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.

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 (4F4K1), 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'-CAGATGCTCTAATATGGACATCTCT-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'-aattCAGATGCGTTAATACGATCCACATCGT-3' and 5'-agctcCAGATGCGTTAATACGATCCACATCGT-3' were annealed and ligated into the EcoRI and HindIII 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% CO2/95% air. For transfection, NIH 3T3 cells were plated at 10^4 cells per cm^2 on 100-mm plates at 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 pSV1 a-globin DNA (18) to control for transfection.

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 protease 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. 2A. 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. 2B 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. 2C). 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.
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
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 not yet understood. 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 p56lck (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 p62TCF, 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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