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# Mutation of the c-*fos* Gene Dyad Symmetry Element Inhibits Serum Inducibility of Transcription In Vivo and the Nuclear Regulatory Factor Binding In Vitro

MICHAEL E. GREENBERG,†* ZAHAVA SIEGFRIED, AND EDWARD B. ZIFF

*Department of Biochemistry and Kaplan Cancer Center, New York University Medical Center, New York, New York 10016*

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**In vitro mutagenesis of a 61-base-pair DNA sequence element that is necessary for induction of the c-*fos* proto-oncogene by growth factors revealed that a small region of dyad symmetry within the sequence element is critical for c-*fos* transcriptional activation. The same c-*fos* dyad symmetry element was found to bind a nuclear protein in vitro, causing a specific mobility shift of this c-*fos* regulatory sequence. An analysis of insertion and deletion mutants established a strict correlation between the ability of the dyad symmetry element to promote serum activation of c-*fos* transcription and in vitro nuclear protein binding. These experiments suggest that the DNA mobility shift assay detects a nuclear protein that mediates growth factor stimulation of c-*fos* expression. In vitro competition experiments indicate that the c-*fos* regulatory factor also binds to sequences within another growth factor-inducible gene, the β-actin gene.**

The c-*fos* proto-oncogene encodes a nuclear protein whose altered expression can lead to cell transformation (5, 6, 27). Recent studies have shown that in many different cell types c-*fos* transcription is rapidly and transiently activated by growth factors as well as by phorbol esters and membrane-depolarizing agents (4, 7, 13, 14, 16, 17, 28, 29). c-*fos* transcriptional activation is one of the earliest known responses to growth factors and occurs independently of new protein synthesis (4, 15, 30). Within 30 min of growth factor addition, c-*fos* transcription is repressed to the undetectable level present in untreated cells (15). This repression is inhibited in the presence of protein synthesis inhibitors, suggesting that it is mediated by a labile protein, possibly by the c-*fos* protein itself (15). When c-*fos* mRNA and protein are expressed constitutively in fibroblasts rather than transiently, the fibroblasts become morphologically transformed (27).

The potential of the c-*fos* protein to induce cell transformation and oncogenesis, its localization to the nucleus, and the exquisite and direct regulation of this gene by growth factors have prompted the hypothesis that c-*fos* may be a nuclear regulator of growth and differentiation events. This view is supported by recent experiments demonstrating that the expression of c-*fos* antisense RNA in fibroblasts inhibits their proliferation (20). Currently, little is known about the specific function of the c-*fos* protein in the nucleus or about the mechanism of c-*fos* transcriptional activation by growth factors.

To begin examining the mechanisms controlling c-*fos* transcriptional activation, efforts have focused on the identification of regulatory sequences within the 5′-flanking regions of the c-*fos* gene that are necessary for its stimulation in fibroblasts (9, 34, 39). By testing the ability of serum to activate human c-*fos* mRNA production in quiescent mouse 3T3 cells that had been transfected with various 5′ deletion mutants of c-*fos* DNA, a 56-base-pair (bp) sequence between −332 and −277 upstream of the c-*fos* mRNA cap site was shown to be essential for serum inducibility (39). This c-*fos* serum response element (SRE) displays sequence homology with several viral and cellular transcriptional enhancers and also has functional properties that have been found to be characteristic of eucaryotic enhancers. The SRE is distinct from other enhancers in that it specifically potentiates transcription in serum-stimulated cells but not in quiescent cells (39). Within the middle of the 56-bp SRE sequence is a 20-bp element that possesses a high degree of dyad symmetry. In several other eucaryotic genes, such sequences have been shown to bind transcriptional regulatory proteins (3, 33, 37, 38).

Using a transient expression assay in which the human c-*fos* gene is expressed in mouse 3T3 cells (39) and an in vitro DNA mobility shift protocol that detects DNA-protein interactions (37), we have identified a nuclear factor(s) that appears to regulate c-*fos* transcriptional activation through binding to the 20-bp dyad symmetry element. We find that the 20-bp sequence is essential for serum stimulation of c-*fos* transcription. Mutations within this region completely abolish the serum inducibility of c-*fos* expression and abrogate in vitro binding of the c-*fos* regulatory factor.

## MATERIALS AND METHODS

**Plasmid constructions.** Plasmids pF4, pF222, pMLV+/F222, and pSV+/F222 were a gift from Richard Treisman (39). pF4 contains a 5.4-kilobase human DNA *Bam*HI fragment encompassing the entire c-*fos* gene inserted into the *Bam*HI site of pUC12. In plasmid pF222, sequences between the pUC12 *Eco*RI site and the c-*fos Apa*I site at position −222 are deleted (39). In pMLV+/F222, a Moloney murine leukemia virus (MoMLV) 3′ long terminal repeat (LTR) fragment (36) is inserted into the 5′ *Eco*RI site of pF222. In pSV40+/F222, a simian virus 40 (SV40) enhancer fragment (41) is inserted into the 5′ *Eco*RI site of pF222. Other plasmids used were provided by U. Nudel (β-actin genomic sequences [32]), Ricardo Dalla Favera (c-*myc* genomic sequences [8]), and James Manley (adenovirus type 2 [Ad2] major late promoter plasmid pHB310 [21]). c-*fos* oligonucle-

* Corresponding author.
† Present address: Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115.

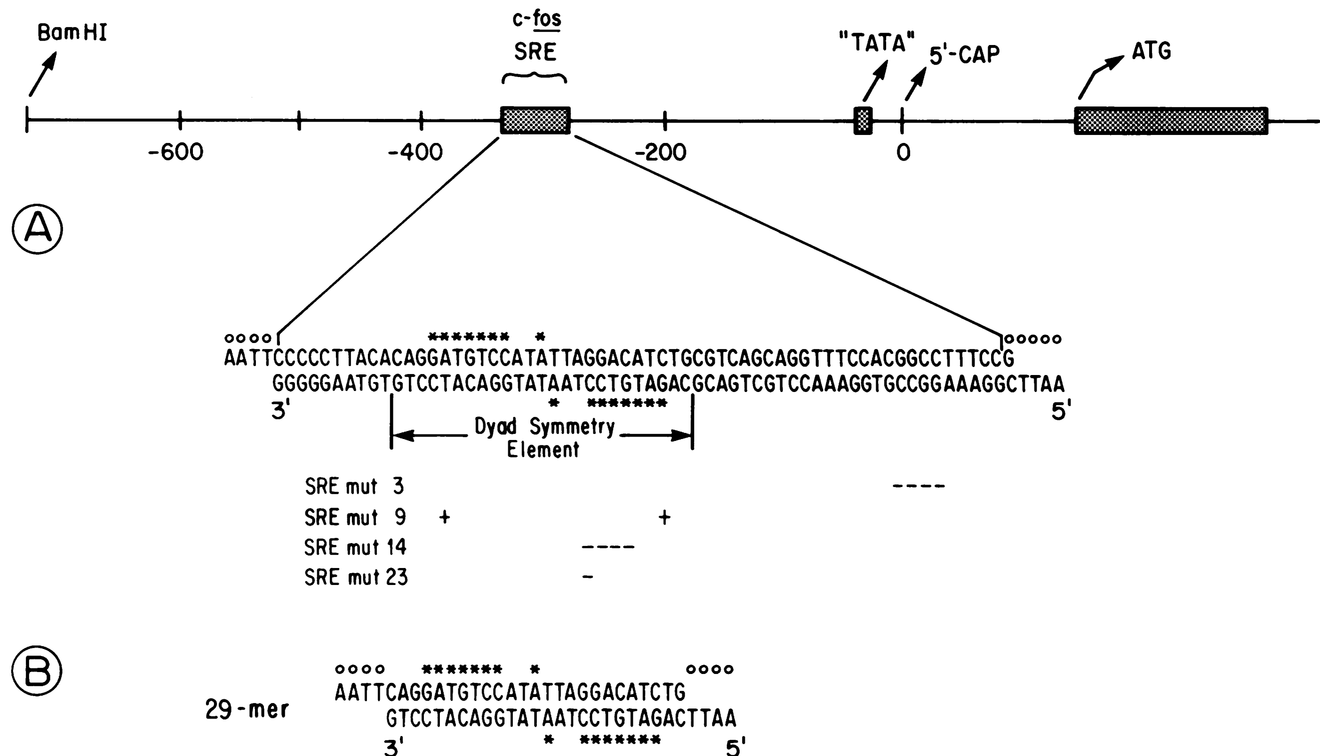


FIG. 1. Structure of human c-*fos* 5' regulatory elements. (A) Position of the 61-bp serum-responsive element between −200 and −400 bp upstream of the c-*fos* mRNA cap site (0 bp) is indicated. *Bam*HI is the site of insertion of c-*fos* DNA into the pUC12 vector. The entire sequence of the synthetic c-*fos* SRE is shown. Mutant SREs are listed below the wild-type SRE sequence. Symbols: +, nucleotide duplicated in the mutant; −, nucleotide that has been deleted. (B) Sequence of the synthetic 29-mer spanning the c-*fos* dyad symmetry element. (A and B) Symbols: ○, nucleotides added to the SRE to generate an *Eco*RI-compatible site; *, nucleotides that form the region of dyad symmetry.

otides shown in Fig. 1 were synthesized by B. Goldschmitt with an Applied Biosystems oligonucleotide synthesizer. The oligonucleotides were purified by electrophoresis on an 8 M urea–8% polyacrylamide sequencing gel. After electroelution the recovered single-stranded DNA was lyophilized and relyophilized four times, and each time the DNA pellets were suspended in 1 ml of $H_2O$. Complementary oligonucleotides, either the 66-mers (Fig. 1A) or the 29-mers (Fig. 1B), were mixed, and the DNA was ethanol precipitated overnight. Pelleted DNA was suspended in $H_2O$ at 10 ng/ml. DNA strands were annealed by heating to 68°C for 10 min, followed by cooling at room temperature for 30 min. The annealed c-*fos* SRE oligonucleotides were cloned into the *Eco*RI site of M13mp8 plasmid DNA. Single-stranded M13 bacteriophage DNA containing the c-*fos* insert was sequenced by the primed synthesis technique (35). The c-*fos* SRE and mutant SREs 3, 9, 14, and 23 (Fig. 1A) were released from M13mp8 by cleavage with *Eco*RI and recloned into the *Eco*RI site of pF222. Restriction enzyme cleavage patterns of the recombinant clones indicated that in each case a single copy of the appropriate SRE had inserted into pF222.

**c-*fos* transient expression assay.** Mouse NIH 3T3 cells were grown in Dulbecco modified Eagle medium (DMEM) with 10% calf serum (CS). At 24 h prior to transfection, cells were split to a density of $4.5 \times 10^5$ per 100-mm dish. The transfection of c-*fos* plasmid DNAs into 3T3 cells and subsequent serum starvation were done as described previously (39). Briefly, cells were transfected with 20 μg of plasmid per plate. After 16 h cells were washed with phosphate-buffered saline and incubated in DMEM with 0.5% CS for 24 h. The medium was removed, and the serum-starved 3T3 cells were stimulated for 0 to 120 min by the readdition of DMEM with 15% CS. Hirt analysis (19) verified that approximately the same amount of DNA was taken up by the 3T3 cells at each time point within a given experiment. Cytoplasmic RNA was isolated from 3T3 cells as described previously (16, 17) and treated with DNase for 20 min at 37°C (12). Human c-*fos* mRNA transcripts were detected by the RNase protection method described previously (39). RNA (20 μg) was hybridized with $^{32}P$-labeled antisense RNA in each reaction. Nuclease-resistant fragments were fractionated on thin 5% polyacrylamide sequencing gels. $^{32}P$-labeled *Hae*III-cut pBR322 DNA was run on all analytical gels as approximate size markers.

**DNA mobility shift binding assay.** The DNA mobility shift protocol was essentially that described previously (3, 37). SRE DNA fragments were released from pF222(SRE) by digestion with *Eco*RI. A 168-bp fragment spanning the region −119 bp to +48 bp in the c-*fos* genomic plasmid pF4 was released by digestion with *Not*I. Digested DNA was fractionated on a 4% acrylamide gel, and the purified fragments were isolated by electroelution. To 5'-end-label the SRE fragments, the DNA was treated with calf alkaline phosphatase, and the dephosphorylated DNA was treated with T4 polynucleotide kinase and [$\gamma$-$^{32}P$]ATP as described previously (25). The specific activity of the labeled fragments was between 50,000 and 250,000 cpm/ng of DNA.

Nuclear extracts were prepared from suspension or monolayer HeLa cell cultures as described (10). For

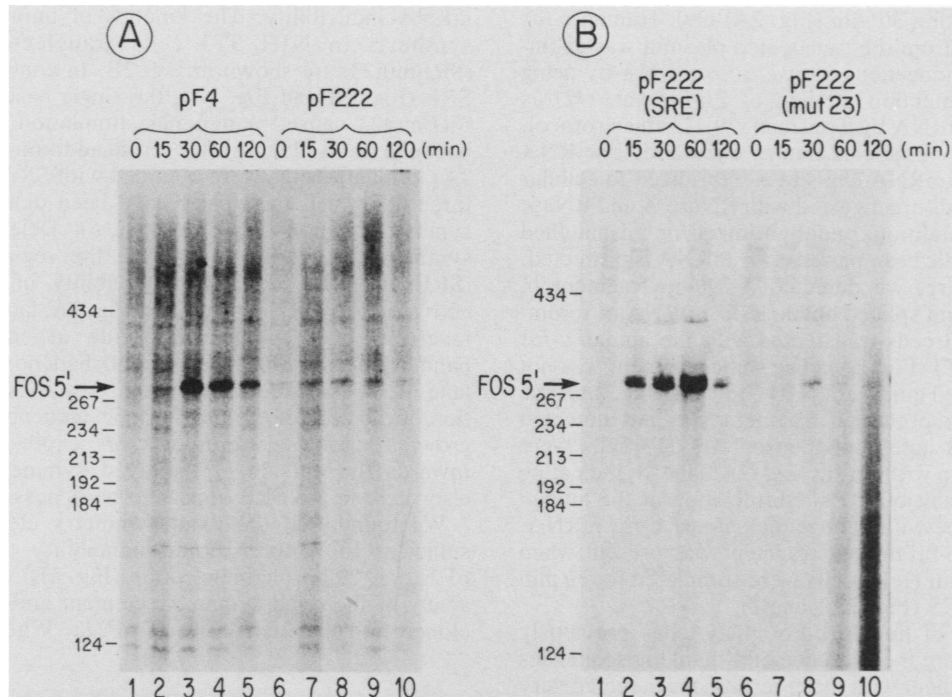


FIG. 2. SRE regulation of human c-*fos* expression in transfected NIH 3T3 cells. (A and B) 3T3 cells were transfected with the indicated human c-*fos* plasmid DNA, serum starved, and stimulated by the addition of 15% CS as described in Materials and Methods. Cytoplasmic RNA was isolated at various times after serum addition (0 to 120 min). Human c-*fos* mRNA was quantitated by the RNase protection method as outlined in Materials and Methods. Fos 5', Protected 296-bp human c-*fos* mRNA fragment. (A) Cells were transfected with the wild-type human c-*fos* genomic clone pF4 (lanes 1 to 5) or the mutant pF222 that lacks all 5' regulatory sequence extending beyond −222 bp of the c-*fos* transcription start site (lanes 6 to 10). (B) Cells were transfected with pF222 containing a wild-type SRE insert (lanes 1 to 5) or with pF222 (SREmut23) (lanes 6 to 10). Sizes are indicated to the left (in base pairs).

monolayer culture extracts, cells were allowed to grow to confluence, and the medium was removed and substituted with fresh DMEM containing 0.5% CS and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4). Cells were serum starved in this medium for 48 h and then left untreated or stimulated for 30 min by the addition of fresh medium containing 15% CS. Nuclear extracts were prepared from both the unstimulated cells and the serum-stimulated cultures by the protocol of Dignam et al. (10). The nuclear extracts were dialyzed for 5 h against BC100 buffer (20 mM HEPES, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 M EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and then frozen at −70°C. For DNA-protein binding reactions, 1 μl of $^{32}$P-labeled DNA (0.2 ng) and 10 μl of $H_2O$ were mixed with 3 μl each of 1.0-mg/ml poly(dI-dC):poly(dI-dC) and 1.0-mg/ml pUC13 DNA. Nuclear extract (10 mg/ml; 0 to 20 μl) was added last, and the binding reaction mixtures were incubated at room temperature for 30 min. For reactions receiving less than 20 μl of nuclear extract, the sample volumes were equalized by the addition of BC100 buffer (10). DNA-protein complexes were analyzed by electrophoresis on 4% acrylamide–0.11% bisacrylamide gels. The electrophoresis conditions have been described previously (3). After electrophoresis at 30 mA for 2 h, gels were fixed in 10% methanol–10% acetic acid for 10 min, dried, and autoradiographed.

## RESULTS

**Synthetic-serum-responsive element restores c-*fos* inducibility.** To better define the sequence requirements for growth factor inducibility of c-*fos* transcription and to develop methods for identifying and characterizing c-*fos* regulatory factors, we synthesized two complementry 66-bp oligonucleotides (Fig. 1A) spanning the 56-bp nucleotide region that is critical for serum inducibility of c-*fos* expression (39). The oligonucleotides were synthesized with *Eco*RI tails, and after annealing the two complementary strands, the SRE was cloned into the *Eco*RI site of the bacterial M13 vector mp8. Sequencing of single-stranded DNA from 29 distinct bacterial plaques yielded one plasmid containing the intact SRE inserted into the plasmid *Eco*RI site. Twenty-eight other clones were sequenced and found to contain the SRE with random insertions or deletions at various sites within its sequence. We suspect that the insertion and deletion mutants were obtained during the synthesis of the oligonucleotide. The wild-type SRE and several of the mutants were used in experiments described below and are shown in Fig. 1A.

We found that serum inducibility was restored when the synthetic wild-type SRE was placed 5' to the c-*fos* transcriptional start site in a mutant c-*fos* genomic clone that lacked this sequence and was not growth factor inducible. Figure 2 shows the results of a transient transfection experiment in which a wild-type c-*fos* genomic plasmid (pF4) containing 750 bp of 5'-flanking sequence, including the intact SRE and the entire c-*fos* coding region, was expressed in mouse 3T3 cells. In this experiment, subconfluent NIH 3T3 cells were transfected with pF4 and then growth arrested in low-serum medium for 24 h. Stimulation of the transfected 3T3 cells by addition of fresh medium containing 15% CS caused rapid and transient accumulation of human c-*fos* mRNA in the

transfected cells within 30 min (Fig. 2A) (39). Human c-*fos* mRNA transcribed from the transfected plasmid was distinguished from the endogenous mouse c-*fos* mRNA by using the SP6 RNase protection method of Zinn et al. (42) as modified for c-*fos* mRNA by Treisman (39). In this protocol, in vitro-transcribed $^{32}$P-labeled human c-*fos* antisense RNA that spans the c-*fos* mRNA cap site is hybridized to cellular RNA, and the hybrids are digested with RNase A and RNase $T_1$. Under these conditions, nonhybridized or mismatched RNA is digested while base-paired c-*fos* mRNA is protected. Using this procedure, we detected a 296-bp fragment of correctly initiated and spliced human c-*fos* mRNA in serum-stimulated NIH 3T3 cells transfected with the human c-*fos* genomic plasmid pF4 (Fig. 2A). The same fragment was not present in transfected quiescent NIH 3T3 cells (Fig. 2A, lane 1). In addition, the protected fragment was not detected when untransfected quiescent mouse NIH 3T3 cells were stimulated for 30 min with serum (Fig. 3A, lane 2), indicating that it is not the result of cross-hybridization of the human c-*fos* antisense probe with endogenous mouse c-*fos* mRNA. In contrast, the 296-bp mRNA fragment was present when serum-starved human HeLa cells were stimulated for 30 min by the addition of CS (Fig. 3A, lane 1).

Serum induction of human c-*fos* mRNA has previously been shown to require the presence of 5'-flanking sequences (9, 34, 39). In agreement with these results, we detected very little serum induction of c-*fos* mRNA in quiescent 3T3 cells transfected with a human c-*fos* genomic plasmid, pF222, that contains a deletion of the entire 5' c-*fos*-flanking sequence beyond 222 bp 5' of the cap site (Fig. 2A). This deleted sequence included the 56-bp SRE that has been shown to be critical for serum induction (39) and approximately 450 bp of additional c-*fos* 5'-flanking sequences. The residual c-*fos* transcriptional activity seen with pF222 (Fig. 2A) suggests that as yet uncharacterized sequences, in addition to the c-*fos* SRE, are probably important for c-*fos* transcription.

When a single copy of the synthetic SRE (Fig. 1A) was cloned into the growth factor-nonresponsive plasmid pF222 at an *Eco*RI site approximately 230 bp 5' to the transcriptional start site, serum stimulation of c-*fos* mRNA was restored. Stimulation of 3T3 cells transfected with plasmid pF222(SRE) resulted in a greater than 50-fold induction of human c-*fos* mRNA production within 30 min. The kinetics of c-*fos* repression that occurred subsequent to the activation of human c-*fos* mRNA synthesis appeared somewhat delayed with pF222(SRE) (Fig. 2B, lanes 1 to 5) compared with the wild-type plasmid pF4 (Fig. 2A, lanes 1 to 5). This difference could be due to the absence of sequences in plasmid pF222(SRE) that are important for c-*fos* transcriptional repression. The DNA sequences controlling c-*fos* repression are not yet defined and may be distinct from the sequences that regulate activation. We conclude from the experiments shown in Fig. 2B that the synthetic SRE is capable of restoring serum inducibility to the c-*fos* gene and that additional sequences that have been deleted from plasmid pF222 (−222 to −750 bp) are not critical for c-*fos* activation.

To determine whether the dyad symmetry element within the SRE is important for serum inducibility, we cloned single copies of several mutant SREs into c-*fos* plasmid pF222 and examined their ability to restore growth factor inducibility to plasmid pF222. The intact dyad symmetry element was critical for growth factor activation of c-*fos* expression inasmuch as a single G:C base pair deletion (SREmut23) or a 4-bp deletion (SREmut14) within the symmetry element completely abolished the ability of the SRE to confer c-*fos* mRNA inducibility. The kinetics of human c-*fos* mRNA synthesis in NIH 3T3 cells transfected with pF222 (SREmut23) are shown in Fig. 2B. In contrast to the intact SRE (Fig. 2B and Fig. 3A), the single base pair deletion in SREmut23 caused a minimal stimulation of c-*fos* mRNA between 0 and 120 min after serum addition (Fig. 2B and Fig. 3A). Similar results were obtained with SREmut14, in which three additional base pairs have been deleted in the dyad symmetry region (Fig. 3A, lane 5). Deletions within the synthetic SRE that were 3' to the region of symmetry (SREmut3) did not affect the ability of the element to activate c-*fos* mRNA synthesis (Fig. 3A, lane 7). In addition, insertion of a single nucleotide at each end of the palindromic sequence (SREmut9) had no effect (Fig. 3A, lane 6). These experiments clearly establish that conservation of the core of the palindromic sequence is essential for growth factor activation of c-*fos* expression. Sequences towards the outside of the dyad symmetry element and elsewhere in the SRE appear to be nonessential.

We found that the dyad symmetry element alone was sufficient to restore serum inducibility to c-*fos* plasmid pF222. A 29-bp oligonucleotide (Fig. 1B) composed exclusively of the dyad symmetry element and *Eco*RI tails was cloned into the *Eco*RI site of pF222. When quiescent 3T3

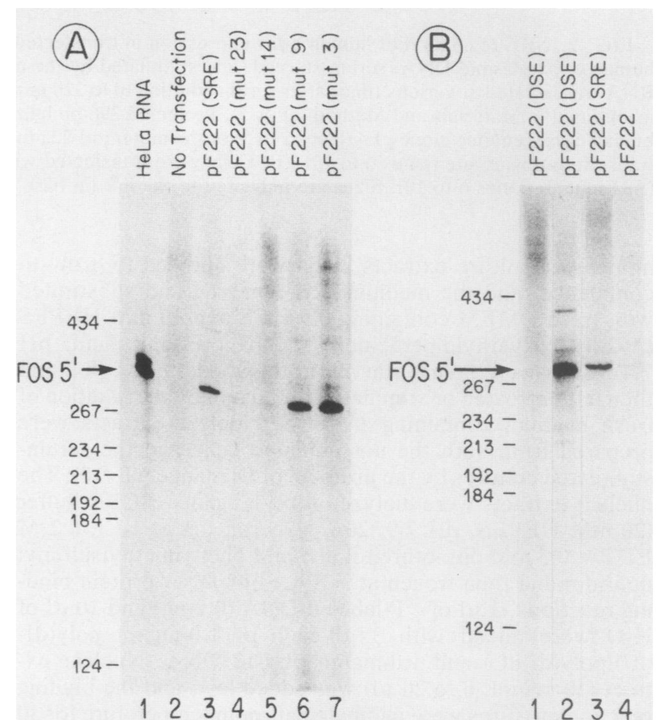


FIG. 3. Dyad symmetry element regulation of human c-*fos* expression in NIH 3T3 cells. Cells were transfected with the indicated human c-*fos* plasmid DNA, serum starved, and stimulated by the addition of 15% CS as described in Materials and Methods. Cytoplasmic mRNA was isolated and the human c-*fos* mRNA quantitated by the RNase protection method outlined in Materials and Methods. Fos 5', Protected 296-bp c-*fos* mRNA fragment. (A) RNA was isolated from serum-starved HeLa cells (lane 1) or 3T3 cells (lanes 2 to 7) that had been stimulated for 30 min with 15% CS. Prior to starvation cells were transfected with no DNA (lanes 1 and 2) or the DNA indicated above each lane. (B) RNA was isolated from serum-starved 3T3 cells (lane 1) or 3T3 cells stimulated for 30 min with 15% CS (lanes 2 to 4). Prior to starvation cells were transfected with the indicated plasmid DNA. Sizes are indicated (in base pairs).

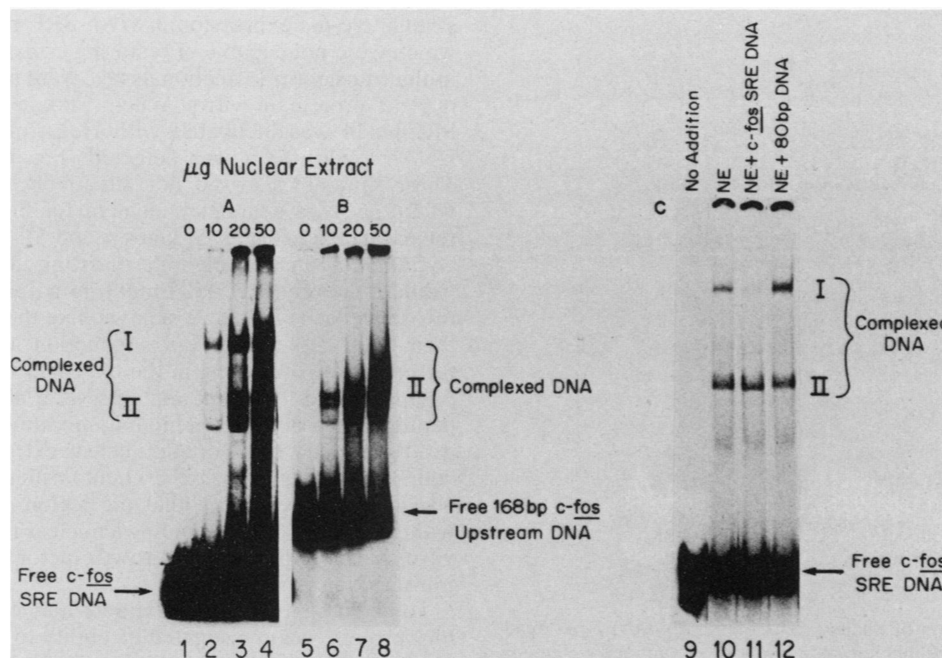


FIG. 4. Detection of an SRE-binding protein in HeLa cell nuclear extracts. $^{32}$P-labeled c-*fos* SRE (A) or $^{32}$P-labeled 168-bp c-*fos* upstream DNA (B) were incubated with increasing amounts of HeLa nuclear extract (0 to 50 μg) in the presence of poly(dI-dC)-poly(dI-dC) and pUC13 DNA as described in Materials and Methods. (C) $^{32}$P-labeled c-*fos* SRE (0.2 ng) was incubated with no extract (lane 9) or 20 μl of HeLa nuclear extract (NE) (lanes 10 to 12) in the presence of no competitor DNA (lanes 9 and 10), 10 ng of unlabeled 61-bp c-*fos* SRE DNA (lane 11), or 15 ng of unlabeled nonhomologous 80-bp DNA (lane 12). poly(dI-dC)-poly(dI-dC) and pUC13 DNA were added to all reaction mixtures as described in Materials and Methods. After incubation for 30 min, all binding reaction mixtures were fractionated on a 4% acrylamide gel, and the free and complexed DNA was visualized by autoradiography.

cells transfected with this plasmid [pF222(DSE)] were stimulated by the addition of 15% CS, significant accumulation of human c-*fos* mRNA was observed within 30 min (Fig. 3B, lanes 1 and 2).

**c-*fos* SRE specifically binds nuclear factors in vitro.** The results presented so far are consistent with the hypothesis that a *trans*-activating factor that mediates the effect of growth factors within the nucleus activates c-*fos* expression by binding to the dyad symmetry element. To identify this factor, we used a sensitive DNA gel electrophoresis assay that has recently been used successfully to detect proteins in crude nuclear extracts that have a high affinity for specific DNA sequences (3, 33, 37). The gel electrophoresis assay is based on the observation that DNA fragments that are bound to proteins migrate through low-ionic-strength polyacrylamide gels more slowly than unbound fragments. $^{32}$P-labeled c-*fos* SRE was mixed, in the presence of increasing amounts of unlabeled nonspecific competitor DNA, with a nuclear extract prepared from growth factor-stimulated HeLa cells that express high levels of c-*fos* mRNA (M. E. Greenberg and E. B. Ziff, unpublished observations). In the absence of nuclear extract, the c-*fos* SRE DNA migrated as a discrete band (Fig. 4A, lane 1). As increasing amounts of nuclear extract were added to the binding reaction mixture, several bands of slower mobility appeared (Fig. 4A, lanes 2 to 4). The intensity of the shifted bands increased proportionally with increased amounts of extract. DNA bands of slower mobility were not detected when the nuclear extract was pretreated with proteinase K (data not shown), suggesting that these bands represent protein-DNA complexes. The sequence specificity of the DNA mobility shifts was established by testing the ability of an excess of various unlabeled DNAs to complete the mobility shift of the $^{32}$P-labeled c-*fos* SRE. The addition of a 50-fold molar excess of unlabeled c-*fos* SRE fragment effectively abolished the mobility shift (Fig. 4C, compare lanes 10 and 11), whereas unlabeled DNA fragments of an unrelated sequence had no effect (lane 12). Not all the DNA bands of slower mobility resulted from the interaction of proteins with a specific DNA sequence. Band II (Fig. 4) appeared to result from a nonspecific protein-DNA interaction, as this band was not effectively competed with by either an excess of unlabeled DNA containing the SRE (Fig. 4C, lane 11) or an unrelated sequence (Fig. 4C, lane 12). In addition, band II was observed when the $^{32}$P-labeled c-*fos* SRE DNA was substituted for in the binding reaction mixture with an unrelated 168-bp c-*fos* upstream sequence (−119 to +48 bp) that spans the mRNA cap site (Fig. 4B, lanes 5 to 8). The specific mobility shift (band I) was not detected when nuclear extracts were incubated with the $^{32}$P-labeled 168-bp c-*fos* upstream sequence (Fig. 4B, lanes 5 to 8).

The observation (Fig. 4 to 6) that only a small percentage of the labeled c-*fos* SRE formed a complex with the protein factor in crude nuclear extracts suggests that this regulatory protein is present at low levels in HeLa cells. Increasing the amount of nuclear extract added to the binding reaction beyond 50 μg resulted in nonspecific protein-DNA interactions which obscured the visualization of the specific c-*fos*-SRE complex. Partial purification of the c-*fos* SRE-binding protein mitigates this problem. When an enriched preparation of the c-*fos* regulatory factor was used in the binding reaction, more than 50% of the labeled DNA was present in the complexed form (data not shown).

To assess the importance of the dyad symmetry element for in vitro binding of nuclear protein to the c-*fos* SRE, shorter oligonucleotides containing just the dyad symmetry

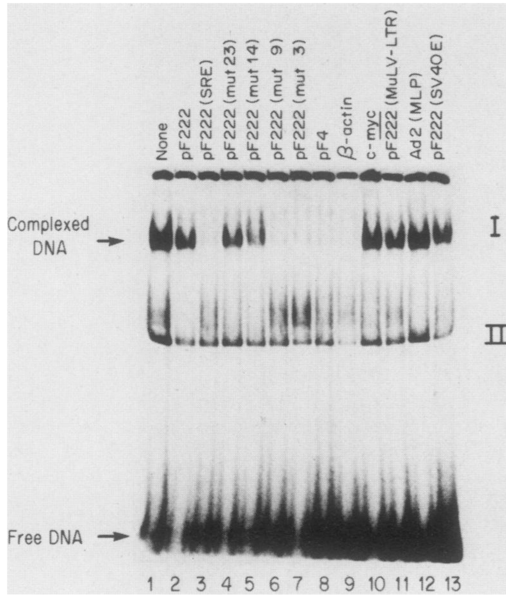


FIG. 5. Competition of nuclear factor binding to the c-*fos* dyad symmetry element. $^{32}$P-labeled 29-bp c-*fos* dyad symmetry element was incubated with HeLa nuclear extract in the presence of an excess of the unlabeled competitor DNAs shown above each lane. The molar ratio of competitor DNA to 29-bp c-*fos* DNA was 100:1. Competitor DNAs were linearized with an appropriate restriction enzyme prior to incubation in the binding reaction mixture. Binding reactions contained no competitor (lane 1) or 2 μg of competitor DNA. Competing DNAs included wild-type c-*fos* genomic plasmid pF4 (lane 8), rat β-actin genomic plasmid (lane 9), human c-*myc* genomic plasmid (lane 10), pF222 containing MoMLV LTR DNA nucleotides 7674 to 8118 (36) (lane 11), Ad2 major late promoter transcription factor-binding element inserted into pBR322 (PHB310) (lane 12), and pF222 containing the SV40 viral enhancer nucleotides 100 to 298 (41) (lane 13).

element were synthesized. The oligomer shown in Fig. 1B was $^{32}$P-labeled and tested for protein binding in the gel mobility shift assay. This 29-bp oligonucleotide is composed exclusively of the inverted palindrome sequence present within the c-*fos* SRE. We found that the 29-mer specifically bound a protein factor(s) present in HeLa nuclear extracts, causing a decrease in the migration of the oligomer (band I; Fig. 5, lane 1). The shift in mobility of the 29-mer (Fig. 5, lane 1, band I) was inhibited by a 100-fold molar excess of c-*fos* genomic plasmid pF4, which contains the inverted palindrome sequence (Fig. 5, lane 8), but not by the mutant plasmid pF222, which lacks the SRE (Fig. 5, lane 2). The DNA mobility shift was unaffected by the addition of double-stranded M13mp8 (Fig. 6, lane 1) but was completely blocked by M13mp8 containing the c-*fos* SRE (Fig. 6, lane 2). These experiments indicate that a protein factor present in crude nuclear extracts from serum-stimulated HeLa cells specifically interacts in vitro with the dyad symmetry element which is essential for growth factor inducibility of c-*fos* expression in vivo. In vitro methylation protection experiments (Z. Siegfried, M. E. Greenberg, and E. B. Ziff, data not shown; Michael Gilman, personal communication) and DNase I digestion studies (40) further demonstrate that a nuclear factor interacts directly with specific nucleotides within this region of dyad symmetry.

Through analysis of mutants we established a strict correlation between the ability of the dyad symmetry element to bind the nuclear protein factor(s) in vitro and its ability to stimulate c-*fos* expression in vivo. SRE mutants 14 and 23, which were not capable of restoring c-*fos* serum inducibility in the transient transfection assay, were also unable to bind nuclear protein in vitro. When $^{32}$P-labeled SREmut23 or SREmut14 was incubated with HeLa nuclear extract, no DNA mobility shift was detected (Fig. 6, lanes 3 and 5). These mutants also did not effectively compete with the wild-type dyad symmetry element for binding of the c-*fos* regulatory factor (Fig. 5, lanes 4 and 5). SRE mutants that contained a single-nucleotide insertion at both ends of the palindromic sequence (SREmut9) or a deletion remote from this sequence (SREmut3) behaved like the wild-type SRE in their ability to restore c-*fos* induction and bound protein factor in vitro, resulting in the expected mobility shift (Fig. 6, lanes 7 and 9). These mutants were also excellent competitors when added to a binding mixture of wild-type c-*fos* dyad symmetry element and nuclear extract (Fig. 5, lanes 6 and 7). Together, these experiments with mutant c-*fos* SREs provide strong evidence that the region of dyad symmetry within the c-*fos* SRE is binding a nuclear regulatory factor in vitro that is important for growth factor induction of c-*fos* transcription in vivo.

To begin characterizing the activities of the c-*fos* regulatory protein, we investigated its ability to bind to regulatory elements present in other viral and cellular genes. The first cellular sequences that we tested in the competition experiments were β-actin and c-*myc* proto-oncogene genomic clones. We have previously shown that β-actin gene transcription is activated over 50-fold in concert with c-*fos* transcription in response to a variety of different growth factors (16). c-*myc* transcription is also activated by growth factors (2, 16, 23), although not as rapidly as c-*fos* and actin. To test the possibility that the regulatory protein that binds to the c-*fos* dyad symmetry element might also bind to actin and c-*myc* genomic sequences, allowing coordinate activation of these genes, we tested whether actin and c-*myc* sequences competed with the $^{32}$P-labeled c-*fos* dyad symmetry element for interaction with the c-*fos* regulatory protein.

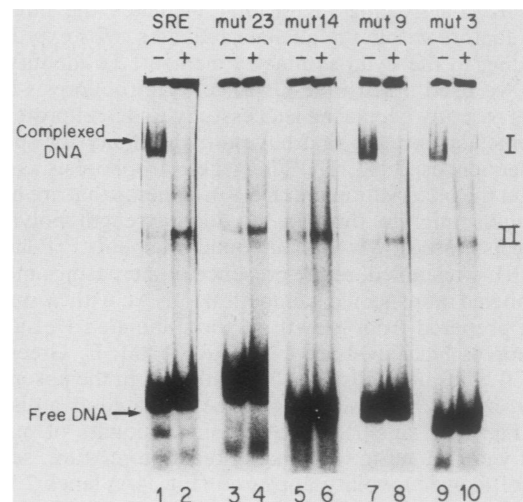


FIG. 6. Analysis of nuclear factor binding to mutant SRE DNA fragments. $^{32}$P-labeled 61-bp c-*fos* SRE (lanes 1 and 2) or mutant SRE DNA fragments (lanes 3 to 10) were incubated with HeLa nuclear extract and 1 μg of double-stranded M13mp8 DNA (lanes 1, 3, 5, 7, and 9) or 1 μg of M13mp8 DNA containing the 61-bp c-*fos* SRE (lanes 2, 4, 6, 8, and 10). After incubation for 30 min, reaction mixtures were fractionated as described in Materials and Methods. Complexed and free c-*fos* DNA was visualized by autoradiography.

The results of these competition experiments were striking; addition of an excess of a full-length rat β-actin genomic clone (32) containing approximately 1,000 bp of 5'-flanking sequence, effectively blocked binding to the c-*fos* dyad symmetry element (Fig. 5, compare lanes 2 and 9). In contrast, a human c-*myc* genomic clone (8) that also contains a long stretch of 5'-flanking sequences had no effect on the binding of the c-*fos* regulatory protein to the c-*fos* inverted palindromic sequence (Fig. 5, lane 10). We conclude from these experiments that the β-actin gene but not the c-*myc* gene contains a sequence element that binds the c-*fos* regulatory factor.

Apart from β-actin, all other sequences that were tested were unable to effectively compete for the binding of the c-*fos* regulatory protein to the inverted palindromic sequence. These include the MoMLV LTR (36) (lane 11), sequences upstream of the Ad2 major late promoter that have been found to bind a nuclear regulatory factor and activate Ad2 late viral transcription in vitro (3) (lane 12), the SV40 enhancer element which binds regulatory factors implicated in stimulating transcription from SV40 early and late genes (11) (lane 13), and the Ad2 E1a enhancer, a sequence that has been shown to enhance transcription of the viral E1a gene (17) (data not shown). These competition experiments suggest that the c-*fos* regulatory factor is distinct from the nuclear regulatory factors that bind to these viral enhancer sequences.

## DISCUSSION

A 56-bp sequence 330 bp 5' to the c-*fos* mRNA cap site is required for serum induction of c-*fos* transcription in a transient expression system (39). In the present study we have established by in vitro mutagenesis that a small region of dyad symmetry within this c-*fos* SRE is essential for growth factor stimulation. A single-nucleotide deletion in the dyad symmetry element was sufficient to abolish serum stimulation of c-*fos* expression. Using a DNA mobility shift assay, we identified a nuclear factor that binds to the c-*fos* dyad symmetry element in vitro. Experiments with several mutant c-*fos* SREs established a strict correlation between nuclear factor binding to the dyad symmetry element and in vivo inducibility. Analysis of additional c-*fos* SRE mutants will be required to strengthen this correlation, but the present experiments support the conclusion that the nuclear factor that binds to the c-*fos* SRE in vitro may mediate growth factor activation of c-*fos* transcription in vivo. The DNA mobility shift assay described here provides a basis for the purification of this c-*fos* regulatory protein.

Our analysis of deletion mutations that disrupt one end of the c-*fos* DSE indicate that an intact symmetrical element is critical for regulatory factor binding. A systematic examination of point mutations to determine which nucleotides within the symmetrical portion of the c-*fos* DSE are important has not yet been done. However, comparison with the homologous DSEs within the β- and γ-actin genes from several species (E. Keller, personal communication) indicates that 12 nucleotides at the center of the DSE are generally conserved and are likely to be important for factor binding. Methylation protection experiments with the c-*fos* DSE (40; M. Gilman, personal communication; Z. Siegfried, M. E. Greenberg, and E. B. Ziff, unpublished observations) support this possibility, indicating that the four guanine residues within the 12-nucleotide DSE core (Fig. 1B) are sites of DNA-protein contact. Both methylation protection experiments and sequence comparison of the c-*fos* and actin DSEs suggest that nucleotide symmetry at the ends of the DSEs is not critical for protein binding and that point mutations are tolerated. Point mutations within the core region which increase the extent of symmetry within the element are also permissive for nuclear factor binding (M. E. Greenberg, unpublished observations).

The symmetry of the SRE binding region (Fig. 1) raises the possibility that the c-*fos* transcriptional activating factor binds to DNA as a dimer. The available evidence suggests that such a regulatory factor dimer is likely to bind within the major groove on one side of the double helix. This is consistent with interference experiments, in which methylation of guanine nucleotides at sites within the major groove blocks protein binding (26). It has been shown (40) that methylation of any of the four guanine nucleotides within the core of the DSE inhibits c-*fos* regulatory factor binding. Since these two pairs of guanine nucleotides are separated by six nucleotides at the center of the DSE, the sites of protein contact with each arm of the DSE must occur on the same side of the double helix. This model is further supported by DNase I protection experiments (40) and by our observation that deletion of one of the guanine nucleotides in the DSE core region results in a loss of DNA-c-*fos* regulatory protein interaction in vitro. The finding that c-*fos* mutants in which one arm of the region of symmetry is altered are also nonresponsive to serum stimulation suggests that if dimer binding does occur, it could be a requirement of activation. In this view, simple binding of the monomer to one half of the symmetry element either would not take place or, if it does, would not be sufficient for c-*fos* activation.

Other models for c-*fos* regulatory protein-DNA interactions can be envisioned, and preliminary experiments suggest that the interaction of proteins with the c-*fos* SRE may be complex. For example, if the SRE-binding protein were the only nuclear factor mediating serum stimulation of c-*fos* transcription, the binding activity might be expected to be absent from extracts of serum-starved HeLa cells. However, the nuclear factor was present not only in extracts from serum-stimulated HeLa cells but also in extracts prepared from serum-starved or asynchronously growing cells, neither of which transcribes the c-*fos* gene in vivo (M. E. Greenberg and E. B. Ziff, unpublished observations). Perhaps additional interacting regulatory factors play a role in c-*fos* transcriptional activation. The interaction between these regulatory proteins might not be recapitulated in the in vitro binding experiments. Alternatively, the c-*fos* regulatory factor defined by the SRE mobility shift could be the key regulatory protein. The biochemical feature that distinguishes the ability of the nuclear factor to bind to the SRE and activate c-*fos* transcription in serum-stimulated HeLa cells as opposed to serum-starved cells could be lost in vitro. For example, as we have suggested previously (15), a growth factor-induced phosphorylation or proteolytic cleavage event may be required to promote the interaction of the c-*fos* regulatory factor with the SRE in vivo. If such a modification were activated in vitro during preparation of the nuclear extract, one could reconcile the observation that extracts from growth factor-arrested HeLa cells contain a nuclear factor that binds to the SRE. To distinguish between various models for c-*fos* transcriptional activation and to specifically establish the function of the nuclear factor that binds to the c-*fos* dyad symmetry element, it will be important to purify this nuclear protein and test its effect on c-*fos* transcription in vitro.

Our initial characterization of the c-*fos* dyad symmetry element-binding protein in crude nuclear extracts has re-

vealed several important properties of the protein. Most significant is the observation that the c-*fos* regulatory protein also binds to a sequence element within the β-actin gene. Sequence analysis and comparison of β-actin genes from several different species, including humans, rats, chickens, and *Drosophila melanogaster* reveals a dyad symmetry element that is homologous to the c-*fos* symmetry element (1, 26, 31; Keller, personal communication). It appears likely that the presence of the β-actin dyad symmetry element in the rat genomic clone accounts for its ability to compete for binding of the c-*fos* regulatory factor. This raises the possibility that the coordinate regulation of c-*fos* and actin expression by growth factors (15, 16) is mediated at least in part by a common nuclear transcription factor which binds to a dyad symmetry element in the 5′-flanking region of these genes and activates their transcription. By differential screening of a cDNA library prepared from 3T3 cells stimulated with serum in the presence of a protein synthesis inhibitor, a set of five additional genes has been identified that are synchronously induced by growth factors with the same time course as c-*fos* and actin (24). An interesting possibility is that all of these genes contain the dyad symmetry element within their 5′-flanking regions and that their expression is controlled by the same regulatory factor. The c-*myc* gene, which in contrast to actin appears not to bind the c-*fos* regulatory factor, is activated with kinetics that are distinct from those seen for c-*fos* and actin (16, 30), consistent with its being controlled by a different regulatory pathway.

The 5′ c-*fos* SRE displays significant sequence homology with several viral enhancers including the MoMLV LTR, the SV40 enhancer, and the polyomavirus enhancer (9, 34, 39). Despite the fact that this homology extends within the region of dyad symmetry, none of the viral enhancer sequences were effective at competing for binding of the c-*fos* regulatory protein. The nuclear factors that bind to these viral enhancers are therefore probably distinct from the c-*fos* dyad symmetry element-binding factor. Whether viral enhancer sequence homology within the c-*fos* SRE is functionally significant remains to be determined. Possibly the nuclear factors that regulate the activity of viral enhancers are structurally related to the c-*fos* regulatory protein. Alternatively, other c-*fos* regulatory proteins may exist that specifically recognize the common sequences within the c-*fos* SRE and the viral enhancers. This possibility is consistent with the observation that c-*fos* transcription has been found to be activated by a variety of agents in addition to growth factors, some of which appear to act by distinct mechanisms (17, 29). A further possibility is that viral enhancer-homologous sequences within the c-*fos* SRE bind c-*fos* transcription-repressing factors.

Further functional analysis of the 5′ regulatory region of the c-*fos* gene and identification of the nuclear factors that interact with these sequences should give increased insight into the biochemical mechanisms by which growth factors and other transmembrane stimuli activate and repress gene expression. The characterization of c-*fos* regulatory factors is of particular interest because of the role of the c-*fos* gene in tumorigenesis. The factors that activate and repress c-*fos* transcription may themselves have the capacity to cause oncogenesis under certain circumstances and could constitute a new class of oncogenes.

During the preparation of this manuscript for publication another report (40) was published that describes the identification of a nuclear protein that interacts with the c-*fos* dyad symmetry element.

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