may stimulate microtubule assembly through RanBPM as well as cytosolic factors such as γTuRC and XMAP215. Furthermore, Ran-GTP could act later to initiate or stabilize spindle formation. The Ran-GTPase–activating protein, RanGAP1, is localized to spindles throughout mitosis (*28*). Tethering RanGAP1 to the spindle could locally activate Ran-GTP hydrolysis to regulate spindle microtubules.

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Identification of a Nuclear Receptor for Bile Acids

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Bile acids are essential for the solubilization and transport of dietary lipids and are the major products of cholesterol catabolism. Results presented here show that bile acids are physiological ligands for the farnesoid X receptor (FXR), an orphan nuclear receptor. When bound to bile acids, FXR repressed transcription of the gene encoding cholesterol 7α-hydroxylase, which is the rate-limiting enzyme in bile acid synthesis, and activated the gene encoding intestinal bile acid–binding protein, which is a candidate bile acid transporter. These results demonstrate a mechanism by which bile acids transcriptionally regulate their biosynthesis and enterohepatic transport.

The enzymatic conversion of cholesterol to bile acids is regulated through feed-forward activation by oxysterols and feedback repression by bile acids (*1*, *2*). Because the feed-forward pathway is mediated by the liver X receptor (LXRα), a nuclear receptor that binds oxysterols (*3*), we speculated that the feedback repression pathway may also be regulated by a nuclear receptor that can bind to bile acids. The orphan receptor FXR (also called RIP14) is an ideal candidate for the bile acid receptor, because it is specifically expressed in tissues where bile acids function (such as the liver, intestine, and kidney); it is evolutionarily related to LXRα; and, like other class II nuclear receptors, it functions as a heterodimer with the retinoid X receptor (RXR) (*4*–*6*). In addition, several isoprenoid lipids can weakly activate FXR at supraphysiological concentrations; however, these compounds do not activate all species of FXR and do not bind as ligands (*4*, *7*). Thus, the identity and physiologic function of FXR ligands have remained unknown.

To test the hypothesis that FXR is the bile acid receptor, murine or human FXR expression plasmids were transfected into monkey kidney CV-1 cells or human hepatoma HepG2 cells. Cells were then treated with a series of bile acid metabolites and screened for the expression of a luciferase reporter gene. The reporter construct contained multiple copies of an inverted repeat response element (IR-1) that binds to FXR-RXR heterodimers (*4*). FXR was strongly activated by bile acids that can regulate gene expression in vivo (Fig. 1A) (*8*, *9*). The activation of FXR was specific and limited to the primary bile acid chenodeoxycholic acid (CDCA) and to a much lesser extent to the secondary bile acids deoxycholic acid (DCA) and lithocholic acid (LCA) (Fig. 1, A through C). Other closely related compounds such as cholesterol, oxysterols, steroid hormones, and other bile acid metabolites were inactive in this assay at concentrations up to 100 μM (Fig. 1) (*10*). The most potent activator, CDCA, had a half-maximal effective concentration (EC_{50}) of 50 μM and 10 μM on murine and human FXR, respectively (Fig. 1B). These concentrations are well within the physiologic intracellular range reported in vivo (*11*). In addition, FXR was the only protein activated by bile acids (Fig. 1C). These results suggest that bile acids are the physiologic ligands of FXR.

After binding to ligand, nuclear receptors undergo a conformational change that increases their affinity for coactivator proteins such as SRC-1, a key step in the assembly of an active transcription complex (*12*). To further demonstrate that bile acids are physiologic ligands for FXR, a mammalian two-hybrid assay (*13*) was used to probe for a functional interaction in vivo between the FXR ligand-binding domain (LBD) and SRC-1. In transfected HEK-293 kidney cells, CDCA treatment promoted the association of FXR LBD and SRC-1 as expected (Fig. 1D). The EC_{50} of this response (20 μM) is similar to that for CDCA-induced transactivation of FXR (Fig. 1B).

The receptor-interacting domain of the co-

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activator SRC-1 has been mapped to a short motif with the amino acid sequence LXXLL, where L is leucine and X is any amino acid. Fragments of SRC-1 or short synthetic peptides containing one LXXLL motif or more bind nuclear receptors in a ligand-dependent manner (*14*). Hence, the association of LXXLL-containing peptides with nuclear receptors can be used to obtain a quantitative measurement of ligand-receptor binding in a simplified biochemical assay. Two experiments were performed to demonstrate that CDCA directly interacts with FXR. In the first experiment, a fluorescence resonance energy transfer (FRET) assay (*15*) measured interactions between the FXR LBD and a fragment of SRC-1 (amino acids 595 through 822) containing three LXXLL motifs (Fig. 1E). In the second experiment, we used an enzyme-linked immunosorbent assay (ELISA) (*16*) to measure the interaction between the FXR LBD and a biotin-labeled peptide containing an LXXLL motif (Fig. 1F). CDCA stimulated binding in both in vitro assays, yielding EC_{50}'s of 10 to 20 μM that correlated well with results from the mammalian two-hybrid (Fig. 1D) and transactivation (Fig. 1B) assays. Derivatives of CDCA conjugated with glycine (GCDCA) or taurine (TCDCA) were also active in both biochemical assays (Fig. 1, E and F) but were inactive in the cell-based assays (Fig. 1, A, B, and D). The

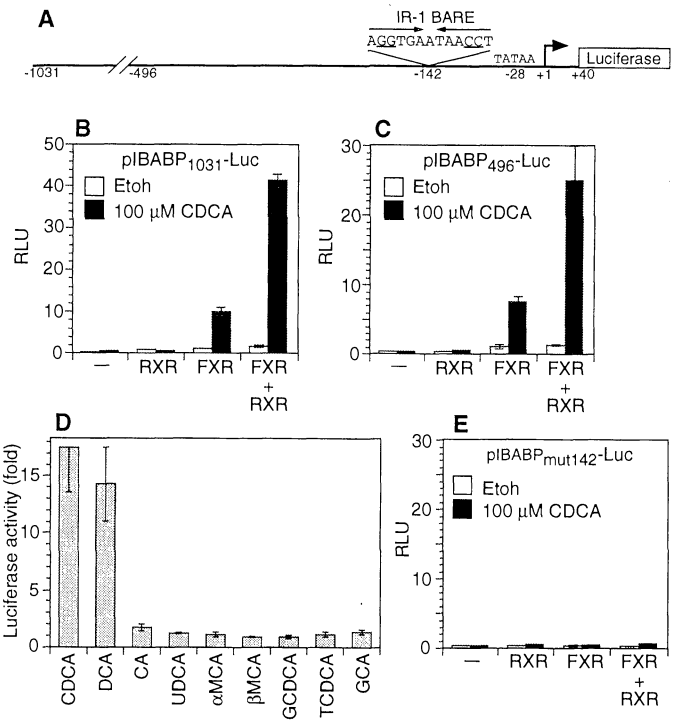
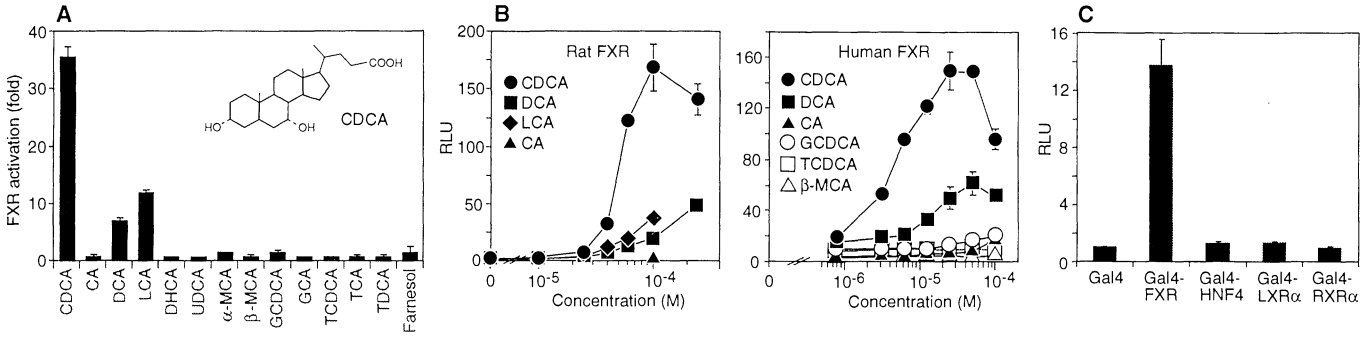


Fig. 2. The I-BABP promoter is activated by FXR and bile acids (*26*). (**A**) Schematic map of the luciferase reporter gene containing the mouse I-BABP promoter used in these studies. The IR-1 sequence that functions as a bile acid response element (BARE) is shown. Cotransfection assays in CV-1 cells demonstrate that the FXR-RXR heterodimer is required for bile acid transactivation of a luciferase reporter containing either 1000 bp (**B**) or 500 bp (**C**) of upstream I-BABP promoter sequence. (**D**) Specificity of bile acids that activate the I-BABP promoter. CV-1 cells were cotransfected with a FXR and RXR expression plasmids and the $pIBABP_{496}$-Luc reporter and treated with 100 μM concentrations of the indicated bile acids. (**E**) Mutation of the IR-1 motif in the I-BABP promoter eliminates activation by FXR and bile acid. The nucleotides in the IR-1 that are mutated are underlined in (A). Etoh, ethanol solvent control.



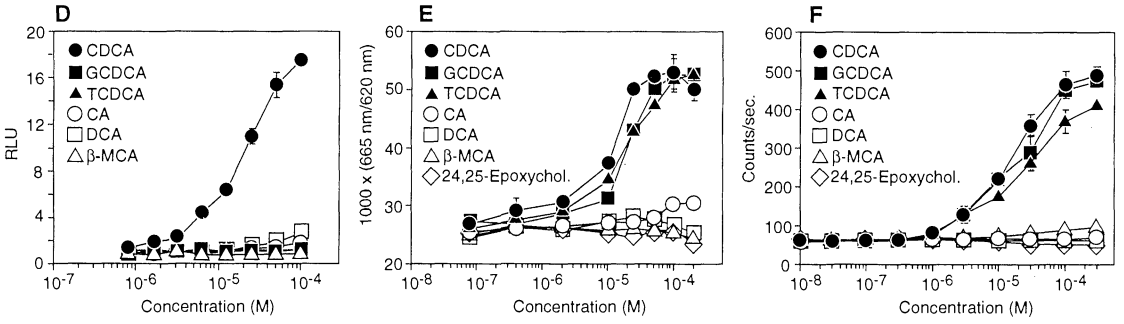


Fig. 1. Bile acids are physiologic ligands for FXR. (**A**) Specificity of bile acids that transactivate FXR. CV-1 cells were cotransfected with a rat FXR expression plasmid and a luciferase reporter gene, treated with 50 μM concentrations of the indicated compounds, and assayed as described (*23*). CDCA, chenodeoxycholic acid; CA, cholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; DHCA, dehydrocholic acid; UDCA, ursodeoxycholic acid; α- and β-MCA; α– and β–muricholic acid; GCDCA, glycochenodeoxycholic acid; GCA, glycocholic acid; TCDCA, taurochenodeoxycholic acid; TCA, taurocholic acid; TDCA, taurodeoxycholic acid. The inset shows the structure of CDCA. (**B**) Bile acid dose response for activation of a reporter gene by rat FXR in CV-1 cells (left panel) or human FXR in HepG2 cells (right panel) (*23*). RLU, relative light units. (**C**) CDCA (50 μM) specifically activates FXR but not other nuclear receptors (HNF4, hepatocyte nuclear factor 4). HepG2 cells were cotransfected as in (B) with Gal4-receptor chimeras in which the DNA binding domain of yeast Gal4 was fused in frame to the ligand binding domain of the indicated nuclear receptor (*23*). (**D**) Bile acids promote association of FXR and SRC-1 in vivo (*24*). (**E** and **F**) Bile acids directly bind to FXR in vitro as analyzed by two coactivator/receptor interaction assays (*25*). 24,25-epoxycholesterol. In (E), FRET measured the ligand-induced binding of europium-labeled FXR to an APC-labeled fragment of SRC-1. In (F), an ELISA measured ligand-induced association of FXR with a biotin-labeled LXXLL peptide.

ability of conjugated bile acids to bind FXR in vitro but not to transactivate FXR in cells is presumably due to the absence in these cells of the ileal bile acid transporter (I-BAT), which is required for conjugated bile acid uptake (*17*). The accompanying paper by Parks *et al.* (*18*) confirms this notion by demonstrating that conjugated bile acids become efficient FXR activators in cells expressing I-BAT. Neither CDCA nor other tested bile acids induced interaction of SRC-1 or LXXLL peptides with other nuclear receptors (such as LXRα, peroxisome proliferator-activated receptor-γ, or RXRα), and ligands for these other receptors did not promote the association of FXR with SRC-1 or LXXLL peptides (*19*). Together, these data provide convincing evidence that bile acids bind to FXR as ligands.

The return of bile acids to the liver is thought to be facilitated by specific binding proteins that transport bile acids across intestinal enterocytes. One of these proteins is the cytosolic intestinal bile acid–binding protein (I-BABP) (*8*). Expression of the gene encoding I-BABP increases in response to bile acids (*20*). To determine whether this response is induced by FXR acting as a bile acid receptor, approximately 1 kb of the 5′-flanking promoter region of the mouse I-BABP gene was linked to a luciferase reporter gene (pIBABP$_{1031}$-Luc) and tested for FXR-specific activation in a cotransfection assay. This portion of the I-BABP promoter is sufficient for proper in vivo expression of I-BABP (*21*) and contains an inverted repeat sequence that may function as an FXR-specific bile acid response element (Fig. 2A). CDCA induced expression of the I-BABP–Luc reporter gene 12-fold in the presence of FXR and 29-fold when FXR was coexpressed with an increased amount of its heterodimeric partner RXR. Truncation of the I-BABP promoter to within 500 base pairs (bp) of the start site (pIBABP$_{496}$-Luc) resulted in a similar CDCA-induced response (Fig. 2C). The rank order of potency of the various bile acids for FXR-specific induction of I-BABP reporter gene expression (Fig. 2D) was identical to that demonstrated in vivo (*8*). Finally, mutation of the IR-1 in the I-BABP promoter completely eliminated activation by CDCA (Fig. 2E). This IR-1 sequence alone drove CDCA- and FXR-dependent activation (*10*).

In addition to regulating their own transport, bile acids also regulate their synthesis by feedback that represses the expression of cholesterol 7α-hydroxylase (Cyp7a), the rate-limiting enzyme (*2*). In keeping with the notion that this regulation is also mediated by FXR, the rank order of bile acids that repress endogenous expression of human Cyp7a protein (Fig. 3A) and mRNA (Fig. 3B) in a hepatocyte-derived cell line was identical to that for binding and activating FXR. In addition, significant repression of a luciferase reporter containing the Cyp7a gene promoter occurred only when FXR, but not other nuclear receptors, was expressed (Fig. 3C). FXR-mediated repression of the Cyp7a promoter by CDCA was dose-dependent and had an EC_{50} of 20 μM (Fig. 3D). Feedback regulation of bile acid synthesis is conserved between human and murine species, and CDCA-mediated repression of the rat Cyp7a promoter also required FXR (*10*).

The above results show that bile acid regulation of I-BABP and Cyp7a gene expression depends on the presence of FXR in a manner consistent with FXR's role as a bile acid receptor. That FXR functions as a bile acid receptor suggests an expanded model for the regulation of cholesterol homeostasis by nuclear receptors (Fig. 4). The accumulation of dietary cholesterol in the liver results in the synthesis of oxysterols that bind to LXRα. LXRα activation in turn enhances the catabolic clearance of cholesterol by increasing bile acid synthesis and excretion. As demonstrated in this work, elevated bile acids regulate their synthesis and transport through their nuclear receptor FXR. Thus, bile acids are able to efficiently repress their further synthesis in the liver and increase the synthesis of transport proteins in the intestine. The discovery of oxysterol and bile acid signaling pathways mediated by nuclear receptors complements the extensive pioneering studies that have defined the intricate feedback and feedforward control of sterol metabolism (*22*). Furthermore, the observation that LXRα and FXR function as key regulators of cholesterol and bile acid homeostasis has important therapeutic implications for the discovery of drugs targeted against these receptors.

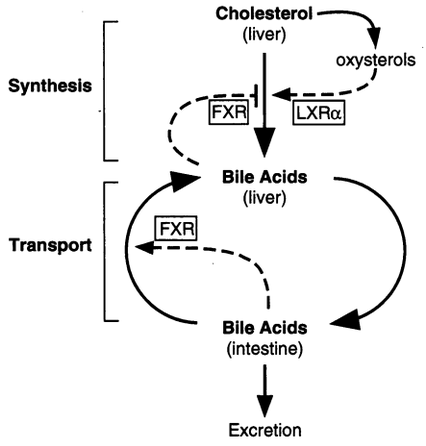
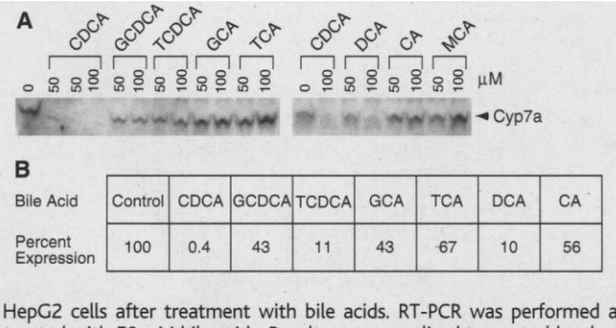


Fig. 4. Model of the transcriptional control of cholesterol and bile acid homeostasis by nuclear receptors.



B

Bile Acid	Control	CDCA	GCDCA	TCDCA	GCA	TCA	DCA	CA
Percent Expression	100	0.4	43	11	43	67	10	56

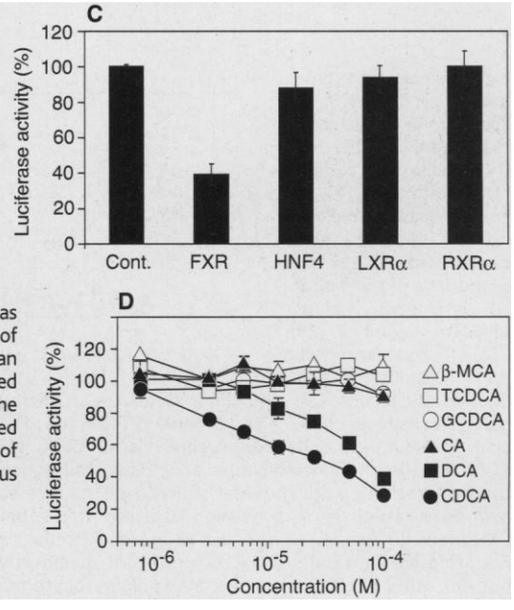


Fig. 3. The Cyp7a promoter is repressed by bile acids and FXR. (**A**) Immunoblot analysis and (**B**) quantitative RT-PCR demonstrate that endogenous human Cyp7a protein and mRNA synthesis are repressed by bile acids. Immunoblots using an antibody to Cyp7a were performed on lysates from human HepG2 cells after treatment with bile acids. RT-PCR was performed as described (*27*) from cells treated with 50 μM bile acids. Results are normalized to control levels of glyceraldehyde phosphate dehydrogenase mRNA. (**C**) CDCA-mediated suppression of human Cyp7a requires FXR. HepG2 cells were cotransfected with expression plasmids for the indicated nuclear receptors and a luciferase reporter plasmid containing nucleotides −716 to +14 of the human Cyp7a promoter (pGL3-CYP7A). Cells were then treated with 10 μM CDCA and analyzed for luciferase activity relative to methanol solvent control (cont.). (**D**) Dose-dependent effects of bile acids on FXR suppression of Cyp7a. Cells were transfected as in (C) and treated with various concentrations of bile acids.

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23. CV-1 cells were cotransfected with a rat FXR expression plasmid, a luciferase reporter construct containing five copies of an IR-1 response element, and a β-galactosidase (β-Gal) expression vector as a marker as described (*4*, *13*). Transfected cells were treated with various compounds (Sigma) for 36 hours and then harvested for luciferase assay. For HepG2 cells cotransfected with human FXR, the luciferase reporter plasmid contained three copies of the IR-1 (AGGTCAATGACCT), and cells were treated for 20 hours with compounds before being harvested. Cotransfections with Gal4-receptor chimeras included a luciferase reporter gene (G5-Luc) containing five copies of the Gal4 DNA binding site. Transfection data were normalized to β-Gal, are expressed relative to ethanol solvent controls as fold induction or relative light units (RLUs), and represent triplicate assays ± SD.
24. HEK-293 cells were transfected with plasmids expressing the chimeric proteins Gal4–SRC-1 (amino acids 583 through 783), FXR (amino acids 105 through 472)–VP16, and the G5-Luc reporter. Luciferase activity was measured as in (*23*).
25. The FXR LBD (amino acids 105 through 472) was fused to the COOH-terminus of glutathione S-transferase (GST), and the resultant GST-FXR protein was expressed in *Escherichia coli* and then purified on glutathione beads. For the FRET assay, a europium-labeled antibody to GST [anti-GST–(Eu)] (Wallac, Gaithersburg, MD) was used to tag GST-FXR. SRC-1 (amino acids 595 through 822) was tagged with hexahistidine, expressed in *E. coli*, purified by metal ion chromatography, biotinylated, and labeled with fluorophore allophycocyanin (APC) (Wallac) conjugated to streptavidin. FRET occurs in solution when ligand-mediated changes in the conformation of FXR increase its affinity for SRC-1, resulting in energy transfer from europium (337 nm excitation and 620 nm emission) to APC (620 nm excitation and 665 nm emission). Results are expressed as a ratio of APC to europium fluorescence (665 nm/620 nm). To each well of a black polypropylene 96-well plate was added 10 nM GST-FXR, 100 nM biotin–SRC-1, anti-GST–(Eu) (0.2 μg/ml), APC-streptavidin (1 μg/ml), and the indicated compound in 100 μl of buffer [100 mM Hepes (pH 7.6), 0.125% CHAPS, and 125 mM NaF]. The reaction was mixed and incubated for 12 hours at 4°C, and fluorescence was measured on a Victor II plate reader (Wallac). For ELISA, 1.5 μM biotin-labeled peptide (amino acid sequence Ile-Leu-Arg-Lys-Leu-Leu-Gln-Glu) was incubated with 100 nM GST-FXR and the indicated compound in 100 μl of buffer [25 mM tris-HCl (pH 7.4) and 150 mM NaCl] in a 96-well plate for 1 hour. The plate was washed and incubated with rabbit antibody to GST, and GST-FXR protein bound to streptavidin was quantitated with a horseradish peroxidase-labeled antibody to rabbit.
26. CV-1 cells were cotransfected as in Fig. 1 with rat FXR and RXRα expression plasmids and with the indicated luciferase reporter genes. To create the reporter genes, the first 1031 bp (pIBABP$_{1031}$-Luc) or 496 bp (pIBABP$_{496}$-Luc) of the mouse I-BABP gene promoter (*21*) were amplified by polymerase chain reaction (PCR) from mouse genomic DNA and ligated into a Luc reporter plasmid (*13*). The mutant reporter (pIBABP$_{mut\text{-}142}$-Luc) was made from the pIBABP$_{496}$-Luc reporter by site-directed mutagenesis within the I-BABP promoter sequence −142 to −130 (Fig. 2A), which converts nucleotides AGGTGAATAACCT to ACCTGAATAAGGT.
27. Human Cyp7a mRNA was quantitated from HepG2 cells that were treated with the indicated compounds using a TaqMan One Step Gold reverse transcriptase (RT) PCR kit (Applied Biosystems/Perkin Elmer). The Cyp7a primers used were CYP7-78: 5′-TGATTTGGGGGATTGCTATA; CYP7-178: 5′-CATACCTGGGCTGTGCTCT; and CYP7-132(FAM): 5′- (6-FAM) TGGTTCACCCGTTTGCCTTCTCCT (TAMRA). Analysis was performed in triplicate parallel assays.

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Bile Acids: Natural Ligands for an Orphan Nuclear Receptor

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Bile acids regulate the transcription of genes that control cholesterol homeostasis through molecular mechanisms that are poorly understood. Physiological concentrations of free and conjugated chenodeoxycholic acid, lithocholic acid, and deoxycholic acid activated the farnesoid X receptor (FXR; NR1H4), an orphan nuclear receptor. As ligands, these bile acids and their conjugates modulated interaction of FXR with a peptide derived from steroid receptor coactivator 1. These results provide evidence for a nuclear bile acid signaling pathway that may regulate cholesterol homeostasis.

Cholesterol homeostasis is achieved through the coordinate regulation of dietary cholesterol uptake, endogenous biosynthesis, and the disposal of cholesterol in the form of bile acids. Bile acids are not simply metabolic by-products, but are essential for appropriate absorption of dietary lipids and also regulate gene transcription. Among the genes regulated by bile acids are cholesterol 7α-hydroxylase (Cyp7a), the rate-limiting enzyme in bile acid biosynthesis (*1*), and the intestinal bile acid–binding protein (I-BABP), a cytosolic protein that serves as a component of the bile acid transport system in the ileal enterocyte (*2*). I-BABP gene expression is induced preferentially by chenodeoxycholic acid (CDCA) relative to other more hydrophilic bile acids (*3*).

To examine whether CDCA mediates its transcriptional effects through an orphan member of the steroid-retinoid-thyroid hormone receptor family (*4*), we used a chimeric receptor system in which the putative ligand-binding domain (LBD) of the human orphan receptor is fused to the DNA binding domain of the yeast transcription factor GAL4 (*5*). In CV-1 cells, CDCA selectively activated FXR [NR1H4] (Fig. 1), an orphan nuclear receptor expressed predominantly in the liver, kidney, intestine, and adrenals (*6*, *7*). This strong activation by CDCA was unanticipated because FXR responds to high concentrations of farnesoids (*6*) and retinoids (*8*).

To further investigate the structure-activ-

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