In vivo regulation of growth hormone-stimulated gene transcription by STAT5b

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Woelfle, Joachim, and Peter Rotwein. In vivo regulation of growth hormone-stimulated gene transcription by STAT5b. Am J Physiol Endocrinol Metab 286: E393–E401, 2004; 10.1152/ajpendo.00389.2003.—The long-term effects of growth hormone (GH) are mediated through coordinated changes in gene expression that are the outcome of interactions between hormone-activated signal transduction pathways and specific feedback loops. Recent studies in mice have implicated the transcription factor STAT5b as part of the GH-regulated somatic growth pathway, because mice lacking this protein showed diminished growth rates. To assess the role of Stat5b in GH-stimulated gene expression, we have delivered modified versions of the protein to the liver of pituitary-deficient male rats by quantitative adenovirus-mediated gene transfer. In pilot studies in cell culture, both constitutive-active and dominant-negative STAT5b showed appropriate binding properties toward a specific DNA response element. After in vivo expression, neither protein prevented nuclear accumulation of STATs 1 and 3 in the liver. Dominant-negative STAT5b completely inhibited GH-stimulated transcription of genes encoding the growth-promoting proteins IGFI-I, IGF-binding protein-3 (IGFBP-3), and acid-labile subunit (ALS), which comprise the major circulating IGF-I complex, and blocked expression of the GH inhibitors SOCS-1, SOCS-2, and CIS, but had little effect on induction of SOCS-3. Constitutive-active STAT5b stimulated robust transcription of IGF-I, ALS, and IGFBP-3 in the absence of hormone but did little to modify GH-mediated activation of SOCS family genes. An adenovirus encoding EGFP was without effect. These results, in addition to establishing STAT5b as one of the key agents of genes encoding the growth-promoting proteins IGF-I, ALS, and IGFBP-3 in the liver, thus coordinated changes in gene expression in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The majority of circulating IGF-I is present in serum in a highly conserved 70-residue secreted protein (3, 21). GH rapidly stimulates IGF-I mRNA and protein expression in the liver and other tissues (2, 5, 15, 23) through acute activation of transcription by mechanisms that remain incompletely characterized (2, 7, 38). In growth-retarded patients with GH insensitivity syndromes and in pituitary-deficient rodents, somatic growth is halted but can be restored by treatment with IGF-I (12, 16, 20, 30, 31).

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Materials. The monoclonal M2 antibody to the FLAG epitope tag was purchased from Sigma (St. Louis, MO), and Alexa 488-conjugated goat anti-mouse IgG was from Molecular Probes (Eugene, OR). Recombinant rat GH was obtained from the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health. Oligonucleotides were synthesized at the Oregon Health and Science University (OHSU) Core Facility in the Department of Molecular Microbiology. Fugene 6 transfection reagent was purchased from Roche (Indianapolis, IN). Cos-7 cells were obtained from the American Type Culture Collection (Manassas, VA) and were incubated in DMEM with 10% fetal calf serum at 37°C in a humidified environment of 95% air-5% CO2. All other chemicals were reagent grade and were obtained from commercial suppliers.

Recombinant plasmids and recombinant adenoviruses. The following plasmids were purchased from commercial suppliers: EGFP-N3 (Clontech, Palo Alto, CA); pShuttle and pAdEasy (Quantum BioTechnologies, Montreal, Canada). The mouse GH receptor in pcDNA3 was a gift from Dr. F. Talamantes (University of California, Santa Cruz, CA), and a cDNA encoding rat STAT5b was from Dr. Christin Carter-Su (University of Michigan, Ann Arbor, MI). The latter was a gift from Dr. F. Talamantes (University of California, Santa Cruz, CA), and a cDNA encoding rat STAT5b was from Dr. Christin Carter-Su (University of Michigan, Ann Arbor, MI). The latter was modified by addition of a FLAG epitope tag at the NH2 terminus of the protein by site-directed mutagenesis, as outlined previously (38). Recombinant expression plasmids and adenoviruses for FLAG-tagged wild-type rat STAT5b, constitutively active STAT5b (N 642 to H), and dominant-negative STAT5b (Y 699 to F) have been described (38), as has a recombinant adenoavirus encoding EGFP (38). All adenoviruses were purified over discontinuous CsCl gradients and titered by optical density. The DNA concentration of a control viral stock measured by optical density with a spectrophotometer was correlated with viral titer determined by plaque assay, and DNA concentrations of other viruses were then related back to the control stock.

Animal studies. Normal male Sprague-Dawley rats (n = 6), 7–8 wk of age, were purchased from Harlan Sprague Dawley (Indianapolis, IN), as were male Sprague-Dawley rats that were hypophysectomized by a transauricular route at age 7 wk. Animals were housed at the OHSU Animal Care Facility on a 12:12-h light-dark schedule, with an enriched environment of 95% air-5% CO2. All studies were approved by the OHSU Animal Care and Use Committee. Hypophysectomized animals were anesthetized with pentobarbital (50 mg/kg ip), and the livers were excised, weighed, and placed on ice. Livers were then used for isolation of proteins and RNA, as outlined in Protein isolation and DNA-protein binding studies and RNA isolation and analysis. Normal rats also were anesthetized with pentobarbital, and their livers were also excised, weighed, placed on ice, and used for protein and RNA isolation.

Cell culture and gene transfer experiments. Cos-7 cells were transfected at ~50% of confluence with expression plasmids for the mouse GH receptor and FLAG-tagged wild-type rat STAT5b (1 μg of DNA for each plasmid per 60-mm culture dish) with Fugene 6, following a protocol from the supplier. After 24 h, cells were washed with PBS, and DMEM plus 1% bovine serum albumin (BSA) were added. After an additional 24 h, recombinant rat GH (final concentration of 45 nM) or vehicle (saline) was added, cells were harvested 30 min later, and nuclear and cytoplasmic proteins were isolated, as previously described (37). In other experiments, Cos-7 cells were first transfected with the mouse GH receptor plasmid and were infected 24 h later with Ad-tTA and either an Ad-STAT5b variant or Ad-EGFP. After an additional 8 h, cells were washed with PBS, and DMEM plus 1% BSA were added. After a further 16 h, recombinant rat GH or vehicle was added, as above, cells were harvested 30 min later, and nuclear proteins were isolated.

Protein isolation and DNA-protein binding studies. Nuclear and cytoplasmic proteins were isolated from Cos-7 cells and from rat liver, as described previously (37, 38). Electrophoretic gel mobility shift assays were performed (34) with 4–15 μg of Cos-7 or rat hepatic nuclear protein extracts and fluorescein-labeled double-stranded oligonucleotides from the STAT5-binding site of the rat β-casein gene (17), the STAT5-binding site of the rat Spi 2.1 gene (34), the high-affinity c-sis inducible element (SIE, which binds STAT1 and STAT3 (10)), or a binding site for specificity protein 1 (Sp1) (19). The top strand of each DNA sequence is shown in Table 1. Nuclear proteins were incubated in buffer containing 25 mM HEPES (pH 7.6), 7.5% glycerol, 60 mM KCl, 5 mM MgCl2, 0.1 μg/ml BSA, 0.1 mM EDTA, 1 mM DTT, and 2 μg poly dIdC for 30 min at 4°C. The double-stranded DNA probe was added (final concn 20 nM) and, after an additional 30 min of incubation at 4°C, samples were loaded onto nondenaturing 4–12% polyacrylamide gels in 0.5× TBE (45 mM Tris, 45 mM boric acid, and 1 mM EDTA, pH 8.3) and electrophoresed at 120 V for 2 h at 20°C. For competition experiments, a 50-fold molar excess of unlabeled double-stranded DNA [either β-casein or Oct-1 (29)] was added to the incubation mixture. For supershift experiments, 1 μg of antibody to the FLAG epitope tag was added to the incubation mixture. Results were detected using a Molecular Image FX and were evaluated with Quantity One software (Bio-Rad).

RNA isolation and analysis. Total liver RNA and hepatic nuclear RNA were isolated as described previously (2). The RNA concentra-

Table 1. Oligonucleotides used in gel mobility shift experiments

<table>
<thead>
<tr>
<th>Transcription Factor</th>
<th>DNA Sequence (Top Strand)</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Casein</td>
<td>STAT5</td>
<td>5’-GAACCTTCTTGAGAATTTAG-3’</td>
</tr>
<tr>
<td>Spi 2.1</td>
<td>STAT5</td>
<td>5’-ACGCTTCATGAAATCTTCTAGAAGATCATCTGACGTGGGCA-3’</td>
</tr>
<tr>
<td>High-affinity SIE</td>
<td>STAT1, STAT3</td>
<td>5’-TGACATTTTCGCCGTTTACGTGA-3’</td>
</tr>
<tr>
<td>Sp1</td>
<td>Sp1</td>
<td>5’-ATTAGCGGAGGGGGGGGAGG-3’</td>
</tr>
</tbody>
</table>

STAT5, signal transducer and activator of transcription 5. SIE, c-sis inducible element; Sp1, specificity protein 1.
tion was determined spectrophotometrically at 260 nm, and its quality was assessed by agarose gel electrophoresis. Aliquots of RNA were pooled from six pituitary-intact rats before the reverse transcription step. RNA (5 μg) was reverse transcribed in a final volume of 20 μl with an RT-PCR kit according to the manufacturer’s instructions (Life Technologies, Carlsbad, CA), with either oligo(dT) primers (for total cellular RNA) or random hexamers (for nuclear RNA). Each PCR reaction contained 0.5 μl of cDNA. Primer sequences are listed in Table 2. The linear range of product amplification was established for each primer pair in pilot studies, so that in final experiments cycle numbers were chosen to reflect the approximate midpoint of the linear phase of amplification. For cDNA derived from whole cell RNA, this varied from 18 to 25 cycles depending on the gene, and for cDNA from nuclear RNA, from 24 to 30 cycles. Results were analyzed on 1.5% agarose gels and were visualized using a Molecular Imager FX. Band intensities were quantified by densitometry with Quantity One software. For all experiments, controls performed in the absence of the RT step yielded no products after PCR, indicating undetectable DNA contamination of RNA.

RESULTS

Analysis of recombinant adenoviruses encoding rat STAT5b and variants. We recently developed a series of recombinant expression plasmids and adenoviruses encoding NH2-terminally FLAG-tagged wild-type rat STAT5b and two variants, a dominant-negative protein, containing substitution of phenylalanine for tyrosine 699, and a constitutive-active version, with alteration of asparagine 642 to histidine. Using these tools, we showed that STAT5b played a central role in the regulation of IGF-I gene transcription by GH in vivo (38). Using the same reagents, we now have sought to determine whether STAT5b is involved in controlling the in vivo expression of other GH-induced genes.

We first tested the DNA-binding properties of recombinant wild-type and modified STAT5b in cultured cells after transient expression. Figure 1A shows results of gel mobility shift experiments with a double-stranded fluorescein-labeled oligonucleotide containing the GH-prolactin response element from the β-casein gene promoter, which binds STAT5b, and nuclear proteins isolated from Cos-7 cells cotransfected with expression plasmids for the mouse GH receptor and wild-type STAT5b and incubated with recombinant rat GH (45 nM) or vehicle for 30 min. GH treatment caused the appearance of a single protein-DNA complex (see lanes 1 and 2) that disappeared after addition of a 50-fold molar excess of unlabeled homologous DNA, but not with a heterologous competitor, Oct-1 (lanes 3 and 4). The presence of recombinant STAT5b in the complex was demonstrated by a supershift with monoclonal anti-FLAG antibody (lane 5).

The DNA-binding properties of the two STAT5b variant proteins were analyzed next by gel mobility shift experiments using nuclear protein extracts from Cos-7 cells transfected with an expression plasmid for the mouse GH receptor and infected with adenoviruses encoding wild-type (Ad-STAT5bWT), dominant-negative (Ad-STAT5bDN), or constitutively active (Ad-STAT5bCA) proteins. As seen in Fig. 1B, a GH-dependent

Table 2. Primers used for RT-PCR of total and nuclear RNA

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location</th>
<th>DNA Sequence</th>
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<tr>
<td>Total RNA</td>
<td>SOCS-1</td>
<td>exon 2</td>
</tr>
<tr>
<td></td>
<td>SOCS-2</td>
<td>exon 1</td>
</tr>
<tr>
<td></td>
<td>SOCS-3</td>
<td>exon 2</td>
</tr>
<tr>
<td></td>
<td>CIS</td>
<td>exon 2</td>
</tr>
<tr>
<td></td>
<td>β-Actin</td>
<td>exon 3</td>
</tr>
<tr>
<td></td>
<td>IGF-1</td>
<td>exon 3</td>
</tr>
<tr>
<td></td>
<td>IGFBP-3</td>
<td>exon 6</td>
</tr>
<tr>
<td></td>
<td>ALS</td>
<td>exon 2</td>
</tr>
<tr>
<td>Nuclear RNA</td>
<td>SOCS-1</td>
<td>exon 2</td>
</tr>
<tr>
<td></td>
<td>SOCS-2</td>
<td>exon 1</td>
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<tr>
<td></td>
<td>SOCS-3</td>
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<tr>
<td></td>
<td>CIS</td>
<td>exon 1</td>
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<tr>
<td></td>
<td>β-Actin</td>
<td>exon 3</td>
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<td></td>
<td>IGF-1</td>
<td>exon 3</td>
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<tr>
<td></td>
<td>IGFBP-3</td>
<td>exon 3</td>
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<tr>
<td></td>
<td>ALS</td>
<td>exon 2</td>
</tr>
</tbody>
</table>

SOCS, suppressor of cytokine signaling; CIS, cytokine-induced SH2 protein; IGFBP-3, IGF-binding protein-3; ALS, acid-labile subunit.
protein-DNA complex of similar mobility could be observed after infection with Ad-EGFP. On the basis of these results and our published studies (38), we conclude that the three STAT5b adenoviruses functioned appropriately.

Hepatic expression of Ad-STAT5b after in vivo infection of pituitary-deficient rats. In depth analysis of the control of gene expression by GH, particularly in the liver, has been hampered by the lack of robust and representative in vitro systems that reproduce the normal patterns of gene regulation seen in vivo. Thus, to characterize potential roles for STAT5b in specific GH-stimulated gene expression, it was necessary first to define experimental conditions in which quantitative in vivo hepatic infection with recombinant adenoviruses could be achieved. Dose-finding studies were performed initially using Ad-EGFP. After intravenous injection of optimal quantities of viruses (2 × 10^10 pfu of Ad-EGFP and 2 × 10^9 pfu of Ad-tTA), nearly 90% of liver cells were infected 48 h later, as demonstrated by analysis of freshly isolated hepatocytes for EGFP (Fig. 2A). Additional pilot experiments showed that similar levels of viral infection produced comparable expression of STAT5b, as seen in Fig. 2B for Ad-STAT5bDN (Ad-tTA is a helper virus that expresses a tetracycline-inhibited transcriptional activator. It induces gene expression from Ad-EGFP, Ad-STAT5bDN, and Ad-STAT5bCA).

Effects of adenovirus infection on GH-induced activation of STATs 1, 3, and 5 in pituitary-deficient rats. GH rapidly activates STATs 1, 3, and 5 in the liver and stimulates their expression by GH, particularly in the liver, has been hampered by the lack of robust and representative in vitro systems that reproduce the normal patterns of gene regulation seen in vivo. Thus, to characterize potential roles for STAT5b in specific GH-stimulated gene expression, it was necessary first to define experimental conditions in which quantitative in vivo hepatic infection with recombinant adenoviruses could be achieved. Dose-finding studies were performed initially using Ad-EGFP. After intravenous injection of optimal quantities of viruses (2 × 10^10 pfu of Ad-EGFP and 2 × 10^9 pfu of Ad-tTA), nearly 90% of liver cells were infected 48 h later, as demonstrated by analysis of freshly isolated hepatocytes for EGFP (Fig. 2A). Additional pilot experiments showed that similar levels of viral infection produced comparable expression of STAT5b, as seen in Fig. 2B for Ad-STAT5bDN (Ad-tTA is a helper virus that expresses a tetracycline-inhibited transcriptional activator. It induces gene expression from Ad-EGFP, Ad-STAT5bDN, and Ad-STAT5bCA).

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Effects of adenovirus infection on GH-induced activation of STATs 1, 3, and 5 in pituitary-deficient rats. GH rapidly activates STATs 1, 3, and 5 in the liver and stimulates their expression by GH, particularly in the liver, has been hampered by the lack of robust and representative in vitro systems that reproduce the normal patterns of gene regulation seen in vivo. Thus, to characterize potential roles for STAT5b in specific GH-stimulated gene expression, it was necessary first to define experimental conditions in which quantitative in vivo hepatic infection with recombinant adenoviruses could be achieved. Dose-finding studies were performed initially using Ad-EGFP. After intravenous injection of optimal quantities of viruses (2 × 10^10 pfu of Ad-EGFP and 2 × 10^9 pfu of Ad-tTA), nearly 90% of liver cells were infected 48 h later, as demonstrated by analysis of freshly isolated hepatocytes for EGFP (Fig. 2A). Additional pilot experiments showed that similar levels of viral infection produced comparable expression of STAT5b, as seen in Fig. 2B for Ad-STAT5bDN (Ad-tTA is a helper virus that expresses a tetracycline-inhibited transcriptional activator. It induces gene expression from Ad-EGFP, Ad-STAT5bDN, and Ad-STAT5bCA).
labeled double-stranded oligonucleotide containing a consensus binding site for the transcription factor Sp1 is not affected by GH in rats infected with any of the recombinant adenoviruses (Fig. 3C). Taken together, the results in Fig. 3 show the selectivity and effectiveness of Ad-STAT5bDN and Ad-STAT5bCA, respectively, to inhibit or mimic STAT5b function in the liver.

Hormonal regulation of gene expression. Members of the SOCS family have been shown to exert inhibitory effects on activation of STAT proteins in response to GH or cytokines and have been proposed to act as part of a classical negative feedback loop (18). In this regard, several investigators have shown that GH can stimulate the gene expression of SOCS-1, -2, -3, and CIS in a variety of cell types, including primary hepatocytes (1, 4, 26, 28, 35), although the biochemical mechanisms have not been established. Pertinent to the current studies are observations in STAT5b knockout mice, where GH treatment failed to increase steady-state mRNA levels for SOCS-2 in the liver (4), thus potentially implicating STAT5b as a key regulator of this SOCS gene.

To address the role of STAT5b in GH-stimulated SOCS gene expression, RNA was isolated from normal male rats, or at various intervals after GH treatment from pituitary-deficient rats infected with Ad-EGFP, Ad-STAT5bDN, or Ad-STAT5bCA for measurement of steady-state mRNA levels by semi-quantitative RT-PCR (for primers see Table 2). As shown in Fig. 4A, GH caused an increase in mRNA abundance for four SOCS family members in Ad-EGFP-infected hypophysectomized rats, but with different kinetics. SOCS-1, SOCS-3, and CIS gene expressions were all stimulated to peak mRNA values within 60 min of GH injection, the earliest time examined, and then declined in abundance by 120 min. In contrast, SOCS-2 mRNA expression did not become detectable until 120 min. Peak levels of transcripts for SOCS-2 were equivalent to values seen under steady-state conditions in pituitary-intact male rats of the same age, whereas expression of SOCS-1, CIS, and SOCS-3 after GH greatly exceeded the low levels measured in intact rats. Ad-STAT5bDN blocked GH-mediated stimulation of SOCS-1, SOCS-2, and CIS gene expression but caused GH-independent accumulation of SOCS-3 mRNA. Ad-STAT5bCA did not alter the GH-regulated pattern of SOCS family gene expressions seen in Ad-EGFP-infected rats, except that an earlier increase in SOCS-2 transcripts was seen after hormone treatment. Steady-state levels of β-actin mRNA were not altered by GH under any circumstance.

In addition to SOCS genes, components of the IGF family are regulated by GH in the liver and in other tissues. IGF-I gene transcription is acutely induced by hormone and, on the basis of our recent observations, appears to require active STAT5b (38). In the circulation, IGF-I is primarily found as a component of a ternary complex that also contains IGFBP-3 and ALS (16). ALS is a GH-regulated liver-enriched gene (24, 25). Hormone treatment readily induces its transcription, potentially through a STAT5 response element in the ALS promoter (25). The role of GH in regulating IGFBP-3 gene expression is less characterized. In some experimental systems, GH exerts a stimulatory effect on IGFBP-3 mRNA abundance that does not appear to require intervening IGF-I (11), whereas in other models, IGF-I seems to be a key inducing agent (6, 11).

To address the role of STAT5b in GH-stimulated ALS and IGFBP-3 gene expression, RNA isolated from saline- and GH-treated adenovirus-infected rats was used in semi-quantitative RT-PCR experiments. As a positive control, IGF-I gene expression also was examined. As seen in Fig. 4B, GH caused a prompt increase in IGF-I mRNA abundance, with transcripts containing exons 3 and 4 being measurable by 120 min in Ad-EGFP rats. Induction of IGF-I mRNA was impaired in rats infected with Ad-STAT5bDN and was markedly enhanced in the absence of GH in animals infected with Ad-STAT5bCA. Neither IGFBP-3 nor ALS mRNA was detectable within 120 min after GH injection in Ad-EGFP- or Ad-STAT5bDN-infected hepatocytes.
fected rats, but transcripts for both genes also were induced in the absence of hormone in animals infected with Ad-STAT5bCA. Peak mRNA values for all three genes reached levels seen under steady-state conditions in pituitary-intact male rats only in animals infected with Ad-STAT5bCA.

**Hormonal control of gene transcription.** On the basis of results seen in Fig. 4, it appears that STAT5b is necessary but not sufficient for GH-dependent gene expression of SOCS-1, SOCS-2, and CIS, as STAT5bDN blocked but STAT5bCA did not enhance their mRNA accumulation after hormone treatment. It appears that STAT5b is both necessary and sufficient for IGF-I, because STAT5bDN prevented hormone-induced expression and STAT5bCA increased IGF-I mRNA abundance in the absence of GH. In contrast, STAT5b seems not to be required for GH-mediated induction of SOCS-3 and could not be fully evaluated for ALS or IGFBP-3, because in the absence of added active STAT5b, acute GH treatment did not induce either transcript within the 120-min time frame studied. To extend these observations in a more mechanistic direction, we next assessed hormone-regulated gene transcription in nuclear RNA by use of a semi-quantitative RT-PCR assay that measures accumulation of nascent nuclear transcripts for each gene (38). In Fig. 5, the schematic gene diagrams to the left of each panel indicate the locations of the gene-specific primers used in these experiments, and their DNA sequences are listed in Table 2.

As seen in Fig. 5A, in pituitary-deficient rats infected with Ad-EGFP, GH caused an increase in gene transcription of all four SOCS family members, but with slightly different kinetics. SOCS-3 was induced within 30 min, and SOCS-1, SOCS-2, and CIS by 60 min after hormone. Infection of rats with Ad-STAT5bDN prevented GH-stimulated induction of SOCS-1, SOCS-2, and CIS but had little effect on SOCS-3 transcription. Ad-STAT5bCA had reproducibly minor effects on SOCS gene transcription. An earlier and more sustained response to GH was observed for SOCS-1 and SOCS-2, with an increase in nascent RNA first being measurable by 30 min instead of 60 min after hormone and being maintained for the entire 120-min experimental period. CIS transcripts also were modestly increased, with an earlier and higher peak of nascent nuclear RNA being observed. By contrast, SOCS-3 gene transcription was minimally altered. β-Actin gene transcription was not regulated by GH.

**Fig. 3.** Activation of STATs 1, 3 and 5 by GH in pituitary-deficient male rats infected with different recombinant adenoviruses. A: time course of induction of STATs 1 and 3 by GH, as measured by gel mobility shift assays using a double-stranded high affinity SIE probe and rat hepatic nuclear protein extracts from adenovirus-infected male hypophysectomized rats. B: time course of induction of active nuclear STATs, as assessed by gel mobility shift assays with a double-stranded DNA probe containing the GH response element from the Spi 2.1 gene and hepatic nuclear protein extracts from adenovirus-infected male hypophysectomized rats. Note that one-third as much nuclear protein was used in samples from rats infected with Ad-STAT5bCA. C: results of gel mobility shift experiments using rat hepatic nuclear extracts from adenovirus-infected male hypophysectomized rats and a double-stranded oligonucleotide containing an Sp1-binding site. In all panels, the large black arrow indicates location of protein-DNA complexes, the white arrow, EGFP, and the small black arrow, free probe. Results are representative of 3 independent experiments.

**Fig. 4.** Regulation of gene expression by GH in pituitary-deficient male rats infected with Ad-EGFP, Ad-STAT5bDN or Ad-STAT5bCA. A: time course studies by semi-quantitative RT-PCR of mRNA for SOCS-1, SOCS-2, SOCS-3, CIS, and β-actin after GH treatment, and in pituitary-intact rats (WT) not treated with GH. Two concentrations of cDNA were used in lanes derived from WT rats, 0.1 and 1 times as much as from hypophysectomized rats. B: time course studies by semi-quantitative RT-PCR of mRNA for IGFBP-3, ALS, and IGF-I after GH treatment, and in pituitary-intact rats (WT) not treated with GH. Results are representative of 3 independent experiments.
IGF-I gene transcription was rapidly and potently induced within 30 min by GH in Ad-EGFP rats, was inhibited by Ad-STAT5bDN, and was enhanced in the absence of hormone by Ad-STAT5bCA. ALS gene transcription was stimulated after 120 min of GH in pituitary-deficient rats infected with Ad-EGFP. This induction in gene expression was blocked by Ad-STAT5bDN. Transcription of the IGFBP-3 gene remained undetectable before or after GH treatment in the presence of either Ad-EGFP or Ad-STAT5bDN. Nascent transcripts for both ALS and IGFBP-3 were stimulated by infection with Ad-STAT5bDN, and GH had little additional enhancing effect.

**DISCUSSION**

The broad effects of GH on somatic growth, tissue differentiation, and intermediary metabolism are mediated in part through coordinated changes in gene expression that are the outcomes of an interplay among several hormone-activated signal transduction pathways and involve both feed-forward and feedback loops. In this report, we have demonstrated the feasibility of specifically targeting STAT5b through use of an adenovirus-mediated gene transfer system in experimental animals to identify which GH-regulated genes are dependent on the actions of this hormone-activated transcription factor.

Our results show that Stat5b is an essential component in the control of transcription by GH for five genes studied, including SOCS-1 and -2, IGF-I, IGFBP-3, and ALS, that it plays a limited role in regulating CIS, and that it has no effect on SOCS-3. We find that dominant-negative STAT5b completely inhibits GH-stimulated SOCS-1, SOCS-2, CIS, IGF-I, ALS, and IGFBP-3 gene transcription, whereas constitutive-active STAT5b could substitute for GH in promoting robust expression of IGF-I, ALS, and IGFBP-3 genes in the absence of hormone and could increase the effects of GH on SOCS-1 and -2, leading to enhanced and more rapid gene activation after hormone treatment. In conjunction with published studies, our observations argue that Stat5b may play both direct and indirect roles in influencing GH-activated gene transcription.

The SOCS family of proteins functions as components of an inhibitory feedback loop that regulates the activity of cytokine receptors (18). Previous analyses in both experimental animals and in cell culture models have demonstrated that mRNA and protein expression of SOCS-1, -2, -3, and CIS are rapidly induced by GH (1, 4, 26, 28, 35), although the biochemical mechanisms responsible have not been established. SOCS-3 gene transcription has been shown to be activated by GH, but not through two putative STAT-binding sites in its proximal promoter, as both have been found to be dispensable for hormone-stimulated gene expression (26). Our observations extend these results, as we show no effects of dominant-negative or constitutive-active STAT5b on GH-activated SOCS-3 transcription. Taken together, these findings indicate that GH stimulates SOCS-3 gene expression through mechanisms independent of STAT5b. Our results also agree with observations of Davey et al. (4), who found that SOCS-3 mRNA was acutely induced by GH in Stat5b-deficient mice, although the extent of induction was less than in wild-type animals. No GH response elements have been definitively identified in the SOCS-1, -2, or CIS genes, and the mechanisms of hormonal regulation also remain uncharacterized. For SOCS-1 and -2, our results indicate a potentiating role for STAT5b in hormone-induced gene transcription, as overexpression of constitutive-active protein enhanced the rate and extent of GH-stimulated gene expression but did not activate transcription in the absence of hormone. These observations, which are consistent with studies performed with mice lacking Stat5b (4), suggest that Stat5b indirectly regulates the SOCS-1 and SOCS-2 genes, possibly through another GH-activated transcription factor that is also dependent on STAT5b.
for either expression or function. In contrast, CIS gene transcription was minimally increased by constitutive-active STAT5b.

Serum levels of IGF-I are stabilized through the formation of a ternary complex containing one molecule of ALS and IGFBP-3 in addition to a single molecule of IGF-1 (16). As shown here, STAT5b is involved in the transcriptional control of the genes encoding all three proteins. All are induced by constitutive-active STAT5b and are not stimulated by GH in the presence of dominant-negative STAT5b. Previous studies have identified a hormone response element in the proximal ALS gene promoter that binds STAT5 and is required for GH-stimulated gene activation (24, 25). A similar mechanism may apply to IGF-I, but to date a GH-regulated STAT5b-binding site has not been identified within the IGF-I locus in any mammalian species, although it has been found in a salmon IGF-I gene (22). In this regard, it is of interest that, despite apparently similar regulation of IGF-I and ALS genes by GH via STAT5b, the kinetics of gene activation in response to hormone differ dramatically. IGF-I gene transcription is induced by 30 min after in vivo GH injection, whereas ALS transcription is markedly slower, being detected only at the 120-min time point. These disparate results suggest that specific modifiers in addition to STAT5b may mediate the transcriptional activation of each gene by GH. Beyond the requirement for active STAT5b identified here, the mechanisms of IGFBP-3 gene activation by GH remain unknown, and under control conditions IGFBP-3 transcription is not detectably stimulated by hormone over the 120-min period evaluated.

GH plays a multifaceted and complex role in mammalian physiology (13). It regulates somatic growth through both direct and indirect pathways, the latter being largely dependent on IGF-I (13, 16, 20, 39); it has important functions in many aspects of glucose, fat, and protein metabolism; and it modulates the immune and cardiovascular systems (13). Our results further establish the central importance of STAT5b in controlling many of the transcriptional effects of GH on target gene expression, at least in the liver, and offer an experimental framework for defining the specific biochemical mechanisms by which other transcription factors mediate hormone-regulated gene activation in distinct tissues in vivo.

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