Synergistic Action of Hepatocyte Nuclear Factors 3 and 6 on *CYP2C12* Gene Expression and Suppression by Growth Hormone-activated STAT5b

PROPOSED MODEL FOR FEMALE-SPECIFIC EXPRESSION OF *CYP2C12* IN ADULT RAT LIVER*

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Growth hormone (GH) exerts sexually dimorphic effects on liver gene transcription through its sex-dependent temporal pattern of pituitary hormone secretion. *CYP2C12* encodes a female-specific rat liver P450 steroid hydroxylase whose expression is activated by continuous GH stimulation of hepatocytes. Presently, we investigated the role of liver-enriched and GH-regulated transcription factors in the activation of *CYP2C12* gene expression in GH-stimulated liver cells. Transcription of a *CYP2C12* promoter-luciferase reporter gene in transfected HepG2 cells was activated 15–40-fold by the liver-enriched hepatocyte nuclear factor (HNF) 3α, HNF3β, and HNF6. Synergistic interactions leading to an ~300-fold activation of the promoter by HNF3β in combination with HNF6 were observed. 5′-Deletion analysis localized the HNF6 response to a single 5′-proximal 96-nucleotide segment. By contrast, the stimulatory effects of HNF3α and HNF3β were attributable to five distinct regions within the 1.6-kilobase *CYP2C12* proximal promoter. GH activation of the signal transducer and transcriptional activator STAT5b, which proceeds efficiently in male but not female rat liver, inhibited *CYP2C12* promoter activation by HNF3β and HNF6, despite the absence of a classical STAT5-binding site. The female-specific pattern of *CYP2C12* expression is thus proposed to reflect the positive synergistic action in female liver of liver-enriched and GH-regulated transcription factors, such as HNF3β and HNF6, coupled with a dominant inhibitory effect of GH-activated STAT5b that is manifest in males.

GH[1] signals to hepatocytes and other target cells via its plasma membrane-bound receptor, which is a member of the cytokine/growth factor receptor superfamily (1). Binding of GH to the GH receptor induces receptor dimerization and activa-

tion of Janus kinase 2 (JAK2), a tyrosine kinase that interacts with the cytoplasmic domain of the GH receptor and phosphorylates both itself and the cytoplasmic domain of the GH receptor on multiple tyrosine residues. These phosphorylated tyrosines, in turn, serve as docking sites for downstream signaling molecules that contain Src homology 2 domains, including STAT transcription factors and insulin receptor substrates 1 and 2 (1, 2). These primary signaling molecules activate secondary messengers, such as diacylglycerol, calcium, and nitric oxide, and enzymes, such as mitogen-activated protein kinase, protein kinase C, phospholipase A$_2$, and phosphatidylinositol 3′-kinase. These GH-stimulated signaling pathways regulate a variety of intracellular events, including gene transcription, metabolite transport, and enzymatic activity, and contribute to the overall regulation by GH of whole body growth and metabolism (3).

The cytochrome P450 gene *CYP2C12* encodes a steroid-disulfate 15β-hydroxylase that is induced in the livers of female rats at puberty. At this same developmental stage, a related P450 gene, *CYP2C11*, which encodes a steroid 16α- and 2α-hydroxylase, is induced in the livers of male rats (4, 5). The differential expression of these two *CYP* genes in male and female rats is established by the sexually dimorphic patterns of pituitary GH secretion (6, 7). In male rats, GH is secreted intermittently to give regular plasma GH peaks of large amplitude (~200 ng/ml) each 3–4 h, followed by trough periods of no detectable GH (8). In contrast, the female rat GH secretory pattern is characterized by more frequent plasma peaks of smaller amplitude, resulting in a near continuous presence of GH in blood at an average level of 40–60 ng/ml (9).

The cellular mechanisms by which the temporal plasma profile of GH regulates *CYP2C11* and *CYP2C12* gene expression in rat liver are only partially understood. We and others have demonstrated that expression of these two genes is regulated by GH at the level of transcription initiation (10, 11). We have further established that a liver-expressed, latent cytoplasmic transcription factor, designated STAT5b, undergoes repeated tyrosine phosphorylation and nuclear translocation in direct response to the male pattern of GH secretion, leading us to propose that STAT5b is a transcriptional activator of male-specific, GH pulse-activated genes such as *CYP2C11* (12, 13). Indeed, targeted disruption of the *Stat5b* gene in male mice leads to GH pulse resistance (14) associated with a selective loss of the male-specific pattern of liver gene expression (15, 16). Further support for this proposal is provided by the correlation between STAT5b nuclear translocation and gender-specific P450 expression in mouse liver (17) and by the presence of *bona fide* STAT5-binding sites in the 5′-flanking region of

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[1] The abbreviations used are: GH, growth hormone; JAK2, Janus kinase 2; STAT, signal transducer and activator of transcription; *CYP2C12* and *2C12*, cytochrome P450 gene *2C12*; GHNF, GH-activated nuclear factor; HNF, hepatocyte nuclear factor; IRE-ABP, insulin response element-A binding protein; C/EBP, CAAT/enhancer-binding protein; nt, nucleotide(s); FBS, fetal bovine serum; LUC, luciferase; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; oligo, oligonucleotide.

several male-specific, GH-dependent liver P450 genes (18).[2]

Several liver-expressed nuclear factors have been proposed to contribute to the female-specific expression of *CYP2C12*. A continuous GH-activated nuclear factor, termed GHNF, was demonstrated to be specifically induced or activated by the female plasma pattern of GH secretion and to bind to multiple sites along the female-specific *CYP2C12* promoter (19). Thus far, however, GHNF has resisted purification and has been only partially characterized. A distinct liver-enriched transcription factor, termed HNF6 (20, 21), whose expression in liver is stimulated by GH, can bind to and enhance transcription from the *CYP2C12* promoter ~3-fold (22). However, HNF6 expression displays only a modest sex difference in rat liver (female/male = 2:1) and by itself cannot account for the striking female-specific expression of *CYP2C12*. The insulin response element-A binding protein (IRE-ABP), a member of the SRY family of transcriptional regulators, was hypothesized to be a transcriptional repressor of *CYP2C12* (23). Indeed, IRE-ABP inhibits activation of *CYP2C12* by the liver transcription factor C/EBPα, and this effect was correlated with the overlapping binding sites for C/EBPα and IRE-ABP in the *CYP2C12* promoter (23). Moreover, one of these sites overlaps with the GHNF-binding site of *CYP2C12* found at nt −231 to −185 (19). However, IRE-ABP has not been shown to be expressed in female liver, and there is no evidence for the regulation of this factor by GH. STAT5b, which is proposed to be a transcriptional activator of male-specific, GH pulse-induced genes (12), was recently proposed to additionally act as a transcriptional repressor of female dominant mouse *Cyp* genes, based on our finding that a subset of such genes is up-regulated in livers of STAT5b-deficient male mice (16). This latter proposal is consistent with our finding that the induction of *CYP2C12* gene expression by the female plasma pattern of GH secretion is associated with a dramatic down-regulation of the STAT5b signaling pathway (12, 24).

This study was carried out to identify both liver-enriched and GH-regulated nuclear factors that may contribute to regulated expression of *CYP2C12*. We demonstrate that the liver-enriched transcription factors HNF3α and HNF3β (25, 26) and HNF6 (20, 21) bind to and strongly activate the *CYP2C12* promoter. We show that these latter two factors act in concert, via distinct sets of binding sites, to synergistically enhance *CYP2C12* gene activation. Additionally, we demonstrate that, when activated by GH, STAT5b and the closely related STAT5a down-regulate HNF3- and HNF6-induced *CYP2C12* gene transcription. Our findings led us to propose a model whereby STAT5 proteins activated in liver *in vivo* by the male pulsatile GH pattern contribute to the sex specificity of *CYP2C12* expression by actively suppressing HNF3- and HNF6-dependent *CYP2C12* transcription in adult male rats.

MATERIALS AND METHODS

Cell Lines—The human hepatoma cell line HepG2, obtained from the American Type Culture Collection repository, was maintained in minimal essential medium containing 10% fetal bovine serum (FBS), 50 units/ml penicillin, and 50 units/ml streptomycin. An SV40-transformed African green monkey kidney cell line, COS-1, was maintained in Dulbecco's modified Eagle's medium containing 10% FBS and 50 units/ml penicillin/streptomycin.

Mammalian Expression Plasmids—Mammalian expression plasmids for rat HNF3α and rat HNF3β, cloned into pNEO-SV40, were provided by Dr. Eseng Lai (Sloan-Kettering Cancer Center, New York) (25, 26). Rat HNF4 expression plasmid pLEN4S was provided by Dr. Frances Sladek (University of California, Riverside, CA) (27). Rat HNF6 expression plasmid pECE-HNF6 was obtained from Dr. Guy Rousseau (University of Louvain Medical School, Brussels, Belgium) (20). Rat GH receptor cDNA, cloned into expression plasmid pcDNAI, was provided
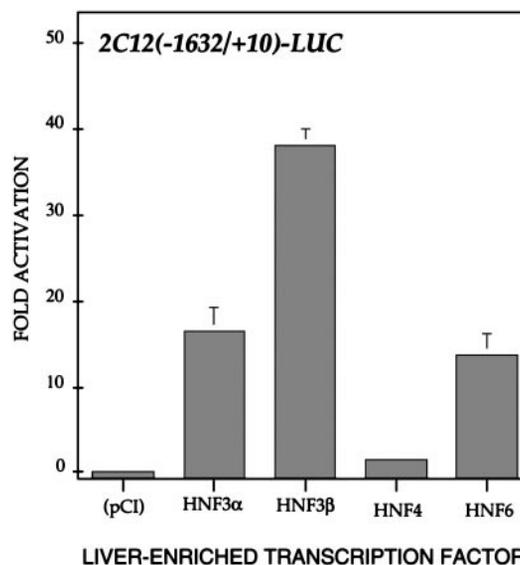
FIG. 1. **Transactivation of *2C12* promoter by liver-enriched transcription factors in HepG2 cells.** Transient transfections were carried out using the *2C12*(−1632/+10)-LUC reporter plasmid in the presence of each of the indicated liver-enriched transcription factor expression plasmids or the control expression plasmid pCI. The pRL-TK *Renilla* luciferase reporter plasmid was included as an internal standard for transfection efficiency. Firefly and *Renilla* luciferase activity values were measured, and normalized luciferase activities were calculated as described under "Materials and Methods." The -fold activation values shown correspond to liver factor-stimulated *2C12* promoter activities relative to *2C12* promoter activity measured in the presence of the pCI control plasmid. Data shown represent mean ± S.E. of three independent transfections.

by Dr. Nils Billestrup (Hagedorn Research Institute, Gentofte, Denmark). Mouse STAT1, STAT3, STAT5a, and STAT5b cDNAs, cloned into expression plasmid pME18S, were obtained from Dr. Alice Mui (DNAX Research Institute of Molecular and Cellular Biology, Inc., Palo Alto, CA). cDNAs encoding tyrosine-to-phenylalanine mutated forms of mouse STAT5a and STAT5b (designated STAT5a-Y694F and STAT5b-Y699F, respectively) and cDNAs encoding serine-to-alanine mutated STAT5 forms (designated STAT5a-S725A and STAT5b-S730A, respectively), all cloned into pcDNAI, were provided by Dr. Hallgeir Rui (Uniformed Services University of the Health Sciences, Bethesda, MD) (28). cDNAs encoding constitutively active forms of mouse STAT5a and STAT5b (designated STAT5a1*6 and STAT5b1*6, respectively) were provided by Dr. Toshiro Kitamura (University of Tokyo) (29) and were subcloned into expression plasmid pCI (Promega). The STAT5-activated luciferase reporter plasmid 4X-pT109-LUC was provided by Dr. Mary Vore (University of Kentucky, Lexington, KY) (30).

2C12 Promoter-LUC Reporter Plasmid Constructions—A *2C12* promoter-firefly luciferase reporter plasmid containing 1632 base pairs of 5′-upstream sequence, designated *2C12*(−1632/+10)-LUC, was constructed as follows. A 1642-base pair DNA fragment corresponding to nt −1632 to +10 of the rat *CYP2C12* gene (nucleotide numbering relative to the *2C12* transcription start site) (RATP4515B1; GenBank™/EBI Data Bank) was synthesized by PCR using the cloned *2C12* promoter as template (plasmid p42) (10) and synthetic oligonucleotides corresponding to nt −1632 to −1614 (ON-477, upstream primer containing a 5′-end *Mlu*I restriction site) and nt +10 to −9 (ON-366, downstream primer containing a 5′-end *Bgl*II restriction site). PCRs were carried out at 94 °C for 1 min, 56 °C for 1.5 min, and 72 °C for 2 min for 30 cycles in a Stratagene RoboCycler. The resultant DNA fragment was double-digested by *Bgl*II and *Mlu*I and then ligated to *Bgl*II/*Mlu*I-digested pGL3-basic (Promega), which encodes a modified firefly luciferase reporter. The sequence was confirmed by DNA sequence analysis.

5′-Deletions of the *2C12*(−1632/+10)-LUC plasmid were prepared by PCR amplification of various lengths of the *2C12* promoter using upstream oligonucleotides (each containing a 5′-end *Mlu*I site) corresponding to the following sequences upstream of the transcription start site: 5′-primer start positions beginning at nt −1398, −1319, −1182, −970, −904, −856, −745, −611, −516, −240, and −96 (see Fig. 2). Primer ON-366 was used as the fixed downstream primer. PCRs using plasmid p42 as template (10) were carried out for 30 cycles consisting of 94 °C for 1 min, 40 to 56 °C for 1.5 min, and 72 °C for 2 min. The
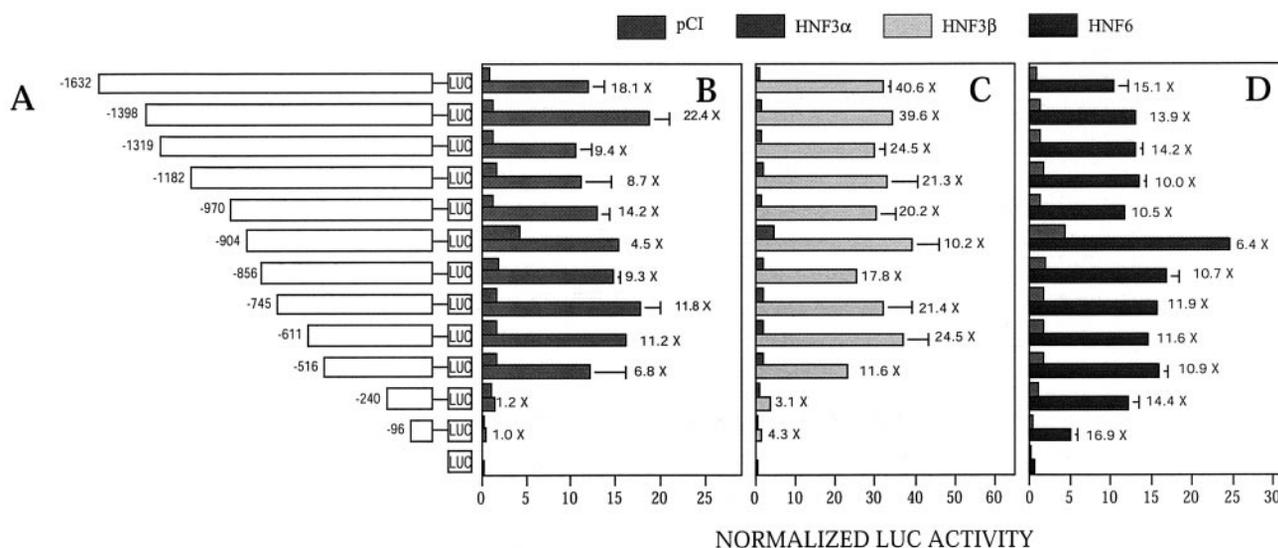
FIG. 2. **Deletion analysis of *2C12* promoter: functional characterization of HNF3- and HNF6-binding sites.** *A*, shown is a schematic representation of the 5′-deleted *2C12* promoter-LUC constructs. *B–D*, each 5′-deleted *2C12* promoter-LUC construct was transiently cotransfected into HepG2 cells with the control expression plasmid (pCI) or with HNF3α (*B*), HNF3β (*C*), or HNF6 (*D*) expression plasmid (200 ng of total pCI or HNF plasmid/well) in the presence of the internal control plasmid pRL-TK. Normalized firefly luciferase activity values were determined. The *bar* values represent mean ± S.E. of three independent series of transfections. *Numbers* to the right of each bar indicate the -fold increase in reporter gene activity stimulated by HNF3α, HNF3β, or HNF6 compared with the pCI control plasmid shown alongside each bar.

resultant PCR products were then cloned into pGL3-basic as described above, and the sequences were confirmed by DNA sequencing.

Transient Transfections—For HepG2 transient transfections, cells were plated in minimal essential medium containing 10% FBS in triplicate in 24-well tissue culture plates (35% confluency; ~90,000 cells/177 mm²/well) and transfected 24 h later using FuGENE™6 transfection reagent as described by the manufacturer (Roche Molecular Biochemicals). For COS-1 transient transfections, similar conditions were used, except that the cells were plated at ~50% confluency and transfected 5 h later. All transfections were carried out using 185 ng of *2C12* promoter-LUC reporter plasmid together with 200 ng of liver-enriched transcription factor expression plasmid or 200 ng of a control expression plasmid (pCI), unless indicated otherwise. When present, 95 ng of GH receptor expression plasmid, 200 ng of STAT expression plasmid, and 90 ng of the STAT-activated reporter plasmid 4X-pT109-LUC (30) were included. In addition, 50 ng of the *Renilla* luciferase reporter plasmid pRL-TK (Promega) was included in each sample as an internal standard for transfection efficiency. Firefly and *Renilla* luciferase activities were determined 20–24 h after the initiation of transfection using the Dual-Luciferase™ reporter assay system (Promega) and a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). Firefly luciferase values were normalized on the basis of the *Renilla* luciferase values. In experiments in which HepG2 cells were treated with GH or prolactin (500 ng/ml, unless specified otherwise), cells were plated in minimal essential medium + 10% FBS. The cells were washed 6 h later to remove the FBS and then incubated overnight (~18 h) in minimal essential medium without serum. Cells were then transfected as described above and were simultaneously treated with either GH or prolactin for a total of 24 h. Normalized luciferase activities were determined as described above. Rat GH and rat prolactin were hormonally pure preparations (SIAFP grade) obtained from Dr. A. Parlow and the National Hormone and Pituitary Program, NIDDK, National Institutes of Health.

Protein Extraction—Total cell extracts were prepared as follows. HepG2 cells were washed once with ice-cold phosphate-buffered saline and then scraped in lysis buffer containing 20 mM HEPES (pH 7.9), 1% Triton X-100, 20% glycerol, 20 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM $Na_2P_2O_7$, 1 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride, 1 μg/ml pepstatin, and 1 μg/ml leupeptin. Crude extracts were aspirated 10 times through a 27-gauge needle, adjusted to 150 mM NaCl, and centrifuged at 13,000 × *g* for 30 min at 4 °C. Supernatants were stored in liquid N_2 until analysis. Protein concentrations were determined using the Bio-Rad Dc detergent protein assay kit. Rat liver nuclear extracts were prepared as described previously (12).

EMSA Analysis—The *CYP2C12* double-stranded oligonucleotides shown in Table I were synthesized by Bio-Synthesis, Inc. (Lewisville, TX) and used as EMSA probes. Other EMSA probes used in this study

include the following: 1) an HNF3 consensus sequence derived from nt −109 to −85 of the transthyretin promoter and mutated to eliminate HNF6 binding (21) (5′-TGA-CTA-A<u>AC</u>-AAA-<u>C</u>A<u>T</u>-TCA-GAA-TCG-3′; probe ON-523, with mutated residues underlined); 2) an HNF3β-specific sequence derived from the transthyretin-2 enhancer site (31) (5′-GGC-CCC-<u>TGT</u>-<u>TCA</u>-<u>AAC</u>-<u>ATG</u>-<u>T</u>CC-TAAT-3′; probe ON-569, with the core HNF3β sequence underlined); and 3) HNF6 consensus sequence derived from nt −109 to −85 of the transthyretin promoter and mutated to eliminate HNF3 binding (21) (5′-TGA-CTA-A<u>AT</u>-CAA-TA<u>T</u>-<u>CGA</u>-GAA-TCA-G-3′; probe ON-527, with mutated residues underlined). The sense strand oligonucleotide of each probe was labeled with [γ-³²P]ATP (PerkinElmer Life Sciences) using T4 polynucleotide kinase (Promega), annealed to the antisense strand, and then purified. Total HepG2 or COS-1 cell extract (10 μg of protein in 5 μl) or rat liver nuclear extract (3 μg of protein in 5 μl) (12) was preincubated for 10 min at room temperature with 9 μl of gel mobility shift buffer (12.5 mM Tris-HCl (pH 7.5) containing 2 μg of poly(dI-dC) (Roche Molecular Biochemicals), 5% glycerol, 1.25 mM MgCl₂, 625 μM EDTA, and 625 μM dithiothreitol). Double-stranded, ³²P-labeled oligonucleotide probe (10 fmol, 1 μl) was then added, and incubation was continued for 20 min at room temperature, followed by a 10-min incubation on ice. After addition of 2 μl of loading dye (30% glycerol, 0.25% bromphenol blue, and 0.25% xylene cyanol), samples were electrophoresed at room temperature through a pre-electrophoresed nondenaturing polyacrylamide gel (5.5% acrylamide and 0.07% bisacrylamide; National Diagnostics, Inc., Atlanta, GA) in 0.5× buffer containing 44.5 mM Trizma (Tris base), 44.5 mM boric acid, and 5 mM EDTA. Unlabeled probe competitions were carried out by including up to a 100-fold molar excess of unlabeled DNA probe in gel mobility shift buffer.

RESULTS

Transcriptional Activation of 2C12 Promoter by Liver Transcription Factors HNF3 and HNF6—GH-dependent expression of *CYP2C12* mRNA requires ongoing protein synthesis (32), suggesting that *CYP2C12* transcription may be regulated by a transcription factor that is itself regulated by GH at the level of gene expression. Because genes coding for several liver-enriched transcription factors are reported to be inducible by GH (*e.g.* Refs. 33 and 34), we examined the role of liver-enriched nuclear factors in *CYP2C12* transactivation as determined by transient transfection into the human hepatoblastoma cell line HepG2. The 5′-flanking sequence of *CYP2C12* (nt −1632 to +10 relative to the transcription start site) was found to have significant basal expression activity when fused to a firefly luciferase reporter gene. This basal activity was increased sig-
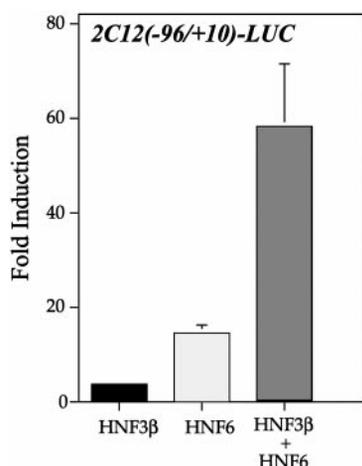
FIG. 3. **HNF3β and HNF6 act on different sites along *2C12*(−96/ +10).** The *2C12*(−96/+10)-LUC reporter plasmid (185 ng) was transfected into HepG2 cells with each of the indicated transcription factor expression plasmids (200 ng each) in the presence of the internal control plasmid pRL-TK. The total amount of transcription factor expression plasmid DNA was adjusted to 400 ng/transfection using pCI (control expression plasmid) in the experiments using HNF3β alone or HNF6 alone. Normalized firefly luciferase activity values were determined, and -fold activation values were then calculated. Data shown represent mean ± S.E. of four independent transfections.

nificantly when the cells were cotransfected with expression plasmids encoding the liver factor HNF3β (39.1 ± 2.0-fold stimulation) or HNF6 (14.6 ± 2.6-fold stimulation) (Fig. 1). HNF3α, a liver-enriched transcription factor that is closely related to HNF3β (25, 26), also transactivated the *2C12* promoter (17.5 ± 2.8-fold increase in luciferase reporter activity) (Fig. 1). By contrast, cotransfection of the liver factor HNF4 (27) did not significantly increase reporter gene activity (Fig. 1), in agreement with an earlier report (35). Control experiments verified that HNF4 was expressed in the transfected HepG2 cells and that it specifically bound its cognate DNA response element (36) in EMSAs (data not shown).

2C12 Promoter Elements Involved in Gene Activation by HNF3 and HNF6—To localize the *2C12* promoter elements responsible for gene activation by HNF3α, HNF3β, and HNF6, 5′-deletions of the *2C12* promoter, designated by their deletion end points relative to the *CYP2C12* transcription start site, were analyzed in transfected HepG2 cells (Fig. 2). The transcriptional activity of *2C12*(−96/+10), the shortest construct examined, was significantly increased by cotransfection of HNF6 (Fig. 2D). The magnitude of the increase (~17-fold) was greater than or similar to that observed with longer *2C12* promoter constructs, indicating that *CYP2C12* sequences downstream of nt −96 are sufficient for maximal HNF6 stimulation. Indeed, a decrease in -fold activation by HNF6 was observed with several of the intermediate length promoter constructs (*e.g.* *2C12*(−904/+10)). This decrease reflects, in part, the higher basal activity of the longer constructs in the absence of cotransfected liver transcription factor (Fig. 2, pCI control plasmid transfections). We conclude that *CYP2C12* promoter elements essential for HNF6 transactivation are exclusively found downstream of nt −96. This conclusion is consistent with the identification of a functional HNF6-binding site ~15 nt upstream of the *CYP2C12* TATA box (*CYP2C12* nt −47 to −38) (22).

In contrast to HNF6, HNF3α did not transactivate *2C12*(−96/+10) or *2C12*(−240/+10) (Fig. 2B). Transactivation by HNF3α was first seen with *2C12*(−516/+10) (6.8-fold activation), indicating the presence of one or more HNF3α responsive element(s) between *2C12* nt −516 and −240. HNF3α
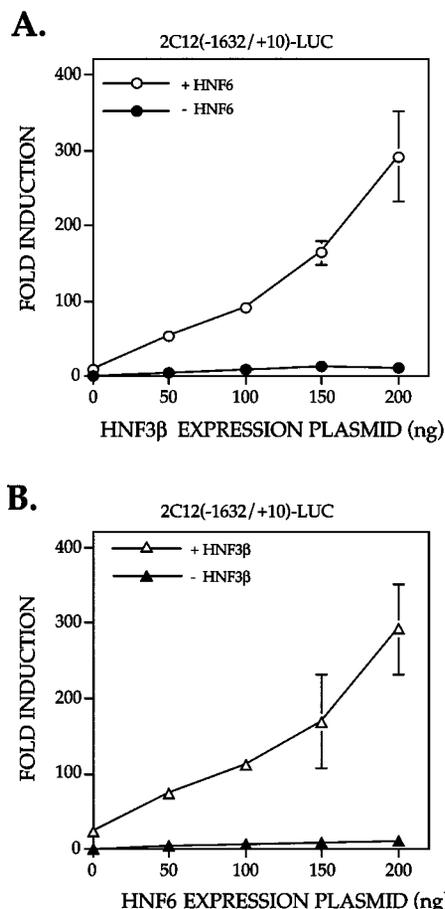
FIG. 4. **Synergistic action of HNF3β and HNF6 on *2C12*(−1632/ +10) promoter activity.** *A*, HepG2 cells were transfected with 0–200 ng of HNF3β expression plasmid either alone (*closed circles*) or in combination with a fixed amount (200 ng) of HNF6 expression plasmid (*open circles*). *B*, cells were transfected with 0–200 ng of HNF6 expression plasmid alone (*closed triangles*) or in combination with a fixed amount (200 ng) of HNF3β expression plasmid (*open triangles*). The total amount of transcription factor expression plasmid DNA was adjusted to 400 ng/transfection using pCI. All samples were also cotransfected with the *2C12*(−1632/+10)-LUC reporter plasmid (185 ng) and the pRL-TK internal control plasmid. Normalized firefly luciferase activity values were determined, and -fold activation values were then calculated. Transfections were repeated twice, and the means ± S.D. of duplicate determinations from a representative experiment are presented. In the experiments shown, using a total of 400 ng of HNF + pCI plasmid DNA, the basal activity of the *CYP2C12* promoter construct was significantly elevated compared with that seen in Fig. 2. Consequently, the -fold induction of the *CYP2C12* promoter by HNF3β and HNF6 was weaker than that observed under the transfection conditions of Fig. 2. All data points include *error bars*, many of which are too small to be seen on the graph.

activation of *CYP2C12* was further increased ~11-, 14-, and 22-fold when constructs extending to nt −611, −970, and −1398 were respectively analyzed. Thus, at least four distinct regions of the *2C12* promoter (−516/−240, −611/−516, −970/ −904, and −1398/−1319) are involved in HNF3α activation of *CYP2C12*. In contrast to HNF3α, HNF3β transactivated *2C12*(−96/+10) moderately (4.3-fold) (Fig. 2C). The transcriptional activation by HNF3β was further increased to 11.6- and 24.5-fold with constructs *2C12*(−516/+10) and *2C12*(−611/ +10), respectively, and maximal activation (39.6-fold) was obtained with *2C12*(−1398/+10) (Fig. 2C). Thus, HNF3β stimulates *2C12* promoter activity via the same four responsive regions transactivated by HNF3α, in addition to the proximal promoter region, nt −96 to +10 (Fig. 2C).

Synergistic Action of HNF3 and HNF6 on CYP2C12 Promoter—HNF3 and HNF6 share related DNA-binding sites (21),

TABLE I
Synthetic oligonucleotides derived from the HNF3 DNA-binding sites of the CYP2C12 promoter

Oligonucleotide (location)[a]	Sequence[b]
A (−587/−563)	5′-cctataaaa**TGTTTACTA**aaaacac-3′
	3′-ggatatttt**ACAAATGAT** ttttgtg-5′
B (−355/−326)	5′-gaa**AAGAAAATA**aggaa**ATGCAAATA**ttag-3′
	3′-ctt**TTCTTTTAT**tcctt**TACGTTTAT**aatc-5′
C (−277/−241)	5′-**TCATAAATAAATA**atttaaaatta**ACTCAAATA**tgtt-3′
	3′-**AGTATTTATTTAT**taaattttaat**TGAGTTTAT**acaa-5′
D (−96/−73)	5′-gag**AGATAAACA**gtggccagatgg-3′
	3′-ctc**TCTATTTGT**caccggtctacc-5′
HNF3 (consensus sequence)	T(A/G)TT(T/G)(G/A)(C/T)T(T/C)

[a] Nucleotide position, numbered relative to the *CYP2C12* transcription start site.

[b] The HNF3-binding sites present in oligos A–D are written in boldface uppercase letters. Underlined sequences on either the sense or the anti-sense strand are compared with the HNF3 consensus recognition sequence (37) with matches of 8/9 (oligo A, each site of oligo B, and oligo D) and either 7/9 or 9/9 (sites of oligo C).

and HNF3 proteins bind weakly to the HNF6-binding site at *CYP2C12* nt −47 to −38 (22). We therefore investigated whether the transcriptional activation of *2C12*(−96/+10) by HNF3β and that by HNF6 are mutually exclusive, which would be consistent with their sharing the same or an overlapping DNA-binding site. Alternatively, transcriptional activation by these two factors might be additive or synergistic, which would suggest distinct binding sites. Fig. 3 shows that coexpression of HNF3β and HNF6 synergistically increased *2C12*(−96/+10) promoter activity. Moreover, the promoter activity obtained exceeded that seen in cells transfected with a double amount of either HNF3β or HNF6 expression plasmid alone (data not shown). These data support the proposal that HNF3β and HNF6 synergistically transactivate *CYP2C12* by acting on different sites along the 96-nt 5′-promoter fragment.

This conclusion was extended in experiments in which the full-length *2C12*(−1632/+10) construct was transfected with variable amounts of HNF3β and HNF6 expression plasmids in the presence or absence of a constant amount of HNF6 or HNF3β, respectively. When varying amounts of HNF3β expression plasmid were combined with a fixed amount of HNF6 (Fig. 4*A*) or vice versa (Fig. 4*B*), *2C12*(−1632/+10) promoter activity was substantially higher at each level of expression plasmid than that obtained in experiments using either expression plasmid alone. Overall, a 300-fold increase in *CYP2C12* promoter activity was obtained in the presence of both liver nuclear factors. Other experiments revealed that HNF3α could similarly stimulate HNF6-dependent *2C12* promoter activity and vice versa (data not shown). Thus, HNF6 can cooperate in a positive, synergistic manner with HNF3α or HNF3β to strongly activate *CYP2C12*.

Multiplicity of HNF3-binding Sites on CYP2C12 Promoter—The observed stimulatory effects of HNF3α and HNF3β on *2C12* promoter activity support the presence of five HNF3-responsive regions. Three of these are downstream of nt −611 and contain at least one consensus HNF3-binding site sequence (37) each (*c.f.* Table I). Four oligonucleotides, which encompass consensus HNF3 motifs identified within regions −611/−516 (oligo A), −516/−240 (oligos B and C), and −96/+10 (oligo D) (Table I and Fig. 5*A*), were synthesized and analyzed by EMSA for the binding of HNF3α and HNF3β proteins expressed in transfected HepG2 cells. Oligos A–D each formed at least one specific EMSA complex with HNF3β-transfected HepG2 cell extracts (Fig. 5*B*, *arrows*). No significant difference in EMSA binding pattern was seen between extracts prepared from HNF3α- and HNF3β-transfected HepG2 cells (Fig. 5*C*, *left panels*, *lane 2 versus lane 3*). Specific HNF3·DNA complexes were confirmed by their increased levels following transfection (*lanes 2* and *3 versus lane 1*; see quantitation in the figure legend) and by the ability of a 100-fold molar excess of unlabeled oligonucleotide to block ^{32}P-labeled complex formation

(*SC lanes* (self-competition)).

Using oligo A (−587/−563), which contains a single consensus HNF3-binding site (Table I), a single specific protein-DNA complex (complex A1) was detected (Fig. 5*C*, *arrow*). This complex was induced 2- and 4-fold in the populations of cells transfected with HNF3α and HNF3β, respectively (*lanes 2* and *3 versus lane 1*; see figure legend). The formation of complex A1 was partially blocked by a 100-fold excess of unlabeled oligo A (*lane 4*) or by a 100-fold excess of unlabeled oligo B, C, or D (*lanes 5–7*, respectively), which were each more effective than oligo A itself. Formation of the oligo A EMSA complex was efficiently blocked by an HNF3β-specific oligonucleotide derived from the transthyretin-2 enhancer (31), but not by an HNF3 consensus sequence mutated to eliminate HNF6 binding (*lanes 9* and *10*). The HNF6 consensus oligonucleotide failed to compete for complex A1 formation (*lane 11*), indicating that the protein that binds to oligo A is HNF3-like.

Oligo B (−355/−326), which contains two consensus HNF3-binding sites (−352/−344 and −338/330) (Table I), gave a strong EMSA complex, designated B1, when incubated with HepG2 cell extracts transfected with HNF3α or HNF3β (Fig. 5*C*, *second left panel*, *lanes 2* and *3*). The increase in complex B1 intensity in the HNF3-transfected cells was only ∼2-fold, a finding that is consistent with the endogenous expression of HNF3 in HepG2 cells, coupled with the fact that only a small percentage of cells in the population take up and express the transfected DNA. The specificity of complex B1 was established by the ability of unlabeled oligo B or C to selectively compete with formation of the ^{32}P-labeled complex (*lanes 5* and *6*). Selective competition by an HNF3β-specific binding site, but not by an HNF6-binding site, was observed, evidencing the binding of HNF3β to oligo B (*lanes 10* and *11*). EMSA analysis was also performed using oligo C (−277/−241), which contains three consensus HNF3-binding sites, including two overlapping sites (−277/−269, −273/−265, and −253/−245) (Table I). One strong complex, migrating faster than complex B1, was observed (Fig. 5*C*, *third panels*). However, this EMSA complex, designated C1, was not increased in intensity in HepG2 cells transfected with HNF3α or HNF3β. Moreover, complex C1 was not competed by oligos A and D and was only partially competed by oligos B and C and by each of the HNF3- and HNF6-binding sites tested (*lanes 5*, *6*, and *9–11*). This suggests that oligo C may bind proteins other than HNF3. Finally, oligo D (−96/−73), which contains a single consensus HNF3-binding site (−93/−85), gave three distinct specific protein-DNA complexes, D1, D2, and D3 (Fig. 5*C*, *lower panels*). Complex D1 was competed most efficiently by oligos B–D and by the HNF3β-specific binding site, but not by the HNF6 sequence (*lanes 5–7*, *10*, and *11*). Partial competition was seen in the case of complexes D2 and D3, further supporting the binding of HNF3 to oligo D.
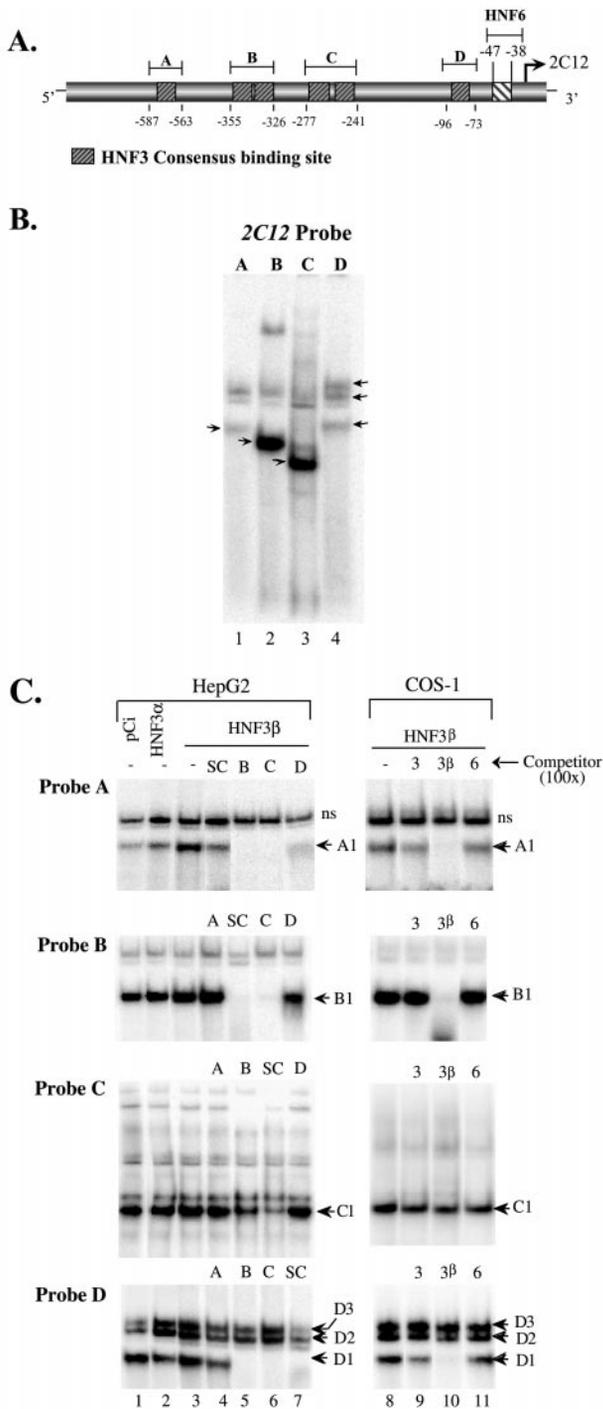
FIG. 5. **EMSA analysis of putative HNF3 DNA-binding sites on *CYP2C12* promoter.** *A*, shown is a schematic presentation of the putative HNF3 DNA-binding sites along the *CYP2C12* promoter. *B*, shown are representative gel shift complexes formed by four consensus HNF3-binding site probes derived from the *CYP2C12* proximal promoter. Oligos A–D (Table I) were ^{32}P-end-labeled, incubated with HNF3β-transfected HepG2 cell extracts (10 μg), and electrophoresed through a 5.5% polyacrylamide gel. *Arrows* indicate specific gel mobility shift complexes. *C*, cells were transfected for 24 h with the control plasmid pCI or with HNF3α or HNF3β expression plasmid (200 ng). Total cell extracts were prepared from transfected HepG2 cells or COS-1 cells, as indicated, as described under "Materials and Methods." Extracts were analyzed by EMSA in the presence or absence of unlabeled self-competing (*SC*) oligonucleotide or in the presence of a cross-competing double-stranded oligonucleotide. Competitor oligonucleotides (as shown at the top of each lane) were present in the binding reaction at a 100-fold excess over the amount of ^{32}P-labeled DNA probe. The HNF3 consensus, HNF3β-specific, and HNF6 competitor oligonucleotide sequences (see "Materials and Methods") are designated *3*, *3β*, and *6*, respectively (*lanes 9–11*). The *CYP2C12* promoter-derived HNF3

Together, these results indicate that HNF3-related proteins bind to at least three distinct sequences within the proximal 600 nt of the *2C12* promoter, represented by oligos A, B, and D. Differences in strength of complex formation are suggested by the large differences in EMSA band intensities (Fig. 5*B*) and by the distinct efficiencies of cross-competition (Fig. 5*C*). Moreover, size differences between the various EMSA complexes (Fig. 5*B*) are suggestive of the binding of distinct HNF3-related proteins, either as monomers or as multimers or perhaps in association with other unidentified nuclear factors. These findings lend support to the 5′-deletion analysis and indicate that the stimulatory effects of HNF3α and HNF3β on *2C12* promoter activity involve multiple HNF3-binding sites.

EMSA analysis of oligos A–D was carried out using nuclear extracts prepared from female and male rat livers to determine whether any of the HNF3-related EMSA complexes exhibit sex-dependent DNA binding patterns that might correlate with the female-specific expression of *CYP2C12*. However, no sex-dependent DNA binding patterns were observed (data not shown).

GH-activated STAT5b Inhibits CYP2C12 Promoter Activity—We next examined whether GH-regulated STAT transcription factors might contribute to the sex-specific expression of *CYP2C12*. We hypothesized that STAT5b, the major GH pulse-responsive STAT form in male rat liver (12, 38), might inhibit HNF3- and/or HNF6-induced *CYP2C12* gene transcription and thereby contribute to the female-specific pattern of expression that characterizes *CYP2C12* in liver *in vivo*. To test this hypothesis, GH signaling was reconstituted in HepG2 cells by cotransfection of expression plasmids encoding the GH receptor and STAT5b. The tyrosine kinase JAK2, required for GH receptor signaling to STAT5b, is endogenously expressed in HepG2 cells at a level sufficient to support STAT5 activation, as demonstrated using a luciferase reporter plasmid driven by four copies of a STAT5-binding site from the rat liver gene *ntcp* (Fig. 6*C* and data not shown). The effect of HNF3β and HNF6 on *2C12*($-1632/+10$) promoter activity, in the presence or absence of GH and STAT5b, was then examined. GH treatment decreased *2C12* promoter activity stimulated by HNF3β (Fig. 6*A*) or by HNF6 (Fig. 6*B*) by ~65–70%. This GH inhibitory effect was dose-dependent and was not observed when using prolactin in place of GH (Tables II and III). The requirement that STAT5b be cotransfected to obtain a strong GH inhibitory response indicates that this inhibition does not proceed via a STAT-independent GH signaling pathway. The small GH-induced decrease in *2C12* promoter activity seen in the absence of STAT5b transfection (Fig. 6, *A* and *B*, *first pair of bars*) may be due to the low endogenous level of STAT5b present in these cells. GH-activated STAT5a, which is >90% identical in sequence to STAT5b, also inhibited HNF3β- and HNF6-stimulated promoter activity (Fig. 7). STAT5a, although much less abundant in liver than STAT5b (16), exhibits the same preferential activation by male plasma GH pulses that characterizes STAT5b (38). Finally, STAT1, which can also be activated by GH in liver cells, but not in a sex-dependent manner (39, 40), did not inhibit *2C12* promoter activity (Fig. 6). These findings

consensus oligonucleotides, designated *A–D* (*lanes 4–7*), are those shown in Table I. *ns*, nonspecific EMSA complex. The following -fold increases in HepG2 cell extract EMSA band intensities were observed upon transfection with HNF3 expression plasmids, as determined by PhosphorImager analysis: complex A1, 2.0 (HNF3α) and 4.0 (HNF3β); complex B1, 1.4 (HNF3α) and 2.1 (HNF3β); complex C1, 1.0 (HNF3α) and 1.2 (HNF3β); and complexes D1, D2, and D3, 0.7, 3.1, and 3.5, respectively (HNF3α), and 1.2, 2.7, and 3.2, respectively (HNF3β). Untransfected COS-1 cells (kidney-derived) were found to have a significant amount of HNF3-like EMSA activity, consistent with an earlier report of substantial HNF3 EMSA activity in kidney tissue extracts (36).
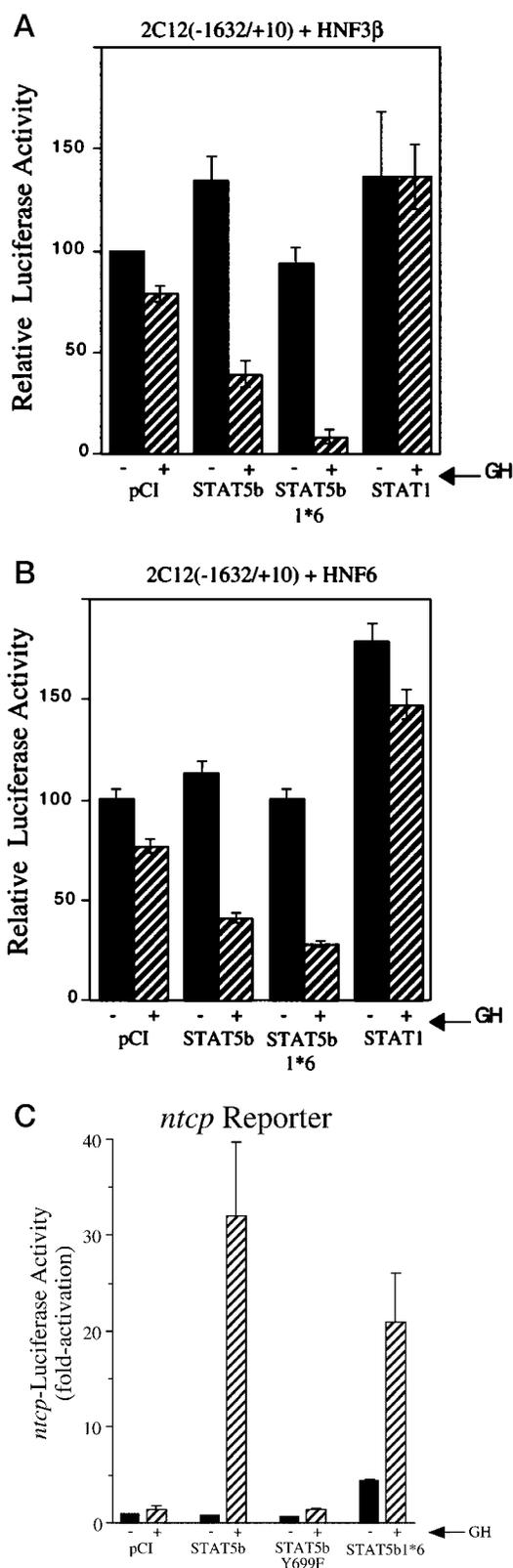
FIG. 6. **Transcriptional inhibition of *CYP2C12* promoter by GH-activated STAT5b.** *A* and *B*, the *2C12*(−1632/+10)-LUC reporter plasmid was transfected into HepG2 cells together with the GH receptor, the indicated liver transcription factor expression plasmids, and the internal control plasmid pRL-TK. STAT expression plasmids were also cotransfected, where indicated. The total amount of HNF + STAT transcription factor expression plasmid was adjusted to 400 ng of DNA/ transfection using pCI. Cells were treated with GH, where indicated, as described under "Materials and Methods." Normalized firefly luciferase activity values were then determined and are expressed relative to the luciferase activity level obtained with HNF3β (*A*) or HNF6 (*B*) alone

TABLE II
GH-activated STAT5b inhibits HNF3β-stimulated CYP2C12 gene transcription in a dose-responsive fashion

HepG2 cells were transfected as described in the legend to Fig. 6*A*, except that the cells were stimulated with either GH or prolactin at the indicated concentrations. Data represent relative mean ± S.D. luciferase activity values, of three replicates.

Expression plasmid	Hormone treatment				
	0	GH (ng/ml)			Prolactin (500 ng/ml)
		40	100	500	
pCI	96 ± 13	96 ± 17	88 ± 17	82 ± 19	112 ± 11
STAT5b	**100**	72 ± 20	63 ± 17	41 ± 11	107 ± 2
STAT5b1*6	98 ± 21	21 ± 6	18 ± 7	19 ± 3	108 ± 11

TABLE III
Effect of GH-activated STAT5b on HNF6-stimulated CYP2C12 promoter activity

HepG2 cells were transfected as described in the legend to Fig. 6*B*, except that the cells were stimulated with either GH or prolactin at the indicated concentrations. Data represent relative mean ± S.D. luciferase activity values of three replicates.

Expression plasmid	Hormone treatment				
	0	GH (ng/ml)			Prolactin (500 ng/ml)
		40	100	500	
pCI	89 ± 3	85 ± 12	79 ± 13	72 ± 16	91 ± 13
STAT5b	**100**	70 ± 6	57 ± 6	44 ± 7	90 ± 4
STAT5b1*6	99 ± 8	21 ± 2	19 ± 1	17 ± 1	89 ± 5

suggest that STAT5a and STAT5b, which are both preferentially activated by GH in male as compared with female rat liver, can contribute to the silencing of *CYP2C12* transcription in adult male rats.

In cultured liver cells, GH treatment under the conditions of continuous hormone stimulation used in the present studies leads to a transient, high level of STAT5b signaling, followed by a persistent, low level of STAT5b activity (24). We therefore examined whether more complete inhibition of *2C12* promoter activity could be obtained by transfection of a constitutively active mutant STAT5b protein, designated STAT5b1*6, which exhibits delayed down-regulation, resulting in a highly stable, persistently activated STAT5b form (29). We first confirmed that, in HepG2 cells, STAT5b1*6 is constitutively active, as judged by its stimulation of the *ntcp*-luciferase reporter in the absence of GH (Fig. 6*C*). Moreover, GH treatment further increased this constitutive transcriptional activity substantially (Fig. 6*C*). Finally, STAT5b1*6 exhibited delayed down-regulation in HepG2 cells compared with wild-type STAT5b when activated by GH (data not shown). Cotransfection of STAT5b1*6 with the *2C12* promoter resulted in a strong inhibition of *2C12* promoter activity stimulated by HNF3β or HNF6 following GH treatment (87 and 79% inhibition of *2C12*(−1632/+10) activity stimulated by HNF3β (Fig. 6*A*) and HNF6 (Fig. 6*B*), respectively; also see Table IV). This GH inhibitory effect was maximal at a physiological concentration of GH (Tables II and III), further supporting the proposed transcriptional repressor role of GH-activated STAT5b on *CYP2C12*.

without GH treatment, which was arbitrarily set at 100. Data shown represent mean ± S.E. of three independent transfections. *C*, GH activates STAT5b transcriptional activity in transfected HepG2 cells. The *ntcp* reporter plasmid 4X-pT109-LUC was transfected into HepG2 cells with each of the indicated STAT expression plasmids in the presence of the GH receptor expression plasmid and pRL-TK. GH treatment was applied for 24 h as described for *A*. Normalized firefly luciferase activity values were determined, and -fold activation values were then calculated. Data shown represent mean ± S.D. of two to three independent transfections.

A. 2C12(-1632/+10) + HNF3β

B. 2C12(-1632/+10) + HNF6

C.
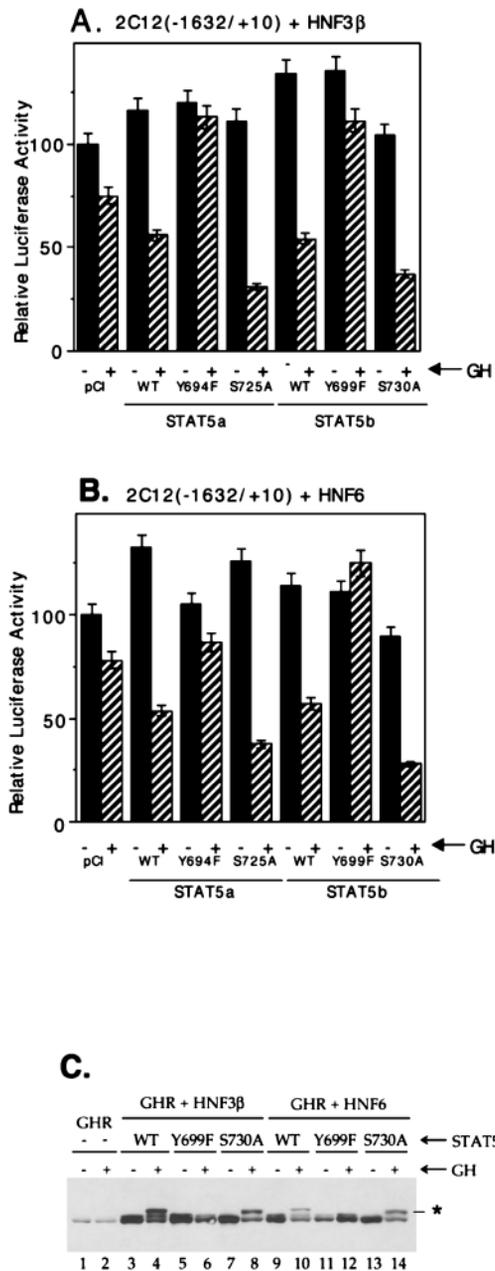
FIG. 7. **Tyrosine phosphorylation, but not serine phosphorylation, of STAT5 proteins is required for GH inhibition of *2C12* promoter.** *A* and *B*, the *2C12*(−1632/+10)-LUC reporter plasmid was transfected into HepG2 cells with the GH receptor (*GHR*), the indicated transcription factor expression plasmids, and the internal control plasmid pRL-TK. STAT expression plasmids were included as indicated. The total amount of HNF + STAT transcription factor expression plasmid was adjusted to 400 ng of DNA/transfection using pCI. GH treatment was for 24 h. Normalized firefly luciferase activity values were determined and are expressed relative to the luciferase activity level obtained with HNF3β (*A*) or HNF6 (*B*) alone without GH treatment, which was arbitrarily set at 100. Data shown represent mean ± S.E. three independent transfections. *C*, shown is a Western blot probed with anti-STAT5b antibody (sc-835, Santa Cruz Biotechnology) indicating the consistency of expression of wild-type (*WT*) STAT5b and of the STAT5b-Y699F and STAT5b-S730A mutants used in the experiments shown in *A* and *B*. The uppermost STAT5b band (indicated by the *asterisk*) corresponds to the GH-activated, tyrosine-phosphorylated form of STAT5b (*lanes 4*, *8*, *10*, and *14*) and was not observed with the STAT5b-Y699F mutant (*lanes 6* and *12*). *Lanes 1* and *2* indicate the presence of an endogenous STAT5-immunoreactive protein that is distinct from STAT5b insofar as it is not converted to the lower mobility tyrosine-phosphorylated form (*asterisk*) upon GH stimulation (*lane 2*). Wild-type STAT5a and its Y694F and S725A mutants also gave similar Western blot signal intensities when transfected into HepG2 cells (data not shown).

TABLE IV
*GH/STAT5b1*6 inhibition of the CYP2C12 promoter: 5'-deletion analysis*

HepG2 cells were transfected as described in the legend to Fig. 6 (*A* and *B*), except that *2C12* promoter constructs of varied length were used, as shown. Cells were cotransfected with the GH receptor, the pRL-TK internal control plasmid, and either HNF3β (part A) or HNF6 (part B) in the absence or presence of STAT5b1*6, as indicated. GH stimulation was for 24 h at 500 ng/ml (last column). Data shown are mean ± S.D. of duplicate analyses based on normalized luciferase activities, with the samples transfected with HNF3β or HNF6 alone (first column) set at 100.

2C12 promoter construct (nt)	Normalized luciferase activity (relative values)		
	Liver transcription factor alone	+STAT5b1*6	
		−GH	+GH
A. HNF3β			
−1632/+10	100 ± 9	101 ± 2	6.8 ± 0.2
−970/+10	100 ± 15	108 ± 10	6.4 ± 0.5
−516/+10	100 ± 2	124 ± 5	7.8 ± 0.2
B. HNF6			
−1632/+10	100 ± 34	150 ± 31	6.8 ± 2.6
−856/+10	100 ± 1	105 ± 4	6.9 ± 0.1
−96/+10	100 ± 9	145 ± 13	5.3 ± 0.4

Tyrosine Phosphorylation, but Not Serine Phosphorylation, of STAT5 Proteins Is Required for GH Inhibition of 2C12 Promoter—GH activation of STAT5 proteins involves tyrosine phosphorylation catalyzed by JAK2, followed by a STAT serine phosphorylation reaction. Mutation of the target tyrosine residue to phenylalanine (STAT5a-Y694F and STAT5b-Y699F) results in a loss of STAT5 transcriptional activity (Fig. 6*C*) (3). Fig. 7 shows that STAT5a-Y694F and STAT5b-Y699F were unable to effect GH inhibition of *2C12*(−1632/+10) promoter activity stimulated by HNF3β or HNF6. Thus, STAT5 tyrosine phosphorylation and/or its downstream activities (STAT5 dimerization, nuclear translocation, DNA-binding activity, or transcriptional activity) are obligatory for GH inhibition of *2C12* promoter activity. By contrast, mutation of the major serine phosphorylation site of STAT5a and STAT5b (STAT5a-S725A and STAT5b-S730A, respectively) (28) did not abrogate the STAT5-dependent GH inhibition of *2C12* promoter activity (Fig. 7). In control analyses, the consistency of STAT5 protein levels was confirmed by Western blotting (Fig. 7*C*).

2C12 Promoter Sequences Required for STAT5b Inhibition—The ability of GH-activated STAT5b to inhibit *2C12* promoter activity stimulated by HNF3β or HNF6 was further investigated using *2C12* promoter-luciferase reporter constructs progressively deleted from the 5'-end. These experiments were carried out using STAT5b1*6, which inhibited *2C12*(−1632/+10) promoter activity by ~90% (Fig. 6). Table IV shows that, in the case of HNF3β-stimulated *2C12* promoter activity, full GH- and STAT5b1*6-dependent inhibition was maintained in promoter constructs deleted to nt −516. Shorter promoter constructs displayed low HNF3β-stimulated activity (Fig. 2*C*) and were not tested. In the case of cells cotransfected with HNF6, STAT5b1*6 suppressed reporter gene activity in a GH-dependent manner with constructs containing as few at 96 nt of 5'-sequence (Table IV). Interestingly, classic STAT5-binding sites (5'-TTC-NNN-GAA-3') are not present in this 96-nt segment and, indeed, are absent from the entire 1.9-kilobase 5'-flanking region of *CYP2C12* (data not shown).

HNF3α, HNF3β, and HNF6 Are Necessary, but Not Sufficient, for High 2C12 Promoter Activity—To further investigate the role of liver-specific transcription factors in *CYP2C12* gene expression, the monkey kidney cell line COS-1 was transfected with *2C12*(−1632/+10) together with expression plasmids encoding HNF3α, HNF3β, or HNF6. In contrast to HepG2 cells, none of these liver-enriched transcription factors activated the *CYP2C12* promoter in COS-1 cells (data not shown). Control

experiments established, however, that all three transcription factors were expressed in the transfected COS-1 cells and were active in EMSAs in binding to their cognate DNA sequences (Fig. 5*C* and data not shown). HNF3α, HNF3β, and HNF6, on their own, are thus not sufficient to activate *2C12* promoter activity, suggesting that additional hepatocyte-specific factors present in HepG2 cells, but absent from COS-1 cells, contribute to *CYP2C12* activation by HNF3α, HNF3β, and HNF6.

DISCUSSION

Liver cytochrome P450 genes *CYP2C11* and *CYP2C12* have served as important models for investigation of the effects of GH on liver gene expression. *CYP* genes are of particular interest, both because of their key role in hepatic drug and steroid metabolism and due to their striking responsiveness to the sex-dependent temporal patterns of plasma GH stimulation (6). *CYP2C11* and *CYP2C12* gene expression is regulated by GH at the level of transcription initiation (10, 11). However, maximal induction of both genes requires several days of GH treatment and, in the case of *CYP2C12*, requires ongoing protein synthesis. Thus, *CYP* gene induction by GH appears to be indirect and mechanistically distinct from the regulation of the c-*fos* and insulin-like growth factor I genes, which are rapidly activated in liver following GH stimulation (41, 42) and do not exhibit the sexual dimorphism characteristic of the *CYP* genes. GH regulatory mechanisms that may potentially contribute to liver *CYP* expression include the direct phosphorylation of pre-existing transcription factors, which may occur as an early event catalyzed by the GH receptor-JAK2 complex (*e.g.* STAT5b activation) (12) or via downstream cascades (*e.g.* mitogen-activated protein kinase activation of the ternary complex factor Elk-1) (43). In addition, these and other GH signaling events may stimulate secondary increases in the transcription of liver-enriched nuclear factors, such as HNF3, HNF6, and C/EBP (33, 34), which could play a role in liver *CYP* expression, perhaps acting in concert with more direct GH regulatory mechanisms.

This study has demonstrated potentially important roles for both liver-specific and GH-regulated transcription factors in controlling *CYP2C12* gene expression. *CYP2C12* promoter activity was found to be strongly activated by the liver-enriched transcription factors HNF3β (and HNF3α) and HNF6, whose actions are synergistic and mediated via distinct DNA-binding sites (Fig. 5*A*). In the case of HNF6, a single responsive region was localized to *CYP2C12* nt −96 to +10, consistent with the HNF6 site at nt −47 to −38 identified by Lahuna *et al.* (22). By contrast, at least five *CYP2C12* promoter regions, identified by 5′-deletion analysis, were shown to contribute to the stimulation of transcription by HNF3β and the related HNF3α. Moreover, the binding of HNF3β to HNF3 consensus sequences present in at least three of these regions was confirmed by EMSA competition analysis. One of these HNF3 sites (oligo D; Fig. 5*A*) is present within the *2C12*(−96/+10) promoter construct at a site distinct from, and upstream of, the HNF6 site (Fig. 5*A*). This latter finding may explain why HNF3β trans-activates this promoter fragment, given its weak binding interaction with the HNF6 site (22).

HNF6 and HNF3β both require GH for maximal expression in liver. In the case of HNF6, hypophysectomy abolishes and GH replacement rapidly restores factor expression, whereas in the case of HNF3β, whose promoter contains a functional HNF6 site (44), a secondary GH-induction response is observed (34). The obligatory requirement of GH for *CYP2C12* expression in female rat liver *in vivo* could thus be mediated by HNF3β and HNF6 working in combination, as suggested by the synergistic, ~300-fold activation of *CYP2C12* seen in the presence of both GH-responsive nuclear factors (Fig. 4). However,
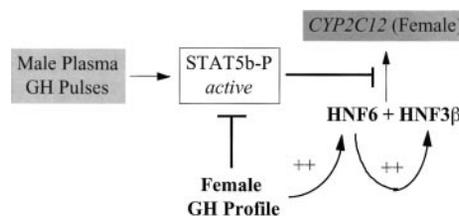
FIG. 8. **Proposed model for role of GH-regulated STAT5b and liver-enriched transcription factors in female-specific expression of *CYP2C12*.** HNF6, which is more abundantly expressed in female than male rat liver in a GH-dependent manner, serves to induce HNF3β, which acts together with HNF6 in a synergistic manner to stimulate *CYP2C12* promoter activity. STAT5b, activated by intermittent plasma GH pulses in adult male rats, is proposed to antagonize the stimulatory action of HNF6 and HNF3β on *CYP2C12* activity. This inhibitory action of STAT5b would not be expected to occur in adult female rat liver, where only a low level of nuclear STAT5b is present as a consequence of the down-regulation of STAT5 signaling. Additional factors that may contribute to the GH-regulated, female-specific expression of *CYP2C12* include GHNF (19) and a factor related to IRE-ABP (23) (see "Discussion").

HNF3β and HNF6 are expressed in both male and female rat livers, albeit at an ~2-fold lower level in males in the case of HNF6, as judged by steady-state mRNA levels and EMSA activity (22). This sex difference in HNF6 expression is clearly too small to account for the >30-fold higher expression of *CYP2C12* in female compared with male rat liver, indicating that additional GH regulatory mechanisms and factors are required to confer the sex-specific pattern of gene expression observed *in vivo*.

One such GH regulatory mechanism may involve STAT5b, a latent cytoplasmic factor that is rapidly activated to a liver nuclear transcription factor in males in direct response to each successive plasma GH pulse (12, 13). By contrast, in female rats, the more continuous pattern of plasma GH stimulation leads to a substantial down-regulation of signaling from the GH receptor to STAT5b, resulting in low female levels of the active liver nuclear factor (38). Our previous finding that, in male STAT5b-deficient mice, certain female-specific, GH-regulated *CYP* genes are derepressed (*i.e.* up-regulated) led us to propose that STAT5b plays a negative regulatory role in the expression of such genes (16). Indeed, we now demonstrate that STAT5b, when activated by GH, can counteract the stimulatory effects of both HNF3β and HNF6 on *CYP2C12* promoter activity (Fig. 8). This effect was not seen with STAT5b-Y699F, whose tyrosine phosphorylation site is abolished by mutation, or with STAT1, a distinct GH-activated STAT protein that does not display the sex-dependent activation that characterizes STAT5b (39). Although the inhibitory effects of GH-activated STAT5b were only 65–70% complete in our studies, this may reflect a limitation of our experimental system, where treatment with continuous GH leads to an initial strong, but transient STAT5b activation, followed by a substantial down-regulation of STAT5b signaling. Of note, we observed more complete (up to 95%) inhibition of HNF3β- and HNF6-stimulated promoter activity when using a STAT5b mutant, STAT5b1*6, which exhibits persistent signaling due to a substantially delayed down-regulation (29).

The synergistic interaction between HNF6 and HNF3β on the *CYP2C12* promoter (Fig. 4) suggests that small differences in the levels of these factors in liver cells *in vivo* (*e.g.* the 2-fold higher expression of HNF6 in females) could conceivably translate into much larger differences in *CYP2C12* gene expression, particularly when coupled to the inhibitory action of GH pulse-activated STAT5b in males. Interestingly, rat liver HNF6 has an apparently short half-life *in vivo* when induced by a pulse of GH (34). It is therefore conceivable that the nuclear levels of

GH Regulation of Female-specific CYP2C12

this liver transcription factor could mirror in time those of GH pulse-activated STAT5b, which would facilitate STAT5b inhibition of any HNF6-stimulated *CYP2C12* gene transcription that might otherwise occur in males. Conversely, the induction of *CYP2C12* transcription that occurs in intact males infused with GH continuously (10) could, in part, reflect the associated ~2-fold increase in HNF6 expression (22), coupled with the marked down-regulation of the inhibitory nuclear STAT5b that is also seen in these animals (12). Additional factors that may contribute to the marked sex-dependent expression of *CYP2C12* include GHNF, a continuous GH-regulated nuclear factor that binds to multiple sites along the *CYP2C12* promoter (19), and factors related to IRE-ABP, which, like STAT5b, can inhibit *CYP2C12* promoter activity, in this case by binding to the proximal promoter at a site that both corresponds to a region of GHNF binding and that overlaps with a site used by C/EBPα in stimulating *CYP2C12* gene expression (23). Further study, including use of intact animal models, will be required to more clearly delineate the respective contributions of each of these proposed mechanisms to GH-regulated *CYP2C12* gene expression in liver *in vivo*.

The mechanism whereby GH-activated STAT5b suppresses the transcriptional activity of the *CYP2C12* proximal promoter, which is devoid of consensus STAT5-binding sites, remains elusive. The demonstration that the STAT5b inhibitory response is fully retained in a promoter construct containing as few as 96 5′-flanking nt (Table IV) suggests that STAT5b might act via a novel negative regulatory element contained within this sequence, perhaps one that corresponds to a generic STAT site (TTN_5AA) that is not a classic STAT5 site (*e.g.* generic STAT sites at *CYP2C12* nt −62 to −54 and nt −29 to −21). Of note, binding sites for STAT5 tetramers that do not correspond to classical STAT5 consensus sequences have recently been described (45). Alternatively, the inhibitory actions of STAT5b on the *CYP2C12* promoter may not require STAT5 DNA binding at all. STAT proteins and other transcription factors can participate in signaling through direct protein-protein interactions without direct binding to the STAT DNA response element (46, 47). Moreover, we have recently demonstrated that STAT5b can mediate the inhibitory effects of GH on nuclear peroxisome proliferator-activated receptor-α (48), which activates liver *CYP4A* genes in response to foreign chemicals classified as peroxisome proliferators (49). Indeed, the inhibition of the transcriptional activity of peroxisome proliferator-activated receptor-α by STAT5b is directed at its NH_2-terminal AF-1 transactivation domain and does not require STAT5b DNA binding (50). Further study will be required to establish whether an analogous mechanism characterizes the inhibitory actions of STAT5b on HNF3β- and HNF6-stimulated *CYP2C12* transcription.

After submission of this manuscript, a report appeared describing a pair of far upstream (−4.1 kilobase) *CYP2C12* STAT5-binding sites that enhance promoter activity in a GH-dependent manner in an *in vivo* female rat liver transfection assay (51). The *CYP2C12* promoter constructs containing these binding sites did not, however, exhibit the female-specific expression that is characteristic of the endogenous *CYP2C12* gene, as they were even more highly expressed when injected into male rat liver. Further study is required to evaluate the role of these upstream sequences and the mechanisms whereby they interact with the more proximal GH-regulated factors and promoter elements presently described to give rise to the female-specific expression pattern that characterizes the endogenous *CYP2C12* gene.

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