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Acetylation of HIV-1 Tat by CBP/P300 Increases Transcription of Integrated HIV-1 Genome and Enhances Binding to Core Histones

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The HIV-1 Tat protein is required for viral replication and is a potent stimulator of viral transcription. Although Tat has been extensively studied in various reductive paradigms, to date there is little information as to how this activator mediates transcription from natural nucleosomally packaged long terminal repeats. Here we show that CREB-binding protein (CBP)/p300 interacts with the HIV-1 Tat protein and serves as a coactivator of Tat-dependent HIV-1 gene expression on an integrated HIV-1 provirus. The site of acetylation of Tat was mapped to the double-lysine motif in a highly conserved region, ^{49}RKKRRQ^{54}, of the basic RNA-binding motif of Tat. Using HLM1 cells (HIV-1^{+}/Tat^{-}), which contain a single copy of full-length HIV-1 provirus with a triple termination codon at the first AUG of the Tat gene, we find that only wild type, and not K50A, K51A, or K50A/K51A alone or in combination of ectopic CBP/p300, is able to produce full-length infectious virions, as measured by p24 gag ELISAs. Tat binds CBP/p300 in the minimal histone acetyltransferase domain (1253–1710) and the binding is stable up to 0.85 M salt wash conditions. Interestingly, wild-type peptide 41–54, and not other Tat peptides, changes the conformation of the CBP/p300 such that it can acquire and bind better to basal factors such as TBP and TFIIB, indicating that Tat may influence the transcription machinery by helping CBP/p300 to recruit new partners into the transcription machinery. Finally, using biotinylated wild-type or acetylated peptides, we find that acetylation decreases Tat's ability to bind the TAR RNA element, as well as to bind basal factors such as TBP, CBP, Core-Pol II, or cyclin T. However, the acetylated Tat peptide is able to bind to core histones on a nucleosome assembled HIV-1 proviral DNA.

INTRODUCTION

The HIV-1 Tat protein is required for viral replication and is a potent stimulator of viral transcription. Tat stimulates viral gene expression through an RNA element in the viral long terminal repeat (LTR). For optimal transactivation of HIV-1 gene expression, Tat requires specific upstream transcription factors, including Sp1 (Jeang *et al.,* 1993), TATA-binding protein (Kashanchi *et al.,* 1994b; Veschambre *et al.,* 1995; Majello *et al.,* 1998), Tat-associated kinase (Herrmann and Rice, 1995; Yang *et al.,* 1996), TFIIH (Garcia-Martinez, *et al.,* 1997; Parada and Roeder, 1996), Tip (Jeang *et al.,* 1993; Henderson *et al.,* 1999), and RNA polymerase II (Cujec *et al.,* 1997; Mavankal *et al.,* 1996). The ability of Tat to regulate viral transcription is related to its ability to interact with the basal transcription complexes responsible for the initiation of transcription including cyclinT_1/cdk9 complex, resulting in a more efficient elongating RNA Pol II complex (O'Keeffe *et al.,* 2000; Romano *et al.,* 1999; Napolitano *et al.,* 1999; Isel and Karn, 1999; Bieniasz *et al.,* 2000; Ramanathan *et al.,* 1999; Ivanov *et al.,* 1999; Chen *et al.,* 1999a; Wimmer *et al.,* 1999; Garriga *et al.,* 1998; Garber *et al.,* 1998; Fujinaga *et al.,* 1998; Wei *et al.,* 1998).

Among the factors associated with basal transcription complexes, CBP (CREB-binding protein) and p300 have emerged as coactivators for various DNA-binding transcription factors. CBP and p300 are large proteins, 2441 and 2414 amino acids, respectively, that have the ability to interact simultaneously with various transcription factors such as nuclear hormone receptors, CREB, c-Jun, v-Jun, c-Myb, v-Myb, Sap-1a, c-Fos, MyoD, YY1, NF-κB, and p53 (Goldman *et al.,* 1997) and with other coactivators such as P/CAF (Blanco *et al.,* 1998; Chakravarti *et al.,* 1999), as well as with basal components of the transcriptional apparatus. Therefore, it is this wide array of functions that have allowed CBP/p300 proteins to be important transcriptional integrators (Shikama *et al.,* 1999). In recent years multiple mechanisms have emerged for the function of CBP/p300. The first mechanism for CBP/p300 activation involves the acetylation of the terminal tails of the core histones by histone acetyltransferase (HAT) and destabilization of histone–DNA interactions, allowing transcription factors access to the promoter region. The second mechanism by which CBP has been suggested to be an activator of transcription is by bridging the gap between upstream DNA-bound transcription factors and

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components of the general transcription machinery. The third possible mechanism is CBP/p300's ability to directly acetylate nonhistone proteins such as p53 (Gu and Roeder, 1997), the erythroid Kruppel-like factor (EKLF, Zhang and Bieker, 1998), the nuclear hormone receptor coactivators ACTR (Chen *et al.,* 1997), and the basal transcription factor TFIIE and TFIIF (Imhof *et al.,* 1997; Martinez-Balbas *et al.,* 1998). In the case of p53, acetylation of the regulatory domain led to a dramatic increase in DNA binding *in vitro,* whereas the acetylation of ACTR by CBP/p300 disrupts the receptor–coactivator interaction, which plays a key role in hormone-induced gene activation (Chen *et al.,* 1999b).

HIV-1 proviral DNA is integrated into host cell chromosomes and packaged into chromatin. The LTR acts as a very strong promoter when analyzed as naked DNA *in vitro* and is silent when integrated into the cellular host genome in the absence of any stimuli (Verdin, 1991; Adams *et al.,* 1994; Van Lint *et al.,* 1996; Marzio *et al.,* 1998; Benkirane *et al.,* 1998). Recently, several reports have shown the existence of an intracellular multiprotein complex that contains Tat, CBP/p300, and P/CAF. It was found that the histone acetyltransferase activity of CBP/p300 and P/CAF is preferentially required for Tat function (Kiernan *et al.,* 1999). CBP/p300 was also recently reported to interact with the HIV-1 Tat protein and serves as a coactivator of Tat-dependent HIV-1 gene expression (Hottiger *et al.,* 1998; Ott *et al.,* 1999). This superinduction has been attributed to the histone acetyltransferase (HAT) activity of CBP/p300 on the integrated HIV-1 promoter.

In this study, we find that Tat is acetylated by CBP/p300 and mapped to the double-lysine motif in a highly conserved region (^{49}RKKRRQ^{54}) of the Tat protein. Using HLM1 (HIV-1^{+}/Tat^{-}) cells, which contain a single copy of full-length HIV-1 provirus with a triple termination codon at the first AUG of the Tat gene, we find that only wild type, and not K50A, K51A, or a double-mutant K50A/K51A alone or in combination with excess CBP/p300, is able to produce full-length infectious virions. Furthermore, mechanistically, the wild-type Tat peptide 41–54, which contains the basic core domain of HIV-1 Tat, changes the conformation of CBP/p300 such that basal factors such as TBP and TFIIB bind better to CBP/p300, indicating that Tat may influence the transcription machinery by helping CBP/p300 to acquire new partners in the transcription machinery.

Tat binds to CBP/p300 minimal HAT domain (1253–1710) and is a stable complex up to 0.85 M salt wash conditions. Acetylation of Tat by CBP/p300 decreases Tat's ability to bind the TAR RNA element *in vitro.* Finally, using biotinylated wild-type or acetylated Tat peptides, we find that acetylation causes a release of Tat from basal factors such as TBP, CBP, or cyclin T. Interestingly, the acetylated Tat peptide is able to bind with higher affinity to core histones on nucleosomal DNA.

RESULTS

HIV-1 Tat is acetylated by the HAT domain of CBP/p300

We initially asked whether a minimal HAT domain of CBP/p300 was capable of acetylating the Tat protein *in vitro.* To answer this question, we constructed a minimal GST–HAT plasmid from a full-length p300 cDNA clone, expressed in *Escherichia coli* and purified using glutathione–agarose beads. When using GST–HAT in a [^{14}C]acetyl coenzyme A exchange reaction, we found that GST–HAT was capable of efficient acetylation of core histones H2A, H2B, H3, and H4 *in vitro* (Fig. 1A, lane 3). We also observed efficient acetylation of purified Tat protein *in vitro,* as shown in Fig. 1A, lane 4. Products shown in Fig. 1A are ^{14}C-acetylated polypeptides that had been separated on 4–20% SDS–PAGE, dried, and exposed to a PhosphorImager cassette. The bottom panel of Fig. 1A shows the Coomassie blue staining of the same gel. It is important to note that we have consistently observed a more efficient acetylation of Tat proteins that do not contain a GST moiety at their N-terminus. Similar efficient *in vitro* acetylations were also observed with histidine- and epitope (influenza)-tagged peptides at the N- or C-terminus of the Tat protein (data not shown).

We next examined the effect of various Tat peptides as substrates in the *in vitro* HAT assay. The results of such an experiment are shown in Fig. 1C, where Tat 41–54 peptide, but not 41–50 peptide (lanes 7–10), was acetylated with GST–HAT. Similar to Fig. 1A, results shown in Fig. 1C are products separated on 4–20% SDS–PAGE, dried, and exposed to a cassette. It is important to note that we have observed reproducible results only when using [^{14}C]acetyl CoA and SDS–PAGE (4–20%) for separation purposes as opposed to [^{3}H]acetyl CoA and filter disks for detection of acetylated small peptides. Peptides of such short lengths do not reproducibly bind to DE52 filter papers and cannot stand rigorous wash conditions. A summary of all the Tat peptides used in the HAT assay is shown in Fig. 1D. It is important to note that peptides such as 65–86, which contains lysine residues, were not acetylated, indicating that the *in vitro* acetylation by the CBP/P300 HAT domain is not a nonspecific reaction. Finally, we used wild-type full-length human HeLa p300 (hp300, a generous gift from R. Shiekhattar), or epitope-tagged recombinant p300 (rp300), and observed efficient acetylation of the Tat protein *in vitro* (Fig. 1E).

Site of Tat acetylation by the HAT domain

The Tat peptide 41–54 contains three lysine residues, one at position 41 and the other two at positions 50 and 51. To determine which lysine residues in the 41–54 peptide were acetylated by GST–HAT, we initially made acetylated 41–54 peptides with acetyl groups at posi-

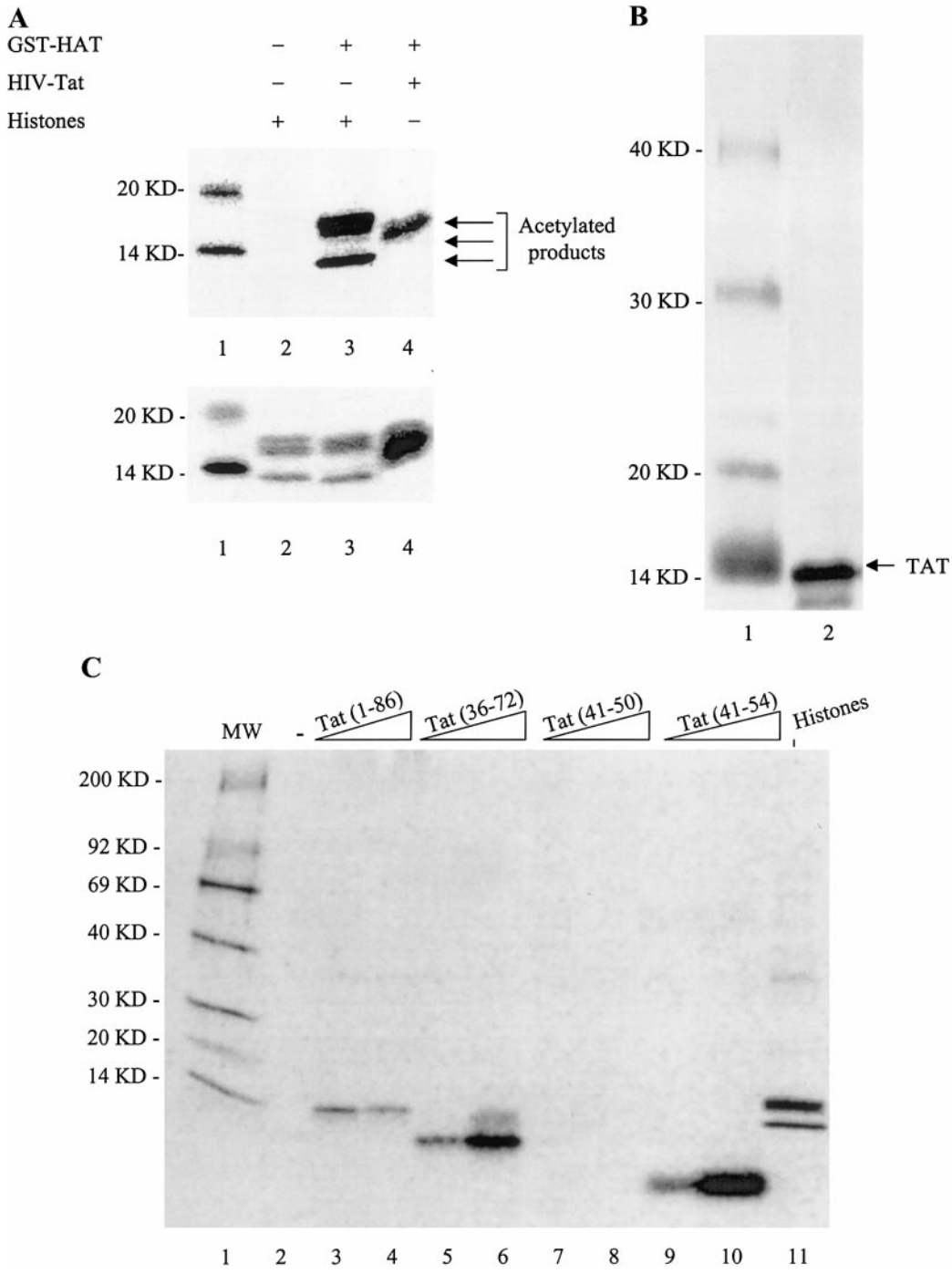


FIG. 1. Acetylation of HIV-1 Tat protein by CBP/p300 *in vitro*. (A, B) The core histones H2A, H2B, H3, and H4 (lanes 2 and 3) and Tat protein (1–86, lane 4) were incubated with or without GST–p300 (HAT domain) and [^{14}C]acetyl-CoA. (A) Acetylated products (lanes 3 and 4) resolved on 4–20% SDS–PAGE, dried, and exposed to a PhosphorImager cassette. (A, bottom) Coomassie blue staining of the gel shown at top. B represents the purified full-length Tat protein (1–86, 1 μg) used in the HAT assay, resolved on a SDS–PAGE gel, and silver stained (31). (C) Tat acetylation site located at the basic RNA-binding domain. Synthesized Tat peptides covering various regions of the Tat protein (lane 3–11) were incubated with GST-p300 and [^{14}C]acetyl CoA and analyzed on 4–20% SDS–PAGE. Lane 2 serves as negative control with no substrate added to the reaction, and lanes 3, 4, and 11 serve as positive controls, where full-length Tat and core histone proteins were added to the reaction. All other reactions were performed with two concentrations (200 and 400 ng) of various peptides. (D) The schematic representation of Tat protein and Tat peptides used in this study and the results of acetylation by GST–p300. (E) Acetylation of HIV-1 TAT by wild-type p300/CBP. (Left) Acetylation of TAT or all four histones with 10 ng of HeLa purified CBP/p300 complexes (hp300). Proteins, BSA, wild-type TAT, or histones were incubated with HeLa CBP/p300, followed by incubation at 37°C for 1 h, spotted on DE52 filter discs, washed, and counted. (Right) A similar experiment with either TAT or free histones incubated with 10 ng of purified epitope-tagged recombinant Baculovirus p300 (rp300). Samples were incubated at 37°C for 1 h and subsequently run on 4–20% SDS–PAGE, dried, and exposed to a PhosphorImager cassette.

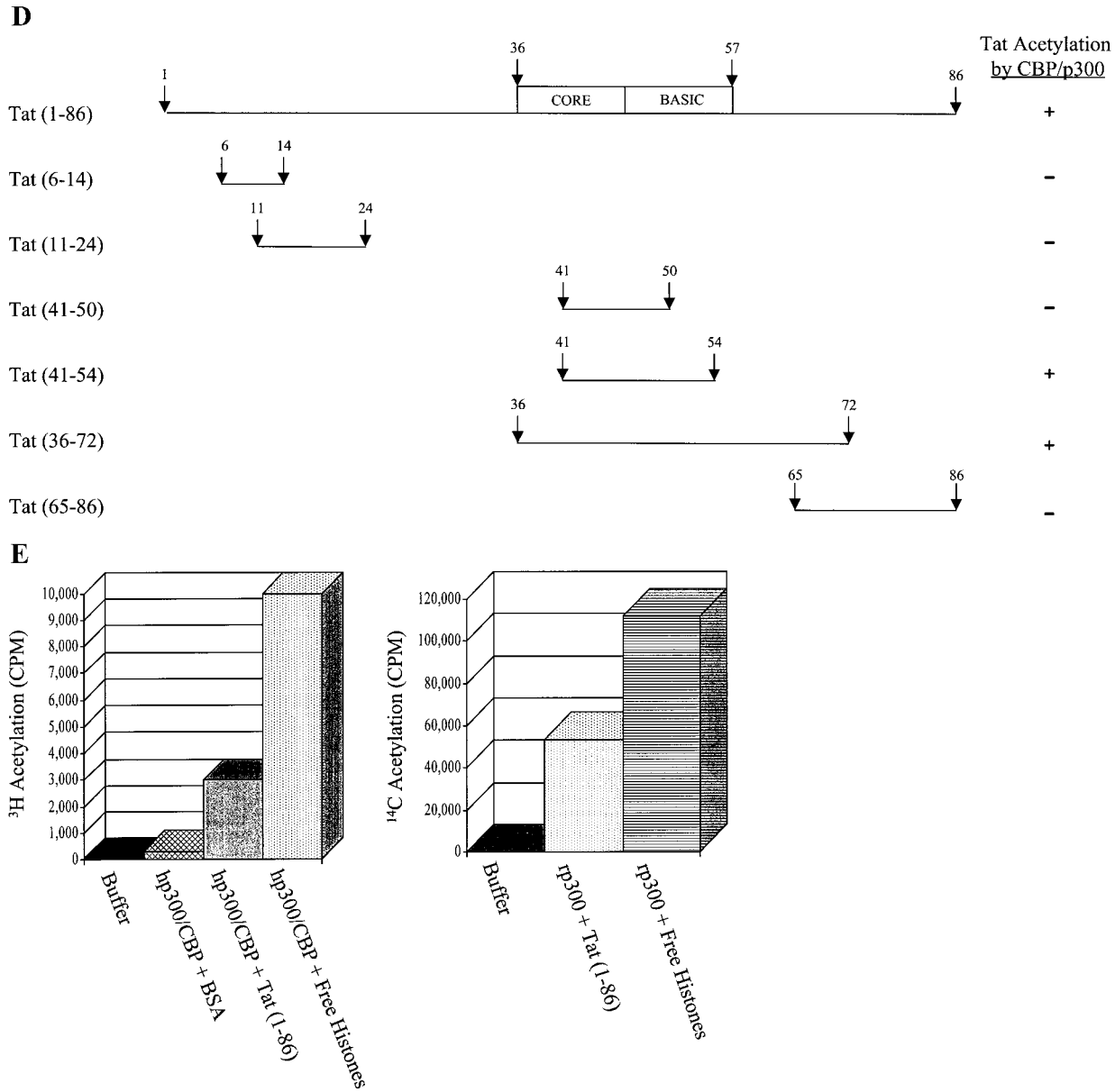


FIG. 1—*Continued*

tions 50, 51, or 50+51. The acetyl groups were put on the peptides during the chemical peptide synthesis. All peptides were subsequently purified on C18 reverse-phase HPLC and dried, and quantitations were determined by protein assay as well as by running small aliquots on 4–20% SDS–PAGE followed by silver stain detection. As can be seen in Fig. 2A, all peptides were efficiently acetylated with GST–HAT, except the double-acetylated 41–54 (lanes 9 and 10). The doubly acetylated 41–54 peptide was, however, very weakly acetylated at lysine 41 position, at a 10-fold excess of GST–HAT concentrations *in vitro,* and similar results were also obtained with a longer peptide, 36–58 (data not shown). Therefore, it may be possible that all three lysines are acetylated, although at very different kinetic rates, depending on the enzyme concentrations used in these assays. To determine which lysine, 50 or 51, was acetylated efficiently *in vitro,* we synthesized a second batch of acetylated peptides starting from position 42–54. The results of such an experiment are shown in Fig. 2B, where peptide 42–54, which had already been acetylated at position 50, was a poor substrate for accepting the new acetyl group at position 51 (lanes 3 and 4). However, the peptide that was acetylated at position 51 could serve as an excellent substrate for acetylation *in vitro.* The doubly acetylated 42–54 peptide could not be acetylated at any concentration of GST–HAT (data not shown). We next examined the effect of Tat acetylation on cells expressing Tat. HeLa/eTat and HeLa/pcep4 lines contain either an epitope-tagged Tat at the C-terminus (HeLa/eTat) or a control

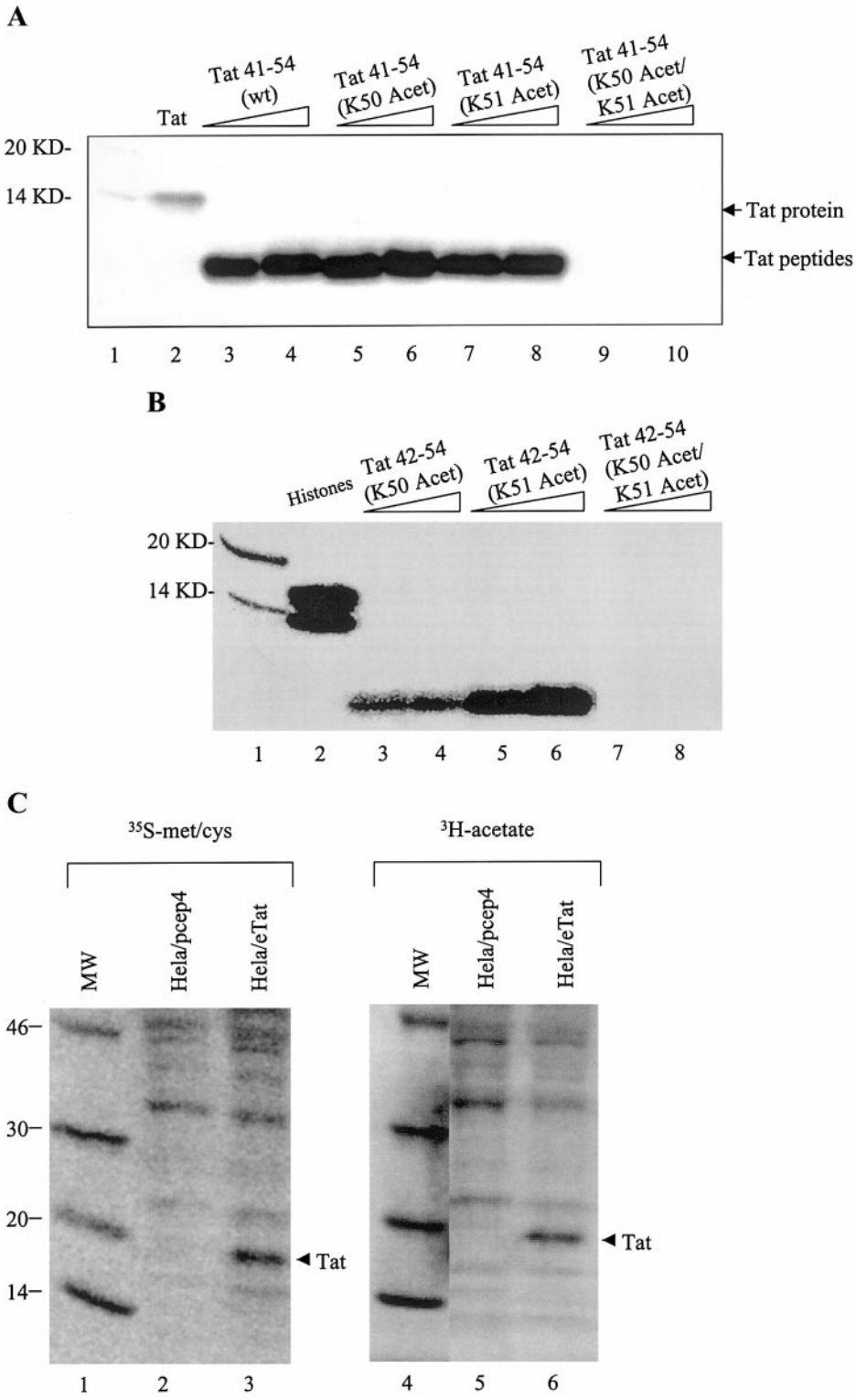


FIG. 2. Tat acetylation sites mapped to double-lysine motifs K50 and K51 in the basic RNA-binding domain. (A, B) Two concentrations (200 and 400 ng) of the Tat peptides (41–54 or 42–54), wild-type or acetylated at lysines position 50, 51, and 50+51, were incubated with GST–p300 and [^{14}C]acetyl CoA, separated on 4–20% SDS–PAGE, and exposed to a PhosphorImager cassette. (C) Both log-phase HeLa/eTat or HeLa/pcep4 cells were labeled with [^{3}H]acetate in DMEM complete medium plus hygromycin (left) or incubated overnight with [^{35}S]methionine/cysteine (right). Nuclear lysates were used for immunoprecipitations on cross-linked monoclonal 12CA5 Ab beads and eluted with a 100-fold excess of influenza peptide. The [^{35}S]methionine/cysteine gel was exposed overnight and the [^{3}H]acetate gel was exposed for 1 week on the PhosphorImager cassette.

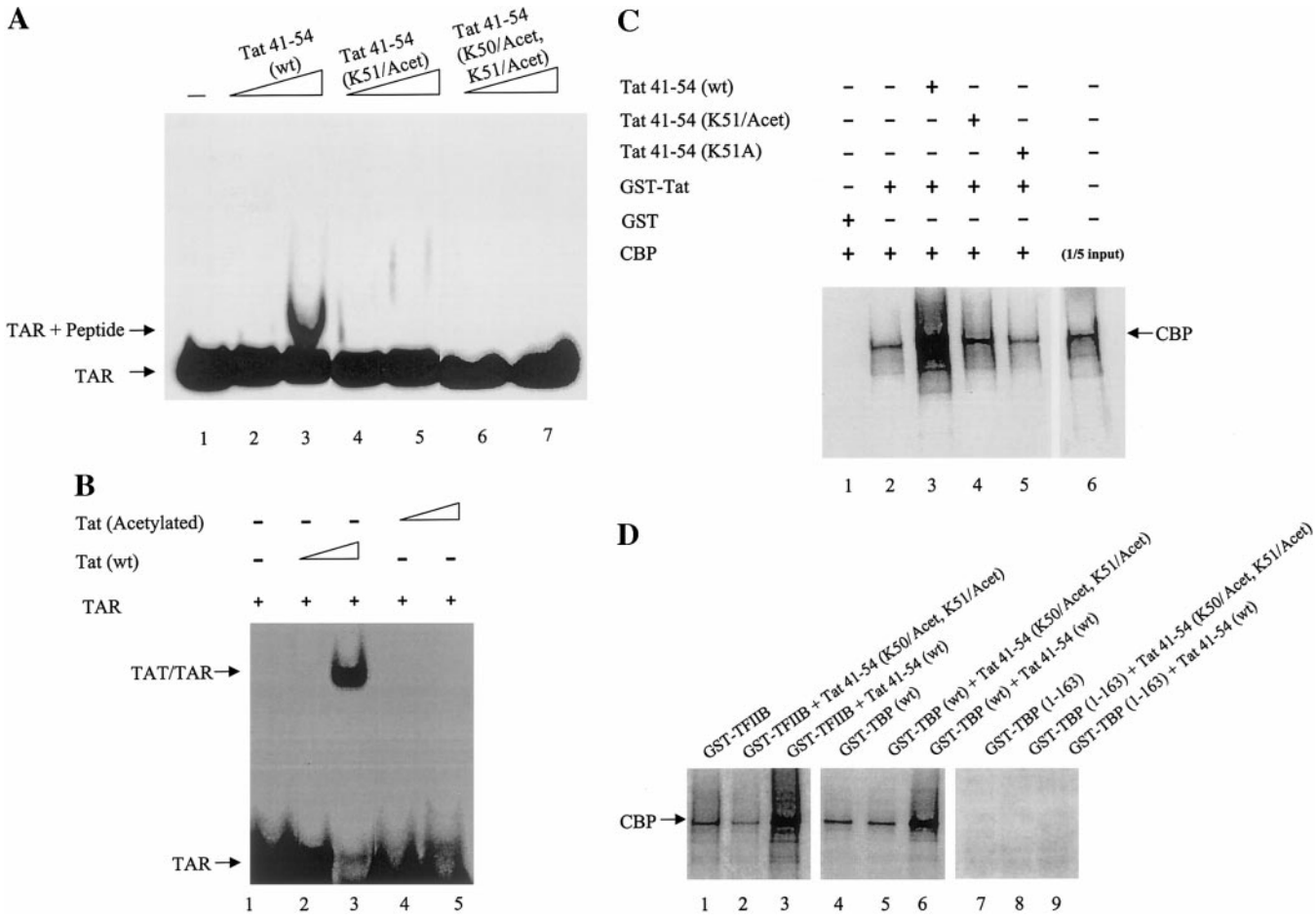


FIG. 3. Acetylated Tat decreases its ability to HIV-1 TAR RNA. (A) The wild-type Tat peptide 41–54 (lanes 2 and 3) and the acetylated Tat peptide 41–54 (lanes 4–7) were incubated with ^{32}P-labeled TAR RNA at room temperature for 30 min and separated on a 6% DNA retardation polyacrylmide gel (Novex). Lane 1 contains TAR RNA alone and lanes 2–7 indicate TAR plus various wild-type or acetylated peptides (2.5 and 5 μg). (B) Wild-type (lanes 2 and 3, 0.5 and 1.0 μg, respectively) or GST–HAT *in vitro* acetylated Tat proteins (1–86, lanes 4 and 5, 0.5 and 1.0 μg, respectively) were incubated with TAR RNA, resolved an a 6% DNA retardation gel, dried, and exposed to a PhosphorImager cassette. (C, D) Tat peptide (41–54) increases the binding of CBP to TBP, TFIIB, and Tat. Tat peptides, wild type or various modified versions (10 μg), were preincubated with ^{35}S-labeled CBP (Promega TNT, 10 μl) for 10 min and subsequently used to bind to 500 ng eluted GST–Tat, GST–TFIIB, GST–TBP (wt), or GST–TBP (1–163, deletion from 164 to 337 of human TBP). After several washes (3×), the bound proteins were resolved by 4–20% SDS–PAGE, dried, and exposed.

backbone vector (HeLa/pcep4). Both cell types have been described previously (Kashanchi *et al.,* 2000). Log-phase-growing cells were labeled with [^{3}H]acetate or [^{35}S]methionine/cysteine, and nuclear extracts were obtained for immunoprecipitations on cross-linked 12CA5 antibody beads. Following binding, Tat was eluted with an excess of competitor peptide and run on a 4–20% gel. The results of such an experiment are shown in Fig. 2C, where acetylated Tat (right column) could be obtained only from HeLa/eTat cells and not from the control pcep4 cells.

Effect of Tat acetylation on TAR and CBP/P300 binding

We next asked whether acetylation of Tat could increase or decrease its affinity for TAR RNA. To address this question, we synthesized labeled TAR RNA, PAGE purified it, and used it in an RNA bandshift assay. The results of such an experiment are shown in Fig. 3A, where wild-type peptide 41–54 was capable of binding to TAR RNA (lane 3). The TAR RNA binding is completely abolished when lysines 50 and 51 are acetylated (lanes 6 and 7). We observed no binding of double-acetylated 50 and 51 peptide to TAR RNA at any peptide concentration (data not shown). Similar results were also observed when Tat protein was acetylated with GST–HAT prior to TAR RNA binding (Fig. 3B, lanes 4 and 5).

We next examined the effect of wild-type or acetylated peptides on CBP/p300 binding *in vitro.* We reasoned that if only wild-type Tat peptide or protein was able to bind to TAR RNA and not the acetylated counterparts, it would then be possible for acetylated Tat to also bind less efficiently to CBP/p300. The results of such an experiment are shown in Fig. 3C, where GST–Tat, but not GST,

was able to bind to ^{35}S-labeled CBP *in vitro.* However, when performing competition experiments with wild-type, acetylated, or alanine-substituted Tat peptides in the same reaction, we found a surprising result, where wild-type 41–54 but not other derivatives was able to enhance the binding of CBP to GST–Tat (compare lanes 3–5). We have found similar results when using immunoaffinity-purified recombinant p300 from Baculovirus-infected cells (data not shown). This unexpected result suggested to us that perhaps the wild-type peptide might change the conformation of CBP/p300 such that it can bind better to other proteins. This interpretation is very likely, since Tat has been shown to dimerize and contact multiple transcription factors on the transcription initiation site. To test that hypothesis, we performed a similar experiment with CBP pretreated with either wild-type or double-acetylated peptide prior to binding to other basal factors such as TFIIB and TBP. Both TFIIB and TBP have previously been shown to bind to CBP/p300 molecules (Sang *et al.,* 1997). The results in Fig. 3D indicate that when CBP is pretreated with only wild-type Tat peptide, it can bind more efficiently to GST–TFIIB or GST–TBP, but not to a GST–TBP (1–163) mutant. The reaction was specific for a possible change of CBP conformation, since pretreatment of GST–TFIIB, TBP, or the mutant TBP with any of the peptides did not increase CBP binding *in vitro* (data not shown). Taken together, the results from TAR binding as well as CBP binding indicate that wild-type and not acetylated Tat binds to the basal transcription machinery and that acetylated Tat might either completely come off the transcriptional complex or simply stay with the elongating RNA Pol II.

Localization of CBP/p300 binding to Tat

It has previously been shown that p300 binds to Tat at core and basic residues (Marzio *et al.,* 1998; Benkirane *et al.,* 1998; Hottiger and Nabel, 1998) and that Tat binds to the HAT, N-terminal, or C-terminal domains of p300. We decided to determine which region of p300 was stably binding to the Tat protein. Initially, we used a series of four GST–CBP mutants (a generous gift from R. Goodman) and three GST–p300 mutants in an *in vitro* TNT-binding assay. Figure 4A is a general diagram that depicts the relationship between CBP and p300 molecules as well as various mutants used in our assays. When binding ^{35}S-labeled Tat to various GST–p300 domains, we found that Tat bound stably to all A, B, and C mutant domains under 0.1 M salt wash conditions (Fig. 4B, lanes 1–4). However, the binding of Tat to p300 was most resistant with the B fragment (aa 744–1540) of GST–p300. Tat retained its binding to this fragment under 0.85 M salt wash conditions (compare lanes 12–14). Similar results were obtained with the GST–CBP HAT domain (data not shown). Therefore, Tat may interact with multiple CBP/p300 domains; however, its ability to tightly associate with the HAT domain may indicate similarity in functions with other viral HAT-binding proteins such as adenovirus E1A or SV40 large T antigen.

Functional activity of mutant Tat proteins on transient transfections

Tat is one of the most powerful viral activators known to date. Tat could stimulate transcription of HIV-1 promoter anywhere from 100- to 1000-fold depending on the assay conditions used. To determine which Tat residues (position 50, 51, or 50+51) were important for HIV-1 promoter activity, we synthesized alanine-substituted vectors and sequenced all three forms of CMV-driven Tat vectors. We subsequently used the Tat mutants in transient transfection assays using CEM (12D7) T-cells. The results of such an experiment are shown in Fig. 5A, where upon electroporation of K50A or K51A with reporter HIV-LTR CAT, we observed a slight drop in transcriptional activity (less than 50%, compare lanes 3–6). However, a more pronounced drop in transactivation was observed with the double-mutant 50 and 51 (~5-fold, lanes 7 and 8). This drop in activation from the 50+51 mutant could not be rescued with ectopic expression of CBP vector (Fig. 5B, lane 4). Similar results were obtained with a CMV–p300 expression vector (data not shown). Finally to conserve the positive charge of the lysine residues, we synthesized Tat protein with arginines at positions 50 and 51 and used the protein to perform CAT assays in CEM cells. The results in Fig. 5C indicate that only lysine residues and not alanine or arginine substitutions at positions 50 and 51 are critical for the observed acetylation effect. Taken together, the transient transfection results indicate that neither K50 nor K51 mutations alone are sufficient to completely lose the Tat transactivation on HIV-1 LTR.

Activation of integrated HIV-1 provirus requires the HAT domain of CBP/P300

We used HLM-1 cells to address whether the interaction of Tat and CBP/p300 plays a role in the activation of integrated proviral HIV-1 sequence *in vivo.* HLM-1 cells (AIDS Research and Reference Reagent Program) were derived from HeLa-T4$^+$ cells containing an integrated copy of the HIV-1 proviral genome with the Tat-defective mutation (termination linker at the first AUG). HLM-1 cells are negative for virus particle production, but can be induced to express high levels of infectious HIV-1 after transfection with Tat-expressing clones or after stimulation with cytokines such as TNF-α or general inducers such as sodium butyrate. The new resulting particles are wild type for infection, RT activity, and integration, but need to be restimulated for the next round of progeny formation. In order to test whether coactivator CBP/p300 plays an important role in the activation of integrated HIV-1, we transfected the HLM-1 cells with Tat and CBP

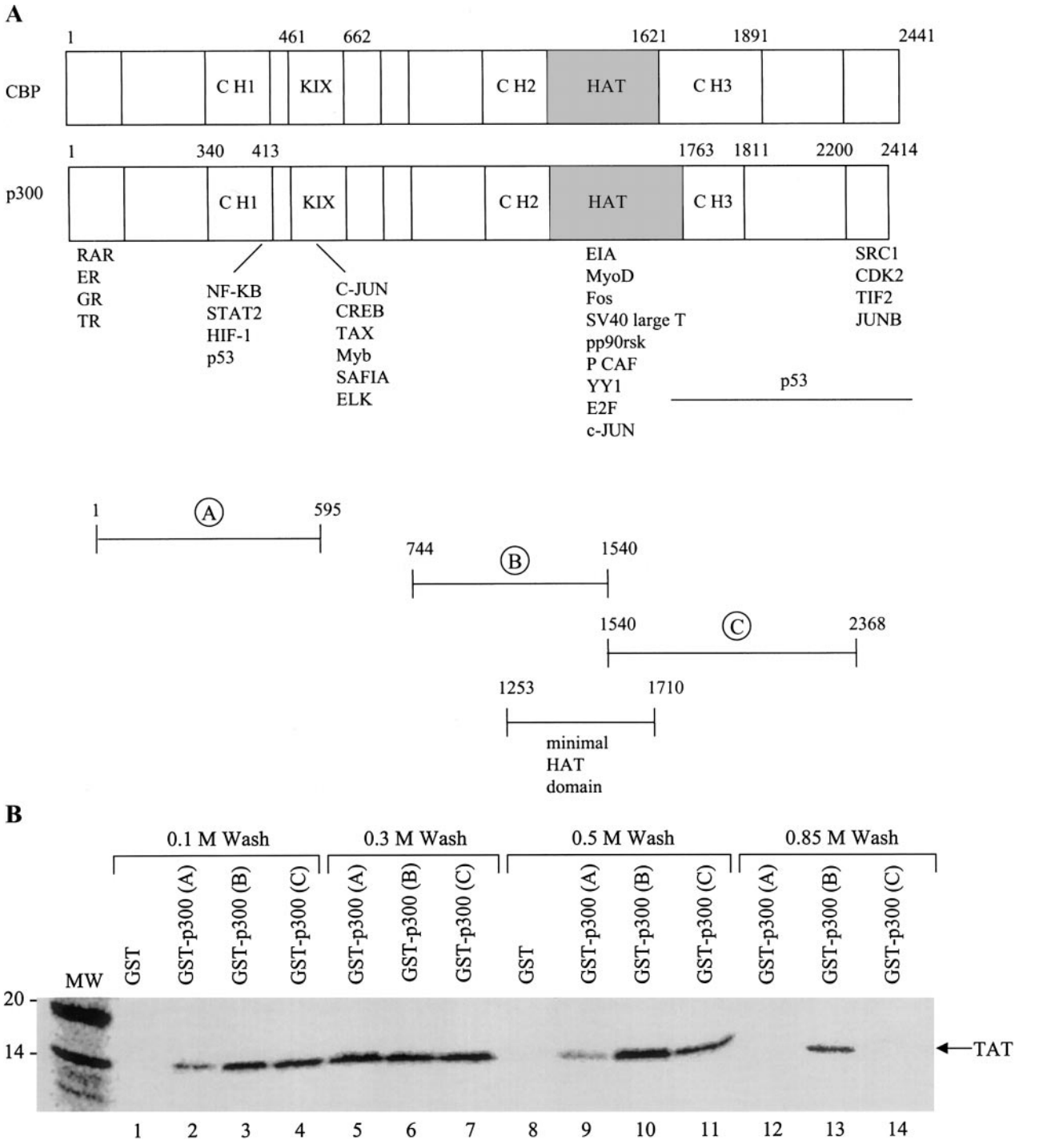
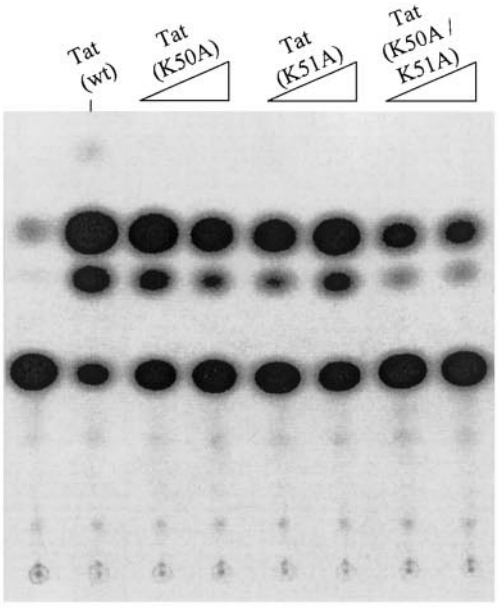


FIG. 4. Binding of Tat to CBP and p300 under various salt conditions. (A) Schematic representation of functional domains in CBP, p300, and the GST–p300 clones containing various domains used here, including GST–p300 A (1–595), GST–p300 B (744–1540), and GST–p300 C (1540–2368). (B) Binding of Tat to p300 under various salt wash conditions. The GST–p300 deletion clones (A, B, and C) were immobilized on glutathione beads from bacterial extracts and incubated with radiolabeled, *in vitro* translated ^{35}S-labeled Tat. The bound proteins were resolved on 4–20% SDS–PAGE after being washed with buffers containing TNE + 0.1, 0.3, 0.5, or 0.85 M salt and 0.1% NP-40. Tat binds to GST–p300 B and C fragments at 0.3 M salt buffer. Tat binds only to GST–p300 B fragment after a 0.85 M salt wash buffer.

and with or without E1A, which has been shown to inhibit the HAT activity of CBP/p300 (Chakravarti *et al.,* 1999). The resulting supernatants were collected at various time points and tested for the production of HIV-1 particles by p24 gag antigen ELISA. The results of such an experiment are shown in Fig. 6A, where ectopic expression of CBP along with Tat activates the viral production by four- to fivefold. Furthermore, the effect of CBP acti-

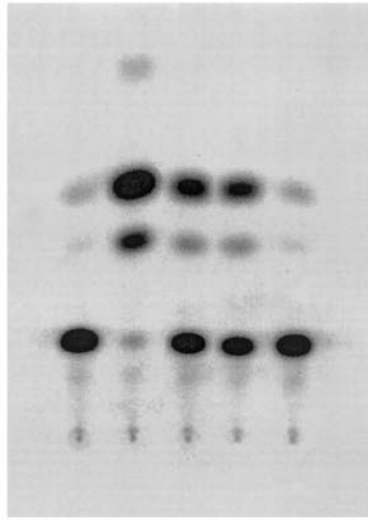
A



Lane:	1	2	3	4	5	6	7	8
% Conversion	1.5	85.0	49.9	43.8	39.1	55.2	14.4	16.6

B

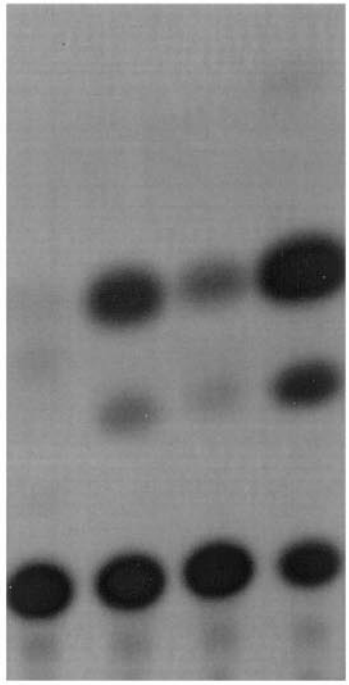
CBP	−	−	−	+	+
Tat (K50A/K51A)	−	−	+	+	−
Tat (wt)	−	+	−	−	−
HIV LTR-CAT	+	+	+	+	+



Lane:	1	2	3	4	5
% Conversion	1.2	98.8	25.2	23.4	1.8

C

TAT (wt)	−	−	−	+
TAT (K50/51R)	−	−	+	−
TAT (K50/51A)	−	+	−	−
HIV LTR-CAT	+	+	+	+



	1	2	3	4
% Conversion	0.9%	17%	9.2%	88%

vation can be reversed using the E1A expression vector. Interestingly, neither E1A nor CBP by itself can activate HIV-1 production, indicating at least in the case of CBP, that CBP exerts its activation effect on the HIV-1 promoter only in the context of chromatin DNA. Therefore, unlike other retroviral activation pathways, such as HTLV-1 (Kashanchi *et al.,* 1998), the HAT domain of CBP/p300 may be a crucial domain in HIV-1 proviral transcriptional activation.

We next examined the effect of the Tat mutants at positions 50 and 51 and a combination 50+51 mutant in HLM1 cells. Results presented in Fig. 6B indicate that point mutations at position 50 or 51 are equally deleterious in activation of the integrated chromatinized HIV-1 DNA. This is in marked contrast to the transfection data (Fig. 5A) where point mutants at 50 or 51 were slightly affected and only the double-mutant 50+51 vector was dramatically reduced in activity. More importantly, ectopic expression of the CBP could not activate the 50 or 51 mutants in these cells. Similar levels of nuclear Tat wild type and mutants were observed in these cells (data not shown). Finally, we performed a titration assay of the Tat 50+51 mutant with CBP and found no induction of the latent virus in these cells (Fig. 6C). Similar results were also obtained with the Tat point mutants 50 or 51 and titration of various concentrations of ectopically expressed CBP (data not shown). Taken together these data indicate that the effect of CBP/p300 is at the level of integrated HIV-1 provirus and that both lysines at position 50 and 51 are equally important for this activation pathway.

Binding target of Tat acetylated peptide

To date there are no clear examples of what exactly happens to a protein once it is acetylated in the transcriptional complex by coactivators such as CBP/p300. In fact, the majority of the existing reports on acetylated proteins discuss what the functional consequence of acetylation may be (Gu and Roeder, 1997) and not so much regarding the proteins or pathways downstream of acetylation. We decided to address this question by utilizing affinity pull-down experiments using wild-type or acetylated 42–54 peptides coupled to a biotin moiety. The biotinylated peptides were incubated with various ^{35}S-labeled proteins or nuclear extracts followed by direct radioactive detection or Western blot for transcription factors involved in HIV-1 transcription. The results of such an experiment are shown in Fig. 7A, where ^{35}S-labeled TBP, CBP, and cyclin T could efficiently bind to wild-type but not acetylated 42–50 peptide. A similar experiment was performed using a purified HeLa core-Pol II fraction (Inostroza *et al.,* 1994; Piras *et al.,* 1994), followed by Western blot for Pol II. Results from Western blots indicated that wild-type and not acetylated peptide was capable of binding to core-Pol II. In an attempt to find substrates that could bind to acetylated peptide, we performed a series of binding assays followed by excision of bound peptides from gels and subjected them to mass spectrometry. The initial material for pull-down assays was whole HIV-1 virus (pDH125, a generous gift from M. Chow and M. Martin) reconstituted *in vitro* with all four histones. The bound complexes were washed with 150 mM salt and 0.5% NP-40, run on SDS–PAGE, and silver-stained, and peptides were excised and subjected to mass spectrometry. The results of such an experiment are shown in Fig. 7B, where acetylated Tat peptide was able to bind to core histones. It is interesting to note that core histones in the absence of DNA did not bind to acetylated Tat, indicating that a fixed conformation of nucleosome is required for Tat to bind to core histones. Therefore, these data collectively suggest that acetylated Tat may leave the initiation complex behind and retain its binding to nucleosomal DNA.

DISCUSSION

In recent years it has become apparent that non-DNA-binding transcriptional coactivators, such as p300 and CBP, that were previously thought to function primarily as "bridging" proteins between DNA-bound transcription factors and the basal transcription complex play a critical regulatory role as integrators of diverse signaling pathways in the selective induction of gene expression. Many examples of such phenomena are exemplified by CBP/p300's interaction with an array of transcription factors including sequence-specific DNA-binding proteins, such as the NF-κB CREB, or activator protein 1 (AP-1) family members, that interact with the promoter and act as

FIG. 5. Mutation of the lysine residues at position 50 and 51 of Tat and their reduced LTR transactivation activity. (A) CAT assays were performed from lysates of transfected CEM (12D7) cells with LTR-CAT (3 μg) and varying concentrations of CMV-Tat mutants (1 and 5 μg). Lanes 1 and 2 represent basal transcription of LTR-CAT and Tat (1 μg) activated transcription, respectively. Lanes 3–8 show transfection of single- or double-mutant Tat constructs into CEM cells. The mutants were pcTat (K50A, lanes 3, and 4), pcTat (K51A, lanes 5 and 6), and pcTat (K50A/K51A, lanes 7 and 8). Cells were transfected by the electroporation method and processed for CAT assay 24 h later. The percentage of CAT conversion is indicated at the bottom of each lane. (B) Cotransfection of pcTat (K50A/K51A) alone or with CBP (2.5 μg) in CEM cells is shown in lanes 4 and 5, respectively. Lanes 1 and 5 serve as negative controls. Titration of either pcTat (K50A/K51A) or CBP plasmids showed similar results, where CBP was not able to rescue the Tat mutant construct (data not shown). (C) Transfection of various proteins along with LTR-CAT reporter into CEM cells. CEM cells were grown to log phase of growth and transfected with purified synthesized TAT 72 proteins of wild type, K 50/51 substituted with R, or K 50/51 substituted with A. A total of 3 μg of LTR-CAT DNA along with 0.5 μg of TAT proteins was electroporated into CEM cells (at 230 V). Extracts were analyzed 24 h after transfection for the presence of CAT enzyme.

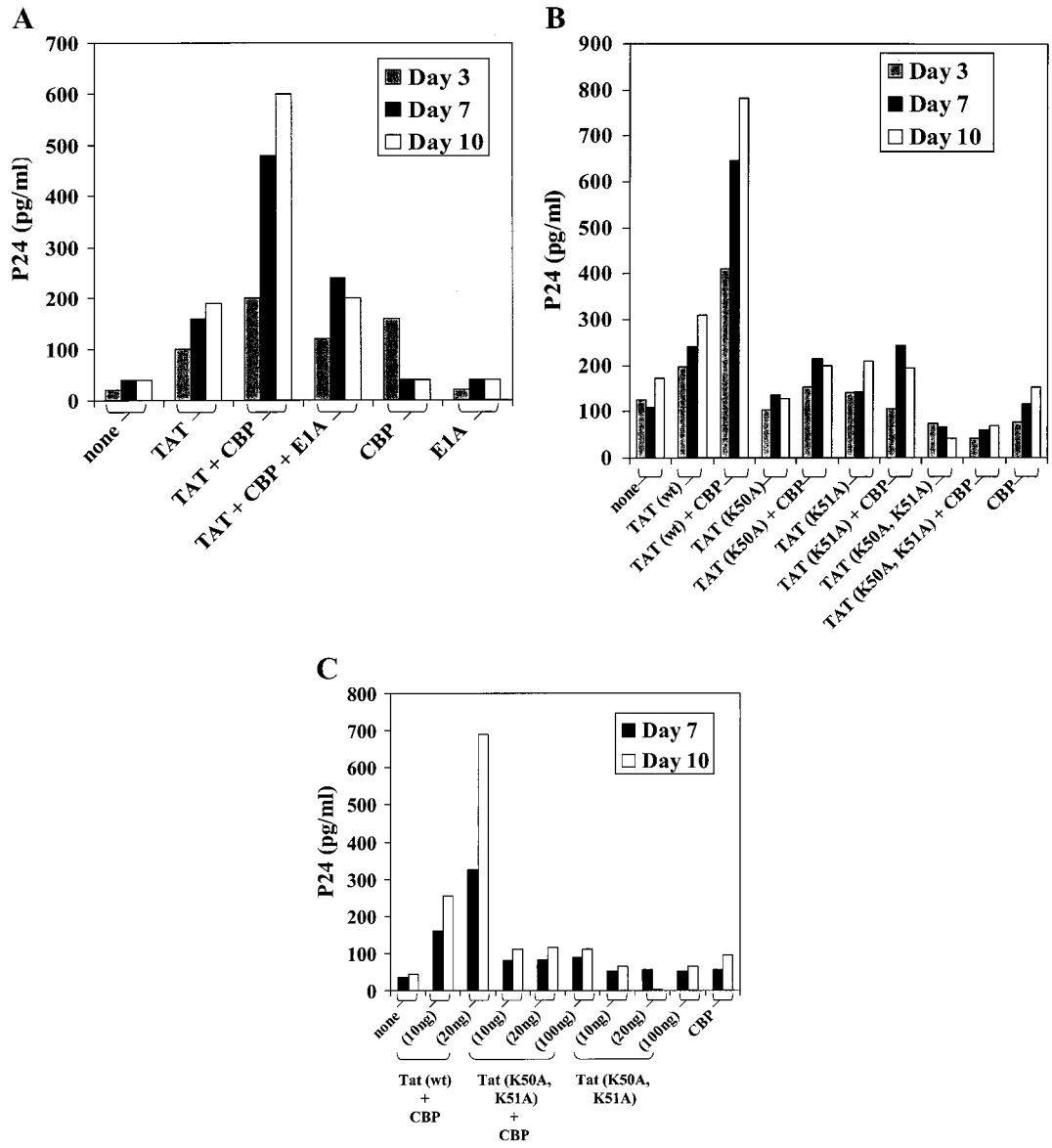


FIG. 6. Both lysines K50 and K51 of Tat play an important role in activation of the proviral integrated HIV-1 DNA. HLM1 cell is a HeLa-derived cell line that contains a wild-type integrated copy of the HIV-1 proviral genome except for the Tat open reading frame. Therefore, HLM1 cells are negative for virus particle production unless they are provided with cytokine signals, such as TNF, or ectopic addition of Tat, as determined by the presence of p24 gag antigen in the supernatant. (A) Coactivation of integrated HIV-1 provirus by Tat and CBP. HLM1 cells were transfected alone with Tat (50 ng), CMV-CBP (6 μg), E1A (6 μg), or in combination with each other by the $CaPO_4$ precipitation method. The p24 gag antigen ELISA was performed from supernatants obtained at days 3, 7, and 10 after transfection. It is interesting to note that cotransfection of Tat plus CBP increased the production of HIV-1 particles in HLM1 cells and E1A was able to reverse the CBP effect, presumably by binding to CBP and inhibiting the HAT activity. (B) HLM1 cells were transfected either alone with wild-type Tat and mutant Tat clones (single-mutants K50A and K51A and double-mutant K50A/K51A) or with CBP into HLM1 cells and p24 gag antigen measured at various time points. (C) Titration of double-mutant Tat (K50A/K51A) with CBP performed in HLM1 cells. Varying concentrations of the Tat double-mutant were transfected either alone or with 6 μg of CBP. Supernatants were collected at days 7 and 10 for the p24 gag ELISA assay. All experiments in A, B, and C were performed twice.

either enhancers or repressors of gene expression during cellular activation. Members of the p300 and CBP family also appear to be present only in higher eukaryotic cells including *Caenorhabditis elegans* and *Drosophila,* and not in the yeast *Saccharomyces cerevisiae.* Thus, p300/CBP-like proteins are likely confined to multicellular organisms where they may fulfill specific functions required for proper growth and development.

Many viruses have evolved mechanisms to control both viral and host transcriptional machinery through CBP/p300. Generic virus infection of cells results in a dramatic hyperacetylation of histones H3 and H4 by CBP/p300, which is localized to target cellular promoters such as IFN-β promoter (Parekh and Maniatis, 1999). Also, both DNA and RNA viruses have evolved means to control CBP/p300 in both activation and repression. For

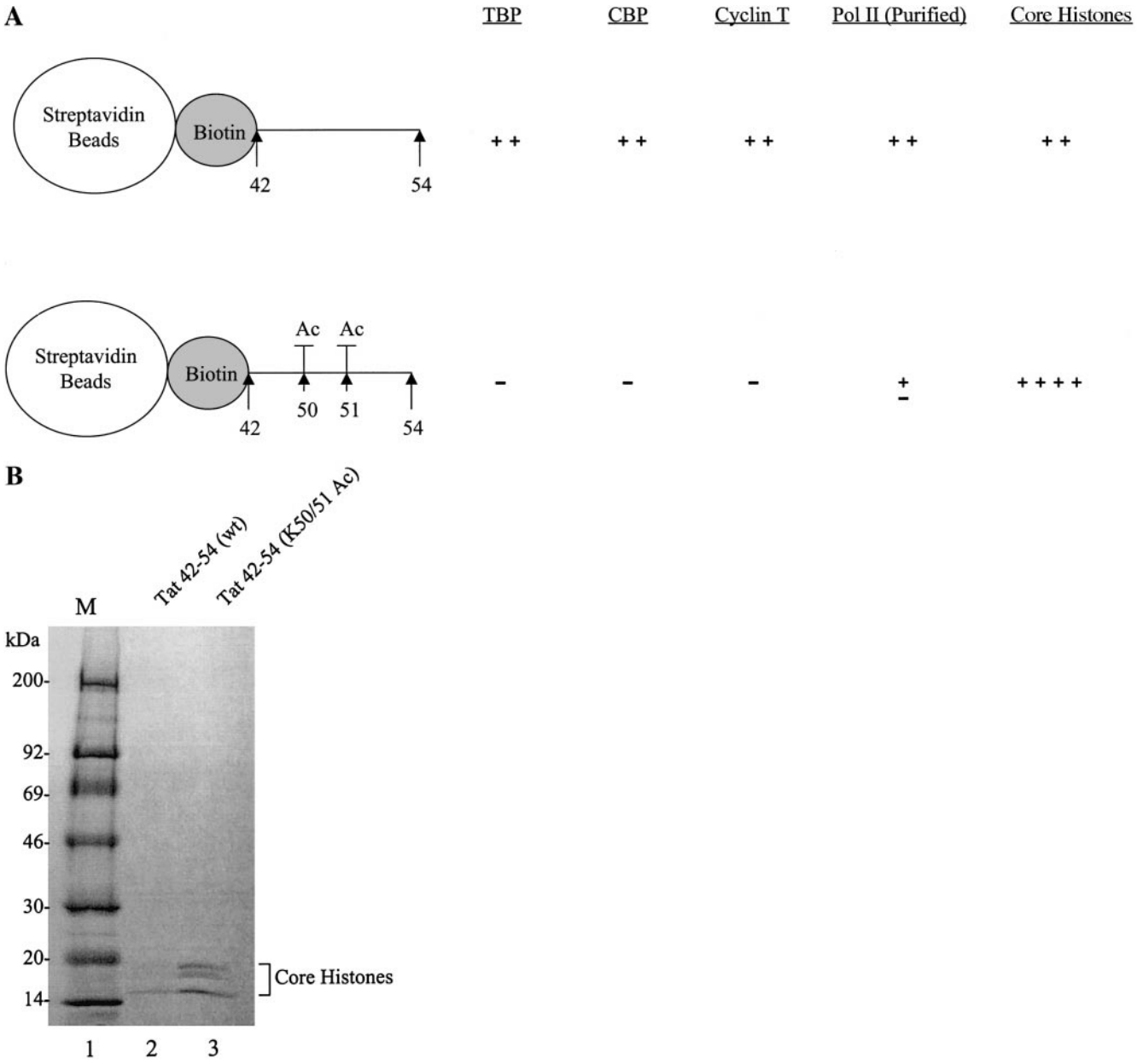


FIG. 7. Binding of acetylated Tat to various transcription factors. (A) Schematic representation of the pull-down experiment and the results of Western blots of the pull-down components with anti-TBP, CBP, cyclin T, and Pol II. The synthesized Tat peptides (42–54) with or without acetylated lysines at positions 50 and 51 were labeled with biotin at the N-terminus. The peptides were incubated with *in vitro* translated, [^{35}S]methionine/cysteine-labeled TBP, CBP, cyclin T, and purified core-HeLa Pol II. Streptavidin–agarose beads were used to pull down the peptide-associated complexes. The pull-down complexes were washed three times and separated on the 4–20% SDS–PAGE gel. For Western blots they were transferred to PVDF membranes and blotted with the antibodies against TBP, CBP, cyclin T, and Pol II. Pull-down experiments with ^{35}S-labeled TBP, CBP, and cyclin T were also performed to confirm the Western blot data. (B) Pull-down complexes from chromatin assembled HIV-1 DNA with the wild-type unacetylated Tat peptide (42–54, lane 2) or double-acetylated Tat peptide (42–54) at positions 50 and 51 lysines (lane 3) were separated on SDS–PAGE, stained with Coomassie blue, excised, subjected to digestion for trypsin digest, and analyzed by mass spectrometry. Western blots for histones H2A, H2B, H3, and H4 were also performed to confirm the mass spectrometry analysis (data not shown).

instance, the oncogenic human papillomavirus type 16 (HPV-16) E6 binds to three regions (C/H1, C/H3, and the C-terminus) of both CBP and p300. Interestingly, HPV-16 E6 inhibits the intrinsic transcriptional activity of CBP/p300 and decreases the ability of p300 to activate p53- and NF-κB-responsive promoter elements (Patel *et al.,* 1999). Similarly, human herpesvirus 8/Kaposi sarcoma-associated virus IRF 1 protein also targets the carboxy-terminal region (aa 1623 to 2414) of the transcriptional coactivator p300 (Burysek *et al.,* 1999). RNA retroviruses such as MMTV, HTLV-I, and HIV have also been noted for regulating p300/CBP. CBP suppresses the responsiveness of the mouse mammary tumor virus (MMTV) promoter to dexamethasone in a dose-dependent fashion

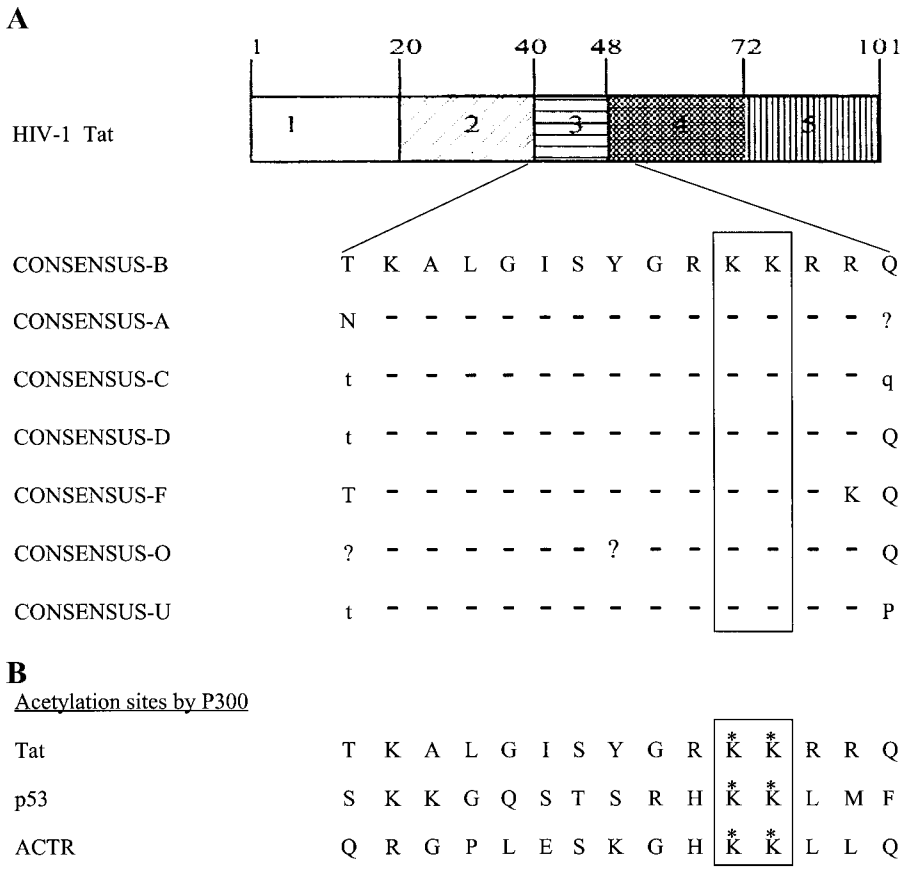


FIG. 8. A general scheme of various Tat domains. (A) Conservation of the double-lysine motif of Tat among various HIV-1 clades. The Tat protein and peptides used in this study were of North American Clade B type. (B) The double-lysine motifs of Tat resemble those in p53 and ACTR proteins, which are also acetylated by CBP/p300 as indicated by asterisks.

(Kino *et al.,* 1999), as well as alleviating NFI-C-mediated repression of MMTV by ectopic expression of p300/CBP (Chaudhry *et al.,* 1999). Transcriptional activation of the HTLV-I sequences by Tax and CBP is induced by reinitiation of transcription (Kashanchi *et al.,* 1998), and cytokine and Tat regulation of HIV transcription requires binding of the p300 coactivator to the promoter region (Hottiger *et al.,* 1998; Hottiger and Nabel, 1998; Benkirane *et al.,* 1998; Marzio *et al.,* 1998; Kiernan *et al.,* 1999; Ott *et al.,* 1999).

CBP/p300 acetylates the core histones as well as nonhistone proteins such as the tumor suppressor protein p53, the hematopoietic transcription factor GATA-1, and the basal transcription factors TFIIE and TFIIF, although their biological functions are still not well understood. To test whether HIV-1 Tat could also be specifically acetylated by CBP/p300, we used the purified full-length Tat (1–86) protein and found that the labeling was completely dependent on the presence of the CBP/p300 HAT domain. The labeling was most efficient with purified Tat and not GST–Tat (1–101), GST–Tat (1–86), GST–Tat (1–72), or 17 other GST–Tat mutants.

When using Tat peptides to pinpoint the amino acid residues important for acetylation, we found that the both lysines in the highly conserved region (^{49}RKKRRQ^{54}) of the basic RNA-binding motif of Tat were acetylated by CBP/p300 (see Fig. 8). Importantly, upon transfection of K50A or K51A Tat vectors with reporter HIV-LTR CAT, we observed a slight drop in transcriptional activity but a more pronounced drop in transactivation with the double-mutant K50A and K51A. This suggested that both lysines together are important in the transient transfection assays. However, when using HLM1 cells, containing integrated virus, we observed that both point mutations at position 50 and 51 were equally deleterious in activation of the integrated chromatinized HIV-1 DNA and that ectopic expression of the CBP could not activate either of the 50 or 51 mutants in these cells. Therefore, we believe that both lysines are equally important for *in vivo* activation of the latent virus.

When examining for the effect of wild-type or acetylated Tat on RNA binding, we found that the acetylated Tat peptide or protein was not able to bind to TAR RNA. This is in marked contrast to other acetylated proteins such as p53, where acetylation increases the DNA-binding activity (Gu and Roeder, 1997). More interestingly, when performing competition experiments with wild-type, acetylated, or alanine-substituted Tat peptides in

the same reaction, we observed that wild-type 41–54 but not other derivatives was able to enhance the binding of CBP to Tat as well as other basal factors such as TFIIB and TBP. This intriguing result indicates that CBP/p300 family members are subject to conformational change upon binding to viral and possibly cellular activators. In support of the change of conformation hypothesis, we have recently obtained preliminary data suggesting that *in vitro* translated CBP in presence of wild-type but not acetylated Tat peptide is susceptible to endoproteinase Glu-C digestion and not to other nucleases such as trypsin, endonuclease Asp-N, or Lys-C proteases.

CBP/p300's ability to acetylate Tat has recently been examined in a chromatin reconstitution experiment. When using purified basal transcription factors NF-κB, SP1, and cdk9/cyclin T in an *in vitro* transcription reaction, where the HIV-1 DNA is chromatinized, we observed no activated transcription *in vitro,* indicating that the mere presence of cdk9/cyclinT is not sufficient to drive RNA pol II through chromatin DNA. However, only in the presence of added minimal p300 HAT domain or wild-type p300 do we find activated transcription on HIV-1 DNA (Deng, unpublished data). Therefore, the role of the cdk9/cyclinT complex in HIV-1 transcription may be to phosphorylate the CTD of RNA pol II and that of p300 may be to acetylate the natural core histones on the HIV-1 genome. To this end, we have mapped the promoter region (−500 to +200) of 26 different HIV-1 clade isolates ranging from subtypes B to O and have observed that all viral isolates have chromatinized DNA *in vivo,* further indicating that the HIV-1 B clade that we have used in this study (in HLM1 cells) was not the only HIV-1 chromatinized template *in vivo.* Finally, our results show that the acetylated Tat decreases Tat's ability to bind the TAR RNA element, as well as to bind basal factors TBP and core-Pol II, but increases the efficiency of binding to core histones and only when assembled as a nucleosomal HIV-1 DNA. This notion may provide a mechanism of how Tat is able to leave the initiation complex behind and to facilitate chromatin modification or remodeling downstream of the transcription initiation site, perhaps by aiding in disruption of the nuc-1 nucleosome. El Kharroubi *et al.* (1998) demonstrated that expression of a functional Tat could alter the chromatin structure downstream of the HIV-1 promoter and that the binding of Tat to TAR (as occurs with Tat K41T) failed to induce chromatin remodeling. Recently, using *in vitro* reconstituted chromatin templates, we found that Tat–p300 interaction increases the acetylation of a nucleosomal histone. Such enhancement of histone acetylation may be due to the finding that the acetylated Tat binds with higher efficiency to nucleosomal DNA and changes the conformation, resulting in the accessibility of histone tails to p300. Acetylation of histones may flag other proteins needed for disruption of nuc-1 and subsequent transcription. Future experiments will determine the nucleosomal position(s) that is affected by acetylated Tat on whole HIV-1 DNA.

MATERIALS AND METHODS

Cell culture and labeling

Log-phase CEM (12D7) T-lymphocytes (Kashanchi *et al.,* 1992, 1994a, 1994b) were grown in culture at 37°C up to 1×10^5 cells/ml in RPMI 1640 medium containing 10% fetal bovine serum treated with a mixture of 1% streptomycin, penicillin, and 1% L-Glutamine (Gibco BRL). HLM-1 cells (AIDS Research and Reference Reagent Program, Catalog No. 2029) were derived from HeLa-T4$^+$ cells integrated with one copy of the HIV-1 genome containing a Tat-defective mutation. The mutation was introduced as a triple termination linker at the first AUG of the Tat gene (Sadaie and Hager, 1994). HLM-1 cells are negative for virus particle production, but can be induced to express noninfectious HIV-1 particles after transfection with Tat cDNA or mitogens such as TNF-α or sodium butyrate. HLM1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 100 μg/ml of G418, plus 1% streptomycin, penicillin, and 1% L-glutamine (Gibco BRL). These cells were always grown to 75% confluency before transfection or passages.

Labeling experiments were performed on log-phase 75% confluent HeLa/eTat or control HeLa/pcep4 cells (Kashanchi *et al.,* 2000). Cells were pulsed for 3 h with [^{3}H]acetate (0.4 mCi, ICN) in complete DMEM plus hygromycin. Cells were also incubated overnight with [^{35}S]methionine/cysteine (0.2 mCi/ml, NEN) in methionine/cysteine-free medium and 10% dialyzed FCS. Nuclear lysates were prepared in 1 ml of lysis buffer (300 mM NaCl, 0.1% NP-40, 10 mM NaH_2PO_4, 1 mM EDTA, pH 8, 1 mM DTT, 50 mM sodium pyrophosphate, 10 mM NaF, 8 mM sodium butrate), and immunoprecipitations were performed on 0.5 mg protein extract. Tat was detected using immunoprecipitation from cellular lysates with monoclonal 12CA5 Ab cross-linked to beads for 3 h and eluted with a 100-fold excess of influenza peptide for 8 h at 4°C. The sequence of peptide used for elution was as follows: ^{N}YPYDVPDYASL^C. Four independent [^{3}H]acetate and [^{35}S]methionine/cysteine labeling experiments were performed with similar results.

Lymphocyte transfection

Lymphocytes (CEM, 12D7) were grown to mid-log phase of growth and were processed for electroporation according to a previously published procedure (Kashanchi *et al.,* 1992). Only one modification was introduced, where cells were electroporated at 230 V and plated in 10 ml of complete RPMI 1640 medium for 18 h prior to harvest and CAT assay.

Transfection and HIV-1 detection of HLM_1 cells

HLM_1 cells were propagated in DMEM (containing 100 μg/ml of G418) and transfected with the plasmid DNAs including Tat, mutant Tat, and CBP using the calcium phosphate method. The transfected cells were washed after 4 h and fresh complete DMEM with 10% fetal bovine was added for the remainder of the experiment. The p24 gag antigen was detected with a standard ELISA kit (Abbott) using the supernatants of transfected cells at days 3, 7, and 10.

Plasmids

HIV-LTR-CAT reporter and eukaryotic Tat expression vectors (pcTat) have been described previously (Hauber *et al.,* 1989; Kashanchi *et al.,* 1994b). Mutants of the lysine residue at position 50 and/or 51 of the Tat expression plasmid were constructed from pcTat, by replacing the *Eco*NI fragment with synthesized mutated oligo adaptor. The following top strands for each mutated construct are indicated: for K50A, 5′GGCAGGGCGAAGCGGAGACAGCGACGAAGACCTCC3′; for K51A, 5′GGCAGGAAGGCGCGGAGACAGCGACGAAGACCTCC3′; and for K50/A+K51/A, 5′GGCAGGGCGGCGCGGAGACAGCGACGAAGACCTCC3′. Eukaryotic expression vectors for CBP E1A have previously been reported (Chakravarti *et al.,* 1999). GST–p300 (HAT) from aa 1197 to 1735 was PCR amplified from a p300 B fragment as well as the p300 C fragment and subcloned into pGEX (more details of subcloning will be provided upon request). The resulting recombinant vector was transformed into *E. coli* DH5a and were grown overnight in 10 ml of LB with 100 μg/ml of ampicillin. A 500-ml LB + ampicillin flask was inoculated with the overnight culture and was grown for 4 h at 37°C. Isopropyl-1-thio-*b*-D-galactopyranoside was added to a final concentration of 0.1 mM to induce fusion protein expression, and the culture was switched to 30°C for an additional 4 h. Cells were collected by centrifugation in a GSA rotor at 5800 *g* for 10 min at 4°C. For sonication, the bacterial pellet was resuspended in 25 ml of phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride and was sonicated (Branson) for 35 pulses at the 3.5 microprobe setting. The resulting mixture was centrifuged in a GSA rotor at 5800 *g* for 10 min at 4°C. A second centrifugation in a SS-34 rotor at 23,500 *g* for 20 min at 4°C clarified the extract of remaining debris. GST–fusion proteins were bound to agarose beads overnight, washed the next day, and run on 4–20% SDS–PAGE for both quality and quantity prior to use in HAT assays.

Baculovirus expression and protein purifications

Sf9 cells were grown to mid-log phase in HyClone HyQ CCM-3 serum-free medium utilizing spinner flask culture methods. For p300 infection, the cells were infected with 11 ml of p300 FLAG virus at 2.0×10^6 cells/ml and then allowed to incubate at 10 rpm for approximately 1 h. After this initial incubation/infection time the spinner plates were turned up to 70 rpm for the remainder of the incubation. The cells were collected via centrifugation 48 h after infection. The cell culture normally yielded approximately 2 ml of PCV from 500 ml of original culture volume, and the cell pellet was processed for further purification. Samples were lysed with lysis buffer, containing 50 mM Tris–Cl, pH 7.4, 120 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF (phosphotyrosine phosphatase inhibitor), 1 mM DTT, and 1 mM PMSF, and processed with 12CA5 monoclonal antibodies for further purifications.

Histone acetyltransferase assay

HAT assays were performed as 30-μl total reactions at 37°C for 60 min in buffer containing 1 μl of purified GST–p300 HAT (1 mg/ml), 200–400 ng substrate proteins or peptides, and 1 μg of histones H2A, H2B, H3, and H4 in 20 mM HEPES–NaOH, pH 7.4, 1 mM dithiothreitol, 10 mM sodium butyric acid, and 1 μl of [^{14}C]acetyl-Coenzyme A (65 mCi/mmol, ICN). Proteins and peptides were resolved on 4–20% or 15% SDS–PAGE. Gels were dried and exposed to a PhosphoImager cassette.

Preparation of TAR RNA and RNA-binding experiments

The plasmid pT7 was constructed from pU3R-III containing a T7 promoter at nucleotide +1 of HIV (Gunnery *et al.,* 1990). pT7 was linearized at nucleotide +82 by digestion with *Hin*dIII and transcribed using T7 RNA polymerase (Promega). TAR RNA was labeled with [α-^{32}P]UTP and was subjected to electrophoresis in a 10% polyacrylamide gel. The major radioactive RNA band was eluted and extracted with phenol/chloroform and precipitated with ethanol.

Gel mobility shift reaction (16-μl final reaction volume) was carried out in binding buffer (10 mM HEPES, pH 7.3; 100 mM KCl; 1 mM $MgCl_2$; 0.5 mM EDTA; 1 mM DTT; and 10% glycerol) and contained 3 ng of labeled TAR RNA as well as 200 ng tRNA as nonspecific competitor. Reactions were incubated for 30 min at room temperature and RNA-bound complexes were separated on a prerun 6% DNA retardation gel (Novex) containing 0.5× TBE buffer, at 7 W for 2.5 h at 4°C.

Peptide synthesis

The biotinylated peptides were prepared on a PAL–PEG–polystyrene resin by continuous-flow solid-phase synthesis on a PerSeptive Biosystems Pioneer synthesizer (Framingham, MA) using HBTU-activated Fmoc amino acids. Side chain protection was as follows: Arg (Pmc), Gln (Trt), Lys (Dde), Ser, and Tyr (Bu^t). Peptide assembly was concluded by N$^\alpha$-acylation with HBTU-activated biotin. The resin-bound peptide was then

treated with 3% hydrazine in DMF for 20 min to selectively remove the Dde groups from the side chain of Lys. The resin was then divided into two equal portions and one-half was subjected to a 1-h treatment with an excess of acetic anhydride in the presence of an equivalent amount of base to acetylate the resulting free N^{ϵ} groups. Both peptides were then separately cleaved from the solid support and simultaneously the remaining side chain was deprotected by reaction with trifluoroacetic acid in the presence of scavengers. Peptide purification was achieved by conventional reverse-phase HPLC on Vydac C18 (Hesperia, CA) in an overall yield of 25–30% based on starting resins. The purity of the two peptides was confirmed by analytical reverse-phase HPLC, capillary zone electrophoresis, and matrix-assisted laser desorption time of flight mass spectrometry. For the non-acetylated peptide, we found MH^+ 1757.5 (calc. MH^+ 1757.1). For the acetylated peptide, we found MH^+ 1840.9 (calc. MH^+ 1840.1).

Synthesis of acetylated peptides at positions 50, 51, and 50+51 was carried out on the ABI 433A Peptide Synthesizer (PE Biosystems, Foster City, CA) using Fastmoc chemistry with *N*$^{\epsilon}$-acetyl-L-lysine, which was purchased from Novabiochem (San Diego, CA). After cleavage and deprotection, the peptides were purified by HPLC (Dionex, Sunnyvale, CA) using an acetonitrile gradient on a C18 reverse-phase column (Pharmacia, Piscataway, NJ). The amount of protein was determined by Bio-Rad protein assay as well as by running small aliquots on 4–20% SDS–PAGE followed by silver staining.

Streptavidin bead pull-down assay

Synthesized Tat (42–54) peptides, labeled with biotin at the N-terminus, and with or without an acetyl group at lysines 50 and 51, were used in the pull-down assays. The biotin-labeled Tat peptides were incubated with the cell extracts in TNE_{50} buffer (100 mM Tris–HCl, pH 7.5; 50 mM NaCl; 1 mM EDTA; 0.1% NP 40) at 4°C overnight. Streptavidin beads (Boehringer Mannheim) were added to the mixture and incubated for 2 h at 4°C. The beads were washed three times with TNE_{150} (100 mM Tris–HCl, pH 7.5, 150 mM NaCl; 1 mM EDTA; 0.1% NP-40). The bound proteins were separated on 4–20% SDS–PAGE and subjected to Western blotting with antibodies against TBP, CBP, cyclin T, and RNA polymerase II (Santa Cruz Inc.; SC-900 (C21) for Pol II, Sc-1211 (451) for CBP, Sc-204 (N12) for TBP, and cyclin T (a generous gift from M. Mathews).

Nucleosome reconstitution by salt dialysis

The core histones were purified from HeLa cells by the method of Simon and Felsenfeld (1979). Chromatins were prepared from high-molecular-weight DNA and plain and purified core histones by dialysis from 1 M NaCl (Imbalzano, 1998; Stein, 1989). Ten micrograms of plasmid DNA of pDH125 (whole HIV-1 genome, a generous gift from M. Cho and M. Martin, NIAID/NIH) was mixed with 5 μl of 5 M NaCl and 2 μl of 10× reconstitution buffer (0.15 M Tris–HCl, pH 7.5; 1 mM DTT; 2 mM EDTA) by pipetting up and down repeatedly. Later, 15 μg of core histones was added in a total volume of 20 μl; the volume was adjusted by adding ddH_2O. Samples were gently flicked in the tube to mix and incubated at 37°C for 20 min. Sequential dilution was carried out by adding 10 μl of 1× reconstitution buffer every 10 min, for 3 h at 37°C. At each time point, samples were mixed by pipetting up and down. An aliquot was run on agarose gels to ensure proper assembly prior to each experiment.

In-gel digestion, mass spectrometry, and protein identification

The in-gel digestion was performed based on a procedure previously described by Fernandez *et al.* (1998). The gel bands of interest were excised from SDS–PAGE and digested with 0.2 μg of trypsin (Promega modified sequencing grade trypsin). The digests were desalted using C_{18} ZipTips (Millipore) according to the manufacturer's protocol. A 1-μl aliquot of sample was taken for peptide mass mapping on a PerSeptive Biosystem DEPRO MALDI–TOF Mass Spectrometer using α-cyano-4-hydroxycinnamic acid as the matrix. Analysis was performed in the linear delayed-extraction mode, with external calibration. Protein identification by mass mapping was performed through the ProFound Web site located at Rockefeller University (prowl.rockefeller.edu).

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