MOLECULAR AND CELLULAR BIOLOGY, Aug. 1990, p. 4243–4255
0270-7306/90/084243-13$02.00/0

Vol. 10, No. 8

Transcriptional Activation and Repression by Fos Are Independent Functions: The C Terminus Represses Immediate-Early Gene Expression via CArG Elements

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Received 19 March 1990/Accepted 11 May 1990

The Fos-Jun complex has been shown to activate transcription through the regulatory element known as the AP-1 binding site. We show that Fos down regulates several immediate-early genes (c-*fos*, Egr-1, and Egr-2) after mitogenic stimulation. Specifically, we demonstrate that the target for this repression is a sequence of the form CC(A/T)$_6$GG, also known as a CArG box. Whereas Fos bound to the AP-1 site through a domain rich in basic amino acids and associated with Jun via a leucine zipper interaction, mutant Fos proteins lacking these structures were still capable of causing repression. Furthermore, Jun neither enhanced nor inhibited down regulation by Fos. Critical residues required for repression are located within the C-terminal 27 amino acids of c-Fos, since v-Fos and C-terminal truncations of c-Fos did not down regulate. In addition, transfer of 180 c-Fos C-terminal amino acids to Jun conferred upon it the ability to repress. Finally, Fra-1, a Fos-related protein which has striking similarity to Fos in its C-terminal 40 amino acids, also down regulated Egr-1 expression. Thus, Fos is a transcriptional regulator that can activate or repress gene expression by way of two separate functional domains that act on distinct regulatory elements.

The behavior of eucaryotic cells is largely determined by complex interactions that occur between the cell and agents, such as polypeptide growth factors, hormones, and neurotransmitters, present in the extracellular environment. Such stimulatory agents elicit long-term changes in cellular phenotype by altering gene expression. Over the last few years, a set of regulatory genes has been defined that functions in coupling the rapid biochemical or biophysical signals initiated by stimulation to phenotypic responses. Transcription of these so-called cellular immediate-early genes is induced extremely rapidly and occurs in the presence of protein synthesis inhibitors (44, 45). Several of these genes encode proteins that are known or putative transcription factors (8, 10, 32, 34, 37, 40, 42, 44, 45, 47, 48, 52, 53, 63, 70, 71, 78, 79, 84). They are thought to initiate an intricate cascade of gene regulation that results in specific biological responses depending on the differentiated state of the stimulated cell and the nature of the stimulus.

In the context of cellular proliferation, a necessary aspect of the immediate-early response is that the expression of genes encoding transcription factors must be repressed rapidly after induction by mitogens. Although the activation of these genes may be required for the initiation of cell growth, their continued expression would be detrimental and could lead to uncontrolled cell division. Indeed, the viral homologs of two cellular immediate-early genes, c-*fos* and c-*jun*, are transforming agents (22, 24, 51).

The c-*fos* gene was initially identified as the cellular homolog of the v-*fos* oncogene, present in two osteogenic murine sarcoma viruses (MuSVs) (22, 24). Both viral and cellular forms of *fos* induce bone tumors in vivo and transform NIH 3T3 cells in vitro (reviewed in reference 16). c-*fos* encodes a nuclear phosphoprotein that forms a noncovalent complex via a leucine zipper interaction with the gene product of c-*jun* (18, 23, 27, 33, 39, 43, 65, 67). Together, Fos and Jun activate transcription through the *cis*-acting DNA element known as the AP-1 binding site (4, 9, 17, 62, 66, 72, 76). Much attention has focused on the mechanism of gene activation by Fos and Jun. Less is known about Fos-mediated repression. It has been suggested that Fos is a negative regulator of its own promoter (73, 82) and that this affect is mediated by an AP-1 site in the 5′ region of the c-*fos* gene (73). However, others (38) have suggested that the c-*fos* serum response element (SRE) is the target for autoregulation.

We were interested in determining whether Fos regulates expression of other cellular immediate-early genes. We chose to study expression of the early growth factor-inducible gene, Egr-1 (78, 79), whose kinetics of induction and subsequent repression are similar to those of c-*fos*. Egr-1, also known as NGFI-A (52), Krox 24 (47), zif268 (10), and TIS 8 (48), is induced by numerous agents that initiate proliferation or differentiation and may also be involved in long-term potentiation in the hippocampus (15). This gene encodes a transcription factor of the C_2H_2 zinc finger class that activates transcription through the target sequence CG CCCCCGC (6, 11; A. Gashler, D. Gius, and V. P. Sukhatme, unpublished observations). Collectively, these data suggest that Egr-1 is likely to serve as a nuclear intermediary in a broad range of signal transduction processes.

In this report, we first show that Egr-1 expression is repressed by Fos after serum stimulation. This model system was then exploited to define in detail both the *cis* and *trans* requirements critical for Fos-mediated repression. Our results provide clear-cut evidence that transcriptional activation and repression by Fos are independent functions. In addition, we find that a Fos-related antigen is also able to repress immediate-early gene expression.

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MATERIALS AND METHODS

Cell culture. NIH 3T3 cells were maintained in Dulbecco modified Eagle medium supplemented with either 0.5, 10, or 20% fetal calf serum (FCS; GIBCO Laboratories) and gentamicin (40 μg/ml). PC12 cells were grown in Dulbecco modified Eagle medium with 5% horse serum and 10% FCS.

Plasmids and DNA preparation. Plasmids $pA_{10}CAT2$, which contains the simian virus 40 minimal promoter, and pSV2CAT are enhancer tester and control vectors that contain the chloramphenicol acetyltransferase (CAT) gene; pCAT3m is a promoter tester plasmid that contains the CAT gene but no simian virus 40 promoter sequences (29). pEgr-1 6.6 has a 6.6-kilobase-pair (kb) *Xba*I fragment cloned into pUC18, which contains the Egr-1 promoter and open reading frame (81). pEgr-1 P1.2 contains sequences from −957 to +248 base pairs (bp) relative to the Egr-1 promoter transcriptional start site. The Egr-1 promoter contains six CArG boxes, and seven additional deletion mutants were constructed by using oligomers and polymerase chain reaction, with the 3′ endpoint for every deletion being +65 bp (X. Cao, C. Tsai-Morris, and V. P. Sukhatme, unpublished data). pE425 contains sequences −425 to +65, including all six CArG boxes; pE395, pE359, pE342, pE125, pE98, and pE70 contain five, four, three, two, one, and no CArG boxes, respectively. The 5′ end is indicated in the plasmid name relative to the transcriptional start site. Plasmids ptkSRE1 and ptkSRE2 contain a synthetic 40-bp oligonucleotide, containing the most distal Egr-1 CArG element (CTAGAGGATCCGAAACGCCATATAAGGAGCAGG AGATCTT). It was synthesized along with its complementary strand on an Applied Biosystems oligonucleotide synthesizer. The oligonucleotides were designed to contain an *Xba*I site at both ends. The two strands were annealed and then inserted into the *Xba*I site of pBLCAT2 (50) to generate ptkSRE1 and a double insert, ptkSRE2. A mutant oligonucleotide (CTAGAGGATCCGAAACGGGATATAACCAGC AGGAGATCTT) was used to generate ptkSREm in a similar manner. CMV-fos and CMV-jun contain the rat c-*fos* and c-*jun* cDNAs expressed from the cytomegalovirus (CMV) early promoter (17). CMV-R is the expression vector containing no open reading frame.

pFos363 contains the c-*fos* promoter placed 5′ of CAT and was a gift from R. Prywes. p29FosCAT has a 29-bp fragment of the c-*fos* promoter which contains the SRE cloned into pBLCAT2 and was a gift from M. Getz (77).

Construction of the *fos* mutant plasmids shown in Fig. 5 and 6 has been previously described (27). A detailed description of the construction of the cDNAs encoding the chimeric proteins FFFJ, JJFF, and JJJF is given by D. R. Cohen and T. Curran (unpublished data). Briefly, three unique restriction sites were introduced into the c-*fos* (rat) and c-*jun* (rat) cDNA sequences (by site-directed mutagenesis) at the 5′ end of the sequence encoding the basic region, between the sequences encoding the basic region and the leucine zipper, and at the 3′ end of the sequence encoding the leucine zipper. Thus, each protein was considered to consist of four regions delineated by the positions of these restriction sites. These sites, together with a unique *Bam*HI site at the 3′ end of each cDNA, facilitated the excision of small cassettes containing one or more of the four domains. The cassettes were exchanged between the two cDNAs to create genes encoding chimeric proteins, which were designated by a four-letter code indicating the origin of each domain of that protein. These constructs were then subcloned into the pCMV vector for use in transient transfection assays. All constructs were tested for protein expression by immunoprecipitation in COS cells and were found to give levels comparable to those obtained with full-length Fos (F. J. Rauscher III and T. Curran, unpublished data).

Transfections and CAT assays. Transfections were performed by calcium phosphate-mediated precipitation, with a total of 30 μg of DNA added to 0.7×10^6 cells per 100-mm dish. For cotransfection experiments, 2 μg of CAT tester DNA was transfected with carrier DNA (pUC9 or CMV-R, a CMV expression plasmid, which gave identical results) or a *trans*-acting effector to equal 30 μg of DNA. Cells were incubated with precipitates overnight and harvested 24 h later. Cell extracts were prepared, and CAT assays were performed as previously described (29). The amount of acetylation was determined by counting the acetylated and nonacetylated forms separated by ascending thin-layer chromatography. All assays were performed in duplicate and repeated at least twice. In the experiments with c-Fos deletion mutants and Fos-Jun chimeras, the construct CMV-βgal was included as an internal control, since in preliminary experiments it was noted that the CMV promoter is not affected by Fos expression.

In vitro binding assays. The Egr-1 sixth CArG box probe was made by annealing two 40-bp complementary oligomers (see above) and ^{32}P end labeled. Cell nuclear extracts were made from BALB/c 3T3 cells rendered quiescent in 0.5% FCS or after serum stimulation for 2 h (25; Cao et al., unpublished data). The binding reaction was performed by incubating 1 to 2 ng of probe with various amounts of 3T3 cell nuclear extracts in binding buffer (25 mM HEPES-NaOH [pH 7.9], 0.1 M KCl, 0.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 20% glycerol) and 2 μg of poly(dI-dC) at 30°C for 30 min. The reaction mixture was loaded onto a 4% polyacrylamide gel and electrophoresed at 200 V for 1.5 to 2 h. Gels were dried and put on film overnight.

RESULTS

c-Fos represses immediate-early gene expression. Transient transfection assays were used to determine the response of the Egr-1 promoter to high serum and cotransfection with a c-Fos expression plasmid (CMV-fos). This plasmid (76) was cotransfected into NIH 3T3 cells with pEgr-1 P1.2, a plasmid that contains 1 kb of Egr-1 promoter sequences placed upstream of the CAT gene (81; Cao et al., unpublished data). NIH 3T3 cells were cotransfected with pEgr-1 P1.2 and control DNA (a CMV expression parent plasmid, CMV-R) or CMV-fos. After 40 h in 0.5% FCS, 20% FCS was added. Cells were harvested after 3, 6, and 9 h in high serum and assayed for Egr-1 expression by monitoring CAT activity. Quiescent NIH 3T3 cells showed no change in the basal level of Egr-1 expression when transfected with control DNA (12-fold normalized to the level of $pA_{10}CAT2$ containing a minimal simian virus 40 promoter) or with the CMV-fos plasmid (13-fold) (Fig. 1). In cotransfection assays with CMV-fos, the level of Egr-1 expression progressively decreased by 1.5-, 3.1-, and 7-fold, respectively (Fig. 1). Since CAT protein is relatively stable, this decrease in promoter activity would be more pronounced if transcription were assessed by an RNA assay.

We also examined the effect of transfecting different concentrations of the CMV-fos expression vector. For simplicity, the transfections were maintained in 10% FCS through the experiment, since the results were identical to those obtained for the 9-h time point after serum induction

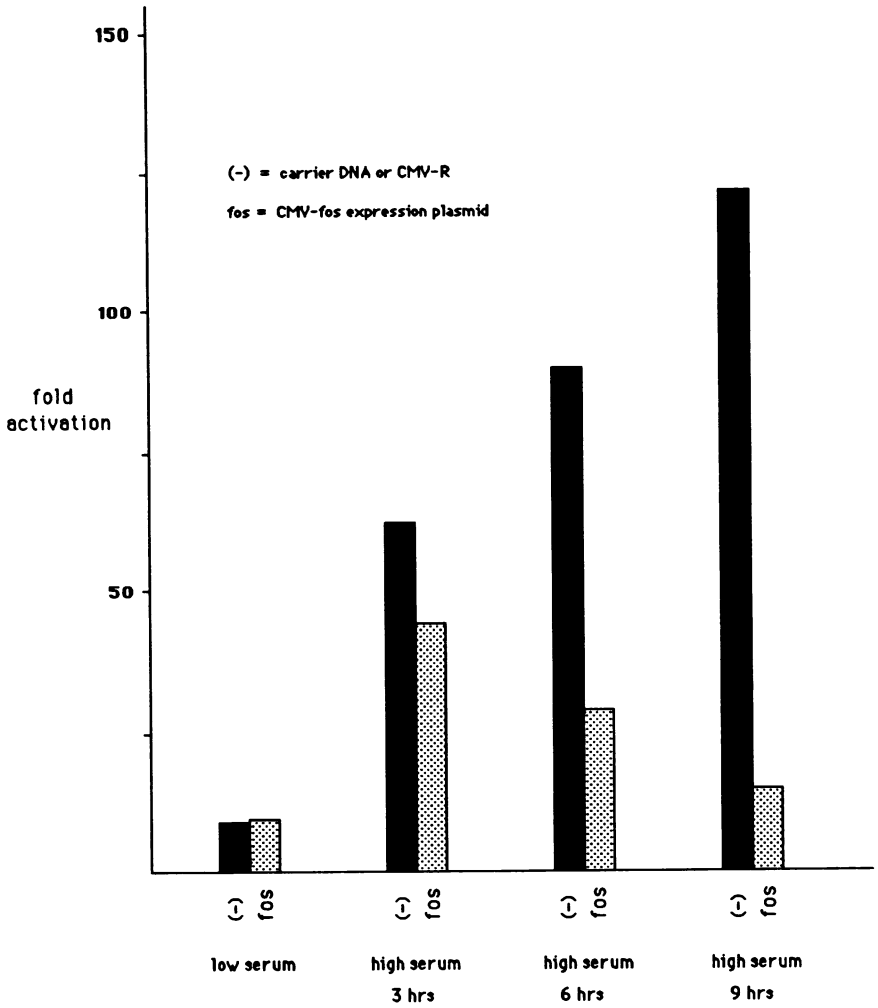


FIG. 1. Activation and Fos-mediated repression of Egr-1 expression at 3, 6, and 9 h after serum stimulation of quiescent cells. NIH 3T3 cells were transfected with 2 μg of pEgr-1 P1.2 and either 28 μg of carrier DNA or 10 μg of CMV-fos and 18 μg of carrier. Cells were maintained in 0.5% FCS until 40 h posttransfection, after which 20% FCS was added and cells were harvested at the indicated time points. Data are presented as percent acetylation normalized to that of $pA_{10}CAT2$. Results represent the average fold activation of at least two separate transfections performed in duplicate. The results of individual transfection varied by less than 25%. Assays were performed as described in the text.

(Fig. 2; Table 1). A 2-μg sample of CAT plasmid (pEgr-1 P1.2) was cotransfected with 2, 6, 10, 16, 22, and 28 μg of CMV-fos, using the CMV empty cassette vector as a filler for carrier DNA. Increasing amounts of cotransfected c-*fos* resulted in a progressive decrease in the level of Egr-1 expression (Fig. 2). These results suggest that Fos-mediated repression of Egr-1 is a titratable nonlinear function of the c-*fos* concentration.

To determine whether c-*fos* repression is specific for fibroblasts in the context of mitogenic stimulation, we examined PC12 cells, in which nerve growth factor (NGF) induces a dramatic induction of c-*fos* (19, 30, 41) and Egr-1 expression (52, 78) with a concomitant induction of neuronal differentiation (83). In cotransfection experiments, a 3.5-fold reduction of Egr-1 expression was observed with CMV-fos (Table 1). These assays were also repeated in low serum, and as seen in NIH 3T3 cells, no change in the level of expression was observed (data not shown). In the presence of NGF, a 3-fold increase in Egr-1 promoter activity was noted, whereas addition of CMV-fos resulted in a 6-fold repression (Table 1). These results are similar to those obtained in NIH 3T3 fibroblasts, suggesting that repression by c-Fos is not restricted to a single cell type and may operate in the context of cellular differentiation. We also examined the effect of the CMV-fos plasmid on two other cellular immediate-early genes, c-*fos* and Egr-2 (7, 8, 37). We tested c-*fos* (pFos363) and Egr-2 (pEgr-2 P16; 64a) and observed down regulation (three- and fourfold, respectively) (Tables 1 and 2). These results suggest a broad role for c-Fos in the negative regulation of cellular immediate-early genes.

The target sequence for c-Fos repression is the CArG box. The Egr-1 upstream regulatory region contains six CArG boxes (55, 56), whereas a single such element is present within the SRE (28, 31, 80) of the c-*fos* promoter (81). By using deletion mutants of the Egr-1 promoter, these elements can be shown to mediate both serum and 12-*O*-tetradecanoylphorbol-13-acetate induction (12; Cao et al., unpublished data). To dissect the *cis*-acting DNA element(s) involved in Fos-mediated repression, we used this series of deletions. These constructs progressively remove sequences upstream of the transcriptional start site until each of the Egr-1 CArG boxes is deleted and only a minimal Egr-1

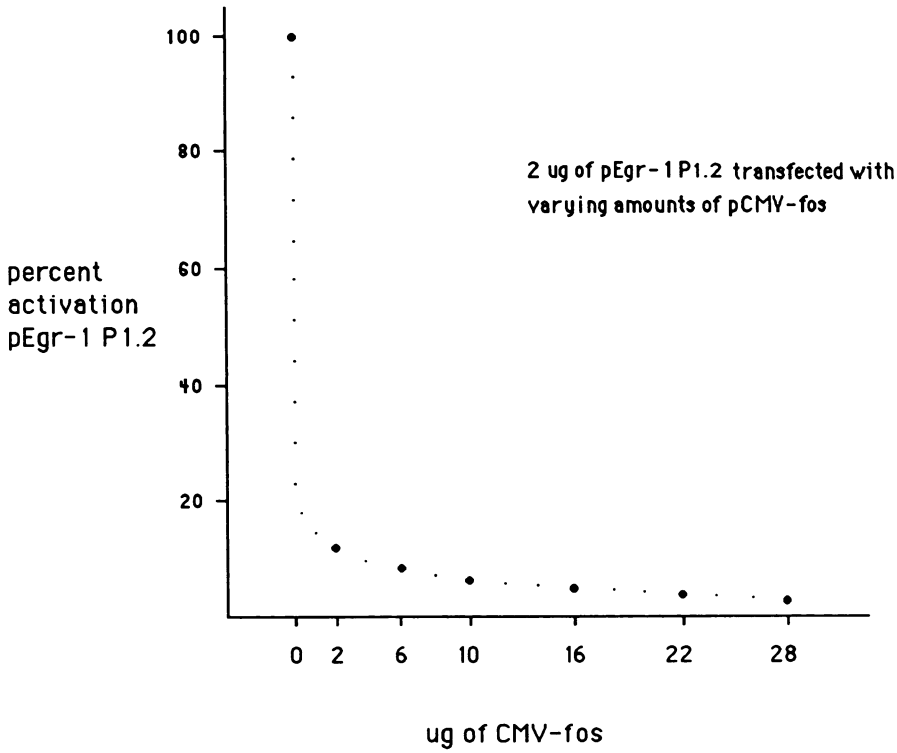


FIG. 2. Effects of increasing concentrations of CMV-fos on Egr-1 expression in NIH 3T3 cells maintained in 10% FCS. For competition experiments, 2 μg of CAT tester plasmid (pEgr-1 P1.2) was cotransfected with increasing amounts of CMV-fos (2, 6, 10, 16, 22, and 28 μg) and a corresponding amount of carrier DNA (CMV empty cassette vector) to equal a total of 30 μg. Data are presented as percent acetylation normalized to that of $pA_{10}CAT2$ (29).

promoter remains (Fig. 3). Plasmids pE425, pE395, pE359, pE342, pE125, pE98, and pE70 contain six, five, four, three, two, one, and no Egr-1 CArG boxes, respectively. The highest levels of both serum induction and c-Fos-mediated repression were observed for pEgr-1 P1.2 and pE425, and a progressive decrease in each was seen as individual CArG boxes were removed (Fig. 4). In particular, we can compare the results for pE98, which contains a single CArG box (threefold repression), with those obtained for pE70. However, these experiments do not exclude the possibility that there are other non-CArG box elements as targets for repression.

TABLE 1. Fos inhibition of Egr-1 and Egr-2 expression in NIH 3T3 and PC12 cell lines

Plasmid	Relative CAT expression[a]					
	NIH 3T3			PC12		
	Serum	Carrier DNA	c-Fos	NGF	Carrier DNA	c-Fos
$pA_{10}CAT2$	LS	1	ND	−	1	ND
pSV2CAT	LS	53	ND	−	18	ND
pEgr-1 P1.2	LS	12	13	−	38	11
pEgr-1 P1.2	HS	143	13	+	108	19
pEgr-2 P16	HS	13	3	−	ND	ND

[a] Normalized to $pA_{10}CAT2$ levels. Assays were performed at 40 h post-transfection, and the average fold activation of several assays is shown. Results from individual transfections varied by less than 25%. A 2-μg sample of tester plasmid was transfected with either 28 μg of carrier DNA or 10 μg of CMV-fos and 18 μg of carrier DNA. LS, Los serum (0.5% FCS); HS, high serum (10% FCS); ND, Not determined. pEgr-2 P16 contains the Egr-2 upstream regulatory region placed upstream of CAT.

To establish definitively whether the CArG element is the target for down regulation, an oligonucleotide containing an Egr-1 CArG box (centered at position −402) was cloned into pBLCAT2, a plasmid containing the minimal thymidine kinase gene promoter placed directly upstream of CAT (50). (There was no particular reason for using the sixth Egr-1 CArG element versus any other.) ptkSRE1 (single insert) and ptkSRE2 (double insert) were expressed at levels four- and eightfold above pBLCAT2. When cotransfected with CMV-fos, 3- and 5.3-fold repression was observed (Fig. 4). Finally, a mutant Egr-1 CArG box (CC and GG interchanged) was used to create ptkSREm. ptkSREm showed neither serum induction nor Fos-mediated transrepression (Fig. 4). We examined whether the c-*fos* SRE alone cloned into pBLCAT2 (p29fosCAT) (77) was a target sequence for c-Fos-mediated repression. Upon cotransfection with CMV-fos, down regulation (fivefold) was observed (Table 2). These results identify a CArG element as the primary target for Fos-mediated repression and indicate that the extended

TABLE 2. Fos inhibition of c-*fox* expression in NIH 3T3 cells

Plasmid	Relative CAT expression[a]	
	Carrier DNA	c-Fos
pBLCAT2	1	1
pSV2CAT	88	ND
pFos363	12	4
p29FosCAT	3	0.5

[a] Normalized to pBLCAT2 levels. Assays were performed at 40 h post-transfection in 10% FCS, and the average fold activation of several assays is shown. ND, Not determined.

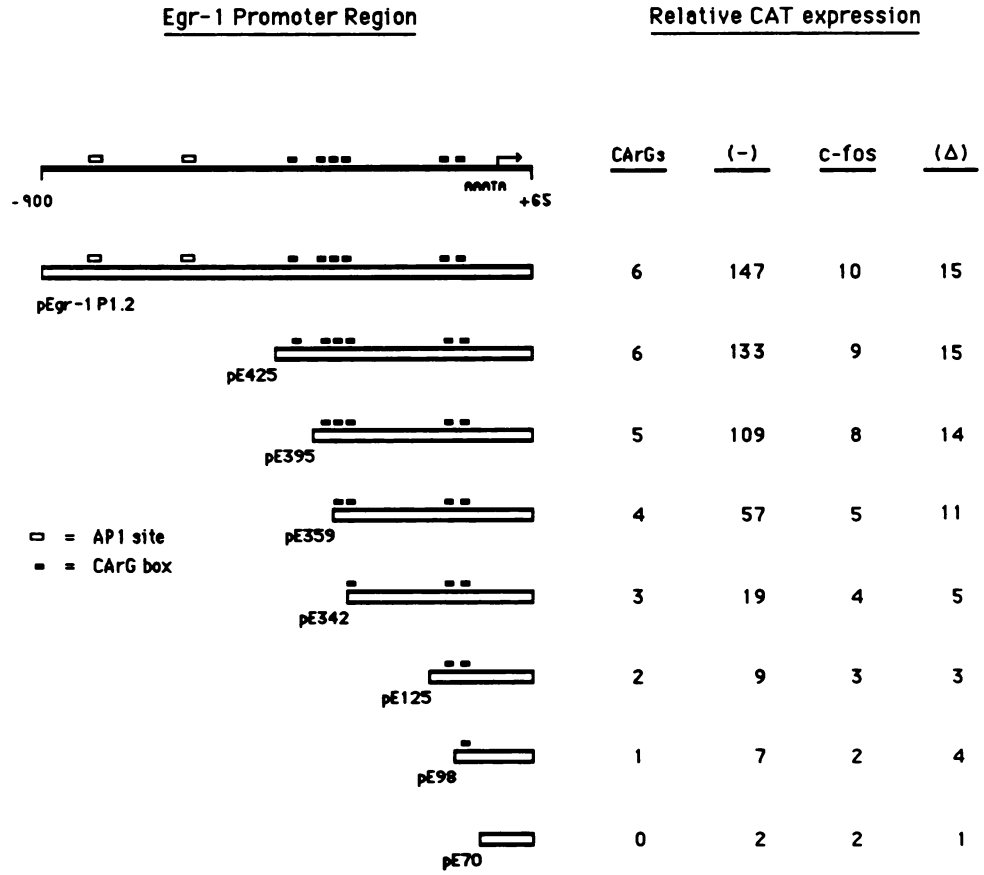


FIG. 3. Effects of c-Fos repression on Egr-1 promoter deletions which progressively remove the six CArG boxes present upstream of the Egr-1 TATA box and transcriptional start site. A series of polymerase chain reaction-generated deletion mutants was constructed as described in Materials and Methods and placed upstream of pCAT3m. These plasmids were transfected in duplicate into NIH 3T3 cells maintained in 10% FCS and harvested 40 h later. Cells were cotransfected with carrier (−) or with CMV-fos (c-fos) DNA to a total of 30 μg. The level of c-Fos-mediated repression is presented as the ratio of Egr-1 expression with carrier to CMV-fos (Δ). Cotransfections were performed at concentrations varying from 5.0×10^5 to 3.0×10^6 cell per 100-mm plate. No change in the extent of c-Fos-mediated repression was observed at various cell densities. The average levels of expression of the Egr-1 deletion mutants are shown normalized to $pA_{10}CAT2$ levels.

dyad symmetry present in the c-*fos* SRE is not critical for this effect.

c-*fos* repression is independent of c-*jun*. Since Fos and Jun interact to activate transcription, we examined whether Jun would cooperate with Fos to repress Egr-1 expression. Initially, we transfected the Egr-1 promoter plasmid with control DNA or with CMV-jun to determine whether Jun would repress Egr-1 expression. No difference in the expression levels of the Egr-1 promoter was observed with CMV-jun (Table 3). Next, we transfected both CMV-fos and increasing concentrations (0 to 18 μg) of CMV-jun; no additional repression of Egr-1 expression was observed (Table 3).

These data suggest that repression of gene transcription by Fos does not require a cooperative interaction of Fos and Jun. To address directly the role of binding to the AP-1 site and dimerization with Jun or with Jun-related proteins in repression, we examined the properties of mutant c-*fos* genes in the repression assay. Fos (179-193) contains a 45-bp deletion which removes the distal two heptads of the leucine zipper (27). Fos L5-P contains a proline substitution for the fifth leucine in the zipper (27) (Fig. 5). Both of these constructs encode Fos proteins that do not dimerize with Jun or interact with the AP-1 binding site (27). Strikingly, Fos-mediated repression was observed with both of these Fos mutants (Fig. 5). Similarly, Fos (139-145), which has a deletion in the basic region and although capable of dimerization with Jun cannot bind to the AP-1 site, repressed Egr-1 transcription as well as wild-type Fos. Thus, dimerization with Jun and binding to the AP-1 site are not required for the down-regulation function of Fos.

The C terminus of c-Fos is necessary and sufficient for repression. To identify the region of Fos required for Egr-1 down regulation, we tested six c-Fos deletion mutants (27) (Fig. 5). An N-terminal deletion mutation of Fos (Fos 102-380) demonstrated the same level of down regulation as full-length Fos (Fos 1-380). Five C-terminal truncation mutations were constructed to progressively remove increasing amounts of carboxy-terminal amino acids. In cotransfection experiments with the Egr-1 reporter plasmid, Fos 1-258 was unable to repress Egr-1 expression. The remaining four C-terminal deletions were also unable to down regulate Egr-1 expression (Fig. 5). A series of fine C-terminal c-Fos truncations was used to define the critical amino acids required for repression. Deletion of as little as 27 amino acids led to a marked fall in repression (sixfold for wild type to twofold; Fig. 6). Furthermore, construct FBJ v-fos also failed to repress. This construct contains sequences which differ from those in c-Fos in the last 48 amino acids. These results indicate that critical sequences necessary for repres-

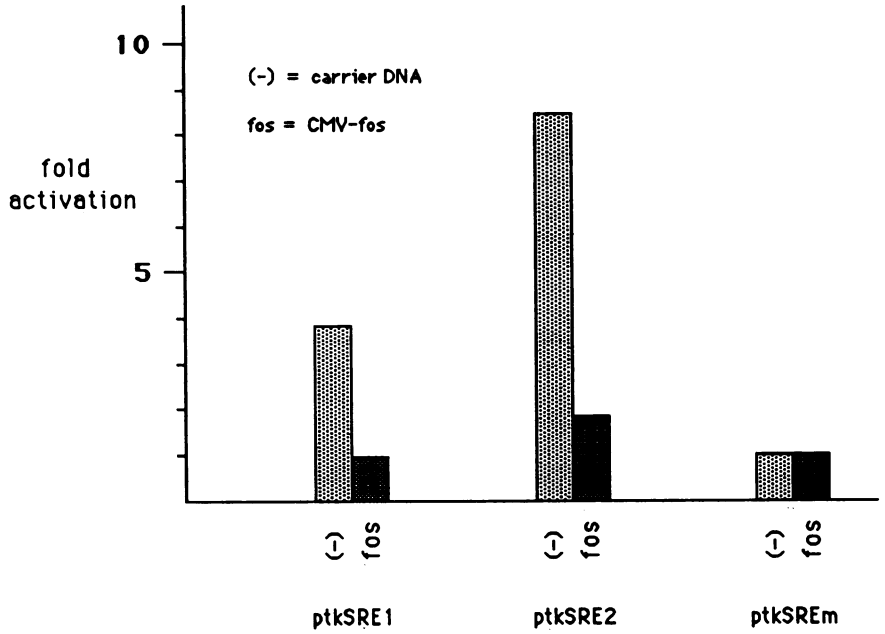


FIG. 4. Identification of the target sequence for c-Fos-mediated Egr-1 repression. ptkSRE1 and ptkSRE2 contain a single and a double insert, respectively, of a 40-bp oligomer that contains the most distal (sixth) Egr-1 CArG box cloned into the *Xba*I site of pBLCAT2. ptkSREm has a 40-bp mutant oligomer in pBLCAT2 that contains a mutant sixth Egr-1 CArG box (see Materials and Methods). pBLCAT2 contains the minimal thymidine kinase gene promoter placed upstream of CAT. Transfections were in NIH 3T3 cells maintained in 10% FCS. A 2-μg sample of pEgr-1 P1.2 was transfected with either 28 μg of carrier DNA or 10 μg of CMV-fos and 18 μg of carrier DNA. Results represent the average fold activation normalized to that of pBLCAT2.

sion are located within the C-terminal 27 amino acids of Fos. These results with the extensive series of deletion Fos constructs as well as those obtained with the leucine zipper and DNA-binding domain mutants also served as controls to show that the Fos repression effect that we observed is not due to toxicity from the CMV expression vectors. Moreover, as discussed in Materials and Methods, protein levels generated from these mutants in COS cells were comparable (within a factor of 2 except for the DNA-binding mutant product, which showed poor reactivity with anti-Fos antibody). Fos-mediated repression was also observed in COS cells with wild-type c-Fos driven by the CMV vector (data not shown).

To determine whether a C-terminal region of Fos is sufficient for down regulation, the properties of a series of chimeric plasmids containing different portions of Fos and Jun were examined (Cohen and Curran, unpublished data).

TABLE 3. Demonstration that Jun neither enhances nor inhibits down regulation of pEgr-1 P1.2 by Fos in NIH 3T3 cells

Plasmid	c-Fos	Relative CAT expression[a]
Carrier	−	127
c-Jun		
2 μg	−	117
2 μg	+	14
7 μg	+	13
12 μg	+	14
18 μg	+	12

[a] Normalized to $pA_{10}CAT2$ levels. Assays were performed at 40 h post-transfection in 10% FCS, and the average fold activation of several assays is shown. The amounts of c-Fos expression vector and pEgr-1 P1.2 reporter plasmids were constant at 2 μg each. − and + represent cotransfection in the absence and presence of CMV-fos, respectively. Results from individual transfections varied by less than 25%.

The rationale for using these Fos-Jun hybrids was that there is no functional or sequence similarity between these two proteins outside of their DNA-binding and leucine zipper domains, which as we have shown play no role in *trans*-repression. As an example, pCMV-FFFJ contains regions 1, 2, and 3 (N terminus, basic region, and leucine zipper) of Fos and the carboxy-terminal domain (region 4) of Jun (Fig. 7).

The Egr-1 promoter was cotransfected with either the CMV parent expression plasmid (pCMV-R), the wild-type c-Fos expression vector pCMV-FFFF (= CMV-fos = Fos 1-380), pCMV-FFFJ, pCMV-JJFF, or pCMV-JJJF. In comparison with transfections with pCMV-R, transfection with pCMV-FFFF (wild-type Fos) resulted in a sixfold repression of Egr-1 expression. pCMV-FFFJ, which removes the carboxy-terminal 180 amino acids of Fos, did not repress, in agreement with our carboxy-terminal deletion mutant data presented above. We also tested pCMV-JJFF (a Jun protein containing the carboxy-terminal 217 amino acids of Fos) and pCMV-JJJF (containing last 180 amino acids of Fos) for their effects on Egr-1 expression. Strikingly, both of these hybrid expression vectors down regulated Egr-1 expression (Fig. 7). These results demonstrate that the sequences sufficient for Fos-mediated repression are contained within the C-terminal 180 amino acids of the c-*fos* open reading frame and that this domain can confer the ability to repress onto a heterologous protein.

fra-1 is a serum inducible, cellular immediate-early gene that encodes a Fos-related antigen (14). Its C-terminal 40 amino acids show extensive similarity (65%) to the Fos C terminus. We therefore asked whether Fra-1 could down regulate Egr-1 expression. Fos and Fra-1 repressed Egr-1 expression in a similar manner (control, 43.2% [^{14}C conversion]; Fos, 7.6%; Fra-1, 7.2%). These results corroborate those obtained with the Fos-Jun chimeras and suggest that

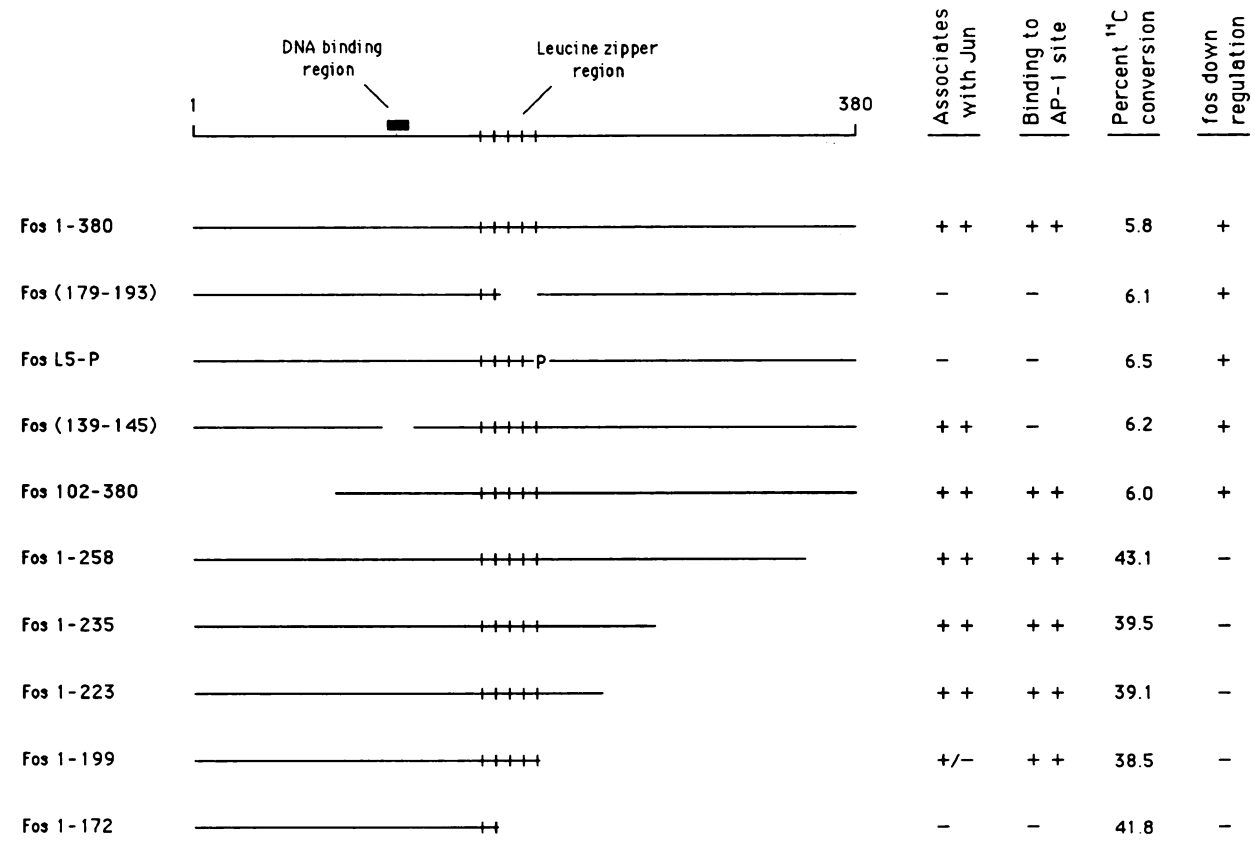


FIG. 5. Analysis of Fos mutants. All c-Fos expression plasmids are transcribed from the CMV promoter. Fos 1-380 is the wild-type rat c-*fos* cDNA. Fos (179-193) contains a 45-bp deletion in the leucine zipper region. Fos L5-P contains a proline substitution for the fifth leucine in the zipper. Fos (139-145) contains a 21-bp deletion in the DNA-binding domain. The remaining six plasmids, which are also expressed from the CMV promoter, are deletions from the N-terminal (Fos 102-380) or the C-terminal end of the Fos open reading frame. For the five C-terminal mutants, translation termination stop linkers were inserted. For cotransfection experiments, 2 μg of pEgr-1 P1.2 was transfected with either 5 μg of parent CMV expression vector or 5 μg of c-Fos mutant plasmid and with 2 μg of a CMV-βgal vector. ^{14}C percent conversion is normalized by β-galactosidase activity.

the conserved C-terminal 40 amino acids of Fos are sufficient for repression.

CArG element gel shift is unchanged after serum stimulation. Genetic results with the Egr-1 promoter indicated that a CArG element is the target sequence for c-Fos-mediated repression. We therefore asked whether Fos itself or Fos-Jun heterodimers bind to this sequence. In vitro-generated Fos and Jun proteins showed no binding to this element (data not shown). We also examined whether an Egr-1 CArG box could interact with cellular factors and whether this interaction was altered after serum stimulation and subsequent down regulation. For these experiments, we isolated nuclear extracts from both quiescent and serum-stimulated NIH 3T3 cells. We performed in vitro DNA-binding assays by gel shift analysis and used an oligonucleotide probe containing a single Egr-1 CArG box.

As increasing amounts of nuclear extract from serum-starved cells were added to the end-labeled oligomer, a DNA-protein complex was identified (Fig. 8A, lanes 1 to 4). These experiments were repeated with nuclear extract from NIH 3T3 cells that were serum stimulated (Fig. 8A, lanes 5 to 8). The gel shift bands obtained from uninduced and induced cell extracts showed a similar pattern. As a control, increasing amounts of unlabeled template were added, and the nucleoprotein complex was progressively abolished in a concentration-dependent manner (Fig. 8B, lanes 3 and 4). These experiments were repeated with another Egr-1 CArG box (Fig. 8B, lanes 5 and 6) and the c-*fos* SRE (Fig. 8B, lanes 7 and 8). A mutant c-*fos* SRE with CC exchanged for GG in the CArG core did not gel shift (X. Cao, Ph.D. thesis, University of Chicago, Chicago, Ill., 1990). Both oligomers competed for the major DNA-protein complex, suggesting that SRE and CArG elements bind a common *trans*-acting factor. These results are in agreement with those of others (5, 35). Recently, we have shown that in vitro-translated serum response factor (SRF) (63) binds to a CArG element in a gel shift assay (X. Cao and V. P. Sukhatme, unpublished data). We also asked whether Fos could participate in the protein complex bound to a CArG element. Gel shift assays were performed with induced nuclear extracts preincubated with anti-Fos antibodies. However, no inhibition of DNA-binding activity was observed (data not shown). Collectively, these data suggest that Fos represses transcription by an indirect effect on the SRE.

DISCUSSION

The identification of cellular immediate-early genes has provided an avenue for the investigation of the molecular basis of cell proliferation. Much effort is now being expended on understanding the structures and functions of the proteins encoded by cellular immediate-early genes, partic-

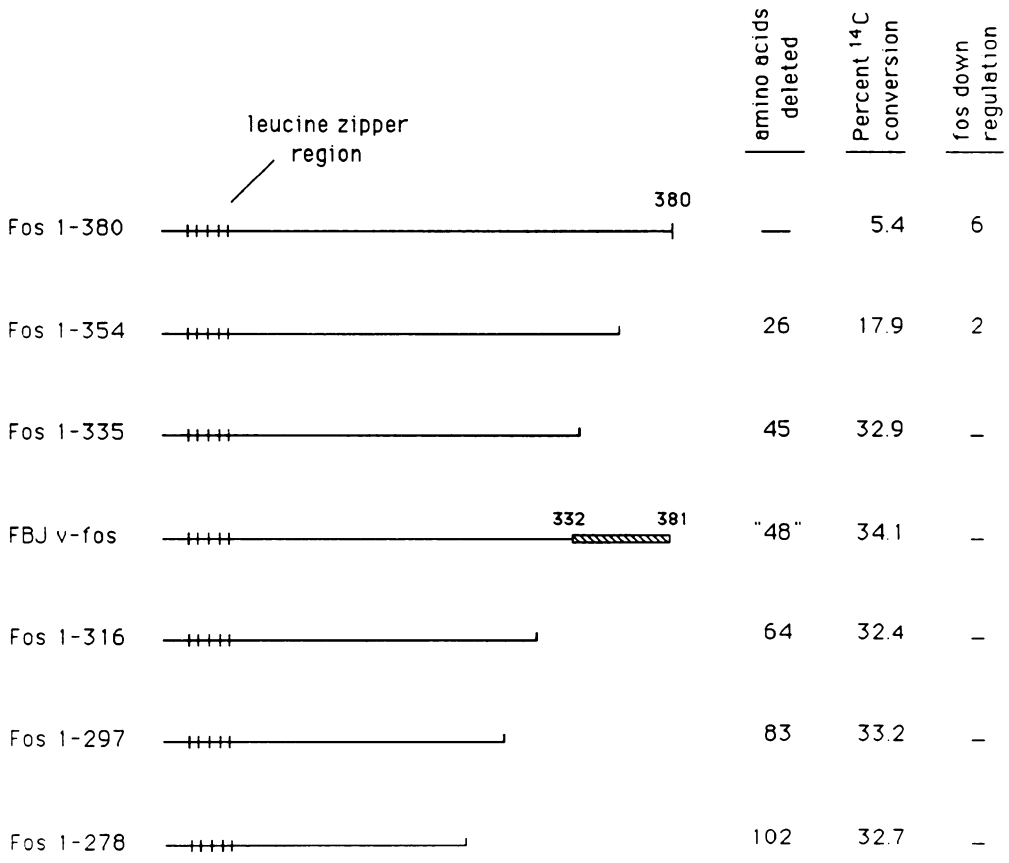


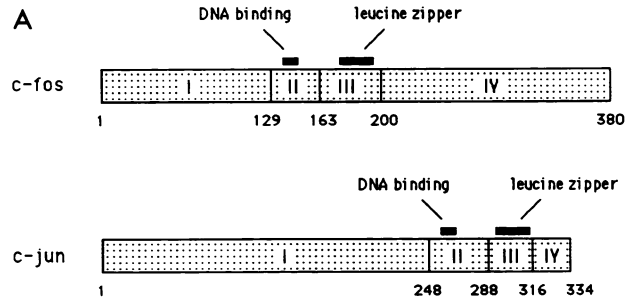
FIG. 6. Analysis of fine C-terminal deletion mutants and FBJ v-fos. All c-Fos expression plasmids are transcribed from the CMV promoter and were used at concentrations indicated in Fig. 5. ^{14}C percent conversion is normalized by β-galactosidase activity.

ularly those that have characteristics of transcriptional regulators. Currently, these include c-*fos*, *fra-1* (14), *fos*-B (84), c-*jun* (42, 65, 70, 71), *jun* B (70), *nur-77*/NGFI-B (34, 53), Egr-1 (10, 47, 52, 78, 79), Egr-2 (8, 37), and SRF (63). It has been suggested that these proteins act, directly or indirectly, to regulate the expression of effector genes that bring about the phenotypic changes required for cellular proliferation. However, the cellular immediate-early response is not restricted to situations involving mitogenic stimulation. Indeed, induction of these genes has been observed in response to differentiation signals, neurotransmitters, and excitation of neurons (reviewed in references 21 and 60). Thus, cellular immediate-early genes may participate in a primordial signal transduction process that has been adapted for use by diverse stimuli in many different cell types. It is possible that the differentiated state of the stimulated cell determines the array of effector genes capable of responding to the proteins encoded by these genes.

Several features of the cellular immediate-early response have suggested that the process is self-limiting. Immediate-early genes and their products are expressed only transiently after induction. Once triggered, the response occurs in the absence of the inducing stimulus or in the presence of blocking agents. After induction, there is a refractory period before a second stimulus will induce the same level of gene activation (58). These features may be necessary aspects of the response, since continuous expression of some immediate-early gene products results in cell transformation (22, 51). Thus, the study of the mechanisms by which these genes are negatively regulated is as critical as understanding the induction process itself.

One of the characteristics of immediate-early gene expression is that the shutoff process requires ongoing protein synthesis (13, 32, 37, 40, 61, 79), suggesting that a newly synthesized protein is required for transcriptional repression. Although this has been explained by the suggestion that a continuously synthesized but labile repressor is required for shutoff, it is equally possible that one or more of the cellular immediate-early products could function as repressors.

When our studies were initiated, it had been suggested that Fos acts as a negative regulator of c-*fos* transcription (38, 73, 82). However, these studies were somewhat contradictory, and the mechanism whereby Fos repressed transcription was unclear. Both an AP-1 site (73) and the SRE (38) in the 5′ region of c-*fos* had been suggested as targets for repression. Interaction between Fos and Jun was felt to be critical in repression (38, 73). Furthermore, although mutations of the c-Fos C terminus were reported to block repression, C-terminal truncations did not (82). Thus, although the results of these studies were in agreement that Fos can act as a repressor, its target sequence was controversial and the mechanism of action was undefined. The experiments described here were designed to resolve these questions by focusing initially on the down regulation of another cellular immediate-early gene, Egr-1. Since the serum induction of Egr-1 is 5- to 10-fold greater than that of c-*fos* (79), this system provides a particularly convenient



B

Relative CAT Expression		
plasmid	% conversion	down regulation
pCMV-R	39.8	-
pCMV-FFFF	6.1	7
pCMV-FFFJ	40.7	-
pCMV-JJFF	6.5	6
pCMV-JJJF	5.9	7

FIG. 7. Analysis of Fos-mediated repression by Fos-Jun chimeras. (A) Schematic representation of Fos and Jun, indicating the four regions of each protein as delineated by the position of the introduced restriction sites in the two cDNAs. Domains were swapped between the two proteins either singly or with one or two adjacent domains. Each chimeric protein created by domain swapping was identified by a four-letter code indicating the origin of the four domains of that protein; for example, Fos is denoted by FFFF, and FFFJ contains the first three regions of Fos fused to region IV (i.e., the C terminus) of Jun. (B) CAT assay results normalized by β-galactosidase activity for a selection of chimeric proteins expressed from the CMV promoter and cotransfected with 5 μg of each effector plasmid, 2 μg of the pEgr-1 P1.2 reporter plasmid, and 2 μg of a CMV-βgal vector.

assay for repressor function. Here we show that (i) Fos functions as a general repressor of immediate-early gene transcription by acting indirectly on CArG-like elements; (ii) this function requires an intact C-terminal region (last 27 amino acids), is independent of Jun, and does not involve the leucine zipper and DNA-binding domain of Fos; and (iii) C-terminal sequences that are conserved and functional in Fos-related genes are also sufficient for mediating repression. Therefore, the cellular immediate-early response is self-limiting at least in part because of a conserved C-terminal domain, present in a subset of immediate-early genes, that acts on the same element that is responsible for induction of transcription. Thus, Fos is a transcriptional regulator that functions in gene activation and repression by way of two independent domains that act on distinct regulatory elements.

The CArG sequence is the target of Fos-mediated repression. The SRE was first defined as the genetic element present in the 5′ region of the c-*fos* gene that mediated transcriptional induction by serum (80). Similar sequences have been identified in the 5′ regions of two other cellular immediate-early genes: Egr-1 and Egr-2. The core consensus element is $CC(A/T)_6GG$ (reviewed by R. H. Treisman, Semin. Cancer Biol., in press), known as a CArG box. Egr-1 has six CArG-like sequences which contribute to transcriptional induction and repression (Fig. 3), whereas Egr-2 has two (7, 64a). Indeed, a single CArG element derived from Egr-1 or c-*fos* placed upstream of a heterologous promoter functions as an activator and repressor in the absence of any AP-1 site (Fig. 4). Thus, the SRE core, or CArG box, is responsible for controlling induction and subsequent repression of cellular immediate-early genes. Very recently, Lucibello et al. (49) and Rivera et al. (68) reported that the SRE of the c-*fos* promoter is the target for repression. The latter report substantiates our finding (obtained with Egr-1 CArG elements) that the extended dyad symmetry present in the c-*fos* SRE is not critical for repression.

These observations provide evidence for cross-talk between immediate-early genes. Our results suggest that Fos will down regulate other immediate-early genes which show kinetics similar to those of Fos if their promoter regions contain CArG elements. Our data on Fra-1-mediated repression suggests an additional mechanism. Since Fra-1 expression is temporally delayed compared with that of Fos (14), it is possible that repression by Fra-1 is important in shutting off transcription of cellular immediate-early genes whose peak occurs later in G_1 or to keep transcriptionally silent those genes initially down regulated by Fos.

The conserved C terminus of Fos mediates repression. Fos interacts with the regulatory element known as the AP-1 site cooperatively with Jun in the form of a heterodimeric protein complex (8, 10, 32, 34, 37, 40, 42, 44, 45, 47, 48, 52, 53, 63, 70, 71, 78, 79, 84). Mutagenesis analysis has revealed that the Fos-Jun association occurs primarily through a leucine zipper interaction which serves to juxtapose regions of each protein rich in basic amino acids that bind to DNA (27, 39). Both Fos and Jun contact each strand of the AP-1 binding site (1). However, dimerization with Jun and binding to the AP-1 site are not required for the down regulation function of Fos (Fig. 5). Furthermore, Fos-mediated repression was neither enhanced nor inhibited by coexpression of Jun (Table 3). These observations agree with those of Rivera et al., who show that a Fos-Jun complex does not bind to the SRE (68), but contrast with those reported recently by others (38, 49). These workers find that Jun can synergize with Fos in down regulating c-*fos* promoter activity. In each case, the conclusion was based on experiments with a single concentration of the effector plasmids used. It was possible that their data differed from ours on Fos-directed repression of the Egr-1 promoter because of subtle differences in the CArG or SRE sequences involved between the *fos* and Egr-1 promoters. Most important, our leucine zipper mutant constructs show that an intact leucine zipper domain is not critical for Fos repression, thus providing strong complementary evidence that interaction with Jun is not involved in this process. These data contrast with and extend the work of Lucibello et al. (49). These authors base their conclusions on data derived by using a plasmid E300 and its mutant derivatives. E300 is a hybrid of the FBR and FBJ strains of MuSV. Moreover, it contains sequences only up to amino acid 316 from FBJ-MuSV/c-Fos. As shown in our results, c-Fos truncations based on wild-type c-Fos of as little as 27 amino acids markedly diminished repression. Further truncations as well as construct FBJ v-fos did not repress (Fig. 5 and 6). In particular, truncation of 64 amino acids (construct Fos 1-316, which is closest to the E300 plasmid of Lucibello

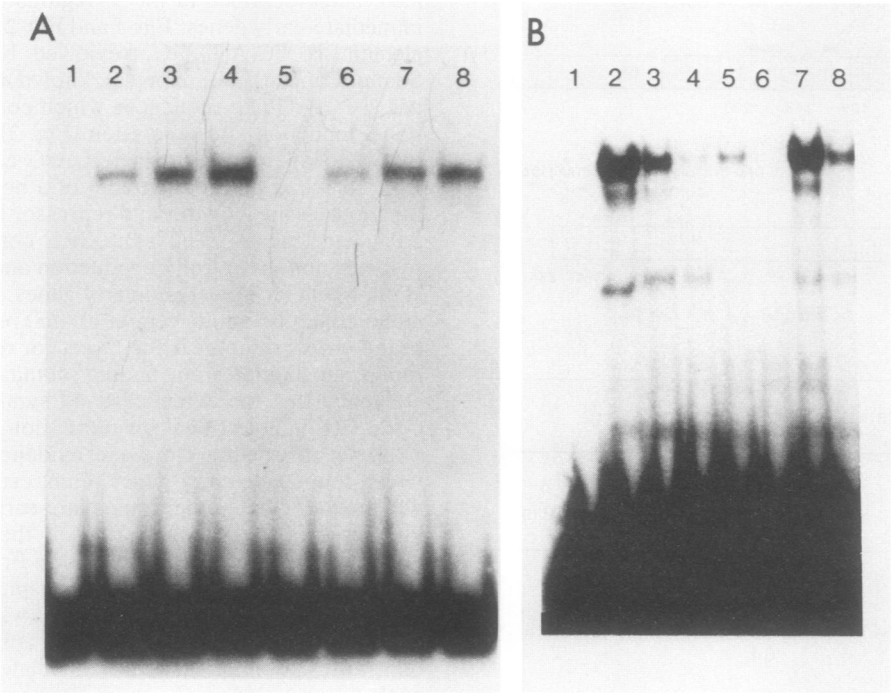


FIG. 8. (A) Gel shift analysis of nucleoprotein complexes formed with the sixth Egr-1 CArG box, contained in an end-labeled 40-mer probe, using NIH 3T3 cell extracts from quiescent and serum-induced cells. Lanes: 1 to 4, 0.5, 5, 10, and 20 μg, respectively, of low-serum NIH 3T3 nuclear cell extract; 5 to 8, 0.5, 5, 10, and 20 μg, respectively, of nuclear extract from serum-stimulated NIH 3T3 cells. (B) Competition gel shifts using the end-labeled 40-mer. Lanes: 1 and 2, 40-mer with no extract and with 20 μg of NIH 3T3 cell (serum-induced) extract, respectively; 3 and 4, 10- and 50-fold molar excess of cold DNA, using the sixth Egr-1 CArG box contained in the 40-mer; 5 and 6, 10- and 50-fold molar excess of cold 50-bp DNA fragment containing the first Egr-1 CArG box; 7 and 8, 5- and 20-fold molar excess of the c-*fos* SRE (350-bp *Pvu*II fragment).

et al. [49]) led to a loss of repression (Fig. 6). Finally, we go beyond the work of Lucibello et al. by showing that our findings with the mutant leucine zipper constructs are supported by our gain-of-function results: transfer of the c-Fos C-terminal 180 amino acids to c-Jun was sufficient to confer repression.

Our C-terminal deletion analysis mapped the critical region necessary for repression to within the last 27 amino acids. Interestingly, this region is highly conserved in several *fos*-related genes (14, 84) and in the chicken c-*fos* gene (57). As shown here, at least one of the *fos*-related genes (*fra-1*) is capable of repressing transcription. Thus, the ability to down regulate immediate-early gene expression may be a general property of the subset of cellular immediate-early genes that are related to *fos*. Several Fos-related proteins are induced with overlapping kinetics in the many and varied circumstances in which Fos is induced (14, 59, 75, 76). All of these proteins contribute to the increase in AP-1 DNA-binding activity that continues for at least 8 h after cell stimulation even after Fos has disappeared (75). Previously, we had noted that the refractory phase for reinduction of Fos expression coincided with the long period of expression of all the Fos-related proteins and not with the relatively shorter period of Fos expression (58, 76). Thus, the down-regulation phenomenon may result from complex interactions among several cellular immediate-early gene products. Repression of transcription and a refractory period for reinduction may be necessary features of this nuclear signal transduction process.

Molecular mechanism of down regulation. Although repression by Fos occurs through CArG elements, the mechanism of action appears to be indirect. In vitro-synthesized Fos does not bind to CArG sequences, and CArG gel shift complexes obtained by using extracts from quiescent or serum-stimulated cells are not inhibited by anti-Fos antibodies. These observations suggest that Fos may have an indirect effect on the CArG element. This could happen in several ways. Fos may affect the posttranslational modification of the SRF (63); the SRF requires phosphorylation for DNA-binding activity (64). Alternatively, Fos may affect some of the other proteins that interact with the SRF (69, 74).

The conserved C terminus of Fos may give some clues to its role in repression. This region of Fos is deleted or mutated in the v-*fos* genes of both the FBJ and FBR strains of MuSV (reviewed in reference 16). Although c-*fos* is capable of inducing cellular transformation (54), a high level of protein expression is required for this activity (46). Interestingly, in these transformed cells the c-Fos protein exists in a partially modified state (18, 46). c-Fos undergoes extensive posttranslational modification, primarily by serine and threonine phosphorylation (18). Indeed, there are several such conserved residues in the C termini of the *fos*-related gene family. The C terminus of c-Fos could act as either the site or signal for phosphorylation. It is possible that only the highly modified form of c-Fos could cause transcriptional repression, since the endogenous c-*fos* gene is not repressed in cells that overexpress partially modified c-Fos (46). Many of the stimuli that cause c-Fos induction also increase the degree of posttranslational modification of Fos (3, 20). Thus, one possibility is that these stimuli lead to the activation of the repressor function of the c-Fos C-

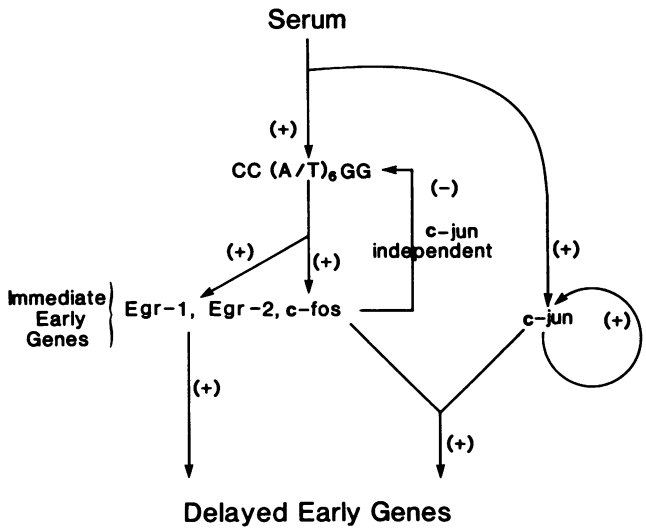


FIG. 9. Schematic representation of early events following mitogenic stimulation. After serum stimulation, a series of immediate-early genes is induced. In the case of c-Fos, Egr-1, and Egr-2, serum induction is mediated through a DNA element with the sequence $CC(A/T)_6GG$. Activation via the CArG box involves a transcriptional complex that includes the SRF (80). Fos, along with Jun, will activate the expression of another set of effector genes referred to as delayed-early genes (nomenclature suggested by D. Nathans). Jun has also been shown to *trans* activate its own expression via a TRE/AP-1 binding site (2). At least one mechanism mediating negative regulation of immediate-early gene expression involves Fos acting in a c-Jun independent manner. The target sequence for both serum induction and Fos-mediated repression is a CArG element.

terminal domain, allowing it to interact with a kinase or a phosphatase that then regulates the activity of the SRF or its associated proteins.

In conclusion, Fos is obviously involved in a complicated feedback loop (Fig. 9). It can activate gene expression in cooperation with Jun as well as repress cellular immediate-early genes in a Jun-independent manner to restore their preinduction transcriptional levels. Our findings also demonstrate that the CArG box is a key *cis* element in the signal transduction pathway utilized by Fos and its related proteins. An understanding of the mechanisms involved in activation and repression provides insight into the control of proliferation and differentiation of eucaryotic cells and enhances our knowledge of how transcriptional factors operate.

ACKNOWLEDGMENTS

This work was supported by the Howard Hughes Medical Institute.

We thank L. Laimins and H. Singh for helpful comments. We also acknowledge gifts of plasmids from R. Prywes, M. Getz, and V. Rangnekar. D.G. and X.C. were supported by Public Health Service grants GM 07183 and DK 13914-20, respectively, from the National Institutes of Health.

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