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Growth Hormone Regulates Ternary Complex Factors and Serum Response Factor Associated with the c-*fos* Serum Response Element*

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For insight into the mechanisms of gene regulation by growth hormone (GH), the regulation of transcription factors associated with the serum response element (SRE) located upstream of c-*fos* was examined. The SRE can mediate induction of reporter expression in response to GH. For insight into the mechanism by which GH regulates transcription factors, regulation of SRE-associated proteins by GH was examined. In nuclear extracts from 3T3-F442A fibroblasts, several SRE-binding complexes were identified by electrophoretic mobility shift assay. GH treatment for 2–10 min transiently increased binding of two complexes; binding returned to control values within 30 min. The two GH-stimulated complexes were supershifted by antibodies against the serum response factor (SRF), indicating that they contained SRF or an antigenically related protein. One of the GH-stimulated complexes was supershifted by antibody against Elk-1, suggesting that it contains a ternary complex factor (TCF) such as Elk-1 in addition to SRF. Induction of binding by GH was lost when the SRF binding site in the SRE was mutated, and mutation of either the SRF or TCF binding site altered the pattern of protein binding to the SRE. Mutation of the SRF or TCF binding site in SRE-luciferase plasmids inhibited the ability of GH to stimulate reporter expression, supporting a role for both SRF and TCF in GH-induced transcription of c-*fos* via the SRE. The TCF family member Elk-1 is capable of mediating GH-stimulated transcription, since GH-stimulated reporter expression was mediated by the transcriptional activation domain of Elk-1. Consistent with this stimulation, GH rapidly and transiently stimulated the serine phosphorylation of Elk-1. The increase was evident within 10 min and subsided after 30 min. Taken together, these data indicate that SRF and TCF contribute to GH-promoted transcription of c-*fos* via the SRE and are consistent with GH-promoted phosphorylation of Elk-1 contributing to GH-promoted transcriptional activation via the SRE.

Recent studies have begun to provide insight into mechanisms by which growth hormone (GH),[1] a major regulator of normal growth, regulates gene expression. Transcription of the proto-oncogene c-*fos* is rapidly stimulated by GH (1, 2). Multiple upstream sequences appear to participate in the regulation of c-*fos* by GH based on deletion analysis (3). Among these, GH-stimulated transcription of reporter constructs has been demonstrated via the serum response element (SRE) (4) and the Sis-inducible element (SIE) (5). The c-*fos* SIE binds signal transducer and activator of transcription (Stat) 1 and Stat 3, and a trimer of the SIE can mediate GH-stimulated transcriptional activation when Stat 3 is overexpressed (5). The SRE is a stronger enhancer, since as a single copy it efficiently mediates transcriptional activation in response to GH (4). Although many proteins have been identified that bind to the c-*fos* SRE (6), their regulation by GH has not been explored.

Transcriptional activation of c-*fos* in response to serum and several growth factors requires binding of the serum response factor (SRF) to the core SRE as a dimer. While SRF binds constitutively to the SRE (7, 8), binding to the SRE has been shown to be rapidly and transiently increased by several growth factors including epidermal growth factor and insulin (9–11). The phosphorylation of SRF on multiple serines and threonines is reported to be regulated and to influence DNA binding kinetics (12–16). However, SRF binding to the SRE is not thought to be sufficient to mediate transcription. Members of the transcription factor family of ternary complex factors (TCF), including Elk-1, SAP-1, and SAP-2/ERP/Net, also bind to the c-*fos* SRE but only in association with SRF (17, 18). Elk-1 is able to mediate transcription via the SRE if SRF is present and if Elk-1 is phosphorylated on serine 383 in its C-terminal transcriptional activation domain (19–21). MAP kinases, including ERK 1, ERK 2, p38, and stress-associated protein kinase, mediate Elk-1 phosphorylation on serine 383 (20, 22, 23). The present study investigates whether and how GH regulates SRF and/or TCFs associated with the SRE by examining the ability of GH to promote: 1) the binding of SRF and TCFs to DNA, 2) the ability of SRF and TCF to mediate expression of reporter constructs, and 3) the phosphorylation of Elk-1.

Both SRF and TCF were found to be essential for GH-stimulated transcriptional activation via the SRE. In addition, GH was found to stimulate Elk-1-mediated transcriptional activation and the serine phosphorylation of Elk-1. These findings

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[1] The abbreviations used are: GH, growth hormone; GHR, GH receptor; SRE, serum response element; SREw, wild type *c-fos* SRE; SRF, serum response factor; mSRF, mutation of the SRF binding site; TCF, ternary complex factor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; CHO, Chinese hamster ovary; EMSA, electrophoretic mobility shift assay; RSV, Rous sarcoma virus; SIE, Sis-inducible element.

identify transcription factors that contribute to GH-promoted transcription of c-*fos* and support a role for serine phosphorylation of a TCF such as Elk-1 in GH-promoted transcriptional activation mediated by the SRE.

EXPERIMENTAL PROCEDURES

Materials—3T3-F442A cells were kindly provided by Dr. H. Green (Harvard University, Boston, MA) and Dr. M. Sonenberg (Sloan-Kettering, NY). Chinese hamster ovary (CHO) cells and CHO cells expressing full-length rat GH receptor (GHR_{1-638}) or GHR lacking the C-terminal half of the cytoplasmic domain (GHR_{1-454}) were provided by Dr. Gunnar Norstedt (Karolinska Inst., Stockholm, Sweden). Recombinant human GH was provided by Eli Lilly. Rabbit polyclonal antibody against the C-terminal sequence (amino acids 46–245) of human SRF (αSRF) and corresponding nonimmune serum were generous gifts from Dr. M. Greenberg (Harvard University, Boston, MA) (15). Affinity-purified polyclonal antibody (100 μg/ml) against a peptide corresponding to amino acids 407–426 of Elk-1 (α-Elk-1(sc)) used for supershifting was obtained from Santa Cruz Biotechnology. For immunoblotting, polyclonal antibodies against a synthetic phospho-serine 383 peptide corresponding to residues 379–392 of Elk-1 (α-P-Elk-1), and polyclonal antibodies against the corresponding nonphosphorylated Elk-1 peptide (αElk-1) were purchased from New England Biolabs. Lipofectamine was purchased from Life Technologies, Inc., luciferin from Promega, and β-galactosidase chemiluminescence reagents from Tropix. The enhanced chemiluminescence (ECL) detection system was purchased from Amersham. Leupeptin, aprotinin, and pepstatin were purchased from Boehringer Mannheim, bovine serum albumin (CRG7) from Intergen, and radioisotopes from NEN Life Science Products.

Cell Culture and Hormone Treatment—3T3-F442A preadipocyte fibroblasts were grown to confluence in Dulbecco's modified Eagle's medium containing 4.5 g/liter glucose and 8% calf serum in an atmosphere of 10% CO_2, 90% air. CHO cells and CHO cells expressing GHR were grown in Ham's F-12 medium containing 0.5 mg/ml G418 and 10% fetal calf serum in an atmosphere of 5% CO_2, 90% air. All media were supplemented with 1 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin. Prior to treatment, cells were deprived of serum overnight in the appropriate medium containing 1% bovine serum albumin instead of serum unless indicated otherwise. Cells were then incubated with GH at 500 ng/ml (22 nM).

Plasmids and Probes—Binding to the c-*fos* SRE was studied by electrophoretic mobility shift assay (EMSA) using oligonucleotides containing the following wild type and mutated sequences (54) where changes from wild type are underlined: Wild type *c-fos* SRE (SREw), 5′-gatcGGATGTCCATATTAGGACATC-3′; mutation of the SRF binding site (mSRF), 5′-gatcGGATGTAAATATTATTACATC-3′; mutation of the TCF binding site (mTCF), 5′-gatcAAATGTCCATATTAGGACATC-3′. To study transcriptional activation, the oligonucleotides were inserted into the *Bam*HI and *Nhe*I sites of TK-Luc, to generate plasmids SREw/Luc, mSRF/Luc, and mTCF/Luc. The reporter plasmid TK-Luc (from Dr. J. Pessin, University of Iowa) contains the herpes simplex virus thymidine kinase minimal promoter driving expression of the luciferase reporter gene (24). Clones were sequenced to verify orientation and sequence. Each plasmid contained a single copy of the wild type or mutated SRE sequence in the correct orientation. The plasmids Gal4/ElkC (25) and 5X Gal/Luc (26) were provided by Dr. C. Der (University of North Carolina). The plasmid RSV-β-galactosidase was provided by Dr. M. Uhler (University of Michigan) and RSV-neo by Dr. Nils Billestrup (Hagedorn Lab, Gentofte, Denmark). The cDNA for Elk-1 was provided by Dr. R. Treisman (Imperial Cancer Res. Fund, London, United Kingdom), and the plasmid CMV-Elk-1 was provided by Dr. Kun-liang Guan (University of Michigan).

Stable and Transient Transfection—3T3-F442A fibroblasts were plated at 10^4 cells/cm^2 on 100-mm plates and stably transfected the next day using lipofectamine, 8 μg/plate of plasmid SREw/Luc, mSRF/Luc, or mTCF/Luc, and RSV-neo (2 μg/plate). Pooled clones were maintained in the presence of 0.6 mg/ml G418. Relative levels of expression of the three SRE/Luc plasmids were comparable. CHO cells expressing GHR_{1-638} were transiently transfected by the calcium phosphate coprecipitation procedure (27) with 2.5 μg of TK-Luc, SREw/Luc, mSRF/Luc, or mTCF/Luc plasmid DNA/35-mm well. In a separate series of experiments, CHO cells expressing GHR_{1-638} (2×10^5 cells/35-mm well) were co-transfected with 5 μg/well of Gal4/ElkC and Gal/Luc DNA and 1 μg RSV β-galactosidase DNA using calcium phosphate (4). 44–48 h after transfection, cells were deprived of serum and treated as indicated. CMV-Elk-1 or the vector pcDNA3 (10 μg/100-mm plate) were transfected into CHO cells expressing GHR_{1-638} or GHR_{1-454} using calcium phosphate. Since GH induces c-*fos* mRNA and stimulates transcriptional activation via the SRE to comparable extents in the CHO cells expressing GHR_{1-638} or GHR_{1-454},[2] these cells were used interchangeably. Unless indicated otherwise, 36 h posttransfection, the cells were incubated in medium containing 0.5% fetal calf serum for 12 h and then incubated in medium containing 1% bovine serum albumin for 5 h prior to treatment as indicated.

EMSA—EMSAs were performed as described previously (28). Briefly, confluent cells were deprived of serum overnight and incubated for the indicated times with hormone, serum, or vehicle at 37 °C. Nuclear extracts were prepared and analyzed as described (28). Binding reactions proceeded for 30 min at 30 °C and were analyzed by nondenaturing polyacrylamide gel electrophoresis followed by autoradiography. In some experiments, nuclear extracts were preincubated for 20 min at room temperature with αSRF (1:10) or corresponding rabbit nonimmune serum (NI, 1:10), or with α-Elk-1(sc) (1:10).

Luciferase Assay—Cell lysates were prepared in reporter lysis buffer (100 mM potassium phosphate, 0.2% Triton X-100, 1 mM dithiothreitol) and luciferase or β-galactosidase activity was measured using an Autolumat Luminometer. The luciferase values from experiments evaluating transcriptional activation via Elk-1 were normalized to β-galactosidase activity. Data are expressed as percentage of values in untreated controls ± S.E. for five independent experiments. Variability in controls was less than 1% among triplicates in each experiment. Analysis of variance with factorical Scheffe F-test was used to analyze data from individual experiments.

Immunoblotting—CHO-GHR cells in 100-mm plates were washed with phosphate-buffered saline and scraped in 0.5 ml of lysis buffer (50 mM Hepes, pH 7.0, 250 mM NaCl, 0.1% Nonidet P-40) containing 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin and leupeptin. Cell lysates were analyzed by immunoblotting as described (5) using α-P-Elk-1 (1:1000). To reprobe blots, the membranes were submerged in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) and incubated at 50 °C for 30 min while shaking. The membranes were then reprobed with αElk-1 (1:1000). The apparent molecular weights indicated are based on prestained molecular weight standards (Life Technologies, Inc.).

RESULTS

GH Rapidly and Transiently Increases Binding of Nuclear Proteins to the SRE—Previous studies indicate that GH significantly induces c-*fos* expression within 30 min (1, 2, 29–31) and that the SRE can mediate stimulation by GH (4). To determine whether such stimulation is accompanied by changes in the binding of nuclear proteins to the c-*fos* SRE, binding was examined by EMSA using nuclear extracts from 3T3-F442A fibroblasts incubated with or without GH (Fig. 1). Several SRE binding complexes were identified in all 3T3-F442A cell extracts tested. They included two slower migrating bands (*A* and *B*) and a diffuse faster migrating complex (*C*). Bands A and B migrate similarly to bands identified by others as the SRE-SRF complex (*band A*), and the ternary complex (*band B*) composed of SRE, SRF, and Elk-1 or another TCF (18, 32, 33). The identity of band C is not known; its intensity varies with cell type (see Fig. 2). *Band D* shows migration of free probe. Complexes A, B, and C were not evident when excess oligonucleotide was present during incubation with nuclear extracts, indicating specificity of binding (data not shown). The presence of SRF in bands A and B is supported by the disappearance of bands A and B and the appearance of a more slowly migrating band when anti-SRF serum (αSRF) is added to nuclear extracts and by co-migration of band A with *in vitro* translated SRF bound to the SRE probe and supershift of that complex with αSRF (data not shown).

When the effect of GH treatment on binding was examined after 30 min, binding of the SRF-containing bands to the SRE was not detectably different from that in untreated cells (Fig. 1*A*, *lane 4 versus 3, bands A and B*). Since changes in binding

[2] T.-W. L. Gong, D.J. Meyer, J. Liao, C.L. Hodge, G.S. Campbell, X. Wang, N. Billestrup, G. Norstedt, C. Carter-Su, J. Schwartz, submitted for publication.

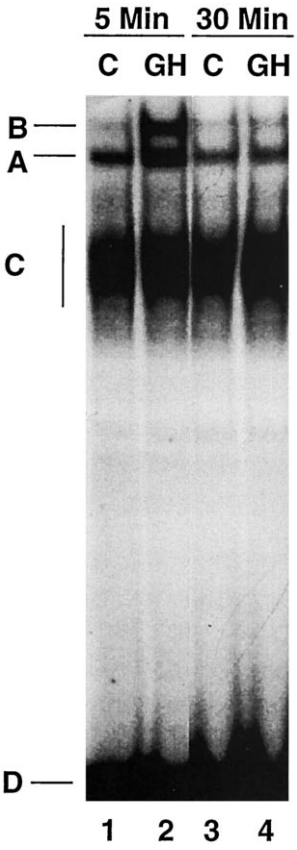

FIG. 1. **GH rapidly and transiently induces binding to the SRE.** Nuclear extracts were prepared from cells treated for 5 min (*lanes 1* and *2*), or 30 min (*lanes 3* and *4*) with vehicle (*C*, *lanes 1* and *3*) or GH (*lanes 2* and *4*). EMSA was performed using SREw as probe. The individual bands representing the SRE-binding complexes are labeled *A*, *B*, and *C* in this and subsequent figures. *D* indicates migration of free probe. This experiment was repeated 4 times.

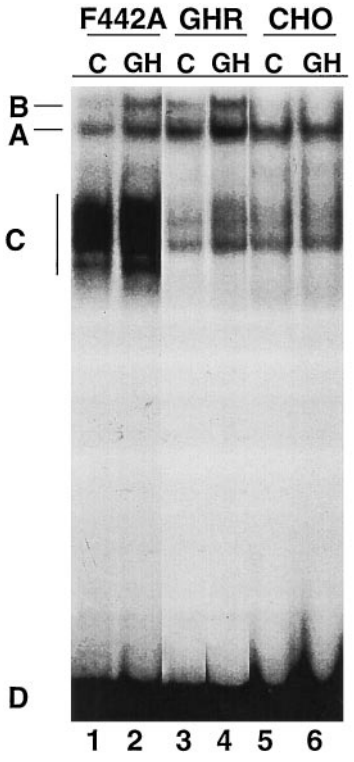

FIG. 2. **GH-induced binding to the SRE occurs in several cell types.** 3T3-F442A fibroblasts (*lanes 1* and *2*), CHO-GHR$_{1-638}$ cells (*GHR*, *lanes 3* and *4*) or nontransfected CHO cells (*CHO*, *lanes 5* and *6*) were incubated for 5 min with vehicle (*C*, *lanes 1*, *3*, and *5*) or GH (*lanes 2*, *4*, and *6*). Nuclear extracts were used for EMSA with the SREw probe.

could precede GH-induced c-*fos* expression, which is evident as early as 15 min, the effect of GH on nuclear protein binding to the SRE was examined at earlier times. A 5 min incubation of cells with GH increased binding of the SRF-containing complexes to the SRE (Fig. 1*A*, *lane 2*, *bands A* and *B*). Similar increases in binding were also observed in extracts incubated with GH for 2 or 10 min (data not shown). By 30 min, the GH-stimulated binding subsided to control levels (Fig. 1*A*, *lane 4*). The intensity of band C generally appeared to increase with GH treatment. These findings indicate that GH rapidly stimulates the binding of several complexes to the SRE and that the stimulation of binding of SRF-containing complexes to the SRE is transient and subsides to control levels within 30 min.

To determine whether GH-induced binding to the SRE is evident in cells other than 3T3-F442A fibroblasts, binding was examined in CHO cells stably expressing full-length GHR (GHR$_{1-638}$), in which GH induces c-*fos* expression.[2] In CHO-GHR cells, GH was found to stimulate binding to the SRE in 5 min (Fig. 2, *lane 4*), whereas the parental CHO cells lacking endogenous GHR failed to respond to GH (*lane 6*). In NIH-3T3 fibroblasts, in which GH can also induce c-*fos* (4, 30), an increase in binding was also observed after 5 min of GH treatment (data not shown). Band C was greatly reduced in CHO and NIH-3T3 cells compared with 3T3-F442A cells but still appeared to increase in CHO-GHR cells with GH treatment.

Mutation of the SRF and TCF Binding Sites Alters GH-stimulated Binding—Identification of bands A and B and their regulation by GH was further investigated by examining the effect of mutations in the SRF or TCF binding sites (see "Experimental Procedures") on GH-induced binding. Mutation of the TCF site (Fig. 3, *lanes 9–12*) prevented formation of band B, but binding of the SRE-SRF complex (*band A*) appeared intact. Mutation of the SRF site eliminated bands A, B, and C (Fig. 3, *lanes 5–8*). Rather, an intermediate band (*D*) consistently appeared that was not stimulated by GH (*lane 7 versus lane 5*). These results provide further support that band A is a complex of the SRE and SRF, and that band B is composed of the complex of the SRE, SRF, and a TCF. In the absence of TCF, SRF binding to the SRE still increased in a GH-dependent fashion (*lane 11 versus 9*). However, in the absence of SRF, the GH-dependent increase in binding of both SRF and TCF was lost (*lane 7*). Consistent with bands A and B containing SRF, the complexes bound to SREw or mTCF were supershifted when nuclear extracts were incubated with αSRF (Fig. 3, *lanes 2, 4, 10,* and *12*). With mSRF, the newly present band D was not supershifted by αSRF (*lanes 6* and *8*), consistent with the mSRF sequence being unable to bind SRF. These observations provide further support for the presence of SRF in the GH-inducible bands A and B.

Mutations in SRF and TCF Binding Sites Impair Transcriptional Activation by GH—To determine whether SRF or TCF are required for induction of c-*fos* via the SRE in response to GH, reporter expression in response to GH was tested in 3T3-F442A cells transfected stably with wild type or mutated SRE-luciferase plasmids. The wild type SRE alone mediated GH-stimulated luciferase expression that was three times higher than control (Fig. 4*A*) consistent with previous reports using a nonheterologous reporter (4). The reporter plasmid alone (TK) consistently failed to respond to GH. Mutation of either the SRF or TCF binding site blocked the ability of GH to stimulate reporter expression. The contribution of SRF and TCF in the GH response was not restricted to 3T3-F442A cells, since similar inhibition of GH-stimulated luciferase expression by mu-

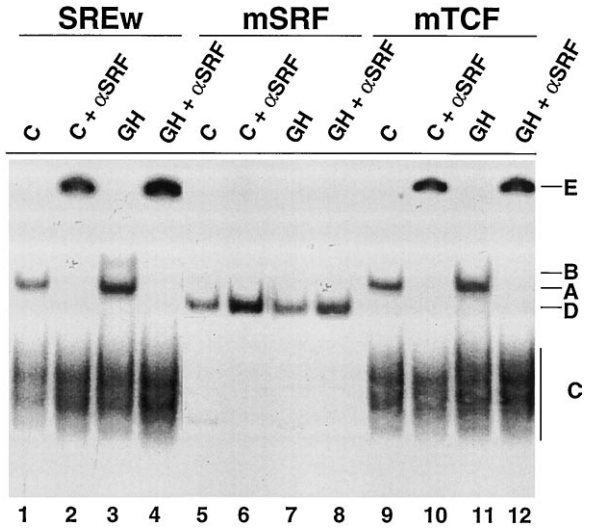

FIG. 3. **Mutation of SRF and TCF binding sites alters GH-induced complexes bound to the SRE.** 3T3-F442A fibroblasts were incubated for 5 min with vehicle (*C*, *lanes 1*, *2*, *5*, *6*, *9*, and *10*) or GH (*lanes 3*, *4*, *7*, *8*, *11*, and *12*). EMSA was performed using as probes SREw (*lanes 1–4*), mSRF (*lanes 5–8*), or mTCF (*lanes 9–15*). Nuclear extracts were preincubated for 20 min at room temperature with (*lanes 2*, *4*, *6*, *8*, *10*, and *12*) or without αSRF. Similar results were obtained in two other experiments.

tation of SRF or TCF binding sites was observed in CHO cells expressing GHR_{1-638} upon transient transfection of wild type or mutated SRE/Luc (Fig. 4*B*). These findings show that the binding of SRF and TCF are essential for GH-stimulated transcriptional activation via the SRE.

GH Stimulates Transcription via Elk-1—For insight into the TCF family member regulated by GH, anti-Elk-1 antibodies were used in EMSA to examine whether Elk-1 was present in the GH-stimulated ternary complex. The endogenous ternary complex in 3T3-F442A cells was found to contain Elk-1 or a related protein, since α-Elk-1 reduced band B in untreated (Fig. 5, *lane 2*) and GH-stimulated cells (*lane 4*). The stimulation of Elk-1 binding by GH was further demonstrated by overexpression of Elk-l in CHO cells expressing GHR. Upon EMSA of nuclear extracts from these cells, GH treatment increased the amount of band B (*lane 7 versus lane 5*) as it had in 3T3-F442A cells. The presence of Elk-1 in band B in control and GH-treated cells was substantiated by reduction of band B upon addition of antibody against Elk-1 (Fig. 5, *lane 6 versus lane 5* and *lane 8 versus lane 7*). A small amount of residual band B in GH-treated cells (*lane 8*) most likely reflects incomplete reaction of α-Elk-1 with the greater amount of Elk-1 bound to the SRE. In the GH-treated cells, the presence of α-Elk-1 also led to appearance of an additional, more slowly migrating band (*lane 8*), consistent with the presence of Elk-1 in the GH-stimulated complex in the CHO-GHR cells. The supershift is visible when Elk-1 is over-expressed, compared with the substantially lower levels of endogenous Elk-1 in 3T3-F442A cells (*lane 4*). Immunoblotting confirmed the expression of comparable amounts of Elk-1 in transfected CHO-GHR cells (data not shown).

Since a TCF is required for GH-promoted reporter expression via the SRE, and since Elk-1 is present in the GH-stimulated ternary complex bound to the SRE, the ability of Elk-1 to mediate GH-stimulated gene expression was examined. CHO-GHR cells were transiently co-transfected with the Elk-Gal expression plasmid Gal4/ElkC encoding the transcriptional activation domain of Elk-1 fused to the Gal4 DNA-binding domain (25), and a Gal-Luc reporter plasmid (5X Gal/Luc) containing 5 consensus Gal4 DNA binding sites upstream of the

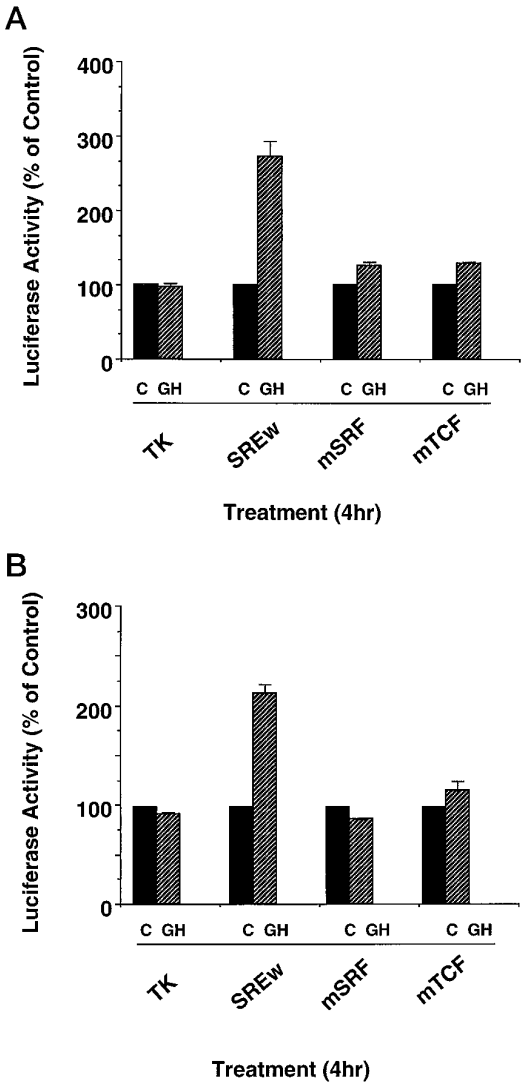

FIG. 4. **Mutations in SRF or TCF binding sites impair transcriptional activation in response to GH.** *A*, 3T3-F442A cells were stably transfected with plasmids SREw/Luc, mSRF/Luc, mTCF/Luc, or vector TK/Luc (*TK*). Cells were incubated with or without GH for 4 h and extracts were analyzed for luciferase expression. *B*, CHO-GHR_{1-638} cells were transiently transfected with the same plasmids as in *A* and were used 48 h later. Cells were tested without (*C*) or with GH for 4 h. Induced luciferase activity is expressed as percent of control (= 100). Data represent means + S.E. for five to six experiments, each performed in triplicate. The stimulation by GH is significant with SREw/Luc ($p < 0.05$). The occasional increase with mSRF or mTCF was not statistically significant in any experiment.

luciferase gene (26). GH produced a consistent stimulation of luciferase expression mediated via the Elk-1 transcriptional activation domain (Fig. 6), indicating that GH increases the transcriptional activation capabilities of Elk-1.

GH Stimulates the Phosphorylation of Elk-1—The phosphorylation of Elk-1 on serine 383 in its C-terminal transactivation domain is required for Elk-1 mediated transcriptional activation in response to growth factors (19, 21, 34). The phosphorylation of Elk-1 is dependent on MAP kinase activation (19, 22, 35). Furthermore, GH is known to activate the MAP kinases ERK 1 and ERK 2 in several cell types including 3T3-F442A fibroblasts and CHO cells expressing GHR_{1-638} and GHR_{1-454} (36–39). Thus the possibility that GH treatment stimulates the phosphorylation of Elk-1 was tested.

The ability of GH to stimulate phosphorylation of Elk-1 was examined by immunoblotting using an antibody that specifically recognizes a peptide from the C-terminal transactivation

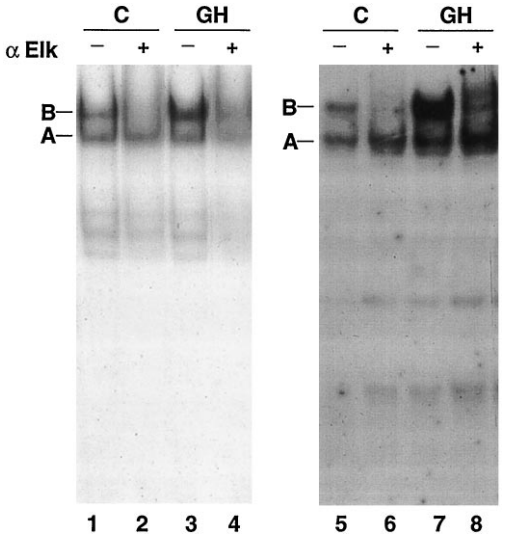

FIG. 5. **Presence of Elk-1 in GH-induced binding complexes.** 3T3-F442A cells (*left panel*) were incubated without (*lanes 1* and *2*) or with (*lanes 3* and *4*) GH for 5 min. Nuclear extracts were treated with (*lanes 2* and *4*) or without (*lanes 1* and *3*) α-Elk-1(sc) and used for EMSA with SREw as probe. CHO cells expressing GHR_{1-454} were transfected with CMV-Elk-1 (*right panel*) and after 7 h of serum deprivation were incubated without (*lanes 5* and *6*) or with (*lanes 7* and *8*) GH for 5 min. Nuclear extracts were preincubated for 20 min at room temperature with (*lanes 6* and *8*) or without (*lanes 5* and *7*) α-Elk-1(sc) and used for EMSA. Comparable levels of Elk-1 expression were verified by immunoblotting (not shown). Similar results were obtained in two experiments.

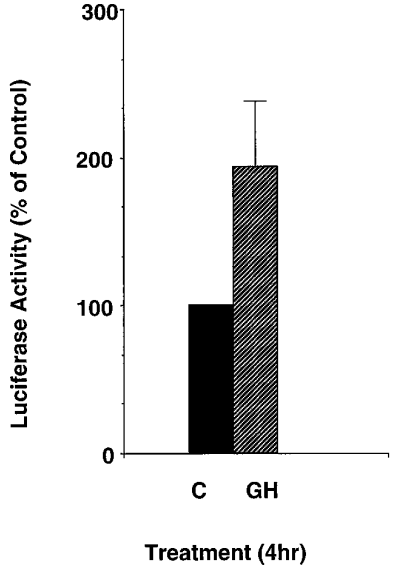

FIG. 6. **GH stimulates Elk-1 mediated transcriptional activation.** CHO-GHR_{1-638} cells were transiently co-transfected with plasmids Gal4/ElkC and 5X Gal/Luc, as well as RSV-β-galactosidase. 24 h later, cells were deprived of serum overnight and then tested without (*C*) or with GH treatment for 4 h. Induced luciferase activity in cell lysates, normalized for β-galactosidase activity, is expressed as percent of control. Data represent means + S.E. for six experiments, each performed in triplicate. The stimulation by GH is statistically significant ($p < 0.05$).

domain of Elk-1 phosphorylated on serine 383 (α-P-Elk-1) (14, 21). Preliminary experiments in 3T3-F442A cells demonstrated that levels of endogenous Elk-1 were too low for detection by immunoblotting with the corresponding antibody against the nonphosphorylated Elk-1 peptide. Therefore, to examine Elk-1 phosphorylation directly, Elk-1 was overexpressed in CHO cells expressing GHR_{1-454}, in which GH stimulates c-*fos* expression and binding to the SRE.[2] Fig. 7 demonstrates that GH increases the amount of serine-phosphorylated Elk-1, while the amount of total Elk-1 is unchanged (*bottom panel*). The increase in phosphorylated Elk-1 is evident in 10 min, increases for 30 min, and subsides by 60 min (Fig. 7). Cells transfected with vector alone showed no evidence of phosphorylated or nonphosphorylated Elk-1 (data not shown). Taken together, these data are consistent with a mechanism in which GH promotes phosphorylation of Elk-1 and transcriptional activation by Elk-1, suggesting contribution of this mechanism to the essential role of TCF in GH-stimulated expression of c-*fos* via the SRE.

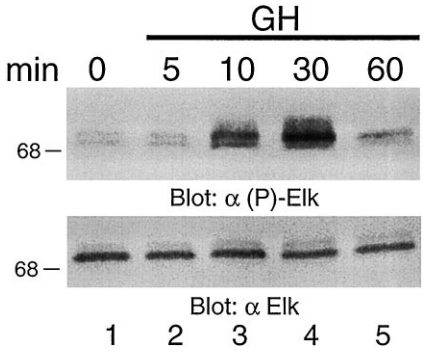

FIG. 7. **GH stimulates the serine phosphorylation of Elk-1.** CHO cells expressing GHR_{1-454} were transfected with the plasmid for CMV-Elk-1. After serum deprivation, cells were treated with GH for the indicated times. Whole cell lysates were analyzed by immunoblotting with α(P)Elk-1 (1:1,000) (*upper panel*) or αElk-1 (1:1,000) (*lower panel*). Molecular weight × 1000 are shown on *left*. This experiment was repeated two times.

DISCUSSION

Essential Role of SRF and TCF in GH-promoted Transcriptional Activation—Since the c-*fos* SRE can mediate transcriptional activation in response to GH, analysis of the regulation by GH of transcription factors associated with the SRE provides insight into general mechanisms for transcriptional activation by this fundamental growth regulator. The present study demonstrates that GH regulates transcription factors associated with the c-*fos* SRE. Such regulation was evident at physiological concentrations of GH, suggesting a role for the SRE and c-*fos* in physiological actions of GH, such as normal growth and differentiation.

Both SRF and TCF are involved in GH-promoted transcriptional activation via the SRE, since mutation of the binding site for either of these factors prevents GH-promoted reporter expression driven exclusively via the SRE. This observation is in agreement with a report that mutations of the SRF and TCF sites attenuated GH-stimulated reporter expression via a fragment the c-*fos* enhancer (−396 to −42 with an inhibitory site from −216 to −81 deleted) (3). The inhibition reported due to the SRE mutations in the context of the promoter fragment was in the range of 30–50%, but in the present study the inhibition is over 95% in the context of the SRE alone. GH-regulated expression of the heterologous reporter TK-luciferase via the SRE alone is in agreement with previous observations that a single copy of the SRE mediated GH stimulation of a human c-*fos* reporter containing 222 base pairs of its promoter (4). Thus, analysis of the SRE alone emphasizes the essential role of SRF and TCF in GH-promoted transcriptional activation.

GH Can Stimulate Binding of SRF and TCF to the SRE—Two SRE-binding complexes were rapidly and transiently stimulated by GH in 2–10 min. The increase in binding returned to control levels within 30 min of GH treatment. Both complexes were constitutively present in 3T3-F442A cells and contained

SRF as indicated by supershift in EMSA with anti-SRF antibodies, comigration with *in vitro* translated SRF, and ablation by mutating the SRF binding site (mSRF). The GH-induced increase in binding involved SRF, based on the finding that induction by GH was lost when the SRF binding site was mutated. One of the two complexes (upper band B) induced by GH contained a TCF as well as SRF, as indicated by its ablation when the TCF site was mutated, by its reduction when extracts were treated with anti-Elk-1 antibodies and by comigration of the complex with over-expressed Elk-1. Whether Elk-1 is the only TCF member present in the endogenous ternary complex has yet to be established. Whereas the binding of SRF and TCF are clearly essential for GH-stimulated transcriptional activation via the SRE, the functional importance of the GH-induced increase in binding of these factors in the stimulation of transcriptional activation has yet to be established.

The phosphorylation of SRF is regulated by serum and is believed to modulate the rate of SRF binding to the SRE (15, 40, 41). Consistent with this, dephosphorylation with alkaline phosphatase reduced the binding of SRF in nuclear extracts from 3T3-F442A cells (data not shown). At present, it is not known whether GH regulates the phosphorylation of SRF. However, such regulation seems likely, since $p90^{RSK}$ is reported to mediate SRF phosphorylation (15, 40–42) and GH stimulates the activity of this kinase in 3T3-F442A cells (43). Regardless of the mechanism for altered binding, the inability of mSRF to mediate transcriptional activation demonstrates that the binding of SRF is required for SRE-mediated transcription in response to GH, as for serum and several growth factors. If increased SRF phosphorylation and binding do contribute to GH-promoted transcriptional activation, they are not sufficient, however, since GH-promoted transcriptional activation was also found to require TCF.

The functional importance of TCF and SRF binding in the regulation of transcriptional activation by GH contrasts with observations using insulin, which also rapidly and transiently stimulates binding of nuclear factors to the SRE in cells overexpressing insulin receptors. Mutation of the SRF or TCF site interfered with binding of proteins to the SRE but not with insulin-stimulated transcriptional activation of reporter constructs (11), suggesting that for insulin the increase in binding is not required for transcriptional activation. Another difference between GH and insulin is that insulin induced a third band not seen with GH (11). These findings suggest that GH and insulin do not utilize identical mechanisms for inducing c-*fos* expression, although both can regulate protein binding to the SRE.

Proteins other than SRF or TCF may associate with the SRE in 3T3-F442A cells, even in the absence of SRF, as suggested by the appearance of a non-SRF-containing band (*D*) bound to mSRF. The binding of such proteins does not appear to be directly regulated by GH under the conditions of these experiments, since band D did not change in extracts from cells incubated with GH. The presence of other SRE-associated proteins is also suggested by the presence of several phosphorylated bands smaller but distinct from SRF in anti-SRF immunoprecipitates of nuclei from 3T3-F442A cells metabolically labeled with ^{32}P.[3] The absence of band C binding to the SRE mutated at the SRF binding site suggests that the presence of band C might require SRF binding or the SRF binding site. The identity of band C, its function and regulation by GH, remains to be determined.

GH Stimulates Serine Phosphorylation of Elk-1—The importance of the TCF site for GH-stimulated transcriptional activation via the SRE suggests that GH might regulate one of the ternary complex factors, which include Elk-1, SAP-1, and SAP-2/ERP/Net, of the Ets family of transcription factors. Responsiveness of Elk-1 to GH was demonstrated by the ability of GH to stimulate reporter expression via the Elk-1 transcriptional activation domain. The stimulation was observed in CHO cells expressing GHR, which demonstrated GH-stimulated binding to the SRE (Fig. 3) and SRE-mediated (Fig. 6*B*), as well as Elk-1-mediated (Fig. 7) transcriptional activation was also observed. Because phosphorylation of the transcriptional activation domain of Elk-1 on serine 383 is required for its function (12, 19, 20), regulation of Elk-1 by GH is likely to involve GH-stimulated phosphorylation of Elk-1. The ability of GH to stimulate the MAP kinases ERK 1 and ERK 2 (37–39, 44) and the ability of ERK 1 and ERK 2 as well as other MAP kinases to phosphorylate serine 383 of Elk-1 (35) support this possibility. Consistent with this possibility, GH was found to increase the appearance of a band recognized by antibody against serine-phosphorylated Elk-1 peptide in cells overexpressing Elk-1. This band comigrated with a band of approximately 70 kDa also recognized by antibody against the nonphosphorylated Elk-1 peptide. Levels of total Elk-1 were unaffected by GH treatment. Taken together, these observations are consistent with GH stimulating the phosphorylation of Elk-1 on serine 383. The levels of endogenous Elk-1 in 3T3-F442A cells were too low for detection by immunoblotting, although the α-P-Elk-1 faintly recognized a GH-stimulated protein that migrated at approximately the same size as the overexpressed Elk-1 (data not shown).

The ability of GH to stimulate serine phosphorylation of Elk-1 was rapid and transient, which is consistent with the rapid and transient stimulation by GH of c-*fos* gene expression (2) of the GH-associated tyrosine kinase Jak2 (45) and of MAP kinase (37, 39, 44). GH-promoted phosphorylation of Elk-1 coincides temporally with epidermal growth factor-stimulated phosphorylation of Elk-1 in COS cells (46) and with epidermal growth factor-stimulated binding of TCF to the E74 DNA probe in astrocytoma cells (47). GHR activation by GH leads to the association with and activation of the tyrosine kinase Jak2 (45) (Fig. 8). This kinase participates in GH-dependent activation of the c-*fos* promoter, as shown in COS cells expressing Jak2, GHR, and a *fos* promoter (−357 to −276)/luciferase plasmid (48). Presumably, GH-promoted activation of Jak2 initiates the activation of the MAP kinases ERK 1 and ERK 2 that leads to the serine phosphorylation of Elk-1 and possibly, via $p90^{RSK}$, of SRF. These events are summarized in a model of c-*fos* regulation by GH (Fig. 8).

Regulation of Transcription Factors by GH—The SRE is one of only a few DNA sequences currently identified that can mediate transcriptional activation in response to GH. γ-activated sequence-like elements (GLE) in genes such as *spi* 2.1 (49), beta casein (50), and CYP 3A10/6β-hydroxylase (51), bind the transcription factor Stat 5 and can mediate reporter expression in response to GH (50–52). While Stat 5 plays a prominent role in the regulation of genes containing GLE sequences by GH, Stat 1 and Stat 3 contribute to the regulation of c-*fos*, binding to the SIE in response to GH (28, 53, 54) and mediating GH-stimulated reporter expression when Stat 3 is overexpressed (5) (Fig. 8). GH is likely to regulate transcription by other mechanisms not yet identified as well. For example, a novel sex-dependent GH-regulated liver nuclear factor, recently reported to interact with the promoter of the liver cytochrome P450 CYP2C12 gene, may regulate gene expression via mechanisms distinct from the GH-pulse activated Stat 5 pathway (55). A novel GH-regulated transcription factor has also

[3] K. Rosenspire and J. Schwartz, unpublished data.

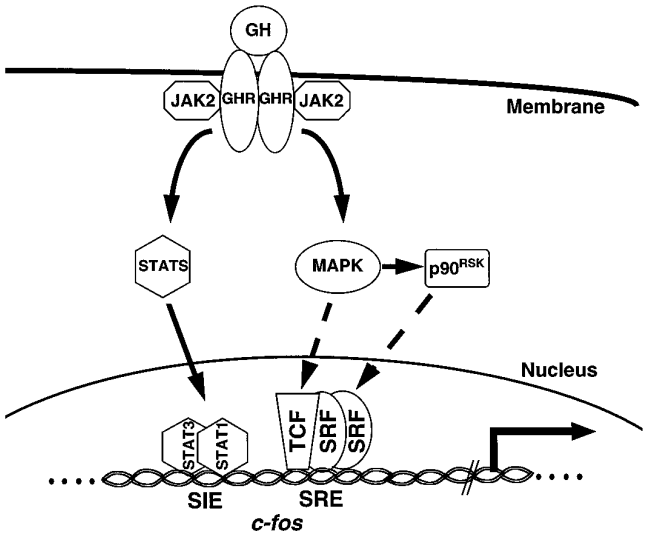

FIG. 8. **Model of c-*fos* regulation by GH through the SRE and SIE.** GH binding to GHR leads to GHR dimerization, association of Jak2 with GHR, and tyrosyl phosphorylation of Jak2 and GHR. GH-induced association of the GHR-Jak2 complex leads to activation of the Ras-MAPK pathway. Activated MAPK phosphorylates a TCF (*e.g.* Elk-1), which leads to transcriptional activation via the SRE. GH may also regulate the phosphorylation of SRF via $p90^{RSK}$. Tyrosyl phosphorylation and binding of Stat 1 and Stat 3 to the SIE in response to GH can lead to transcriptional activation. The *solid arrows* indicate pathways regulated by GH; *dotted arrows* indicate known pathways not yet shown to be regulated by GH. Possible interactions among pathways are not shown.

been reported to associate with the *spi* 2.1 promoter (56). GH has also been reported to stimulate the synthesis and binding of the transcription factors C/EBPβ and -δ (57). The present study indicates that GH regulates two transcription factors associated with the c-*fos* SRE, SRF, and Elk-1 or another TCF, which contribute to GH-dependent gene expression. These findings support a mechanism involving MAP kinase-mediated serine phosphorylation of Elk-1 or related protein by which GH stimulates transcriptional activation (Fig. 8).

The *c-fos* regulatory elements are well characterized and include sequences responsive to growth factors, protein kinase C, intracellular calcium, and cAMP (58–60). The present findings do not exclude the possibility that sequences other than the SRE in the c-*fos* promoter also participate in mediating a physiological response to GH. GH-stimulated reporter expression appears to be relatively less dependent on the SRE when other c-*fos* regulatory sequences are also present, since SRE mutations in the context of the *fos* promoter produced less inhibition of GH stimulation than the same mutations in the context of the SRE alone (3). This is consistent with the concept that multiple regulatory sequences participate in GH-promoted c-*fos* regulation. Examination of the regulation of c-*fos* by GH using 5′ mutations and deletions in specific regulatory elements of the c-*fos* enhancer showed that the combined presence of the SRE, SIE, and AP-1 sites is required for full induction by GH (3). The involvement of multiple elements in the physiological regulation of c-*fos* is also supported by transgenic mouse studies that demonstrate the requirement for multiple control elements for normal c-*fos* expression *in vivo* (61). Therefore, it is likely that other sequences in the c-*fos* regulatory region will work in coordination with the SRE to induce c-*fos* in response to GH. Identifying possible interactions of SRE-associated proteins with other regulatory sequences and their associated proteins in response to GH will offer insight into the complexity of mechanisms by which GH regulates gene transcription.

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