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Calcium and Growth Factor Pathways of c-*fos* Transcriptional Activation Require Distinct Upstream Regulatory Sequences

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Transcription of the c-*fos* proto-oncogene is rapidly induced in the rat pheochromocytoma PC12 cell line by a wide variety of stimuli, including polypeptide growth factors, phorbol esters, and calcium ion fluxes. We have mapped the upstream sequence requirements for this activation in PC12 cells by analysis of promoter deletion mutants in a transient expression assay. Two distinct pathways of c-*fos* induction are defined that differ in their requirement for *cis*-acting DNA sequences. Calcium activation of c-*fos* transcription is dependent on a DNA element located approximately 60 base pairs upstream of the transcription start site. This region is highly conserved between human, mouse, and chicken c-*fos* genes and contains a sequence that resembles the consensus for a cyclic AMP response element. The dyad symmetry element at position −300, which is necessary for serum responsiveness of c-*fos*, appears to be unimportant for calcium activation of the gene. The dyad symmetry element is, however, an essential *cis*-acting sequence for c-*fos* inducibility by nerve growth factor, epidermal growth factor, fibroblast growth factor, and the phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate. Studies in vivo and in vitro with various mutants of the dyad symmetry element indicate that c-*fos* activation by polypeptide growth factors and 12-*O*-tetradecanoyl phorbol-13-acetate is mediated by a common transcription factor, and that this factor is identical to the previously described serum response factor. In vitro DNA-binding assays suggest that the quantity of serum response factor-binding activity remains unchanged during c-*fos* transcriptional activation.

Expression of the c-*fos* proto-oncogene is rapidly and transiently activated in a variety of mammalian cell types by a diverse set of stimuli, including serum, specific polypeptide growth factors, phorbol esters, neurotransmitters, and agents that elevate intracellular calcium and cyclic AMP (cAMP) (3, 5, 6, 12, 13, 15, 16, 23, 24, 29, 32–34). c-*fos* induction occurs at the transcriptional level (15) and is not only independent of new protein synthesis but also greatly amplified in the presence of protein synthesis inhibitors (5, 13).

Current evidence suggests that c-*fos* plays a central role in mediating nuclear responses to signals acting at the cell surface. The exquisite control exercised over the expression of this gene has made it a model system for the study of the molecular mechanisms of gene regulation and signal transduction in eucaryotic cells.

Deletion analysis of the upstream regions of the human c-*fos* (c-*fos*^{H}) gene has defined a sequence, located between nucleotides −332 and −277 relative to the cap site, that appears to be essential for serum inducibility of the gene when transfected into NIH 3T3 cells (42). A 20-base-pair (bp) dyad symmetry element (DSE) within this serum response element (SRE) interacts in a sequence-specific manner with a DNA-binding protein, serum response factor (SRF), found in mammalian cell nuclear extracts (11, 14, 43). A synthetic copy of the DSE restores serum inducibility when inserted into c-*fos* genes lacking the activating element (14, 43). These findings implicate the interaction between the DSE and its cognate protein, SRF, in the activation of c-*fos* transcription by serum. Initial results suggest that SRF binding activity appears to be present at similar levels in serum-starved and serum-stimulated HeLa cells (14, 43). In A431 cells, however, inducible binding to the c-*fos* DSE has been observed with epidermal growth factor (EGF) stimulation, but not with other agents (36). Controversy thus exists concerning the inducibility of SRF binding.

The cytoskeletal actin genes are coinduced with c-*fos* by serum and growth factors (9, 15). A sequence bearing strong homology to the c-*fos* DSE is present in the actin gene promoter, competes specifically for binding to SRF, and appears to mediate serum responsiveness of this gene (14, 30). However, some agents that activate c-*fos*, notably those that require the presence of extracellular calcium such as potassium chloride depolarization of the cell membrane, do not stimulate actin transcription (16). Thus the presence of the DSE per se does not account for the observed complexity of c-*fos* regulation, suggesting that the control of c-*fos* transcription involves more than one *cis*-acting regulatory element.

In this paper we present evidence for the existence of two distinct pathways of activation of c-*fos*^{H} by external stimuli. One pathway is dependent on the DSE and mediates the response to growth factors and phorbol ester. The other pathway mediates transcriptional activation by the second messenger calcium and shows a novel requirement for a promoter sequence located at around position −60 in the c-*fos* gene. Neither mode of activation involves a detectable change in the quantity or the characteristics of binding of the regulatory protein SRF to the c-*fos* DSE.

MATERIALS AND METHODS

Plasmid constructions. pF4, pF222, and pF123 and internal deletion plasmids pF370/124, pF327/124, pF276/124, and pF262/124 were gifts from Richard Treisman and have been described previously (42). pF4 contains a 5.4-kilobase human genomic *Bam*HI fragment, encompassing the entire c-*fos* gene, inserted into the *Bam*HI site of pUC12. In pF222, sequences between the pUC12 *Eco*RI site and the c-*fos* *Apa*I

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site at position −222 are deleted and the *Eco*RI site has been regained by end repair (42). pF222DSE contains a 29-mer synthetic oligonucleotide spanning the c-*fos* DSE inserted into the *Eco*RI site of pF222 (14). pF123 $(DSE)_2$ contains two copies of the DSE oligonucleotide inserted into the *Eco*RI site of pF123. Deletion plasmids pF178, pF156, pF145, pF139, pF65, pF57, pF52, and pF42 were constructed as follows. Parent plasmids pF222 and pF123 were linearized with *Eco*RI and digested with exonuclease III and exonuclease VII for various times. The ends were made blunt with T4 DNA polymerase and ligated to octamer *Eco*RI linkers. Subsequent digestion of the mixture with *Eco*RI and *Hin*dIII released fragments containing the entire c-*fos* gene with various lengths of upstream sequence; the fragments were gel isolated and cloned into the vector pUC19, opened with *Eco*RI and *Hin*dIII in its polylinker. These constructs contain the c-*fos* gene in the opposite orientation to that found in the parent plasmids. Deletion endpoints were determined by DNA sequencing.

The control alpha-globin plasmid pSV-alpha-1 was a gift from Ann-Bin Shyu and was derived as follows. The *Bgl*I-*Xba*I fragment from plasmid *pi*-SVHP-alpha-2 (42), containing the human alpha-globin gene driven by the simian virus 40 early promoter and enhancer, was subcloned into vector pT7/T3-18 (Bethesda Research Laboratories, Inc.) opened with *Pst*I and *Xba*I, the *Bgl*I and *Pst*I ends having been filled in with Klenow DNA polymerase.

Cell culture. PC12 cells were grown in 10% CO_2 in Dulbecco modified Eagle medium containing 10% heat-inactivated horse serum, 5% fetal bovine serum, 0.01% penicillin, and 0.01% streptomycin. All sera were purchased from Sigma Chemical Co.

Transient expression assay. One day before transfection, PC12 cells were passaged onto 100-mm tissue culture dishes freshly coated with 1 mg of poly-L-lysine per ml at a density of 4×10^6 to 5×10^6 cells per plate. Then 20 μg of the test plasmid and 5 μg of the internal control alpha-globin plasmid (pSV-alpha-1) were transfected by the calcium phosphate technique described previously (42) with the following modifications. Precipitate was added to the cells at room temperature and left for 20 min with gentle shaking, followed by the addition of Dulbecco modified Eagle medium containing 5% fetal bovine serum. After 5 h of incubation in 5% CO_2, the cells were shocked with a solution of 25% glycerol in HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline (pH 7.0) for 2 min and washed with phosphate-buffered saline (pH 7.4). The cells were then incubated in complete growth medium (see above) in 10% CO_2 until stimulation and RNA isolation.

At 40 to 48 h after transfection, the cells were stimulated as described in the figure legends with EGF (3 ng/ml), nerve growth factor (NGF) (50 ng/ml), fibroblast growth factor (FGF) (50 ng/ml), 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (0.3 μg/ml), KCl (32 mM), $BaCl_2$ (1.0 mM), or ionophore A23187 (10 μg/ml). Concentrations in parentheses refer to the final concentration of the agent in conditioned growth medium. Where indicated in the figure legends, anisomycin was added 30 min before the stimulating agent to a final concentration of 100 μmol/liter. NGF was purchased from the Research Foundation (Albany, N.Y.), EGF and FGF were from Collaborative Research (Lexington, Mass.), and v-*sis* platelet-derived growth factor (PDGF) was from Amgen Biological (Thousand Oaks, Calif.). KCl, $BaCl_2$, forskolin, and TPA were purchased from Sigma Chemical Co., and ionophore A23187 was from Boehringer Mannheim Biochemicals.

Isolation and analysis of RNA. Total cytoplasmic RNA was prepared (15, 16) and RNase protection was performed (14, 42) as previously described. PC12 cytoplasmic RNA (30 μg) was hybridized to the c-*fos* and alpha-globin antisense probe in each reaction. RNase-resistant fragments were separated on 6% polyacrylamide–8 M urea gels and visualized by autoradiography.

Nuclear run-on transcription assay. Experimental procedures for the in vitro nuclear run-on transcription assay and sources of cloned plasmid DNAs used as hybridization probes for *fos*, *raf*, and beta-actin have been described in detail previously (12, 15).

DNA mobility shift assay. Whole cell extracts for the in vitro DNA-binding assay were prepared by a rapid procedure described previously (45), with the following modifications. PC12 cells were grown to approximately 70% confluence in 100-mm dishes, stimulated as described above for the transient expression assay, and scraped into 2 ml of phosphate-buffered saline before pelleting in 1.5-ml microcentrifuge tubes. The protein concentrations of the extracts were determined by the Bio-Rad protein assay (Bio-Rad Laboratories). In some experiments (see text), protease and phosphatase inhibitors were added to the 0.4 M salt buffer before cell lysis. The protease inhibitors have been described previously (44). Phosphatase inhibitors used were 0.1 mM sodium vanadate, 0.1 mM ammonium molybdate, and 50 mM sodium fluoride, all purchased from Aldrich Chemical Co.

The synthetic double-stranded oligonucleotide probe, corresponding to the sequence of the c-*fos* DSE with 5′ *Eco*RI overhangs, has been previously described (14). The probe was labeled with ^{32}P-labeled gamma-ATP and polynucleotide kinase, and a specific activity of 10^6 cpm/ng of DNA was typically obtained. Binding reactions were performed in a total volume of 22 μl containing 55 mM NaCl, 6 mM HEPES (pH 7.9), 10% glycerol, 0.06 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 0.3 mM dithiothreitol, 1 mM $MgCl_2$, 5 mM spermidine, 1 μg of sonicated pUC19 DNA, 5 μg of *Escherichia coli* tRNA, and 5 μg of pdN_6 (Pharmacia Fine Chemicals) as nonspecific carrier DNA. Each binding reaction contained 0.1 ng of labeled probe and 15 to 20 μg of extract protein. Complexed and free probes were separated on nondenaturing 4% polyacrylamide gels as previously described (14) and visualized by autoradiography.

RESULTS

Novel pathway of c-*fos* induction independent of the DSE. Expression of the c-*fos* gene in the rat pheochromocytoma PC12 cell line is induced by numerous agents, including NGF, EGF, FGF, TPA, calcium ionophore A23187, elevated extracellular KCl concentration, $BaCl_2$, cAMP, and forskolin. These inducers act initially on different cellular targets, but the mechanisms by which their signals are subsequently transduced to the c-*fos* gene in the nucleus are unclear. As a first step toward understanding these processes we have determined the upstream sequences of the c-*fos* gene required for its activation by these agents. Our results indicate that inducers fall into two groups that utilize distinct pathways to activate c-*fos* transcription.

To determine the promoter requirements for c-*fos* induction, 5′ deletion mutants of the human c-*fos* gene were transfected into PC12 cells and stimulated with one of a variety of agents, and c-*fos* mRNA was assayed by RNase protection with a labeled anti-sense RNA probe complemen-

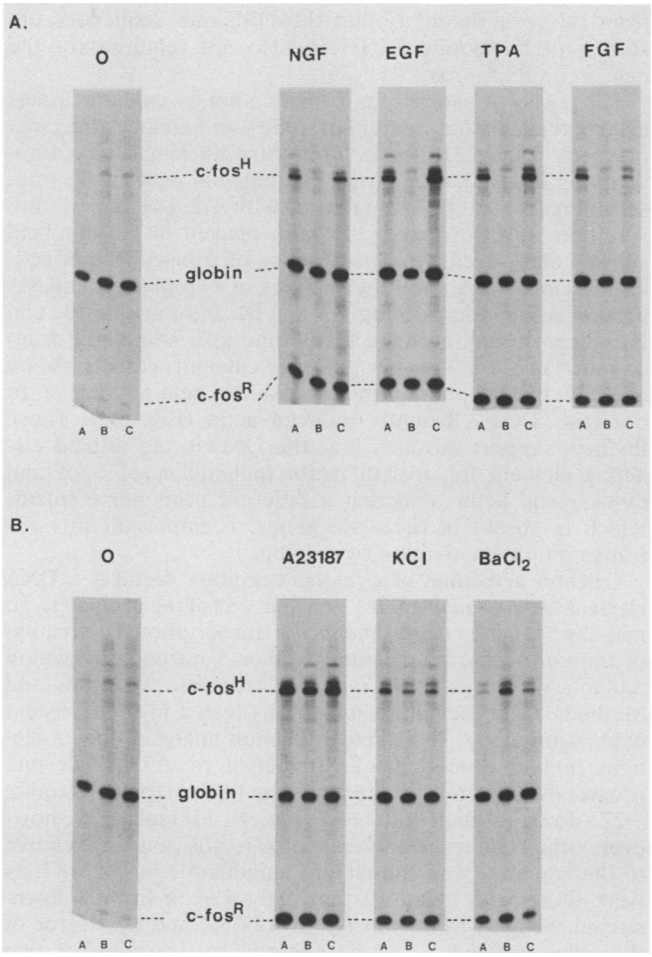

FIG. 1. Analysis of DSE requirement for c-*fos* induction by growth factors, TPA, and calcium agonists. PC12 cells were transfected with plasmid pF4 (lane A), pF222 (lanes B), or pF222DSE (lanes C) and the internal control plasmid pSV-alpha-1. (A) Cells were left unstimulated (O) or were stimulated for 30 min with NGF, EGF, TPA, or FGF as described in Materials and Methods. (B) Cells were left unstimulated (O) or were stimulated for 60 min with A23187, potassium chloride, or barium chloride as described in Materials and Methods. Cytoplasmic RNA was prepared and quantitated for c-*fos* and alpha-globin mRNA by using the RNase protection assay described in Materials and Methods. Probe fragments specifically protected by transcripts from the transfected human gene (c-*fos*^{H}), the endogenous rat gene (c-*fos*^{R}), and the internal control alpha-globin gene (globin), were separated by gel electrophoresis and visualized by autoradiography.

tary to, and spanning the initiation site of, the human gene. The correctly initiated and spliced exogenous gene transcript protects a 296-nucleotide fragment of the probe, whereas the endogenous c-*fos* mRNA protects only a 65-nucleotide fragment, as a result of sequence nonidentity between the human and rat genes (Fig. 1 and data not shown). The level of c-*fos* mRNA transcribed from both the transfected human gene and the endogenous rat gene was very low in unstimulated, asynchronously growing PC12 cells (Fig. 1). The endogenous signal (c-*fos*^{R}) was markedly elevated (at least 30-fold by densitometry) upon treatment with NGF, EGF, FGF, TPA, A23187, KCl, and $BaCl_2$. All of these agents also caused activation of the wild-type human c-*fos* gene on plasmid pF4, which carries 750 bp of upstream sequence (Fig. 1, lanes A). Plasmid pF222, which carries a c-*fos*^{H} gene with a 5′ deletion to nucleotide −222 relative to the cap site, was no longer activated by the growth factors NGF, EGF, and FGF or the phorbol ester TPA (Fig. 1A, lanes B). This is not because of inadequate stimulation of the cells by these agents, since the endogenous rat c-*fos* gene was strongly induced. Furthermore, the transfection efficiency in these experiments appeared to be relatively constant; the expression of the cotransfected control plasmid, which carries the alpha-globin gene driven by the simian virus 40 enhancer, remained unchanged. These results suggest that sequences 5′ of position −222 are important for responsiveness to growth factors and TPA.

The DSE at nucleotide −300 is essential for serum responsiveness of the c-*fos* gene (14, 42). In addition, a region at around nucleotide −340, just upstream of the DSE, binds a factor in vitro that is induced in fibroblast cells after treatment with *sis*-conditioned medium (18). A sequence immediately downstream of the DSE closely resembles the consensus sequence for the binding site of AP1, a transcription factor implicated in the TPA responsiveness of several cellular and viral genes (1, 26). These sequences are schematically diagrammed in Fig. 8. To determine whether, among these DNA elements, the DSE is the one critical for growth factor and TPA inducibility of c-*fos*, we tested the ability of agents to stimulate plasmid pF222DSE. This plasmid was identical to pF222, except that a synthetic oligonucleotide copy of the DSE was inserted at position −222. pF222DSE, in contrast to pF222, was fully inducible by EGF, NGF, FGF, and TPA (Fig. 1A, lanes C). Similarly, plasmid pF123, which was deleted to nucleotide −123 relative to the transcription start site and was unresponsive to growth factors and TPA, regained growth factor and TPA inducibility when the DSE was inserted at nucleotide −123 (data not shown). Internal deletions of the c-*fos* gene between nucleotides −124 and −262 and between nucleotides −124 and −276, which left intact the DSE, also did not impair NGF inducibility (Fig. 2). In contrast, internal deletion plasmids pF327/124 and pF370/124 were totally unresponsive to NGF. Both of these internal deletions remove the DSE, but pF327/124 leaves intact the site of inducible binding reported by Hayes et al. (18). Thus the DSE appears to be the critical upstream regulatory sequence, whereas the other two elements are either redundant or play no role in normal c-*fos* regulation. Sequences between the DSE and nucleotide −124 are also not essential. As in the case of serum, therefore, growth factors and TPA activate c-*fos* expression via a mechanism dependent on the presence of the DSE in *cis*.

In contrast to the polypeptide growth factors and TPA, calcium agonists stimulate c-*fos* expression in a DSE-independent fashion. Depolarization of the PC12 cell membrane by elevated KCl results in c-*fos* transcriptional activation that is dependent on the presence of extracellular calcium and the functioning of voltage-dependent membrane calcium channels (16). $BaCl_2$ also appears to act through the voltage-dependent calcium channels and may mimic calcium directly (7). A23187 is an ionophore which permeabilizes the cell membrane to calcium influx. These three different agents that activate the calcium second messenger pathway stimulated c-*fos* expression to a similar extent for pF4, pF222, and pF222DSE (Fig. 1B). The slight variation in inducibility between these plasmids in Fig. 1B was not consistently seen in repeated experiments. Thus the transient transfection assays indicate that activation by KCl is independent of the DSE, as is activation by $BaCl_2$ and calcium ionophore A23187. Pertinent to this conclusion is the observation that

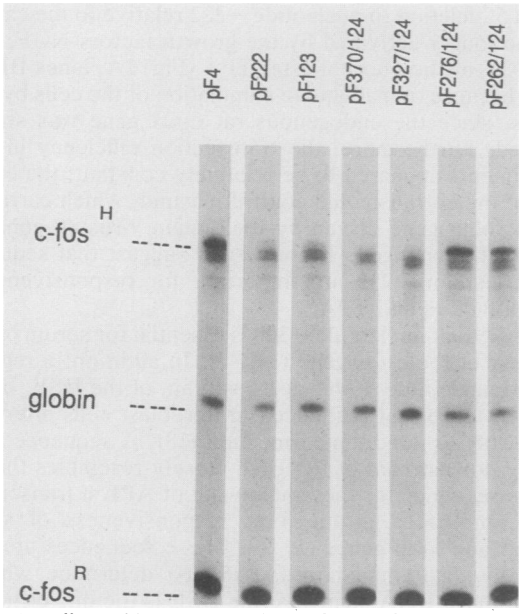

FIG. 2. Effect of internal deletions of the c-*fos* upstream region on inducibility by NGF. PC12 cells were transfected with the internal control plasmid pSV-alpha-1 and deletion mutants of the c-*fos* gene as indicated and stimulated with NGF for 30 min. Levels of cytoplasmic mRNA transcribed from the transfected human c-*fos* gene (c-fos^H), the endogenous rat gene (c-fos^R), and the control alpha-globin gene (globin) were assayed as described in the legend to Fig. 1.

calcium agonists do not stimulate transcription of the actin gene (16) (see below), a gene that contains the DSE and is responsive to growth factors. This group of agents therefore appears to induce c-*fos* mRNA by a novel pathway that is distinct from that utilized by the growth factors and TPA. Preliminary results also indicate that forskolin, an activator of adenylate cyclase, stimulates c-*fos* transcription without a requirement for the DSE (data not shown).

Calcium activates c-*fos* expression at the transcriptional level. The finding that calcium signals can activate c-*fos* expression independently of the presence of the DSE suggests that a distinct calcium regulatory DNA element lying downstream of nucleotide −222 is involved in the transduction pathway. Two alternative explanations, however, required exclusion. First, calcium may cause stabilization of the labile c-*fos* message, thus raising the basal level of c-*fos* cytoplasmic mRNA. Second, the kinetics of c-*fos* activation by calcium may differ from those resulting from growth factor stimulation, and a DSE dependence of this activation may be missed by the analysis of a single time point. To address these questions we transfected PC12 cells with the same plasmids used in Fig. 1 and stimulated them with A23187 over a prolonged time course (Fig. 3A). c-*fos* induction by calcium was rapid and transient for both endogenous and exogenous messages. c-*fos* mRNA was elevated by 30 min, peaked at 60 to 120 min, and subsequently decayed despite the continued presence of A23187. The kinetics of activation were slower than those observed with serum and growth factors, for which peak mRNA accumulation occurs at 30 to 60 min (12, 15, 16) (data not shown). The calcium activation time course profiles were identical for the three plasmids pF4, pF222, and pF222DSE, confirming that the DSE and other sequences upstream of nucleotide −222 are unimportant in the calcium response. A further conclusion from this experiment is that the DSE and sequences upstream of nucleotide −222 are also not required for the deactivation of c-*fos*.

The transient nature of c-*fos* activation by calcium argues against the idea that calcium in some way stabilizes the c-*fos* message. Figure 3B shows an in vitro nuclear run-on transcription assay that confirms that calcium acts at the transcriptional level. On stimulation with A23187, c-*fos* transcription was elevated by 15 min, peaked at 60 min, and subsequently declined. The kinetics of transcriptional activation closely matched the kinetics of cytoplasmic mRNA accumulation (compare Fig. 3A and B). Barium chloride and KCl depolarization of the membrane also stimulated transcription of c-*fos* (Fig. 3C). All three calcium agonists had no effect on transcription of the cytoskeletal beta-actin gene. In contrast, NGF efficiently induced actin (Fig. 3C). These findings support the idea that the DSE is the critical *cis*-acting element for growth factor inducibility of c-*fos* and cytoskeletal actins and that a different promoter element, which is absent in the actin genes, is important in c-*fos* transcriptional activation by calcium.

Calcium activation of c-*fos* transcription requires a DNA element between nucleotides −65 and −57 of the promoter. To map the sequence requirements for transcriptional activation of the c-*fos* gene by calcium, further 5′ promoter deletion mutants of the gene were constructed (see Materials and Methods), and their inducibility was tested in the transient expression assay. In a typical deletion analysis, the exogenous human gene remained inducible by A23187 despite progressive deletions of the upstream region from nucleotide −222 down to nucleotide −65 (Fig. 4). Mutant pF57, however, which had its promoter deleted to position −57 relative to the cap site, was completely uninducible by A23187, as were those with deletions that extend even further downstream. pF123 was responsive to A23187, and the degree of this responsiveness was not altered by insertion of two copies of the DSE at the 5′ deletion endpoint [pF123$(DSE)_2$]. Deletion analysis with KCl depolarization or $BaCl_2$ as an alternative stimulating agent of the calcium pathway gave identical results, although the overall magnitude of c-*fos* induction by these agents was slightly lower than that with A23187 (data not shown). With all three agents, inducibility was retained despite progressive deletions to nucleotide −65 but was completely abolished with a further truncation to nucleotide −57. Furthermore, there was no c-*fos* transcription from any of these plasmids in unstimulated PC12 cells (data not shown). These findings define an upstream element in the c-*fos* gene essential for calcium activation, the 5′ border of which lies between coordinates −65 and −57. This region contains a sequence closely related to the consensus sequence for the cAMP response element (31). More rigorous analysis is required to define the calcium response element and its relationship, if any, to this putative cAMP regulatory sequence.

In some experiments, the wild-type plasmid pF4, which carries 750 bp of upstream sequence, appeared to be activated by A23187 to a greater degree than pF222 and plasmids carrying deeper deletions, raising the possibility that additional sequences upstream of nucleotide −222 may play a minor role in calcium responsiveness. This sequence is unlikely to be the DSE, since inserting the DSE at position −222 or −123 did not enhance A23187 inducibility (Fig. 1B, 3, and 4). The reproducibility and significance of this observation are under further investigation.

In a recent study with A431 and HeLa cells (10), a c-*fos*-CAT fusion gene carrying 2,000 bp of c-*fos* upstream

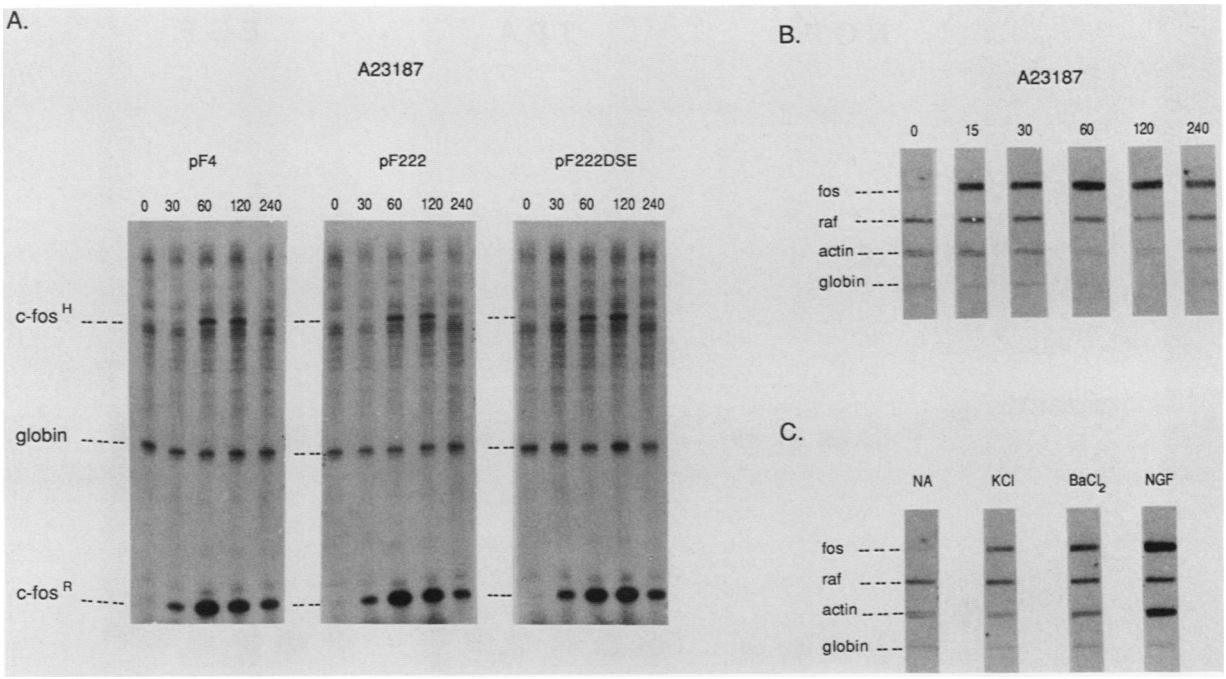

FIG. 3. Kinetics of c-*fos* induction by A23187. (A) Kinetics of mRNA accumulation. PC12 cells were transfected as indicated with plasmid pF4, pF222, or pF222DSE and the internal control plasmid pSV-alpha-1. Cells were then stimulated with A23187 for the indicated times (in minutes). Levels of mRNA transcribed from the transfected human gene (c-*fos*^{H}), the endogenous rat gene (c-*fos*^{R}), and the alpha-globin gene (globin) were assayed as described in the legend to Fig. 1. (B) Kinetics of transcriptional activation. Untransfected PC12 cells were stimulated with A23187 for the indicated amounts of time (in minutes). (C) c-*fos* transcriptional activation by potassium chloride, barium chloride, and NGF. PC12 cells were stimulated for 30 min by KCl, $BaCl_2$, or NGF as indicated (NA, no addition). Nuclei were isolated, and nascent RNA transcripts were labeled in an in vitro nuclear run-on transcription assay. Labeled nuclear RNA was hybridized to specific *fos*, *raf*, alpha-globin, and beta-actin DNA probes immobilized on nitrocellulose, and radioactivity was visualized by autoradiography as described previously (12, 15).

sequence showed no activation after 30 min of A23187 treatment in a transient transfection assay. However, our time course experiments (Fig. 3) indicated that the c-*fos* mRNA response to A23187 was only just beginning at 30 min. Furthermore, the relatively high basal level of CAT gene mRNA suggests that the reporter CAT gene transcript is more stable than the c-*fos* message, and the transient stimulation of transcription may be difficult to detect against this background. The possibility of a cell-type difference in mechanism of calcium regulation also cannot be excluded.

Growth factor and TPA activation of c-*fos* transcription through a common final pathway. Although it was not essential for calcium activation of c-*fos*, the presence of the DSE was absolutely required for c-*fos* induction by NGF, EGF, FGF, and TPA in PC12 cells. These agents are known to act initially on different cellular targets: specific cell surface receptors for growth factors and protein kinase C for TPA. It is of interest therefore to know at what level these signals converge along the transduction pathways leading to c-*fos* transcriptional induction. Specifically, we examined whether a common transcription factor mediates activation by these different agents, or whether multiple different nuclear factors that recognize the DSE are involved.

It has been shown previously that there is a direct correlation between the ability of mutant DSEs to confer serum responsiveness in vivo and their ability to bind a specific HeLa nuclear factor SRF in vitro (14). If SRF also mediates the response to polypeptide growth factors and TPA in PC12 cells, an identical pattern and correlation of mutant inducibility in vivo and SRF binding in vitro would be predicted.

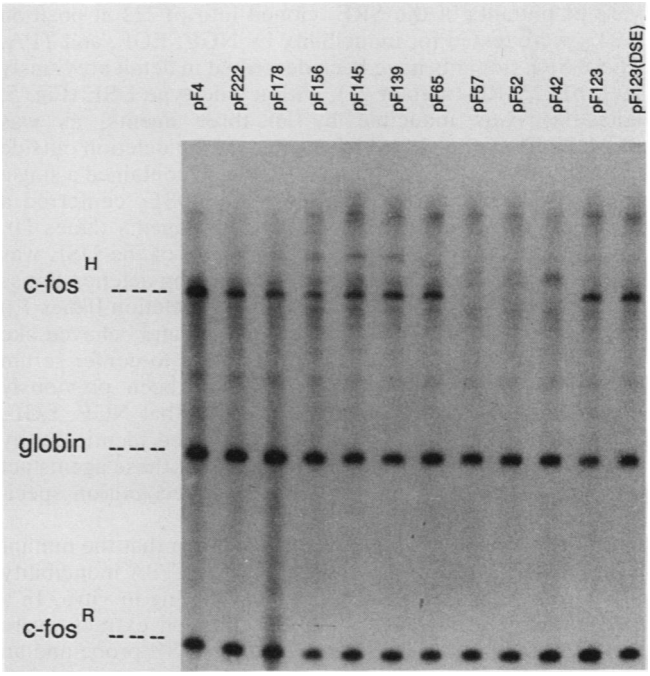

FIG. 4. Analysis of upstream sequence requirements for calcium activation of c-*fos*. PC12 cells were transfected with the internal control plasmid pSV-alpha-1 and the wild-type human c-*fos* plasmid pF4 or 5′ deletion mutants pF222, pF178, pF156, pF145, pF139, pF65, pF57, pF52, pF42, pF123, or pF123(DSE)$_2$, as indicated. Cells were stimulated with A23187 for 60 min, and c-*fos* and alpha-globin mRNA was assayed as described in the legend to Fig. 1.

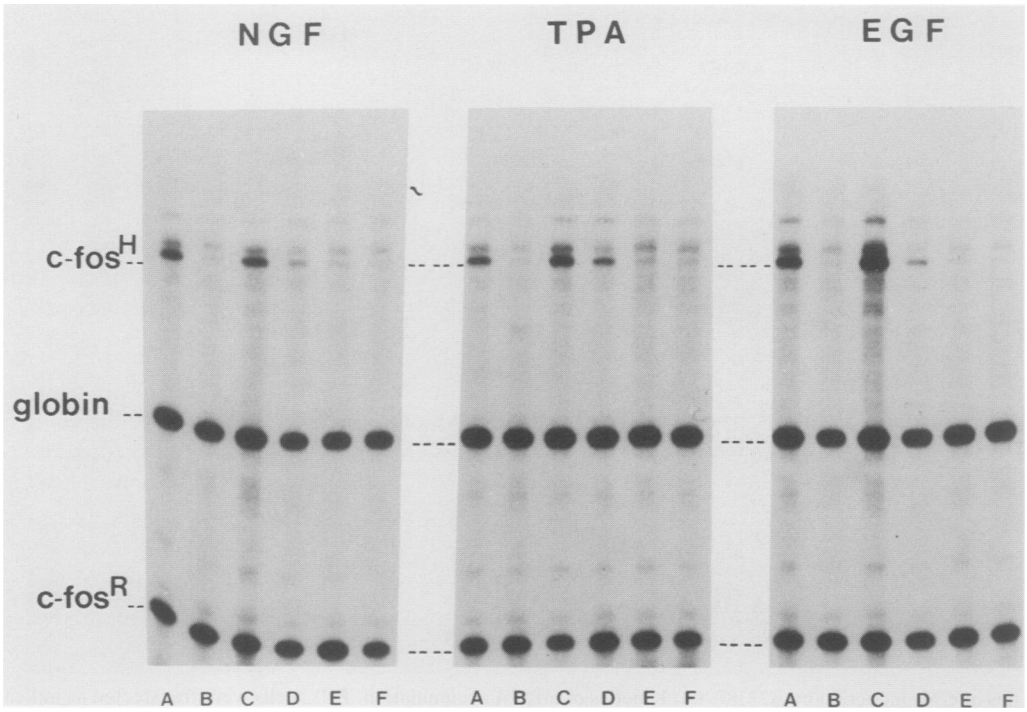

FIG. 5. Effect of DSE mutations on c-*fos* inducibility by growth factors and TPA. PC12 cells were transfected with internal control plasmid pSV-alpha-1 and one of the following plasmids (lanes): A, pF222 (SRE mutant 21), carrying the wild-type DSE; B, pF222, lacking the DSE; C, pF222 (SRE mutant 3); D, pF222 (SRE mutant 9); E, pF222 (SRE mutant 14); and F, pF222 (SRE mutant 23). The SRE mutants are described in the text, and their construction has been detailed previously (16). Cells were stimulated for 30 min with NGF, EGF, or TPA as indicated, and c-*fos* and globin mRNA were assayed as described in the legend to Fig. 1.

Figure 5 shows a transient transfection assay in which various mutants of the SRE, cloned into pF222 at position −222, were tested for inducibility by NGF, EGF, and TPA. These SRE mutants have been described in detail previously (14). pF222SRE (mutant 21), with a wild-type DSE (Fig. 5, lanes A), was inducible by all three agents, as was pF222SRE (mutant 3), which carried a 3-bp deletion outside the DSE (lanes C). SRE (mutant 9), which contained a single base pair addition in both arms of the DSE, conferred a lesser degree of inducibility by all three agents (lanes D). Two SRE mutants in which the right arm of the DSE was disrupted (mutant 14, which contained a 4-bp deletion [lanes E], and mutant 23, which carried a 1-bp deletion [lanes F]) were not activated by NGF, EGF, or TPA and behaved like pF222 (lanes B). The same order of ability to confer serum inducibility by these mutant SREs has been previously observed in NIH 3T3 cells (14). The fact that NGF, EGF, TPA, and serum inducibility are all affected identically by each particular DSE mutation suggests that these agents act via a common transcription factor with recognition specificity for this sequence.

In further support of this, we have shown that the mutant SREs that fail to confer growth factor and TPA inducibility in vivo also have no affinity for SRF binding in vitro. In a DNA mobility shift assay, PC12 whole cell extracts were incubated with a labeled oligonucleotide DSE probe and an unlabeled competitor plasmid, and the complexed and free forms of the probe were separated by nondenaturing polyacrylamide gel electrophoresis (Fig. 6). The specific complex (labeled C) ran with the same mobility as that shown by a complex of the DSE probe with SRF from HeLa cells (data not shown). pF222 in 50-fold excess did not compete with the specific complex, since plasmid pF222 lacks the DSE. However, pF222 carrying SRE (mutant 21) or SRE (mutant 3), which are efficiently inducible in vivo, but not SRE (mutant 23) or SRE (mutant 14), both of which are uninducible, did compete. SRE (mutant 9) showed an intermediate degree of competitiveness for SRF binding, consistent with its weak ability to confer growth factor and TPA inducibility in vivo. Our analysis of DSE mutants in vitro and in vivo suggests that the same nuclear factor, SRF, mediates the activation of c-*fos* transcription by growth factors and TPA in PC12 cells.

c-*fos* transcriptional activation not accompanied by changes in SRF-binding activity. One model for transcriptional induction of a gene involves the activation of the DNA-binding capacity of a positive transcription regulatory factor. The validity of such a model with regard to c-*fos* regulation is controversial. We measured DSE-binding activity in PC12 cells, before and after stimulation of c-*fos* transcription by six different agents, and found no changes in any case. A DNA mobility shift assay was employed, with a labeled synthetic 29-mer oligonucleotide probe containing the DSE sequence and whole cell extracts prepared by a rapid lysis procedure (45) (see Materials and Methods). This method for rapid extract preparation has been employed to address the possibility that a lack of inducibility reflects alterations of the binding protein that take place during cell lysis or extract preparation. This extraction procedure has been successfully used to detect induction of heat shock transcription factor DNA-binding activity within minutes of heat shock (45). Unstimulated PC12 cells contained readily detectable levels of SRF-binding activity, and there was no significant change after stimulation by NGF, FGF, or EGF (Fig. 6B). Not surprisingly, there was also no change after stimulation by KCl depolarization, barium chloride, or forskolin, agents

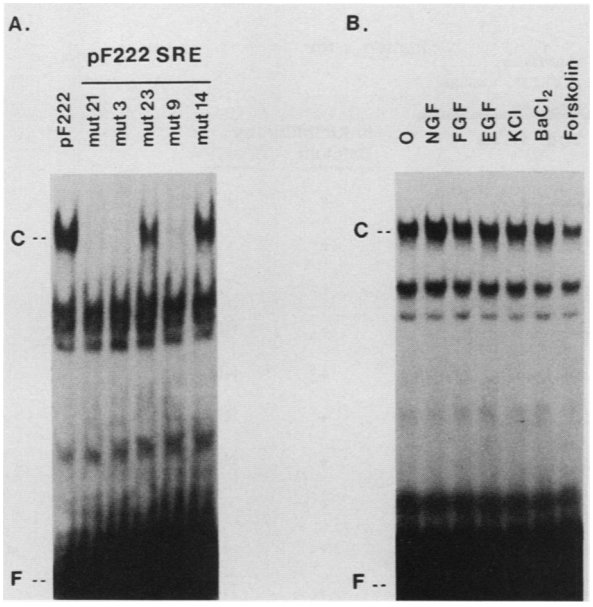

FIG. 6. (A) DSE mutants which fail to confer growth factor inducibility do not compete for SRF binding. Whole cell extracts were prepared from unstimulated PC12 cells and incubated with a ^{32}P-labeled DSE oligonucleotide probe in an in vitro DNA mobility shift assay as described in Materials and Methods. Unlabeled competitor plasmids, pF222 (SRE mutants 21, 3, 23, 9, or 14) or pF222, were added to the binding reaction as indicated in a 50-fold molar excess over probe. The complex (C) representing specific binding to the DSE probe was separated from the free probe (F) by gel electrophoresis. Radioactivity was visualized by autoradiography. (B) c-*fos* activation is not accompanied by an increase in DSE-binding activity. PC12 cells were left unstimulated (O) or were stimulated for 30 min with NGF, FGF, or EGF or for 60 min with potassium chloride, barium chloride, or forskolin as indicated. Whole cell extracts were prepared and DSE-binding activity was assayed as in panel A.

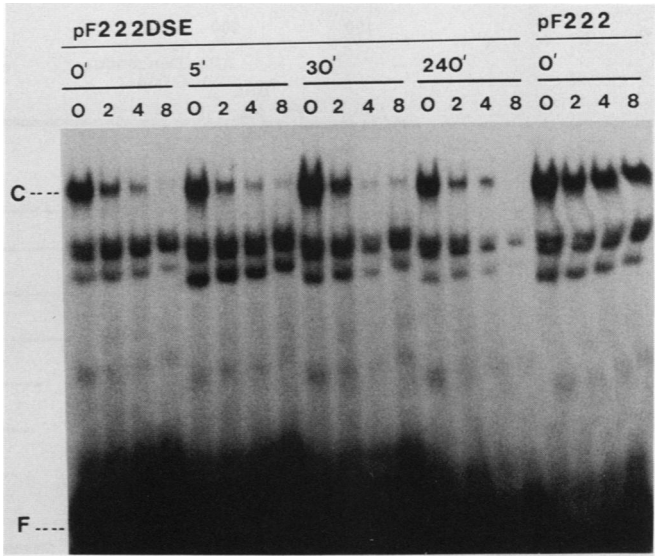

FIG. 7. Off-rate analysis of SRF-DSE binding during the course of NGF stimulation. Whole cell extracts were prepared from PC12 cells which were unstimulated (O'), or stimulated with NGF for 5, 30, or 240 min as indicated. After incubation with the labeled DSE probe in the standard binding reaction, unlabeled competitor plasmid pF222DSE or pF222 was added as indicated in 50-fold molar excess. Samples of the binding reaction were removed immediately before addition of the competitor (O) and at 2, 4, and 8 min after addition of the competitor. The level of specific binding to the probe remaining at these times was quantitated by the DNA gel mobility shift assay as described in the legend to Fig. 6.

which act independently of the DSE. RNase protection analysis of c-*fos* mRNA, performed in parallel, verified that in these experiments the expected stimulation of c-*fos* transcription occurred (data not shown).

To address the possibility that transient changes in SRF-binding activity may be missed when assaying a single time point, its level was measured over a full time course of stimulation from 0 to 240 min with several c-*fos*-inducing agents. No significant change in DSE-binding activity was seen during 240 min of stimulation of PC12 cells with NGF, EGF, TPA, or KCl, irrespective of whether the extracts were prepared in the presence or absence of protease and phosphatase inhibitors (data not shown; see Materials and Methods). Protein synthesis inhibitors are well known to magnify c-*fos* induction, at least partly at the transcriptional level (13). However, the quantity of SRF-binding activity in PC12 cells did not change during the course of NGF stimulation, even when c-*fos* transcription was superinduced in the presence of anisomycin, a stringent inhibitor of protein synthesis. A previous study detected inducible binding to the DSE after EGF treatment of A431 cells (36). We have repeated this experiment on A431 cells over an extended time course by using the procedure for rapid extract preparation (45). Under these conditions a constitutively high level of SRF-binding activity was observed, whether or not the extracts were prepared in the presence of phosphatase and protease inhibitors (data not shown).

The constitutive expression of SRF-binding activity suggests that gene induction does not occur by activation of the ability of SRF to bind to the c-*fos* DSE. We therefore investigated whether more subtle changes in the binding of SRF to the DSE occur as a result of growth factor stimulation of c-*fos*. An off-rate analysis was used to determine whether the affinity of DSE-SRF binding is altered by NGF stimulation (Fig. 7). PC12 cell extracts were prepared before and at various times after NGF treatment and incubated with the DSE probe. After equilibration of binding, a cold competitor, pF222DSE, was added in 50-fold molar excess over the probe. Samples of the combined binding reaction were then taken at 2-min intervals. The rate of decay of the level of the specific labeled complex thus gives a measure of the off rate and the affinity of SRF-DSE binding. The half-life of binding to the DSE probe was not detectably different in extracts derived from unstimulated PC12 cells and from cells stimulated with NGF for 5, 30, or 240 min (half-life was approximately 1 min) (Fig. 7). The addition of pF222, which did not contain the DSE, failed to compete for binding to the labeled probe. The results of this experiment indicate that if a posttranslational modification of SRF does occur after growth factor stimulation, it does so without significantly altering the affinity of its binding to the DSE.

The rate of migration of a DNA-protein complex in a DNA gel mobility assay may be affected by a change in the size (19) or by a posttranslational modification (40) of the protein component. We asked whether SRF is altered in such a way by looking for a change in the mobility of the SRF-DSE complex with NGF stimulation. When extracts from PC12 cells, stimulated for various periods of time with NGF, were bound to DSE probe and electrophoresed for 6 h, no alteration in the mobility of the specific complex was ob-

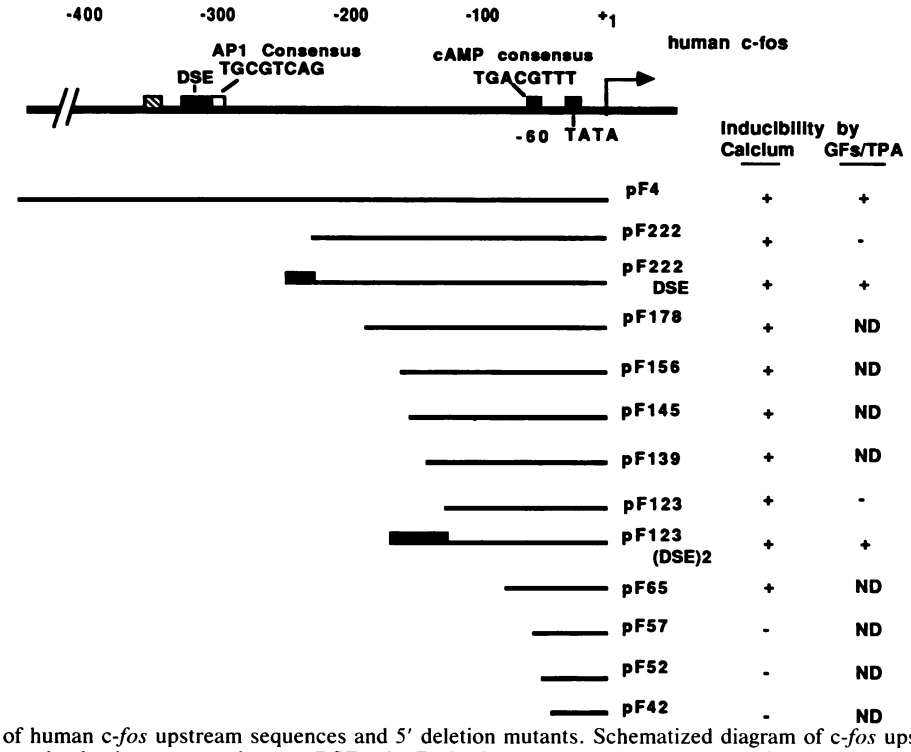

FIG. 8. Summary of human c-*fos* upstream sequences and 5′ deletion mutants. Schematized diagram of c-*fos* upstream sequences: the regions corresponding to the dyad symmetry element (DSE), the TATA box (TATA), the sequences with homology to the AP1-binding site, and the cAMP response element, are shown as filled boxes. The hatched box upstream of the DSE represents the in vitro DNA-binding site of a factor inducible by *sis*-conditioned medium (18) (see text). The arrow represents the transcription start site. The 5′ deletion mutants of the c-*fos* gene used in this study are shown in scale. In pF222DSE and pF123(DSE)$_2$, the DSEs are represented as filled boxes inserted at the 5′ ends of the deletions. The inducibility of these mutants by growth factors (GFs) and TPA and calcium agonists (calcium) is summarized. ND, Not determined experimentally.

served (data not shown). With the DNA gel mobility assay we were unable to detect any change in SRF activity, either quantitative or qualitative, during the course of c-*fos* transcriptional induction.

DISCUSSION

Novel sequence requirement for transcriptional activation by calcium. The calcium cation is an important intracellular second messenger, especially in excitable cells such as neurons and myocytes. Classical studies have established that transient fluxes of calcium between intracellular and extracellular compartments are involved in the regulation of such diverse processes as neurotransmitter release (22) and excitation-contraction coupling of myofibrils (41). More recently it has been demonstrated that changes in intracellular calcium can affect gene expression. For instance, depolarization of PC12 cells by elevated extracellular KCl causes c-*fos* induction at the transcriptional level. This is dependent on the presence of extracellular calcium and is abolished by pharmacological blockade of voltage-dependent calcium channels (16, 33). Furthermore, drugs that inhibit calmodulin, a protein that binds calcium and mediates at least some of its intracellular effects, either block or attenuate c-*fos* activation, suggesting that this ligand-receptor interaction may be involved in the signal pathway (33).

We have identified a promoter region approximately 60 bp upstream of the transcription start site of c-*fos* that is essential for transcriptional induction of the gene by calcium agonists (Fig. 8). The activation by ionophore A23187, KCl depolarization, and $BaCl_2$ is blocked by adding the calcium-chelating agent EGTA to the culture medium (M. E. Greenberg, M. Sheng, and S. Dougan, unpublished results). The region required for calcium responsiveness contains a short DNA sequence that is conserved between mouse, human, and chicken c-*fos* genes; it also contains an in vitro binding site for a protein found in crude nuclear extracts of mammalian cells (10, 11). These observations lend support to the idea that a calcium-responsive DNA element exists at this position.

Regulation of transcription by calcium ionophore has been previously described for a set of genes that is also markedly induced by glucose starvation. A fragment from the upstream region of one of these genes confers A23187 inducibility to heterologous fusion genes (28, 38). This calcium-responsive element, however, differs in several respects from that present in the c-*fos* promoter. First, transcriptional activation peaks at 3 to 4 h after A23187 treatment and is sustained (38), whereas the c-*fos* response peaks at approximately 60 min and is transient. Second, A23187 stimulation of the glucose-responsive genes is blocked by protein synthesis inhibitors, whereas c-*fos* induction is independent of new translation. These findings are consistent with a model in which calcium flux initiates a hierarchial cascade of gene regulatory events, with the activation of c-*fos* playing an early and central role. Subsequently, the product of the c-*fos* gene may directly or indirectly control the expression of other calcium-responsive genes such as the glucose-responsive family. The fact that calcium can stimulate c-*fos* transcription, promptly and without the need for new protein

synthesis, suggests that the intracellular signal transduction pathway for calcium is acting directly on this gene. These findings earmark c-*fos* as a model system for the study of ion flux and its effects on transcription. Recently, genes which are induced by KCl depolarization with kinetics similar to c-*fos* have been described (25), and it will be of interest to know whether they are subject to the same mechanisms of regulatory control as c-*fos*.

Previous studies with *c-fos*-CAT fusion genes have implicated the nucleotide −60 region in the control of the basal activity of the c-*fos* promoter (10, 11). Our results indicate, however, that this region has an inducible function with respect to calcium activation of c-*fos* transcription. The basal activity of this element may be due to stimulation by low levels of calcium in quiescent cells or may reflect calcium-dependent activation in a small subpopulation of these cells. With CAT gene fusions, the serum-inducible DSE has also been shown to contribute to basal promoter activity in the unstimulated cell (8, 30). Thus the identification of a calcium response element in the nucleotide −60 region would be completely consistent with these earlier findings.

The promoter region required for calcium inducibility of c-*fos* contains a sequence between nucleotides −62 and −54, (5′-TGACGTTT-3′), which bears resemblance to the consensus sequence for the cAMP response element (5′-TGACGTCA-3′) found upstream of several cellular genes (31). Our findings therefore raise the intriguing possibility that this element may mediate c-*fos* response to both calcium and cAMP second messengers, and that other genes regulated by cAMP may also be controlled by calcium. More rigorous definition of the calcium and cAMP response elements of the c-*fos* gene is necessary to address these questions.

SRF is the common target for multiple growth factor signals. We have shown that NGF, EGF, FGF, and TPA activate c-*fos* transcription in PC12 cells in a DSE-dependent fashion. Furthermore, mutational analysis of the DSE both in vivo and in vitro suggests that a common transcription factor, SRF, mediates the action of all these agents. The fact that serum shares this pathway is presumably by virtue of the growth factors it contains. By analogy one might expect that growth factors which are active on other cell types may also induce c-*fos* through the DSE. Indeed, purified v-*sis* protein, the product of the viral oncogene coding for the alpha subunit of PDGF, requires the presence of the DSE to activate c-*fos* in NIH 3T3 cells (M. E. Greenberg, unpublished results), and EGF induction of c-*fos* is DSE dependent in HeLa cells (10). SRF therefore appears to belong to a class of transcription factors, which includes AP2 (21), that are able to mediate the transcriptional response to more than one type of external signal. In addition, SRF is a member of the growing family of transcription factors which seem to transduce the effects of TPA. Currently these include AP1 (1, 21), AP2 (21), AP3 (4), and NF-kappaB (27, 39).

Growth factors act on specific cell surface receptors, whereas TPA acts as an agonist of diacylglycerol to stimulate protein kinase C. Presumably, these initially disparate signals converge at some stage, either at the point of SRF activation or at an earlier stage in the pathway. Although the exact signal transduction mechanism for c-*fos* induction by growth factors or TPA is unclear, there is good reason to consider protein phosphorylation in this regard. EGF and FGF activate receptor-associated tyrosine kinases, and TPA stimulates protein kinase C (for a recent review, see reference 20). Although the NGF receptor appears not to have protein kinase activity, there is evidence that NGF causes the rapid phosphorylation of various cellular proteins (17). The binding of certain growth factors to their receptors also results in increased phosphoinositide turnover and consequent stimulation of protein kinase C by diacylglycerol and the release of intracellular calcium by inositol 1,4,5-triphosphate (for reviews, see references 2 and 35). It is likely therefore that distinct networks of phosphorylation events initiated by different growth factors may overlap at one or several points. With regard to c-*fos* transcriptional activation, we have shown that these signals converge on a common transcription factor, SRF, and its interaction with the DSE. Our in vitro binding experiments suggest that induction is not due to activation of the DNA-binding ability of SRF but leave unanswered the question of whether a modification of the protein occurs upon growth factor stimulation. The recent purification of SRF (37, 44) and the cloning of its gene will undoubtedly facilitate understanding of the molecular action of SRF and the mechanism by which it is activated as a transcription factor during c-*fos* stimulation.

Although the DSE is essential for growth factor induction of c-*fos*, we have not excluded the possibility that sequences further downstream of nucleotide −123 are also required (Fig. 8). In particular, the nucleotide −60 element that is critical for calcium activation may also play a role in growth factor and TPA stimulation. An internal deletion of the nucleotide −60 region that leaves intact the DSE has been shown to impair the response of the gene to EGF and TPA in HeLa cells (10). The function of the nucleotide −60 element in growth factor activation of c-*fos* in PC12 cells remains to be determined.

Two distinct pathways for c-*fos* transcriptional activation. In this paper we have shown that two distinct DNA elements are involved in c-*fos* induction in PC12 cells by growth factors and calcium flux. However, these two pathways differ in more than just their promoter requirements. Growth factors and TPA activate c-*fos* with significantly faster kinetics, and with a higher efficiency, than agonists of the calcium pathway (7, 12, 16; M. Sheng, S. Dougan, and M. E. Greenberg, unpublished results). Furthermore, agents which act via the DSE-dependent pathway cause a greater degree of posttranslational modification of the induced *fos* protein (7). It is tempting to speculate that these two pathways of c-*fos* activation have evolved to control different sets of c-*fos* effector functions. On the one hand, responses to major differentiation and mitogenic stimuli (growth factors and phorbol esters) may be mediated by c-*fos* induction via the DSE-dependent pathway. Second messenger signals like calcium, on the other hand, which are probably more important in end-differentiated cells such as neurons, mediate more subtle cellular responses by modulating c-*fos* activity through the nucleotide −60 element.

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