Heterodimer formation between CREB and JUN proteins

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DNA binding protein families have been identified that contain a leucine zipper dimerization motif preceded by a conserved, highly basic domain involved in direct specific interaction with DNA. Members of two of these families, the Jun and Fos related proteins, have been shown to directly interact and form heterodimeric complexes. A third such family known as the CREB or ATF proteins, bind to a sequence element present in promoters from a number of viral and cellular genes; this element can confer cAMP-inducibility and E1A-inducibility of transcription. In this report we show that one member of the CREB family can efficiently form a heterodimeric complex with the cJun protein. The DNA binding specificity of the heterodimer was indistinguishable from CREB alone. Transfection studies in undifferentiated F9 cells suggest that the CREB/cJun heterodimer can form *in vivo*, but that the complex does not activate transcription. The heterodimer formation between CREB and Jun proteins is highly specific; only one of the two CREB proteins would heterodimerize with cJun and it would not form dimers with JunB or cFos. The interaction of members of these two families of proteins increases the repertoire of possible regulatory complexes that could play an important role in the regulation of transcription of specific cellular genes.

Introduction

The products of the proto-oncogenes Jun and Fos bind as a heterodimeric complex to a DNA sequence element known as the TRE or AP1 binding site (5' TGAGTCA 3') (Rauscher *et al.*, 1988a, 1988b; Franza *et al.*, 1988; Chiu *et al.*, 1988; Sassone-Corsi *et al.*, 1988). This element can confer response of a promoter to a large number of varied stimuli such as phorbol esters, serum, nerve growth factor, the calcium ionophore A23187 and a number of oncogenes such as *ras*, *src* and PyMT (Angel *et al.*, 1987; Lee *et al.*, 1987; Franza *et al.*, 1988; Rauscher *et al.*, 1988a; Imler *et al.*, 1988; Wasylyk *et al.*, 1988; Schonthal *et al.*, 1988). The Jun protein itself can bind as a homodimer to the AP1 site but with a relatively low apparent affinity; however, in the presence of Fos protein Jun/Fos heterodimers are preferred with an accompanying increase in AP1 binding affinity (Rauscher *et al.*, 1988c; Franza *et al.*, 1988; Chiu *et al.*, 1988; Sassone-Corsi *et al.*, 1988; Halazonetis *et al.*, 1988). Jun related proteins (JunB, JunD) have been identified (Ryder *et al.*, 1988; Ryder *et al.*, 1989; Hirai *et al.*, 1989) whose binding to the AP1 site is enhanced by heterodimerization with Fos (Nakabeppu *et al.*, 1988). Fos related (Fos-B, Fra-1) products have also been identified and these products can also form hetero-

dimers with Jun (Zerial *et al.*, 1989, Cohen *et al.*, 1989). In all cases, the Fos and Jun family members contain a structural motif, the 'leucine zipper' that mediates dimer formation. The motif is immediately preceded by a similar, highly basic domain which has been shown by a series of mutagenesis experiments to be the domain that is responsible for specific protein-DNA interaction (Landshulz *et al.*, 1989; Gentz *et al.*, 1989; Schuermann *et al.*, 1988; Turner & Tjian, 1989; Kouzarides & Ziff, 1988). Proteins containing these two continuous motifs have been termed 'bZIP' proteins (Vinson *et al.*, 1989).

Other families of nuclear proteins have been identified that contain bZIP motifs. One such family is the CREB/ATF family of proteins, whose members are characterised as being able to specifically bind to a DNA sequence element that differs from the AP1 site by insertion of a single nucleotide (Montminy *et al.*, 1986; Lee *et al.*, 1987). This element (5' TGACGTCA 3') often referred to as the CRE, confers response of a promoter to elevated levels of cAMP (Montminy *et al.*, 1986). Two human cDNA clones encoding different members of this family have been isolated, one from a placental cDNA library (Hoeffler *et al.*, 1988) and the other from a brain library (Maekawa *et al.*, 1989). In addition a rat cDNA clone has been isolated that is very similar to the placental clone (Gonzalez *et al.*, 1989). Outside of the bZIP domain the products are entirely different, although each contains a consensus protein kinase A phosphorylation site possibly involved in regulation of its activity by cAMP. In addition to conferring response to cAMP the CREB or ATF binding site has also been implicated in being able to mediate a response to the viral E1A trans-activator protein although different CRE sites appear to vary in their ability to be regulated by these different stimuli (Kanei Ishii & Ishii, 1989; Lee *et al.*, 1989; Deutsch *et al.*, 1988). This possibly indicates that different members of the family may be involved in each response.

In light of the findings that Jun and Fos family members can efficiently form heterodimers through interaction of their respective bZIP domains, we investigated the possibility of similar interactions between members of the Jun and CREB families. We report here that specific members of these families can indeed form a heterodimeric complex which efficiently binds to a specific target DNA sequence.

Results

CREB and cJun associate in vitro

Complex formation between Jun and Fos monomers has been established by studies using Fos and Jun proteins synthesised *in vitro* in rabbit reticulocyte lysates

(Rauscher *et al.*, 1988c; Franza *et al.*, 1988; Chiu *et al.*, 1988; Sassoni-Corsi *et al.*, 1988; Halazonetis *et al.*, 1988). We employed a similar strategy to investigate the possible interactions between CREB and other bZIP containing proteins. DNA fragments containing the coding regions of the two CREB proteins were obtained by PCR amplification from a human cDNA library. The fragments were linked to the bacteriophage T7 promoter to allow *in vitro* synthesis of CREB RNA. For clarity, we refer to the product of the sequence equivalent to the previously described placental cDNA (Hoeffler *et al.*, 1985) as CREB1 and the product of the sequence equivalent to the previously described brain cDNA as CREB2 (Maekawa *et al.*, 1989).

The synthetic RNAs were translated in a rabbit reticulocyte lysate and the products assayed by gel retardation for their ability to specifically interact with the CRE element. *In vitro* synthesis of CREB1 protein generated one major and two minor faster migrating complexes (Figure 1a, lane 2). The abundance of the two faster complexes varied quite considerably and are most likely caused by the production of truncated products due to internal translation initiation. In order to investigate dimer formation, a severely truncated version of CREB1 was also synthesised; this product, DL1, lacked the N-terminal half of CREB1 but included the bZIP motif (amino acid residues 236–326). DL1 generated a very prominent, rapidly migrating complex (lane 1). Both CREB1 and DL1 bind as homodimers; simultaneous synthesis of both proteins resulted in a new binding complex of intermediate mobility (lane 3) consistent with the formation of a heterodimer between a CREB1 and DL1 monomer. CREB1, however, does not appear to functionally dimerize with the other CREB protein, CREB2. Translation of full length CREB2 gave rise to a retarded complex (lane 4). Synthesis of CREB2 and DL1 together, however, did not result in heterodimeric complexes (lane 5). A faint complex was seen migrating at the position of the CREB1/DL1 heterodimer. This is probably due to endogeneous CRE
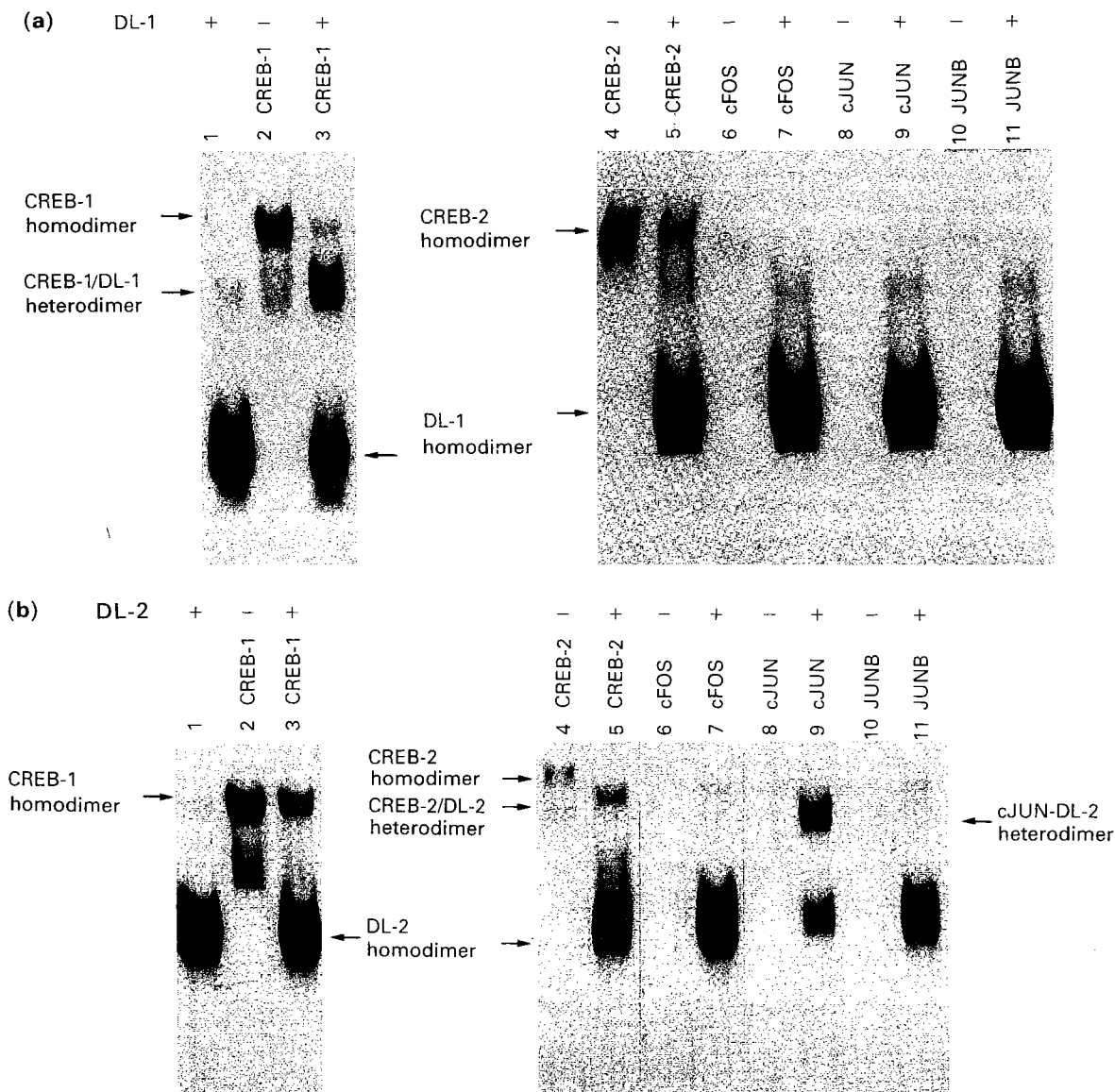

Figure 1 Gel retardation assay of CREB1 and CREB2 binding to the CRE either alone or in the presence of other co-translated proteins. Unlabelled translation products were incubated with [^{32}P]-labelled oligonucleotide containing the fibronectin CRE/ATF binding site. (a) The various bZIP proteins were either synthesised alone or together with DL1, a truncated version of CREB1 that only contains the basic DNA binding and leucine zipper domains. (b) The bZIP proteins were either synthesised alone or together with DL2, a truncated version of CREB2, containing the bZIP motif

binding activity present in the rabbit reticulocyte lysate which can dimerize with the DL1 product. The amount of endogenous CREB binding activity was found to vary with different batches of lysate but in most cases was quite significant. In order to alleviate this problem and thus simplify results obtained with protein synthesised from added CREB RNA, we routinely precleared the lysate with a biotinylated probe containing a CRE element as described in Materials and methods. Often, however, a small amount of endogenous activity remained.

We next tested whether DL1 could form heterodimers with members of the Jun and Fos families. No evidence of heterodimer formation with cJun, JunB or cFos was obtained (lane 7, 9 and 11). Synthesis of these products alone did not result in any detectable, unique complexes (lanes 6, 8 and 10). The very weak complex seen in all three lanes is again explained by the presence of small amounts of endogenous binding activity remaining in the lysate.

A similar analysis was carried out with the product of the CREB2 gene (Figure 1b). An N-terminal truncated version of CREB2, DL2 (amino acids 337–399 which includes the bZIP motif) was synthesised and shown to bind to the CRE sequence (lane 1). Simultaneous synthesis of CREB2 and DL2 generated a heterodimer complex migrating at a position intermediate between the CREB2 and DL2 homodimer complexes (compare lanes 1, 4 and 5); this was accompanied by a loss of the CREB2 homodimer due to the presence of an excess of DL2. In contrast no heterodimeric complex was observed following co-translation of DL2 and CREB1 (compare lanes 1, 2 and 3) confirming our earlier conclusion that these two different CREB proteins cannot form a DNA binding heterodimer. We also detected no interaction between DL2 and JunB or cFos protein (lanes 7 and 11). However, a heterodimeric complex between DL2 and the cJun protein was observed (lane 9). Heterodimerization appeared to be efficient since the cJun monomer competed efficiently with the DL2 monomer for dimer formation.

DNA binding specificity of the CREB2/cJun heterodimer

As shown in Figure 1b, the CREB2/cJun heterodimer could clearly bind to the CRE site. Since CREB2 and cJun have different binding specificities, the former preferentially binding to the CRE site and the latter to the AP1 site, we investigated both the specificity and binding affinity of the heterodimeric complex. A number of different promoters contain CRE consensus binding sites. However, CREB protein binds to these sites with slightly different apparent affinities as judged by cross-competition analysis (Hardy & Shenk, 1986). We took advantage of this to compare the specificity of the CREB2/CREB2 and the CREB2/cJun dimers. Complexes formed on a DNA probe containing the fibronectin CRE/ATF sequence were competed with increasing concentrations of cold competitor DNAs containing CRE/ATF sites from four different promoter origins, namely the fibronectin, somatostatin, human chorionic gonadotropin (hcg) and adenovirus E4 promoters (Figure 2). An identical hierarchy of competition was seen with the DL2/DL2 homodimer and the DL2/cJun heterodimer. In both cases, the fibronectin site was the most efficient competitor followed by E4, hcg and somatostatin. This specificity was also seen with CREB2 and CREB1 homodimers (data not shown). Neither complex was efficiently competed by the SV40 AP1 site (Figure 2) except at very high molar excess. The heterodimer therefore, showed CRE specificity. In addition, off-rate analysis of both complexes showed that the stability of each was identical (data not shown).

Dimerization with Fos stimulates cJun binding to the AP1 site whereas dimerization with CREB2 stimulates its binding to the CRE (Figure 1b). In the presence of all three proteins, the cJun/Fos heterodimer appears to be favoured. Fos, cJun and an excess of DL2 RNA were simultaneously translated and the extract analyzed for binding to the E4 CRE/ATF or SV40 AP1 probes (Figure 3). DL2/cJun dimer formation which was readily seen in the absence of Fos (lane 4) was not evident when Fos was present (lane 1). Neither the co-translation of cJun and cFos RNAs (lane 2) nor the translation of cJun alone (lane 3) resulted in any specific complex with this probe. In contrast when assayed on the SV40 AP1 probe, translation of all three RNAs resulted in a specific complex (lane 6) that co-migrated with the cJun/Fos heterodimer (lane 7) obtained by synthesis of cJun and Fos in the absence of DL2. The cJun/Fos complex, therefore, was able to form despite the presence of excess DL2 product, suggesting preference for formation of the cJun/Fos heterodimer over the
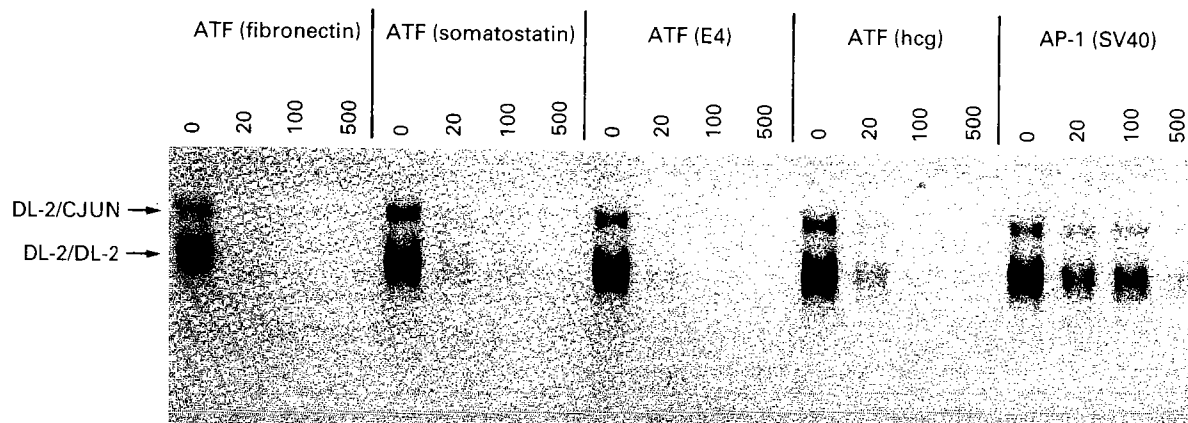

Figure 2 Specificity of DL2 homodimer and DL2/cJun heterodimer binding. Gel retardation competitor analysis of co-translated DL2 and cJun proteins bound to the fibronectin CRE/ATF containing oligonucleotide. Various concentrations of unlabelled oligonucleotides were added; the nature of the competing DNA and its molar excess over the [^{32}P]-labelled probe is indicated
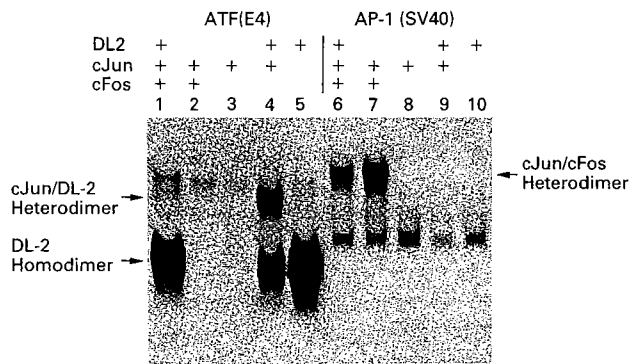
Figure 3 Preferential dimerization of cJun protein with cFos over CREB2. DL2, cJun and cFos proteins were either synthesised alone or together and the resulting complexes analyzed by gel retardation on either the adenovirus E4 CRE/ATF containing probe or the SV40 AP1 site containing probe. The proteins that were co-synthesised are indicated above each lane

cJun/CREB heterodimer. As suggested by the competition experiments described earlier, neither the cJun/DL2 heterodimer nor the DL2 homodimer could form a detectable complex on the AP1 probe (lanes 9, 10).

Coimmunoprecipitation of CREB2 and cJun proteins

The interaction between cJun, Fos and CREB proteins was investigated using an immunoprecipitation assay. An antibody that specifically recognizes a C-terminal peptide of the cJun protein was used to immunoprecipitate proteins from *in vitro* translation extracts (Figure 4). Translation of the synthetic cJun RNA gave rise to a major immunoprecipitable product of 43 kd and some minor products (lane 1). Whereas cFos protein alone was not immunoprecipitated by the anti-
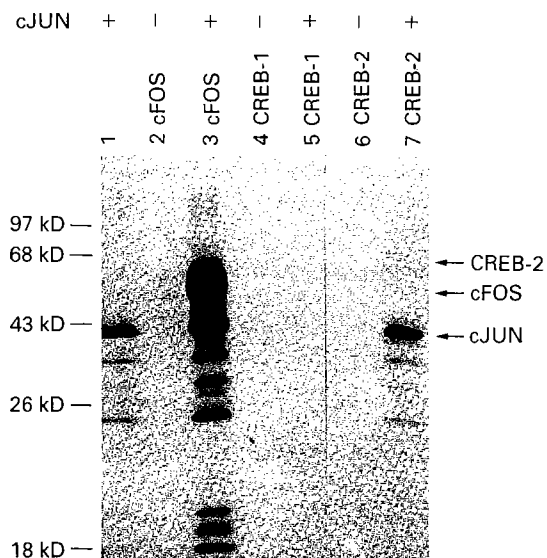

Figure 4 Association of CREB2 and cJun *in vitro.* cFos, CREB1 or CREB2 RNAs were translated *in vitro* either alone or together with cJun RNA. The translated products were then immunoprecipitated with an antibody that recognizes a C-terminal peptide of cJun. In all cases except lane 5, the proteins were labelled with [^{35}S]methionine. In the case of lane 5, the CREB1 and cJun proteins were synthesized separately and then mixed; CREB1 was S-35 labelled but cJun was unlabelled. The mixture was heated at 55°C for 15 min, cooled and immunoprecipitated

body (lane 2), it was precipitated when synthesized together with Jun protein (lane 3). The antibody, therefore, efficiently recognized the cJun/Fos heterodimer. CREB1 protein was neither immunoprecipitated when synthesized alone (lane 4) nor together with cJun (lane 5) confirming our conclusion based on the gel retardation assay that cJun/CREB1 heterodimers cannot form. In lane 5, unlabelled cJun protein was synthesized since CREB1 and cJun migrate with similar mobilities and are therefore difficult to distinguish. In contrast, CREB2 protein, migrating with an apparent molecular size of 68 kd, was precipitated but only following its co-translation with cJun (compare lanes 6 and 7). The CREB2 protein was poorly translated compared to cJun, hence its relatively low abundance. This result confirms our conclusion that cJun and CREB2 proteins can directly interact.

Interaction between CREB2 and cJun in transfected F9 cells

While cJun/Fos heterodimers could form when the two proteins were translated separately and subsequently mixed, efficient dimerization between CREB2 and cJun was only detected when the proteins were co-translated. Separately translated CREB2 and cJun efficiently formed heterodimers only if they were first incubated at 55°C. A question posed by this result is whether such heterodimers could efficiently form *in vivo*. In order to address this question and to begin to investigate the possible significance of CREB2/cJun heterodimer formation, gene transfer experiments were performed. Undifferentiated F9 cells were chosen as recipients for these transfers because of their low endogenous cJun levels (Kryske *et al.*, 1987). cDNAs encoding CREB and cJun proteins were transfected into F9 cells either individually or together, and their effect on expression from promoters containing multiple AP1 binding sites or CRE/ATF sites tested (Figure 5). Transfection of either cJun or JunB resulted in a modest (about 3-fold) stimulation of expression from the chimeric luciferase gene containing the AP1 site promoter (Figure 5a). A similar stimulation was obtained following transfection of the rat cFos encoding cDNA. As reported by many other groups, co-transfection of cJun and cFos resulted in a much higher stimulation than that obtained with either gene alone (Lucibello *et al.*, 1988; Schonthal *et al.*, 1988; Schuermann *et al.*, 1989; Sassoni-Corsi *et al.*, 1988). In contrast however, co-transfection of cJun and CREB2 gave no significant stimulation suggesting that CREB2 could interfere with the stimulation seen with cJun alone. Similarly, co-transfection with cJun, cFos and CREB2 resulted in a significantly lower level of stimulation than that obtained with cJun and cFos in the absence of CREB2. This result is in variance with the *in vitro* binding data which suggested that when all three products were synthesized, cJun/Fos heterodimers were preferred over CREB2/cJun dimers. The reason for the apparent discrepancy is not known but is under investigation. CREB2 did not interfere with JunB stimulation and neither cJun nor JunB were affected by co-transfection with the CREB1 cDNA. Thus the results are fully consistent with the possibility that CREB2 and cJun can indeed form heterodimers *in vivo* and that such heterodimers are functionally inactive, at least in the assay used. No interference was seen following the
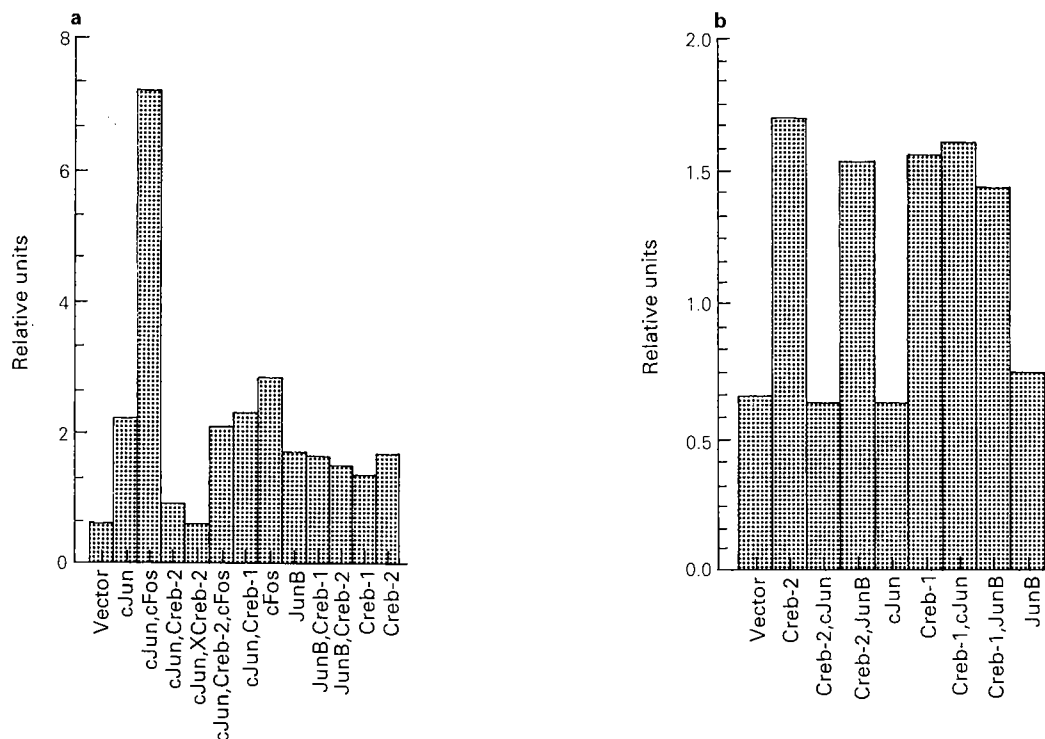
Figure 5 Transcriptional stimulation by transfected CREB, Jun and Fos cDNAs into undifferentiated F9 cells. Undifferentiated F9 cells were transiently transfected with expression plasmids containing either CREB1, CREB2, cJun, JunB or Fos cDNAs. In each case 10 μg of each of the indicated plasmids was transfected except for the case of XCREB2, where a 10-fold excess of CREB2 was used. The effect of introducing these plasmids on expression of either a co-transfected $(AP1)_5$-TK-luciferase (**a**) or $(CRE)_3$-TK-luciferase (**b**) test gene was assayed. The experiment was performed at least 3 times with essentially identical results

co-transfection of cDNAs whose products could not be demonstrated to dimerize *in vitro*. Surprisingly, some stimulation was observed following the transfection into these cells of either CREB1 or CREB2 alone, an unexpected result considering that these proteins bind very poorly to the AP1 site *in vitro*. The significance of this stimulation is not yet clear.

The effect of these genes on expression from a CRE/ATF containing promoter was also consistent with the *in vivo* formation of an inactive CREB2/cJun heterodimer. The introduction of CREB1 or CREB2 cDNAs into F9 cells resulted in a modest but significant and consistent stimulation (Figure 5b). In contrast, neither cJun nor JunB had any effect. However, cJun interfered with the CREB2 stimulation, co-transfection of both genes giving no increase in expression over that seen with the CRE-luciferase vector alone. cJun could not interfere with CREB1 mediated stimulation and JunB had no effect on the stimulation seen with either CREB1 or CREB2. Thus the only interaction observed was with the products that could form heterodimers *in vitro*.

Discussion

The main conclusion from this report is that CREB2 and cJun polypeptides can efficiently form heterodimers. Such heterodimers formed *in vitro* could specifically bind to the CRE DNA element and were stable to immunoprecipitation. Additionally, transfection studies into F9 cells, strongly suggested that these heterodimers could also form *in vivo*. The significance of this dimer formation is not yet clear although it obviously expands the repertoire of sequence-specific DNA binding complexes that could respond to certain cellular and

environmental stimuli and regulate expression of specific genes. This possibility is particularly intriguing with respect to the CREB2/cJun heterodimer since the individual component proteins have been associated with the transcriptional response to stimuli that affect two different signalling pathways in the cell. The cJun protein, together with cFos, binds to the AP1 site which mediates a transcriptional activation response to signals that stimulate protein kinase C activity (Lee *et al.*, 1987; Angel *et al.*, 1987). In contrast, CREB2 binds to the CRE element which mediates response of a promoter to elevated protein kinase A activity, usually accomplished by elevated levels of intracellular cAMP (Montminy *et al.*, 1986). The exact role of these factors in these two transcriptional responses is not yet clear. CREB1, which also binds to the CRE element, has been shown to be the target for kinase A phosphorylation *in vitro*; this phosphorylation is necessary for the factor to activate transcription (Gonzalez *et al.*, 1989). CREB2 also has a potential protein kinase A phosphorylation site (Maekawa *et al.*, 1989) and although it has not been shown directly, it is reasonable to assume that transcriptional activation by CREB2 is also modulated by kinase A mediated phosphorylation. In contrast, an increase in the phosphorylation state of cJun in response to protein kinase C activation has not been demonstrated. It will be interesting to determine the effects of these two kinase pathways on the activity of the CREB2/cJun heterodimer.

Transfection studies in undifferentiated F9 cells suggested that the CREB2/cJun heterodimer complex is non-functional as an activating complex; when both proteins are synthesized in the same cell stimulation from either a CRE or an AP1-binding site containing promoter was not detected, even though synthesis of

either protein individually did result in stimulated levels of transcription. However, what is not clear is whether this complex is non-functional in all cell types or in response to various stimuli. Under certain conditions it could function as an activator. Alternatively, the complex may play a significant role in the cell as a transcriptional repressor possibly by interfering with the binding of other complexes or by sequestering each protein and thus preventing either homodimer formation or alternative heterodimer formation. Indeed there is every reason to believe that the expression of many genes is regulated by interplay between both positively and negatively acting factors (for review see Levine & Manley, 1989). It is also very clear, particularly from studies in prokaryotes, that many transcription factors or complexes that have been shown to repress transcription can also function as activators. Thus, depending upon the context of the binding site and environment of the cell, it is feasible that the CREB2/cJun heterodimer can both repress and activate transcription. These possibilities are currently being investigated.

Unexpectedly, the CREB2/cJun dimer did not appear to have a novel DNA binding specificity; rather, binding of the heterodimer was indistinguishable from that of a CREB homodimer. Specifically binding was at least 10-fold more efficient to the four different CRE elements tested than to the SV40 AP1 binding site. However, the effect of sequences that flank the CRE core element has not been rigorously tested. The importance of flanking sequences to binding specificity was highlighted by a recent study demonstrating that binding of a cJun/Fos heterodimer to a CRE element was affected at least 10-fold by the nature of the nucleotides at positions 1 and 10 which flank the 7 bp core sequence (Nakabeppu & Nathans, 1989). The cJun/Fos heterodimer will efficiently bind to a CRE when A and T residues are present at these positions, respectively. The CRE containing probes that were used in our study to investigate cJun/Fos and CREB2/cJun binding did not contain the 1A and 10T residues, probably explaining why we saw very inefficient binding of cJun/Fos to any of our CRE probes. The observed binding properties of the CREB2/cJun dimer suggests that CREB2 is the more important component in determining its specificity.

The physical interaction of members of the CREB and cJun families of proteins appears to be very restricted and the only case where interaction was detected was with CREB2 and cJun. Thus, although cJun can dimerize with other Jun family members as well as with Fos protein, CREB2 is far more selective. Selectively of leucine zipper proteins with respect to dimerization is commonly seen and a number of zipper swap experiments have demonstrated that the selectivity resides in the zipper region (Kouzarides & Ziff, 1989; Sellers & Struhl, 1989; Neuberg et al., 1989). However, the structural basis for the selectivity is presently unclear but must depend upon the nature of the amino acid residues located between the leucine repeats. Homology between the zipper regions is obviously not a good indicator since Fos monomers fail to dimerize. CREB2 is more similar in its zipper domain to cJun than is CREB1 (44% versus 23%); however it is equally similar to the zipper domain of JunB to which it fails to dimerize. Detailed mutational and physical analysis will

be needed before an understanding of the features which govern dimerization is reached.

Materials and methods

Plasmids

The taq polymerase chain reaction (PCR) was used to amplify and clone sequences containing part of the entire coding regions of CREB (Hoeffler et al., 1988) and CRE/BP1 (Maekawa et al., 1989). The DNA used for the amplification was a human cDNA library from MG63 cells in the pscDM8 plasmid vector (a gift from S.T. Whiteside and S.E.Y. Goodbourn). The full length and truncated CREB sequences are referred to as CREB1 and DL1 and the full length and truncated CRE/BP1 sequences as CREB2 and DL2 respectively. In the case of CREB1, DL1 and DL2 the sequences were inserted downstream of the human β globin initiation codon contained in the vector T7βsal (Norman et al., 1988). This vector (a gift from R. Treisman) contains the bacteriophage T7 promoter upstream of the β globin 5' untranslated leader sequence. The CREB2 sequences were inserted into the bluescript vector downstream of the T7 promoter. DL1 and DL2 contained amino acid residues 236–326 and 337–399 of CREB1 and CREB2 respectively. In all cases the cloned fragments were sequenced to ensure that alterations generated during the PCR reaction were not present. The cJun (a gift from R. Tjian) (Bohmann et al., 1987), mouse JunB (a gift from R. Bravo) (Zerial et al., 1988) and rat cFos (a gift from T. Curran) (Curran et al., 1987) cDNA containing plasmids have been previously described.

For transfection experiments, cDNAs were cloned into the vector PJ8.6 (a gift from J. Morgenstern and H. Land) which contains the F2 promoter that functions efficiently in undifferentiated F9 cells (Barklis et al., 1986), and the SV40 intron and polyadenylation site. The reporter plasmids were constructed by inserting either 3 copies of the fibronectin CRE containing oligonucleotide or 5 copies of the SV40 AP1 site containing oligonucleotide upstream of the firefly luciferase coding region fused at its 5' end to the basal promoter (−39 to +15) of the herpesvirus thymidine kinase gene (Visvanathan & Goodbourn, 1989).

Oligonucleotides

The double stranded oligonucleotides used for the binding reactions were as follows:

CRE Fibronectin:
5'GATCCCCCGTGACGTCACCCGGGAGCCGGATC3'

CRE Somatostatin:
5'GATCCTGACGTCAGCCAAGGATC3'

ATF E4:
5'AAGCTTCTAAAAAATGACGTAACGGAAGCTT3'

CRE hcg:
5'AGCTTGACGTCATGGTAAAAATTGACGTCATGGTAAGCT3'

AP1 SV40:
5'GATCCTTGCTGACTAATTGAGATGGATG3'

In vitro transcription and translation

Plasmids containing various cDNA sequences downstream of the bacteriophage T7 promoter were linearized and capped RNA synthesized using T7 polymerase. Templates were transcribed with 1 mM ATP, UTP and CTP, 100 μM GTP and 0.5 mM of the cap analogue m7GpppG. The resulting transcripts were translated in pretreated rabbit reticulocyte lysates with unlabelled or [35S]-labelled methionine (Promega). The lysate was pretreated to deplete it of endogenous ATF/CREB

binding activity by incubation for 30 min at 4°C with a bio-tinylated probe containing the fibronectin ATF binding site coupled to avidin agarose. The supernatant cleared by centrifugation was either used immediately for translation or stored at −70°C.

Gel retardation assay

5 μl of the translation extract were incubated with 0.1 ng of appropriate [^{32}P] end-labelled, double-stranded oligonucleotide probe in a total volume of 25 μl of buffer containing 25 μM HEPES pH 7.9, 150 mM KCl, 1 mM EDTA, 5 mM dithiothreitol (DTT), 10 × Denhardts reagent, 0.25 μg of poly(dI-dC) and 0.25 μg of poly(dA-dT). Incubation was at room temperature for 15 min and the mixtures were then separated on 6% non-denaturing polyacrylamide gels at room temperature at 12.5 V cm^{-1}.

Immunoprecipitation of translation products

20 μl of translation extract were diluted to 100 μl in E1A buffer (Harlow et al., 1986) to give a final concentration of 50 mM HEPES pH 7.0, 250 mM NaCl and 0.1% Nonidet P-40 (NP-40). 1 μl of an anti-Jun peptide antibody (raised against a peptide comprising of the last 12 residues of cJun; a gift from H. Hurst) was added and the mixture incubated at 4°C overnight. 10 μl of a saturated solution of protein A sepharose was added and incubation with agitation continued for 2 h at 4°C. The immune complexes were washed three times with 1.0 ml of E1A buffer and once with E1A buffer minus NP-40. The complexes were resuspended in gel sample buffer and electrophoresed on 10% SDS polyacrylamide gels. The gels were fixed, soaked in Amplify (Amersham) and autoradiographed.

Cell culture and DNA transfection

F9 EC cells were grown in Dulbecco's modification of Eagles medium supplemented with 10% (v/v) foetal calf serum and antibiotics on tissue culture plates coated with 0.1% porcine skin gelatin. Every third day the cells were subcultured 1:10 with the aid of a 10 ml syringe and a 19 gauge needle. After 15 passages the cells were discarded. Prior to transfection the cells were split 1:10 and the calcium phosphate DNA co-precipitate added 5 hours later. The plates were harvested 40 h after transfection.

Luciferase assays

Each plate of transfected cells was washed twice with PBS and 150 microlitres of lysis buffer (0.65% NP-40, 10 mM Tris (pH 8.0), 1 mM EDTA and 150 mM NaCl) was added. After two minutes when only the nuclei were visible under the microscope, the lysate was transferred to a microfuge tube and cooled on ice. The cooled tubes were spun for one minute and the supernatant transferred to a fresh tube. Luminometer cuvettes were filled with 350 microlitres of Luciferase reaction buffer (25 mM Glycylglycine pH 7.8, 5 mM ATP (pH 8.0), and 15 mM MgSO$_4$) and 20 microlitres of lysate. An LKB 1251 luminometer was used to inject 33 microlitres of 3 mM Luciferin and measure the peak activities. Each transfection extract was assayed four times and the average taken.

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Note added in proof: We have subsequently found that the DL-2/JUN heterodimer has a higher affinity for the collagenase AP-1 binding site than the DL-2/DL-2 homodimer.