

# STAT5b-deficient Mice Are Growth Hormone Pulse-resistant

## ROLE OF STAT5b IN SEX-SPECIFIC LIVER P450 EXPRESSION\*

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**The signal transducer and transcriptional activator STAT5b is required to maintain the adult male pattern of liver gene expression and whole body pubertal growth rates, as demonstrated by the loss of these growth hormone (GH) pulse-dependent responses in mice with a targeted disruption of the STAT5b gene. The present study investigates whether these phenotypes of STAT5b-deficient mice result from impaired intracellular GH signaling associated with a loss of GH pulse responsiveness, as contrasted with a feminization of the pituitary GH secretory profile leading to the observed feminization of body growth and liver gene expression. Pulsatile GH replacement in hypophysectomized mice stimulated body weight gain in wild-type but not in STAT5b-deficient mice. Expression of the male-specific liver P450 enzyme CYP2D9, which is reduced to female levels in hypophysectomized male mice, was restored to male levels by GH pulse replacement in wild-type but not in STAT5b-deficient mice. Similarly, a female-specific liver CYP2B P450 enzyme that was up-regulated to female levels following hypophysectomy of males was suppressed to normal basal male levels by GH pulses only in wild-type hypophysectomized mice. Finally, urinary excretion of the male-specific, GH pulse-induced major urinary protein was restored to normal male levels following pulsatile GH treatment only in the case of wild-type hypophysectomized mice. STAT5b-deficient mice are thus GH pulse-resistant, supporting the proposed role of STAT5b as a key intracellular mediator of the stimulatory effects of plasma GH pulses on the male pattern of liver gene expression.**

Growth hormone (GH)<sup>1</sup> has diverse effects on metabolism and growth that can result from the direct effects of GH on gene expression, or may be indirectly mediated by factors such as insulin-like growth factor I (1). In addition, the temporal patterns of pituitary GH secretion differ in males and females in

many species, resulting in the expression of some genes predominantly in males and other genes predominantly in females (2–4). In male rats, plasma GH pulses are separated by periods of >2 h during which there is negligible GH detectable in blood. In contrast, the female plasma GH pattern is characterized by a more continuous presence of GH or, in some species such as mice, by more frequent pulses of GH (3, 5).

GH binds to cell surface receptors that dimerize upon hormone binding and subsequently activate the receptor-associated tyrosine kinase JAK2. JAK2 in turn phosphorylates itself and the GH receptor on multiple tyrosine residues. STAT proteins are then recruited to the GH receptor/JAK2 complex and are phosphorylated on tyrosine and subsequently on serine. STAT proteins activated in this manner form homo- or heterodimers and translocate to the nucleus where they bind to target sites in GH-responsive genes (6, 7). STAT5 proteins in the liver are intermittently, and repeatedly, activated in response to GH pulses (8, 9). GH also activates liver STAT1 and STAT3, but the activation of these STATs is largely independent of the temporal pattern of GH in blood (10).

STAT5a and STAT5b are encoded by different genes; however, they share >90% amino acid identity. While initially referred to as mammary gland factor because of the role in mediating the effects of prolactin on the expression of  $\beta$ -casein in the mammary gland (11, 12), mammary gland factor was renamed STAT5 when the cDNA was cloned and shown to be a member of the STAT family of transcription factors (13). Subsequently, two separate genes were identified and shown to be expressed in a wide range of tissues (14–17). STAT5a and STAT5b are now known to be differentially expressed in various tissues, and to have independent and distinct as well as common functions (18–20). STAT5b accounts for ~90% of the STAT5 in the liver; however, STAT5b and STAT5a are both required for the constitutive expression of certain GH-regulated liver cytochrome P450 enzymes (20).

The involvement of STAT5b in GH-pulse responsive liver gene expression was recognized from studies in which liver STAT5b tyrosine phosphorylation, nuclear localization, and DNA binding were temporally correlated with plasma GH pulses and with the pattern of male-specific liver gene expression (8). Further confirmation came from the finding that certain GH pulse-regulated, male-specific liver P450 genes contain STAT5 response elements (21), from the recent demonstration that STAT5b nuclear localization correlates with the gender-specific expression of P450 genes in wild-type and estrogen receptor- $\alpha$ -deficient mice (22), and from our analysis of STAT5b-deficient mice, which displayed loss of male-specific pubertal body growth and male-specific liver gene expression (23). However, it is unclear from these studies whether GH target tissues such as liver become unresponsive to pulsatile GH as a direct consequence of the loss of STAT5b,

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<sup>1</sup> The abbreviations used are: GH, growth hormone; STAT, signal transducer and activator of transcription; MUP, major urinary proteins; EMSA, electrophoretic mobility shift assay; CYP, cytochrome P450; T<sub>4</sub>, thyroxine; BSA, bovine serum albumin; JAK2, Janus tyrosine kinase 2.

or whether an alteration in the pattern of pituitary GH secretion in these animals leads to the observed phenotypes.

In the present study, we address this question by directly examining the effects of exogenous GH pulses given to mice where the major source of endogenous GH has been eliminated by hypophysectomy. The effects of pulsatile GH replacement on body weight gain and on the gender-specific expression of genes in the liver were analyzed. Pulsatile GH replacement in wild-type but not STAT5b-deficient mice is shown to restore expression of genes normally expressed in intact male mice. Additionally, GH pulse replacement is shown to increase overall body weight gain in wild-type but not in STAT5b-deficient mice. These data strongly support our proposal that the STAT5b pathway directly mediates physiological signaling in hepatocytes in response to pulsatile GH stimulation.

#### EXPERIMENTAL PROCEDURES

**Animals**—Male outcrossed 129 × BALB/c wild-type and STAT5b gene-disrupted mice (23) were hypophysectomized at 4–17 weeks of age and maintained on a 12-h light, 12-h dark schedule with free access to food and drinking water supplemented with 5% glucose. After a recovery period of at least 7.5 weeks, the mice were given pulsatile GH replacement by injection. GH (2 µg/g body weight) was injected intraperitoneally at 12-h intervals for 7 days, either alone or combined with T<sub>4</sub> (0.2 µg/ml added to the 5% glucose/drinking water to give an approximate dose of 20 ng of T<sub>4</sub>/g of body weight/day). This hormone replacement regimen has been shown to re-establish male-specific patterns of liver P450 gene expression in GH-deficient mice (24). Administration of T<sub>4</sub> in combination with GH is required for expression of MUP proteins (25). Urine was collected and body weights were monitored during the hormone replacement period. The mice were maintained without hormone treatment for another 3–6 weeks, at which time they were killed to collect untreated hypophysectomized livers, or were given GH pulse replacement without T<sub>4</sub> as described above. Mice were killed by CO<sub>2</sub> asphyxiation, and the tissues were snap-frozen in liquid nitrogen and stored at –80 °C until use. Animal procedures were approved by the Ruakura Animal Ethics Committee operating under the guidelines of the New Zealand National Animal Ethics Advisory Committee.

**Hypophysectomy**—Mice were anesthetized using a mixture of ketamine and rompun (intraperitoneal injection of 120 µg of ketamine hydrochloride (Bristol-Meyers Co.) and 12 µg of xylozine hydrochloride (Rompun, Bayer NZ Ltd., Auckland, NZ) per g of body weight) and hypophysectomized by the parapharyngeal route (26). Successful hypophysectomy was verified by monitoring body weights and major urinary proteins in urine, and by post mortem inspection of the base of the skull.

**MUP Analysis**—Urine was collected from mice before and after hypophysectomy and following hormone replacement. Samples (0.5 µl) were run on 12% SDS-polyacrylamide gels, which were stained with Coomassie Blue. MUP protein (major ~20-kDa protein band) was quantified by densitometry using a Molecular Dynamics densitometer. Samples that contained high levels of MUP were diluted as required to enable quantification.

**Preparation of Mouse Liver Homogenates and Cytosolic and Microsomal Proteins**—A total tissue homogenate was prepared by homogenizing liver (~1 g) in 10 ml of homogenizing buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 250 mM sucrose, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 100 µM phenylmethanesulfonyl fluoride) and centrifuging at 9,000 rpm for 15 min. Microsomal pellets were separated from the cytosolic supernatant by ultracentrifugation (100,000 × g for 1 h), suspended in 0.1 M KP<sub>i</sub> buffer, 0.1 mM EDTA, 20% glycerol, pH 7.4, and stored at –80 °C until use. Cytosolic and total tissue homogenate protein concentrations were determined using the Bio-Rad DC detergent protein assay kit with bovine serum albumin as a standard. Microsomal protein concentrations were determined using the Bradford assay kit (Sigma).

**Antibodies**—Mouse monoclonal anti-STAT1 (S21120) and anti-STAT3 (S21320) antibodies were purchased from Transduction Laboratories (Lexington, KY). Rabbit polyclonal anti-STAT5a (sc-1081) and anti-STAT5b (sc-835) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). These STAT5 antibodies were shown to be essentially specific for STAT5a and STAT5b, respectively, under our Western blotting conditions (27). Rabbit polyclonal anti-rat CYP2B1 (28) and rabbit polyclonal anti-mouse CYP2D9 antibody, obtained from Dr. M. Negishi (NIEHS, National Institutes of Health, Research Triangle Park, NC)

(29), were used for the Western blot analysis of mouse microsomal CYP2B and CYP2D proteins, respectively.

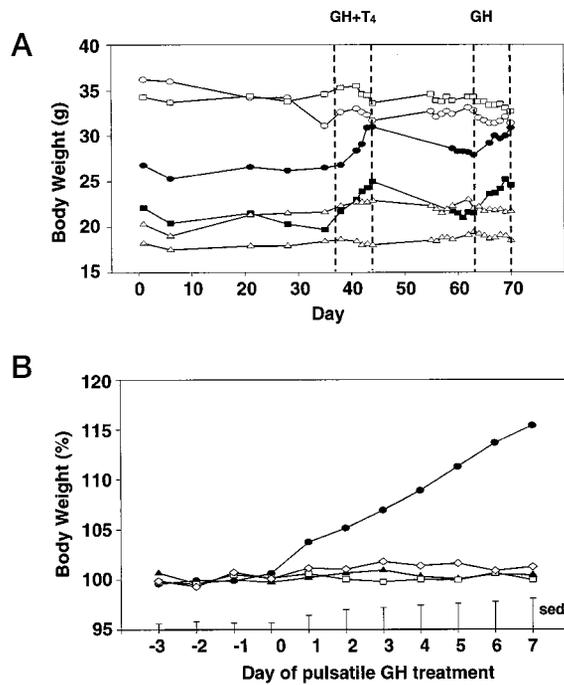
**Western Blotting**—Liver cytosolic proteins (40 µg) or microsomal proteins (20 µg) were electrophoresed through standard Laemmli SDS-polyacrylamide gels (10% gels for STAT proteins; 8% gels for mouse microsomal CYP proteins), transferred to nitrocellulose membranes and then probed with anti-STAT or anti-CYP antibodies. Membranes were blocked for 1 h at 37 °C with 3% nonfat dry milk and 1% BSA in a high Tween buffer (0.3% Tween 20 in phosphate-buffered saline) for anti-STAT1 and anti-STAT3, or with 2% Blotto and 2% BSA in TST buffer (10 mM Tris-HCl, pH 7.5, 0.1% Tween 20, 50 mM NaCl) for probing with anti-STAT5a and anti-STAT5b. For microsomal CYP Western blotting, membranes were blocked for 2 h at 37 °C with 3% nonfat dry milk and 1% BSA in TST (10 mM Tris-HCl, pH 7.5, 0.1 M NaCl, and 0.1% Tween 20). Primary antibodies were diluted 1/3000 (STAT antibodies) or 1/5000 (CYP antibodies) and incubated with the membranes for 1 h at 37 °C. Antibody binding was detected by enhanced chemiluminescence using the ECL kit from Amersham Corp. Bands visualized on x-ray film were scanned using a Cannon IX-4015 scanner and Ofoto scanning software. Nitrocellulose membranes were stripped for 20 min at 50 °C (62.5 mM Tris-HCl, pH 7.6, 2% SDS, 50 mM 2-mercaptoethanol) before reprobing.

**EMSA Analysis**—Total liver homogenate protein (15 µg) 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 10 fmol of DNA probe, 2 µg of poly(dI-dC) (Roche Molecular Biochemicals), 5% glycerol, 1.25 mM MgCl<sub>2</sub>, 625 µM EDTA, and 625 µM dithiothreitol). A double-stranded oligonucleotide probe containing the STAT5 response element of the rat β-casein promoter (nucleotides –101 to –80, 5'-GGA-CTT-CTT-GGA-ATT-AAG-GGA-3') was end-labeled on one strand with <sup>32</sup>P using T4 kinase. The probe was incubated for 20 min at room temperature and 10 min on ice, to stabilize the STAT5-DNA gel-shift complex (9), then added to the protein-buffer mix. For supershift analysis, an additional 10-min incubation in the presence of STAT antibodies was carried out after the addition of the labeled DNA probe. Antibodies used were anti-STAT5a (sc-1081x) and anti-STAT5b (sc-835) from Santa-Cruz. Following 30 min of pre-electrophoresis, the samples were electrophoresed through non-denaturing polyacrylamide gels (5.5% acrylamide, 0.07% bisacrylamide) (National Diagnostics, Atlanta, GA) in 0.5× TBE (44.5 mM Trizma base, 44.5 mM boric acid, 5 mM EDTA) for 20 min in the cold room at 120 V, then at room temperature. DNA-protein complexes were visualized by PhosphorImager analysis.

#### RESULTS

**Pulsatile GH Replacement Stimulates Growth of Hypophysectomized Wild-type but not hypophysectomized STAT5b-deficient Mice**—Wild-type and STAT5b-deficient male mice were hypophysectomized to eliminate endogenous pituitary GH. Successful hypophysectomy was demonstrated by the cessation of body weight gain (Fig. 1) and by the loss of MUP protein excretion in urine (see below), and was verified by post mortem inspection of the base of the skull and by the substantial decrease in serum prolactin at sacrifice (data not shown). In wild-type mice, resumption of growth, evidenced by body weight gain, was stimulated by hormone replacement therapy in the form of pulsatile GH combined with T<sub>4</sub> treatment, or alternatively, pulsatile GH alone (Fig. 1, A, closed symbols, and B). In STAT5b-deficient mice, however, GH did not stimulate body weight gain (Fig. 1).

**MUP Excretion Is Reduced following Hypophysectomy and Restored following GH Pulse Replacement in Wild-type but Not in STAT5b-deficient Mice**—MUPs, which are synthesized in the liver in response to GH and other hormonal factors (25), are excreted in urine at approximately 3-fold higher levels in male than female mice (30). MUP excretion in wild-type male mice was markedly reduced following hypophysectomy (Fig. 2), in agreement with earlier reports in hypophysectomized or GH-deficient mice (25, 30, 31). STAT5b-deficient males excreted low levels of MUPs, and these were further reduced following hypophysectomy. Pulsatile GH replacement in combination with T<sub>4</sub> in wild-type hypophysectomized mice increased MUP protein excretion to the original levels of intact male mice. By

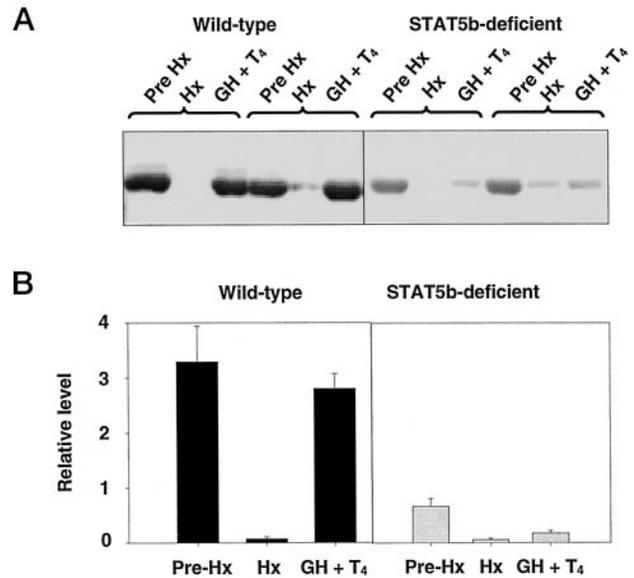


**FIG. 1. Effect of GH pulses on body weight gain in hypophysectomized male wild-type and STAT5b-deficient mice.** *A*, shown are body weights of representative individual wild-type (solid symbols) and STAT5b-deficient (open symbols) male mice following hypophysectomy, and during two separate 7-day periods of hormone replacement (pulsatile GH + T<sub>4</sub> from days 37–44 and pulsatile GH alone from days 63–70). Day 0 corresponds to ~3 weeks after hypophysectomy. *B*, changes in body weights during a 7-day period when hypophysectomized male mice were either untreated or were treated twice daily with pulses of GH pulses as described under “Experimental Procedures” (days 0–7). The averages and maximum standard errors of the differences between the means (*sed*), are shown for GH-treated wild-type (●; *n* = 10), untreated wild-type (▲; *n* = 7), GH-treated STAT5b-deficient (□; *n* = 9), and untreated STAT5b-deficient (◇; *n* = 6) male mice.

contrast, this same hormonal regimen had essentially no effect in STAT5b-deficient mice (Fig. 2*B*).

**Expression of STAT Proteins in the Liver Is Not Affected by Hypophysectomy**—Western blots of liver homogenates probed with anti-STAT antibodies confirmed the selective absence of STAT5b protein and showed that hypophysectomy did not alter the expression of STAT1, STAT3, STAT5a, or STAT5b proteins in wild-type (Fig. 3*A*, lane 6 versus lanes 1 and 2) or in STAT5b-deficient mice (Fig. 3*A*, lane 7 versus lanes 8–10). GH pulse replacement in hypophysectomized wild-type mice induced the appearance of a slower migrating STAT5b band (Fig. 3*A*, lanes 3–5), which we previously identified as a tyrosine-phosphorylated STAT5b form (10). Similar to our earlier observations (23), the levels of STAT1 were higher in STAT5b-deficient compared with wild-type mice (Fig. 3*A*, lanes 7–14 versus lanes 1–6), and these levels were not altered by hypophysectomy (lane 7 versus lanes 8–10).

EMSA analysis using the STAT5-binding sequence of the rat  $\beta$ -casein promoter (32) showed that there was negligible STAT5 DNA binding activity in liver extracts from both wild-type and STAT5b-deficient hypophysectomized mice (Fig. 3*B*, lanes 1–3 and 9–11). This finding is in accord with the requirement of pituitary GH for STAT5 tyrosine phosphorylation and DNA binding activity indicated by studies carried out in the rat liver model (8). GH replacement strongly increased DNA binding in hypophysectomized wild-type mice (Fig. 3*B*, lanes 4–6). A significant, albeit weaker STAT5 EMSA activity was obtained in hypophysectomized STAT5b-deficient mice after GH pulse treatment (Fig. 3*B*, lanes 12 and 13). In wild-type mice these EMSA bands were partially supershifted by anti-STAT5a

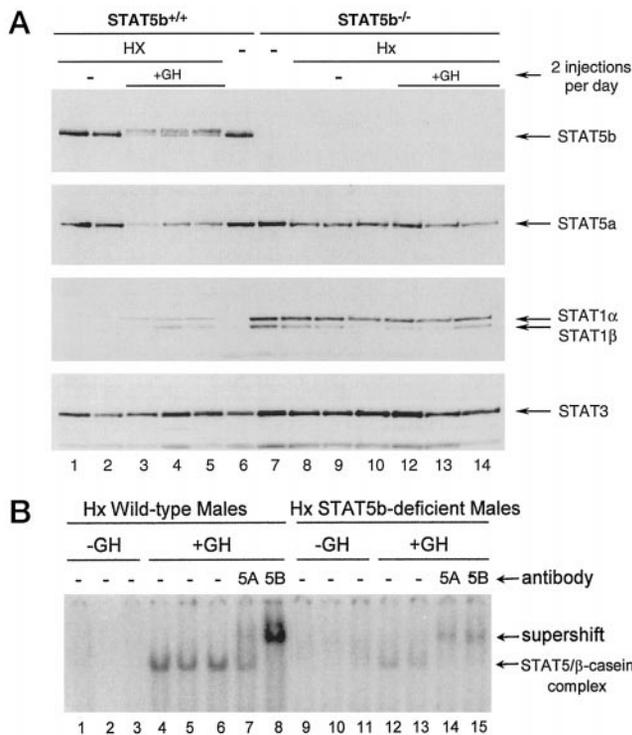


**FIG. 2. Pulsatile GH combined with thyroxine treatment restore MUP expression in hypophysectomized male wild-type mice but not in STAT5b-deficient mice.** *A*, MUP proteins (~20 kDa) excreted in urine obtained from individual mice were analyzed on 12% SDS gels. Shown are gels of representative urine samples after staining with Coomassie Blue. *B*, relative amounts of MUP proteins in wild-type (*n* = 3) and STAT5b-deficient mice (*n* = 4) were quantified using densitometry. Bars indicate standard errors of the means. Urinary samples from wild-type and STAT5b-deficient mice were collected prior to hypophysectomy (Pre-Hx), after hypophysectomy (Hx), and following 7 days of hormone replacement (GH + T<sub>4</sub>).

antibodies (Fig. 3*B*, lane 7) and completely supershifted by anti-STAT5b antibodies (Fig. 3*B*, lane 8). These supershifted bands primarily contain STAT5b homodimers, but also include STAT5a homodimers and STAT5a-STAT5b heterodimers (20). The weak STAT5-DNA complex formed by liver extracts from STAT5b-deficient mice given GH pulse replacement was completely supershifted by anti-STAT5a antibody (Fig. 3*B*, lane 14), consistent with the complex corresponding to a STAT5a-STAT5a homodimer that is formed in livers deficient in STAT5b. The supershift with anti-STAT5b antibodies seen in STAT5b-deficient liver extracts (Fig. 3*B*, lane 15) is indicative of cross-reactivity between this antibody and STAT5a under conditions of EMSA analysis, as discussed elsewhere (27).

**GH Pulse Replacement Reverses Loss of Male-predominant CYP2D9 in Hypophysectomized Wild-type Mice but Not in Hypophysectomized STAT5b-deficient Mice**—CYP2D9 is expressed at severalfold higher levels in males compared with females (Fig. 4*A*, band *b*, lanes 1–3 versus lanes 6 and 7). Moreover, livers of STAT5b-deficient male mice express levels of CYP2D9 that are much lower than wild-type males and are similar to those in wild-type females (lanes 4 and 5 and lanes 6 and 7). Wild-type hypophysectomized mice also expressed low levels of CYP2D9 (Fig. 4*B*, band *b*, lanes 2–4), but expression was substantially restored following GH pulse replacement (Fig. 4*B*, lanes 5–8). In contrast, the low levels of CYP2D9 in hypophysectomized STAT5b-deficient male mice were not restored by GH pulse replacement (Fig. 4*C*, band *b*, lanes 7–9 versus lanes 2–6).

**Female-specific CYP2B Enzyme Is Down-regulated by GH Pulses in Hypophysectomized Male Wild-type but Not STAT5b-deficient Mice**—CYP2B (band *b*) is expressed specifically in female mouse liver (Fig. 5*A*, lanes 6–7 versus lanes 1–3). Hypophysectomy of wild-type male mice increased CYP2B (band *b*) to female levels (Fig. 5*B*, lanes 4–6), supporting the proposal that expression of this protein is negatively regulated by pituitary GH (33). CYP2B (band *b*) is also expressed at an elevated

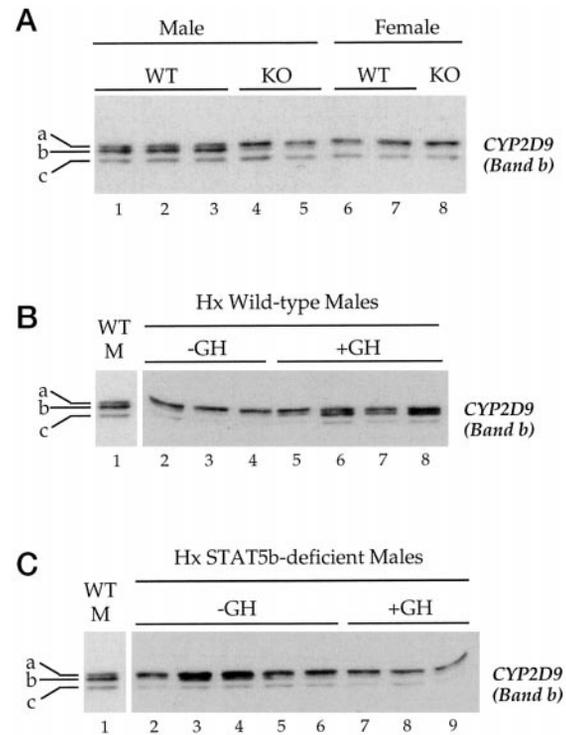


**FIG. 3. Expression of STAT proteins (A) and STAT5 EMSA activity (B) in hypophysectomized wild-type and STAT5b-deficient male mice.** A, total liver homogenates (40  $\mu$ g) were analyzed on Western blots probed sequentially with the indicated anti-STAT antibodies. The livers were from hypophysectomized (*Hx*) or intact control (–) wild-type and STAT5b-deficient mice. Hypophysectomized mice were untreated (– *GH*) or were treated with pulsatile GH replacement for 7 days (+ *GH*). STAT1 $\alpha$  (upper band of doublet) corresponds to the DNA-binding active form of STAT1. STAT5b doublet seen in lanes 3–5 is comprised of non-phosphorylated (lower band) and tyrosine-phosphorylated (upper band) STAT5b forms. The corresponding non-phosphorylated and tyrosine-phosphorylated forms of the other STATs do not resolve well on these gels. B, EMSA of total liver homogenates using the STAT5 binding site from the rat  $\beta$ -casein promoter as a probe. Anti-STAT5a (5a) and STAT5b (5b) antibodies were used to supershift EMSA complexes containing STAT5a (lanes 7 and 14) or STAT5b (lanes 8 and 15). The STAT5/ $\beta$ -casein DNA complexes and the supershifted bands are marked with arrows. Some immune cross-reactivity between STAT5b antibodies and STAT5a present in the STAT5b-deficient liver samples is evident (lane 15) (see “Results”).

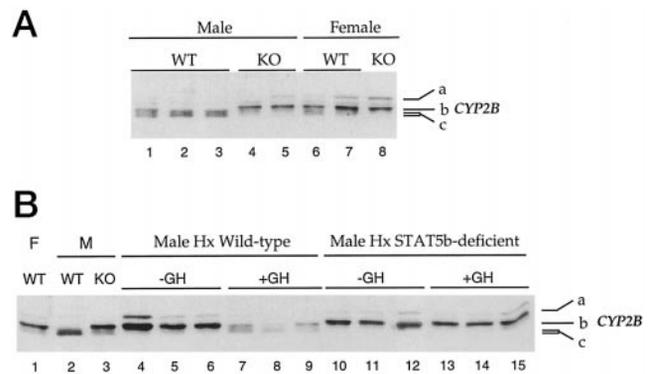
level in both intact and hypophysectomized male STAT5b-deficient mice (Fig. 5, A, lanes 4 and 5; B, lanes 10–12). GH pulse replacement eliminated the expression of CYP2B (band b) in wild-type males; however, there was continued expression of this CYP following GH pulse replacement in STAT5b-deficient males (Fig. 5B, lanes 7–9 versus lanes 13–15).

#### DISCUSSION

Our previous studies with STAT5b-deficient mice indicated that STAT5b is required for multiple biological processes, and that several of the phenotypic defects characteristic of STAT5b-deficient mice are associated with the loss of male-specific, sexually dimorphic liver gene expression (20, 23). However, it could not be determined from these studies whether the observed liver phenotype reflects a direct requirement for STAT5b for maintenance of the male liver expression profile, or alternatively, whether the pattern of pituitary GH secretion in STAT5b-deficient mice is altered to a more frequent secretory pattern, such as is present in females; such a change in pituitary GH secretion would alone be sufficient to induce the feminization of liver gene expression profiles and body growth rates that characterizes STAT5b-deficient mice. Direct analysis of mouse plasma GH profiles has been reported by one



**FIG. 4. Effect of hypophysectomy and pulsatile GH replacement on CYP2D9 gene expression in wild-type and STAT5b-deficient mice.** A, liver microsomal proteins from individual male and female, wild-type (WT) and STAT5b-deficient (KO, knockout) mice were analyzed by Western blotting for the expression of CYP2D9 (Band b). CYP2D9 is expressed predominantly in males. Panels B and C, Western blot analysis, using anti-CYP2D9 antibodies, of liver microsomes prepared from individual hypophysectomized (*Hx*) wild-type and STAT5b-deficient male mice that were untreated (– *GH*) or were given pulsatile GH replacement treatment (+ *GH*), as indicated.



**FIG. 5. Effect of hypophysectomy and pulsatile GH replacement on CYP2B gene expression in wild-type and STAT5b-deficient mice.** A, Western blot analysis of liver microsomal proteins from male and female wild-type (WT) and STAT5b-deficient (KO) mice probed with anti-CYP2B antibodies. CYP2B band b is expressed predominantly in female mice. B, Western blot analysis of male hypophysectomized (*Hx*) wild-type and STAT5b-deficient mice (KO) that were untreated (– *GH*) or given pulsatile GH replacement (+ *GH*), as indicated.

group (5); however, this type of analysis presents serious technical difficulties owing to the small blood vessels, low blood volume, difficulty in maintaining an open catheter for blood sampling in mice, and the requirement that the mice not be stressed during the sampling process. Moreover, in view of the potential role of STAT5b in the established feedback effects of GH on the hypothalamic regulation of pituitary GH secretion (34), direct measurements of plasma GH profiles in STAT5b-deficient mice would not provide an unambiguous answer to

the question of whether the loss of liver STAT5b *per se* is responsible for the observed feminization of liver P450 profiles and body weight gain in STAT5b-deficient mice. We therefore undertook in the present study an alternative approach, in which the pituitary was surgically removed to eliminate the major source of endogenous plasma GH, enabling us to evaluate the intrinsic responsiveness of the liver to plasma GH pulses applied exogenously.

The effects of hypophysectomy and GH pulse replacement on body growth and liver gene expression obtained in wild-type mice were in full agreement with earlier reports (1, 25, 35–37). First, there was no increase in the body weights of any of the mice following hypophysectomy. Second, GH pulse replacement, with or without T<sub>4</sub>, stimulated a dramatic and immediate resumption of body weight gain in wild-type mice. In contrast, hormone replacement did not stimulate an increase in body weight in STAT5b-deficient mice. This finding provides strong support for our proposal that there is impaired GH signaling in STAT5b-deficient mice and that STAT5b mediates body growth stimulated by the male pattern of pulsatile plasma GH. Further study is required to determine the extent to which this STAT5b dependence of body growth involves the liver acting in concert with other tissues that mediate the whole body growth response.

Studies on the excretion of MUPs in urine provide support for the critical importance of STAT5b in intracellular hepatocyte signaling. MUPs are a family proteins of ~20 kDa that are expressed in and secreted by the liver. There are 35–40 MUP genes and pseudogenes in mice (38, 39), and the encoded MUP proteins are thought to contribute to scent marking of territories (40). About 3-fold higher levels of MUPs are excreted in male compared with female mouse urine, and the high level expression of some of these genes is induced by pulsatile GH and suppressed by GH administered continuously. The expression of other MUP genes is induced to a smaller degree by pulsatile GH (30, 31, 41). We previously reported that STAT5b-deficient male mice have MUP levels that are lower than wild-type male mice and are similar to those in wild-type female mice (23). However, the MUP levels in urine of female STAT5b-deficient mice were lower than in the corresponding female wild-type control mice in that study, suggesting that STAT5b might also be generally involved in intracellular GH signaling, and not solely in signaling in response to GH pulses. Because T<sub>4</sub> is also required for the expression of MUP proteins (25), T<sub>4</sub> was added to the drinking water when we evaluated the responsiveness of urinary MUP levels to GH pulse replacement. Pulsatile GH in combination with T<sub>4</sub> replacement stimulated restoration of urinary MUP levels in wild-type but not in STAT5b-deficient mice (Fig. 2), in support of a role for STAT5b in mediating hepatic responses to pulsatile GH at the level of MUP gene expression.

The third observation implicating STAT5b in pulsatile GH signaling pathways comes from cytochrome P450 gene expression studies. We showed previously that the selective loss of male-specific liver cytochrome P450 gene expression in STAT5b-deficient males is coupled with expression of cytochrome P450 genes that are normally expressed only in females (20, 23). Removal of the pituitary gland followed by pulsatile GH stimulation of the liver was presently shown to restore expression of the male-specific liver enzyme CYP2D9 while suppressing the expression of a female-specific CYP2B enzyme in wild-type, but not STAT5b-deficient hypophysectomized male mice (Figs. 4 and 5). Presumably, STAT5b acts in a positive manner to stimulate expression of CYP2D9 and other male-expressed liver CYPs by *trans*-activating STAT5 response elements found in the 5'-flanking DNA of this group of genes

(21).<sup>2</sup> The mechanism responsible for the apparent negative regulation by STAT5b of certain female-expressed liver CYPs (e.g. CYP2B, band b; Fig. 5 and Ref. 20) is uncertain, but could involve “negative STAT5 response elements” or perhaps other, more complex mechanisms. Further studies will be required to elucidate the detailed molecular mechanisms through which STAT5b mediates the sexually dimorphic regulation of liver CYP expression.

Although GH pulse treatment did not restore the normal male pattern of body weight gain and MUP and CYP gene expression in STAT5b-deficient mice, it did activate STAT5a in livers of these animals, as demonstrated by EMSA analysis (Fig. 3B). Accordingly, GH receptor, JAK2 tyrosine kinase, and other cellular factors required for GH pulsed-induced liver STAT activation are present and functional in the livers of STAT5b-deficient mice. This finding provides further support for our proposal that it is the deficiency in STAT5b *per se* that gives rise to the observed GH pulse-resistance phenotype of these animals. STAT5a, when activated by GH pulses in livers of these mice, may either be intrinsically ineffective with respect to stimulating and maintaining a male pattern of liver gene expression, or perhaps may be present at a level that is too low (20) to effectively carry out these STAT5b-stimulated responses. STAT5a does, however, contribute to the expression of certain female-expressed CYPs, perhaps via a mechanism that involves heterodimerization with STAT5b (20).

The present study establishes that STAT5b-deficient mice exhibit a GH pulse resistance phenotype that leads to the observed major alterations in sex-dependent liver CYP gene expression and pubertal body growth rates. However, this finding does not rule out the possibility that STAT5b may additionally help regulate the hypothalamic control of pituitary GH secretion (42), and that the pattern of pituitary GH secretion may consequently be altered in STAT5b-deficient mice. Indeed, we have reported increases in plasma GH levels in STAT5b-deficient mice at sacrifice (23). Moreover, decreased somatostatin mRNA in hypothalamic periventricular nuclei has been observed in STAT5b-deficient mice, providing indirect evidence that pituitary GH secretion may be increased in these animals (43). Further study will be required to elucidate the role of STAT5b in these and other physiologically important hormonal regulatory circuits.

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