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Growth Hormone, but Not Prolactin, Maintains Low-Level Activation of STAT5a and STAT5b in Female Rat Liver

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ABSTRACT

STAT5b, a member of the signal transducers and activators of transcription family, is activated in rat liver in response to the intermittent (pulsatile) plasma pattern of GH that is characteristic of adult males. Previous studies have shown that the near-continuous plasma GH pattern of adult female rats is associated with a dramatic down-regulation of the STAT5 activation pathway. The present study demonstrates the presence of a low-level STAT5 DNA-binding activity in adult female rat liver and investigates the hormonal factors required for its maintenance. PRL is not responsible for this low-level STAT5 activity, as demonstrated in experiments involving estrus cycle monitoring (to investigate a possible role of the proestrus PRL surge), implantation of bromocriptine pellets (to eliminate PRL release by the pituitary), and direct injection of purified PRL. Rather, the low-level STAT5 activity is shown to result from chronic plasma GH stimulation, as demonstrated by GH infusion studies carried out in hypophysectomized rats. Furthermore, gel mobility supershift experiments demonstrate that the same STAT5-containing DNA-binding complexes are formed by both male and female adult rat liver extracts, albeit at approximately 10- to 20-fold lower levels by the female extracts. This DNA-binding activity is primarily comprised of STAT5b but also contains STAT5a, which is shown to be preferentially activated by the male plasma GH pattern in a manner similar to STAT5b. Thus, the dominance of activated STAT5b, compared with STAT5a, in the strong DNA-binding complexes formed in adult male rat liver nuclear extracts, is a reflection of the relative abundance in liver of the two STAT5 forms and is not attributable to an intrinsic, preferential activation of STAT5b by plasma GH pulses. The physiological significance of the low-level activated STAT5a and STAT5b seen in female rat liver and its effects on liver gene expression are uncertain but may involve the activation of female-expressed cytochromes P450 and other liver genes. (*Endocrinology* **140:** 5126–5135, 1999)

THE SIGNAL TRANSDUCER and activator of transcription protein STAT5 mediates the effects of a variety of cytokines, growth factors, and hormones on liver and other target tissues. Two distinct forms of STAT5, designated STAT5a and STAT5b, are encoded by separate genes and have high sequence similarity (>90%) (1, 2) but distinct physiological functions (3–5). STAT5a is the major STAT5 form that becomes activated by PRL-induced tyrosine phosphorylation in the mammary gland, where it is required for adult mammary gland development and lactogenesis (4). In contrast, STAT5b is the major STAT5 form in the liver (5–7), where it is activated to a high level in response to stimulation by the intermittent GH profile (8–10) that is characteristic of adult male rats but not by the near-continuous pattern of GH stimulation that characterizes adult female rats (11). Liver STAT5b is implicated in the transcriptional activation of GH pulse-inducible male-specific cytochrome P450 genes (3, 5, 11), several of which have STAT5 response elements in their 5′ flank (S. H. Park and D. J. Waxman, unpublished) (12). Conversely, the near-continuous GH pattern characteristic of adult female rats results in a dramatic decrease in the nuclear levels and DNA-binding activity of liver STAT5 (11) by a mechanism that involves an apparent increased dephosphorylation of both STAT5 and the GH receptor-JAK2 kinase signaling complex (13). Although the presence of activated STAT5 in rat liver nuclei is largely restricted to adult male rats, in some individual female rats, liver STAT5 protein is present in the nucleus, albeit at a much lower level than in males (11). This observation takes on added significance with our recent finding in a *Stat5a* knockout mouse model, that STAT5a, in addition to STAT5b, is required for expression in female mouse liver of a subset of female-specific, GH-regulated P450s (5).

Rat PRL, administered at a replacement dose to hypophysectomized rats, does not induce activation of STAT5 in the liver (6, 11). Other investigators have reported a marginal response to supraphysiological PRL levels in hypophysectomized rats, as assessed from the increase in STAT5 DNA-binding activity by gel electrophoretic mobility shift analysis (EMSA) using the *Spi2.1* promoter STAT5-binding site as probe (14). By contrast, induction of a suckling response in postpartum female rats, which results in a large increase in circulating PRL, is accompanied by strong activation of liver STAT5 (15). The unresponsiveness of liver STAT5 to PRL seen in hypophysectomized rats (11) could result from the down-regulation of liver PRL receptor that accompanies hypophysectomy (16). This unresponsiveness of liver STAT5 to PRL could additionally reflect the fact that, in liver, PRL receptor is primarily expressed as a short form (inactive, with respect to signaling to STAT5) that can inhibit PRL signaling to STAT5 by a dominant-negative mechanism. In contrast, in

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PRL-responsive mammary tissue, PRL receptor is primarily expressed in its fully active long form (17).

The observation that STAT5 becomes activated in female rat liver, in response to suckling (15), raises several questions. First, are the low levels of active STAT5 seen in the livers of some female rats activated in a PRL-dependent manner? Is this activation estrus cycle-dependent in a manner that follows the PRL surge (18, 19) that occurs on the afternoon of proestrus? Moreover, given that there are two STAT5 forms expressed in liver, is one form (*e.g.* STAT5b) preferentially activated by GH, whereas the other is preferentially activated by PRL? Are there differences in the extent to which GH and PRL induce the formation of homodimeric *vs.* heterodimeric STAT5a and STAT5b DNA-binding complexes in the liver? These and related questions are addressed in the present study, where we show that GH, but not PRL, is responsible for the low-level of liver STAT5 activity observed in adult female rats. The implications of these findings are discussed in the context of the roles proposed for STAT5b and STAT5a in regulating the GH-dependent sexual dimorphism of liver gene expression.

Materials and Methods

Animal treatments

Adult male and female Fischer 344 rats (Taconic Farms, Inc., Germantown, NY; and Charles River Laboratories, Inc. Wilmington MA) were either untreated or were hypophysectomized by the supplier, with follow-up care provided as previously described (20). Where indicated, female Sprague Dawley rats of the same age were used. Animals were maintained at the Laboratory for Animal Care Facility at Boston University (lights on from 0700–1900 h). All animal procedures were approved by the Boston University Institutional Animal Care and Use Committee. Hypophysectomized animals were maintained for at least 2 weeks after surgery, to ensure completeness of surgery (as evidenced by the absence of body weight gain).

Bromocriptine pellets (bromocriptine mesylate formulated with biodegradable carrier binder; Innovative Research of America, Sarasota, FL) were administered to intact female rats (135–160 g) at a dose of 15 mg/rat (2 pellets, 7.5 mg each). Pellets were implanted sc on the backs of animals under Ketamine anesthesia. Vehicle control animals received placebo pellets. Animals were killed 7 days after pellet implantation. Plasma PRL levels at the time of sacrifice were determined by standard RIA methods in assays performed by Dr. M. Vore (University of Kentucky, Lexington, KY).

Rat PRL (hormonally pure grade, NIDDK-rPRL-B-8-SIAFP, National Hormone and Pituitary Program, NIDDK) was injected into intact female rats ip at a dose of 12.5 or 50 μg/100 g BW. Rats were killed 30 min later, and the liver was removed for biochemical analysis. Rat GH (hormonally pure rat GH, rGH-B-14-SIAFP, National Hormone and Pituitary Program, NIDDK) was administered by continuous infusion at 2 μg/100 g BW·h for 1, 3, or 7 days using an Alzet osmotic minipump (model 2001; Alzet Corp., Palo Alto, CA). This dose of GH was previously shown to be a physiologic replacement dose when given to hypophysectomized rats, insofar as it gives a plasma GH level of approximately 35–40 ng/ml and thus mimics the near-continuous GH profile of adult female rats (21). Minipumps were implanted sc on the backs of hypophysectomized rats under Ketamine anesthesia. Estrus cycles were monitored in adult female rats (140–150 g), over 4 cycles, by vaginal smears. Rats were killed on the afternoon of proestrus or diestrus (2 h before lights out).

Preparation of whole-liver homogenates

Approximately 200–400 mg frozen rat liver tissue was homogenized at 4 C in a Dounce tissue grinder (10 strokes) in 2 ml 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 (5 μg/ml aprotinin, 0.1 mM phenylmethanesulfonyl fluoride, 10 mM NaF, 1 mM $NaVO_3$, and 5 μg/ml leupeptin). Samples were centrifuged at 9000 rpm for 20 min at 4 C in a Sorvall RC 5C centrifuge. Supernatants were aliquoted, snap-frozen in liquid nitrogen, and stored at −80 C. Little STAT5 DNA-binding activity (by EMSA) was present in the pellet fraction (data not shown).

Preparation of liver nuclear extracts

Nuclear extracts were prepared from individual, freshly excised rat livers using established methods (22) with the addition of the phosphatase inhibitors sodium fluoride (10 mM) and sodium orthovanadate (1 mM) in the homogenization and nuclear lysis buffers and with the inclusion of a mixture of protease inhibitors, as described elsewhere (11).

EMSA

Whole-liver homogenates (30 μg) were adjusted to a total vol of 11 μl, then added to 0.5-ml microfuge tubes on ice containing 2 μl EMSA buffer (20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 50 mM Tris, pH 7.5) plus 1 μl containing 2 μg of poly(deoxyinosinic-deoxycytidylic)acid (Roche Molecular Biochemicals, Indianapolis, IN), and incubated for 10 min at room temperature. Double-stranded ^{32}P-labeled oligonucleotide probe (1 μl, 10 fmol) was then added to give a total vol of 15 μl. The mixture was incubated for 20 min at room temperature and then for an additional 10 min on ice. Loading dye (2 μl of 30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol) was added, and the mixture was loaded onto a nondenaturing acrylamide gel (5.5% acrylamide, 0.07% bis-acrylamide) (National Diagnostics, Atlanta, GA) in 0.5× TBE (44.5 mM Tris, 44.5 mM boric acid, 5 mM EDTA) which had been pre-run at 4 C for 30 min at 100 V. The gel was run at 100 V in 0.5× TBE, first at 4 C for 20 min, and then for 160 min at room temperature (23). For supershift analyses, anti-STAT5 antibodies were added 20 min after addition of the ^{32}P-labeled probe, incubated at room temperature for 10 min, then placed on ice for 10 min. Antibodies used were anti-STAT5a and anti-STAT5b (sc-1081 and sc-835, respectively; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Gels were dried and exposed to phosphorimager plates for 1–3 days, followed by analysis on a Molecular Dynamics, Inc. (Sunnyvale, CA) PhosphorImager with quantitation using ImageQuant software. The relative intensity of STAT5 EMSA bands observed in female rat liver was expressed as a percentage of the average maximal STAT5 EMSA activity seen in male rat liver. Background activities corresponding to the residual signal, in the presence of a 500-fold excess of unlabeled self-competitor oligonucleotide probe, was taken as background and subtracted from each sample.

The STAT5/mammary gland factor response element of the rat β-casein promoter (nucleotides −101 to −80) 5′-GGA-CTT-CTT-GGA-ATT-AAG-GGA-3′ (sense strand) was used as STAT5-binding probe in EMSA analyses. The sense strand was end-labeled with ^{32}P using T4 polynucleotide kinase, annealed to the antisense strand, and then purified on a BioSpin30 Chromatography Column (Bio-Rad Laboratories, Inc. Hercules, CA).

Western blotting

Whole-liver homogenates (40 μg) were electrophoresed through Laemmli SDS/PAGE gels (7.5%) 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 nitocellulose and probed with either anti-STAT5a or anti-STAT5b antibody (sc-1081 and sc-835, respectively; Santa Cruz Biotechnology, Inc.). Blocking and probing conditions were as previously described (11). Detection on x-ray film was by enhanced chemiluminescence using Amersham Pharmacia Biotech ECL reagents (Amersham Pharmacia Biotech, Arlington Heights, IL).

Plasmids

pRc/CMV expression plasmids encoding rat STAT5a and rat STAT5b were provided by Drs. J. Rosen and L. Yu-Lee, respectively (Baylor College of Medicine, Houston, TX) (24, 25). Rat GH receptor complementary DNA (cDNA) (cloned into the expression plasmid pcDNAI) and mouse JAK2 tyrosine kinase (cloned into the expression plasmid

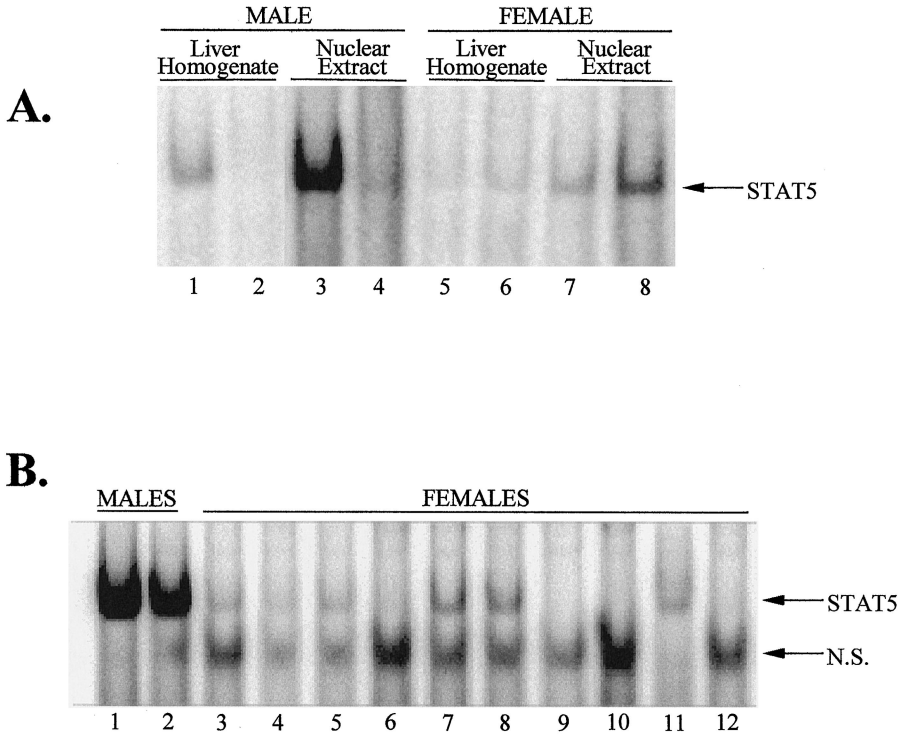

FIG. 1. EMSA analysis of STAT5 activity in male and female rat liver. A, Comparison of STAT5 DNA-binding activity in whole-liver homogenates and liver nuclear extracts. Two male and two female rats were killed, and half of each liver was used to prepare whole-liver homogenates and half to prepare liver nuclear extracts, as described in *Materials and Methods*. Gel shift analysis was performed with the β-casein probe, which formed a distinct STAT5-DNA complex. Lanes 1 and 3; 2 and 4; 5 and 7; and 6 and 8 correspond to four individual rats. B, Low-level STAT5 activity is present in some individual female livers. Whole-liver homogenates were prepared from individual female rats and subjected to gel shift analysis. Lanes 1 and 2 are liver homogenates from male rats with high levels of activated STAT5. Lanes 3–12 correspond to liver homogenates from individual females. N.S., Nonspecific EMSA band present in a variable level in liver samples. This band was not supershifted by anti-STAT5 antibody and was not competed by 50-fold molar excess of unlabeled EMSA probe (data not shown).

pRK5) were provided by Dr. N. Billestrup (Hagedorn Research Institute, Denmark) (26) and Dr. J. Ihle (St. Jude Children's Research Hospital, Memphis, TN), respectively (27).

Cell culture and transfections

COS-1 cells were maintained in DMEM containing 10% FCS. Transfection of COS-1 cells grown in 6-well tissue culture plates (9.4 cm^2/well) was carried out using Fugene6 (Roche Molecular Biochemicals). Three microliters of Fugene6 reagent was mixed with 72 μl serum-free DMEM (per well) and incubated for 30 min at room temperature. The mixture was added in a dropwise manner into a 1.5-ml microfuge tube containing plasmid DNA and was incubated for 15 min at room temperature. Subsequently, the mixture was added to one well of a 6-well plate containing COS-1 cells in DMEM + 10% FBS, then incubated overnight at 37 C. The media was then removed and replaced with serum-free DMEM containing 500 ng/ml rat GH and incubated for 30 min at room temperature. The GH-containing media was removed, and cells were washed with ice-cold PBS, followed by addition of 250 μl of 1× Passive Lysis Buffer (Promega Corp., Madison, WI). Cells were incubated for 15–30 min at 4 C (with shaking every 5 min), then harvested, and centrifuged for 5 min at 4 C. The supernatant was aliquoted, snap-frozen in liquid nitrogen, and stored at −80 C.

Transfections were performed using the following amounts of plasmid DNA per well of a 6-well tissue culture plate: 500 ng rat STAT5a or rat STAT5b expression plasmid (or a mixture of both STAT5 forms, as described in the text), 500 ng GH receptor expression plasmid, and 20 ng JAK2 kinase expression plasmid. Renilla luciferase expression plasmid (pRL-TK, 150 ng) (Promega Corp.) was added, as internal control, to assay for transfection efficiency. The total amount of DNA was adjusted to 2 μg/well using sonicated salmon sperm DNA (Stratagene, La Jolla, CA).

Results

Whole-liver homogenates to assay for STAT5 activity

Because preparation of rat liver nuclear extracts is a labor- and time-intensive procedure, we developed a simpler and more rapid method to assay for liver STAT5 DNA-binding activity in an EMSA using whole-liver homogenates. Individual rats were killed, and portions of each liver were used to prepare liver nuclear extracts from fresh tissue, or alternatively, whole-liver homogenates from frozen tissue. Fig. 1A shows that individual adult male rat livers showing high STAT5 DNA-binding activity in nuclear extracts[1] also show high activity in whole-liver homogenates (lanes 1 and 3). Similarly, adult female livers exhibiting differences in STAT5 activity in nuclear extracts (lane 7 *vs.* 8) exhibited corresponding differences when liver homogenates were assayed (lane 5 *vs.* 6). Accordingly, liver STAT5 activity, using a β-casein STAT5-binding site as probe, can be reliably assayed using whole-liver homogenates. The higher absolute STAT5 activity seen in the nuclear extracts reflects the concentration of STAT5 in the nucleus after GH stimulation (28) and the fact that the nuclear extracts represent a partially purified protein fraction (22).

A low-level of activated STAT5 is present in some female livers

We previously observed a low level of STAT5 protein in nuclear extracts of some individual female rats (11). Fig. 1A demonstrates that STAT5 DNA-binding activity is present in individual female rat liver samples, albeit at a level that is low compared with that found in STAT5-positive male livers. We further investigated this female STAT5 EMSA activity using whole-liver extracts prepared from 10 individual adult female rats. Activated STAT5 was present in some females, albeit at a significantly lower level than the maximal STAT5 activation level seen in males (Fig. 1B; lanes 7, 8, 11 *vs.* 1, and 2). In other females, STAT5 activity was at or below the limit

[1] These livers are derived from rats killed at the time of a plasma GH pulse (unpublished experiments).

of detection (*e.g.* lanes 4, 6, 9, and 12). The specificity of the low-level STAT5 EMSA signal seen in female rat liver was confirmed by supershift with anti-STAT5 antibody (*e.g.* Fig. 5) and by competition with a 50-fold molar excess of unlabeled β-casein DNA probe (data not shown). Quantitation of the STAT5 activity in these and a large number of other adult female livers revealed an average STAT5 EMSA activity level in female rat liver of 9.6 ± 1.7% (mean ± SE, n = 31), compared with the maximal male STAT5 EMSA activity (100%), with 28 of 31 female livers showing less than 15% of the maximal STAT5 activity seen in males. Western blot analysis of STAT5b protein, under conditions where the phosphorylated and nonphosphorylated STAT5b band are separated (23), confirmed these findings, insofar as a small amount of the tyrosine-phosphorylated STAT5 could be detected in the female liver samples showing the highest STAT5 EMSA activity (data not shown).

No correlation between presence of low-level STAT5 activity and proestrus PRL surge

In view of our earlier finding that intermittent plasma GH pulses rapidly activate liver STAT5 in adult male rats (11), and given the suggestion that a postpartum suckling-induced PRL surge may activate STAT5 in adult female rat liver (15), we investigated the possibility that the physiological pulses in circulating PRL levels that occur naturally on the afternoon of proestrus (18, 19, 29) may stimulate the low-level STAT5 activity seen in adult female rats. To investigate this hypothesis, estrus cycles were monitored in rats over a 2-week period. One group of animals was killed on the afternoon of proestrus (2 h before lights out), and a second group was killed on the afternoon of diestrus. EMSA analysis, using whole-liver extracts, did not reveal any apparent differences in the STAT5 activity of each group (Fig. 2A, lanes 3–6 *vs.* 7–9). Two rats from each group showed the characteristic low-level STAT5 activity (lanes 3 and 5, and lanes 8 and 9, respectively), whereas one rat from each group showed very little or no STAT5 activity (lanes 4 and 7). Analysis of the quantitated STAT5 EMSA signals revealed no correlation with the stage of the estrus cycle (data not shown). We conclude that the low-level STAT5 activation seen in these rats is not a consequence of the preovulatory PRL surge that occurs during proestrus. Of note, one rat, killed at proestrus, exhibited an uncharacteristically high level of activated STAT5 (lane 6).

Low-level STAT5 activity is not abolished by bromocriptine treatment

To confirm these results and to rule out the possibility that the comparatively high level of activated STAT5 seen in one individual (Fig. 2A, lane 6) was attributable to PRL stimulation, rats were implanted with sc bromocriptine pellets. Bromocriptine is a potent dopamine agonist that prevents release of PRL from the anterior pituitary gland and thereby suppresses circulating PRL levels (30, 31). Rats were killed 7 days later, and liver extracts were prepared. Quantitation of STAT5 EMSA activity in liver extracts prepared from these female rats showed no correlation between bromocriptine treatment and the presence (Fig. 2B; lanes 4, 8, 9, and 11) or the absence (lanes 5–7, and 10) of a low-level STAT5 activity signal (bromocriptine-treated rats: 6.1 ± 1.7%, mean ± SE STAT5 EMSA activity, compared with male liver STAT5 positive control; placebo-treated rats: 5.9 ± 1.1%, mean ± SE; n = 6 rats/group). RIA for PRL confirmed that the bromocriptine pellets dramatically decreased circulating PRL levels (bromocriptine-treated rats: 23.4 ± 4.1 ng

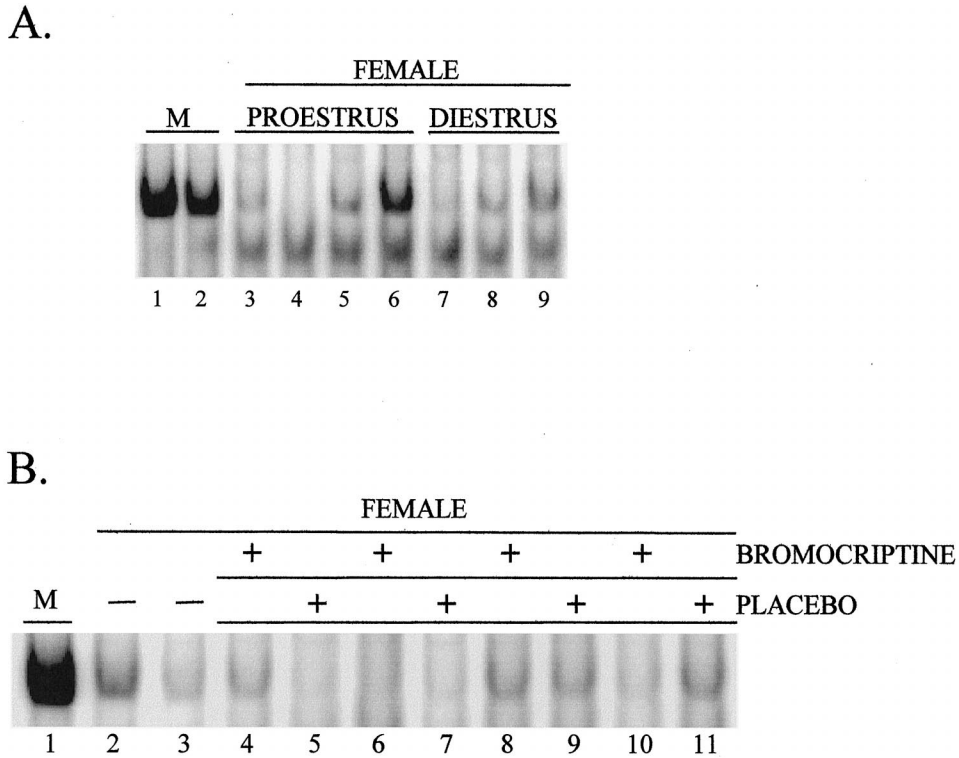

FIG. 2. Relationship between PRL status and liver STAT5 EMSA activity. A, Low-level STAT5 activation in females does not correlate with the proestrus PRL surge. Estrus cycles of individual females were monitored by vaginal smears over 4 cycles. Animals were killed on the afternoon (2 h before lights out) of proestrus (lanes 3–6) or diestrus (lanes 7–9). B, Low-level activated STAT5 is not abolished by suppression of circulating plasma PRL. Female rats were surgically implanted with bromocriptine or placebo pellets for 7 days. Suppression of circulating PRL was confirmed by RIA analysis of blood plasma (see text). Shown is an EMSA analysis of individual rat liver extracts. M, Individual male rat whole-liver homogenates.

PRL/ml, mean ± SE; placebo-treated rats: 230 ± 107 ng PRL/ml, mean ± SE; n = 6 rats/group).

STAT5 activation is not induced by a pulse of exogenous rat PRL

The intrinsic responsiveness of liver STAT5 to activation by PRL was assessed by administration of purified PRL to adult female rats by ip injection at two different doses (12.5 and 50 μg/100 g BW). These doses were chosen to be high, compared with a dose of GH (3 μg/100 g BW) that was previously shown to give a peak GH plasma level of approximately 225 ng/ml, which approximates that of a normal adult male rat GH pulse (21) and can efficiently activate STAT5 in hypophysectomized rat liver (6, 11). EMSA analysis of whole-liver extracts revealed that STAT5 DNA-binding activity was not significantly elevated by PRL at either dose in rats killed 30 min after PRL injection (Fig. 3). Because earlier studies pointing to a PRL-responsive liver STAT5 used Sprague Dawley rats (15), compared with Fischer 344 rats in this study, we repeated the experiment in female Sprague Dawley rats. Fig. 3B shows that injection of 50 μg PRL/100 g BW did not induce STAT5 EMSA activity in adult female Sprague Dawley rats. To ascertain whether PRL injection stimulates liver STAT5 activation that is transient or, alternatively, is slower than the activation of liver STAT5 after GH treatment of hypophysectomized rats (11), the effect of PRL (50 μg PRL ip/100 g BW) on STAT5 EMSA activity was evaluated at times ranging from 5 min to 2 h after PRL injection. No significant PRL-dependent increase in STAT5 EMSA activity was observed (data not shown).

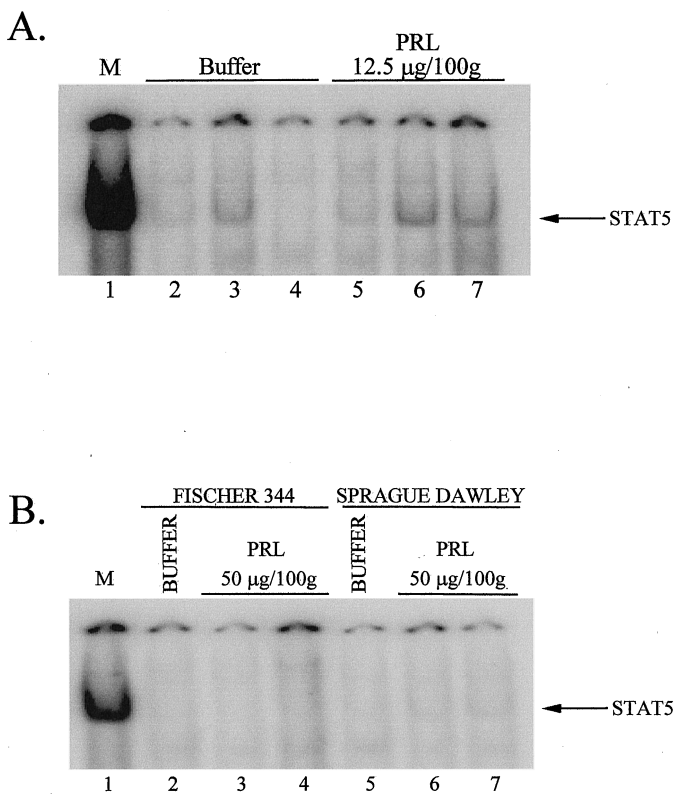

FIG. 3. PRL treatment and liver STAT5 EMSA activity. A, Exogenous PRL does not increase STAT5 activity. Rat PRL was administered ip at a dose of 12.5 μg/100 g BW into intact female rats. Individual animals were killed 30 min later, and liver extracts were analyzed for STAT5 activity by EMSA. B, Experiment as in A, but PRL was administered ip at a high dose (50 μg/100 g BW) into intact females of two different rat strains (Fisher 344 and Sprague Dawley, as indicated).

Hypophysectomy abolishes, and continuous GH replacement restores, low-level liver STAT5 activity

Given our finding that circulating PRL does not stimulate the low-level STAT5 activity seen in female rat liver, and given the possibility that other hormonal factors may activate STAT5 in the liver (32), we investigated whether the low-level STAT5 activity was attributable to a pituitary hormone. Male and female rats were hypophysectomized, and whole-liver extracts were prepared and assayed for STAT5 activity. As seen in the EMSA analysis presented in Fig. 4A, liver STAT5 activity was abolished by hypophysectomy, both in males and females, indicating that the low-level STAT5 activity seen in intact female rats is either directly or indirectly supported by factor(s) released by the pituitary.

We next investigated whether STAT5 activity could be restored in the hypophysectomized rats by GH replacement in a continuous pattern, to mimic the near-continuous plasma GH stimulation that is characteristic of adult female rats. Osmotic minipumps, delivering GH at 2 μg/100 g BW·h, were implanted into hypophysectomized rats for 1 day, 3 days, or 7 days. Fig. 4B shows that these treatments stimulate a low-level STAT5 activity, as seen by EMSA analysis of whole-liver extracts. A comparatively high level of STAT5 activity was observed after 1 day of continuous GH treatment (lanes 7 and 8 *vs.* hypophysectomized controls in lanes 4–6). A lower STAT5 activity level was observed after 3 and 7 days GH treatment, which gave band intensities similar to that of intact female rats (lanes 9–13 and 17 and 18 *vs.* lanes 2 and 3). We conclude that the near-continuous plasma GH profile of adult female rats is both necessary and sufficient to support the low-level STAT5 activity exhibited by adult female rats.

Low-level of activated STAT5 in female rat liver is primarily STAT5b and has the same STAT composition as the DNA-binding complex present in male liver

STAT5a and STAT5b exhibit distinguishable DNA-binding specificities (33, 34) and may contribute differentially to the activation of GH-regulated, sexually dimorphic liver gene products (5). STAT5b is the dominant STAT5 form present in both rat and mouse liver (5–7). Accordingly, it was important to determine whether the low-level STAT5 activity seen in female rat liver corresponds to STAT5a, STAT5b, or a mixture of both STAT5 forms. To accomplish this, STAT5 DNA-binding activity was assayed by EMSA, and supershift analysis of the STAT5 composition of the DNA-protein complexes was performed (Fig. 5) using antibodies shown to be STAT5 form-specific under the conditions of Western blot analysis (see Fig. 6). As a positive control for each STAT5 protein, rat STAT5a and STAT5b expression plasmids were transfected into COS-1 cells (which are deficient in both STAT5 forms) together with expression plasmids for the other components required for GH-induced STAT5 activation, namely GH receptor and JAK2 tyrosine kinase. STAT5

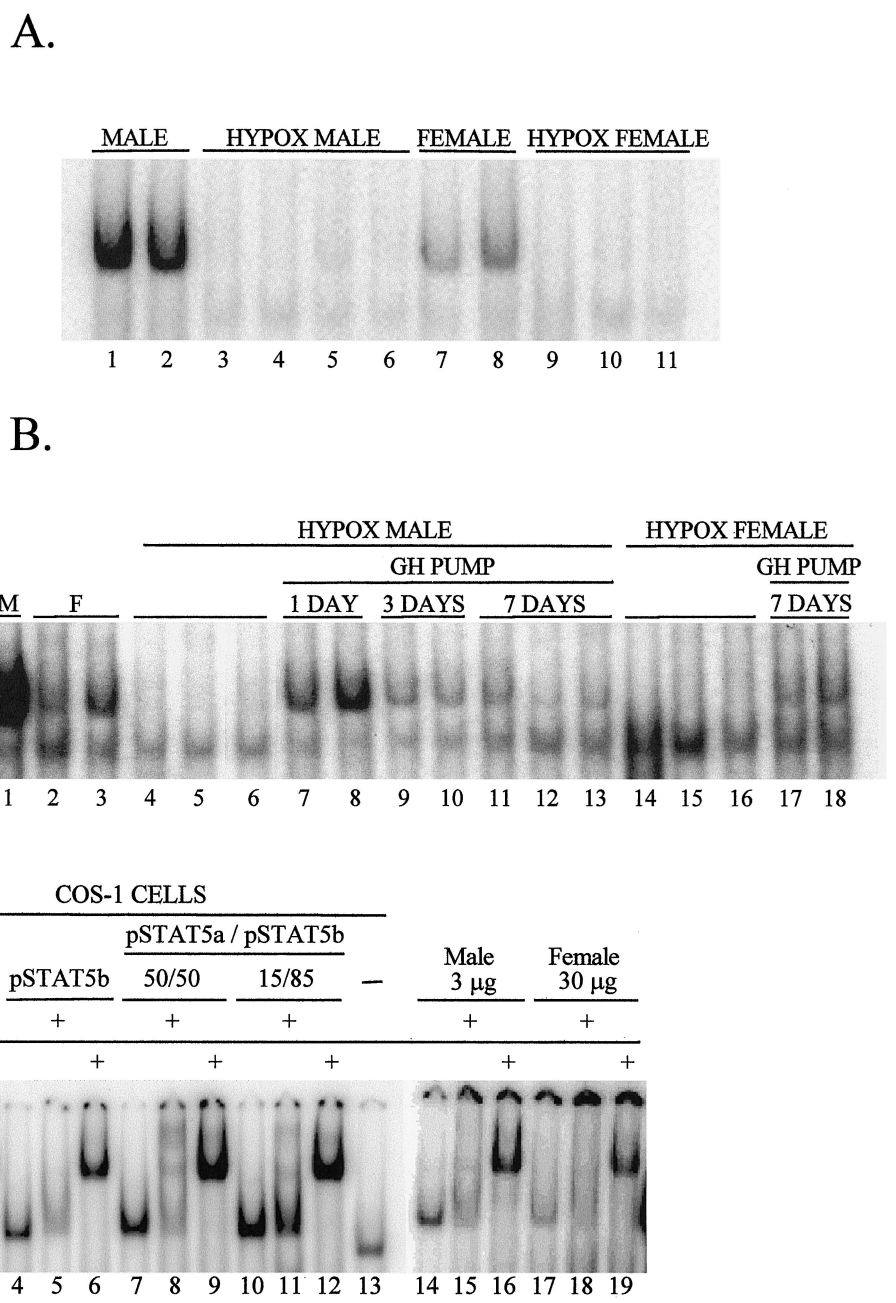

FIG. 4. Effect of hypophysectomy and continuous GH replacement on STAT5 activity. A, Hypophysectomized (HYPOX) rats do not exhibit STAT5 EMSA activity. Hypophysectomized male and female rat livers were assayed for STAT5 EMSA activity, compared with intact male and female controls. B, Low-level STAT5b activity is restored in hypophysectomized rats administered GH in a continuous manner. Hypophysectomized male and female rats were implanted sc with osmotic minipumps containing GH, which is released at a rate of 2 μg/100 g BW·h. Rats were killed after 1, 3, or 7 days of continuous GH treatment.

FIG. 5. STAT5-DNA complex formed by male and female rat liver extract is composed predominantly of STAT5b. Male and female liver homogenates were supershifted with antibodies (AB) to rat STAT5a and rat STAT5b (lanes 14–19). As control, COS-1 cells were transfected with expression plasmids for: 1) STAT5a; 2) STAT5b; 3) both STAT5 forms at a weight ratio of 50% STAT5a and 50% STAT5b; or 4) at a ratio of 15% STAT5a and 85% STAT5b. Cells were cotransfected with GH receptor and JAK2 kinase expression plasmids then treated with rat GH (500 ng/ml) for 30 min, as described in *Methods and Materials*. COS-1 cells not transfected with STAT5 plasmids and treated with GH are shown in lane 13. Supershift analysis with antibody (AB) to STAT5a or STAT5b was done as indicated. s.s., Supershifted STAT5 EMSA complexes. pSTAT, STAT plasmid.

plasmids were cotransfected at a weight ratio of 50/50 (250 ng STAT5a/250 ng STAT5b) or 15/85 (75 ng STAT5a/425 ng STAT5b), the latter being similar to the measured ratio of STAT5a to STAT5b messenger RNA in liver (5). Transfected cells were given a 30-min pulse of GH to activate STAT5 before EMSA analysis of cell extracts.

Cells transfected with the STAT5a and STAT5b expression plasmids gave a distinct gel shift band (Fig. 5; lanes 1, 4, 7, and 10) that was absent from untransfected cells (lane 13)[2]. The STAT5a band was 3-fold more intense and migrated

[2] In COS-1 cells transfected with expression plasmids for JAK2 and GHR, but not STAT5a or STAT5b, GH stimulation yielded a faster-migrating complex (lane 13). This complex was supershifted with a STAT1 antibody (data not shown), indicating that it corresponds to endogenous STAT1 that is activated by GH and can bind the rat β-casein STAT5 response element (6).

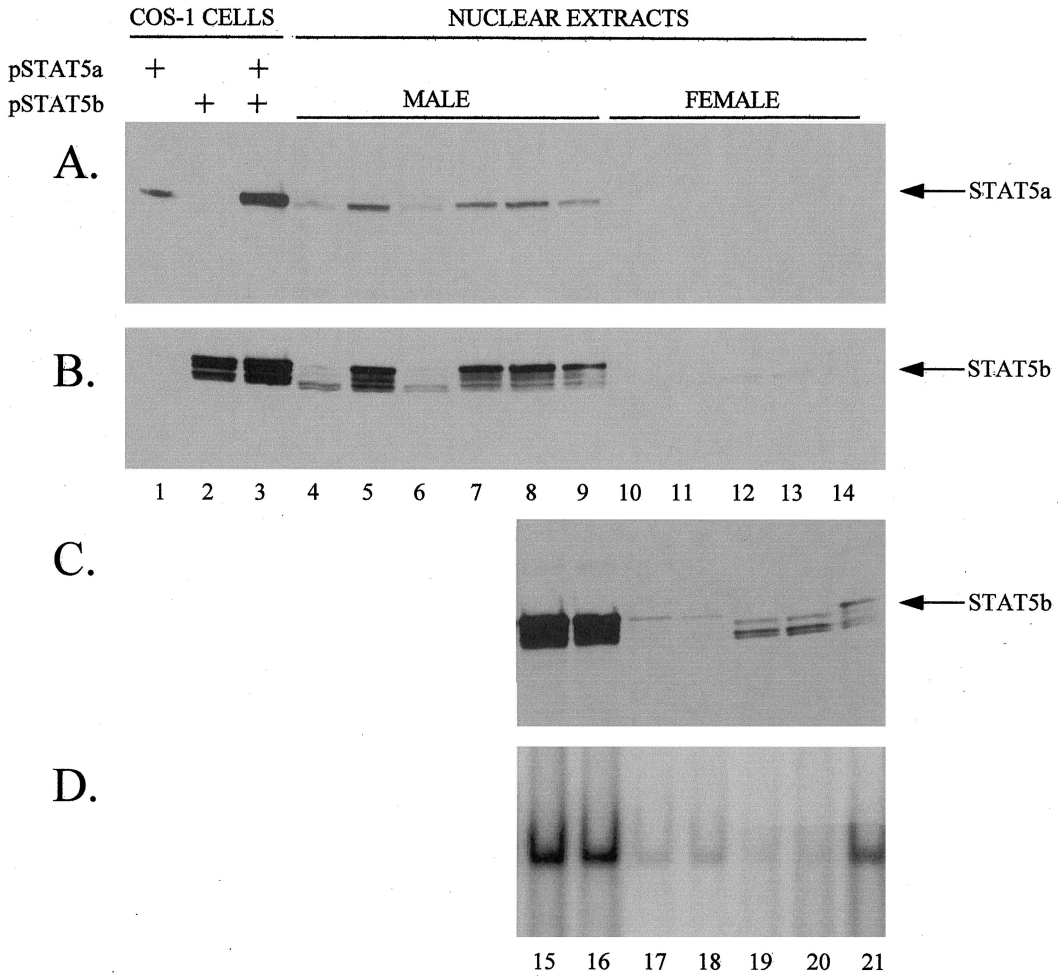

FIG. 6. Western blot analysis of liver nuclear extracts showing STAT5a and STAT5b are both present in the nucleus of male rat liver at a much higher level than in female rat liver. Nuclear extract samples from individual male and female rats were Western blotted with an anti-STAT5a antibody (A). The blot was stripped and subsequently probed with an antibody to STAT5b (B). Specificity of the two antibodies was verified by running authentic standards for STAT5a and STAT5b (COS-1 cells transfected with expression plasmids for rat STAT5a, rat STAT5b, or both; lanes 1–3). The uppermost STAT5b band seen in B, lanes 2–9, corresponds to the tyrosine + serine-diphosphorylated STAT5b (11, 23). Shown in C is a longer exposure of lanes 8–14, probed with the same anti-STAT5b antibody as in B. A very small amount of the diphosphorylated form of STAT5b is seen in the female nuclear samples in lanes 17, 18, and 21. EMSA analysis using B-caseine probe was performed on these samples (D).

slightly more slowly than the STAT5b band (lane 1 *vs.* 4; also see Ref. 5). In extracts of cells transfected with STAT5a alone, STAT5a antibody supershifted the DNA-protein complex to give a slowly migrating pair of bands (s.s.). STAT5b antibody supershifted the STAT5a and STAT5b complexes equally well (lanes 3 and 6), evidencing cross-reactivity between the STAT5b antibody and the STAT5a DNA-binding complex[3]. Conversely, in extracts of cells transfected with STAT5b, whereas there was a clear supershift with the STAT5b antibody (lane 6), STAT5a antibody disrupted the STAT5b DNA-binding complex, perhaps by steric hindrance of the DNA-binding region of the cDNA-expressed STAT5b (lane 5 *vs.* control in lane 4).

With extracts of COS-1 cells transfected with a mixture of STAT5a and STAT5b, STAT5b antibody gave a single distinct supershifted band, as expected (lanes 9 and 12). By contrast, STAT5a antibody gave three distinct complexes (lanes 8 and 11): a partially disrupted STAT5-DNA complex that corresponded to the STAT5b complex (lane 5) and the much more slow-moving pair of supershifted bands characteristic of STAT5a (lane 2). Based on these supershift patterns, the STAT5 DNA-binding complex in these cotransfected cells is likely composed of a mixture of STAT5 homodimers and heterodimers.

The same STAT5 supershift analyses were performed on whole-liver extracts prepared from male rats (3 μg protein) and female rats (30 μg protein) (Fig. 5, lanes 14–19). In both cases, STAT5a antibody primarily disrupted the DNA-binding complex and yielded only a small amount of the slow-

[3] The lower STAT5 form-specificity of the STAT5a and STAT5b antibodies seen by EMSA supershift (Fig. 5) compared with Western blotting (Fig. 6) may relate to the fact that minor cross-reactivities between STAT5 forms may be lost during the extensive washing of the Western blots. In addition, denaturation of the STAT5 antigen during Western blotting may lead to a loss of cross-reactive antigenic determinants detected by EMSA.

moving supershift bands (lanes 15 and 18), whereas a distinct supershift was formed with the STAT5b antibody (lanes 16 and, 19). These patterns are very similar to those seen in lanes 11 and 12, with extracts of COS-1 cells transfected with STAT5a and STAT5b in a ratio (15/85) similar to that present in liver. From these results, we can draw two conclusions. First, the STAT5 composition of the liver DNA-binding complex is the same, or very similar, between males and females, although the *number* of active DNA-binding STAT5 molecules is much greater in male liver (3 μg *vs.* 30 μg loadings in lanes 14–16 *vs.* 17–19). Second, STAT5b is the primary STAT5 form that is activated and binds DNA in both male and female rat liver. However, STAT5a also seems to become activated and binds DNA as a minor component, in accordance with its much lower abundance in liver tissue.

STAT5a and STAT5b are both found predominantly in nuclei prepared from male, but not female, rat liver

The EMSA studies presented in Fig. 5 indicate that STAT5a and STAT5b are activated in rat liver in rough proportion to their relative abundance, in both males and females. This, in turn, suggests that STAT5a may exhibit the same sexually dimorphic pattern of activation (male > female) that is seen for the more abundant STAT5b form in rat liver. This hypothesis was evaluated by Western blot analysis of liver nuclear extracts prepared from a series of individual male and female rats. First, the specificity of the STAT5a and STAT5b antibodies used in these experiments was verified by analyzing rat STAT5 standards that were prepared by transfection of COS-1 cells with expression plasmids for either rat STAT5a, rat STAT5b, or a mixture of both STAT5 forms. As shown in Fig. 6, each antibody reacted specifically with its cognate STAT5 form (A, lane 1 *vs.* B, lane 2). Next, we compared the expression in male *vs.* female liver nuclear extracts of each STAT5 form. STAT5a and STAT5b were both found predominantly in male liver nuclear extracts (lanes 4–9), compared with female liver nuclear extracts (lanes 10–14). Furthermore, individual male nuclear samples that contain STAT5b at a lower level and in the faster migrating nontyrosine-phosphorylated form (lanes 4 and 6) also contain less STAT5a than the other male liver samples[4]. In control experiments, STAT5 analysis of cytosolic extracts from these same male and female rats revealed equal (or very similar) protein levels in each sex for both STAT5 forms (data not shown). These findings, together with the EMSA supershift analysis shown in Fig. 5, make it unlikely that the low liver STAT5 EMSA activity seen in individual female rats is the result of a selective or a preferential activation of the less abundant STAT5a protein.

Exposure of the STAT5b Western blot for a prolonged period of time revealed a low level of STAT5b protein in the female nuclear samples (Fig. 6C, lanes 17–21). Three samples exhibited the slower migrating, tyrosine-phosphorylated STAT5b form (lanes 17, 18, and 21), whereas two liver samples consisted predominantly of the faster-migrating nontyrosine-phosphorylated STAT5b forms (lanes 19 and 20). EMSA analysis (Fig. 6D) of the same female liver nuclear samples revealed that the liver nuclei containing tyrosine-phosphorylated STAT5b exhibited greater DNA-binding activity, with one sample (lane 21) showing the highest activity. The presence of nontyrosine-phosphorylated STAT5b in two of the nuclear extracts (lanes 19 and 20) may be attributable to contamination of the cytosolic fraction during sample preparation or perhaps may result from the dephosphorylation of nuclear STAT5b that is ongoing in continuous GH-stimulated liver cells (13).

Discussion

In the rodent model, GH is released from the pituitary gland into the bloodstream in a temporal pattern that is markedly sex-dependent (9, 10). In males, GH is released in an intermittent fashion, and the resultant pulsatile plasma GH pattern activates the transcription of male-specific genes. In females, the pituitary secretion of GH is more frequent and results in a near-continuous plasma GH pattern that activates transcription of several female-specific genes (35). Prototypical examples of such genes include the cytochrome P450 genes *CYP2C11* and *CYP2C12*, which encode liver-expressed steroid hydroxylase enzymes that are respectively expressed in male and female rat liver. Previous studies have shown that the male-specific intermittent GH plasma profile is both necessary and sufficient to activate *CYP2C11* and to suppress *CYP2C12* gene expression at the level of transcription initiation. Conversely, the near-continuous GH profile present in females is both necessary and sufficient to suppress *CYP2C11* transcription and activate the transcription of *CYP2C12* (36, 37). An important clue to understanding the factors necessary to transduce to these liver-expressed genes the information encoded within the sex-dependent plasma GH profiles was provided by our previous studies using rat hepatocyte nuclear protein extracts (11), where an intermittent (male) plasma GH profile was shown to be required for the intracellular signaling molecule STAT5 to become activated, translocate to the nucleus, and bind DNA. Exposure to continuous plasma GH levels, however, inhibits and perhaps desensitizes this activation pathway, such that STAT5 largely remains in the cytosol and inactive, with respect to DNA binding and gene transcription. The present study extends these findings in several ways and establishes that: 1) both forms of STAT5 (STAT5a and STAT5b) are activated much more extensively in male than in female rat liver (Fig. 6); 2) female rat liver contains a low level of active STAT5 that has a STAT5 form composition (5b > 5a) similar to that found in male rat liver (Fig. 5); and 3) the female plasma pattern of continuous plasma GH stimulation, but not PRL exposure, is required to maintain this female pattern of low STAT5 activity (Fig. 4).

We initially considered whether PRL might be the endocrine factor responsible for stimulating the low-level STAT5 activity seen in female rat liver. PRL exhibits a physiologically important activation of STAT5 in the mammary gland (38) that is important for lactogenesis and mammary gland differentiation (4). Moreover, beginning at day 30 of age, rat plasma PRL levels are much higher in females than in males

[4] In contrast to STAT5b, which is comprised of multiple bands that differ in their phosphorylation status (23), the tyrosine-phosphorylated and nontyrosine-phosphorylated forms of STAT5a do not resolve on Western blots.

(39). During the 4-day rat estrus cycle, rats exhibit a dramatic surge in plasma PRL (peaks as high as 500–600 ng/ml) on the afternoon of proestrus. This well-characterized secretory event occurs in rodents, but not in primates, and consists of three distinct phases [an early sharp peak, followed by a prolonged plateau, and finally a termination phase (29)]. Additionally, STAT5 can be activated in postpartum female rat liver in response to suckling, which induces a striking elevation in plasma PRL levels (15). However, suckling can also stimulate the release of GH (40), raising the question as to whether GH or PRL is responsible for the strong activation of liver STAT5 seen in suckled female rats. We therefore examined the potential role of PRL in stimulating the low-level STAT5 activity seen in adult (nonsuckled) females. Three independent experiments presented in this study indicate that this low-level female liver STAT5 activity is not associated with stimulation by plasma PRL. First, no correlation was observed between the animal's PRL status and the presence or absence of the low-level liver STAT5 activity at the time of proestrus. Second, treatment of rats with bromocriptine under conditions where pituitary PRL secretion is suppressed did not abolish the low liver STAT5 activity. Finally, liver STAT5 seems to be intrinsically unresponsive to PRL, not only in hypophysectomized rats (11), but also in intact female rats, insofar as exogenous PRL administration was ineffective at activating liver STAT5 above background levels. This unresponsiveness may be explained by the finding that, whereas the long form of PRL receptor is expressed in the liver and mammary gland in roughly equal amounts, PRL receptor short form dominates in the liver but is only a minor component in mammary tissue (17). PRL receptor short form can exert a dominant-negative phenotype, with respect to STAT activation, presumably by heterodimerizing with the receptor's signaling-active long form, precluding a high level of PRL-dependent STAT5 activation in liver tissue (17). Nevertheless, exogenous administration of PRL can up-regulate the expression of some liver genes, such as μ-class subunits of glutathione *S*-transferase (41). Conceivably, this induction may occur by a PRL signaling mechanism that is independent of STAT5.

The loss of female liver STAT5 activity after hypophysectomy, and its restoration by continuous GH replacement using osmotic mini-pumps (Fig. 4), provides strong evidence that GH, rather than PRL, is the key factor responsible for the low-level STAT5 activity seen in female rat liver. These findings are supported by recent cell culture model studies carried out in the rat liver-derived cell line CWSV-1, where continuous GH treatment down-regulates STAT5b activity, to about 10–15% of its peak level, by a mechanism that apparently involves enhanced dephosphorylation of both the GH receptor-JAK2 kinase complex and STAT5b. Moreover, this low-level STAT5b activity can be maintained indefinitely, provided that the liver cells are continuously stimulated with GH (13).

Although previous studies of liver nuclear extracts, prepared from continuous GH-treated hypophysectomized female rats, suggested that nuclear STAT5 protein levels were fully suppressed after 3 days of GH treatment (11), the EMSA assay for STAT5 activity used in the present study is more sensitive at detecting low levels of STAT5 activity, which in individual female rats, ranged as high as approximately 15% of the peak levels present in GH pulse-stimulated male rats. Comparison of female liver STAT5 activity levels in whole-liver extracts and nuclear extracts revealed a similar profile between livers (Fig. 1A), suggesting that the low-level of STAT5 that becomes activated in female liver translocates to the nucleus and does not remain sequestered in the cytosol in a form that is inactive, with respect to transcriptional activation of STAT5-dependent target genes. Male-specific, STAT5b-dependent (3, 5) liver genes are typically expressed in females at much lower than 5–10% of the levels found in males (35), suggesting that a minimum threshold of STAT5 activity, not met in female rats, is required to support their expression.

In an occasional adult female liver sample, we observed a level of activated STAT5 that is greater than the average (approximately 10%) relative intensity of a male GH pulse-activated STAT5 signal (*e.g.* Fig. 2A, lane 6). Conceivably, this may reflect activation by cytokines or growth factors that are also known to activate STAT5 (42). We cannot rule out the possibility that other factors, such as the high nocturnal peaks of plasma GH that characterize female rats at about 5–6 weeks of age (43), may also activate liver STAT5 in females. Further investigation is required to understand the physiological significance of the low-level activation of STAT5 demonstrated here in adult female rats and its effects on liver gene expression. One possible role, suggested by our recent studies in *Stat5a* gene knockout mice, involves the activation of certain GH-regulated, female-specific P450 genes, including a female-specific *Cyp2b* gene (5).

STAT5b protein (6, 7) and messenger RNA (5) are an estimated 10- to 20-fold more abundant than STAT5a in both rat and mouse liver. Moreover, supershift experiments, carried out in mouse (5) and rat (Fig. 5) liver extracts, indicate that the GH pulse-stimulated male STAT5 activity is predominantly in the form of STAT5b homodimers (5). Given our present finding that STAT5a is also activated and translocates to the nucleus in a male-specific manner (Fig. 6A), it is likely that predominance of STAT5b homodimers in the males is not attributable to an intrinsic, preferential activation of STAT5b, compared with STAT5a, by male GH pulses, but rather is a reflection of the greater abundance in liver of STAT5b, compared with STAT5a. Hence, it is likely that STAT5b-STAT5b homodimers and STAT5a-STAT5b heterodimers can both form in a male-specific manner. Our finding that, in *Stat5a* knockout mice, expression of male-specific cytochrome P450 genes is not blocked (5), may reflect the fact that STAT5a-STAT5b heterodimers constitute only a small fraction of the activated liver STAT5 population, rather than an inability of STAT5a-STAT5b heterodimers *per se* to support transcription of male-expressed liver genes.

We previously suggested that the down-regulation of liver STAT5 activity in response to the near-continuous adult female plasma GH profile could lead to a relative abundance in female liver of STAT5a-STAT5b heterodimeric complexes and, hence, the potential to regulate the expression of female-specific genes (5). Indeed, targeted disruption of either *Stat5a* or *Stat5b* leads to a selective loss of certain female-specific liver cytochrome P450 genes (5), suggesting that heterodimeric STAT5a-STAT5b complexes are specifically required for their expression. The present studies show, however, that whereas this hypothesis may be correct, there is little difference in the ratio of activated

STAT5b to activated STAT5a between male and female rat liver. Consequently, though the loss of expression of certain female-specific *Cyp* genes in *Stat5a* and *Stat5b* knockout mice could very well result from the inability to form STAT5a-STAT5b heterodimers in liver cells of these animals, the mechanistic basis for the proposed specificity of such heterodimers for regulating *Cyp* gene expression in females remains unknown.

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