Characterization of Distinct Stat5b Binding Sites That Mediate Growth Hormone-stimulated IGF-I Gene Transcription*

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A key agent in the anabolic actions of growth hormone (GH) is insulin-like growth factor-I (IGF-I), a 70-amino acid secreted protein with direct effects on somatic growth and tissue maintenance and repair. GH rapidly and potently stimulates IGF-I gene transcription by mechanisms independent of new protein synthesis, and recent studies have linked the transcription factor Stat5b to a regulatory network connecting the activated GH receptor on the cell membrane to the IGF-I gene in the nucleus. Here we analyze two distinct conserved GH response elements in the rat IGF-I locus that contain paired Stat5b sites. Each response element binds Stat5b in vivo in a GH-dependent way, as assessed by chromatin immunoprecipitation assays, and consists of one high affinity and one lower affinity Stat5b site, as determined by both qualitative and quantitative protein-DNA binding studies. In biochemical reconstitution experiments, both response elements are able to mediate GH-stimulated and Stat5b-dependent transcription when fused to a reporter gene containing either the major IGF-I promoter or a minimal neutral promoter, although the paired Stat5b sites located in the second IGF-I intron were more than twice as effective as the response element that mapped −73 kb 5′ to the IGF-I exon 1. Taken together, our results define the initial molecular architecture of a complicated GH-regulated transcriptional pathway, and suggest that apparently redundant hormone response elements provide a mechanism for amplifying GH action at a physiologically important target gene.

The actions of growth hormone (GH)3 on somatic growth and intermediary metabolism have been intertwined with the effects of insulin-like growth factor-I (IGF-I) ever since the somatomedin hypothesis was first proposed nearly 50 years ago (1). Much is now known about the physiology, biochemistry, and signal transduction pathways of both proteins (2, 3), and their interdependent roles in regulating growth during childhood, stimulating tissue regeneration in the adult, and maintaining tissue integrity during aging have been both confirmed and refined in intervening years (4–6).

GH initiates its physiological effects after binding to its membrane-bound receptor, leading to activation of Jak2, a receptor-associated intracellular tyrosine-protein kinase (2), and setting into motion a series of protein phosphorylation steps that stimulate the activity of several transcription factors, including Stats1, 3, 5a, and 5b, among others (7, 8). It has been known for over a dozen years that GH potently induces IGF-I gene transcription (9), causing the sustained accumulation of IGF-I mRNAs and protein (9, 10), yet the signaling pathways connecting the GH receptor and cytoplasmic Jak2 to the nuclear IGF-I gene have been elucidated only recently (2, 11). Contributing to this challenge is the fact that the IGF-I gene is more complicated than would have been predicted from its simple protein sequence. In mammals, the gene is transcribed from tandem promoters, each with distinct leader exons, and its initial transcripts undergo both differential RNA splicing and alternative polyadenylation to yield multiple mature mRNA species encoding distinct IGF-I precursor proteins that are secreted and processed into a single mature bioactive 70-residue IGF-I (12).

Studies from our laboratory have implicated the transcription factor Stat5b as a key component in acute GH-stimulated IGF-I gene activation in rats (11), thus extending previous observations pointing to effects of Stat5b and to a lesser extent Stat5a on regulating postnatal somatic growth in mice (13, 14). More recently, a key role for Stat5b in normal growth in humans was inferred through identification of an individual with a homozygous point mutation in the Stat5b gene, profound short stature, absent clinical responses to exogenous GH, and diminished expression of IGF-I (15). At the same time we reported the characterization of a putative GH response element in the second intron of the rat IGF-I gene, termed HS7, that contains tandem Stat5 binding sites and functions as a cis-regulatory region capable of mediating GH-induced and Stat5b-mediated IGF-I gene transcription (16). Here we use a combination of in vivo analyses in GH-deficient rats, biochemical reconstitution experiments in tissue culture cells, and protein-DNA binding studies in vitro to evaluate a second potential GH response element in the IGF-I locus. We show that this DNA segment also binds Stat5b in a GH-dependent way, and can mediate GH-activated IGF-I gene transcription. Our observations begin to elucidate the molecular architecture of a complex transcriptional pathway and suggest that redundant hormone response elements provide a mechanism for sustaining and amplifying GH action at a physiologically critical target gene.

EXPERIMENTAL PROCEDURES

Materials—Antibodies to Stat5 (C17X) were from Santa Cruz Biotechnology (Santa Cruz, CA), anti-FLAG (M2) was from Sigma, and anti-T7 from Novagen (San Diego, CA). Recombinant rat GH was purchased from the National Hormone and Pituitary Program, NIDDK,
National Institutes of Health. Oligonucleotides were synthesized at the OHSU Core Facility in the Department of Molecular Microbiology and Immunology. Transit-LT1 was from Mirus (Madison, WI), the Quick PCR DNA purification kit was from Qiagen (Valencia, CA), and protein A-agarose beads were from Sigma. All other chemicals were reagent grade and were purchased from commercial suppliers.

Recombinant Plasmids—An expression plasmid for the mouse GH receptor in pcDNA3 has been described previously, as have expression plasmids for wild-type and constitutively active rat Stat5b (Stat5b
N642H), each modified with an N-terminal FLAG epitope tag (11).

Cell Culture, Transient Transfections, and Reporter Gene Assays—COS-7 cells (ATCC CRL-1651) were incubated in antibiotic-free Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, and were co-transfected with expression plasmids for the mouse GH receptor and wild-type or constitutively active rat Stat5b, using serum-free medium was added containing 1% bovine serum albumin for GH receptor and wild-type or constitutively active rat Stat5b, using serum, and were co-transfected with expression plasmids for the mouse GH receptor and wild-type or constitutively active rat Stat5b, using serum-free medium was added containing 1% bovine serum albumin for

GH receptor and wild-type or constitutively active rat Stat5b, using serum-free medium was added containing 1% bovine serum albumin for

**TABLE 1**

| Gene | Location | DNA sequence | Product size (bp) |
|------|----------|--------------|------------------|
| IGF-1 | Intron 2 | 5'-GCATGTGTCCTCAGAAAGGTGAGA | 200 |
| IGF-1 | Intron 3 | 5'-CAGTACGAAAGATGTTA | 319 |
| IGF-1 | Intron 3 | 5'-AGCGTCGAGGAAGTAGATGTT | 351 |
| Far 5' | Top strand | 5'-GTCGACAGAGAAATGAGTGG | 202 |
| ALS | Promoter | 5'-GAGTATTTGCCAGCGCCAGCTC | 282 |
| Sox-3 | Promoter | 5'-CCTAGAAGCTGGCAGCAGCT | 382 |
| Cis | Promoter | 5'-GGTCAACGTAGGAGCCTC | 267 |
| β-Actin | Exon 3 | 5'-GTTAAGGGTGGTTAGTGCAGA | 298 |
| β-Actin | Intron 3 | 5'-AGAGAAAGGCGTGTCAGAAGTT | 400 |

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**DNA-Protein Binding Studies**—Electrophoretic mobility shift assays were performed as indicated (11, 17), with COS-7 or rat hepatic nuclear proteins and 5'-IRDye™700-labeled double-stranded oligonucleotides from rat HS7 GHRE-1 (top strand, 5'-GGCCTCTCTCTGGAAGAAG-3'); HS7 GHRE-2 (top strand, 5'-CTGTCGTCTGCTAGATGAAAG-3'); 5' distal region RE-1 (top strand, 5'-AGTCAAGAAGGATTCTAAGAATCCTGCAAGC-3'); 5' distal region RE-2 (top strand, 5'-GCCCTTTTTTCTTTTATTAGGATTAGAA-3'); or with a binding site for Sp1 (top strand, 5'-ATTGCCATGGGCAGGAGGACG-3'). After incubation of proteins and DNA for 30 min at 4°C, products were separated by electrophoresis through non-denaturing 4 or 5% polyacrylamide gels in 1X Tris borate/EDTA (90 mM Tris, 90 mM boric acid, and 2 mM EDTA, pH 8.3) at 200 V for 25–35 min at 20°C. Results were analyzed using an Odyssey infrared imaging system and v1.2 analysis software.

**RNA Analysis**—Hepatic nuclear RNA was isolated as described (9). RNA concentrations were determined spectrophotometrically at 260 nm, and assessed by agarose gel electrophoresis. Nuclear RNA (5 μg) was reverse-transcribed with random hexamers in a final volume of 20 μl using an RT-PCR kit (Invitrogen). PCR reactions were then performed with 0.5 μl of cDNA (11). Primer sequences are listed in Tables 1 and 2. The linear range of product amplification was established in pilot studies for each primer pair, and the cycle number that reflected the approximate midpoint was used in final experiments. This varied from 24 to 28 cycles. Results were quantified by densitometry after electrophoresis through 1.5% agarose gels. All experiments were performed on at least four separate occasions with comparable results. **Chromatin Immunoprecipitation Assays (ChIP)**—Initial steps were modified from published protocols, as described (16, 18). For each time point a 200-ng fragment of rat liver was minced and incubated at 20°C in 10 ml of Dulbecco’s modified Eagle’s medium plus 1% formaldehyde barbitol (50 mg/kg intraperitoneally), and sacrificed. Liver nuclear proteins were isolated as described (17) and RNA and DNA as outlined below. All procedures were approved by the OHSU Committee on Animal Care and Use.
on a rotating platform for 15 min, followed by addition of 1.5 ml of 1 M glycine and incubation for an additional 5 min. After centrifugation at 200 × g for 5 min at 20 °C, the pellet was suspended in 1 ml of phosphate-buffered saline, transferred to a 2-ml Dounce, and homogenized with 10 strokes of a tight-fitting pestle. After brief centrifugation in a microcentrifuge, the supernatant was diluted to 4 ml with immunoprecipitation buffer (20 mM Tris-HCl, 5 mM EDTA, 1% SDS pH 8.1 plus protease inhibitors), and incubated for 15 min at 4 °C. Each sample was sonicated at 4 °C using a total of 5 pulses for 15 s each of a Branson micro-tip sonicator at 14,000 setting 5 interspersed with 30-s incubations on ice. After centrifugation at 14,000 × g for 10 min at 4 °C in a microcentrifuge, the supernatant was diluted to 4 ml with immunoprecipitation buffer (20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA pH 8.1 plus protease inhibitors), and 1-ml aliquots were used for immunoprecipitation (16, 18), with 3 μl of the Stat5 C17X antibody and 45 μl of a 50% slurry of protein A-agarose beads. DNA was extracted using the QiaQuick PCR DNA purification kit, following a protocol from the supplier, and was suspended in 100 μl of 10 mM Tris-HCl, 1 mM EDTA pH 8.0. PCR reactions were performed with 1 μl of DNA using the primer pairs listed in Table 1. The linear range of product amplification was established for each primer pair in pilot studies, and the cycle number that reflected the approximate mid-range of product amplification was established for each primer pair.

All experiments were performed on at least three separate occasions with comparable results.

RESULTS

Identification of a Second GH-regulated Stat5 Binding Site within the Rat IGF-I Gene—In recent studies, we characterized a DNA segment within the second intron of the rat IGF-I gene that was able to confer GH responsiveness to the major IGF-I promoter in vitro, and that could bind Stat5b in vivo in a GH-dependent way (16). More recently, by a shotgun cloning approach, a different putative Stat5 binding element was mapped to the human IGF-I locus ~77 kb 5’ to exon 1 (19). This DNA fragment when fused to reporter genes containing otherwise neutral promoters could confer responsiveness to constitutively active Stat5a or Stat5b. As this DNA sequence was conserved among several mammalian species (19), we sought to determine whether it was a cis-acting element in a GH-regulated IGF-I gene transcriptional pathway. As a first step, we asked whether this DNA element was a bona fide GH-activated Stat5b binding site in vivo. Shown in Fig. 1 is a map of the rat IGF-I locus, illustrating the six exons of the gene, its two promoters, P1 and P2, and the locations of HS7, our previously defined Stat5b binding site and GH response element (16), and the rat homologue of the 5’ distal region identified in the human IGF-I locus (19).

We used ChIP experiments to determine if the 5’ distal region was capable of binding Stat5b in vivo in a GH-dependent way. As seen in Fig. 2A, little Stat5b could be detected in hepatic chromatin from GH-deficient rats in association with any of the genes illustrated in the absence of hormone. Within 30 min of systemic GH injection, Stat5b was found at the IGF-I HS7 site, and binding persisted for the 60-min duration of the experiment. Identical kinetics of induction of Stat5b binding were observed on chromatin at the IGF-I 5’ distal region, and at the promoters of two other known GH-activated and Stat5b-regulated genes, acid labile subunit (ALS) and Cis (20–22), while putative Stat sites in the Socs-3 promoter remained unoccupied by Stat5b, as did a region of the non-GH-regulated β-actin gene, and intron 3 of the rat IGF-I gene.

GH-stimulated gene transcription was assessed in parallel with ChIP assays, using nuclear RNA isolated from the same livers to confirm that there were functional consequences of hormone treatment. The accumulation of nascent transcripts was measured by semi-quantitative RT-PCR assay. As pictured in Fig. 2B, transcription directed by the IGF-I promoters was stimulated within 30 min of systemic GH treatment and remained enhanced at 60 min. A similar rate and extent of activation was seen for ALS, Cis, and Socs-3 genes, while β-actin gene expression remained constant. Thus, in vivo GH treatment rapidly stimulates binding of Stat5b to at least two sites within the IGF-I locus, coincident with potent stimulation of IGF-I gene transcription.

Response Element 1 of the 5’ Distal Region Can Mediate GH-activated and Stat5b-dependent Gene Transcription—The 5’ distal region of the rat IGF-I locus contains two potential Stat5b binding sites, here termed RE-1 and RE-2, that are separated by 228 nt of intervening DNA. We evaluated the transcriptional properties of the 5’ distal region and RE-1 and RE-2 by performing reconstitution experiments in COS-7 cells with luciferase reporter genes containing IGF-I P1 without or with either a 352-nt segment of the 5’ distal region or 825 nt HS7. Cells were transiently co-transfected with expression plasmids for the mouse GH receptor and wild-type rat Stat5b, and individual luciferase reporter genes. As seen in Fig. 3, GH treatment had no effect on IGF P1 as shown previously (16), stimulated a 4-fold increase in gene expression of IGF P1 plus HS7, and ~2-fold rise in reporter activity of IGF P1 when the 5’ distal region was present.
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To extend this observation of GH-stimulated gene transcription via the 5' distal region of the IGF-I locus, additional experiments were performed with luciferase reporter genes containing a minimal thymidine kinase promoter. As pictured in Fig. 4, in the presence of HS7, GH and Stat5b induced a nearly 8-fold increase in gene transcription, whereas with a fusion gene containing the 5' distal region, GH and Stat5b stimulated a ~3-fold rise in luciferase activity. The importance of the two REs for hormonal regulation was next examined after engineering point mutations into each site that would potentially eliminate binding of Stat5b. As also shown in Fig. 4, a ~3-fold increase in gene expression by GH and Stat5b was maintained when RE-2 was modified but the hormonal response declined to nearly background levels when either RE-1 or both sites were mutated. Thus, based on these results, HS7 appears to be a more potent GH response element than the 5' distal region, potentially because in HS7 both Stat5b binding sites are needed for full transcriptional activity (16), while in the 5' region, only RE-1 appears necessary for GH-induced gene transcription in the presence of Stat5b.

DNA Binding Properties of RE-1 and RE-2—We used gel mobility shift experiments to determine if Stat5b could bind directly to RE-1 or RE-2 of the 5' distal region. Fig. 5 shows results using IR-labeled double-stranded oligonucleotides for each element, and nuclear protein extracts from COS-7 cells transiently transfected with expression plasmids for the mouse GH receptor and either wild-type or constitutively-active rat Stat5b, and treated with GH or vehicle for 60 min. As depicted in the figure, GH induced binding of wild-type Stat5b to both DNA probes, with two discrete protein-DNA complexes being formed for each oligonucleotide. Binding of Stat5b to RE-1 appeared to be stronger than to RE-2, particularly to the complex showing faster mobility. Constitutively active Stat5b was able to bind to both oligonucleotides in the absence of hormone. Competition experiments using either homologous DNA or an unrelated sequence confirmed specific binding to each protein-DNA complex, whereas antibody supershift experiments showed that Stat5b interacted with each oligonucleotide.

We next evaluated binding of endogenous liver proteins to the putative REs in the 5' distal element in comparison to GHRE-1 and GHRE-2.
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FIGURE 5. Binding of Stat 5b to response elements 1 and 2 of the 5′ distal region. Results of gel-mobility shift assays using IR-labeled double-stranded oligonucleotides for either RE-1 or RE-2 of the 5′ distal region, and nuclear protein extracts from COS-7 cells transfected with expression plasmids encoding the mouse GH receptor and either wild-type (WT) or constitutively active (CA) N-terminal FLAG-tagged rat Stat5b. Cells transfected with Stat5bWT were incubated without serum for 4 h followed by addition of rat GH (40 nM) or vehicle for 60 min. Arrows delineate protein-DNA complexes, which are not observed in the absence of Stat5b (lanes 1). In cells transfected with Stat5bWT, protein-DNA binding is seen only after GH treatment (lanes 3 versus 2), whereas in cells expressing constitutively active Stat5b, binding is observed in the absence of hormone (lanes 4–8). Lanes 3 and 6 demonstrate specific protein-DNA binding as evidenced by competition with homologous oligonucleotides (lanes 5) but not DNA containing an Oct-1 recognition site (lanes 6). Lanes 7 show that an antibody to the FLAG epitope causes a supershift of each DNA-protein complex (arrowhead), whereas lanes 8 demonstrate that an irrelevant antibody to T7 does not. Unbound probe has been electrophoresed beyond the bottom of each gel.

FIGURE 6. In vivo GH treatment induces binding of rat hepatic nuclear proteins to response elements 1 and 2 of the 5′ distal region and HS7 GHRE 1 and 2. Results of gel-mobility shift assays using IR-labeled double-stranded oligonucleotides for either RE-1 or RE-2 of the 5′ distal region, GHRE-1 or GHRE-2 of HS7, or Sp1, and nuclear protein extracts (5 μg) from GH-deficient male rats after systemic GH treatment for 0, 15, 30, or 60 min. Arrows indicate protein-DNA complexes, and arrowheads unbound probe.

FIGURE 7. Cross-competition among rat IGF-I Stat5b sites. Gel mobility shift experiments were performed with IR-labeled double-stranded probe RE-1, nuclear protein extracts from COS-7 cells transfected with expression plasmids for the mouse GH receptor and wild-type N-terminal FLAG-tagged rat Stat5b, and treated with recombinant rat GH (40 nM) for 60 min, and unlabeled competitor DNAs at 5- and 50-fold molar excess. Arrows indicate protein-DNA complexes. Unbound probe has been electrophoresed beyond the bottom of each gel.

of HS7. Illustrated in Fig. 6 are results of time course experiments using hepatic nuclear protein extracts from GH-deficient rats acutely treated with hormone. Inducible binding was observed with all four DNA probes beginning by 15 min after systemic GH injection, and reached a maximum by 30 to 60 min. Under the conditions used in these experiments, binding to RE-1 and GHRE-1 oligonucleotides was stronger than for RE-2 or GHRE-2, implying that the latter are lower affinity sites. A similar kinetic pattern of first order, and then three inducible protein-DNA complexes was observed for RE-1, RE-2, and GHRE-1 DNA, whereas only a single complex was seen for GHRE-2. As a control, gel mobility shift assays were performed with a double-stranded probe that recognizes members of the Sp1 family of transcription factors (23), which are not regulated by GH. As expected, protein-DNA binding was constant over the same time course, demonstrating that both the quantity and quality of nuclear protein extracts were comparable at each time point after hormone treatment.

RE-1 Is a Very High Affinity Binding Site for Stat5b—The relative affinity for Stat5b of RE-1 of the 5′ distal region was examined using COS-7 nuclear proteins isolated at 60 min after GH, and a variety of unlabeled competitor oligonucleotides. As shown in Fig. 7, unlabeled RE-1 competed avidly with itself, causing an 80% decline in both protein-DNA complexes at 5× excess molar concentration, whereas RE-2, GHRE-1, and GHRE-2 were only effective at 50× molar excess, and the unrelated binding site for Oct-1 was inert.

Quantitative gel-mobility shift assays were performed next to determine the affinity of Stat5b for both RE-1 of the 5′-region and GHRE-1 of HS7. Nuclear extracts from COS-7 cells expressing constitutively active Stat5b were used as the source of recombinant protein, and DNA-protein binding reactions were performed with a constant quantity of nuclear protein (2.8 μg) and a 50-fold concentration range of IR-labeled double-stranded oligonucleotides (0.5–25 nM, Fig. 8). For each probe, binding was saturable, with an EC50 of ~1 nM for RE-1 and ~4 nM for GHRE-1 (compared with Figs. 5 and 7, where a doublet was seen, only a single protein-DNA complex was observed for RE-1 in Fig. 8 because...
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DISCUSSION

The GH-IGF-I pathway plays a central role in the control of postnatal somatic growth in humans and in nearly all other mammals (4–6). Despite considerable interest in this pathway with regard to human physiology and health, the biochemical mechanisms by which GH regulates IGF-I expression have proven to be difficult to unravel. Only recently has experimental evidence emerged demonstrating that Stat5b is a key intermediate in the steps between binding of GH to its cell membrane-spanning receptor and onset of IGF-I gene transcription in the nucleus (11). By a combination of approaches we previously identified and analyzed a DNA segment termed HS7 in the second intron of the rat IGF-I gene that contains tandem Stat5 binding sites, and showed that it functions as a cis-regulatory region capable of mediating GH-induced IGF-I gene transcription (16). Now we have characterized a second putative Stat5 binding segment in the rat IGF-I locus that maps 73 kb 5′ of exon 1, and nearly 77 kb from HS7. We find that GH treatment also rapidly stimulated binding of Stat5b to chromatin at this 5′ distal region coincident with the onset of IGF-I gene transcription in vivo, and demonstrate that similar to HS7 this DNA segment conferred responsiveness to the major IGF-I promoter and to a neutral promoter in cell-based reconstitution experiments. Like HS7, the 5′ distal region appears to contain tandem Stat5 binding sites with widely different affinities for Stat5b, but unlike HS7, only one of the two sites appears essential for GH- and Stat5b-regulated gene expression, an observation that may account for the weaker actions of the 5′ distal region as a transcriptional enhancer in cell-based reconstitution assays. In aggregate, these results begin to elucidate the complex molecular architecture of the signal transduction pathway responsible for GH-activated IGF-I gene transcription.

The biological effects of GH are multifactorial and include early activation of target genes that are regulated by distinct signaling steps involving both pre-existing and newly synthesized intermediates (2). Stats are latent transcription factors that are present in the cytoplasm in advance of hormonal stimulation (25). Previous studies have demonstrated that GH can rapidly activate Stats 1, 3, 5a, and 5b both in vivo and in cell culture by stimulating their tyrosine phosphorylation, dimerization, and nuclear translocation (7), and have supported the idea that together these proteins mediate a substantial fraction of the transcriptional effects of GH (26). In addition to now showing that Stat5b binds to DNA in chromatin within at least two distinct regions in the IGF-I locus, we demonstrate that the promoters for both ALS and CIS contain bona fide Stat5b binding sites that become occupied in chromatin in a GH-dependent way coincident with their gene activation. In contrast, although Socs-3 is also a GH-inducible gene (11, 27–30), similar sequences within its proximal promoter did not bind Stat5b in vivo, results consistent with previous observations showing that these sites are dispensable for hormone-stimulated transcription in cell-based promoter-reporter assays (27).

Thus, through the combination of ChIP electrophoresis was performed for a shorter time to visualize unbound probe DNA). The calculated $K_d$ of 0.82 nM for GHRE-1 was very similar to values reported for the binding of Stat1 to a variety of high affinity sites (∼1 nM, Ref. 24), whereas the $K_d$ for 5′ RE-1 of 0.02 nM was significantly higher. In contrast, using the same experimental approach we were unable to calculate the $K_d$ of binding of Stat5b to either RE-2 or GHRE-2, indicating that they are lower affinity sites. Taken together, the results in Figs. 5–8 show that RE-1 and RE-2 of the 5′ distal region, and GHRE-1 and -2 of HS7 are bona fide Stat5b binding elements, and that the 5′ distal region and HS7 each contain one higher affinity and one lower affinity Stat5b site.

FIGURE 8. Assessing affinity of binding of Stat5b to site 1 of the 5′ distal region and HS7 GHRE-1. Quantitative gel-mobility shift experiments were performed as described under “Experimental Procedures” with varying concentrations of IR-labeled double-stranded oligonucleotides for RE-1 (A) or GHRE-1 (B), and 2.8 μg of nuclear protein from COS-7 cells transfected with an expression plasmids encoding constitutively active N-terminal FLAG-tagged rat Stat5b. DNA binding was quantified with a LiCor Odyssey infrared scanner and v1.2 analysis software, and results were plotted as shown. Representative experiments are shown in the left panels, binding curves are illustrated in the center panels, and Scatchard plots are displayed in the right panels.
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experiments with hepatic chromatin and measurements of GH-stimulated in vivo transcription, we are able to discriminate between true and false GH-regulated Stat5b response elements.

Stat proteins typically bind as dimers to specific sites on DNA, which optimally consist of the nucleotide sequence 5′-TTCN₃GAA-3′, where N is any deoxyribonucleotide and n ranges from 2–4 (31). Through use of an iterative DNA binding assay, both Soldaini et al. (32) and Ehret et al. (33) have shown for Stat5 that n = 3, which is the same spacing observed in the paired Stat5 sites found in both the 5′ distal region and in HS7. Soldaini et al. additionally attempted to discriminate between optimal DNA sequences for Stat5a and Stat5b, proteins that are 91% identical overall and 97% identical in their nucleotide binding domains (34). Not surprisingly, the core binding sequences could not be distinguished from each other, although Stat5a was shown to bind not only as a dimer to this core element but also as a tetramer to DNA sequences that contained two less than optimal cores in tandem (32). In contrast, in the same assays Stat5b only recognized optimal core sequences and only bound to DNA as a dimer (32). Other studies addressing binding of Stat5 to the human Cis promoter also have concluded that Stat5a can form tetramers on DNA, while Stat5b cannot (22). Given these published observations, it seems unlikely that either of the two discrete protein-DNA complexes seen with RE-1 and with RE-2 probes in Figs. 5 and 7 represent Stat5b tetramers, although as supported by results of antibody super-shift experiments, both bands contain Stat5b. Additional studies will be needed to identify all of the proteins present in each complex. Similarly, the nature of the complexes formed with hepatic nuclear proteins will need to be characterized.

RE-1 of the 5′ distal region is identical in its core Stat5 binding sequence to a site in the proximal HNF-6 promoter that is responsible for gene activation by GH (35), whereas RE-2 matches both a site in the prolactin-induced a hs1 casein promoter (36) and GHRE-2 of HS7 (16). Along with results of gel-mobility shift experiments presented in Figs. 5 and 6, these observations suggest that both RE-1 and RE-2 of the 5′ distal region are bona fide Stat5b sites. Yet, our studies to address functional effects of RE-1 and RE-2, which are separated by 228 nucleotides in the linear DNA sequence, have indicated that only RE-1 is active, at least as assessed by promoter-reporter gene experiments. As these latter studies did not examine binding of Stat5b in chromatin, it remains a formal possibility that cooperative interactions do take place between the RE-1 and RE-2 sites, as long as RE-1 retains high affinity binding of Stat5b.

The identification of two widely spaced potential Stat5b-binding GH response elements in the IGF-I locus raises several questions regarding mechanisms of GH-stimulated IGF-I gene transcription. First, do both regions contribute to hormone-regulated IGF-I gene expression? We do not know if either HS7 or the 5′ distal region becomes physically associated with the two IGF-I promoters just prior to onset of GH-stimulated IGF-I gene transcription. The interaction of a distant enhancer with its cognate promoter has been characterized for several other genes recently (37–40), leading to the idea of inducible looping of DNA in chromatin as part of the process of gene activation. This remains to be tested for the putative enhancers in the IGF-I locus. Second, what co-activators interact with Stat5b to mediate GH-stimulated IGF-I gene transcription? At present, we know little about the assembly of transcriptional cofactors at either site, although in preliminary studies we find that acetylation of core histones accompanies GH-induction of binding of Stat5b to HS7 (data not shown). CBP, p300 (41), SRC-1 (42), Nmi (43), and the glucocorticoid receptor (44, 45) are among several characterized Stat5-binding proteins, yet none have been demonstrated to physically interact with the IGF-I locus on chromatin, or to play a role in GH-stimulated IGF-I gene transcription. Third, do additional GH response elements exist in the IGF-I gene? Both HS7 and the 5′ distal region were initially identified by functional studies. HS7 was shown to undergo a reversible alteration in chromatin structure upon systemic GH treatment (9), while the 5′ distal region was characterized by a shotgun cloning strategy as a chromosomal segment that conferred responsiveness to constitutively active Stat5a in promoter-reporter gene assays (19). Evaluation of the nucleotide sequences of the human, rat, and mouse IGF-I loci reveals the presence of over 90 potential single Stat5 consensus sites within the 200 kb including each gene and flanking DNA, with more than a dozen paired sites in each species (data not shown). Although several other potential single and paired sites demonstrate conservation across species, except for HS7 and the 5′ distal region, none have been examined for GH-stimulated binding of Stat5 in chromatin or in vitro, and none have been tested in functional studies.

GH and IGF-I are critical for normal somatic growth during childhood and play key roles in tissue repair and regeneration in the adult (2–5, 46, 47). By contrast, there is a dark side to this pathway with implications for increased cancer risk and progression (48), and for enhanced tissue senescence and aging (49–51). Any future potential therapeutic interventions to maximize the safe use of these potent agents will require a detailed understanding of the biochemical and molecular basis of their actions.

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