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The functional versatility of CREM is determined by its modular structure

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The CREM gene (cAMP-responsive element modulator) generates both activators and repressors of cAMP-induced transcription by alternative splicing. We determined the exon structure of the CREM gene and have identified new isoforms. We show that CREM isoforms with different structural characteristics are generated by the shuffling of exons to produce proteins with various combinations of functional domains. CREM proteins bind efficiently to CREs and here we demonstrate that the various isoforms heterodimerize *in vivo* with each other and with CREB. The two alternative DNA binding domains of CREM, which are differentially spliced in the various isoforms, show distinct binding efficiencies, while CREMα/CREB heterodimers exhibit stronger binding than CREMβ/CREB heterodimers to a consensus CRE *in vitro*. We identify the protein domains involved in activation function and find that the phosphorylation domain and a single glutamine-rich domain are sufficient for activation. A minimal CREM repressor, containing only the b-Zip motif, efficiently antagonizes cAMP-induced transcription. In addition, phosphorylation may reduce repressor function, as a CREMβ mutant carrying a mutation of the serine phosphoacceptor site ($CREM\beta_{68}$) represses more efficiently than the wild-type CREMβ.

Key words: alternative splicing/gene regulation/multiexonic structure/signal transduction/transcription factor

Introduction

The cAMP-dependent signal transduction pathway is characterized by its specific protein kinase [protein kinase A (PKA)] and its ultimate target for transcriptional control, the CRE (cAMP-responsive element) (for reviews see Habener, 1990; Ziff, 1990; Borrelli *et al.*, 1992). A consensus CRE site is constituted by an 8 bp palindromic sequence (TGACGTCA) (Comb *et al.*, 1986; Andrisani *et al.*, 1987; Delegeane *et al.*, 1987; Sassone-Corsi, 1988). Changes in the intracellular levels of cAMP directly control the activity of PKA (McKnight *et al.*, 1988). PKA, in turn, has a wide range of protein targets which include nuclear transcription factors (Habener, 1990; Ziff, 1990; Borrelli *et al.*, 1992).

A number of cDNAs encoding CRE binding factors have been isolated (Hoeffler *et al.*, 1988; Gonzalez *et al.*, 1989; Hai *et al.*, 1989; Maekawa *et al.*, 1989; Ivashkiv *et al.*, 1990; Foulkes *et al.*, 1991a; Hsu *et al.*, 1991). All belong to the b-Zip [basic motif/leucine zipper (LZ)] family of proteins (Landschulz *et al.*, 1988; Busch and Sassone-Corsi, 1990). The first cDNAs to be characterized encoded CREB (CRE binding protein) (Hoeffler *et al.*, 1988; Gonzalez *et al.*, 1989). The leucine zipper is responsible for the dimerization of the protein and dimerization is a prerequisite for DNA binding (Dwarki *et al.*, 1989).

Various protein domains cooperate to elicit the CREB transcriptional activation function. The first, defined as KID domain or P-box (for reviews see Habener, 1990; Brindle and Montminy, 1992), contains several consensus phosphorylation sites. Flanking this domain are two regions rich in glutamine residues. Glutamine-rich regions have been found in the activation domains of other factors, such as Oct1, Oct2 and Sp1 (Courey and Tjian, 1988; Tanaka and Herr, 1990). Phosphorylation by PKA is necessary for transcriptional activation by CREB via a serine residue at position 133 (Gonzalez and Montminy, 1989; Lee *et al.*, 1990; Yamamoto *et al.*, 1988, 1990). However, the presence of other phosphorylation sites strongly suggests a more complex regulation (Flint and Jones, 1991; Sheng *et al.*, 1991; Borrelli *et al.*, 1992; R.de Groot and P.Sassone-Corsi, in preparation). In addition, the motif DLSSD, C-terminal to the serine 133 residue, appears to cooperate with the PKA site to elicit transcriptional activation (Gonzalez *et al.*, 1991). The current model to explain the CREB activation domain function is based upon allosteric conformational changes mediated by phosphorylation of the KID domain. In this model, phosphorylation triggers the activation of the glutamine-rich domains by a distant conformational change (Gonzalez *et al.*, 1991).

The isolation of the CREM gene constitutes an important advance in the understanding of cAMP-regulated transcription (Foulkes *et al.*, 1991a). A remarkable feature of CREM is that it encodes two alternative b-Zip domains (DBDI and DBDII). In contrast to CREB, CREM expression appears to be finely regulated, both transcriptionally and post-transcriptionally; various isoforms are produced in a cell- and tissue-specific manner. The CREM products share extensive homology with CREB, especially in the DNA binding domains and the phosphorylation region, and also specifically bind to CREs. However, CREMα, β and γ proteins block the transcriptional activation obtained by the joint action of CREB and the catalytic subunit of PKA (Foulkes *et al.*, 1991a,b). As well as antagonists, the CREM gene also encodes an activator of cAMP-dependent transcription, CREMτ (Foulkes *et al.*, 1992). CREMτ differs from the CREM antagonists by the coordinate insertion of two glutamine-rich domains which confer transcriptional activation function on the protein. We have demonstrated a splicing-dependent reversal in CREM function which represents an important example of developmental modulation in gene expression. During spermatogenesis there is a functional switch in CREM

expression, from low levels of antagonists to high levels of the activator CREMτ (Foulkes *et al.*, 1992). In addition, we have recently demonstrated that the CREMτ transcript, by the use of an internal AUG codon, can alternatively encode an N-terminally truncated CREMτ protein, S-CREM (Delmas *et al.*, 1992). Interestingly, this form functions as a repressor of cAMP-induced transcription. Thus, by two distinct mechanisms, a single gene encodes both activators and repressors of cAMP-mediated transcription (Foulkes and Sassone-Corsi, 1992).

Here we report the characterization of the structure and corresponding function of both the CREM repressor proteins and activators. We identify two new CREM isoforms, CREMτ1 and CREMτ2. Each form contains only one glutamine-rich (Q) domain. We have determined the coding exons of the CREM gene and we are able to correlate exon structure with structural and functional modules of the protein. We demonstrate differences between DBDI and DBDII, both in DNA binding specificity and in heterodimerization capacity. We show that CREM isoforms can heterodimerize with each other and with CREB *in vivo*. Using GAL4 fusion constructs and by deletion analysis we have identified the domains of the CREM proteins responsible for activator and repressor function. Finally, we have investigated the importance in both activation and repression of a serine phosphoacceptor site for protein kinase A.

Results

Modular structure of CREM

The genomic organization of the CREM gene shows a multi-exonic structure. By comparing genomic and cDNA clone sequences and by RNase protection mapping, we identify eight CREM coding exons (Figure 1A; in preparation). For the purpose of this paper we outline the exonic structure of the CREM gene which is relevant to the results reported here. The N-terminal exon of 118 bp contains the putative ATG initiation codon and is present in all the known CREM isoforms. The second exon encodes the first glutamine-rich domain of CREMτ, In.1. The two downstream exons encode the phosphorylation domain or P-Box. The second glutamine-rich domain of CREMτ, In.2, is encoded by a 189 bp exon. A short exon of 36 bp, which encodes the γ-domain, is present in all the isoforms, except CREMγ (Foulkes *et al.*, 1991a). Finally, the two DNA binding domains are encoded by the terminal 3′ exon as shown in Figure 1A. Within this exon there is an alternative splice acceptor site which lies downstream of DBDI and is used to splice the second DNA binding domain, DBDII, into the coding sequence (Foulkes *et al.*, 1991a). Both DBDI and DBDII share the same 5′ sequence encoded by the 3′ end of the penultimate exon.

The functional domains of the CREM proteins are encoded by distinct exons. Thus, shuffling of the domains by alternative splicing in various combinations generates proteins with different functions. The highly related CREB gene has a very similar genomic structure (Waeber *et al.*, 1991; Ruppert *et al.*, 1992) and also generates multiple isoforms by alternative splicing. However, unlike CREM, CREB has been shown to produce only a transcriptional activator and no repressor; in addition, the functional significance of the other isoforms remains poorly understood (Waeber *et al.*, 1991; Ruppert *et al.*, 1992).

In order to assess the full functional repertoire of the CREM gene, we have systematically analysed the range of CREM mRNA isoforms by RT-PCR and RNase protection analyses (N.S.Foulkes and P.Sassone-Corsi, in preparation). Here, as a representative example, we report the RT-PCR analysis of mRNAs from seven different cell lines using primers specific to the phosphorylation and DNA binding domains (primers A and C; Figure 1A). The ratio between the bands corresponding to CREMα, β and γ and CREMτ as well as the overall quantity of CREM mRNA varies extensively between various cell lines. Although this analysis is not fully quantitative it suggests strongly that there is a wide variation in CREM expression in different cell types (Figure 1B). This is in contrast to CREB which appears to be expressed at an equivalent level in all the cell types used in this analysis (Figure 1B). It is interesting to note that the pattern of CREM expression is different between GC and GH_3 cells, which are both derived from the pituitary somatotroph cell lineage.

CREMτ contains two glutamine-rich domains which were shown to be coordinately inserted; the presence of these additional structural modules confers activation function on this factor (Foulkes *et al.*, 1992). We wished to determine whether exons In.1 and In.2 can also be found individually in CREM isoforms. When we performed RT-PCR using primers specific to In.1 and In.2 (primers E and F respectively, see Figure 1A) in combination with primers complementary to flanking exons (primers D and G, Figure 1A), we identified two new splicing variants (Figure 1C). By hybridization and sequence analysis (data not shown) we determined that they are identical to CREMβ except that they each incorporate a single glutamine-rich domain: either In.1, which generates CREMτ1, or In.2 which generates CREMτ2. The relative levels of CREMτ1 and CREMτ2 transcripts appear to vary in a cell-specific manner (Figure 1C; and data not shown). We present the data here as an indication of the qualitative changes in CREM mRNA composition in various cell types; a complete quantitative report is in preparation.

DNA binding modules: differential specificity of DBDI and DBDII

The CREM gene encodes two DNA binding domains (Foulkes *et al.*, 1991a; see also Figure 1A), each containing a basic region and leucine zipper (b-Zip) motif (Figure 2A). The CREMα transcript encodes both of these domains, however, only DBDI is translated. Other isoforms encode the second domain, DBDII (Foulkes *et al.*, 1991a). Comparison of the basic region and LZ of CREB with CREM DBDI and DBDII reveals that DBDI shows 95% identity with CREB and that DBDII shows 75% identity, the differences being mostly within the LZ domain (Foulkes *et al.*, 1991a; see also Figure 2A). To determine precisely the binding specificity of DBDI compared with DBDII, bacterially synthesized CREMα and CREMβ proteins (Delmas *et al.*, 1992) were tested for their ability to bind several different CRE sequences in a gel retardation assay (Figure 2C). Oligonucleotides were synthesized which correspond to known CRE sites found in 10 different genes. The core CRE sequence consists of an 8 bp palindrome and in these oligonucleotides 5 bp flanking the core sequence of each CRE were included (see Table I). The consensus core CRE sequence is TGACGTCA but many of the sequences listed show sequence variation either at the 5′ or

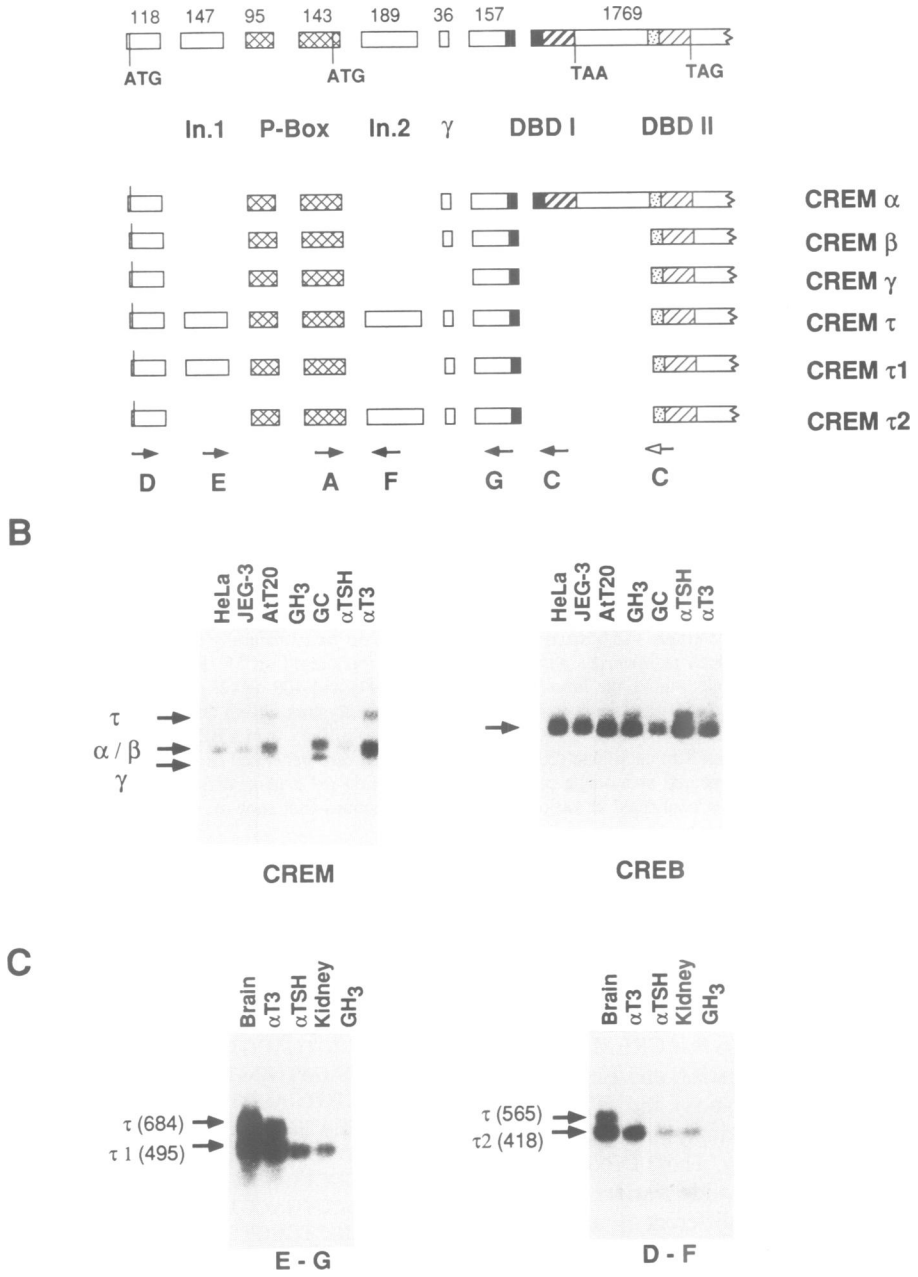

Fig. 1. CREM multi-exonic structure: the basis of functional versatility. (**A**) Schematic representation of CREM exon structure and transcripts. On the top row, numbers indicate the size (bp) of the corresponding exons, below are labelled the functional domains, In.1, In.2 (glutamine-rich domains), P-Box (phosphorylation domain), γ and b-Zip domains (DBDI and DBDII). Cross-hatched boxes are the phosphorylation domain exons while the hatched boxes represent the leucine zipper portion of the two DNA binding domains. The solid black boxes represent the basic region of DBDI and the shared 5′ portion of the basic region of DBDII. The stippled box shows the 3′ portion of the basic domain of DBDII. The positions of the initiation (ATG) and termination codons (TAA and TAG) are indicated. The position of the alternative ATG used to generate S-CREM (Delmas *et al.*, 1992) is also shown. Beneath is represented the exon composition of each of the CREM mRNA isoforms. Below, the positions and direction of the priming sites for each of the oligonucleotides used in the RT-PCR analysis are indicated by arrows (see also Materials and methods and panels B and C, this figure). A hollow arrowhead shows a second priming site of oligonucleotide C within DBDII. Here, the oligonucleotide is partially mismatched with the CREM mRNA and empirically, we consistently fail to detect fragments corresponding to CREMα, being amplified from this site (Foulkes *et al.*, 1991a). (**B**) RT-PCR analysis of CREM RNA in seven different cell lines using primers A and C (see panel A; Foulkes *et al.*, 1991a, 1992). Duplicate Southern blots of the PCRs were probed with CREM and CREB-specific cDNA probes (see Materials and methods). For the CREM analysis, arrows indicate bands corresponding to the CREMα/β (336 bp) and CREMγ (300 bp) antagonist mRNA isoforms and the CREMτ activator (525 bp). An arrow also indicates the band corresponding to the uniformly expressed CREB transcript. The cell lines corresponding to each lane are labelled above: JEG-3 human choriocarcinoma cells, HeLa, the GH_3 and GC somatotropic/lactotropic pituitary cell lines, the corticotropic AtT-20 cell line, the thyrotropic cell line: αTSH (Akerblom *et al.*, 1990) and the gonadotropic cell line: αT3 (Windle *et al.*, 1990). (**C**) RT-PCR analysis of five RNAs from different tissues and cell lines (see panels A and B, this figure). Primers E and G (left hand panel) corresponding to sites in the In.1 exon and the penultimate exon amplify, in addition to the CREMτ isoform (684 bp amplified fragment), a fragment which corresponds to exclusion of the In.2 exon (495 bp: CREM τ1). Using primers D and F (right hand panel), corresponding to sites in the ATG exon and In.2, in addition to a fragment amplified from CREMτ (565 bp, this band can clearly be seen on a longer exposure), a smaller fragment of 418 bp corresponds to an isoform lacking In.1 i.e. CREMτ2. Although RT-PCR reactions alone are not sufficient to accurately assess the relative quantities of the different isoforms, for a given set of primers it is evident that there is significant variation in the ratio of the CREMτ, CREMτ1 and CREMτ2 between different cells and tissues. Consistent with data reported here and from previous analyses (panel B, this figure and Foulkes *et al.*, 1991a), the somatotropic/lactotropic pituitary cell line GH_3 does not exhibit detectable levels of CREM mRNA although in common with the other cell lines it has normal CREB expression.

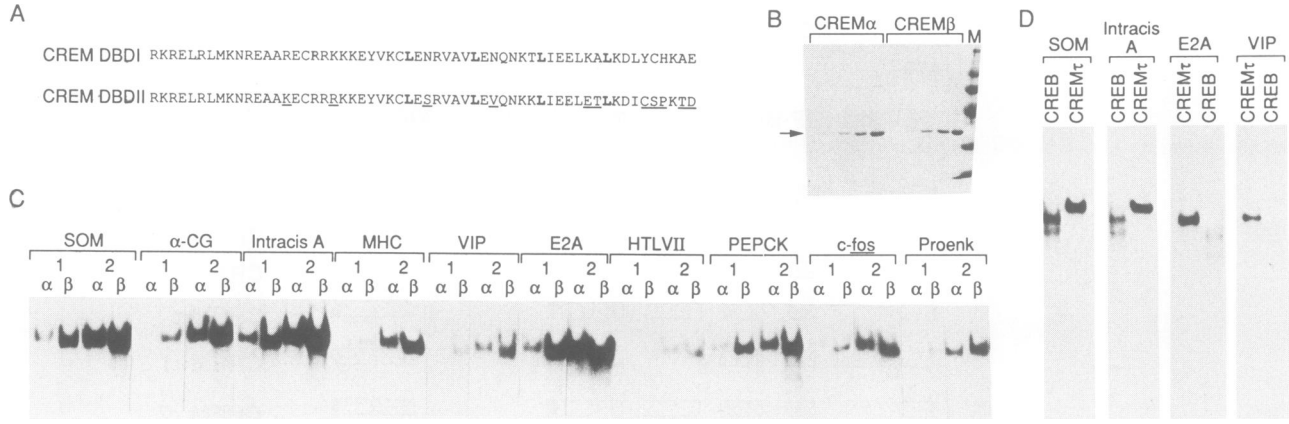

Fig. 2. Differential binding of CREMα and CREMβ to cAMP-responsive elements. (**A**) Sequence of the two alternatively spliced b-Zip motifs of CREM, DBDI and DBDII. CREMα protein contains DBDI and CREMβ protein contains DBDII. Residues underlined in DBDII are not conserved in DBDI. The four leucine residues of the LZ are shown in bold type face (see also Foulkes *et al.*, 1991a). (**B**) SDS−PAGE gel showing bacterially purified CREM proteins. Increasing concentrations (50, 100, 200, 400 and 800 ng) of CREMα and CREMβ were electrophoresed on an 11% denaturing gel to determine the purity and concentration of the proteins. The CREM proteins have identical molecular weights. The prestained molecular weight markers are shown in lane M. The proteins were purified as described previously (Delmas *et al.*, 1992). (**C**) Gel retardation experiments illustrating the binding of bacterially synthesized CREMα and CREMβ to 10 naturally occurring CRE sites. The proteins were incubated with excess end-labelled probes as described in Materials and methods and electrophoresed on 5% PAGE gels containing 0.25×TBE buffer. The CRE probes used (see Table I) are indicated above the lanes. Binding of 50 ng (1) and 400 ng (2) of CREMα and CREMβ to each CRE is shown. (**D**) Comparative analysis by gel retardation of the binding efficiency of the two activators CREB and CREMτ to various CRE sites. CREMτ, as predicted by the data obtained using CREMα and CREMβ (C), binds with higher affinity than CREB, especially to the lower affinity sites (E2A and VIP). The same amount of protein was used in each lane (200 ng). The proteins were generated in bacteria and their quality tested on SDS−PAGE, as shown in panel B; although CREB appeared as a single protein, on a retardation gel it generated two bands. The higher affinity of CREMτ with respect to CREB for most CREs has been confirmed at various protein concentrations (not shown).

3′ end. To verify the purity and the concentration of the CREM bacterial proteins, increasing concentrations were loaded on to a denaturing SDS−PAGE gel (Figure 2B). We then tested the binding of increasing amounts of CREMα and CREMβ proteins to excess end-labelled CRE oligonucleotides (Figure 2C). The specificity of binding varies among the different CREs so that CREM proteins bind strongly to the somatostatin, IntracisA and E2A CREs, but only weakly to the MHC II (X-box) and HTLVII CREs (Figure 2C). However, most strikingly, in all cases CREMβ binds more strongly than CREMα. Thus, DBDII recognizes more efficiently than DBDI a wide variety of naturally occurring CRE sites which have different 5′ and 3′ flanking sequences. DBDI has a high identity with the CREB b-Zip domain and we found that, likewise, CREB binds to various CREs with lower affinity than CREMτ, which contains DBDII (Figure 2D). We then tested whether there is a difference in binding affinity between CREMα/CREB and CREMβ/CREB heterodimers. Increasing amounts of bacterially synthesized CREMα and CREMβ proteins were allowed to heterodimerize to a CREBcore peptide of 63 amino acids (corresponding to the CREB b-Zip domain; see also Foulkes *et al.*, 1991a) and bind to an end-labelled somatostatin CRE oligonucleotide (Figure 3A, lanes 1−7). More CREMα/CREB heterodimers bind to the CRE than CREMβ/CREB heterodimers. We confirmed this result by carrying out the converse experiment, in which the concentration of the CREM proteins was kept constant while the amount of CREBcore peptide increased (lanes 8−13). These data indicate that CREMα heterodimerizes more efficiently with CREB, possibly due to the higher sequence identity in the b-Zip region. Alternatively, the intrinsic binding capacity of the two heterodimers may be different.

Table I. Sequences of the 10 CREs used in Figure 2

CRE sequence	Corresponding gene
TTGGC**TGACGTCA**GAGAG	somatostatin
AAAAT**TGACGTCA**TGGTA	α-CG
TCCCG**TGACGTCA**TCTGG	IntracisA
AACAG**ATGCGTCA**TCTCA	MHC (X-box)
TACTG**TGACGTCT**TTCAG	VIP
TCTGA**TGACGTAT**TTCAG	E2A
GGCCC**TGACGTCC**CTCCC	HTLVII LTR
GCCCC**TTACGTCA**GAGGC	PEPCK
GCCCG**TGACGTTT**ACACT	c-*fos*
GGGCC**TGCGTCAG**CTGCA	proenkephalin

Sequences of one strand of the 10 oligonucleotides synthesized and used in gel retardation assays (Figure 2C and D), which correspond to naturally occurring CRE sites. The eight base CRE sequence is shown in bold type. The name of the corresponding gene is given (see Borrelli *et al.*, 1992 and references therein).

CREM heterodimerizes in vivo

We wished to determine whether CREM proteins can be present *in vivo* as homodimers and/or heterodimers. We co-transfected COS cells with CREMτ, CREB, S-CREMα and S-CREMγ expression vectors (see Figure 6A for description of S-CREM; Delmas *et al.*, 1992) and analysed the CRE binding activity present in the corresponding whole cell extracts (Figure 3B). Although gel retardation assays demonstrate binding of proteins to specific DNA sequences, they also provide important information on the different protein−DNA complexes which can be formed. Truncated CREM constructs (S-CREM) were used in order to visualize clearly heterodimer complexes. The presence of intermediate mobility complexes is consistent with heterodimer formation

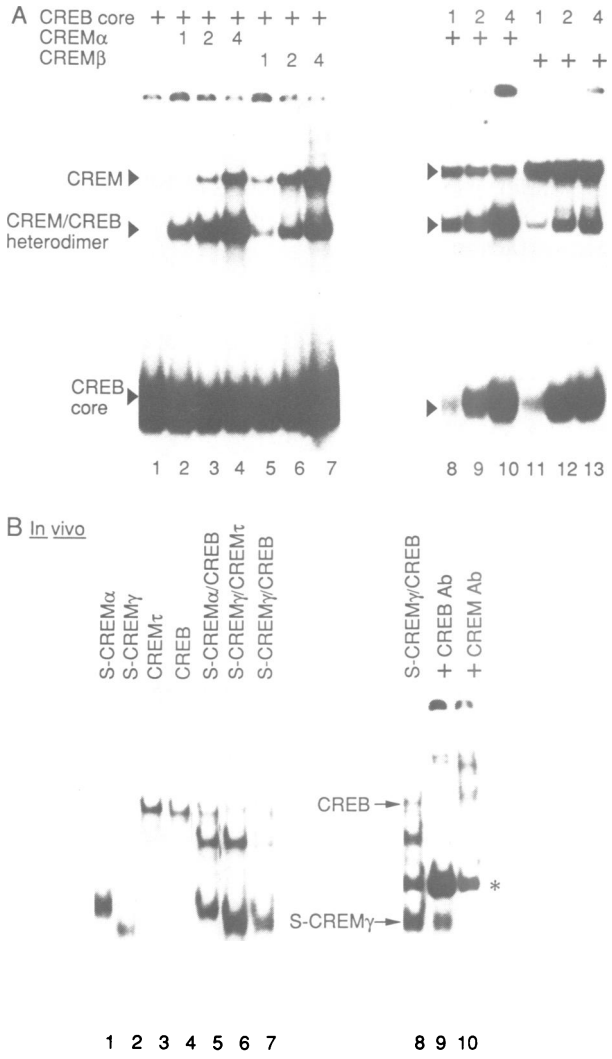

Fig. 3. Heterodimerization of CREM isoforms *in vitro* and *in vivo*. (**A**) CREMα/CREB heterodimers exhibit more binding than CREMβ/CREB heterodimers to a consensus CRE site *in vitro*. Bacterially synthesized CREMα and CREMβ were allowed to heterodimerize with a 63 amino acid CREBcore peptide, which contains the b-Zip motif of CREB (Foulkes *et al.*, 1991a), and then the complexes, bound to a labelled CRE probe, were visualized on a 4% gel. Lane 1, CREBcore; lanes 2–4, CREBcore and increasing amounts of CREMα (1, 2 and 4 μg); lanes 5–7, CREBcore and increasing amounts of CREMβ (1, 2 and 4 μg). The inverse experiment was also carried out, using increasing amounts of CREBcore with constant amounts of bacterially synthesized CREMα and CREMβ proteins; lanes 8–10, CREMα and CREBcore, lanes 11–13, CREMβ and CREBcore. (**B**) *In vivo* heterodimerization of CREM isoforms. Gel retardation assays were carried out using COS whole cell extracts. COS cells were transiently transfected with various CREM or CREB expression vectors and 10 μg of whole cell extracts were allowed to bind to γ-^{32}P-labelled somatostain CRE probe and the complexes analysed on a 4% gel (lanes 1–10). The expression vectors transfected are shown above each lane. Heterodimer complexes migrate with intermediate mobility. To verify that the intermediate mobility complex corresponds to the hetetrodimer complex, either anti-CREM-specific or anti-CREB-specific antibodies (see Materials and methods) were added to the reaction before the addition of the labelled CRE probe. The results for S-CREMγ/CREB only are shown in lanes 8–10. In lane 8, in the absence of antibody, a CREB complex, S-CREMγ complex and the intermediate heterodimeric mobility complex is seen. Following the addition of anti-CREB-specific antibody, both the intermediate band and the CREB-specific bands are supershifted (lane 9); likewise, in the presence of anti-CREM-specific antibody, the intermediate band and the CREM-specific bands are supershifted (lane 10). A non-specific band is shown by an asterisk.

between S-CREMα and CREB (lane 5), between S-CREMγ and both CREMτ (lane 6) and CREB (lane 7). Homodimer complexes are also formed. To verify that the intermediate bands are the expected heterodimer complexes, anti-CREB-specific and anti-CREM-specific antibodies were used to supershift the observed complexes (Figure 3B, lanes 8–10). These antibodies were unfortunately not efficient in revealing the presence of the various dimers in immunoprecipitation assays (not shown). Consistent with *in vitro* data, the S-CREMα/CREB heterodimer complex is more evident than the S-CREMγ/CREB complex. In addition, S-CREMγ/CREMτ heterodimers exhibit stronger binding than S-CREMγ/CREB heterodimers, further supporting the notion that heterodimers with higher sequence similarity in their b-Zip motifs heterodimerize and/or bind more efficiently.

Two distinct exons encode the glutamine-rich activation domains

CREMτ, like CREB, is an activator of cAMP-induced transcription (Foulkes *et al.*, 1992). Interestingly, the activation obtained with CREMτ is consistently greater than for CREB (Foulkes *et al.*, 1992). To test whether this difference could be a function of the higher efficiency in CREMτ DNA binding (Figure 2D), or due to other distinct structural modules, we generated GAL4–CREM fusion proteins. G4CREMτ contains the 254 N-terminal amino acids of CREMτ, excluding the b-Zip domain, fused in-frame downstream from the GAL4(1–147) DNA binding domain (Figure 4A). G4CREBΔLZ is an equivalent fusion of the N-terminal region of the CREB gene to the DBD domain of GAL4 (Sheng *et al.*, 1991). Transfections were carried out in JEG-3 cells using a reporter plasmid, GAL4–CAT, which carries five GAL4 binding sites upstream from the adenovirus-2 E1b TATA-box and CAT gene sequences (Lillie and Green, 1989). In this system there is no interference from endogenous CRE binding proteins and no background of PKA-induced transcription. In the absence of PKA, the level of CREMτ induced expression is low but consistently 3-fold higher than CREB induced expression (Table II). These data suggest that transcriptional activation by CREMτ is not as dependent on PKA as compared with CREB. This is further supported by mutation of the PKA phosphoacceptor site (see Figure 4C). Cotransfection with pCαEV, which encodes the PKA catalytic subunit (McKnight *et al.*, 1988; Mellon *et al.*, 1989), dramatically increases activation in both cases, and again G4CREMτ exerts a more powerful effect than G4CREBΔLZ (Figure 4B and Table II). We considered whether the two glutamine-rich domains of CREMτ, encoded by two distinct exons (In.1 and In.2 in Figure 1A; Q1 and Q2 in Figure 4A), could act in an additive or synergistic manner. Furthermore, because alternatively spliced isoforms exist which contain only one of the two insertions (CREMτ1 and CREMτ2, see Figure 1), it suggests that the function of a single Q-domain may be of physiological significance. GAL4 fusions were created using either the N-terminal 191 amino acids of CREMτ1 or the N-terminal 205 amino acids of CREMτ2 fused C-terminally to the GAL4 (1–147) sequence (Figure 4A). Both fusion proteins appear to be able to activate transcription from the

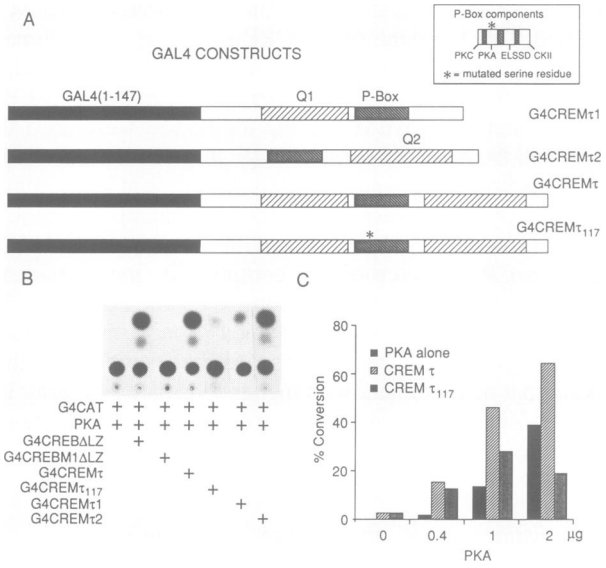

Fig. 4. Activity of CREM transcriptional activators in transient transfection assays. **(A)** GAL4−CREM (G4CREM) constructs. CREMτ, CREMτ1 and CREMτ2, were fused C-terminally to the GAL4 DNA binding domain (black box, amino acids 1−147), as described in Materials and methods (see also Sheng *et al.*, 1991). In all cases the N-terminal region of CREM, excluding the CREM b-Zip motif, was used to create the fusion product. The putative phosphoacceptor sites for PKC, PKA and CKII kinases as well as the acidic ELSSD domain are represented as the P-box (dark, hatched box) and the glutamine-rich domains (Q) as crossed boxes. G4CREMτ_{117} contains a serine to alanine mutation in the consensus PKA phosphoacceptor site. **(B)** Representative CAT assays of a JEG-3 transfection experiment showing CREM activator function. The activation potential of G4CREMτ_{117} is compared with G4CREMτ, G4CREMτ1, G4CREMτ2, G4CREBΔLZ and G4CREBM1ΔLZ in the presence of PKA. The plasmids used for each transfection are shown below each lane. Further data are presented in Table II. **(C)** Mutation of the serine 117 phosphoacceptor site in CREMτ. A representative histogram of a CAT assay from a JEG-3 transfection assay showing the effect of cotransfecting increasing amounts of pCαEV vector (encoding the PKA catalytic subunit) with a constant amount (4 μg) of either CREMτ or CREMτ_{117}. The black bars show the increase in endogenous CAT activity with increasing amounts of transfected PKA, the stippled bars represent cotransfection with CREMτ and the striped bars cotransfection with CREMτ_{117}. Variation did not exceed 15%.

GAL4−CAT reporter gene. G4CREMτ1 shows 1.5- to 2-fold less activity than G4CREMτ2 (Figure 4B). However, the combined activation potential of each domain is equivalent to activation by CREMτ (Table II). It is interesting to speculate why In.2 alone should elicit a stronger activation than In.1 alone. It could be that the difference reflects the importance of the conformation given by the tertiary structure.

Role of phosphorylation in CREMτ function

Inactivation of the protein kinase A site of CREB by mutating the serine 133 abolishes CREB transcriptional function (Gonzalez and Montminy, 1989; Lee *et al.*, 1990; Sheng *et al.*, 1991). We tested the effect of mutating the equivalent CREMτ serine, serine 117, to a glutamic acid residue. The corresponding plasmid, pSVCREMτ_{117}, as well as CREMτ, were cotransfected with pSomCAT and increasing amounts of pCαEV (which encodes the catalytic subunit of PKA; McKnight *et al.*, 1988; Figure 4C). CAT activity increases 4- to 5-fold in the presence of CREMτ, as previously reported (Foulkes *et al.*, 1992). The serine to glutamic acid mutation reduces CREMτ_{117} activity substantially. In the absence of PKA, transfection of the mutant plasmid resulted in a very low level of expression, in the same order of magnitude as for wild-type CREMτ. Increasing the amounts of pCαEV and cotransfecting with a constant amount of mutant plasmid demonstrates that the activation potential of CREMτ_{117} is severely reduced but not eliminated (Figure 4C). Equivalent results were obtained with a CREMτ mutant carrying a serine to alanine mutation at position 117 (data not shown). To determine whether the basal activity is simply due to endogenous CRE activating factors, we constructed a G4CREMτ_{117} fusion (Figure 4A) and compared the activity of this mutant with G4CREBM1ΔLZ, which carries the equivalent mutation at serine 133 (Sheng *et al.*, 1991). We found that the activity of G4CREMτ_{117} is reduced 14- to 17-fold compared with G4CREMτ. However, this mutant retains some activity, unlike G4CREBM1ΔLZ which is completely inactive

Table II. Activities of GAL4 fusion constructs and truncated CREM constructs

GAL4 fusion constructs	% conversion		Truncated CREM antagonists	% conversion	
	+PKA	−PKA		400 ng	1 μg
G4CAT	0.89 (±0.07)	0.76 (±0.05)	pSomCAT	23.0 (±2.1)	23.0 (±2.1)
G4CREBΔLZ	54.4 (±5.2)	1.5 (±0.1)	CREMα	5.9 (±0.5)	1.5 (±0.1)
G4CREBM1ΔLZ	1.3 (±0.1)	ND	CREMβ	4.7 (±0.4)	1.0 (±0.08)
G4CREMτ	78.0 (±6.9)	5.1 (±0.4)	CREMβ_{68}	1.7 (±0.1)	0.57 (±0.04)
G4CREMτ_{117}	4.6 (±0.4)	ND	S-CREMβ	1.7 (±0.1)	0.51 (±0.04)
G4CREMτ1	20.8 (±1.9)	1.7 (±0.1)	S-CREMβ_A	2.2 (±0.2)	0.68 (±0.05)
G4CREMτ2	51.0 (±4.8)	3.8 (±0.4)	S-CREMβ_D	1.6 (±0.1)	1.0 (±0.1)
			S-CREMβ_{AC}	2.2 (±0.2)	1.2 (±0.1)
			S-CREMβ_{D68}	2.5 (±0.2)	1.3 (±0.1)

Values represent the percentage conversion of chloramphenicol to its acetylated form, determined by CAT assays following transfection in JEG-3 cells. The data shown are the mean results from five different experiments. (ND, not determined.) The left panel illustrates the activation potential of various GAL4−CREM and GAL4−CREB (Sheng *et al.*, 1991) fusion proteins in the presence or absence of PKA. All the constructs contain the N-termini of CREM or CREB, excluding the b-Zip motifs, fused in-frame to the GAL4(1−147) DNA binding domain. G4CREBM1ΔLZ (Sheng *et al.*, 1991) and G4CREMτ_{117} contain a mutation at the serine phosphoacceptor site for PKA. The values for G4CAT represent the background level in the presence of reporter plasmid only. The right hand panel illustrates the antagonistic effect of different S-CREMβ constructs (see Figure 6) as well as CREMβ constructs carrying a mutation of the putative serine phosphoacceptor site, serine 68. 400 ng or 1 μg of CREM expression vector was cotransfected with PKA (2 μg of pCαEV plasmid) and pSomCAT reporter plasmid. The values for pSomCAT represent the level of endogenous cAMP-induced expression. Standard deviation is given.

(Figure 4B and Table II). Thus, in contrast to CREB, mutating the serine 117 phosphoacceptor site of CREMτ does not entirely abolish CREMτ transcriptional activity. It is interesting to note that a naturally occurring truncated form of CREMτ, S-CREM, which contains one Q-domain and no P-box, acts as a repressor of cAMP-induced transcription (Delmas *et al.*, 1992). This suggests the importance of the phosphorylation domain in activation function because CREMτ2 , which contains the same Q-domain as S-CREM, but also contains the P-box, is an efficient activator of transcription (Figure 4B; see Discussion).

Dominant repression by CREM antagonists

Different levels of both the activator and repressor forms of CREM are copresent in a large number of cell types tested (Figure 1 and in preparation), suggesting that the expression of each isoform is strictly regulated and that the combination of CREM isoforms present in a particular cell has important consequences in determining the cell's response to cAMP. Thus, we carried out experiments to determine whether we could detect any differences in activity between the different repressors. When we transfected cells (JEG-3, HeLa or F9) with CREMα, CREMβ or CREMγ, in the presence of PKA and a CRE reporter plasmid, pSomCAT (Sassone-Corsi *et al.*, 1988), we found that all the repressors antagonize endogenous PKA-induced activity to an equivalent extent (Table III and data not shown). Experiments using different reporters, containing either the c-*fos* (Sassone-Corsi *et al.*, 1988) or the α-CG (Delegeane *et al.*, 1987) CREs produced similar results (Table III). A reporter containing a non-functional, mutated CRE was used as control (Table III).

Because of the high similarity between CREMα and CREB DNA binding domains and CREMβ and CREMτ DNA binding domains, we tested whether CREMα might repress CREB activity more effectively than CREMβ, and likewise whether CREMβ might repress CREMτ activity more effectively than CREMα. Cotransfection of CREMτ with either CREMα or CREMβ results in efficient repression of activity even at substoichiometric amounts of repressor. The levels of repression appear similar irrespective of which isoform is transfected (Figure 5A). Similarily, CREMα and CREMβ repress CREB activity to the same extent (Figure 5B). This is interesting, as there is a clear difference in binding and heterodimerization potential between CREMα and CREMβ (see Figures 2 and 3).

These data were further verified by examining the dominance relationship of the GAL4 fusion expression plasmids. Constructs were made using the 5′ regions of CREMβ and CREMγ fused to the GAL4 binding domain, as described (see legend to Figure 4A). The reporter plasmid in this case was GAL4CAT. Analogous results were obtained: G4CREMα/β and G4CREMγ fusion proteins repress G4CREMτ and G4CREB to the same extent and are dominant at substoichiometric amounts with respect to activator proteins (Figure 5C and not shown). CREMβ and CREMγ are identical in sequence except for the 36 bp γ-domain which is absent in CREMγ (Foulkes *et al.*, 1991a). The function of the γ-domain remains unclear, however, we note the presence of a serine residue which could constitute a phosphoacceptor site.

Role of phosphorylation in repressor activity

To determine whether phosphorylation plays a role in repressor activity, we mutated the serine residue to glutamic acid at position 68 in CREMβ, which is equivalent to the serine 117 in CREMτ and part of a consensus PKA site (see also Figure 5C). Interestingly, we observed a small but consistent increase in antagonistic function when mutant CREMβ_{68} was transfected compared with CREMβ (Figure 5D; Table II). When the expression vectors pSVCREMβ and pSVCREMβ_{68} are cotransfected with CREB or CREMτ expression vectors, the mutated repressor appears to down-regulate more strongly (data not shown). A GAL4 fusion, G4CREMβ_{68}, in which the serine 68 is mutated also showed an increase in repressor activity as compared with G4CREMβ, using a GAL4−CAT reporter plasmid (see Figure 5C). This indicates that phosphorylation by PKA may decrease the efficiency of CREM antagonistic function.

Minimal repressor structure

The importance of sequences surrounding the serine phosphoacceptor site, the so-called kinase inducible domain (KID), in CREB activation function has been demonstrated (Lee *et al.*, 1990; Gonzalez *et al.*, 1991; Sheng *et al.*,

Table III. All CREM antagonists are efficient repressors of cAMP-dependent transcription

	pSomCAT		α-CG−CAT		c-*fos*CRE−CAT		c-*fos*CRE*−CAT	
	−PKA	+PKA	−PKA	+PKA	−PKA	+PKA	−PKA	+PKA
	1.0	16.3	1.0	15.3	1.0	10.2	1.0	1.7
CREMα S	0.9	1.4	1.0	1.2	1.2	1.1	0.9	2.1
AS	1.0	15.7	0.9	14.8	0.8	9.5	ND	ND
CREMβ S	0.8	1.2	1.2	1.1	1.1	1.2	1.0	1.0
AS	1.2	15.8	1.2	16.2	1.4	11.2	ND	ND
CREMγ S	1.2	1.2	1.3	1.2	0.7	1.1	0.9	1.4

Values represent fold induction. 1.0 indicates CAT activity obtained with the reporter plasmid transfected alone. (ND, not determined.) Data from several transfection experiments in human choriocarcinoma JEG-3 cells are presented. The first row of data represents transfections which include only the reporter plasmid with or without the PKA expression vector. Variability in the results is < 15%. Analogous data were obtained in transfections in HeLa and F9 cells (not shown). The reporter plasmids have already been described: pSomCAT (Foulkes *et al.*, 1991a), α-CG−CAT (the α-chorionic gonadotropin CRE region cloned in the same position as in pSomCAT, Delegeane *et al.*, 1987). c-*fos*CRE−CAT and c-*fos*CRE*−CAT contain the human c-*fos*CRE located at −60 in the promoter. CRE* indicates a mutated CRE which is not cAMP-inducible and does not bind CREM and CREB proteins (Sassone-Corsi *et al.*, 1988). Both sense (S) and antisense (AS) expression plasmids were tested.

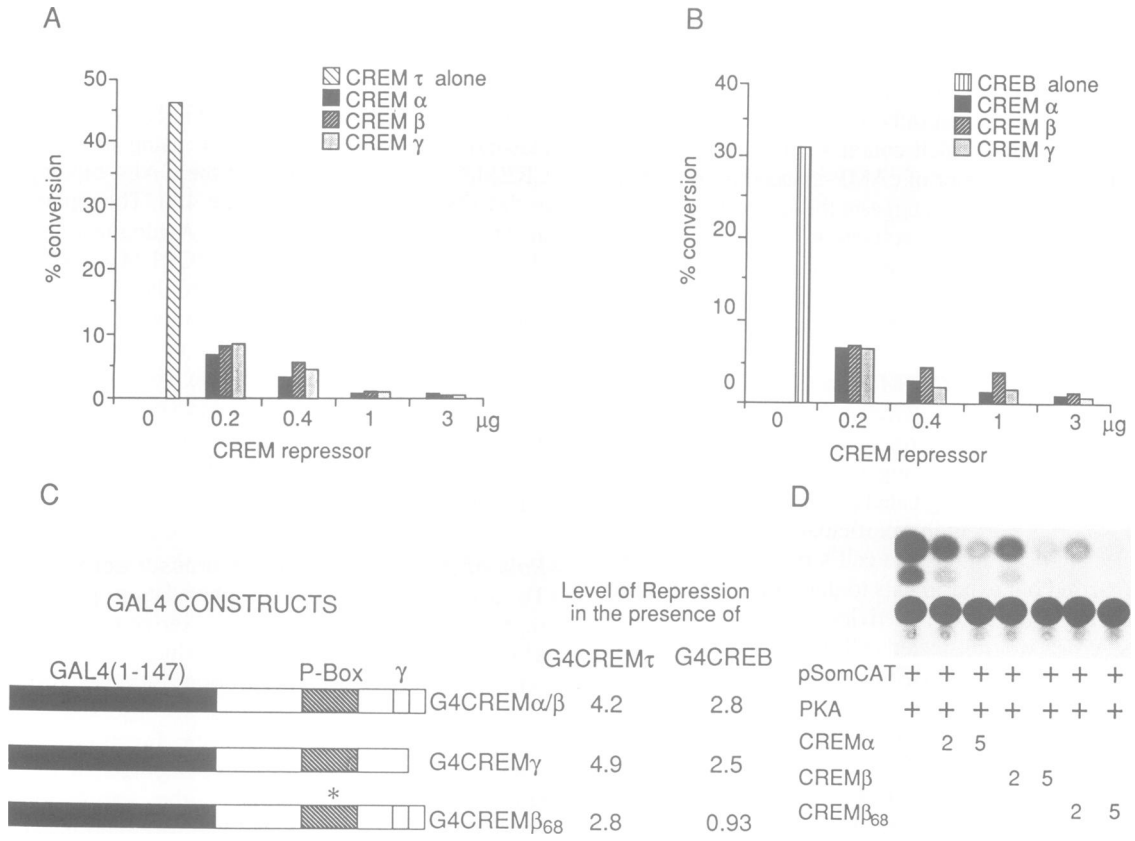

Fig. 5. Analysis of the activity of various CREM repressor forms. (**A** and **B**) Histograms showing mean results of CAT assays from transfection experiments in JEG-3 cells. Different concentrations of CREMα, CREMβ and CREMγ expression vectors (200 ng to 3 μg) were cotransfected with pCαEV (2 μg) and with either CREMτ (1 μg; A) or CREB (1 μg; B) activators. The black bars represent the results for CREMα, the striped bar represents the results for CREMβ and the stippled bars represent the results for CREMγ. Values for the activation by CREMτ (1 μg) and CREB (1 μg) in the absence of CREM repressors are also given. Variation did not exceed 15%. (**C**) GAL4−CREM repressor function. The structure of the G4CREM repressors is shown (see legend to Figure 4A) and the corresponding activity is given. CREMα and CREMβ differ only in their C-terminal DNA binding domains therefore G4CREMα and G4CREMβ are identical (G4CREMα/β). The γ-domain is absent in the CREMγ isoform. G4CREMβ_{68} carries a serine to glutamic acid mutation (TCA to GAA) in the consensus PKA phosphoacceptor site. 1 μg of each GAL repressor construct was cotransfected with 1 μg of activator expression plasmid (G4CREMτ or G4CREBΔLZ). The values shown represent the data from four experiments. (**D**) CREMβ_{68} is an efficient repressor of cAMP-induced transcription. Representative CAT assay of a JEG-3 transfection experiment showing CREM repressor function. The antagonistic potential of CREMβ_{68} is compared with CREMα and CREMβ, in the presence of PKA. The plasmids used for each transfection are shown below each lane. Further data are presented in Table II.

1991). A critical region of 16 amino acids, including both the PKA site and a short acidic (DLSSD) motif, was defined by deletion mutation studies as essential for CREB activity (Lee *et al.*, 1990; Gonzalez *et al.*, 1991). The CREM gene encodes a similar acidic domain, ELSSD, which is also present four amino acids downstream from the PKA site. Strikingly, this domain, as well as the P-box, are not only present in the CREM activator isoforms, but also in the repressors; thus, these elements alone are not able to confer activation function.

To generate a minimal repressor, we first deleted the entire 5′ region of CREMα, CREMβ and CREMγ, using two in-frame *Nco*I sites, one at the 5′ end and a second 5′ to the DBD domain, generating S-CREMα, S-CREMβ and S-CREMγ, respectively (Figure 6A and not shown; see also Delmas *et al.*, 1992). These short forms, when expressed in bacteria or synthesized *in vitro*, efficiently dimerize and bind CRE sequences (Figure 6B). Different motifs were then added back to S-CREMβ: the PKA motif generating S-CREMβ_A; the PKA motif and the ELSSD motif generating S-CREMβ_D; the PKA and the PKC motifs generating S-CREMβ_{AC}; a mutated (serine to glutamic acid) ELSSD motif generating S-CREMβ_{D68} (see Materials and methods and Figure 6A). Remarkably, all the constructs showed significant repressing ability, in different cell types and using different reporter plasmids (Figure 6C, and data not shown), either of endogenous CRE-induced transcription or in cotransfection experiments with either CREMτ or CREB activators. It appears that S-CREMβ is a more efficient repressor than CREMβ itself. Interestingly, adding back the different phosphorylation motifs does not significantly alter repressor function (Table II). There is no significant difference in activity between S-CREMβ_A, S-CREMβ_D, S-CREMβ_{D68}, S-CREMβ_{AC} and S-CREMβ, suggesting that the structural conformation of the NH_2-region is not crucial either for heterodimerization or for DNA binding. To verify that in these experiments the same amount of repressor protein is present, COS cells were transfected in duplicate with the various repressor constructs. Immunoprecipitation experiments were performed using a CREM-specific antibody, Abγ (Delmas *et al.*, 1992), following [^{35}S]methionine labelling of the cells. Figure 6D shows that

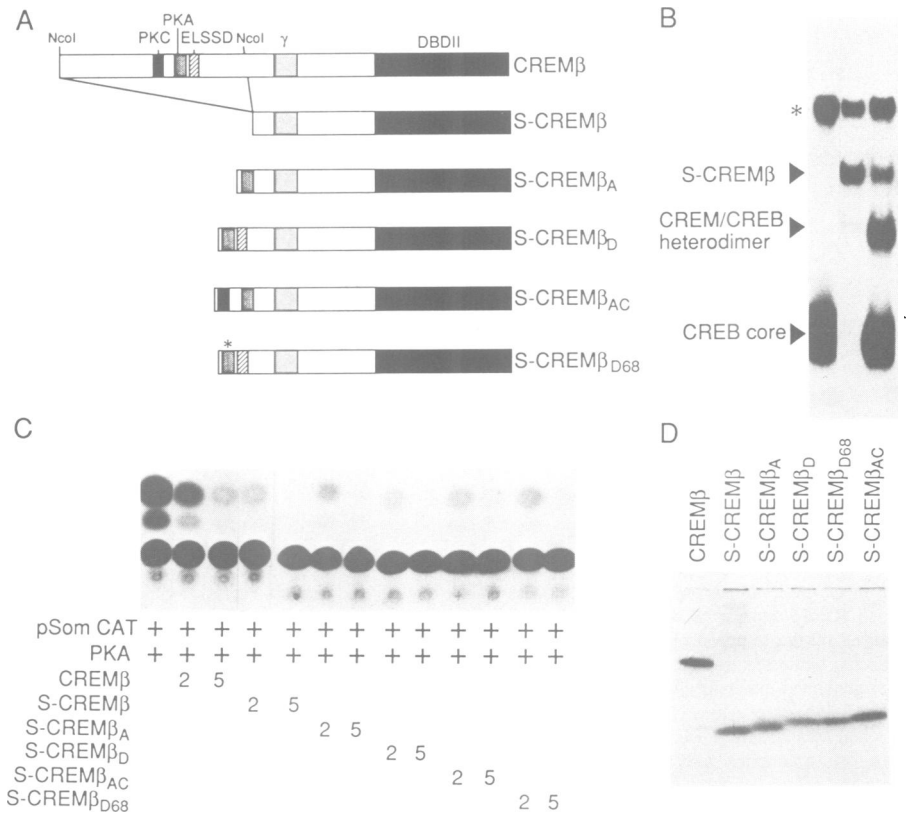

Fig. 6. Minimal CREM repressor. (**A**) S-CREMβ was constructed by deleting an N-terminal *Nco*I fragment which includes the P-box region (see Materials and methods; Delmas *et al.*, 1992). Synthetic double-stranded oligonucleotides with *Nco*I ends containing either the PKA motif (amino acids 65–71), the PKA motif and the ELSSD acidic motif (amino acids 65–79) or the PKC motif and the PKA motif (amino acids 56–71) as well as a PKA + ELSSD fragment containing a mutation of the serine 68 residue to glutamic acid (TCA to GAA) were inserted into the *Nco*I site of the S-CREMβ construct. (**B**) A truncated form of CREM repressor, S-CREMβ, binds a somatostatin CRE efficiently and heterodimerizes with CREB *in vitro*. S-CREMβ was synthesized by *in vitro* transcription–translation, as previously described (Foulkes *et al.*, 1991a; Delmas *et al.*, 1992). A 63 amino acid CREBcore synthetic peptide corresponding to the b-Zip motif of CREB (Foulkes *et al.*, 1991a) was used for the heterodimerization experiment. Lane 1, binding of CREBcore homodimer, lane 2, binding of S-CREMβ, lane 3, binding of CREBcore/S-CREMβ heterodimer. A non-specific band, formed by lysate endogenous proteins, is shown by an asterisk. (**C**) The b-Zip motif of CREM is sufficient for repression. Various S-CREMβ constructs were tested for their repressor activity in the presence of PKA following transfection in JEG-3 cells. All the truncated constructs repress more efficiently than the full-length CREMβ protein. Values from an average of five experiments are given in Table II. (**D**) Immunoprecipitation of *in vivo* labelled CREM repressors. COS cells were labelled with [^{35}S]methionine 36 h after transfection with various CREM expression vectors. The proteins were immunoprecipitated using an anti-CREM-specific antibody, Abγ, previously described (Delmas *et al.*, 1992), and electrophoresed on a 15% SDS–PAGE denaturing gel. Lane 1, CREMβ; lane 2, S-CREMβ; lane 3, S-CREMβ_A; lane 4, S-CREMβ_D; lane 5, S-CREMβ_{D68} and lane 6, S-CREMβ_{AC}.

the same amount of protein is immunoprecipitated in each case, indicating that the observed effect is not due to variations in repressor levels but is intrinsic to the protein itself.

DNA binding is a prerequisite for repressor function

To establish whether DNA binding is required for the repression function exerted by the CREM antagonists, we have generated an insertion mutant of CREMβ, CREMβm4, carrying an insertion of four amino acids in the basic region (see Figure 7A). This mutant contains the intact leucine zipper motif so that its dimerization capacity is not affected (Gentz *et al.*, 1989; Cohen and Curran, 1990; not shown); however, we show that the CREMβm4 protein, whether generated *in vivo* (in transfected COS cells) or *in vitro* (by coupled transcription–translation), is unable to bind to a consensus CRE site (Figure 7B). In mixing experiments (lanes 1 and 5) we also show that the heterodimers CREMβ/CREMβm4 do not bind the CRE, both *in vitro* and *in vivo*. Indeed, there is a clear decrease in the formation of the specific complex, indicating that the heterodimers are formed but are unable to bind. This is somewhat expected from previous studies on Fos/Jun, which demonstrated the requirement of the two intact basic domains for binding of the dimer (Neuberg *et al.*, 1989; Turner and Tjian, 1989; Ransone *et al.*, 1990).

We then tested the transregulatory activity of CREMβm4 in a transfection assay, using a reporter containing the cAMP-responsive α-chorionic gonadotropin promoter (Delegeane *et al.*, 1987). The results show that CREMβm4 differs from CREMβ (Figure 7C, lanes 3–10) since it no longer represses cAMP-induced transcription at substoichiometric amounts (lanes 11–18). However, when high levels of the pSVCREMβm4 expression vector are transfected, repression is observed but at much reduced levels compared with the wild-type CREMβ protein (Figure 7C and not shown). Interestingly, we found that the impaired repression function of this CREM mutant varies

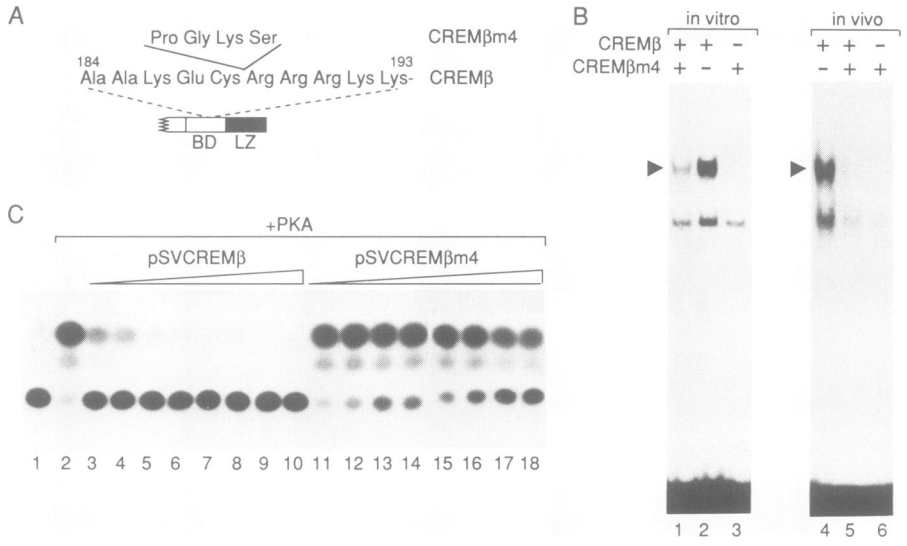

Fig. 7. Insertional mutation in the basic domain of CREMβ eliminates DNA binding and reduces repressor function. (**A**) Schematic representation of the DNA binding domain of CREMβ and partial sequence of the basic region, between positions 184 and 193. Insertion of the four amino acids -Pro-Gly-Lys-Ser- was achieved by the insertion of a synthetic oligonucleotide in a naturally occurring *SalI* site, generating the mutant CREMβm4. Only the basic region is affected while the leucine zipper is intact, allowing normal dimerization (Gentz *et al.*, 1989; Cohen and Curran, 1990; and not shown). (**B**) CREMβm4 generated either *in vitro* or *in vivo* does not bind to a CRE. Lanes 1 and 2: CREMβ and CREMβm4 were synthesized by *in vitro* transcription−translation, as previously described (Foulkes *et al.*, 1991a; Delmas *et al.*, 1992). Gel retardation assay using unlabelled protein and [γ-^{32}P]ATP-labelled somatostatin CRE probe; lanes 1−3: assay with *in vitro* generated proteins; lane 2, CREMβ complex; lane 3, CREMβm4, which does not form a complex with the DNA probe; lane 1, mixed CREMβ and CREMβm4 shows a decreased formation of the complex. Lanes 4−6: the coding sequences for CREMβ and CREMβm4 were cloned in the same expression vector pSG5 (Foulkes *et al.*, 1991a) and COS cells were transfected as described in Materials and methods. Nuclear extracts were prepared from the transfected cells and DNA binding was tested on a CRE. Also in this *in vivo* assay CREMβm4 shows no binding activity. (**C**) CREMβm4 transregulatory function is tested using the cAMP-inducible α-chorionic gonadotropin−CAT reporter (Delegeane *et al.*, 1987). CREMβm4 is a very weak repressor of α-CG−CAT induced transcription (lanes 11−18) when compared with the wild-type CREMβ protein (lanes 3−10). The effect of CREMβm4 appears to be dependent on the reporter used (not shown). The CREM expression vectors were transfected with increasing amounts (specifically, 0.1, 0.2, 0.3, 0.5, 1, 1.5, 2 and 4 μg), in the presence of 0.2 μg of pCαEV encoding the catalytic subunit of the PKA, following transfection in JEG-3 cells. We have controlled that the same amount of the two proteins was made in the transfected cells and that the proteins are translocated into the nucleus with the same efficiency (not shown). A representative CAT assay is shown demonstrating the effect of both pSVCREMβ and pSVCREMβm4 on endogenous activity.

with the cAMP-inducible reporter used (not shown). These results suggest that CREMβ repression function is not simply mediated by the titration of the activator and formation of heterodimers which do not bind to CREs. Instead, efficient repression requires the binding of both the repressor homodimers and inactive heterodimers to CREs, thereby inhibiting the DNA binding of the activator homodimers.

Discussion

The flexibility of the CREM structure is reflected in the number of CREM isoforms generated in a cell- and tissue-specific manner. Alternative splicing of the primary transcript results in the inclusion or exclusion of exons which in turn alters the characteristic of the protein produced, possibly modulating its physiological and functional role. Strikingly, CREM contains two alternative DNA binding domains which are differentially spliced in the various isoforms. By determining the binding affinity of CREMα (which contains the first DNA binding domain, DBDI) and CREMβ (which contains the second domain, DBDII) to 10 naturally occurring CRE sites, we show that CREM isoforms carrying DBDII have higher affinity for all the sites tested (Figure 2). Interestingly, in *in vitro* heterodimerization and binding studies, more CREMα/CREB heterodimers bind to a CRE than CREMβ/CREB heterodimers. This suggests that heterodimers with almost identical b-Zip motifs dimerize and/or bind more efficiently than heterodimers with less closely related domains. We did not detect any differences in antagonist function between CREMα, CREMβ and CREMγ. It is possible, however, that in the context of multiple promoter elements and higher order transcriptional complexes these proteins may show functional differences. We have shown that CREM proteins can homodimerize and heterodimerize *in vivo* (Figure 3B). It appears that CREM repressors act both by homodimerizing and heterodimerizing with CREMτ or CREB to form antagonistic dimers. First, antagonism by CREM is obtained at substoichiometric amounts with respect to the activator, and, secondly, a minimal S-CREMβ protein containing only the b-Zip motif is a strong antagonist. A mutation of the repressor DNA binding domain severely reduces antagonistic function implying that binding of both repressor homodimers and heterodimers to CREs is a prerequisite for efficient repressor function. Antagonism of transcriptional activators by substoichiometric amounts of specific repressors is common among regulators which act as dimers; in these cases the heterodimer is non-functional since only one activation domain is available. This is the case for the factors mTFE3 (Roman *et al.*, 1991), FosB (Nakabeppu and Nathans, 1991; Yen *et al.*, 1991), LIP (Descombes and Schibler, 1991), I-POU (Treacy *et al.*, 1992), c-Jun (Granger-Schnarr *et al.*, 1992) and others (Foulkes and Sassone-Corsi, 1992). We have recently shown that CREM antagonists can also block transcriptional activation, simply as homodimers, by occupation of a regulatory site (Masquilier and Sassone-

Corsi, 1992). In this distinct case, CREM represses Jun-mediated transactivation although unable to dimerize with Jun proteins.

We have identified CREM isoforms which contain only one of the glutamine-rich domains in CREMτ, which are also a feature of the activation domains of some other transcription factors (Courey and Tjian, 1988). These CREM protein products also act as activators of CRE-mediated transcription (Figures 1 and 4). The effect of the presence of both domains appears additive and indicates that the modularity of the activation domains confers flexibility on CREM activation potential. The GAL4 fusion experiments demonstrate that the spacing and orientation between the activation domains and the DNA binding domain is not crucial for activator function and that each activation domain acts as an individual module; this suggests that these domains interact independently with other components of the transcriptional machinery. Mutational and deletional analyses of CREB (Lee *et al.*, 1990; Flint and Jones, 1991; Gonzalez *et al.*, 1991) have identified domains which are important for CREB function. There are, however, some conflicting data: the importance of the first Q-domain is ambiguous. A 67 amino acid deletion of this region results in a CREB mutant with ~7-fold less activity than wild-type (Gonzalez *et al.*, 1991), while a 91 amino acid N-terminal deletion generated by Lee *et al.* (1990) had little or no effect. In the case of CREM, the naturally occurring isoform, CREMτ2, which contains only the second Q-domain, is essentially similar to the N-terminal CREB mutants. CREMτ2 has <2-fold reduced activity compared with CREMτ and, thus, is still an efficient activator of cAMP-responsive transcription (Figure 4). It is interesting that another activator of the CRE/ATF family, ATF-1 (Hurst *et al.*, 1991; Rehfuss *et al.*, 1991), naturally contains only one Q-domain, corresponding to that encoded by In.2 in CREMτ and CREMτ2 (Rehfuss *et al.*, 1991). The N-terminus of ATF-1 differs significantly from either CREB or CREM and it has been suggested that it might specify the basal transactivation level (PKA independent), which is ~2-fold less for ATF-1 than for CREB (Hurst *et al.*, 1991). We also found a difference in the basal level of transactivation between CREMτ and CREB (Figure 5); however, in contrast to ATF-1, CREMτ basal activity is higher than CREB. The significance of the N-terminus remains unclear as CREMτ shows 47.4% sequence similarity with CREB in the first 40 amino acids, while only eight N-terminal amino acids of ATF-1 are identical to CREB (24.2%).

Mutational data from studies of CREB have illustrated the relative importance of a number of other regions required for maximal activity. The most striking effect is obtained with a mutation of the serine 133 residue to either alanine (conservative change) or to glutamic acid (to maintain the negative charge) which results in a total loss of CREB activation function. In addition, an acidic region 3′ to the serine residue, DLSSD (amino acids 140−144) is also essential for CREB activity. In contrast, a highly acidic region upstream, which also contains consensus sites for CKII kinases, is entirely dispensible (Lee *et al.*, 1990; Gonzalez *et al.*, 1991). We determined the importance of equivalent regions in CREMβ. This is of particular interest because of the presence of these motifs in CREM proteins with antagonistic function. We have used both GAL4 constructs and the native isoforms in our analysis and have also generated truncated CREM repressors which lack the entire N-terminus of the protein. To the truncated CREMβ protein we added, without altering the spacing between domains, the various phosphorylation and activation motifs (Figure 6). Interestingly, we found that these regions are dispensible for CREMβ repressor function and that the minimal repressor appears to be slightly more efficient than the wild-type protein in antagonizing cAMP-induced transcription. One possible function of the N-terminal region of CREM proteins could include interactions with other components of the transcriptional machinery. To verify this hypothesis, additional experiments involving more complex promoter structures and other nuclear components are required.

All the CREM mRNA isoforms so far characterized contain a phosphorylation domain which is encoded by two exons (see Figure 1A). While the P-box in CREB is absolutely required for transactivation potential, mutation of the equivalent serine residue in CREMτ does not entirely abolish its function (Figure 4). Even more strikingly, mutating the same serine in the CREMβ isoform appears to increase its antagonistic function (Figure 5). This suggests that phosphorylation of CREM repressors by PKA actually reduces their repressing ability. Thus, it appears that the effect of PKA is to increase activation function and to decrease antagonist function.

The importance of CREM as a mediator of the cAMP signal transduction pathway is clearly evident. CREM products act as transcription factors to regulate cAMP-responsive genes in both a positive and negative manner (Foulkes and Sassone-Corsi, 1992). In addition, the number of naturally occurring CREM isoforms which are expressed in a cell- and tissue-specific manner, and which show differences in activity, suggest that CREM is intricately involved in the modulation and fine tuning of the cell's response to external signals of the cAMP pathway. The exon structure of the CREM gene is organized so that each exon (or group of exons in the case of the P-box) encodes a functional module of the corresponding protein and, in turn, the combination of these modules determines the characteristics and activity of the corresponding isoform. This results in a diversity of CREM transcription factors which act in various combinations, depending on the cell type, to regulate gene expression. Finally, the modularity of CREM structure may also be a clue to its evolutionary origin, reflecting its assembly from the duplicated subunits of ancestral genes.

Materials and methods

RT-PCR analysis

Aliquots (1 μg) of total RNA were analysed by RT-PCR essentially as described previously (Foulkes *et al.*, 1991a). The sequences of primers used in this analysis are as follows (see also Figure 1B):
primer D (ATG exon) 5′-AGGACAAATGTAAGGCAAATGACC-3′;
primer E (In.1 specific) 5′-CCACATCCATCGGTTATTCAA-3′;
primer F (In.2 specific) 5′-CAGATCCTGGGTTAGAAATC-3′;
primer G (penultimate 3′ exon) 5′-GGGGACTGTGCAGGCTTCCT-3′.
Primers A and C which are also complementary to the CREB mRNA sequence have already been reported (Foulkes *et al.*, 1991a). Southern blots of PCR products were hybridized with full-length CREM and CREB cDNA probes.

Plasmid constructs

pSomCAT contains the bacterial chloramphenicol acetyltransferase gene cloned 3′ to the herpes thymidine kinase (tk) −109/+52 promoter region;

the consensus CRE element of the rat somatostatin gene is cloned upstream from the tk promoter (Sassone-Corsi *et al.*, 1988). α-CG (α-chorionic gonadotropin)−CAT has already been described and contains two CREs naturally occurring in the promoter (Delegeane *et al.*, 1987).

GAL4 fusion (G4CREM) constructs. The vector pG4MpolyII, which contains the GAL4 DBD (1−147) with T7 and SV40 early promoters upstream and a multicloning site downstream, was used for cloning and expression. All G4 constructs were made by digesting pG4MpolyII with *Cla*I, end-filling with Klenow enzyme and then digesting with *Sac*I. Blunt-ended *Not*I and *Sac*I fragments from the CREM expression vectors (Foulkes *et al.*, 1991a) were then cloned into this vector so that the CREM sequences are downstream of, and in phase with, the GAL4 sequences. The *Not*I site of CREM is upstream from the translational start site and there is a unique *Sac*I site 5′ to the DBD in the represssor cDNAs. In the case of CREMτ and CREMτ2 there is a second *Sac*I site in In.2 (Foulkes *et al.*, 1992) therefore *Not*I−partial *Sac*I fragments of 799 and 652 bp, respectively, were used.

CREM bacterial expression vectors were constructed as previously described (Delmas *et al.*, 1992). Mutations of CREMτ serine 117 or CREMβ serine 68 to alanine or glutamic acid were constructed by oligonucleotide directed site mutagenesis (Kunkel, 1985) using a bacteriophage M13 mutagenesis system (Bio-Rad, Richmond, CA). The following oligonucleotides were used:

AGACCCGCATATAGAA serine to alanine;

AGACCCGAATATAGAA serine to glutamic acid.

The mutations introduced were verified by sequencing. CREM truncated constructs (S-CREM) were generated by deletion of an *Nco*I fragment which spans the N-terminus of CREM, between amino acid positions +3 and +103 in CREMα and CREMβ and +3 to +111 in CREMγ. S-CREMβ derivatives were constructed by linearizing S-CREMβ and ligating double stranded oligonucleotides carrying *Nco*I ends. All the constructs were verified by sequencing. The sequence of the sense strand and corresponding name of the construct is as follows:

CATGGCACGAAGACCCTCATATAGAAAAAC S-CREMβ_A;

CATGGCACGAAGACCCGCATATAGAAAAATACTGAATGAACT-TTCCTCTGATAC S-CREMβ_{D68};

CATGGCACGAAGACCCTCATATAGAAAAATACTGAATGAACT-TTCCTCTGATAC S-CREMβ_D;

CATGGATTCGCATAAACGTAGAGAAATTCTTTCACGAAGACC-CTCATATAGAAAAAC S-CREMβ_{AC}.

CREMβm4 was contructed by the insertion of the oligodeoxynucleotide: 5′-TGCCCGGGGAAGAGT-3′ in the *Sal*I site present in the CREMβ sequence at position 962.

Preparation of whole cell extracts

COS cells were transfected, 1 h after passaging the cells, with a total of 8 μg DNA per 5 cm plate. 24 h later the cells were washed and a further 24 h later the cells were harvested by scraping in ice-cold PBS medium. The cells were pelleted by centrifugation, resuspended in lysis buffer (400 mM KCl, 10 mM Tris pH 8, 15% glycerol, 1 mM DTT, 0.5 mM DMSF) and were then frozen in liquid nitrogen and thawed on ice, three times (Andrews and Faller, 1991). Following centrifugation, the supernatant was stored at −80°C. Protein concentration was determined using the Bradford assay.

Gel retardation assays

In vitro transcription−translation and gel retardation assays were performed as previously described (Foulkes *et al.*, 1991a,b). The sequences of one strand of the CRE elements used are given in Table I. The double stranded oligonucleotides were labelled with [γ-^{32}P]ATP and purified from 6% polyacrylamide gels. Unless otherwise stated a consensus somatostatin CRE probe was used in all gel retardation assays. In *in vitro* heterodimerization experiments with CREBcore peptide, the samples were heated to 95°C for 5 min and allowed to cool to room temperature, to allow heterodimers to form, before addition of the probe. For supershift experiments with anti-CREM and anti-CREB-specific antibodies, 1 μl of antibody was added to the COS extract and the reaction was incubated for 20 min at room temperature before addition of the probe.

Cells, transfections and CAT assays

All cells were grown as suggested by the suppliers (ATCC). The αTSH and αT3 cells were a gift from P.L.Mellon (University of California, San Diego) and were grown as described (Akerblom *et al.*, 1990; Windle *et al.*, 1990). The AtT-20 cell line in Figure 1B correspond to catalogue number CRL1795. JEG-3 human choriocarcinoma and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and were transfected by the calcium phosphate coprecipitation technique. COS cells were maintained in DMEM supplemented with 5% fetal calf serum. Cells were transfected with 10 μg of total plasmid DNA, unless otherwise specified. CREM and CREB cDNA sequences have been previously described (Gonzalez *et al.*, 1989; Foulkes *et al.*, 1991a, 1992). pCαEV encodes the catalytic subunit of the mouse PKA gene and is a gift from S.G.McKnight (Washington University, Seattle). CAT activity was assayed by standard methods. Chloramphenicol acetylation was determined by TLC and was quantified by liquid scintillation counting of the TLC plate ^{14}C spots.

Immunoprecipitations

Transfections for immunoprecipitation studies were carried out in COS cells. ^{35}S-labelling of the cultured cells and immunoprecipitations were performed as described (Delmas *et al.*, 1992).

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