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Plasma Growth Hormone Pulse Activation of Hepatic JAK-STAT5 Signaling: Developmental Regulation and Role in Male-Specific Liver Gene Expression

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ABSTRACT

The intracellular signaling molecule STAT5 is activated in rat liver by the intermittent male plasma GH pattern to a 10-fold higher level than by the more continuous pattern of plasma GH stimulation seen in females. Individual adult male rats are presently shown to exhibit large differences in liver STAT5 DNA-binding activity, which correlates with the presence of significant levels of GH in plasma at the time of liver excision. Examination of STAT5 activity as a function of postnatal development revealed that these intermittent pulses of liver STAT5 activity are first observed at 5 weeks of age, when plasma GH pulsation first begins and expression of male-specific, GH pulse-activated liver genes, including *CYP2C11,* first occurs. Prepubertal rats exhibited low liver STAT5 activity, likely a consequence of the absence of high plasma GH pulses in these animals. Proteins required for GH activation of STAT5 are expressed in liver before puberty, and correspondingly, STAT5 can be precociously activated by exogenous administration of GH pulses given to 2-week-old rats, albeit with a lower sensitivity to GH than is seen in hypophysectomized adult rats. However, this precocious activation of STAT5, via twice daily administration of GH for 7 days, did not lead to *CYP2C11* expression or masculinization of hepatic enzyme profiles, unlike in GH pulse-stimulated hypophysectomized adult rats. Based on these findings we conclude: 1) liver STAT5 is repeatedly activated in adult male rats in direct response to the intermittent pattern of plasma GH stimulation; 2) the developmental onset of this STAT5 activation pattern supports the proposed requirement of STAT5 transcriptional activity for male-specific, GH pulse-regulated hepatic gene expression; and 3) the activation of STAT5 is, by itself, not sufficient to impart the adult male pattern of liver gene expression, suggesting a requirement for additional liver factors that are absent in prepubertal rats. (*Endocrinology* **141:** 3245–3255, 2000)

GH IS A pituitary polypeptide hormone that regulates a variety of physiological processes (1, 2). In rodents, GH regulates hepatic genes that are expressed in a sex-specific manner, including cytochrome P450 (CYP) genes involved in steroid hydroxylation (3). Prototypical examples of sex-specific, GH-regulated genes include *CYP2C11,* which is expressed exclusively in livers of adult male rats, and *CYP2C12* which is expressed in adult female but not male rats. This sex-specificity of gene expression occurs at the level of transcription initiation (4, 5) and is a consequence of sex differences in the temporal pattern of GH release from the pituitary. In adult male rats, GH is released from the pituitary in an intermittent manner such that there are pulses of GH in the plasma (200–300 ng/ml) each approximately 3.5 h separated by a trough period of no detectable GH (≤2 ng/ml). In adult female rats, GH release from the pituitary is more frequent, resulting in a more persistent presence of circulating GH at an average plasma level of approximately 40 ng/ml (2, 6–8).

Recent investigations focusing on the mechanism by which hepatocytes discriminate between circulating plasma GH patterns have implicated the transcription factor STAT5b as an important intracellular mediator of GH pulse-activated, male-specific liver gene expression (9–11). STAT5b and the closely related STAT5a (>90% identical) (12, 13) belong to a family of Signal Transducers and Activators of Transcription that mediate the effects of a broad range of cytokines, growth factors, and hormones on various target tissues, including the liver (14). Exogenous administration of GH to hypophysectomized (GH-depleted) rats leads to the rapid activation of cytoplasmic liver STAT5[1] to yield a tyrosine phosphorylated nuclear dimer of STAT5 that has DNA-binding and transcriptional activity (11, 15). By contrast, treatment of hypophysectomized rats with GH administered continuously, *i.e.* in a female-like manner, effects a dramatic decrease in liver STAT5 activity (11, 16). STAT5a and STAT5b are both activated by male plasma GH pulses (16), although STAT5b, but not STAT5a, is obligatory for maintenance of the male-pattern of hepatic gene expression, as demonstrated in gene knockout studies (10, 17, 18).

The kinetics of STAT5b activation following GH pulse stimulation have been elucidated using the cell line CWSV-1, an SV40-immortalized rat hepatocyte-derived cell line that is responsive to GH (19). Application of intermittent GH pulses, but not continuous GH treatment, strongly activates STAT5b, which is the major STAT5 form present in these cells (20) and in liver (17). Repeated application of GH pulses to the cells stimulates repeated cycles of STAT5b activation via tyrosine phosphorylation and nuclear translocation, followed by deactivation via tyrosine dephosphorylation and return to the cytosol (21). Full STAT5b responsiveness to a

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[1] Liver STAT5 is predominantly comprised of STAT5b, but includes the less abundant STAT5a. Both STAT forms contribute to the EMSA activities measured in this study (16, 17).

second GH pulse requires a minimum off-period of 2.5 h (20), similar to the off-time between successive GH pulses seen in adult male rats *in vivo* (6, 7). This responsiveness of STAT5b to GH pulses applied in cell culture or given to hypophysectomized rats supports the proposal (11) that the substantially higher STAT5 activity in male compared with female rat liver is a direct reflection of the activation of STAT5b by physiological male GH pulses. A more direct evaluation of this hypothesis requires the examination of the temporal relationship in intact male rats between the occurrence of a plasma GH pulse and the presence of liver STAT5 in its active form. This would help establish whether STAT5b is repeatedly activated by the endogenous male-specific plasma GH pattern *per se*, or alternatively, whether the high STAT5b activity seen in male liver is due to other endogenous male-specific factors.

The onset of the sexual dimorphism of pituitary GH secretion during development is well characterized (22, 23). Before puberty, GH is present at low levels in the plasma of both male and female rats, and consequently, GH-responsive, sexually dimorphic hepatic genes are expressed at a low level (*CYP2C12*) or not at all (*CYP2C11*). Beginning at puberty (approximately 5 weeks postnatal in the rat), male rats exhibit their characteristic pulsatile plasma GH pattern leading to expression of *CYP2C11* and loss of the low prepubertal levels of *CYP2C12* (24–26). STAT5b is proposed to contribute to the GH-regulated expression of these and other sexually dimorphic liver genes (27); however, the expression of STAT5b and its activation during the course of male postnatal development have not been investigated. If STAT5b is indeed an intracellular mediator of the effects of plasma GH pulses on male-specific liver genes, then changes in liver STAT5b activity would be expected to accompany changes in circulating GH during postnatal development. To investigate these issues, we presently address the following questions. Does liver STAT5 activity vary in direct response to the occurrence of a plasma GH pulse? Do changes in liver STAT5 activity correlate with the developmental onset of *CYP2C11* expression? Are the factors required for STAT5 signal transduction, including STAT5a, STAT5b, and the tyrosine kinase JAK2, expressed before puberty, or is the expression of these factors itself dependent on pubertal GH stimulation? Finally, is the activation of hepatic STAT5 by plasma GH pulses sufficient to activate *CYP2C11* and confer a male pattern of hepatic gene expression? Our findings lead us to conclude that liver STAT5 is temporally activated in response to successive plasma GH pulses and is developmentally activated in parallel to *CYP2C11* gene expression. However, STAT5 activation alone, although necessary, is not itself sufficient to induce an adult male pattern of liver gene expression.

Materials and Methods

Administration of GH to prepubertal rats

Litters of 1-week-old Fischer 344 rats were purchased from Taconic Farms, Inc. (Germantown, NY) and housed for 1 week at the Boston University Animal Care Facility. Rat GH (rGH-B-14-SIAFP, obtained from Dr. A. Parlow and the National Hormone and Pituitary Program, NIDDK) was subsequently administered to the 2-week-old pups at a dose of either 3 or 50 μg GH/100 g BW, ip, and the animals killed 30 min later. In other studies, GH was given by sc injection twice daily (0800 h and 2000 h) for 1, 2, 4, or 7 days at a dose of 50 μg GH/100 g BW/injection. Mother rats were removed from their cages, their pups weighed and injected with GH, and the mother subsequently returned to the cage and suckling allowed to resume. Pups were killed either 12 h after the last GH injection, or were given one additional GH injection on the last morning of the GH treatment period and killed 30 min later. This latter time point was chosen because liver STAT5 activity is near-maximally induced within 30 min in GH-injected hypophysectomized rats (11). Livers were removed, snap-frozen in liquid nitrogen, and transferred to −80 C for storage.

STAT5 activity analysis in rat liver homogenates

Rat liver homogenates were prepared from frozen liver tissue and electrophoretic mobility shift analysis (EMSA) assays were performed using a STAT5-specific DNA probe derived from the promoter of the rat β-casein gene, as previously described (16, 20). Approximately 200–400 mg of frozen rat liver tissue was homogenized at 4 C in 2 ml of ice-cold homogenization buffer (10 mM Tris pH 7.6, 1 mM EDTA, 250 mM sucrose) containing a mixture of protease inhibitors and phosphatase inhibitors. Samples were centrifuged at 9,000 rpm for 20 min at 4 C. Supernatants were aliquoted, snap-frozen in liquid nitrogen, and stored at −80 C. The validity of using liver homogenates prepared in this manner to assay liver nuclear STAT5 activity has been previously established (16). This assay measures both STAT5a and STAT5b, although STAT5b is the more abundant contributor to total hepatic STAT5 EMSA activity. EMSA gels were dried and exposed to phosphorimager plates for 1–3 days. Analyses were done on a Molecular Dynamics, Inc. PhosphorImager (Sunnyvale, CA) with quantitation using ImageQuant software (16). Background values (typically corresponding to 2–5% of a maximal male liver STAT5 signal) were determined based on the average of 2–4 blank regions from each gel and were subtracted from all samples on the gel to yield net activity values. These values were then expressed as a percentage of a standard high STAT5 activity male rat liver sample or the average of several such male rat liver samples.

Statistical analyses were performed using Prism GraphPad Software, Inc. Linear and nonlinear (rectangular hyperbola) regressions were performed on the same data set. The saturation curve shown (see Fig. 2C) was drawn by the computer from the nonlinear regression analysis performed.

GH RIA

Blood was withdrawn from individual rats by cardiac puncture and placed at 20–22 C for 20 min. Samples were centrifuged for 20 min at 4 C to obtain plasma, which was stored at −20 C. Rat plasma GH levels were subsequently determined by RIA performed in the laboratory of Dr. Gloria Tannenbaum (McGill University, Montréal, Canada) using standard protocols (28). Standard curves obtained with this assay are linear up to 320 ng/ml and the least detectable concentration of plasma GH is 1.2 ng/ml.

Microsomal testosterone hydroxylation assay

Cytochrome P450-dependent microsomal metabolism of testosterone was assayed at 37 C with shaking (29). Incubations contained 20 μg of rat liver microsomal protein in 0.2 ml containing 100 mM Tris buffer, pH 7.6, 0.5 mM $MgCl_2$, and ^{14}C-labeled testosterone (10 nmol, ~100,000 cpm; Amersham Pharmacia Biotech, Arlington Heights, IL). Reactions were initiated by the addition of 0.98 mM NADPH and terminated 10 min later by the addition of 1 ml ethyl acetate. Testosterone and hydroxytestosterone metabolites were extracted with ethyl acetate and then chromatographed on silica gel TLC plates developed sequentially in solvent A [methylene chloride/acetone (80:20, vol/vol)] and then solvent B [chloroform/ethyl acetate/ethyl alcohol (70:17.5:12.5, vol/vol/v)] (29). TLC places were exposed to Molecular Dynamics, Inc. Phosphoimager plates for 48 h and the radioactivity content and molar abundance of each individual testosterone metabolite then quantitated using ImageQuant software.

Western blotting

Liver microsomes (40 μg) or whole liver homogenates (40 μg) were electrophoresed through Laemmli SDS gels (7.5% gels) run at constant

current and a starting voltage of 75 V, with cross-over to a constant voltage of 170 V. Gels were electrotransferred to nitrocellulose and probed with the following antibodies: anti-STAT5a, anti-STAT5b and anti-JAK2 (antibodies sc-1081, sc-835, sc-294, respectively, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or anti-CYP2C11 (generously provided by Dr. J. Capdevilla, Vanderbilt University, Nashville, TN). Blocking and antibody probing conditions were as previously described (11). Detection on x-ray film was accomplished by enhanced chemiluminescence using ECL reagents (Amersham Pharmacia Biotech).

Results

Differential activation of liver STAT5 in individual male rats in direct response to a plasma GH pulse

Administration of exogenous, male-like GH pulses to hypophysectomized rats stimulates the tyrosine phosphorylation, nuclear translocation, and DNA-binding activity of liver STAT5 (11). We investigated whether the activation of liver STAT5 seen in this exogenous GH replacement model is representative of the activation of STAT5 that occurs in intact male rats under the influence of natural plasma GH pulses. Specifically, we examined whether liver STAT5 activity varies in direct relation to the occurrence of a plasma GH pulse at the time of liver excision. A group of 8 individual 10- to 12-week-old male rats were killed and their livers analyzed for STAT5 EMSA activity. As shown in Fig. 1, the individual male rats exhibited large differences in hepatic STAT5 activity (panels A and B). Furthermore, GH RIA analysis of plasma obtained from the same rats revealed a direct relationship between the presence of significant amounts of GH (*e.g.* rats 1, 3, 4, 7, and 8; Fig. 1C) and the occurrence of a strong liver STAT5 EMSA signal (c.f., Fig. 1B)

To further investigate the relationship between liver STAT5 activity and circulating plasma GH levels, we assayed liver STAT5 activity and plasma GH levels in several additional groups of rats. The overall results obtained (n = 29 male rats) are summarized in Fig. 2. STAT5 activity values were calculated relative to the average value of 4 livers[2] showing a typical high STAT5 activity profile (livers 19, 22, 25, 27; Fig. 2A). A correlation between liver STAT5 activity (panel A) and plasma GH at the time of liver excision (panel B) was apparent, both when analyzed by linear regression analysis (r = 0.84; data not shown), and when a nonlinear regression analysis (rectangular hyperbola) was performed (r = 0.85) (Fig. 2C). STAT5 activation is shown to plateau with increasing plasma GH concentration (B_{max} = 163.2% relative STAT5 activity). A similar correlation (r = 0.84) was seen when the high outlier data point (liver 29) was removed (B_{max} = 104%). These results indicate that liver STAT5 activity reaches a maximal level beyond which there is diminished response to further increases in plasma GH levels. This maximal STAT5 activity level may reflect saturation of GH receptors present on the cell surface and/or depletion of the unphosphorylated STAT5 pool present in the cytosol.

To distinguish basal liver STAT5 activity from the GH pulse-inducible liver STAT5 activity, individual male rats were designated plasma GH positive where plasma GH levels at the time of liver excision were >3.7 ng/ml, corresponding to 3-fold above the least detectable GH concentration under the conditions of the RIA (28). Rats having plasma GH values below this level were designated GH pulse-negative, and presumably correspond to animals killed between plasma GH pulses. Analysis of the 29 adult male samples using these criteria revealed (Table 1) that 28 individuals exhibited a direct correlation between liver STAT5 activity and the occurrence of a GH pulse: 17 plasma GH-positive rats all showed substantial liver STAT5 activity [*i.e.* activity ≥ the average STAT5 activity level of 9.6 ± 1.7% seen in adult female rats (16)]; and 11 rats showed low STAT5 activity and low GH levels. The one rat that did not fit this general pattern (rat 20; Fig. 2C, circled data point) showed a high plasma GH level (34 ng/ml) but very low STAT5 activity (1.4%).

A further examination of these data reveals that none of the

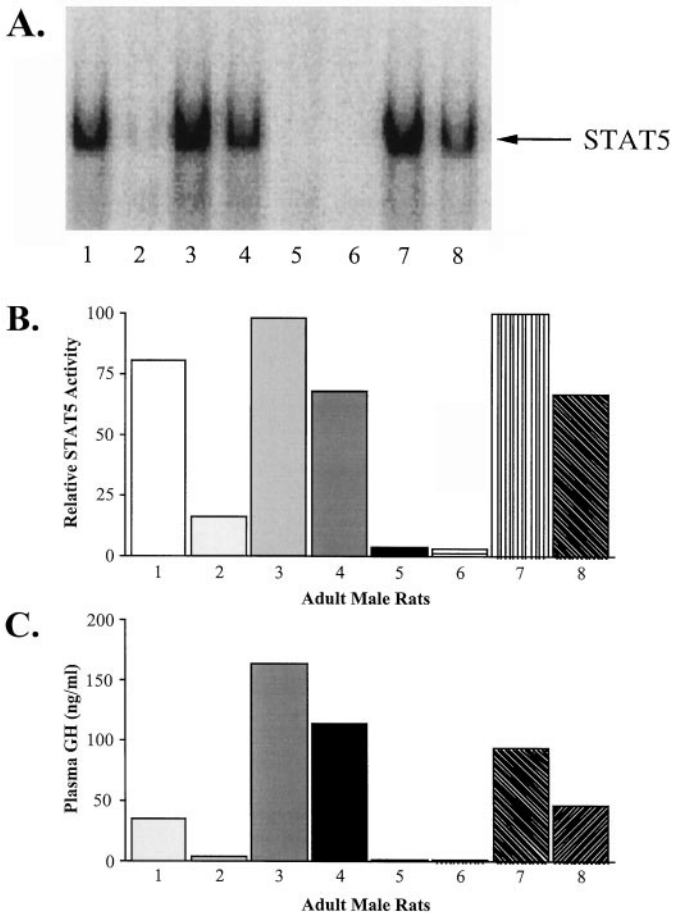

FIG. 1. Liver STAT5 DNA-binding activity and plasma GH levels in individual adult male rats. A, Whole liver homogenates prepared from eight 10- to 12-week-old male rats were prepared as previously described (16), and 30 μg was analyzed by EMSA using a β-casein probe, which forms a distinct STAT5 protein-DNA complex (*arrow*). Shown is a phosphoimage of the EMSA gel. B, Intensities of STAT5 EMSA bands shown in panel A were quantitated by exposure to phosphorimager plates and the analyzed using ImageQuant software. The STAT5 activity of each liver is expressed as a percentage of the highest intensity seen (lane 3) among the eight individual livers after correction for background, as described in *Materials and Methods*. C, Plasma GH levels at time of liver excision were determined using blood samples removed from each of the individual rats shown in panels A and B.

[2] Liver 29, which exhibited an unusually high liver STAT5 activity, was not included in setting the 100% relative STAT5 activity value.

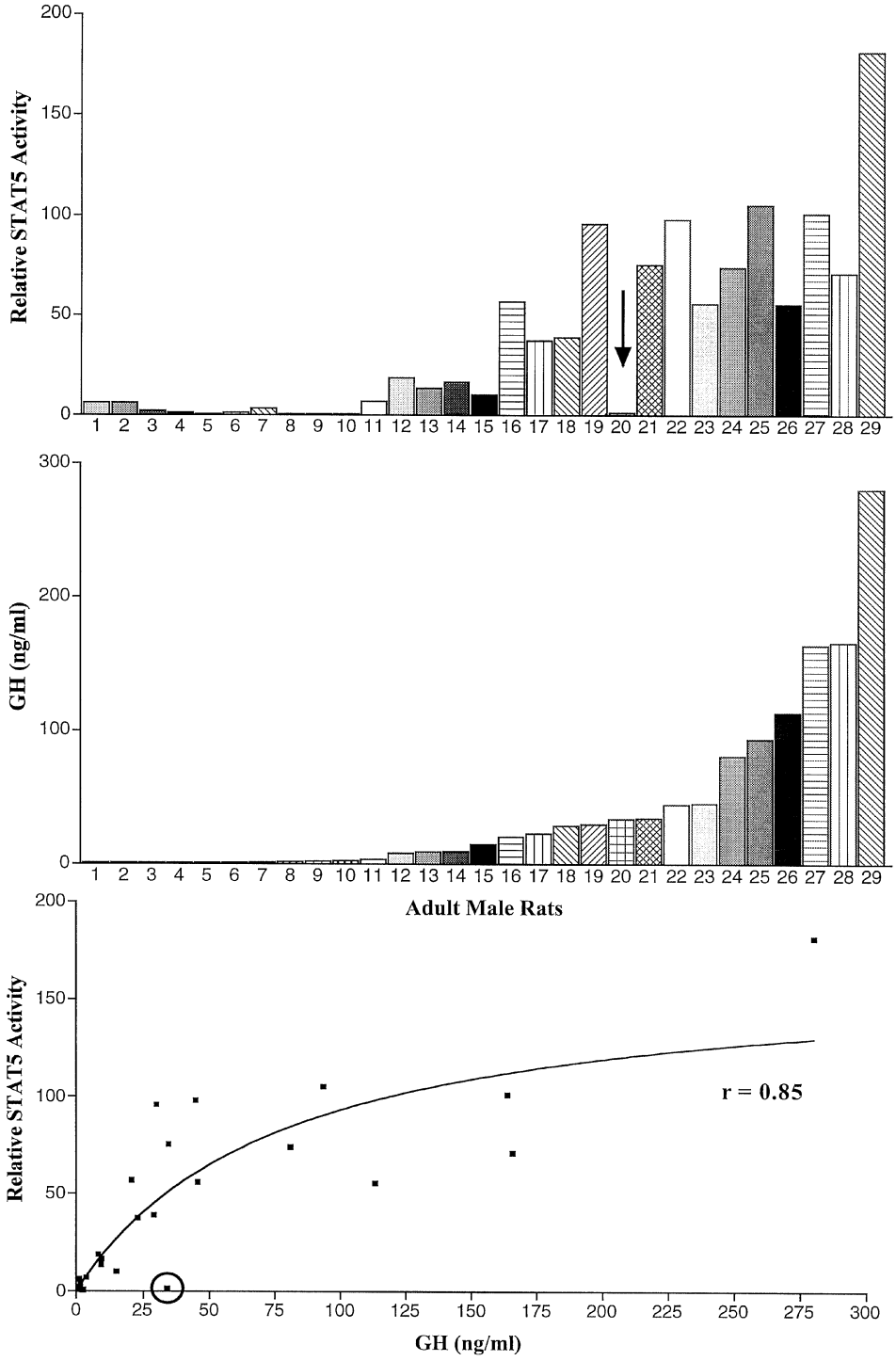

FIG. 2. Relationship between liver STAT5 activity and circulating plasma GH levels in 29 individual adult male rats. A, STAT5 DNA-binding activity was assayed by EMSA for 29 individual adult male rats as described in Fig. 1. Individual animals are numbered on the *x*-axis and are ordered according to increasing plasma GH levels (c.f., panel B). One rat (*arrow*) exhibited no liver STAT5 activity, despite a significant level of plasma GH at the time of liver removal (see panel B; also see data point circled in panel C). B, Plasma GH levels at time of liver removal for the same group of rats shown in Panel A. C, Correlation of the relative STAT5 activity *vs.* plasma GH (ng/ml). The data were fit to a rectangular hyperbola using nonlinear regression analysis and GraphPad Software, Inc. Prism software. *Data point circled* corresponds to a plasma GH-positive rat with a low STAT5 EMSA activity.

STAT5-positive livers was GH negative (Fig. 2; Table 1). Furthermore, no correlation (r = 0.02) between liver STAT5 activity and plasma GH level was seen among the 11 samples in the low STAT5 and low plasma GH level group. Also, the STAT5 activity level of the low plasma GH group (2.8 ± 0.8%) is significantly lower than that of adult females determined earlier using the same methodology (9.6 ± 1.7%) (16). The absence of a correlation between STAT5 activity and plasma GH level in the low plasma GH group suggests that a threshold level of circulating GH is required for activation of STAT5. The apparent threshold GH level, 3.7 ng/ml, compares favorably with the reported K_d of the GH-GH receptor complex [2.2 ng/ml; (30)]. The positive correlation in individual adult male rats between liver STAT5 activity and the presence of significant levels of GH (>3.7 ng/ml) at the time of liver excision provides strong support for the earlier proposal, based on hypophysectomized rat studies (11), that liver STAT5 directly responds to each successive plasma GH

pulse, and thus undergoes repeated cycles of activation and nuclear translocation in intact adult male rats.

Activation of STAT5 during postnatal development

Expression of the male-specific, GH pulse-activated *CYP2C11* is not detected in rat liver until 4.5–5 weeks of age (25), *i.e.* the time of onset of the plasma GH pulses that characterize pubertal and adult male rats. Prepubertal rats are characterized by a more continuous presence of low-levels of plasma GH (22, 23), which supports expression at a low level of the adult female rat P450 form CYP2C12 in both male and female rats at 3–4 wk of age (25). At puberty, when pulsatile pituitary GH secretion begins, *CYP2C11* gene expression begins. To ascertain whether there is a correlation between the developmental onset of sex-specific GH profiles (and consequently, *CYP2C11* expression) and liver STAT5 activation, liver homogenates prepared from individual male rats killed at different time points after birth were assayed for STAT5 EMSA activity (Fig. 3). Low liver STAT5 activity was seen in rats aged 4 days or 2 weeks. Liver STAT5 activity in 4-week-old rats was somewhat higher, but still low compared with that of pubertal and adult rats, and with no apparent dependence on the plasma GH concentration (Table 2). The 4-week-old rats exhibited low GH levels (ranging from 1.5–12 ng/ml) except for one sample which had an unusually high level of plasma GH (359 ng/ml) but little STAT5 activity (Fig. 3A, lane 17). Beginning at week 5, the differential activation of liver STAT5 in individual male rats was seen, and this activation correlated with the presence of GH in plasma at the time of liver excision (Fig. 3B, lanes 2–9). This plasma GH-dependent activation of STAT5 was also observed in rats aged 8 and 12 weeks (Fig. 3C; Table 2), as seen earlier for the larger group of adult males (Fig. 2). Western blot analysis of these same liver samples revealed a striking increase in CYP2C11 protein beginning at 5 wk (Fig. 4), which paralleled the onset of the pulsatile STAT5 activation profile (Fig. 3).

TABLE 1. STAT5 activity *vs.* plasma GH levels in 29 individual adult male rats

	STAT5 positive	STAT5 negative	Total
GH positive	17/29	1/29	18/29
GH negative	0/29	11/29	11/29
Total	17/29	12/29	

Quantitation of STAT5 EMSA activity was as described in *Materials and Methods* and shown in Fig. 2. Individual rats were designated liver STAT5 positive if EMSA activity was ≥9.6% of maximal activity seen in adult males and GH pulse positive if plasma GH levels were ≥3.7 ng/ml (see text for further details).

STAT5a, STAT5b, and JAK2 are expressed throughout postnatal development

We next examined whether the low STAT5 DNA-binding activity seen in prepubertal rat liver reflects a deficiency of either STAT5a or STAT5b, both of which contribute to the STAT5 activity signal in rat liver (16). We also examined JAK2 protein levels because this tyrosine kinase catalyzes the

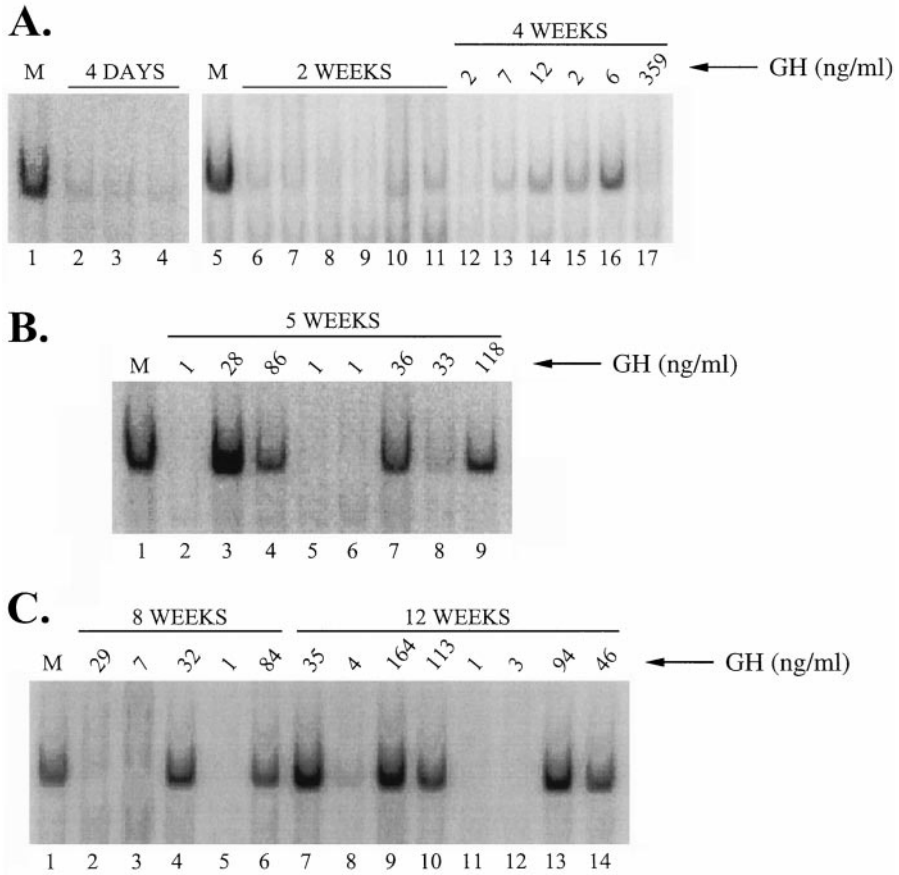

FIG. 3. Liver STAT5 DNA-binding activity during postnatal development. Livers obtained from postnatal male rats aged 4 days to 12 weeks were analyzed for STAT5 activity by EMSA as described in *Materials and Methods*. Corresponding plasma GH levels measured for each rat are indicated above each lane. Blood samples could not be obtained from the 4-day-old and 2-week-old pups due to their small size. Included as a reference on the *left* of each EMSA gel is an adult male sample (M) that exhibits high STAT5 DNA-binding activity.

GH-dependent activation of STAT5 (31, 32). Western blotting of liver homogenates prepared from rats killed at different developmental ages revealed the expression of all three proteins as early as postnatal day 4, and at levels that were maintained at least through 8 wk of development (Fig. 5). Prepubertal expression of these proteins is thus independent of the plasma GH pulses that occur in pubertal and adult male rats. Accordingly, given that GH receptor is also expressed in prepubertal rat liver (33), the low-level liver STAT5 activity seen in rats aged 4 weeks and younger is suggested to result from the absence of a sufficiently strong stimulatory plasma GH pulse in these animals, rather than the absence of the protein factors required for activation of the STAT5 signaling cascade.

Intrinsic responsiveness of liver STAT5 to GH pulses in prepubertal rats

Given the presence of JAK2, STAT5a, STAT5b, and GH receptor in liver before the pubertal onset of plasma GH pulses, we investigated the intrinsic responsiveness of STAT5 to exogenous GH pulses given to prepubertal rats. Two doses of GH were used in this study: 3 μg GH/100 g BW, which gives a physiologic plasma GH level of approximately 225 ng/ml when administered to hypophysectomized adult rats (34, 35), and 50 μg GH/100 g BW, which corresponds to a supraphysiologic GH dose. Treatment of prepubertal rats (2–3 weeks old) with the exogenous pulse of GH led to the activation of liver STAT5 within 30 min (Fig. 6). This response confirms the presence of functional GH receptor, JAK2 kinase and STAT5 in prepubertal rat liver, and indicates that the activation of STAT5 by GH does not require additional factors that are expressed following puberty. Treatment of the prepubertal rats with GH at 50 μg/100 g BW stimulated activation of liver STAT5 (55 ± 8% of adult male control, mean ± SEM, n = 5; Fig. 6, lanes 14–18 *vs.* 4 ± 1.3% STAT5 activity in sham-injected controls, mean ± SEM, n = 5, Fig. 6, lanes 5–9) ($P < 0.05$). Administration of a physiologic GH dose of 3 μg/100 g BW also led to a significant, albeit less-pronounced, activation of STAT5 (16 ± 2.2% of adult male control, mean ± SEM, n = 4, Fig. 6, lanes 10–13; *vs.* 4 ± 1.3% in sham-injected controls, mean ± SEM, n = 5, lanes 5–9) ($P < 0.05$). This finding is in contrast to our earlier investigations in hypophysectomized rats (11) where GH at a replacement dose of 3 μg/100 g BW stimulated maximal STAT5 activation. We conclude that the STAT5 signaling cascade is intrinsically functional and responsive to GH in prepubertal rat liver, albeit with a requirement for higher GH concentrations to achieve maximal STAT5 activation compared with hypophysectomized adult rats.

TABLE 2. Comparison of relative liver STAT5 activity in rats aged 4–12 wk with high and low circulating plasma GH levels

Age (weeks)	Relative STAT5 Activity	
4 days	4.1 ± 0.7 (3)	
2	8.0 ± 2.0 (6)	
	High GH	Low GH
4	19.3 ± 8.9 (4)	11.7 ± 10.8 (2)
5	66.5 ± 24.0 (5)	3.3 ± 0.2 (3)
8	37.6 ± 12.4 (4)	9.7 (1)
12	69.2 ± 13.5 (6)	5.9 ± 2.9 (2)

Quantitation of STAT5 EMSA activity (Fig. 3) was as described in *Materials and Methods.* Individual rats were grouped according to whether the plasma GH level at the time of liver removal was high (>3.7 ng/ml) or low (<3.7 ng/ml). STAT5 EMSA activity values shown (mean ± SEM (n) are relative to the same high activity male standard used in Fig. 3. Differences between the high and low GH 4-week groups were not statistically significant ($P > 0.05$). Also shown (*top two lines*) are relative liver STAT5 activities in rats aged 4 days and 2 weeks, where blood samples were not obtained for GH analysis.

Prepubertal rats administered exogenous GH pulses do not express CYP2C11 *precociously*

Twice-daily administration of GH to hypophysectomized (GH-depleted) adult male or female rats for 7 days at a dose of 35–85 μg GH/100 g BW stimulates expression of near-normal adult male levels of liver *CYP2C11* (36). Liver STAT5 activity is *necessary* for expression of adult male-specific liver genes regulated by intermittent plasma GH pulses (9, 10), but it is not known whether liver STAT5 activity is *sufficient* for this expression. Our finding that GH-stimulated STAT5 signaling in prepubertal rat liver is intrinsically functional (Fig. 6) provided the opportunity to investigate the sufficiency of STAT5 activity for male-specific liver gene expression.

Two-week-old pups were administered GH by sc injection twice-daily (0800 h and 2000 h) for periods ranging from 1 to 7 days at a dose of 50 μg GH/100 g BW. Pups were killed 12 h after the last GH injection. This hormone injection regimen is effective in restoring normal adult male levels of liver CYP2C11 mRNA and activity in hypophysectomized adult rats (36). Moreover, this dose of GH activates STAT5 in prepubertal rats to a level (Fig. 6) that is more than sufficient to restore *CYP2C11* expression in hypophysectomized adults (see *Discussion*). Western blot analysis of liver microsomes prepared from the

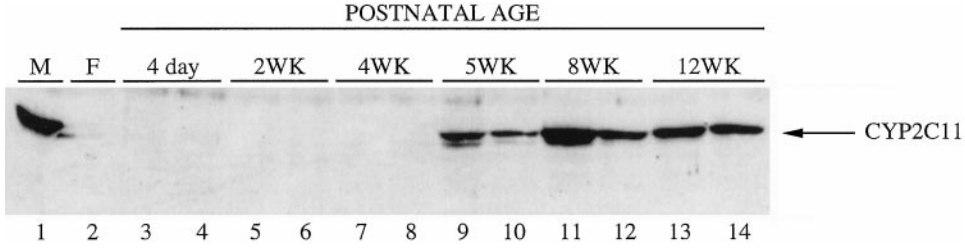

FIG. 4. Induction of CYP2C11 protein in male rat liver during postnatal development. Shown is a Western blot analyzing CYP2C11 protein in whole liver homogenates from male rats aged 4 days to 12 weeks (lanes 3 to 14). Lanes 1 and 2 include positive and negative controls for CYP2C11 protein, corresponding to whole liver homogenates from an adult male (M) and an adult female (F), respectively.

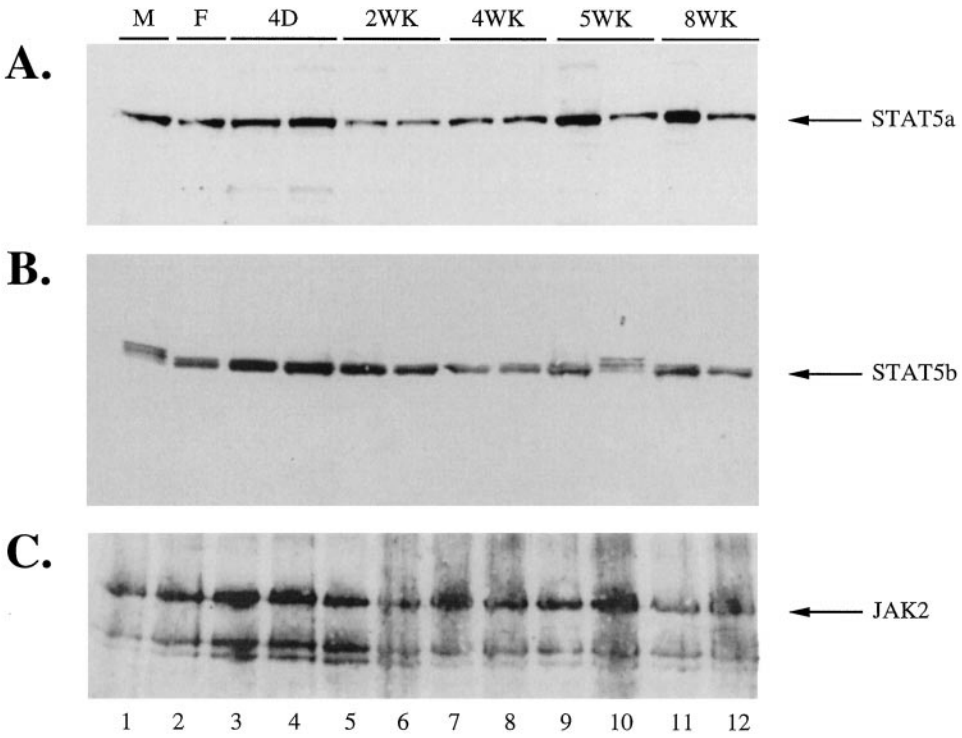

FIG. 5. STAT5a, STAT5b, and JAK2 protein in male rat liver during postnatal development. Shown is a Western blot analyzing whole liver homogenates prepared from male rats aged 4 days to 8 weeks (lanes 3 to 12). Blots were probed with antibodies to STAT5a, STAT5b, and JAK2 kinase, as indicated. Adult male (M) and adult female (F) livers are shown for reference in lanes 1 and 2. Liver samples with high STAT5 activity exhibit a characteristic, lower mobility STAT5b band (*e.g.* lanes 1, 10), which corresponds to STAT5b phosphorylated on both tyrosine and serine (20, 38).

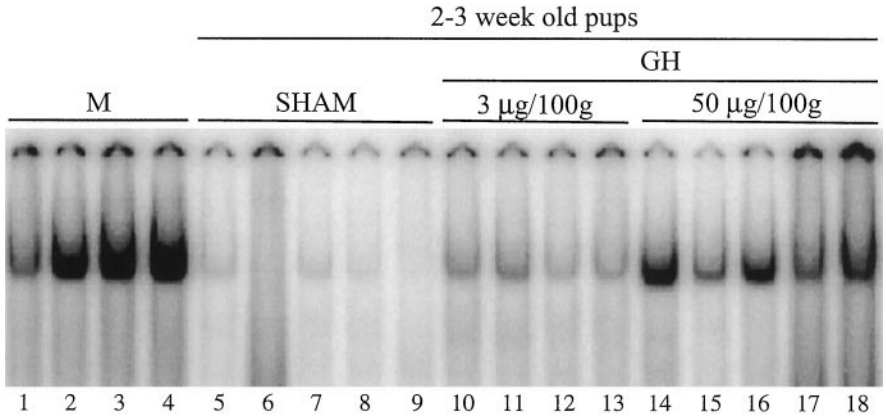

FIG. 6. Precocious activation of STAT5 DNA-binding in prepubertal rats by exogenous GH injection. Prepubertal male rats 2 to 3 weeks old were administered GH at either 3 μg/100 g BW, ip, or 50 μg/100 g BW, ip, and killed 30 min later. Shown is an EMSA analysis of STAT5 DNA-binding activity in liver homogenates prepared from individual rats. Quantitation revealed relative STAT5 EMSA activities of 100 $\pm$ 22 (adult males, lanes 1–4), 4 $\pm$ 1.3 (pups sham-injected with vehicle; lanes 5–9), 16 $\pm$ 2.2 (3 μg GH dose, lanes 10–13) and 55 $\pm$ 8 (50 μg GH dose, lanes 14–18).

GH pulse-treated pups revealed no induced expression of CYP2C11 protein (Fig. 7A). This finding was confirmed by enzymatic analysis of liver microsomal, P450-dependent testosterone hydroxylase activities. Whereas adult male rats exhibited high CYP2C11-dependent liver microsomal testosterone 2α- and 16α-hydroxylase activity, GH pulse-treatment of prepubertal pups did not increase these activities above that of the very low levels seen in sham-treated pups (Table 3). Thus, in contrast to the GH pulse responsiveness of hypophysectomized adult rats, hepatic enzyme profiles were not masculinized by GH pulse injection into prepubertal rats. Analysis of female-dependent, GH-regulated hepatic microsomal activities revealed no significant changes in testosterone 7α-hydroxylase (CYP2A1-dependent) and steroid 5α-reductase activities (Table 3).

The effectiveness of the twice daily GH treatment regimen with respect to repeated activation of STAT5 over the course of the 7-day treatment period was examined in pups given one additional GH injection 12 h after the previous injection, and then killed 30 min later. As shown in Fig. 7, B and C, pups treated with GH for either 2 or 7 days and killed 12 h after final injection showed little residual STAT5 activity, which is a consequence of STAT5 being dephosphorylated and returned to the cytosol (20, 21) during the intervening 12 h. However, pups given one additional injection of GH and killed 30 min later clearly

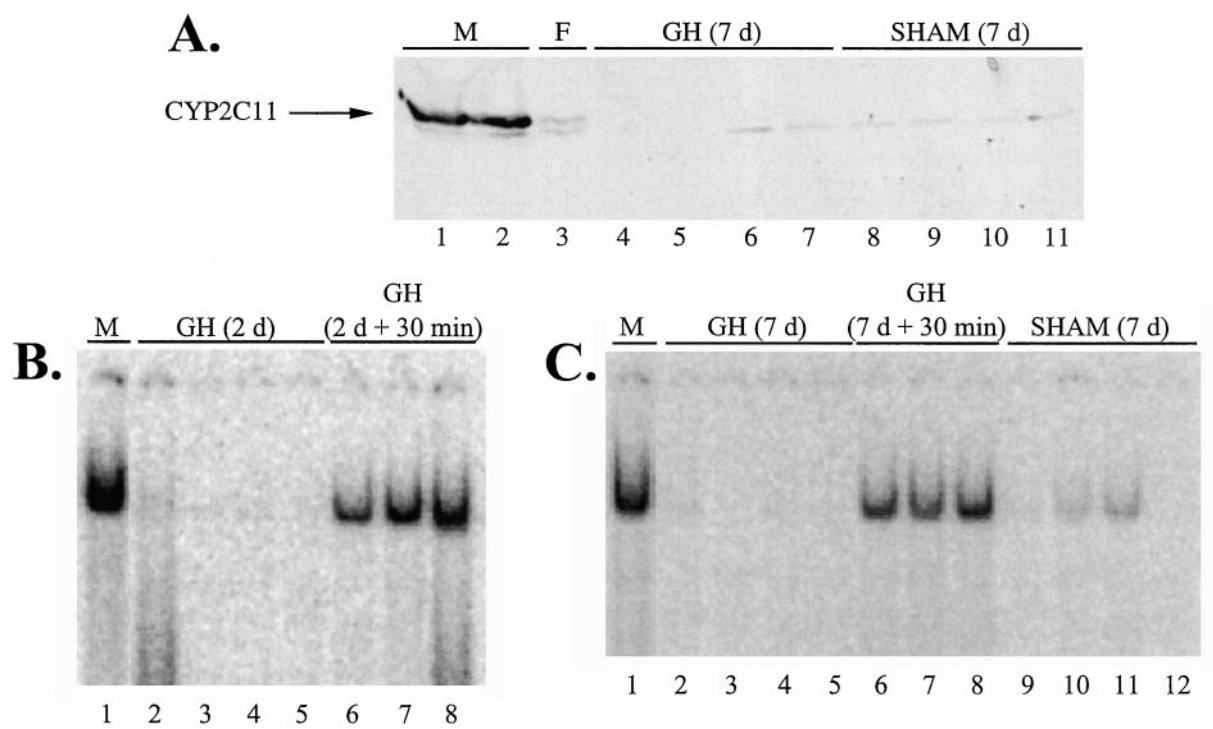

FIG. 7. Impact of GH injection on STAT5 DNA-binding activity and *CYP2C11* expression in prepubertal rats. A, Shown is a Western blot of liver microsomes prepared from individual livers probed with anti-CYP2C11 antibody. GH was administered to 2 wk old rats by sc injection given twice daily (0800 h and 2000 h) for 7 days (7 d) at a dose of 50 μg/100 g BW per injection (males, lanes 4–5; females, lanes 6–7). Animals were killed 12 h after the last injection. Four rats given twice daily vehicle injections served as controls (SHAM) (males, lanes 8–9; females, lanes 10–11). Included are adult male liver microsomes (lanes 1, 2; M) and liver microsomes prepared from an adult female rat (lane 3; F), which respectively serve as positive and negative controls for CYP2C11 protein. Faint band beneath the major band in lanes 1 and 2 (also seen in lane 3 and in lanes 6–11) is nonspecific. B and C, EMSA assay to verify the activation of STAT5 in liver *in vivo* following GH injection. Whole liver homogenates prepared from prepubertal rats treated with GH twice daily, as described in panel A, for either 2 days (2 d) (panel B, lanes 2–5) or 7 days (7 d) (panel C, lanes 2–5) were analyzed by EMSA for STAT5 DNA-binding activity. To ensure that multiple injections into prepubertal rats resulted in repeated STAT5 activation, some of the rats were given one additional GH injection 30 min before liver removal after completing a series of either 2 days or 7 days of twice daily GH injection (2 d + 30 min and 7 d + 30 min) (panel B, lanes 6–8 and panel C, lanes 6–8). This additional GH injection was given 12 h after the prior GH injection. Control pups were injected twice daily with buffer for 7 days ("sham"; panel C, lanes 9–12).

TABLE 3. Impact of prepubertal GH replacement on sex-dependent liver microsomal testosterone hydroxylase activities

Testosterone Metabolite[a]	Adult Males (2)[b]	Adult Female (1)	Pups +GH 7 Days (8)	Pups +SHAM 7 days (8)
		pmol/min/μg ± SEM		
2αOH-T (CYP2C11)	2.39 ± 0.14	≤0.13	0.14 ± 0.01	0.09 ± 0.02
16αOH-T (CYP2C11)	2.69 ± 0.32	≤0.21	0.06 ± 0.01	0.11 ± 0.03
7αOH-T (CYP2A1)	1.78 ± 0.29	3.60	2.35 ± 0.43	4.21 ± 0.90
5αH-T	1.27 ± 0.27	15.60	0.52 ± 0.11	0.52 ± 0.16

Two-week-old pups were administered GH twice-daily sc for 7 days at a dose of 50 μg/100 g BW as described in *Materials and Methods*. Liver microsomes were prepared and ^{14}C-testosterone hydroxylase assay performed and metabolites quantitated as described in *Materials and Methods*.

[a] Hydroxylation of testosterone (T) at each of the indicated positions is selectively catalyzed by each of the specified liver CYP enzymes in adult rat liver. Steroid 5α-reductase (5αH-T) is a GH-regulated, female-specific non-P450 enzyme.

[b] Number of livers assayed in each group is shown in *parentheses*.

showed high levels of liver STAT5 activity, indicating that STAT5 is repeatedly activated by the exogenous GH pulses over the course of the 7-day study.

Analysis of body growth revealed no significant differences in absolute weights or growth rates between sham and GH-treated pups over the 7-day hormone treatment period. GH-treated pups showed an average daily weight gain of 2.04 ± 0.12 g (mean ± SEM, n = 9) compared with 1.72 ± 0.14 g for sham-injected rats (n = 6) ($P < 0.05$). This supports a previous report that the rapid body growth of rats from 2 to 3 weeks of age is not further stimulated by twice-daily injection of recombinant human GH (300 μg/100 g BW) (37).

Discussion

Previous investigations have shown that liver STAT5 can be rapidly activated in hypophysectomized adult rats in-

jected with a physiological replacement dose of GH (11). Furthermore, administration of GH in a continuous manner, to mimic the female plasma GH pattern, resulted in a dramatic decrease in the level of activated STAT5 in the liver (11, 16). To evaluate the relevance of these hypophysectomized rat model studies for intact male rats, where the liver is repeatedly stimulated by a plasma GH pulse each approximately 3.5 h, we presently investigated the response of liver STAT5 to endogenous plasma GH pulses. A striking correlation was seen between the liver's STAT5 activation status and the presence of GH in plasma at the time of liver removal. Rats killed at the time of high plasma GH, *i.e.* during a plasma GH pulse, had high levels of liver STAT5 activity, and conversely, rats killed when plasma GH is low, *i.e.* during a GH interpulse interval, showed low STAT5 activity. These findings provide strong support for our earlier proposal, based on the hypophysectomized rat model (11), that STAT5 becomes activated in direct response to a plasma GH pulse by a sequence of events that involves STAT5 tyrosine phosphorylation and nuclear translocation and that this initial activation enables STAT5 to bind to and transactivate promoter sites adjacent to STAT5-responsive genes, including male-specific *CYP* genes. Subsequently, during the time interval between plasma GH pulses, STAT5 is proposed to be deactivated by dephosphorylation, leading to its return to the cytosol, where it awaits a subsequent round of GH pulse-induced activation and signaling to the nucleus (21).

Of the 29 adult male rats examined in the present study, one individual did not exhibit the correlation between liver STAT5 activity and circulating GH shown by all of the other rats. The high plasma GH and low STAT5 activity seen in this rat (Fig. 2 and Table 1) could correspond to a situation where the liver is excised very early during a GH pulse, *i.e.* before there has been time for efficient assembly of the GH receptor/JAK2 kinase complex and STAT5 tyrosine phosphorylation. Indeed, in hypophysectomized rats given GH by ip injection, liver STAT5 tyrosine phosphorylation does not occur until 5–10 min after GH administration (38). The small number of rats that did not fit the correlation between liver STAT5 activity and the presence of significant GH in plasma (1 out of 18 GH positive individuals) (Table 1) is consistent with the activation of liver STAT5 being a relatively rapid event. All of the rats killed at the time when plasma GH was low ($\leq$3.7 ng/ml) displayed low STAT5 activity, suggesting this group corresponds to animals killed during a plasma GH interpulse interval ("trough period"), at which time STAT5 molecules activated by the prior plasma GH pulse have already been dephosphorylated and returned back to the cytosol. Furthermore, the average liver STAT5 activity in females (9.6 $\pm$ 1.7%) (16) is substantially higher than that of GH-negative males (2.8 $\pm$ 0.8%). This indicates that the STAT5 activity of female rats is significant, albeit much lower than the peak level of STAT5 activity obtained in male rats stimulated by a plasma GH pulse. Accordingly, STAT5, when activated by GH in adult female liver, could contribute to the regulation of liver gene expression. Examples of this regulation may include a female-specific, GH-regulated hepatic CYP2B enzyme, which requires both STAT5a and STAT5b for full expression in adult female mice (17), and the liver enriched transcription factor HNF6, which is transcriptionally activated by GH in rats in a STAT5-dependent manner (39).

The absence of a correlation in the low STAT5 activity group between plasma GH level and liver STAT5 activity ($r = 0.2$) suggests that the low basal liver STAT5 activity in male rat liver may not be due to pulsatile plasma GH stimulation but may result from stimulation of STAT5 signaling by cytokines or other endogenous factors. Together, these findings suggest that a threshold plasma GH pulse level, which is $\geq$ 3.7 ng/ml, is required for efficient STAT5 activation. Limitations of these correlative observations include the fact that the precise threshold for male-specific, GH pulse-dependent STAT5 activation cannot be determined and the uncertainty of whether the plasma GH values assayed for individual rats correspond to samplings taken during the "upswing" or "downswing" phase of the GH pulse. Further investigations, including direct monitoring of the temporal relationship between plasma GH profiles and liver STAT5 activation patterns in individual rats, will be necessary to address these points.

The repeated activation and deactivation of STAT5 in the liver raises the possibility that GH pulse-activated, male expressed genes, such as *CYP2C11*, may be transcribed in an intermittent, or pulsatile, manner in direct relation to the intermittent presence of STAT5 transcription factor in its active form in the nucleus. In an alternative model, suggested by the observed transcriptional inhibitory potential of STAT5b in some systems (40–43), STAT5 could act to repress *CYP2C11* transcription, such that the inactivation of nuclear STAT5 at the conclusion of a plasma GH pulse serves as the stimulus that leads to *CYP2C11* derepression and transcriptional activation. If this latter model is correct, then the temporal profile of *CYP2C11* transcription initiation would correlate negatively with the liver's STAT5 activation status and plasma GH levels. However, given the role of STAT5b as a positive regulator of male-specific liver *CYP* gene expression evident from *STAT5* knockout mouse studies (10), the latter model seems unlikely. Other models are possible, however, including the *indirect* involvement of STAT5 in transcription of male-specific GH-regulated liver genes. The potential role of STAT5 as an indirect mediator of *CYP2C11* expression is supported by the fact that a minimum of 2–3 days of GH pulse-treatment of hypophysectomized rats is required to restore *CYP2C11* expression (4, 44). Because GH pulses activate liver STAT5 rapidly, within 15 min (11), this finding indicates that additional GH-dependent liver factors must be expressed before the transcriptional activation of *CYP2C11* can occur. In agreement with this model, GH-activated STAT5 appears to be required, either directly or indirectly, for the expression of the liver-enriched transcription factors HNF6, HNF4, and HNF3β (39, 45). These liver factors, in turn, may contribute to the expression of male-specific, liver expressed P450 genes, such as *CYP2C11*, perhaps acting in concert with STAT5. According to this model, transcription of genes such as *CYP2C11* would be dependent on, but not necessarily temporally related to the plasma GH profile and nuclear STAT5 status of the liver. Further studies, including transcription initiation analysis in individual male livers that differ in STAT5 activation status, will be required to distinguish between these and other potential regulatory mechanisms.

The presence in prepubertal rats of the key protein factors required for GH-stimulated STAT5 signaling, namely GH receptor (33), JAK2, STAT5a, and STAT5b (Fig. 5) provided a unique opportunity to investigate the sufficiency of STAT5 DNA-binding activity for *CYP2C11* expression in male rats. The precocious activation of liver STAT5 in prepubertal rats required supraphysiological GH doses, suggesting that mechanism(s) exist to moderate the responsiveness of prepubertal rats to GH and thereby maintain hepatic STAT5 activity at a low level. These mechanisms could include: more efficient sequestration by plasma GH binding protein or enhanced plasma GH clearance; a lower abundance of liver GH receptors (33); and less efficient STAT5 activation or enhanced STAT5 dephosphorylation in prepubertal compared with adolescent and adult rats. Although twice-daily GH pulse treatment of 2-week-old rats for 7 days resulted in the repeated activation of STAT5, it did not lead to an induction of *CYP2C11* gene expression. This finding was further confirmed by the lack of masculinization of hepatic enzyme profiles, evaluated by microsomal testosterone hydroxylase activity, and by the lack of significant additional weight gain in GH-treated compared with sham-injected immature rats. The ineffectiveness of exogenous GH pulses with respect to prepubertal *CYP2C11* activation cannot be explained by the somewhat lower than maximal liver STAT5 activity that we obtained (55% of adult male level), insofar as even a low GH dose (*e.g.* 1 μg GH/100 g BW, corresponding to 25% of a normal, physiological GH peak) induces full expression of *CYP2C11* in hypophysectomized adult rats (34), even though liver STAT5 is only partially activated at this GH dose in the same hypophysectomized rat model (38). Rather, the absence of *CYP2C11* expression under conditions where liver STAT5 is repeatedly activated over a 7-day period (Fig. 7) suggests that prepubertal rat liver may be intrinsically unresponsive to STAT5-stimulated gene expression. Additionally, postpubertal liver factors other than STAT5 alone may be required for efficient gene induction in the case of *CYP2C11* and other male-expressed genes.

The liver-enriched transcription factors that presumably cooperate with STAT5 to achieve the male-specific pattern of liver gene expression which characterizes *CYP2C11* and other sexually dimorphic, GH-regulated P450 genes are not known. Potential candidates include the liver-enriched transcription factors HNF1β, HNF3β and DBP, whose mRNA levels are very low during early postnatal periods compared with adults (46) and whose absence could conceivably be a determinant of the unresponsiveness of *CYP2C11* to precocious activation by liver STAT5. Furthermore, other developmentally regulated factors may be required. For example, circulating androgen is required for full expression of *CYP2C11*. Birth-castrated rats do not express CYP2C11 protein or activity at adulthood (25, 26, 47), and full expression requires androgen replacement during both prepubertal and postpubertal periods (47). However, this androgen requirement is generally presumed to be a consequence of the effects of sex-steroids on GH-releasing hormone and somatostatin in the hypothalamus, leading to regulation of the circulating GH pattern, rather than a direct consequence of sex-steroid action on the liver (48–50). Nevertheless, given our findings regarding the insufficiency of GH pulse-activated STAT5 for stimulating male *CYP* expression, one cannot rule out the possibility that androgen-dependent factors other than pulsatile GH act in concert with STAT5 to stimulate the male-specific pattern of liver gene transcription.

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