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E1A directly binds and regulates the P/CAF acetyltransferase

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The P/CAF protein has intrinsic histone acetyltransferase (HAT) activity and is capable of binding the transcriptional co-activator CBP. Here we show that P/CAF can regulate transcription and that this function is independent of its binding to CBP. The HAT domain of P/CAF has transcriptional activation potential in yeast. In mammalian cells P/CAF can stimulate transcription of the RSV promoter, using the activity of its HAT domain. We show that the adenovirus protein E1A targets P/CAF and sequesters its transcriptional activity. Binding of E1A to P/CAF is direct, independent of CBP and requires residues within E1A conserved region 1. We find that the P/CAF binding residues in E1A are within a motif shown to be essential for efficient disruption of myogenesis by E1A. The fact that E1A can directly bind and regulate the activity of P/CAF, independently of its regulation of CBP, highlights an important role for P/CAF in the process of cell differentiation.

Keywords: acetyltransferase/E1A/myogenesis/P/CAF/transcription

Introduction

In eukaryotes, transcription of mRNA-encoding class II genes involves the ordered recruitment of general factors and the RNA pol II holoenzyme into the basal transcription pre-initiation complex (Orphanides *et al*., 1996; Roeder, 1996). This assembly then gives rise to specific, but low-level gene transcription. This relatively inefficient process is stimulated by transcriptional activators which bind to specific DNA sequences outside of the core promoter (Tjian and Maniatis, 1994). Sequence-specific activators work in conjunction with other factors which do not contact DNA directly. These factors are termed co-activators and are recruited to promoters via their interaction with the DNA-bound transcription factors (Pugh and Tjian, 1990; Guarente, 1995).

CBP and p300 are distinct but functionally related co-activator proteins, involved in both proliferative and differentiating pathways, and which interact with numerous sequence-specific transcription factors including CREB (Chrivia *et al*., 1993), c-*Fos* (Bannister and Kouzarides, 1995), c-*Jun* (Bannister *et al*., 1995), c-*Myb* (Dai *et al*., 1996), Stat1 and 2 (Bhattacharya *et al*., 1996), MyoD (Eckner *et al*., 1996), NFκB p65 (Perkins *et al*., 1997), E2F1 (Trouche *et al*., 1996), p53 (Gu and Roeder, 1997) and nuclear hormone receptors (Kamei *et al*., 1996). In addition, CBP/p300 also complex with other co-activators such as ACTR (Chen *et al*., 1997) and SRC-1 (Smith *et al*., 1996; Yao *et al*., 1996), with the adenoviral transforming protein E1A (Eckner *et al*., 1994) and with the P/CAF protein (Yang *et al*., 1996).

The mechanism for the CBP/p300-mediated stimulation of transcription is not yet clear. The CBP/p300 proteins contain multiple activation domains and can contact the basal transcription factors TBP and TFIIB (Kwok *et al*., 1994; Yuan *et al*., 1996). Consequently, it was suggested that the CBP/p300 proteins increase transcription by bridging sequence-specific activators to the basal transcriptional machinery, thereby stabilizing the pre-initiation complex whilst adding to it additional activation domains (Kwok *et al*., 1994). However, the overall picture is more complex because of the recent observation that both CBP and p300 possess intrinsic nucleosomal histone acetyltransferase (HAT) activity (Bannister and Kouzarides, 1996; Ogryzko *et al*., 1996). Moreover, other co-activators which complex with CBP/p300, such as P/CAF (Yang *et al*., 1996), ACTR (Chen *et al*., 1997) and SRC-1 (Spencer *et al*., 1997), themselves contain intrinsic nucleosomal HAT activity.

In vivo the transcriptional machinery has to transcribe DNA which is tightly associated with histones in nucleosomal arrays (reviewed in Grunstein, 1997). Packaging DNA into this type of structure represses transcription. Stimulation (or more precisely, derepression) of transcription requires remodelling of the chromatin, and correlates with the acetylation of specific lysines within the N-termini of nucleosomal histones (reviewed by Loidl, 1994). This acetylation neutralizes the basic charge of the lysines and causes a repositioning of the histone N-termini whereupon new protein–protein contacts are initiated. The net result is a weakening of the nucleosomal and higher order protein–DNA structure. *In vivo* a strong correlation exists between the hyperacetylation of histone N-termini and active genes (Hebbes *et al*., 1988; Turner and O'Neill, 1995), and conversely, between hypoacetylation of histones and transcriptionally inactive genes (reviewed by Pazin and Kadonaga, 1997). Thus, it seems likely that co-activator proteins with intrinsic HAT activity would use this activity to stimulate transcription. Interestingly, the known co-activators capable of acetylating nucleosomal histones, CBP/p300, P/CAF, ATCR/SRC-1 and TAFII250, show varied substrate specificity (Bannister and Kouzarides, 1996; Mizzen *et al*., 1996; Yang *et al*., 1996; Chen *et al*., 1997; Spencer *et al*., 1997) suggesting a mechanism to fine-tune transcriptional responses.

E1A protein is encoded by the small DNA tumour

virus, adenovirus. By interaction with cellular proteins E1A pushes cells through their cell cycle, thereby facilitating virus reproduction (for reviews see Moran, 1993; Jones, 1995). A consequence of prolonged E1A expression within mammalian cells is cellular transformation. For this effect, E1A requires two distinct domains within its N-terminus. One domain binds members of the pocket-containing protein family, the most characterized of which is the Rb tumour suppressor protein (Moran, 1993; Jones, 1995). The second domain binds members of the CBP/p300 family of co-activators (Moran, 1993; Jones, 1995). Via these interactions, E1A relieves growth suppression and inhibits cellular differentiation, for example myogenesis.

CBP/p300-mediated transcriptional activity can be abrogated by E1A (Arany *et al.*, 1995; Bannister and Kouzarides, 1995; Lundblad *et al.*, 1995). The mechanism by which this repression occurs may involve sequestration of CBP/p300 away from the promoter. Alternatively, E1A may displace a CBP/p300-associated factor which is required for efficient activation of transcription. Since E1A displaces the CBP/p300-associated factor P/CAF from the co-activator complex (Yang *et al.*, 1996), it is possible that P/CAF displacement results in a loss of CBP/p300-dependent transcription.

P/CAF was originally identified as a CBP/p300-binding protein by virtue of its sequence similarity to a yeast HAT, namely yGCN5 (Yang *et al.*, 1996). The proteins show considerable sequence conservation, especially in two regions; one of these regions is responsible for full HAT activity in yGCN5 and the second is required for yGCN5 to bind the yADA2 co-factor (Yang *et al.*, 1996; Candau *et al.*, 1997; Wang *et al.*, 1997). Indeed, a human homologue of yADA2 has been identified (hADA2; Candau *et al.*, 1996) which may be important for P/CAF function. *In vitro* yGCN5 efficiently acetylates free histones in solution, but does not recognize nucleosomal histones as substrate (Brownell *et al.*, 1996; Yang *et al.*, 1996). However, *in vivo*, yGCN5 is found in a large multisubunit complex containing yADA2 and yADA3 (Candau and Berger, 1996; Grant *et al.*, 1997). Within this complex, yGCN5 is the HAT enzymatic component and now acetylates nucleosomal histones (Grant *et al.*, 1997). In contrast, P/CAF has an intrinsic ability to acetylate nucleosomal histones *in vitro*, even though it has retained the ADA2 binding site (Yang *et al.*, 1996). It is possible that *in vivo*, hADA2 may alter the specificity of P/CAF's nucleosomal HAT activity.

Since P/CAF itself has intrinsic nucleosomal HAT activity, we asked whether P/CAF is able to stimulate transcription. Here we present evidence that P/CAF is indeed a transcriptional activator protein. Its ability to stimulate transcription is independent of CBP/p300 but is dependent on P/CAF's intrinsic HAT activity. The adenoviral E1A oncoprotein binds directly to P/CAF and abrogates P/CAF-induced transcription. These results indicate that E1A can bind and regulate independently the activity of two different HATs, namely CBP and P/CAF. Moreover, we find that E1A mutated in a previously characterized motif (Sandmöller *et al.*, 1996), which is essential for E1A inhibition of myogenesis, has reduced binding to P/CAF. Since P/CAF itself is essential for myogenesis (Puri *et al.*, 1998) our results are consistent with a model in which E1A, at least in part, represses myogenesis by inactivating the functions of P/CAF.

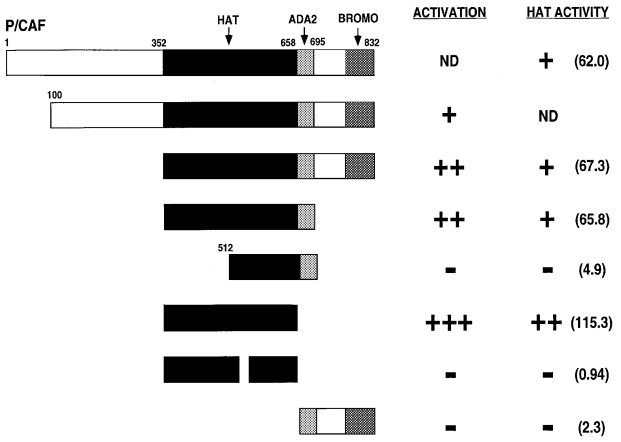

Fig. 1. The P/CAF HAT domain has activation potential in yeast. Various P/CAF domains were fused to the LexA DNA-binding domain and transformed into yeast. P/CAF-HATΔ contains a 20 amino acid deletion (Δ528–547). The reporter contains LexA DNA-binding sites upstream of the bacterial *lacZ* gene. β-galactosidase activity was determined by standard techniques. Activity is indicated by degree with '+' signs, with '+++' indicating highest activity (deep blue colour in <15 min in a filter *lacZ* assay) and '–' indicating background activity (remaining white after 5 h). The LexA–P/CAF fusions were all expressed to equivalent levels, as determined by Western blotting (data not shown). The same P/CAF domains were expressed as GST fusions and their intrinsic HAT activity measured by liquid HAT assays. An equivalent amount of each GST–P/CAF fusion was assayed (~500 ng) as determined by Coomassie Blue staining. HAT activity for each fusion was normalized relative to the activity for GST alone (expressed as fold over GST).

Results

P/CAF stimulates transcription independently of CBP

Many different transcription factors recruit CBP to promoters, resulting in enhanced transcription. The currently held model is that these transcription factors may also recruit P/CAF to promoters by virtue of its interaction with CBP. We wanted to establish whether P/CAF had activator functions independent of CBP. Therefore, we sought to identify the region of P/CAF harbouring HAT enzyme activity and then to ask whether the activity is sufficient to activate transcription, independently of CBP. To this end, we expressed various domains of P/CAF as GST fusions and tested whether they could acetylate free histones in solution assays. Figure 1 clearly shows that P/CAF 352–658 has full enzyme activity. GST fusions which do not include this region have no HAT activity. Thus, we refer to P/CAF 352–658 as the P/CAF HAT domain. These data are in very close agreement with the recently reported minimum HAT domain of yGCN5, which shares significant homology with P/CAF (Candau *et al.*, 1997). An in-frame deletion of amino acids 528–547 within the HAT domain completely abrogates HAT activity (Figure 1).

Given the strong correlation that exists between the acetylation state of chromatin and transcriptionally active DNA, we asked whether the HAT activity residing within P/CAF is sufficient to stimulate transcription. To ensure that we were monitoring CBP-independent HAT activity,

we asked whether P/CAF could stimulate transcription in yeast where there is no CBP. Figure 1 shows that P/CAF can stimulate transcription when fused to the LexA DNA binding domain, and that the domain carrying HAT activity (352–658) is sufficient for this function. Moreover, a functional HAT activity is essential to activate transcription since the deletion in the HAT domain, which abrogates HAT activity, also completely abrogates its ability to activate transcription.

Having established that P/CAF has a CBP-independent transcriptional activation potential, we sought to establish whether P/CAF could activate transcription of mammalian promoters. Previous CBP–P/CAF binding studies (Yang *et al.*, 1996) had strongly implicated the N-terminus of P/CAF as the region which binds CBP. To ensure that our assays would monitor CBP-independent P/CAF activity, we generated a P/CAF mutant (P/CAF-CT) lacking the N-terminal CBP-binding residues. Figure 2B shows that deletion of the P/CAF N-terminus does indeed abrogate the binding of CBP to P/CAF.

Since P/CAF is likely to be recruited to the promoter by as yet unidentified DNA-bound transcription factors, we asked whether P/CAF could stimulate complex viral promoters. Figure 2C shows that P/CAF can stimulate the RSV-LTR in transient transfection assays in MRC-5 cells. Other viral promoters, such as the HIV-LTR, are also activated by P/CAF (data not shown). This activation capacity is independent of CBP, since P/CAF-CT is sufficient for this activity. Indeed, a minimal P/CAF HAT domain (amino acids 352–652) is sufficient for this effect, albeit with a slightly reduced efficiency (~60% of full-length activity). The observed 3- to 5-fold activation by P/CAF is very similar to the levels of induction observed with CBP on a variety of promoters (Eckner, 1996 and references therein). The HAT activity of P/CAF is essential for the stimulation of the RSV promoter since a deletion in the HAT domain that destroys HAT activity (Figure 1) completely abrogates its ability to stimulate transcription (Figure 2C). These results establish that P/CAF has the ability to stimulate transcription via its HAT domain and that this stimulation is independent of its ability to bind CBP.

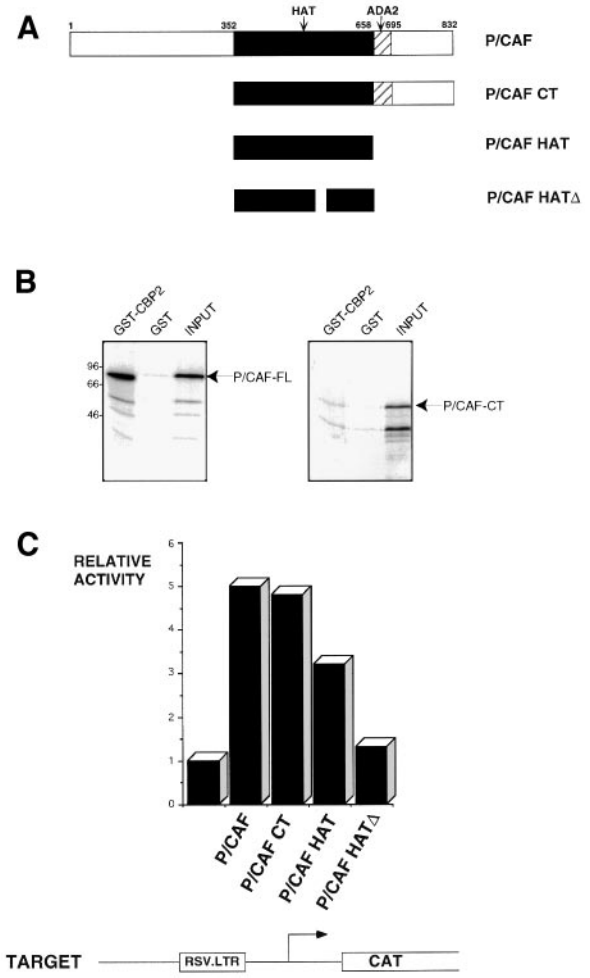

Fig. 2. P/CAF stimulates transcription from the RSV-CAT reporter, independently of CBP. (**A**) A schematic representation of the P/CAF constructs used, showing the position of the HAT domain, the ADA2 binding domain (based on homology to GCN5) and the 20 amino acid deletion (Δ528–547) in P/CAF-HATΔ. (**B**) A P/CAF construct deleted of its N-terminus no longer binds CBP *in vitro*. Either GST or GST–CBP2 was incubated with *in vitro* translated, ^{35}S-radiolabelled P/CAF-FL (left panel) or P/CAF-CT (right panel) and subjected to GST pull-down. Lane 1 in each case shows 25% of the P/CAF input. (**C**) MRC5 cells were transfected with 2 μg of the RSV-CAT reporter and 2 μg of either P/CAF, P/CAF-CT, P/CAF-HAT, P/CAF-HATΔ or equivalent empty vector. Whole-cell extracts were used in CAT assays and the results quantified on a phosphoimager. The activity derived from the RSV-CAT reporter plus empty vector was normalized to 1.0 and the other activities expressed relative to this. All the P/CAF proteins were expressed to similar levels, as determined by Western blotting (data not shown). The data shown is an average of at least three independent transfections.

E1A directly contacts P/CAF

The viral E1A protein and P/CAF bind to overlapping sites within CBP. Consequently, E1A can displace P/CAF from CBP (Yang *et al.*, 1996). This displacement has been proposed to be the mechanism behind the antagonistic effect of P/CAF and E1A on cell-cycle progression (Yang *et al.*, 1996). However, given that P/CAF has CBP-independent co-activator function, we considered the possibility that E1A may directly target and inactivate P/CAF function. We first asked if E1A could contact P/CAF directly and independently of CBP. Various E1A constructs were expressed as GST fusion proteins and we determined whether they could bind *in vitro* translated and radiolabelled P/CAF protein. Figure 3A shows that P/CAF binds efficiently to GST–E1A but not to GST alone. The E1A N-terminus (amino acids 1–90) encompassing CR1 (amino acids 40–80) is sufficient for this interaction. P/CAF does not bind to E1A CR2 or CR3. An E1A N-terminal mutant (E1A 1–90 CBP MUT, Figure 3A, lane 6), which no longer binds to CBP (Wong and Ziff, 1994; Bannister and Kouzarides, 1995; Trouche and Kouzarides, 1996) still binds P/CAF efficiently. This indicates that the *in vitro* translated P/CAF is not binding to the GST–E1A via CBP present in the lysate. Indeed, the contact between P/CAF and E1A is direct rather than via any other intermediary proteins present in the lysate, since GST–P/CAF binds efficiently to a bacterially expressed and radiolabelled E1A 1–90 domain (Figure 3B). This is further supported by the observation that E1A interacts with P/CAF in yeast (in a two-hybrid interaction assay) where there is no CBP (Figure 3C).

The interaction between P/CAF and E1A can also be observed *in vivo* by employing a co-immunoprecipitation

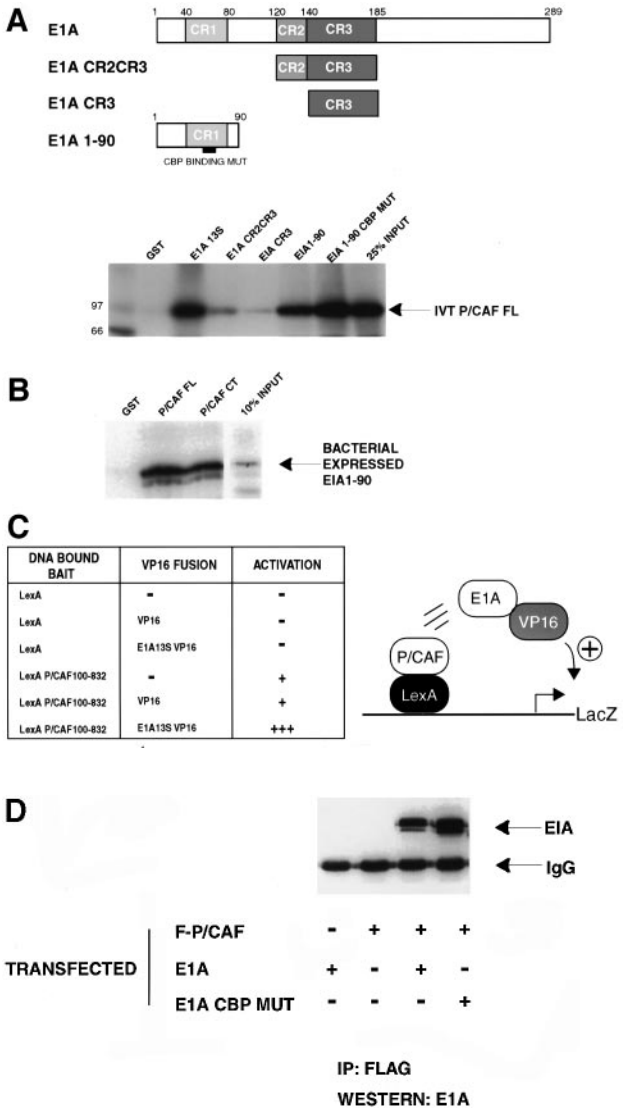

Fig. 3. P/CAF interacts with E1A *in vitro* and *in vivo.* (**A**) GST–E1A binds P/CAF *in vitro.* A schematic representation of E1A 13S showing the position of conserved regions 1, 2 and 3 (CR1, 2 and 3) and the mutation in the CBP binding site (CBP MUT). The various GST fusions as indicated were incubated with *in vitro* translated, ^{35}S-radiolabelled P/CAF and subjected to GST pull-down. Lane 8 shows 25% of the P/CAF input. (**B**) E1A1-90 binds P/CAF directly. Bacterially expressed GST, GST–P/CAF or GST–P/CAF-CT were incubated with bacterially expressed, ^{32}P-labelled E1A1-90 protein and subjected to GST pull-down. (**C**) P/CAF interacts with E1A in a yeast two-hybrid assay. Either the LexA–DBD alone or a LexA–P/CAF100-832 fusion were co-transformed into yeast cells with E1A 13S fused to the VP16 activation domain, or pVP16 alone. β-galactosidase activity was determined by standard techniques and quantified relative to the activity of LexA-P/CAF100-832 alone ('–' means no detectable β-gal activity, '+' represents the activity of LexA–P/CAF100-832 (see Figure 1) and '+++' represents a 300% increase in activity). Expression of LexA–P/CAF100-832 and VP16–E1A13S, in the appropriate yeast strains, was confirmed by Western blotting (data not shown). (**D**) E1A co-immunoprecipitates with P/CAF from extracts of transfected U2OS cells. Cells were transfected with 10 μg each of pCX–P/CAF (expresses Flag-tagged P/CAF) and pBJ9Ω–E1A12S (or the E1A12S–CBP binding mutant) as indicated. Whole-cell extracts were precipitated with the M2 (αFlag) antibody and the presence of E1A in the immunoprecipitates was visualized by Western blot analysis with the M73 (αE1A) antibody. Expression of the appropriate proteins in each extract was confirmed by Western blotting of the cell lysates with the M2 (αFlag) and M73 (αE1A) antibodies (data not shown).

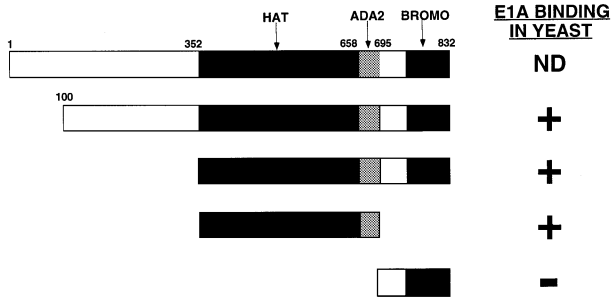

Fig. 4. The P/CAF HAT domain binds E1A in a yeast two-hybrid interaction. Various LexA–P/CAF fusions were co-transformed into yeast cells with either a VP16–E1A13S fusion or VP16 alone. β-galactosidase activity was determined by standard techniques. The intrinsic activity (with VP16 alone) was normalized for each construct and the activity with VP16–E1A13S expressed relative to this ('–' means no increase in activity and '+' an ~300% increase in activity). The LexA–P/CAF fusions were all expressed to equivalent levels, as determined by Western blotting (data not shown). Expression of VP16–E1A13S, in the appropriate yeast strains, was confirmed by Western blotting (data not shown).

approach. E1A 12S or an E1A12S CBP-binding mutant (E1A CBP MUT) and Flag-tagged P/CAF were transfected together into U2OS cells. An anti-Flag antibody was used to immunoprecipitate Flag-tagged P/CAF from whole-cell extracts and then anti-E1A antibodies were used to detect E1A (or the E1A CBP MUT) following Western blotting of the immunoprecipitate (Figure 3D, lanes 3 and 4). Both E1A and E1A CBP MUT were efficiently co-immunoprecipitated by the anti-Flag antibodies indicating that (i) P/CAF and E1A form a complex *in vivo* and (ii) CBP does not mediate this interaction. The interaction was not observed when either P/CAF or E1A were transfected alone (Figure 3D, lanes 1 and 2). The P/CAF–E1A interaction is also seen if we immunoprecipitate E1A and Western blot for P/CAF (data not shown).

A yeast two-hybrid assay was employed to determine which region of P/CAF contacted E1A. Various deletions of P/CAF were expressed in yeast as LexA DBD fusions and we asked whether they were capable of interacting with an E1A.VP16 fusion. Figure 4 shows that the N-terminus of P/CAF (1–352) or the C-terminal residues containing the bromodomain (695–832) are not required for E1A binding. A central P/CAF domain, containing the HAT and ADA2 sites, is sufficient to bind E1A. The same results were obtained in *in vitro* binding assays employing GST–P/CAF and *in vitro* translated and radiolabelled E1A (data not shown).

Binding of E1A to P/CAF does not affect P/CAF HAT activity

Given that E1A binds the HAT domain of P/CAF, we next sought to determine whether E1A binding affected the intrinsic P/CAF HAT activity. GST–P/CAF was incubated with a 10-fold molar excess of either GST–E1A or GST alone (Figure 5A) under conditions which allow efficient E1A binding to P/CAF. In a liquid HAT assay, the activity of GST–P/CAF was not affected by the presence of excess E1A (Figure 5A).

By employing an IP-HAT assay approach, we also observed no effect of E1A on P/CAF HAT activity derived from mammalian cell-expressed P/CAF. Flag-tagged P/CAF, in the presence or absence of E1A, was transiently

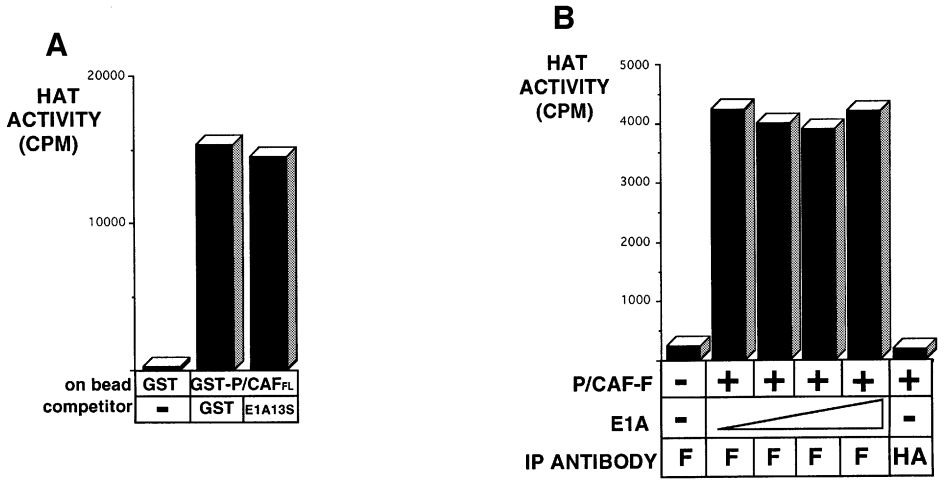

Fig. 5. Binding of E1A to P/CAF does not affect P/CAF HAT activity *in vitro* or *in vivo.* (**A**) Approximately 500 ng of GST–P/CAF was pre-incubated with a 10-fold molar excess of either GST or GST–E1A13S. The HAT activity of the GST–P/CAF or a GST control was then measured in a liquid HAT assay. (**B**) U2OS cells were transfected with 10 μg of pCX–P/CAF (expresses Flag-tagged P/CAF) and increasing amounts of pBJ9Ω–E1A12S (0, 5, 10 and 20 μg). Whole-cell extracts were precipitated with M2 αFlag antibody (F) and the immune complexes tested for their ability to acetylate free histones. The HAT activity associated with the M2 antibody in the absence of transfected P/CAF is shown in column 1 and the activity associated with a non-specific antibody (HA) is shown in column 6.

transfected into U2OS cells. Exogenous P/CAF was then immunoprecipitated with an anti-Flag antibody and the HAT activity present within the immunopellet was determined using a liquid HAT assay. Significant activity was pelleted with the Flag antibody only when P/CAF was transfected (Figure 5B). A control antibody (anti-HA) did not immunoprecipitate HAT activity. Co-transfection of E1A with P/CAF had no effect on the level of P/CAF HAT activity, even at the highest concentration of E1A employed (Figure 5B).

E1A represses P/CAF-mediated activation

Although we observed no effect of E1A on P/CAF's HAT activity, we wished to know whether E1A could affect the functional activity of P/CAF. More specifically, we asked whether E1A affected P/CAF's ability to stimulate the RSV promoter. Figure 6A clearly demonstrates that E1A efficiently abrogates the ability of P/CAF to stimulate this promoter. Moreover, the CR1 domain of E1A is essential for E1A's repression of P/CAF, since deletion of this region severely abrogates E1A's ability to suppress the P/CAF dependent activation (Figure 6A). An E1A mutant that no longer binds CBP represses P/CAF-induced activation to the same extent as wild-type E1A 12S (data not shown) indicating that under these conditions E1A does not repress P/CAF via an interaction with CBP.

Since the HAT domain of P/CAF is sufficient to activate the RSV promoter (Figure 2) we next sought to determine whether E1A is able to abrogate this activity. Figure 6B shows that E1A efficiently abrogates the ability of the P/CAF HAT domain to activate transcription from the RSV promoter, indicating that the P/CAF HAT domain contains determinants which allows targeting by E1A. This is in perfect agreement with our P/CAF–E1A interaction assays (Figures 3C and D, and 4).

It has been known for some time that both the CBP/p300 and Rb families of proteins are involved in myogenesis. Whilst the work presented here was in progress, it was reported that P/CAF activity is also essential for muscle-specific enhancer function and muscle differentiation (Puri *et al.*, 1998). The E1A protein can block myogenesis and Sandmöller *et al.* (1996) have identified a motif within the CR1 region of E1A (amino acids 55–60) which is required for E1A's ability to block myogenesis. E1A proteins containing mutations within this motif still interact with CBP/p300 and Rb (Sandmöller *et al.*, 1996). Given P/CAF binds the CR1 domain of E1A (Figure 3), we sought to establish whether the motif (amino acids 55–60) within E1A CR1 which is required for E1A's anti-myogenic effect, is also required for binding P/CAF. A co-immunoprecipitation approach was used to test for P/CAF binding to an E1A protein mutated in this region (E1A E55 mutant). Wild-type E1A 12S, E1A E55 mutant, or a control E1A 12S Rb binding mutant (E1A RB MUT) were transfected along with Flag-tagged P/CAF into U2OS cells. An anti-E1A antibody was used to immunoprecipitate the E1A proteins from whole-cell extracts and then an anti-Flag antibody was used to detect P/CAF following Western blotting of the immunoprecipitate. Figure 7B shows that E1A 12S and E1A 12S RB MUT efficiently bind to P/CAF but that the E1A E55 mutant is severely compromised in P/CAF binding. Densitometric analysis of this data showed that P/CAF binding was reduced by >75% (data not shown). In all cases, the levels of P/CAF and E1A proteins were equivalent (Figure 7; data not shown). These results indicate that residues 55–60 within CR1 are involved in binding P/CAF. These residues are not involved in binding CBP/p300 or Rb (Sandmoller *et al.*, 1996; data not shown). Thus, the disruption of P/CAF binding correlates with the ability of E1A to suppress myogenesis.

Discussion

Here we show that P/CAF has the ability to stimulate gene transcription in a CBP-independent manner and that this activity requires P/CAF's intrinsic HAT activity. The requirement for P/CAF HAT activity to stimulate transcription is in excellent agreement with similar data obtained using a yeast homologue of P/CAF, namely

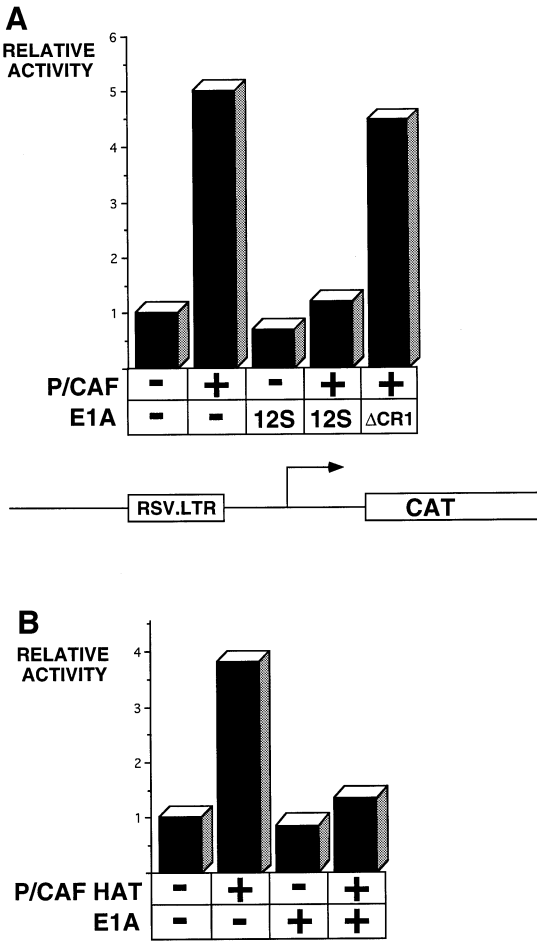

Fig. 6. E1A represses P/CAF-mediated activation. (**A**) MRC5 cells were transfected with 2 μg of the RSV-CAT reporter, 2 μg pCX–P/CAF or equivalent empty vector and 2 μg of either pBJ9Ω–E1A12S or the empty pBJ9Ω vector. Whole-cell extracts were used in CAT assays and the results quantified on a phosphoimager. The activity derived from the RSV-CAT reporter plus empty vectors was normalized to 1.0 and the other activities expressed relative to this. The E1A12S and E1A12S–ΔCR1 proteins were expressed to similar levels, as determined by Western blotting (data not shown). The data shown are an average of at least three independent transfections. (**B**) The experiment was performed essentially as in (A), except 2 μg of the P/CAF–HAT construct (described in Figure 2), which does not bind CBP, was used instead of full-length P/CAF.

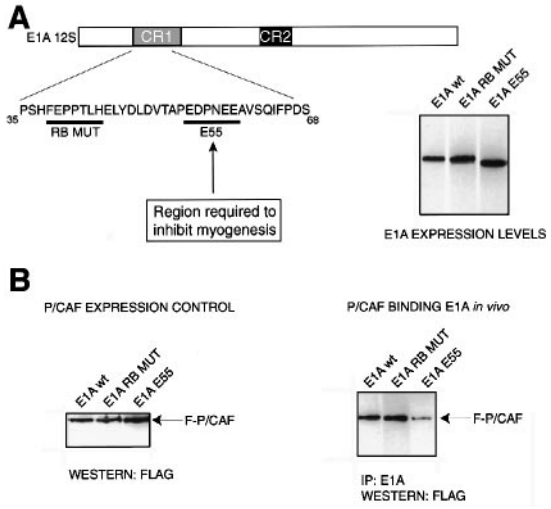

Fig. 7. P/CAF binds to E1A residues that are required for E1A to block myogenesis. (**A**) A schematic representation of E1A12S showing the positions of CR1 and CR2, the RB binding site mutation (RB MUT) and the region mutated within E1A E55. Equal expression of E1A wild type, E1A RB MUT and E1A E55, after transfection of the relevant expression plasmids into U2OS cells, was confirmed by Western blotting with the M73 (αE1A) antibody. E1A E55 binds Rb and CBP at levels equivalent to E1A wild type (Sandmöller *et al.*, 1996; data not shown). (**B**) U2OS cells were transfected with 10 μg each of pCX–P/CAF and pBJ9Ω–E1A12S (or E1A RB MUT or E1A E55) as indicated. Whole-cell extracts were precipitated with 2 μg of the M73 (αE1A) antibody and the presence of P/CAF in the immunoprecipitates was visualized by Western blot analysis with the M2 (αFLAG) antibody (right panel). Prior to immunoprecipitation, 2% of the lysates were removed and Western blotted with the M2 (αFLAG) antibody to confirm that P/CAF expression in the three lysates was equivalent (left panel). The three E1A proteins were expressed to equivalent levels (Figure 7A; data not shown). The experiment was repeated and the same decrease in P/CAF binding to E55 observed. The panels display non-contiguous lanes from the same Western blot of a single experiment.

yGCN5 (Kuo *et al.*, 1998; Wang *et al.*, 1998). Our results establish P/CAF as a co-activator protein, whose activity may be required for the stimulation of certain promoters.

The fact that P/CAF and CBP are likely to act independently as co-activators raises the question of promoter selectivity. It is possible that these two HATs are recruited to different promoters by distinct sets of promoter-targeting transcription factors. Indeed, our preliminary analysis of cellular promoter targets for P/CAF indicates that P/CAF is unlikely to be a 'promiscuous' co-activator like CBP. Several of the cellular promoters stimulated by CBP are not affected by P/CAF (data not shown).

The need for two independent HAT activities may result from the fact that CBP and P/CAF preferentially acetylate distinct histones *in vitro* (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996; Yang *et al.*, 1996). This may lead to differential 'activated' states necessary for some promoters but not others. However, the fact that a proportion of P/CAF protein is found complexed with CBP (Yang *et al.*, 1996) suggests that some promoters may require the combined stimulatory effect of both HATs. This combinatorial HAT activity may lead to synergistic activation of transcription.

Another complexity to the target specificity of these HATs is raised by the recent observation that non-histone proteins are substrates for HAT activity. The fact that transcription factors such as p53 (Gu and Roeder, 1997) and basal factors such as TFIIE (Imhof *et al.*, 1997) are acetylated by CBP and P/CAF, suggests that HATs may stimulate transcription by regulating the activity of promoter-bound targets. If such mechanisms operate, then it is conceivable that P/CAF may have a different and more limited target specificity than CBP, leading to the activation of a specific subset of promoters.

Adenovirus requires cellular proliferation for its own replication. Consequently, one of its proteins, the transforming protein E1A, has evolved to steer cells through the cell cycle. E1A uses two distinct but essential domains to transform mammalian cells (Moran, 1993; Jones, 1995). One domain directs binding to the RB-pocket protein family resulting in loss of growth suppression, whilst the second domain directs binding to the p300/CBP family of coactivators, leading to a loss of p300/CBP-induced differentiation, for example myogenesis. Here we have shown that E1A can also efficiently block P/CAF-mediated

transcription independently of CBP. Exactly how E1A blocks P/CAF transcriptional activity is not clear. Since E1A binds to the P/CAF HAT domain, the most simple model would involve E1A inactivating the HAT enzymatic activity. However, this does not appear to be the case as the P/CAF–E1A complex retains full HAT activity (Figure 5). This is consistent with the observation that the CBP–E1A complex also retains full HAT activity (Bannister and Kouzarides, 1996; data not shown) even though E1A represses CBP's ability to activate transcription. One model would be that E1A masks the binding or recognition for a non-histone substrate of P/CAF HAT activity. An alternative, but not mutually exclusive explanation is that E1A sequesters P/CAF away from the promoter. In this scenario, the E1A binding site in P/CAF may overlap with the binding site for transcription factors that target P/CAF to the promoter. This model of promoter sequestration has been proposed for the ability of E1A to inactivate CBP function. For CBP this model is bolstered by the fact that the E1A binding site is also the binding site for a variety of transcription factors which recruit CBP to the promoter (reviewed in Eckner, 1996). The E1A binding site in CBP is situated proximally to the HAT domain, a situation reflected in P/CAF (Figure 4). Thus, the region overlapping the HAT domain of P/CAF is likely to harbour the binding site for the transcription factors which recruit P/CAF to the promoter. Yeast two-hybrid screens are currently being used to identify such promoter targeting factors.

Many factors influence the process of muscle differentiation, including the Rb family of proteins, CBP/p300 and the transcription factor MyoD. E1A actively represses muscle differentiation, at least in part, by targeting Rb and CBP/p300. Recently, P/CAF was also shown to be essential for stimulating the myogenic pathway (Puri *et al.*, 1998). E1A can repress P/CAF-mediated muscle-specific enhancer activation and myogenic differentiation (Puri *et al.*, 1998). Mutants of E1A have already been characterized which fail to block myogenesis efficiently, even though they still retained the ability to contact CBP/p300 and Rb (Sandmöller *et al.*, 1996). Furthermore, a region within E1A (amino acids 40–61) which does not bind CBP/p300 or Rb is necessary and sufficient for the repression of muscle-specific enhancers (Sandmöller *et al.*, 1996). In particular, a six amino acid motif (amino acids 55–60) is necessary for this effect. Here we show that mutagenesis of E1A residues 55–60 disrupts the binding to P/CAF. The fact that this mutant does not completely abolish binding to P/CAF may be an indication that a second P/CAF binding site exists in E1A CR1. The incomplete loss of binding is also consistent with the biological data which show that mutating amino acids 55–60 in E1A CR1 is only partially effective in disrupting the myogenic blockage (Sandmoller *et al*., 1996). Taken together these data strongly suggest that the E1A E55 mutant is unable to override the process of muscle differentiation because it has lost the capacity to associate stably with the P/CAF protein. Thus P/CAF must have an important role to play in differentiation since a viral oncoprotein, E1A, has evolved to contact and regulate its activity directly.

Materials and methods

HAT and IP-HAT assays

Liquid HAT and IP-HAT assays were performed essentially as described previously (Bannister and Kouzarides, 1996).

Cell culture, transfections and CAT assays

U2OS cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and grown at 37°C, 5% CO_2. MRC5 cells (ECACC No. 84101801) were maintained in minimum essential media with non-essential amino acids and 10% FCS and grown at 37°C, 5% CO_2. Cells were transfected by the calcium phosphate co-precipitation method and CAT assays performed according to standard methods.

In vivo expression plasmids

pCX–P/CAF has been described previously (Yang *et al.*, 1996). P/CAF-CT and P/CAF-HAT were cloned from pCX–P/CAF, by PCR, into the pcDNA3 vector (Invitrogen). The P/CAF-HATΔ mutant (deletion of amino acids 528–547) was made from P/CAF-HAT using the Quick Mutagenesis Kit (Stratagene) and checked by DNA sequencing. Both P/CAF-HAT and P/CAF-HATΔ have an N-terminal Gal4 epitope. All Ad5 E1A12S constructs were expressed from plasmid pBJ9Ω, an RSV-driven expression vector (gift from H.Land). The Ad5 E1A12S CBP and Rb binding mutants have been described previously (Bannister and Kouzarides, 1995; Trouche and Kouzarides, 1996; Trouche *et al.*, 1996). The CBP mutant contains a deletion of amino acids 64–68 and has been shown to be defective in binding CBP *in vivo* (Wong and Ziff, 1994). The Rb mutant has amino acids 38–44 converted to alanine and has been shown to not bind to Rb *in vivo* (Trouche and Kouzarides, 1996). The E55 mutant was made from pBJ9ΩAd5 E1A12S using the Quick Mutagenesis Kit (Stratagene) and checked by DNA sequencing. The E55 mutant has amino acids 55–60 mutated to alanine. This six amino acid motif has been shown to be essential for E1A to block myogenesis efficiently and mutations in this region do not affect E1A binding to Rb or CBP/p300 (Sandmöller *et al.*, 1996). It was confirmed that the E1A E55 mutant bound to Rb and CBP at levels equivalent to wild type (data not shown).

GST fusion proteins

Various domains of P/CAF and Ad5 E1A were cloned into the pGex-2TK vector (provided by W.Kaelin) using PCR. pGex-4T2-P–CAF-FL was a gift from Pino Santarelli and Maurizia Caruso. pGex2TKP–CBP2 has been described previously (Bannister and Kouzarides, 1995). Recombinant proteins were expressed in and purified from *Escherichia coli* XA90 as reported previously (Bannister *et al.*, 1991).

In vitro translations and pull-down assays

In vitro translations and GST pull-downs were performed essentially as described previously (Hagemeier *et al.*, 1993). The buffer used for the pull-downs was a variation of Z′ (25mM HEPES pH 7.5, 12.5 mM $MgCl_2$, 20% glycerol, 0.1% NP-40, 250 mM KCl). For the direct interaction experiment, pGexE1A1-90 was radiolabelled with ^{32}P according to Kaelin *et al.* (1992) and cleaved from GST with thrombin (Sigma).

Immunoprecipitations and Western analysis

Co-immunoprecipitations and Western analysis were performed, essentially as described previously (Lavender *et al.*, 1997) but with several modifications. 15 cm dishes of U2OS cells were transfected with 10 μg of each expression vector and lysed in 1 ml of IPH lysis buffer (Bannister and Kouzarides, 1996) 36–48 h post-transfection. Immunoprecipitations were performed using standard procedures. Antibodies used were α-Flag (M2 affinity gel and M2 monoclonal antibody, Kodak), α-E1A (M73, Santa Cruz) and α-HA (12CA5, Boehringer Mannheim). For the immunoprecipitations in Figure 7, the washes were performed in IPH buffer with 500 mM salt.

Yeast plasmids, activation and two-hybrid assays

Various deletions of P/CAF were cloned, by PCR, into the LexA DNA binding domain vector pBTM116 (Vojtek *et al*., 1993). pVP16 E1A 13S and pVP16 E1A1-90 were cloned by PCR into the pVP16 vector (Vojtek *et al.*, 1993). The activation and two-hybrid assays were performed in yeast strain L40. Yeast transformation was carried out as described previously (Kaiser *et al.*, 1994) and β-galactosidase assays were performed using standard techniques.

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