Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 5448–5452, June 1991
Biochemistry

Transcriptional antagonist cAMP-responsive element modulator (CREM) down-regulates c-*fos* cAMP-induced expression

(early-response gene/antagonist cAMP-responsive element modulator/gene regulation)

NICHOLAS S. FOULKES, BRID M. LAOIDE, FLORENCE SCHLOTTER, AND PAOLO SASSONE-CORSI*

Laboratoire de Génétique Moléculaire des Eucaryotes du Centre National de la Recherche Scientifique, Unité 184 de l'Institut National de la Santé et de la Recherche Médicale, Faculté de Médecine, 11 rue Humann, 67085 Strasbourg Cedex, France

Communicated by Pierre Chambon, March 20, 1991

ABSTRACT **Protooncogene c-*fos* is induced by activation of adenylate cyclase through the major cAMP-responsive element (CRE) centered at position −60 of the promoter. cAMP induction is followed by a rapid decrease in transcriptional rate, reminiscent of down-regulation after serum stimulation. Fos protein is known to negatively autoregulate serum-induced transcription of c-*fos* promoter, but whether Fos is responsible for down-regulation of cAMP-induced transcription is unclear. Here we show that Fos is unable to down-regulate CRE-mediated activation. We present evidence that the transcriptional antagonist CRE modulator (CREM) can bind to c-*fos* CRE and heterodimerize with activator CRE-binding protein, thereby blocking cAMP induction. Furthermore, expression of antisense CREM enhances c-*fos* basal and cAMP-induced transcription. CREM does not antagonize serum-induced transcription; therefore, we conclude that down-regulation of c-*fos* is exerted by different effectors, depending upon which signal transduction pathway is activated. We speculate that, by its c-*fos* down-regulatory function, CREM may act as an antioncogene.**

c-*fos* protooncogene is the prototype of the early-response class of genes. Its transcription rapidly increases upon activation of intracellular signal-transduction pathways by growth factors, phorbol esters, neurotransmitters, and agonists of adenylate cyclase (1). c-*fos* is induced by pharmacological activators of two cytoplasmic protein kinases, the Ca^{2+} and lipid-dependent protein kinase C (PKC) and the cAMP-dependent protein kinase A (PKA). The molecular mechanisms involved in c-*fos* transcriptional induction have been studied in detail. Induction by growth factors and phorbol esters is mediated by an upstream promoter sequence (serum-responsive element, SRE) centered at position −300 (2–4), which binds the transcription factor SRF (5, 6). The SRE is the molecular target of the PKC signal-transduction pathway and is not responsive to induction by cAMP (1, 3, 5). The rapid induction by serum is followed by a decrease in c-*fos* transcriptional rate, which is due to a negative autoregulatory function of Fos protein (7, 8).

Induction of the cAMP signal-transduction pathway significantly increases c-*fos* transcription (9). The protein-synthesis inhibitor cyclohexemide does not inhibit c-*fos* cAMP-induced expression, indicating that the nuclear factors required for c-*fos* cAMP-induced expression are present before activation of adenylate cyclase. Indeed, several studies have demonstrated that the transcriptional activator cAMP-responsive element (CRE)-binding protein (CREB) (10, 11) is responsible for c-*fos* stimulation upon cAMP induction (12–14). CREB protein binds as a dimer to CREs and exerts its transcriptional regulatory function upon several genes when in a phosphorylated form (15).

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The major element responsive to cAMP in the c-*fos* promoter is centered around position −60 with respect to initiation of transcription (12–14) and contains the sequence TGACGTTT, which, although different from the canonical CRE consensus TGACGTCA (15–18), binds CREB.

At least 10 different genes encode CRE-binding proteins (19, 20). Some of these were first identified as being involved in the trans-activation process exerted by the early products of the adenovirus *E1A* oncogene (16–18) and have been termed activating transcription factors (ATFs). These genes belong to the family of transcription factors having a leucine-zipper dimerization motif (21) that includes Fos, Jun, C/EBP, and GCN4 proteins (22).

We have recently characterized another member of the CRE-binding protein family, named CRE modulator (CREM), which is particularly striking because it presents two alternative DNA-binding domains that are differentially used in various tissues by cell-specific alternative splicing (23). The differential expression of CREM contrasts with the ubiquitous presence of CREB transcripts (20, 23), indicating that although CREB may function as a "housekeeping" CRE activator, CREM is likely to modulate the final CRE response.

In this report we show that CREM proteins down-regulate cAMP-induced transcription of the c-*fos* promoter. The c-*fos* CRE, centered at position −60, is efficiently recognized by the CREM products that antagonize the transcriptional activation exerted by CREB. Antisense CREM blocks endogenous CREM function and inhibits the down-regulation of c-*fos* cAMP-induced transcription. We also show that the down-regulation exerted by the Fos protein upon serum-induced expression of c-*fos* (7) is specific because Fos is unable to down-regulate cAMP-induced transcription. Likewise, CREM products do not affect serum-induced expression mediated by the SRE. We speculate that CREM might act as a molecular effector of the observed decrease in c-*fos* expression after cAMP transcription induction.

MATERIALS AND METHODS

Plasmids. FC4 and FC8 have been described (7, 12). They contain c-*fos* promoter fragments −404 to +42 and −220 to +42, respectively (+1 is the start site of transcription), which are cloned upstream of the chloramphenicol acetyltransferase (CAT) reporter gene. Additional c-*fos*/CAT plasmids have been used, such as FC1, FC2, and FC3 (7), obtaining results equivalent to FC4. c-*fos* CRE–tk–CAT contains a synthetic oligodeoxynucleotide corresponding to the c-*fos* CRE sequence (TGACGTTT) cloned upstream from a herpes

Abbreviations: CRE, cAMP-responsive element; CREB, CRE-binding protein; CREM, CRE modulator; PKC, protein kinase C; PKA, protein kinase A; CAT, chloramphenicol acetyltransferase; tk, thymidine kinase; SRE, serum-responsive element.

*To whom reprint requests should be addressed.

thymidine kinase (tk) promoter/CAT fusion gene (12). pCαEV is an expression plasmid for the catalytic subunit of mouse PKA (24) and is a gift from S. G. McKnight (University of Washington, Seattle). The pSVCREMα, -β, and -γ (sense and antisense) plasmids have been described elsewhere (23). pSVCREB contains rat CREB cDNA (15) cloned into expression vector pSG5 (23). Plasmids GAL4–VP16–F and UAS–tk–CAT have already been described (25). BK28 expression plasmid carries the c-*fos* cDNA sequence (7).

***In Vitro* Translation and Gel-Retardation Assays.** Proteins were synthesized *in vitro* by using a rabbit reticulocyte translation system (Promega) and labeled with [^{35}S]methionine. Products were analyzed on an 11% polyacrylamide/SDS gel, and the gel was fixed with acetic acid/methanol and dried before autoradiography. *In vitro*-synthesized unlabeled proteins were used in gel-retardation assays to determine binding to the c-*fos* −60 CRE (fos) (5′-AGCTTGAGCCCGTGACGTTTACACTA-3′) and to the rat somatostatin CRE (som) (5′-AAGCTTGCATGACGTCAGACAG-3′). Three microliters of translation product was added to 3 μg of poly[d(I-C)] in TM buffer (50 mM Tris hydrochloride, pH 7.9/12.5 mM $MgCl_2$/1 mM EDTA/1 mM dithiothreitol/20% glycerol) in a 20-μl final volume. [γ-^{32}P]ATP end-labeled DNA was added, and the reaction was incubated for 20 min at room temperature. DNA–protein complexes were resolved on a 4% (Fig. 1*B*) or 5% polyacrylamide gel (Fig. 1*C*) containing 0.25× TBE (1× TBE = 50 mM Tris borate, pH 8.3/1 mM EDTA). The CREBcore is a 63-amino acid synthetic peptide corresponding to the CREB dimerization and DNA-binding domain (amino acid positions 264–326, ref. 11) and is a gift from R. Frank (European Molecular Biology Laboratory, Heidelberg). Mixing experiments (Fig. 1*C*) were done at room temperature for 10 min. Before mixing, the core peptide was incubated at 60°C for 1 min.

Transfections and Cell Treatments. Transfections of JEG-3 human choriocarcinoma or NIH 3T3 fibroblast cells and CAT assays were done as described (23). Ten micrograms of total plasmid DNA per 10-cm plate was used in each transfection. One microgram of reporter plasmid was included in each transfection sample together with 2 μg of each other plasmid, unless otherwise stated. CAT activity was quantified by liquid scintillation counting of the TLC plate ^{14}C spots. Transfected cells were treated with 20 μM forskolin, 1 mM dibutyryladenosine 3′,5′-cyclic monophosphate or cholera toxin at 100 ng/ml. Induction by serum was obtained by first depriving cells of serum for 24–36 hr and then adding fresh medium containing 20% fetal calf serum for 3 hr before harvesting (7). All experiments were repeated several times, and the variability was <10%.

RESULTS

Fos Does Not Down-Regulate CRE-Mediated Induction. We transfected JEG-3 or NIH 3T3 cells with FC8, a c-*fos* CAT reporter plasmid carrying the c-*fos* promoter region (−220 to +42). In the absence of cAMP induction, expression levels of the reporter CAT gene were barely detectable (Fig. 1*A*, lane 1). When the transfected cells were treated with forskolin, an agonist of adenylate cyclase that increases intracellular cAMP, the fusion gene was activated through the c-*fos* −60 CRE element (Fig. 1*A*, lane 2). Cotransfection with BK28, a proven Fos expression vector (7), has no effect either on the basal level of transcription (lane 3) or on induced levels (lane 4). Analogous results were obtained by using FC4, which contains c-*fos* promoter sequences up to position −404 (data not shown; ref. 7). Thus, Fos protein is unable to down-regulate either the basal or cAMP-induced levels of transcription directed from the CRE-reporter gene.

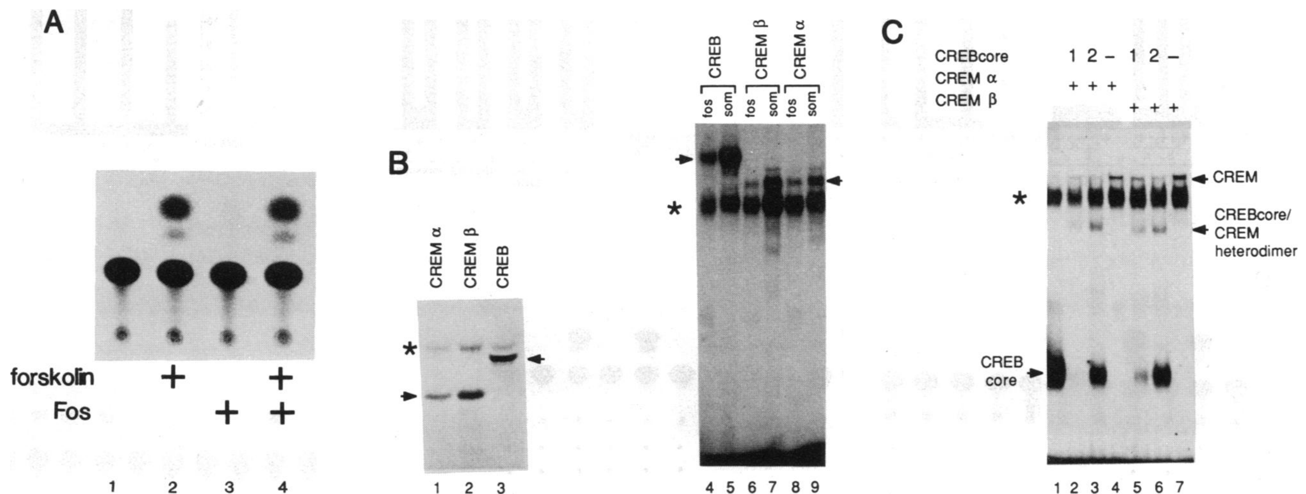

FIG. 1. (*A*) Fos does not down-regulate cAMP-induced c-*fos* transcription. CAT assays from JEG-3 cells transfected with FC8, a c-*fos*/CAT construct. For lanes 2 and 4, cells were treated with forskolin, and for lanes 3 and 4, cells were cotransfected with the BK28 Fos expression plasmid (7). In lane 1 cells were transfected with FC8 without forskolin treatment. Similar results were obtained using NIH 3T3 cells (data not shown). (*B*) *In vitro* translation of CREM products. Autoradiograph of a polyacrylamide/SDS gel showing CREMα, CREMβ, and CREB *in vitro*-translated proteins. Arrows indicate specific products. A star indicates a nonspecific product generated from endogenous RNA of the lysate. In lanes 1 and 2 CREMα and CREMβ show the expected size of 26 kDa (23), while lane 3 shows the 43-kDa band corresponding to CREB (15, 23). Lanes: 4–9, gel-retardation assays to test binding of *in vitro*-synthesized unlabeled proteins to the c-*fos* −60 CRE (fos) and to the rat somatostatin CRE (som). Bands corresponding to specific binding by CREB (lanes 4 and 5), CREMβ (lanes 6 and 7), and CREMα (lanes 8 and 9) are labeled by arrows. Presence of a nonspecific complex is due to endogenous lysate-binding proteins and is indicated by the star (15, 23). (*C*) Heterodimer formation between CREM and CREBcore peptide studied with gel-retardation assays with c-*fos* CRE. Lanes: 1, binding of CREBcore peptide (20 ng) with reticulocyte lysate; 4, binding of CREMα; 7, binding of CREMβ. In lanes 2 and 3, CREBcore (1 and 2, 5 ng and 10 ng, respectively) was premixed with *in vitro*-synthesized CREMα (as in lane 4), whereas in lanes 5 and 6, CREBcore was premixed with *in vitro*-synthesized CREMβ. The intermediate mobility complex is indicated as CREBcore/CREM heterodimer. Appearance of a greater amount of heterodimer is accompanied by corresponding decrease in quantity of CREM-specific homodimer. Increase in intensity of CREBcore complex is not linearly proportional to the increase in peptide concentration due to the optimum in dimerization potential of these peptides (see ref. 26). The nonspecific band is indicated by a star.

The recently identified CREM proteins share sequence homologies with CREB and have been shown to bind to a consensus CRE sequence (23). Expression of the CREM gene produces different isoforms with different DNA-binding domains (23). One of these isoforms, CREMα, has a basic region and leucine-zipper motif very similar to the equivalent region of the CREB protein, whereas the DNA-binding region of the CREMβ isoform is slightly less conserved. We synthesized CREMα, CREMβ, and CREB *in vitro* (Fig. 1*B*, lanes 1–3) to investigate whether the c-*fos* CRE (noncanonical TGACGTTT sequence) could also act as a CREM target and to compare efficiency of binding with the canonical somatostatin CRE sequence. Both CREM products (Fig. 1*B*, lanes 6 and 8) and CREB (lane 4) recognize the c-*fos* −60 element, although they do not appear to bind as efficiently to this site as to the somatostatin CRE element. CREMβ shows a larger difference in affinity for the two sites than CREMα. To determine whether CREM proteins might heterodimerize with CREB we used a synthetically generated peptide corresponding to amino acids 264–326 of CREB protein (CREBcore, see refs. 11 and 23). This region of the protein contains the CREB dimerization motif and DNA-binding domain, binds efficiently to the c-*fos* CRE, and can be identified as a fast-mobility band in a gel-retardation assay (Fig. 1*C*, lane 1), clearly different in mobility from the CRE-binding CREM protein complexes (lanes 4 and 7). Mixing more CREBcore with *in vitro*-generated CREM proteins decreases homodimer–complex formation; a corresponding increase in an intermediate mobility band is consistent with formation of CRE-binding heterodimers (Fig. 1*C*, lanes 2, 3, 5, and 6; see also ref. 23).

CREM Specifically Antagonizes CRE-Mediated Activation. In Fig. 2 we report experiments that demonstrate that CREM products efficiently down-regulate cAMP-induced transcription by c-*fos* promoter. The transcription of various c-*fos* promoter CAT reporter plasmids was analyzed. In Fig. 2*A* we report data concerning FC4, which contains the serum-responsive element and CRE sites, and FC8, which only contains the CRE. Using FC1, a construct containing up to −2000 of the c-*fos* promoter (7), we obtained data equivalent to FC4 (data not shown). In the first analysis JEG-3 cells were transfected with these constructs and were then treated with serum. Transcription was induced only for FC4, which is consistent with the presence of the SRE in this construct (Fig. 2*A*). Cotransfection with the sense CREMα expression plasmid did not affect the basal level of transcription from FC4 or FC8 (Fig. 2*A*); the serum-induced transcription of FC4 was similarly unaffected. In the same assay Fos protein acts as a potent down-regulator of serum-induced transcription (refs. 7 and 8, and data not shown).

When transfected JEG-3 cells were treated with forskolin, strong induction of both FC4 and FC8 transcription was seen

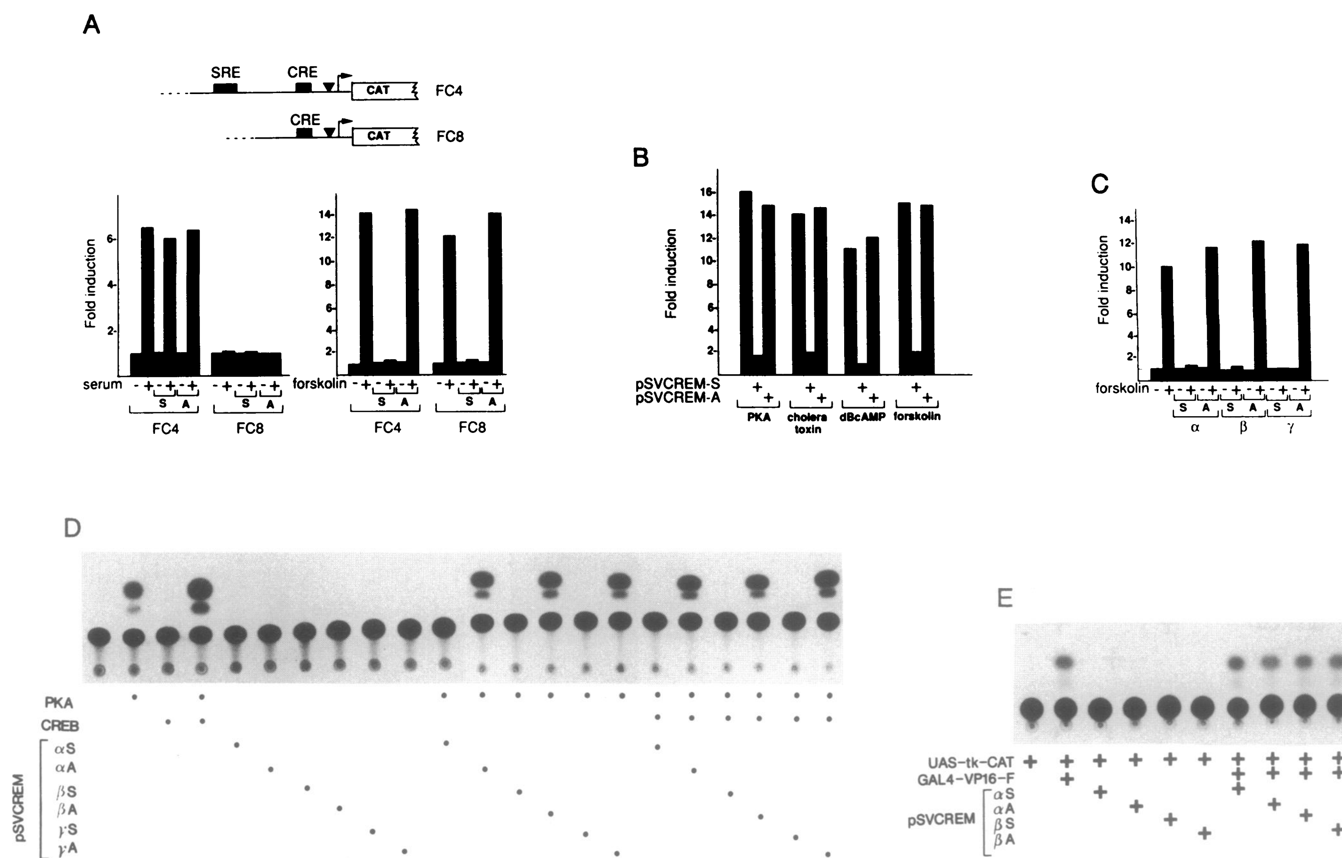

FIG. 2. CREM down-regulates cAMP-induced c-*fos* transcription. (*A*) At top is a schematic representation of the two c-*fos* promoter reporter constructs. Positions of SRE and CRE are indicated by filled boxes. A filled triangle represents the TATA box, and an arrow represents the start site of transcription. (*A–C*) Histograms summarizing CAT-assay results. CAT activity was quantified by measuring percentage of chloramphenicol acetylated and is expressed as fold induction. Reporter plasmids in transfections were FC4 and FC8 (*A*) or the c-*fos* CRE–tk–CAT reporter plasmid (*C*). Cotransfections included expression plasmid for catalytic subunit of PKA: pCαEV, pSVCREM sense (S) and antisense (A) expression plasmids encoding the α isoform (*A* and *B*) or the α, β, and γ isoforms of CREM (*C*, see ref. 23 for details on CREM isoforms). Transfected cells were induced with forskolin, dibutyryladenosine 3′,5′-cyclic monophosphate (dBcAMP), cholera toxin, or by serum. (*D*) CAT assay results from JEG-3 cells transfected with the c-*fos* CRE–tk–CAT reporter plasmid in combination with pCαEV, a CREB expression vector (pSVCREB) and the sense (S) and antisense (A) pSVCREM α, β, and γ expression plasmids (see ref. 23). (*E*) Activation by fusion transactivator GAL4-VP16-F (25) of the reporter UAS–tk–CAT is not affected by CREM. Cotransfections were with the sense and antisense pSVCREM expression plasmids.

(Fig. 2 *A* and *B*). In contrast to serum induction, cotransfection with the sense CREMα expression vector dramatically decreases cAMP-mediated induction (Fig. 2 *A* and *B*). Transfection with the CREMα sense expression vector also down-regulates CRE-mediated activation achieved by treatment with dibutyryladenosine 3′,5′-cyclic monophosphate and cholera toxin or by cotransfection with an expression vector (pCαEV) encoding the catalytic subunit of PKA (Fig. 2*B*). In all these experiments expression of antisense CREM has no significant effect upon transcription of the reporter plasmids. It is worth stressing that the amount of CREM expression vector transfected in this kind of experiment (Fig. 2) is relatively low. Higher amounts of CREM provoke a different response, as shown later (see Fig. 3).

The CAT reporter plasmid c-*fos* CRE–tk–CAT contains the c-*fos* −60 CRE sequence cloned upstream from the tk promoter. Forskolin induction of this plasmid is also blocked by cotransfection with sense expression plasmids for all three CREM isoforms (α, β, and γ) to a similar extent (Fig. 2*C*). Significantly, cotransfection with the CREM expression plasmids also blocks induction achieved by coexpression of CREB and PKA (Fig. 2*D*). These results were also confirmed by using a reporter plasmid containing the entire functional c-*fos* promoter, such as FC4 (data not shown). Antisense expression vectors for all three forms fail to down-regulate (Fig. 2*C*) and, indeed, consistently seem to elevate (Fig. 3, see below) the level of induced transcription. We further confirmed the specificity of CREM antagonism for CRE-mediated activation by testing the GAL4–VP16 transactivation function on a yeast upstream activation sequence/tk–CAT reporter plasmid (UAS–tk–CAT; Fig. 2*E*) (25). No effect of cotransfecting CREM sense or antisense expression plasmids was seen in this case.

Antisense CREM Blocks c-*fos* Down-Regulation. As shown above, expression of all three CREM isoforms specifically down-regulates cAMP induction mediated by the c-*fos* −60 CRE. To assess the contribution of endogenous CREM to the down-regulation of c-*fos* transcription, FC8 was cotransfected with increased amounts of CREMα antisense expression plasmid to block the expression of endogenous CREM (Fig. 3). CRE-mediated activation was achieved by cotransfection with the pCαEV expression plasmid, the product of which activates endogenous CREB.

Increasing the amount of transfected CREM antisense expression vector causes a significant rise in the basal transcription rate of the reporter gene (Fig. 3). The amount of transfected antisense CREM used in this approach is significantly higher than in previous experiments (Fig. 2). In control experiments, in which a sense CREM expression plasmid was used, no such effect was observed. This result directly implicates CREM in the negative regulation of basal transcription. Indeed, it has previously been shown that the −60 element is important for maintaining basal c-*fos* transcription and that upon cAMP induction its function is converted to that of a CRE (13, 14, 16, 27).

Cotransfection of antisense CREM with pCαEV also increases the induced level of transcription (Fig. 3). In this case, however, when a significant amount of antisense CREM construct was transfected, we observe a decrease in transcriptional activation. This result is consistent with the observation that, at high concentrations, promoter excess of the cotransfected plasmid can sequester factors necessary for activity of the transfected promoter (this titration effect has been already observed, refs. 23 and 28). This process may decrease expression of the protein kinase catalytic subunit. Increased amounts of transfected sense CREM expression plasmids increase repression of transcription.

These data strongly indicate that endogenous CREM is significant in the down-regulation of cAMP-induced c-*fos* transcription.

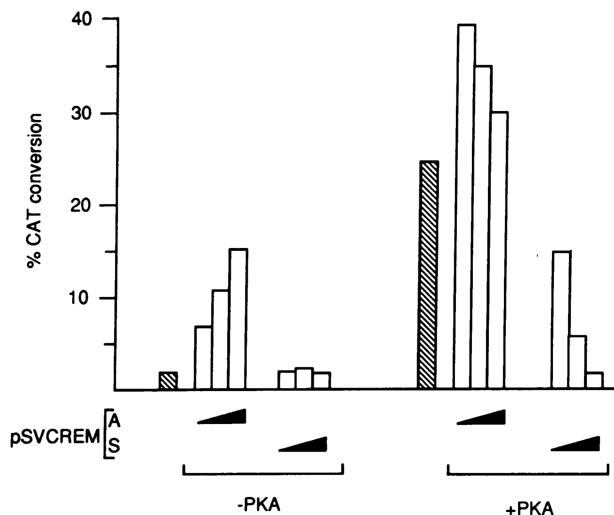

FIG. 3. Antisense CREM elevates c-*fos* activation. Increased amounts of pSVCREMα sense (S) and antisense (A) plasmids were cotransfected with the FC8 reporter plasmid with or without pCαEV (± PKA). CAT assay results are summarized in histogram form as percentage conversion by CAT of chloramphenicol to acetylated form. Open bars show transfections that included the pSVCREM expression plasmids, whereas hatched bars show level of CAT activity from FC8 with or without pCαEV. Equivalent data were obtained with FC4 as reporter plasmid (data not shown). From left to right, in each transfection series, amount of transfected pSVCREM plasmid increases as 2 μg, 4 μg, and 8 μg. One microgram of FC8 and 2 μg of pCαEV were used in each case.

DISCUSSION

c-*fos* transcription increases rapidly upon induction of the cAMP signal-transduction pathway. However, this increase is transient and is followed by a sharp decrease in c-*fos* expression. The data reported here show that the CREM gene products are likely to play a role for the observed, but previously unexplained, decrease in transcription of the c-*fos* gene. The Fos protein, known to be involved in negative regulation of the c-*fos* gene upon serum stimulation (7, 8), is not involved in this process. Similarly, we show that CREM does not down-regulate serum-induced expression.

The c-*fos* promoter contains a CRE site positioned at −60. Upon cAMP induction the nuclear factor CREB activates transcription from this site (12–15). Our results show that *in vitro*-generated CREM and CREB can form heterodimers that bind to this site. Furthermore, we show *in vivo* that CREM antagonizes the activation function of CREB. This fact suggests either that CREM/CREB heterodimers can bind to the c-*fos* CRE but are unable to activate transcription or that nonactivating CREM homodimers compete for the same binding sites as CREB.

We have reported the existence of CREM isoforms with different DNA-binding domains (23). It is conceivable that the CREM products could have different affinities for various CRE sites. The observed difference between CREMα and β in their relative binding to the CREs (fos and som in Fig. 1*B*) might indicate this phenomenon. By conferring different affinities for various CRE sites, the alternative use of DNA-binding domains could provide a way to control the spectrum of genes that the CREM products may regulate.

That the decrease in c-*fos* cAMP-induced transcription is delayed in the presence of cyclohexamide has been reported (9), suggesting that a *de novo* synthesized protein is involved in this down-regulation. Our preliminary data indicate no significant increase in level of CREM mRNA during cAMP induction (unpublished results). This finding suggests that CREM expression may be under translational control.

An important result reported here shows that antisense CREM expression in cotransfection assays releases inhibition of c-*fos*-directed transcription in transfected cells (Fig. 3). These data clearly indicate a significant contribution of endogenous CREM to the down-regulation of both basal and cAMP-induced c-*fos* transcription. Whether CREM alone is responsible for the c-*fos* down-regulation after cAMP induction *in vivo* is still unclear. Our observations lead us to speculate that CREM functions as an antioncogene, by specifically blocking expression of an oncogene.

In conclusion, our results indicate that the transcriptional inhibition, which follows induction of several early-response genes, appears regulated by several distinct effectors. Protooncogene c-*fos*, the prototype of this class of genes, is the target of down-regulation by both Fos and CREM, which specifically act depending on which signal-transduction pathway is first activated. Furthermore, our results underline the complex interplay that exists between the main signal-transduction pathways.

We thank E. Borrelli, R. Breathnach, S. G. McKnight, R. Frank, J. P. Montmayeur, E. Benusiglio, and D. Masquilier for gifts of materials and discussions. We thank M. Acker, J. L. Weickert, F. Ruffenach, A. Staub, and B. Boulay for technical help. N.S.F. is supported by a European Molecular Biology Organization long-term fellowship; B.M.L. is the recipient of an Association Recherche Sur le Cancer fellowship. This work is supported by grants from Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, and Association Recherche Sur le Cancer.

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