THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 46, Issue of November 16, pp. 43031–43039, 2001

Printed in U.S.A.

Inhibitory Cross-talk between STAT5b and Liver Nuclear Factor HNF3β

IMPACT ON THE REGULATION OF GROWTH HORMONE PULSE-STIMULATED, MALE-SPECIFIC LIVER CYTOCHROME P-450 GENE EXPRESSION*

Received for publication, August 9, 2001, and in revised form, September 6, 2001
Published, JBC Papers in Press, September 18, 2001, DOI 10.1074/jbc.M107597200

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STAT5b is repeatedly activated in rodent liver by the male pattern of intermittent plasma growth hormone (GH) stimulation and is required to maintain the GH pulse-regulated, male-specific pattern of liver gene expression. We presently investigate the interactions between STAT5b and hepatocyte-enriched nuclear factors (HNFs) that contribute to regulation of GH pulse-inducible, male-specific liver cytochrome P-450 (CYP) genes. STAT5 binding sites were identified in the 5′-flank of the adult male-expressed genes *CYP2A2* (nucleotides −2255 to −2247), *CYP4A2* (nucleotides −1872 to −1864), and *CYP2C11* (nucleotides −1150 to −1142). STAT5-DNA complexes were formed by each *CYP* sequence with nuclear extract from GH pulse-activated male, but not female, rat liver. The *CYP2C11* STAT5 site, which is flanked by HNF3 consensus sequences, conferred STAT5b-inducible reporter gene activity in GH-treated HepG2 cells. *trans*-Activation of the intact *CYP2C11* promoter (1.8-kilobase 5′-flank) was strongly induced by the liver nuclear factors HNF1α and HNF3β but, unexpectedly, was inhibited by GH-activated STAT5b. This STAT5b inhibitory effect could be reversed by HNF1α and reflects a functional antagonism between STAT5b and HNF3β, as evidenced by the inhibition of HNF3β DNA binding and transcriptional activity by STAT5b. HNF3β, in turn, inhibited STAT5b by a novel mechanism that leads to suppression of GH-inducible STAT5b tyrosine phosphorylation, DNA binding activity, and transcriptional activity. The potential for GH-activated STAT5b to stimulate male-specific liver *CYP* expression can thus be modulated by HNF3β, highlighting the complex interrelationship between STAT5b and liver transcription factors controlling expression of GH-regulated *CYP* genes.

Growth hormone (GH)[1] is secreted by the pituitary gland in a sex-dependent manner in both rodents (1) and humans (2). In male rats, GH is released into circulation every ~3–3.5 h, giving a repeated, pulsatile plasma hormone pattern (3), which contrasts to the more frequent, nearly continuous profile of pituitary GH secretion seen in adult females. These sexually dimorphic plasma GH patterns directly regulate the sexually dimorphic pattern of liver gene expression, in particular, liver cytochrome P-450 (CYP) gene expression (4, 5). Well studied examples of sex-specific, GH-regulated liver CYPs include the male-specific androgen 16α- and 2α-hydroxylase CYP2C11, which is strongly induced at puberty in male but not female rat liver, and the steroid sulfate 15β-hydroxylase CYP2C12, which is exclusively expressed in adult female rat liver (6, 7). The sexual dimorphism of liver with respect to expression of these and other sex-dependent liver CYPs, including CYP2A2 and CYP4A2 (both male-specific in their expression), is regulated by GH at the level of transcription initiation (8, 9).

GH signaling is initiated by GH receptor dimerization at the cell surface, leading to activation of the GH receptor-associated tyrosine kinase JAK2. JAK2, in turn, activates multiple intracellular signaling proteins (10–12). One such protein, the transcription factor STAT5b, displays a unique GH pulse responsiveness in rat liver (13). The intermittent stimulation of liver cells by successive plasma GH pulses triggers a rapid and repeated tyrosine phosphorylation and nuclear translocation of STAT5b in adult male rats. By contrast, in adult female rats, the more continuous pattern of GH exposure down-regulates GH receptor-JAK2 activity, leading to a low steady-state level of the transcriptionally active, nuclear STAT5b (14–16). A close correlation between STAT5b nuclear translocation and sex-specific liver gene expression is also seen in mouse liver (17). Targeted gene disruption studies establish that STAT5b, but not the closely related (>90% identical) STAT5a, plays a critical role in the physiological, sex-dependent responses of the liver to GH (18). Thus, STAT5b-deficient male mice exhibit a loss of sexually dimorphic liver CYP gene expression in addition to a loss of pulsatile GH-stimulated pubertal growth (19–21). By contrast, STAT5a is a principal and obligate mediator of mammopoietic and lactogenic signaling stimulated by prolactin in the mammary gland (22).

Although the above studies demonstrate an important role for STAT5b in maintaining the male-specific pattern of liver gene expression, the molecular details of the gene regulatory mechanisms whereby STAT5b contributes to the sexual dimorphism of the liver have remained elusive. In the case of the female-specific *CYP2C12* gene, GH-activated STAT5b is proposed to interfere with the strong, synergistic *trans*-activation of promoter activity effected by two liver-enriched transcription factors, HNF6 and HNF3β (23). Additional mechanisms contributing to the GH-regulated, sexual dimorphic expression of *CYP2C12* have also been proposed (24–27). In the case of the male-expressed *CYP2C11*, an initial promoter analysis revealed the presence of two negative regulatory regions ("silenc-

* This work was supported in part by Grant DK33765 from the National Institutes of Health (to D. J. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: GH, growth hormone; CYP, cytochrome P-450; STAT, signal transducer and activator of transcription; Luc, luciferase; nt, nucleotide; HNF, hepatocyte nuclear factor; EMSA, electrophoretic mobility shift assay; C/EBP, CCAAT/enhancer-binding protein.

ers") (28), but the identification of other functional elements and the potential role of STAT5b and specific liver transcription factors in regulating either basal on hormone-dependent transcription of this gene have not been described.

Several liver transcription factors contribute to the liver-specific expression of hepatic CYPs. These include the variant homeodomain protein HNF1, CCAAT/enhancer-binding proteins (C/EBPs), the winged helix factor HNF3, the nuclear receptor HNF4, and the one-cut homeoprotein HNF6 (29). These liver transcription factors function in unique combinations, often synergistically, to activate liver-expressed genes via a complex array of interactions. For example, *HNF6* expression is stimulated by HNF4 and inhibited by C/EBPα (30), whereas HNF3β positively regulates the expression of *HNF4* and *HNF1*α and their downstream targets (31). Other studies have shown that the *HNF6* gene can be regulated by GH, resulting in its sex-dependent expression by a mechanism involving STAT5b and HNF4 (32). As noted above, GH-dependent and liver-specific expression of *CYP2C12* in adult female rats is proposed to reflect cooperative regulation by HNF3β and HNF6 (23) as well as by HNF4 and HNF6 (27). Although the general importance of these transcription factor cascades in liver-specific gene expression is well established, only limited information is available regarding their particular role in the GH-regulation of sex-specific liver *CYP* gene transcription. Unique combinations of factors and interactions between GH-regulated STAT and liver-expressed regulators appear likely and may be required to establish and maintain the liver-specific and sexually dimorphic profiles of *CYP* gene expression.

The present study investigates the influence of GH-activated STAT5b on the expression of *CYP2C11* and its regulation by liver-enriched transcription factors. Our findings demonstrate that *2C11* gene expression is subject to regulation by GH-activated STAT5b in a manner that is modulated by two liver transcription factors that *trans*-activate the *2C11* promoter, HNF3β and HNF1α. Moreover, novel inhibitory cross-talk between HNF3β and GH-activated STAT5b is described. The implications of these findings are discussed in the context of current models for the sex-dependent regulation of *CYP2C11* expression by plasma GH pulses.

MATERIALS AND METHODS

Antibodies—Rabbit polyclonal anti-STAT5b antibody (sc-835) raised against STAT5b residues 776–786 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal anti-phosphotyrosine-STAT5b, raised against a synthetic Tyr(P)^{699} peptide (keyhole limpet hemocyanin (KLH)-coupled), was purchased from Cell Signaling Technology (Beverly, MA). Goat polyclonal anti-mouse HNF3β (sc-9187x) and anti-human HNF3α (sc-6553x), raised against peptides mapping near the C terminus, were purchased from the same vendor. Anti-phosphotyrosine monoclonal antibody 4G10 was from Upstate Biotechnology Inc. (Lake Placid, NY). Rabbit polyclonal anti-HNF3β antibody used for Western blotting and rabbit polyclonal anti-STAT5b antibody used for immunoprecipitation were generously provided by Dr. R. Costa (University of Chicago, Chicago, IL) and Dr. L. Hennighausen (NIDDK, National Institutes of Health, Bethesda, MD), respectively.

Expression and Reporter Plasmids—Expression plasmids for mouse STAT5b (Dr. A. Mui; DNAX Corp., Palo Alto, CA), mouse STAT5b-Y699F (Dr. H. Rui, Uniformed Services University of the Health Sciences, Bethesda, MD), rat GH receptor (Dr. N. Billestrup, Hagedon Research Institute, Gentofe, Denmark), HNF1α (Dr. F. Gonzalez, NCI, National Institutes of Health, Bethesda, MD), HNF6 (Drs. F. Lemaigre and G. Rousseau, University of Louvain Medical School, Brussels, Belgium), and HNF3α and HNF3β (Dr. E. Lai, Memorial Sloan-Kettering Cancer Center, New York) were obtained from the indicated individuals. The STAT5 *ntcp* reporter plasmid 4x-pT109-Luc, containing four copies of a naturally occurring STAT5 response element, and the HNF3 reporter (6xHNF3)-Cdx-2-Luc were respectively provided by Drs. M. Vore (University of Kentucky, Lexington, KY) and R. Costa (University of Illinois, Chicago, IL).

2C11 Promoter Plasmids—Segments of the *2C11* 5′-flank were prepared by polymerase chain reaction amplification using Fischer 344 rat genomic DNA as template and then subcloned into the *Sac*I and *Xho*I sites of the promoterless luciferase reporter plasmid pGL3-basic (Promega) with the assistance of D. Ding of this laboratory. Polymerase chain reactions were carried out for 30 cycles consisting of 94 °C for 1 min, 48 °C for 1 min, and 72 °C for 2 min. Sense primers were as follows: 5′-GGC ATA AAG TGG TGG AT-3′ (nts −1769 to −1753), 5′-GGA GGT GCC TGT TCT GG-3′ (nts −1208 to −1194), 5′-GTC ACT TCA GAG GTT-3′ (nts −1182 to −1168), 5′-GTT ATT CCC GCA TTC TC-3′ (nts −968 to −952), and 5′-GGG GGT GCC TTA GTT GG-3′ (nts −633 to −617). Sense primers were paired with a common antisense primer, 5′-GCA GCC TTC CTC AGG GAG-3′ (nts +22 to +5), to generate five corresponding *2C11* promoter reporter constructs: −1769/2C11-Luc, −1208/2C11-Luc, −1182/2C11-Luc, −968/2C11-Luc, and −633/2C11-Luc. −1533/2C11-Luc was generated from −1769/2C11-Luc by digestion with *Sac*I and *Sac*II, followed by filling in and religation. Sequence analysis of the *2C11* promoter constructs revealed the presence of only one of the two GCTA repeats at nts −47 to −40 reported in GenBank™ (accession number XB79081). This sequence was present in amplified genomic DNA from three independent Fischer 344 rat genomic DNA polymerase chain reactions. *2C11* promoter nucleotide positions were numbered according to GenBank™ XB79081 after removing the four nucleotides corresponding to the extra GCTA sequence.

Luciferase reporters containing a single copy of the wild-type or mutated *2C11* STAT site (STAT5/2C11-Luc and STAT5mut/2C11-Luc, respectively) were prepared as follows. Complementary oligonucleotides corresponding to wild-type or mutated *2C11* nts −1159 to −1138 (see Fig. 2*A*) and containing *Xba*I and *Bgl*II site adapters were subcloned into the corresponding restriction sites of the luciferase reporter pGL3 promoter. A reporter plasmid containing two tandem copies of the *2C11* STAT5 site subcloned into pGL3 promoter 2x STAT5/2C11-Luc was kindly provided by Dr. Y. Jounaidi of this laboratory. Wild-type and mutated sequences were verified by DNA sequence analysis.

Animal Treatments and Nuclear Extract Preparation—Untreated adult Fischer 344 rats and hypophysectomized rats treated with rat GH, rat prolactin, or lipopolysaccharide were described earlier (13). Nuclear extracts were prepared from freshly excised liver tissue using standard methods and stored frozen at −80 °C (33).

Cell Culture and Transient Transfection—HepG2 human hepatoma and COS-1 cells were maintained in Dulbecco's modified essential medium containing 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. For transient transfections, HepG2 cells were seeded at a density of 1.3×10^5 cells/well in 24-well plates. The cells were transfected with FuGENE 6™ reagent (Roche Molecular Biochemicals). FuGENE 6-DNA complexes were prepared as described in the manufacturer's protocol at a ratio of 1.3 to 1 (FuGENE 6:DNA, v/w). Typically, each well of a 24-well tissue culture plate received a total of 800 ng of DNA: 50–200 ng of luciferase reporter plasmid (200 ng of *2C11* promoter-Luc or 50 ng of either STAT5/2C11-Luc, 4x-pT109-Luc, or (6x-HNF3)-Cdx-2-Luc), 100 ng of GH receptor, 200 ng of STAT5b, and 200 ng HNF expression plasmid. pRL-tk-Luc plasmid (*Renilla* luciferase; 50 ng) was included in all transfections as an internal control for transfection efficiency. 24 h after transfection, the cells either were treated with 200 ng/ml rat GH for 18–20 h or were left untreated. Cell lysates were prepared by shaking the cells in 150 μl of 1× Promega lysis buffer for 10 min at room temperature. Firefly and *Renilla* luciferase activities were measured using a dual reporter assay system (Promega) and a Monolight 2010 luminometer (Analytical Luminesence Laboratory, San Diego, CA). Firefly luciferase activity values were divided by *Renilla* luciferase activity values to obtain normalized luciferase activities (mean ± SD values for $n = 3$ separate transfections, unless indicated otherwise). Relative luciferase activities were then calculated to facilitate comparisons between samples within a given experiment. Individual group comparisons were examined for statistical significance using the two-tailed Student's t test ($p < 0.05$).

Cell extracts were prepared for EMSA and Western blot analysis as follows. COS-1 and HepG2 cells were seeded on 35-mm tissue culture dishes at ~60% confluency and incubated overnight. A total of 3.2 μg of plasmid DNA containing GH receptor, STAT5b, and/or HNF3β expression plasmid and *Renilla* luciferase was transfected using FuGENE 6, as specified in each figure legend. 36 h later, the cells were either stimulated with 200 ng/ml GH for 30 min or were left untreated. The cells were washed twice with ice-cold phosphate-buffered saline and scraped with 100 μl of 1× Promega lysis buffer containing 1 mM sodium orthovanadate, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 2 μg/ml antipain, and 0.1 mM phenylmethylsulfonyl fluoride. The cell extracts were incubated in a cold room for 30 min with shaking and then centrifuged for

A. STAT5 DNA-binding sites in male-specific CYP genes

CYP genes	Location	STAT5 site
CYP2A2	Promoter (-1872 to –1864)	5'-gtt-caa-TTC-CCA-GAA-cca-ca-3'
CYP2C11	Promoter (-1150 to –1142)	5'-gca-aac-att-TTC-CAT-GAA-aaa-a-3'
CYP4A2	Promoter (-2255 to –2247)	5'-tgt-gtt-TTC-CTG-GAA-ttc-tt-3'

B. EMSA

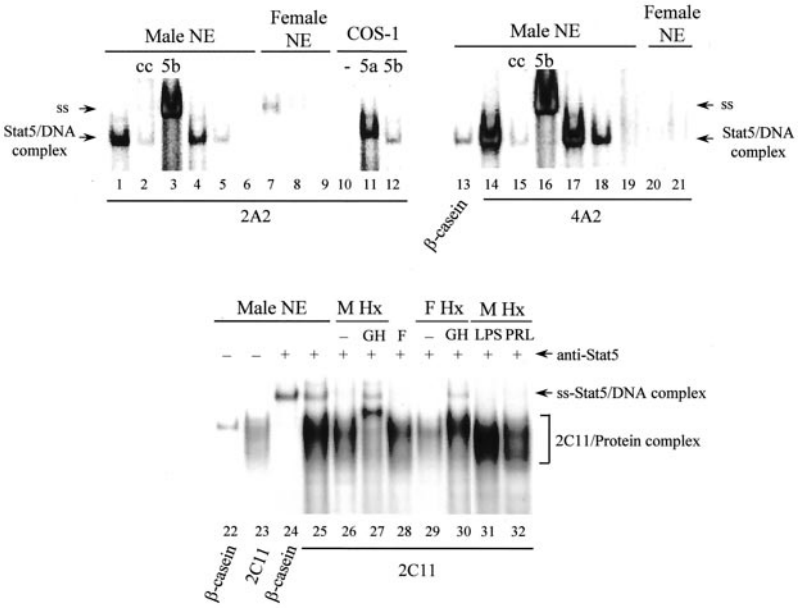

FIG. 1. **EMSA analysis of STAT5 sites in male-specific CYP promoters.** *A*, STAT5 consensus core sequences (*capital letters*) located in the promoters of *CYP2A2, CYP4A2*, and *CYP2C11* at the indicated positions are shown in the context of the complete sequences of the EMSA probes used in *B*. *B*, EMSA analysis using liver nuclear extract (*NE*) prepared from intact or hypophysectomized (*Hx*) male (*M*) and female (*F*) rats, as indicated. Hypophysectomized rats were treated with GH, lipopolysaccharide (*LPS*), or prolactin (*PRL*) as noted. Male nuclear extracts were prepared from intact rats killed during a GH pulse secretory period, when STAT5b is active and nuclear (*lanes 1*, *4*, *14*, *17*, and *22–25*); from rats killed between plasma GH pulses, when STAT5b is cytoplasmic and inactive (*lanes 6* and *19*); or from rats killed late in a GH pulse, when the nuclear STAT5b pool has been partially deactivated by dephosphorylation (*lanes 5* and *18*). EMSA samples shown in *lanes 10–12* used extracts of GH-treated COS-1 cells that were untransfected (*lane 10*) or were transfected with GH receptor and either STAT5a or SAT5b (*lanes 11* and *12*). EMSA analysis was carried out in the presence of polyclonal anti-STAT5b antibody (1 μl) to visualize the STAT5-dependent DNA binding activity as a supershifted band (*lanes 3*, *16*, and *24–32*) or in the presence of a 100-fold excess of unlabeled probe (*cc*, cold competition) to confirm the specificity of the EMSA complex (*lanes 2* and *15*).

30 min at 15,000 × *g*. The supernatants were snap-frozen in liquid N_2 and stored at −80 °C. Protein concentrations were determined with Bio-Rad Dc detergent protein assay kit with bovine serum albumin as a standard.

EMSA Assays—Cell extract (10 μg) was incubated for 10 min in EMSA reaction buffer, consisting of 3% glycerol, 700 μM $MgCl_2$, 350 μM EDTA, 350 mM dithiothreitol, 7 mM Tris-HCl, pH 7.5, and 2 μg of poly(dI-dC) (Roche Molecular Biochemicals). 10 fmol of ^{32}P-end-labeled, double-stranded oligonucleotide probe (~30,000 cpm) was then added and incubated for 20 min at room temperature then 10 min on ice, followed by the addition of 2 μl of loading buffer (30% glycerol, 0.25% bromphenol blue, 0.25% xylene cyanol). For STAT5b supershift analysis, rabbit polyclonal anti-STAT5b antibody was added 10 min after addition of the ^{32}P-labeled DNA probe, followed by an additional 10-min incubation. For HNF3β supershift/complex disruption analysis, goat polyclonal anti-HNF3β antibody was incubated with the sample for 10 min prior to addition of the labeled DNA probe. The samples were electrophoresed for 3–4 h at 4 °C through a 5.5% nondenaturing gel in 0.5× TBE buffer (for the *2C11* STAT5 site probe; see below) or in 0.25× TBE buffer (for the *2C11* HNF3-STAT5 probe) following a 30-min pre-electrophoresis step. The gels were exposed to PhosphorImager plates overnight and visualized using a Molecular Dynamics PhosphorImager and ImageQuant software (Sunnyvale, CA).

EMSA analysis was performed using the following DNA probes: STAT5 site probes from rat *CYP2C11*, *CYP2A2*, and *CYP4A2* genes, as listed in Fig. 1A; the *β-casein* STAT5 probe used earlier (13); rat *CYP2C11* HNF3-STAT5 site, nts −1174 to −1138 (sense: 5′-(g)aga ggt taa tta **aat gca aac aat T**TC CAT GAA aaaa-3′; antisense: 5′-(g)tttt TTC ATG GAA aat gtt tgc att taa acc tct-3′); and mutated HNF3-STAT5 probe. Mutations were introduced either in the HNF3 binding site (aa tgc aaa catt *to* aa tga att gatt-3′) or the STAT5 binding site (TTC CAT GAA to Tat CAT GAA) of the wild-type HNF3-STAT5 probe. Nucleotides corresponding to a STAT5 consensus site are shown as capital letters; those corresponding to an HNF3 consensus site are shown in bold type, with the mutated residues underlined.

Western Blotting and Immunoprecipitation—Cell extracts (20 μg) were electrophoresed on 7.5% Laemmli SDS gels, electrotransferred onto nitrocellulose membranes, and then probed with anti-Tyr(P)^{699} STAT5b antibody, as described in the manufacturer's protocol, or with anti-STATb5 antibody. For immunoprecipitation with anti-STAT5b antibody, cell extract (70 μg) was preimmune-cleared for 1 h at 4 °C in a total volume of 200 μl of IP buffer (10 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride) containing 20 μl of 50% protein A-Sepharose beads and 1 μg of rabbit anti-mouse IgG. Protein A-Sepharose beads were removed by centrifugation, and anti-STATb5 antiserum was added to the precleared cell lysate and incubated for 3 h on ice. Immune complexes were collected by centrifugation after a further 1 h of incubation with 20 μl of 50% protein A-Sepharose beads at 4 °C, washed three times with 300 μl IP buffer, and resuspended in 30 μl of 1.5× SDS gel sample buffer. The samples were then analyzed on Western blots probed with anti-phosphotyrosine antibody 4G10 using blocking and probing conditions described earlier (13). Antibody binding was visualized on x-ray film by enhanced chemiluminescence using the ECL kit from Amersham Pharmacia Biotech. To reprobe with rabbit polyclonal HNF3β antibody, nitrocellulose membranes were heated in stripping buffer (62.5 mM Tris-HCl, pH 7.6, 2% SDS, 50 mM 2-mercaptoethanol) for 20 min at 50 °C. Membranes were blocked in Solution I (0.3% Tween 20 in 1× phosphate-buffered saline) containing 1% bovine serum albumin and 1% nonfat dry milk for 1 h at 37 °C and then incubated overnight at 4 °C with anti-HNF3β serum (diluted 1:4000 dilution in blocking solution). The results are presented in figures prepared from gray scale scans of portions of the x-ray films of each blot. Scans were obtained using a Cannon IX-4015 scanner and Ofoto scanning software.

RESULTS

Occurrence of STAT5 Sites in 5′-Flanking DNA of Male-specific, GH-regulated CYP Genes—Computer analysis revealed the presence of STAT5 consensus sites (TTC NNN GAA) in the 5′-flanking DNA of *CYP* genes *2A2, 2C11*, and *4A2* (Fig. 1*A*). Each of these *CYPs* is expressed in rat liver in a male-specific manner and is regulated by the temporal pattern of

pulsatile plasma GH stimulation (see the introduction). No STAT5 consensus sites were found in the 5′-flank of numerous other rat *CYP* genes, including CYPs 1A1, 1A2, 2A1, 2B2, 2C6, 2E1, and 4A1, which are not subject to male-specific, GH pulse regulation (data not shown). To ascertain whether the *CYP* STAT5 sites are functional in STAT5 binding, EMSA analyses were carried out using liver nuclear extracts prepared from adult male rats killed at the time of a plasma GH pulse when STAT5b is active and nuclear (15). Fig. 1*B* shows the formation of a single DNA-protein complex by the *CYP2A2* (*lanes 1* and *4*) and *CYP4A2* STAT5 site probes (*lanes 14* and *17*). These complexes were strongly competed by unlabeled probe (*lanes 2* and *15*) and were supershifted by anti-STAT5b antibody (*lanes 3* and *16*). Little or no specific complex was formed with liver nuclear extracts prepared from female livers (*lanes 7–9* and *lanes 20* and *21*) or from livers of male rats killed between GH pulses (*lanes 6* and *19*) when STAT5b is primarily cytoplasmic and inactive (15). Protein-DNA complexes of the same mobility were formed by male liver nuclear extracts incubated with an established *β-casein* STAT5 probe and with extracts of GH-treated COS-1 cells transfected with STAT5b and GH receptor (*lanes 13* and *12*, respectively, and data not shown). These complexes were distinguished from the complex formed by STAT5a, which migrates more slowly (*e.g. lane 11 versus lanes 1*, *4*, and *12*). STAT5a is much less abundant than STAT5b in liver tissue (20).

In contrast to the discrete STAT5b-containing complex formed by the *CYP2A2* and *CYP4A2* STAT5 site probes, the *CYP2C11* STAT5 site probe formed a diffuse protein-DNA complex when incubated with male rat liver nuclear extract (Fig. 1*B*, *lane 23*). Supershift analysis confirmed the presence of STAT5b protein in the complex (*lane 25*). Formation of the STAT5 supershifted complex was male-specific (*lane 25 versus lane 28*) and could be induced by treatment of hypophysectomized rats with a pulse of GH (*lane 27 versus lane 26* and *lane 30 versus lane 29*). Complex formation was not induced by treatment of rats with lipopolysaccharide or prolactin (*lanes 31* and *32*), neither of which activates liver STAT5 (13). Mutation of the core STAT5 binding site abolished STAT5 binding to the *2C11* promoter probe, whereas mutation of the adjacent upstream sequence was without effect (Fig. 2*A*).

To determine whether the STAT5 site is functional in mediating GH-stimulated reporter gene activity, luciferase reporter constructs driven by the *2C11* STAT5 site were transfected into HepG2 cells together with expression plasmids for GH receptor and STAT5b. GH stimulated a 1.8-fold increase in luciferase reporter activity driven by the wild-type *2C11* STAT5 site but not when the STAT5 core sequence contained a mutated STAT5 site (Fig. 2*B*, *left panel*). This modest increase was observed in three independent experiments but did not reach statistical significance. However, GH-stimulated reporter activity was significantly increased, by ~3-fold, by adding a second copy of the isolated *2C11* STAT5 site. GH had no effect in cells transfected with the inactive STAT5b tyrosine phosphorylation site mutant, STAT5b-Y699F (Fig. 2*B*, *right panel*). We conclude that GH-activated STAT5b binds to the STAT5 site of *2C11* in a functional, transcriptionally active manner.

GH-activated STAT5b Inhibits trans-Activation of Intact 2C11 Promoter—To further characterize the GH dependence of *2C11* transcriptional activity, we prepared six luciferase reporter constructs containing various lengths of *2C11* promoter sequence, ranging from 1769 to 633 nts of 5′-flanking DNA and extending to nt +22 relative to the transcription start site. These constructs were individually transfected into HepG2 cells together with GH receptor and STAT5b to establish a robust GH signaling pathway. Each *2C11* promoter construct

A. STAT5-DNA binding activity

Probe	Sequence	STAT5 EMSA Activity
wt 2C11	5'-gca-aac-att-**TTC**-CAT-**GAA**-aaa-a-3'	+
Single-mt 2C11	5'-gca-aac-att-**TAT**-CAT-**GAA**-aaa-a-3'	–
Double-mt 2C11	5'-gca-aac-att- **TAT**-CAT-**TTA**-aaa-a-3'	–
Upstream-mt 2C11	5'-gct-ggg-att-**TTC**-CAT-**GAA**-aaa-a-3'	+

B. 2C11/STAT5-Luciferase Reporter Activity

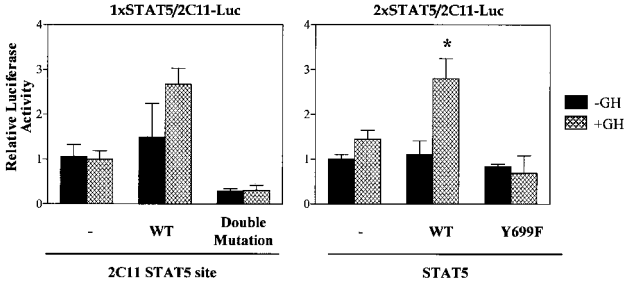

FIG. 2. **GH-stimulated 2C11 STAT5 site reporter gene activity.** *A*, STAT5 DNA binding activity of the *2C11* STAT5 site EMSA probe incorporating either wild type (*wt*) or one of the indicated mutant (*mt*) STAT5 site sequences. STAT5 binding activity was evaluated by EMSA supershift analysis, as in Fig. 1*B*. *B*, STAT5/2C11-Luc reporter (wild type or double mutant; c.f. *panel A*) was transfected into HepG2 cells in the presence of STAT5 and GH receptor expression plasmids. The cells were treated with 200 ng/ml GH for 18–20 h or were left untreated. Normalized luciferase activity (Firefly/*Renilla* luciferase) was determined and set at 1 for control samples (empty reporter plasmid, *left panel*; no STAT5b, *right panel*) in the absence of GH treatment (relative luciferase activity). *Right panel*, cells were transfected with 2x-STAT5/2C11-Luc reporter plasmid, together with GH receptor and wild-type STAT5b or STAT5b-Y699F. *Asterisk*, $p < 0.05$ compared with the corresponding −GH control.

exhibited significant basal expression in the absence of GH treatment, ranging from 3- to 15-fold higher than the empty pGL3-basic reporter plasmid (Fig. 3). A strong decrease in basal promoter activity was observed with −1208/2C11-Luc compared with −1182/2C11-Luc, indicating the presence of a negative regulatory element, or silencer, between nts −1208 and −1182, supporting a previous report of a silencer between nts −1226 and −1184 (28). Unexpectedly, *2C11* promoter activity was decreased by 50–80% by GH treatment in constructs containing at least 968 nts of promoter sequence (Fig. 3). The GH-dependent inhibition of −968/2C11-Luc transcription demonstrates that the STAT5 site at nts −1150 to −1142 (Fig. 1*A*) is not required for this inhibition. No GH-dependent inhibition of promoter activity was observed in cells transfected with the STAT5b tyrosine phosphorylation site mutant, STAT5b-Y699F (data not shown).

Transcription Activation of 2C11 Promoter by HNF1α and HNF3β—We next investigated whether the inhibitory effect of STAT5b on *2C11* promoter activity can be modulated by co-expression of one or more liver-enriched transcription factors. An initial screen of the effects of the nine liver factors (HNF1α, HNF1β, HNF3α, HNF3β, HNF4, HNF6, C/EBPα, C/EBPβ, and DBP) on basal *2C11* promoter activity revealed a substantial *trans*-activation by HNF1α and HNF3β (Fig. 4, *A* and *B*). HNF6 moderately *trans*-activated *2C11* promoter activity (~2-fold increase), whereas the other factors tested, including HNF1β and HNF3α, had little or no effect (data not shown). Maximal activation of the *2C11* promoter by HNF1α was observed with −1182/2C11-Luc (~40-fold), whereas maximal activation by HNF3β was seen with −1769/2C11-Luc (~12-fold).

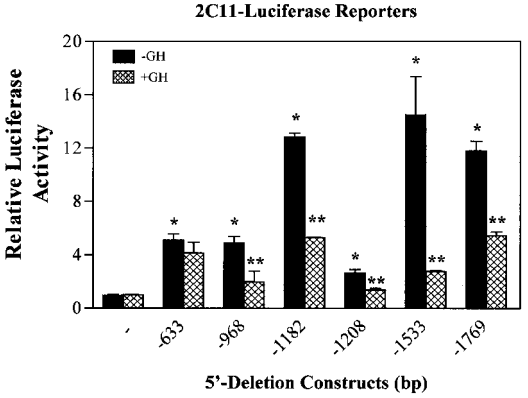

FIG. 3. **Effect of GH-activated STAT5b on 2C11 promoter activity: 5′-deletion series.** HepG2 cells were transfected with the indicated 5′-deleted *2C11* promoter-Luc reporter constructs together with expression plasmids encoding STAT5b and GH receptor, as described under "Materials and Methods." 24 h after transfection, the cells were either treated with 200 ng/ml GH for 18–20 h or were left untreated. Normalized Firefly luciferase activity was determined. The data shown are relative luciferase activities with the activity of empty pGL3-basic plasmid set at 1. *Single asterisk*, $p < 0.05$ compared with empty pGL3-basic; *double asterisk*, $p < 0.05$ compared with the corresponding −GH control.

5′-Deletion analysis revealed that the HNF1α-responsive sequences are primarily localized to two promoter regions, spanning nts −633 to +22 and −968 to −633. *trans*-Activation of the *2C11* promoter by HNF3β was first seen with −968/2C11-Luc (~2-fold) and was further increased, to ~6-fold, with −1182/2C11-Luc. HNF3β-stimulated promoter activity was substantially reduced by inclusion of the silencer element present in −1208/2C11-Luc. This decrease was partially reversed with −1533/2C11-Luc and was fully reversed with −1769/2C11-Luc, the longest construct examined, indicating a strong site of HNF3β *trans*-activation between nts −1533 and −1769. Thus, at least four promoter regions (−1769 to −1533, −1533 to −1208, −1182 to −968, and −968 to −633) contribute to HNF3β *trans*-activation of *CYP2C11*. Two of these regions are also the most responsive to GH-dependent STAT5b inhibition of basal promoter activity (Fig. 3).

We next examined whether HNF1α and HNF3β can cooperatively interact with each other or with STAT5b to regulate *2C11* promoter activity. Co-transfection of HNF3β with HNF1α stimulated transcription of each of the 2C11-Luc constructs additively and in a manner consistent with the activation patterns exhibited by the individual factors (Fig. 4*C* and Fig. 5). Further examination of −1769/2C11-Luc revealed that when STAT5b and GH receptor were additionally present, GH treatment decreased HNF3β-stimulated *2C11* promoter activity but had no effect on HNF1α-stimulated promoter activity (Fig. 5). The addition of HNF1α to the combination of HNF3β and STAT5b reduced but did not eliminate, the extent to which *2C11* promoter activity was inhibited by GH-activated STAT5b (~50% inhibition in the presence of HNF1α *versus* ~85% inhibition in its absence). This suggests that HNF1α may compete with HNF3β to block the GH- and STAT5b-dependent inhibition of *2C11* activity.

STAT5b Inhibition of HNF3β DNA Binding and Transcriptional Activity—The mechanism whereby STAT5b inhibits HNF3β-stimulated *2C11* promoter activity was further characterized by examining the effects of STAT5b on HNF3β DNA binding activity. EMSA analysis using a *2C11* probe that encompasses a consensus HNF3 site (−1162 to −1150) and the immediately adjacent STAT5 site (−1150 to −1142) (HNF3-STAT5 probe; nts −1174 to −1138) revealed two EMSA complexes, designated complex I and complex II, in HepG2 cell

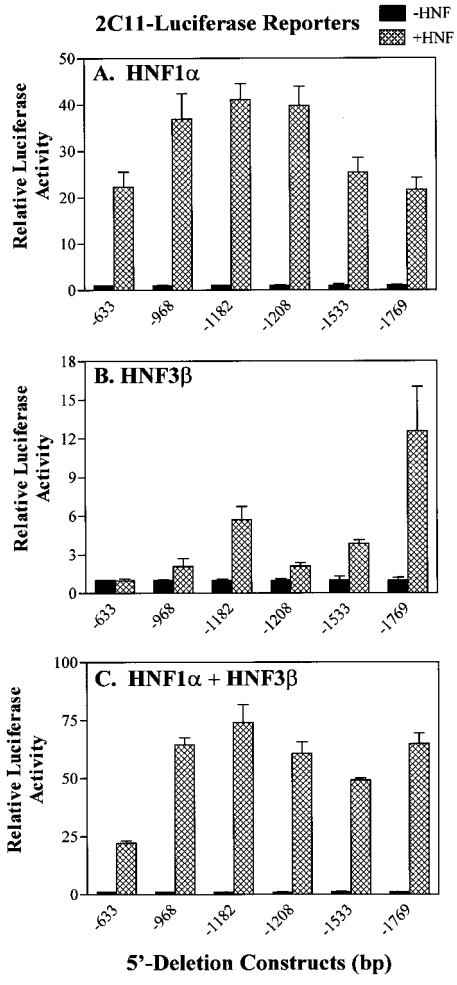

FIG. 4. **Transcriptional activation of the 2C11 promoter by HNF1α and HNF3α.** The indicated 5′-deleted *2C11* promoter constructs were transfected into HepG2 cells together with HNF1α (*A*), HNF3β (*B*), or HNF1α and HNF3β in combination (*C*). Normalized firefly luciferase activity was determined (mean ± S.D., $n = 4$) and is shown relative to the activity of each *2C11*-Luc reporter in the absence of HNF expression plasmid (*i.e.* fold induction).

extracts (Fig. 6*A*, *lane 1*). Both complexes were less intense with COS-1 cell extracts (Fig. 6*B*, *lane 4 versus lane 1*), and both were substantially decreased in intensity by 100-fold molar excess of unlabeled probe (data not shown). Complex I includes HNF3-related protein, as indicated by the partial inhibitory effect of anti-HNF3α and anti-HNF3β antibody on complex formation. This inhibition was accompanied by an increase in mobility of the residual DNA-bound protein complex (Fig. 6, *A*, *lanes 2–4*, and *B*, *lanes 2* and *3*; see quantitation in figure legend). The presence of HNF3 in complex I is supported by the substantial decrease in complex I intensity upon mutation of the core HNF3 binding site (Fig. 6*C*, *lanes 9* and *10 versus lanes 1* and *2*).

Given the substantially lower level of endogenous HNF3-related proteins in COS-1 cells compared with HepG2 cells (Fig. 6*B*), COS-1 cells were used to investigate the effects of GH-activated STAT5b on HNF3β binding to the *2C11* promoter probe. COS-1 cells were transfected with HNF3β, with STAT5b and GH receptor, or with all three factors in combination. Transfection of HNF3β (verified by Western blotting; see Fig. 8*B*) did not increase the intensity of EMSA complex I in COS-1 cell extracts (data not shown). However, upon transfection with STAT5b and stimulation with GH, the HNF3-containing complex I was partially replaced by a STAT5b-containing DNA

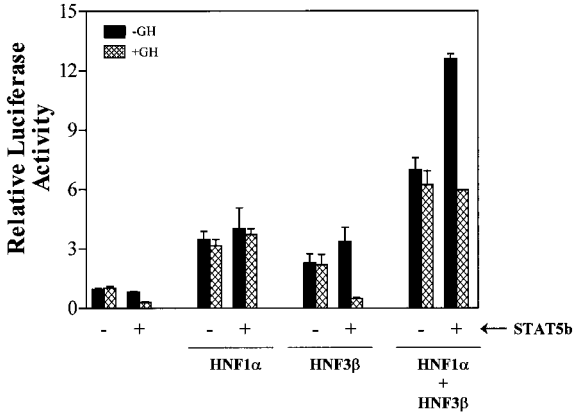

FIG. 5. **Effect of HNF1α and HNF3β on GH- and STAT5-dependent inhibition of 2C11 promoter activity.** The reporter −1769/2C11-Luc was transfected into HepG2 cells together with a single expression plasmid (STAT5b, HNF1α, or HNF3β), two expression plasmids (STAT5b with HNF1α or HNF3β; HNF3β with HNF1α; HNF3β with HNF1α), or three expression plasmids (STAT5b, HNF1α, and HNF3β), all in the presence of GH receptor expression plasmid. Normalized luciferase activity was determined, and the activity of the reporter in the absence of STAT5b or HNF factor was set at 1. The extent to which HNF1α blocked the inhibitory action of STAT5b on HNF3β-stimulated reporter activity varied with the ratio of HNF1α to HNF3β and STAT5b (data not shown). The lower fold activation by HNF1α and HNF3β shown in this figure compared with Fig. 4 reflects the elevated basal luciferase reporter activity seen in cells transfected with the higher total amount of DNA required for this experiment (1 μg/well of a 24-well plate; total DNA normalized with sonicated salmon sperm DNA).

complex of similar mobility (supershiftable with STAT5b antibody; Fig. 6*C*, *lane 5 versus lane 4*). This effect of STAT5b was more apparent in experiments using the HNF3 site-mutated EMSA probe (*lanes 12* and *13 versus lane 11*). In control experiments, STAT5b antibody had no effect on complex I in the absence of STAT5b transfection (data not shown). Interestingly, co-transfection of STAT5b with HNF3β inhibited formation of both the STAT5-DNA complex and the HNF3-DNA complex in a GH-dependent manner (Fig. 6*C*, *lane 8 versus lane 7*, and data not shown). This suggests that neither factor binds efficiently to the *2C11* HNF3-STAT5 probe when HNF3β and GH-activated STAT5b are present simultaneously.

Because the STAT5 and HNF3 consensus binding sites are immediately adjacent on the *2C11* EMSA probe, with one overlapping nucleotide, the reduced HNF3-DNA binding activity seen in the presence of GH-activated STAT5b could, in principle, result from steric hindrance between STAT5b and HNF3β for binding to their respective sites. However, mutation of the STAT5 site, although leading to the expected loss of supershiftable STAT5b binding seen on the wild-type STAT5 site probes (Fig. 6*C*, *lane 21 versus lanes 5* and *13*), did not restore HNF3β DNA binding activity in cells co-transfected with STAT5b (*lane 24*). This suggests that STAT5b and HNF3β interact in an inhibitory manner that is unrelated to their binding to adjacent sites on the *2C11* promoter.

To test this hypothesis, we investigated whether GH-activated STAT5b inhibits HNF3β transcriptional activity when assayed using a reporter construct that does not contain STAT5 binding sites. HepG2 cells were transfected with HNF3β and a luciferase reporter driven by six copies of an isolated HNF3 binding site, derived from the *Cdx*-2 gene (34). This reporter is specifically *trans*-activated by HNF3α and HNF3β but not by HNF6 (Fig. 7*A*), which can bind to a subset of promoter sequences in common with HNF3β (35). Moreover, STAT5b inhibited HNF3β-stimulated transcription of the reporter in a GH and dose-dependent manner (Fig. 7*B*), despite the absence of STAT5 binding sites. Control experiments ver-

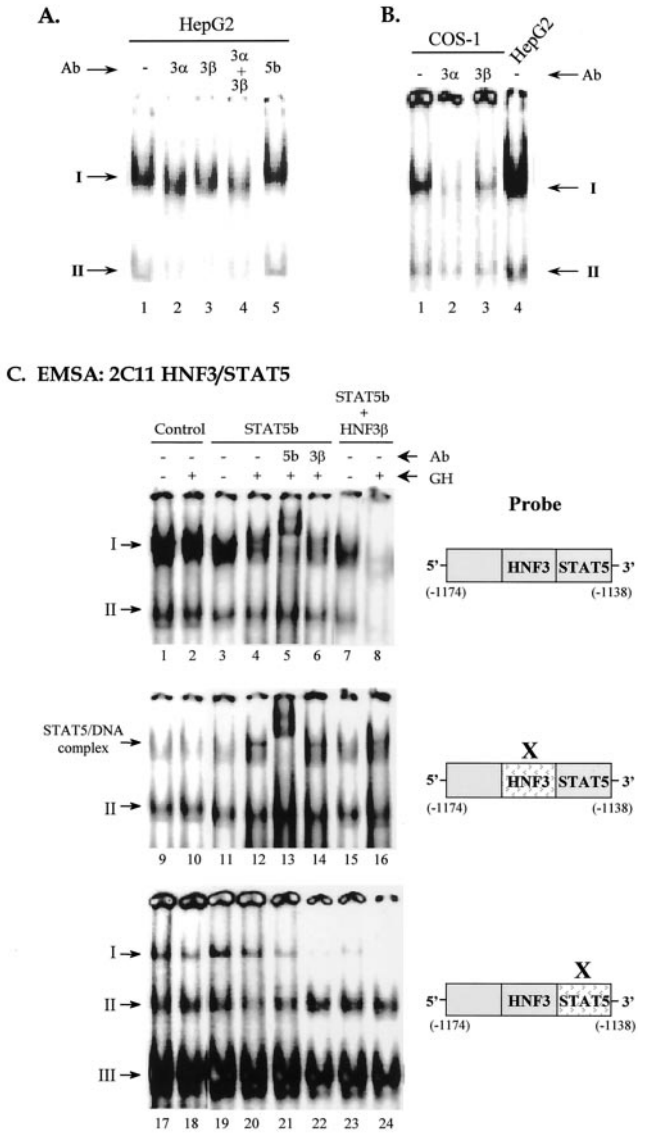

FIG. 6. **EMSA analysis of HNF3 and STAT5 DNA binding activities using 2C11 HNF3-STAT5 probe.** Expression plasmids (200 ng) encoding STAT5b or HNF3β (as indicated) were transfected into HepG2 cells (*A*) or COS-1 cells (*B* and *C*) grown in 35-mm culture dishes. 30–36 h later, the cells were treated with GH for 30 min as indicated. The cell extracts were analyzed by EMSA using the *2C11* HNF3-STAT5 probe (*A* and *B* and *lanes 1–8* of *C*) or the *2C11* HNF3-STAT5 probe containing mutations in the HNF3 site (*panel C*, *lanes 9–16*) or the STAT5 site (*panel C*, *lanes 17–24*). Where indicated, the samples were incubated with antibody (*Ab*) to HNF3 proteins (3α or 3β, as indicated) or STAT5b (5b) prior to EMSA analysis. *A* and *B*, partial inhibition of the HNF3-containing EMSA complex I by anti-HNF3 antibodies (42, 50 and 63% inhibition, respectively, in HepG2 cells the presence of HNF3α, HNF3β, and HNF3α/3β antibodies). *C*, the STAT5-dependent EMSA complex co-migrates with the HNF3-containing complex I and is more easily detected when the HNF3 site is mutated (*lane 12 versus lane 4*). Mutation of the STAT5 site (*lanes 17–24*) significantly decreased the HNF3-containing complex I while increasing the intensity of complex II and revealing a new complex, complex III.

ified the dose-dependent expression of STAT5b protein (Fig. 7*C*) and DNA binding activity (Fig. 7*D*). Together, these findings suggest that the GH- and STAT5b-dependent inhibition of HNF3β-stimulated *2C11* promoter activity (Fig. 5) results from a loss of HNF3β DNA binding activity and, consequently, a loss of HNF3β-stimulated *2C11* transcription. Similarly, the inhibition by GH-activated STAT5b of basal *2C11* promoter activity (Fig. 3) is suggested to reflect inhibition by STAT5b of the endogenous HNF3β present in the HepG2 cells used in those studies (Fig. 6*A*).

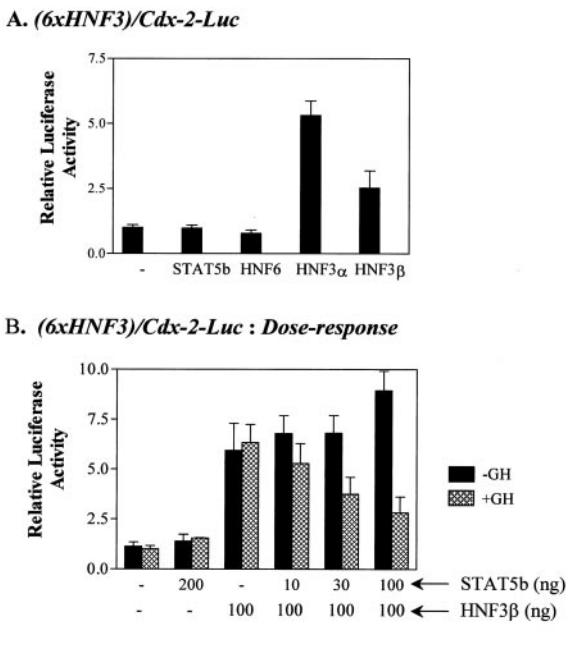

FIG. 7. **Inhibitory effect of GH-activated STAT5b on HNF3β-stimulated (6xHNF3)/Cdx-2-Luc reporter activity.** HepG2 cells were transfected with (6xHNF3)/*Cdx-2*-Luc reporter plasmid in the presence of the indicated expression plasmids. The data shown are relative luciferase activities compared with samples in the absence of STAT5b or HNF factors. *A*, *trans*-activation of (6xHNF3)/*Cdx*-2-Luc by HNF3α and HNF3β but not by STAT5b or HNF6. *B*, Dose-dependent inhibition of (6xHNF3)/*Cdx*-2-Luc activity in the presence of increasing amounts of STAT5b expression plasmid (10–100 ng) together with a fixed amount of HNF3β and GH receptor expression plasmid (100 ng each). GH treatment was for 30 min. *C*, plasmid dose dependence of STAT5b protein expression in HepG2 cells co-transfected with GH receptor and 300 ng of HNF3β expression plasmid and stimulated with GH for 30 min. Shown is a STAT5b Western blot (*top panel*) with quantitation of STAT5b band intensities (*bottom panel*; mean ± range, $n = 2$). STAT5b and its phosphorylated forms were not resolved on the Western blot shown. *D*, the same cell extracts shown in *C* were assayed for STAT5 EMSA activity using a β-casein STAT5 probe. *Cont*, control.

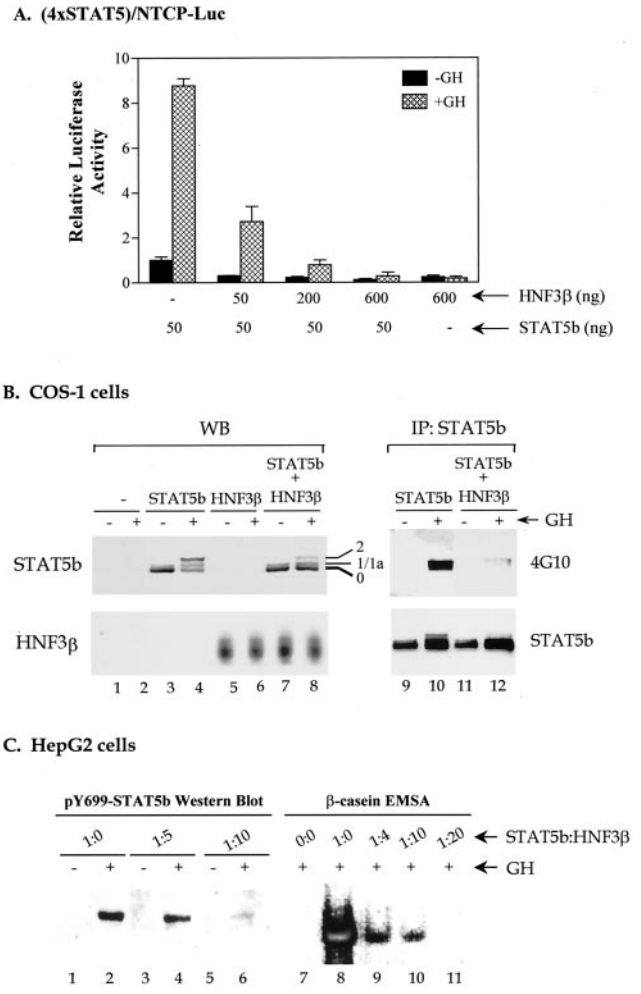

FIG. 8. **HNF3β inhibition of STAT5b activation and transcriptional activity.** *A*, HepG2 cells were transfected with the STAT5 *ntcp* reporter 4x-pT109-Luc in the presence of STAT5b and GH receptor and increasing amounts of HNF3β expression plasmid. The cells were stimulated with GH overnight. Relative luciferase activity is presented with the unstimulated activity in the absence of HNF3β set at 1. *B*, COS-1 cells were transfected with STAT5b, HNF3β, or STAT5b in combination with HNF3β (1:1 plasmid weight ratio; 200 ng of each plasmid) together with GH receptor expression plasmid. The cells were treated for 30 min with 200 ng/ml GH beginning 36 h after transfection in serum-free Dulbecco's modified essential medium. The cell extracts were analyzed directly on Western blots (*lanes 1–8*) or were immunoprecipitated with anti-STAT5b antibody (*lanes 9–12*). The blots were probed sequentially with anti-STAT5b and anti-HNF3β antibodies (*left panel*) or with anti-phosphotyrosine (4G10) and anti-STAT5b antibodies (*right panel*), as indicated. The differentially phosphorylated STAT5b protein bands are partially resolved (*band 0*, unphosphorylated; *bands 1* and *1a*, STAT5b phosphorylated on tyrosine and serine, respectively; *band 2*, STAT5b phosphorylation on both tyrosine and serine (49)). *C*, cell extracts from HepG2 cells transfected with STAT5b and HNF3β at the indicated plasmid weight ratios were analyzed by anti-Tyr(P)^{699}-STAT5b Western blotting (*lanes 1–6*) or by EMSA using a STAT5 *β-casein* probe (*lanes 7–11*). STAT5b plasmid was fixed at 50 ng for the Western blot samples and 20 ng for the EMSA samples.

HNF3β Inhibits STAT5b Transcriptional Activity by Blocking STAT5b Tyrosine Phosphorylation—We next investigated whether the inhibitory interactions between HNF3β and STAT5b are mutual, as judged by the effects of HNF3β on GH-activated STAT5b transcriptional activity. STAT5b activity was assayed in HepG2 cells co-transfected with GH receptor and a STAT5 reporter containing four tandem copies of a natural STAT5 site derived from the GH-responsive *ntcp* gene (36). As shown in Fig. 8*A*, GH stimulated a 9-fold increase in STAT5b-dependent *ntcp* reporter activity. Moreover, HNF3β

inhibited this STAT5b-stimulated transcriptional response in a dose-dependent manner, despite the absence of HNF3 binding sites in the *ntcp* reporter. In control experiments, transfection of another liver transcription factor, HNF1α, had no effect on STAT5b reporter activity (data not shown).

To ascertain the mechanism for this inhibitory effect of HNF3β, we investigated whether HNF3β interferes with GH-stimulated STAT5b activation. Fig. 8*B* shows that HNF3β significantly inhibited STAT5b activation, as demonstrated in transfected COS-1 cells by the decreased conversion of unphosphorylated STAT5b (*band 0*, *lane 4*) to the lower mobility, tyrosine-phosphorylated form of STAT5b (*band 2*; compare *lane 8 versus lane 4*). Moreover, a large decrease in STAT5b tyrosine phosphorylation was revealed by anti-phosphotyrosine 4G10 Western blotting (*lane 12 versus lane 10*). HNF3β inhibition of STAT5b tyrosine phosphorylation was also observed in HepG2 cells, as shown by Western blotting using anti-Tyr(P)^{699}-STAT5 antibody (Fig. 8*C*, *lane 2 versus lanes 4* and *6*). This inhibition resulted in a dose-dependent decrease in STAT5 DNA binding activity, assayed by EMSA using a β-casein probe (*lane 8 versus lanes 9–11*). HNF3β thus inhibits STAT5b-dependent transcription by a mechanism that targets the initial, GH receptor-dependent STAT5b tyrosine phosphorylation step.

DISCUSSION

The present study investigates the role of STAT5b and of liver-enriched transcription factors in regulating the GH-dependent and liver-specific expression of *CYP2C11*. STAT5 binding sites were identified in three male-specific liver *CYP* promoters, and in the case of *2C11*, the isolated binding site was shown to confer GH-inducible, STAT5b-dependent reporter activity when fused to a heterologous promoter. These findings support the role of STAT5b in maintaining the male-specific profile of liver gene expression that was proposed earlier, based on the repeated tyrosine phosphorylation and nuclear translocation of liver STAT5b in direct response to each male plasma GH pulse (13, 15, 16) and on the selective loss of male-specific *CYP* expression in STAT5b-deficient mice (19–21). Analysis of the STAT5b responsiveness of the intact *2C11* promoter revealed, however, a GH- and STAT5b-dependent decrease in promoter activity (Fig. 3). This unexpected effect of STAT5b was shown to involve its mutually inhibitory cross-talk with the liver transcription factor HNF3β, which, together with HNF1α, can strongly *trans*-activate *2C11* promoter activity (Fig. 4). These findings highlight the complex interrelationship between STAT5b and liver-enriched transcription factors that contribute to the transcriptional activity of GH-regulated liver *CYP* genes. Conceivably, the inhibitory action of STAT5b on HNF3β-stimulated *2C11* transcription could contribute to the silencing of the *2C11* gene in female rat liver, where STAT5b is activated in a nearly continuous manner, albeit at a low level (14).

Several liver-enriched transcription factors participate in a complex cross-regulatory network with STAT5b (30). HNF4, acting in concert with STAT5b, activates the *HNF6* gene, whereas HNF6, in turn, stimulates expression of *HNF3β* and *HNF4*. This regulatory network contributes to the expression of GH-inducible liver *CYP* genes, with HNF3β and GH-regulated HNF6 activating the female-specific *CYP2C12* by binding to distinct promoter sites (23, 25), and HNF3β and HNF1α, but not HNF6, strongly *trans*-activating *CYP2C11* (Fig. 4). Our finding that STAT5b can inhibit HNF3β-inducible *2C11* expression raises the question of whether GH pulse-activated STAT5b might repress *2C11* transcription, such that the inactivation of STAT5b at the conclusion of each GH pulse (15, 16) could serve as a stimulus to de-repress and thereby activate the *2C11* gene. This possibility is not likely, however, given the positive regulatory role of STAT5b evidenced by the loss of male-specific liver *CYP* expression in STAT5b-deficient mice (19). An alternative hypothesis, consistent with a positive regulatory role of STAT5b, is that the high concentrations of active, nuclear STAT5b found in male rat hepatocytes early during a plasma GH pulse directly stimulate *2C11* expression. By contrast, in females, the persistence of a low level of nuclear STAT5b may serve as a negative regulatory signal by counteracting the *2C11* gene activation potential of HNF3β. According to this model, nuclear STAT5b would reach the threshold level required to *trans*-activate *2C11* in male but not female liver. This activation may synergize with the action of HNF1α, which *trans*-activates the *2C11* promoter (Fig. 4*A*) and can partially reverse the inhibitory effects of STAT5b on HNF3β-stimulated *2C11* transcription (Fig. 5). Of note, in the present studies of the *2C11* promoter, HepG2 cells were treated with GH continuously, a treatment that mimics the female plasma GH pattern. Efforts to stimulate a pulsatile pattern of STAT5b activation in HepG2 cells were hampered by the slow deactivation of STAT5b in this cell line. This precluded a determination of whether the *2C11* promoter can be stimulated by STAT5b when the STAT is activated in a pulsatile manner, as occurs in male rat liver *in vivo*.

HNF3β and STAT5b were shown to exhibit mutual inhibitory cross-talk, as revealed by our studies on the interactions of these two factors on a *2C11* promoter fragment that contains immediately adjacent HNF3β and STAT5 binding sites. Further investigation revealed, however, that direct DNA binding is not required for this mutual inhibition. The inhibitory cross-talk between HNF3β and STAT5b could conceivably involve direct protein interactions between the two transcription factors; however, no such interaction was detectable in co-immunoprecipitation experiments.[2] In agreement with this finding, the inhibition of STAT5b transcriptional activity by HNF3β was shown to involve a novel mechanism whereby HNF3β blocks STAT5b activation at the level of STAT5b tyrosine phosphorylation rather than by inhibiting STAT5b DNA binding through direct protein-protein interactions. Possible mechanisms for this intriguing effect of HNF3β on STAT5b activation include HNF3β-inducible expression of a negative regulator of GH receptor/tyrosine kinase JAK2 signaling, such as a cytosolic phosphotyrosine phosphatase (37) or a SOCS/CIS protein, several of which can strongly inhibit GH receptor-dependent signaling to STAT5b (38, 39).

The present finding that STAT5b can inhibit HNF3β transcriptional activity in the absence of a STAT5 DNA-binding site (Fig. 7) helps explain our earlier finding that STAT5b inhibits HNF3β- and HNF6-stimulated *CYP2C12* transcription even in promoter constructs devoid of recognizable STAT5 sites (23). The mechanism for this inhibitory effect of STAT5b is unknown. Inhibitory effects of STAT5b have been observed with several other transcription factors, including the nuclear receptor PPARα, where inhibition is mediated by the N-terminal AF1 transcriptional domain of the nuclear receptor (40, 41), and the ubiquitous factor NFκB, which is inhibited by STAT5b by competition for the limiting co-activator p300/CBP (42). Interestingly, an inhibitory NFκB site located immediately downstream of the TATAA box of *2C11* (−2 to +8) mediates down-regulation of *2C11* promoter activity in cells stimulated with interleukin-1 (43); however, it is not known whether GH-activated STAT5b is able to counteract that inhibition and thereby stimulate *2C11* expression.

[2] S.-H. Park and D. J. Waxman, unpublished results.

STAT5b regulates target gene expression by transcriptionally activating promoters containing γ-interferon-activated sequences matching the consensus sequence TTC-N_3-GAA. Promoters containing adjacent γ-interferon-activated sequence-like motifs have been shown to bind two STAT5 dimers that interact through their N-terminal region to form a tetrameric STAT5 complex (44, 45). STAT5 tetramerization may confer functional cooperativity between adjacent STAT5 binding sites by increasing the level of occupancy of both sites above a threshold level required for efficient enhancer activity. In the case of *2C11*, the STAT5 site that we characterized (TTC-$(N)_3$-GAA; −1150 to −1142) is flanked by two generic STAT sites (TT-$(N)_5$-AA, at nts −1169 to −1161 and at −1132 to −1124), with a 9–10-nt spacing between the central STAT5 site and each of the adjacent STAT sites. HNF3 consensus sequences at nts −1162 to −1150 and at nts −1137 and −1126 partially overlap the generic STAT sites and separate them from the central STAT5 site. Although the inhibitory cross-talk between HNF3β and STAT5b does not require factor DNA-binding sites, as noted above, the close spacing, indeed the overlap of the HNF3β and STAT5 sites in the case of *2C11*, could nevertheless serve to enhance the inhibitory cross-talk by increasing competition for STAT5b DNA binding. Conceivably, high levels of active STAT5b (such as are present in a GH pulse-stimulated male liver) may be required to overcome the inhibitory action of HNF3β, leading to STAT5b activation of *2C11* gene expression. This activation could be mediated by the STAT5 site at nts −1150 to −1142 identified in the present study, perhaps in concert with the adjacent generic STAT sites. Uncharacterized STAT5 sites elsewhere in the *2C11* promoter or elsewhere in the *2C11* gene might also be involved. STAT5b is known to bind cryptic STAT5 response elements that occur as adjacent pairs but do not match the established TTC-N_3-GAA STAT5 consensus sequence (46).

Although STAT5b clearly plays an essential role in GH-dependent expression of male-expressed *CYPs* and certain other liver gene products, as demonstrated in the mouse knockout studies noted above (19, 20), additional factors are likely to be required to achieve the male-specific pattern of liver *CYP* gene expression. This conclusion is supported by the rapid activation of liver STAT5 (within 10–15 min) in hypophysectomized rats given a single injection of GH (13, 33), in contrast to the repeated pulsatile GH stimulation (over at least 2–3 days) that is required to restore male-specific liver *2C11* gene expression in the same animal model (8, 47). A further indication of the requirement for additional factors is the relatively modest stimulatory effect observed with the *2C11* STAT5 response element in the present study and that of the hamster *CYP3A10* promoter in an earlier report (48). Moreover, precocious activation of STAT5b in prepubertal rats administered exogenous pulses of GH for 7 days is not sufficient to activate *2C11* gene expression, pointing to a requirement for additional liver factors that are absent in prepubertal rats (15). Further study is required to identify these factors and to establish the molecular details and underlying mechanisms whereby GH and its sexually dimorphic secretory patterns induce the sex-dependent expression of *2C11* and other liver *CYP* genes.

Acknowledgments—We thank Drs. A. Mui, H. Rui, N. Billestrup, F. Gonzalez, F. Lemaigre, G. Rousseau, E. Lai, M. Vore, L. Hennighausen, and R. Costa for providing plasmid DNAs and antibodies.

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