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Both the basic region and the 'leucine zipper' domain of the cyclic AMP response element binding (CREB) protein are essential for transcriptional activation

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Second messengers like cAMP can activate the transcription of genes containing consensus cAMP response element (CRE). A 43 kd nuclear phosphoprotein previously identified as the cAMP response element binding (CREB) protein has been shown to bind as a dimer to CRE and activate gene transcription. The rat and human CREB protein contain the 'leucine zipper' motif. We have analyzed the role of both leucine zipper domain and the amino-terminal basic region by making site-specific mutations. Our results show that the first three leucines in the leucine zipper domain are essential for efficient dimer formation. Mutations of two consecutive leucines in the leucine zipper domain completely abolish the ability to form dimers. Mutant CREB protein unable to form homodimers is also unable to bind to DNA. In contrast, however, mutations, in the DNA binding region had no effect on dimer formation but were unable to bind to CRE sites or activate transcription. We propose that CREB protein functions by forming homodimers which bind to CRE and activate transcription. Furthermore, the CREB protein needs to be phosphorylated before activating transcription. Finally, we show that the CREB basic region mutant acts as a *trans*-dominant transcriptional suppressor of wild-type CREB function.

Key words: CRE/CREB protein/DNA binding domain/ leucine zipper/transactivation

Introduction

Transcription of a number of cellular genes is activated by second messengers like cAMP and phorbol esters that act through specific protein kinases. Induction of cellular genes by cAMP requires both: (i) the presence of a consensus cAMP response element (CRE; 5'TGACGTCA 3') (Comb *et al.*, 1986; Montminy *et al.*, 1986; Delegeane *et al.*, 1987; Hurst and Jones, 1987); Hardy and Shenk, 1988; Lin and Green, 1988; Sassone-Corsi, 1988; Sassone-Corsi *et al.*, 1988a) and (ii) the catalytic subunit of cAMP dependent protein kinase (A kinase) (Montminy and Bilezikjian, 1987; Riabowl *et al.*, 1988; Mellon *et al.*, 1989). Upon addition of agonists of adenylate cyclase, the A kinase is thought to phosphorylate a 43 kd nuclear transcription factor that binds to CRE and activates transcription (Yamamoto *et al.*, 1988; Jones and Jones, 1989). The 43 kd protein referred to as cAMP response element binding (CREB) protein binds as a dimer to CRE sequences in the rat somatostatin gene (Yamamoto *et al.*, 1988). Furthermore, the transcriptional efficacy of CREB protein is modulated by A kinase mediated phosphorylation (Montminy and Bilezikjian, 1987; Yamamoto *et al.*, 1988). Recently cDNAs encoding CREB protein have been isolated from human and rat cells (Hoeffler *et al.*, 1988; Gonzalez *et al.*, 1989). The deduced amino acid sequence has several characteristic features of the leucine zipper class of transcription factors (Landschulz *et al.*, 1988a; Johnson and McKnight, 1989). Members of this class include nuclear oncoproteins *fos, myc, jun* AP-1 and the yeast transcriptional factors GCN4, YAP-1, enhancer binding protein (C/EBP), and more recently, CREB related proteins MXB/CRE-BP-1 (Hope and Struhl, 1987; Halazonetis *et al.*, 1988; Kouzarides and Ziff, 1988; Nakabeppu *et al.*, 1988; Sassone-Corsi *et al.*, 1988b; Dang *et al.*, 1989; Gentz *et al.*, 1989; Landschulz *et al.*, 1989; Maekawa *et al.*, 1989; Moye-Rowley *et al.*, 1989; Ransone *et al.*, 1989; Schuermann *et al.*, 1989; Turner and Tjian, 1989). The leucine zipper domain of the CREB protein is located near the carboxyl terminus and contains four leucines spaced seven amino acid residues apart (Hoeffler *et al.*, 1988; Gonzalez *et al.*, 1989). Like other leucine zipper containing proteins, immediately preceding the leucine zipper is a region of basic amino acids that are implicated in DNA binding (Johnson and McKnight, 1989). A comparison between CREB protein and another transcription factor, AP-1, the product of mouse c-*jun* gene revealed a 61% amino acid identity in the putative DNA binding regions (Gonzalez *et al.*, 1989). This arginine and lysine-rich region (the basic region) also shares substantial identity with the basic domain of the GCN4 protein previously shown to be related to *jun*/AP-1 protein (Vogt *et al.*, 1987).

It is now generally accepted that transcriptional modulation by *fos*, *jun* (AP-1), C/EBP and GCN4 require the formation of either a homodimer or heterodimer. For instance, in the case of *jun*–*fos* heterodimer formation, the two proteins associate via their leucine zipper domain which brings their basic regions in apposition, thus forming the DNA binding site of the protein (Kouzarides and Ziff, 1989). If more than one leucine residue constituting the heptad in the leucine zipper domain of *fos* are mutated, there is no heterodimer formation or DNA binding and consequently no transcriptional activation (Ransone *et al.*, 1989; L.Ransone and P.Wamsley, personal communication). Because transcriptional activation by CREB protein has an obligatory requirement for dimer formation, we have undertaken a study of the role of leucine zipper domain and the basic region in this protein (Yamamoto *et al.*, 1988). Our results with site-directed mutational analysis indicate that the leucine zipper domain is essential for CREB protein homodimer formation with individual leucines contributing to varying degrees towards stable homodimer formation. Furthermore, we show that mutations in the basic region which have no effect on dimer formation completely abolish DNA binding. Finally, we demonstrate that mutations in the CREB protein

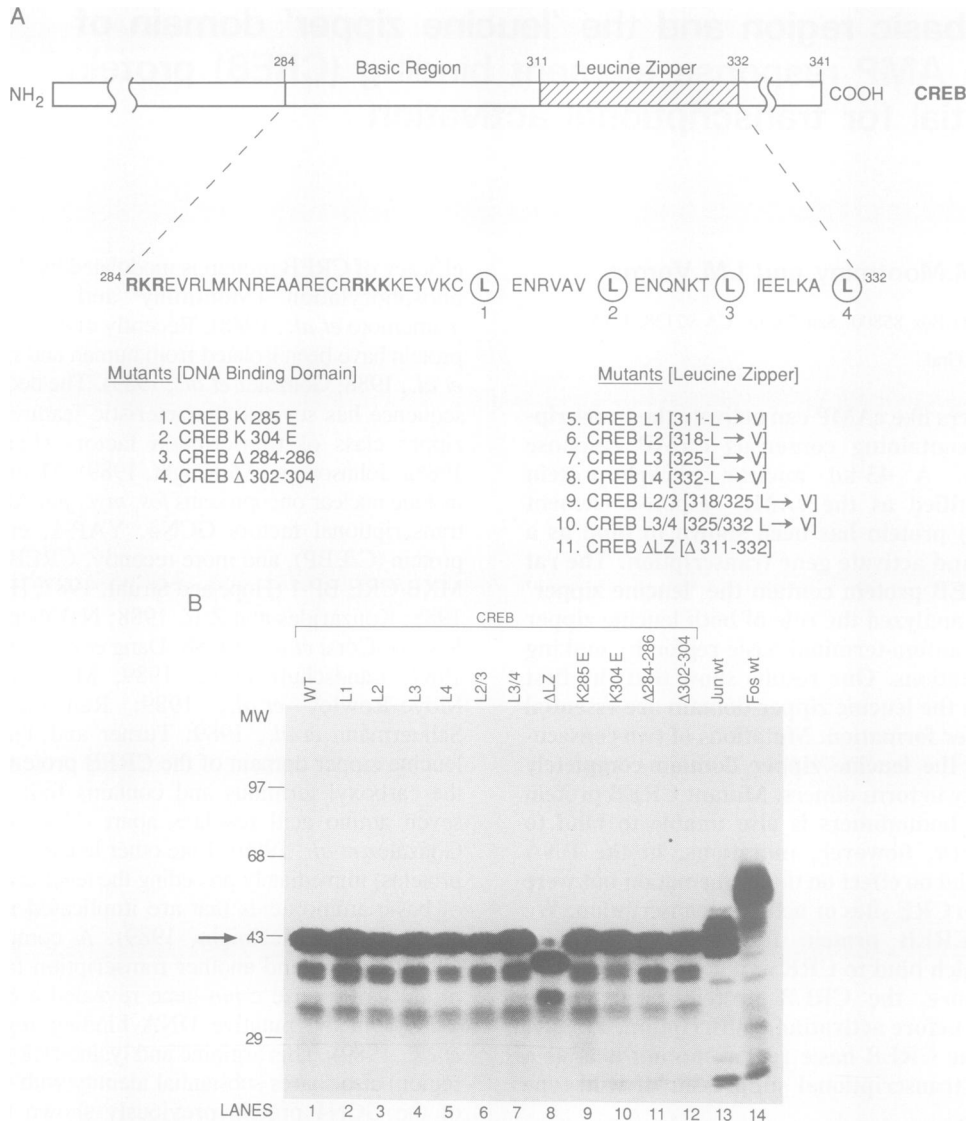

Fig. 1. (**A**) Structure of rat CREB protein. Schematic representation of the rat CREB protein as described by Gonzalez *et al.* (1989). The basic region and leucine repeat domains are shown. The various mutants of the basic region and leucine repeats are listed. The amino acids undergoing change are shown in bold. (**B**) *In vitro* translation products of CREB, CREB mutants, *jun* and *fos*. SDS−polyacrylamide gel electrophoresis of [^{35}S]-methionine-labeled *in vitro* translated proteins. One microgram of RNA prepared by transcription of cDNA was translated in the presence of [^{35}S]-methionine. Translation products were subjected to 10% SDS−PAGE. The positions of standard mol. wt protein markers are indicated. Five microliters of each of the translated products were loaded on the gel.

that influence its ability to either form dimers or bind to DNA are unable to activate transcription of promoters containing CRE sites.

Results

The leucine zipper is required for dimer formation

It has been postulated that the leucine zipper domain allows monomeric polypeptides to associate and form dimers. Presumably this protein association is facilitated by hydrophobic interactions among the leucine residues of the monomers. Figure 1A shows a diagrammatic sketch of the leucine zipper domain and the basic region of the rat CREB protein. To substantiate the role of leucine residues in the formation of CREB protein dimers, we generated two types of constructs: (i) the complete leucine zipper of the CREB protein was deleted (CREB ΔLZ), and (ii) both individual and consecutive leucine residues in the leucine zipper were mutated to valine (Figure 1A). To assay dimer formation, the wild-type and mutant CREB proteins were synthesized in an *in vitro* translation system using cRNAs generated from cDNAs as previously described (Ransone *et al.*, 1989). Analysis of dimer formation was carried out by glutaraldehyde cross-linking followed by analysis on SDS−PAGE which distinguishes the monomer from a cross-linked dimer. Figure 1B shows the *in vitro* translation profile of the wild-type CREB protein (lane 1) and mutants (lanes 2−7). A mutant of CREB protein where the entire leucine zipper (CREB ΔLZ; deletion of amino acid residues 311−332) is also shown (lane 8). The faster mobility of CREB ΔLZ protein is consistent with its shorter size. Figure 1B also displays the *in vitro* translational profile of mutants in the basic region (lanes 9−12). In most cases a prominent 43 kd band corresponding to the expected size of CREB protein can be identified (lanes 1−7 and 9−12). The amount of the CREB protein made by the wild-type and different mutants

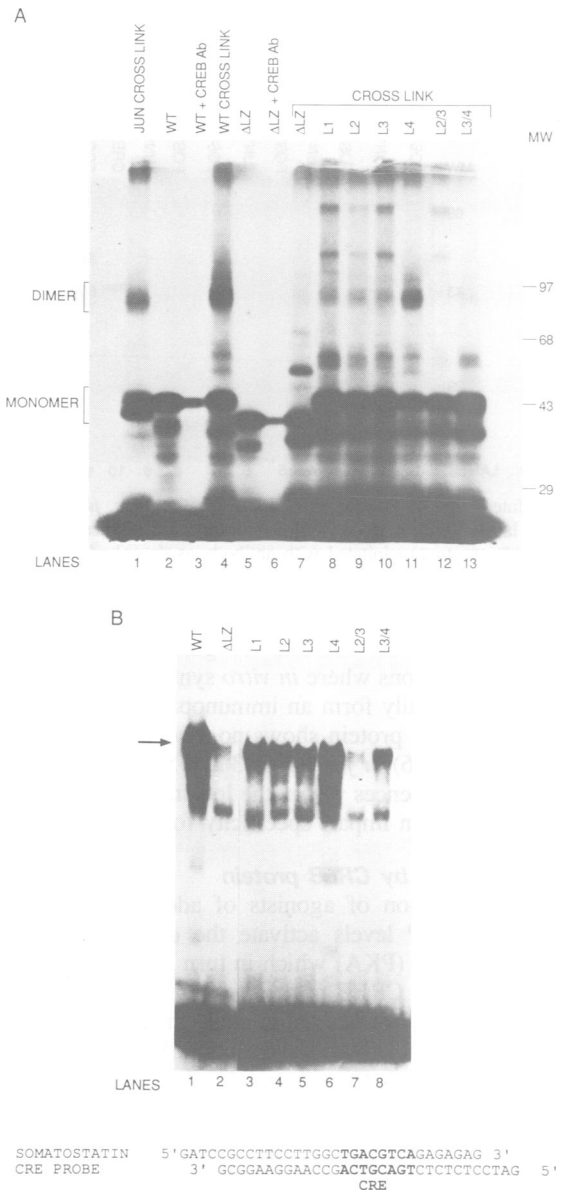

Fig. 2. (**A**) Dimer formation of wild-type and leucine zipper mutants of CREB protein. **Lanes 1, 4, 7–13**, glutaraldehyde cross-linking of c-*jun*, CREB WT, CREB ΔLZ, CREB L1, CREB L2, CREB L3, CREB L4, CREB L2/3, CREB L3/4: **lane 2,** *in vitro* translated product of CREB WT; **lane 3**, immunoprecipitated CREB WT translated product with CREB antibody; **lane 5**, *in vitro* translated product of CREB ΔLZ mutant; **lane 6**, immunoprecipitated CREB ΔLZ-translated product with CREB antibody. Monomer and dimer positions have been indicated. The ratios of the monomer to dimer formation for wild-type CREB (**lane 4**) is 63:37; L1 (**lane 8**) 92:8; L2 (**lane 9**) 91:9; L3 (**lane 10**) 91:9; L4 (**lane 11**) 70:30; L2/3 (**lane 12**) 100: < 1; L3/4 (**lane 13**) 93:7 as measured by densitometric tracing of the autoradiograms. (**B**) DNA binding with CREB leucine zipper mutants: gel retardation analysis. ^{32}P-Labeled somatostatin CRE was incubated prior to gel electrophoresis and autoradiography with unlabeled translation products of wild-type and leucine zipper mutants of CREB. The CREB–DNA complex is indicated as an arrow. Annealed somatostatin CRE oligo used for labeling is indicated at the bottom.

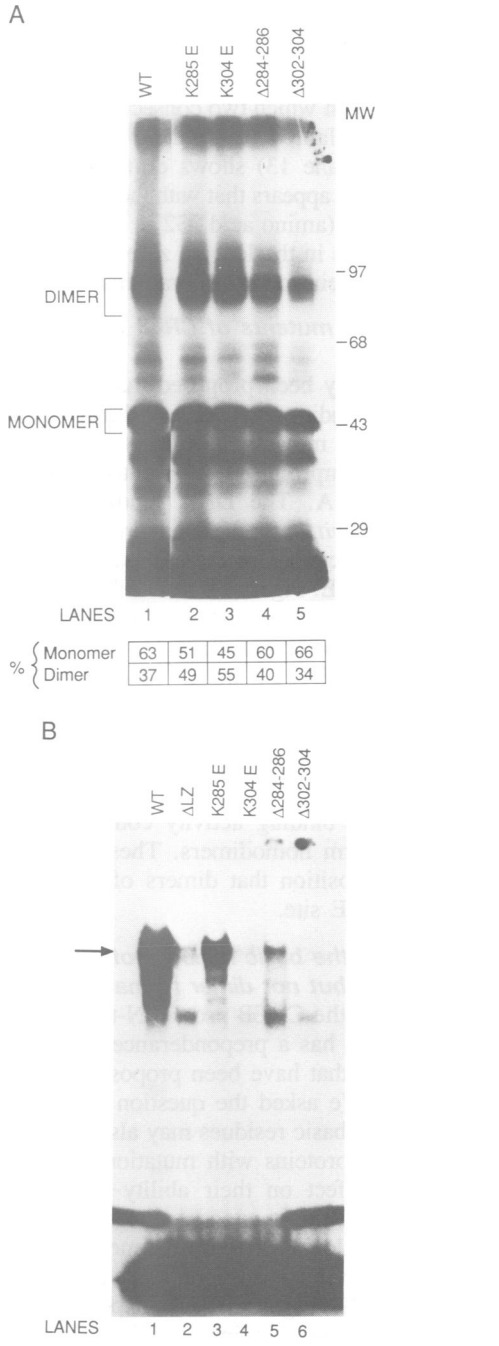

Fig. 3. (**A**) Effect of amino acid substitutions or deletions in the basic region on dimerization. Monomer and dimer positions and their ratios are indicated. (**B**) DNA binding with DNA binding domain mutants of CREB protein: gel retardation analysis. ^{32}P-labeled somatostatin CRE was incubated prior to gel electrophoresis and autoradiography with unlabeled translation products of wild-type and DNA binding domain mutants of CREB. The CREB–DNA complex is indicated as an arrow.

is comparable. Because the mutations do not affect the translational efficacy, the comparisons of the *in vitro* translated products are valid.

To test the effect of mutations of the leucine residues in the leucine zipper domain on the formation of homodimers we performed cross-linking experiments. The various CREB proteins were treated with glutaraldehyde and analyzed by PAGE. The wild-type CREB protein (Figure 2A; lane 4) forms a dimer of ~85–90 kd while the CREB ΔLZ protein (lane 7) is unable to form a dimer. These results suggest the requirement of leucine zippers for dimer formation. Figure 2A (lanes 8–10) also shows that mutants in which the first three leucine residues are individually mutated to valine form dimers with substantially reduced efficiency

(< 10% of the wild-type level). However, a mutation of the last leucine, L4 (lane 11) has much less effect on the ability of this mutant to form homodimers. In contrast, the double leucine mutant in which two consecutive leucines have been mutated L2/3 (lane 12) is unable to form dimers, while mutant L3/4 (lane 13) shows diminished ability to form dimers. Thus, it appears that with the exception of the fourth leucine residue (amino acid 332 in Figure 1A) most of the leucine residues in the leucine zipper domain are essential for CREB protein to form homodimers.

Leucine zipper mutants of CREB protein are unable to bind to CRE

It has previously been proposed that CREB protein binds to its cognate binding site CRE as a dimer (Yamamoto *et al.*, 1988). Having mutants of CREB protein where dimer formation is compromised, we wanted to study their ability to bind to DNA. The DNA binding assay consists of binding of *in vitro* synthesized protein with end-labeled oligonucleotide containing the CRE site and analysis by non-denaturing PAGE. Figure 2B shows that CREB ΔLZ protein has little or no binding to labeled CRE (lane 2) as compared with the wild-type protein (lane 1). The three single leucine residue mutants L1, L2 and L3 have considerably reduced binding while the L4 mutant, which can form dimers with wild-type efficiency (Figure 2A, lane 11) had DNA binding patterns similar to that of the wild-type protein. The double mutants of leucine residues L2/3 and L3/4 show much reduced DNA binding activity commensurate with their inability to form homodimers. These results reaffirm the previous supposition that dimers of CREB protein favor binding to CRE site.

Mutations in the basic region profoundly influence DNA binding but not dimer formation

The region of the CREB protein N-terminal of the leucine zipper domain has a preponderance of basic amino acids (nearly 50%) that have been proposed to interact directly with DNA. We asked the question whether mutations in some of these basic residues may also influence binding to CRE. CREB proteins with mutations in the basic region showed no effect on their ability to form homodimers (Figure 3A). Thus it appears that the major function of leucine zipper domain is to form dimers. However, mutation or deletion of the basic amino acids profoundly influences binding to CRE (Figure 3B). As expected, the CREB ΔLZ protein shows no binding to CRE (Figure 3B; lane 2) because it is unable to form homodimers. Mutation of Lys285 to glutamic acid reduces binding to CRE considerably (Figure 3B, lane 3), while mutation of Lys304 to glutamic acid completely abolishes DNA binding (lane 4). Deletion of amino acids 284–286 or amino acids 302–304 drastically diminish the ability of CREB protein to bind to its cognate DNA sequence (lanes 5 and 6). These results clearly demonstrate that the basic region of the CREB protein is directly involved in the binding to CRE.

CREB protein does not form heterodimers with jun or fos proteins

Because of the close similarity of CREB protein and *jun* AP-1 binding sites (CRE = TGACGTCA; TRE = TGAC/GTCA) and amino acid sequence identities in their DNA binding region, we wanted to test whether CREB protein would associate with either *fos* or *jun* proteins and form an immuno-

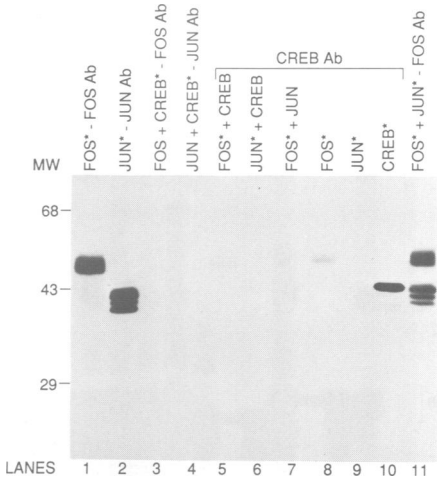

Fig. 4. Interaction of *fos*, *jun* and CREB proteins. *Fos*, *jun* and CREB proteins labeled with cold or [^{35}S]methionine were synthesized *in vitro*, mixed and immunoprecipitated with antibody as described in Materials and methods. Positions of standard mol. wt protein markers are indicated. * Indicates that protein used is labeled with [^{35}S]methionine.

precipitable complex. The data in Figure 4 demonstrate that under the conditions where *in vitro* synthesized *jun* and *fos* proteins can readily form an immunoprecipitable complex (lane 11), CREB protein shows no association with either *jun* (lanes 4 and 6) or *fos* (lanes 3 and 5) proteins. Thus it appears that sequences within the leucine zipper domain of the CREB protein impart specificity for dimer formation.

Transactivation by CREB protein

Following addition of agonists of adenylate cyclase the increased cAMP levels activate the catalytic subunit of protein kinase A (PKA) which in turn has been postulated to phosphorylate CREB protein to initiate transcription by binding to CRE. We wanted to study the ability of various CREB mutants to activate transcription. We used a transient transfection assay system where a CRE–CAT reporter gene (somatostatin CRE–CAT Δ–71) co-transfected with CREB expression vector and the catalytic subunit of PKA (an assay developed by Gonzalez and Montminy, 1989). Figure 5A shows that the induction of CAT activity from the reporter plasmid CRE–CAT is dependent on the presence of catalytic subunit of protein kinase (PKA; compare lanes 2 and 3). The CREB mutant L1 that shows both reduced dimer formation (Figure 2A) and binding to CRE (Figure 2B) also shows a 3-fold reduction in CAT activity (lane 4). The basic region mutants K304E (lane 5), Δ284–286 (lane 7) and Δ302–304 (lane 8) that form dimers (Figure 3A) but show little or no DNA binding (Figure 3B) are unable to activate the reporter plasmid as judged by lack of CAT activity. Mutant K285E (lane 6) which shows some DNA binding activity (Figure 3B) exhibits a reduced ability for transactivation (lane 6). As would be expected on the basis of its inability to form even homodimers, the CREB ΔLZ mutant has no transcriptional activity (lane 9). The effect of mutants of CREB protein in leucine zipper domain on their ability to activate transcription were also tested. As expected, mutants L2 and L3 which show reduced dimer formation and DNA binding activity have also reduced transcriptional activation (Figure 5B, lanes 4 and 5). Similarly, double leucine residue mutants, L2/3 (lane 7) and L3/4 (lane 8) show much reduced ability for transactivation.

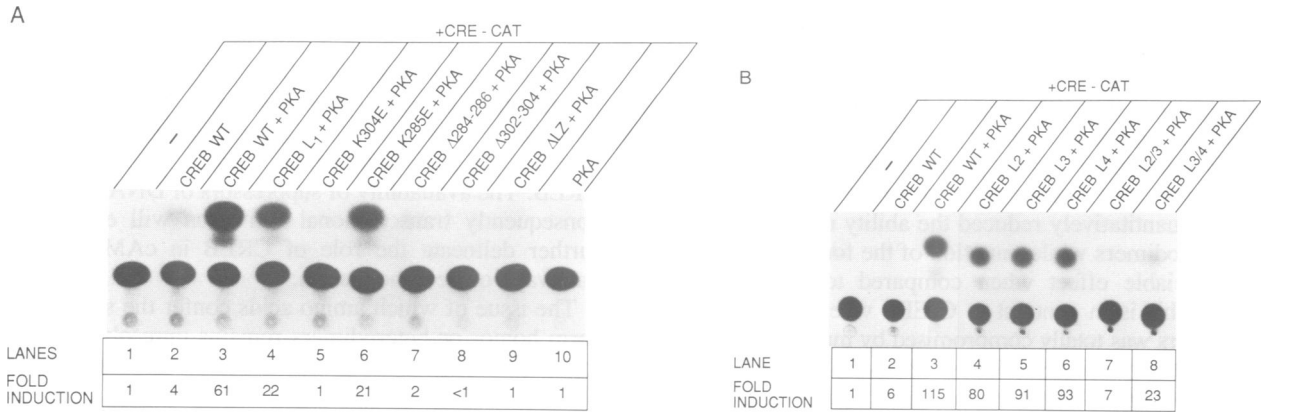

Fig. 5. (**A**) Transactivation of CRE−CAT reporter gene by CREB WT and mutants. Transfection experiments were done in embryonal carcinoma F9 cells according to the assay developed by G.Gonzalez and M.Montminy (unpublished results). Four micrograms of CRE−CAT was co-transfected with 10 μg of CREB WT expression vector, or 10 μg of CREB WT or CREB mutant and 10 μg of PKA plasmid. As a control PKA plasmid alone was also co-transfected. The total amount of DNA transfected was always 25 μg. CAT assays were performed as described in Materials and methods. Fold induction of CAT activity is indicated. (**B**) Transactivation of CRE−CAT reporter gene by CREB WT and leucine zipper mutants. Transfection experiments were done in embryonal carcinoma F9 cells. Experimental protocol is described in Materials and methods and **A**. Fold induction of CAT activity is indicated.

We, therefore, conclude that the ability of CREB protein to form dimers and binding to DNA is directly reflected in its ability to activate transcription.

Transdominant suppression of CREB transactivation

Mutational analysis of the basic region of CREB protein demonstrated that mutation made in this region (CREB K304E) (Figure 3A, lane 3) had no effect on homodimer formation, but was unable to bind to DNA and activate transcription. We asked whether this CREB basic region mutant would act as transdominant suppressor of wild-type CREB function. CREB K304E, which is unable to bind to DNA alone (see Figure 6A, lane 3) was unable to significantly suppress the DNA binding of the wild-type protein in a gel shift competition assay (compare lane 2 with lanes 4−6) at any ratio of wild-type to mutant protein. However, when a similar experiment was performed in a transient transfection assay system which measures the functional ability of CREB protein to activate transcription, the results were different (Figure 6B). CREB K304E suppressed transactivation of CREB almost 3-fold when co-transfected at either a 1:1 (lane 5) or a 1:2 (lane 6) wild-type:mutant ratio. This discrepancy in the results with DNA binding and transactivation may be explained by the ability of CREB heterodimers (mutant:wild-type), as opposed to mutant homodimers (mutant:mutant) to bind to a DNA half site in an *in vitro* gel shift DNA binding assay. When these complexes are formed *in vivo*, they are able to bind to the target DNA sequence, but are unable to function as transcriptional transactivators. Thus, CREB K304E is a dominant negative mutant, however, it exerts its effect not at the level of direct DNA binding, but rather in the transcriptional activation pathway, at the level of transcriptional transactivation.

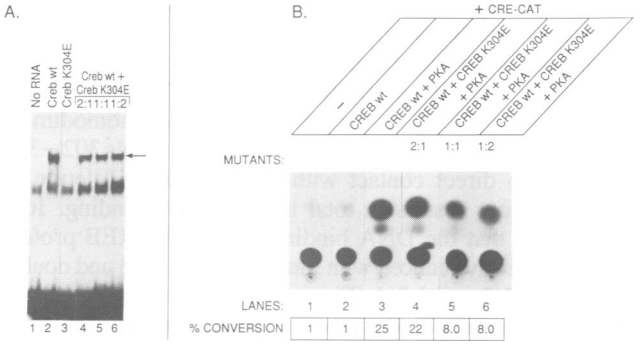

Fig. 6. (**A**) Transdominant suppression by CREB DNA binding mutant. DNA binding with CREB WT and various ratios of WT and CREB K304E. ^{32}P-Labeled somatostatin CRE was incubated prior to gel electrophoresis and autoradiography with unlabeled translation products of wild-type and CREB K304E. The CREB complex is indicated as an arrow. (**B**) Transactivation of CRE−CAT reporter gene by CREB WT and various ratios of WT to CREB K304E. Transfection experiments were done in embryonal carcinoma F9 cells. Experimental protocol is described in Materials and methods and Figure 5A. Percentage conversion of CAT activity is indicated.

Discussion

Eukaryotic cells rely on two principal signal transduction pathways to respond to external stimulus. One pathway is mediated by the participation of protein kinase C, while the other employs the activation of adenylate cyclase. In both cases, the signal is relayed to the nucleus, culminating in activation of gene transcription (Sassone-Corsi *et al.*, 1988c). A number of transcription factors which bind to DNA in sequence-specific manner have been identified (Mitchell and Tjian, 1989). A 43 kd transcription factor referred to as the CREB protein is phosphorylated when forskolin is added to the cells (Montminy and Bilezikjian, 1988). The phosphorylated form of CREB protein presumably binds to promoters containing CRE and initiates transcription. We have analyzed the rat CREB protein by mutational analysis to delineate the regions required for dimerization, DNA binding and transcriptional activation.

CREB protein belongs to the growing family of transcription factors that contain the leucine zipper motif (Johnson and McKnight, 1989). These proteins contain a region rich in basic amino acids immediately followed at the C terminus by a conserved heptad of leucine repeats. Mutational analysis of other leucine zipper containing proteins shows that the stretch of basic amino acids is

involved in DNA binding while the leucine repeat element is required for homo- or heterodimer formation (Ransone *et al.*, 1989; Landschulz *et al.*, 1989). Like the enhancer binding protein (C/EBP), the CREB protein has four leucine residues as compared to *jun* or *fos* with five leucine residues in the leucine zipper domain (Landschulz *et al.*, 1988b). We have shown that mutations of the first three individual leucines quantitatively reduced the ability of the protein to form homodimers while mutation of the fourth leucine had no appreciable effect when compared to the wild-type protein. This is in contrast to C/EBP where the ability to form dimers was totally compromised by mutations in either of the first two leucine residues (Landschulz *et al.*, 1989). On the other hand, mutations in either single or consecutive leucine residues in *jun* protein had no effect on either homodimer formation or heterodimer formation with *fos* (Ransone *et al.*, 1989). Like C/EBP, however, CREB proteins with mutations of two consecutive leucine residues (L2/3 or L3/4, Figure 2A) are unable to form homodimers. Since the amino acid sequences between the leucine residues of CREB protein, C/EBP and *jun* are quite different, it is likely that they exert a substantial influence on the formation of homo- or heterodimers.

Mutations in the basic region of the CREB protein drastically reduce its ability to bind to CRE but do not significantly decrease the ability to form homodimers (Figure 3). It appears that basic region amino acids 302–304 must be in direct contact with DNA because deletion of these residues results in total loss of DNA binding. It is interesting that the DNA binding ability of CREB protein is quantitatively reduced with mutants L1, L2, L3 and double mutants L2/3 and L3/4 (Figure 2B) suggesting that dimer formation is required prior to DNA binding. Thus, in accordance with the data on C/EBP or *jun* homodimers or *jun*–*fos* heterodimers, the role of the leucine zipper domain is to bring the two polypeptide chains of CREB protein in association as dimers to facilitate binding to CRE sites.

The sequence of events leading to activation of promoters containing CRE sites by CREB protein envisages that upon addition of agonists of adenylate cyclase, the catalytic domain of PKA is activated to phosphorylate CREB which in turn binds to DNA (CRE) as a homodimer and initiates transcription (Yamamoto *et al.*, 1988). We have studied the effect of various leucine zipper and DNA binding mutants of CREB protein on their ability to influence transactivation. The data presented in Figure 5 demonstrates that transactivation by CREB protein has an absolute requirement for (i) phosphorylation by PKA; (ii) the formation of homodimers; and (iii) binding to CRE. All the mutants which were unable to bind to DNA (e.g. Δ302–304) or were unable to form homodimers (e.g. L2/3) showed no transcriptional activity as judged by CAT activity (Figure 5).

The dominant negative mutant of CREB might be acting at the level of transcriptional transactivation. We assume that this mutant can form DNA binding heterodimers with wild-type CREB which can be detected by gel shift analysis as shown in Figure 6A, but is unable to form an active transcription complex capable of transactivating the CRE–CAT construct *in vivo* (Figure 6B). At a 1:1 ratio of wild-type to mutant CREB, we would expect only one sixth of the dimers to be wild-type, whereas at 1:2 the frequency would be only 1:12. The decrease in transactivation at these ratios is commensurate with the expected results. Thus, in a protein such as CREB, which is exclusively homodimeric, a derivative capable of interacting with wild-type polypeptide will be inhibitory if it causes the formation of non-functional multimers (Herkowitz, 1987) as shown in Figure 6B. The dominant negative mutant of CREB could provide information on the *in vivo* function of CREB. The availability of suppressors of DNA binding and consequently transcriptional activation will enable us to further delineate the role of CREB in cAMP mediated pathways of gene activation.

The issue of which amino acids confer the specificity to form homo- or heterodimers remains unresolved. Clearly leucine residues in the leucine zipper domain are important, but there must be other contributing factors. The mere presence of the leucine heptad repeat is not sufficient to specify dimer formation because *fos* is unable to form homodimers (Nakabeppu *et al.*, 1988; Halazontis *et al.*, 1988). Inability of CREB protein to associate with either *fos* or *jun* (Figure 4) supports the notion that specificity for dimer (homo or hetero) formation resides in the leucine zipper domain. It is, however, formally possible that sequences outside the leucine zipper region contribute by preventing dimer formation. To test these ideas, experiments are in progress in which the entire leucine zipper domain of CREB protein has been substituted with the leucine zipper domain of *jun* or *fos* proteins.

Materials and methods

Cell culture

Undifferentiated embryonal carcinoma F9 cells (Strickland and Mahdavi, 1978) were grown on 10 cm dishes in DMEM containing 10% calf serum.

DNA transfection and transient expression assays

Embryonal carcinoma F9 cells were plated in DMEM, 10% calf serum at 1×10^6 cells/10 cm tissue culture dish, 24 h before DNA transfection. Cells were transfected by the calcium phosphate co-precipitation technique (Sassone-Corsi and Verma, 1987) and exposed to the precipitate for 12 h. After washing with phosphate-buffered saline (PBS) fresh medium was added, and cells were harvested after 24 h. When <25 μg of specific DNA was used for 10 cm culture dish, pGEM 3 plasmid DNA was added to give 25 μg of total DNA. CAT activity was determined as described by Gorman *et al.* (1982).

DNA manipulations

Standard DNA recombinant methodology was employed (Maniatis *et al.*, 1982). Oligonucleotide-directed mutagenesis was utilized to generate site-specific mutants in the rat CREB leucine zipper domain and DNA binding domain (Kunkel, 1985). The rat CREB cDNA clone (Gonzalez *et al.*, 1989) was cloned into M13mp19 in the positive orientation. Oligodeoxynucleotides complementary to the regions containing the specific leucines and the amino acids to be altered were synthesized. They are:

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                 981                          964
CREB L1          5'TCT GTT CTC TAC ACA TTT3'
                          VAL

                 1002                         985
CREB L2          5'TTG GTT TTC AAC CAC TGC3'
                          VAL

                 1023                         1006
CREB L3          5'CTC CTC AAT CAC TGT TTT3'
                          VAL

                 1041                         1024
CREB L4          5'GTC CTT AAC TGC TTT TAG3'
                          VAL

                 1020                                                  985
CREB L2/3        5'CTC AAT CAC TGT TTT GTT TTG GTT TTC AAC CAC TGC3'
                          VAL                          VAL
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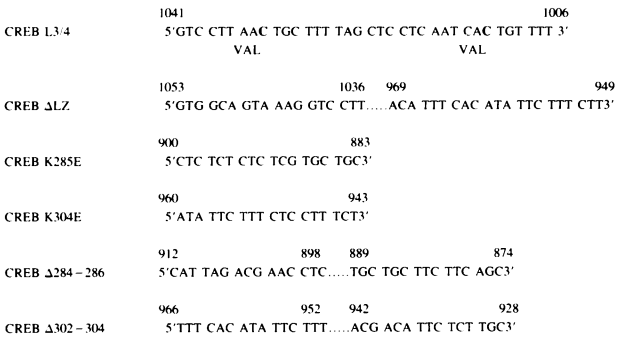

The CREB ΔLZ, Δ284−286, Δ302−304 oligonucleotides contains sequences on either side of the amino acids to be deleted. This resulted in an in-frame deletion of the entire region of interest. 2−3 pmol of each oligonucleotide were hybridized to 0.1 pmol of uracil containing DNA which was prepared as described by passage of the phage through CJ236, an *Escherichia coli* strain which contains the *dut,ung* double mutation (Kunkel, 1985). The complementary strand of DNA was synthesized in the presence of T4 DNA polymerase and T4 DNA ligase. The double-stranded DNA was transformed into competent MV1190 cells (Biorad), plaques were isolated, and screened by sequencing using the Sequenase kit (US Biochemicals).

Recombinant plasmids

Full length rat CREB cDNA clone (Gonzalez *et al.*, 1989) and the various CREB mutants generated were subcloned in pGEM4 (Promega) and placed under the control of the bacteriophage T7 promoter. Expression vectors used in transient transfection experiments were generated by subcloning rat CREB cDNA clone and the mutants under the control of murine sarcoma virus FBJ long terminal repeat and SV40 early poly(A) addition site (Van Beveren *et al.*, 1983). CRE−CAT reporter plasmid has been described (Montminy *et al.*, 1986). It contains the somatostatin gene promoter from positions (−71 to +53) linked to the CAT structural gene. Expression vector for the catalytic subunit of PKA (pSKG4) was a kind gift from Steve K.Hanks (Maldonado and Hanks, 1988). This clone contains human cDNA coding for cAMP dependent protein kinase catalytic subunit type under the control of the early SV40 regulatory promoter region and the SV40 poly(A) addition site (Okayama and Berg, 1983).

In vitro transcription and translation

Prior to *in vitro* transcription, all plasmids were linearized with *Eco*RI. Four micrograms of each linearized plasmid was transcribed *in vitro* using T7 polymerase in a final reaction volume of 100 μl as previously described (Sassone-Corsi *et al.*, 1988b). After a 2 h incubation at 37°C, RQ1 DNase I was added to a concentration of 1 U/μg of DNA, and the reaction incubated for an additional 20 min. The mRNA was extracted with phenol, and then with chloroform, precipitated with ethanol and sodium acetate, and resuspended in half of the original reaction volume.

RNA was translated *in vitro* with a micrococcal nuclease-treated, methonine-free rabbit reticulocyte lysate in a 50 μl reaction volume as directed by the supplier (Promega). Translation products were stored at −80°C. [^{35}S]Methionine-labeled translation products were analyzed by SDS−PAGE (10%) as described by Laemmli (1970). Pre-stained high mol. wt markers (Bethesda Research Laboratories) were used for mol. wt standards. After electrophoresis, the gel was fixed with 10% acetic acid/30% methanol, treated with 1 M salicylic acid (Sigma) and autoradiographed.

In vitro protein association studies

For *in vitro* protein association assays, equal volumes of reticulocyte lysate (5 μl each) containing [^{35}S]methionine-labeled *fos*, *jun* or CREB was combined and incubated at 30°C for 30 min as described by Sassone-Corsi *et al.* (1988b). Protein complexes were then mixed with 1 μl of antibody. The antigen−antibody complexes were collected by the addition of Pansorbin (Calbiochem-Behring), the immunocomplexes were washed in RIPA buffer (10 mM Tris−HCl pH 7.5, 150 mM NaCl, 1% NP40, 1% deoxycholate, 0.1% SDS) and prepared for SDS−PAGE as described (Barber and Verma, 1987). The immunoprecipitated proteins were separated on 10% PAGE gels and prepared for autoradiography as described above. Cross-linking with glutaraldehyde was performed as described by Dang *et al.* (1989). Briefly, 5−10 μl of reticulocyte lysate containing [^{35}S]methionine-labeled CREB protein was incubated with 0.005% glutaraldehyde in 75 mM sodium phosphate, pH 7.0, at 20°C for 30 min. Samples were immediately loaded on SDS−polyacrylamide gels and prepared for autoradiography.

Gel shift analysis

Gel retardation assays were performed as described (Sassone-Corsi *et al.*, 1988b). Two microliters of the *in vitro* translated proteins were incubated with 2 μg of poly(dI-dC) (Sigma) labeled probe in TM buffer (50 mM Tris−HCl pH 7.9, 12.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol) for 20 min at room temperature in a 20 μl final volume. A synthetic 32 bp oligonucleotide containing the somatostatin CRE was end labeled with [γ-^{32}P]ATP, with T4 polynucleotide kinase. Approximately 0.1 ng of ^{32}P-labeled DNA (20 000 c.p.m.) was incubated with the proteins. DNA−protein complexes were resolved on a 4% polyacrylamide gel (38:2 acrylamide:bisacrylamide) in 0.25 × TBE, (50 mM Tris-borate, pH 8.3, 1 mM EDTA). The gels were dried and autoradiographed with intensifying screens at −70°C.

Antibodies

CREB antibody w39 has been described (Gonzalez *et al.*, 1989). Monoclonal antibodies against the N peptide of c-*fos* (amino acids 4−17) was prepared by De Togni *et al.* (1988). Affinity purified anti-*jun* antibody 4882 against the mouse c-*jun* peptide (6-24) was prepared by W.W.Lamph.

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