Cholesterol conversion to bile acids occurs via the “classic” (neutral) or the “alternative” (acidic) bile acid biosynthesis pathways. Sterol 12α-hydroxylase/CYP8b1 is the specific enzyme required for cholic acid synthesis. The levels of this enzyme determine the ratio of cholic acid to chenodeoxycholic acid and thus the hydrophobicity of the circulating bile acid pool. Expression of the 12α-hydroxylase gene is tightly down-regulated by hydrophobic bile acids. In this study, we report the characterization of two DNA elements that are required for both the 12α-hydroxylase promoter activity and bile acid-mediated regulation. Mutation of these elements suppresses 12α-hydroxylase promoter activity. Mutations of any other part of the promoter do not alter substantially the promoter activity or alter regulation by bile acids relative to the wild type promoter. These two DNA elements bind α1-fetoprotein transcription factor (FTF), a member of the nuclear receptor family. We also show that overexpression of FTF in a non-liver cell line activates the sterol 12α-hydroxylase promoter. These studies demonstrate the crucial role of FTF for the expression and regulation of a critical gene in the bile acid biosynthetic pathways.

Proper control of intracellular and circulating cholesterol levels is essential for maintaining cholesterol homeostasis, since metabolic disarrangement can lead to many degenerative conditions with a probable genetic component, such as atherosclerosis, cholestasis, and cholesterol gallstone disease. Because nearly 50% of the body cholesterol is catabolized to bile acids, this pathway plays an important role in the cholesterol homeostasis of mammals. Current evidence suggests a decreased bile acid output as the major contributing factor in the metabolic disarray of the circulating bile acid pool. This leads to an abnormal ratio of cholesterol to bile acids and lecithin, which is a major risk factor for cholesterol gallstone formation (1).

Cholesterol conversion to bile acids occurs via the “classic” (neutral) or the “alternative” (acidic) bile acid biosynthesis pathways (2). Cholic acid and chenodeoxycholic acid (CDCA) are the end products of these pathways (Fig. 1) and the major primary bile acids found in most vertebrates. Cholic acid is hydroxylated at position 12α, whereas CDCA is not. There are three enzymes that play major regulatory roles in these two pathways. Cholesterol 7α-hydroxylase/CYP7a1 (7α-hydroxylase) is the rate-limiting enzyme in the classic pathway. Sterol 27-hydroxylase/CYP27 is the first enzyme in the alternative pathway. Sterol 12α-hydroxylase/CYP8b1 (12α-hydroxylase) is the specific enzyme for cholic acid synthesis and determines the ratio of cholic acid to chenodeoxycholic acid and thus the hydrophobicity of the circulating pool.

The altered ratio of cholic to CDCA has been postulated to play a role in cholesterol gallstone formation (3). Suppression of 12α-hydroxylase by specific inhibitors has been suggested as a possible therapeutic strategy for dissolution of cholesterol gallstones (3). Because chenodeoxycholic acid is a more potent suppressor of HMG-CoA reductase and 7α-hydroxylase than cholic acid (3, 4), the relative activity of 12α-hydroxylase may play an important role in the regulation of hepatic cholesterol homeostasis. The alteration of cholic/CDCA ratio affects biliary cholesterol and phospholipid secretion, thus altering intestinal cholesterol absorption and receptor-mediated lipoprotein uptake by the hepatocyte (4).

It is well documented that bile acids exert negative feedback regulation on their own synthesis (5). Interruption of the enteroheptic circulation, by biliary diversion or by feeding bile acid-binding resins (cholestyramine), enhances cholesterol and bile acid synthesis (6). Conversely, feeding bile acids suppresses bile acid and cholesterol synthesis (7). Bile acids negatively regulate the transcription of the 7α-hydroxylase gene, which controls output from the classic pathway (8). Two bile acid response elements have been localized within the 5′-flanking region of the rat gene (9, 10), but the factors that mediate regulation have not been characterized. It remains to be demonstrated whether these elements are indeed involved in this regulation. Similarly, bile acids also down-regulate transcription of the sterol 27-hydroxylase gene (11), and it has been reported that hepatocyte nuclear factor 1 (HNF-1) is involved in this regulation (12). However, the molecular mechanisms involved in that regulation are not well defined.

More recently, it has been shown that a transcriptional fac-
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**CHOLESTEROL**

**27-HYDROXY CHOLESTEROL**

**CLASSIC OR NEUTRAL PATHWAY**

7α-HYDROXY CHOLESTEROL

**Sterol 12α-hydroxylase**

**ALTERNATIVE OR ACIDIC PATHWAY**

**CHENODEOXYCHOLIC ACID**

**CHOLIC ACID**

**FIG. 1. Bile acid biosynthetic pathways.** A highly schematic depiction of the bile acid metabolic pathways is shown, and the key enzymes for both pathways are noted. Conversion of cholesterol to 7α-hydroxycholesterol by 7α-hydroxylase is the initial and rate-limiting step in the classic pathway. Hydroxylation at position 27 is the initial step of the acidic pathway. Cholic acid and chenodeoxycholic acid are the major products of these pathways and are commonly referred to as the primary bile acids.

ator, named the CYP7A promoter-binding factor (CPF), is required for the expression of the 7α-hydroxylase gene (13). This factor is a member of the Ftz-F1 family of the class IV orphan nuclear receptor superfamly (14). CPF binds to the region of the 7α-hydroxylase promoter previously characterized as a bile acid response element (10), which suggests that CPF might play a role in the bile acid-mediated down-regulation of 7α-hydroxylase transcription.

The cDNAs and genes encoding the rabbit, rat, and human 12α-hydroxylase enzyme have been cloned (15–17), and studies of the molecular basis of its regulation are now feasible. It is expressed exclusively in the liver, since it corresponds to a critical role of FTF for 12α-hydroxylase promoter activity. These studies demonstrate the crucial role of FTF in the bile acid biosynthetic pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—Reagents used in DNA cloning and sequencing were from New England Biolabs, Roche Molecular Biochemicals, U.S. Biochemical Corp., or Life Technologies, Inc. Common laboratory chemicals were from Fisher, Sigma, or Bio-Rad. The luciferase promoterless vector, pGL3-Basic, was purchased from Promega. Oligonucleotides were prepared in the Medical College of Virginia DNA Synthesis Facility by the phosphoramidite method on an automated DNA synthesizer. pCI-FTF, an expression plasmid that contains the human FTF cDNA in the expression vector pCI (Promega), was a generous gift from Dr. Belanger (19). pCMX, a plasmid for expression in mammalian cells and in vitro, contains the cytomegalovirus and the T7 promoters and was a gift from Dr. Ronald M. Evans. Anti-FTF antibodies were a gift from Dr. David W. Russell and were raised against a peptide corresponding to amino acids 180–1197 of the DNA binding domain.

**General Methods**—Standard recombinant DNA procedures were carried out essentially as described (8). DNA sequencing was done by the dideoxy chain termination method using DNA fragments subcloned into M13 vectors or with double-stranded clones and the universal primer or sequence-specific primers with reagents from U.S. Biochemical Corp. Genomic Cloning and Characterization—A rat genomic library was obtained from CLONTECH. The library was plated and screened with a double-stranded probe made by reverse transcriptase-polymerase chain reaction and contained a 200-nt fragment from the 5′-end of the rabbit 12α-hydroxylase cDNA. The polymerase chain reaction primers were synthesized based on published DNA sequence (22). DNA sequencing was done by the dideoxy chain termination method using DNA fragments subcloned into M13 vectors or with double-stranded clones and the universal primer or sequence-specific primers with reagents from U.S. Biochemical Corp.

**Preparation of Chimeric CYP3A10 Promoter/Luciferase Reporter Constructs**—PGL3-R12α-865 was prepared by placing a 903-nucleotide SacI–SacI fragment containing nucleotides +865 to +37 into the SacI site of pGL3-Basic (Promega). The three 5′-deletion constructs PGL3-R12α-289, PGL3-R12α-163, and PGL3-R12α-106 were prepared by polymerase chain reaction using a specific 5′ primer and a common 3′ primer corresponding to nucleotides 20–37. Mutation constructs were generated by oligonucleotide-directed mutagenesis in M13 (23) or by using the QuikChange site-directed mutagenesis kit (Stratagene). All

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*J. D. Tugwood, I. Issemann, and S. Green, GenBank™ accession number M81385.*
constructs were confirmed by DNA sequencing. 

**Transient Transfection and Luciferase Assays—**HepG2 and CV-1 cells were obtained from American Type Culture Collection. Both cell types were transfected with Lipofectin (Life Technologies, Inc.), using 1.5 μg of total DNA in 35-mm plates. HepG2 were transfected with 25 ng of test plasmid and 5 ng of pCMV-Gal (a plasmid containing the human cytomegalovirus promoter in from of the bacteria galactosidase gene) to normalize for transfection efficiencies. CV-1 cells were transfected with 200 ng of test plasmid, 50 ng of pCMV-Gal, and the indicated amounts of pCI-FTF, an expression plasmid that contains the rat FTF (19). Transcription/translation of cDNAs encoding FTF or of the growth hormone receptor (GHR) as a control was performed using the TNT T7-coupled rabbit reticulocyte lysate system according to the manufacturer’s protocol. Average values are for the number of experiments indicated.

*In Vitro Transcription/Translation and HepG2 Nuclear Extracts—*Transcription/translation of cDNAs encoding FTF or of the growth hormone receptor (GHR) as a control was performed using the TNT T7-coupled rabbit reticulocyte lysate system according to the manufacturer’s protocol (Promega). HepG2 nuclear extracts were prepared as indicated (24).

**Electrophoretic Mobility Shift Analysis—**DNA binding reactions were set up in 50 mM KCl, 20 mM Tris-HCl (pH 8.0), 0.2 mM EDTA, 4% Ficoll, 1.0 μg of poly (dI–dC), 4 μl of the translated protein, and a 1500-fold molar excess of an irrelevant single-stranded DNA, in a final volume of 20 μl on ice. After a 15-min incubation, 320 fmol of the indicated 32P-labeled DNA probes (~2 × 10^6 cpm) were added. All probes were adjusted to the same specific radioactivity. After incubation for 20 min on ice, samples were loaded onto a 4.5% polyacrylamide gel and subjected to electrophoresis at 4 °C. Gels were dried and exposed to XAR-5 (Eastman Kodak Co.). For supershift experiments, 1 μl of the crude serum was used.

**RESULTS**

The DNA sequence of the 5′-flanking region of the 12α-hydroxylase gene is shown in Fig. 2. The transcriptional initiation site was located by primer extension techniques (data not shown) and is numbered +1. This sequence contains a TATA box-like element, two consensus binding sites for HNF-3, a liver-specific DNA-binding protein (16), and two sterol regulatory elements (SREs) that are implicated in cholesterol-mediated regulation of the transcription of several genes (17). One stretch of DNA located between nucleotides –63 and –48 contains two potential sites for the liver-specific nuclear receptor FTF (19).

To functionally characterize the 12α-hydroxylase promoter, we prepared a chimeric gene (pGL3-R12α-865, as shown in Fig. 3) by fusing the SceI–SceI fragment (Fig. 2), which contains 865 base pairs of the 5′-flanking region and 33 base pairs of 5′-untranslated region, to the coding region of the luciferase gene. We then used HepG2 cells as recipient cells to transfect this chimeric gene. After transfection, cells were treated with or without 100 μM CDCA, which was shown to suppress expression of the endogenous gene in primary hepatocytes (data not shown), mimicking the bile acid-mediated regulation that has been described in vivo (18). Cells were harvested for luciferase and galactosidase activities as explained under “Experimental Procedures.” As shown in Fig. 3, pGL3-R12α-865 had promoter activity (100-fold above background levels) that was regulated approximately 5-fold upon the addition of CDCA.

To narrow down the promoter region involved in both transcriptional activity and bile acid-mediated regulation, we made the three 5′-deletion constructs shown in Fig. 3. All three constructs showed regulated activity, indicating that all of the DNA elements required for both promoter activity and bile acid-mediated regulation are located in the first 106 nucleotides of the 12α-hydroxylase promoter.

To further characterize the 12α-hydroxylase promoter, we mutated the first 106 nucleotides in blocks of approximately 20 nucleotides each as shown in Fig. 2 (brackets A–G). The results of these experiments are shown in Fig. 4A, and the actual mutations introduced in mutants D, E, and F are shown in Fig. 4B. All mutants exhibited promoter activity and bile acid-mediated regulation, except when nucleotides –62 to –49 were mutated. This region contains two putative elements with homology to the rat FTF site, a member of the *Drosophila* orphan transcription factor family.
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**Fig. 3. Expression and bile acid-mediated regulation of 5'-deleted 12α-hydroxylase promoter/luciferase constructs in HepG2 cells.**
The DNA fragment from the 5’-flanking region of the rat CYPb1 gene is indicated by a thin line with the Xho I site as a reference point. The luciferase coding sequence is denoted by the hatched bar, and the TATA-like box is indicated. A scheme of the different 5’ deletion constructs used is shown. HepG2 cells were transfected with the indicated construct and treated with chenodeoxycholic acid as described under “Experimental Procedures.” Relative transcription was determined by normalizing luciferase activity to β-galactosidase activity. The data were normalized to the activity produced by the construct containing the longest promoter fragment, pGL3-R12α-865, in cells grown in the absence of CDCA and represent the averages of n experiments ± S.D. bp, base pairs.

receptor fushi tarazu F1 (Ftz-F1) (25), and we named them FTF sites. These elements are also homologous to the recently described CPF site for the 7α-hydroxylase gene (26), another nuclear receptor of the same family. These homologies are shown in Fig. 5.

Based on these homologies, we hypothesized that there are two FTF elements within the 12α-hydroxylase promoter located at positions −63 to −55 and −56 to −48. The data in Fig. 4A show that both mutants D and F, which have the two potential FTF sites independently mutated, and mutant E, which has both sites mutated, had very low, if detectable, activities. This suggests that both elements are required for activity.

Fig. 6 shows that *in vitro* synthesized FTF binds to the 12α-hydroxylase promoter (lanes 2 and 3). When *in vitro* made FTF was preincubated with antibodies raised against a peptide corresponding to the DNA binding domain of FTF, binding was mostly abolished (lane 5), demonstrating that the protein binding to the 12α-hydroxylase probe is in fact FTF. Preimmune serum did not produce any effect (lane 4). Mutations of 5 nucleotides (−62 to −58) within the first site diminished binding about 5-fold (lanes 7 and 8). Mutation of 4 nucleotides (−52 to −49) within the second putative site also diminished binding by about 5-fold (Fig. 6A, lanes 13 and 14). Mutation of 8 nucleotides (−56 to −49) that modifies both sites abolished binding completely (lanes 10 and 11). As a negative control, we used *in vitro* synthesized GHR (lanes 1, 6, 9, and 12).

To determine the specificity of the binding and if the 7α-hydroxylase CPF site binds to the same family, we performed the competition experiment shown in Fig. 6B. Wild type probe competed as expected (lanes 3–5). A probe made from the rat 7α-hydroxylase promoter sequence (−150 to −131) containing the described CPF site (13) competed even better than the 12α-hydroxylase probe itself (lanes 6–8), suggesting that the 7α-hydroxylase CPF site has higher affinity for FTF than the 12α-hydroxylase sites. A mutated 12α-hydroxylase fragment (mutant E), with nucleotides mutated in both FTF sites, did not compete for binding (lanes 9–11), confirming the specificity of the binding.

To demonstrate that the protein binding to the 12α-hydroxylase FTF sites exists in liver cells, HepG2 nuclear extracts were used for binding experiments (Fig. 6C, lanes 7–9). The binding activity found in HepG2 cells was also inhibited by incubation with a specific antibody against FTF (lane 9), and the DNA-protein complexes formed exhibited mobility identical to that of the complex formed by *in vitro* made FTF (lanes 4–6).

As a control, we used a probe from the rat 7α-hydroxylase promoter known to bind CPF, a highly homologous protein to FTF (13). Both *in vitro* made FTF and HepG2 nuclear extracts bind the 7α-hydroxylase probe, and that binding was specific as indicated by the fact that anti-FTF antibodies also prevented binding (Fig. 6C, lanes 10–18). The 7α-hydroxylase probe binds FTF at higher affinity that the 12α-hydroxylase probe, in agreement with the competition experiments shown in Fig. 6B.

To further demonstrate the key role of FTF for 12α-hydroxylase promoter activity, we overexpressed FTF in the kidney cell line CV-1 (Fig. 7). Since 12α-hydroxylase is expressed only in the liver, pGL3-R12α-865 was inactive in CV-1 cells as expected. However, when we cotransfected pCI-FTF, pGL3-R12α-865 became active. The level of activation was nearly 13-fold when 200 ng of pCI-FTF was used. As a control, we used pGL3-Basic and pGL3-R12α-865 mutant E, which had no promoter activity in HepG2 cells (Fig. 4). The activities from these two plasmids were the same as pGL3-R12α-865 when no pCI-FTF was included in the transfection. Overexpression of FTF produced only a slight activation (2–3-fold) of both the promoterless plasmid pGL3-Basic and the pGL3-R12α-865 mutant E. For comparison, a construct containing the rat 7α-hydroxylase promoter was activated only 8-fold when 2-fold more pCI-FTF (400 ng) was cotransfected (data not shown), suggesting a greater activity of FTF for the 12α-hydroxylase promoter than the 7α-hydroxylase promoter.

**DISCUSSION**

The data presented in this study demonstrate that FTF is a factor required for 12α-hydroxylase expression. Several lines of evidence support this conclusion. First, mutagenesis of either of its two binding sites abolished promoter activity completely in HepG2 cells (Fig. 4); therefore, both sites are required for promoter activity. Second, *in vitro*-made FTF binds specifically to these sites (Fig. 6), and the same binding activity was observed in nuclear extracts prepared from HepG2 cells. Third, expression of FTF in a non-liver cell line activates the 12α-hydroxylase promoter, which is otherwise inactive (Fig. 7). Additionally, this study also demonstrates that all of the DNA elements required for the bile acid-mediated regulation of 12α-hydroxylase promoter activity are located in the first 106 nucleotides of the 5’-flanking region (Fig. 3).

The 12α-hydroxylase promoter sequence also has two other potential regulatory elements. Two putative HNF-3 sites are located at approximately nucleotides −455 and −385 (Fig. 2). HNF-3 sites are found in the promoter of some liver-specific genes and are required for the expression of those genes (27). Additionally, two SRE sites are located at approximately nucleotides −315 and −328. SREs are regulatory elements found in cholesterol and fatty acid-regulated genes (28). However,
neither the HNF-3 sites nor the SRE sites seem to be required for either promoter activity or regulation by bile acids, since deletion of these sites has very little effect, if any (Fig. 3). It is possible that several members of the Ftz-F1 family have similar activity on genes involved in bile acid biosynthesis. In fact, it has been shown that at least both CPF and CPF variant 1 (another member of the same family) were active on the 7α-hydroxylase promoter (13). This suggests a crucial role of this family of factors in the control of bile acid biosynthesis.

An interesting issue is the apparent existence of two FTF binding sites within the 12α-hydroxylase promoter. These two FTF sites were located based on homology with other FTF sites, and they overlap by the last 2 nucleotides of the first site based on published consensus sequences (13, 19, 25). Although DNA probes containing an individual mutation of either site still bind FTF weakly (Fig. 6A), both sites are required for an active promoter (Fig. 4A). Nuclear receptors of the Ftz-F1 family are known to bind as monomers (19), and given the migration pattern of the protein-probe complex, only one protein molecule seemed to bind per probe molecule. Thus, mutant D, which has only the first site mutated (Fig. 4B), has some binding capability (Fig. 6A) but has no promoter activity (Fig. 4A). Mutant F, which has 4 nucleotides mutated within the second site, has a binding capability similar to that of mutant D (Fig. 6A) and has some promoter activity (Fig. 4A), but much lower than wild type. Mutant E, which has 8 nucleotides mutated across both sites, lost all binding (Fig. 6A) and promoter activity. The most likely explanation to this issue is that binding of FTF to the 12α-hydroxylase promoter site requires more nucleotides than the binding of FTF or homologous factors to other promoters, and in fact, there may be only one extended binding site. It is possible that several members of the Ftz-F1 family have similar activity on genes involved in bile acid biosynthesis. In fact, it has been shown that at least both CPF and CPF variant 1 (another member of the same family) were active on the 7α-hydroxylase promoter (13). This suggests a crucial role of this family of factors in the control of bile acid biosynthesis.

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Besides the similarity between the 12α-hydroxylase and 7α-hydroxylase promoters in the requirement for FTF or a homologous factor such as CPF for its expression, these two genes
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Fig. 6. FTF binds to the 12α-hydroxylase promoter. Gel shift experiments were performed as described under “Experimental Procedures,” using the indicated amounts of in vitro synthesized FTF protein or in vitro made GHR as a control. In A, five different probes were used, the wild type 12α-hydroxylase sequence from nucleotides −62 to −58 (lanes 1–5) and the three indicated mutants (lanes 6–14). Preimmune or immune serum against FTF was obtained and used as indicated under “Experimental Procedures” (lanes 4, 5, 18, and 19). An arrow points to the retarded band. The sequence containing the two FTF sites (indicated by dashed boxes) is shown at the bottom, with the nucleotides mutated in mutants D, E, and F noted by brackets. In B, increasing amounts of the indicated competitor DNA was used. In C, both in vitro FTF (ivFTF) and in vitro GHR (ivGHR) as a control and HepG2 nuclear extracts were used for binding.

FTF is required for bile acid regulation of their transcription. In experiments using HepG2 cells and with CDCA, the 7α-hydroxylase promoter required exogenous farnesol X receptor in order to show bile acid-mediated regulation of its activity (20), whereas the 12α-hydroxylase promoter did not show this requirement (Figs. 3 and 4).
Fig. 7. Overexpression of FTF provides 12α-hydroxylase promoter activity in a non-liver cell line. pCI-FTF was cotransfected in CV-1 cells together with pGL3-Basic, pGL3-R12α-865, or pGL3-R12α-865 mutant E (Fig. 3) with nucleotides 56 to 49 mutated. Cells were harvested and analyzed for luciferase and β-galactosidase activities as described under “Experimental Procedures.” The data were normalized to the activity of each construct in the absence of pCI-FTF and represent the averages of three experiments. Error bars denote the S.D. value.

Although this study was not specifically directed to study the factors involved in the bile acid-mediated regulation of 12α-hydroxylation expression, our data strongly suggest that FTF is also implicated in that regulation. This is based on the fact that only mutations within the FTF binding sites abolished regulation. All deletion promoter constructs (Fig. 3) and all block mutant constructs (Fig. 4) are regulated by bile acids except for the two constructs that abolished FTF binding. It could be argued that elimination of FTF binding renders the promoter inactive, and no conclusion could be drawn about a its regulation. However, mutation of the rest of the promoter did not alter bile acid-mediated regulation, and therefore the FTF sites should be involved in the regulation either directly or indirectly. Whether the FTF binding sites or FTF itself is capable of interacting with a bile acid-regulated factor is still unknown. In conclusion, this study demonstrates the key role of FTF or its homologues in the regulation of bile acid biosynthesis and should help to elucidate the molecular mechanisms involved in the bile acid-mediated down-regulation of gene transcription, a process poorly understood to date.

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