Cloning, Expression and Regulation of CYP3A10, a Hamster Liver Cytochrome P450 Involved in Lithocholic Acid and Steroid 6β-Hydroxylation: a Dissertation

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Cloning, Expression And Regulation Of CYP3A10, A Hamster Liver Cytochrome P450 Involved In Lithocholic Acid And Steroid 6β-Hydroxylation

A Dissertation Presented

by

Jose Manuel Teixeira

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in Partial Fulfillment of the requirements for the degree of:

Doctor of Philosophy in Biochemistry and Molecular Biology

January, 1994
Cloning, Expression And Regulation Of CYP3A10, A Hamster Liver Cytochrome P450 Involved In Lithocholic Acid And Steroid 6β-Hydroxylation

A Thesis
By
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January, 1994
Dedicated

In Loving Memory To

My Father Who So Longed To See This Day

And To

My Mother For Her Love And Support
ACKNOWLEDGEMENTS

I would like to thank the following people for their help and support.

**Gregorio Gil**, for allowing me to do the following work in his lab and for teaching me how to be a scientist.

**Erica Selva**, for help with the initial characterization of the λ genomic clone of the CYP3A10 gene, for many helpful discussions regarding many aspects of biology and life and for that occasional shoulder to cry on. I cannot thank her enough for the many times I needed a friend and she was there.

**Carlos B. Hirschberg**, for being that scientific father figure everyone needs at various times.

All my fellow coworkers in the lab, especially **Diego Haro, Diane Magada, Roya Bashirzadeh, Carmen Barillas, Denise Karaoglu and Marisa Ramirez**, for the many inspired moments of laughter and kindness that I will cherish. I will remember you always.

**Denise, Annette and Karen** for all the good times. You have made my days in the department a lot more fun.

I am very grateful to **Craig Ferris** from the Department of Psychiatry, University of Massachusetts Medical School, without whose help the neonatal castrations could not have been done and for helpful discussions.

I'd like to acknowledge the **March of Dimes Research Foundation** for four years of generous support.
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 Portions of this thesis have been published in:


ABSTRACT

Bile acid metabolism is integrally involved in cholesterol homeostasis in mammals because it is the major means by which cholesterol is eliminated from the body. We have undertaken an effort to study the molecular mechanisms underlying the regulation of bile acid metabolism by isolating and characterizing the cDNA and gene for an enzyme that hydroxylates lithocholic acid (LCA) at position 6β, lithocholic acid 6β-hydroxylase; the first bile acid-induced gene reported. LCA is a very hydrophobic, toxic bile acid formed from chenodeoxycholic acid in the gut lumen upon reduction of the 7α-hydroxy group by microbial enzymes. The proper elimination of LCA is essential for maintenance of the bile acid pool and for prevention of cholestasis which results from LCA precipitating in the cannaculi of the liver when its concentration is high.

The LCA 6β-hydroxylase cDNA was isolated by differential hybridization of hamster liver libraries prepared from animals fed either a cholic acid enriched diet or a cholestipol-rich chow and was named CYP3A10 based on its homology with other cytochrome P450s (P450) in family 3A. We found that CYP3A10 was essentially expressed only in males. A statistical analysis of RNA from young males fed with cholic acid and normal chow showed that the cholic acid induction was about 50% at the RNA level.

We determined the biological nature of the protein encoded by CYP3A10 by expression of the cDNA in COS cells. Microsomes prepared from transfected cells were assayed with LCA as a substrate and found to hydroxylate LCA predominantly at position 6β. We examined whether CYP3A10 could hydroxylate other steroid compounds by assays with testosterone, progesterone and androstenedione and found that, although 6β-hydroxylase (as well as others) activity was observed with all three, LCA was the preferred substrate based on kinetic analysis.
A developmental time course of CYP3A10 expression in males showed little expression before puberty, a striking induction of expression at puberty and a fourfold induction thereafter through adulthood. We then examined the male-specific expression of CYP3A10 in hamster liver. We disrupted the pattern of GH secretion in male hamsters by hypophysectomy, neonatal glutamate treatment and by continuous infusion of GH via osmotic minipumps (to mimic the female pattern of GH secretion) and found no significant effect on CYP3A10 expression. Conversely, in females, hypophysectomy and neonatal glutamate treatment significantly induced CYP3A10 expression 5- to 10-fold. Additionally, when females treated neonatally with glutamate were injected twice daily with GH as adults (to mimic the male pattern of GH secretion), the levels of CYP3A10 expression were not significantly different from those of normal males. These results led us to conclude that the pattern of GH secretion in males does not control the male-specific expression of CYP3A10 but that in females expression can be induced by altering the tonic secretion of GH. No significant effect on CYP3A10 expression was observed by castration of adult males, indicating that circulating androgens were not required for expression. We found that gonadal hormones (e.g. estrogen and progesterone) do not have a suppressive effect on CYP3A10 expression in females since ovariectomy did not induce expression.

Many genes are "imprinted" neonatally by exposure to a given effector for developmental-, tissue- or sexually regulated expression. We investigated whether neonatal androgen exposure was required for male-specific expression of CYP3A10 by castrating hamsters neonatally and determining the level of CYP3A10 expression in adulthood. Our results indicate that androgens are required neonatally for CYP3A10 expression since no expression was observed in neonatally castrated hamsters. We were unable to induce expression in neonatally castrated hamsters by either GH or testosterone injections. These results suggest several notable points 1) that CYP3A10 expression is programmed neonatally by androgen exposure; 2) that androgens exert their effect directly
on the liver and not via the hypothalamus; 3) that neither testosterone nor GH can restore CYP3A10 expression when males have not been exposed to androgens neonatally; and 4) that in experimental conditions, females can be induced to express CYP3A10, which indicates that there are two modes for regulating expression: by "imprinting" in males and by GH and testosterone in females.

We are now studying the molecular mechanisms involved in the bile acid-mediated induction and the male-specific expression of CYP3A10. We have cloned approximately 8 kb of 5' flanking DNA from a hamster genomic library and sequenced about 1 kb of proximal DNA. Primer extension and S1 digestion analyses indicate that the mRNA for CYP3A10 has multiple transcription initiation sites clustered about 90 bp from the initiator methionine codon. We have also prepared CYP3A10 promoter/lacZ chimeric constructs to begin delineating the cis-acting elements controlling CYP3A10 expression and regulation. We used H2.35 cells as recipients because they are a mouse hepatocyte cell line that has been transformed with a temperature sensitive SV40. These cells can be grown at the permissive temperature and can be induced to behave like liver cells, the differentiated condition, by switching to a nonpermissive temperature. We have found that the construct with 1 kb of proximal CYP3A10 5' flanking DNA was able to express the reporter gene at higher levels under differentiated conditions, which were consistent with higher expression of an albumin promoter/lacZ construct, upon switching the cells to the more liver phenotype.

The system characterized and described here is ideally suited for dissecting the molecular details governing bile acid-mediated regulation and sexually dimorphic expression of liver genes. Very little is known about both these very important biological phenomena. Much could be learned about transcriptional regulation of liver genes by investigating the cis-elements and trans-acting factors mediating regulation of CYP3A10 expression.
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List of Abbreviations Used

1 x SSC, 150 mM NaCl/15 mM NaCitrate
6α-hydroxylase, lithocholic acid 6α-hydroxylase
6β-hydroxylase, lithocholic acid 6β-hydroxylase
7α-hydroxylase, cholesterol 7α-monooxygenase
bp, base pair(s)
BM, basal medium
CMV, cytomegalovirus
DMEM, Dulbecco’s modified Eagle’s medium
DTT, dithiothreitol
EDTA, ethylenediaminetetraacetic acid
GH, growth hormone
HEPES, N-2-hydroxyethylpiperazine-N’-2-ethane-sulfonic acid
HMG CoA, 3-hydroxy-3-methylglutaryl-coenzyme A
HPLC, high performance liquid chromatography
Hx, Hypophysectomy
Inr, initiator element
kb, kilobase(s)
LCA, lithocholic acid
LDL, low density lipoprotein
MSG, monosodium glutamte
Ovx, Ovariectomy
P450, cytochrome P450
P450 reductase, NADPH-dependent cytochrome P450 reductase
RIA, radioimmunoassay
SDS, sodium dodecyl sulfate
S.E.M., standard error of the mean
SFM, serum free media
SV40, simian virus 40
TBP, TATA-Binding Protein
TLC, thin layer chromatography
CHAPTER I
Introduction
Cholesterol is an essential constituent of animal cells. It plays a vital role in biological membranes by introducing rigidity in the lipid bilayer while enhancing its flexibility and mechanical stability. It is also the immediate precursor of a number of steroid hormones, vitamin D and bile acids. In mammals, cholesterol homeostasis is maintained by the conjunction of three finely tuned metabolic pathways (see Figure 1). In the first pathway, cholesterol is synthesized de novo from acetate precursors (Goldstein and Brown, 1990). This biosynthetic pathway is regulated largely by the activity of 3-hydroxy, 3-methylglutaryl CoA (HMG-CoA) reductase, the rate-limiting enzyme controlled by end-product suppression that catalyzes the synthesis of mevalonate from HMG-CoA. All cells are inherently able to synthesize cholesterol by this complex but well-studied metabolic process (Brown and Goldstein, 1980). However, most cellular cholesterol is absorbed from the diet via the activity of low density lipoprotein (LDL) receptors which internalize cholesterol-laden lipoprotein particles from the blood (Brown and Goldstein, 1986). This is the second pathway by which mammals maintain cholesterol homeostasis. It is high levels of serum LDL particles that cause atherosclerotic plaques, a major cause of coronary disease and its consequent morbidity and mortality. The third pathway is a catabolic pathway whereby cholesterol is converted to bile acids in the liver by the activity of cytochrome P450s (P450) (Danielsson and Sjövall, 1975).

The P450 superfamily is a group of hemeproteins that catalyze the oxidation of a variety of endogenous and exogenous substrates, often with broad and overlapping specificities, in concert with NADPH, NADPH-dependent cytochrome P450 reductase and molecular oxygen (Gonzalez, 1989). They were identified in microsomal fractions by a reduced pigment with an absorption band with a $\lambda_{\text{max}}$ at 450 nm after binding carbon
Cholesterol homeostasis is controlled by 3 metabolic pathways. 1) De novo synthesis of cholesterol is feedback inhibited primarily at the level of transcription of the HMG CoA reductase gene and to a lesser extent at the level of transcription of the HMG CoA synthase gene. 2) Uptake of exogenous dietary cholesterol via the activity of the LDL receptor is reduced by excess cholesterol inhibiting transcription of the LDL receptor gene. 3) Bile acid biosynthesis is increased by excess cholesterol exerting a positive effect on the activity of cholesterol 7α-hydroxylase, which catalyzes the first and rate-limiting step in the conversion of cholesterol to bile acids.

Adapted from Goldstein and Brown (Goldstein and Brown, 1990). Figure 1. The Pathways Regulating Cholesterol Homeostasis in Mammals.
Acetyl CoA + Acetoacetyl CoA

Synthase

HMG CoA

Reducease

Mevalonate

Squalene

Cholesterol

LDL Receptor

Steroid Hormones
Vitamin D
Lipoproteins
Bile Acids

Cholesterol 7α-Hydroxylase

Plasma LDL
monoxide. Subsequent analyses indicated that multiple forms of P450s existed in humans and other species. Greater than 220 genes encoding P450s have been described in both eukaryotes and prokaryotes and are classified by sequence homology into several families (Nelson et al., 1993). Many P450s are involved in very different metabolic pathways, such as steroid hormone metabolism (Gonzalez, 1989), drug detoxification (Shimada and Guengerich, 1985; Guengerich, 1987; Shaw et al., 1989) and bile acid synthesis (Björkhem and Danielsson, 1974; Björkhem, 1985). Some P450s are subject to complex endocrine controls that render their expression tissue-, developmental- and/or sex specific (Guengerich, 1991; Guengerich, 1991; Paine, 1991; Porter and Coon, 1991). The molecular mechanisms involved in the regulation of P450 expression are under intense scrutiny but definitive paradigms are sorely lacking.

Among the systems of special interest to us is the involvement of P450s in cholesterol homeostasis; specifically in converting cholesterol to bile acids for their subsequent elimination from the body. Since mammals do not possess enzymes that degrade cholesterol, it must be eliminated via the bile acid metabolic pathway (Dietschy, 1984); 80-90% of intravenously injected cholesterol is catabolized to bile acids (Björkhem, 1985). Bile acids are also involved in the emulsification and passive transport of dietary fat and lipophilic vitamins from the gut lumen into the intestinal absorptive cells. They activate digestive enzymes such as pancreatic lipase and cholesterol esterase. Additionally, atherosclerosis, gallstone disease, and some lipid storage diseases may be affected by the rate of cholesterol elimination via bile acid biosynthesis and excretion (Björkhem, 1985).

The first and rate-limiting step in the conversion of cholesterol to bile acids is catalyzed by cholesterol 7α-hydroxylase (7α-hydroxylase), a liver P450 that is subject to feedback suppression by high levels of bile acids. (see Figure 2) (Russell and Setchell, 1992).
Figure 2. Bile Acid Metabolic Pathway. A highly schematic depiction of the bile acid metabolic pathway is shown. Conversion of cholesterol to 7α-hydroxycholesterol by 7α-hydroxylase is the initial and rate limiting step in bile acid synthesis. 7α-Hydroxycholesterol undergoes subsequent enzymatic conversions, that have been omitted, in its route to becoming the primary bile acids, cholic acid and chenodeoxycholic acid. Secondary bile acids, lithocholic acid and deoxycholic acid, are synthesized in the gut lumen from the primary bile acids by microbial enzymes and are efficiently reabsorbed into the enterohepatic circulation. In the liver these bile acids can be further hydroxylated for facilitated excretion. Shown are the enzymes and known metabolites involved in the 6-hydroxylation of secondary bile acids.
Cholesterol $\rightarrow$ 7α-hydroxycholesterol

Cholic Acid

Cholesterol 7α-hydroxylase

3α,6β,7α,12α-tetrahydroxy-5β-cholan-24-oic acid

Cholic Acid 6β-Hydroxylase

Chenodeoxycholic Acid

Microbial dehydroxylases

Lithocholic Acid

Lithocholic Acid 6β-Hydroxylase

Murideoxycholic Acid

Lithocholic Acid 6α-Hydroxylase

Hyodeoxycholic Acid
Conversely, high levels of cholesterol induce the activity of 7α-hydroxylase. A major advance in the effort to lower human serum cholesterol levels was accomplished by feeding patients bile acid-binding resins which effectively activate 7α-hydroxylase (Dietschy and Wilson, 1970). The genes encoding rat and human 7α-hydroxylase have recently been cloned and exciting progress is being made in the effort to understand the molecular details governing bile acid biosynthesis (Chiang et al., 1990; Jelinek et al., 1990; Jelinek and Russell, 1990; Li et al., 1990; Ramirez et al., 1993).

Cholic acid and chenodeoxycholic acid are the primary bile acids synthesized by a number of hydroxylations of the steroid nucleus and side chain of the cholesterol molecule. These hydroxylations are catalyzed by different liver microsomal cytochrome P450s. Primary bile acids are then converted in the gut lumen by microbial enzymes to secondary bile acids such as lithocholic acid (LCA) and deoxycholic acid by reduction of the 7α-hydroxyl group (Danielsson and Sjövall, 1975).

Cloning, Expression and Regulation of Lithocholic Acid 6β-Hydroxylase

LCA is a toxic bile acid that has been shown to cause cholestasis in experimental animals (Miyai et al., 1975). Indeed, a clinical hallmark of cholestasis is the dramatic increase in serum bile acids, including LCA. The deleterious effects of this accumulation of lithocholic acid, as well as others, can be attenuated by two detoxification pathways found in the liver: oxidative reactions, mainly hydroxylations, and conjugation with sulfuric or glucuronic acids (Summerfield et al., 1976). Hydroxylation of bile acids makes them more hydrophilic and thus easily excreted in urine or feces. LCA can be hydroxylated at position 6 (see Figure 2), either below the plane (α) or above the plane (β), by the enzymes LCA 6α-hydroxylase (6α-hydroxylase) and LCA 6β-hydroxylase (6β-
hydroxylase) (Hofman, 1988). In rats, the major hydroxylated product of LCA is the 6\(\beta\)-metabolite, murideoxycholic acid (Zimniak et al., 1989).

The perturbation of bile acid metabolism can be a consequence of biliary obstruction and alcoholism (Summerfield et al., 1976; Bremmelgaard and Sjövall, 1980), pregnancy (Thomassen, 1979), fetal development (Strandvik and Wikström, 1982), liver disease and unknown genetic factors (Shoda et al., 1990) whereby the levels of 6-hydroxylated products of bile acids are dramatically elevated in serum and urine. The general evidence for this observation is in clinical cholestasis where the 6-hydroxylation of different bile acids leads to their 6\(\alpha\)- and 6\(\beta\)-derivatives. These bile acids are found in substantial amounts in the urine and serum of patients with cholestasis and are not found in normal healthy individuals. It would seem logical that 6-hydroxylation can act as a bile acid induced mechanism for excretion of excess bile acids due to biliary obstruction and liver dysfunction.

In order to better understand how the synthesis and catabolism of bile acids is regulated, and thus, develop a means for studying bile acid metabolism and the perturbation thereof, we are undertaking an effort to study the regulation of different enzymes involved in bile acid metabolism. We have reported the isolation and nucleotide sequence of a full-length cDNA for the enzyme LCA 6\(\beta\)-hydroxylase (Teixeira and Gil, 1991). It was isolated using differential hybridization techniques with RNA prepared from livers of hamsters fed either a normal diet or a diet supplemented with 0.5% (w/w) cholic acid. Computer analysis of the sequence revealed that its deduced amino acid sequence was highly homologous to P450 family 3 proteins. This homology approaches 70% with other members of the family previously isolated from human, rat and rabbit. Because of this homology, the isolated cDNA has been named CYP3A10 according to the recommended nomenclature system (Nelson et al., 1993).
We found that by RNA blot analysis the size of the message, 2.1 kb, corresponds to that predicted from the sequence and that expression of CYP3A10 mRNA is much higher in males than in females. In young males (28 days old) CYP3A10 mRNA was induced by cholic acid feeding. In females, in addition to the much lower expression of CYP3A10, its RNA is not regulated by cholic acid.

To determine the nature of the biological activity encoded by CYP3A10, we performed transient expression experiments in COS cells. We placed CYP3A10 cDNA in the expression vector pCMV that contains the CMV promoter as well as the simian virus 40 (SV40) origin of replication. Microsomes were prepared from the transfected cells and shown to exhibit LCA 6-hydroxylase activity. Consistent with the observed induction of CYP3A10 RNA in male hamster liver by cholic acid feeding, we also observed an induction in 6β-hydroxylation of LCA by microsomes prepared from cholic acid-fed hamster liver.

A more rigorous kinetic analysis of CYP3A10 activity indicates that although LCA may be the preferred substrate ($K_M = 25 \mu M$, $V_{max} = 164 \text{ pmol/min per mg}$) other steroids are efficiently modified by microsomes prepared from COS cells transfected with the CYP3A10 cDNA. We have reported that androstenedione, testosterone and progesterone are converted to their 6β-hydroxy (and to a lesser extent other) metabolites in vitro by expressed CYP3A10 (Chang et al., 1993).
Gonadal Signals that Control the Male-specific Expression of Lithocholic Acid 6β-Hydroxylase, CYP3A10

The sex-specific expression of some liver genes, including but not limited to P450s, appears to be regulated by permutations in growth hormone (GH) and/or gonadal hormone exposure (reviewed in Roy and Chatterjee, 1983; Zaphiropoulos et al., 1989 and Waxman, 1992). In rats, gonadal hormones are thought to exert their effects on the liver by two different modes. Firstly, neonatal androgen exposure affects liver sexual dimorphism indirectly by a poorly understood mechanism called "imprinting" whereby genes may be turned on or off according to the sex of the individual. In the neonate, gonadal hormones program the hypothalamus to direct the pattern of GH release from the pituitary. When adult, males will have a pulsatile profile of GH release with no detectable serum GH, at times, and females will have a relatively stable pattern of GH release and continuous, albeit lower, levels of serum GH. Secondly, adult androgen or estrogen exposure may influence the liver directly or, again, indirectly via the gonadal-hypothalamus-pituitary-liver axis. It is the resultant pattern of GH release, either male- or female-specific and/or the levels of circulating gonadal hormones that are thought to control sexually dimorphic expression of liver genes.

One of the better studied examples of liver P450 sexually dimorphic expression is that of CYP2C11, the major male rat androgen 16α- and 2α-hydroxylase. CYP2C11 is a constitutive male-specific rat liver P450 whose expression is induced at puberty and is dependent on a male-specific pattern of pulsatile GH release from the pituitary. However, CYP2C11 expression can be experimentally induced in females by androgen exposure and suppressed in males by estrogen administration (Janeczko et al., 1990). Some controversy has ensued of late over the role of growth hormone in controlling P450 expression from recent experiments done with dwarf rats, which have no detectable serum
Figure 3. Gonadal Hormones Can Exert Their Effects on Sex-specific Expression Directly on the Liver or Else Via the Gonadal-Hypothalamus-Pituitary-Liver Axis. Neonatal androgens can "imprint" the liver to express male-specific genes in the adult. The hypothalamus is programmed by neonatal androgens to direct the pituitary to release growth hormone in a male-specific, pulsatile pattern or a female-specific, continuous pattern. It is the resulting pattern of growth hormone secretion that controls sexual dimorphic expression of many genes in the liver. In the adult, circulating gonadal hormones can also control liver expression directly either by induction or suppression.
growth hormone, indicating GH has little or no effect on male- or female-specific P450 expression (Bullock et al., 1991). In light of these results, experiments were done, which suggest that, perhaps, the lack of serum GH for extended periods, as found in the pulsatile pattern of male GH release, is necessary for male-specific expression (Waxman et al., 1991). In contrast, CYP2C12 is a female-specific, rat liver steroid sulfate 15β-hydroxylase whose expression is induced at puberty and is controlled by the obligate female pattern of GH release from the pituitary (Kato et al., 1986; MacGeoch et al., 1987). Additionally, several other sex-specific P450s are not regulated at all by GH from results of experiments performed with hypophysectomized (Hx) rats (Waxman et al., 1988; McClellan-Green et al., 1989). The molecular mechanisms governing sexually dimorphic expression have yet to be elucidated.

In order to begin addressing the nature of the in vivo effector of male-specific expression of CYP3A10, we have determined the developmental time course of expression in male and female hamsters and found that the gene was turned on at puberty in males and rose to its highest level at sexual maturity. In females, there was essentially no expression at any age. We have performed experiments with adult hypophysectomized males and have shown that hypophysectomy does not appear to alter the level of CYP3A10 expression in males. Additionally, no significant difference in CYP3A10 expression was observed when GH was administered by osmotic minipumps to males, which mimics the female pattern of GH secretion. We have also performed experiments with animals treated neonatally with glutamate. Neonatal administration of glutamate partially destroys the hypothalamus thus reducing the level of circulating growth hormone. Glutamate treated female hamsters had much higher levels of CYP3A10 mRNA expression when adult and, most interestingly, that level of expression was not significantly different from male levels when similarly treated females were injected with GH twice daily, mimicking the pattern of male growth
hormone release. No detectable difference was seen in corresponding experiments with males.

Experiments were also done to determine whether the lack of expression in females is due to suppressive effects of estrogen or to the absence of circulating testosterone. Estradiol when injected into adult males does not reduce the level of expression to female levels and ovariectomy of females does not result in CYP3A10 expression. Females, when treated with daily injections of testosterone, expressed CYP3A10 at a level twofold higher than untreated females.

Adult castration does not affect the levels of CYP3A10 mRNA expression in males, which indicates that circulating gonadal hormones are not required. Experiments with animals castrated neonatally indicate that the level of CYP3A10 mRNA is greatly reduced when these animals reach adulthood. These results suggest that perhaps sometime before puberty, the gene is turned on in males by the action of gonadal hormones, acting directly on the liver, and that later removal of the gonadal hormone source does not affect that expression.

**Cloning and Characterization of the CYP3A10 Promoter**

Regulation of gene expression can be achieved at several points along the protein biosynthetic pathway, including but not limited to, transcription, mRNA processing and transport, translation and covalent modification. The first and arguably the most important means of regulation is at the transcriptional level where the regulation of a number of both eukaryotic and prokaryotic genes has been analyzed at the molecular level in very fine detail. Transcriptional regulation is achieved by the exquisitely choreographed interplay of DNA binding proteins with each other and their cognate binding sites on the DNA
molecule. The molecular mechanisms involved in the regulatory processes governing P450 expression are now being investigated by a number of laboratories and their initial results are proving very interesting.

One of the better studied examples of regulated liver P450s involved in bile acid metabolism is that of cholesterol 7α-hydroxylase, which performs the first and rate-limiting step in the conversion of cholesterol to bile acids (Danielsson and Sjövall, 1975; Björkhem, 1985). In mammals, the bile acid biosynthetic pathway is the major route for elimination of cholesterol from the enterohepatic circulation (Dietschy, 1984; Björkhem, 1985). Understanding the molecular mechanisms involved in regulation of cholesterol 7α-hydroxylase is of critical importance since the rate of accumulation of cholesterol is directly related to the activity of this enzyme. Cholesterol 7α-hydroxylase is subject to diurnal variation, feedback suppression by bile acids and up regulation by cholesterol. Some of the molecular players involved in the diurnal variation have been characterized by Lavery and Schibler and include the albumin D-element binding protein (DBP) with its cognate binding site centered around -225 bp. At this site, the predominant binding activity is an evening-specific complex of which DBP is a component (Lavery and Schibler, 1993).

A combination of transgenic mice and tissue culture systems have been used by others in our laboratory to delineate important DNA regions of the cholesterol 7α-hydroxylase (7α-hydroxylase) gene for bile acid-mediated suppression and cholesterol-mediated induction of chimeric reporter constructs. For example, by using a temperature-sensitive SV40 transformed mouse hepatocyte cell line, H2.35, that can be induced at will to display a liver phenotype, they have shown that a liver-specific enhancer, about 7 kb upstream in the rat gene, is required for full expression of a proximal 342 bp 7α-hydroxylase promoter/reporter construct (Ramirez et al., 1993). They have also shown, with the 7α-hydroxylase enhancer functionally substituted with another liver-specific
enhancer, that low density lipoprotein (LDL) can induce expression of the 342 bp promoter/reporter construct twofold, which is consistent with the observed induction in vivo. These results indicate that the cis-acting elements necessary for LDL-mediated regulation of 7α-hydroxylase are within those 342 bp (Ramirez et al., 1993).

In order to elucidate the molecular mechanisms involved in the bile acid-mediated induction and male-specific regulation of CYP3A10 transcription we have cloned and begun to characterize 8 kb of the 5' flanking region of CYP3A10. We have mapped the CYP3A10 transcription initiation site by primer extension and S1 digestion analyses and have sequenced 1 kb of proximal promoter DNA. Finally, using CYP3A10 promoter/lacZ chimeric reporter constructs transfected into a temperature-sensitive SV40 transformed hepatocyte cell line, we have shown liver-specific induction of reporter expression when the cells are grown under conditions that promote a liver-specific phenotype.

With this thesis, we have established a system for dissecting the molecular details of sexually dimorphic expression of a liver gene in bile acid metabolism and its bile acid-mediated induction. Since most of the body cholesterol is catabolized to bile acid, the bile acid biosynthetic pathway plays an important role in cholesterol homeostasis of mammals. Comprehending the endocrine factors that regulate the complex, sexually dimorphic patterns of postnatal development that characterize liver gene expression and the molecular mechanisms by which these factors operate will undoubtedly lead to a better understanding of the liver and its phenotypic expression, in general, and bile acid metabolism, in particular.
CHAPTER II:
Experimental Procedures
Materials

$^{32}$P-labeled nucleotides were purchased from New England Nuclear, and $^{14}$C-lithocholic acid from Amersham. Thin layer chromatography plates were from Merck. Reagents used in cDNA cloning and sequencing were from New England Biolabs, Boehringer Mannheim, United States Biochemicals or Gibco/BRL. Subtractive hybridizations were done with reagents from Invitrogen, San Diego, CA. Authentic murideoxycholic acid was purchased from Steraloids, Wilton, NH. and other bile acids from Sigma. Common laboratory chemicals were from Fisher, Sigma or BioRad. Steroid hormones were purchased from Sigma. Human Growth hormone (2 IU/mg) was either from Sigma or the National Hormone and Pituitary Program. Growth hormone and testosterone radioimmunoassay (RIA) kits, as well as nylon membrane, were from ICN Biomedicals. Nitrocellulose was purchased from Millipore, Bedford, MA. Osmotic minipumps were supplied by Alza Inc., Palo Alto, CA and implanted according to the manufacturer's instructions. Golden Syrian hamsters were purchased from Charles River Laboratories, Wilmington, MA. DEAE-dextran was from Pharmacia. Colestipol was purchased from Upjohn, Kalamazoo, MI. The lacZ promoterless vector, pNASSB, was purchased from Clontech and pGem and Bluescript vectors were from Promega and Stratagene, respectively. S1 nuclease was purchased from Boehringer Mannheim and reverse transcriptase was from Life Science. Oligonucleotides were supplied by Oligos, Etc. or prepared in our department by the phosphoramidite method on an automated DNA synthesizer.

General Experimental Procedures

Standard recombinant DNA procedures were carried out essentially as described (Sambrook et al., 1989). DNA probes were labeled by random hexanucleotide priming
(Feinberg and Vogelstein, 1983) or by primer extension with M13 clones and the universal primer (Church and Gilbert, 1984). DNA sequencing was done by the dideoxy chain termination method (Sanger et al., 1977) with DNA fragments subcloned into M13 vectors or with double stranded clones and the universal primer or sequence specific primers with reagents from United States Biochemicals. Protein was quantified by the method of Bradford (Bradford, 1976). Total cytochrome P450s were quantified as described (Andersson et al., 1985). RIAs for testosterone and GH quantification were performed according to the instructions provided by the manufacturer on serum samples collected at time of death.

**Preparation of RNA and Microsomes from Liver**

Golden Syrian hamsters were maintained on a 12 h light/12 h dark cycle, fed either regular laboratory chow or chow supplemented with 0.5% (w/w) cholic acid or 4% (w/w) colestipol for five days or subject to hormonal treatments (see below) and were killed at the middle of the dark cycle. Their livers were removed and used to prepare total cellular RNA by guanidinium thiocyanate homogenization followed by centrifugation through a cesium chloride cushion (Chirgwin et al., 1979). Liver microsomes were prepared by differential centrifugation from another portion of the livers of four weeks old hamsters treated with diets as for RNA preparation. The liver was homogenized in 5 volumes/g homogenization buffer (10 mM Tris Acetate pH 7.4, 1 mM EDTA and 0.25 M sucrose) with 10 strokes of a Dounce homogenizer. The homogenate was centrifuged at 27,000 x g for 20 min at 4 °C. The supernatant was centrifuged at 100,000 x g for 1 h at 4 °C in a refrigerated ultracentrifuge. The resultant pellet was resuspended in 1/5 the original volume of homogenization buffer and quickly frozen for later use.
Northern and Slot Blot Analyses

Northern blot hybridizations were performed essentially as described (Sambrook et al., 1989). Briefly, 10 μg aliquots of RNA were denatured at 65 °C with glyoxal and dimethyl sulfoxide and electrophoresed overnight through an agarose gel buffered with circulating 10 mM Na2PO4, pH 7 and blotted onto a nylon membrane overnight. When slot blot analyses were done, 10 μg of RNA was denatured in an equal volume of 18% formaldehyde/10 x SSC at 65 °C and spotted onto nylon. Blots were dried in a vacuum oven at 80 °C and prehybridized in a 50% formamide hybridization solution at 42 °C. After prehybridization, 32P-labelled probes were added to the solution at 2 x 10^6 cpm/ml and hybridized overnight. The blots were washed at 65 °C with 0.1 x SSC plus 0.1% SDS and exposed to x-ray film at -70 °C with intensifying screens. A βetagen™ blot analyzer or a Molecular Dynamics PhosphorImager™ was used to directly measure cpm per band or slot.

Preparation, Subtraction and Screening of cDNA Libraries

Poly(A)+ RNA (10 μg) was selected from total RNA by 2 cycles of oligo d(T)-cellulose chromatography (Chirgwin et al., 1979) and used to prepare cDNA libraries from the livers of 6 week old male hamsters fed colestipol (library A) or cholic acid (library B) as described above. After second strand synthesis, BstXI non-palindromic linkers were added by ligation, excess linkers removed and cDNAs ranging in size from 1-8 kb size selected by agarose gel electrophoresis. The selected cDNAs were eluted and ligated to pcDNAII, a plasmid vector that contains the M13 origin of replication to facilitate single strand synthesis (Duguid et al., 1988). The libraries were transformed into DH1α' E. coli cells by electroporation (Potter et al., 1984) generating 2 x 10^6 clones each before amplification.
Libraries were subtracted according to the method of Duguid (Duguid et al., 1988). Briefly, single-stranded DNA from library B was biotinylated with photobiotin and UV irradiation and hybridized for 24 h with the single-stranded DNA from library A at 68 °C. After hybridization was complete, clones common to both libraries were precipitated by addition of streptavidin. The subtracted library was transformed into DH1α E. coli cells by electroporation (Potter et al., 1984) and used for screening.

The subtracted library was plated onto nitrocellulose and replicas of each plate were made. Cells were lysed and the DNA denatured and immobilized on the filter. Filters were pre-hybridized overnight in 50% formamide at 42 °C as described (Sambrook et al., 1989). One set of replicas was probed with hexamer primed, 32P-labelled cDNA library A subtracted with cholic acid-fed hamster liver mRNA. The other set was probed with cDNA library B subtracted with colestipol-fed hamster liver mRNA and labelled as above with equal specific activity. After hybridization, filters were washed in 0.1 x SSC, 0.1% SDS at 65 °C and exposed to x-ray film. Plasmids were prepared from putative positives displaying different signal intensities. These were labelled by random hexanucleotide priming and used as a probe for RNA blots prepared with RNA from colestipol-fed and cholic acid-fed animals. Screening was completed when one clone, pFR29, displayed approximately 4-fold induction with RNA from cholic acid-fed animals versus RNA from normal or colestipol-fed animals.

Subcloning, Sequencing and Analysis of FR29

pFR29-1 was digested at sites astride the BstX I cloning site of pcDNAIII with BamH I and Xho I and the 1.1 kb fragment inserted into M13 vectors. After Sanger dideoxy sequencing (Sanger et al., 1977), a 5'-end 200-bp fragment was isolated by digestion with BstNI and Hinf I and used as a probe to re-screen library B to obtain the full-length cDNA. The complete cDNA, pFR29-2, was subcloned by partial digestion with
EcoR I and the 2 kb fragment inserted into the EcoR I site of pCMV (pFR29-5), an expression vector that contains the cytomegalovirus (CMV) promoter and facilitates expression of the cDNA in mammalian cells (Andersson et al., 1989). Complete sequencing of both strands of the full-length cDNA was accomplished by subcloning several fragments into M13 for Sanger dideoxy sequencing DNA analyses were performed with Microgenie™ software from Beckman Instruments or with MacVector™ from IBI.

Transient Expression in COS cells

COS cells were transfected by the DEAE-dextran method (Sambrook et al., 1989). The essentials or modifications are as follows. COS cells were seeded on day 0 at a density of 10^6 cells/100 mm plate in Dulbecco's Modified Eagle Medium (DMEM), 10% fetal calf serum and 20 mM N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES). The following day, cells were transfected for 30 min with 10 µg of pFR29-5, pCMV and/or NADPH-cytochrome P450 reductase cDNA, inserted in the same pCMV vector, in 500 µg/ml DEAE-dextran. Cells were then incubated at 37 °C for 3 h in media supplemented with 100 µM chloroquine and followed by a 4 min shock with 20% (v/v) glycerol media. After 48 h incubation at 37 °C, the cells were washed in ice-cold phosphate buffered saline and harvested. Microsomes were prepared from the collected cells as for liver except that the first centrifugation was done at 2,000 x g.

Assay of Lithocholic Acid and Androstenedione 6-Hydroxylase Activity

Lithocholic acid was prepared for substrate delivery by diluting 7 nmoles of 14 Ci/mole [14C-carboxy]lithocholic acid in 20 µl of 1 mg dilauroyl phosphatidylcholine/ml toluene, dried down under N₂, and resuspended in 250 mM KPO₄, pH 7. Incubations were done under linear conditions in 250 mM KPO₄, pH 7 (for lithocholic acid) or 100
mM HEPES, pH 7.4, 0.1 mM EDTA (for androstenedione) at 37 °C with 1 mM NADPH and microsomes from COS cells for 1 h or liver microsomes for 20 min. Reactions were stopped by dilution with 500 μl ethanol, acidified with 20 μl 0.1 N HCl and extracted with 5 ml ethyl ether (Danielsson, 1973). The ether phase was transferred, dried down under N₂ and resuspended in ethanol for spotting on silica gel G thin layer chromatography (TLC) plates or for reversed phase high performance liquid chromatography (HPLC). The plates were chromatographed with authentic standards in a sealed tank with iso-octane:ethyl acetate:acetic acid (5:5:1) (Eneroth, 1963), for lithocholic acid, methylene chloride:ethanol (97:3) then ethyl acetate:chloroform (1:1), for androstenedione (Waxman et al., 1991), dried and exposed to x-ray film overnight. Radioactive spots were excised from the plate and counted in a liquid scintillation counter or else quantified by a βetagen blot analyzer equipped to count ¹⁴C. HPLC was done essentially as described (Zimniak et al., 1989) to confirm the lithocholic acid 6β-metabolite. Briefly, the samples were chromatographed on a C18 column with 5 mM KPO₄, pH 5 and a 60 min, 50 - 85% methanol gradient and collected. Detection of the murideoxycholic acid standard was done at 214 nm with a Waters 441 detector and the radioactive reaction products were detected by scintillation counting.

Hormonal Treatments of Hamsters

Hypophysectomy (Hx), adult castration (Cx) and ovariectomy (Ovx) were performed by the supplier at 6 weeks of age. The animals were allowed at least 2 weeks to recover before any additional manipulations. Neonatal glutamate treatment was done as described elsewhere (Shapiro et al., 1989); the details are such that glutamate was dissolved in water and injected subcutaneously on days 5, 7 and 9 after birth at a dose of 4 mg/g body weight. To mimic the female pattern of GH release, GH dissolved in GH buffer (30 mM NaHCO₃ pH 9.3, 150 mM NaCl, 0.1 mg/ml BSA), was supplied to males
via osmotic minipumps at a dose of 36 μg/day for 5 days before harvest. Hx females were similarly treated but received 70 μg/day of GH. To mimic the male pattern of GH release, GH, in GH buffer, was injected subcutaneously at a dose of 70 μg once in the morning and again a minimum of 5 hours later. Estradiol valerate, 125 μg in propylene glycol, was administered to intact adult males by a single subcutaneous injection 7 days before death (Janeczko et al., 1990). Testosterone propionate (2 mg dose in propylene glycol) was administered to adult intact females and to neonatally castrated males by subcutaneous injections daily for 5 days before death (Squires and Negishi, 1988). Neonatal castration was performed on day 4. Male neonates were identified by their ano-genital distance and placed on ice. A ventral incision was made and the testes were removed. The animals were returned to the mother and allowed to reach 8 weeks of age before hormonal treatments.

**Genomic Cloning**

A hamster genomic library was prepared in λGEM-11 as described (Sambrook *et al.*, 1989). The library was plated and screened with a single-stranded probe containing a 200 nt fragment from the 5' end of the CYP3A10 cDNA (Teixeira and Gil, 1991) prepared in M13. Southern blot analysis was done to characterize that λ clone. The entire insert was placed in pGem4 and an approximately 8 kb fragment that extended from the 5' Sal I site to the EcoN 1 site (see Figure 17) in the 5' untranslated region of the CYP3A10 cDNA was subcloned into a Bluescript based vector. Sequencing was done with M13 vectors prepared from convenient restriction digests of a 2 kb Hind III fragment that extends from -819 bp in the 5' flanking region to a site in the first intron. Sequencing across and upstream of the Hind III site was done with sequence specific primers.
Primer Extension and S1 Digestion Analyses

Primer extensions were performed with 20 μg of total hamster liver RNA, or tRNA as a control, and a 40 nucleotide primer (40mer) labelled with [γ-32P]-ATP and polynucleotide kinase which corresponds to the sequences from +6 to +45 of the first exon (5' CAGCAGCACCAGGTTTCTATAGAAAGTTGGGATCAGC 3'). Briefly, RNA was coprecipitated with the 5' end-labelled primer, resuspended in 10 μl of 10 mM Tris, pH 8, 1 mM EDTA, 1.25 M KCl and heated at 68 °C for 20 min. 23 μl of primer extension mix (20 mM Tris, pH 8.7, 10 mM MgCl₂, 10 mM DTT, 0.33 mM dNTPs) was added with 1 unit of reverse transcriptase and incubated for 30 min at 42 °C. The reaction was ethanol precipitated, resuspended in formamide-dyes and boiled before loading on a 6%/7 M urea polyacrylamide sequencing gel. The gel was run for 2 h at 60 W, dried and exposed overnight at -70 °C with intensifying screens.

S1 nuclease digestion analyses were done with a single stranded probe prepared from an M13 vector containing the Hind III fragment used for sequencing as described above and the same 40mer primer used for primer extensions. 20 μg of total hamster liver RNA was heated at 65 °C for 5 min and hybridized overnight at 45 °C with the S1 probe in a total volume of 50 μl hybridization solution that contained 40 mM piperazine-N,N'-bis[2-ethanesulfonic acid], pH 6.4, 1 mM EDTA and 400 mM NaCl. S1 nuclease digestion was carried out the next day at 25 °C for 1 h by adding 250 μl S1 digestion buffer (250 mM NaCl, 20 mM potassium acetate, pH 4.5, 1 mM Zn(SO₄)₂) and 200 units of S1 nuclease. The reaction was stopped by adding 100 μl S1 stop buffer (3 M ammonium acetate, 2.5 mM EDTA, 200 μg tRNA), ethanol precipitated and resuspended in formamide with dyes for gel electrophoresis as above.
Preparation of Chimeric CYP3A10 Promoter/Reporter Constructs

Chimeric CYP3A10 promoter/lacZ reporter constructs were prepared in the following manner. pGal3A10-893 was prepared by digesting the genomic clone with EcoN I, blunt-ending, digesting with Hind III and inserting the 0.9 kb Hind III-EcoN I into the Hind III-Sma I site of a Bluescript-based vector and called pHN306. pHN306 was then digested with Hind III and Sac I, generating a 0.9 kb genomic DNA fragment, blunt-ended and inserted into the blunt-ended Xho I site of pNASSβ to create pGal3A10-893. pGal3A10-8kb was also prepared in two steps. A 7 kb Sal I-Hind III fragment from the genomic clone was isolated and ligated to Sal I-Hind III digested pHN306 to generate pSN306. pSN306 was digested with Sal I and Sac I and blunt-ended. The 8 kb fragment was isolated and ligated to Xho I-digested and blunt-ended pNASSβ to generate pGal3A10-8kb. pGalAEP was created by digesting pAN/T2B (kindly provided by Dr. Ken Zaret, Brown University) with Nhe I and Hinc II, blunt-ending and isolating the 2.8 kb fragment, and then inserting it into the Xho I-digested and blunt-ended pNASSβ. pGalAE3A10-893 (a plasmid containing the albumin enhancer in front of 893 bp of the CYP3A10 promoter) was prepared by first creating pGalAE by digesting pAN/T2B with Nhe I and BamH I, adding adapters to both ends that contained Xho I and Sfi I sites in the 5' end and Kpn I and Acc 651 sites in the 3' end, blunt-ending and inserting it into the Xho I, blunt-ended pNASSβ. pHN306 was digested with Hind III and BamH I, blunt-ended and the 0.9 kb fragment isolated and inserted into Acc 651-digested and blunt-ended pGalAE to produce pGalAE3A10-893.

Transfection of H2.35 Cells and Assays for Reporter Expression

H2.35 cells, a temperature sensitive SV40 transformed mouse hepatocyte cell line, were made available to us by the generosity of Dr. Ken Zaret at Brown University (Zaret et
The cells are grown and maintained at 33 °C, the permissive temperature, in basal medium (BM; DMEM supplemented with 4% fetal calf serum, 0.2 μM dexamethasone and antibiotics). Transient transfections were done with 2 X 10⁶ cells in 100 mm plastic dishes and 15 μg of plasmid DNA with 1 μg pSV-CAT as a control by the calcium phosphate coprecipitation method (Chen and Okayama, 1988). The following day the media was changed 2 h before the cells were trypsinized and replated. One fraction (1/3) of the cells was replated on plastic and grown at 33 °C in the same media as above for two days. The remainder of the transfected cells was seeded on plates prepared with rat type I collagen (Zaret et al., 1988) and grown at the nonpermissive temperature, 39 °C, in SFM (serum free media) for two days, conditions which promote a more liver-like phenotype (Liu et al., 1991). The plates were rinsed with phosphate buffered saline and harvested by scraping for cells grown on plastic and by treatment with 1 mg/ml collagenase for cells grown on collagen. The cells were pelleted in microcentrifuge tubes and processed for β-galactosidase and chloramphenicol acetyltransferase (CAT) assays.

β-Galactosidase activity in the cell extracts was measured by colorimetric analysis with o-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate. Pelleted cells were resuspended in 100 μl of 250 mM Tris, pH 7.4 and disrupted by three cycles of freezing and thawing. Cellular debris was removed by centrifugation and 30 μl of the supernatant was added to 270 μl of a mixture containing 89 mM NaP04, pH 7.5, 264 μg ONPG, 1 mM MgCl₂, and 40 mM β-mercaptoethanol. The reaction was performed at 37 °C for 15 min, stopped with 500 μl of 1 M Na₂CO₃ and the resulting mixture’s optical density read in a spectrophotometer at 420 nm. CAT activities were measured by a method described in detail elsewhere (Gorman et al., 1982) after heating the extracts for 5 min at 65 °C. β-Galactosidase activity was normalized to CAT activity to control for transfection efficiencies.
CHAPTER III

Results
Cloning, Expression and Regulation CYP3A10

Using differential hybridization techniques with libraries prepared from hamsters fed either a cholic acid-rich diet or a colestipol diet (see "Experimental Procedures") we have isolated a hamster liver cDNA (initially called pFR29) whose expression is induced by cholic acid feeding. Figure 4 is a schematic representation of the isolated cDNAs and the strategy used to determine its nucleotide sequence. The complete nucleotide sequence of pFR29-2 and the predicted amino acid sequence of the protein is shown in Figure 5. pFR29 encodes a 503 amino acid protein with 76 nucleotides in the 5' untranslated region and 430 nucleotides in the 3' untranslated region. Its deduced amino acid sequence contains a putative heme-binding domain present in all mammalian P450s characterized to date (Gonzalez, 1989) as well as the residues that direct the specificity of P450s for steroids (Gotoh and Fujii-Kuriyama, 1989).

Computer analysis of the sequence revealed that the deduced amino acid sequence of pFR29 was highly homologous to P450 family 3 proteins. This homology approaches 70% with other members of the family previously isolated from human, rat and rabbit. In Figure 6, the alignment of the amino acid sequence of FR29 is shown with that of CYP3A1/pcn1, the first of the P450 family 3 proteins whose cDNA was isolated (Gonzalez et al., 1985). Because of this homology, the protein encoded by the isolated cDNA has been named CYP3A10 according to the recommended nomenclature system (Nelson et al., 1993).

RNA blot analysis was performed in order to determine the extent of regulation by cholic acid of the steady state level of CYP3A10 mRNA. The autoradiogram of a typical experiment is shown in Figure 7. The size of the message, 2.1 kb, corresponds to that
Figure 4. Restriction Endonuclease Map and Sequencing Strategy for FR29. The scale at the top designates the nucleotide positions (in kilobases) relative to the first nucleotide of the full-length cDNA. The thick black line represents the 503 amino acid coding region and the 3' and 5' untranslated regions are represented by a thin line. The restriction endonuclease sites used for subcloning into M13 are indicated. The regions encompassing the initial cDNA isolated (pFR29-1) and the full-length (pFR29-2) are indicated below the restriction map. The arrows indicate the direction and extent of a given sequencing experiment. Both strands of the entire cDNA were sequenced by the dideoxy chain termination method.
Figure 5. **Nucleotide Sequence of the cDNA Corresponding to the Hamster Lithocholic Acid 6β-Hydroxylase mRNA and Predicted Amino Acid Sequence of the Protein.** The nucleotides are numbered on the right hand side; nucleotide 1 has been arbitrarily defined as the A of the initiator codon. The 5' untranslated region is numbered negatively and the nucleotides are in small case letters as are those of the 3' untranslated region. The last nucleotide is the first A of the poly(A) tail. The amino acid sequence is shown below the nucleotide sequence and numbered with the initiator methionine as the first residue. The amino acid residues of the putative heme binding domain, characteristic of and highly homologous in all higher eukaryotic P450s characterized to date (Gonzalez, 1989), is underlined. The residues starred *with an asterisk* are those that have been implicated in directing the specificity of cytochrome P450s for steroid substrates (Gotoh and Fujii-Kuriyama, 1989).
Figure 6. Amino Acid Alignment of CYP3A10/Lithocholic Acid 6β-Hydroxylase with that of CYP3A1/pcn1. Computer analysis of the amino acid sequence encoded by FR29 revealed that it shares a high degree of homology with that of P450 Family 3 proteins. Shown is the alignment of lithocholic acid 6β-hydroxylase with that of rat pcn1, a well characterized Family 3 protein. They share identity (indicated by a l) in 69% of their residues and only one gap in lithocholic acid 6β-hydroxylase was necessary for maximal alignment. The overall homology between rat pcn1 and lithocholic acid 6β-hydroxylase, considering both identical and conserved (indicated by a :) amino acids increases to 81%.
Figure 7. Regulation of Lithocholic Acid 6β-Hydroxylase mRNA. RNA was prepared from the indicated sources and analyzed by Northern analysis as described in Experimental Procedures. λHind III-digested DNA was used as standards and visualized by ethidium bromide staining and the relative mobilities of the fragments are indicated on the right. Panel A: single-stranded, 32P-labelled probes complementary to lithocholic acid 6β-hydroxylase mRNA were prepared as described in Experimental Procedures from 5’ EcoRI fragment (nt -76 to 186) of the cDNA and used to detect CYP3A10 RNA. Exposure to x-ray film was done for 2 h at -70 °C with intensifying screens. Panel B: a parallel blot containing the same RNAs was treated as above but hybridized with a single-stranded actin probe (Ma et al., 1986) and exposed for 4 h to film as above.
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<tr>
<th>Age in weeks</th>
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**A.**

CYP3A12

- 14
- 9.4
- 6.6
- 4.4

B.

Aclar

- 2.3
- 2.0

- 5.0

- 2.0
predicted from the sequence. Regulation of CYP3A10 was determined by measuring the cpm per band with a βetascope analyzer to ensure accuracy and normalizing to the hybridizable actin message quantified in the same manner (Figure 7B). Expression of CYP3A10 mRNA is much higher (about 50-fold) in males (lanes 1-8) than in females (lanes 9-16), in fact, only after exposure of the blot for four days (versus 2 h for the males) can CYP3A10 mRNA be detected in female RNA (data not shown). In young males (28 days old) CYP3A10 mRNA was induced approximately 3-fold by cholic acid feeding (lanes 7 and 8) in this particular experiment (see other experiments below). There was a slight induction (20%) by cholic acid in males at 7 weeks of age (lanes 5 and 6) and no regulation at other ages (lanes 1-4). In females, in addition to the much lower expression of CYP3A10, its RNA is not regulated by cholic acid feeding at any age.

Since family 3 proteins are highly homologous, at least in rat, we eliminated the possibility that our probes might be hybridizing to more than one RNA species under the conditions we normally do the RNA blot analyses (50% formamide hybridization solution and 0.1 x SSC, 65 °C washes) by performing a Southern analysis (Figure 8) with hamster genomic DNA. When we probed one blot with the 5' end, EcoR I fragment (bp -76 to 186) of the CYP3A10 cDNA (panel A), two bands were visualized. This is not unexpected since we have detected an EcoR I site within an intron (unpublished results) which begins 73 bp (see Figure 5) into the coding region of pFR29-2. When a parallel blot was probed with the 3' end, Sca I fragment (bp 1718 to 2010) of the FR29 cDNA (panel B), we observed a single band in each lane. This probe was generated from the 3' untranslated region of the cDNA where one would not expect there to be an intron. This clearly demonstrates that the conditions we used to do the RNA blot analyses were such that only the product of one gene was being detected. Additionally, when the same analysis was done under lower stringency conditions (35% formamide hybridization
Figure 8. **Southern Analysis of Hamster Genomic DNA with 5'-End and 3'-End CYP3A10 Probes.** Hamster genomic DNA was digested with EcoR I (lanes 1, 3) and Xba I (lanes 2, 4), electrophoresed through an agarose gel, denatured, neutralized and transferred to a nylon membrane. The blots were hybridized overnight in 50% formamide solution at 42 °C with single stranded, $^{32}$P-labelled probes (see Experimental Procedures) prepared from the 5' EcoR I, nt -76 - 186, (panel A) or the 3' Sca I fragment, nt 1718 - 2010, (panel B) of FR29 and washed at 65 °C, 0.1 x SSC. The blots were exposed to Xray film overnight at -70 °C with intensifying screens. The relative mobilities of λ/Hind III digested standards are indicated.
A
5' end probe

B
3' end probe

kb

23 -

9.4 -

6.6 -

4.4 -

2.3 -

2.0 -

1 2

3 4
solution and 2 x SSC, 55 °C washes), an innumerable amount of bands were visualized (data not shown), presumably due to hybridization with genes that are homologous to CYP3A10, including other cytochrome P450 family 3 members.

To determine the nature of the biological activity encoded by CYP3A10, we performed transient expression experiments in COS cells (see Figure 9). We placed CYP3A10 cDNA in the expression vector pCMV (Andersson et al., 1989) that contains the CMV promoter as well as the simian virus 40 (SV40) origin of replication. We transfected COS cells with either the parental vector pCMV, another construct that contains the P450 reductase cDNA placed in pCMV, our construct containing the CYP3A10 cDNA or both CYP3A10 and P450 reductase cDNAs. About 48 h after transfection, cells were harvested and microsomes were prepared and assayed for LCA 6-hydroxylase activity (see "Experimental Procedures"). The reaction products were identified by comparing their migration distances in a TLC system with those of authentic 6α-hydroxy (hyodeoxycholic acid) and 6β-hydroxy lithocholic acid (murideoxycholic acid). COS cells transfected with vector DNA (lane 1) or with P450 reductase cDNA alone (lane 2) produced no activity that converts LCA. Cells transfected with CYP3A10 cDNA (lane 3) converted 4% of the LCA used in the assay to a product that migrated with authentic murideoxycholic acid. Cells transfected with both CYP3A10 and P450 reductase cDNAs (lane 4) converted about 15% of the added substrate to the same product. Two other products were visualized and have yet to be identified. Reversed-phase HPLC confirmed that the major radioactive product of the CYP3A10, reductase-transfected COS cells microsomes assay was murideoxycholic acid with 5% of total cpm loaded recovered as such (data not shown).

Figure 9 also shows the LCA hydroxylase activity of liver microsomes from adult hamsters of either sex, fed with a control diet or the same diet supplemented with cholic
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**Lithocholic Acid**

6β-Hydroxy

6α-Hydroxy

**Origin**
Figure 9. Lithocholic Acid 6β-Hydroxylase Activity in Transfected COS cells and in Hamster Liver Microsomes. Lithocholic acid 6β-hydroxylase activity was measured as described in Experimental Procedures with 200 μg microsomal protein prepared from COS cells transfected with the indicated DNA or with 20 μg hamster liver microsomal protein from the indicated sources. The zones corresponding to the migration distance of authentic standards are indicated on the left. The specific activity of 6β-hydroxylase was 0.001, 0.001, 0.014, 0.089, 0.339, 0.544, 0.076, and 0.090 nmoles/min per mg of protein (lanes 1-8 respectively).
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<th>Transfected COS Cells</th>
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<tr>
<td>CYP1A10</td>
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<td>P450 Reductase</td>
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Lithocholic Acid —

6α-Hydroxy —

5α-Hydroxy —

Origin —
acid (lanes 5-8). Cholic acid feeding, in male hamsters, induced 6β-hydroxylase activity by approximately 50-100% (lanes 5 and 6), consistent with the observed induction of CYP3A10 RNA (see Figure 7, lanes 7 and 8). There was no observable induction of 6β-hydroxylase activity in females (lanes 7 and 8). Additionally, there was an induction of 6α-hydroxylase activity by cholic acid feeding.

CYP3A10-transfected COS cell microsomes also hydroxylated other substrates, specifically the steroid hormones androstenedione, progesterone and testosterone (Chang et al., 1993). Kinetic analyses were performed to compare directly the efficiency of CYP3A10-catalyzed steroid-hormone versus bile acid 6β-hydroxylation. Apparent $K_M$ and $V_{\text{max}}$ values of 75 μM and 132 pmol/min per mg respectively were determined for androstenedione 6β-hydroxylation, and values of 25 μM and 164 pmol/min per mg respectively were determined for LCA 6β-hydroxylation (see Figure 10). CYP3A10 exhibits a 3.7 fold higher efficiency ($V_{\text{max}}/K_M$) for metabolism of LCA when compared with androstenedione.

A statistical analysis of the cholic acid-mediated induction of CYP3A10 RNA and lithocholic acid 6β-hydroxylase activity was done, the results of which are shown in Figure 11. In this experiment, 4 week old hamsters, n = 15, were fed either standard laboratory chow or the same diet supplemented with 0.5% cholic acid for 5 days and their livers were harvested for RNA and microsomes as described in Experimental Procedures. Slot blot analyses and 6β-hydroxylase assays were done with RNA and microsomes from the individual animals. We observed a 68% increase in CYP3A10 mRNA (solid bars) and a 110% increase in lithocholic acid 6β-hydroxylase activity (stippled bars), p < 0.025 and p < 0.0005 respectively. We also observed an 130% induction of lithocholic acid 6α-hydroxylase (data not shown). The induction in RNA was less than the 3-fold observed in
Figure 10. Steady-state Kinetic Analysis of Androstenedione 6β-Hydroxylation and Lithocholic Acid 6β-Hydroxylation Catalyzed by CYP3A10. Microsomes prepared from COS cells cotransfected with CYP3A10 and P450 reductase cDNAs in pCMV expression vectors were used for a kinetic analysis of 6β-hydroxylation of lithocholic acid and androstenedione. Experiments were performed as outlined in "Experimental Procedures". The data are from representative experiments and are shown in the form velocity plotted against substrate concentration. The inset shows the same data plotted in the form of Lineweaver-Burke double-reciprocal plots. The $K_M$ and $V_{max}$ were determined graphically from these data: androstenedione 6β-(AND-6β) hydroxylation, $K_M = 75$ μM and $V_{max} = 132$ pmol/min per mg; lithocholic acid 6β-(LCA-6β) hydroxylation, $K_M = 25$ μM and $V_{max} = 164$ pmol/min per mg.
Figure 11. Induction of CYP3A10 mRNA and Lithocholic Acid 6β-Hydroxylase Activity by Cholic Acid Feeding. RNA and microsomes were prepared from liver as described in Experimental Procedures from individual hamsters, 15 of which were fed a standard laboratory diet and 15 fed the same diet supplemented with 0.5% cholic acid. The level of CYP3A10 RNA was quantified by slot blot analysis with a βetagen (see "Experimental Procedures") and normalized to the amount of hybridizable actin RNA and shown in arbitrary units (solid bar). Lithocholic acid 6β-hydroxylase activity in liver microsomes from the same animals was assayed as described in "Experimental Procedures" with 100 μg protein and shown in nmoles/min·mg (stippled bar). Error bars represent the standard error of the mean. The inductions by cholic acid feeding were statistically significant (p < 0.025 for RNA; p < 0.0005 for activity).
A bar graph comparing the standard diet and the 0.5% cholic acid diet. The graph shows that the 0.5% cholic acid diet has a higher value than the standard diet.
Figure 7, which depicts the results of an experiment with a pool of RNA from three animals. We believe this is due to the high variability of CYP3A10 expression observed in individual animals which necessitated experimentation with a large number of animals and individual analysis to obtain statistically significant data.

**Hormonal Regulation of Male-specific Expression**

We began our study of the regulatory mechanisms underlying the male-specific expression of 6β-hydroxylase in hamster liver by determining its level of expression throughout the development of the hamster. We quantified the amount of 6β-hydroxylase mRNA from the livers of animals at the indicated age and sex by using a 6β-hydroxylase-specific cDNA fragment as a probe under stringent conditions in our slot blot or northern analyses of total RNA for all our experiments and normalizing the level of steady-state 6β-hydroxylase mRNA with that of actin mRNA (see "Experimental Procedures"). The results of the experiment to determine the ontology of 6β-hydroxylase expression is shown in Figure 12. Several important findings were extracted from this experiment. The first was that at two weeks of age there is little detectable 6β-hydroxylase mRNA in either sex. At the onset of puberty, four weeks in the hamster, there is a dramatic increase in 6β-hydroxylase mRNA in male hamsters. In females, however, the level of 6β-hydroxylase mRNA was still very low and remained so through sexual maturity at 10-12 weeks of age. Male levels gradually increased through 10 weeks and reached levels 20- to 60-times greater than female levels. On the basis of these results we chose to perform our subsequent experiments on animals at 8 weeks of age.

To assess what role, if any, circulating GH played in 6β-hydroxylase expression we manipulated the levels of plasma GH and the patterns of GH secretion in male and female hamsters; the results are shown in Figure 13. Normal male and female levels of 6β-
Figure 12. Developmental Time Course of CYP3A10/6β-Hydroxylase Expression. Total RNA was prepared from hamster liver harvested at the indicated age (n=6). The RNA (10 μg) was applied to nylon with a slot blot apparatus, hybridized to CYP3A10- or Actin-specific probes and quantified (see "Experimental Procedures"). The CYP3A10/Actin RNA ratios are shown and the error bars represent standard error of the mean (S.E.M.).
Figure 13. Effect of Adult Hypophysectomy and/or Growth Hormone Administration on CYP3A10/6β-Hydroxylase Expression.

Panel A: Hypophysectomized and normal golden Syrian hamsters were supplied at 8 weeks of age. Animal treatments are described in detail in "Experimental Procedures". Animals (n=6 unless otherwise indicated) were treated as follows: Normal males, lane 1; Normal males plus growth hormone (GH) via implanted osmotic minipumps, lane 2; Hypophysectomized (Hx, n=10) males, lane 3; Normal females, lane 4; Hx females (n=10), lane 5; Hx females (n=4) plus GH via implanted osmotic minipumps, lane 6. RNA was prepared and analyzed by slot blot analysis as in Figure 12. The normalized ratios of CYP3A10/Actin RNA are shown on the left and error bars represent S.E.M. * Significantly higher than untreated females (lane 4) and Hx females treated with GH (lane 6), p<0.05.

Panel B: Total RNA was pooled from the individual animals used in Panel A and analyzed by northern analysis (see "Experimental Procedures"). The lane descriptions are as above for Panel A. The blots were exposed overnight at -70 °C with intensifying screens.
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<td>+ GH Pumps</td>
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B

CYP3A10 mRNA

Actin mRNA
hydroxylase expression are shown in Panel A (lane 1 and lane 4, respectively). Disruption of the male pattern of circulating GH either by hypophysectomy (lane 2) or by continuous GH administration via osmotic minipumps (lane 3) to mimic the female pattern of GH release, had little effect, if any, on 6β-hydroxylase levels of expression. To ensure that the minipumps were releasing GH as expected, we measured the levels of serum GH by RIA. The serum GH concentration in normal males ranged from zero to about 5 ng/ml but the males implanted with the GH-releasing minipumps had levels of circulating serum GH, 40-80 ng/ml, similar to that found in female rats, 25-75 ng/ml. In normal male rats GH levels can reach 150 ng/ml but periods with little detectable serum GH are more common (Waxman et al., 1990). We also found that hypophysectomy of males and subsequent treatment with GH by infusion had no significant effect on expression (see Table 1).

In females, hypophysectomy led to a 10-fold increase in 6β-hydroxylase expression (lane 5) over that of untreated females (lane 4) although that level of expression was still lower than that of normal males (lane 1) or similarly treated males (lane 2). Furthermore, mimicking the female pattern of GH release from the pituitary via osmotic minipumps implanted in another group of hypophysectomized females (lane 6) was sufficient to lower 6β-hydroxylase expression to that of normal females (lane 4). In Panel B, the RNA from the individual animals used in the slot blot analyses of Panel A was pooled for northern analysis to assess the integrity of the RNA and to visualize the aggregate differences in expression of the groups discussed in Panel A.

We then investigated whether disruption of the arcuate nucleus of the hypothalamus neonatally by glutamate (MSG) injections, with its resulting lack of GH release from the pituitary (Millard et al., 1982), affected 6β-hydroxylase expression in the adult. The results of those experiments are shown in Figure 14. Panel A represents the results of slot blot analyses done with the indicated RNAs. In males, the effect of MSG injections (lane
2) on the levels of 6β-hydroxylase mRNA when compared with normal males (lane 1) is not significantly different (0.1<p<0.375). Additionally, mimicking the male pattern of GH secretion by GH injections (lane 3) did not significantly alter the expression of 6β-hydroxylase mRNA in MSG-treated males. Females treated neonatally with MSG normally had 4- to 9-fold (depending on the experiment, see Figure 14 and Table 1) higher levels of 6β-hydroxylase mRNA (lane 5) when compared with that of untreated females (lane 4), p<0.05. These results were expected given the results of the experiments with hypophysectomized females (see figure 13). When another group of neonatally MSG-treated females was injected with GH twice daily to mimic the male pattern of circulating GH (lane 6), the levels of 6β-hydroxylase were 16-fold higher than that of untreated females (lane 4), p<0.01, and threefold higher than that of neonatally MSG-treated females without GH injections (lane 5), p<0.05, but not significantly different from that of any of the male groups (lanes 1, 2 and 3). Panel B is the visualization of pooled RNA from the groups in Panel A analyzed by northern analysis.

We next assessed whether circulating gonadal hormones were necessary either for expression in males or suppression in females and the results are depicted in Figure 15. Panel A represent the results of slot blot analyses performed with the indicated RNAs. When we compared the level of 6β-hydroxylase mRNA in 8 week old males castrated at 6 weeks of age (lane 2) with that of normal males (lane 1) we found that there was no significant difference. To ensure the completeness of the castration, we measured the serum testosterone concentration at the time of death by RIA and found that the level in castrated males was essentially undetectable, less than 0.05 ng/ml, whereas that of normal males was about 1 ng/ml. Male hamsters injected with estrogen (lane 3) also had levels of 6β-hydroxylase mRNA similar to that of untreated males (lane 1). On the other hand, ovariectomized females (lane 5) had lower levels of 6β-hydroxylase mRNA, half as much, when compared to normal females (lane 4), p<0.05. Ovariectomized females injected
Figure 14. Effect of Neonatal Glutamate Treatment and Growth Hormone Injections on CYP3A10/6β-Hydroxylase Expression.

Panel A: Hamsters were treated neonatally (n=6 in each group, see "Experimental Procedures") with saline solution (males, lane 1; females, lane 4) or glutamate (males, lane 2; females, lane 5). One group of glutamate-treated hamsters was injected with growth hormone twice daily at 8 weeks of age (males, lane 3; females lane 6). Total RNA was prepared from livers of adult hamsters and processed as in Figure 12. The normalized ratios of CYP3A10/Actin RNA are shown and error bars represent S.E.M. a,b,c Significantly different from other female groups, in this particular experiment, p<0.05.

Panel B: Total RNA was pooled from the individual animals used in Panel A and analyzed by northern analysis (see "Experimental Procedures"). The lane descriptions are as above for Panel A. The blots were exposed overnight at -70 °C with intensifying screens.
Male  Female
-  +  +  -  +  +  MSG Treated
-  -  +  -  -  +  +GH Injections

A

B

CYP3A10 mRNA/Actin RNA

1  2  3  4  5  6

CYP3A10 mRNA

Actin mRNA
Figure 15. Effects of Castration and Gonadal Hormone Administration in the Adult Hamster on CYP3A10/6β-Hydroxylase Expression.

Panel A: Total RNA was prepared from livers of adult hamsters (n=6 unless indicated otherwise) at 8 weeks of age and processed as in Figure 12. Male animals were normal (N), lane 1; castrated (Cx), lane 2; or injected with estradiol (E2), lane 3. Females were normal (N), lane 4; ovariectomized (Ovx), lane 5; or ovariectomized and injected with testosterone (T), lane 6 (see "Experimental Procedures"). The normalized ratios of CYP3A10/Actin RNA are shown on the left and error bars represent S.E.M. * significantly higher than normal females, lane 4, p<0.05

Panel B: Total RNA was pooled from the individual animals used in Panel A and analyzed by northern analysis (see "Experimental Procedures"). The lane descriptions are as above for Panel A. The blots were exposed overnight at -70 °C with intensifying screens.
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CYP3A10 RNA/Actin RNA

B

1 2 3 4 5 6

CYP3A10 mRNA

Actin mRNA
with testosterone (lane 6) had 5-fold higher levels of 6β-hydroxylase mRNA when compared with that of normal females (lane 4), p<0.05 and 10-fold higher when compared with untreated castrated females (lane 5). Panel B is a northern blot analysis of the pooled RNAs from Panel A.

Lastly, we determined whether neonatal exposure of males to gonadal hormones was necessary for the high level of 6β-hydroxylase expression when adult (Figure 16). Male hamsters were castrated 4 days after birth and allowed to reach 8 weeks of age. The animals were divided into 3 groups of four animals for different treatments and compared with normal male expression (lane 1). The first group was that of untreated, neonatally castrated hamsters (lane 2) in which the level of expression was essentially that of females. In the second group (lane 3), which was treated with daily injections of testosterone for 5 days prior to death, the level of 6β-hydroxylase expression was significantly higher (p<0.005) than that of untreated, neonatally castrated males but did not approach the level of expression in normal males. GH administration to neonatally castrated animals (lane 4) did not appreciably alter the level of expression from that of untreated animals, p<0.375.

CYP3A10 Promoter Cloning and Expression of Chimeric Report Constructs

Having established that the male-specific expression of CYP3A10 in hamster liver is the result of neonatal androgen exposure and that GH can induce expression in females, experimentally, we began investigating the molecular mechanisms involved in neonatal "imprinting" of liver gene expression. A hamster genomic library was prepared in λGEM-11 phage and screened with the 5' end of the CYP3A10 cDNA. We isolated one clone, which we chose to characterize further, that contained approximately 15 kb of the
Figure 16. Effects of Neonatal Castration and Hormone Treatment on CYP3A10/6β-Hydroxylase Expression.

Panel A: Male hamsters (n=4 in each group, see "Experimental Procedures") were castrated four days after birth and treated with the indicated hormones at 8 weeks of age. Total RNA was prepared from livers of adult hamsters and processed as in Figure 12. CYP3A10 expression in uncastrated males (N) is shown in lane 1. Neonatally castrated hamsters were untreated (U, lane 2), injected with testosterone (T, lane 3), or injected with growth hormone (GH, lane 4). The normalized ratios of CYP3A10/Actin RNA are shown and error bars represent S.E.M. *Significantly different from untreated castrated males, p<0.005.

Panel B: Total RNA was pooled from the individual animals used in Panel A and analyzed by northern analysis (see "Experimental Procedures"). The lane descriptions are as above for Panel A. The blots were exposed overnight at -70 °C with intensifying screens.
CYP3A10 mRNA
Actin mRNA
CYP3A10 gene. Southern blot analyses were done to characterize that clone and revealed that it contained approximately 8 kb of the 5' flanking region of the CYP3A10 gene. An endonuclease restriction map of the isolated DNA is shown in Figure 17 with convenient restriction sites indicated. The transcription initiation site and the first exon/intron boundary were determined as discussed below in figure 19 and 18, respectively.

We sequenced approximately 1.1 kb of the genomic clone using M13 clones and the universal primer or else double stranded plasmids with sequence-specific primers (see "Experimental Procedures") and the results are shown in Figure 18. The first intron begins 71 bp downstream of the initiator methionine codon. Computer analysis of the sequence revealed multiple regions of homology to the consensus binding site of HNF3, a liver-specific DNA binding protein (Jackson et al., 1993). We found no consensus TATA or CAAT box sequences in the regions where one would expect to find them (see below), 5' of the transcription initiation sites. However, we did find a consensus initiator element (Inr) which can be bound by TFII-I in TATA-less promoters at the transcription initiation site. TATA-binding protein (TBP) binds cooperatively with TFII-I to Inr-containing TATA-less promoters, functionally generating an alternate TFII-D pathway for preinitiation complex assembly (Roeder, 1991). The site we found in the CYP3A10 promoter (CTGGGTCTC) is identical to the HIV-1 initiator (Roy et al., 1993).

We performed primer extension and S1 nuclease digestion analyses (see "Experimental Procedures") to map the 5' end of the CYP3A10 message. Figure 19 shows the results of those experiments, as well as a schematic diagram depicting the products of the reactions. The probe used for the S1 analysis was prepared with the same primer that was used for the primer extensions and for sequencing so that the products of the S1 digestion and the primer extension would be identical in length. The sequencing ladder was run alongside the primer extension and S1 digestion experiments to pinpoint the exact
Figure 17. **Restriction Endonuclease Map of the CYP3A10 Genomic Clone.** A schematic representation of approximately 15 kb of CYP3A10 Genomic DNA, cloned from a hamster genomic library in λ (see "Experimental Procedures") is shown. The scale at the top represents the nucleotide positions (in kilobases) relative to the transcription initiation site which is indicated by a bent arrow (see Figure 19). The coding region of the first exon is represented by a box and extends for 71 bp. The probe used for screening is indicated by a starred line and corresponds to the 5' EcoR I fragment of the CYP3A10 cDNA, nt -76 to 186 (see Figure 5). Restriction endonuclease sites that were useful in subcloning are indicated.
Figure 18. **CYP3A10 Proximal Promoter Sequence.** Sequencing was performed as explained in "Experimental Procedures". Nucleotides are numbered on the right with +1 indicating the first transcription initiation site (see figure 19) and nucleotides upstream negatively numbered. The transcription initiation sites are indicated by arrows and a putative TFII-I-binding Inr site (Roy et al., 1993) is indicated by a *double underline*. The restriction endonuclease site used for subcloning are *boxed* and the identity of the site indicated *underneath*. The predicted single letter amino acids are shown *below* the first exon and lower case letters indicate nucleotides that are in the first intron. The sequence corresponding to the 40mer used as a primer for the primer extension analysis and for preparing the S1 digestion probe are *underlined* with a *thick line*. 
Figure 19. Localization of the Transcription Initiation Site by Primer Extension and S1 Digestion Analyses. Total RNA was prepared and primer extension and S1 digestion analyses were performed as described in "Experimental Procedures". Experiments were done with RNA from the livers of hamsters of the indicated sex (males, lanes 1 and 6; females, lanes 2 and 5) or with control tRNA (lanes 3 and 4). The primer used for the primer extension was the same as that used for preparing the S1 probe. A sequencing experiment, shown on the right (lanes 7-10), was also done with the same primer and run on the same gel to identify the precise nucleotides where transcription of the CYP3A10 mRNA begins. The arrows on the sequence interpreted from the sequencing ladder indicate those nucleotides that were revealed by the more sensitive primer extension experiment. The schematic diagram is shown depicting the primer extension product and the S1 nuclease probe, which are not drawn to scale. Asterisks indicate the $^{32}$P-label at the 5' end of the primer in the primer extension experiment and the uniformly labelled S1 probe.
Figure 20. **Schematic Diagram of the Chimeric CYP3A10 Promoter/lacZ Reporter Constructs for Use in Promoter Analysis.** The 8 kb genomic clone is shown at the top for reference with restriction sites used for plasmid construction indicated. Constructs were prepared as described in "Experimental Procedures". The DNA fragment from the CYP3A10 promoter is shown as a solid bar. The stippled bar represents the lacZ reporter DNA. The albumin enhancer and albumin promoter were cloned from pAN/T2B (Zaret et al., 1988) as described in "Experimental Procedures" and are shown as an open bar and a shaded bar, respectively.
nucleotide, or in this case nucleotides, that correspond to the first ribonucleotides of the mRNA. The product sizes ranged from 140 nt at the longest to 133 nt at the shortest. As expected we saw a significantly higher level of S1 digestion and primer extension products with RNA prepared from male hamster liver. No products were seen with control tRNA.

Having concluded that we had cloned the transcription initiation site, we prepared chimeric CYP3A10 promoter/lacZ reporter constructs to investigate whether the 5' flanking region of CYP3A10 contained the necessary DNA sequences for the liver-specific, sexually dimorphic and bile acid-mediated regulation of its expression. Figure 20 shows, schematically, those constructs and the 8 kb fragment of 5' flanking region for reference. The first construct, pGal3A10-8kb contains the complete 8 kb of 5' flanking, Sal I-EcoN I fragment of CYP3A10 in front of reporter lacZ coding sequence. pGal3A10-893 was made with the 893 bp Hind III-EcoN I fragment of the proximal promoter. The construct containing the albumin enhancer 5' of the 893 bp Hind III-EcoN I fragment, pGalAE-3A10-893, was made to exclude the possibility that expression required a liver specific enhancer, which may not be within that small fragment, as is the case with the albumin and cholesterol 7α-hydroxylase genes (Ramirez et al., 1993). Lastly, the albumin enhancer/albumin promoter lacZ fusion construct, pGalAEP, was made to ensure that conditions were optimal for liver-specific expression in H2.35 cells.

H2.35 cells were chosen as recipients for the promoter studies because of the relative ease of maintenance and because they have been successfully used to show liver-specific expression and cholesterol-mediated regulation of another gene being studied in our laboratory, cholesterol 7α-hydroxylase. H2.35 cells are a temperature sensitive SV40 transformed mouse hepatocyte cell line that when maintained at the permissive temperature (33 °C) show a fibroblast-like phenotype. When the cells are switched to nonpermissive or differentiating conditions (39 °C, plated on collagen and maintained in SFM, a hormonally
defined media), they display a more liver-like phenotype and express liver-specific genes at higher levels (Zaret et al., 1988)

When we transfected pGal3A10-893, which contains the Hind III-EcoN I fragment of the CYP3A10 promoter in front of the bacterial lacZ gene, into H2.35 cells and switched them to the differentiating conditions we observed an induction in reporter expression twofold higher than when the cells were maintained under permissive conditions (see Figure 21). This level of induction was similar to that observed with the pGalAEP, which expresses higher levels of the reporter when H2.35 cells are switched to differentiating conditions (Zaret et al., 1988), and suggests that the sequences necessary for liver-specific expression of CYP3A10 are within the 893 bp Hind III-EcoN I fragment and, most importantly, that H2.35 cells may be useful for investigating the molecular mechanisms regulating CYP3A10 expression.
Figure 21. Expression of a CYP3A10 Promoter/lacZ Fusion Gene in Transfected H2.35 Liver Cells. H2.35 cells were transfected with a -893 bp CYP3A10 promoter fragment/lacZ fusion gene (pGal3A10-893), an albumin enhancer/CYP3A10 -893 bp/lacZ gene (pGalAE3A10-893) an albumin enhancer/albumin promoter/lacZ fusion gene (pGalAEP) or the promoterless vector in conjunction with pSVCAT as a control by the calcium phosphate coprecipitation method. Cells were grown under differentiating conditions (39 °C on collagen) or nondifferentiating conditions (33 °C on plastic) for two days and harvested for β-galactosidase and CAT activities. The average β-galactosidase activity from two transfections was normalized to CAT activity and shown when cells are grown under differentiating conditions versus nondifferentiating. Activity from the promoterless vector was approximately 30% higher on plastic, indicating that the vector did not contribute to differentiated expression.
CHAPTER IV
Discussion
In this thesis, I report the isolation and characterization of the cDNA encoding the hamster liver lithocholic acid 6β-hydroxylase (Teixeira and Gil, 1991), the in vivo effector regulating its male-specific expression (Teixeira and Gil, 1994), and the cloning and characterization of its 5' flanking region (manuscript in preparation). This thesis has established a system for studying the molecular mechanisms governing the bile acid-mediated induction and male-specific expression of a gene that is crucial for maintaining the bile acid pool and, consequently, cholesterol homeostasis in mammals.

**cDNA Cloning and Characterization**

The cDNA, isolated by differential hybridization, has been identified as 6β-hydroxylase based on its ability to program the synthesis of active enzyme upon transfection of the cDNA when placed after the CMV promoter in COS cells (Figure 9). Other circumstantial evidence supports this finding. First, by amino acid sequence homology, the isolated cDNA CYP3A10 belongs to family 3 of the P450 gene superfamily (Figure 6), and all the members of that family characterized to date catalyze hydroxylation of different steroid substrates at position 6 (Gonzalez, 1989). Second, the major hydroxylation product of LCA in male hamsters is at position 6 (Figure 9) as it is in both rats (Zimniak et al., 1989), and humans (Radominska et al., 1990). Third, our results in hamster agree with previous reports that cholic acid feeding to rats (Danielsson, 1973) induced LCA 6β-hydroxylase activity in rat liver microsomes. We have extended those observations by demonstrating a concomitant increase in message for CYP3A10 associated with increased LCA 6β-hydroxylase activity, indicating the induction is at the pre-translational level.

It would not be unexpected for CYP3A10 to have other natural substrates since many P450s have broad substrate specificities and absolute identification of the preferred or
physiological substrate of any P450s may prove a formidable task. However, we have determined the $K_M$ for lithocholic acid in hamster liver microsomes to be approximately 3 μM (data not shown), within the concentration range reported for LCA in rat liver (Kurtz et al., 1982). We have also compared the kinetics of 6β-hydroxylase when expressed in COS cells with the substrates lithocholic acid, $K_M = 25 \mu$M, and androstenedione, $K_M = 75 \mu$M, and found that lithocholic acid was the preferred substrate. We, therefore, fully expect LCA to be a physiological substrate for CYP3A10.

Several new insights into bile acid metabolism are deduced from these studies. First, in this study, we unequivocally characterized LCA 6β-hydroxylase as a P450 family 3 protein, CYP3A10. This was done based on the degree of homology with other family 3 proteins (references found in (Nelson et al., 1993). Previous studies hypothesized that 6β-hydroxylation of LCA could be catalyzed by a P450 family 2 or 3 protein, based on immunological data (Zimniak et al., 1991). At the amino acid level, CYP3A10 is roughly 70% homologous with the other known members of this family. Due to its activity on lithocholic acid, we believe CYP3A10 is probably a new member of P450 family 3 rather than the hamster homolog of a previously characterized gene. The best characterized members of the family, CYP3A1/pcn1 and CYP3A5/pcn3, prefer steroid hormone substrates and are not the rat LCA 6β-hydroxylase (Radominska et al., 1990). Absolute identification of CYP3A10 as nonorthologous to other family 3 proteins characterized to date would necessitate the isolation of the rat LCA 6β-hydroxylase cDNA.

Second, our results suggest that in females lithocholic acid 6β-hydroxylase activity is not highly expressed. The level of CYP3A10/6β-hydroxylase mRNA is 50-fold higher in male versus females hamsters (Figure 5), although the level of 6β-hydroxylase activity is only 5-fold lower in females (Figure 7), which agrees with the 2-fold lower 6β-hydroxylase activity in females versus males described in rats (Zimniak et al., 1991).
Interestingly, the level of chenodeoxycholic acid, the immediate precursor of LCA, is significantly higher in female rat liver homogenate (Yousef et al., 1973; Kurtz et al., 1982). This is probably due to the ability of male rat liver to readily convert chenodeoxycholic acid to a more excretable metabolite (Yousef et al., 1973). Sex differences in the enzymes involved in bile acid metabolism have been reported for conjugation (Barnes et al., 1979) oxidoreduction (Björkhem, 1985) and bile acid binding proteins in the liver (LeBlanc and Waxman, 1990) and it certainly would not be surprising if that were the case for 6β-hydroxylase. The sex differences we have demonstrated in hamsters, 50-fold higher level of 6β-hydroxylase message and fivefold higher 6β-hydroxylase activity in males, are significantly different. The physiological significance of these sex differences in 6β-hydroxylase and bile acid metabolism is unknown, but one could speculate that in males 6β-hydroxylase is required for some steroid hormone hydroxylation which is not required in females.

A third insight derived from these studies relates to the cholic acid-mediated induction of CYP3A10/6β-hydroxylase mRNA, the first reported induction by bile acids. In young male hamsters, 6β-hydroxylase mRNA is induced by cholic acid feeding (Figure 5 and 8) and no induction thereafter (Figure 5). This mRNA induction is reflected in the 6β-hydroxylase activity (Figure 7 and 8). Furthermore, cholic acid feeding to hamsters also induces 6α-hydroxylase activity (Fig 7). Since hydroxylation of LCA is the major pathway of elimination of this toxic bile acid, the induction of both LCA hydroxylases suggests that feeding of cholic acid increases the level of LCA in the hepatocytes, which also suggests that a pathway that converts cholic acid into chenodeoxycholic acid may exist: If this is the case one would expect that chenodeoxycholic acid feeding of hamsters also induces 6β-hydroxylase, since chenodeoxycholic acid is the direct precursor of LCA (see Figure 1). However, preliminary experiments indicate that chenodeoxycholic acid feeding to hamsters suppresses CYP3A10/6β-hydroxylase RNA, in agreement with a
previous report that indicates chenodeoxycholic acid feeding suppresses LCA 6β-hydroxylase activity in rat liver microsomes (Danie1sson, 1973). Alternatively, deoxycholic acid (the 7α-dehydroxylated form of cholic acid) may get converted to LCA by a 12α-dehydroxylation reaction.

**Male-specific Expression of CYP3A10**

Sexual development in mammals occurs by sequential, ordered and relatively simple processes. In males, the product of the SRY gene on the Y chromosome directs the embryo to develop testes which in turn secrete testosterone to elicit development of male secondary sex characteristics collectively known as the male phenotype. In the absence of testosterone, as is the case in XX females and XY females lacking SRY, the normal, default female phenotype develops (Lovell-Badge, 1992). Two of the sexually dimorphic target organs of testosterone-mediated development are the brain and liver. In the neonatal brain, testosterone is converted to estrogen by aromatase activity and it is that estrogen that influences hypothalamic structures to direct the pituitary to release GH in the male-characteristic, pulsatile pattern. Circulating neonatal estrogen in females is absorbed by fetal estrogen binding proteins and so does not affect the brain, resulting in the female-characteristic, tonic pattern of GH release from the pituitary (MacLuskey and Naftolin, 1981). In the liver, sexual dimorphism can be controlled by three distinct but often overlapping mechanisms: 1) by androgen "imprinting" during a critical period in development, 2) by circulating gonadal hormones in the adult and 3) by the pattern of GH secretion (Bardin and Catterall, 1981; Westin et al., 1992).

In the present study, we have uncoupled the gonadal-hypothalamic-pituitary-liver axis to show that the sexually dimorphic expression of 6β-hydroxylase is not regulated by the pattern of GH secretion in males. The results from female experiments taken together
with what we have seen with males lead us to conclude that 6β-hydroxylase can be regulated by two distinct and independent pathways. We believe that in males 6β-hydroxylase expression is somehow turned on in liver by a mechanism independent of the hypothalamus since glutamate treatment of neonates does not significantly alter levels of expression in male hamster liver. Neonatal androgens are absolutely required since neonatal castration of males abolishes 6β-hydroxylase expression in the adult. Once the liver is "imprinted" for male-specific expression, adult gonadal hormones and GH are no longer required. In females, 6β-hydroxylase expression can be induced to essentially male levels by mimicking the pattern of male GH secretion, independent of circulating gonadal hormones or neonatal "imprinting". Preliminary results of a Southern experiment with Msp I- and Hpa II-digested hamster genomic DNA indicate that "imprinting" of the CYP3A10 gene does not involve methylation. Msp I and Hpa II are isoschizomers that digest the same site, CCGG, but recognize different methylation patterns.

Here we report the result of our efforts to identify the in vivo effector of male-specific expression of 6β-hydroxylase, a hamster liver P450 that hydroxylates lithocholic acid, as well as other steroid compounds, predominantly at the 6β position (Teixeira and Gil, 1991; Chang et al., 1993). The results of the experiments discussed in this report are compiled in Table 1. We have found that adult castration of males has no significant effect on the high level of 6β-hydroxylase expression normally observed in male hamster liver (Figure 4). This indicates that circulating testosterone is not necessary for that expression. On the other hand, in ovariectomized females that were injected daily with testosterone just before the harvesting of their livers, there was a 10-fold increase in 6β-hydroxylase expression. Ovariectomy of females alone did not alter the level of 6β-hydroxylase thus indicating that the lack of expression in females was not due to a suppressive effect of estrogen.
### TABLE 1

*Regulation of CYP3A10 mRNA Expression*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>100 ± 25</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>+ GH infusion</td>
<td>83 ± 15</td>
<td>nd</td>
</tr>
<tr>
<td>+ Estrogen injections</td>
<td>71 ± 21</td>
<td>nd</td>
</tr>
<tr>
<td>Adult Castration</td>
<td>85 ± 15</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>+ Testosterone injections</td>
<td>nd</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>Hypophysectomy</td>
<td>73 ± 5</td>
<td>18 ± 8</td>
</tr>
<tr>
<td>+ GH infusion</td>
<td>113 ± 57</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Neonatal Glutamate Treatment</td>
<td>66 ± 15(^b)</td>
<td>18 ± 6</td>
</tr>
<tr>
<td>+ GH injections</td>
<td>86 ± 18</td>
<td>66 ± 20</td>
</tr>
<tr>
<td>Neonatal Castration</td>
<td>0</td>
<td>nd</td>
</tr>
<tr>
<td>+ Testosterone injections</td>
<td>7 ± 5</td>
<td>nd</td>
</tr>
<tr>
<td>+ GH injections</td>
<td>3 ± 1</td>
<td>nd</td>
</tr>
</tbody>
</table>

\(^a\) RNA analysis and animal treatments are described in "Experimental Procedures". The values have been normalized to untreated males and are shown with S.E.M.

\(^b\) Not significantly different from normal males.

nd; not determined.
We have also shown that neither adult hypophysectomy nor continuous GH infusion significantly alters the level of 6\(\beta\)-hydroxylase expression in males (see Table 1). This is in stark contrast to what appears to be the case for CYP2C11 and a growing list of other rat liver proteins so regulated (Waxman, 1992; Westin et al., 1992). Interestingly, when hypophysectomy was performed on female hamsters, 6\(\beta\)-hydroxylase expression was significantly elevated. Furthermore, GH infusion treatment of hypophysectomized females reversed that induction so that the level of expression of 6\(\beta\)-hydroxylase was characteristically female again.

Glutamate treatment of neonates rather specifically destroys the arcuate nucleus of the hypothalamus, essentially abolishing GH secretion from the pituitary (Shapiro et al., 1989). Male neonates treated with glutamate expressed 6\(\beta\)-hydroxylase at levels that were not significantly different from untreated males. In females, however, there was a significant increase in expression. Mimicking the pattern of male GH secretion, by injecting glutamate-treated females with twice daily injections of GH, resulted in levels of 6\(\beta\)-hydroxylase expression that were not significantly different from male levels of expression. These results provide some support for the experimental results observed with hypophysectomy, given the observed induction with hypophysectomy was similar to that found with glutamate treatment. It appears that the pattern of GH secretion plays no significant role in 6\(\beta\)-hydroxylase expression in male hamster liver but in females, whose hypothalamus has been ablated, mimicking the male pattern of GH secretion does render the level of 6\(\beta\)-hydroxylase expression male-specific. The physiological significance, if any, of these observations which indicate 6\(\beta\)-hydroxylase can be expressed in females by altering the pattern of circulating GH, is unclear. Perhaps, under certain pathological circumstances in which the pattern of GH secretion is altered in females, 6\(\beta\)-hydroxylase is expressed.
We were able to significantly lower the expression of 6β-hydroxylase by neonatal castration. In those animals, the level of expression was essentially that of females. Further treatment of those animals either with testosterone or GH injections could not restore male levels of expression. It seems likely that neonatal testosterone is necessary during the "critical period" for neonatal "imprinting" of male specific expression of 6β-hydroxylase. Presumably, without testosterone, the male hamster liver and hypothalamus were essentially those of a female by default, hence the female characteristically low level of expression.

Having dissected the distinct and independent roles of testosterone and GH in the male-specific expression of CYP3A10/6β-hydroxylase, we now have a system to study the molecular details involved in its sexually dimorphic expression in the liver. We have begun investigating the molecular mechanisms involved in both the testosterone-mediated induction found in neonatal males and the GH-mediated regulation found in females of 6β-hydroxylase expression in a tissue culture system amenable for that purpose. The results of these efforts will lead, eventually, to a better understanding of liver sexual dimorphism.

**Cloning of the 5' Flanking and Expression of Chimeric Promoter/Reporter Constructs**

The P450 superfamily of proteins are remarkable both for the breadth of substrates on which they are active and —most interesting to us— for the complex, and sometimes obscure, molecular mechanisms regulating their expression (Gonzalez, 1989; Zaphiropoulos *et al.*, 1989; Guengerich, 1991; Guengerich, 1991; Paine, 1991; Porter and Coon, 1991; Waxman, 1992). Many P450s are often liver-specific but may be expressed in other tissues, as well or instead. They can be induced or suppressed by both exogenous and endogenous compounds that are usually subject to their catalytic dispositions. They
may be programmed for expression by development, diurnal variation, and according to the sex of the individual. For this thesis, we have cloned, expressed and characterized the cDNA encoding CYP3A10, a P450 that hydroxylates lithocholic acid at position 6β in hamster liver (Teixeira and Gil, 1991; Chang et al., 1993). We have determined the in vivo effector regulating its male-specific expression (Teixeira and Gil, 1993), and developed the tools necessary to begin investigating the molecular mechanisms governing the bile acid-mediated induction and the sexually dimorphic expression of CYP3A10. The molecular details involved in these two modes of regulation are unknown and will provide critical information regarding the bile acid catabolic pathway which is so important for cholesterol homeostasis in mammals.

We report here that the 893 bp Hind III-EcoN I fragment from the CYP3A10 promoter is capable of establishing liver-specific expression of a reporter with a lacZ fusion construct transfected into H2.35 cells when those cells are induced to display a liver phenotype (see Figure 21). These results suggest that the H2.35 cell culture system may be suitable for dissecting the molecular details involved in the sexually dimorphic and bile acid-induced expression of CYP3A10. It will be necessary to determine whether these cells will accurately reproduce the correct physiological environment for regulated expression of CYP3A10. H2.35 cells have been successfully used to characterize the different DNA elements and nuclear factors that are required for the liver-specific expression of the albumin gene (Zaret et al., 1990; Liu et al., 1991) and were used by others in our own laboratory to characterize the LDL-mediated regulation of cholesterol 7α-hydroxylase (Ramirez et al., 1993). These cells are propagated in a "de-differentiated" state at the permissive temperature (33 °C) and can be induced to the "differentiated" state, at will, by growing them at the non-permissive temperature (39 °C) on a collagen matrix and in a serum-free, hormonally-defined medium. It should be a trivial matter to adjust the
medium with hormonal factors such as GH and testosterone to define optimal conditions for regulated expression of CYP3A10 to determine the DNA elements required.

Liver cells, in culture, are notorious for not reproducing liver-specific functions and it may be necessary to try another approach for determining important DNA elements in the CYP3A10 promoter. Although much more tedious and time-consuming, transgenic mice may provide a suitable alternative should cultured cells fail to reproduce regulated expression. With this approach, chimeric promoter/reporter gene constructs are introduced into the germ line of mice where the male and female physiological environment exists naturally. Transgenic mice were used to show that, in GH-deficient dwarf mice, the male-specific expression of α₂u-globulin, a GH pattern-dependent rat liver protein, could easily be manipulated by the pattern of GH administration (Norstedt and Palmiter, 1984). The cis-elements and the trans-acting factors involved in that regulation were not investigated.

The DNA elements involved in the regulated expression of CYP3A10 will be identified by 5' deletions and point mutations of its promoter and by using heterologous promoters in the culture system (or transgenics) described above. Once the cis-elements are characterized, identification of the trans-acting factors involved in the sexually dimorphic and bile acid-induced expression of CYP3A10 will be done largely by DNA-binding studies that include DNase I footprinting and gel shift analysis (Gil et al., 1988; Smith et al., 1988). DNase I footprinting will be done to define more precisely the nucleotides involved in regulation with fragments that are identified as important in the experiments described above. In this assay, nuclear extracts are incubated with labelled DNA to allow proteins to recognize and bind to sequences in the DNA. DNase I is then added to generate a nested series of semi-random digestion fragments. Cleavage sites are displayed by gel electrophoresis and, in the presence of bound protein, a footprint or DNase I protected region is observed. This assay can also be used to follow a purification
scheme for isolating putative sequence-specific DNA binding proteins. To study the male-specific expression of CYP3A10, for example, nuclear extracts will be prepared from male and female hamster liver and incubated with a DNA fragment that confers male-specific expression of a reporter gene. Presumably, there will be different footprints from the two extracts representing the trans-acting factor(s) involved in the male-specific expression of CYP3A10.

Elucidating the molecular mechanisms involved in the sexually dimorphic and the bile acid-mediated induction expression of CYP3A10 should engender a better understanding of the liver's role in bile acid metabolism and cholesterol homeostasis. It will provide important clues for understanding the hereditary factors responsible for neonates born with cholestasis and what predisposes others, when adult, to develop cholestasis.

In this thesis we have cloned and characterized a hamster hepatic gene, CYP3A10, which encodes lithocholic acid 6β-hydroxylase, a P450 involved in bile acid metabolism. Its expression is induced by bile acid feeding and is male-specific, a result of neonatal androgen exposure; female hamsters can be persuaded, experimentally, to express CYP3A10 by manipulating the pattern of GH secretion. We have established a tissue culture system with H2.35 cells and chimeric CYP3A10 promoter/lacZ reporter constructs to investigate molecular mechanisms involved in the sexually dimorphic and bile acid-induced expression of CYP3A10.
CHAPTER V

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