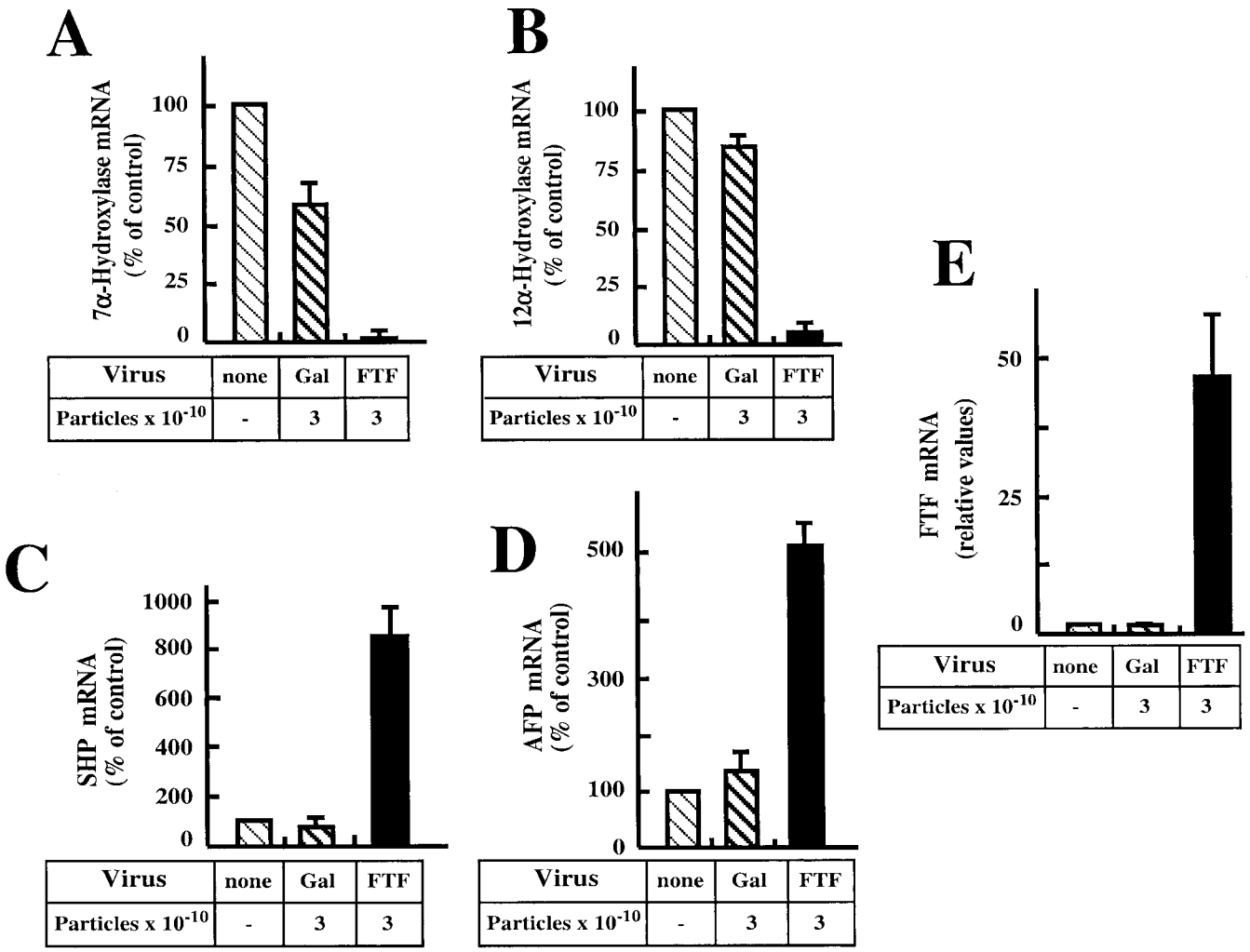

FIG. 4. **FTF overexpression suppresses the expression of bile acid biosynthesis genes, but induces SHP and AFP expression in C57BL/6 mice.** Male mice (three to five/group) where injected with the indicated viruses. 5 days later, they were killed, and their livers were harvested. Total RNA was isolated from individual livers and used in Q-RT-PCR experiments to quantify the indicated mRNAs as described under "Experimental Procedures." Values represent the means ± S.D. of the indicated mRNA/actin ratios from three to five mice/group. *Gal*, adenovirus control containing the bacterial galactosidase coding sequence in front of the CMV promoter.

two proteins were included in the assay, HNF-4 competed FTF out from the 12α-hydroxylase promoter in a concentration-dependent manner (*lanes 3–6*). When the rat AFP probe was used, there was only ~2-fold competition (*lanes 9–12*), indicating that HNF-4 can prevent FTF from binding to the 12α-hydroxylase promoter in a specific manner, at least under the *in vitro* experimental conditions. This experiment was performed with probes from the rat promoter because it is the best 12α-hydroxylase promoter characterized to date (9, 10, 26, 33). However, this arrangement of FTF and HNF-4 sites in genes down-regulated by bile acids seems to be highly conserved. Fig. 7*B* shows that the overlapping FTF/HNF-4 site is present in both 7α- and 12α-hydroxylase promoters in the three species in which these promoters have been characterized.

To characterize whether a similar phenomenon occurs *in vivo* that could potentially explain the 12α-hydroxylase expression phenotype in $FTF^{+/-}$ mice, we performed ChIP experiments using chromatin from wild-type and $FTF^{+/-}$ mice and an anti-HNF-4 polyclonal antibody. Available anti-FTF antibodies do not work in immunoprecipitation experiments, and they are not suitable for ChIP assays. This technique allows the identification of factor(s) that bind to a certain DNA-binding site in a particular promoter *in vivo*, and it can be used in a quantitative manner (34, 35). The co-immunoprecipitated chromatin fragments were amplified with primers flanking the FTF/HNF-4 site of the 12α-hydroxylase promoter. Another set of primers flanking the HNF-4 site in the apoC-III gene was used as a control. This apoC-III promoter site binds HNF-4, but not bind FTF (36). As expected, the anti-HNF-4 antibody (but not preimmune IgG) co-immunoprecipitated the HNF-4-binding element in the apoC-III gene (Fig. 8, *lower panel*, compare *lanes 2* and *3* and *lanes 5* and *6*). The FTF DNA-binding element of the 12α-hydroxylase promoter was specifically co-immunoprecipitated by the anti-HNF-4 antibody, but not by preimmune IgG (*upper panel*, compare *lanes 1–3* and *lanes 4–6*). Most important, the amount of the FTF DNA-binding element coprecipitated by the anti-HNF-4 antibody was much higher in $FTF^{+/-}$ mice than in wild-type mice, but only for the 12α-hydroxylase promoter FTF/HNF-4 site (*upper panel*, compare *lanes 3* and *6*) and not for the apoC-III promoter HNF-4 site (*lower panel*, compare *lanes 3* and *6*). We performed PCRs with the total input chromatin (*lanes 7* and *8*) and total DNA (*lane 9*) as controls. No-IgG controls were also performed (*lanes 1* and *4*) in addition to a no-template control (*lane 10*). These observations support the notion that HNF-4 is capable of competing with FTF for binding to the FTF/HNF-4-binding element present in the 12α-hydroxylase promoter. We have been unable to amplify by PCR the FTF site in the 7α-hydroxylase promoter presumably due to the high A/T content of the surrounding nucleotides.

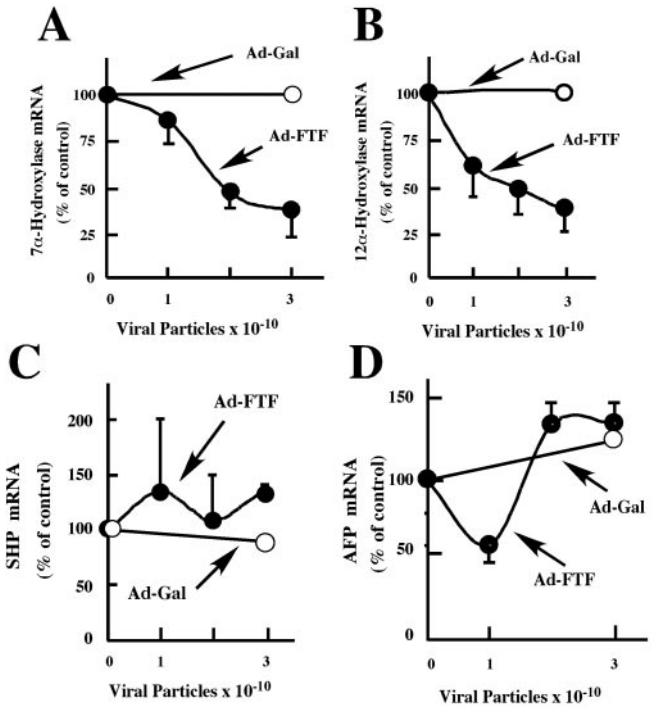

FIG. 5. **FTF overexpression suppresses the expression of bile acid biosynthesis genes in C57BL/6 mice.** The experimental conditions were similar to the conditions described in the legend to Fig. 4, except for the number of virus particles used and that the mice were killed 3 days after infection. Values represent the means ± S.D. of the indicated mRNA/actin ratios from three to five mice/group. *Ad-Gal*, adenovirus control containing the bacterial galactosidase coding sequence in front of the CMV promoter.

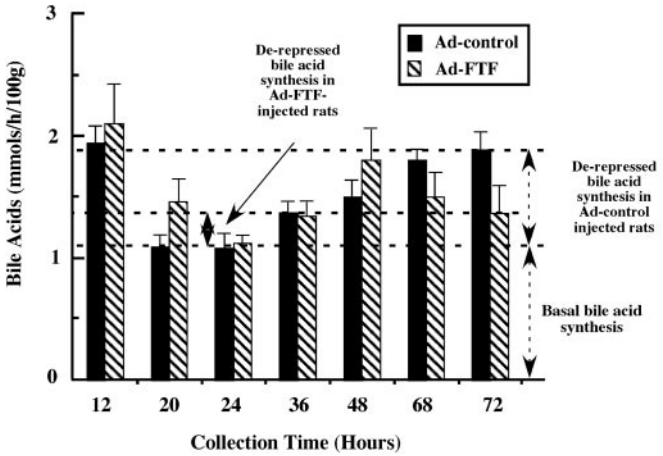

FIG. 6. **FTF overexpression suppresses bile acid synthesis in wild-type bile fistula rats.** Bile fistulation was performed in control virus (*Ad-control*)-injected (1.5×10^{11} particles, $n = 3$) and Ad-FTF-injected (1.5×10^{11} particles, $n = 3$) rats. Bile was collected at the indicated times, and bile acids were quantified.

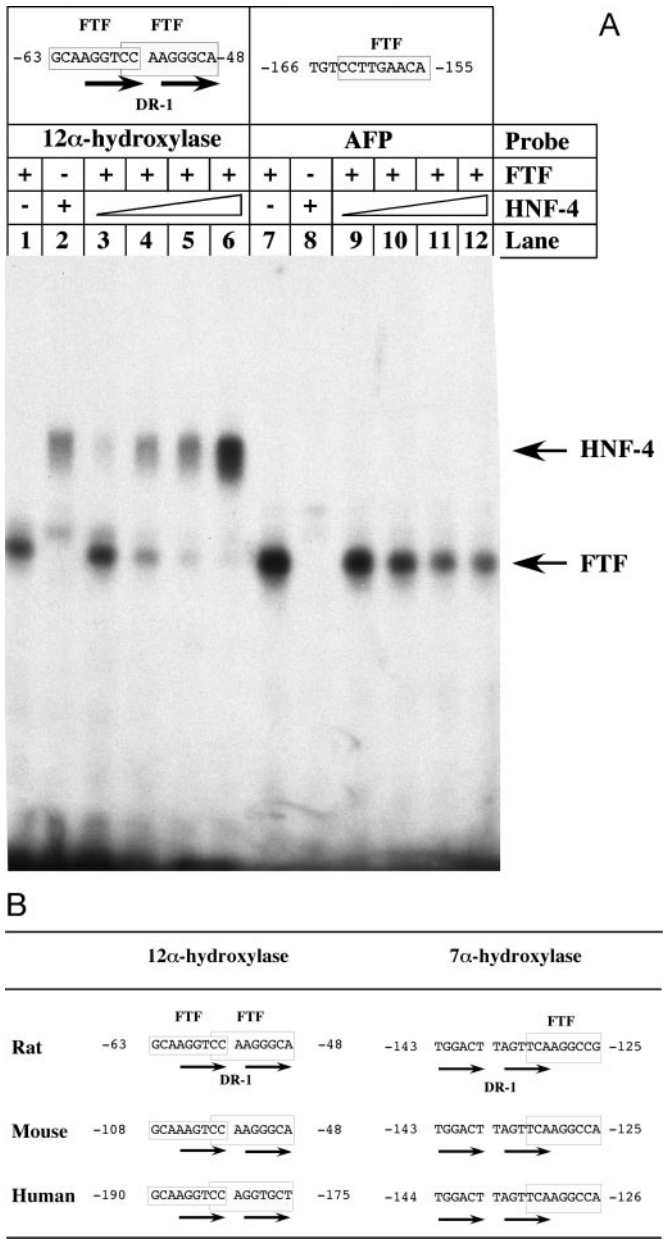

FIG. 7. **HNF-4 competes out FTF from binding to the 12α-hydroxylase promoter, but not to the AFP promoter.** *A*, gel shift experiments were performed as described under "Experimental Procedures" using FTF and HNF-4 proteins made *in vitro* and the indicated rat probes. The DNA sequences with the FTF- and HNF-4-binding motifs for the two probes are shown at the top. The proteins used were 0.25 μl of FTF (*lanes 1* and *7*) and 1 μl of HNF-4 (*lanes 2* and *8*). In *lanes 3–6* and *9–12*, a mixture of the two proteins was used, with a constant amount of FTF (0.25 μl) and increasing amounts of HNF-4 (0.25, 1, 2, and 4 μl, respectively). The concentrations of both proteins were indistinguishable based on ^{35}S incorporation into both proteins in parallel synthesis reactions performed in the presence of [^{35}S]methionine. *B*, shown are the nucleotide sequences of the FTF/HNF-4-binding sites located in the rat, mouse, and human 7α- and 12α-hydroxylase promoters. The FTF sites are *boxed*, and the HNF-4 DR-1 sites are indicated by *arrows*.

DISCUSSION

During the last few years, it has become apparent that several nuclear receptors play crucial roles in the transcription and bile acid-mediated regulation of genes involved in bile acid metabolism. Receptors such as liver X receptor, farnesoid X receptor, SHP, HNF-4, and FTF are directly implicated in the regulatory processes that control bile acid synthesis. Whereas knockout mice have revealed key aspects of these regulatory processes for the first four of these receptors, to date, there are no animal models that show the role that FTF plays *in vivo* in bile acid metabolism. All of the published data implicating FTF in this process have been obtained in tissue culture models. In this study, we used three animal models to characterize the role of FTF in bile acid biosynthesis and, in particular, in the expression of two key genes involved in this process, *i.e.* the 7α- and 12α-hydroxylase genes. All three models show the surprising result that FTF, a nuclear receptor believed to work as an activator of gene transcription, acts as a suppressor of 7α- and 12α-hydroxylase expression and bile acid biosynthesis.

Several lines of evidence support the conclusion that, in bile

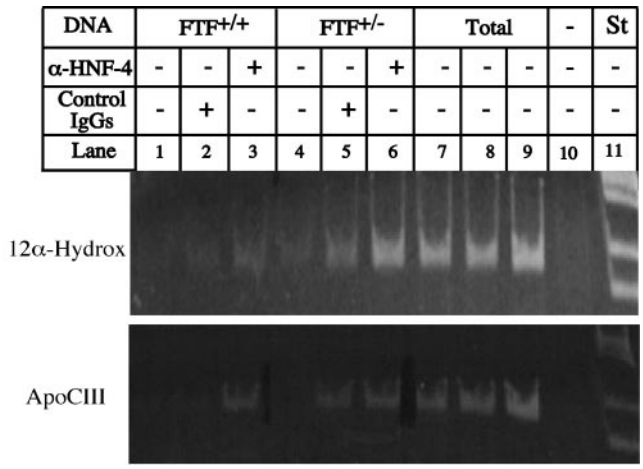

FIG. 8. **HNF-4 differentially binds to the 12α-hydroxylase promoter *in vivo*.** Liver chromatin from wild-type or $FTF^{+/-}$ mice was prepared and used in ChIP experiments as described under "Experimental Procedures." The anti-HNF-4 antibody or preimmune IgG was used where indicated (*lanes 2*, *3*, *5*, and *6*). No-antibody controls were also included (*lanes 1* and *4*). Total chromatin from both mice was used (*lanes 7* and *8*), and mouse genomic DNA (*lane 9*) was used as a positive control. A no-template negative control was also included (*lane 10*). HaeIII-cut ΦX174 DNAs were used as size markers (*lane 11*). *St*, standards; *12α-Hydrox*, 12α-hydroxylase promoter.

acid biosynthesis, FTF acts as a negative factor of 7α- and 12α-hydroxylase transcription rather than as a positive one, as was described by several laboratories, including our own, based on experiments performed in tissue culture (5, 8–12). The first line of evidence came from $FTF^{+/-}$ mice. Not only do these mice express 7α- and 12α-hydroxylases at 3–7-fold higher levels compared with wild-type mice (Fig. 2), but this higher expression is reflected in a 33% increase in gallbladder total bile acids and bile with a higher cholic acid/muricholic acid ratio, reflecting the higher 12α-hydroxylase expression (Fig. 3). Perhaps most important, this apparent higher bile acid biosynthesis activity in $FTF^{+/-}$ mice is reflected in a 13% reduction in plasma cholesterol levels. This change, albeit relatively modest, is of very high biological significance given the tight and complex regulation to which plasma cholesterol levels are subjected. As a reference, deletion of one low density lipoprotein receptor allele, a protein directly involved in plasma cholesterol clearance, results in a 33% increase in plasma cholesterol levels (37).

The second line of evidence came from a mouse model overexpressing FTF obtained by infecting mice with an adenovirus that contains the human FTF cDNA driven by the CMV promoter. This model showed the opposite phenotype to $FTF^{+/-}$ mice. Some aspects of the phenotype of these mice suggest potential mechanisms of action to explain the FTF-mediated suppression of 7α- and 12α-hydroxylases. 5-Day infection experiments showed an opposite effect of FTF overexpression on the expression of bile acid biosynthesis genes, *i.e.* 7α- and 12α-hydroxylases, compared with the expression of other genes with FTF sites in their promoters such as SHP and AFP (Fig. 4). The FTF-mediated activation of SHP expression has been proposed to be part of the mechanism of action by which bile acids down-regulate expression of the 12α-hydroxylase gene based on promoter studies in tissue culture (12). This increase in SHP expression upon FTF overexpression could explain, at least in part, the suppression of the 7α- and 12α-hydroxylase genes because SHP is believed to be a repressor of 7α- and 12α-hydroxylase transcription through its interaction with FTF (11, 12). It should be mentioned that, although SHP has been shown to be capable of heterodimerizing and suppressing HNF-4 activity (38), SHP does not suppress HNF-4-activated 12α-hydroxylase promoter activity (33). Interestingly, however, shorter infection times did not activate SHP expression, but still suppressed 7α- and 12α-hydroxylase expression (Fig. 5), although not as much as the 5-day infection (3-fold compared with 10-fold at 5 days). This lack of SHP activation in the 3-day infection experiments is important in attempting to explain the mechanism of action of FTF on 7α- and 12α-hydroxylase expression. The FTF-mediated increase in SHP expression by itself cannot explain the decrease in 7α- and 12α-hydroxylase mRNAs because SHP expression did not increase in the 3-day infection experiments, and it suggests that FTF has two effects on 7α- and 12α-hydroxylase expression, *i.e.* displacement of HNF-4 from the FTF/HNF-4 sites in the 7α- and 12α-hydroxylase promoters and an increase in SHP expression, which in turn suppress FTF activity and hence 7α- and 12α-hydroxylase transcription.

Another important clue for this mechanism of action came from the observation that the differential effect of FTF overexpression on different target genes seen at 5 days post-infection correlates with the primary structures of the FTF recognition sites on the promoters of those genes. The FTF sites present in both the 7α-hydroxylase (32) and 12α-hydroxylase (9, 10) promoters overlap (more or less) DR-1 sequences capable of binding to HNF-4. In the 12α-hydroxylase promoter, the overlap of the two sites is almost complete (Fig. 7), and the two factors cannot bind to the same molecules simultaneously. However, the other two promoters, SHP (19) and AFP (7), do not contain DR-1 sequences overlapping the FTF sites, and they do not bind HNF-4. This suggested to us that perhaps HNF-4 and FTF could displace each other, depending upon the relative abundance of the two factors and the affinities for their recognition sites, from the 12α-hydroxylase promoter and perhaps also from the 7α-hydroxylase promoter, explaining the FTF-mediated suppression of their expression in the absence of increased SHP expression. It should be pointed out that $FTF^{+/-}$ mice expressed SHP at about the same levels as wild-type mice (data not shown). The fact that HNF-4 is capable of preventing FTF from binding to the 12α-hydroxylase promoter *in vitro* supports this hypothesis (Fig. 7*A*).

It is important to note that the arrangement of overlapping FTF and HNF-4 sites in bile acid-regulated genes seems to be highly conserved (Fig. 7*B*). The FTF/HNF-4 sites within the 7α-hydroxylase promoter are almost identical in rats (32), mice (39), and humans (5); only the last nucleotide varies. In the 12α-hydroxylase promoter, the overlapping FTF and HNF-4 sites are also highly conserved, and they differ by only one nucleotide between rats (9, 10) and mice (40). The human 12α-hydroxylase promoter is less well characterized, although an overlapping FTF/HNF-4 site in the 5′-untranslated region of the gene (+200) has also been characterized (41). By sequence inspection, we have localized another FTF/HNF-4 site within the promoter region of the human 12α-hydroxylase gene (at a position similar to the other sites) that is also highly conserved in the rat and mouse sequences (Fig. 7*B*) and that binds HNF-4 *in vivo* according to ChIP experiments performed with HepG2 cells (data non shown). This overlapping FTF/HNF-4 site could provide the specificity expected so that only certain genes with FTF sites in their promoters are down-regulated by bile acids, but others such as SHP with simple FTF sites are not. The displacement mechanism suggested by this study could then play a key role in the bile acid-mediated down-regulation of gene transcription. More direct evidence for this potential displacement of FTF by HNF-4 came from *in vivo* experiments using a ChIP approach. The FTF/HNF-4 site within the 12α-hydroxylase promoter had more HNF-4 bound in $FTF^{+/-}$ mice than in wild-type mice (Fig. 8), which could explain, at least in

part, the higher expression of 12α-hydroxylase and perhaps of 7α-hydroxylase in FTF$^{+/-}$ mice. However, HNF-4 bound equally to its site within the apoC-III promoter. Final evidence should come from similar ChIP experiments performed with anti-FTF antibodies. Unfortunately, currently available antibodies are not suitable for immunoprecipitation.

The third line of evidence that FTF works as a suppressor of bile acid synthesis *in vivo* is a direct measurement of bile acid synthesis in a rat bile fistula model. Rats injected with Ad-FTF showed 50% lower bile acid synthesis after a bile fistula was performed to prevent suppression of bile acid synthesis by the returning bile acids compared with rats injected with control virus (Fig. 6). This also indicates that the acidic bile acid biosynthesis pathway does not compensate for the additional synthesis, emphasizing the key role that FTF plays in bile acid synthesis and regulation.

Interestingly, another key gene involved in cholesterol homeostasis, HMG-CoA reductase, was also induced in FTF$^{+/-}$ mice (Fig. 2). To date, no FTF sites have been localized in the reductase promoter. However, the involvement of FTF in reductase transcription suggested by this experiment could potentially explain the loss of bile acid-mediated regulation of HMG-CoA expression in the SHP-null mouse (42). This result also suggests that the effect of diminished FTF expression in the FTF$^{+/-}$ mouse on 12α-hydroxylase expression is direct rather than indirect and cannot be the result of the increased HMG-CoA reductase expression because cholesterol down-regulates the expression of the 12α-hydroxylase gene (43), in contrast to our observation (Fig. 2).

These novel findings emphasize the key role that FTF plays in bile acid synthesis *in vivo*, which has not been shown to date. They also suggest a potentially new mode of regulation of gene transcription, *i.e.* transcription factor displacement. Furthermore, FTF could play a role in the bile acid-mediated regulation of gene transcription if FTF expression, nuclear translocation, or binding activity is increased by bile acids. This potential mechanism would be, at least in part, SHP-independent. It should be pointed out that SHP-null mice still exhibit bile acid-mediated regulation of 7α- and 12α-hydroxylase expression (42, 44), and the potential alternative mechanism of bile acid-mediated regulation suggested here, displacement of HNF-4 by FTF, could be part of the SHP-independent mode of regulation. Interestingly, bile acids induce FTF expression in human enterocytes (17), and feeding rats chenodeoxycholic acid induces FTF expression in the liver (45). To date, however, we have been unable to show any alteration in FTF expression in mouse liver or DNA binding activity upon cholic acid feeding, the major primary bile acid in both humans and rodents, or upon cholestyramine feeding, a bile acid sequestrant. In summary, we propose that FTF has two synergetic effects on bile acid biosynthesis: 1) it occupies its recognition site within the 7α- and 12α-hydroxylase promoters, which is otherwise occupied by HNF-4; and 2) it activates SHP expression (12, 19), and then there is enough SHP to heterodimerize and suppress FTF, resulting in lower 7α- and 12α-hydroxylase transcription.

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