

Involvement of chromatin and histone deacetylation in SV40 T antigen transcription regulation

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ABSTRACT

Simian Virus 40 (SV40) large T antigen (T Ag) is a multifunctional viral oncoprotein that regulates viral and cellular transcriptional activity. However, the mechanisms by which such regulation occurs remain unclear. Here we show that T antigen represses CBP-mediated transcriptional activity. This repression is concomitant with histone H3 deacetylation and is TSA sensitive. Moreover, our results demonstrate that T antigen interacts with HDAC1 *in vitro* in an Rb-independent manner. In addition, the overexpression of HDAC1 cooperates with T antigen to antagonize CBP transactivation function and correlates with chromatin deacetylation of the TK promoter. Finally, decreasing HDAC1 levels with small interfering RNA (siRNA) partially abolishes T antigen-induced repression. These findings highlight the importance of the histone acetylation/deacetylation balance in the cellular transformation mediated by oncoviral proteins.

INTRODUCTION

Simian virus 40 (SV40) large T antigen (T Ag) is a 708-amino-acid multifunctional oncoviral protein involved in numerous viral and cellular processes, including viral replication, transcriptional activation and repression, and blockade of differentiation and cell transformation (1). T antigen can be post-translationally modified by phosphorylation, glycosylation, adenylation and ADP ribosylation. Moreover, T antigen can be acetylated by CBP in a p53-dependent manner (2), although the functional consequences of this modification remain largely unknown. The ability of T Ag to transform cells depends on complex interactions between viral

oncoproteins and the various intracellular proteins involved in cell control (3) and transcription regulation, such as p53 (4,5), pRb and the Rb-related proteins p107 and p130 (6–9) and CBP/p300 (10–13). T antigen requires the LXCXE motif to interact with the pRb protein family. Two regions in the C-terminal part of the T antigen protein are required for p53 binding. Finally, the regions of T antigen required for CBP/p300 interaction overlap these p53-binding regions. Other oncoviral proteins, such as E1A, also require CBP/p300 and p53 targeting for cell transformation.

Histone acetylation at the N-terminal tails is a regulatory mechanism that controls gene expression (14,15). Histone acetylation levels inside cells are maintained through the coordinated action of histone acetyltransferases (HATs) and deacetylases (HDACs). CBP/p300, a coactivator protein (16) involved in both proliferative and differentiating pathways, contains HAT activity (17,18). CBP/p300 is ubiquitously expressed and regulates a broad spectrum of biological activities such as proliferation, differentiation, cell cycle control and apoptosis. To accomplish such processes, CBP/p300 must be recruited to a specific promoter via interactions with a sequence-specific transcription factor, including CREB, c-Myb, MyoD, E2F1, p53, nuclear hormone receptors, etc.

In mammalian cells, HDAC1 and HDAC2 are found in multiprotein complexes. These have been implicated as corepressors that associate with different factors such as Rb, Mad, MeCP2, etc., in repressing transcription.

Several reports have shown that viral oncoproteins select HAT enzymes as cellular partners, in some cases disrupting enzymatic activity. E1A can increase, decrease or redirect CBP/p300 HAT activity (19–21); while CBP, by interacting with T antigen increases its HAT activity (22). In addition, CBP, p300 and P/CAF acetylate several viral oncoproteins such as AdE1A (23,24) and T antigen (2), although the consequences of these modifications remain to be elucidated. Oncoviral proteins also target HDAC complexes: the adenovirus E1B-55K protein

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interacts with a mSin3A-histone deacetylase 1 complex (25); HIV Tat displaces HDAC1, which is bound to SATB1, leading to increased acetylation of promoters *in vivo* (26); the Epstein-barr virus nuclear antigen 3C interacts with HDAC1 to repress transcription (27); the E7 oncoprotein from papilloma virus type 16 (HPV16) interacts with HDAC activity, resulting in the stimulation of cell growth (28).

T antigen affects transcription levels of the cAMP-responsive promoter, which is modulated *in vivo* by p300 in REV2 cells. Moreover, by interacting with CBP/p300, T antigen abrogates CBP/p300-mediated transcriptional activity (11,12), although the mechanism involved remains unclear. Here we examine the mechanisms underlying the repressive behavior of T antigen. Our results not only show that T antigen repression is alleviated by increasing cellular acetylation levels via trichostatin A (TSA) treatment, but also that it acts concomitantly with histone H3 deacetylation. Moreover, we have demonstrated that T antigen interacts *in vitro* with HDAC1 in an Rb-independent manner. The data presented in this study link chromatin modification to T antigen transcriptional regulatory potential.

MATERIALS AND METHODS

Constructs

PSG5-T antigen, PSG5-T antigen K1 and PSG5-T antigen PVU-1 plasmids were kindly provided by Dr J. DeCaprio. pcDNA3Gal4-HAT-CBP2, pcDNA3Gal4-HAT, pcDNA3Gal4-CBP (FL) and pcDNA3-HDAC1-Flag constructs have been previously described (29) and were kindly provided by Dr T. Kouzarides. The Gal4-TK-luciferase and Gal4-hsp70- β gal reporters were supplied by Dr J. Bernues. The pSUPER-Control-siRNA was kindly provided by Dr E. Marti.

Cell cultures, transfections and reporter gene assays

HeLa, CV1 and CV1COS cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal calf serum (FCS) and grown at 37°C, 5% CO₂. Cells were transfected by a standard calcium phosphate coprecipitation (for luciferase and β galactosidase assays) or with a Jet-PEI Kit (Polyplus-Transfection, Illkirch, France) (for ChIPs and siRNA assays) with 0.1–2 μ g (as indicated) of either expression vector and with 2 μ g reporter plasmid. The cells were then washed in PBS after 14 h and harvested 36 h after transfection (except when indicated). They were subsequently washed once in phosphate-buffered saline (PBS). Luciferase and β galactosidase assays were performed using a 'Promega Dual Kit' and a 'Promega Galactosidase Kit' respectively, following the manufacturer's instructions. The TSA treatment was conducted at 100 nM for 18 h.

Cell extract preparation and protein content estimation

Total cell extracts were prepared as follows: cells were lysed in 1 ml of buffer A (50 mM Tris, pH 8, 150 mM NaCl, 0.5% NP40, 10 μ g/ml of aprotinin and leupeptin,

1 mM phenylmethylsulfonyl fluoride, 50 mM NaF and 10 mM Na₂-B-glycerophosphate) by maintaining the cells for 20 min on ice; cell debris were removed by centrifugation at 12 000 \times g for 10 min at 4°C. Protein content was measured by using the 'BIO-RAD protein assay reagent' following the manufacturer's instructions.

Chromatin immunoprecipitation analysis (ChIPs)

HeLa cells were treated with 1% formaldehyde at room temperature for 15 min. The reaction was stopped by the addition of glycine to a final concentration of 125 mM. Cells were washed once in ice-cold PBS, once in buffer I (0.25% triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM Hepes pH 6.5) and in buffer II (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM Hepes pH 6.5) at 4°C for 10 min. The pellet was then resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.1 μ g/ml protease inhibitors, 1 mM PMSF) and sonicated on ice until the cross-linked chromatin was sheared to an average DNA fragment length of 0.2–0.5 kbp. After centrifugation (10–45 min 12 000 \times g) soluble cross-linked chromatin was diluted 1:10 in immunoprecipitation (IP) buffer (1% triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris pH 8 and protease inhibitors), divided into aliquots and stored at –80°C. Chromatin preparations were pre-cleared by incubation with Protein A-Sepharose solution: 30 μ g pre-immune serum (35 μ g/ μ l), 2 μ g herring sperm DNA (1 μ g/ μ l), 50 μ l Protein A-Sepharose CL4B (Amersham-Pharmacia) 10% in TE buffer (50 μ g/500 μ l TE) in IP buffer for 2 h at 4°C under rotation. The protein A-Sepharose was removed by centrifugation; the pre-cleared chromatin was immunoprecipitated by incubation with the antibody O/N at 4°C. The immunoprecipitates were washed in buffers TSE I (0.1% SDS, 1% Triton X-100, 2 Mm EDTA, 20 mM Tris-HCl pH 8, 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 Mm EDTA, 20 mM Tris-HCl pH 8, 500 mM NaCl), buffer III (0.25 M LiCl, 1% NP40, 1% Deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8), and three times with Tris-EDTA (TE) buffer. Washed pellets were eluted in 300 μ l of a solution containing 1% SDS, 0.1 M NaHCO₃. Eluted pellets were de-cross-linked O/N at 65°C, and then purified in 50 μ l TE buffer using a DNA purification kit (Amersham-Pharmacia). The DNA was analyzed by PCR. The antibodies used were as follows: rabbit polyclonal antibody against Acetyl H3 (k9, k14) and Poly-acetyl H4 (K5, K8, K12, K16) (Upstate) and HDAC1 (Abcam).

PCR analysis

PCR analysis was performed as described by Breiling and colleagues (30). PCR products measured 220–330 bp and were resolved by agarose-gel electrophoresis and revealed by staining with ethidium bromide. Decreasing amounts of input DNA (10, 1, 0.1 and 0.01%) were used to determine the linear range of the PCR reactions for each primer pair and IP. The following primer pairs were used: 5'-CATGTCGATCCGCTCGG-3' and 5'-CTTTATGTTTTGGCGTCTTCCA-3', for TK-luc promoter and 5'-CCCCAAAGGTTGCCTGGCGG-3'

and 5'CAAGATGAGAGGAGGCTGGG-3' for PDGF β -receptor promoter, provided by TIB MOLBIOL (Germany).

ChIP quantification

PCR gel bands were quantified with QuantityOne software (Bio-Rad). Values from immunoprecipitated samples were subsequently divided by that corresponding to 1% input sample (the PCR signals of this input sample were not saturated). Bound/input values were then averaged from multiple (at least three) PCR reactions from independent experiments. All data in the figures correspond to percentages of the 1% input sample.

In vitro translations, recombinant proteins and pull-down assays

In vitro translations and GST pull-downs were performed essentially as described previously (31). GST and GST fusion proteins were expressed in *Escherichia coli* XA90 using the pGEX (Pharmacia) vector system, for 4 h following the addition of 0.1 mM IPTG at 30°C. Purification from crude bacterial lysates was performed as previously described (17). PGEX-HDAC1 has been described elsewhere (17,31). The buffer used for the pull-downs was a variation of Z' (25 mM HEPES pH 7.5, 12.5 mM MgCl₂, 20% glycerol, 0.1% NP40, 250 mM KCl).

Cell proliferation assays

Doubling time and saturation density were measured using a modification of a previously described method (32). CV1 and CV1COS cells were seeded at a density of 4×10^4 cells/60 mm plate in CS (10%)-containing DMEM. This time point was considered as day 1. The remaining cultures were incubated with 10% serum-containing DMEM and replenished every day. Cells were trypsinized and counted by a hemocytometer every day.

siRNA interference

Target sequences for small interfering RNAs (siRNAs) for HDAC1 are available upon request. The oligonucleotides were cloned into a pSUPER vector (OligoEngine) at the *Bgl*II and *Hind*III sites. The siRNAs were transfected twice using Jet-*Pei*TM (Polyplus-Transfection, Illkirch, France) at 12 h intervals. Target sequences for control small interfering RNAs (c-siRNA) were as follows: (forward) CCAGCGCTACGGCTGGCAC; (reverse) GTGCCAGCCGTAGCGCTGG, neither of which cross-hybridize with any human sequence.

RESULTS

T antigen represses the transcriptional activity of CBP

Several independent findings indicate that SV40 T antigen represses the transcriptional activity of CBP (FL) on the thymidine kinase (TK) promoter (11,12). On the other hand, our previous results show that T antigen cooperates

with HAT-CBP2 to activate the hsp70 promoter (22). This apparent discrepancy may stem from (i) the dual role of T antigen as a promoter-dependent corepressor and coactivator or (ii) the differential effects of T antigen on CBP (FL) and HAT-CBP2. To investigate these possibilities, we first analyzed whether T antigen represses the transcriptional activity of HAT-CBP2 on the TK promoter. To this end, transient transfections into HeLa cells were performed using HAT and CBP2 (which contains the T antigen interaction region) domains of CBP fused to the Gal4 DNA-binding domain (GAL-HAT-CBP2). The transactivation activity due to the fusion protein was measured with a luciferase reporter vector containing five Gal4 DNA-binding sites upstream of the TK promoter (see Figure 1). T antigen repressed the transcriptional activity mediated by GAL-HAT-CBP2 (Figure 1A). Efficient repression depends on the presence of the CBP2 domain (T antigen-binding region), since the transcriptional activity of the GAL-HAT fusion protein lacking the T antigen-interacting region was only slightly (19%) repressed (Figure 1B). As previously reported, CBP (FL) activity on the TK promoter was also repressed by T antigen (11,12) (Figure 1C). These results indicate not only that T antigen may work as a repressor of CBP (FL) and HAT-CBP2, but also that it requires the T antigen-CBP interacting domain (CBP2).

These data also suggest that T antigen may play a dual role as a positive or negative regulator of CBP transcriptional activity in a promoter-dependent manner. To confirm this hypothesis, we analyzed whether T antigen acts in tandem with CBP (FL) on the hsp70 promoter to activate transcription. Figure 1D shows that overexpression of T antigen clearly increases the CBP-mediated transcriptional activity on the hsp70 promoter.

We wanted to better determine the molecular mechanisms involved in T antigen-mediated repression. To this end, we repeated the experiment in Figure 1A in the presence of the histone deacetylase inhibitor TSA. The results in Figure 2 show that the T antigen-induced repression of HAT-CBP2 activity is partially abolished (by 50–55%). These data suggest that histone deacetylase activity plays a role in T antigen-mediated repression.

Transcriptional repression of CBP by T antigen correlates with chromatin deacetylation

To assess whether histone deacetylation is involved in T antigen-mediated repression, we analyzed the chromatin acetylation status on the TK promoter using chromatin immunoprecipitation assays (ChIPs). To this end, HeLa cells were transfected with the TK promoter (containing Gal 4 binding sites) and Gal-CBP in the presence or absence of T antigen, as indicated in Figure 3A. Cross-linked and sonicated chromatin was immunoprecipitated using an antibody directed against acetylated histone H3 (lysines 9 and 14) and against acetylated histone H4 (lysines 5, 8, 12 and 16). The precipitated DNA was analyzed by PCR with primers

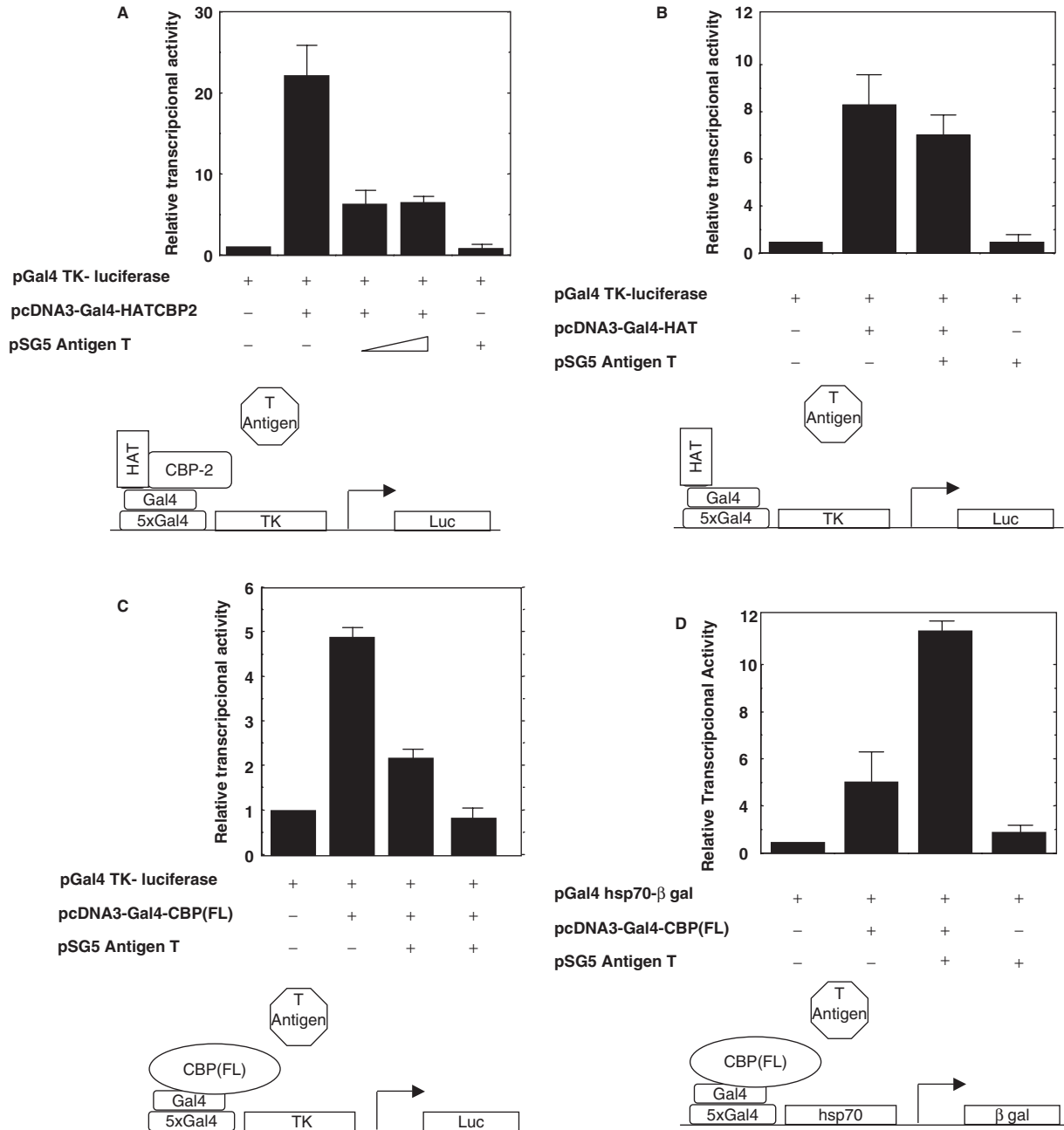


Figure 1. T antigen regulates CBP-mediated transcriptional activation. (A) HeLa cells were transfected with 2 μg of the Gal4-TK-luciferase reporter, 2 μg of pcDNA3-Gal-HAT-CBP2, 1 and 2 μg of pSG5-T antigen or the empty vector and 1 μg of renilla reporter vector. Whole cell extracts were used in the luciferase-renilla assay. The activity derived from the Gal4-TK-luciferase reporter was normalized to 1.0 and the other activities are expressed relative to this. The data represent an average of at least four independent transfections. (B) HeLa cells were transfected as in (A), but the pcDNA3-Gal-HAT expression vector was used instead of pcDNA3-Gal-HAT-CBP2. (C) HeLa cells were transfected with 2 μg of the Gal4-TK-luciferase reporter, 2 μg of pcDNA3-Gal-CBP (FL), 2 μg of pSG5-T antigen or the empty vector and 1 μg of renilla reporter vector. Whole cell extracts were used in the luciferase-renilla assay. The transcriptional activities are expressed as in (A). The data represent an average of at least four independent transfections. (D) HeLa cells were transfected with 2 μg of the Gal4-hsp70-βgal reporter, 2 μg of pcDNA3-Gal-CBP (FL), 2 μg of pSG5-T antigen or the empty vector and 1 μg of renilla reporter vector. Whole cell extracts were used in the β galactosidase and renilla assays. The activity derived from the Gal4-hsp70-βgal reporter was normalized to 1.0 and the other activities are expressed relative to this. The data represent an average of at least three independent transfections. A schematic representation of the constructs used is shown at the bottom of each figure.

spanning the TK promoter and the luciferase coding gene region. The resulting data were normalized relative to the 1% (unsaturated) input. The results shown in Figure 3A and B indicate that Gal-CBP activation (lane 2) correlates

with an increase (2–3 fold) in H3 acetylation (Figure 3A and B) as well as a slight increase in H4 acetylation (lane 2) (Figure 3A and B). The addition of T antigen (lane 3) leads to the clear deacetylation of both H3 and H4

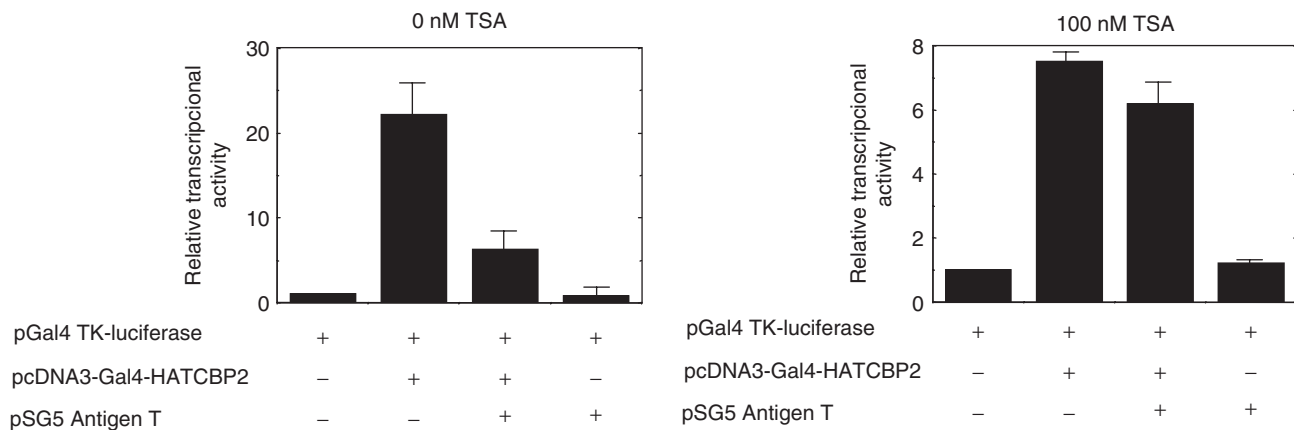


Figure 2. T antigen-mediated repression is TSA sensitive. HeLa cells were transfected as in Figure 1A and 100 nM TSA was added 18 h before collection. Whole cell extracts were prepared and used in the luciferase/renilla assay as indicated above. The data represent an average of at least four independent transfections.

to basal levels (Figure 3A and B). No PCR products were detected in extracts subjected to immunoprecipitation with non-specific antiserum (mock) (Figure 3A). These data confirm the previous hypothesis that histone deacetylation acts as a mechanism involved in T antigen repression.

Finally, we wanted to test whether histone deacetylation is involved in the T antigen-mediated repression of a natural promoter. To this end we analyzed the chromatin acetylation status on the PDGF β -receptor promoter. It has been previously shown (33) that T antigen represses this promoter in the absence of pRb and p53 in Saos-2 cells stably expressing T antigen, probably by interfering with the transcriptional activity of Sp1, which utilizes CBP as a coactivator. Thus, we first tested whether histone deacetylation was involved in T antigen-mediated repression of the PDGF β -receptor by testing the chromatin acetylation status on this promoter in CV1 and CV1COS cells. Figure 3C shows a clear decrease (4–5 fold) in H3 as well as a slight decrease (2 fold) in H4 acetylation in CV1COS compared with CV1 cells. No PCR products were detected in extracts subjected to immunoprecipitation with non-specific antiserum. These results suggest that histone deacetylation is involved in T antigen repression *in vivo*.

T antigen interacts with Histone Deacetylase 1 (HDAC1) *in vitro*

Based on our previous data, we investigated whether T antigen physically interacts with histone deacetylase enzymes. Thus, we therefore performed *in vitro* pull-down assays using 35-S-labeled T antigen. In these experiments, different bacterially expressed GST-HDACs (Figure 4A) were incubated with 35-S-labeled T antigen. Reaction mixtures were precipitated using GST-resin and the interacting 35-S-T antigen was detected radiographically. As shown in Figure 4B, HDAC1 clearly interacts with T antigen *in vitro*. Under the same experimental conditions, HDAC1 bound neither to an unrelated GST-protein fusion (data not shown), nor to GST (Figure 4B). It has been previously shown that T antigen interacts with

retinoblastoma products (pRb), thereby inducing cell-cycle progression. On the other hand, several reports have shown that pRb interacts with HDAC1 to mediate transcriptional repression on gene promoters involved in the G1/S phase transition of the cell cycle (34). Thus, we decided to assess whether the T antigen-HDAC1 interaction that we detected was mediated by pRb. To this end, we repeated the previous experiments using two well-characterized T antigen mutants that do not bind pRb (T antigen K1 and PVU-1, see Methods). Figure 4B shows clear T antigen K1, PVU-1 and HDAC1 interacting signals.

We then sought to determine whether T antigen could bridge CBP and HDAC1 cofactors. To this end, GST-CBP was bound to 35-S-labeled T antigen using an *in vitro* pull-down assay. The complex GST-CBP-T antigen as well as the recombinant GST-CBP were incubated with 35-S-labeled HDAC1. The reaction mixtures were precipitated using GST-resin and the interacting HDAC1 was detected either radiographically or via western blot (data not shown). Figure 4C shows that T antigen is capable of bridging CBP and HDAC1. It is worth noting that in the absence of T antigen, GST-CBP interacts weakly with HDAC1. However, previously bound T antigen clearly increases this interaction, suggesting that T antigen helps to recruit HDAC1 to the CBP-regulated promoters.

Afterwards, we analyzed whether T antigen and HDAC1 also interact *in vivo* by conducting co-immunoprecipitation experiments using CV1COS cells, and by two-hybrids analysis, however, no clear interaction was observed.

HDAC1 cooperates with T antigen to antagonize the transcriptional activity of CBP

Our previous results showed that T antigen not only represses the transcriptional activity of CBP via histone H3 deacetylation, but that it also interacts with HDAC1 *in vitro*. We therefore tested the ability of HDAC1 to cooperate with T antigen in repressing transcription and decreasing histone H3 and H4 acetylation levels. To this

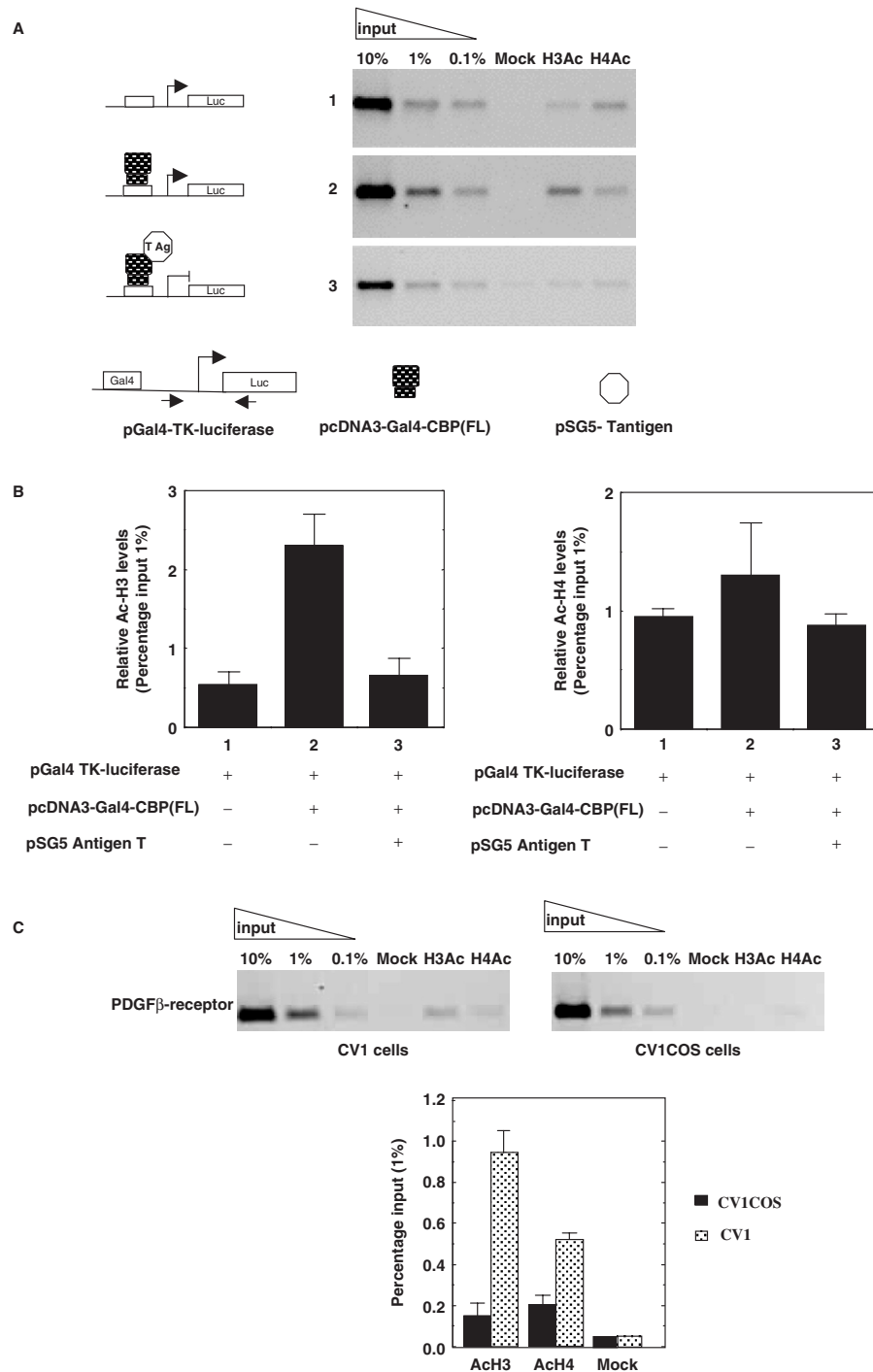


Figure 3. T antigen repression correlates with histone H3 deacetylation. (A) HeLa cells were transfected with 2 μ g of Gal4-TK-luciferase reporter (lane 1), 2 μ g of Gal-CBP (FL) (lane 2) and 2 μ g of pSG5-T antigen (lane 3) and comparative ChIP analysis were performed in parallel with the same number of cells using antibodies that specifically recognize acetylated histone H3 (K 9 and 14) and poly-acetylated histone H4. The immunoprecipitates were analyzed by quantitative PCR using specific primers for the TK promoter. PCR products were resolved in agarose gels and quantified with QuantityOne software (Bio-Rad). A schematic representation of the TK-luciferase promoter and the position of the primers used for ChIP are shown at the bottom of the figure. (B) Quantification of PCR products from (A) was conducted as described in Materials and Methods. The values on the y axis represent the amount of immunoprecipitated DNA as a percentage of the 1% input sample. Relative histone modification levels were calculated from three independent experiments. (C) Comparative ChIP analysis performed in parallel with the same number of CV1 and CV1COS cells using antibodies that specifically recognize acetylated histone H3 (K 9 and 14) and poly-acetylated histone H4. The immunoprecipitates were analyzed by quantitative PCR using specific primers for the PDGF β -receptor promoter. PCR products were resolved in agarose gels and quantified as described in (B). Relative histone modification levels were calculated from two independent experiments.

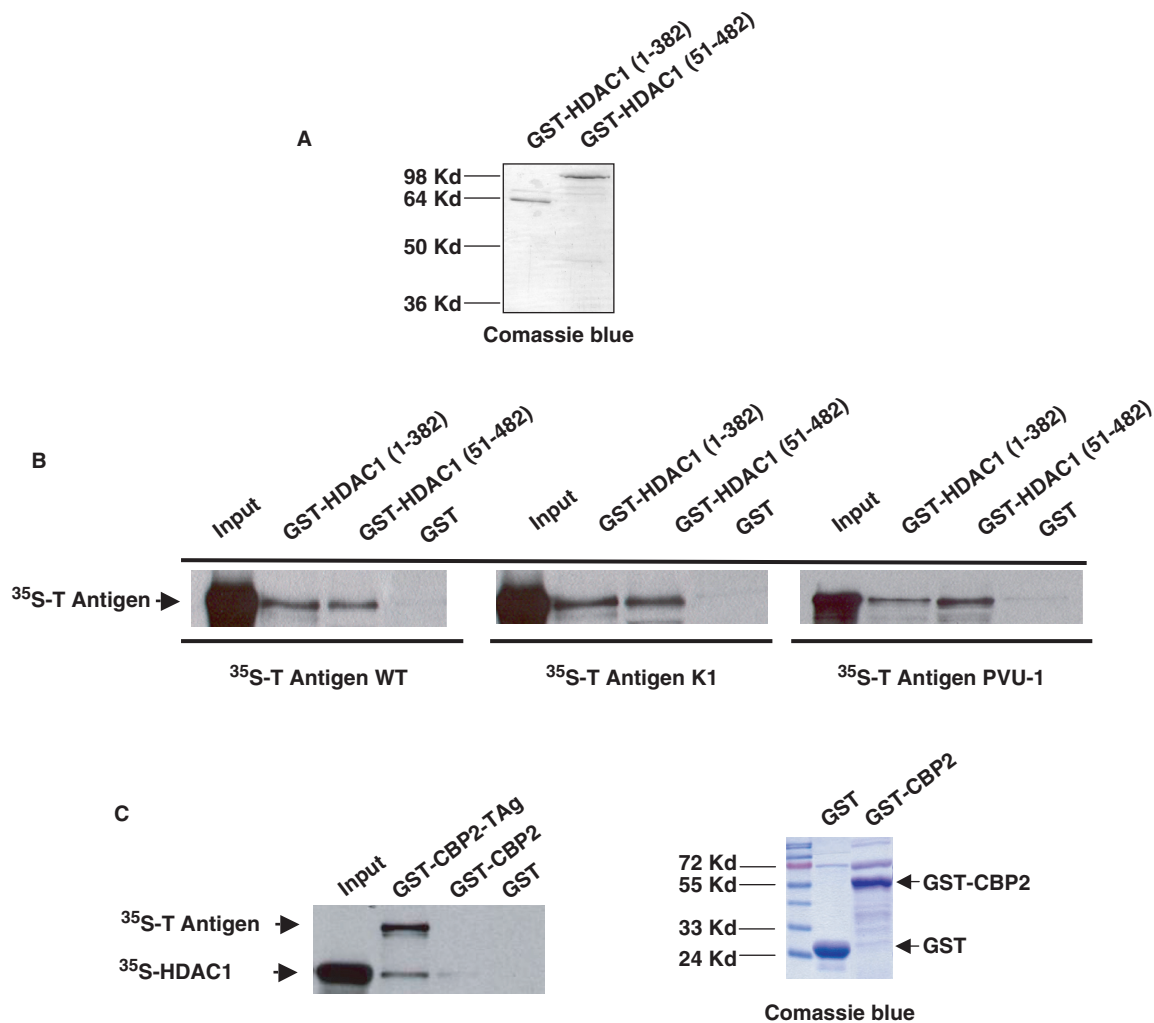


Figure 4. T antigen interacts with HDAC1 *in vitro*. (A) Protein gel of GST-HDAC1(51-482) and GST-HDAC1(1-382) fusion proteins expressed in *E. coli* and used in the *in vitro* pull-down experiment in (B). (B) GST, GST-HDAC1(1-382) and GST-HDAC1(51-482) fusion proteins were expressed in *E. coli*. Binding of the fusion proteins to T antigen WT and pRb-binding mutants [K1 (E107K) and PVU-1 (dl-107-112Y)] *in vitro* was determined using an *in vitro* pull-down experiment (see Material and Methods). Input represents 50% of total T antigen input. The arrow indicates the T antigen or T antigen mutant's position in the gel. (C) ³⁵S-labeled T antigen was bound to the GST-CBP2 fusion protein. Binding of the complex GST-CBP2-TAg, GST-CBP2 and GST to ³⁵S-labeled HDAC1 *in vitro* was determined by an *in vitro* pull-down experiment (left panel). The position of T antigen and HDAC1 in the gel is indicated with arrows. Protein gel of GST-CBP2 and GST proteins expressed in *E. coli* and used in the *in vitro* pull-down experiment (right panel).

end, transient transfections into HeLa cells were performed using Gal-CBP, T antigen and the TK promoter fused to the luciferase reporter gene (as described above) overexpressing (or not) HDAC1. T antigen repression and histone acetylation levels were subsequently analyzed by luciferase and ChIPs assays, respectively. Our results show that overexpression of HDAC1 results in increased T antigen-induced transcriptional repression (Figure 5A), as well as in decreased histone H3 and H4 acetylation levels, even under basal acetylation conditions (lane 4) (Figure 5B). This process is concomitant with HDAC1 recruitment to the promoter (lanes 3 and 4) (Figure 5B). Only a partial transcriptional repression (24%) by HDAC1 alone was observed (Figure 5A, lane 5), consistently with the ability of HDAC1 to interact weakly with CBP (see Figure 4C).

To further analyze the necessity of HDAC1 for mediating T antigen repression *in vivo*, we performed transcriptional assays (as indicated above) in the presence of small interfering RNAs (siRNAs), which partially block the expression of HDAC1 (HDAC1-siRNA) following an extended expression period (Figure 5D) and control siRNA (c-siRNA). The results in Figure 5C show that in the presence of HDAC1 siRNA, T antigen-mediated repression is partially reduced (from 60 to 30%). siRNA control did not affect T antigen-induced repression. The T antigen expression levels were not affected by the presence of the siRNAs (Figure 5E). Based on these results, we would suggest that HDAC1 contributes to T antigen-mediated repression *in vivo*, although we cannot discard the participation of other deacetylases or co-repressors in this process.

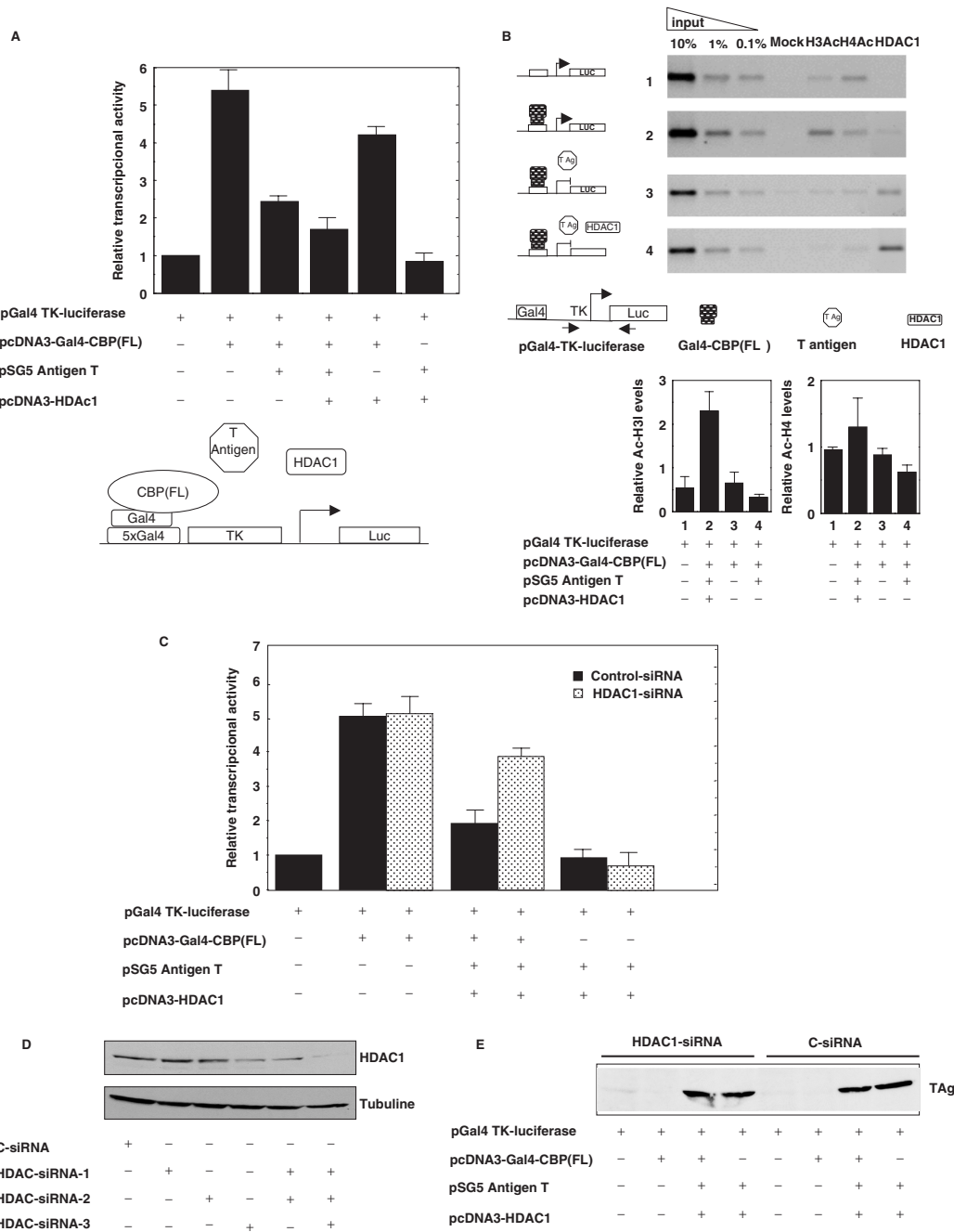


Figure 5. HDAC1 acts in tandem with T antigen to antagonize the transcriptional activity of CBP. **(A)** HeLa cells were transfected with 2 μ g of the Gal4-TK-luciferase reporter, 2 μ g of Gal-CBP (FL), 2 μ g of pSG5-T antigen, 1 μ g of pcDNA3-HDAC1 and 1 μ g of renilla reporter vector. Whole cell extracts were used in the luciferase-renilla assay. The activity derived from the Gal4-TK-luciferase reporter was normalized to 1.0 and the other activities are expressed relative to this. The data represent an average of at least five independent transfections. A schematic representation of the constructs used is shown at the bottom of the figure. **(B)** HeLa cells were transfected with 2 μ g of Gal4-TK-luciferase reporter (lane 1), 2 μ g of Gal-CBP (FL) (lane 2), 2 μ g of pSG5-T antigen (lane 3) and 1 μ g of pcDNA3-HDAC1 (lane 4) and comparative ChIP analysis was performed in parallel with the same number of cells using antibodies that specifically recognize acetylated histone H3 (K 9 and 14), poly-acetylated histone H4 and acetyl-H4 ChIPs. The immunoprecipitates were analyzed by quantitative PCR as in Figure 3A. Quantification of the acetyl-H3 and acetyl-H4 ChIPs bands (as described in Materials and Methods) is shown at the bottom part of the figure. **(C)** HeLa cells were transfected with 2 μ g of the Gal4-TK-luciferase reporter, 1 μ g of renilla reporter, 2 μ g of Gal-CBP (FL), 2 μ g of pSG5-T antigen and 1 μ g of pcDNA3-HDAC1 together with 8 μ g of HDAC1(1, 2 and 3) or control siRNA vectors. Total cell extracts were prepared 48 h after transfection and used in the luciferase-renilla assay. The activity derived from the Gal4-TK-luciferase reporter in the presence of the control siRNA vectors was normalized to 1.0 and the other activities are expressed relative to this. The diagrams show the relative protein levels obtained from three independent experiments. **(D)** HeLa cells were transfected with 8 μ g of control siRNA or different HDAC1 siRNA (1, 2 or 3) alone or in combination. Total cells extracts were prepared 80 h after transfection and the levels of endogenous HDAC1 protein were detected by western blot analysis. The decrease of HDAC1 levels reached 90%; however, when the HDAC1 levels were analyzed 48 h after transfection the observed decrease was modest (20–30%) (data not shown). The experiment in (C) was performed 48 h after transfection due to the low activation of TK promoter by Gal-CBP after that time. **(E)** HeLa cells were transfected as in (A) and the levels of T antigen protein in the presence of control siRNA or HDAC1 siRNA were analyzed by Western blot.

Table 1. Summary of T Antigen repression

Sensitivity To TSA	H3 Deacetylation	H4 Deacetylation	HDAC1 Overexpression	HDAC1 SiRNA	T Ag- HDAC1 interaction
++	++	+/-	+	+	+ (<i>in vitro</i>)

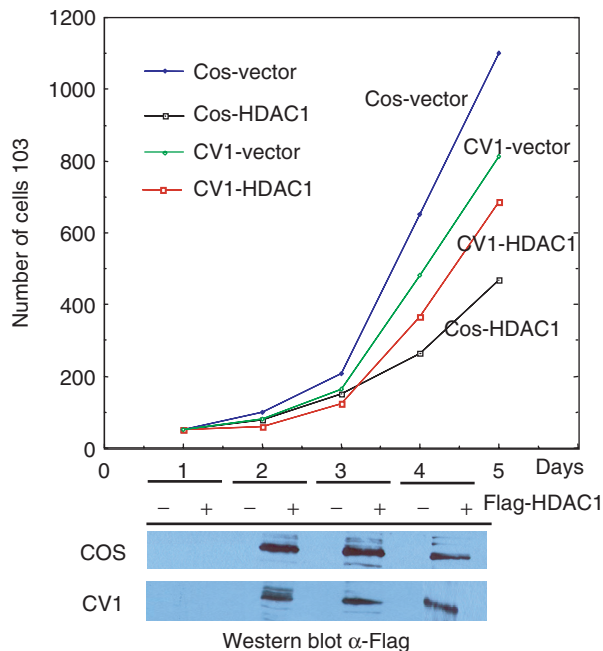


Figure 6. HDAC1 levels affect cell growth. Doubling times of CV1 and CV1COS cells transfected with 2 μ g of pcDNA3-Flag-HDAC1, or the vector alone, were measured as described in the Material and Methods section. Doubling time and the Flag-HDAC1 levels were determined by counting cells every day and by western blot analysis, respectively. Results shown represent the mean \pm SD of three independent experiments, in which each sample was analyzed in triplicate.

Following CV1 SV40 infection, T antigen induces the stimulation of cell growth for which it requires CBP interaction (3). As our results suggest that CBP-mediated transcriptional activity may be repressed by T antigen, we decided to analyze whether HDAC1 levels could affect T antigen-stimulated cell growth. To this end, CV1 and CV1COS cells were transfected with Flag-HDAC1, or with the vector alone. We then analyzed the doubling time of the cells as well as Flag-HDAC1 levels over four days after transfection. The results (Figure 6) show a clear decrease in cell growth in CV1COS cells, suggesting that HDAC1 levels affect T antigen-induced cell growth stimulation.

DISCUSSION

In this report, we have shown that T antigen represses CBP-mediated transcriptional activity. This repression is concomitant with histone H3 deacetylation and is TSA sensitive. On the other hand, our results indicate that T antigen interacts with HDAC1 *in vitro* and overexpression of HDAC1 not only acts in tandem with

T antigen to antagonize CBP transactivation function, but also that it correlates with chromatin deacetylation on the TK and PDGF β -receptor promoters. Finally, decreasing the HDAC1 levels by use of siRNAs partially abolishes the repression induced by T antigen. The results from these experiments are summarized in Table 1.

Our data suggest that acetylase and deacetylase activities play key roles in the viral growth promoted by T antigen, revealing that the latter interacts with both HAT (11,12,22) and HDAC activities. Given that they have opposite roles in transcriptional regulation, the interaction of these two activities through T antigen might appear paradoxical. However, we believe that T antigen stimulates growth by altering gene expression patterns; thus, the activation of one gene set might be as necessary as the repression of another.

The possible presence of both activities in one complex might facilitate more efficient and rapid changes in promoter acetylation. In fact, recent studies have shown that HAT and HDAC may be found in the same complexes. For example, HDAC1 may interact with the HAT enzymes p300 (35) or P/CAF (36), leading to greater control of global histone acetylation inside the cell. Another possibility is that HAT and HDAC reciprocally modulate their enzymatic activity or T antigen function by cross-acetylation/deacetylation. In this context, we must mention that T antigen exists as an acetylated protein *in vivo*. p53 targets T antigen for acetylation by CBP (2). HDAC-T antigen interaction could favor T antigen deacetylation, thereby changing its regulatory capabilities.

Other viral proteins have also been found to associate with HDAC activities [E7 interacts with the Mi2-NRD complex (28)], and in particular with the HDAC1 enzyme, such as the adenovirus-2 E1B-55K protein (25) and the Epstein-Barr virus nuclear antigen 3C (27). As well as the retroviral Tax (37,38) and Tat (39–41) proteins, the T antigen viral oncoprotein can associate with both HDAC and HAT enzymes. This suggests that the control of acetylation/deacetylation equilibrium is a critical step in viral-induced cell transformation.

Our results suggest that acetylase and deacetylase activities play key roles in the viral growth promoted by T antigen. Indeed, our findings show that a viral oncoprotein may manipulate gene expression by interacting with both acetyltransferase and deacetylase activities. These observations underscore the relevance of both the acetylation/deacetylation pathways as well as chromatin status, in cell proliferation and viral infection.

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