Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 4226–4230, May 1992
Biochemistry

Alternative usage of initiation codons in mRNA encoding the cAMP-responsive-element modulator generates regulators with opposite functions

(gene regulation/nuclear factor/translational control)

VÉRONIQUE DELMAS, BRID M. LAOIDE, DENIS MASQUILIER, ROLF P. DE GROOT, NICHOLAS S. FOULKES, AND PAOLO SASSONE-CORSI*

Laboratoire De Génétique Moléculaire des Eucaryotes, Centre National de la Recherche Scientifique, U184 Institut National de la Santé et de la Recherche Médicale, Institut de Chimie Biologique, Faculté de Medecine, 11, rue Humann, 67085 Strasbourg, France

Communicated by Robert P. Perry, January 23, 1992 (received for review December 23, 1991)

ABSTRACT **The cAMP-responsive-element modulator (CREM) gene encodes both antagonists (CREM$\alpha/\beta/\gamma$) and an activator (CREMτ) of cAMP-responsive transcription by alternative splicing. In adult mouse brain a predominant 21-kDa protein, not corresponding to any previously characterized transcript, is detected with specific CREM antibodies. A developmental switch occurs in brain as expression changes at birth from CREMα/β to the 21-kDa protein. We show that the 21-kDa protein corresponds to S-CREM (short CREM), a protein produced by the use of an internal AUG initiation codon in the CREMτ transcript. S-CREM shares with the other CREM proteins the basic DNA-binding and leucine-zipper dimerization domain. S-CREM functions as a transcriptional repressor of cAMP-induced transcription. Thus, two proteins with opposite functions are generated by alternative translation using two AUG codons within the same reading frame.**

The signal transduction pathway that involves adenylyl cyclase has, as nuclear targets, a number of transcription factors that interact with promoter sequences termed cAMP-responsive elements (CREs). Several genes encoding CRE-binding proteins have been isolated (1, 2), all of whom belong to the leucine-zipper superfamily of transcription factors. We have recently cloned the CRE modulator (CREM) gene (3). The CREM gene was isolated from a mouse pituitary cDNA library screened at low stringency with oligonucleotides corresponding to the leucine zipper and basic region of CREB. The most striking feature of the CREM cDNA is the presence of two DNA-binding domains. Four mRNA isoforms were identified that appear to be obtained by differential cell-specific splicing. Alternative usage of the two DNA-binding domains was demonstrated in various tissues and cell types, where quite different patterns of expression were found (3, 4). Three major isoforms were initially characterized, which revealed alternative usage of the two DNA-binding domains (α and β isoforms), as well as a small deletion of 12 amino acids (γ isoform). The strict cell- and tissue-specific expression of CREM is indicative of a pivotal function in the regulation of cell-specific cAMP response. The CREM products share extensive homology with the transcriptional activator CREB (CRE binding protein, ref. 1), especially in the DNA-binding domains and the phosphorylation domain. However, two glutamine-rich regions involved in the transcriptional activation function are absent in CREMα, -β, and -γ (3). Indeed, in transfection experiments using CRE reporter plasmids, it was demonstrated that CREM proteins block the transcriptional activation obtained by the joint action of CREB and the catalytic subunit of the cAMP-dependent protein kinase A (5). As well as encoding antagonists, the CREM gene also encodes an activator of cAMP-dependent transcription, CREMτ (4). CREMτ contains the insertions of two glutamine-rich domains that confer transcriptional activation function on the protein.

In this report we present evidence of translational regulation of CREM expression. By alternative usage of two translation initiation codons in CREMτ, two proteins are produced. Particularly striking is the fact that the small form, S-CREM, generated by the use of an internal AUG codon, acts as a repressor of cAMP-induced transcription. Thus, two proteins are generated from a single transcript that elicit opposite functions. S-CREM is abundant in adult brain tissues and its production appears to be developmentally regulated. An additional mechanism operates in CREM, therefore, to generate both an antagonist and an activator of cAMP-induced transcription.

MATERIALS AND METHODS

Plasmids. CREM bacterial expression vectors were constructed by inserting *Nco* I–*Bam*HI full-length cDNA fragments (3, 4) into a pET11d vector (6). S-CREM was generated by deletion of an *Nco* I fragment that spans the N-terminal portion of the CREMτ protein, between amino acid positions +3 and +152; in the pET11 plasmids the ATG codon at position +1 of CREM (3) is not present, so that the +3 ATG corresponds to the initiation codon. The expression vector for the catalytic subunit of protein kinase A, pCαEV, has been described (5). CREMτ-ATC was generated by using a Bio-Rad mutagenesis kit. CREM cDNAs were cloned into the expression vector pSG5, as described (3). While pSom-CAT (3) and α-CG (7) reporters contain the isolated CREs from the respective promoters inserted upstream of the thymidine kinase–chloramphenicol acetyltransferase (CAT) fusion gene (3, 7), the c-*fos* and enkephalin gene reporters contain regions of the respective promoter upstream of the CAT gene [c-*fos*, from −220 to +42 (8); enkephalin gene, from −193 to +210 (9)].

Transfections and CAT Assays. Transfections for both Western blot analysis and immunoprecipitation studies were carried out in COS cells. ^{35}S labeling of the cultured cells and immunoprecipitations were performed as described (10). Transfections were performed with human choriocarcinoma JEG-3 cells as described (3). For each transfection 400 ng of pCαEV and 2 μg of reporter plasmid were used. CAT assays were performed as described (3). The results from the trans-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: CRE, cAMP-responsive element; CREM, CRE modulator; CAT, chloramphenicol acetyltransferase.

*To whom reprint requests should be addressed.

fections were reproducible and a variation of no more than 15% was observed.

Protein Expression and Gel Shift Assays. Bacterial proteins were expressed and purified as described (11). *In vitro* transcriptions/translations and gel shift retardation assays were performed as described (3). A synthetic 18-base-pair oligonucleotide containing the rat somatostatin CRE (5'-CTTGGCTGACGTCAGAGA-3') was used as a DNA probe. One milligram of total brain extract protein was loaded for SDS/PAGE; the proteins were eluted from the gel as described (12).

Western Blot Assays. We raised a polyclonal antibody (Abγ) against the γ peptide (amino acid sequence NEETDLAPSHMAA; see ref. 3) to which a cysteine was added at the C terminus to allow conjugation to ovalbumin by *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester. Two hundred micrograms of peptide–ovalbumin was injected into rabbits, and antisera were collected 20 days later. Total brains (or heads in the case of 16-day embryos) were resuspended in 10 vol of Laemmli buffer, extracts were prepared, protein concentration was determined, and samples were prepared for SDS/PAGE. Western analysis was carried out with a 1:2000 dilution of Abγ and with a 1:10,000 dilution of peroxidase-conjugated F(ab')$_2$ fragment of donkey anti-rabbit immunoglobulin. Protein–antibody complexes were visualized with blotting detection reagents (30 μl/cm^2; Amersham) and filters were exposed for 10 sec to 5 min.

RESULTS

Pattern of Expression of CREM Proteins in Mouse Brain. To characterize the CREM proteins present in various tissues and at different times of mouse development, we generated a specific antibody against a peptide corresponding to the γ domain, a 12-amino acid segment deleted in the CREMγ isoform (3). This antibody, Abγ, is specific for CREM because the γ domain has no counterpart in all the described CRE/ATF factors (1, 2) and does not recognize CREB (data not shown). By Western blot analysis we examined CREM protein distribution in liver, spleen, kidney, pituitary, hypothalamus, and testis. We observed the predicted CREM proteins, corresponding in size to the translation of the various isoform transcripts (data not shown; V.D., B.M.L., and P.S.-C., unpublished observations). However, in adult brain we encountered an unexpected pattern. Two predominant bands of 70–80 kDa and 21 kDa were detected (Fig. 1*A*, lane 1), which do not correspond to any predicted CREM product (3, 4). As controls in our Western assays, we used various CREM proteins generated in bacteria. One of these, termed S-CREM, is produced by a deletion between two *Nco* I sites (corresponding to two ATG codons at amino acids +3 and +152; see ref. 4 and Fig. 2*A*) that removes the N-terminal 149 amino acids in CREMτ. Interestingly, S-CREM comigrated with the brain p21 band (Fig. 1*A*, compare lanes 1 and 2). To confirm whether the 21-kDa immunoreactive product binds to a CRE, we eluted the protein from the gel and used it in a gel mobility-shift assay. The eluted protein bound to a canonical CRE sequence and the protein–DNA complex comigrated with that formed by S-CREM (Fig. 1*B*, compare lanes 1 and 4). The protein(s) constituting the 70–80 kDa band in the Western did not bind the CRE (Fig. 1*B*, lane 3). Thus, under denaturing and nondenaturing conditions, the 21-kDa protein comigrates exactly with S-CREM. Together with the specific immunoreactivity, these data strongly indicate that the 21-kDa protein is a bona fide CREM protein.

The 21-kDa protein is developmentally regulated in brain. It was not detectable in prenatal brain tissues (16- and 20-day embryos), whereas a band comigrating with the CREMβ protein generated in bacteria was visible (Fig. 1*C*, lanes 1 and 2). After birth, an increasing amount of the 21-kDa protein

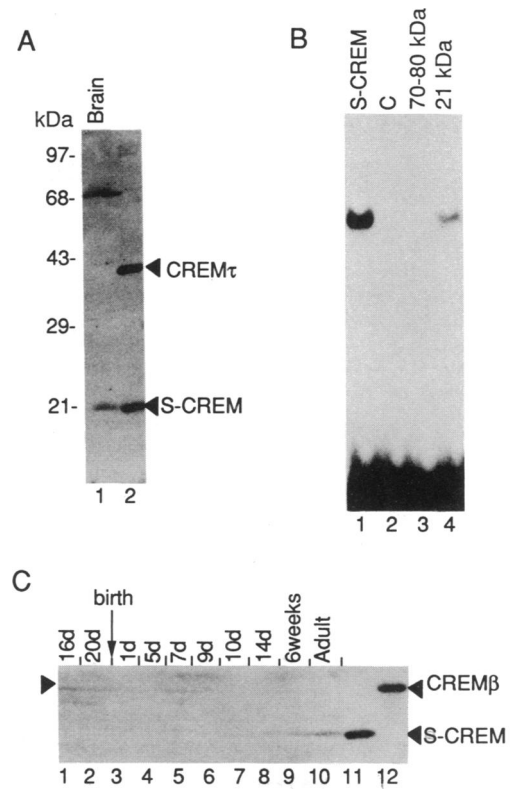

FIG. 1. A 21-kDa immunoreactive CREM protein in mouse brain. (*A*) Western blot analysis of a brain protein extract from adult mouse. CREM-specific antibody Abγ detected two bands of 70–80 kDa and 21 kDa (lane 1). As controls, bacterially generated CREM proteins were mixed and loaded on the same gel (lane 2). S-CREM corresponds to a deleted CREMτ (4), in which a *Nco* I fragment encoding 149 amino acids has been deleted. (*B*) Gel shift analysis using a somatostatin CRE probe. Lane 1, binding with bacterial S-CREM; lane 2, binding with proteins extracted from a gel equivalent to the one in *A*, in a position where no CREM-immunoreactive bands were detected; lane 3, binding with proteins eluted from the 70- to 80-kDa band in lane 1 of *A*; lane 4, binding with CREM-immunoreactive proteins eluted from the 21-kDa band in lane 1 of *A*. (*C*) Developmental expression pattern of the brain 21-kDa protein in mouse. Lanes 1 and 2, before birth (16 and 20 days of gestation); lanes 3–10, after birth; lanes 11 and 12, bacterial CREMβ and S-CREM proteins.

was detected, with a concomitant decrease in the band corresponding to CREMα/β (lanes 3–10). The 21-kDa protein is predominant in the adult. These results imply a developmental modulation in the expression of CREM proteins in brain.

An Internal AUG Is Used to Generate S-CREM. We wished to determine the origin of the 21-kDa protein. Although this protein is a strong candidate to be a CREM product, as it contains the γ domain and binds to a CRE (see Fig. 1), no CREM transcript has been detected to date that could encode a 21-kDa CRE-binding protein that includes a γ domain (refs. 1 and 2; data not shown). The identical migration of S-CREM and the 21-kDa protein led us to consider the possibility that both initiated at the same AUG at amino acid position 152 in CREMτ, which constitutes a good Kozak consensus sequence (Fig. 2*A* and ref. 13). Thus, the use of an alternative AUG in the CREMτ transcript could constitute a mechanism for generating this new CREM product, with possibly altered activity. To test this hypothesis we first translated CREMτ mRNA in an *in vitro* reticulocyte lysate system. Synthesis using this single transcript does indeed generate two protein products, one corresponding to CREMτ and the other with the same size as S-CREM (Fig. 2*B*, lanes 1, 4, and 6). When mRNA from the CREB transcript was used no smaller product was visible (lane 3). The amino acid sequence of

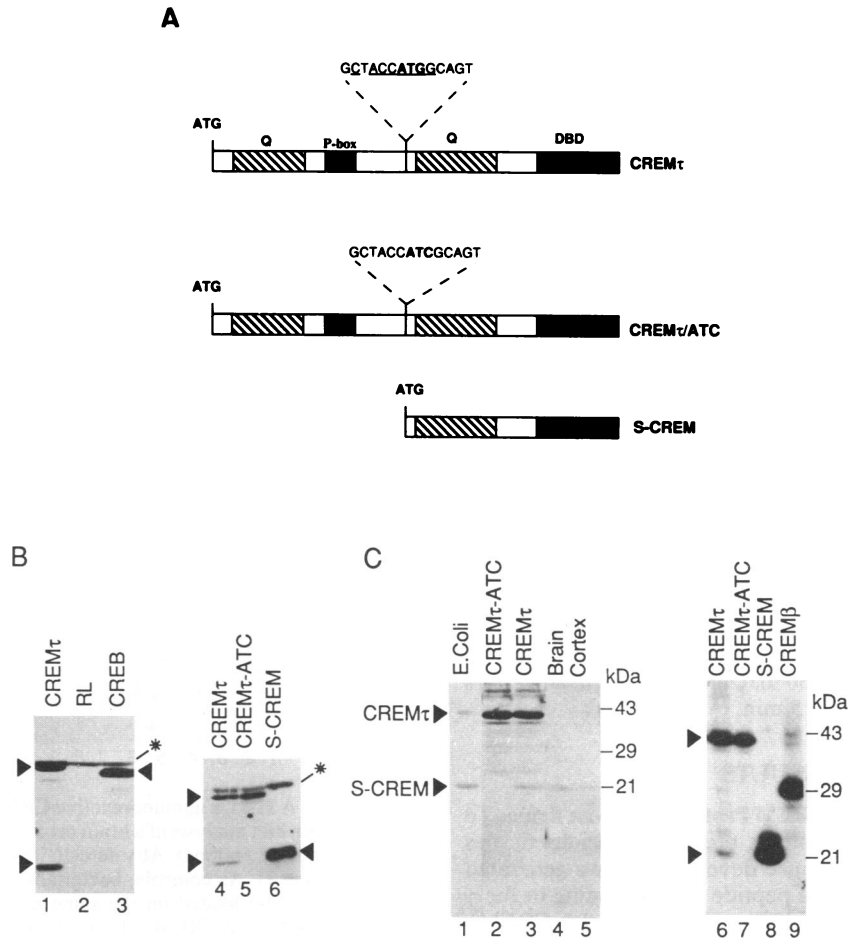

FIG. 2. An internal AUG is used as an initiation codon in CREM mRNA. (*A*) Schematic representation of the CREMτ protein (4) and of the CREMτ-ATC mutant engineered by *in vitro* mutagenesis. Initiation from the internal AUG generates S-CREM. The nucleotide sequence of the region containing the internal ATG is shown. The Kozak consensus is underlined and the ATG and mutated ATC are highlighted. Positions of the various protein domains of CREMτ are as shown (DBD, DNA-binding and dimerization domain; Q, glutamine-rich domains; P-box, phosphorylation acceptor sites; see also ref. 3). (*B*) *In vitro* generated CREM and CREB proteins. Lanes 1 and 4, proteins generated by using an *in vitro* synthesized CREMτ transcript; lane 3, CREB; lane 5, CREMτ-ATC; lane 6, S-CREM; lane 2, translation control (RL) with no specific mRNA. Asterisks denote a nonspecific translation product (3, 4). Arrowheads indicate the positions of the various CREM and CREB proteins. (*C*) Analysis of *in vivo* synthesized CREM proteins. Lanes 2–5, Western blot analysis; lanes 6–9, immunoprecipitation after ^{35}S labeling of the cells. Lanes 2, 3, and 6–9 show proteins from transfected cultured COS cells; lanes 4 and 5 show the 21-kDa protein from brain and cortex, respectively. Lane 1 shows bacterially produced CREMτ and S-CREM proteins as controls.

CREB is homologous to that of CREMτ in the domain containing the putative AUG initiation codon, but the methionine in CREM is replaced by a valine residue in CREB (1, 3). In addition, mRNA synthesized from S-CREM DNA generates a protein that comigrates with the smaller proteins produced with CREMτ mRNA (compare lanes 4 and 6). To unambiguously determine whether the use of the internal AUG could be responsible for the generation of S-CREM, we mutated the ATG sequence in the CREMτ cDNA to ATC, encoding an isoleucine instead of a methionine (CREMτ-ATC in Fig. 2*A*). RNA from CREMτ-ATC failed to generate the S-CREM protein (lane 5), demonstrating the requirement of the internal AUG for initiation. Next, we determined whether internal initiation could occur *in vivo*, and thus explain the 21-kDa protein present in adult brain tissues. We transfected cultured COS cells with expression plasmids containing either wild-type CREMτ, CREMτ-ATC, or the S-CREM coding sequences. The products were scored either by Western blot (Fig. 2*C*, lanes 1–5) or by immunoprecipitation of ^{35}S labeled cells (lanes 6–9). The results confirm that the internal AUG is responsible for the production of S-CREM *in vivo*. Note that S-CREM shows the same size whether it is generated *in vitro*, in bacteria, or in transfected cells and that it comigrates exactly with the 21-kDa product from mouse brain (Fig. 2*B*, lanes 1, 4, and 6; and compare Fig. 2*C*, lanes 1 and 3–5). Taking into account all these data, we postulate that S-CREM and the developmentally expressed 21-kDa brain protein are the same.

S-CREM Is a Repressor. We next analyzed the function of S-CREM. Using bacterially produced proteins, we demonstrated that S-CREM efficiently bound to a CRE (Fig. 1*B* and Fig. 3*A*, lane 2) and heterodimerizes with CREMτ (Fig. 3*A*, lane 3). This was expected because of the integrity of the S-CREM basic/leucine zipper domain. S-CREM lacks the phosphorylation domain and one glutamine-rich region (see Fig. 2*A*), both of which are required for transcriptional activation potential (4, 14); hence we tested its function in transcriptional regulation. Transfection experiments using JEG-3 cells demonstrated that S-CREM efficiently represses cAMP-induced transcription (Fig. 3 *B–D*). At the concentrations used, S-CREM down-regulated PKA-induced transcription as efficiently as the previously described antagonist CREMβ (Fig. 3*B*, compare lanes 3–5 and 8–10; see ref. 3). Transfection experiments with low amounts of expression plasmid indicated that S-CREM was slightly less powerful than CREMβ in down-regulating cAMP-induced transcrip-

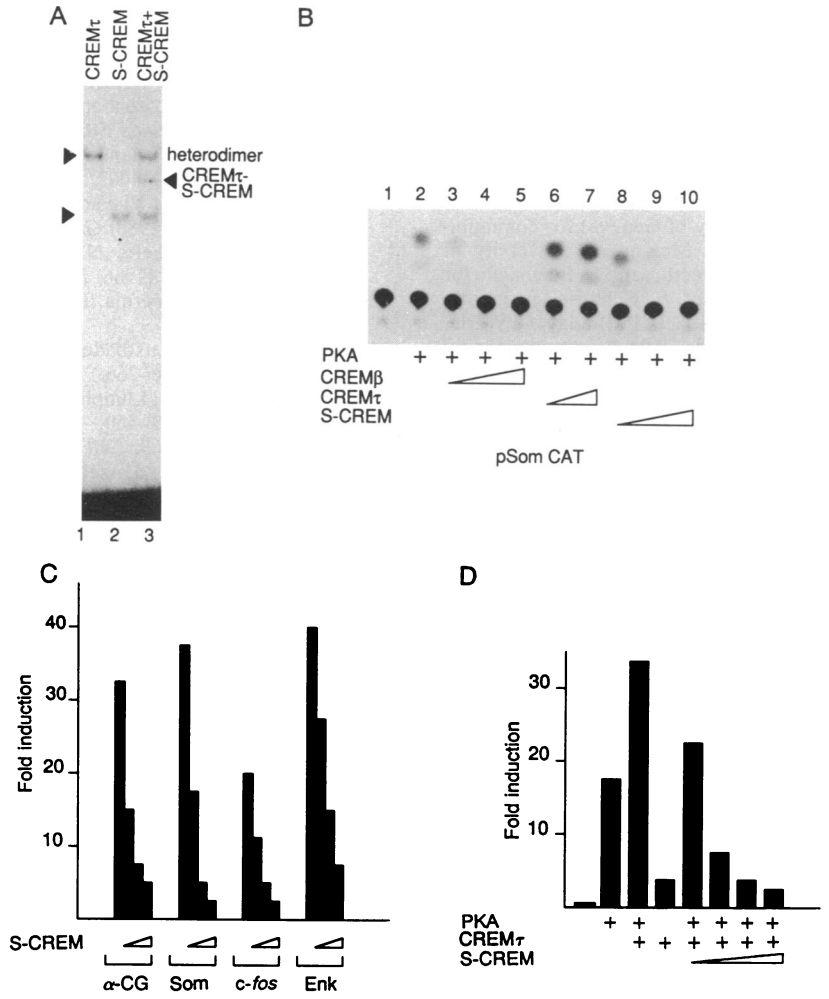

FIG. 3. S-CREM acts as a CRE-binding transcriptional repressor. (*A*) Gel shift analysis using a somatostatin CRE probe and bacterially produced CREMτ and S-CREM. Both CREM proteins efficiently bind to the CRE (lanes 1 and 2) and heterodimerize (lane 3, arrowhead). (*B*) Representative transfection in JEG-3 cells showing that S-CREM represses protein kinase A (PKA)-induced activation of pSomCAT (3). Compare lane 2 with lanes 8–10. In the same experiments CREMβ antagonizes (lanes 3–5; see ref. 3) and CREMτ stimulates (lanes 6 and 7; see ref. 4) PKA-mediated activation. Increasing amounts of each CREM expression plasmid were as follows: 200 ng (lanes 3, 6, and 8), 400 ng (lanes 4, 7, and 9), and 1 μg (lanes 5 and 10). (*C*) S-CREM represses transcription from various cAMP-inducible promoters; α-CG (human α chorionic gonadotropin; ref. 7); Som (rat somatostatin); c-*fos* (human c-*fos* protooncogene; ref. 8); Enk (human proenkephalin; ref. 9). In each case the first bar indicates the PKA-induced level with respect to the basal level (defined as 1). The three subsequent bars show the effect of increasing amounts of cotransfected S-CREM (400 ng, 1 μg, and 2 μg, respectively). (*D*) Effect of cotransfection of S-CREM with exogenous CREMτ. S-CREM represses transcriptional activation obtained with coexpression of PKA and CREMτ. CREMτ amount was kept constant (1 μg) while S-CREM was increased (200 ng, 400 ng, 1 μg, and 2 μg).

tion (data not shown). In the same assay, CREMτ activated CRE-mediated transcription (lanes 6 and 7). S-CREM repressed cAMP-induced transcription from four different cAMP-inducible promoters (Fig. 3*C*), indicating its wide range of activity. Finally, we tested the effect of S-CREM on exogenous CREMτ activity, by cotransfection of both expression vectors, and also observed a down-regulation in this case (Fig. 3*D*).

DISCUSSION

We present evidence that by alternative usage of translation initiation sites, a single CREM mRNA generates both an activator and a repressor. It is puzzling that CREM, which already makes extensive use of differential splicing to generate both an activator and antagonists (3, 4), utilizes an additional mechanism to generate factors with opposite functions. It is important to note, however, that S-CREM is distinct from the previously described antagonist CREM forms. It does not contain the phosphoacceptor sites (P-box) but instead contains a single glutamine-rich domain (Fig. 2*A*). This is in contrast to the CREM$\alpha/\beta/\gamma$ antagonists, which contain the P-box but no glutamine-rich domains (3). It is reasonable to hypothesize that the structural differences between these various down-regulators have functional significance and that these proteins may interact differently with other components of the transcriptional machinery. In addition, the differential presence of the P-box among these various CREM down-regulators suggests that some of them might be modulated in their function by phosphorylation.

The observations reported here describe a mechanism by which one gene can generate both activator and repressor proteins. In other genes the possibility of generating both activators and repressors is achieved by alternative splicing (15). In this report we show that alternative initiation constitutes an important level of regulation in the production of a transcription factor with a specific function. Internal initiation has been described for other genes: for example, the oncogenes *int-2* (16) and *pim-1* (17) and the androgen receptor gene *Tfm* (18). Interestingly, alternative initiation has recently been described for another leucine-zipper regulatory protein, the liver activator protein (LAP) (19). In LAP, an

internal AUG is used to produce LIP, a repressor protein. The major difference between LAP and CREM is that LAP is an intronless gene, whereas CREM has a multiexonic structure (N.S.F., unpublished data), which, by differential splicing, is already the basis for extensive functional modulation.

V.D. and B.M.L. contributed equally to the work presented in this paper. We thank U. Schibler (University of Geneva) for communicating data prior to publication, G. S. McKnight (University of Washington) for the pCαEV expression vector, and E. Benusiglio for discussions. The technical assistance of F. Schlotter is greatly appreciated. We thank A. Staub, F. Ruffenach, B. Boulay, C. Werlé, G. Duval, Y. Lutz, and M. Acker for help. B.M.L. is supported by a Human Frontier Science Program long-term fellowship. R. dG. is supported by a Dutch Cancer Society fellowship. N.S.F. is supported by a European Molecular Biology Organization long-term fellowship. This work was supported by grants from the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Association pour la Recherche sur le Cancer, and Rhone-Poulenc-Rorer.

1. Hoeffler, J. P., Meyer, T. E., Yun, Y., Jameson, J. L. & Habener, J. F. (1988) *Science* **242,** 1430–1433.
2. Hai, T.-Y., Liu, F., Coukos, W. J. & Green, M. R. (1989) *Genes Dev.* **3,** 2083–2090.
3. Foulkes, N. S., Borrelli, E. & Sassone-Corsi, P. (1991) *Cell* **64,** 739–749.
4. Foulkes, N. S., Mellstrom, B., Benusiglio, E. & Sassone-Corsi, P. (1992) *Nature (London)* **355,** 80–84.
5. Mellon, P. L., Clegg, C. H., Correll, L. A. & McKnight, G. S. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 4887–4891.
6. Studier, F. W., Rosenberg, A. M., Dunn, J. J. & Dubendorff, J. W. (1990) *Methods Enzymol.* **185,** 60–89.
7. Delegeane, A., Ferland, L. H. & Mellon, P. L. (1987) *Mol. Cell. Biol.* **7,** 3994–4002.
8. Sassone-Corsi, P., Visvader, J., Ferland, L. H., Mellon, P. L. & Verma, I. M. (1988) *Genes Dev.* **2,** 1529–1538.
9. Comb, M., Birnberg, N. C., Seasholtz, A., Herbert, E. & Goodman, H. M. (1986) *Nature (London)* **323,** 353–356.
10. Barber, J. R. & Verma, I. M. (1987) *Mol. Cell. Biol.* **7,** 2201–2211.
11. Hoeffler, J. P., Lustbader, J. W. & Chen, C.-Y. (1991) *Mol. Endocrinol.* **5,** 256–266.
12. Sassone-Corsi, P., Lamph, W. W., Kamps, M. & Verma, I. M. (1988) *Cell* **54,** 553–560.
13. Kozak, M. (1989) *J. Cell Biol.* **108,** 229–241.
14. Gonzalez, G. A., Menzel, P., Leonard, J., Fischer, W. H. & Montminy, M. R. (1991) *Mol. Cell. Biol.* **11,** 1306–1312.
15. Foulkes, N. S. & Sassone-Corsi, P. (1992) *Cell,* **68,** 411–414.
16. Acland, P., Dixon, M., Peters, G. & Dickson, C. (1991) *Nature (London)* **343,** 662–665.
17. Saris, C. J. M., Domen, J. & Berns, A. (1991) *EMBO J.* **10,** 655–664.
18. Gaspar, M. L., Meo, T., Bourgarel, P., Guenet, J. L. & Tosi, M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 8606–8610.
19. Descombes, P. & Schibler, U. (1991) *Cell* **67,** 569–579.