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Inducible binding of a factor to the c-*fos* regulatory region

(signal transduction)

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ABSTRACT **The c-*fos* gene is rapidly and transiently activated in quiescent BALB/c-3T3 cells in response to serum, platelet-derived growth factor or conditioned medium from v-*sis*-transformed cells. This activation occurs at the level of transcription and in the absence of new protein synthesis. Using a gel electrophoresis DNA-binding assay, we have found a DNA-binding activity in BALB/c-3T3 cells that is induced within 20 min of treatment with conditioned medium from v-*sis*-transformed cells. A DNA methylation interference assay has shown that this factor binds to a sequence approximately 346 base pairs upstream of the transcription initiation site of the human c-*fos* gene. Insulin, epidermal growth factor, and phorbol 12-myristate 13-acetate fail to induce this DNA-binding factor. Protein synthesis inhibitors do not block the induction of this activity. We propose that this factor preexists in an inactive form in quiescent cells and that its binding activity is activated in response to appropriate extracellular inducers.**

Polypeptide growth factors that bind to cell surface receptors can promptly regulate specific gene transcription in the nucleus (1, 2). The induction of gene expression by growth factors is required for the mitogenic response (3, 4). However, the biochemical details of the signaling pathways that lead from the receptor to the activation of specific gene transcription in the nucleus are incomplete. Many oncogenes are derived from cellular genes that encode components of growth factor-regulated signal transduction pathways. Some oncogenes themselves, such as v-*sis*, encode growth factors (5). Other oncogenes, such as v-*erbB* and v-*fms*, appear to encode parts of growth factor receptors (6, 7). The *ras* family of oncogenes encodes proteins that bear striking similarity to the guanine nucleotide-binding proteins that are involved in receptor-mediated signal transduction at the membrane (8). The expression of still other oncogenes, such as c-*myc* and c-*fos*, is regulated by the binding of growth factors to cell surface receptors (9–13). Thus, it is clear that the chronic activation of growth factor-regulated metabolic pathways by oncogenes is an important step in cell transformation. However, it is as yet unclear which of the many immediate intracellular responses to growth factors lead to the activation of transcription.

Within minutes of growth factor addition to cells, changes such as autophosphorylation of the receptor, an increase in phosphatidylinositol turnover, a rise in intracellular pH and Ca^{2+} concentration, and a rearrangement of actin cables can be observed (2). Any or all of these events could transmit signals to nuclear proteins that regulate gene transcription. Alternatively, the receptor, or fragments thereof, could be internalized, migrate to the nucleus, and activate specific genes.

One approach to dissecting the signal transduction pathway is to start at the level of the gene and then attempt to trace the signaling pathway backward, out to the cytoplasm. This approach has several advantages over working from the cytoplasm inward. One is that the starting point, the activation of new gene transcription, is an event that is known to be important for the mitogenic response to growth factors (3, 13). A second advantage is that in retracing the pathway, important target substrates for protein kinases might be identified. Growth factors activate many cellular kinases, each of which has many substrates (2). Identifying the substrates for a given protein kinase and then determining which substrate is critical for nuclear signaling is a formidable task. It should be easier to identify a potential kinase for a given phosphoprotein substrate than vice versa.

To initiate this type of analysis, we have studied the regulation of the cellular *fos* gene. c-*fos* is promptly and transiently induced by platelet-derived growth factor (PDGF) (12–14). As judged by nuclear run-off transcription assays, c-*fos* transcription starts to increase within 5 min of PDGF addition to BALB/c-3T3 cells (13). The rate of transcription peaks in 30 min and returns to basal levels 1–2 hr after stimulation. The induction of c-*fos* transcription by PDGF does not require new protein synthesis. In fact, PDGF superinduces c-*fos* at the transcriptional level in the presence of protein-synthesis inhibitors. The superinduction of c-*fos* is mediated both by a 10-fold increase in the rate of initiation and by a several hour increase in the duration of transcription (15).

The upstream region of c-*fos* contains an element which can function as an inducible enhancer (16–18). Although it is unclear exactly how cellular enhancers function to increase transcription, it appears that the binding of cellular transcription factors to these enhancer sequences is required for enhancer function (19, 20). An inducible enhancer such as that of the c-*fos* gene is likely to have its function controlled by a trans-acting cellular factor whose activity is regulated at the level of DNA binding. Such a trans-acting factor would be an intermediate in the signal transduction pathway from a growth factor receptor to the cell nucleus and might be an important substrate for growth factor-activated protein kinases. Therefore, we have looked for cellular factors present in BALB/c-3T3 cells that can specifically bind to the c-*fos* enhancer region in a regulated manner. In this paper we report the finding of an inducible DNA-binding factor in BALB/c-3T3 cells that binds to a sequence located 346 base pairs (bp) upstream of the human c-*fos* gene.

MATERIALS AND METHODS

Cell Culture and Growth Factors. All experiments were performed on BALB/c-3T3 cells, subclone A31, which were grown in Dulbecco's modified Eagle's (DME) medium/10% bovine calf serum. After the cells reached confluence, the

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Abbreviations: DME medium, Dulbecco's modified Eagle's medium; PDGF, platelet-derived growth factor; SCM, v-*sis*-conditioned medium; PMA, phorbol 12-myristate 13-acetate.

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medium was changed to DME medium/5% human platelet-poor plasma (21). Experiments were initiated the following day.

Murine epidermal growth factor (receptor grade) was obtained from Collaborative Research (Waltham, MA). Porcine insulin, phorbol 12-myristate 13-acetate (PMA), and cycloheximide were obtained from Calbiochem. Anisomycin was obtained from Sigma. v-*sis*-conditioned medium (SCM) was produced from a NRK-simian sarcoma virus (SSV) nonproducer cell line (22, 23). NRK-SSV cells were grown to confluence in DME medium/10% bovine calf serum, washed, and incubated for 48 hr in DME medium alone. At a 1- to 2-fold concentration, this medium gave a maximum induction of c-*fos* gene expression and replicative DNA synthesis in BALB/c-3T3 cells. All additions were made onto cells in DME medium/5% human platelet-poor plasma.

Plasmids and Probes. All *fos* sequences used were subcloned out of the human genomic c-*fos* clone pc-*fos*-human-1 (ATCC 41042) into the vector pGEM1 (Promega Biotec, Madison, WI). The 585-bp fragment extending from the *Xho* I site at −711 to the *Xma* III site at −126 was subcloned between the *Sal* I and *Sma* I sites of pGEM1. Digestion of this plasmid with *Pst* I liberates a 352-bp fragment extending from the *Pst* I site at −361 to the *Pst* I site upstream of the *Sal* I–*Xho* I junction in the polylinker. This is fragment B (Fig. 1). The 235-bp fragment extending from the *Pst* I site at −361 to the *Xma* III site at −126 was subcloned between the *Pst* I and the *Sma* I sites of pGEM1. Digestion of this plasmid with *Hin*dIII and *Eco*RI liberates the entire insert and polylinker. This is fragment A (Fig. 1). Fragment C is the 250-bp *Eco*RI–*Bst*NI fragment of pGEM1. The end-labeled probe, which includes the 96-bp *Pst* I–*Bst*NI fragment from −361 to −265, was prepared by labeling the *Pst* I–*Xma* III subclone at the *Hin*dIII site next to the *Pst* I site in the polylinker, either using reverse transcriptase and [α-^{32}P]dNTPs or using polynucleotide kinase and [γ-^{32}P]ATP. Digestion with *Bst*NI liberated a 109-bp radiolabeled fragment, which was electrophoretically purified and used as a probe in band-shift assays as described below.

Whole-Cell Extracts. Whole-cell extracts were prepared according to Manley *et al.* (24). All operations were performed at 4°C. In brief, cells were harvested in phosphate-buffered saline and pelleted. The cell pellet was resuspended in 4 packed-cell volumes of 10 mM Tris, pH 7.9/1 mM EDTA/1 mM dithiothreitol, and the cells were incubated 20 min on ice. The swollen cells were homogenized eight strokes in a Dounce homogenizer with a type B pestle and mixed with 1 packed-cell volume of 50 mM Tris, pH 7.9/10 mM $MgCl_2$/1 mM dithiothreitol/25% (wt/vol) sucrose/50% (vol/vol) glycerol. One packed-cell volume of saturated $(NH_4)_2SO_4$ was added dropwise while the mixture was stirred, and it was stirred an additional 30 min. The mixture was centrifuged for 3 hr at 43,000 rpm in an SW50.1 rotor. The upper 90% of the supernatant was collected and 0.33 g of $(NH_4)_2SO_4$ was added per ml of fluid. The mixture was stirred an additional 30 min after the salt had dissolved. The mixture was centrifuged for 20 min at 11,000 rpm in an SS-34 rotor, and the pellet was dissolved in 1 packed-cell volume of 10 mM Hepes, pH 7.9/100 mM KCl/1 mM EDTA/1 mM dithiothreitol/20% glycerol/0.5 mM phenylmethylsulfonyl fluoride. The solution was dialyzed against two changes of 100 volumes each of the same buffer. The extract was divided into aliquots and was stored at −80°C. Frozen extracts retained binding activity for at least 6 months. Protein concentrations were determined by the Bio-Rad protein assay.

Gel Electrophoresis DNA-Binding Assay. Whole-cell extracts were used for band-shift assays essentially as described by Carthew *et al.* (25). In brief, the end-labeled probe (0.15 ng, 7000 dpm), whole-cell extract, and 1 μg of poly(dI-dC)·poly(dI-dC) were incubated for 30 min at 30°C in a final volume of 10–15 μl of 10 mM Hepes, pH 7.9/50 mM NaCl/1 mM dithiothreitol/1 mM EDTA/10% (vol/vol) glycerol. Competitor DNAs and the labeled probe were added to the binding reaction simultaneously. Samples were layered onto 4% acrylamide gels (acrylamide/bisacrylamide, 80:1) and electrophoresed at 10 V/cm in 6.7 mM Tris·HCl, pH 7.9/3.3 mM sodium acetate/1 mM EDTA, with buffer recirculation between the electrode chambers at 50 ml/min. Gels were fixed in 10% acetic acid/10% methanol, dried, and exposed to x-ray film.

Methylation Interference of Binding. The end-labeled probe was treated with dimethyl sulfate according to Maxam and Gilbert (26). Methylated probe was incubated with whole-cell extract prepared from cells treated with SCM for 30 min. The sample was electrophoresed as above, and the wet gel was exposed to x-ray film. Individual bands were excised from the gel, and the radiolabeled DNA was isolated by electrophoresis onto an NA45 membrane (Schleicher & Schuell) in the presence of 0.1% sodium dodecyl sulfate. Radiolabeled DNA from each band was cleaved with piperidine (26) and fractionated on a sequencing gel. The identity of methylated residues was confirmed by dideoxy chain-termination sequencing.

RESULTS

To identify specific DNA-binding proteins, we have used the sensitive band-shift technique (25–29). This assay takes advantage of the fact that a protein–DNA complex will migrate with a characteristically slower mobility than a free DNA fragment when electrophoresed through a low-ionic-strength polyacrylamide gel. Thus, when unfractionated nuclear or whole-cell extracts are incubated with end-labeled DNA fragments and electrophoresed through the appropriate gel system, a series of bands appears that migrate more slowly than free DNA. Some of the bands seen on such gels are due to nonspecific DNA binding proteins. Such nonspecific binding can be reduced by including simple-sequence carrier DNA such as poly(dI-dC)·poly(dI-dC) in the binding reaction mixture. Bands representing specific DNA binding can be identified by virtue of the fact that their intensity is decreased by adding excess homologous, but not excess nonhomologous, DNA to the binding reaction.

The results of a band-shift gel where a DNA probe extending from positions −361 to −265 of the c-*fos* promoter has been incubated with extracts of growth factor-treated BALB/c-3T3 cells are shown in Fig. 1. Although many bands can be seen, the top two bands (labeled B1 and B2) are consistently seen as sequence-specific complexes. The binding specificity was assessed by including unlabeled competitor DNAs in the binding reactions. Adding as a competitor an unlabeled c-*fos* upstream fragment (fragment A) that spans the entire length of the labeled DNA probe diminishes the amounts of the B1 and B2 bands observed. Similar amounts of competitor DNA from a fragment that lies upstream of the probe (fragment B) or from fragment C from the vector backbone do not effectively compete for the DNA-binding factor in complex B2. Some of the binding in band B1 is diminished by these nonhomologous competitor DNAs. Experiments below, however, indicate that both of these complexes reflect binding to the same sequence. Carthew *et al.* (25) have observed a similar type of complex that competes, but is specific, in studies of factors that bind to the adenovirus major late promoter. We suspect that the upper band represents a modified and less avidly binding form of the same factor which is responsible for B2. Formation of the B2 complex cannot be diminished by the addition of DNA fragments from the promoters of the β-globin genes, the adenovirus pIX promoter, the major late promoter of adenovirus, the Moloney leukemia virus long terminal re-

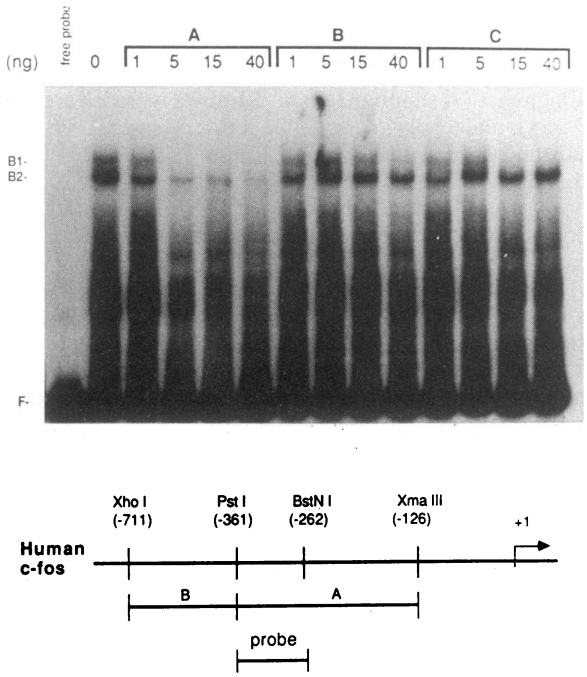

FIG. 1. Specific binding of a cellular factor to the c-*fos* 5′-flanking region. Whole-cell extracts were prepared from cells treated with SCM for 30 min. End-labeled probe was incubated with 6 μg of extract in the presence of competitor DNA fragments and was electrophoresed through band-shift gels. The amount of competitor DNA fragment is indicated above each lane. The locations of fragments A and B with respect to the c-*fos* gene and the probe are indicated diagrammatically. Fragment C was from the vector pGEM1 (see *Materials and Methods*). B1 and B2, complexes that diminish in intensity when fragment A competes with the probe; F, position of the free probe.

peat, or the γ-fibrinogen promoter. This result indicates that this factor is not a general transcription factor but has specificity for the c-*fos* gene. Some of the lower bands in Fig. 1 are also affected by specific competitor DNAs. However, they are not consistently observed in different extract preparations and do not bind to an oligonucleotide containing the binding site of the B1 and B2 complexes.

Since c-*fos* transcription is induced by growth factors, we looked for inducibility of this DNA-binding activity. Conditioned medium from v-*sis*-transformed cells induces the appearance of this binding activity as shown in Fig. 2. This medium contains a v-*sis*-encoded protein which is similar to PDGF in that it binds to the PDGF receptor, has mitogenic activity, and reacts with anti-PDGF antibody (22, 23, 30, 31). This conditioned medium induces the c-*fos* gene in a manner kinetically identical to that of PDGF (unpublished results). Confluent, quiescent BALB/c-3T3 cells contain little of this c-*fos* promoter DNA-binding activity. Within 20 min of addition of SCM, an approximately 30-fold increase in the level of this specific DNA-binding activity can be found in whole cell extracts of the SCM-treated cells as measured by scanning densitometry. By 3 hr, the amount of *fos*-promoter binding activity present in the extracts has diminished significantly from its peak. Thus, this DNA-binding activity shows a rapid and transient induction similar to that seen for transcription of the c-*fos* gene.

By using a methylation-interference assay, we have been able to identify the site where this factor binds the c-*fos* DNA fragment (20). The results of this experiment are shown in Fig. 3. The −361 to −262 fragment of the c-*fos* promoter was partially methylated by reaction with dimethyl sulfate. This end-labeled, methylated DNA was used to run a preparative band-shift gel. Labeled DNA was then isolated from com-

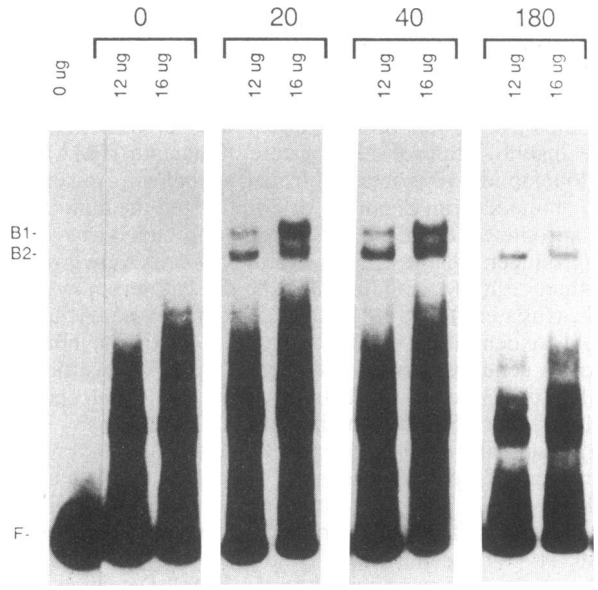

FIG. 2. Time course of induction of binding activity. Confluent, quiescent BALB/c-3T3 cells were treated with SCM for the indicated times (in min), and whole-cell extracts were prepared and analyzed for binding activity. The amount of extract used is indicated above each lane. F, position of the free probe; B1 and B2, complexes that diminish in intensity when fragment A competes with the probe.

plexes B1 and B2 and from the free probe. These DNAs were cleaved with piperidine and electrophoresed through DNA sequencing gels. The resulting autoradiograph gives a pattern of bands corresponding to guanine residues in the sequence. Some adenine residues are also partially methylated. Where methylation of a guanine residue has interfered with the binding of the factor to the probe, the lanes with the bound

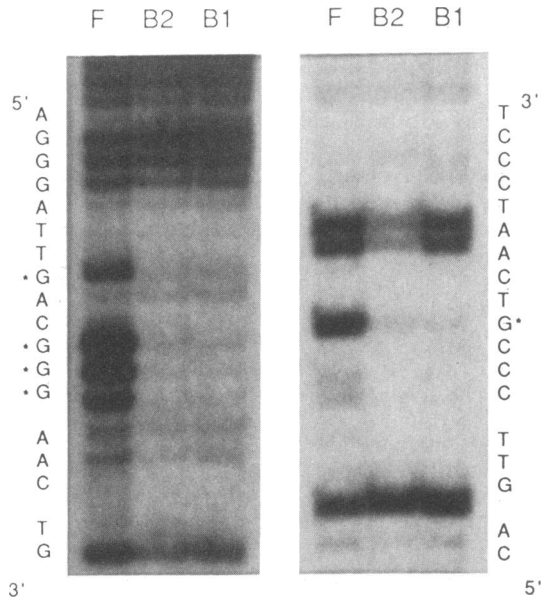

FIG. 3. Methylation interference of binding to the c-*fos* upstream region. End-labeled probe was treated with dimethyl sulfate and incubated with whole-cell extract from SCM-treated cells, and the sample was fractionated by electrophoresis. Methylated DNA was isolated from complexes B1 and B2 (see Fig. 1) and from free probe, cleaved with piperidine, and fractionated on a sequencing gel. The sequence indicated extends from −352 to −335 of the human c-*fos* gene. The complex from which each DNA was isolated is indicated above each lane. (*Left*) Probe labeled on the noncoding strand. (*Right*) Probe labeled on the coding strand. F, free probe; *, methylated guanine residues that interfere with binding of the factor.

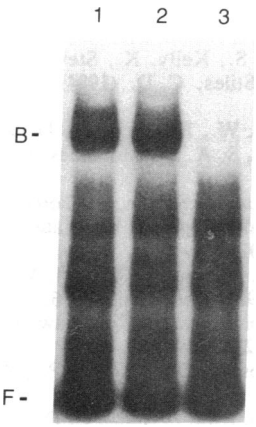

FIG. 4. Competition of specific binding with synthetic oligonucleotides. Binding reactions were done with 8 μg of extract from SCM-treated cells in the presence of a 125-fold molar excess of double-stranded synthetic oligonucleotides. Lane 1, no competitor added. Lane 2, competition with a 28-bp sequence that includes residues −321 to −298 of the human c-*fos* gene. Lane 3, competition with a 21-bp sequence that includes residues −351 to −337 of the human c-*fos* gene. F, position of free probe; B, position of the B1 and B2 complexes (see Fig. 1).

complexes have a diminished guanine band relative to the lane with the free probe. From Fig. 3, it can be seen that this factor binds to the sequence, 5′-CCCGTC-3′, located at position −346 of the c-*fos* gene. All the methylated guanine residues of both DNA strands interfere with factor binding at this site. Two additional adenine residues may also be involved at the binding site. The interference pattern is the same for both the B1 and B2 complexes. Evidence of interference of binding was detected only at this sequence and at no other location in the probe. To further confirm that this sequence is the factor binding site, a pair of complementary 21-residue oligonucleotides including 15 residues of the c-*fos* sequence spanning the region of methylation interference was synthesized. This synthetic fragment specifically competes with the probe for B1 and B2 complex formation as shown in Fig. 4. A competitor oligonucleotide made homologous to a sequence of the probe that does not show methylation interference fails to compete with the probe for this binding. The oligonucleotide that contains the binding site will also specifically bind to the inducible factor when it is used directly as the labeled probe in the gel-shift assay (data not shown). The 6-bp sequence, CCCGTC, is not necessarily sufficient for factor binding. Because the methylation interference assay primarily detects guanine residues at contact sites, the actual binding-recognition site is likely to include some A·T pairs flanking this central hexamer.

The induction of this c-*fos* binding factor is inducer specific. Band-shift assays done with extracts from confluent, quiescent BALB/c-3T3 cells treated with EGF, insulin, the tumor promoter PMA, or SCM are shown in Fig. 5*A*. Only the SCM-stimulated cells appear to contain the B2-complex binding activity. Heat-treated platelet extracts (32), which contain significant amounts of PDGF, also induce the appearance of this binding activity (data not shown). Control experiments show that another DNA-binding factor which binds to the −320 to −299 c-*fos* sequence shows less than a 2-fold change in binding activity when quiescent cells are treated with SCM, PMA, or epidermal growth factor (see *Discussion*). It is not surprising that extracts from insulin- or epidermal growth factor-treated cells fail to contain the inducible c-*fos* DNA-binding activity since these agents do not induce transcription of the c-*fos* gene under the conditions of this experiment (13). However, the tumor promoter

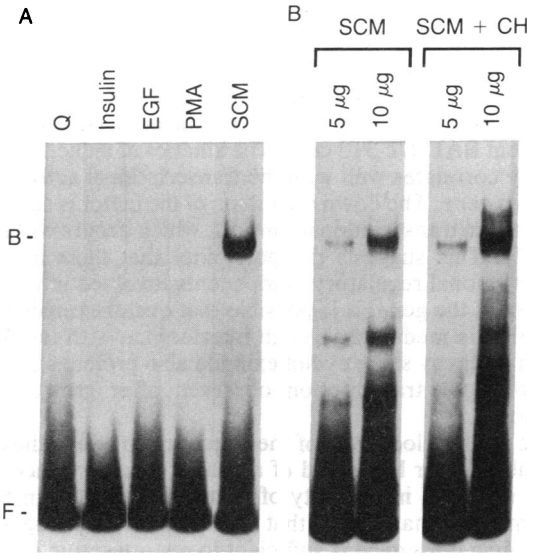

FIG. 5. Induction of c-*fos* promoter binding activity by growth factors. Confluent, quiescent BALB/c-3T3 cells were treated for 30 min with growth factors, and whole-cell extracts were prepared and analyzed for binding activity. (*A*) Cells were treated as follows: Q (quiescent cells), no additions; insulin, 5 μg of porcine insulin per ml; EGF, 100 ng of murine submaxillary gland epidermal growth factor per ml; PMA, 100 ng of phorbol 12-myristate 13-acetate per ml; SCM, v-*sis* conditioned medium. Each lane contains 5 μg of whole-cell extract. (*B*) Extracts were prepared from cells treated for 30 min with SCM alone or with SCM and cycloheximide (10 μg/ml). The amount of extract used is shown above each lane. CH, cycloheximide; EGF, epidermal growth factor.

PMA does induce the c-*fos* gene at the transcriptional level (13, 33) to an extent similar to that seen with SCM or platelet extracts. There are at least two interpretations of this result. One is that PMA induces this factor only slightly and that it is below the threshold level of detection. A second possibility, which we favor, is that PMA acts by way of another DNA-binding activity that can activate an enhancer located elsewhere in the c-*fos* locus.

Fig. 5*B* shows that the *fos* promoter binding activity is inducible even in the absence of protein synthesis. BALB/c-3T3 cells were treated with both SCM and cycloheximide (10 μg/ml). Under these conditions, protein synthesis is inhibited by >90% and the rate of c-*fos* transcription is superinduced by 10-fold (ref. 15 and unpublished results), whereas scanning densitometry measures only a 2-fold superinduction in c-*fos* binding activity. Similar results are obtained using anisomycin as a protein synthesis inhibitor (>95% inhibition of protein synthesis). This result, coupled with the rapid time course of induction, suggests that the factor preexists in quiescent cells and is not newly synthesized after growth factor treatment. Thus, it is likely that SCM addition to the cells stimulates a conversion of the factor from an inactive to an active DNA-binding form through an as yet unknown mechanism. In addition, this experiment indicates that the superinduction of transcription caused by cycloheximide cannot be quantitatively accounted for by a corresponding increase in the level of this DNA-binding activity.

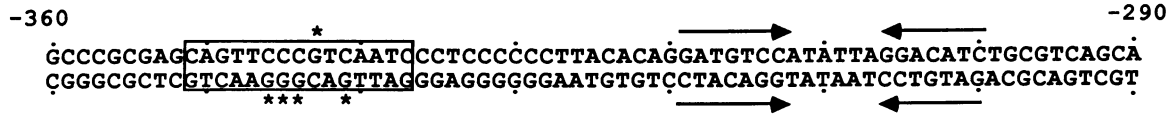

FIG. 6. Sequences of the human c-*fos* upstream regulatory region. Numbers indicate position relative to the site of initiation of transcription. □, Synthetic oligonucleotide that binds SCM-inducible factor; *, methylated guanine residues that interfere with binding of the factor; ⟶, palindromic sequence implicated in serum induction of c-*fos*.

DISCUSSION

We have found an SCM-inducible DNA-binding factor that binds to a sequence 346 bp upstream of the c-*fos* transcription initiation site. The appearance of this DNA-binding activity in cell extracts occurs within 20 min of SCM addition to quiescent BALB/c-3T3 cells. The kinetics of induction of this activity correlates well with the transcriptional activation of the c-*fos* gene. The down regulation of the factor is somewhat slower than transcriptional shut-off, which occurs within 1 hr (13, 15). This suggests the possibility that there are other transcriptional regulatory components involved in the deactivation of the gene. It is possible that cycloheximide superinduction is mediated through interference with the deactivation pathway since cycloheximide also prolongs the duration of c-*fos* transcription observed after growth factor addition (15).

The relative locations of the sequence to which the SCM-inducible factor binds and of a palindromic sequence implicated in serum inducibility of c-*fos* are indicated in Fig. 6. Treisman (34) has shown that the −320 to −299 fragment of the human c-*fos* gene is sufficient to restore serum inducibility to an uninducible deletion mutant of the c-*fos* promoter. Gilman *et al.* (18) and Treisman (34) have found a constitutive factor from HeLa and WEHI-231 cells that binds to the palindromic sequence in this fragment. However, the −320 to −299 fragment does not bind to the SCM-inducible factor suggesting that the −346 site is not required for serum induction of *fos*. Experiments that we have performed with BALB/c-3T3 cell extracts confirm that the −320 to −299 binding factor is constitutively present while the −346 factor is induced by SCM (data not shown). It seems unlikely that the −346 sequence is not involved in the regulation of c-*fos* since it binds to an inducible factor and shows absolute conservation between human and mouse (17, 35). A possible explanation for the functions of this sequence could lie in the fact that the c-*fos* gene is capable of responding to a wide range of external stimuli including serum, PDGF, epidermal growth factor, nerve growth factor, membrane depolarization, PMA, poly(rI-rC), and Ca^{2+} influx (10, 11, 13, 36, 37). Each of these agents, rather than activating the same enhancer element in the c-*fos* gene, could activate an independent responder element or set of elements. This implies that c-*fos* transcription is controlled by multiple independent enhancer elements, each activated by distinct signals. Our observation that PMA does not induce the −346 binding factor but does activate c-*fos* transcription is consistent with this idea. PMA induction of *fos* could be mediated through a sequence elsewhere in the c-*fos* locus.

The inducible DNA-binding factor which we have found may be an intermediate in the signaling pathway which couples a cell surface receptor to specific gene regulation in the cell nucleus. As such, it is potentially a substrate for growth factor-regulated protein kinases and may be useful in elucidating the metabolic pathways which lead from the cell surface to the nucleus.

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