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The serum response element can mediate induction of c-*fos* by growth hormone

(c-*fos* promoter/physiological concentrations of growth hormone/serum growth factors/synergism)

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ABSTRACT **The c-*fos* protooncogene is transcriptionally activated by a wide variety of agents including serum, growth factors, and phorbol esters. This induction is rapid and transient and is mediated through a number of identified promoter elements. Growth hormone (GH) is also known to induce transcription of c-*fos* in a variety of cell types including NIH 3T3 fibroblasts and 3T3-F442A preadipocytes. To identify DNA sequences in the c-*fos* gene regulated by GH, this study sought to determine whether induction of c-*fos* by GH involves previously identified c-*fos* promoter elements. A plasmid containing a growth factor-sensitive fragment of the upstream region of the c-*fos* promoter from −361 to −264 bp was tested for GH sensitivity. The fragment was cloned upstream of a human c-*fos* reporter [designated *FOS* by Human Gene Mapping 11 (1991)], which included basal promoter elements. In transiently transfected mouse NIH 3T3 fibroblasts, the promoter fragment conferred GH sensitivity on the human c-*fos* reporter. To identify a specific GH-sensitive DNA sequence in this promoter, a serum response element (SRE)-reporter plasmid was tested and found to be stimulated by GH. GH was effective in inducing expression through the SRE over a range of physiological GH concentrations. Since GH was recently found to synergize with serum factors in inducing c-*fos* transcription, the effect of GH and serum on SRE function was examined for insight into the mechanism for such synergism. The combined effect of GH and serum to induce reporter expression through the SRE was greater than the added effects of GH and serum, indicating that the synergism between GH and serum in inducing c-*fos* involves the SRE sequence. These studies identify the SRE as one specific DNA sequence in the c-*fos* promoter functionally regulated by GH. It is notable that GH is effective at physiological concentrations. Furthermore, synergism in c-*fos* induction between GH and serum factors is evident through the SRE.**

Molecular events associated with the effects of growth hormone (GH) are just beginning to be elucidated. Among these events, GH rapidly and transiently increases expression of the protooncogene c-*fos* in a variety of cell types, including 3T3-F442A cells and NIH 3T3 fibroblasts (1–5). The gene product Fos has been implicated as a transcription factor that regulates genes important in cell growth and differentiation (6, 7). Hence, c-*fos* may play a focal role early in GH signaling and participate in the growth-promoting actions of GH.

At present, a DNA sequence in the c-*fos* promoter which is responsive to GH has not been identified. To determine whether a GH-responsive sequence could be identified within the c-*fos* promoter, this study examined the ability of GH to stimulate c-*fos* promoter-reporter plasmids. Since mechanistic features of the action of GH, a "classical" growth-promoting hormone, are reminiscent of the actions of other growth factors, the focus of this study included sequences from −350 to −295 bp upstream of the transcription start site, in which at least three regions have been identified that are responsive to growth factors. The serum response element (SRE; also called dyad symmetry element, DSE) mediates transcriptional stimulation by serum and a variety of growth factors (8–11). Other sites in this region include an AP-1 site immediately 3′ of the SRE (12) and a region known as the SIF element, which lies 5′ of the SRE and binds a factor inducible specifically by *sis*/platelet-derived growth factor in BALB/c 3T3 cells and by epidermal growth factor in A431 cells (13–15).

An appealing aspect of studying the induction of c-*fos* by GH in fibroblasts is that it occurs at GH concentrations in the physiological range. Hence, this study provides a context not only in which to identify a DNA sequence responsive to GH but in which to evaluate the role of such a sequence in GH action and to identify such functions in cells regulated by physiological levels of a major growth-promoting hormone.

MATERIALS AND METHODS

Plasmids. The plasmid 222FOS, containing the entire human c-*fos* transcription unit and 222 bp of promoter sequence, and the plasmid PB4/222 (F4K1), containing the sequence from −361 to −264 (13) upstream of 222FOS, have been described (14). The plasmid DSE/222 (KB/KX) contains a SRE/DSE oligonucleotide in the indirect orientation (sequence, 5′-CAGATGTCCTAATATGGACATCCTCT-AG-3′) upstream of 222FOS, as described (14). The plasmid MSRE contains in the indirect orientation one copy of a mutated SRE which fails to bind the serum response factor (16). The oligonucleotides 5′-aattCAGATGTGGTAATAC-CACATCCTG-3′ and 5′-agcttCAGGATGTGGATATTAC-CACATCTG-3′ were annealed and ligated into the *Eco*RI and *Hin*dIII sites of 222FOS. Clones were sequenced to verify orientation and sequence.

Cell Culture, Transfections, and Growth Factor Treatment. NIH 3T3 fibroblasts and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g of glucose per liter and 10% calf serum in an atmosphere of 10% CO_2/90% air. For transfection, NIH 3T3 cells were plated at 10^4 cells per cm^2 on 100-mm plates ≈24 h prior to transfection. DNA was introduced by the calcium phosphate coprecipitation procedure (17) with 20 μg of c-*fos* plasmid DNA and 1 μg of pSVA1 α-globin DNA (18) to control for trans-

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Abbreviations: GH, growth hormone; SRE, serum response element; SIF, *sis*-inducible factor; IL, interleukin.

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fection efficiency. At 64–68 h after transfection, cells were deprived of serum using DMEM with 1% bovine serum albumin for 16–18 h and then treated as indicated. Human GH (provided by Genentech) was added at various concentrations directly to the conditioned medium. Controls received equivalent volumes of vehicle. Serum was administered by the addition of fresh DMEM containing the indicated concentration of serum (0.5–20%). Treated cells were incubated for 30 min at 37°C prior to harvesting.

RNA Preparation and Analysis. Total RNA was isolated by the guanidine isothiocyanate/cesium chloride method as described (1) or by the acid/phenol method (19) for Northern blot analysis. The Northern blot (1) and RNase protection (14) analyses were performed essentially as described. For RNase protection, RNA (30–40 μg) was denatured and incubated with RNA probes for c-*fos* and α-globin (200,000 cpm each) overnight at 55°C. The plasmids AHfos and SP6-α-globin used to generate RNA probes for analysis by RNase protection have been described (14, 18). Digestion with RNase A (40 μg/ml) and RNase T1 (2 μg/ml) for 1 h was followed by the addition of proteinase K. Double-stranded RNA products were precipitated, resuspended in loading buffer, and denatured at 80°C for 5 min. Samples were subjected to electrophoresis on a 6% polyacrylamide/urea gel; gels were dried and used in autoradiography. The estimated sizes of the transcripts studied were 280 nt for human c-*fos*, verified by running HeLa cell RNA on each gel as a reference, 125 nt for the α-globin control, and 65 nt for the endogenous mouse c-*fos*. Autoradiograms exposed in a linear range were quantified by laser-scanning densitometry and c-*fos* signals were normalized to those of the control probe.

RESULTS

A Growth Factor-Sensitive Fragment of the c-*fos* Promoter Mediates a Response to GH. To elucidate the mechanism for transcriptional stimulation of c-*fos* by GH, a growth factor-sensitive fragment of the human c-*fos* promoter was analyzed for its ability to confer responsiveness to GH. The fragment of the c-*fos* promoter (14) derived from the sequence −361 to −264 bp, containing the SRE and the flanking SIF element and AP-1 site, upstream of the human c-*fos* reporter gene 222FOS (diagrammed in Fig. 1) was found to be responsive to GH.

GH stimulated the expression of the human c-*fos* reporter through the −361 to −264 promoter fragment in mouse NIH 3T3 fibroblasts transiently transfected with the plasmid PB4/222 (Fig. 1, lane C, upper arrow). HeLa cell RNA was used as a reference for the human c-*fos* transcript (lane A, upper arrow). As expected, expression of the reporter was minimal in quiescent NIH 3T3 control cells (lane B, upper arrow) and was strongly stimulated by serum (lane D, upper arrow). A nonspecific band evident in lanes B–D appears just above the human c-*fos* transcript. The transfected α-globin transcript (middle arrow, lanes B–D) indicates comparable introduction of transfected DNA. The analysis by RNase protection also reveals, in the same cells, the endogenous mouse c-*fos* transcript (lower arrow), since the human c-*fos* probe hybridizes with a small fragment of the mouse c-*fos* gene as well as with the transfected human c-*fos* DNA. The endogenous mouse c-*fos* transcript was also induced by GH (lane C, lower arrow) to a lesser extent than by serum (lane D) as reported previously (1). This suggested that the DNA sequence from −361 to −264 in the human c-*fos* promoter confers sensitivity to GH in this system.

The SRE Mediates Induction of c-*fos* by GH. Since the −361 to −264 promoter fragment contains the SRE, which is highly responsive to growth factors, the effect of GH was tested by using a plasmid containing the SRE alone. The plasmid DSE/222 contains an oligonucleotide containing the sequence of the SRE in the c-*fos* promoter, in the indirect orientation, directly upstream of the 222FOS reporter, as indicated in the diagram in Fig. 2*A*. GH stimulated reporter expression through the SRE alone (lane C, upper arrow) in cells transfected with the SRE-containing plasmid. In quiescent control cells (lane A) containing an equivalent level of transfected DNA, expression of the reporter was undetectable. In cells treated with 10% calf serum (lane B), stimulation of expression of human c-*fos* through the SRE is also evident. Endogenous mouse c-*fos* transcripts (lower arrow) show stimulation by GH and serum comparable to that seen for the human c-*fos* reporter.

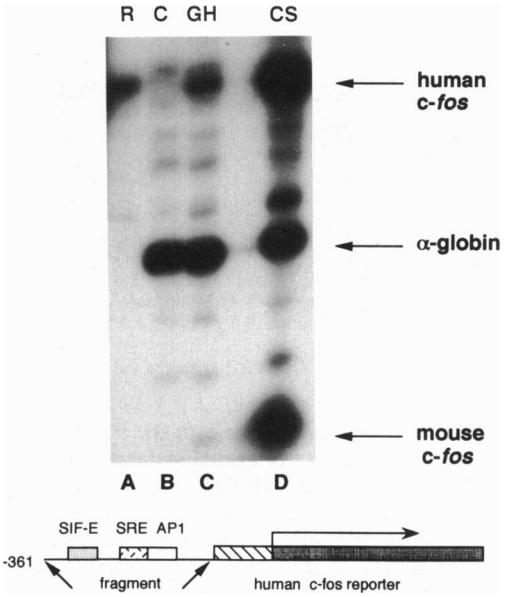

FIG. 1. GH induces c-*fos* expression through a fragment (−361 to −264) of the c-*fos* promoter. NIH 3T3 cells were transiently transfected with plasmid PB4/222, indicated schematically in the diagram at the bottom. Test plasmid contains a fragment of the human c-*fos* promoter from −361 to −264 bp, upstream of a reporter containing the human c-*fos* coding region (shaded area) and 222 bp of upstream sequence (hatched area). Cells were also transfected with α-globin DNA to control for transfection efficiency. At 64 h after transfection, quiescent cells were stimulated with GH (500 ng/ml) (lane C), 20% calf serum (lane D), or vehicle (control; lane B) for 30 min. Total RNA was isolated and analyzed by RNase protection. Positions of the human c-*fos* (upper arrow), α-globin (middle arrow), and endogenous mouse c-*fos* (lower arrow) protected fragments are indicated. HeLa cell RNA (lane A) provides reference for the human c-*fos* transcripts in this and subsequent figures.

Fig. 2*B* shows that the reporter plasmid 222FOS, which contains basal elements, does not respond to GH. While stimulation of the endogenous mouse transcript by GH is clearly visible (lower arrow, lane C), the reporter alone failed to respond to GH in the same cells (upper arrow, lane C). The α-globin bands (middle arrows) indicate that comparable amounts of the plasmid were successfully transfected into the cells. Similarly, although serum responsiveness of the endogenous mouse c-*fos* gene is clearly intact in these cells (lane D, lower arrow), serum failed to stimulate the reporter (lane D, upper arrow). To verify that induction by GH was dependent on the functional SRE, a plasmid (MSRE) containing a mutated SRE sequence that fails to bind serum response factor was tested (Fig. 2*C*). Neither GH (lane C) nor serum (lane D) induced expression of the reporter driven by the mutated SRE (upper arrow), although the endogenous c-*fos* was stimulated by GH and serum (lower arrow). Taken together, these data indicate that the GH-sensitive region of the c-*fos* promoter is not present in the basal elements of 222FOS and resides in the SRE.

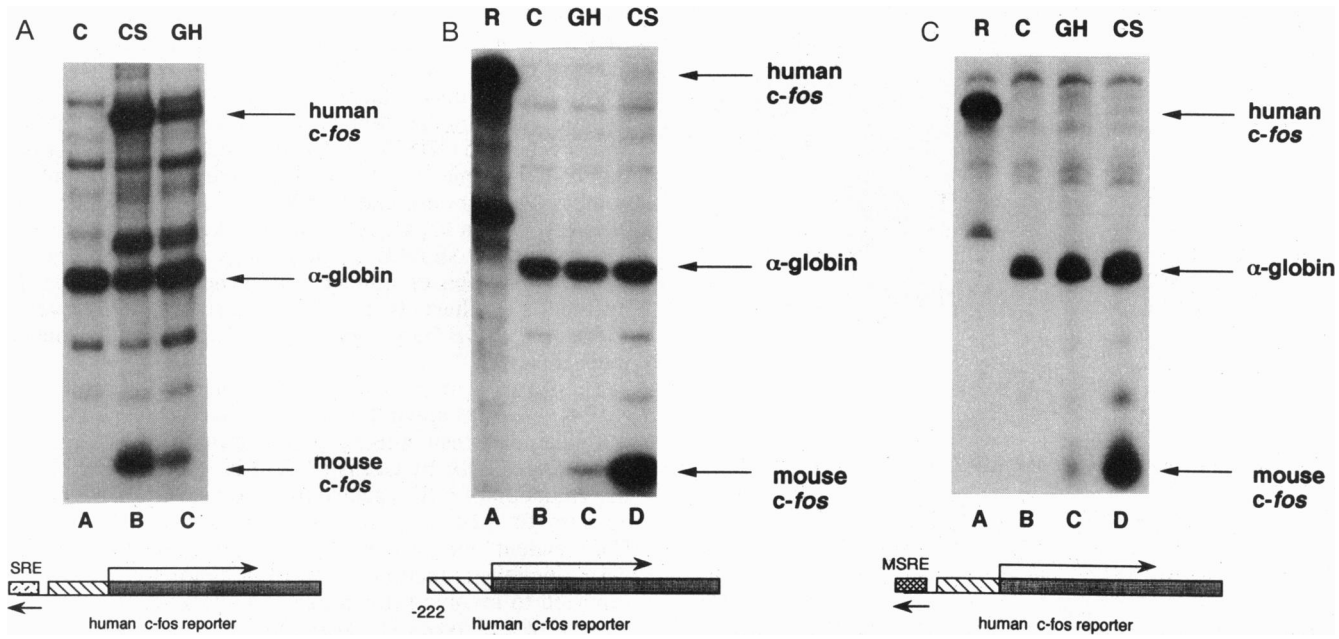

FIG. 2. GH induces c-*fos* expression through the SRE. (*A*) Stimulation by GH of SRE-FOS. NIH 3T3 cells were transiently transfected with a reporter plasmid driven solely by the SRE (DSE/222; diagrammed at the bottom) and were treated and analyzed as described in Fig. 1. Lanes: A, quiescent control cells; B, cells treated with 10% calf serum; C, response to GH (500 ng/ml). (*B*) Lack of induction of the reporter gene 222FOS. Lanes: A, HeLa cells; B, quiescent control cells; C, growth hormone (500 ng/ml); D, 10% calf serum. (*C*) Lack of induction of a mutant SRE-FOS plasmid. In cells transfected with 222FOS driven by a mutated SRE (MSRE), treatment with GH (500 ng/ml) (lane C), 10% calf serum (lane D), or vehicle (control, lane B) failed to elicit human c-*fos* expression, although mouse c-*fos* was stimulated. Lane A, HeLa cells. Positions of human c-*fos* (upper arrow), α-globin (middle arrow), and endogenous mouse c-*fos* (lower arrow) protected fragments are indicated.

Physiological Concentrations of GH Regulate c-*fos* Through the SRE. While previous studies have shown that the SRE responds to a variety of growth factors and some hormones, these agents have been tested at high concentrations and/or in cells overexpressing receptors (e.g., see ref. 10). In contrast, in the experiments shown above, the NIH 3T3 cells responded to GH at a concentration of 500 ng/ml, which is in the physiological range for GH secretory episodes in rodents (20, 21). To determine whether GH was effective at even lower concentrations, the sensitivity of the SRE-reporter plasmid was tested at concentrations of GH ranging from 50 to 500 ng/ml. Fig. 3 (lanes B–E) shows that the SRE mediates human c-*fos* induction at a GH concentration as low as 50 ng/ml (lane C, upper arrow). Concentrations of GH from 50 to 1000 ng/ml increasingly stimulated the transfected human c-*fos* and the endogenous mouse c-*fos* in the same cells (lanes C–E; data not shown). This is especially impressive since NIH 3T3 cells are at least 10 times less sensitive to GH than other cells in which GH induces c-*fos* (2).

The SRE Participates in Synergism Between GH and Serum. One recently identified feature of GH action is its synergism with serum factors in inducing c-*fos* transcription in 3T3-F442A fibroblasts (22). It is thought that synergism of GH with other growth factors might be an important component of the physiological effects of GH. To determine whether the synergism between GH and serum might occur in cells other than the highly sensitive 3T3-F442A fibroblasts, the combined effect of GH and serum was examined in the NIH 3T3 cells. Northern blot analysis indicates that synergism between GH and serum in inducing c-*fos* expression is evident in the NIH 3T3 cells (Fig. 4). GH alone elicited a modest induction of c-*fos* when tested at 500 ng/ml (lane B). When the GH was added in combination with 1% calf serum (lane D), the induction of c-*fos* was greater than the added individual effects of GH and 1% calf serum, indicating that synergism between GH and serum occurred in the NIH 3T3 fibroblasts.

To determine whether synergism in induction of c-*fos* involved the SRE, the SRE-driven reporter plasmid (DSE/222) was tested for its ability to respond synergistically to GH and serum in the NIH 3T3 cells. Under the conditions of these

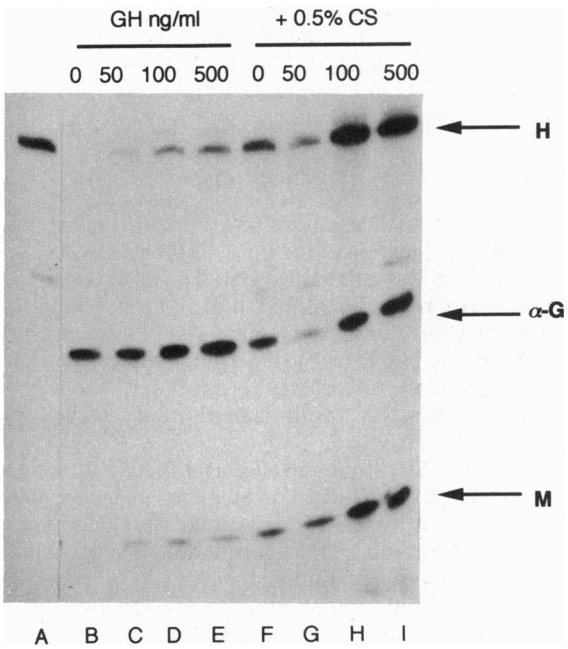

FIG. 3. SRE responds to physiological concentrations of GH and exhibits synergism between GH and serum. NIH 3T3 cells transiently transfected with the SRE reporter plasmid DSE/222 were treated with the indicated concentrations of GH (ng/ml) in the absence (lanes B–E) or presence (lanes F–I) of 0.5% calf serum and were analyzed as described for Fig. 1. Lane A, reference lane. Positions of the human c-*fos* (H, upper arrow), α-globin (α-G, middle arrow), and endogenous mouse c-*fos* (M, lower arrow) protected fragments are indicated.

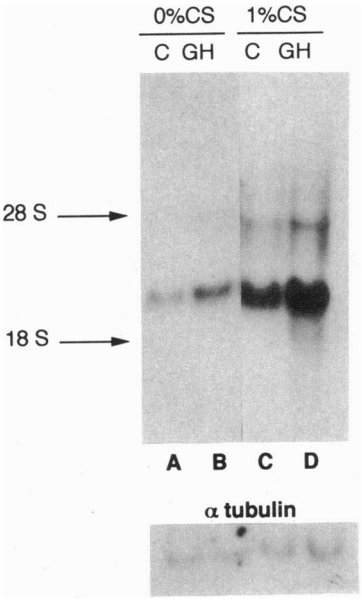

FIG. 4. GH synergizes with serum in inducing c-*fos* expression in NIH 3T3 cells. (*Upper*) Quiescent fibroblasts were treated without (lanes A and C) or with (lanes B and D) GH (500 ng/ml) in the absence (lanes A and B) or presence (lanes C and D) of calf serum (1%) for 30 min. Total RNA was prepared and analyzed by Northern blot analysis. Positions of 28S and 18S rRNA are indicated. (*Lower*) Results of hybridization of the blot with cDNA for rat α-tubulin (1) to control for loading.

experiments, cells are deprived of serum by using DMEM containing 1% bovine serum albumin, and subsequent incubation with low concentrations of calf serum elicits a modest stimulation of c-*fos* (Fig. 3, lane F; Fig. 4, lane C). However, the combined effect of GH and 0.5% serum in inducing c-*fos* through the SRE, adjusted for transfection efficiency, was ≈2-fold greater than the sum of their individual effects (Fig. 3, lanes F–I). This suggests that the SRE mediates at least part of the synergism between GH and other serum growth factors in increasing c-*fos* expression.

DISCUSSION

Analysis of the induction of c-*fos* transcription by GH facilitated identification of the c-*fos* SRE as a DNA sequence whose function is regulated by GH. The SRE/DSE was the first regulatory region identified in the c-*fos* promoter (8, 23, 24) and has been shown to mediate stimulation of c-*fos* not only by serum but also by a variety of agents including epidermal growth factor, nerve growth factor, platelet-derived growth factor, insulin, and phorbol esters (9–11, 25, 26).

In regulating SRE function, GH is effective at concentrations in the physiological range. This suggests that the induction of c-*fos* by GH can contribute to regulation by GH of normal cell growth and differentiation. This possibility is strengthened by observations that GH is a major factor required for the differentiation of 3T3-F442A preadipocytes to adipocytes (27, 28), that GH stimulates c-*fos* transcription in 3T3-F442A cells (1), and that Fos protein, in conjunction with Jun, regulates transcription of the differentiation-dependent gene for adipocyte P2, a lipid binding protein, in 3T3-F442A cells (5, 6).

The binding of nuclear proteins to the SRE is evident in nuclear extracts from both GH-treated and untreated NIH 3T3 or 3T3-F442A fibroblasts, as evaluated by electrophoretic mobility shift assay (D.J.M. and J.S., unpublished observations). This is not surprising, since the serum response factor is reported to be constitutively bound to the SRE and cannot be regulated by growth factors in most of the systems tested so far (15, 25).

The SRE is quite distinct from a DNA sequence in the hepatic gene *Spi-2.1*, which has been shown to confer responsiveness to GH (29). A 45-bp sequence in the 5′ flanking region of this gene, which encodes a serine protease inhibitor, confers GH responsiveness on a heterologous promoter and reporter gene in hepatocytes when linked in tandem copies. This sequence showed no homology to the documented (GenBank data base) sequence for the human c-*fos* gene. The difference in these two GH responsive DNA sequences suggests that GH may regulate gene expression by multiple mechanisms.

The mechanism by which GH induces c-*fos* through the SRE is as yet unknown. It is unlikely that this response to GH is mediated by insulin-like growth factor 1 (IGF-1), since the induction of c-*fos* by GH is evident in <30 min, well before the induction of IGF-1 gene expression by GH (3). The SRE appears to mediate c-*fos* transcription by protein kinase C-dependent and protein kinase C-independent pathways (26). Since the induction of c-*fos* expression by GH has been reported to involve protein kinase C-dependent events in a variety of cell types (1, 3–5), protein kinase C-dependent events may participate in the response of the SRE to GH.

Signaling mechanisms involved in responsiveness of the SRE to GH may be related to those for other members of the GH/cytokine/hematopoietic receptor superfamily, since ligands for at least three other members of this receptor family, interleukin 2 (IL-2), IL-3, and erythropoietin, also stimulate c-*fos* via the SRE in lymphoid cells (30). Recent studies indicate that at least some of these receptor family members, including receptors for GH (31–33), IL-2 (34), and erythropoietin (35), when activated by ligand, stimulate tyrosyl phosphorylation of their receptors and other cellular proteins. In addition, formation of complexes with cellular tyrosine kinases by the GH receptor, the IL-2 receptor, the prolactin receptor, and the erythropoietin receptor has been demonstrated (31–36). The kinase associated with the IL-2 receptor has been identified as $p56^{lck}$ (34). Thus, it is tempting to speculate that tyrosine kinase-mediated events may participate in the mechanism by which GH regulates c-*fos* through the SRE. At least some of the multiple transcription factors that have been shown to associate with the SRE (8), including serum response factor and the ternary complex factor $p62^{TCF}$, are known to be phosphorylated, and kinases such as casein kinase II and microtubule-associated protein kinase (MAP kinase) appear to be involved (37–39). In this regard, it is of interest that GH has been shown to activate MAP kinase activity and to promote tyrosyl phosphorylation of MAP kinases in 3T3-F442A cells (40, 41).

The present findings do not preclude the possibility that other sequences in the c-*fos* promoter also participate in mediating a response to GH. The present data indicate that GH does not induce c-*fos* through the basal sequences between −222 and the transcription start site. The possibility that other sequences are involved is strengthened by preliminary observations that GH induces SIF binding activity in nuclear extracts from 3T3-F442A cells (D.J.M., C. Hoban, B.H.C., and J.S., unpublished observation), suggesting a role for the SIF binding sequence in mediating a GH response. Furthermore, GH induced AP-1 binding activity in nuclear extracts from osteoblasts (4), suggesting involvement of AP-1 sites in response to GH. Whether such sequences participate in induction of c-*fos* by GH, whether they cooperate with each other and, indeed, whether they also contribute to synergism between GH and other growth factors remains to be determined. The mediation of the GH response by the SRE may thus be only one aspect of a more complex regulatory mechanism still to be elucidated.

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