Growth hormone activates gene transcription of the serine protease inhibitors (SPI) 2.1 and 2.2 by an unknown mechanism. In order to define the promoter regions responsible for this effect and to characterize the transcription factors involved, we have performed gel electrophoresis mobility shift assays on nuclear extracts from cells lines transfected with growth hormone receptor cDNA. We have identified a 9-base pair DNA element, the SPI-GLE 1, which forms a complex with nuclear proteins following activation by growth hormone and which, when placed upstream of a minimal thymidine kinase promoter, drives chimeric adenovirus expression in a growth hormone-dependent fashion. This element is similar to those from several genes regulated by other cytokines including interferon. The growth hormone-induced complexes formed were dependent on tyrosine phosphorylation but did not contain the interferon-activated transcription factor Stat 91. Competition studies with oligonucleotides similar to murine interleukin-2 receptor and murine interleukins (IL) 2-7, and granulocyte- and granulocyte-macrophage colony-stimulating factor Stat 91. Competition studies with oligonucleotides similar to SPI-GLE 1 reveal the sequence of a consensus element that specifically binds growth hormone-regulated nuclear proteins.

Growth hormone (GH) exerts its cellular effects as a result of binding to a specific receptor (1, 2). This receptor is a member of the cytokine receptor superfamily, which includes the receptors for prolactin, erythropoietin, tumor necrosis factor α, interleukins (IL) 2-7, and granulocyte- and granulocyte-macrophage colony-stimulating factors (3, 4). The receptors for interferon-α and interferon-γ also show structural homologies to the cytokine receptors (5, 6). The members of the cytokine receptor superfamily are characterized by (i) their possession of a single putative membrane spanning domain, (ii) defined sequence homologies in their extracellular domains and the region of their intracellular domains immediately juxtaposed to the membrane, and (iii) the absence of a consensus sequence for protein kinase activity. In addition, the receptors for GH, erythropoietin, IL-3, IL-6, leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, and interferon-γ have been demonstrated to interact with tyrosine kinases of the Janus kinase family (7-11). These shared properties may be fundamental to the biology of the cytokine receptors and, as such, probably dictate that the receptors share similar mechanisms of signal transduction. However, some mechanisms directing hormone specificity are likely to exist. In this paper, we have investigated the mechanism and specificity of regulation of transcription from the serine protease inhibitor (SPI) 2.1 and 2.2 promoters following GH stimulation. We have defined a 9-bp region of the SPI growth hormone response element (GHRE) described by Yoon et al. (12), which shares homology with elements that recognize the interferon-γ-activated transcription factor Stat 91 and which binds nuclear proteins in a growth hormone-dependent fashion. We term this element, which has the sequence TT CT G AG AA, the SPI-γ-activated sequence (GAS)-like element I (SPI-GLE 1).

**MATERIALS AND METHODS**

**Cell Culture and Preparation of Nuclear Extracts—Buffalo rat liver cells were cultured in Dulbecco’s minimal essential medium (Life Technologies, Inc.), and Chinese hamster ovary cells were cultured in F-12 Medium (Life Technologies, Inc.) (both containing 10% fetal calf serum (Life Technologies, Inc.) and 50 units/ml penicillin, 50 units/ml streptomycin (Life Technologies, Inc.)). Before the addition of 100 ng recombinant human (h) GH (Pharmacia) or 25 µg/ml mouse interferon-γ (Boehringer Mannheim), the cells were starved of fetal calf serum for 26-20 h. After treatment, the cultures (1-2 x 10^6 cells/l) were centrifuged to remove unbound GH (Centrifugation). The cell pellets were harvested in cold buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 6 mM MgCl₂, 1 mM dithiothreitol, 0.4 µM phenylmethylsulfonyl fluoride and 0.1 mM Na₂VO₃) and Dounce homogenized. The nuclear pellet, after centrifugation, was resuspended in cold buffer (20% glycerol, 0.1 mM Na,VO₃) and incubated on ice for 30 min. The nuclear extract was then collected into 10 ml of buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 6 mM MgCl₂, 1 mM dithiothreitol, 0.4 µM phenylmethylsulfonyl fluoride and 0.1 mM Na₂VO₃) and incubated on ice for 30 min. The nuclear extract was finally collected by centrifugation.

**Gel Electrophoresis Mobility Shift Assay—Gel electrophoretic mobility shift assays (GEMSA) were performed according to standard protocols (14). Nuclear extracts were incubated with 5P-labeled double-stranded SPI-GHRE (5AGTACCAGCTCTCAGTAACACATGAGATA) or double-stranded oligonucleotides (core sequence shown in boldface) in a buffer containing 20% Ficoll, 60 µg/ml HEPES pH 7.9, 20 mM MgCl₂, 0.2 mM EDTA, 0.2 µM GABACTCAGTCTGAGAA-AGAAATCATCAGTCTGCCCATG) or double-stranded oligonucleotides (core sequence shown in boldface) in a buffer containing 20% Ficoll, 60 µg/ml HEPES pH 7.9, 20 mM Tris, pH 7.9, 0.5 mM EDTA, 5 mM dithiothreitol, and 5 µg of poly(dI-dC). Antibody-induced supershift analyses with nuclear extracts were performed as described (15).

**GEMSA Competition Studies—** For competition studies, we used unlabeled double-stranded SPI-GHRE (5AGTACCAGCTCTCAGTAACACATGAGATA) or double-stranded oligonucleotides representing different parts of the SPI-GHRE (7-(TACTAATCCATGTTC), 8-(GATC-TACGCTTCTACTAATCCATGWCTG), 15-(GATCTACGCTTCTACTAATCCATGWCTG), or double-stranded oligonucleotides representing various GAS-like DNA elements (see Fig. 2 for references) (human GAP, TATTACTCAA, human FcRyI, TTTCCCGAAA; human ICSPB, TTTCCCGAAA; hu-
man IPF-53, ATTTCTAGAAA; human IRF-1, TTTCCCCGAAA; mouse IRF-1, TTTCCCCGAAA; mouse Ly6E, ATTTCTGTAAG; mouse mig, CTACTATAGA).

Assembly of Reporter Constructs—Double-stranded oligonucleotides containing three tandem SPI-GLE 1 sequences (CTAGTGTTTCTAGAAAAGCTGGCTGAGAAATGACGTTTCGAGAAAT) were ligated, using standard techniques (14), into the XbaI site of pBLCAT2, a reporter plasmid containing a thymidine kinase minimal promotor adjacent to the chloramphenicol acetyltransferase cDNA (16).

Cell Transfection and Chloramphenicol Acetyl Transferase Assay—These were carried out as described previously (17). Briefly, BRL-4 cells gated, using standard techniques (14), into the XbaI site of pBLCAT2, cells were washed with phosphate-buffered saline and then scraped into iced, using standard techniques (14), into the XbaI site of pBLCAT2, cells were incubated with DOTAP/DNA for 12 h, and then the medium was changed to serum-free Dulbecco's minimal essential medium containing 100 nM hGH. After a further 12 h, cells were washed with phosphate-buffered saline and then scraped into 0.25 M Tris, pH 8.0. Following three rounds of freeze-thaw lysis and heat treatment at 65°C for 10 min, extracts were centrifuged to remove cell debris and then assayed for chloramphenicol acetyl transferase activity. 20 µg of protein was incubated with 3 µl of [3H]chloramphenicol (Amersham Corp., 50-62 mCi/mmol, 25 pCi/ml) and 25 µg of butyryl coenzyme A (Boehringer Mannheim) for 3 h at 37°C. Butyrylated chloramphenicol was then extracted with xylene and detected using a Wallac scintillation counter.

RESULTS

Gel Electrophoresis Mobility Shift Assays with 45-bp SPI-GHRE Probe—It has previously been reported that GH induces the binding of proteins extracted from rat liver nuclei to a 45-bp DNA element (SPI-GHRE) in the 5′ flank of the serine protease inhibitor 2.1 gene (12). To facilitate investigation of the mechanism of GH regulation of gene transcription, we first wished to reproduce this effect using cultured cell lines. We, therefore, prepared nuclear extracts from Chinese hamster ovary (CHO-K1) and buffalo rat liver (BRL-3A) cells that had been stably transfected with rat GH receptor cDNA and shown to respond to hGH (17). These cell lines will hereafter be referred to as CHO-4 and BRL-4. The extracts were subjected to GEMSMA with a [32P]-labeled oligonucleotide probe consisting of a single copy of the SPI-GHRE. We observed a hGH-dependent formation of two complexes, A and B (Fig. 1). The GH-dependent complexes first appeared at 5 min, reached a maximum between 10 and 15 min, and were decreased in intensity by 60 min. That the addition of an excess (50-fold molar ratio) of unlabeled SPI-GHRE oligonucleotide, but not of an unrelated DNA (pSV2-Luc), resulted in a loss of electrophoretic mobility shift, indicates that the observed DNA binding was specific for the probe used (data not shown). Similar results were observed in both cell lines.

Definition of the GH-activated Complex-binding Domain of SPI-GHRE—The 45-bp SPI-GHRE contains two regions that share homology to the GAS DNA element (Fig. 2). We termed these two regions SPI-GLE 1 (−129 to −118 bp) and SPI-GLE 2 (−144 to −136 bp). In order to define minimal regions of the SPI-GHRE required for the response to GH, we used several double-stranded oligonucleotides representing discrete parts of the SPI-GHRE (including both SPI-GLE 1 and SPI-GLE 2) as competitors in GEMSMA experiments with the 45-bp SPI-GHRE probe (Fig. 3). We found that the oligonucleotides corresponding to SPI-GLE 1 were the most efficient inhibitors of complex formation (Fig. 3) abolishing both complexes A and B. Oligonucleotides covering SPI-GLE 2 abolished complex A but not complex B. Since SPI-GLE 1 appeared to have a higher affinity for GH-activated proteins, further experimentation was focused on this element.

Gel Electrophoresis Mobility Shift Assays with a SPI-GLE 1 Probe—To determine whether SPI-GLE 1 alone was sufficient for formation of GH-induced DNA-binding complexes, we la-

![Fig. 1. GH activation of SPI-GHRE binding activity in BRL-4 cell nuclear extracts (panel A) and CHO-4 cells (panel B). BRL-4 cells grown in 30-mm cell culture dishes to 75% confluence and then washed twice with phosphate-buffered saline. Transfections were carried out in serum-free Dulbecco's minimal essential medium using DOTAP (Boehringer Mannheim) and 1.25 µg of COS density gradient purified plasmid DNA/well. Cells were incubated with DOTAP/DNA for 12 h, and then the medium was changed to serum-free Dulbecco's minimal essential medium containing 100 nM hGH. After a further 12 h, cells were washed with phosphate-buffered saline and then scraped into 0.25 M Tris, pH 8.0. Following three rounds of freeze-thaw lysis and heat treatment at 65°C for 10 min, extracts were centrifuged to remove cell debris and then assayed for chloramphenicol acetyl transferase activity. 0.25 mg of protein was incubated with 3 µl of [3H]chloramphenicol (Amersham Corp., 50-62 mCi/mmol, 25 pCi/ml) and 25 µg of butyryl coenzyme A (Boehringer Mannheim) for 3 h at 37°C. Butyrylated chloramphenicol was then extracted with xylene and detected using a Wallac scintillation counter.

![Fig. 2. Lineup between SPI-GAS-like element, interferon-γ response elements (a, Ref. 24), a GAS-like element from the c-fos promoter, milk protein binding factor elements, a GAS-like element from the β casein promoter (b, Ref. 28), interleukin-4 nuclear-activated factor binding elements (c, Refs. 25 and 26), and interleukin-6-activated acute phase response factor binding elements (d, Ref. 27).]
placed three copies of the 9-bp SPI-GLE1 upstream of a minimal thymidine kinase promoter driving chloramphenicol acetyltransferase cDNA expression. This construct, when transiently transfected into BRL-4 cells, gave a 5-6-fold GH-dependent enhancement of chloramphenolic acetyltransferase expression (Fig. 5), which was similar to the degree of enhancement seen when a construct containing the SPI-GHRE was used (17). A chloramphenicol acetyltransferase reporter construct (16) containing the minimal thymidine promoter alone gave no such GH-dependent effects. These results demonstrate that SPI-GLE1 is able to confer a functional response to GH and enhance transcription of the SPI genes.

The GH-activated SPI-GLE1 Binding Protein Does Not Require Protein Synthesis for Activation—The time-dependent activation of nuclear protein-binding to SPI-GLE1 is consistent with GH activating preexisting but latent transcription factors rather than increasing their expression. This is the case for several other cytokine molecules (18, 19). We tested this hypothesis by inhibiting cellular protein synthesis with cycloheximide (50 μg/ml) for 15 min before the addition of hGH. These conditions (dose and time) have previously been demonstrated to completely inhibit cellular protein synthesis (20). As observed in Fig. 6, cycloheximide had no effect on the ability of GH to induce the protein-binding to SPI-GLE1 in nuclear extracts of CHO-4 cells.

Phosphorylation Is Required for Activation of the SPI-GLE1 Binding Protein—The hormone/cytokine-induced activation of latent transcription factors has previously been demonstrated to be mediated by tyrosine phosphorylation (18, 19, 20). In order to determine the involvement of phosphorylation in GH induction of nuclear protein binding to the SPI promoter, we made nuclear extracts from CHO-4 cells co-incubated with 100 nm hGH and 100 nm staurosporine for 15 min (Fig. 6). Such treatment with staurosporine has previously been demonstrated to prevent GH-induced phosphorylation of proteins in 3T3 adipocytes (21) and inhibit GH stimulation of lipid synthesis (22). No SPI-GLE1 binding activity was detected in nuclear extracts prepared from cells treated with staurosporine. Thus it seems that activation of cellular kinases and cellular protein phosphorylation are required for transcription factor activation. To investigate whether tyrosine phosphorylation of transcription factors is necessary for their GH-activated binding to SPI-GLE1, we incubated nuclear extracts from GH-treated CHO-4 cells with tyrosine-specific phosphatase 1B (UBI). This treatment specifically removed SPI-GLE1 binding activity from nuclear extracts of GH treated cells (Fig. 7). Further evidence for the importance of phosphorylation for protein binding to SPI-GLE1 is provided by the reduction of intensity of the band corresponding to the GH-dependent complex seen when nuclear extracts are preincubated with an antiphosphotyrosine monoclonal antibody but not with a control antibody (antichymotrypsin; data not shown).

The Complexes Between Nuclear Proteins and SPI-GLE1 or IRF1 Probes from Interferon-γ or GH-treated Cells Are Differ-
ent—Interferon-γ stimulation of cells induces the phosphorylation and subsequently the binding of Stat 91 (STAT 1α) to the GAS DNA element (18). It is possible that GH could also induce Stat 91 binding to SPI-GLE 1. In order to compare the GH-activated SPI-GLE 1 binding complex with interferon-γ-activated Stat 91, nuclear extracts were prepared from BRL-4 cells treated with recombinant mouse interferon-γ or hGH and subjected to GEMSA with a SPI-GLE 1 or IRF-1 probe (24). The GH-activated transcription complex migrated more slowly under gel electrophoresis than the interferon-γ-activated Stat 91 (Fig. 7) with both SPI-GLE 1 and IRF 1 probes. To determine whether Stat 91 is present in both the GH- and interferon-γ-stimulated complexes, we performed supershift analyses with four different anti-Stat 91 antisera produced against four separate GST fusion peptides corresponding to amino acids 2–66, 515–607, 609–716, and 715–750 (26). We were unable to supershift the GH-stimulated complex with any of these antisera, although they were able to recognize an interferon-γ-stimulated complex with either the SPI-GLE 1 or IRF1 probes (Fig. 7; data shown for the antisera raised against amino acids 2–66). These results clearly show that the GH-induced DNA-binding complex is specific to GH and antigenically distinct from that generated by interferon-γ.

DNA Binding Specificity for GH-activated Nuclear Proteins—Some degree of DNA-binding specificity must exist if cytokine activation of the Stat 91-like transcription factor family is to have nonoverlapping transcriptional effects. We have investigated this using oligonucleotide competitors with sequences representing various members of the GAS family in the GEMSA analyses of nuclear extracts from GH-treated CHO-4 cells (See Fig. 8 for GEMSA analyses and Fig. 2 for sequences).

The elements used included those from the GBP, FcγR1, ICSBP, IFP-53 IRF-1, Ly6E, and Mig genes. These experiments define a palindromic recognition sequence for DNA-binding of GH-activated nuclear proteins.

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\begin{align*}
TTCCCA & \quad \text{SEQUENCE 1} \\
TGT & \quad \text{GAA}
\end{align*}
\]

The principal difference is the greater conservation of the palindrome in the SPI-GLE 1. This requirement for a palindromic sequence is supported by the observation that SPI-GLE 2 does not bind nuclear proteins in GEMSA analyses (data not shown). In addition the GAS's absolute requirement for a cytosine at position four is relaxed to a pyrimidine in the SPI-GLE 1.

DISCUSSION

Growth hormone is a member of a family of cytokines and growth factors that exert their cellular effects as a result of binding to specific membrane-bound receptors (3, 4, 5, 6). These cytokine receptors, in addition to having a number of common structural features, may also share related signal transduction pathways. The pathway for interferon-γ may be considered to represent the paradigm. Following ligand binding, interferon-γ
causes the activation of members of the Janus kinase family. These in turn activate, by phosphorylation, the transcription factor Stat 91. Phosphorylated Stat 91 is then translocated to the nucleus where it binds to specific y-activated DNA sequences (CTAGTGTTCTGAGAATGACAAGTTCTGAGAAAGTACACTCTGAGAAT) and another, the previously mentioned SPI-GHRE, more distal between positions -175 and -114 (23). SPI-GLE 1 lies within the distal region, and since it is functionally active when placed upstream of a minimal thymidine kinase promoter-driving chloramphenicol acetyltransferase, cDNA expression is likely to be the major contributor to its GH responsiveness. The mechanism of GH regulation of SPI expression via the proximal promoter region is unknown, although the identification of several C/EBP binding sites raises the possibility that this transcription factor family may mediate GH responses in some way (23) and may also explain why SPI mRNA levels are almost undetectable in transformed cell lines (17).

DNA elements similar to SPI-GLE 1 are also found in promoters activated by interferon-γ (24), interleukin-4 (25, 26) interleukin-6 (27), and prolactin (28) (all hormones binding to members of the cytokine receptor family). The SPI-GHRE contains a second GAS-like element upstream of SPI-GLE 1 (Fig. 9). This element showed no binding activity for nuclear proteins in GEMSA analyses. However the fact that it is disrupted by a 42-bp insertion in the GH refractory SPI 2.3 promoter indicates that it may have a role in vivo. This is interesting in light of the recent discovery that both the prolactin-regulated β casein (29, 30) and the interferon-γ-regulated mig genes (31) contain tandem repeated GAS-like DNA elements that mediate transcription regulation. The two GAS-like elements in the β casein promoter that bind the mammary gland factor are separated by a binding site for the transcription factor YY1 (32). A sequence similar to the YY1 binding consensus, CCATnT, also separates the GAS elements in the SPI-GHRE. This fact, together with the observation that BRL cells transiently co-transfected with a rabbit prolactin receptor expression plasmid and pSPI-CAT and treated with lactogenic hormone express chloramphenicol acetyltransferase activity,2 indicates that prolactin and GH may share similar mechanisms of transcription enhancement. We are currently investigating the possibility...

2 T. J. J. Wood, unpublished observations.
that, as is the case for prolactin, GH enhances transcription by overcoming YY1-dependent transcription repression.

The time frame of appearance and the resistance to inhibition by cycloheximide treatment of nuclear proteins binding to the SPI promoter are consistent with GH activating preexisting but latent transcription factors rather than increasing their expression. Our results support the theory that this activation is caused by tyrosine phosphorylation of transcription factors. GH also induces tyrosine phosphorylation of microtubule-associated protein kinase (33) and the GH receptor itself, and it is reasonable to speculate that all of these phosphorylations are mediated by the physical association of Janus kinase 2 to the receptor (7). It is interesting that Stat 91 activation by interferon-γ activates a phosphorylated tyrosine residue located in the carboxyl terminus (34) of the receptor. This phosphorylated tyrosine residue is also involved in the physical association of Stat 91 with the interferon-γ receptor.

The Janus kinases are required for the activation of the interferon-γ-activated, GAS-binding transcription factor Stat 91. This activation, which results from phosphorylation of a single tyrosine, leads to DNA binding via the formation of Stat 91 homodimers (11, 35–38). Since Janus kinase 2 associates with the growth hormone receptor (7), one may postulate that GH also induces the phosphorylation of Stat 91. Indeed, during the preparation of this manuscript several groups reported the activation by GH of a transcription factor sharing antigenic determinants with Stat 91 and the binding of a Stat 91-like protein to the GAS-like SIE in the c-fos promoter (39–42). We have similarly tried to demonstrate the involvement of Stat 91 or a Stat 91-like molecule in the GH regulation of the SPI gene via SPI-GLE 1. We have, however, obtained and describe here several lines of evidence suggestive of a unique GH-induced factor mediating signal transduction via the SPI-GLE 1. (i) The electrophoretic mobility of the GH-induced factor binding to SPI-GLE 1 is retarded in comparison to interferon-γ-stimulated binding to the same element, suggestive of a higher molecular weight. (ii) The GH-induced factor binding to SPI-GLE 1 is not supershifted by several different polyclonal Stat 91 antisera in a gel electrophoresis mobility shift assay despite the fact that we observed a supershift with interferon-γ-stimulated cells, and (iii) GH but not interferon-γ is able to drive chloramphenical acetyltransferase expression from the SPI-GLE 1. Since Stat 91 and the growth hormone-activated transcription factor described here share rather similar DNA binding specificities, it seems likely that they are related, belonging to a family of cytokine-activated Stat 91-like transcription factors. This hypothesis is consistent with results demonstrating that the IL-6-activated transcription factor APRF shares sequence similarities with Stat 91 (43), and a recent report describing the existence of a family of Stat proteins with distinct but overlapping hormonal specificities (44). As yet, there is very little evidence to indicate how related transcription factors binding to related DNA elements could maintain hormonal specificity. It is possible that the small differences between DNA binding affinities seen in vitro may be much larger in vivo, a contention supported by the GH-activated DNA-binding complex's sensitivity to salt concentration in a gel mobility shift assay. Additional factors not seen in vitro but capable of dictating specificity of transcription enhancement in vivo may be present in cytokine-activated transcription complexes. Alternatively the activation of different Stat molecules may show different kinetics as supported by the observation that IL-6 activates APRF more rapidly and for a longer period of time than Stat 91 (27).

In conclusion, we have identified a γ-activated sequence-like DNA element in the growth hormone-regulated serine protease inhibitor 2.1 and 2.2 genes that is capable of mediating GH effects even in nondifferentiated cell lines by specifically interacting with one or more unique GH-activated tyrosine-phosphorylated transcription factors. This element does not act alone in the GH-dependent regulation of SPI expression, but since it is sufficient to confer GH responsiveness on a heterologous promoter, can be regarded as a paradigmatic growth hormone responsive DNA elements and will, therefore, be of value for the continued study of GH's mechanism of gene regulation.
REFERENCES


