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CREB represses transcription of fos promoter: role of phosphorylation

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We have studied the regulation of protooncogene fos following serum induction. We show that un- or hypo-phosphorylated form of transcription factor cyclic AMP response element binding (CREB) protein represses the transcription of fos promoter. The negative regulation by CREB is alleviated if it is phosphorylated at serine 133 by the catalytic subunit of protein kinase A (PKA). A DNA binding mutant of CREB is unable to suppress transcription of the fos promoter. However, mutation in the cyclic AMP responsive element (CRE) at -60 or AP-1 binding site at -290, known to bind to CREB, does not appear to be involved in repression. Serum induction of dyad symmetry element (DSE) linked reporter gene is also repressed by unmodified CREB, which can be relieved following phosphorylation by PKA. We propose that posttranslational modification of CREB regulates serum inducibility of fos promoter.

Protooncogene fos is induced by a wide variety of stimuli to the cell ranging from mitogens to differentiation-specific agents, pharmacological drugs, stress, etc. (Verma 1986). Most of these inducers operate by activating protein kinase C or adenylate cyclase signal transduction pathways (Sassone-Corsi et al., 1988a). Located upstream of the initiation site of human fos mRNA are principally two DNA motifs implicated in the induction of the fos gene (Treismann, 1985, 1986; Verma and Sassone-Corsi, 1987; Rivera and Greenberg, 1990). These are (1) the DSE located between nucleotides -296 to -322, which mediates the induction of the fos gene by agents known to activate protein kinase C pathway, and (2) the CRE located at -60, which is the target of agents activating adenylate cyclase pathway (Sassone-Corsi et al., 1988b; Gilman, 1988; Fisch et al., 1989). Additionally, there are other elements like AP-1 (-296), sis-condition medium inducible element (-340), and retinoblastoma control element (RCE, -70 to -120) which contribute to the complex regulation of the fos gene (Hayes et al., 1987; Curran and Franza, 1988; Robbins et al., 1990).

Both viral and cellular fos proteins are members of the leucine zipper family and associate with members of the jun family to form heterodimers (Chiu et al., 1988; Landschulz et al., 1988; Sassone-Corsi et al., 1988c). Fos protein by itself is unable to bind to DNA in a sequence specific manner, but together with jun protein binds to TPA-responsive element (TRE) very readily (Kouzarides and Ziff, 1988; Sassone-Corsi et al., 1988e). Furthermore, fos and jun together activate the transcription of genes linked to TRE (Chiu et al., 1988; Sassone-Corsi et al., 1988c). The expression of fos and jun genes is tightly regulated. For instance, phosphorylated form of fos protein shuts off the transcription from

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fos promoter (Lucibello et al., 1988; Sassone-Corsi et al., 1988d; Schonthal et al., 1988; Ofir et al., 1990). Fos collaborates with jun to augment the transcription of jun gene which contains TRE. Furthermore, junB, a member of the jun family, is able to repress the transcription of c-jun gene (Schutte et al., 1989). More recently, we have shown that unphosphorylated form of CREB suppresses the transcription of c-jun promoter (Lamph et al., 1990). However, phosphorylation of CREB at Ser133 relieves this repression and activates transcription of c-jun gene (Lamph et al., 1990). Here we report that the serum inducibility of fos promoter is repressed by transcription factor CREB. Repression is relieved upon phosphorylation of CREB protein by the catalytic subunit of cAMP-dependent PKA. Repression by CREB requires intact DNA binding domain, but does not act through any known CRE or AP-1 sites in the fos promoter. Additionally, serum induction of a reporter gene linked to DSE is also repressed by CREB, and this repression is also alleviated following co-transfection with PKA.

Materials and methods

Cell culture and DNA transfection

NIH 3T3 cells were cultured in Dulbecco-Vogt modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were seeded 24 hours prior to transfection at 5×10^5 cells per 10 cm tissue culture dish in DMEM with 10% FBS. The cells were transfected by the calcium-phosphate co-precipitation technique and exposed to the precipitate for 12–16 hours. After transfection, the cells were washed with PBS and grown in DMEM. The transfected cells were cultured for an additional 24 hours and then either harvested (serum-starved conditions) or treated with 20% FBS in DMEM for two hours prior to harvesting the cells (serum-stimulated conditions). Discrepancies in the total amount of DNA transfected per plate were compensated for by the addition of carrier DNA (pGEM-4). CAT activity was determined as described (Gorman et al., 1982). All samples for CAT assay were standardized for protein content.

DNA manipulation

Standard DNA recombinant methodology was employed (Maniatis et al., 1982). Oligonucleotide directed mutagenesis was utilized to generate site specific mutants in the fos promoter (Kunkel, 1985). The 404 bp Hind III fragment of the promoter sequence in FC4 (Deschamps et al., 1985) was cloned in M13MP19. Oligodeoxynucleotides complementary to the site to be altered (AP-1 at −290 bp and CRE at −60 bp) were synthesized. The oligos were:

Ap-1

−281 −307

5′ GGAAACCTGCGGATGTAGATGTCCTAA 3′

−60CRE

−51 −68

5′ GTGTAAGTTTAACGGGCT 3′

The 404 bp fragment of the fos promoter (−404 bp) and the mutants generated (−60*CRE; −290 *AP-1; −60*CRE, −290 *AP-1) were subcloned in PBL-CAT 3. The other plasmids utilized from transfection have been described: FC4 (Deschamps et al., 1985), pBK-CREB (Dwarki et al., 1990), PBK-CREB/K304E (Dwarki et al., 1990), M1CREB (Gonzalez and Montminy, 1989). Expression vector for the catalytic subunit of PKA (pSKG4) (Maldonado and Hanks, 1988) was a kind gift from Steve Hanks, and DSE-TK-CAT (Fujii et al., 1989) was a gift from Masahiro Fujii.

Results

Transcriptional repression of fos promoter by CREB

Figure 1 shows that serum induction of FC4 (−404 to +42 of the c-fos promoter linked to marker chloramphenicol acetyl transferase [CAT] gene) is repressed by cotransfection with a construct capable of synthesizing CREB protein (compare lanes 2 and 4). Little or no repression was observed by DNA binding mutant of CREB (CREB K304E; lane 5). Cotransfection with catalytic subunit of PKA with CREB, however, relieved repression (compare lanes 4 and 6) to a level observed with serum alone (compare lanes 2 and 6). To ensure that relief of suppression by PKA is due to phosphorylation of CREB, we used a phosphorylation deficient mutant M1 CREB (Ser 133 is mutated to Ala, according to Gonzalez and Montminy, 1989). As expected, M1 CREB was able to suppress serum induction (lane 8), but unlike the wild type CREB, did not relieve repression upon co-transfection with PKA (lane 9). As an internal control for transfection efficiency, we used SV

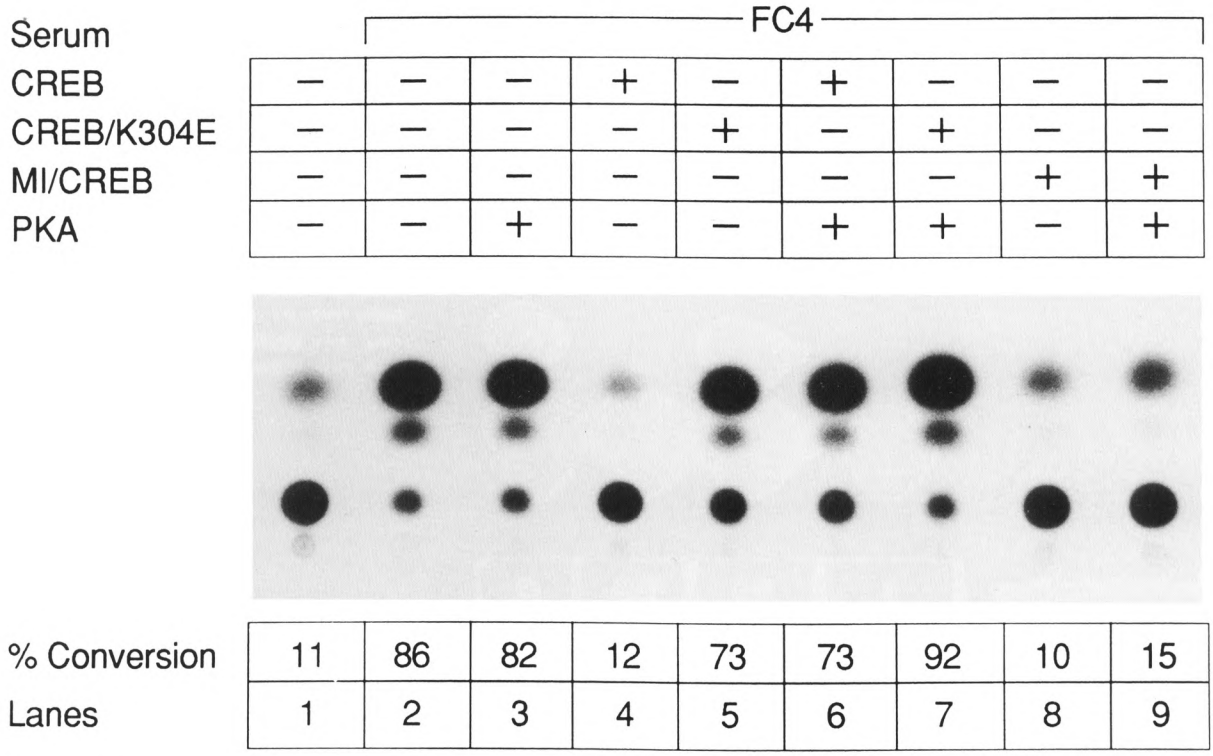

Figure 1. Repression of c-fos promoter-CAT reporter construct by CREB. NIH 3T3 cells were transiently transfected and starved for 24 hr (lane 1) or stimulated with 20% serum (lanes 2–9). The plasmids transfected in each lane are indicated. The c-fos promoter CAT reporter (FC4; 4 μg) was transfected in all samples. The amounts of plasmid transfected were: CREB, 6 μg; CREB K304E, 6 μg; M1CREB, 6 μg; PKA, 4 μg. The percent converted was determined by scintillation counting of the modified and unmodified forms of chloramphenicol. Six independent experiments were carried out.

40-β-galactosidase construct, which showed no fluctuation of activity in the presence of either CREB or CREB+PKA. Thus, CREB does not act as a general inhibitor of transcription by binding to some basal transcription factor.

CRE and AP-1 binding sites are not involved in repression

Since DNA binding mutant CREB K304E was unable to repress transcription (Fig. 1, lane 5), we argued that direct binding of CREB to specific DNA motifs in fos promoter may be required. Therefore, we mutated the −60 CRE as well as −290 AP-1 binding sites, both of which have been shown to bind to CREB in vitro (Gonzalez et al., 1989; data not shown). The mutated CRE and AP-1 sites did not bind to CREB (data not shown); yet when they were introduced into the FC4 promoter, repression by CREB remained unabated (Table 1).

DSE is required for repression by CREB

Because fos induction by serum is mediated by DSE, we next asked if CREB can repress induction of DSE-linked reporter gene. Figure 2 shows a 10-fold increase in CAT activity following serum induction (lane 2). Co-transfection with

Table 1. Repression by CREB protein does not require intact CRE site.

NIH 3T3 cells were starved for 24 hr and then stimulated with 20% serum. Four μg of each of the FOS-CAT constructs indicated were transfected alone or with 6 μg of CREB. The percentage of chloramphenicol converted was determined as described in Figure 1.

	CAT activity (% conversion)	
FOS-CAT constructs	−CREB	+CREB
−404	12	1
−60*CRE	10	1
−290*AP-1	52	4
−60*CRE, −290*AP-1	16	1

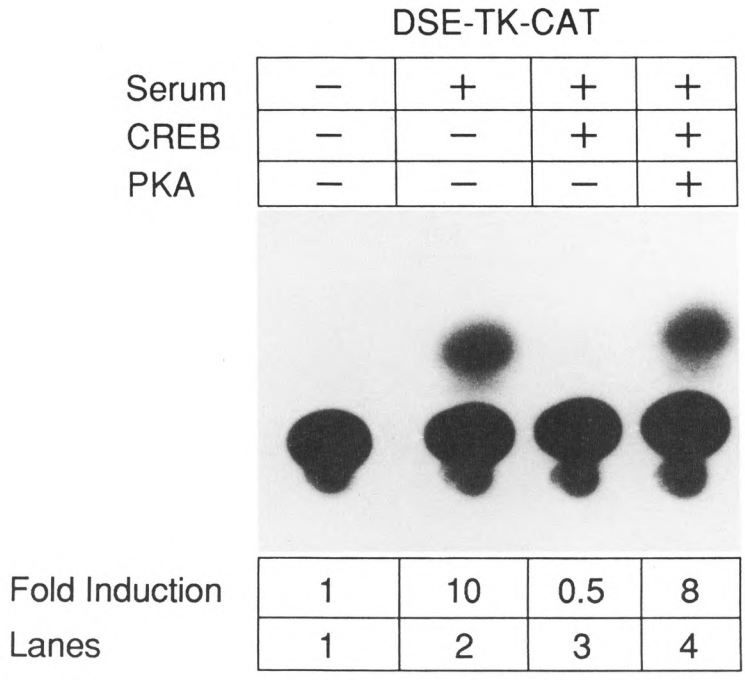

Figure 2. Repression by CREB is mediated through the DSE. The transfected NIH 3T3 cells were starved for 24 hr (lane 1) or stimulated with 20% serum, lanes 2–4. The plasmids transfected in each lane are indicated. The DSE-TK-CAT reporter (6 μg) was transfected in all samples. Six μg CREB were transfected in lanes 3 and 4; 4 μg PKA were transfected in lane 4. The percent converted was determined as described in Figure 1. Fold induction was calculated as the induction over starved level. Four independent experiments were carried out.

CREB once again completely suppressed induction (lane 3), which was relieved upon co-transfection with PKA (lane 4). Thus, CREB is able to repress fos induction by serum.

Discussion

We have shown that the un- or hypo-phosphorylated form of the transcription factor CREB is a negative regulator of transcription of fos promoter. Although the DNA binding mutant of CREB is unable to repress fos transcription, apparently it does not require either the intact CRE or AP-1 site in the fos promoter. Furthermore, serum stimulation of DSE Tk-CAT is also suppressed by CREB, indicating the lack of requirement of a classical CREB response element. How does CREB then act as a negative regulator?

Dual function of CREB

CREB protein is a member of the leucine zipper family and functions as a homodimer (Yamamoto et al., 1988; Dwarki et al., 1990). Presumably the dimeric form binds to its cognate sequence efficiently, but transactivation is dependent on its phosphorylation at serine residue 133 (Gonzalez and Montminy, 1989). The phosphorylated CREB undergoes an allosteric transformation which allows it to interact with the transcriptional machinery. It was therefore a surprise that hypo- or unphosphorylated form of CREB acts as a negative regulator of fos transcription. Suppression of jun promoter by CREB could be explained because of its binding to AP-1 site in the c-jun promoter, since addition of excess CRE oligonucleotide prevented this association and relieved repression (Lamph et al., 1990). In the case of fos repression, mutation in either CRE or AP-1 binding sites does not appear to have any effect (Table 1). We propose that CREB associates with a factor required for binding to DSE. Because CREB DNA binding mutant is unable to repress fos transcription, we presume that the heterodimer of the cellular factor with CREB is unable to bind to DSE. This hypothesis at first glance appears to be unlikely, because CREB does not bind to DSE. It is possible, however, that CREB can bind to DSE only as a heterodimer with another protein. This could be analogous to fos which cannot bind to TRE alone, but as a heterodimer with jun binds efficiently to TRE. Following phosphorylation, the CREB protein undergoes allosteric changes to prevent association with cellular factors. Thus CREB can act as a negative or positive regulator, depending on the state of its phosphorylation.

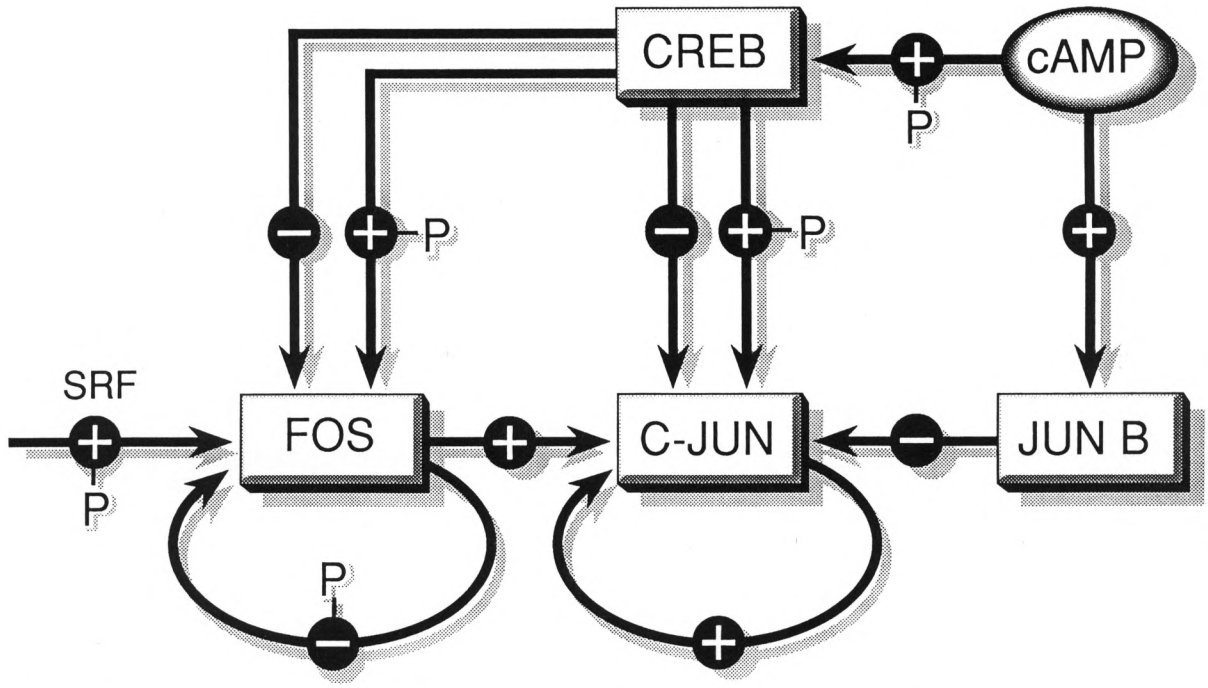

Figure 3. Proposed scheme of regulation of fos-jun genes by posttranslational modification.

Role of phosphorylation in modulating transcription

Expression of many early response genes is induced in the absence of protein synthesis, suggesting that modification of existing proteins leads to transcriptional induction and subsequent regulation. Figure 3 schematizes some observations regarding the complex regulation of fos-jun genes following induction. Protooncogene fos is induced only if the serum response factor (SRF) is phosphorylated (Norman et al., 1988). Following its synthesis, the fos protein is rapidly modified, primarily due to phosphoesterification of serine residues at its C-terminus (Barber and Verma, 1987). The hyperphosphorylated form of fos suppresses transcription from the c-fos promoter (Sassone-Corsi et al., 1988d; Ofir et al., 1990). The fos protein augments the c-jun gene transcription, presumably by forming a heterodimer with jun, which then binds to the AP-1 site in the c-jun promoter. Interestingly, phosphorylation of specific sites of jun protein by protein kinase C diminishes DNA binding and transactivation (T. Hunter, personal communication). Unphosphorylated form of CREB suppresses c-jun transcription, which can be relieved following phosphorylation by PKA (Lamph et al., 1990). One might therefore expect that in the presence of cyclic AMP, CREB is phosphorylated, and thus c-jun should be induced. Yet cyclic AMP has not been shown to induce c-jun gene. Part of the explanation may be that cAMP induces junB, which is a negative regulator of transcription of c-jun gene (Angel et al., 1988). We propose that phosphorylation of key transcriptional factors can modulate their activity either negatively or positively.

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