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Characterization of the FTF/HNF-4 Sites Within the 7Alpha- and the 12Alpha-Hydroxylase Promoters Involved in the Bile Acid-Mediated Transcription of their Regulation

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CHARACTERIZATION OF THE FTF/HNF-4 SITES WITHIN THE 7ALPHA- AND
THE 12ALPHA-HYDROXYLASE PROMOTERS INVOLVED IN THE BILE ACID-
MEDIATED TRANSCRIPTION OF THEIR REGULATION

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

by

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DEDICATION

To my Ma
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ABSTRACT

CHARACTERIZATION OF THE FTF/HNF-4 SITES WITHIN THE 7 ALPHA- AND THE 12 ALPHA-HYDROXYLASE PROMOTERS INVOLVED IN THE BILE ACID-MEDIATED REGULATION OF THEIR TRANSCRIPTION

By Preeti Pramanik, MS

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2006

Major Director: Dr Gregorio Gil
Professor, Department of Biochemistry

Bile acids regulate their own synthesis through a feedback regulatory mechanism of mainly two enzymes in the classic pathway, the 7α-hydroxylase and the 12α-hydroxylase. In the early 1990’s it was shown that the regulatory responses of 7α-hydroxylase are mediated at the transcriptional level and since then many positive and negative transcription factors that mediate regulatory response have been identified. An important finding was that the transcription factors regulating the expression of 7α- and 12α-hydroxylase genes are nuclear receptors.
One of the first nuclear receptors identified to play a role in the transcription of the 7α-hydroxylase gene was HNF-4 since then many nuclear receptors have been identified that are involved in regulating the 7α- and 12α-hydroxylase genes. Among them the most important ones are FTF and HNF-4 which has been shown to play crucial roles in the transcription and regulation by bile acids.

In this study we demonstrate the importance of FTF and HNF-4 independent of each other in the transcription and bile acid-mediated regulation of the 7α- and 12α-hydroxylase enzymes by creating promoter mutants that would either bind FTF or HNF-4. Once the binding studies were established we performed tissue culture experiments to confirm the promoter activity and bile acid-mediated regulation with the respective promoter mutant constructs.

The data from this study shows that HNF-4 is important for 7α-hydroxylase promoter activity but is not required and importantly we show that HNF-4 is not a required for the bile acid-mediated regulation of the 7α-hydroxylase. We present data which suggests that FTF is absolutely required for the promoter activity and bile acid-mediated regulation of 7α-hydroxylase. With respect to the 12α-hydroxylase show that both FTF and HNF-4 are absolutely required for promoter activity.

In this study we present evidence that since the bile acid responsive elements (BARE) are similar within both the 7α- and 12α-hydroxylase promoters one can be exchanged for the other maintaining both activity and bile acid-mediated regulation.
INTRODUCTION

Cholesterol

The cell membrane of most vertebrates, including humans, contains a single major sterol species known as cholesterol. It was first isolated from gallstones in 1784 and ever since then it has been the fascination of scientists in every field of research. It is found in the external cellular membranes and in the layers that make up the myelin sheaths in the central and peripheral nervous systems. In the plasma membrane, the cholesterol molecules are intercalated between the phospholipid molecules to maintain membrane fluidity. Cholesterol is synthesized in eukaryotes from acetate precursors (1) and is the precursor of all other steroids in the body such as, corticosteroids, sex hormones, bile acids and vitamin D. Although cholesterol performs important functions in the mammalian cells, it has been observed that defects in cholesterol metabolism results in hypercholesterolemia, atherosclerosis and cholesterol gallstone disease. Therefore cholesterol homeostasis is very important.

There are four metabolic pathways for regulating cholesterol homeostasis in the liver, first is the de novo synthesis of cholesterol from acetyl coenzyme A (1). Second is the uptake of serum cholesterol esters by low density lipoprotein (LDL) receptor
mediated endocytosis (2). The third pathway in the maintenance of cholesterol homoeostasis involves catabolism to bile acids and the final pathway involves secretion of cholesterol into the bile.

Of the total cholesterol catabolized approximately, 50% of cholesterol is catabolized to bile acids in the mammalian liver, 10% is used for synthesis of steroid hormones and the remaining 40% is removed together with bile acids and phospholipids via bilary excretion in stool (3).

**Bile Acid Biosynthesis**

Conversion of cholesterol to bile acids in the liver occurs via four different routes and more than 17 enzymes located in the endoplasmic reticulum, mitochondria, cytosol, and peroxisomes participate in bile acid synthesis (4). The most studied pathways are the "classic" (neutral) and the "alternative" (acidic) pathways (Fig. 1). The major end products of these pathways in humans, also known as primary bile acids are chenodeoxycholic acid, which has two hydroxyl groups at positions 3α and 7α, and cholic acid, which has three hydroxyl groups at positions 3α, 7α, 12α and is thus more hydrophobic than chenodeoxycholic acid. The composition of bile acids in the bile affects the secretion of cholesterol and phospholipids from the liver as well as synthesis.
Fig. 1 - Schematic representation of bile acid biosynthetic pathways.
of cholesterol and bile acids (3).

*The classic (neutral) bile acid biosynthetic pathway:*

The first step of this pathway, which is also the rate-limiting step, is catalyzed by the enzyme cholesterol 7α-hydroxylase (7α-hydroxylase) also known as CYP7A1 (4). Cholesterol 7α-hydroxylase is a microsomal cytochrome P450 isozyme expressed exclusively in the liver hepatocytes and converts cholesterol to 7α-hydroxycholesterol. In the next step, membrane bound microsomal 3β-hydroxy-C27-steroid oxidoreductase oxidizes the 3β-hydroxyl group to 3-oxo group and allows shifting of the double bond from position 5 to 4 in the steroid nucleus to form 7α-hydroxy-4-cholesten-3-one. This is a common precursor for both cholic acid and chenodeoxycholic acid. Then 7α-hydroxy-4-cholesten-3-one is acted upon by the microsomal enzyme sterol 12α-hydroxylase (12α-hydroxylase) also known as CYP8B1, which is the specific enzyme for synthesis of cholic acid, to form 7α, 12α-dihydroxy-4-cholesten-3-one, a precursor of cholic acid. The cytosolic enzymes, 3-oxosteriod-5β-reductase and 3α-hydroxysteroid dehydrogenase catalyze the reduction of 7α, 12α-dihydroxy-4-cholesten-3-one into 5β-cholestan-3α, 7α, 12α-triol. Alternatively, these two cytosolic enzymes, 3-oxosteriod-5β-reductase and 3α-hydroxysteroid dehydrogenase, can also catalyze the reduction of 7α-hydroxy-4-cholesten-3-one into 5β-cholestan-3α, 7α-diol, a precursor for chenodeoxycholic acid.
The products of ring modification undergo oxidation and shortening of the sterol side chain in the subsequent steps, catalyzed by the enzyme sterol 27-hydroxylase/CYP27A1, to form oxidized bile acid intermediates. These intermediates exit in the mitochondria and undergo side chain cleavage by a variety of enzymes located in the peroxisome, mitochondria and endoplasmic reticulum, which results in the formation of cholic acid and chenodeoxycholic acid. Before secretion into the bile ducts, the primary bile acids undergo conjugation with either taurine or glycine to increase their hydrophilicity and solubility which makes them impermeable to cell membranes.

*The Alternative (acidic) Pathway:*

The initial step in this pathway, which is also the rate-limiting step is catalysed by sterol 27-hydroxylase, a mitochondrial cytochrome P450 mono-oxygenase to form 27-hydroxysterol and 3β-hydroxy-5-cholestenoic acid (4). Sterol 27-hydroxysterol can be hydroxylated by microsomal oxysterol 7α-hydroxylase to 7α, 27-dihydroxycholesterol. The product of this hydroxylation can undergo ring modification similar to those of the neutral pathway. The main end product of this pathway after oxidation cleavage of the side chain is chenodeoxycholic acid (5), although some studies suggest that cholic acid is also produced by the acidic pathway (6, 7).
The newly formed primary bile acids are acted upon by anaerobic bacteria in the gut to convert them into secondary and tertiary bile acids (8). The pool of diversified bile acids in the lumen of the small intestine ensures complete emulsification of dietary lipids, fat-soluble vitamins and cholesterol.

Decreased bile acid output is the major factor in the production of lithogenic bile, which leads to an abnormal ratio of cholesterol to bile acids and lecithin, a major risk factor for cholesterol gallstone formation. Since bile acids act as powerful detergents to facilitate the absorption of lipids, fat-soluble vitamins and cholesterol, proper control of bile acid synthesis is necessary and takes place mainly, at the transcriptional level (4).

**Regulation of Bile Acid Synthesis**

Accumulation of large quantities of hydrophobic bile acids in the hepatocytes is very toxic and therefore a tight control of bile acid synthesis and transport is necessary. This is brought about by negative feedback regulation of mainly two enzymes, cholesterol 7α-hydroxylase, which is the rate-limiting enzyme in the classic pathway and sterol 12α-hydroxylase, the specific enzyme for cholic acid synthesis (3, 4).
It was originally reported that the 7α-hydroxylase gene deficient mice die within few days of birth due to liver failure, vitamin deficiencies, and lipid malabsorption (9). Supplementing vitamins and bile acids to nursing mothers prevented death. These mice had 75% less bile acid pool and they reversed their pathologic phenotype (10), and started producing bile acid via different pathways in which oxysterols rather than cholesterol serve as substrate. Recently, it was reported that the 7α-hydroxylase gene deficient mice survive and the survival of pups to adulthood is >65%, when they were fed normal rodent chow (11). The reason for these observations is unclear and all of the 7α-hydroxylase gene deficient mice were hypercholesterolemic.

The second important enzyme in the control of bile acid synthesis is sterol 12α-hydroxylase, a cytochrome P450 that catalyses the hydroxylation of the steroid nucleus at position 12. The specific activity, mRNA levels and the transcriptional activity of 12α-hydroxylase is inhibited by bile acids and stimulated by cholestyramine (7). Sterol 12α-hydroxylase is thus 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 loss of 12α-hydroxylase activity eliminates synthesis of cholic acid (12) that leads to increased hydrophilicity of bile acids and hence less absorption of dietary cholesterol and higher hepatic cholesterol synthesis. Alteration in the rate of cholic acid and deoxycholic acid ratio has been implicated in the formation of cholesterol gallstones.
Nuclear Receptors

It has been recognized that the regulation of bile acid synthesis takes place, mostly at the transcriptional level and the transcription factors responsible for regulating the genes involved are nuclear receptors (4, 5). Many nuclear receptors are thought to play crucial roles in regulating the genes involved in bile acid synthesis and cholesterol metabolism. These nuclear receptors are present in the enterohepatic and peripheral tissues and are activated by micromolar concentrations of lipids, bile acids and steroids. The elements recognized by nuclear receptors involved in the transactivation of genes in bile acid synthesis have been identified as hexameric repeats of AGGTCA sequence with 1, 4, 5, nucleotide spacing. These are also known as DR-1, DR-4, and DR-5 (13).

The structure of nuclear receptors consists of an activation function domain-1, a highly conserved DNA binding domain in the N-terminal region, followed by the Hinge region, a moderately conserved ligand-binding domain and activation function domain-2 in the C-terminal region (14). There are two cysteine-coordinated Zn$^{2+}$ motifs in the DNA-binding domain which are involved in DNA binding and dimerization. The ligand-binding domain, in addition to determining ligand-specificity, contains a ligand-inducible transactivation function, and is also involved in the dimerization and interaction with
coregulators (15). Upon ligand-binding the nuclear receptors undergo conformational change, release the corepressors and recruit the coactivators (16).

Many nuclear receptors have no identified ligands for activation and are referred to as orphan nuclear receptors (14). Most of the ligands for nuclear receptors bind hydrophobic molecules to fit in the ligand-binding domain. The unique structure of the hydrophobic bile acids make them excellent candidates as endogenous ligands for orphan nuclear receptors (17). Many ligands for the nuclear receptors involved in the regulation of the above genes have recently been identified. Next we will review some of the key nuclear receptors involved in bile acid synthesis.

**Farnesiod X Receptor (FXR):** FXR is expressed in the liver, gut, kidneys and the adrenal cortex and activates transcription in response to micromolar amounts of farnesol (18). FXR binds to an inverted repeat separated by one base pair (IR1), although it also binds weakly to DR4 and DR5 motifs (19). Several studies indicate that FXR is activated by bile acids and functions as bile acid receptor to repress 7α-hydroxylase transcription (20, 21).

Mice deficient in FXR express high levels of 7α-hydroxylase and 12α-hydroxylase mRNA, which results in increased synthesis of bile acids (22). These mice
accumulate bile acid in the plasma due to decreased levels of ABCB11, which transports bile acids from the hepatocytes into the bile (23). The FXR knockout mice are also hypertriglyceridemic due to decreased expression of apolipoprotein CII, which is required for triglyceride metabolism (24).

Other target genes for FXR includes many ABC transporters for efflux of bile acids (23), and phospholipid transport protein for reverse cholesterol transport (25).

**Liver X Receptor (LXR):** There are two LXR genes in mammals, LXRα and LXRβ. It has been reported that LXRα plays a role in regulating cholesterol metabolism. Upon cholesterol accumulation in the hepatocytes, oxysterols activate LXRα, which heterodimerizes with RXR and binds to the DR-4 response element in the 7α-hydroxylase promoter (26). Another study reported that mice lacking LXRα, but having an intact LXRβ, do not induce 7α-hydroxylase expression in response to cholesterol feeding (27). Mice lacking both LXRα and LXRβ show severe phenotype (28). These studies established that LXRα is the cholesterol sensor responsible for feed-forward regulation of 7α-hydroxylase expression. However, it is important to note that although LXRα induces 7α-hydroxylase transcription in rats, the LXR element is either missing in species that do not respond to cholesterol (human and rabbit), or is present but nonfunctional in other nonresponding species (hamster) (29).
LXR also plays a critical role in integrating the pathways of cholesterol synthesis and metabolism by regulating expression of sterol regulatory element binding protein-1c (SREBP-1c) gene (30). LXR stimulates the transcription of genes encoding cholesterol efflux proteins, ABCA1, ABCG5, and AGCG8 transporters and helps in the direct secretion of sterol from the liver (31) (32). There are numerous other genes involved in lipid metabolism that are targets of LXR (33), these include apolipoprotein E, ABCG1, phospholipid transfer protein (25), etc.

**α-Fetoprotein Transcription Factor (FTF):** Also known as LRH-1 (liver receptor homologue-1) and CYP7A promoter-binding factor (CPF) (34), or NR5A2 (35). It belongs to Fushi-tarazu factor-1 (Ftz-F1) family of nuclear receptors, and plays crucial roles in steriodogenesis, liver growth, endocrine development and differentiation. The Ftz-F1β (NR5A2) gene encodes α-FTF and its homologs, rat FTF (36), human CPF (34), hepatitis B virus enhancer 1 factor (37), human FTF (38), mouse LRH (39). These variants differ in their N-terminal amino acid sequences and C-terminal truncation due to differential promoter usage and alternative mRNA splicing (37). Members of the Ftz-F1 family bind as monomers to their response elements (29).

FTF is expressed in the liver, intestine and the pancreas. It shares sequence identity with steroidogenic factor-1 (SF-1), a nuclear receptor which directs tissue-
specific expression of genes involved in gonadal and adrenal steroidogenesis (40). The binding site of FTF has been identified in many genes which includes, 7α-hydroxylase (20), 12α-hydroxylase (41), SF-1 (42), SHP (43), HBV (44), HNF3β, HNF-4α (45), etc. It has intrinsic transcriptional activity. Recently, phosphotidyl inositols phosphates have been identified as the ligand for FTF (46).

It has been suggested that FTF functions as a competence factor for sterol regulation of mouse 7α-hydroxylase and human cholesterol ester transfer protein (CEPT) gene by LXR (47). FTF also induces multidrug-resistant protein-3 (Mrp-3) gene, involved in excretion of bile acids across basal lateral membrane into portal blood (48). There are a number of other genes like, HNF-3β (45), HNF-4α (45), HNF-1α (45), adiponectin (ACDC) (49), ABST protein, ABCG5, and ABCG8 (50), steroidogenic acute regulatory protein (StAR) (51), scavenger receptor-BI (SR-BI), leutinizing hormone receptor (LHR) (52), 3β-HSD, 11β-hydroxylase (53), 17,20-lyase (51), secretory phospholipase A2 (54), carboxyl ester lipase (55), etc, that are regulated by FTF binding.

**Hepatocyte Nuclear Factor-4α (HNF-4α):** The most abundant orphan nuclear receptor in the liver binds to the DR-1 motif and regulates several liver specific and non-specific genes. It has constitutive activity and is able to transactivate gene with (56) or without ligand binding (57). HNF-4α binds to the DR-1 site in 7α-hydroxylase and 12α-
hydroxylase, and also binds 27α-hydroxylase promoter. HNF-4α binding stimulates the 7α-hydroxylase promoter (21). It has been reported that HNF-4α mediates bile acid repression of 12α-hydroxylase transcription (58). Phosphorylation of the DNA binding domain of HNF-4α by protein kinase A reduces its transactivation activity (59).

Disruption of HNF-4α gene in mice is embryonic lethal because HNF-4α is critical for early liver development and differentiation. Mice with induced hepatic deficiency of HNF-4 have markedly reduced expression of 7α-hydroxylase (60). The expression of oxysterol 7α-hydroxylase and 12α-hydroxylase genes is reduced to very low levels.

There are a number of liver-specific genes involved in lipid metabolism, like ApoAl, ApoB and ApoCIII (61) whose expression is regulated by HNF-4α. HNF-4α binds as homodimer to the DR-1 motif and regulates their expression. HNF-4α also regulates transcription of a number of other genes including, glucokinase (62), pancreatic-β cell (63), HMG-CoA synthase (64), PPARα (65), PXR (66), guanylyl cyclase (67), macrophage stimulating protein (68), biliary glycoprotein (69), SREBP-1c (70), SREBP-2 (71), α1-fetoprotein (72), etc.
Short Heterodimer Partner (SHP): A unique nuclear receptor, which lacks the DNA binding domain but contains a receptor-interacting domain and a repressor domain (45). It inhibits transactivation activity of HNF-4α (73), FTF (74), RAR (75), CAR (76), PPAR (77), etc. SHP represses nuclear receptor activity by two mechanisms. First, it competes with other nuclear receptors for coactivators and second, SHP represses nuclear receptors directly by its repressor function located at the C-terminal (73).

The promoter for the SHP gene contains an FXR response element composed of inverted repeats separated by single base pair (78). In addition to the FXR binding site in the SHP promoter, there is a functional FTF response element (46, 78).

Regulation of Cholesterol 7α-Hydroxylase Expression

Early studies showed that increasing the bile acid pool by bile acid feeding suppresses bile acid synthesis (79), whereas depletion of the bile acid pool by biliary diversion or bile acid sequestrant feeding increases bile acid biosynthesis (80). The molecular mechanisms responsible for regulation of cholesterol 7α-hydroxylase by bile acids and cholesterol have been the intense focus of many research groups in the past few years.
Bile acids negatively regulate the transcription of 7α-hydroxylase gene in the classic pathway. It was reported that the rat cholesterol 7α-hydroxylase promoter contains two bile acid response elements, BARE I and BARE II between nucleotides -149 to +32 (3). Although, BARE I is an LXRα response element there is no evidence for its involvement in bile acid mediated regulation. The BARE II contains an overlapping HNF-4 and FTF binding sites from nucleotides -146 to -127 (Fig. 2) (20). HNF-4 binds to the DR-1 site and the direct repeat motif contains AG(G/T)TCA-like sequence.

A number of mechanisms have been proposed for the suppression 7α-hydroxylase gene in response to bile acid accumulation and the majority of the mechanisms implicate nuclear receptors. It has been reported that bile acids bind to FXR (81-83). FXR then heterodimerizes with retinoid X receptor (RXR) and binds to an inverted repeat of 6 nucleotides separated by one nucleotide (IR-1), and activates expression of SHP (78). SHP heterodimerizes with FTF, the major activator of 7α-hydroxylase promoter (34, 78, 82), resulting in the repression of 7α-hydroxylase transcription. FTF binds to the GC-rich sequence TCAAGGCG (84) in the 7α-hydroxylase promoter and is required for the transcriptional activation of the 7α-hydroxylase promoter in response to cholesterol accumulation (34, 78, 82).
Fig. 2 - Similarities between the bile acid-regulatory sites within the 7α- and the 12α-hydroxylase promoters. The FTF sites noted by solid line boxes and the DR-1 sites are noted by arrows.
This is consistent with FXR \(^{-} \) studies, which reports that FXR deficient mice accumulate high levels of 7α-hydroxylase and 12α-hydroxylase; this results in increased bile acid synthesis. Cholic acid feeding to these mice neither reduces 7α-hydroxylase and 12α-hydroxylase levels nor increases SHP levels. These findings suggest a mechanism that indirectly suppresses 7α-hydroxylase and 12α-hydroxylase gene transcription by bile acids through induction of SHP (85).

Experiments in two independent lines of SHP knockout mice reveal only modest elevations in the levels of 7α-hydroxylase and 12α-hydroxylase gene expression (86), which is consistent with SHP repressing bile acid synthesis. However, 7α-hydroxylase and 12α-hydroxylase levels fall when these mice are fed bile acids but not when specific FXR agonist (GW4064) is administered to these mice. These results indicate that, additional SHP-independent pathways exit, which are capable of suppressing bile acid synthesis.

Another mechanism that has recently been proposed for the down-regulation of 7α-hydroxylase transcription is the involvement of c-Jun N-terminal kinase (JNK). In this mechanism, the JNK pathway is activated by bile acids, which in turn activates c-Jun resulting in higher SHP transcription (87).
An alternate SHP-independent mechanism proposed for the suppression of 7α-hydroxylase transcription by bile acids is by inhibition of the transactivation potential of hepatocyte nuclear factor-4α (HNF-4) (88).

The fourth mechanism, which also implicates HNF-4 for the regulation of 7α-hydroxylase transcription by bile acids, is that bile acids impair the recruitment of PPAR-γ coactivator-1α (PCG-1α) and cAMP-responsive element-binding protein by HNF-4 (88).

**Regulation of Sterol 12α-Hydroxylase Expression**

The other enzyme in the bile acid biosynthetic pathway also regulated by bile acids is 12α-hydroxylase (7), which also requires FTF for promoter activity (41). The rat 12α-hydroxylase promoter contains a TATA box-like element, two consensus binding sites for HNF-3, two sterol regulatory elements (SREs) between nucleotides -233 to -206 (36) and two overlapping sites (Fig. 2) for liver-specific nuclear receptor FTF between nucleotides -64 to +48 (41).

The FTF sites within this promoter are absolutely required for bile acid-mediated regulation. Previous studies from our lab implicate the interaction between FTF and SHP
in the bile acid mediated suppression of 12α-hydroxylase promoter. The distinctive domains required for the interaction of FTF and SHP has also been identified and studies suggest that FTF requires an intact SHP protein for the interaction between them in vivo. SHP prevents binding of FTF to its binding sites within the 12α-hydroxylase promoter, providing a mechanism of action for SHP-mediated suppression of 12α-hydroxylase transcription (74).

Sterol 12α-hydroxylase gene knockout mice have increased chenodeoxycholic acid, murichlolic acid, bile acid synthesis and pool size, and cholesterol synthesis (12). The highly hydrophilic bile in these mice may derepress the cholesterol 7α-hydroxylase gene. It is also suggested that lack of cholic acid may decrease intestinal cholesterol absorption and hence stimulates de novo cholesterol synthesis in the liver (17).

The two FTF sites in the 12α-hydroxylase promoter overlap with a direct repeat (DR-1), which is a functional PPAR binding site (89) between nucleotides -78 to -61 (Fig. 2). This is similar to the 7α-hydroxylase promoter which also contains a DR-1 element overlapping with the FTF binding site (Fig. 2) and it has been shown that HNF-4 indeed does bind to and activate the 7α-hydroxylase promoter (90).

The DR-1 is the binding site for HNF-4 among other nuclear receptors, and it has
been hypothesized that HNF-4 is capable of competing with FTF for binding to the
FTF/HNF-4 binding element in the 12α-hydroxylase promoter (93). The 7α- and 12α-
hydroxylase expression is higher in FTF\(^{+/−}\) mice than in the wild type suggesting that FTF
might be acting a negative factor of both. These mice not only have 3-7 fold higher 7α-
and 12α- hydroxylase expression but their higher expression is reflected in a robust
increase in total bile acids in the gallbladder with a higher ratio of cholic acid/murocholic
acid ratio, reflecting higher 12α- hydroxylase expression in the FTF\(^{+/−}\) mice (91, 92).
FTF overexpression experiments in mice suggest that FTF has two effects on 7α- and
12α- hydroxylase expression. First, it displaces HNF-4 from the FTF/HNF-4 site in 7α-
and 12α- hydroxylase promoters and second it activates increased expression of SHP
which suppresses FTF activity and hence transcription of 7α- and 12α- hydroxylase (92).

However, studies with short term overexpression of FTF using transgenic mice
(TgF35) (91) carrying an FTF transgene controlled by the inducible MTI promoter
showed 6-and 18-fold increase in mRNA levels of 7α-hydroxylase and SHP, respectively. This result pointed to an indirect mechanism for higher 7α-hydroxylase
mRNA in the FTF\(^{+/−}\) mice and also supported the FTF activating effect on 7α-
hydroxylase gene activity (91). This difference in result might be attributable to the
technique used to overexpress FTF in the transgenic mice (TgF35).
RATIONAL FOR THIS WORK

From the studies referenced in the introduction, there is evidence for either FTF or HNF-4 to be involved in the bile acid mediated regulation of 7α- and 12α-hydroxylase. A number of promoters have been identified which contain either FTF or HNF-4 site that bind FTF as monomer and HNF-4 as homodimers, but they are not regulated by bile acids. We hypothesize that the FTF/ HNF-4 sites in the 7α- and 12α-hydroxylase promoters are unique and that FTF/HNF-4 DNA elements that mediate bile acid regulation bind as FTF/HNF-4 heterodimer.

Our lab has previously reported the importance of FTF binding for 7α- hydroxylase and 12α-hydroxylase promoter activity and bile acid mediated regulation (82, 92). These studies were done by creating promoter mutants that would bind FTF but not HNF-4 in both the rat 7α-hydroxylase and rat 12α-hydroxylase promoter. However, a mutant that would bind HNF-4 but not FTF was not created. Such a mutant could help to elucidate whether HNF-4 is involved in the 7α-hydroxylase and 12α-hydroxylase promoter activity as well as bile acid-mediated regulation, since the DR-1 is the binding site for HNF-4 and it is a receptor know to interact with SHP (73).

As discussed in the introduction, both 7α-hydroxylase and 12α-hydroxylase
promoters’ bile acid mediated regulatory elements are quite similar, as expected for two genes whose transcription is coordinately regulated by bile acids (7). In both promoters, the HNF-4 site overlaps the FTF site (92) and there are two functional FTF sites in the 12α-hydroxylase promoter and apparently only one in the 7α-hydroxylase promoter (Fig. 2) (34). We hypothesize that since the bile acid regulatory sites in both the 7α- and 12α-hydroxylase promoters are similar one site can be exchanged for the other.

Finally, in vivo overexpression of HNF-4 in mouse using adenovirus system will demonstrate whether HNF-4 is involved in the transcription of 7α-hydroxylase and 12α-hydroxylase genes.
MATERIALS AND METHODS

Materials

Common laboratory chemicals were from Fisher, Sigma, Gibco & Bio-Rad. DNA cloning and sequencing reagents were from New England Biolabs, Boehringer Mannheim, US Biochemical Corp. and Gibco BRL. $^{32}$P-ATP was purchased from ICN biomedical. Oligonucleotides were purchased from Integrated DNA Technologies. The luciferase promoter-less vector pGL3-Basic was purchased from Promega. Anti-FTF antibody was prepared against carboxy terminal peptide of the rabbit FTF sequence and was affinity-purified before use (in vitro studies). Anti-HNF-4 and anti-HDAC-1 antibodies were obtained from Santa Cruz Biotechnology. Monoclonal anti-myc antibody was obtained from Sigma.

Preparation of Chimeric 7α- and 12α-hydroxylase Promoter-Luciferase Reporter Constructs

The pGL3-R7α-342 was prepared previously in our lab by PCR and contains -342 to +59 nucleotides, ligated to into the Smal site of pGL3-Basic vector. The three 7α-hydroxylase promoter constructs used for the experiments were made using a
QuickChange site-directed mutagenesis kit (Stratagene) and the corresponding oligonucleotides shown in Table I.

**QuickChange site-directed mutagenesis method:** This procedure utilizes a template which is a double stranded DNA with an insert of interest and two oligonucleotide primers that are complementary to the template and contains the desired mutation. The two oligonucleotide primers, opposite to the template are extended during temperature cycling by *Pfu turbo* DNA polymerase. The mutated plasmid generated was incubated with 1 µl of Dpn I for 1 hr at 37°C. The Dpn I endonuclease is specific for methylated and hemimethylated DNA, and was used to digest the parent DNA and select the mutated plasmid. The plasmid DNA containing the desired mutation was transformed into DH5α cells, grown and sequenced to confirm the sequence of the mutant.

The pGL3-R12α-865 was prepared in our lab by placing a 903-nucleotide Sac1- Sac1 fragment containing nucleotides -865 to +37 into the Sac1 site of pGL3-Basic vector from Promega. The 12α-hydroxylase promoter constructs, DR-1 mutant and FTF mutant used in the experiments were generated by using QuickChange site-directed mutagenesis kit (Stratagene), as described above.
**MUTAGENESIS OLIGONUCLEOTIDES**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Mutagenesis Oligonucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>7α-Hyd DR-1 Mut (1)</td>
<td>CGC TCT TCT GAG ACT ACA ACC TTA GTC CAA GGC CCG G</td>
</tr>
<tr>
<td>7α-Hyd DR-1 Mut (2)</td>
<td>TTC TGA GAC TAT TGA ATT AGT TCA AGG CCG GGT A</td>
</tr>
<tr>
<td>7α-Hyd FTF Mut</td>
<td>CTA TGG ACT TAG TTC AAC AAT GGG TAA TGC TAT TTT TTT C</td>
</tr>
<tr>
<td>12α-Hyd DR-1 Mut</td>
<td>GCT ATT TAC CAC GTC CgG ACT TAG TTC AAG GCC CAA TAT TAT G</td>
</tr>
<tr>
<td>12α-Hyd FTF Mut</td>
<td>CTA TGG ACT TAG TCC AAC aat GAA TAT TAT GGG CCG</td>
</tr>
<tr>
<td>12α-7α FTF Mut</td>
<td>CCA GAC TAT GGA CTT AGT TCA Aca atG aGG GCC CAG CCA ACT TAT C</td>
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**OLIGONUCLEOTIDES USED FOR EMSA**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>EMSA Probes</th>
</tr>
</thead>
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<tr>
<td>7α-Hyd DR-1 Mut (1)</td>
<td>CTA GGA GAC TAC aac CTT AGT TCA AGG CCG GGT A</td>
</tr>
<tr>
<td>7α-Hyd DR-1 Mut (2)</td>
<td>TTC TGA GAC TAT TGA ATT AGT TCA AGG CCG GGT A</td>
</tr>
<tr>
<td>7α-Hyd FTF Mut</td>
<td>CTA GGA GAC TAT GGA CTT AGT TCA Aca atG GGT A</td>
</tr>
<tr>
<td>12α-Hyd DR-1 Mut</td>
<td>GCT ATT TAC CAC GTC CgG aCT TAG TTC AAG GCC CAA TAT TAT G</td>
</tr>
<tr>
<td>12α-Hyd FTF Mut</td>
<td>AGT CCA GAC TAT GGA CTT AGT TCA Aca atG AAT ATT ATG GGC CGT TTG</td>
</tr>
<tr>
<td>12α-7α</td>
<td>CCA GAC TAT GGA CTT AGT TCA AAG CCG TGG GAA CAG C</td>
</tr>
<tr>
<td>12α-7α FTF Mut</td>
<td>AGC TCA CAG ACT ATG GAC TTA GTC CAA caa tCG TT</td>
</tr>
</tbody>
</table>

Table 1- Sequences of mutagenesis oligonucleotides and EMSA probes used in the experiments.
The 12α-hydroxylase promoter construct 12α/7α FTF mutant was generated by oligonucleotide-directed mutagenesis in M13. Mutagenesis oligonucleotide was phosphorylated using 5 mM ATP T₄ polynucleotide kinase, annealed to M13 template and extended for 2 hrs at 14°C using the Klenow fragment of DNA polymerase I. The above reaction was diluted 1:10 and 3 µl was used to transform TG-1 competent cells. The transformation was screened for positive clones and 4 positive clones were grown overnight and the M13 DNA was isolated and sequenced. The DNA for a positive clone was resuspended in 20 µl of water and extension reaction was carried out using 2.5 mM dNTP, oligonucleotide sequence 5'-GAAACGACCCTCAACGTCCT-3', and the Klenow fragment of DNA polymerase I. The DNA was cut to yield the SacI-SacI fragment (approximately 900bp). The fragment was purified by electro-elution technique. The SacI-SacI fragment was ligated to the pGL3 basic vector cut at the SacI site. Ligation reaction was carried out overnight at 14°C. The pGL3-Sac-I ligated DNA was transformed into DH5α-cells. Colonies were picked, DNA isolated and sequenced to confirm mutation.

**Transformation of Recombinant Plasmids**

One hundred microliters of DH5α competent cells were transformed using the PCR product or ligation mixture with 0.8% polyethylene glycol (PEG), 10% of 50 mM
KCL, 25 mM MgCl$_2$, and 15 mM CaCl$_2$. The mixture was incubated for 20 mins in ice followed by 10 mins at room temperature. One ml of Luria Broth (LB) was added to the above mixture and incubated for 45 mins at 37°C, followed by centrifugation at 5000 rpm for 5 mins. The supernatent was discarded and the pellet resuspended in 200 μl of LB, the cells were then plated on LB containing 50 μg/ml ampicillin plates. The plates were incubated overnight in a 37°C incubator.

**Mini-preparation of Plasmid**

Three ml of LB containing 50 μg/ml of ampicillin was inoculated with a single bacterial colony and grown overnight in a 37°C shaker. After centrifugation for 5 mins the supernatent was discarded and the pellet was resuspended in 1ml of 10 mM NaCl and transferred into micro-centrifuge tube. Cells were pelleted at 10,000 rpm for 1 min and the supernatent was discarded. The pellet was resuspended in 150 μl of 25 mM Tris-HCl [pH 8] 10 mM EDTA, 50 mM glucose, 4 mg/ml lysozyme. Following 5 mins incubation at room temperatue 300 μl of 0.2 M NaOH and 1% SDS was added to the sample. After 5 mins, 225 μl of 5 M KAc was added and incubated for 5 mins then spun for 5 mins at 10,000 rpm. The supernatent was transferred into another tube and the DNA was extracted with equal volume of phenol/chloroform and ether to remove proteins followed by 3 M NaAc and 95% ethanol precipitation at -20°C for 30 mins. Sample was
centrifuged for 10 mins at 10,000 rpm the pellet was dried and resuspended in 50 μl of TE (10 mM Tris-HCl [pH 7.6], 1 mM EDTA) containing 20 μg/ml of RNase. Appropriate enzyme digestion was done using 2 μl of DNA, to confirm the presence and orientation of the fragment.

**Plasmid DNA Preparation**

Plasmid DNA was prepared as described by Sambrook et al, 1989. Single bacterial colony was inoculated into 1 L of LB containing 50 μg/ml of ampicillin and grown overnight in a 37 °C shaker. The culture was centrifuged at 4200 rpm for 10 mins and the supernatent was discarded. The pellet was resuspended in 9 ml of 50 mM Tris-HCl (pH 7.5), 40 mM EDTA, 25% sucrose (w/v), lysozyme in a final concentration of 0.3 mg/ml. Following 5 mins incubation at room temperature, 0.1M EDTA and 4 mg of RNase was added and incubated for 5 mins at room temperature. To the plasmid mixture was added 14.5 ml Triton solution, dropwise and centrifuged at 25,000 rpm for 45 mins to pellet the chromosomal DNA.

The supernatent was transferred into a 50 ml polypropylene tube and the weight of the supernatent was adjusted to 30.17 g with TE. To the sample tube was added 28.14 g of ultra pure grade CsCl along with 4.5 ml of 10 mg/ml ethidium bromide and mixed
thoroughly. The sample was transferred into polyallomer quick seal tube through a 60 ml syringe using 16 gauge needle. The tube was sealed according to manufacturer's instruction and centrifuged overnight at 45,000 rpm.

Following centrifugation the tube was illuminated with long wave U.V. light to locate the plasmid band. The tube was first punctured on top for venting and then from side below to collect the lowest DNA band without disturbing any other bands containing chromosomal DNA, using a 10 ml syringe with a 16 gauge needle. Ethidium bromide was removed from the sample by extraction with equal volume of water-saturated butanol until ethidium bromide was completely eliminated.

The sample was dialyzed against 4 L of TE containing 30 mM sodium acetate for 2-3 hrs to remove CsCl. The plasmid DNA was extracted with phenol/chloroform as described above and appropriate digestions were done to confirm that the right plasmid was obtained. All the plasmids were confirmed by sequencing.

**Electrophoretic Mobility Shift Analysis and In vitro transcription/translation**

Binding reactions were done using 20 mM Tris-HCL (pH 8), 0.2 mM EDTA, 4% Ficoll, 1 µg of poly dl-dC, 4 µl of protein, 1500-fold molar excess of irrelevent single
stranded DNA, in a final volume of 20 μl. After 20 mins incubation on ice 350 fmol of indicated $^{32}$P labelled DNA probe was added, followed by 20 mins incubation on ice. Samples were then loaded on a 4.5% polyacrylamide gel and subjected to electrophoresis at 180 volts, at 4°C. For supershift assays 1 μl of antibody was added followed by 20 mins incubation on ice. The gels were dried and exposed to X-OMAT/ XAR autoradiography films from Kodak. *In vitro* proteins used were prepared using the TNT T7 coupled reticulocyte lysate system (Promega) according to manufacturer's protocol.

**Transient Transfection and Luciferase Asssays**

Primary hepatocytes were isolated from adult male Sprague-Dawley rats and prepared with collagenase perfusion-technique of Bissell and Guzelian. Hepatocytes (8.5 \times 10^5) were plated onto 35 mm Falcon Primera culture dishes. Prior to plating, cells were judged for >90% viability using trypan blue exclusion. Cells were incubated in 2 ml serum-free William’s E medium supplemented with 1 μM thyroxine, 0.1 μM dexamethasone, 0.25 units/ml insulin, and 100 units/ml penicillin, glutamine in a 5% CO$_2$ atmosphere at 37°C. Six hours after plating, hepatocytes were transfected with Lipofectin (Invitrogen) using 1 μg of total DNA, 100 ng of test plasmid, 2 ng of pCMV Gal (a plasmid containing human cytomegalovirus [CMV] promoter in front of the bacterial β-galactosidase gene) to normalize transfection efficiencies and 5 ng of pRSV-FXR.
DNA was removed 16 hrs after transfection and 50 µM taurocholic acid was added. Cells were harvested after 24 hrs and the luciferase and β-galactosidase assay was performed using a kit from Tropix (Bedford, MA) according to manufacture’s protocol. All transfections were done in duplicate. Background activity (pGL3-Basic) was subtracted in each case.

Human hepatocellular blastoma cells HepG-2 were obtained from American Type Culture Collection. HepG2 cells were transfected by the calcium phosphate method using 2 µg total DNA. HepG2 cells were transfected with 100 ng of test plasmid, 2 ng of pCMV-Gal to normalize transfection efficiencies. The DNA was removed 16 hrs after transfection and 50 µM chenodeoxycholic acid was added. The cells were harvested 48 hours later and the luciferase and β-galactosidase assays were performed using Tropix (Bedford, MA) kit according to manufacture’s protocol.

**Liver Nuclear Extracts Preparation**

Livers were obtained by harvesting mice (3 mice per group), rinsed in 1x ice cold SSC solution then placed in Thomas Tissue Grinder with 25 ml of homogenizing buffer (2 M sucrose, 25 mM KCL, 10 mM Hepes [pH 7.6], 1 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 1 mM DTT, 10% glycerol, 10 mM NaF, 0.5% milk). The livers
were homogenized, filtered through a cheese cloth and centrifuged for 45 mins at 26,000 rpm. The nuclei was resuspended in 2 ml of nuclear lysis buffer (100 mM KCl, 10 mM Hepes [pH 7.6] 0.1 mM EDTA, 3 mM MgCl₂, 10% glycerol, 5 mM NaF, 0.1 mM PMSF, 1 mM DTT) and centrifuged at 9000 rpm for 10 mins. The nuclei were freezed in liquid nitrogen, resuspended in 250 µl (per gram of liver) of nuclear lysis buffer and made 0.4 M NaCl. The sample tubes were centrifuged for 1 hr at 45,000 rpm. The supernatant was dialyzed twice in dialysis buffer at 4°C and the samples were quantified for protein concentration by the Bradford method.

Adenovirus Preparation and Propagation

The adenovirus constructs used in this study was obtained through the Massey Cancer Center Shared Resource Facility of Virginia Commonwealth University. The pZeroTG/CMV-hHNF-4-myc-His needed to create the first adenovirus in the study was prepared as follows. A 1.4-Kb fragment of the hHNF-4 was obtained by PCR using the T7 promoter primer and the appropriate oligonucleotide complementary to the 3’ end of the HNF-4 coding region. The HNF-4 cDNA was used as the template. After blunt ending and fragment purification the fragment was inserted in frame into pcDNA3.1/mycHisA opened with EcoRV. An EcoRV fragment generated from hHNF-4-myc fragment was cloned into the EcoRV site of pZeroTG/CMV. The resulting
pZeroTG/CMV-hHNF-4-myc plasmid was cotransfected with Clal-linearized pTG/CMV (containing the entire Ad5dl324 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 in (93).

**RNA Isolation and Quantification**

Total liver RNA was isolated by using the SV total RNA isolation kit (Promega) according to manufacture’s instructions. The 7α-hydroxylase, 12α-hydroxylase, LDL receptor, HMG CoA reductase, Apo-ClIII, Apo-AI, mRNA’s were quantified by quantitative reverse transcription (Q-RT)-PCR using together the primers and probes shown in Table II and the Brilliant Q-RT-PCR Core Reagent kit (Startagene) probes with a DNA Engine Opticon2 (MJ Research Inc). All the values were normalized to the levels of actin mRNA, quantified also by Q-RT-PCR using TaqMan probes.

**Western Blot Analysis**
Three micrograms of liver nuclear protein was loaded onto 10% SDS-polyacrylamide gel, transferred onto nitrocellulose membrane, blocked for 1-2hrs in 5% milk in TBST and incubated overnight at 4°C with anti-HNF-4 (1:1000), anti-myc (1:1000), and anti-HDAC-1 (1:1000) antibodies. The membranes were washed 3 times in 5% milk in TBST for 10 mins each, and incubated in appropriate secondary antibody (1:25,000) for 1 hr, after which the membranes were washed 3 times in 5% milk in TBST for 10 mins each. The membranes were then processed with Western Lightning Chemiluminescence Reagent Plus Kit (Perkins Elmer Life Sciences) according to manufacture’s recommendations.
<table>
<thead>
<tr>
<th>Oligo</th>
<th>Gene</th>
<th>GenBank™ No.</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
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<td>m7α-hydroxylase</td>
<td>NM_007824</td>
<td>CAGGGCTCCTGATCATTTGAAATAAGCTCC</td>
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<tr>
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<td>mLDL-receptor</td>
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<td>mβ-actin</td>
<td>NM_007393</td>
<td>CCTGCGTCTGGACCTGGCTGGC</td>
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</tbody>
</table>

Table 2- Primers and Probes used in the Q-RT-PCR quantification of RNAs.
RESULTS

In order to further characterize the FTF and HNF-4 sites in the 7α- and 12α-hydroxylase we set out to create promoter mutants, that would bind only FTF or HNF-4 and test their activity and regulation by bile acids. We first created mutants that should bind either FTF or HNF-4 and checked whether they indeed bind only FTF or HNF-4 using in vitro made proteins. Once this was established, we moved to make the mutants in the chimeric 7α- and 12α-hydroxylase promoter-luciferase reporter constructs and test them for activity and bile acid mediated regulation.

Figure 3 shows a gel retardation assay using the 7α- and 12α-hydroxylase FTF/HNF-4 site as probes using in vitro synthesized proteins. The FTF-, and HNF-4-DNA complexes migrate at approximately the same position with both probes, although the binding affinity of FTF and HNF-4 are different. We also used liver nuclear extracts with the 12α-hydroxylase probe in the same experiment to check whether nuclear extracts bind to this probe similarly to the in vitro made proteins. Interestingly, the binding and migration of FTF and HNF-4 using liver nuclear extracts with the 12α-hydroxylase probe is very different (Fig. 3, lane 5), when compared to the in vitro made proteins where FTF is thought to bind as monomer and HNF-4 binds as dimer, suggesting that FTF and HNF-4 might be forming a heterodimer.
Fig. 3 - FTF and HNF-4 bind to both the 7α- and 12α- hydroxylase promoters and the migration of FTF and HNF-4 is different when liver nuclear extracts are used.

A gel shift experiment as described in "Materials and Methods" using in vitro made FTF, HNF-4 and liver nuclear extracts, and the indicated rat probes. The proteins used were 1 μl of FTF, 4 μl of HNF-4, and 3 μg of liver nuclear extract.
7α-Hydroxylase Promoter Mutants

To analyze the role of DR-1 in the 7α-hydroxylase promoter we created two DR-1 mutants, 7α-Hyd DR-1 Mut 1 and Mut 2, the first DR-1 repeat was mutated leaving the FTF site virtually unmodified (Fig. 4 and 5, lower panel). To characterize the altered binding of HNF-4 and to check the binding of FTF to these two mutants, we performed supershift assays using the 7α-hydroxylase wild-type and mutant probes and in vitro made HNF-4, FTF, and GHR. Figure 4 shows that, as expected, the wild-type probe binds both HNF-4 and FTF but with different affinities. The HNF-4 and FTF bands were confirmed by supershifting the respective bands using anti-HNF-4 antibody and anti-FTF antibody. As expected the two DR-1 mutants do not bind HNF-4 at all but they differ in their ability to bind FTF. The reason for creating two DR-1 mutants was that the 7α-Hyd DR-1 Mut 1 probe binds FTF with lesser affinity as compared to the wild type probe (Fig. 4, lanes 2 and 9) whereas the 7α-Hyd DR-1 Mut 2 binds FTF similar to wild type or even better, (Fig. 5, lanes 2 and 9).

In the third mutant, 7α-Hyd FTF we mutated four nucleotides within the FTF site leaving the DR-1 site unchanged (Fig. 6, lower panel). This mutant probe was not capable of binding to FTF but HNF-4 binding was similar to wild-type probe (Fig. 6,
<table>
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<th>Probe</th>
<th>7α-Hyd Wild Type</th>
<th>7α-Hyd DR-1 Mut 1</th>
</tr>
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<tr>
<td>In vitro Protein</td>
<td>G</td>
<td>FTF</td>
</tr>
<tr>
<td>Control Antibody</td>
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<td>+</td>
</tr>
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<td>FTF Antibody</td>
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</tr>
<tr>
<td>HNF-4 Antibody</td>
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**Fig. 4** - Mutation in the DR-1 site completely abolishes HNF-4 binding and reduces binding of FTF in the 7α-hydroxylase promoter.

Super shift experiment performed as described in "Materials and Methods" using the indicated probes and in vitro synthesized FTF or HNF-4 proteins or in vitro synthesized GHR as control. The lower panel shows the probes used, with the mutated nucleotides in lower case.
Fig. 5 - Mutation in the DR-1 site completely abolishes HNF-4 binding and increases binding of FTF in the 7α-hydroxylase promoter. Super shift experiment performed as described in “Materials and Methods” using the indicated probes and in vitro synthesized FTF or HNF-4 proteins or in vitro synthesized GHR as control. The lower panel shows the probes used, with the mutated nucleotides in lower case.
Fig. 6 - Mutation in the FTF site of the 7α-hydroxylase promoter completely abolishes FTF binding but has no effect on HNF-4 binding. Supershift experiment performed using the indicated probes and *in vitro* synthesized FTF or HNF-4 proteins or *in vitro* synthesized GHR as control. The lower panel shows the probes used, with the mutated nucleotides in lower case.
lanes 5 and 12). As a negative control we used *in vitro* made GHR, which as expected, did not bind any of the probes.

To study the promoter activity and bile acid mediated regulation, we tested the two DR-1 constructs, pGL3-7α/DR-1 Mut 1 and 2 and the pGL3-7α/FTF construct in tissue culture. We transfected them into primary hepatocytes that were incubated with or without 50 μM taurocholic acid. As expected, the two DR-1 constructs had promoter activity although very less as compared to the wild type construct. Both of these mutants were regulated by bile acids (Fig. 7). Most importantly, the pGL3-7α/FTF construct had minimal promoter activity but was not regulated by taurocholic acid, which support earlier studies (81) that suggests that FTF is required for the regulation of 7α-hydroxylase.

**12α-Hydroxylase Promoter Mutants**

In the introduction the importance of FTF binding for 12α-hydroxylase promoter activity and bile acid mediated regulation have been explained (41, 74). These studies were done by creating mutants that would bind FTF but not HNF-4 in the rat 12α-hydroxylase promoter. However, a mutant that would bind HNF-4 but not FTF was not
**Fig. 7 - Expression and bile acid-mediated regulation of 7α-hydroxylase promoter/luciferase DR-1 and FTF mutants in primary hepatocytes.**

Primary rat hepatocytes were transfected with the indicated constructs and treated with taurocholic acid as described in “Materials and Methods”. The data were normalized to the activity produced by the wild type construct, pGL312α-342, in cells grown in the absence of taurocholic acid and represent the averages of n experiments ± S.D. Lowercase refers to the promoter sections mutated as shown in the figure.
created which could provide important insights whether HNF-4 is involved in the bile acid regulation of the 12α-hydroxylase promoter.

To accomplish this, we used the 12α-hydroxylase FTF consensus mutant (74) as the template to create the 12α-hydroxylase FTF Mut 1 (Fig. 8, lower panel). To construct this mutant we first re-created the 7α-hydroxylase DR-1 site in the FTF consensus and then mutated the FTF site. This mutant probe does not bind FTF, as confirmed by performing supershift assay using anti-FTF antibody (Fig. 8, lanes 4 and 11). HNF-4 binding is also less using this mutant probe (Fig. 8, lanes 5 and 12), probably due to mutations in the actual 12α-hydroxylase DR-1 site.

When tested in cell culture, this promoter construct pGL312α-865/FTF Mut 1 had no detectable activity which suggests that FTF is required for the 12α-hydroxylase promoter activity and HNF-4 binding alone is not sufficient for activity of the 12α-hydroxylase promoter.

The BARE Site in the 12α-Hydroxylase Promoter can be Substituted by the BARE Site of the 7α-Hydroxylase Promoter
Fig. 8 - Mutation in the FTF site of the 12α-hydroxylase promoter completely abolishes FTF binding and significantly reduces binding of HNF-4. A Supershift experiment performed using the indicated probes, antibodies, and in vitro synthesized FTF or HNF-4 proteins or in vitro synthesized GHR as control. The lower panel shows the probes used, with the mutated nucleotides in lower case.
Fig. 9. Expression and bile acid-mediated regulation of 12α-hydroxylase promoter/luciferase DR-1 and FTF mutants in Hep G2 cells.

Hep G2 cells were transfected with the indicated constructs and treated with chenodeoxycholic acid as described in "Materials and Methods". The data were normalized to the activity produced by the wild type construct, pGL312α-865, in cells grown in the absence of chenodeoxycholic acid and represent the averages of n experiments ± S.D. Lowercase refers to the promoter sections mutated as shown in the figure.
In the 12α-hydroxylase FTF Mut 1 shown above (Fig. 9) the HNF-4 site was placed 5’ region of its wild type position in the 12α-hydroxylase promoter and thus, the results obtained might have been affected by this shifting. So the next approach was to create a promoter mutant that had the 7α-hydroxylase BARE exactly on the 12α-hydroxylase BARE. This was achieved by placing the 7α-hydroxylase FTF/HNF-4 site on the exact FTF/HNF-4 site of the 12α-hydroxylase promoter (Fig. 10, lower panel). This mutant should also allow us to study whether the 7α-hydroxylase and the 12α-hydroxylase BARE’s are exchangeable. To check and confirm the binding of FTF and HNF-4, supershift assay was performed with the respective antibodies using in vitro made FTF and HNF-4 with the wild-type and the 12α-Hyd/7α Site Substitution probes. As expected, FTF and HNF-4 bound to the wild-type probe as well as to the 12α-Hyd/7α Site Substitution probe. Although the HNF-4 binding to the 12α-Hyd/7α Site Substitution probe was similar to the wild-type probe (Fig. 10, lanes 5 and 12), FTF binding was much more pronounced as compared to the 12α-Hyd wild type probe (Fig. 10, lanes 2 and 9).
Interestingly, when this promoter mutant was tested in tissue culture the promoter activity and the bile acid mediated regulation of this construct was similar to the 12α-hydroxylase wild-type promoter construct (Fig. 12).

The next step in this study was to create a FTF mutant in the 12α-hydroxylase promoter by mutating the FTF site within the 12α-Hyd/7α substitution probe (Fig. 11, lower panel). To check and confirm the binding of FTF and HNF-4 we performed a supershift assay using in vitro made GHR, FTF, and HNF-4. Figure 11 shows that FTF does not bind to this mutant probe whereas the HNF-4 binding was similar to the 12α-Hyd/7α substitution probe.

Tissue culture experiments performed using this mutant had no detectable activity (Fig. 12), which once again confirms that FTF is crucial for 12α-hydroxylase promoter activity. By creating this mutant we rule out that HNF-4 binding alone can mediate bile acid mediated regulation of 12α-hydroxylase at least in tissue culture (81) (92).
Fig. 10. - FTF and HNF-4 both bind to the 12α-Hyd 7α Site Substitution probe, in which the BARE of the 7α-hydroxylase is placed on the BARE of the 12α-hydroxylase promoter. Gel shift experiment performed using the indicated probes, antibodies, and *in vitro* synthesized FTF or HNF-4 proteins or *in vitro* synthesized GHR as control. The shaded area shows the site substitution.
Fig. 11 - Mutation within the FTF site of the 12α-Hyd 7α Site Sub completely abolishes FTF binding and significantly reduces binding of HNF-4 in the 12α-hydroxylase promoter. Gel shift experiment performed using the indicated probes, antibodies, and in vitro synthesized FTF or HNF-4 proteins or in vitro synthesized GHR as control.
Fig. 12 - Expression and bile acid-mediated regulation of the 12α-hydroxylase promoter/luciferase 12α-Hyd 7α Site Sub and the 12α-Hyd 7α Site Sub FTF Mut in HepG2 cells. HepG2 cells were transfected with the indicated constructs and treated with chenodeoxycholic acid as described in “Materials and Methods”. The data were normalized to the activity produced by the wild type construct, pGL312a-865, in cells grown in the absence of chenodeoxycholic acid and represent the averages of n experiments ± S.D. Lower case refers to the mutated promoter sections and the highlighted area shows the substitution.
In vivo Overexpression Studies

To demonstrate the involvement of HNF-4 in the transcription of key genes implicated in bile acid biosynthesis and regulation we carried out in vivo overexpression studies in mouse using adenovirus system. We used adenovirus carrying either HNF-4-myc or HNF-4 cDNA driven by the CMV promoter. In all the experiments, a control adenovirus containing the bacterial galactosidase coding sequence in front of the CMV promoter (Ad-Gal) was also injected.

In the first experiment male mice were injected with either $5 \times 10^{10}$ or $3 \times 10^{10}$ viral particles of an adenovirus containing human HNF-4-myc cDNA in front of the CMV promoter (Ad-HNF-4-myc). Five days later these two sets of mice together with the control mice that had been injected with $5 \times 10^{10}$ viral particles of Ad-Gal were sacrificed and their livers harvested. The body weight of these animals did not change as a result of viral injections, and all the mice had similar weights between 22 and 25 g. Liver RNA was isolated and used to quantify 7α-, 12α- hydroxylase, LDL receptor, HMG Co-A, Apo AI, and Apo CIII mRNAs by TaqMan Q-RT-PCR. Actin RNA was
quantified and used to normalize RNA values. Interestingly, the $12\alpha$-hydroxylase mRNA was suppressed approximately 5- and 10-fold with the lower and higher injection of Ad-HNF-4-myc, respectively. Whereas the $7\alpha$-hydroxylase mRNA was minimally affected by the lower dose injection but there was no effect with the higher dose of the Ad-HNF-4-myc (Table 3 B).

The adenovirus infection with Ad-HNF-4-myc described above produced considerable liver toxicity based on the plasma levels of liver marker enzymes such as alkaline phosphate, alanine aminotransferase and aspartate aminotransferase (Table 3 A).

We performed a similar overexpression experiment with $4 \times 10^{10}$ viral particles and the harvested the mice after 4 and 5 days. Quantification of the $12\alpha$-hydroxylase mRNA by TaqMan Q-RT-PCR showed approximately 10 fold suppression in the group of mice that were harvested after 4 days of injection. The $12\alpha$-hydroxylase mRNA quantification of the other group which was harvested after 5 days showed less suppression (~ 5-fold) (Table 3 B).
Table 3. HNF-4-myc overexpression suppresses the 12α-hydroxylase gene in mice.

A, Clinical laboratory report to analyze the changes in total cholesterol, and liver enzymes B, 12α-hydroxylase, 7α-hydroxylase, LDL receptor, HMG CoA reductase, Apo AI, and Apo CIII mRNA quantification by RT-PCR as described in “Materials and Methods”.

<table>
<thead>
<tr>
<th>Condition</th>
<th>12α-Hyd</th>
<th>7α-Hyd</th>
<th>LDL RT</th>
<th>HMG Red</th>
<th>Apo AI</th>
<th>Apo CIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-gal (5X10^10)</td>
<td>100 ± 6</td>
<td>100 ± 6</td>
<td>100 ± 10</td>
<td>129.5 ± 31</td>
<td>100 ± 3</td>
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<td>4 Days</td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>HNF-4(5X10^10)</td>
<td>0.12 ± 0.04</td>
<td>100 ± 31</td>
<td>11 ± 2</td>
<td>100 ± 35</td>
<td>99 ± 28</td>
<td>8.7 ± 3.2</td>
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<td>4 Days</td>
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<tr>
<td>HNF-4(3X10^10)</td>
<td>20 ± 12</td>
<td>97.5 ± 19</td>
<td>17 ± 6</td>
<td>100 ± 26</td>
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### A

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<td>ALK</td>
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<td>AST</td>
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<tr>
<td>β-gal (4x10^10)</td>
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<td>34 ± 4</td>
<td>48 ± 3</td>
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<td>4 Days</td>
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<tr>
<td>HNF4-myc (4x10^10)</td>
<td>636 ± 182</td>
<td>7343 ± 301</td>
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<tr>
<td>4 Days</td>
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<tr>
<td>β-gal (4x10^10)</td>
<td>183 ± 3</td>
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<td>48 ± 2</td>
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<td>5 Days</td>
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<td></td>
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<tr>
<td>HNF4-myc (4x10^10)</td>
<td>639 ± 340</td>
<td>2019 ± 480</td>
<td>1129 ± 511</td>
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<tr>
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### B

<table>
<thead>
<tr>
<th>Condition</th>
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<tbody>
<tr>
<td>Wild Type</td>
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<tr>
<td>β-gal (4x10^10)</td>
<td>96 ± 0.5</td>
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<tr>
<td>HNF4-myc (4x10^10)</td>
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<td>β-gal (4x10^10)</td>
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<td>HNF4-myc (4x10^10)</td>
<td>20 ± 3</td>
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<td>5 Days</td>
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</tbody>
</table>

### C

![Western blotting using liver nuclear extracts prepared from mice injected with gal (4 x 10^10) and HNF-4-myc adenovirus and (4 x 10^10) for 4 and 5 days.](image)

**Fig. 13.** HNF-4-myc overexpression suppresses the 12α-hydroxylase gene in mice.

A. Clinical laboratory report to analyze the changes in total cholesterol, and liver enzymes B. 12α-hydroxylase mRNA quantification by RT-PCR as described in “Materials and Methods”. C. Western blotting using liver nuclear extracts prepared from mice injected with gal (4 x 10^10) and HNF-4-myc adenovirus and (4 x 10^10) for 4 and 5 days.
However, under this condition also the liver marker enzymes were still affected by the virus infection (Fig. 13 A) and the marked increase in the liver enzymes might influence the mRNA level of 12α-hydroxylase gene.

To analyze the amount of HNF-4 and myc in the liver we performed western blotting with liver nuclear extracts prepared from the livers of the harvested mice. In vitro HNF-4 was used as a positive control to identify the actual HNF-4 band, which suggests that these two bands might correspond to the abnormal processing of the overexpressed protein. The 4 days Ad-HNF-4-myc injected liver nuclear extract show two different bands with anti-HNF-4 antibody, and none of which correspond to the actual HNF-4 band. The 5 days Ad-HNF-4-myc injected liver nuclear extract showed only one partial band not corresponding to the actual HNF-4 band (Fig. 13 C). The myc was much higher in the 4 days Ad-HNF-4-myc injected liver nuclear extract than in the 5 days Ad-HNF-4-myc injected liver nuclear extract (Fig. 13 C).

In an attempt to avoid this toxicity, we performed another overexpression experiment but with Ad-HNF-4 without the myc tag. We injected two different amounts of Ad-HNF-4, 1 x 10^{11} and 4 x 10^{10}, sacrificed the mice after 4 days and harvested the livers. Control Ad-Gal virus was injected to another set of mice in this group. Quite
### Liver Enzymes

<table>
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<tr>
<th>Condition</th>
<th>ALK (IU/L)</th>
<th>ALT (IU/L)</th>
<th>AST (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type 4 Days</td>
<td>109 ± 12</td>
<td>38 ± 3</td>
<td>52 ± 3</td>
</tr>
<tr>
<td>β-gal (4x10^6) 4 Days</td>
<td>107 ± 8</td>
<td>45 ± 9</td>
<td>53 ± 4</td>
</tr>
<tr>
<td>HNF-4 (1X10^11) 4 Days</td>
<td>441 ± 51</td>
<td>1481 ± 398</td>
<td>1443 ± 348</td>
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<tr>
<td>HNF-4 (4X10^9) 4 Days</td>
<td>120 ± 4</td>
<td>95 ± 49</td>
<td>102 ± 39</td>
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### 12α-hydroxylase mRNA

<table>
<thead>
<tr>
<th>Condition</th>
<th>12α-hydroxylase mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>β-gal (1X10^11) 4 Days</td>
<td>104 ± 1.2</td>
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<tr>
<td>HNF-4 (1X10^11) 4 Days</td>
<td>14 ± 1.5</td>
</tr>
<tr>
<td>HNF-4 (4X10^9) 4 Days</td>
<td>129 ± 0.2</td>
</tr>
</tbody>
</table>

### Western Blotting

**Fig. 14.** HNF-4 overexpression suppresses 12α-hydroxylase gene in mice.

A. Clinical laboratory report to analyze the changes in total cholesterol, triglycerides, HDL and liver enzymes. B. 12α-hydroxylase mRNA quantification by RT-PCR as described in “Materials and Methods”. C. Western blotting using liver nuclear extracts prepared from wild type mice, mice injected with gal (4 x 10^10) and HNF-4 adenovirus (1 x 10^11) and (4 x 10^10) for 4 days.
interestingly, there was suppression of 12α-hydroxylase mRNA with only the higher amount of the Ad-HNF-4 and with the lower amount of the Ad-HNF-4 there was an increase in the 12α-hydroxylase mRNA level. The level of 12α-hydroxylase mRNA in Ad-Gal was slightly increased. These overexpression study results with the AdHNF-4-myc and AdHNF-4, does suggest that HNF-4 might play an important role in regulating the 12α-hydroxylase gene transcription.

The adenovirus infection with 4 x 10^{10} produced limited liver toxicity, based on the liver marker enzymes but infection with 1 x 10^{11} produced considerable toxicity (Fig.14 A). The suppression of 12α-hydroxylase mRNA might be attributed to the increased liver toxicity and hence more experiments needs to be done with lower amounts of virus to confirm the above results.

The increase in HNF-4 protein was analyzed by performing a western blot using liver nuclear extracts prepared from adenovirus infected mice (Fig.14 C). The blot shows that there is ~10 fold increase in HNF-4 protein in the liver extracts of mice injected with 1 x 10^{11} viral particles as compared to the non-injected wild-type mice.

A potential explanation for the observation made after HNF-4 overexpression is that the HNF-4 might be acting as a dominant negative HNF-4 mutant due either to the
myc tag and/or to some potential mutation introduced in the overexpression constructs and hence the lower levels of mRNA of target genes. To characterize whether indeed that was the case we used the human apo CIII gene promoter which contains a well characterized HNF-4 binding site (57). We co-transfected this promoter with different amounts (Table 4) of either pZeroHNF-4-myc or pZeroHNF-4 expression plasmids. Table 4 shows that both expression plasmids caused a strong activation of the apo CIII promoter. The activation of apo CIII promoter was much more pronounced when the pZeroHNF-4-myc plasmid was used as compared to the pZeroHNF-4 plasmid (Table 4). These results, together with the high levels of liver enzyme observed in the plasma (Table 3 and Fig. 14) suggests that the suppression caused by overexpression of HNF-4 in mice is due to the inflammatory effect of the virus and not by the overexpressed genes acting as dominant negative mutants.
Table 4 - ApoCIII promoter activity is elevated by both HNF-4-myc and HNF-4. HepG2 cells were co-transfected with increasing amounts of expression vectors producing HNF-4-myc or HNF-4 (amounts indicated in the table) together with the wild-type ApoCIII promoter-luciferase reporter, pLZ854. Cells were harvested 36 hrs post transfection.

<table>
<thead>
<tr>
<th>Reporter Plasmid</th>
<th>Expression Plasmid</th>
<th>Promoter Activity</th>
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</thead>
<tbody>
<tr>
<td>pLZ854/ApoCIII</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>pZeroHNF-4-myc 10ng</td>
<td>947 ± 149 (2)</td>
<td></td>
</tr>
<tr>
<td>pZeroHNF-4-myc 25ng</td>
<td>1148 ± 536 (2)</td>
<td></td>
</tr>
<tr>
<td>pZeroHNF-4-myc 100ng</td>
<td>3021 ± 1102 (2)</td>
<td></td>
</tr>
<tr>
<td>pZeroHNF-4 10ng</td>
<td>56 ± 14 (2)</td>
<td></td>
</tr>
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<td>pZeroHNF-4 25ng</td>
<td>342 ± 98 (2)</td>
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<tr>
<td>pZeroHNF-4 100ng</td>
<td>1117 ± 486 (2)</td>
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DISCUSSION

Bile acids regulate their own synthesis through a feedback regulatory pathway. In the early 1990's it was shown that the regulatory responses of 7α-hydroxylase are mediated at the transcriptional level (4, 94) and since that time, the transcription factors that mediate positive and negative feedback regulation of bile acid synthesis have been identified. An important finding was that the transcription factors regulating the expression of 7α- and 12α-hydroxylase genes are nuclear receptors (95).

One of the first nuclear receptors identified to play a role in the transcription of the 7α-hydroxylase gene was HNF-4 (96), since then many nuclear receptors have been identified that are involved in regulating the 7α-hydroxylase gene. Among them the most important is FTF which has been shown not only to be required for the transcription of the 7α-hydroxylase gene (36) but also for its regulation by bile acids through dimerization with SHP (78).

The second important enzyme in the neutral pathway is the 12α-hydroxylase and the expression of this gene is also regulated by bile acids and cholesterol (7). Our laboratory first showed that FTF is essential for 12α-hydroxylase transcription (41) and is also required for bile acid-mediated suppression by the FTF/SHP pathway (74).
In previous studies, to determine the roles of HNF-4 and FTF on the transcription and regulation of 7α-hydroxylase gene experiments were performed by creating promoter mutants by either mutating the DR-1 site or the FTF site. Results from these tissue culture studies indicated that FTF is essential for activity and bile acid-mediated regulation of the 7α-hydroxylase promoter (81). However, in these studies binding of HNF-4 or FTF to the respective mutants was not shown and it was unclear whether FTF was able to bind to the DR-1 mutant and whether HNF-4 was able to bind to the FTF mutant. Similarly, to characterize the 12α-hydroxylase promoter studies were performed by creating promoter mutants within the 12α-hydroxylase promoter lacking the DR-1 site (74). These promoter mutants had no detectable activity suggesting that HNF-4 binding is crucial for the activity and perhaps regulation of 12α-hydroxylase expression (74). However, all the mutants that did not bind HNF-4 could not bind FTF either. No mutant could be created that would bind HNF-4 but not FTF in these studies, and thus the role of each of these factors could not be studied independent of each other.

The first objective of this study was to further explore the role of both FTF and HNF-4 independent of each other in the transcription and bile acid-mediated regulation of the 7α- and 12α-hydroxylase genes, by creating promoter mutants that would bind only HNF-4 or FTF. Secondly, due to the similarities between the BARE’s of the 7α- and 12α-hydroxylase, we wanted to study whether one site can be exchanged for the other.
Finally, to investigate the role of HNF-4 in the 12α-hydroxylase expression *in vivo* we performed adenovirus overexpression studies in mice.

In the experiments shown in this thesis, we were able to create 7α-hydroxylase promoter mutants that bound either HNF-4 or FTF only (Fig. 4, 5, 6). Our studies show that within the 7α-hydroxylase promoter the HNF-4 site is important for activity (although not absolutely required) but is not required for bile acid-mediated regulation. The two DR-1 mutants in the 7α-hydroxylase promoter (Fig. 7) show lower activity (30%-40%) as compared to the wild type promoter, but most importantly these results indicate that the promoter is still regulated similar to the wild type promoter by bile acids. These results are in agreement with a recent publication that shows *in vivo* studies preformed in a conditional HNF-4 knockout mice in which the expression of 7α-hydroxylase mRNA is reduced in the dark cycle, indicating that HNF-4 controls 7α-hydroxylase gene expression (97). The FTF mutant created within the 7α-hydroxylase promoter binds HNF-4 similar to the wild type promoter (Fig. 6) but when tested in tissue culture this promoter mutant showed a dramatic reduction in promoter activity and no regulation by bile acids (Fig. 7), suggesting that FTF acts as a crucial factor for the promoter activity and bile acid-mediated regulation of 7α-hydroxylase transcription. In summary HNF-4 is important for 7α-hydroxylase expression but is not required, whereas FTF is essentially required.
With respect to the 12α-hydroxylase promoter we were successful in creating a promoter mutant that would bind HNF-4 but not FTF within the 12α-hydroxylase promoter, called 12α-Hyd FTF Mut (Fig. 8), to analyze the role of HNF-4 in 12α-hydroxylase expression. We started by performing tissue culture experiments with the 12α-Hyd-DR-1 Mut/FTF consensus promoter construct which does not bind HNF-4 but binds FTF (74). Results from these experiments indicate that HNF-4 binding is crucial for 12α-hydroxylase promoter activity (Fig. 9) since this promoter showed no activity. Next we performed tissue experiments using the 12α-Hyd FTF Mut (this mutant binds HNF-4 but not FTF), and results show no activity of the 12α-hydroxylase promoter indicating that FTF is required for activity. Thus, in the 12α-hydroxylase gene, both FTF and HNF-4 are required for transcription (Fig. 9).

Due to the overlapping of their binding sites (Fig. 2) FTF and HNF-4 could not bind simultaneously to the same 12α-hydroxylase promoter molecule if they bind as a monomer (FTF) and as a dimer (HNF-4), which is the current belief for other FTF-activated genes such as AFP (36) and HNF-4 activated genes such as apoCIII (61). However, the heterodimer FTF/HNF-4 could bind since the 5’ FTF site (GCAAGGTCC) would be spaced by one nucleotide (A) from the HNF-4 3’ repeat (AGGGCA) (Fig. 2). This arrangement for FTF/HNF-4 is similar to most nuclear receptor binding sites, which consists of two repeats, either direct or reverse, spaced by 1 or 5 nucleotides depending on
the specific receptor and gene (13). We have some evidence that the binding of FTF and HNF-4 to the 12α-hydroxylase promoter is different when liver nuclear extracts are used, than when we use *in vitro* made proteins (Fig. 3). When liver nuclear extracts were used the DNA-protein complexes migrates much slower than FTF monomer, which suggests that FTF/HNF-4 might be binding to the 12α-hydroxylase promoter as heterodimer.

Since the BARE of 7α- and 12α-hydroxylase promoter are quite similar as expected for the two genes that are coordinately regulated by bile acids in this report we provide evidence that one site can be exchanged for the other. We substituted the BARE site in the 12α-hydroxylase promoter by the BARE site of the 7α-hydroxylase promoter (Fig. 10). Experiments performed in tissue culture using this promoter construct had very similar promoter activity and bile acid-mediated regulation as the wild type 12α-hydroxylase promoter construct, suggesting that one site can indeed be exchanged for the other (Fig. 12). We then created a mutant in the "hybrid" promoter which will eliminate the FTF site but still bind HNF-4 (Fig. 11), called 12α-Hyd/7α Site Substitution FTF Mut and tested this mutant in tissue culture. The results from the 12α-Hyd/7α Site Substitution FTF Mut, once again confirms that FTF is absolutely required for the promoter activity and bile acid-mediated regulation of 12α-hydroxylase (Fig. 12).
To characterize the role of HNF-4 in vivo we performed overexpression studies in mice using an adenovirus system. The data from the HNF-4-myc and HNF-4 viral injection studies suggests that overexpression of HNF-4 might suppress 12α-hydroxylase mRNA but has minimal effect on 7α-hydroxylase mRNA (Table 3). A potential explanation for this result is that HNF-4 needs to be post-translationally modified (like phosphorylation) to be active. Overproduction of HNF-4 thus might be acting as a dominant negative factor since the over expressed HNF-4 might not be post-translationally modified by overwhelming the modification machinery. Another explanation is the high liver toxicity of the injected virus as evidenced by elevated liver enzymes, might have affected the results and hence more experiments need to be done to show which one of these two possibilities is correct, and then study whether HNF-4 is indeed involved in suppressing the 12α-hydroxylase mRNA.

In summary, this study draws the following conclusions:

1. HNF-4 is important for transcription of 7α-hydroxylase, although it is not required.

2. HNF-4 does not appear to be required for the bile acid-mediated regulation of 7α-hydroxylase.
3. Both FTF and HNF-4 are absolutely required for the expression of 12α-hydroxylase.

4. The 7α-hydroxylase and the 12α-hydroxylase FTF/HNF-4 sites can be exchanged for each other, maintaining both activity and regulation.
REFERENCES


VITA

Preeti Pramanik was born and brought up in India. She is currently a permanent resident of the United States of America and resides in Richmond, Virginia with her husband.

She has her bachelor’s and a master’s degree from Nagpur University, Nagpur, India. She also worked as a marketing and sales executive in GlaxoSmithkline India Ltd for approximately 2 years.