A growth hormone (GH)-inducible nuclear factor (GHINF) from rat liver has been purified to near homogeneity. On SDS-polyacrylamide gel electrophoresis and UV-cross-linking, a major band of ~70 kDa and a minor band of ~93 kDa are detected in the purified fraction. DNase I footprinting using purified GHINF yields a protected region of ~149–115 on the rat serine protease inhibitor 2.1 (Spi 2.1) promoter encompassed within the growth hormone response element (GHRE). Mutational analysis demonstrated that GHINF binds synergistically to two γ-interferon-activated sites (GAS) within the GHRE, with the 3' element being the pivotal binding domain. Functional assays show that both GAS elements are necessary for full GH response. GHINF has no immunoreactivity with either a C-terminal Stat1 antibody or an N-terminal Stat3 antibody, while cross-reacting with a C-terminal Stat5 monoclonal antibody. GHINF will bind to two GAS elements from the Stat5 binding region of the β-casein gene. These studies indicate that GHINF is a Stat5-related factor binding synergistically to two GAS elements to activate Spi 2.1 transcription.

Great strides have been made in the last year toward understanding the mechanisms of cytokine and growth factor signal transduction. These extracellular signaling proteins include growth hormone (GH),1 prolactin, interleukins (IL), interferons, granulocyte-macrophage colony stimulating factor, and colony stimulating factor 1. The binding of these polypeptides to their specific surface receptors in target cells is followed by a cascade of events activating the Jak-STAT pathway. In this process, the Janus kinase (Jak) family of tyrosine kinases, known to be associated with these receptors, are activated and tyrosine-phosphorylated. These kinases, in turn, presumably activate a family of latent cytoplasmic proteins known as signal transducers and activators of transcription (STAT), through phosphorylation of tyrosine residues. The activated STAT proteins are then translocated to the nucleus where they, by themselves or in combination with otherwise weak DNA-binding proteins, bind to specific response elements on responsive genes and activate transcription (1). Six of these STAT proteins have been identified to date. Some of the STAT proteins are highly specific in their response to individual cytokines (e.g. Stat2 for interferon-α), while others appear to be involved in multiple pathways (2). The STAT proteins recognize response elements that share homology with the γ-interferon activation site (GAS) recognized by Stat1 (1).

The involvement of Jak-STAT pathways in GH signal transduction has been evidenced recently. Jak2 has been shown to be associated with GH receptors following GH binding with phosphorylation of both Jak2 and the GH receptor and subsequent activation of signal transduction (3). Further, it has been observed that GH treatment appears to activate several STAT proteins resulting in their phosphorylation. This has been noted both in cultured cell systems (4–7) and in liver (8, 9), a known target organ for GH action. The association of these STAT protein activations with altered GH-responsive gene transcription is, however, less certain.

Our own investigations into the mechanism of GH-responsive gene expression in the rat liver have centered on the serine protease inhibitor (Spi) 2.1 gene. It is, to date, the best characterized physiological system for studying GH action. Spi 2.1 expression is greatly reduced by hypophysectomy and can be restored to 40% of its normal level by the administration of GH alone. Full restoration requires the synergistic action of GH, thyroxine, corticosterone, and dihydrotestosterone (10). Its rapid induction by GH is direct and not mediated by insulin-like growth factor I,2 another GH early response gene (11). We have previously characterized a GH response element, GHRE, extending from –147 to –103 in the 5'-flanking region of the Spi 2.1 gene that is responsible for its induction by GH and detected an inducible nuclear factor(s) in rat liver, designated as GHINF, which binds to the GHRE in a state-specific manner (12). Appearance of this binding activity following GH treatment of hypophysectomized rats requires no new protein synthesis (12) suggesting that post-translational modification of an extant factor is required. We recently demonstrated that the critical modification of GHINF is that of tyrosine phosphorylation, which is required for its binding to the GHRE (13). Within the GHRE, we and others have noted the presence of...
two GAS elements (11, 14). To examine the function of these GAS elements and to further characterize GHINF, we undertook purification of GHINF from rat liver. These studies indicate that GHINF interacts synergistically with two GAS elements in the Spi 2.1 promoter for stimulating transcription and that GHINF has antigenic similarity to Stat5.

EXPERIMENTAL PROCEDURES

GHINF Purification—Normal Sprague-Dawley rats were injected with 150 μg of human GH (Genentech, South San Francisco, CA)/100 g body weight, intravenously, and livers were removed 1 h later. All subsequent work was performed at 4 °C. Crude nuclear extracts were prepared (13) and subjected to four successive column chromatography steps: heparin-Sepharose CL-6B (Pharmacia Biotech Inc.), salmon sperm DNA-Sepharose, sequence-specific GHRE-Sepharose, and agarose-conjugated anti-phosphotyrosine (UBI, Lake Placid, NY). Salmon sperm DNA (Pharmacia) was sonicated and phenol-extracted according to standard protocols, then ethanol-precipitated twice, washed in coupling buffer, and suspended in coupling buffer (13). An insertion of 85 β-casein was used to cover the sequence containing a second GAS-like element. Appropriate primers were also synthesized for duplex formation and extension reactions.

Plasmid Constructions—The construction of Spi 2.1 (–275/+85) into the HindIII/PstI sites of the parent plasmid pCAT(An), designated here as Spi-A-CAT, was described previously (12). The construction of GHRE mutations in place of the wild type sequence in Spi-A-CAT was as follows.

For each GHRE mutation, two PCRs were performed with appropriate primers to generate a fragment extending from the HindIII site at –275 to the mutation/restriction site and a second fragment extending from the mutation/restriction site to PstI site at +85 of the template Spi-A-CAT plasmid. The resultant PCR products were purified with the Qiaquick PCR product purification kit (Qiagen Inc., Chatsworth, CA), restriction-digested with appropriate enzymes, purified again, and quantitated. The template plasmid was digested with HindIII/PstI to remove the –275/+85 fragment, treated with calf intestinal phosphatase, and gel-purified with Prep-A-Gene (Bio-Rad). A triple ligation incorporating the two PCR products and the template vector was performed. The complete plasmid containing each mutation was transformed into Escherichia coli RR1 cells. With the exception of the mutation/restriction site, the resultant clones were identical with the original template plasmid. Mutations were confirmed by sequencing according to the manufacturer’s protocol (Sequenase Version 2.0, United States Biochemical Corp.). The plasmids generated in this manner were designated Spi-B-CAT, Spi-C-CAT, Spi-D-CAT, Spi-E-CAT, and Spi-F-CAT to reflect, respectively, mutations B, C, D, E, and F as listed in Table I.

Functional Assays—Functional assays in primary rat hepatocytes were carried out as described previously (17). Primary hepatocytes were isolated from male Sprague-Dawley rats (180–240 g) using the collagenase perfusing method (18). After a 6-h attachment period, transfection was performed using Lipofectin reagent (Life Technologies, Inc.) in modified Williams E medium with 27.5 mM glucose for 12–14 h. Cells were then cultured for 48 h in the presence or absence of 0.5 μg/ml GH. In addition, for the first 24 h, 500 μg/ml Matrigel (Life Technologies, Inc.) was added to the medium. At the end of 48 h, the cells were harvested for chloramphenicol acetyltransferase (CAT) assay. Results were expressed as percent conversion of chloramphenicol acetyltransferase to chloramphenicol acetylase determined by direct scintillation counting. Each experiment was repeated three times with freshly isolated hepatocytes.

Table I

| Probe | Description | Mutations in Spi 2.1 | CAT Activity (%) |
|-------|-------------|---------------------|------------------|
| Spi-A | Wild type   |                     | 100              |
| Spi-B | B mutation  |                     | 60               |
| Spi-C | C mutation  |                     | 40               |
| Spi-D | D mutation  |                     | 30               |
| Spi-E | E mutation  |                     | 20               |
| Spi-F | F mutation  |                     | 10               |

Table II

| Probe | Description | Mutations in Spi 2.1 | CAT Activity (%) |
|-------|-------------|---------------------|------------------|
| Probe A | Wild type   |                     | 100              |
| Probe B | B mutation  |                     | 60               |
| Probe C | C mutation  |                     | 40               |
| Probe D | D mutation  |                     | 30               |
| Probe E | E mutation  |                     | 20               |
| Probe F | F mutation  |                     | 10               |
GH Action via a Stat5-like Factor Binding to Two GAS Sites

Purification of GHINF

| Stage                  | Total activity (units) | Specific activity | Purification | Recovery |
|------------------------|------------------------|-------------------|--------------|----------|
|                        | units/μg protein       |                   | %            |          |
| Crude extract          | 49,600 units/310 mg    | 0.16              |              |          |
| Heparin Seph           | 29,920 units/94 mg     | 0.32              | 2×           | 60%      |
| SS-DNA-Seph            | 14,750 units/4 mg      | 1                 | 6×           | 30%      |
| GHRE-Seph              | 3,000 units/2 μg       | ~1,500            | ~9,000×      | 6%       |
| Anti-Tyr(P)-agarose    | 315 units/~0.3 μg      | ~1,050            | ~7,000×      | 1%       |

* A unit of binding activity for GHINF is defined as the amount of GHINF that, under our standard EMSA conditions in the presence of 20 fmol of labeled GHRE probe, retards 1 fmol of labeled GHRE.

* Quantity of protein estimated from fixed quantities of molecular mass standards on silver-stained SDS-PAGE gels compared to purified fractions.

RESULTS

Purified GHINF Interacts with the Spi 2.1 GHRE—GHINF was identified as a DNA binding activity that interacts with sequences of the Spi 2.1 gene critical to its response to GH. Binding is GH-dependent and occurs rapidly following GH treatment of hypophysectomized animals. Given the potential importance of this activity to the GH-stimulated transcription of the Spi 2.1 gene, we initiated an effort to purify it. Table III presents the results of a purification of GHINF from the livers of normal rats treated with GH. GHINF activity was followed by EMSA using the Spi 2.1 GHRE as probe during successive stages of purification (Fig. 1). GHINF eluted from both heparin-Sepharose and salmon sperm DNA-Sepharose columns at 0.3 M KCl. Major purification was achieved by DNA-affinity chromatography using a GHRE-Sepharose matrix. GHINF eluted from this column at 0.6 M KCl with an estimated purification factor of ~9000-fold over the crude extract. Further purification took advantage of the fact that activated GHINF is tyrosine-phosphorylated (13). The DNA affinity-purified material was chromatographed on an agarose-conjugated anti-phosphotyrosine column and eluted with 2 μg o-phospho-L-tyrosine. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of this eluted fraction shows that GHINF has been purified to near homogeneity (Fig. 1B). The predominant band found in both affinity- and immunopurified GHINF had an apparent molecular mass of ~93 kDa. A minor band at ~70 kDa was also routinely observed in preparations. That these two bands are molecular mass of 93 kDa. A minor band at ~70 kDa was also routinely observed in preparations. That these two bands are

To delineate the boundaries of the Spi 2.1 gene sequences bound by GHINF, DNase I footprinting was performed using affinity-purified GHINF and various crude liver nuclear extracts. With affinity-purified GHINF, only one domain of protection is seen on examination of the fragment from ~192 to +85 of the Spi 2.1 gene (Fig. 2, lane 5). This protected region extends from ~149 to ~115. The same footprint, with somewhat less protection from ~149 to ~138, is apparent in extracts of normal rats (lane 3), normal rats treated with GH (lane 4), or hypophysectomized rats treated with GH (lane 2). It is absent in extracts from untreated hypophysectomized rats (lane 1). This binding activity is therefore GH state-specific.

Mutational Analysis of GHRE Binding—Given the unexpectedly large size of the DNase I footprint obtained with purified GHINF, we were interested in determining whether bases throughout the region were critical for binding. The effects of the GHRE mutations listed in Table I on GHINF binding are shown in Fig. 3A. We had noted previously the presence of two GAS elements in the Spi 2.1 GHRE (11). The significance of both elements for GHINF binding is suggested by the results of this analysis. Mutations B and F flank the two GAS elements and had no effect on GHINF binding (lanes B and F). Mutation C disrupts the 5' GAS element and greatly diminishes GHINF binding (lane C). Mutations D and E are mutations of the TTC or GAA of the 3' GAS element. Both abolished all binding (lanes D and E). Mutations of the intervening 3 base pairs in the 3' GAS element (G and H) did not affect binding (lanes G and H). A point mutation of the A in GAA of the 3' GAS element to a C (mutation I) abolished almost all binding. However, mutation of the corresponding A in the 5' GAS element led to only a decrease in binding (mutation J). These results suggest that both GAS elements are important for GHINF binding, and that the 3' GAS element interacts more strongly than the 5' GAS element. This conclusion is supported by the results of competition experiments (Fig. 3B). The mutation containing an intact 3' GAS element (mutation C) competed effectively for binding to the wild type GHRE. On the other hand, mutations with intact 5' GAS elements (mutations D and E) competed only slightly. These results suggest that the pivotal recognition sequence for GHINF is likely to be TTC-NNNGAA which is found in the 3' GAS element. The 5' GAS element contains a 5 out 6 match to the consensus sequence: TTCNNNTA, and appears to be necessary for the assembly of the intact complex as seen on EMSA.

Functional Assays of GHRE Mutations—To correlate in vitro GHINF binding with its physiological significance in transcriptional regulation, we constructed Spi-CAT plasmids containing mutations B, C, D, E, and F and tested their functional responses to GH in primary hepatocytes. Previous studies using hepatocytes to study GH action have been limited by their responses to GH in primary hepatocytes. Previous studies using hepatocytes to study GH action have been limited by their attenuated responses to GH (12). We have shown recently that addition of Matrigel to the culture medium of hepatocytes following DNA transfection significantly enhances their responses to extracellular stimuli, including GH (21). In addition, culturing hepatocytes under high glucose conditions (27.5 mM) obviated the need for co-transfection of GH receptor cDNA (22). The results of functional assays performed under these conditions are shown in Fig. 4. Transfection of the wild type Spi-A-CAT leads to a 20-fold induction of CAT activity by GH. Mutations of the TTC (Spi-D-CAT) or the GAA (Spi-E-CAT) in the 3' GAS element abolished the GH response entirely. Mutation of the 5' GAS element substantially reduced the response to GH (Spi-C-CAT). Thus, both GAS elements appear to be necessary for full functional response to GH.

While GHINF binding as shown on EMSA does not require the sequence upstream of the 5' GAS element, mutation of that sequence (Spi-B-CAT) led to a dramatic reduction of CAT activity in response to GH. This result suggests that another factor binding 5' to GHINF may be important for the GH response. However, mutation downstream of the 3' GAS element did not lead to any diminution of the GH response (Spi-F-CAT). This indicates that only the region delineated by...
GH Action via a Stat5-like Factor Binding to Two GAS Sites

Fig. 1. Purification and characterization of GHINF. A, GHINF binding activity at successive stages of purification. EMSA was performed as described previously (13). Decreasing amounts of poly(dI-dC) were added to fractions from successive stages of purification. No poly(dI-dC) was added in the affinity- or immunopurified fractions. The following amounts of protein were added in each reaction: crude extract (Cr), 6 μg; heparin-Sepharose fraction (He), 6 μg; salmon sperm DNA-Sepharose fraction (sd), 3 μg; affinity-purified fraction (Af), ~2 ng; immunopurified fraction (Im), ~1 ng. B, SDS-PAGE of affinity- and immunopurified GHINF. SDS-PAGE (10%) was performed according to standard protocols (36). Silver staining was performed as described previously (37). Low molecular size SDS-PAGE standards (Bio-Rad Laboratories) were used in estimating the sizes. The heavy arrow indicates the position of the dominant band seen at ~93 kDa. The minor band at ~70 kDa is indicated by the light arrow. Shown are the affinity- (Af) and immunopurified (Im) fractions. C, UV-cross-linking of affinity-purified GHINF. GHRE was labeled with deoxy-GTP as the radioactive nucleotide to a specific activity of $1 \times 10^8$ cpm/μg. Prestained low molecular size SDS-PAGE standards (Bio-Rad Laboratories) were used in estimating sizes. Two bands, corresponding to ~93 kDa and ~70 kDa, were cross-linked to the GHRE.

DNase I footprinting is involved in GH activation of Spi 2.1 transcription.

GHINF is Distinct from Stat1 and Stat3—Recent evidence indicates that GH is capable of inducing the DNA binding activity of both Stat1 and Stat3 (5, 6, 8). To test the possible relationship of GHINF to these factors, we tested the binding of GHINF to the high affinity SIE from the promoter of c-fos. This oligonucleotide has previously been shown to bind both Stat1 and Stat3 (8, 19). GHINF does not bind to this probe (Fig. 5, lanes 3 and 6). To demonstrate the effectiveness of this probe, EMSA was performed with crude liver nuclear extracts from hypophysectomized rats without and with GH treatment. GH treatment stimulates the appearance of several binding activities in liver that recognize the SIE probe (lanes 1 and 2). These complexes most likely represent a homodimer of Stat1, a homodimer of Stat3, and a heterodimer of Stat1 and Stat3 (19). Addition of AbN, an antibody that cross-reacts with both Stat1 and Stat3, to the reaction led to a disruption of all three complexes and formation of a slower migrating supershifted complex (lane 5). In contrast, addition of AbN did not alter the migration of the GHINF-GHRE complex when GHRE was used as a probe (lane 8). The inability of GHINF to recognize the SIE probe or AbN under the same conditions and the unique migration of the GHINF-GHRE complex compared to Stat1 and -3 indicate that GHINF is neither one of these factors. This conclusion is further supported by the inability of an N-terminal Stat1 antibody (13), a C-terminal Stat1 antibody (Fig. 6A), and AbN (Fig. 6B) to recognize GHINF on immunoblotting although crude hepatic extracts clearly contain immunoreactive species recognized by these antibodies.

GHINF and Stat5—We have shown in Fig. 3 that the pivotal recognition sequence for GHINF is TTCNNNGAA. This site is identical with the binding site for Stat5, which was recently identified as a prolactin-responsive STAT protein (20). To determine whether GHINF was related to Stat5, we performed immunoblotting with GHINF and a C-terminal monoclonal antibody to Stat5. Further, AbN has also been shown in recent additional studies to also cross-react with Stat5 (19, 23), so that use of AbN as an antibody should provide information about similarities to Stat5 as well as Stat1 and -3. As noted previously, GHINF showed no reactivity toward AbN (Fig. 6B) although there are several species in crude extracts which this antibody recognizes. However, both the ~93-kDa and ~70-kDa bands of affinity-purified GHINF cross-reacted with the monoclonal Stat5 antibody, indicating an immunological relationship (Fig. 6C).

To further explore the relationship between GHINF and Stat5, we examined GHINF binding to a known Stat5 binding element. EMSA was performed with two probes from the PRE

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region of the rat β-casein promoter. The PRE formed a band with purified GHINF; however, this band migrated with a faster mobility than the complex formed with the GHRE (Fig. 7, lane 4). Examination of the β-casein promoter in the region of the PRE revealed a second GAS-like element located 7 bases upstream. This element is conserved at the same position in the promoters of the rat, mouse, rabbit, and cow β-casein genes. We therefore probed with an oligonucleotide corresponding to this “long” β-casein sequence. In this case, a diffuse complex with mobility between that of the PRE and that of the GHRE complex was seen (lane 3).

To examine another element which has similar architecture and which has known GH-state specific binding, we examined GHINF binding to the GRR of FcγRI (4). GHINF also binds to both the short (lane 2) and long (lane 1) forms of GRR from FcγRI. As with the β-casein probes, the complexes formed are qualitatively different from that of GHRE. The 3’ fragment of the GRR forms a faster moving complex, and the full-length GRR appears to form a diffuse complex. In contrast to binding observed seen with both the PRE and the short GRR, an oligonucleotide corresponding to only the 3’ GAS site of GHRE (SPI-GLE-1) did not lead to the formation of a DNA-protein complex with purified GHINF under our conditions (lane 6), suggesting that sequences flanking the PRE influenced binding. Together, these results suggest that the GHINF is a Stat5-like protein that interacts synergistically with two GAS elements for binding and function.

**DISCUSSION**

We have purified GHINF, a DNA binding activity of rat liver that recognizes the GHRE of the Spi 2.1 gene, to near homo-
GAS element, SPI-GLE-1, shows failure of binding of a GHRE fragment that contains only the 3\'24908 leading to the loss of function for the GAS site. This relative preference is also supported by competition studies, which showed that only one oligonucleotide with an intact 3\' site was able to compete for binding. The 5\' site contains a functional mismatch when compared to the 3\' site (TTCCNNNTAA instead of TTCCNNNNGAA), which may account for its lower affinity for GHINF. With the exception of the 5\' A in the 3\' GAS element, which is critical for GHINF binding, the relative importance of the remaining nucleotides in these palindromic half-sites and the significance of the spacing between them remains to be determined. However, it is clear from these binding studies that both sites are necessary for efficient formation of the GHINF-GHRE complex.

These observations correlate well with functional assays in primary hepatocytes. Mutations of the TTC or GAA of the 3\' GAS site that led to ablation of GHINF binding on EMSA led to a total loss of GH-induced CAT activity upon transfection. Transfection of constructs with the mutated 5\' site that showed weak GHINF binding on EMSA led to reduced GH-induced CAT activity. Thus, the two GAS sites appear to function synergistically to support the GH response.

While mutation of the sequence upstream of the 5\' GAS element led to no change in GHINF binding on EMSA, transfection of the construct with this mutation led to a greatly diminished GH response. This observation suggests that interaction with an accessory protein(s) binding to this region is required for normal GH response. We have observed that a Spi-147/+85-CAT construct did not support a GH response, consistent with a role for a factor binding upstream of -147 (data not shown). In contrast, transfection of a mutation downstream of the 3\' GAS site that did not affect GHINF binding on EMSA led to a GH response that is comparable to that of the wild type. These functional data also correlate well with DNase I footprinting results: almost the entire region protected from DNase I is necessary for normal GH response. The stronger protection of the 3\' site evident in the crude extract footprint supports the suggestion that the 3\' site is the pivotal GHINF binding site.

Interestingly, Silva et al. (14) reported a GH-responsive factor in crude rat liver extracts that requires only the 3\' GAS element in the GHRE (SPI-GLE-1) for binding. We did not observe any binding using the same oligonucleotide element and purified GHINF in EMSA. In their functional assays performed in CHO cells stably transfected with GH receptor, a construct containing 3 copies of the 3\' GAS element was shown to be sufficient to confer a 5- to 6-fold GH response to a heterologous promoter. Thus, the DNA binding activity these authors detected may reflect weak binding of GHINF to the 3\' GAS element. We show here that in primary hepatocytes, one copy of the 3\' GAS element in its native promoter (Spi-C-CAT) was sufficient to confer partial GH responsiveness. However, two copies are required for maximal GH induction. In addition, we show that the combined presence of Matrigel and high glucose concentration in the culture medium dramatically enhanced the ability of the cultured hepatocytes to respond to GH. This strategy facilitated evaluation of more subtle differences in promoter structure. The 20-fold GH induction observed with Spi-A-CAT compares favorably with the induction of Spi 2.1 mRNA in GH-deficient rats treated with GH.

The organization of the GHRE of the Spi 2.1 gene appears to parallel that of the GRR of the Fc\gammaRI gene (24). GRR contains a 3\' GAS palindromic sequence, TTCCNNNGAA, and, although
not noted by the authors, a 5'-GAS-like element that contains one mismatch (TTCNNNGAT). In functional assays, the ability of the 3' fragment alone to respond to interferon-γ was only 25% of that of the intact GRR. The 5' fragment alone was essentially inactive. In EMSA, only the 3' fragment was able to assemble complexes, while no complexes were observed with the 5' fragment. Thus, it appears that synergism between two GAS elements may also be important for interferon-γ induction from the GRR.

The GRR has also been shown to bind to a GH-stimulated factor in extracts of IM-9 lymphocytes (4). Given the similarities of the architecture of GRR to that of GHRE, GHINF might factor in extracts of IM-9 lymphocytes (4). Given the similarity from the GRR.

(23). Wood et al. (1994) reported that the GHINF, forming a complex with a faster mobility. Both GHINF and the IM-9 factor migrate to ~93 kDa on SDS-PAGE. While the IM-9 factor has been reported to cross-react with a C-terminal Stat1 antibody, GHINF does not. In spite of this difference, they are likely to be similar proteins.

We have shown that while GHINF is neither Stat1 nor Stat3, it is capable of binding to the PRE that is recognized by Stat5. Its possible relationship with Stat5 is further demonstrated by its cross-reactivity to a C-terminal Stat5 monoclonal antibody on an immunoblot. However, it shows no cross-reactivity with AbN, either on an immunoblot or in EMSA when GHRE is used as a probe, although this antibody does cross-react with Stat5 in some studies (19, 23). Nor does it cross-react with AbN in EMSA when PRE is used as a probe (data not shown). Thus, while GHINF shares antigenic determinants with the C-terminal sequence of Stat5, its N-terminal sequence is sufficiently different from those of Stat1, -3, and -5 such that AbN does not recognize it.

GH and prolactin both belong to the GH/prolactin/placental lactogen gene family. Both GH and prolactin receptors are characterized by similar structural features as members of the cytokine receptor superfamily. Both receptors are known to have associated Jak2 activities upon ligand binding (3, 25). Recent reports indicate that other ligands binding to receptors of this cytokine receptor superfamily transduce signals through Stat5 isoforms or homologs: IL-3, IL-5, and granulocyte-macrophage colony stimulating factor in mouse mast cells (26, 27), IL-3, IL-5, and granulocyte-macrophage colony stimulating factor in mouse mast cells (28).

The involvement of Stat5 in transducing GH signals, however, is less clear. Prolactin was not able to stimulate expression of Spi 2.1 and/or insulin-like growth factor I mRNA under conditions that produced a GH response (29, 30). Although GH can activate Stat5 (7), it does not induce transcription of a β-casein construct in transfection assays even if Stat5 is co-transfected along with GH receptor (23). Thus, Stat5 alone is not sufficient to confer GH responsiveness to the β-casein gene (23). Wood et al. (7) reported that a rat liver SPI-GLE-I binding complex could be supershifted by polyclonal Stat5 antisera.

They were, however, unable to supershift this complex in its entirety even after increasing the ratio of antisera to nuclear proteins. They suggested that in addition to Stat5, other, as yet uncharacterized, transcription factors are activated by GH in rat liver.

Using Stat5 CDNA as a probe, Stat5 mRNA has been found in several tissues in sheep, but not in liver (20). An examination of cellular distribution of Stat5a and Stat5b, by nuclease protection assay, did not reveal their presence in liver (28). However, using a PCR product generated from a murine thymocyte library as probe, Stat5 mRNA was demonstrated in several murine tissues, including liver (26).

Stat5 shares DNA binding and transactivation potential with Stat3 (31). In luteinized granulosa cells, prolactin, but not GH, regulates the transcription of the acute phase response gene, α2-macroglobulin (32). While GH does activate Stat3 in the rat liver, it does not induce the transcription of Spi 2.2, an acute phase-responsive gene and a homolog of Spi 2.1 (33). It is interesting to note that in the Spi 2.2 promoter, the region corresponding to the 5' GAS element in Spi 2.1 is disrupted twice with additional sequences (34). This may explain why Spi 2.2 does not respond to GH.

Purified GHINF protects a region on the Spi 2.1 promoter encompassing two GAS elements. EMSA studies demonstrate that GHINF is capable of binding two GAS elements from either the Spi 2.1, β-casein, or FcɛRI promoters. The occurrence of serial repeats of STAT binding elements and their relevance to the mechanism of enhancement of transcription by STAT proteins has been noted by others (14, 35). We present evidence here that GHINF, in binding synergistically to two GAS elements on Spi 2.1, together with an accessory protein(s) binding to their flanking sequences, initiates GH-responsive transcription. The exact relationship of GHINF to Stat5 must await amino acid sequence information from a larger scale purification.

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REFERENCES

1. Darnell, J. E., Kerr, I. M., and Stark, G. R. (1994) Science 264, 1415-1421
2. Ihe, J., and Kerr, I. M. (1995) Trends Genet. 11, 69–74
3. Argetsinger, L. S., Campbell, G. S., Yang, X. N., Witthuhn, B. A., Silvennoinen, O., Ihe, J. N., and Carter-Su, C. (1993) Cell 74, 237-244
4. Finbloom, D. S., Petriconi, E. F., Hackett, R. H., David, M., Feldman, G. M., Garasaki, K., Filach, E. Weber, M. J., Thorer, M. O., Silva, C. M., and Larner, A. C. (1994) Mol. Cell. Biol. 14, 2113–2118
5. Meyer, D. J., Campbell, G. S., Cochran, B. H., Argetsinger, L. S., Larner, A. C., Finbloom, D. S., Carter-Su, C., and Schwartz, J. (1994) J. Biol. Chem. 269, 4701–4704
6. Campbell, G. S., Meyer, D. J., Raz, R., Levy, D. E., Schwartz, J., and Carter-Su, C. (1995) J. Biol. Chem. 270, 3974–3979
7. Wood, T. J., Silva, D., Lobie, P. E., Pircher, T. J., Goulleux, F., Waka, H., Gustafsson, J. A., Groner, B., Norstedt, G., and Haldosen, L. A. (1995) J. Biol. Chem. 270, 9448–9453
8. Gronowski, A. M., and Rotwein, P. (1994) J. Biol. Chem. 269, 7874–7878
9. Gronowski, A. M., Zhong, Z., Wen, Z. L., Thomas, M. J., Darnell, J. E., and Rotwein, P. (1995) Mol. Endocrinol. 9, 171–177
10. Berry, S. A., Manthei, R. D., and Seelig, S. (1986) Endocrinology 119, 2290–2296
11. Thomas, M. J., Gronowski, A. M., Berry, S. A., Bergad, P. L., and Rotwein, P. (1995) Mol. Cell. Biol. 15, 12–18
12. Yoon, J.-B., Berry, S. A., Seelig, S., and Towle, H. C. (1990) J. Biol. Chem. 265, 19947–19954
13. Berry, S. A., Bergad, P. L., Whaley, C. D., and Towle, H. C. (1994) Mol. Endocrinol. 8, 1714–1719
14. Dialva, S., Wood, T. J., Schindler, C., Lobie, P. E., and Norstedt, G. (1994) J. Biol. Chem. 269, 26208–26214
15. Kadonaga, J. T. (1991) Methods Enzymol. 208, 10–23
16. Ausubel, F., Brent, R., Kingston, R. M., Moore, D., Seidman, J., Smith, J., and Struhl, K. (1994) Current Protocols in Molecular Biology, Sect. 12.5, Wiley Interscience, New York
17. Shih, H., and Towle, H. (1990) Biol. Chem. 267, 13222–13228
18. Ausubel, F., Brent, R., Kingston, R. M., Moore, D., Seidman, J., Smith, J., and Struhl, K. (1994) Current Protocols in Molecular Biology, Sect. 12.4, Wiley Interscience, New York
19. Raz, R., Durbin, J. E., and Levy, D. E. (1994) J. Biol. Chem. 269, 24391–24395
20. Wakao, H., Goulleux, F., and Groner, B. (1994) EMBO J. 13, 2182–2191
21. Shih, H., and Towle, H. (1995) BioTechniques 18, 813–816
22. Niimi, S., Hayakawa, T., Tanaka, A., and Ichihara, A. (1991) Endocrinology 128, 2734–2739
23. Goulleux, F., Pallard, C., Dusante-Fourt, I., Waka, H., Haldosen, L. A., Norstedt, G., Levy, D., and Groner, B. (1995) EMBO J. 14, 2005–2013
24. Pearse, R. N., Feinman, R., Shaah, K., Darnell, J. E., and Ravetch, J. V. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 4314–4318
25. Campbell, G. S., Argetsinger, L. S., Ihe, J. N., Kely, P. A., Rillena, J. A., and Carter-Su, C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5232–5236
26. Azam, M., Erdument-Bromage, H., Kreider, B., Xia, M., Queller, F., Basu, R., Sarris, C., Tempst, P., Ihe, J., and Schindler, C. (1995) EMBO J. 14, 1402–1411
27. Barahmandpour, F., Meinke, A., Eilers, A., Gouilleux, F., Groner, B., and Decker, T. (1995) FEBS Lett. 360, 29–33
28. Mui, A. L. F., Wakao, H., O’Farrell, A. M., Harada, N., and Miyajima, A. (1995) EMBO J. 14, 1166–1175
29. Warren, W. C., Munie, G. E., and Glenn, K. C. (1993) Mol. Cell. Endocrinol. 98, 27–32
30. Kleinberg, D., Catanese, V., Newman, C., and Feldman, M. (1990) Endocrinology 126, 3274–3276
31. Standke, G., Meier, V., and Groner, B. (1994) Mol. Endocrinol. 8, 469–477
32. Gaddy-Kurten, D., and Richards, J. (1991) Mol. Endocrinol. 5, 1280–1291
33. Schwarzenberg, S. J., Yoon, J.-B., Seelig, S., and Berry, S. A. (1992) Am. J. Physiol. 265, C1144–C1148
34. Pages, G., Rouayrenc, J. F., Rossi, V., Le Cam, G., Mariller, M., Szpirer, J., Szpirer, C., Levan, G., and Le Cam, A. (1990) Gene (Amst.) 94, 273–282
35. Wong, P., Severs, C., Guyer, N., and Wright, T. (1994) Mol. Cell. Biol. 14, 914–922
36. Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J., and Struhl, K. (1994) Current Protocols in Molecular Biology, Sect 10.2, Wiley Interscience, New York
37. Berry, S. A., and Seelig, S. (1984) Endocrinology 115, 1164–1170
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