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# A STAT Factor Mediates the Sexually Dimorphic Regulation of Hepatic Cytochrome P450 3A10/Lithocholic Acid 6β-Hydroxylase Gene Expression by Growth Hormone

ASHA SUBRAMANIAN, JOSE TEIXEIRA,† JIANMING WANG, AND GREGORIO GIL*

*Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01655*

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**Adult male rodents have a pulsatile profile of growth hormone (GH) release, whereas female rodents have a relatively steady-state pattern with uniform, albeit lower levels of GH. The expression of a number of sexually differentiated hepatic proteins is primarily determined by these plasma GH profiles and only secondarily regulated by gonadal hormones. An important subset of these sexually dimorphic proteins is cytochrome P450s. CYP3A10/6β-hydroxylase is a cytochrome P450 that catalyzes the 6β-hydroxylation of lithocholic acid. CYP3A10/6β-hydroxylase is expressed only in male hamsters; however, mimicking the male GH secretion pattern in females induces expression of the gene to male levels. Using chimeric CYP3A10/6β-hydroxylase promoter/luciferase reporter genes transfected into hamster primary hepatocytes, we have shown a GH-mediated induction of promoter activity. A combination of 5′-deletion constructs, heterologous promoter constructs, and specific mutagenesis was used to localize the DNA element involved in the GH-mediated regulation of CYP3A10/6β-hydroxylase promoter activity, which resembles a STAT binding site. Footprint and gel shift analyses confirmed that the expression of the protein binding to this site is regulated by GH and that the DNA-protein complex can be partially supershifted by anti-STAT-5 antibodies. This protein is 50% more abundant in male than in female hamster livers, is absent in hypophysectomized female livers, and is restored when hypophysectomized females are injected with GH in a manner that masculinizes female hamsters in terms of CYP3A10/6β-hydroxylase expression. The system characterized and described here is ideally suited for dissecting the molecular details governing the sexually dimorphic expression of liver-specific genes.**

The expression of a number of sexually differentiated hepatic proteins is primarily determined by plasma growth hormone (GH) profiles and only secondarily regulated by gonadal hormones through their effects on the hypothalamo-pituitary axis and its control of GH secretion (reviewed in references 32, 50, and 46). In rats, gonadal hormones are thought to exert their effects on the liver by two different mechanisms: first by a direct neonatal androgen effect, and second by their effect on the hypothalamus to direct the pattern of GH release from the pituitary. Adult males have a pulsatile profile of GH release, with serum levels becoming undetectable at times and with peaks of 200 to 300 ng/ml, and females have a relatively steady-state pattern of GH release, with uniform, albeit lower levels of serum GH (10 to 20 ng/ml). An important subset of these sexually differentiated hepatic proteins is cytochrome P450s (P450), which are a superfamily of heme-thiolate proteins that metabolize a variety of endogenous and exogenous substrates. They have characteristic, broad but overlapping substrate specificities and perform vital biochemical reactions, including drug detoxification and steroid hormone and bile acid synthesis. More than 220 genes encoding P450s have been found in both eukaryotes and prokaryotes and are classified by sequence homology into several families (28). Many of these families are under complex endocrine controls, which render their expression tissue, developmental stage, and/or sex specific (15, 16, 29, 30).

GH initiates its biological actions by binding to a specific cell surface receptor (20). Hormone binding induces GH receptor dimerization (8), and the complex binds to and activates JAK2 (2), an intracellular protein tyrosine kinase that can also interact with receptors for gamma interferon and other members of the cytokine/hematopoietin receptor family (39). Although the unique and specific pathways by which GH regulates target gene expression have not been defined, some information has recently become available on the DNA elements and *trans*-acting factors involved in the GH-mediated activation of two genes, the serine protease inhibitor (Spi) 2.1 (19, 44) and the c-*fos* (14) genes. The promoters of both of these genes contain a gamma interferon-activated sequence (GAS) that consists of a $TT(N)_5AA$ motif (9), which mediates the transcriptional activation of these genes by GH. The proteins that bind to GAS elements, named STAT (for signal transducer and activator of transcription) proteins, were first identified by studies of the gamma interferon signal transduction pathway and were shown to undergo tyrosine phosphorylation and nuclear translocation in response to receptor activation (9). From the results of studies using antibodies raised against previously characterized STAT factors, it has been suggested that STAT-3, a protein that mediates signalling pathways activated by epidermal growth factor, interleukin-6, and lipopolysaccharide (34, 51), binds to the c-*fos* GH response element (14) and that STAT-1, STAT-3, and STAT-4 do not bind to the Spi 2.1 GH response element (44). On the other hand, in two other studies, STAT-1 (24) and STAT-5 (49) have been shown to bind to the Spi 2.1 GH response element. Furthermore, it has been shown that GH activates tyrosine phosphorylation of at least

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School, 55 Lake Ave. North, Worcester, MA 01655. Phone: (508) 856-6138. Fax: (508) 856-2391.

† Present address: Pediatric Surgical Research Laboratories, Massachusetts General Hospital, Boston, MA 02114.

eight nuclear proteins, including STAT-1 (13). All of these data strongly suggest that several *trans*-acting factors mediate transcriptional activation by GH, and hence it is reasonable to hypothesize that the factor(s) involved in the sex-specific expression of liver proteins must be different from the above-mentioned STAT proteins, since the Spi 2.1 and c-*fos* genes are expressed both in males and in females.

We have previously reported the isolation of a hamster liver P450, CYP3A10/6β-hydroxylase, which catalyzes the 6β hydroxylation of lithocholic acid (LCA) as well as some steroid hormones (43, 5) and is expressed at 50-fold-higher levels in males than in females (43). 6β-Hydroxylation of LCA represents the major pathway for LCA catabolism and is important for maintenance of the bile acid pool and for prevention of cholestasis (26). The present study aims to elucidate the molecular mechanisms involved in the male-specific expression of CYP3A10/6β-hydroxylase. First, we show that GH is the primary hormone involved in the sex-specific expression of CYP3A10/6β-hydroxylase. We then cloned the 5′-flanking region of the CYP3A10/6β-hydroxylase gene. Using chimeric CYP3A10/6β-hydroxylase promoter/luciferase (Luc) reporter constructs transfected into hamster primary hepatocytes, we show a GH-mediated induction of CYP3A10/6β-hydroxylase promoter activity. Further using this system, we have localized a DNA element that mediates this transcriptional activation of the CYP3A10/6β-hydroxylase gene by GH. This element has the characteristics of a GAS element, and the factor that binds to it is absent in hypophysectomized female hamster nuclear extracts but is restored upon injections of GH in a manner that mimics the male pattern of GH secretion, consistent with the observed induction of CYP3A10/6β-hydroxylase expression to levels in males. The DNA-protein complex can be partially supershifted by anti-STAT-5 antibodies, supporting the idea that the factors involved in the sexually dimorphic expression of CYP3A10/6β-hydroxylase belong to the STAT family of transcriptional factors. This system should prove to be very useful in dissecting the molecular mechanisms involved in the sexually dimorphic expression of liver-specific genes.

## MATERIALS AND METHODS

**Materials.** $^{32}$P-labeled nucleotides were purchased from New England Nuclear. Reagents used in DNA cloning and sequencing were from New England Biolabs, Boehringer Mannheim, U.S. Biochemical Corp., or Gibco/BRL. Common laboratory chemicals were from Fisher, Sigma, or Bio-Rad. Human GH (2 IU/mg) was from either Sigma or the National Hormone and Pituitary Program. Golden Syrian hamsters were purchased from Charles River Laboratories, Wilmington, Mass. The Luc promoterless vector pGL2-Basic and pGem4 were purchased from Promega, and pBluescript SK+ was from Stratagene. S1 nuclease was purchased from Boehringer Mannheim, and reverse transcriptase was from Life Science, Miami, Fla. Affinity-purified anti-STAT antibodies were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, Calif. Anti-STAT-1 was raised against a peptide corresponding to amino acids 716 to 739 of human STAT-1 (p91); anti-STAT-3 was raised against a peptide corresponding to amino acids 750 to 769 of mouse STAT-3; anti-STAT-5 was raised against a peptide corresponding to amino acids 765 to 781 of mouse STAT-5b. Oligonucleotides were prepared in our department by the phosphoramidite method on an automated DNA synthesizer.

**General experimental procedures.** Standard recombinant DNA procedures, RNA isolation, blotting, and hybridization were carried out essentially as described elsewhere (35). DNA probes were labeled by random hexanucleotide priming (10) or by primer extension with M13 clones and the universal primer (7). DNA sequencing was done by the dideoxy-chain termination method (36) with DNA fragments subcloned into M13 vectors or with double-stranded clones and the universal primer or sequence-specific primers with reagents from U.S. Biochemical Corp. Protein was quantified by the method of Bradford (4).

**Genomic cloning.** A hamster genomic library was prepared in λGEM-11 as described elsewhere (35). The library was plated and screened with a single-stranded probe containing a 200-nucleotide (nt) fragment from the 5′ end of the CYP3A10/6β-hydroxylase cDNA (43) prepared in M13. Southern blot analysis was done to characterize the 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 *Eco*NI site (Fig. 1) in the 5′ untranslated region of the CYP3A10/6β-hydroxylase cDNA was subcloned into a pBluescript SK+ to generate pBS-6βgene. Sequencing was done with M13 vectors prepared from convenient restriction digests of a 2-kb *Hin*dIII fragment that extends from −822 bp in the 5′-flanking region to a site in the first intron.

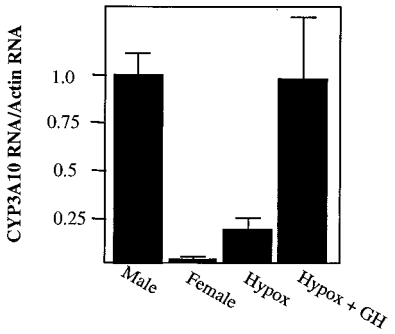



FIG. 1. Effect of adult hypophysectomy and/or GH administration on CYP3A10/6β-hydroxylase expression. Hypophysectomized (Hypox) and normal golden Syrian hamsters (five animals per group) were supplied at 8 weeks of age. Animal treatments are described in detail in Materials and Methods; total RNA was prepared and analyzed by slot blot analysis using a 182-nt single-stranded probe that has been shown to be specific for CYP3A10/6β-hydroxylase (43) and a human β-actin probe (22) as a control. The normalized CYP3A10/6β-hydroxylase/actin RNA ratios are shown on the left, and error bars represent standard errors of the means.

**Primer extension and S1 nuclease analyses.** Primer extensions were performed with 20 μg of total hamster liver RNA, or tRNA as a control, and a 40-nt primer (40-mer) labeled with [γ-$^{32}$P]ATP and polynucleotide kinase which corresponds to the sequences from +6 to +45 of the first exon (5′ CAGCAGCAC CCAGGTTTCTATAGAAAGGTTGGGGATCAGC 3′). Briefly, RNA was coprecipitated with the 5′-end-labeled 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. Twenty-three microliters of primer extension mix (20 mM Tris [pH 8.7], 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.33 mM deoxynucleoside triphosphates) was added with 1 U of reverse transcriptase, and the mixture was incubated for 30 min at 42°C. The reaction mixture was ethanol precipitated, resuspended in formamide dyes, and boiled before being loaded on a 6% polyacrylamide–7 M urea sequencing gel. The gel was run for 2 h at 60 W, dried, and exposed overnight at −70°C with intensifying screens.

S1 nuclease analyses were done with a single-stranded probe prepared from an M13 vector containing the *Hin*dIII fragment used for sequencing as described above and the same 40-mer primer used for primer extensions. Twenty micrograms 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 of hybridization solution that contained 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; 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 of S1 digestion buffer [250 mM NaCl, 20 mM potassium acetate (pH 4.5), 1 mM $Zn(SO_4)_2$] and 200 U of S1 nuclease. The reaction was stopped by adding 100 μl of S1 stop buffer (3 M ammonium acetate, 2.5 mM EDTA, 200 μg of tRNA), and the mixture was ethanol precipitated and resuspended in formamide with dyes for gel electrophoresis as described above.

**Preparation of chimeric CYP3A10 promoter/Luc reporter constructs.** Chimeric CYP3A10 promoter/Luc reporter constructs were prepared in the following manner. First, a plasmid named pHN306 was prepared by digesting pBS-6βgene with *Eco*NI, blunt ending, digesting with *Hin*dIII, and inserting the 0.9-kb *Hin*dIII-*Eco*NI fragment into the *Hin*dIII-*Sma*I site of pBluescript SK+. pHN306 was then digested with *Hin*dIII and *Bam*HI, generating a 0.9-kb genomic DNA fragment, blunt ended, and inserted into the blunt-ended *Xho*I site of pGL2-Basic to create pLuc6β-821. pLucAEP was created by digesting pAN/T2B (kindly provided by Ken Zaret, Brown University) with *Nhe*I and *Hin*cII, blunt ending and isolating the 2.8-kb fragment, and then inserting it into the *Xho*I-digested and blunt-ended pGL2-Basic. pLuc6β-438 and pLuc6β-137 were prepared by inserting the 518-bp *Ava*I-*Bam*HI or the 217-bp *Eco*RI-*Bam*HI fragment from pHN306 into the *Xho*I site of pGL2-Basic. All other 6β-hydroxylase promoter constructs were prepared by amplifying the corresponding 6β-hydroxylase promoter fragment by PCR and then blunt ending with T4 DNA polymerase and inserting the fragments into the *Xho*I site of pGL2-Basic. Plasmids pLuc6β-821 (Mut-101/-78) and pLuc6β-821 (Mut-91/-83) were created by oligonucleotide-directed mutagenesis as described previously (40). pLucAP was created by inserting the mouse albumin promoter (*Nco*I-*Hin*cII fragment from pAN/T2B) into the *Xho*I-digested and blunt-ended pGL2-Basic. The heterologous albumin promoter plasmids used in the experiments shown in Fig. 6 were created by inserting the corresponding synthesized fragments from the

CYP3A10/6β-hydroxylase promoter into the *Sma*I site of pLucAP. The number and orientation of the inserted fragments were determined by DNA sequencing.

**Isolation and transfection of primary hepatocytes.** Hepatocytes were prepared for primary culture by perfusion of isolated female hamster livers. Procedures were carried out under aseptic conditions, and all solutions were filter sterilized through 0.22-μm-pore-size Millipore filters. On day 0, 6- to 8-week-old female hamsters weighing between 100 and 150 g were anesthetized with an intraperitoneal injection of pentobarbitol (Nembutal) sodium (10 mg/100 g of body weight). The hepatic portal vein was cannulated, and perfusion was initiated by using 250 ml of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (10 mM HEPES, 142 mM NaCl, 6.7 mM KCl [pH 7.4]) containing heparin (1,000 USP units/100 ml) and EGTA (0.66 mM) to remove blood and $Ca^{2+}$. The liver was perfused for an additional 8 min with recirculation of 100 ml of the above-described buffer to which collagenase (0.05%) and $CaCl_2$ (11.6 mM) had been added. Following perfusion, dispersion of cells was accomplished by finely mincing the liver with scissors. Viable cell counts were performed with trypan blue, and cells were subjected to isodensity Percoll centrifugation (18), which results in the isolation of single, viable liver parenchymal cells. Primary cultures were initiated by seeding $8 \times 10^5$ viable cells onto 60-mm-diameter Corning plastic tissue culture dishes containing 4 ml of medium (1:1 mixture of Dulbecco modified Eagle medium and Ham's F12 medium supplemented with 10% fetal bovine serum, 100 U of penicillin per ml, and 0.1 mg of streptomycin per ml) and were incubated at 37°C in a humidified atmosphere containing 95% air and 5% $CO_2$. These tissue culture dishes had been precoated with rat tail collagen overnight and then allowed to calibrate with 4 ml of the above-described medium in an incubator at 37°C in the presence of humidified 95% air and 5% $CO_2$ for at least 2 h prior to seeding. Four hours after plating, when the cells had adhered to the collagen matrix, fresh medium was added, and cultures were incubated at 37°C in a humidified atmosphere containing 97% air and 3% $CO_2$. An hour later, cells were cotransfected by the calcium phosphate coprecipitation method as modified by Chen and Okayama (6), using 8 μg of the test plasmid, 1 μg of pCMV-RGHR (a plasmid containing the rabbit GH receptor cDNA in pCMV [1]), and 1 μg of pCMV-Gal (a plasmid containing the *Escherichia coli lacZ* gene in pCMV), and were incubated with the DNA overnight at 37°C in 97% air–3% $CO_2$. On day 1, the DNA was washed off the cells by rinsing the cells twice with 1× phosphate-buffered saline, and cultures were refed with a chemically defined serum-free medium (William's E) supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), insulin (0.25 U/ml), triiodothyronine (1 μM), and dexamethasone (0.1 μM). GH (500 ng/ml unless otherwise indicated) was added to the appropriate dishes. On day 2, fresh medium was added along with GH. On day 3, i.e., 48 h after GH treatment (except for the time course experiment shown in Fig. 4), cells were harvested and assayed for Luc activity by using a kit from Promega and for β-galactosidase activity by colorimetric analysis using *o*-nitrophenyl-β-D-galactopyranoside as the substrate. Luc activity was normalized to β-galactosidase activity to control for transfection efficiency.

**Experimental animals.** Golden Syrian hamsters were maintained on a 12-h light/12-h dark cycle, fed regular laboratory chow, and killed at the middle of the dark cycle. Hypophysectomy was performed by the supplier, and the animals were allowed at least 2 weeks to recover before any additional manipulations. To mimic the male pattern of GH release, GH, in GH buffer (30 mM $NaHCO_3$ [pH 9.3], 150 mM NaCl), was injected subcutaneously at a dose of 600 ng/g of body weight every 12 h for 3 days. Total RNA was isolated from 0.5 g of liver, and the remaining liver was used for the isolation of nuclear extract by the Schibler method (12). All animal experiments were conducted in accordance with the principles and procedures of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

**DNase I footprinting and gel mobility shift analysis.** [$^{32}$P]DNA probes for footprinting analysis were prepared by phosphorylation of the *Nhe*I-*Hin*dIII fragment from pHN306 with [$^{32}$P]ATP and T4 polynucleotide kinase and digestion with *Ava*I. Probes were incubated with nuclear extracts and digested with DNase I as described previously (11). Gel mobility shift analysis was performed as described previously (11), using as probes the following oligonucleotides: CYP3A10/6β-hydroxylase GH regulatory element probe (GTTACCCAAGTTC CTGGAAGCGTG) and Oct-1 binding element (CTAGCCGAATGCAAAT CACT).

## RESULTS

**Role of circulating GH in the sex-specific expression of CYP3A10/6β-hydroxylase.** In previous studies, we observed that expression of the CYP3A10/6β-hydroxylase gene is male specific (43). To assess what role, if any, circulating GH played in CYP3A10/6β-hydroxylase gene expression, we manipulated the levels of GH secretion in female hamsters and compared the level of mRNA expression with that in normal males; the results are shown in Fig. 1. Normal female levels of CYP3A10/6β-hydroxylase mRNA are 50- to 100-fold lower than normal male levels. Disruption of the female pattern of circulating GH by hypophysectomy increased the level of CYP3A10/6β-hydroxylase mRNA up to 22% of the level in normal males, and the level of circulating GH was undetectable. Furthermore, mimicking the male pattern of GH release from the pituitary via subcutaneous injections of GH twice daily for 3 days to another group of hypophysectomized females was sufficient to increase the CYP3A10/6β-hydroxylase mRNA level to that seen in normal males. This result prompted us to use this system to unravel the molecular mechanisms involved in GH-mediated sex-specific gene expression.

**CYP3A10/6β-hydroxylase promoter cloning.** A hamster genomic library was prepared in λGEM-11 phage and screened with the 5′ end of the CYP3A10/6β-hydroxylase cDNA. We isolated one clone, which we chose to characterize further, that contained approximately 15 kb of the CYP3A10/6β-hydroxylase gene. Southern blot analyses were done to characterize this clone, which revealed that it contained approximately 8 kb of the 5′-flanking region of the CYP3A10/6β-hydroxylase gene. An endonuclease restriction map of the isolated DNA is shown in Fig. 2, with convenient restriction sites indicated.

We sequenced approximately 1.1 kb of the genomic clone, using M13 clones and the universal primer or using double-stranded plasmids with sequence-specific primers (see Materials and Methods), and the results are shown in Fig. 2. The first intron begins 71 bp downstream of the initiator methionine codon. Analysis of the sequence revealed multiple regions of homology to the consensus binding site of HNF3, a liver-specific DNA-binding protein (17). 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 binds cooperatively with TFII-I to Inr-containing TATA-less promoters, functionally generating an alternate TFII-D pathway for preinitiation complex assembly (31). The Inr site that we found in the CYP3A10/6β-hydroxylase promoter (CTGGGTCTC) is identical to the human immunodeficiency virus type 1 initiator (32).

We performed primer extension and S1 nuclease digestion analyses to map the 5′ end of the CYP3A10/6β-hydroxylase message. Figure 3 shows the results of these 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 extension and for sequencing, so that the products of S1 digestion and primer extension would be identical in length. The sequencing ladder was run alongside primer extension and S1 digestion products to pinpoint the exact 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 livers. No primer extension products were seen with female RNA or tRNA.

**Localization of a CYP3A10/6β-hydroxylase GH regulatory region.** Having concluded that we had cloned the transcription initiation site, chimeric CYP3A10/6β-hydroxylase promoter/Luc reporter constructs were prepared to determine whether the 5′-flanking region of CYP3A10/6β-hydroxylase contained the DNA sequences necessary for transcription and for GH-mediated regulation of transcription. We started with a construct containing 821 nt of the 5′-flanking region and used hamster primary hepatocytes as host cells for transfection. The plasmid to be tested was cotransfected with pCMV-RGHR (a plasmid that contains the GH receptor in an expression vector in which transcription is driven by the human cytomegalovirus

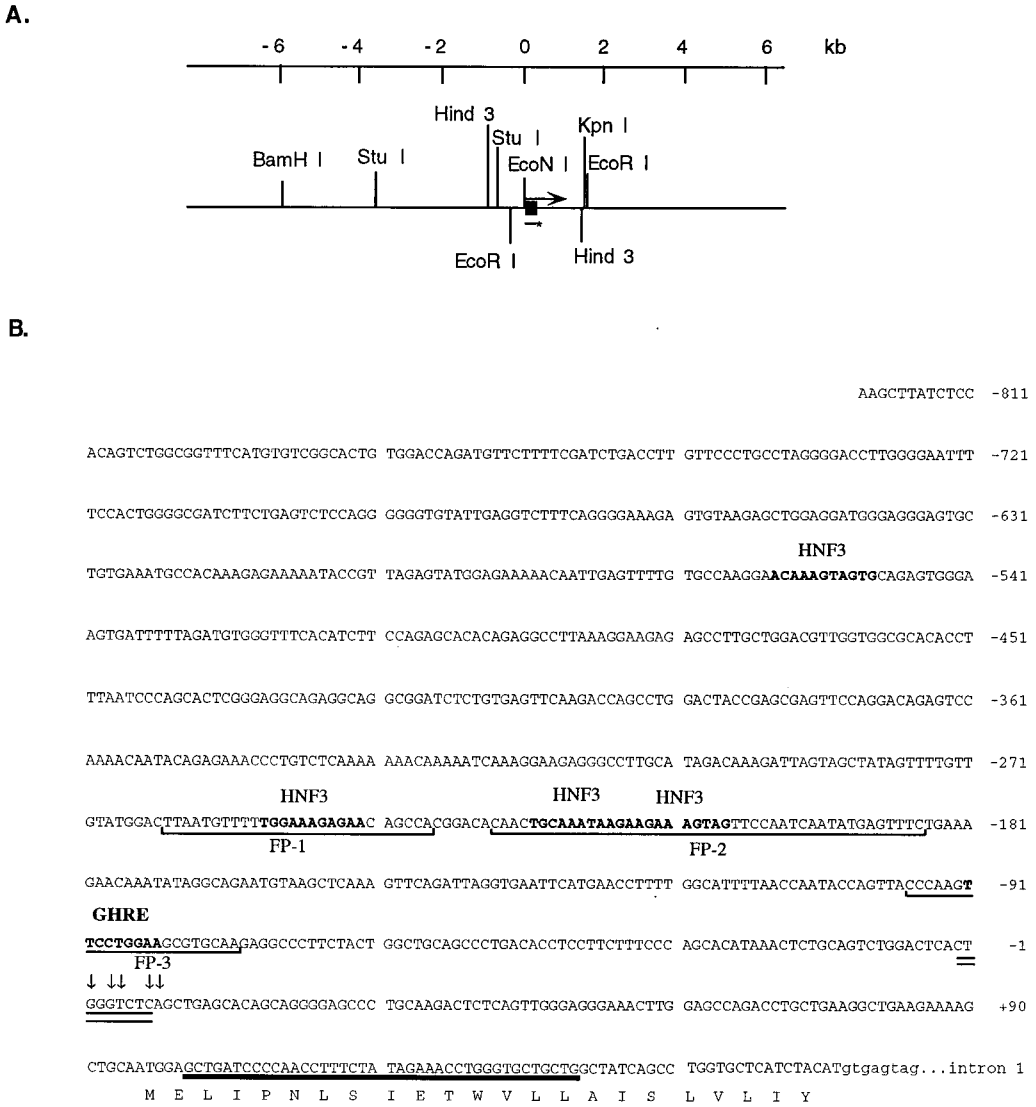



FIG. 2. Restriction endonuclease map of the CYP3A10/6β-hydroxylase genomic clone and sequence of the CYP3A10/6β-hydroxylase proximal promoter. (A) Schematic representation of approximately 15 kb of CYP3A10 genomic DNA. The scale at the top represents the nucleotide positions relative to the transcription initiation site, which is indicated by a bent arrow. 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′ *Eco*RI fragment of the CYP3A10/6β-hydroxylase cDNA, nt −76 to 186. Restriction endonuclease sites that were useful in subcloning are indicated. (B) Nucleotide sequence of the CYP3A10/6β-hydroxylase promoter. Nucleotides are numbered on the right, with +1 indicating the first transcription initiation site (see Fig. 3); nucleotides upstream are negatively numbered. The transcription initiation sites are indicated by arrows, and a putative TFII-I-binding Inr site (33) is indicated by a double underline. The predicted single-letter amino acids are shown below the first exon, and lowercase letters indicate nucleotides that are in the first intron. The sequence corresponding to the 40-mer used as a primer for the primer extension analysis and for preparing the S1 digestion probe is underlined with a thick line. Potential HNF3 binding sites are in boldface, and regions protected by liver nuclear proteins against DNase I digestion (see Fig. 8) are underlined. The functionally identified CYPGHRE (GHRE) is also in boldface.

major immediate-early gene promoter [45]) to ensure that GH became internalized and with pCMV-Gal to normalize for transfection efficiency. Experiments performed without co-transfecting the GH receptor expression plasmid showed a wide variation among individual experiments, presumably as a result of partial degradation of the GH receptor during hepatocyte isolation and/or loss of expression of the endogenous receptor during the course of the experiment. To establish optimal conditions, a GH concentration curve and a time course for GH induction were performed (Fig. 4). Induction of CYP3A10/6β-hydroxylase promoter activity reflected increasing concentrations of added GH, reaching a plateau at approximately 50 ng of GH per ml (Fig. 4A) with a GH incubation time of 25 h (Fig. 4B). To demonstrate the specificity of this regulation, a construct containing the mouse albumin promoter/enhancer in front of Luc, pLucAEP, was transfected under identical conditions, and the transfected cells were incubated with the same GH concentrations. As can be seen in Fig. 4A, no activation was observed under any condition tested.

To localize the putative DNA element(s) involved in the GH-mediated regulation of CYP3A10/6β-hydroxylase promoter activity, we constructed a series of 5′-deletion constructs as shown in Fig. 5. Hamster primary hepatocytes were transfected with each plasmid and incubated in the presence or absence of 500 ng of GH per ml. GH addition induced promoter activity in all constructs except pLuc6β-62. Also, there

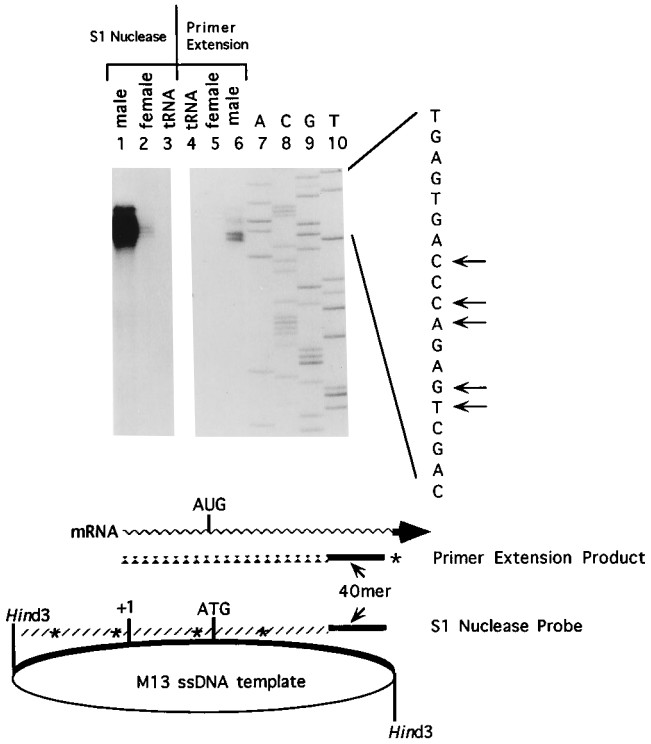



FIG. 3. Localization of the transcription initiation site by primer extension and S1 digestion analyses. Total RNA was prepared and primer extension and S1 digestion analyses were performed as described in Materials and Methods. Experiments were done with RNA from the livers of hamsters of the indicated sex (males, lanes 1 and 6; females, lanes 2 and 5) or with control tRNA (lanes 3 and 4). The primer used for primer extension was the same as that used for preparing the S1 probe. A sequencing experiment, shown on the right (lanes 7 to 10), was also done with the same primer and run on the same gel to identify the precise nucleotides where transcription of the CYP3A10/6β-hydroxylase 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 depicts 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 labeled S1 probe.

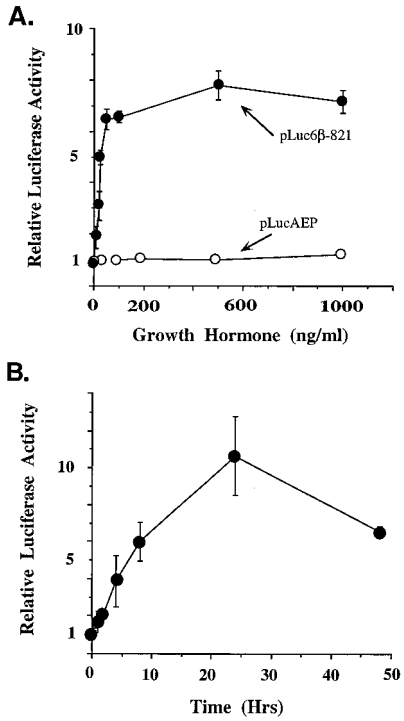


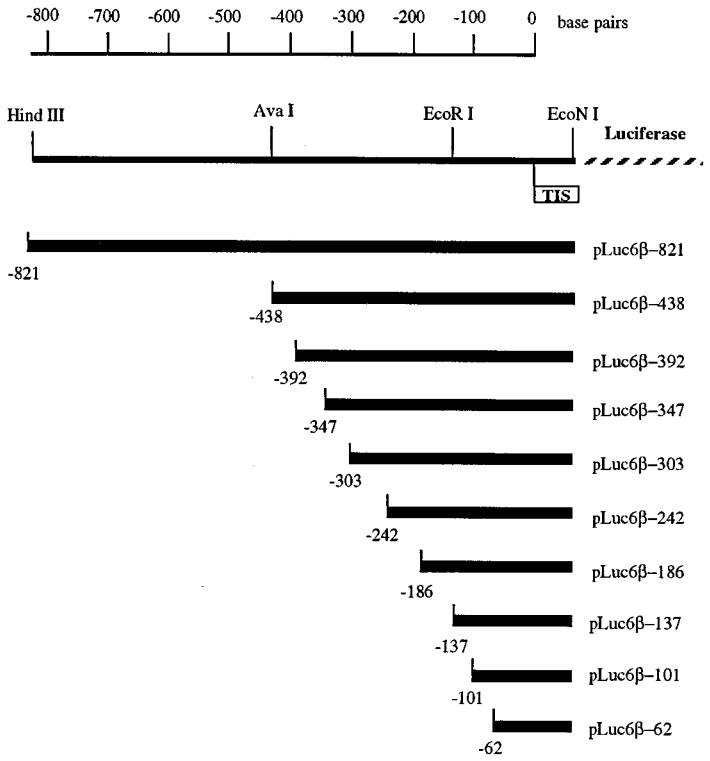

FIG. 4. GH concentration curve and time course for GH induction of CYP3A10/6β-hydroxylase promoter activity. Female hamster primary hepatocytes were cotransfected with pLuc6β-821, pCMV-RGHR, and pCMV-Gal on day 1 as described in Materials and Methods. Transfected cells were then subjected to the indicated concentrations of GH (A) or 500 ng of GH per ml of medium for the indicated times (B). All cells were harvested on day 3. Average values for three experiments relative to the values for cells grown in the absence of GH are shown. Bars indicate the standard errors of the means.

was a partial loss of GH activation in cells transfected with constructs containing 303 nt or less of the CYP3A10/6β-hydroxylase 5′-flanking region. This result suggested that a putative CYP3A10/6β-hydroxylase GH regulatory element (CYPGHRE) is located at positions −101 to −62, and perhaps others are located between −303 and −101.

To test whether a CYPGHRE is located between −101 and −62, we created a chimeric gene by inserting the −101 to −53 fragment from the CYP3A10/6β-hydroxylase promoter into a plasmid that contains the mouse albumin promoter in front of the Luc gene as a reporter (pLucAP). Both constructs were analyzed for the ability to provide GH-mediated regulation of promoter activity, using the system described above. Figure 6 shows the average results of six experiments performed with each construct. As expected, the albumin promoter alone was not regulated by GH, but when three copies of the −101 to −53 fragment from the CYP3A10/6β-hydroxylase promoter were present, albumin promoter activity was induced 2.9-fold. To further narrow down the location of such an element, we made three similar plasmids, each containing two copies of either a −101 to −78, −87 to 68, or −77 to −53 fragment from the CYP3A10/6β-hydroxylase promoter (Fig. 6), and analyzed them as described above. Only the construct containing the −101 to −78 fragment showed significant activation upon GH addition, indicating that this fragment contains a CYPGHRE.

Thus, from the 5′-deletion constructs (Fig. 5) and heterologous promoter constructs (Fig. 6), we localized a 24-nt fragment necessary and sufficient for GH-mediated regulation of CYP3A10/6β-hydroxylase promoter activity. The question remained as to whether this fragment contained the only CYPGHRE present in the 821 bp of the CYP3A10/6β-hydroxylase 5′-flanking region that we studied, since constructs which contained less than 303 bp of the CYP3A10/6β-hydroxylase 5′-flanking region were not activated by GH as much as the pLuc6β-821 construct. To address this question, we mutated the −78 to −101 fragment by site-directed mutagenesis, in the context of the longest construct that we studied (pLuc6β-821). Figure 7 shows that this mutation completely abolished the GH-mediated activation of promoter activity compared with the wild-type construct pLuc6β-821. This result indicates that the only CYPGHRE present within the 821 bp of CYP3A10/6β-hydroxylase promoter analyzed is at positions −78 to −101. Visual inspection of this nucleotide sequence revealed a 9-nt palindrome (TTCCTGGAA) with the characteristics of a GAS element. To test whether this sequence is the CYP3A10/6β-hydroxylase CYPGHRE, we mutated these 9 nt and analyzed the resulting construct for its ability to mediate GH regulation. As shown in Fig. 7, expression from this mutant CYP3A10/6β-hydroxylase promoter construct was not regulated by GH.

**Characterization of the nuclear protein that binds to the CYPGHRE.** To localize regions within the CYP3A10/6β-hydroxylase promoter that may bind nuclear proteins involved in




| Promoter Activity (Luc/β-gal) - GH | Promoter Activity (Luc/β-gal) + GH | Regulation Fold |
|---|---|---|
| 100 | 673 ± 36 | 6.7 (n = 14) |
| 69 ± 9 | 378 ± 35 | 5.5 (n = 8) |
| 51 ± 5 | 224 ± 14 | 4.4 (n = 5) |
| 96 ± 17 | 439 ± 55 | 4.6 (n = 5) |
| 106 ± 13 | 567 ± 66 | 5.4 (n = 5) |
| 116 ± 22 | 455 ± 68 | 3.9 (n = 5) |
| 105 ± 16 | 364 ± 56 | 3.5 (n = 5) |
| 35 ± 7 | 83 ± 3 | 2.4 (n = 8) |
| 26 ± 4 | 87 ± 8 | 3.4 (n = 7) |
| 12 ± 1 | 17 ± 2 | 1.4 (n = 7) |

FIG. 5. Expression of 5′-deleted CYP3A10/6β-hydroxylase promoter/Luc constructs in hamster primary hepatocytes. The DNA fragment (thin bar) from the 5′-flanking region of the hamster CYP3A10/6β-hydroxylase gene is shown with the restriction sites used for plasmid construction (see Materials and Methods). The Luc coding sequence is denoted by the hatched bar, and the putative transcription initiation site is indicated. A scheme of the different chimeric genes used is shown. Female hamster primary hepatocytes were transfected with the indicated construct and treated with GH as described in Materials and Methods. Relative transcription was determined by normalizing Luc activity to β-galactosidase (β-Gal) activity. The data were normalized to the activity produced by the construct containing the longest promoter fragment, pLuc6β-821, in cells grown in the absence of GH and represent the averages of *n* experiments ± standard errors of the means.

promoting or modulating transcription of the CYP3A10/6β-hydroxylase gene, we performed DNase I footprinting experiments with its promoter (Fig. 8). In the presence of a male hamster liver nuclear extract, three regions of the promoter were protected from DNase I; these regions were arbitrarily named FP-1 to FP-3 and localized by running a sequencing reaction on the same gel (data not shown) as indicated in Fig. 2B. FP-1 and FP-2 are centered around potential HNF3 binding sites. The third region, FP-3, is centered around the functionally localized CYPGHRE, suggesting that a nuclear protein involved in the GH-mediated regulation of CYP3A10/6β-hydroxylase gene transcription binds to the CYPGHRE located between nt −83 and −91. These three protected regions were the only footprints found in the entire 821 bp of CYP3A10/6β-hydroxylase promoter analyzed (Fig. 8 and data not shown).

To further characterize the nuclear protein that binds to the CYPGHRE, gel shift assays were performed with a 24-bp (−101 to −78) fragment that contains the CYPGHRE as a probe (Fig. 9A). Liver nuclear extracts were prepared from the same hamsters used in the experiment shown in Fig. 1. A retarded band was observed when male or female extracts were used, indicating that a nuclear protein that binds to the CYPGHRE is present in both male (lanes 1 to 3) and female (lanes 4 to 6) extracts. The specificity of the binding to CYPGHRE was demonstrated by competing for binding with a 100-fold molar excess of either the unlabeled wild-type oligonucleotide (lane 13) or an unlabeled mutant oligonucleotide in which the CYPGHRE (−83 to −91) had been mutated. Binding was consistently higher in several experiments in which male extracts were used than in experiments using female extracts (50% in the experiment shown). In hypophysectomized female hamsters, binding was completely absent (lanes 7 to 9) and was restored upon subcutaneous injection of GH for 3 days, a treatment that masculinized female hamsters in terms of CYP3A10/6β-hydroxylase expression (Fig. 1). As a control, a similar experiment was performed with a DNA fragment containing the Oct-1 sequence as a probe (Fig. 9B). Binding was similar with all four extracts.

Since the characterized DNA-binding element has the characteristics of a GAS element, and since different STAT factors have been suggested to be involved in the GH-mediated activation of gene transcription (14, 24, 49), we performed supershift experiments with antibodies raised against STAT-1, STAT-3, and STAT-5 peptides (Fig. 10). As a control, we used antialbumin antibodies (lanes 2 and 9). Anti-STAT-1 (lanes 3 and 10) and anti-STAT-3 (lanes 4 and 11) showed no specific effect on the shifted band. Anti-STAT-5 partially supershifted the DNA-protein complex in both male (lanes 5 to 7) and female (lanes 12 to 14) hamster liver extracts. We were unable to completely supershift the retarded band even when we used 10-fold more anti-STAT-5 than shown.

## DISCUSSION

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

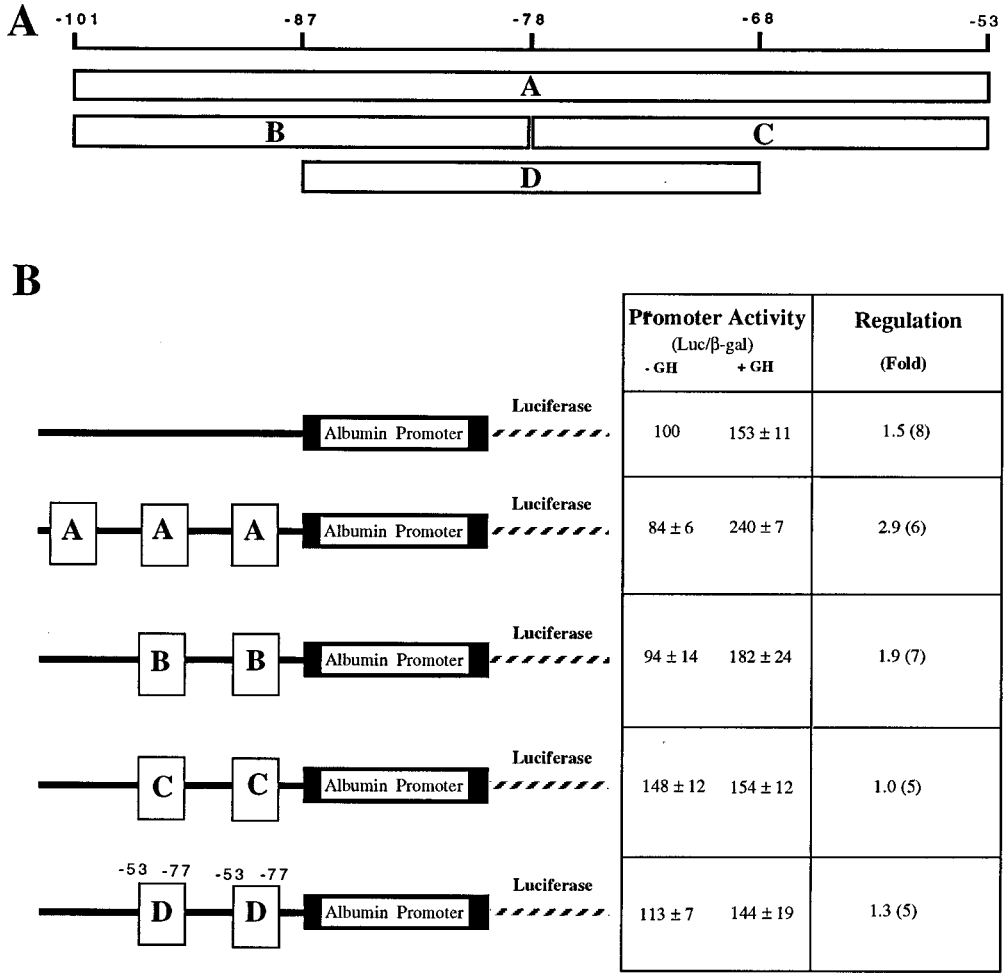
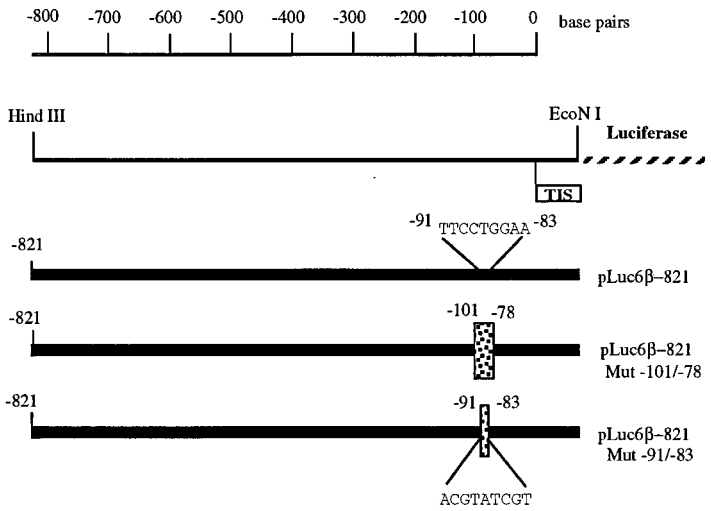



FIG. 6. Expression of CYP3A10/6β-hydroxylase/albumin promoter/Luc constructs in hamster primary hepatocytes. (A) Schematic representation of the four fragments from the CYP3A10/6β-hydroxylase promoter inserted into pLucAP. (B) Each resultant construct is shown schematically and was transfected into primary hepatocytes and incubated with or without GH as indicated in Materials and Methods. The data are shown in the same form as in Fig. 5.

develop testes, which in turn secrete testosterone to elicit development of male secondary sexual 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 (21). Two 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 this 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 hence does not affect the brain, resulting in the female-characteristic, tonic pattern of GH release from the pituitary (23). In the liver, sexual dimorphism of gene expression can be controlled by three distinct but often overlapping mechanisms: (i) by androgen imprinting during a critical period in development, (ii) by circulating gonadal hormones in the adult, and (iii) by the pattern of GH secretion (3, 48). The last is perhaps the most common mechanism involved in the sex-specific expression of a subset of P450s that catalyze the hydroxylation of a variety of steroid hormones (46).

In this study, we have classified the hamster CYP3A10/6β-hydroxylase gene in this last group. This gene encodes a protein that among other activities, catalyzes the hydroxylation, at position 6β, of LCA, testosterone, androstenedione, and progesterone (5) and is expressed only in males. By manipulating the pattern of GH secretion in female hamsters to mimic that of males (by hypophysectomy followed by GH injection), expression of CYP3A10/6β-hydroxylase could be induced to levels seen in normal males (Fig. 1). This result strongly suggests that GH is involved in the male-specific transcription of the CYP3A10/6β-hydroxylase gene. The same result was observed when instead of hypophysectomy, the pattern of GH secretion was altered by treating neonates with monosodium glutamate, a treatment that destroys the arcuate nucleus of the hypothalamus, abolishing GH secretion from the pituitary (38), followed by GH injections (42). Interestingly, hypophysectomized and monosodium glutamate-treated female hamsters showed a remarkable induction of CYP3A10/6β-hydroxylase expression (25% of normal male expression), which indicates that GH, under the conditions of female GH secretion, may also act as a suppressor of gene transcription.

The molecular mechanisms involved in the GH-mediated regulation of gene transcription are only now starting to be unraveled. GH regulatory elements have been located in two genes, Spi 2.1 (19, 44) and c-*fos* (14), but both of these genes are expressed in both sexes, and hypophysectomy of rats abol-




| Promoter Activity (Luc/β-gal) - GH | + GH | Regulation Fold |
|---|---|---|
| 100 | 720 ± 63 | 7.2 (n = 5) |
| 325 ± 46 | 423 ± 55 | 1.3 (n = 5) |
| 166 ± 49 | 212 ± 36 | 1.3 (n = 3) |

FIG. 7. Expression of mutant CYP3A10/6β-hydroxylase promoter/Luc constructs in hamster primary hepatocytes. pLuc-821 Mut-101/-78 and pLuc-821 Mut-91/-83 were created as indicated in Materials and Methods. The wild-type sequence of the CYPGHRE and the mutated version are indicated. Each resultant plasmid and pLuc6β-821 (as a standard) were independently transfected into primary hepatocytes and incubated with or without GH as indicated in Materials and Methods. The data are shown in the same form as in Fig. 5.

ishes expression of both genes, indicating that GH acts as an activator of the transcription of these two genes. Thus, it seems unlikely that the factors and mechanisms involved in the GH-mediated regulation of transcription of the Spi 2.1 and c-*fos* genes and of the CYP3A10/6β-hydroxylase gene would be the same. This prompted us to establish a system suitable for studying GH-mediated regulation of the CYP3A10/6β-hydroxylase promoter, which could lead to a better understanding of the mechanisms involved in the sexually dimorphic expression of liver genes.

Since GH-mediated regulation of hepatic P450 sexual dimorphism has not been described, we started by establishing a hamster primary hepatocyte tissue culture system that, upon transfection with chimeric genes containing 821 bp of the CYP3A10/6β-hydroxylase promoter in front of a reporter gene (Luc) and subsequent incubation with GH, could help in detecting variations in reporter gene expression. The maximal activation obtained was between 7- and 10-fold (Fig. 4), which is quantitatively similar to what we observed in vivo, approximately 5-fold, between GH-depleted (hypophysectomized) and GH-injected hamsters (Fig. 1). It is also worth noting that the GH concentration necessary for maximal activation (approximately 50 ng/ml) is within the range of the circulating GH level in male rats, which peaks at approximately 225 ng/ml (47). Attempts to establish conditions that would reproduce the GH-mediated suppression of CYP3A10/6β-hydroxylase transcription observed in vivo (normal female hamsters versus hypophysectomized females) have been unsuccessful to date.

We used a combination of 5′-deletion analysis of the CYP3A10/6β-hydroxylase promoter (Fig. 5), heterologous promoters (Fig. 6), and specific mutagenesis (Fig. 7) to demonstrate that 9 nt at positions −91 to −83 which are characteristic of a GAS element (9) mediate the activation of the CYP3A10/6β-hydroxylase promoter by GH and that this element is the only one involved in GH regulation within the 821 bp of CYP3A10/6β-hydroxylase promoter analyzed. This set of experiments also suggests that the *trans*-acting factor that binds to the CYPGHRE interacts with factors that bind to DNA elements located between nt −303 and −101 because different deletion constructs of this region quantitatively diminished the GH-mediated regulation of the CYP3A10/6β-hydroxylase promoter, in spite of the presence of a unique CYPGHRE at positions −91 to −83. Interestingly, three putative HNF3 binding sites are located between nt −252 and −206 (Fig. 2), and these regions are protected against DNase I by liver nuclear extracts (Fig. 8). DNase I footprinting analysis also showed a protected region around the CYPGHRE.

Functional characterization of *cis* elements involved in GH-mediated sexually dimorphic gene expression has not been previously described, and our study of the CYPGHRE is the first step toward characterizing the molecular mechanisms involved in the sexually dimorphic expression of P450 genes in particular and of liver genes in general. Recently, GH response elements have been characterized in two other genes, Spi 2.1 (19, 44) and c-*fos* (14, 25). The GH response element within the Spi 2.1 gene has been narrowed down to a 45-bp fragment that contains two GAS elements, whereas the c-*fos* GH response element contains one GAS site. The GAS elements in both of these genes bind nuclear proteins that are induced upon injection of GH into hypophysectomized rats (14, 24), but these two genes are not sexually dimorphic.

Supershifted experiments performed with the c-*fos* GH response element and with antibodies raised against STAT-1 and STAT-3 indicate that these (or other closely related) proteins are likely to be involved in the GH-mediated regulation of c-*fos* gene transcription (14, 24), whereas the same approach failed to show any association between STAT-1, -3, or -4 and the Spi 2.1 GH response element (44). In two other studies, STAT-1 (24) and STAT-5 (49) have been shown, also by supershift experiments, to bind to the Spi 2.1 GH response element. STAT-5 cDNA was also capable of facilitating the activation of a GH reporter system based on the Spi 2.1 GH response element (49). Attempts in our laboratory to supershift the retarded band shown with the CYPGHRE by using antibodies against STAT-1 or STAT-3 have failed (Fig. 10), despite the fact that we were able to reproduce the published data by using the GH response element from the c-*fos* promoter and anti-STAT-3 antibodies (data not shown). Antibodies raised against STAT-5 partially supershifted the CYPGHRE-protein complex (Fig. 10), although attempts to fully supershift the com-

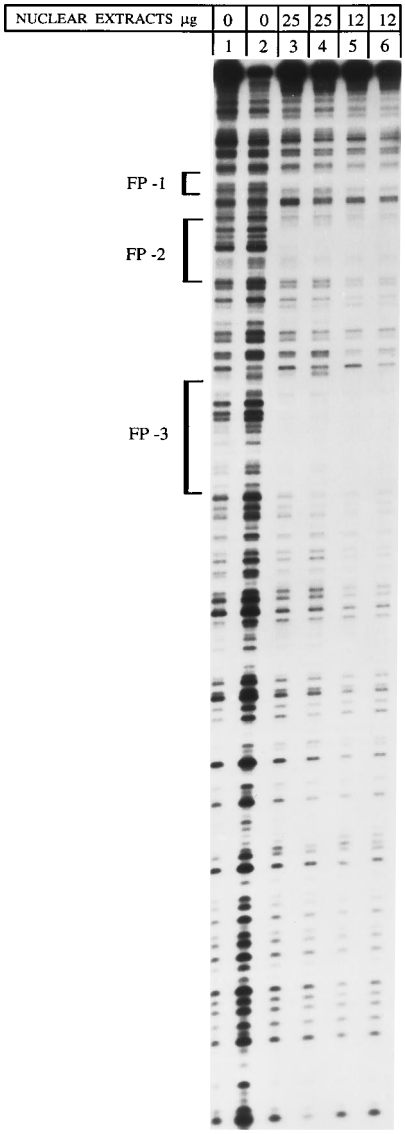



FIG. 8. DNase I footprint analysis of the CYP3A10/6β-hydroxylase promoter. A noncoding-strand probe was labeled at position +74 with $^{32}P$ by phosphorylation of the *Nhe*I-*Hin*dIII fragment from pHN306 and a second digestion with *Ava*I. It was incubated in the presence of the indicated amounts of hamster liver nuclear protein and subjected to partial digestion with 2 ng (lane 1), 5 ng (lane 2), 15 ng (lane 3), 25 ng (lane 4), 5 ng (lane 5), or 10 ng (lane 6) of DNase followed by denaturing polyacrylamide gel electrophoresis as described in Materials and Methods. The dried gel was exposed to X-ray film for 16 h at −70°C with an intensifying screen. To assign the protected regions, sequencing reactions were performed and run on the same gel (data not shown). The positions of the three protected regions are shown to the left of each footprint.

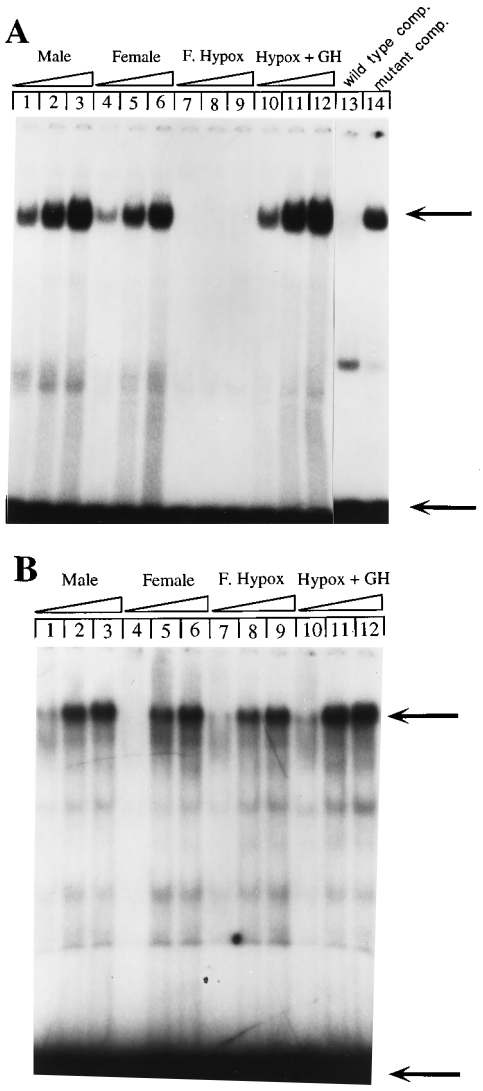



FIG. 9. Binding of liver nuclear proteins from male and female hamsters is abolished by hypophysectomy and restored by GH injections. Gel shift experiments were performed as described in Materials and Methods, using increasing amounts (1, 2, and 3 μg of protein) of hamster liver nuclear extracts from males (lanes 1 to 3), females (lanes 4 to 6), hypophysectomized females (F. Hypox; lanes 7 to 9), or hypophysectomized females injected with GH as indicated in Materials and Methods (Hypox + GH; lanes 10 to 12). In panel A, the −101 to −78 fragment from the CYP3A10/6β-hydroxylase promoter was used as a probe. To show the specificity of binding to the CYPGHRE, 100-fold molar excess of unlabeled probe (lane 13) or 100-fold molar excess of unlabeled fragment containing the same mutation at positions −91 to −83 as indicated in Fig. 7 (lane 14) was used as a competitor (comp.). Arrows point to the retarded band (top) and the free probe (bottom). In panel B, the probe was the Oct-1 binding element. Arrows indicate the major retarded band (top) and the free probe (bottom).

plex failed, suggesting that other transcriptional factors in addition to STAT-5 bind to the CYPGHRE. The idea that more than one STAT factor is regulated by GH is also supported by the fact that GH rapidly stimulates the tyrosine phosphorylation of at least eight nuclear proteins (13), a protein modification involved in the activation of STAT proteins (9). Furthermore, the transcriptional activation of the vasoactive intestinal peptide gene by neuropoietic cytokines is mediated by a GAS element that is identical to the CYPGHRE (41). Since the tissue distribution, sexual dimorphism, and regulatory cytokines for the vasoactive intestinal peptide and CYP3A10/6β-hydroxylase genes are very different, it is reasonable to suggest that different members of the STAT family of proteins may bind to the same site.

A question raised by this study is how such different levels of CYP3A10/6β-hydroxylase gene expression (at least 50-fold; Fig. 1) can be explained by a modest change in binding (2-fold; Fig. 9A) to the characterized CYPGHRE in male and female extracts. There are many possible explanations, one of them being that perhaps there are other elements either upstream of the 821 bp studied or within one of the introns. However, the correlation between manipulation of circulating GH in female hamsters, levels of CYP3A10/6β-hydroxylase mRNA, and

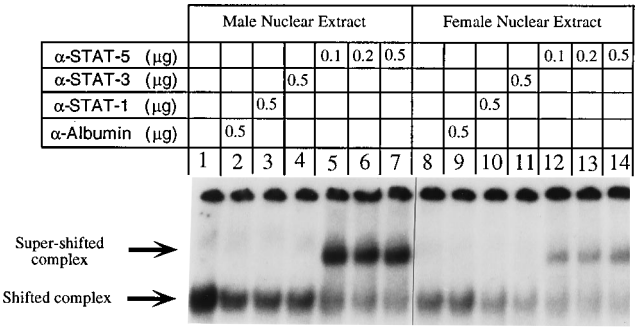



FIG. 10. Antibodies to STAT-5 partially supershift the CYPGHRE-nuclear protein complexes. Two-microgram aliquots of nuclear extracts prepared from male or female hamster livers were used in a gel shift experiment performed as described in Materials and Methods, in the absence of any antibody (lane 1 and 8) or in the presence of 0.5 μg of antialbumin antibodies (lanes 2 and 9), 0.5 μg of anti-STAT-1 (lanes 3 and 10), 0.5 μg of anti-STAT-3 (lanes 4 and 11), or increasing amounts (0.1, 0.2, and 0.5 μg) of anti-STAT-5 (lanes 5 to 7 and 12 to 14). Only the upper part of the gel is shown.

binding to the characterized CYPGHRE makes this CYPGHRE a likely candidate involved in mediating the sexually dimorphic expression of the CYP3A10/6β-hydroxylase gene. Several putative molecular mechanisms involved in the male-specific expression of the CYP3A10/6β-hydroxylase gene could explain our observations. There could be two CYPGHRE-binding proteins, one acting as an activator of transcription and the other acting as a suppressor. This hypothesis is supported by experiments shown in Fig. 1 and 9. Hypophysectomized females that showed no binding to the CYPGHRE (Fig. 9) expressed the CYP3A10/6β-hydroxylase gene at much higher levels than normal female hamsters (Fig. 1), suggesting that the female pattern of GH secretion (continuous) inhibits the basal level of CYP3A10/6β-hydroxylase expression seen in the absence of GH (hypophysectomized hamsters). Injection of GH in a manner that reproduces the male pattern of GH secretion induces the level of CYP3A10/6β-hydroxylase mRNA and binding to the CYPGHRE to male levels through activation of a specific and yet uncharacterized STAT-like protein. It is interesting that two murine STAT-5 cDNAs have been isolated (27), and whether they are functionally different is unknown. The putative proteins encoded by both cDNAs are recognized by the antibody used in our supershift experiment (Fig. 10). Several variations of this putative mechanism are possible as well. For example, in vivo, STAT-5 could bind to the characterized CYPGHRE as a monomer or as a dimer, in which one form is an activator and the other one is a suppressor, this conformational change being triggered by the different modes of GH secretion. An example of a similar mechanism already exists: the *Drosophila* transcriptional factor Krüppel binds as a monomer that activates transcription and as a dimer that binds to the same DNA element but is a potent repressor (37). Another possible variation is that a unique factor, probably STAT-5, binds to the characterized CYPGHRE in both males and females, but in the presence of other sex-specific regulatory factors, brought to the CYP3A10/6β-hydroxylase promoter either by DNA-protein interactions to either an upstream or downstream element or by protein-protein interactions, it functions either as an enhancer or as a repressor.

Elucidating the molecular mechanisms involved in the sexually dimorphic expression of CYP3A10/6β-hydroxylase should lead to a better understanding of the role of the liver in bile acid metabolism, cholesterol homeostasis, and many other sexually dimorphic liver specific processes.

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