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CBP-induced stimulation of c-Fos activity is abrogated by E1A

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The CBP protein stimulates transcription of cAMP-responsive genes by binding to the phosphorylated activation domain of the CREB transcription factor. Here we show that CBP stimulates transcription of Fos/Jun activity in F9 cells and that this response is mediated, at least partly, via c-Fos. We show that CBP binds c-Fos in a phosphorylation-independent manner *in vitro*, using a domain distinct from that required to bind CREB. When this CBP domain is linked to the activation domain of VP16 it can stimulate GAL4–Fos activity *in vivo*. The domain of CBP that binds c-Fos is also used to contact the E1A protein. We therefore asked whether the documented repression of AP1 activity by E1A is due to sequestration of CBP from c-Fos. We show that E1A 12S can repress c-Fos activation functions. The use of E1A mutants indicates that binding of CBP, but not RB, to E1A is essential for E1A-mediated repression. These data support a model whereby E1A can modulate AP1 activity by directly competing for the CBP co-activator protein.

Keywords: AP-1/CBP/c-Fos/c-Jun/E1a/transcription

Introduction

Binding sites for the AP1 family of proteins have been identified in a variety of promoters. The c-Fos and c-Jun proteins are the prototypes for the family of factors which bind the AP1 site and activate transcription. Activity of the c-Fos protein has been linked to both the proliferative and differentiating pathways (Müller and Wagner, 1984; Jenuwein *et al.*, 1985; Mitchell *et al.*, 1985), suggesting that it can mediate a variety of responses depending on the stimulus and the promoter context.

Structure–function analysis of c-Fos has revealed that it has a DNA binding domain of the bZIP family which allows it to heterodimerize with c-Jun and bind DNA (Halazonetis *et al.*, 1988; Kouzarides and Ziff, 1988, 1989; Nakabeppu *et al.*, 1988; Sassone-Corsi *et al.*, 1988). In addition, c-Fos has a number of transcriptional activation domains (Abate *et al.*, 1991; Sutherland *et al.*, 1992; Brown *et al.*, 1995). One of these domains has a motif, HOB1, which is also present in a c-Jun activation domain. The activation functions of the c-Fos HOB1 motif are stimulated by phosphorylation carried out by the MAP kinase superfamily of enzymes and are regulated by Ha-Ras (Bannister *et al.*, 1994; Deng and Karin, 1994). The c-Jun HOB1 motif is similarly regulated. Ha-Ras and UV stimulate its activity by inducing phosphorylation of HOB1 by MAP kinase-related, stress-activated protein kinases (Binétruy *et al.*, 1991; Dérijard *et al.*, 1994; Kyriakis *et al.*, 1994). Recently an inhibitor domain has been identified in c-Fos which negatively regulates activity of the HOB1 motif (Brown *et al.*, 1995).

The activation functions of c-Fos are also likely to be mediated by protein–protein interactions. So far one target of c-Fos has been identified. This is the TATA box binding protein (TBP), which is part of the multisubunit basal factor TFIID. A specific motif in c-Fos, the TBM, is required for TBP binding and for transcriptional activation (Metz *et al.*, 1994).

The adenovirus E1A protein has the capacity to modulate AP1-site-containing promoters. The form of regulation imposed is dependent on promoter context. E1A will stimulate the c-Jun promoter by enhancing DNA binding functions at the AP1 site (Van Dam *et al.*, 1990; De Groot *et al.*, 1991; Kitabayashi *et al.*, 1991a). This stimulatory response of E1A requires, in addition to the AP1 site, a distinct element, RERE, present within the c-Jun promoter (Kitabayashi *et al.*, 1991b). In contrast, E1A can repress the activity of the collagenase promoter via an AP1 site (Frisch *et al.*, 1990; Offringa *et al.*, 1990; Van Dam *et al.*, 1990). In this case E1A does not appear to affect the DNA binding capacity of the AP1 site complex, suggesting that it directly affects the transcriptional activation functions of the complex. The mechanism by which this repression is brought about is not yet understood.

Recently a protein, CBP, has been identified which mediates the activation functions of the CREB transcription factor. The CBP protein will only bind a form of CREB which is phosphorylated at Ser133, present within its activation domain (Chrivia *et al.*, 1993). Since CBP can also contact the basal factor TFIIB, it is considered to be an 'adaptor' protein between upstream enhancer binding CREB protein and the general transcriptional machinery. The CBP protein and a highly related family member, p300, (Eckner *et al.*, 1994), have been shown to contact the transforming protein E1A (Arany *et al.*, 1995; Lundblad *et al.*, 1995). Loss of p300 binding to E1A correlates with loss of E1A-induced immortalization and cell cycle activation (for a review see Moran, 1993).

Microinjection experiments have suggested that CBP may be involved in signalling cascades which lead to activation of distinct elements. In particular, the TPA responsive element (TRE) and the serum response element (SRE) are repressed by microinjection of CBP antibodies (Arias *et al.*, 1994). This prompted us to examine whether CBP could function as a co-activator for a member of the AP1 family, the c-Fos protein. Here we show that CBP can stimulate activity of the Fos–Jun complex *in vivo* and that part of this response is mediated through the direct interaction of CBP with c-Fos. Given that E1A can also

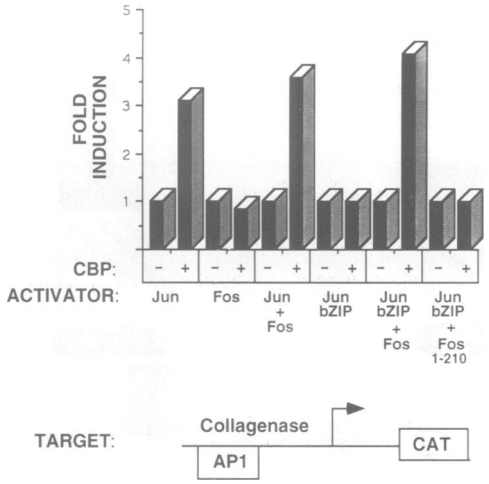

Fig. 1. CBP stimulates c-Fos-dependent activity from an AP-1 site. F9 teratocarcinoma cells were transiently transfected with 2.5 μg coll–CAT (residues –73 to +63 of the collagenase promoter fused next to CAT cDNA) and 1 μg indicated effector (expressed from the SV40 promoter-driven pHK vector). Also co-transfected were 4 μg RSV.CBP (+) or 4 μg empty RSV vector (–), as indicated. Following a CAT assay the results were quantitated using a PhosphoImager. In each case the activity of the effector in the absence of CBP is normalized to a value of 1.

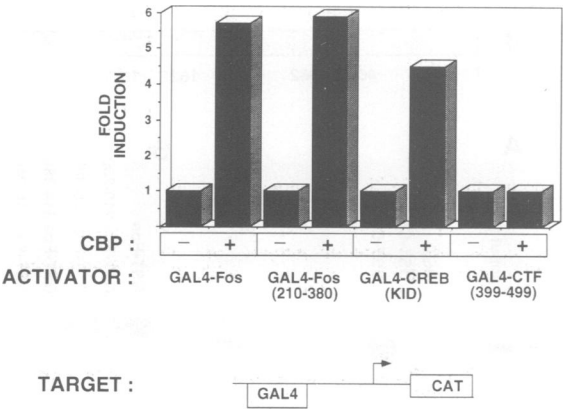

Fig. 2. The C-terminus of c-Fos is sufficient for transcriptional enhancement by CBP. U2OS human osteosarcoma cells were co-transfected with 1 μg SV40 promoter-expressed Gal fusions (from plasmid pHKG) as indicated and 4 μg target reporter, G_5E1BCAT. Also co-transfected along with each activator were 4 μg RSV.CBP (+) or 4 μg empty RSV vector (–), as indicated. In each case the activity of the effector in the absence of CBP is normalized to a value of 1. The CREB KID domain corresponds to two copies of the PKA kinase-inducible domain of CREB (102–151).

bind CBP, we tested the possibility that E1A can repress AP1 activity by sequestering CBP from c-Fos. We find that, indeed, E1A represses c-Fos activation capacity and that this function requires an intact CBP binding site in the E1A protein.

Results

CBP stimulates c-Fos activity

We set out to establish whether the CBP protein would act as a co-activator for Fos–Jun activity. Figure 1 shows that the ability of Fos–Jun to stimulate the AP1 site-containing collagenase promoter is stimulated more than 3-fold by CBP in F9 cells. This level of CBP stimulation is comparable with the already characterized stimulation of CREB activity (Chrivia *et al.*, 1993). The stimulatory effect of CBP can be seen even when only c-Jun is used as the activator, which is consistent with the fact that c-Jun can bind CBP (Arias *et al.*, 1994; unpublished results). To test whether CBP also augments c-Fos activity we transfected c-Fos along with a plasmid expressing just the DNA binding domain of c-Jun, to allow c-Fos to bind the AP1 site. Figure 1 shows that the activity of the c-Fos–Jun bZIP combination is stimulated by CBP, whereas c-Fos or Jun bZIP activity is not. These results suggest that CBP can stimulate c-Fos activity. When the C-terminal activation domains of c-Fos are removed the remaining sequences (Fos 1–210) do not respond to CBP stimulation.

To provide further support for the c-Jun-independent stimulation of c-Fos activity by CBP we used GAL4–Fos fusions. Figure 2 shows that GAL4–Fos activity is stimulated by CBP and that c-Fos residues 210–380, implicated in the CBP response in F9 cells (Figure 1), are sufficient for the CBP response. The level of CBP stimulation of GAL4–Fos is equivalent to that observed for GAL4–CREB. The CBP protein is not a general inducer of transcription, since it will not stimulate the activity of the CTF activation domain (Figure 2).

CBP binds directly to c-Fos

We next wanted to establish whether stimulation of c-Fos activity by CBP resulted from a direct interaction between these two proteins. The CBP protein has two domains which have been shown to bind transcription factors: residues 461–662 (designated CBP1) are required to bind CREB (Chrivia *et al.*, 1993), whereas sequence 1621–1877 (designated CBP2) is required to bind E1A (Arany *et al.*, 1994; Eckner *et al.*, 1994). We fused each of these two domains (CBP1 or CBP2) onto GST protein and asked whether c-Fos would bind to either region. Figure 3A shows that c-Fos translated *in vitro* can bind very efficiently to GST–CBP2, whereas binding to GST–CBP1 is only slightly above the background interaction with GST. The contact between c-Fos and CBP is direct, rather than via an intermediary protein present in the lysate, since GST–Fos binds efficiently to a bacterially expressed and radiolabelled CBP2 domain (Figure 3B).

These results indicate that, unlike the CREB protein, which requires phosphorylation before it can bind CBP (Chrivia *et al.*, 1993), the c-Fos protein can bind CBP in an unphosphorylated form. To verify that the conditions used in our GST pull-down experiments were discriminative and specific we asked whether *in vitro* translated CREB would bind GST–CBP in a phosphorylation-dependent manner. Figure 3C shows that, as predicted, protein kinase A (PKA) phosphorylated CREB will bind the CBP1 domain, but not the CBP2 domain. Unphosphorylated CREB will bind neither CBP1 nor CBP2. These results confirm that the specificity of c-Fos for the CBP2 domain is not shared by CREB and that, unlike CREB, c-Fos does not need prior phosphorylation to bind the CBP2 domain. Further evidence for the specificity of this assay comes from the fact that another transcription factor, Elk1, does not interact with either CBP1 or CBP2 (Figure 3D).

Dissection of the c-Fos protein indicates that the C-

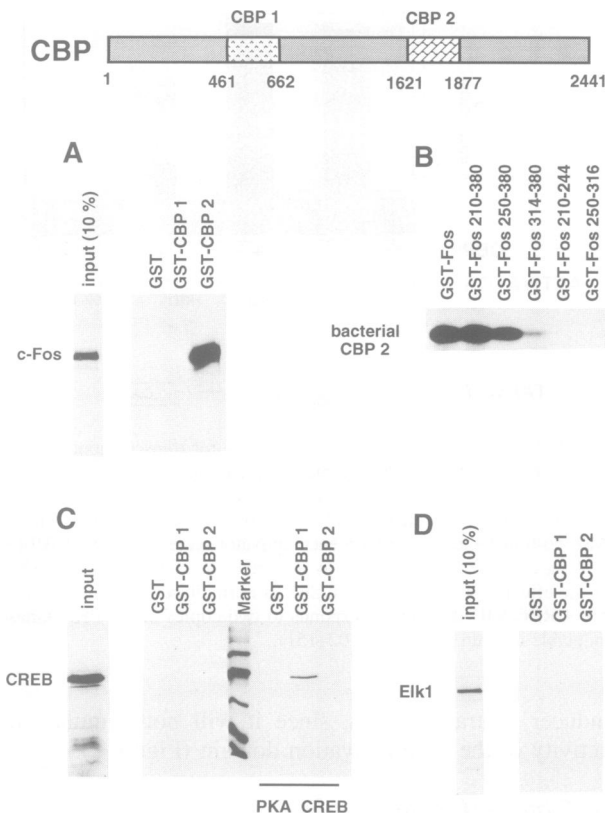

Fig. 3. CBP binds c-Fos and CREB *in vitro*. (**A**) c-Fos interacts with CBP. GST, GST–CBP1 or GST–CBP2 were incubated with *in vitro* translated radiolabelled c-Fos and subjected to GST pull-down. (**B**) CBP2 binds c-Fos directly. Bacterially expressed GST–Fos or various GST–Fos deletions as indicated were incubated with bacterially expressed, ^{32}P-labelled CBP2 protein and subjected to GST pull-down. (**C**) CBP1 binds PKA phosphorylated CREB. GST, GST–CBP1 or GST–CBP2 were incubated with *in vitro* translated radiolabelled CREB and subjected to GST pull-down. Where indicated, following *in vitro* translation CREB was incubated with PKA and ATP prior to GST pull-down. (**D**) Elk1 does not bind CBP1 or CBP2. A GST pull-down was performed as described for (A) except Elk1 was the radiolabelled input protein.

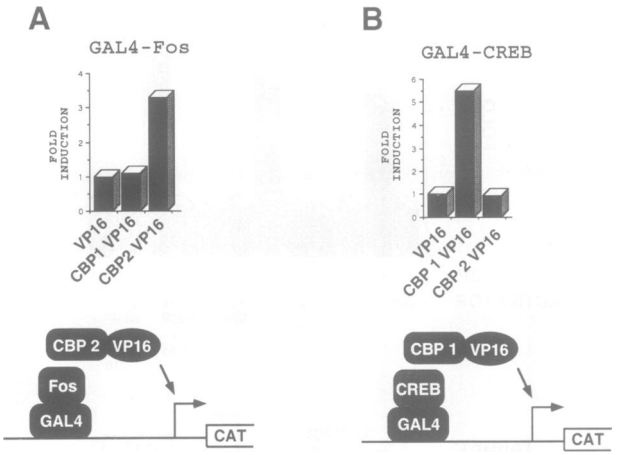

Fig. 4. *In vivo* two hybrid interaction between c-Fos and CBP. U2OS human osteosarcoma cells were co-transfected with 1 μg pHKGal4-Fos (**A**) or 1 μg pHKGal4-CREB KID (**B**) and 4 μg G_5E1BCAT as reporter and, where indicated, with 2 μg pHK3nVP16 (expressing the VP16 activation domain), 2 μg pHK3nCBP1VP16 (expressing $CBP_{461-662}$ in-frame with the VP16 AD) or 2 μg pHK3nCBP2VP16 (expressing $CBP_{1621-1877}$ in-frame with the VP16 AD). Following a CAT assay the results were quantitated using a PhosphorImager. The activity of Gal4–Fos (A) or Gal4NCREB KID (B) in the presence of the VP16 AD is normalized to a value of 1.

terminus of c-Fos (residues 210–380) is sufficient for CBP binding (Figure 3B). This is consistent with the *in vivo* data presented in Figures 2 and 3, which show that the c-Fos C-terminus is required and sufficient for the response to CBP stimulation. The c-Fos sequence required for CBP binding spans the activation modules FAM4 and FAM5 (Sutherland *et al.*, 1992; Brown *et al.*, 1995), present within residues 250–380. However, individually these two modules do not bind CBP efficiently: FAM5 (314–380) binds weakly, whereas FAM4 (250–316) does not bind at all. These data suggest that the binding domain for CBP is bipartite, requiring the co-operation of modules FAM4 and FAM5, which co-operate to activate transcription.

Having established that a specific domain of CBP (CBP2) can directly contact an activation domain of c-Fos *in vitro*, we set out provide evidence that this interaction is required and sufficient for *in vivo* stimulation. To this end we fused the CBP1 or CBP2 domain of CBP to the activation domain (AD) of VP16 and asked if either could stimulate GAL4–Fos activation capacity in U2OS cells. Figure 4A shows that in this 'two hybrid' *in vivo* interaction assay the CBP2–VP16 AD fusion stimulates GAL4–Fos activity specifically. This stimulation is not observed with the CBP1–VP16 AD fusion (which does not bind c-Fos) or when the VP16 AD alone is used in this assay (Figure 4A).

To provide evidence that CBP2 is not a promiscuously activating domain *in vivo* we carried out the same two hybrid interaction experiments in U2OS cells, this time with GAL4–CREB as the target. Figure 4B shows that CBP2–VP16 AD does not activate GAL4–CREB, which is consistent with the fact that CBP2 does not interact with CREB. In contrast, the CBP1 domain, which can bind CREB, is able to stimulate GAL4–CREB activity in the context of the CBP1–VP16 AD fusion. Since no exogenous PKA has been added, this result also suggests that U2OS cells contain sufficient active PKA to phosphorylate CREB and thereby allow the interaction with CBP1. In F9 cells, which have a very low level of endogenous active PKA, this reaction is strictly dependent upon the addition of exogenous PKA (data not shown; Chrivia *et al.*, 1993).

Collectively these results show that CBP has a domain, CBP2, which directly contacts unphosphorylated c-Fos *in vitro* and which is sufficient for interaction with c-Fos *in vivo*.

E1A represses c-Fos activity in a CBP-dependent manner

The E1A protein has the capacity to repress transcription of the collagenase AP1 site. This repression is dependent on the intactness of the CR1 domain of E1A, which contains a binding site for CBP (Offringa *et al.*, 1990). In the light of the experiments described here, showing that CBP functions as an adaptor for c-Fos activity, we asked whether E1A-induced repression of AP1 activity was due to the sequestration of CBP from c-Fos. We therefore asked whether E1A has the capacity to repress the activity of a GAL4–Fos fusion in a way similar to

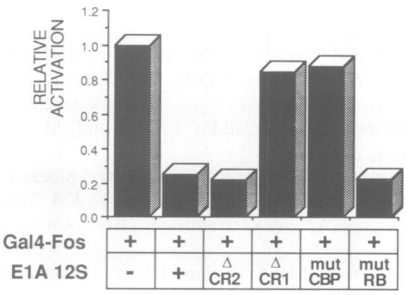

Fig. 5. E1A represses Fos transcriptional activity in a CBP-dependent manner. U2OS human osteosarcoma cells were co-transfected with 1 μg RSV.Gal4-Fos and 4 μg G_5E1bCAT reporter. Also co-transfected were 2 μg RSV.E1A 12S (+), 2 μg empty RSV vector (–) or 2 μg indicated mutant E1A 12S. The activity of Gal4–Fos in the absence of E1A is normalized to a value of 1. All the E1A proteins were expressed to similar levels, as determined by Western blotting.

that reported for the repression of AP1 activity. Figure 5 shows that, indeed, E1A can repress GAL4–Fos activity. This effect can be seen even after CBP-induced stimulation of c-Fos activity (data not shown). The repression requires sequences previously shown to be essential for repression of AP1 activity; it requires the presence of the CR1 domain of E1A, but is unaffected by deletion of CR2 (Figure 5).

We next asked whether the requirement for the E1A CR1 domain reflected a requirement for binding to CBP. To address this question we used a mutant of E1A within CR1 (Sub 1032; Smith and Ziff, 1988) which affects binding to p300 (Wong and Ziff, 1994) and to CBP (data not shown), but does not affect binding to RB (Wong and Ziff, 1994; data not shown). Figure 5 shows that this mutant, E1AmutCBP, is severely impaired in c-Fos repression. In contrast, another mutation of CR1 sequences, E1AmutRB (Sub 1085; Smith and Ziff, 1988) which affects the binding of RB, but not p300 or CBP (Wong and Ziff, 1994; data not shown), does not affect the ability of E1A to repress c-Fos activation functions. These data indicate that the binding of CBP to E1A correlates with the ability of E1A to repress c-Fos activation functions.

Discussion

We show here that the CBP protein can stimulate the activation functions of the c-Fos protein. A domain of CBP, previously characterized as an E1A binding region, is sufficient to mediate c-Fos binding *in vitro* and *in vivo*. The region of c-Fos contacted by CBP is an activation domain. Loss of CBP binding correlates with loss of c-Fos transcriptional activation capacity. These results suggest that CBP acts as a co-activator for a specific activation domain within c-Fos.

The CBP protein has been shown to function as a co-activator protein for the CREB transcription factor. We found that the characteristics of CREB stimulation by CBP are different from those of c-Fos stimulation. Firstly, CBP uses distinct domains to contact CREB and c-Fos. Secondly, CBP will only bind a PKA phosphorylated form of the CREB activation domain, but will bind an unphosphorylated activation domain of c-Fos. Thus the CBP–CREB contact is regulated by cAMP-mediated pathways, whereas the CBP–Fos contact is not. We cannot, at this point, rule out the possibility that phosphorylation of a distinct activation domain in c-Fos mediates binding to a different domain of CBP.

The mechanism by which CBP stimulates Fos–Jun activity is yet to be established. The CBP protein is thought of as an adaptor protein between CREB and the basal machinery, since it has the capacity to contact both CREB and the basal factor TFIIB. This model may also be true for Fos–Jun-mediated stimulation. However, an important distinction between CREB and c-Fos is that the latter binds to the region of CBP (CBP2) which can also contact TFIIB (Kwok *et al.*, 1994). Considering that the CBP2 domain is relatively large (250 residues), it is perfectly possible that the TFIIB binding residues are distinct from those required to bind c-Fos and that CBP2 may be capable of contacting c-Fos and TFIIB simultaneously. Experiments to address this issue are in progress.

The c-Fos protein has a number of independently acting activation domains which are composed of co-operating activation modules, FAMs. High affinity binding of CBP to c-Fos requires the combination of FAM4 and FAM5, which activate transcription synergistically when linked together next to the GAL4 DNA binding domain. We have therefore scrutinized the FAM4 and FAM5 sequences for the presence of similarity to the N-terminus of E1A, which also contains a binding site for CBP. We find FAM4 and FAM5 possess a similar sequence (SVPDMD and TYPEAE respectively) which bears a resemblance to a sequence in E1A CR1 (IFPDSV), which when deleted abolishes p300 binding (Wong and Ziff, 1994). Further experiments will establish the significance, if any, of this similarity. It is worthwhile noting that the FAM4/FAM5 combination is also required to bind the TBP protein (Metz *et al.*, 1994). It will be interesting to examine whether TBP and CBP can contact c-Fos simultaneously and whether the binding to these two proteins leads to co-operation in transcriptional activation.

The ability of E1A to repress the AP1 activity of the collagenase promoter is well documented (Frisch *et al.*, 1990; Offringa *et al.*, 1990; Van Dam *et al.*, 1990). Here we provide evidence for the mechanism of this repression. We show that E1A can repress the activation functions of a protein, c-Fos, which stimulates transcription from the collagenase AP1 site. Repression by E1A results from the sequestration of a protein, CBP, required for c-Fos-mediated activation. Since E1A and c-Fos contact a similar domain of CBP (CBP2; Figure 3), it is possible that E1A functions by masking the c-Fos binding region of CBP. Thus the repressive effect of E1A on the AP1 site is likely to result from the removal of the CBP adaptor from the Fos–Jun complex.

The activity of c-Fos and c-Jun has been implicated in both the proliferative and differentiating pathways. Thus the AP1 site is likely to be present within the promoter of genes involved in proliferation as well as differentiation. Adenovirus requires the cellular proliferative pathways to be functional, since it needs them for its own replication. Consequently, it can be argued that E1A targets for repression AP1-containing promoters which are not necessary for proliferation. Indeed, these promoters may regulate gene functions that are antagonistic to proliferation, since they dictate differentiation-specific events. The activity of these 'non-proliferation'-specific AP1-containing pro-

moters may be dependent on the CBP adaptor protein. If this is the case, E1A may be able to sequester CBP and thus co-ordinately switch off a number of genes whose activity is deleterious to the life cycle of the adenovirus.

E1A-mediated immortalization of cells in culture requires binding of CBP. The data reported here suggest that these immortalizing functions are related to the ability of E1A to sequester CBP from c-Fos. Thus by silencing the activity of certain AP1-containing promoters, E1A may be able to specifically steer the cell into proliferative pathways.

Materials and methods

Cell cultures, transfections and CAT assays

U2OS human osteosarcoma cells were maintained in DMEM supplemented with 10% fetal calf serum and grown at 37°C (5% CO_2). F9 cells were grown and maintained as reported previously (Bannister *et al.*, 1994). Both cell lines were transfected using the calcium phosphate co-precipitation technique. Extracts from transfected cells were then used for CAT assays. The CAT assays were quantitated with a PhosphorImager. For transfections involving analysis of E1A-mediated repression cells were seeded to ~30% confluency.

In vivo expression plasmids

For the Gal4 fusion experiments various domains of c-Fos or other transcription factors were cloned into pHKG (Sutherland *et al.*, 1992) using PCR or engineered restriction sites. pHKG has the GAL4(1–147) DBD under the control of a SV40 promoter. The reporter construct for Gal4 fusions was G_5E1b.CAT. For activation of the collagenase promoter construct (containing bases –73 to +63 of the collagenase gene fused next to the CAT DNA coding sequence; a gift from H.J.Rahmsdorf) the c-Fos and c-Jun cDNAs were expressed from the pHK vector (described above), which lacks the Gal4 DBD. To express transcription factor domains which do not contain a nuclear localization sequence (NLS) DNA was cloned into pHKnt (which contains the nucleoplasmin NLS) using PCR or engineered restriction sites. CBP was expressed under the control of a RSV promoter and was a gift from R.Goodman. All Ad5 E1A12S constructs were expressed from plasmid pBJ9Ω, which is a RSV-driven expression vector (a gift from H.Land). The E1A12S and the ΔCR1 and ΔCR2 mutants were gifts from H.Land. The E1A-mut CBP (a deletion of amino acids 64–68) and E1A-mut RB (conversion of amino acids 38–44 to alanine) constructs were made by PCR and checked by DNA sequence analysis. For the E1A repression experiments Gal4–Fos was subcloned into plasmid pBJ9Ω using standard cloning procedures.

In vitro translation of proteins

c-Fos cDNA was expressed from pING14, an SP6 transcription vector (S.Inglis, unpublished results). The Elk-1 *in vitro* expression plasmid was a gift from P.Shaw. All *in vitro* translation products were generated using the TNT coupled transcription–translation system as outlined by the manufacturer (Promega). For PKA labelling of the CREB translation product 10 μl of the final reticulocyte lysate containing ^{35}S-labelled CREB was made 10 μM with respect to ATP, 20 U PKA (Sigma) were added and the reaction incubated at 30°C for 15 min.

GST fusion proteins and pull-down assay

Various domains of c-Fos or CBP were cloned into the relevant pGex vector (Pharmacia) using PCR or engineered restriction sites. Recombinant proteins were expressed in and purified from *Escherichia coli* as reported previously (Bannister *et al.*, 1991). Pull-down assays were performed as described previously (Hagemeier *et al.*, 1993).

^{32}P-Labelling of GST fusion proteins

Proteins to be ^{32}P-labelled were cloned into pGex-2TK (kindly provided by W.Kaelin). This plasmid expresses a fusion protein which contains a PKA phosphorylation site directly adjacent to the C-terminus of the thrombin cleavage site. The proteins were labelled according to Kaelin *et al.* (1992).

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