The acetyltransferase activity of CBP stimulates transcription

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The CBP co-activator protein possesses an intrinsic acetyltransferase (AT) activity capable of acetylating nucleosomal histones, as well as other proteins such as the transcription factors TFIIE and TFIIIF. In addition, CBP associates with two other TSSs, P/CAF and SRC1. We set out to establish whether the intrinsic AT activity of CBP contributes to transcriptional activation. We show that a region of CBP, encompassing the previously defined histone AT (HAT) domain, can stimulate transcription when tethered to a promoter. The stimulatory effect of this activation domain shows some promoter preference and is dependent on AT activity. Analysis of 14 point mutations reveals a direct correlation between CBP's ability to acetylate histones *in vitro* and to activate transcription *in vivo*. We also find that the HAT domains of CBP and P/CAF share sequence similarity. Four conserved motifs are identified, three of which are analogous to motifs A, B and D, found in other *N*-acetyltransferases. The fourth motif, termed E, is unique to CBP and P/CAF. Mutagenesis shows that all four motifs in CBP contribute to its HAT activity *in vitro* and its ability to activate transcription *in vivo*. These results demonstrate that the AT activity of CBP is directly involved in stimulating gene transcription. The identification of specific HAT domain motifs, conserved between CBP and P/CAF, should facilitate the identification of other members of this AT family.

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Introduction

Acetylation of the N-terminal tails of histones has been known for many years to be a process correlating with transcriptional activation (Hebbes *et al.*, 1988; Loidl, 1994; Turner and O'Neill, 1995). Until recently the mechanisms underlying this stimulation remained obscure, but the recent identification of nuclear histone acetyltransferases (HATs) has begun to shed light on the process.

There are now four families of nuclear proteins which have been shown to possess HAT activity. These are GCN5 and P/CAF (Brownell *et al.*, 1996; Yang *et al.*, 1996), CBP and p300 (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996), SRC1 and ACTR (Chen *et al.*, 1997; Spencer *et al.*, 1997) and TAF_{II}250 (Mizzen *et al.*, 1996). All proteins have a direct role in transcriptional regulation. In yeast, GCN5 is a co-activator for GCN4 (Georgakopoulos and Thireas, 1992), CBP and p300 are co-activators for a variety of different transcription factors (Bannister and Kouzarides, 1995; Bannister *et al.*, 1995; Chakravarti *et al.*, 1996; Janknecht and Nordheim, 1996; Trouche *et al.*, 1996; Yuan *et al.*, 1996), SRC1 and ACTR are co-activators for nuclear receptor proteins (Chen *et al.*, 1997; Spencer *et al.*, 1997) and TAF_{II}250 is a component of the TATA box binding TFIID complex (Burley and Roeder, 1996).

The domain that mediates enzyme activity within each of the acetyltransferases is relatively large, of the order of several hundred residues. Surprisingly, no sequence similarity has been detected between them. This suggests that different acetyltransferases may carry out distinct functions. Indeed, the fact that CBP is able to form complexes with other proteins with similar enzymatic activities, such as P/CAF and SRC1 (Yao *et al.*, 1996; Chen *et al.*, 1997; Spencer *et al.*, 1997), suggests that a combination of acetyltransferase activities may be required for certain functions.

One of the mechanisms by which these enzymes could function differently is by having distinct histone targets. *In vitro*, P/CAF and GCN5 acetylate H3 and H4 (Kuo *et al.*, 1996; Yang *et al.*, 1996) whereas CBP can acetylate all four histones equally well (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996). A separate possibility is that acetyltransferases have distinct non-histone targets. The recent demonstration that the p53 protein can be acetylated by CBP and that this modification stimulates p53 DNA binding capacity, suggests that these enzymes can stimulate transcription via non-histone specific pathways (Gu and Roeder, 1997). In addition, CBP, P/CAF and TAF_{II}250 can all acetylate the basal transcription factor TFIIE- β and the RAP30/74 subunits of TFIIIF, at least *in vitro* (Imhof *et al.*, 1997). Since the enzymatic domains of the aforementioned acetyltransferases were identified using histones as substrates, we refer to these domains as HAT domains. However, given the widening substrate specificity of CBP, P/CAF and TAF_{II}250, we now refer to their intrinsic enzymatic activity as acetyltransferase (AT) activity.

All of the questions raised above are based on the assumption that the AT activity present within these nuclear proteins is directly responsible for stimulating transcription *in vivo*. We addressed this point by investigating whether the AT domain of CBP is sufficient to stimulate

transcription and whether acetylation is responsible for transcriptional stimulation. Here we show that acetylation directly correlates with transactivation, and show that two of the AT families, exemplified by CBP and P/CAF, respectively, have a related HAT domain.

Results

We set out to establish whether the intrinsic AT activity of CBP is sufficient to activate transcription. CBP is known to possess an activation domain at its C-terminus and is capable of binding proteins (P/CAF and SRC1) which carry their own AT activity. To avoid interference by these domains, we separated the CBP HAT domain away from the rest of the protein and investigated whether it would activate transcription independently as a GAL4-fusion (Figure 1A). Figure 1B shows that the GAL4-CBP HAT domain can stimulate transcription from the adenovirus major late (AdML) promoter. Activation is dependent on the HAT domain being tethered to the promoter, since the AdML promoter is not activated in the absence of upstream GAL4 DNA-binding sites (data not shown).

To assess whether AT activity is required for transactivation, we introduced an 18 residue in-frame deletion (amino acids 1458–1475) within the CBP HAT domain (Figure 1A). When tested on the G5AdML promoter, this mutant (CBP-HAT Δ) shows no detectable transactivation capacity *in vivo* (Figure 1B) and has no detectable *in vitro* histone AT activity when precipitated with a GAL4-specific antibody (Figure 1C). Since the wild-type and mutant HAT domains are expressed equally well in these cells (data not shown), these results suggest that AT activity is required for the transactivation capacity of the HAT domain.

Adjacent to the CPB HAT domain lies a cysteine-rich region (CBP2) which harbours the binding site for a variety of regulatory transcription factors (MyoD, E2F, c-Fos), the basal factor TFIIB and the P/CAF protein. In contrast to the HAT domain, the CBP2 domain does not activate transcription of G5AdML (GAL4-CBP2, Figure 1B). However, the CBP2 domain augments the activation capacity of the CBP HAT domain, as shown by GAL4-CBP-HAT-CBP2 which contains the two contiguous domains fused to GAL4 (Figure 1B). The activation capacity provided by CBP2 is unable to function in the absence of AT activity; a deletion within the HAT domain which eliminates *in vitro* histone AT activity [GAL4-CBP-HAT Δ -CBP2 (Figure 1C)] completely abolishes the activation potential of GAL4-CBP-HAT-CBP2 (Figure 1B). Together, these results indicate that CBP contains an activation domain within residues 1099–1877. The activation capacity of this region is eliminated when the intrinsic AT activity is lost.

Proteins which form part of the basal machinery (TFIIE and TFIIIF) are *in vitro* targets for acetylation by CBP (Imhof *et al.*, 1997). To establish whether the stimulating effect of the CBP acetyltransferase domain was directed against these components (rather than histones), we asked whether GAL4-CBP-HAT could stimulate transcription in a reconstituted *in vitro* transcription system, that lacks histones but is dependent on TFIIE and TFIIIF. Figure 1D shows that GAL4-CBP-HAT is unable to activate tran-

scription of the AdML promoter under these *in vitro* conditions, whereas a GAL4-Sp1 protein is competent in doing so. The GAL4-CBP-HAT protein used in this assay was fully active in an *in vitro* histone acetylation assay (data not shown). Under the experimental conditions employed, the quantity of GAL4-CBP-HAT and GAL4-Sp1 used in the transcription assay was sufficient to saturate the reporter's GAL4 DNA-binding sites. In a separate set of experiments, we quantitatively acetylated TFIIE *in vitro* and found that the acetylation did not increase TFIIE-dependent *in vitro* transcription (data not shown). These results suggest that acetylation of TFIIE and TFIIIF is unlikely to be the cause of the transcriptional stimulation of the AdML promoter observed *in vivo* (Figure 1B).

The activation capacity of the CBP acetyltransferase domain shows some promoter selectivity *in vivo*. Figure 2A shows that, in the same cell-type (U2OS cells) and under the same conditions, GAL4-CBP-HAT is able to activate the AdML and E4 promoters of adenovirus but not the SV40 promoter or the adenovirus E1B promoter. Direct comparison of the AdML and E1B promoters in 293T cells indicates that this is not a cell-type-specific effect (Figure 2B). Examination of the sequences of these minimal promoters revealed that the TATA-box sequences within them varied in sequence. This raised the possibility that acetylated components of the basal machinery (such as TFIIE and TFIIIF) may either be missing or be present in a conformationally inadequate fashion on certain promoters, resulting in the inability of the CBP acetyltransferase to stimulate transcription. To explore the possibility that the TATA-box sequence was responsible for the observed selectivity, we replaced the TATA-box of the CBP-unresponsive E1B promoter (GGGTATATAAT) with that of the CBP-responsive AdML promoter (GGCTATAAAG). Figure 2B shows that the AdML TATA box did not confer GAL4-CBP-HAT inducibility to the E1B promoter. This was not due to inactivity of the chimeric E1B/AdML TATA promoter since a GAL4-Fos fusion protein efficiently activated all three reporters (data not shown). These results suggest that the TATA-box-associated factors are not responsible for the selectivity. This interpretation is reinforced by the experiments in Figure 2C which show that the T-cell-specific CD4 promoter, which lacks a TATA box, is efficiently stimulated by GAL4-CBP-HAT when GAL4 sites are introduced upstream. The activation capacity is entirely due to the action of the CBP acetyltransferase activity, since GAL4-CBP-HAT Δ is unable to activate the CD4 promoter. Collectively, these results argue against TFIIE and TFIIIF being the targets for the stimulating effect of the CBP acetyltransferase activity. We cannot exclude the possibility that as yet unidentified acetylation-sensitive factors associated with certain promoters are responsible for the stimulating effect. However, our data are consistent with histones being the target for the CBP acetyltransferase, since the AdML promoter is activated by the CBP HAT domain *in vivo* but not *in vitro*.

Examination of the AT domains of recently identified ATs has not revealed any overt sequence similarity between them. Indeed, a BLAST search using CBP does not identify the other ATs, P/CAF, SRC1 or TAF_{II}250. However, careful scrutiny of the relevant HAT domains of CBP and P/CAF

Fig. 1. The HAT domain of CBP activates transcription *in vivo* but not *in vitro*. (A) A schematic representation of the CBP protein. Indicated above are the factors which have been shown to bind CBP. Also indicated are important features of the CBP protein; cys, ZZ and TAZ are putative zinc-finger regions, Bromo is the bromodomain and HAT is the histone acetyltransferase domain. Shown below the CBP schematic are schematics of the constructs used; GAL4 is the GAL4-DBD, CBP-HATΔ has an in-frame deletion of amino acids 1458–1475 within the CBP HAT domain. (B) CBP co-operates with the CBP HAT domain to activate transcription. 2 μg of G5AdML CAT reporter plasmid were transfected into U2OS cells along with 2 μg of the indicated effector plasmid. Extracts from these cells were then used in CAT assays. The activity derived from the GAL4-DBD was normalized to 1.0 and the activity of the other effectors is expressed relative to this. (C) An IP-HAT assay was performed using an anti-GAL4 antibody on extracts derived from (B). The background HAT activity associated with the GAL4-DBD was normalized to 1.0 and the activity of the other GAL4-fusions is expressed relative to this. (D) GAL4-DBD, GAL4-Sp1 or GAL4-CBP-HAT were analysed in a reconstituted *in vitro* transcription assay. The position of the accurately initiated transcript is indicated by an arrow. All GAL4 DNA-binding proteins were in excess relative to the DNA-binding sites. The GAL4-CBP-HAT was fully active in an *in vitro* histone acetylation assay (data not shown).

revealed considerable sequence similarity over a 100 residue stretch (Figure 3). Over this region of CBP there is 16% identity and 32% similarity with the P/CAF sequence. Although this falls below the detection level for a computer-based search, a number of features of the similarity suggest that it is functionally relevant. First, there are no major gaps introduced in the sequence. Secondly, the conserved residues coincide with motifs found conserved between *N*-acetyltransferases and the P/CAF-related HAT, GCN5 (Neuwald and Landsman, 1997). Aligning the AT domains derived from *N*-acetyltransferases has identified four conserved motifs A, B, C and D. Three of these motifs (A, B and D) are also

conserved in the alignment between CBP and P/CAF. In addition, a motif not found in other *N*-acetyltransferases (which we now refer to as motif E; Figure 3) resides at the N-terminus of the CBP and P/CAF HAT domains. This motif must be important for the AT activity of CBP, since the deletion in Figure 1 (GAL4-CBP-HAT Δ), which compromises *in vitro* histone AT activity, overlaps motif E.

Having identified a region of CBP that may represent the catalytic domain of the AT activity, we introduced point mutations in this sequence with a view to addressing two questions: (i) whether AT activity correlates with transcriptional activation; and (ii) whether conservation between CBP and P/CAF reflects a conservation of residues required for AT activity. Fourteen point mutations were introduced into CBP in both conserved and non-conserved residues, relative to P/CAF. The mutant CBP HAT domains were linked to the GAL4 DNA-binding domain and transfected into U2OS cells. Using a GAL4-specific antibody in Western blots, we first ensured that all mutants were expressed at comparable levels (data not shown). The GAL4 antibody was then used to immunoprecipitate the GAL4-CBP-HAT mutants and their ability to acetylate histones *in vitro* was assessed. Figure 4A shows that eight point mutations almost completely abolish the HAT activity of CBP (Y1467, H1471, E1479, D1522, L1532, L1538, Y1540 and F1541), whereas six mutations still allow substantial histone AT activity (I1456, Q1490, K1510, I1516, Q1526 and E1529). When the 14 mutants are tested for transactivation capacity of the AdML promoter (Figure 4B), the results show a remarkable correlation between the ability of the point mutants to activate transcription and the ability to acetylate histones (compare Figure 4A and B). The eight mutants with reduced histone AT activity also have reduced activation potential (Y1467, H1471, E1479, D1522, L1532, L1538, Y1540 and F1541), whereas the six mutants which retained substantial histone AT activity also possess significant activation potential (I1456, Q1490, K1510, I1516, Q1526 and E1529). These results clearly demonstrate a direct correlation between *in vitro* HAT activity and transcriptional stimulation *in vivo*. The mutagenesis results indicate that residues spanning almost the entire length of the CBP-P/CAF homology are essential for HAT activity and transactiv-

Fig. 2. The HAT domain of CBP activates transcription in a promoter-specific manner. (A) Promoter specificity. 2 μ g of the indicated reporter plasmid were transfected into U2OS cells along with 2 μ g of either CMV-promoter-driven GAL4-DBD or CMV-promoter-driven GAL4-CBP-HAT. Extracts from these cells were then used in CAT assays. For each reporter, the activity derived from the GAL4-DBD was normalized to 1.0 and the activity of GAL4-CBP-HAT is expressed relative to this. (B) Promoter specificity is not attributable to the TATA box. A similar analysis to that described in (A) was performed in 293T cells. The E1B/AdML-promoter-containing plasmid is identical to the CBP-HAT-unresponsive E1B-containing plasmid except that its TATA box has been replaced with that from the CBP-responsive AdML-promoter-containing plasmid. (C) The CBP HAT domain activates a TATA-less promoter. Jurkat cells were transfected with 10 μ g of the T-cell-specific CD4 promoter, which has GAL4 DNA-binding sites integrated upstream, and which drives the synthesis of mRNA encoding luciferase. Along with this reporter, 2 μ g of GAL4-DBD, GAL4-CBP-HAT or GAL4-CBP-HAT Δ was also transfected into the cells. In all cases, 2 μ g of an internal control plasmid, CMV- β -galactosidase, were also included. The activity derived from the GAL4-DBD was normalized to 1.0 and the activities of GAL4-CBP-HAT and GAL4-CBP-HAT Δ are expressed relative to this (following normalization to the β -galactosidase units).

Fig. 3. CBP and P/CAF HAT domains have sequence similarities. Alignment of the mouse CBP and human P/CAF HAT domains showing the conserved motifs identified in other *N*-acetyltransferases (A, B, D) as well as the newly identified motif E. The mutated residues in CBP and their positions are indicated. Conserved residues are shaded.

Fig. 4. CBP HAT activity is required for transactivation. (A) Fourteen point mutations were introduced into GAL4-CBP-HAT and transfected into U2OS cells. After immunoprecipitation of the GAL4-CBP-HAT mutants with an anti-GAL4 DNA-binding domain antibody, the precipitates were tested for their *in vitro* HAT activity. The HAT activity associated with the GAL4-DBD is indicated as Backg. The mutated residues and their position in the CBP sequence are indicated. (B) 3 μ g of wild-type GAL4-CBP-HAT domain mutants were co-transfected along with 3 μ g of G5AdML CAT reporter plasmid into U2OS cells. Extracts were made from these cells and then used in a CAT assay. The CAT activity is expressed as a percentage of the wild-type GAL4-CBP-HAT.

Fig. 5. CBP and P/CAF have conserved motifs. (A) Alignment of the mouse CBP and human P/CAF HAT domains showing the conserved motifs identified in other *N*-acetyltransferases (A, B and D). Below the motifs, the conserved sequence derived from an alignment of *N*-acetyltransferases (Neuwald and Landsman, 1997) is shown. Residues mutated in CBP and P/CAF are indicated by an asterisk. Conserved residues are shaded. (B) Three point mutations were introduced into the GAL4-P/CAF-HAT domain and transfected into U2OS cells. IP-HAT assays from transfected cells were performed as described for Figure 4. The mutated residues and their positions in P/CAF are indicated.

ation. We have also examined selected point mutants for their ability to acetylate recombinant TFIIIE- β . Our results indicate that the residues required for the acetylation of histones are also required for the acetylation of TFIIIE- β (data not shown).

All eight point mutants which compromise the *in vitro* HAT activity fall within one or other of the four motifs identified in the alignment in Figure 3 (Y1467 and H1471 in motif E, E1479 in motif D, D1522 in motif A, and L1532, L1538, Y1540 and F1541 in motif B). In contrast, four of the six mutations which do not affect HAT activity (I1456, Q1490, Q1526 and E1529) lie in the non-conserved regions. The correlation provides evidence that the conserved residues are important for HAT activity. The exceptions are two residues, K1510 and I1516, which are conserved within motif A but which are apparently not affected by a substitution to alanine. This may signify that these residues are not essential for histone AT activity, or that a substitution to alanine represents a conservative change.

Motifs D, A and B of CBP contain residues conserved in *N*-acetyltransferases. Figure 5A shows that the six CBP residues sensitive to mutagenesis are conserved in the *N*-acetyltransferase family. If the similarity between CBP and P/CAF represents conservation of residues required

for AT activity, we would expect that mutagenesis of analogous P/CAF residues would also affect AT activity. We tested this prediction by introducing three mutations in the P/CAF HAT domain and investigating what effect they had on P/CAF's *in vitro* HAT activity. Figure 5B shows that mutagenesis of two P/CAF residues (L606 and F617) severely affects *in vitro* HAT activity, consistent with the results of mutating the analogous residues in CBP (L1532 and F1541; Figure 5A). In contrast, mutating a non-conserved P/CAF residue (H600) had no effect on HAT activity, as predicted by the mutation of the analogous (non-conserved) residue in CBP (Q1526; Figure 4A). Collectively, the results in Figures 4 and 5 provide an experimental validation of the similarity between the HAT domains of CBP and P/CAF, and define sequence motifs essential for AT activity. Moreover, the results indicate a direct link between AT activity and the ability to activate transcription *in vivo*.

Discussion

The region of CBP having intrinsic AT activity (1099–1758) lies within an independently acting transcriptional activation domain (1099–1877). The activation capacity of this domain is absolutely dependent on AT activity, but its potency is augmented by adjacent sequences, which contain binding sites for numerous factors. These results are consistent with the analysis of yeast GCN5 which shows that a deletion within the HAT domain eliminates HAT activity and transactivation (Candau *et al.*, 1997).

The AT-containing activation domain of CBP can only stimulate transcription from certain promoters. There are several possibilities that may account for this preference. First, the chromatin state of the different reporters may be distinct, leading to a selective sensitivity to acetylation. Indeed, we note that in the various reporters, the distances between the GAL4 binding sites and the start point of transcription do differ slightly. This would most likely be reflected by the chromatin architecture on the different promoters. Secondly, AT activity may need the co-operation of a distinct factor to stimulate transcription, which is present only in some of the reporters. Thirdly, the AT activity of the CBP HAT domain may be directed against a target other than histones, which may only be present on a subset of promoters. If this third scenario is correct, our results support the conclusion that TFIIE and TFIIIF are unlikely to be the factors involved. The experiments shown in Figures 1 and 2 are, however, most consistent with a differential chromatinized state of the promoters. The most direct evidence comes from the fact that the AT activity of CBP cannot activate transcription *in vitro*, but can do so *in vivo*. Given the evidence that transfected plasmid DNA does get rapidly chromatinized (Reeves *et al.*, 1985; Pullner *et al.*, 1996; Stanfield-Oakley and Griffith, 1996), the results point to chromatin as the likely target. However, we cannot exclude the possibility that a factor, yet to be identified, is acetylated by CBP and that this factor is missing in the *in vitro* transcription system we have used.

Whichever scenario is operational, the apparent selectivity for promoters may reflect an intrinsic characteristic of AT activity. This could explain the need for distinct AT proteins (P/CAF and SRC1) to be associated with CBP.

If the AT activity of each protein had a distinct role (such as stimulating specific promoters), then a complex of several ATs may be more globally effective.

The activation potential of the CBP HAT domain, assayed on the AdML promoter, is clearly dependent on AT activity. This is strongly suggested by the absolute correlation between stimulation of transcription and the preservation of AT activity exhibited by the point mutants. The mutagenesis does not identify a discreet catalytic site essential for activity. Instead, multiple mutations, over a region of almost 100 residues, compromise AT activity equally well. Indeed, all deleterious mutations result in the near complete loss of activity. This points to a relatively structural domain, with specific sub-domains coming together to make up an enzymatically active site.

The sequence similarity between the CBP and P/CAF HAT domains identifies some of the motifs which, in combination, make up an active HAT domain. Mutagenesis of residues within these motifs abolishes activity almost completely, suggesting that they are all required for function. An alignment of *N*-acetyltransferases derived from various species has revealed four conserved motifs (A–D), which are interspersed with sequence of non-identity (Neuwald and Landsman, 1997). Motifs A and B have previously been proposed to represent the acetyl Co A binding site (Tercero *et al.*, 1992). CBP and P/CAF contain the motifs A, B and D but not C. However, CBP and P/CAF have an additional motif (E) that has no counterpart in *N*-acetyltransferases, and may therefore represent a sub-domain essential for a subset of ATs. Scrutiny of other recently identified ATs, such as TAF_{II}250 (Mizzen *et al.*, 1996) and SRC1 (Spencer *et al.*, 1997), does not reveal extensive sequence similarity with the alignment shown in Figure 3. Although similarity with some of the motifs is detectable, the significance of this remains to be established by mutational analysis.

The similarity in the structure of the CBP and P/CAF HAT domains suggests that they belong in the same 'family' of HAT domains. It is too early to tell whether other AT proteins, such as SRC1 and TAF_{II}250, will be in a similar or distinct family. Structural analysis of these domains is required to resolve the issue. However, the identification of specific motifs between CBP and P/CAF, previously considered to be unrelated, should aid the search for other members of this AT family.

Materials and methods

Recombinant DNA

The GAL4–CBP HAT fusion construct was made by cloning the HAT domain of CBP (1098–1758) between the *Xba*I and *Sac*I restriction sites of pcDNA3 vector (Stratagene) containing the GAL4–DBD (1–147) (pcDNA3–GAL4–DBD; GAL4–DBD amino acids 1–147 were cloned into pcDNA3 as a *Hind*III–*Xba*I PCR amplicon). The GAL4–P/CAF–HAT construct was made by cloning the HAT domain of P/CAF (amino acids 352–658) between the *Xba*I and *Sac*I restriction sites of pcDNA3–GAL4–DBD. The GAL4–CBP–HAT–CBP2 was made by introducing an *Eco*RI–*Not*I CBP2 fragment (amino acids 1680–1877) into an *Eco*RI- and *Not*I-digested pcDNA3–GAL4–CBP–HAT construct generating pcDNA3–GAL4–CBP–HAT–CBP2. The GAL4–CBP2 plasmid was constructed by cloning an *Xba*I-ended CBP2 PCR fragment (1620–1877) of CBP into the *Xba*I site of pcDNA3–GAL4–DBD. The GAL4–CBP–HATΔ and GAL4–CBP–HATΔ–CBP2 fusion constructs were made by deleting amino acids 1458–1475 of the CBP HAT domain by employing the Stratagene site-directed mutagenesis kit according to the manufacturer's instructions (see below). The vectors used as a reporter on the

CAT assays contain five GAL4 binding sites cloned upstream of the promoters E1B, SV40, E4 and AdML, which were gifts from M.Green, A.Berk, M.Carey and H.Stunnenberg, respectively. CD4 core promoter sequences [positions -42 to +20; (Salmon *et al.*, 1993)] were cloned into the *SmaI* and *BglIII* sites of pGL2-Basic (Promega). Five GAL4 binding sites were cloned upstream of the promoter from position -59 to -157.

Cell culture, transfections and reporter gene assay

U2OS cells were maintained in DMEM with 10% fetal calf serum (FCS). The cells were kept as monolayers and split 1:6 every 3 days. They were transfected using the standard calcium phosphate co-precipitation method (Van der Eb and Graham, 1980), with 2 or 3 µg (as indicated) of either CMV promoter driven GAL4-DBD or CMV-promoter driven GAL4-CBP-HAT or GAL4-P/CAF-HAT and 2 or 3 µg (as indicated) of CAT-reporter plasmid (G5E1B, G5SV40, G5E4 and G5AdML, described above). The cells were washed after 14 h and harvested 36 h after transfection. After that, they were washed once in phosphate-buffered saline (PBS), resuspended in 100 µl 0.25 M Tris pH 8.0 and lysed by freezing and thawing three times (liquid nitrogen/37°C). Supernatants were clarified by centrifugation (5 min, 12 000 g). CAT assays were done as described previously (Hagemeier *et al.*, 1992) using 50 µl of the extracts. The results were quantitated on a PhosphorImager. Western blots were probed with an antibody against GAL4 DNA-binding domain (Santa Cruz, Biotechnology) at 1 µg/ml to check the level of protein expressed by each vector. Jurkat cells were cultivated in RPMI medium containing 10% FCS (PAN systems). Cells were collected at densities of $5-8 \times 10^5$ per ml and washed once in PBS. Approximately 1.6×10^7 Jurkat cells were mixed with 10 µg of CD4/Gal luciferase reporter, 2 µg of GAL4(1-147) or GAL4-CBP-HAT expression plasmids and 2 µg of CMV-β-galactosidase expression plasmid in 400 µl of RPMI medium lacking serum. After 20 min, cells were electroporated in 0.4 cm cuvettes at 960 µF/250 V (Gene Pulser, Bio-Rad). After an additional 20 min, the cells were re-seeded in 10 ml RPMI medium containing 10% FCS. Phorbol-12-myristate (10 ng/ml) (PMA, Sigma) in dimethyl sulfoxide was added 4 h after electroporation. Thirty-six to 40 h after electroporation cells were harvested, washed once in PBS and lysed in 150 µl luciferase lysis buffer (Promega) for 20 min at room temperature. Supernatants were clarified by centrifugation (2 min, 12 000 g), 40 µl were mixed with 100 µl luciferase assay solution (Promega) and analysed in a Topcounter (Canberra-Packard). β-galactosidase activity was determined by incubating 30 µl of the supernatant with 270 µl β-galactosidase assay solution (Sambrook *et al.*, 1989) and measurement of OD₄₂₀. Luciferase units were normalized with β-galactosidase values.

In vitro transcription assay

Transcription reactions included 50 ng *SmaI*-linearized templates, pAdML-G-less, containing the AdML core promoter and five upstream GAL4-binding sites, with the *SmaI* site being located immediately downstream of the 390 bp G-free region, and 90 ng pUC18 as carrier DNA. Reactions were performed under standard conditions as detailed in Kaiser *et al.* (1995), and included 10 ng of recombinant TFIIB, 1.5 µl of TFIID, (DE52-fraction, 0.4 mg/ml protein), 10 ng of recombinant TFIIE-α, 5–10 ng of recombinant TFIIE-β, 1.0 µl of TFIIIF/TFIIH (DE52, 0.3 mg/ml protein, cf. Kaiser *et al.*, 1995), 2.0 µl of RNA polymerase II obtained from HeLa nuclear extracts and enriched on phosphocellulose (0.5 M KCl eluate) and DE52 (0.3 M KCl step eluate), 80 ng of PC4 and 30 ng of GAL94-Sp1 (comprising the Q1 transcriptional activation domain as defined in Courey and Tjian, 1988) expressed in, and purified from *Escherichia coli*. GAL-CBP-HAT and GAL-CBP-HATΔ were over-expressed in, and purified from, 293T cells and were then analysed in DNA-binding and acetylation assays (data not shown). The amount of GAL4-CBP-HAT added to the purified transcription system was saturating with respect to template binding.

Mutagenesis

The GAL4-CBP-HAT mutants were made using the Quick mutagenesis Stratagene kit following the manufacturer's instructions, using 50 ng of the plasmid template, GAL4-CBP-HAT and GAL4-P/CAF-HAT (described above) and 125 ng of each primer, in a final volume of 50 µl. Mutants were identified by DNA sequencing.

IP-HAT assay

After transfection of the GAL4-CBP-HAT and GAL4-P/CAF-HAT constructs, extracts were made using 250 µl of IPH buffer (Bannister and Kouzarides, 1996). The lysis mixture was incubated on ice for 20 min and cleared by centrifugation at 12 000 g for 10 min at 4°C.

Three mg of a GAL4 DNA-binding domain antibody were used to precipitate the GAL4 constructs for 3 h at 4°C with rotation, then 15 µl of protein A/protein G-Sepharose beads (a 50:50 mix) were added and incubated overnight at 4°C with rotation. The immune complexes were pelleted by gentle centrifugation and washed 3 times with 1 ml of lysis buffer IPH. After the final wash, the buffer was aspirated down to 30 µl and the liquid HAT assay was done as described (Bannister and Kouzarides, 1996).

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