**Distinctive Roles of STAT5a and STAT5b in Sexual Dimorphism of Hepatic P450 Gene Expression**

**IMPACT OF Stat5a GENE DISRUPTION†**

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Stat5b gene disruption leads to an apparent growth hormone (GH) pulse insensitivity associated with loss of male-characteristic body growth rates and male-specific liver gene expression (Udy, G. B., Towers, R. P., Snell, R. G., Wilkins, R. J., Park, S. H., Ram, P. A., Waxman, D. J., and Davey, H. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7239–7244). In the present study, disruption of the mouse Stat5a gene, whose coding sequence is ~90% identical to the Stat5b gene, resulted in no loss of expression in male mice of several sex-dependent, GH-regulated liver cytochrome P450 (CYP) enzymes. By contrast, the loss of Stat5b feminized the livers of males by decreasing expression of male-specific CYPs (CYP2D9 and testosterone 16α-hydroxylase) while increasing to female levels several female-predominant liver CYPs (CYP3A, CYP2B, and testosterone 6β-hydroxylase). Since Stat5a is thus nonessential for these male GH responses, Stat5b homodimers, but not Stat5a-Stat5b heterodimers, probably mediate the sexually dimorphic effects of male GH pulses on liver CYP expression. In female mice, however, disruption of either Stat5a or Stat5b led to striking decreases in several liver CYP-catalyzed testosterone hydroxylase activities. Stat5a or Stat5b gene disruption also led to the loss of a female-specific, GH-regulated hepatic CYP2B2 enzyme. Stat5a, which is much less abundant in liver than Stat5b, and Stat5b are therefore both required for constitutive expression in female but not male mouse liver of certain GH-regulated CYP steroid hydroxylases, suggesting that STAT5 protein heterodimerization is an important determinant of the sex-dependent and gene-specific effects that GH has on the liver.

The cytochrome P450s (CYPs)† are a superfamily of heme proteins that hydroxylate steroid hormones and other endogenous chemicals as well as numerous drugs and environmental carcinogens. CYPs are highly expressed in liver, where they are subject to complex hormonal regulation and sex-dependent expression. Prototypic examples of sex-specific liver CYPs in the rat model are the male-specific CYP2C11 (testosterone 16α- and 2α-hydroxylase) and the female-specific CYP2C12 (steroid sulfate 15β-hydroxylase) (1). Marked sex-dependent differences in hepatic CYP profiles are also seen in the mouse, where CYP2D9 (a testosterone 16α-hydroxylase) and CYP2A4 (a testosterone 15α-hydroxylase) are expressed in males and females, respectively, in certain strains (2, 3). Sexually dimorphic expression of a mouse CYP2B testosterone 16α-hydroxylase has also been described (4–6). The expression of these sexually dimorphic liver steroid hydroxylase CYPs is primarily determined by the sexual dimorphism of plasma growth hormone (GH) profiles (2, 7–9). Intermittent plasma GH pulses, a characteristic of adult male rats, induce expression of male-specific CYP proteins and their associated steroid hydroxylase activities, while the near continuous presence of GH in the plasma of adult female rats induces expression of female-specific and female-dominant liver CYP proteins (1, 10). The plasma GH pattern in mice is pulsatile in both sexes; however, sex-specific responses of liver CYPs to plasma GH profiles can be discerned by the distinct frequency of pulsation in males (interpulse interval of ~2.5 h) and females (interpulse interval <1 h) (11).

Pulsatile GH, but not continuous GH, strongly activates the signal transducer and transcriptional activator STAT5 in rat liver (12). STAT5 was originally identified in lactating mammary gland as a prolactin-inducible transcription factor (13). Subsequently, two highly conserved (~90% identical in coding sequence) STAT5 genes, Stat5a and Stat5b, were identified and found to be expressed in many tissues (14–17). Both STAT5 forms can be activated in tissue culture by multiple cytokines and growth factors, including GH, erythropoietin, epidermal growth factor, and various interleukins (14, 18–21). STAT5 proteins thus have the potential to contribute to multiple signaling pathways associated with cell growth and differentiation. The proposed mediation by STAT5 of GH pulse-regulated, sexually dimorphic liver gene expression (12) is supported by the finding of a functional STAT5 response element in the promoter region of several male-specific GH pulse-regulated genes (22). In addition, targeted disruption of Stat5b leads to a major loss of multiple, sexually differentiated responses associated with pulsatile GH secretion (23), demonstrating that this GH pulse-activatable transcription factor (12) is essential for maintaining sexually dimorphic body growth rates and liver gene expression.

During mammary gland differentiation, STAT5a and STAT5b both undergo prolactin-inducible tyrosine phosphorylation and bind as a heterodimeric STAT5a-STAT5b complex at γ-interferon activation-like regulatory sites (TTCNNGAA), such as that found in the mouse β-casein promoter (13, 24).

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‡ The abbreviations used are: CYP, cytochrome P450; STAT, signal transducer and activator of transcription; EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction; GH, growth hormone; bp, base pair(s).

‡ S. H. Park and D. J. Waxman, unpublished observations.
Stat5a gene disruption has demonstrated that STAT5a is critically required to activate and/or repress yet unknown target genes that promote mammary gland differentiation and lactogenesis (25). Mammary gland Stat5b can only partially fill this function and then again only after repeated hormonal stimulation through multiple pregnancies and episodes of suckling (26). Although targeted disruption of Stat5b leads to the loss of multiple sexually differentiated responses governed by a pulsatile plasma GH profile (23), it is uncertain whether this loss reflects a requirement for heterodimeric STAT5a-STAT5b complexes or perhaps an absolute requirement for homodimeric STAT5b-STAT5b complexes for GH pulse-regulated liver gene expression. STAT5a and STAT5b exhibit differences with respect to their tissue distribution (14, 15) and DNA binding specificities (27) and in the sequences of their COOH-terminal transcription activation domain (28). These two STATs could thus have distinct functions with respect to their role in GH signaling and its impact on the sexual dimorphism of liver gene expression.

To address the role of STAT5a in the GH-regulated sexual dimorphism of liver gene expression, we have examined the effects of Stat5a gene disruption on hepatic CYP enzyme activities and protein expression. 129J congenic Stat5b−/− mice were also examined to verify and extend using an inbred mouse congenic 129J congenic ES cells) were mated with 129J females. The Stat5b−/− progeny were then bred to obtain the 129J congenic Stat5b−/− and Stat5b−/+ mice used for these experiments. Experiments using Stat5a−/− mice were carried out using littermates obtained from 129J × Black Swiss outbred mice. In all cases, comparisons of wild-type and Stat5-deficient mice were made between littermates obtained from the crossing of heterozygote siblings or of heterozygote females with wild-type males. Efforts to obtain liver tissue from 129J congenic gene-disrupted mice has been unsuccess- ful, since these animals proved very difficult to breed. Although the possibility cannot formally be excluded that a 129J-derived regulatory determinant of liver CYP gene expression is tightly linked to the Stat5 locus and contributes to some of the liver CYP profile differences between wild-type and Stat5a−/− 129J × Black Swiss mice, this is considered unlikely.

Preparation of Mouse Liver Homogenates, Cytosol, and Microsomes—Mouse liver tissues were snap-frozen in liquid nitrogen and stored at −80 °C until use. Liver (about 1 g) was homogenized in 10 ml of homogenizing buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 250 mM sucrose) with the addition of phosphatase inhibitors (1 mM sodium orthovannadate and 10 mM sodium fluoride) and a protease inhibitor (100 μg phenylmethylsulfonyl fluoride) and centrifuged at 9000 rpm for 15 min to obtain a total tissue homogenate. Ultracentrifugation at 100,000 × g for 1 h was carried out to separate microsomal pellets and the cytosolic supernatant. Microsomal pellets were suspended in KPi buffer, pH 7.4, containing 0.1 mM EDTA and 20% glycerol, and stored at −80 °C until use. Microsomal protein concentrations were determined using the Bio-Rad DC detergent protein assay kit using bovine serum albumin as a standard.

Antibodies—Rabbit polyclonal antibodies raised against mouse STAT5a amino acids 774–793 and mouse STAT5b amino acids 776–786 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) (sc-1081 and sc-853, respectively). These STAT5B antibodies were shown to be specific for STAT5a or STAT5b, respectively, under our Western blotting conditions. Some cross-reactivity was apparent in electrophoretic mobility shift assay (EMSA) supershift analysis (see Fig. 2B). Mouse monoclonal anti-STAT1 and anti-STAT3 antibodies purchased from Transduction Laboratories were raised against STAT1 amino acids 591–731 (S214/218) and STAT3 amino acids 1–178 (S233/236) and recognized STAT1 and STAT3 complexes or perhaps an absolute requirement for microsomal CYP protein separation, and 8% gels for mouse microsomal CYP protein separation), 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 (Blotto) and 1% bovine serum albumin 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% bovine serum albumin in TST buffer (10 mM Tris-HCl, pH 7.5, 0.1% Tween 20, 100 mM NaCl) for probing with anti-STAT5a and anti-STAT5b. For microsomal CYP Western blotting, membranes were blocked with 3% Blotto and 1% bovine serum albumin in TST buffer. Incubations with primary antibodies were carried out for 1 h at 37 °C at dilutions of 1:5000. Antibody binding was visualized on x-ray film by enhanced chemiluminescence using the ECL kit from Amersham Pharmacia Biotech. Nitrocellulose membranes were reprobed after stripping in 62.5 mM Tris-HCl, pH 7.6, 2% SDS, 50 mM 2-mercaptoethanol for 20 min at 50 °C. Results are presented in figures prepared from grayscale scans of portions of the x-ray films of each blot. Scans were obtained using a Cannon IX-4015 scanner outfitted with Ofoto scanning software.

Microsomal Testosterone Hydroxylation Assay—Microsomal metabolism of testosterone was assayed as described previously (32) using 25 μg of mouse liver microsomal protein incubated in 0.2 ml containing 50 mM Tris buffer, pH 7.6, 3 mM MgCl2, and 4C-labeled testosterone (10 nmol, ~100,000 cpm). Reactions were initiated by the addition of 0.36 mM NADPH and terminated 20 min later by the addition of 1 ml of ethyl acetate. Testosterone and hydroxytestosterone metabolites were extracted with ethyl acetate and then chromatographed on silica gel TLC plates developed in solvent A (methanol/chloroform/methanol/acetone [80:20, v/v,v/v]) and then solvent B (chloroform/ethanol/acetic acid/ethanol (70:15:7.5:12.5, v/v/v/v)). TLC plates were exposed overnight to PhosphorImager plates (Molecular Dynamics) and analyzed using Molecular Dynamics phosphorimager instrument and ImageQuant software (Sunnyvale, CA). In order to assess the homogeneity and identity of individual radiolabeled testosterone metabolites, radioactive spots of interest were cut from the aluminum-backed TLC plates, and the 4C-labeled monohydroxytestosterone metabolites were then eluted with ethyl acetate. Unlabeled authentic hydroxytestosterone metabolites were then individually co-injected with each of the 4C-metabolites to verify co-migration in two independent TLC solvent systems (32).

EMSA—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) (Boehringer Mannheim), 5% glycerol, 1.25 mM MgCl2, 625 μM EDTA, and 625 μM dithiothreitol). Double-stranded oligonucleotide probe con- taining the STAT5a response element of the rat β-casein promoter (nucleotides −101 to −80; 5′-GGA-CTT-CTT-GGA-ATT-AAG-GGA-3′) was 32P-end-labeled on one strand using T4 kinase and then incubated with the protein sample for 20 min at room temperature and then 10 min on ice to stabilize the STAT5a-DNA gel shift complex (33). 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. Samples were electrophoresed in a cold room through a nondenaturing polyacrylamide gel (5.5% acrylamide, 0.07% bisacrylamide) (National Diagnostics, Atlanta, GA) in 0.5 × TBE buffer (44.5 mM Trizma-base, 44.5 mM boric acid, 5 mM EDTA) following a 30-min preelectrophoresis step. After electrophoresis of the samples into the gel for 20 min at 120 V, the gel apparatus was moved to room temperature to increase the speed of protein migration. In some cases, the electrophoresis time was increased and/or the percentages of acrylamide and bisacrylamide gel were increased to 6.5% and 0.08%, respectively, to increase the resolution of the STAT5-containing EMSA complexes.

RNA Isolation and Reverse Transcription Polymerase Chain Reaction (PCR)—Total RNA was isolated from ~100 ng of mouse liver using TRIZOL Reagent (Life Technologies, Inc.). First strand cDNA was syn-
RESULTS

Stat5a Gene Disruption—Liver cytosols prepared from Stat5a/−/− mice were assayed for the expression of individual STAT proteins by Western blotting. Wild-type mice showed similar levels of liver cytosolic Stat5a protein in both males and females, with some differences between individual animals apparent (Fig. 1, lanes 1, 2, and 7–9). No STAT5a protein was detected in the Stat5a/−/− mice of either sex. Reprobing with antibodies to other STAT proteins revealed that STAT5b, STAT1, and STAT3 protein levels were not significantly changed by the Stat5a gene disruption. This contrasts to the increase in liver STAT1 in Stat5b/−/− mice (23). STAT3 was present in liver at a significantly higher level in females than in males in the 129J × Black Swiss mice used in the present study (Fig. 1, bottom panel, lanes 7–13 versus lanes 1–6). No sex difference in hepatic STAT3 levels was seen, however, in 129J or in 129J × BALB/c mice (data not shown).

Our previous study of Stat5b/−/− mice (23) was carried out using 129J × BALB/c outbred mice. Since there are significant strain differences in the patterns of CYP enzyme expression in mouse liver (4, 34, 35), we generated Stat5b/−/− mice in the 129J inbred strain in order to eliminate random genetic contributions from the 129J × BALB/c outcrossed background to individual animal variation. Examination of the Stat5b/−/−/129J congenic mice revealed effects of Stat5b disruption on liver STAT protein expression similar to those seen earlier in outbred mice; STAT1 levels were increased, while the expression of STAT3 and STAT5a was unchanged (data not shown).

Characterization of STAT5a and STAT5b DNA Binding Activity in Stat5 Gene-Disrupted Mouse Liver—We next used a STAT5-binding DNA probe from the rat β-casein gene in an EMSA of total liver homogenates to examine the functional (DNA binding) activity of STAT5b protein in Stat5a/−/− mice. STAT5 protein present in Stat5a/−/− mouse liver was active in this DNA binding assay (Fig. 2A, lane 1). Given the absence of STAT5a protein in these livers, we conclude that the DNA complex detected is composed of STAT5b-STAT5b homodimers. These homodimers migrated distinctly faster than STAT5a homodimers, which are present in liver homogenates prepared from Stat5b/−/− mice (lanes 4 and 5). A corresponding fast mobility complex was formed by a GH pulse-activated rat liver homogenate (lane 8), in agreement with earlier studies indicating that STAT5b is the major STAT5 protein in this tissue (36, 37). The major complex observed in wild-type mice migrated at an intermediate mobility compared with that present in Stat5a/−/− and Stat5b/−/− mice (lanes 2, 3, 6, and 7), indicating the presence of STAT5a-STAT5b heterodimers.

The presence of STAT5 homo- or heterodimers in these liver samples was further investigated by supershift analysis using anti-STAT5a and anti-STAT5b antibodies (Fig. 2B). Anti-STAT5b antibody fully supershifted the STAT5b-containing DNA complex present in Stat5a/−/− mice (Fig. 2B, lane 9). A supershift complex of similar mobility was obtained in wild-type mouse liver (lanes 6 and 12) and in male rat liver (lane 3), suggesting that these tissues contain homodimeric STAT5b-STAT5b complexes in addition to the intermediate mobility STAT5a-STAT5b heterodimeric complexes evident from Fig. 2A. Some cross-reactivity of this anti-STAT5b antibody with STAT5a-STAT5a homodimers was apparent, however, as revealed by the more rapidly migrating supershift complex obtained with Stat5b/−/− mouse liver samples (lane 15). This latter complex was not present in significant amounts in wild-type mouse liver (lanes 6 and 12) or male rat liver (lane 3), indicating that the majority of activated STAT5a is complexed as a heterodimer with STAT5b in wild-type liver. Anti-STAT5a antibody yielded a nearly complete supershift of the STAT5a-STAT5a complexes present in Stat5b/−/− mouse liver (lane 14) but only a partial supershift of the STAT5a-containing complexes present in wild-type mice (lanes 5 and 11) or in male rat liver (lane 2). The supershift pattern obtained with anti-STAT5a antibody is consistent with the presence of both STAT5b-STAT5b homodimer and STAT5a-STAT5b heterodimer, as demonstrated for GH-activated STAT5a and STAT5b standards expressed in extracts of transfected COS-1 cells; STAT5a antibody completely supershifted STAT5a-STAT5a EMSA complexes, while it partially shifted STAT5a-STAT5b and STAT5b-STAT5b complexes formed by the transfected COS-1 cell extracts (data not shown). This cross-
In our earlier Stat5b−/− mice (A) in the absence or presence of supershifting anti-Stat5a and anti-Stat5b antibodies (B). Liver extracts were prepared from individual mice and then analyzed by EMSA for Stat5 DNA binding activity as described under "Materials and Methods." A, a distinct mobility difference between Stat5a-Stat5a homodimers and Stat5b-Stat5b heterodimers is apparent from a comparison of lanes 4 and 5 versus lanes 6 and 7. A GH pulse-activated male rat liver extract is shown in lane 8 for comparison. B, supershift analysis using anti-Stat5a and anti-Stat5b antibodies revealed some immune cross-reactivity between the Stat5a and Stat5b antibodies (see text). Male rat total liver homogenates were used as a positive control (lanes 1–3). Lane 14 shows that the anti-Stat5a antibody can completely supershift the Stat5a-Stat5a homodimer that is present in the Stat5b−/− male mouse liver. Arrows with asterisks at the right mark supershifted protein-DNA complexes. Note distinct mobilities of each of the nonsupershifted complexes shown in lanes 4, 7, 10, and 13 as a function of the presence or absence of Stat5a or Stat5b (cf. panel A).

Expression of Sex-dependent CYPs in Stat5a−/− Male Mice—Experiments were carried out to ascertain whether Stat5a is required for expression of sex-dependent liver CYP enzymes. To achieve this objective, we first examined the patterns of CYP enzyme expression in male and female Stat5a−/− and Stat5b−/− mice, using the diagnostic CYP substrate testosterone (32). Liver CYP enzyme patterns are known to differ significantly between individual strains of mice (2, 34, 35). We observed several differences in the sex-dependence of liver microsomal testosterone hydroxylation in the two mouse strains used in this study, 129J × Black Swiss for Stat5a−/− and 129J inbred for Stat5b−/−. Three female-dominant hydroxysteroid metabolites were formed in wild-type 129J × Black Swiss mouse liver microsomal incubations (2α-OH, 6α-OH (Fig. 3A) and 7α-OH (Table I)), while only a single female-dominant hydroxysteroid metabolite (6β-OH; Fig. 3B) was formed by wild-type 129J mouse liver microsomes (Table I). In the case of 129J × Balb/c outbred mice, two female-dominant hydroxysteroid metabolites (6α-OH and 6β-OH) were formed (Table I). In addition, 129J inbred mouse liver microsomes formed a male-specific 16α-OH-testosterone metabolite that was not observed in the 129J × Balb/c outbred mice used in our earlier Stat5b−/− studies (Fig. 3B; Table I). This latter strain difference is the result of a repression of a female-specific, CYP2B-dependent testosterone 16α-hydroxylase in 129J females (4).

In male 129J mice, the loss of Stat5b increased the female-dominant testosterone 6β-hydroxylase enzyme activity up to the level of wild-type females (Fig. 3B). The same response to the loss of Stat5b was seen for two CYP activities that have a female-dominant expression profile in 129J × Balb/c mice, testosterone 6α-hydroxylase and testosterone 6β-hydroxylase (Table I). By contrast, the loss of Stat5a did not increase expression of any of the female-dominant CYP enzyme activities assayed (testosterone 2α- and 6α-hydroxylase) in affected males (Fig. 3A; Table I). None of the female-dominant testosterone hydroxylase activities in female mouse liver was affected by Stat5a or Stat5b gene disruption (Table I). We conclude that the loss of Stat5a in male mouse liver does not lead to the increase in female-dominant CYP enzyme activities that occurs in response to the loss of Stat5b.

A similar conclusion can be drawn based on the effects of Stat5a and Stat5b gene disruption on sex-dependent CYP3A and CYP2B protein expression. Western blot analysis indicated that the female-dominant liver CYP3A protein band b was increased in Stat5b−/− males (Fig. 4B, lanes 5–7 versus lanes 1–4), in agreement with the increase in CYP3A-diagnostic testosterone 6β-hydroxylase activity (Fig. 3B). Two other liver CYP proteins that are female-dominant in 129J × Balb/c mice, CYP2B band a and CYP2B band b, were also increased in Stat5b−/− males to the much higher female levels (Fig. 5A, lanes 5–8 versus lanes 1–4; cf. lane 9). By contrast, none of the female-dominant CYP proteins, i.e., CYP3A band b and CYP2B band b, was increased in Stat5a−/− male mice (Fig. 4A, lanes 4–7 versus lanes 1–3; Fig. 5B, lanes 4–7 versus lanes 1–3).3 Stat5b−/− male mice also exhibited a significant (albeit partial) loss of male-specific testosterone 16α-hydroxylase activity (Fig. 3B) and its associated CYP2D band b (Fig. 6B, lanes 5–8 versus lanes 1–4). In contrast, expression of the male-specific liver CYP2D band b was unchanged in Stat5a−/− male mice (Fig. 6A, lanes 4–7 versus lanes 1–3) (Table II).

We conclude that Stat5b gene disruption feminizes the livers of male mice by increasing the expression of female-dominant CYP3A and CYP2B proteins to the level of wild-type female mice, while partially decreasing the expression of male-dominant testosterone 6α-hydroxylase and testosterone 6β-hydroxylase (Table I). By contrast, the loss of Stat5a did not increase expression of any of the female-dominant CYP enzyme activities assayed (testosterone 2α- and 6α-hydroxylase) in affected males (Fig. 3A; Table I). None of the female-dominant testosterone hydroxylase activities in female mouse liver was affected by Stat5a or Stat5b gene disruption (Table I). We conclude that the loss of Stat5a in male mouse liver does not lead to the increase in female-dominant CYP enzyme activities that occurs in response to the loss of Stat5b.

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CYP Expression in STAT5a Knockout Mice

**FIG. 3. Influence of STAT5a and STAT5b gene disruption on sex-dependent liver microsomal testosterone hydroxylase activities.** Testosterone hydroxylase activities were determined in liver microsomes prepared from wild-type mice (filled bars) and STAT5a−/− or STAT5b−/− mice (speckled bars). Hepatic microsomal proteins (25 µg) were incubated with 14C-labeled testosterone in the presence of NADPH as described under “Materials and Methods.” Specific activities shown are mean ± S.E. for n = 5 individual mice per group. A single asterisk designates significant differences from wild-type at p ≤ 0.05. The double asterisks represent significant differences from males at p ≤ 0.05.

**TABLE I**

| Hydroxysterone metabolite | Male Wild type | Female Wild type | Male Knockout | Female Knockout |
|---------------------------|---------------|-----------------|---------------|-----------------|
| STAT5a−/− (129J × Black Swiss) |               |                 |               |                 |
| 2α-OH                    | +             | ++              | ++            | ++              |
| 6α-OH                    | +             | ++              | ++            | ++              |
| 7α-OH                    | + +          | ++              | ++            | ++              |

| STAT5b−/− (129J) |             |                 |               |                 |
| 6β-OH                  | ++           | +++             | +++           | +++             |
| 16α-OH                 | +++          | +               | −             | −               |

| STAT5b−/− (129J × BALB/c) |             |                 |               |                 |
| 6α-OH                  | +++          | +++             | +++           | +++             |
| 6β-OH                  | +++          | +++             | +++           | +++             |

* Partial elevation in enzyme activity was seen in some individual mice but did not reach statistical significance (p ≤ 0.15 compared to wild-type males).

are dispensable for the male-specific pattern of liver CYP expression.

**Selective Loss in Female Mice of Select CYP Enzyme Activities upon Disruption of STAT5a or STAT5b**—We next investigated whether either of the STAT5 proteins is required to maintain normal CYP enzyme and protein expression in female mouse liver. Although disruption of the STAT5a or STAT5b genes had no effect in males on any of the sex-independent CYP activities examined (Fig. 7; Table III), in females the loss of STAT5a or STAT5b did result in a marked loss of some, but not all, sex-independent testosterone hydroxylase activities. Thus, liver microsomal testosterone 16α-hydroxylation was decreased in both female STAT5a−/− mice and in female STAT5b−/− mice (129J × BALB/c), while testosterone 6α-hydroxylation was decreased in STAT5b−/− females (129J strain) (Fig. 7). Other sex-independent testosterone hydroxylases were unaffected (e.g. 6β-hydroxylase and 2α-hydroxylase; left panels of Fig. 7). Although testosterone 16α-hydroxylation is a male-specific enzyme activity in 129J inbred mice (Fig. 3B), this activity is high in both females and males in wild-type 129J outcrossed mice (filled bars, Fig. 7). This latter finding is consistent with the characterization of the female mouse liver testosterone 16α-hydroxylase enzyme as a female-dominant P450 whose repression in 129J females is inherited as an autosomal recessive trait (4). Moreover, the selective loss of testosterone 16α-hydroxylase activity in female mouse liver in response to the loss of either STAT5a or STAT5b (Table III, last column; Fig. 7) indicates that both STAT5 forms are required to maintain full expression of this constitutively expressed CYP gene product in female but not male mice. Similarly, the selective loss in female 129J Stat5b−/− mouse liver of testosterone 6α-hydroxylase activity (Fig. 7B, right panel) indicates a requirement for both STAT5 proteins to maintain expression of this P450 enzyme in females.

**Loss of Female-specific CYP2B Protein in Female STAT5a+−/− Mice**—The mouse CYP2B subfamily contains several liver-expressed proteins, including at least one whose expression is female-predominant and growth hormone-regulated (6). In view of the GH dependence of this CYP2B enzyme, we examined whether STAT5a or STAT5b disruption impacts on its expression. Western blot analysis revealed three CYP2B cross-reactive proteins in 129J × Black Swiss mouse liver microsomes: band a, which is sex-independent in this mouse strain, or albeit variable in individual mice (Fig. 5B); band b, which is female-specific; and band c, which in some experiments could be resolved to give two bands (Figs. 5, A and C). In male mice, STAT5a gene disruption did not cause any notable change in the level of the sex-independent CYP2B band a or any increase in the female-specific CYP2B band b (Fig. 5B), as indicated above. By contrast, a substantial loss of the female-specific band b was seen in five of six female Stat5a−/− mice (Fig. 5C, lanes 7–11 versus lanes 2–6; Table II, and data not shown). This response of CYP2B band b to STAT5a gene disruption in females is in sharp contrast to the lack of an effect of STAT5a gene disruption on other female-specific or female-dominant liver CYP proteins or activities, such as CYP3A band b (Fig. 4A) or testosterone 2α- and 6α-hydroxylase activity (Fig. 3A). CYP2B band b was not expressed in 129J male or female mouse liver, precluding a determination of the impact of Stat5b gene disruption on its expression in this strain. In 129J × BALB/c outbred mice, CYP2B bands a and b were both expressed as female-dominant forms (Fig. 5A); band a remained at the same level of expression in female Stat5b−/− mice, while band b was partially decreased in five of seven female Stat5b−/− mice (data not shown).

Relative Expression Levels of STAT5a and STAT5b mRNA in
Fig. 4. CYP3A protein expression in Stat5a−/− and Stat5b−/− mice. Shown are Western blots of mouse liver microsomes (20 μg) prepared from wild-type and Stat5a−/− (A) and Stat5b−/− mice (B) using an anti-CYP3A antibody. Three immune cross-reactive CYP3A bands are seen. The lower-immunoreactive band a was significantly elevated in Stat5b−/− male mice (C). The detection a, b, and c bands in panel B, in part due to the fuzzy nature of the bands seen. Band b was somewhat difficult to distinguish from band c in panel B, in part due to the fuzzy nature of the bands seen on this Western blot.

Fig. 5. Effect of Stat5a gene disruption on hepatic microsomal CYP2B protein expression. Shown are Western blots of male and female mouse liver microsomes from the indicated strains, probed using anti-CYP2B polyclonal antibody. Three immune cross-reactive CYP2B bands are seen (bands a, b, and c). CYP2B band b is female-specific in wild-type 129 × BALB/c mice (A) but is expressed in both sexes in 129J × Black Swiss mice (B). CYP2B band b is female-specific in both strains.

STAT5a and STAT5b mRNA in total mouse liver RNA using a reverse transcription PCR method. Since the two STAT5 mRNAs are ~90% identical, we used an assay that incorporates an NcoI restriction digestion step to distinguish a PCR-amplified STAT5a cDNA fragment, which contains an NcoI site, from the corresponding STAT5b cDNA, which does not (see "Materials and Methods"). Results obtained from five individual 129J × Black Swiss mouse livers are shown in Fig. 8A. NcoI digestion of the PCR fragments revealed that the majority of the STAT5 cDNA was derived from STAT5b mRNA in both male (Fig. 8A) and female 129J × Black Swiss mice (data not shown), as indicated by the minor fraction that was digested to yield the STAT5b-derived 310- and 240-bp NcoI fragments. In control experiments, NcoI fully digested STAT5a cDNA amplified from a cloned STAT5a plasmid using the same PCR primers, while there was no digestion of a corresponding STAT5b cDNA fragment (data not shown). Quantitation of the ratio of digested to undigested fragments revealed that STAT5b corresponded to 95–96% of the total STAT5 mRNA in both male and female 129J and 129J × Black Swiss mice and to ~90% of the total STAT5 mRNA in 129J × BALB/c mice (Fig. 8B and data not shown).

DISCUSSION

The present study demonstrates that STAT5a and STAT5b both play important roles in the maintenance of sexually dimorphic liver CYP gene expression in the mouse model. The two highly conserved (~90% identical) STAT5 proteins, STAT5a and STAT5b, were shown to be activated in mouse liver to form both homodimers (STAT5a-STAT5a; STAT5b-STAT5b) and heterodimers (STAT5a-STAT5b). STAT5b alone, most likely in the form of a STAT5b-STAT5b homodimeric complex, was found to be required to maintain the male-specific pattern of GH pulse-stimulated liver Cyp expression. This was apparent from the loss of the male-specific CYP2D9 band b and the increase in several female-predominant P450s in Stat5b−/− but not Stat5a−/− male mouse liver (CYP3A band b and CYP2B band b). By contrast, in female mouse liver, STAT5a and STAT5b were both required for full expression of several CYP proteins and activities, including a female-specific CYP2B protein (band b; Fig. 5C), which apparently corresponds to the female-specific, GH-regulated mouse liver CYP2B protein with the same relative mobility described elsewhere (6). This requirement of both STAT5a and STAT5b in female mouse liver suggests that STAT5a-STAT5b heterodimers play a unique regulatory role in the female. This contrasts with the role proposed for STAT5b homodimers in male liver in mediating...
Stat5a and Stat5b gene disruption on sex-dependent P450 protein levels

Expression of the indicated hepatic microsomal CYP proteins was determined by Western blotting (e.g. as shown in Figs. 4–6).

| CYP3A, band b (female-specific) | Male | Female |
|---------------------------------|------|--------|
| Wild type | – | ++ |
| Stat5a<sup>−/−</sup> (129J × Black Swiss) | – | ++ |
| Stat5b<sup>−/−</sup> (129J) | +++ | +++ |
| CYP2B, band b (female-specific) | – | +++ |
| Wild type | – | +++ |
| Stat5a<sup>−/−</sup> (129J × Black Swiss) | – | – |
| Stat5b<sup>−/−</sup> (129J × BALB/c) | +++ | +++ |
| CYP2D9, band b (male-dominant) | – | +++ |
| Wild type | – | – |
| Stat5a<sup>−/−</sup> (129J × Black Swiss) | – | ++ |
| Stat5b<sup>−/−</sup> (129J) | + | + |
| Stat5b<sup>−/−</sup> (129J × BALB/c) | – | – |

GH pulse regulation of sexually dimorphic liver CYPs. Although a definitive cause-and-effect relationship is not established by these findings, the loss of male-specific liver gene expression in Stat5b<sup>−−</sup> but not Stat5a<sup>−−</sup> mice could indicate a direct effect of, and a specific requirement for, Stat5b-STAT5b homodimers to regulate expression of male GH pulse-induced Cyp genes. Alternatively, it is possible that other STAT5-containing complexes (STAT5a-STAT5b and STAT5a-STAT5a) could be intrinsically capable of regulating the male-expressed Cyp genes but might simply not be present in sufficient amounts in Stat5b<sup>−−</sup> male mouse liver to satisfy the threshold requirements with respect to transmission to the nucleus of a male, pulsatile plasma GH signal.

Strain-dependent Expression of Mouse Liver CYP Testosterone Hydroxylases—In contrast to the rat, where at least 24 hepatic CYP forms have been extensively characterized at a molecular and regulatory level and CYP gene-specific catalytic and immunochemical probes are widely available (39), the characterization of individual murine members of the CYP enzyme system is far less advanced, and in many instances the relationship of specific mouse P450 proteins with specific Cyp genes is uncertain. Moreover, unlike in the rat, there are major strain differences in Cyp gene expression patterns in the mouse (4, 34, 35) (Tables I and III). For example, whereas testosterone 15α-hydroxylase activity has been associated with the female-specific CYP2A4, and testosterone 16α-hydroxylase activity represents the male-specific CYP2D9 in some mouse strains, these P450 enzyme activities are also associated with other CYP gene products and consequently lose their sex-dependence in other mouse strains (Table III) (4, 31). For example, testosterone 16α-hydroxylase is not only catalyzed by CYP2D9, but is also catalyzed by a female-expressed CYP2B enzyme that is expressed in select mouse strains (4, 5, 40, 41).

Accordingly, the high level of liver microsomal testosterone 16α-hydroxylase activity seen in both male and female wild-type 129J × Black Swiss and 129J × BALB/c mice (versus the male-specific expression of testosterone 16α-hydroxylase activity in 129J mice) (4, 34, 35) probably results from the combined expression in the outcrossed strains of the male-specific CYP2D9 band b (e.g. Fig. 6) and the female-specific CYP2B, band b (Fig. 5). CYP2B band b is not expressed in 129J mice (data not shown), in agreement with the repression via an autosomal recessive trait of the female-predominant testosterone 16α-hydroxylase CYP enzyme in 129J females (4). Consequently, the selective loss in female, but not male, Stat5a and Stat5b knockout mice of certain sex-independent testosterone hydroxylase activities, namely 16α-hydroxylase (129J), is likely to reflect a loss of female-specific CYP gene products rather than a loss in the female of sex-independent CYP gene products. Further progress in linking the individual mouse liver microsomal testosterone hydroxylase activities of each strain with their corresponding Cyp genes will be necessary in order to further investigate at the gene-regulatory level the differential effects of STAT5a-STAT5b heterodimers versus STAT5b-STAT5b homodimers on liver CYP gene expression that we hypothesize to occur on the
basis of our findings. Identification and further investigation of the specific Cyp genes that may be regulated in this manner is likely to be a formidable task, in view of the complexity of the mouse Cyp gene superfamily (cf. three distinct classes comprised of 16 closely related mouse Cyp2b genes, only two of which have been identified (42)).

**Proposed Role of Hormone-induced Activation and Dimerization of STAT5a and STAT5b**—In mammary gland, STAT5a and STAT5b are both activated by prolactin (24). The generation of mice in which the genes encoding prolactin receptor (43), STAT5a (25), and STAT5b (23) have been individually inactivated has demonstrated that in mammary gland the het-

| Hydroxytestosterone Metabolite | Male | Female |
|--------------------------------|------|--------|
| 6α-OH | NC* | NC     |
| 15α-OH | NC | NC     |
| 16β-OH | NC | NC     |
| 16α-OH | NC | NC     |
| 2α-OH | NC | NC     |
| 7α-OH | NC | NC     |
| 15α-OH | NC | NC     |
| 16α-OH | NC | NC     |

* NC, no change in enzyme level in response to either Stat5a or Stat5b gene disruption.

**Fig. 7. Effect of Stat5a**<sup>−/−</sup> or Stat5b**<sup>−/−</sup> on sex-independent liver testosterone hydroxylase activities.** Testosterone hydroxylase activities were determined in liver microsomes prepared from wild-type mice (filled bars) and Stat5a**<sup>−/−</sup> or Stat5b**<sup>−/−</sup> mice (speckled bars) as described under “Materials and Methods.” Specific activities shown are mean ± S.E. for n = 5 individual mice per group. A single asterisk represents significant differences from wild type (p < 0.05). Disruption of Stat5a or Stat5b is seen to decrease expression in female but not male mouse liver of some but not other sex-dependent, CYP-catalyzed testosterone hydroxylase activities.

**Fig. 8. Reverse transcription PCR analysis of liver STAT5 mRNA levels.** 2 μg of total RNA isolated from five individual male 129J × Black Swiss mouse livers was reverse-transcribed and then PCR-amplified with STAT5 primers that do not distinguish between STAT5a and STAT5b, as described under “Materials and Methods.” A, the amplified PCR products (550 bp), with or without NcoI digestion, as indicated, were electrophoresed in an ethidium bromide-stained gel. The 550-bp STAT5b cDNA fragment does not contain an NcoI site, whereas the STAT5a cDNA is digested by NcoI to yield fragments of 240 and 310 bp. B, quantitation of the fractions of total STAT5 cDNA that correspond to STAT5a (310- plus 240-bp fragments) and STAT5b (550-bp fragment) after NcoI digestion, as determined by integration of band intensities. In control experiments not shown, the corresponding 550-bp cDNA amplified from cloned STAT5 plasmid DNA was shown to be fully digested with NcoI in the case of STAT5a and not at all digested with NcoI in the case of STAT5b.

**Fig. 7.** Effect of Stat5a**<sup>−/−</sup> or Stat5b**<sup>−/−</sup> on sex-independent liver testosterone hydroxylase activities. Testosterone hydroxylase activities were determined in liver microsomes prepared from wild-type mice (filled bars) and Stat5a**<sup>−/−</sup> or Stat5b**<sup>−/−</sup> mice (speckled bars) as described under “Materials and Methods.” Specific activities shown are mean ± S.E. for n = 5 individual mice per group. A single asterisk represents significant differences from wild type (p < 0.05). Disruption of Stat5a or Stat5b is seen to decrease expression in female but not male mouse liver of some but not other sex-dependent, CYP-catalyzed testosterone hydroxylase activities.

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**TABLE III**

| Effect of Stat5a and Stat5b gene disruption on sex-independent liver microsomal CYP-catalyzed testosterone hydroxylase activities |
|---------------------------------------------------------------|
| Hydroxytestosterone Metabolite | Male | Female |
|--------------------------------|------|--------|
| 6α-OH | NC* | NC     |
| 15α-OH | NC | NC     |
| 16β-OH | NC | NC     |
| 16α-OH | NC | NC     |
| 2α-OH | NC | NC     |
| 7α-OH | NC | NC     |
| 15α-OH | NC | NC     |
| 16α-OH | NC | NC     |
| 2α-OH | NC | NC     |
| 7α-OH | NC | NC     |
| 15α-OH | NC | NC     |
| 16α-OH | NC | NC     |

* NC, no change in enzyme level in response to either Stat5a or Stat5b gene disruption.

**Fig. 7. Effect of Stat5a**<sup>−/−</sup> or Stat5b**<sup>−/−</sup> on sex-independent liver testosterone hydroxylase activities. Testosterone hydroxylase activities were determined in liver microsomes prepared from wild-type mice (filled bars) and Stat5a**<sup>−/−</sup> or Stat5b**<sup>−/−</sup> mice (speckled bars) as described under “Materials and Methods.” Specific activities shown are mean ± S.E. for n = 5 individual mice per group. A single asterisk represents significant differences from wild type (p < 0.05). Disruption of Stat5a or Stat5b is seen to decrease expression in female but not male mouse liver of some but not other sex-dependent, CYP-catalyzed testosterone hydroxylase activities.

**Fig. 8.** Reverse transcription PCR analysis of liver STAT5 mRNA levels. 2 μg of total RNA isolated from five individual male 129J × Black Swiss mouse livers was reverse-transcribed and then PCR-amplified with STAT5 primers that do not distinguish between STAT5a and STAT5b, as described under “Materials and Methods.” A, the amplified PCR products (550 bp), with or without NcoI digestion, as indicated, were electrophoresed in an ethidium bromide-stained gel. The 550-bp STAT5b cDNA fragment does not contain an NcoI site, whereas the STAT5a cDNA is digested by NcoI to yield fragments of 240 and 310 bp. B, quantitation of the fractions of total STAT5 cDNA that correspond to STAT5a (310- plus 240-bp fragments) and STAT5b (550-bp fragment) after NcoI digestion, as determined by integration of band intensities. In control experiments not shown, the corresponding 550-bp cDNA amplified from cloned STAT5 plasmid DNA was shown to be fully digested with NcoI in the case of STAT5a and not at all digested with NcoI in the case of STAT5b.

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|---------------------------------------------------------------|
| Hydroxytestosterone Metabolite | Male | Female |
|--------------------------------|------|--------|
| 6α-OH | NC* | NC     |
| 15α-OH | NC | NC     |
| 16β-OH | NC | NC     |
| 16α-OH | NC | NC     |
| 2α-OH | NC | NC     |
| 7α-OH | NC | NC     |
| 15α-OH | NC | NC     |
| 16α-OH | NC | NC     |
| 2α-OH | NC | NC     |
| 7α-OH | NC | NC     |
| 15α-OH | NC | NC     |
| 16α-OH | NC | NC     |

* NC, no change in enzyme level in response to either Stat5a or Stat5b gene disruption.

**Fig. 7.** Effect of Stat5a**<sup>−/−</sup> or Stat5b**<sup>−/−</sup> on sex-independent liver testosterone hydroxylase activities. Testosterone hydroxylase activities were determined in liver microsomes prepared from wild-type mice (filled bars) and Stat5a**<sup>−/−</sup> or Stat5b**<sup>−/−</sup> mice (speckled bars) as described under “Materials and Methods.” Specific activities shown are mean ± S.E. for n = 5 individual mice per group. A single asterisk represents significant differences from wild type (p < 0.05). Disruption of Stat5a or Stat5b is seen to decrease expression in female but not male mouse liver of some but not other sex-dependent, CYP-catalyzed testosterone hydroxylase activities.

**Fig. 8.** Reverse transcription PCR analysis of liver STAT5 mRNA levels. 2 μg of total RNA isolated from five individual male 129J × Black Swiss mouse livers was reverse-transcribed and then PCR-amplified with STAT5 primers that do not distinguish between STAT5a and STAT5b, as described under “Materials and Methods.” A, the amplified PCR products (550 bp), with or without NcoI digestion, as indicated, were electrophoresed in an ethidium bromide-stained gel. The 550-bp STAT5b cDNA fragment does not contain an NcoI site, whereas the STAT5a cDNA is digested by NcoI to yield fragments of 240 and 310 bp. B, quantitation of the fractions of total STAT5 cDNA that correspond to STAT5a (310- plus 240-bp fragments) and STAT5b (550-bp fragment) after NcoI digestion, as determined by integration of band intensities. In control experiments not shown, the corresponding 550-bp cDNA amplified from cloned STAT5 plasmid DNA was shown to be fully digested with NcoI in the case of STAT5a and not at all digested with NcoI in the case of STAT5b.
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erodimeric STAT5a-STAT5b complex is the principal mediator of mammapoietic and lactogenic signaling (44). Since STAT5a and STAT5b can thus be activated by prolactin, in addition to GH, the decreased expression of certain Cyp genes in both Stat5a<sup>−/−</sup> and Stat5b<sup>−/−</sup> female mouse liver described in the present study could conceivably reflect a loss of STAT5a- and STAT5b-mediated liver prolactin signaling. However, while the long form of prolactin receptor is expressed in liver tissue, the short form of the receptor is much more abundant. Moreover, prolactin receptor short form can exert a dominant-negative phenotype with respect to STAT activation, such that prolactin-dependent STAT5 activation is not achieved in liver tissue (12, 45, 46). Accordingly, female Cyp gene expression in the liver is not likely to be regulated by prolactin-induced STAT5a-STAT5b activation and heterodimerization, but rather by GH or perhaps other cytokines that can also activate STAT5a and STAT5b (47–49). The factors that regulate the extent of dimerization between STAT5a and STAT5b in the target tissue is likely to be a key factor. In liver tissue, STAT5a appears to be the more minor expressed STAT5 form, both in the rat (36, 37) and in the mouse (Fig. 8). Accordingly, the ratio of activated STAT5b to STAT5a may be much greater than 1, such that homodimeric STAT5b complexes dominate, particularly in males, where the pulsatile plasma GH profile activates STAT5b much more efficiently than in females (12). It is conceivable, however, that in female liver activated heterodimeric STAT5a-STAT5b complexes may be relatively abundant as a consequence of the down-regulation of STAT5b activation in response to the female-characteristic, nearly continuous plasma GH profile (12, 33, 50). Thus, whereas the abundance of activated, homodimeric STAT5b complexes may serve to maintain sexually dimorphic GH responses in male liver, a heterodimeric STAT5a-STAT5b complex in female liver could contribute to the expression of certain female-specific Cyp genes, such as that which encodes CYP2B<sub>b</sub>. Implications for GH-regulated CYP Gene Expression—In males, but not females, Stat5b gene disruption increased female-dominant testosterone hydroxylase enzyme activity and protein expression (Tables I and II). This elevation of normally female-dominant liver enzyme levels suggests that STAT5b may negatively regulate some GH-regulated liver-expressed genes in addition to its demonstrated positive effects on the transcriptional activation of certain male GH pulse-stimulated genes (22).<sup>2</sup> This model is in accord with other studies based on an analysis of CYP enzyme patterns in GH-deficient <i>lit/lit</i> mice, where it is shown that the low expression in male mouse liver of several female-specific, GH-regulated CYP enzymes is at least in part due to the suppressive effects of male GH pulses (2, 6). Precedent for an inhibitory effect of activated STAT5b on gene expression is provided by the transcriptional inhibition by prolactin-activated STAT5b of interferon-regulatory factor-1 (51) and by the inhibitory effects of GH-activated STAT5b on gene transcription stimulated by cross-talk with the nuclear receptor PPARα (52). Thus, while GH pulse-induced expression of male-specific Cyp genes such as rat CYP2C11 or mouse Cyp2d9 may require direct STAT5b-DNA interactions, we hypothesize that GH-dependent Cyp gene products that are female-expressed (<i>e.g.</i> rat CYP2C12 or mouse CYP2B<sub>b</sub>, band b) may in part be regulated by interactions between STAT5b and other factors (<i>e.g.</i> a hypothetically repressed of the female-expressed Cyp genes). These interactions could lead to inhibition of expression in the male, giving rise to the observed female-specific pattern of gene expression.

Distinct intracellular signaling pathways are activated by a nearly continuous (female) compared with an intermittent (male) pattern of plasma GH stimulation and have been implicated in the sex-dependent expression of certain Cyp genes in rat liver (1). These include the JAK/STAT5b pathway in the case of males (12) and pathway(s) that may involve a novel GH-regulated nuclear factor, termed GHNF (53) and perhaps also phospholipase A2 signaling (54) in females. In addition, the present study demonstrates that in female mouse liver STAT5a and STAT5b are both required for expression and thus may be important regulators, of certain female-specific CYP enzymes. The interactions of STAT5 with female-expressed Cyp genes may be direct, e.g. could involve STAT5a-STAT5b DNA-protein complexes, or may be indirect. Conceivably, Cyp genes, even within the same gene subfamily, may respond to distinct GH-dependent signaling pathways. Alternatively, a single GH-activated signaling pathway may regulate different Cyp genes, in some cases leading to activation and in other cases leading to inhibition of Cyp expression. Further study will be required to elucidate the multiple GH-activated signaling pathways that activate members of this multigene family and to establish the precise roles that STAT5a and STAT5b play in their expression.

After completion of the present study, Teglund et al. (55) confirmed our earlier report (23) that Stat5b gene disruption leads to loss of the GH pulse-regulated male pattern of postpubertal body growth rate and liver gene expression. They also reported the lack of an effect of Stat5a gene disruption on male-specific CYP2D expression, in agreement with the present study.

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