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# Enhancement of the p300 HAT Activity by HIV-1 Tat on Chromatin DNA

Longwen Deng,* Dai Wang,* Cynthia de la Fuente,* Lai Wang,* Hong Li,† Chee Gun Lee,† Robert Donnelly,‡ John D. Wade,§ Paul Lambert,¶ and Fatah Kashanchi‖[1]

†*Department of Biochemistry and Molecular Biology and* ‡*Department of Pathology,* **UMDNJ–New Jersey Medical School, Newark, New Jersey 07103;* §*Howard Florey Institute, University of Melbourne, Parkville, Victoria 3052, Australia;* ¶*Flinders University of South Australia School of Medicine, Bedford Park, South Australia 5042, Australia; and* ‖*George Washington University School of Medicine, 2300 Eye Street, NW, Ross Hall, Room 552, Washington DC 20037*

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HIV-1 Tat is able to form a ternary complex with P/CAF and p300 and increase the affinity for CDK9/P-TEFb CTD kinase complex. Our previous study demonstrated that Tat binds to p300/CBP in the minimal HAT domain (aa 1253–1790) and that the interaction results in a change of conformation on p300/CBP. Here, we show that the Tat–p300 interaction increases the HAT activity of p300 on histone H4 that is associated with nucleosomal DNA and not with free histones. Nucleosomal histone H4 was acetylated on lysines 8, 12, and 16. Acetylation of H4 was inhibited by Lys-coenzyme A (CoA), a selective inhibitor of p300 acetyltransferase activity. Unexpectedly, we also found that Tat could autoacetylate itself, which was specific to lysine residues 41 and 71. Peptides lacking these two lysines could not enhance the HAT activity of p300. Comparison of the sequences of Tat with other HIV-1 clades and HAT containing transcription factors indicated sequence identity in the acetyl-CoA binding motif A, KGXG. Furthermore, when utilizing an *in vitro* transcription assay, as well as a Tat mutant virus, we found that ectopic expression of only wild-type Tat in the presence of p300, and not a lysine 41 Tat mutant, could activate HIV-1 chromatin DNA, as evidenced by the absence of HIV-1 virion antigen. Therefore, transcription of integrated viral DNA *in vivo* requires the HAT activity of coactivators that are modulated by Tat to derepress the HIV-1 chromatin structure and aid in activated transcription. 

## INTRODUCTION

The HIV-1 Tat is required for viral replication and is a potent stimulator of viral transcription. Gene expression from the viral long terminal repeat (LTR) is several hundredfold higher in the presence of Tat. The ability of Tat to regulate viral transcription is related to its ability to bind to TAR (*trans*-activation responsive) and interact with cdk9/cyclinT1 complex, resulting in a more efficient elongation of the RNA Pol II complex (O'Keeffe *et al.,* 2000; Romano *et al.,* 1999; Napolitano *et al.,* 1999; Isel and Karn, 1999; Bieniasz *et al.,* 1999; Ramanathan *et al.,* 1999; Ivanov *et al.,* 1999; Chen *et al.,* 1999; Wimmer *et al.,* 1999; Garriga *et al.,* 1998; Garber *et al.,* 1998; Fujinaga *et al.,* 1998; Wei *et al.,* 1998). For optimal transactivation of HIV-1 gene expression and high viral load, Tat also requires specific upstream transcription factors, including Sp1 (Jeang *et al.,* 1993), TATA binding protein (Kashanchi *et al.,* 1994; Veschambre *et al.,* 1995; Majello *et al.,* 1998), Tat-associated kinase (TAK) (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).

HIV-1 proviral DNA is integrated into the host cell chromosomes and packaged into chromatin. The LTR, which acts as a very strong promoter when analyzed as naked DNA *in vitro,* is silent when integrated into the cellular host genome in the absence of any stimuli (Verdin, 1991; Van Lint *et al.,* 1996; Marzio *et al.,* 1998; Benkirane *et al.,* 1998). Therefore, physiological mechanisms for proviral transcription account for the activation of chromatin-associated viral genomes.

It is known that the four histones, which comprise the nucleosome core (H2A, H2B, H3, and H4), participate in the transcriptional regulation of numerous genes (Owen-Hughes and Workman, 1994). The core histones undergo several posttranslational modifications including phosphorylation, methylation, ADP-ribosylation, ubiquitination, and/or acetylation. The reversible acetylation of the E-amino groups of the lysine residues present in the amino-terminal domain of the core histones is the modification most strongly linked with transcriptional activity. Two classes of enzymes for histone acetylation have been identified. Cytoplasmic type-B histone acetyltransferases (HATs) are thought to acetylate free histones that subsequently are assembled into chromatin, while nuclear type-A histone acetytransferases are thought to carry out transcription-related acetylation of chromosomal histones (Roth and Allis, 1996; Strahl and Allis, 2000). Many type-A HATs have been identified from the

[1] To whom correspondence and reprint requests should be addressed. Fax: (202) 994-1780. E-mail: bcmfxk@gwumc.edu.

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transcriptional regulatory proteins, such as GCN5, CBP/p300, P/CAF (human homologue to GCN5), $TAF_{II}250$, Tip60, and nuclear hormone receptor ACTR (Sterner and Berger, 2000).

CBP and p300 are two highly homologous proteins that behave as transcriptional coactivators and cooperate with a number of sequence-specific transcription factors. It has been suggested that CBP/p300 acts as a central integrator of transcriptional signals from various signal transduction pathways (Kamei *et al.,* 1996). In addition, CBP/p300 has intrinsic HAT activity and is able to cooperate with transcription factors through chromatin remodeling or the modification of histone and nonhistone proteins. The HAT activity of p300/CBP is also regulated by other factors such as viral proteins. For instance, it has been reported that the HAT activity of CBP/p300 is stimulated by interaction with E1A or phosphorylation by cdk2/cyclin E complex (Ait-Si-Ali *et al.,* 1998).

Many lines of evidence indicate a relationship between histone acetylation and gene activity. For example in *Drosophila,* equalization of transcription from the sex chromosomes is achieved by a twofold upregulation of transcription from the male X chromosome that is associated with acetylation of H4 at lysine 16. Chromatin immunoprecipitations of the activated gene with the antibodies against the specific acetylation sites in H3 and H4 reveal an enrichment of transcribed sequences. Moreover, recent evidence suggests that acetylation of specific lysine residues in the amino-termini of the core histones by different HAT enzymes plays a fundamental role in transcriptional regulation (Strahl and Allis, 2000). For instance, transcription-linked acetylation, catalyzed by the GCN5 family of HATs, shows a preference for lysine 14 of H3 and 8 of H4 *in vitro,* although an expanded set of lysine residues is likely to be used *in vivo.* P/CAF primarily acetylates lysine 14 of H3 and less efficiently acetylates lysine 8 of H4. CBP/p300 acetylates all histones, but preferentially acetylates lysines 14 and 18 of H3 and lysines 5 and 8 of H4 with nucleosomal substrates (Schiltz *et al.,* 1999). These distinct patterns of acetylation by the CBP/p300 and P/CAF may contribute to their differential roles in transcriptional regulation. Collectively, these data support the theory that acetylation on the N-termini of core histones is important for transcriptional regulation.

Recently, several reports have provided evidence for the existence of an intracellular multiprotein complex that contains Tat, CBP/p300, and P/CAF (Kiernan *et al.,* 1999; Hottiger *et al.,* 1998). HIV-1 Tat is able to form a ternary complex with P/CAF and p300 to stimulate the ability of Tat to activate LTR-dependent transcription. Moreover, the formation of such a complex containing Tat and p300 targets these proteins to the viral promoter. The histone acetyltransferase activity of CBP/p300 and P/CAF is preferentially required for Tat function (Kiernan *et al.,* 1999). However, Tat is also capable of forming complexes with a component of TFIID, the $TAF_{II}250$ (Weissman *et al.,* 1998) and Tip60 (Creaven *et al.,* 1999). In these cases, Tat-$TAF_{II}250$ and Tat-Tip60 do not affect the transcription from the LTR, but interfere with the transcriptional activity of these cellular genes. It is postulated that different targets of HATs by Tat have different consequences. The interaction of Tat with CBP/p300 and P/CAF stimulates its ability to transactivate LTR-dependent transcription, while $TAF_{II}250$ or Tat-Tip60 interaction controls the transcription of cellular genes (Creaven *et al.,* 1999).

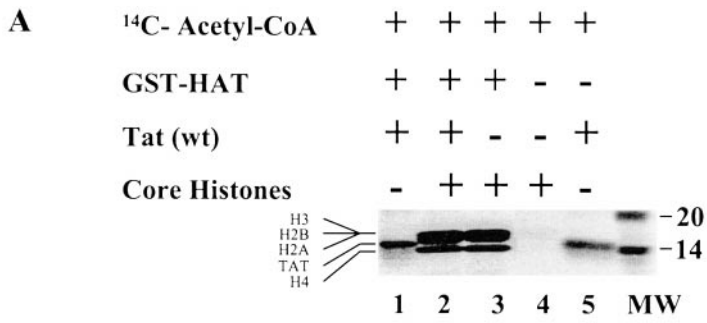
In addition to histone acetylation, some HATs are known to modify certain nonhistone transcription-related proteins, such as p53, E2F, and high-mobility-group chromatin proteins. Similar to certain cellular transcription factors, Tat is also acetylated by CBP/p300 and P/CAF (Kiernan *et al.,* 1999; Ott *et al.,* 1999). P/CAF-acetylated Tat was found to have an increased affinity for CDK9/P-TEFb CTD kinase complex, suggesting that this acetylation event enhances transcriptional elongation by the hyperphosphorylation of the RNA polymerase II CTD.

Our previous work indicated that acetylation of Tat by CBP/p300 decreased Tat's ability to bind to the TAR RNA element and allowed better binding to core histones on an assembled chromatin template (Deng *et al.,* 2000). Tat binds to the minimal HAT domain (aa 1253–1790) of CBP/p300 and the interaction results in a change of conformation for CBP/p300. In this study, we asked whether Tat affects the intrinsic HAT activity of p300. We found that Tat–p300 interaction increases the HAT activity of p300 on histone H4 associated with nucleosomal DNA. The acetylation can specifically be blocked by a synthetic inhibitor of p300 acetyltransferase activity, namely, Lys–coenzyme A (CoA). Interestingly, we also found that Tat autoacetylates itself on lysines 41 and 71. Tat alone cannot acetylate core histones, but enhances p300 HAT activity through its autoacetylation. Furthermore, using *in vitro* transcription assays, we found that Tat–p300 interaction increases the transcription from an *in vitro* assembled whole HIV-1 viral or nucleosomal LTR DNA. Taken together, these observations suggest a possible mechanism by which Tat and the HAT activity of CBP/p300 play a critical role in the transcriptional activation of the integrated HIV-1 provirus.

## RESULTS

### Autoacetylation of HIV-1 Tat

We have previously shown that CBP/p300 was able to acetylate HIV-1 Tat at positions 50 and 51 and that the acetylation was critical for transcription of integrated HIV-1 chromatin DNA and not transfected unorganized DNA (Deng *et al.,* 2000). Upon close examination of the Tat protein in acetylation experiments, we recently found that Tat was able to autoacetylate itself, albeit less effi-

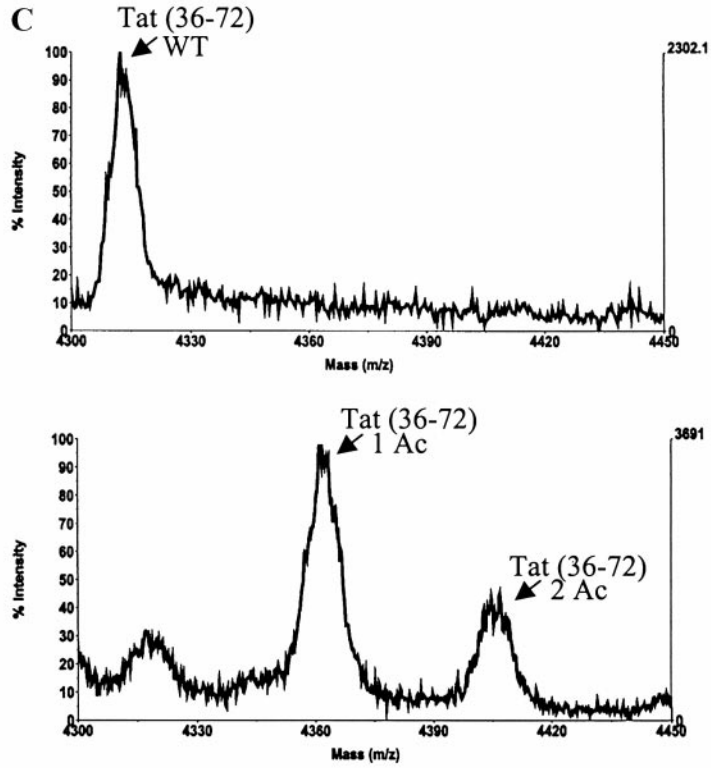



B

| | | 14C Counts | |
|---|---|---|---|
| | | + GST-HAT | Autoacetylation |
| Tat (1-86) | | 118,231 | 16,532 |
| Tat (36-72) | | 109,791 | 15,974 |
| Tat (38-70) | | 75,053 | 1,243 |
| Tat (42-72) | | 80,918 | 2,183 |
| Tat (42-70) | | 89,609 | 144 |
| Tat (36-72; K41A, K71A) | | 61,826 | 235 |
| Tat (1-72) | | 194,017 | 22,499 |
| Tat (1-72; K41A, K71A) | | 163,003 | 1,426 |

FIG. 1. Autoacetylation of HIV-1 Tat. (A) The core histones (H2A, H2B, H3, and H4) and purified Tat protein (1–86) were incubated with or without GST–HAT and [$^{14}$C]acetyl-CoA. The acetylated products were resolved on a 4–20% SDS/PAGE, dried, and exposed to a PhosphorImager cassette. Band intensities were quantitated using Molecular Dynamics software. Tat was efficiently acetylated by GST–HAT (lane 1, 126,508 cpm), as well as autoacetylation of the purified wild-type Tat in the absence of GST–HAT (lane 5, 18,308 cpm). (B) Summary of the autoacetylation results using purified Tat protein and various synthesized Tat peptides. The purified Tat (1–86) and (1–72) mutant proteins and peptides were incubated with [$^{14}$C]acetyl-CoA in the presence or absence of GST–HAT. The acetylated products were separated on a SDS/PAGE, exposed, and counted as mentioned above. The relative radioactivity of autoacetylated Tat peptides with and without GST–HAT is shown in the right-hand columns.

ciently, in the absence of exogenous CBP/p300 HAT domain. The results of such an experiment are shown in Fig. 1A, where Tat was acetylated efficiently by GST–HAT (lane 1, 126,508 cpm), as well as autoacetylation of the wild-type Tat in absence of the p300 HAT domain (lane 5, 18,308 cpm). To determine which region of Tat was required for autoacetylation, we used a series of Tat synthesized peptides in an *in vitro* acetylation reaction. The HAT acetylation reaction consisted of either substrate Tat proteins (400 ng) or Tat peptides (200 ng) and a mixture of histones H2A, H2B, H3, and H4 (1 $\mu$g total) alone ("Autoacetylation") or in the presence of GST–p300 HAT (2 $\mu$l, 1 mg/ml, "+ GST–HAT"), in acetylation buffer with [$^{14}$C]acetyl-Coenzyme A (1 $\mu$l). All reactions were incubated at 37°C for 60 min and resolved on a 4–20% or 15% SDS/PAGE, dried, and exposed to a PhosphorImager cassette. As seen in Fig. 1B, the minimum Tat domain that was able to autocetylate itself efficiently was the Tat 36–72 peptide. Other control peptides that were missing lysines 41 or 71 were inefficient in autoacetylation reactions (peptides 38–70 and 42–72), and another control peptide, 42–70, was completely inactive in autoacetylation experiments. Similar results were obtained with a synthetic Tat protein (1–72), where mutation of K41 and K71 abolished the autoacetylation. It is important to note that all of the Tat peptides and proteins tested




D

| | | |
|---|---|---|
| HIV-1 Tat | : | C Q V C F M T K G L G I S Y G R K K R R Q R R |
| ATF2 human | : | V T N G D T V K G H G S G L V R T Q S E E S R |
| PCAF human | : | V T S N E Q V K G Y G T H L M N H L K E Y H I |
| GCN5 yeast | : | I S S T E Q V R G Y G A H L M N H L K D Y V R |
| HAT1 yeast | : | I F P P Y Q N K G H G S C L Y E A I I Q S W L |
| HAT tetrahymena | : | V T A N E Q V R G Y G T R L M N K F K D H M Q |

HIV-1 Tat Clades

| | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Consensus-B | C | Q | V | C | F | M | T | K | G | L | G | I | S | Y | G | R | K | K | R | R | Q | R | R |
| Consensus-A | - | - | - | - | - | l | n | - | - | - | - | - | - | - | - | - | - | - | - | - | ? | - | - |
| Consensus-C | - | l | - | - | - | q | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Consensus-D | - | - | - | - | - | i | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Consensus-E | - | - | L | - | - | L | K | - | - | - | - | - | - | - | - | - | - | - | - | K | H | - | - |
| Consensus-F | - | - | w | - | - | t | - | - | - | - | - | - | - | - | - | - | - | - | - | ? | - | - | h |
| Consensus-G | - | - | ? | - | - | L | N | - | - | - | - | - | - | - | - | - | - | - | - | - | ? | - | H |
| Consensus-M | - | L | - | - | - | q | T | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Consensus-N | - | - | L | - | - | - | R | - | - | - | - | - | - | - | - | - | - | - | - | - | p | - | - |
| Consensus-O | - | Y | - | - | - | ? | ? | - | - | - | - | - | - | ? | - | - | - | - | - | · | ? | ? | ? |

FIG. 1. *Continued.* (C) MALDI-TOF mass spectrometry of peptide Tat (36–72) (top) and autoacetylated Tat (36–72) (in the absence of GST–HAT) (bottom). Ac represents the number of acetyl groups added to the Tat peptide. (D) Sequence alignment of various transcription and coactivator factors including the GCN5-related histone acetyltransferase family members that contain the conserved acetyl-CoA binding motif A sequence KGXG. Alignment of various HIV-1 Tat consensus sequences from known HIV-1 clades and their conservation in the KGXG sequence.

contained lysines 50 and 51, which were efficiently acetylated by the p300 HAT domain (Fig. 1B, left column). To further test whether autoacetylation could occur *in vitro,* we performed an autoacetylation assay with Tat (36–72) peptide along with cold acetyl-CoA and subjected the peptides to matrix-assisted laser desorption-time of flight (MALDI-TOF) mass spectrometry analysis. The results of such an experiment are shown in Fig. 1C, where the

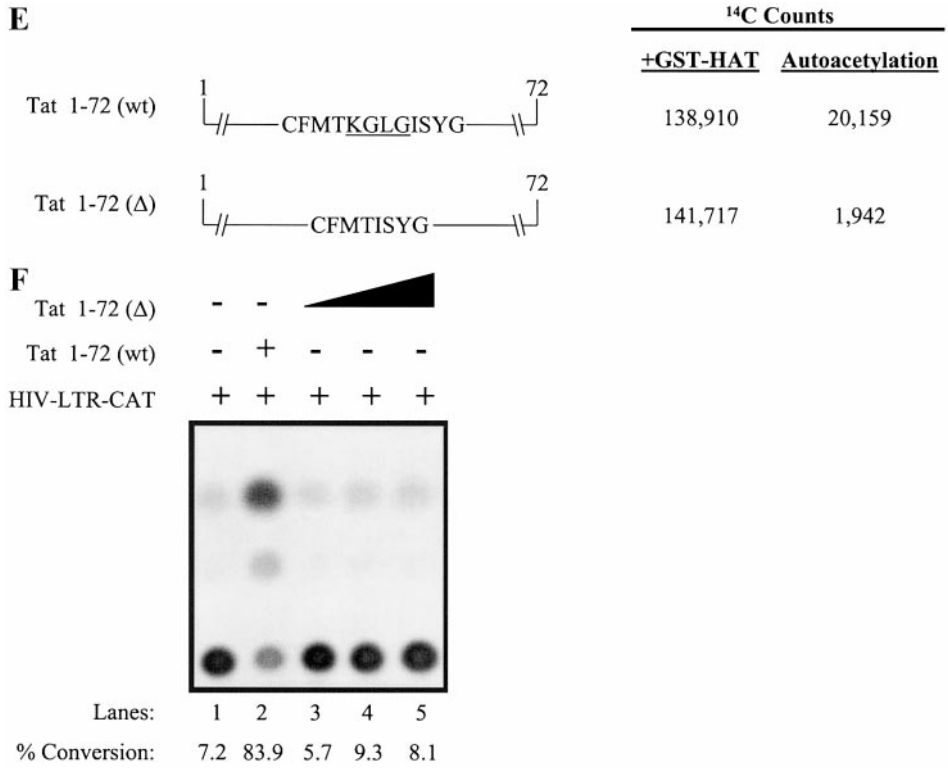



FIG. 1. *Continued.* (E) Similar to B, where 1 $\mu$g of each protein was used for either autoacetylation or acetylation with GST–HAT. (F) Transfection of CEM cells with HIV-1 LTR-CAT (3 $\mu$g) in the presence or absence of Tat protein. Lane 1 contained no Tat, lane 2 contained DNA plus 1 $\mu$g of the purified Tat (1–72) protein, and lanes 3–5 contained DNA plus 1, 3, and 5 $\mu$g of the mutant Tat (1–72) protein in the transfection mix. Cells were electroporated with both the DNA and the protein at the same time, incubated at 37°C overnight, and lysed for CAT assay the next day.

acetylated Tat showed two predominant populations, a major peak of the acetylated peptide at lysine 41 or 71 (1 Ac) and the other diacetylated peak showing both acetylated lysines (2 Ac, Bottom). Interestingly, the comparison of the sequences of Tat (34–56) with other known HAT containing transcription factors from human to yeast showed sequence identity in motif A, KGXG, which is the binding site to acetyl-CoA. Similar sites were also 100% conserved in all of the HIV-1 consensus clades sequenced to date (Fig. 1D). Finally, to determine whether the KGLG present in Tat sequence was essential for autoacetylation, we synthesized a wild-type and a Tat (1–72) deletion mutant. Both proteins were purified using reverse-phase HPLC following synthesis, and quantified by BCA assay as well by running a small aliquot on SDS/PAGE for quality control. The results in Fig. 1E show that when the KGLG sequence was deleted, autoacetylation was also abolished, and as expected the protein still served as a good substrate for GST–HAT. Importantly, when assaying for *in vivo* function (Kashanchi *et al.,* 1992), only the wild-type protein could activate transcription in a transient transfection experiment (Fig. 1F), indicating that the KGLG sequence was essential for Tat activated transcription. A Western blot from the transfected CEM cells showed equal Tat protein recovery, indicating that both the mutant and the wild-type Tat proteins were able to translocate into the nucleus (data not shown).

### Tat enhances the nucleosomal H4 acetylation by p300

It has previously been shown that p300 binds to Tat at both core and basic residues (Marzio *et al.,* 1998; Benkirane *et al.,* 1998; Hottiger *et al.,* 1998). Our previous work indicated that Tat interacts with multiple p300 domains, with the strongest binding to the minimal HAT domain (aa 1253–1790) (Deng *et al.,* in press). To assess whether Tat may affect the intrinsic HAT activity of p300 *in vitro,* we used free core histones as substrates in a HAT assay. Following the reaction, the histones were resolved on a 4–20% SDS/PAGE, and the $^{14}$C-labeled products were detected after autoradiography. Bacterially expressed GST–p300 (HAT domain) could efficiently acetylate all core histones (H2A, H2B, H3, and H4) and Tat alone did not acetylate free histones (Fig. 2A, lanes 1 and 7). However, a more interesting pattern emerged from the nucleosomal assembled DNA substrates. Using nucleosomal histones assembled with full-length HIV-1 DNA (pDH125), acetylation of only H2A and H2B histones was observed (lane 2). Interestingly, when using salmon sperm nucleosomal DNA as a substrate, GST–p300 (HAT

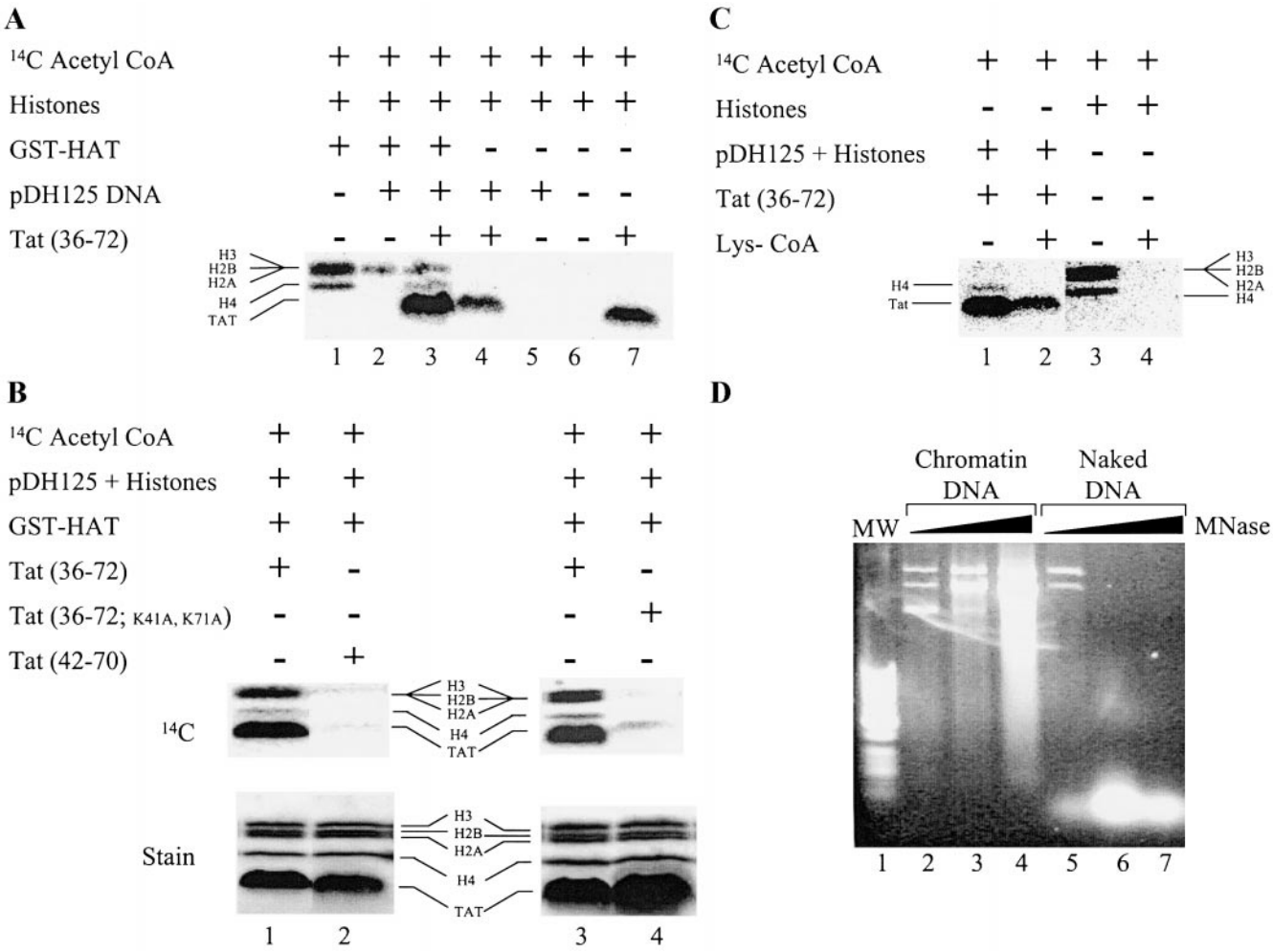



FIG. 2. Tat-enhanced acetylation of histone H4 by the p300 HAT domain. (A) Free core histones and the assembled nucleosomal histones were acetylated by GST–HAT in the presence or absence of Tat. The $^{14}$C-labeled acetylated products were separated on a 4–20% SDS/PAGE and exposed to a PhosphorImager cassette. Substrates for the HAT assay were as follows: Lane 1, free core histones; lanes 2–5, assembled nucleosomal histones with a mono- and T-tropic HIV-1 whole-genome plasmid DNA (pDH125). Following nucleosomal assembly of the pDH125 plasmid DNA, samples were mixed with or without histones and sequentially diluted from 1 to 0.1 M with salt buffer. Samples were separated on a 1% agarose gel and stained with ethidium bromide for quality control prior to the experiment (data not shown). Lanes 6 and 7 represent controls in the presence or absence of Tat, indicating that Tat alone cannot acetylate free histones. (B) Nucleosomal histones assembled with pDH125 plasmid DNA were acetylated by GST–HAT in the presence of wild-type Tat (36–72) (lanes 1 and 3), Tat (42–70) (lane 2), or Tat (36–72) mutant (lane 4). Reactions were separated on a 4–20% SDS/PAGE, stained first with Coomassie blue (bottom) and then dried and exposed to a PhosphorImager cassette (top). (C) Effects of Lys-CoA on inhibition of H4 acetylation. Free core histones (lanes 3 and 4) were incubated with GST–HAT and 5 $\mu$M Lys-CoA and a 10-fold reduction in the acetylation of all histones were observed. At the same concentration, there was a complete nucleosomal inhibition of H4 acetylation (lanes 1 and 2) by Lys-CoA. Histone H4 was reduced 8-fold (from 2368 to 291 cpm) and Tat by 3-fold (from 44,759 to 14,881). (D) MNase digestion of the nucleosomal and naked DNA. Aliquots of the assembled pDH125 chromatin DNA (100 $\mu$l, ~5 $\mu$g DNA) were digested by micrococcal nuclease for 15 min (lane 2), 45 min (lane 3), and 65 min (lane 4). As control, aliquots of naked plasmid pDH125 DNA (lane 5, 6, and 7) were also digested under the same conditions as the assembled pDH125 chromatin DNA. The digested DNAs were precipitated and run on a 1.2% agarose gel and stained with ethidium bromide.

domain) exhibited no acetylation on this complex (data not shown), indicating that the choice of DNA for chromatin assembly *in vitro* makes a significant difference in the outcome of the acetylation results. Possible explanations for the observed difference could be at the level of DNA structure, supercoiled plasmid DNA vs random oligomers of A, B, and Z forms present in salmon sperm DNA, and possible requirement of linker histones, such as histone H1, H5, or HMG proteins, for higher order chromatin structure in long linear DNA.

Interestingly, when we incubated GST–p300 (HAT) with HIV-1 nucleosomal histones in the presence of Tat, we observed a high level of histone H4 acetylation (lane 3). Tat by itself was not able to acetylate the HIV-1 nucleosomal histones in the absence of p300 HAT domain (lane 4). We therefore attributed the enhanced acetylation of

p300 HAT activity on nucleosomal H4 to be associated with the presence of the Tat peptide. To determine whether autoacetylation of Tat was important for the acetylation of nucleosomal H4, we incubated the nucleosomal HIV-1 DNA with wild-type Tat (36–72), Tat (42–70), or Tat (36–72) double 41/71 mutant (K to A substitution), along with GST–p300 HAT. The results of such an experiment are shown in Fig. 2B, where p300 HAT along with only wild type and not the 42–70 or double-lysine mutant was able to acetylate nucleosomal H4. Finally, we utilized the effect of a potent p300 inhibitor *in vitro* and found that Lys-CoA, as expected (Lau *et al.,* 2000), inhibited the acetylation of free core histones by 10-fold (Fig. 2C, lane 3 and 4). More importantly, at an $IC_{50}$ of approximately 5 $\mu$M, there was a complete nucleosomal inhibition of the H4 acetylation (lanes 1 and 2), indicating that the Tat-enhanced p300 acetylation activity can be inhibited by HAT inhibitors. As a control we consistently performed the MNase assay on naked vs assembled DNA prior to HAT assays to ensure chromatin assembly. As can be seen in Fig. 2D assembled DNA is much more resistant to MNase treatment than naked DNA. Finally and of particular interest, a P/CAF inhibitor, H3-CoA-20 (Lau *et al.,* 2000), inhibited full-length Tat autoacetylation ($IC_{50}$ of 30 $\mu$M, ~70%) in a nucleosomal HAT assay and the overall rate of acetylation of free Tat (36–72) showed a first-order rate constant at 20 $\mu$M acetyl-CoA, which is about 1000- to 10,000-fold slower than p300 or P/CAF *in vitro* (data not shown).

## Increased acetylation of the H4 lysine residues in the presence of p300 HAT and Tat

To address whether H4 lysine residues were acetylated in the presence of Tat, we utilized an immunoprecipitation assay with ChIP grade anti-acetyl-H4 antibodies. The results of such an experiment are shown in Fig. 3A, where polyclonal antibodies against H4 were able to pull down H4 from a chromatin assembled DNA (lane 2). As expected the p300 HAT domain efficiently acetylated the free histones as evident in the H4 immunoprecipitates (lane 3). To address which H4 lysines on nucleosome DNA were acetylated by Tat–p300 interaction, we used antibodies specific against lysine 5, 8, 12, and 16 in immunoprecipitation (IP) reactions following the HAT assay. Antibodies used for IP were first evaluated on a SDS/PAGE for quality and amounts (heavy and light chains) prior to the immunoprecipitations. The immunoprecipitated H4 was then resolved on a SDS/PAGE, and the HAT activity was detected following autoradiography. Figure 3B shows the histogram for the IP reaction, where three antibodies were able to immunoprecipitate the histone H4 off of the chromatin DNA, and lysines 8, 12, and 16 were all acetylated to varying degrees. Interestingly, lysine 16, which has previously been correlated with increased transcription (Strahl and Allis, 2000), was

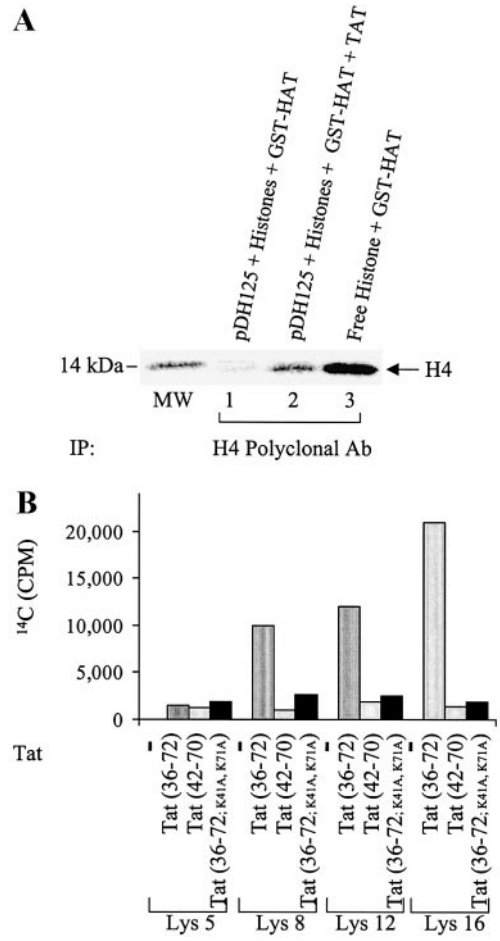



FIG. 3. Increased acetylation of H4 lysines in the presence of p300–HAT and Tat. (A) Nucleosomal histones assembled with pDH125 plasmid DNA were acetylated by GST–HAT in the presence (lane 2) or absence (lane 1) of Tat. Samples were immunoprecipitated with ChIP grade anti-acetyl-H4 antibodies. Immunoprecipitated $^{14}$C-labeled H4 lysines were separated on 4–20% SDS/PAGE, dried, and exposed. (B) Nucleosomal histones were immunoprecipitated with antibodies specific against lysine 5, 8, 12, and 16 following the HAT assay in the presence of Tat (36–72) wild-type, Tat (36–72) mutant, or Tat (42–70). The quality of all antibodies was determined by running a small amount (100 ng) on SDS/PAGE and staining for heavy and light chains prior to immunoprecipitations. Immunoprecipitates were separated on a 4–20% SDS–PAGE, dried, and exposed. Radioactive bands were counted using Molecular Dynamics software.

the most acetylated residue in the presence of wild-type Tat and not the double-mutant Tat.

## Histone acetylation increases transcription of HIV-1 chromatin DNA *in vitro*

*In vivo,* acetylation of histone H4 at lysines 8 and 16 correlates well with increased transcriptional activity (Strahl and Allis, 2000). The ability of Tat to enhance the acetylation of nucleosome histone H4 allowed us to directly test whether the Tat–p300 interaction would increase the transcription from the chromatin LTR *in vitro.* Nucleosomes were assembled on biotinylated HIV-1 LTR

DNA template first and then immobilized using streptavidin agarose beads (Fig. 4A). The immobilized chromatin LTR was washed in acetylation buffer and used as a substrate for acetylation by p300 in the presence or in the absence of Tat. Following the acetylation reaction, the template was washed in transcription buffer and used in an *in vitro* run-off transcription reaction. Assembly of the nucleosome led to a tight repression of the HIV LTR transcription (Fig. 4B, lane 2), and not the nonnucleosomal DNA (lane 1). Addition of the wild-type Tat (36–72), and not the double-mutant, prior to the transcription reaction increased the transcription from the chromatin template (lanes 3–5), presumably by its ability to enhance the acetylation of nucleosomal H4. Addition of Tat (36–72) wild-type, Tat mutant, or 42–70 peptides during the transcription did not enhance the transcription of nucleosomal DNA (data not shown). Finally, to assess whether acetylation of histone H4 perturbed the nucleosome, we performed pull-down experiments on pre- and posttranscription templates. As shown in Fig. 4C, all histones remained bound to DNA before and after *in vitro* transcription reaction, indicating that acetylation of histone H4 does not perturb the nucleosomal structure nor does it aid in the removal of core histones from DNA (lanes 3 and 4). Taken together, these observations indicate that Tat with its association with the p300 HAT domain acetylates histone H4, which aids in the transcription of repressed chromatin.

### Effect of the Tat lysine mutation on viral replication

We used HLM-1 cells to address whether the interaction of Tat and p300 plays a role in the activation of integrated proviral HIV-1 sequence *in vivo.* HLM-1 cells were derived from HeLa-T4+ cells containing an integrated copy of the HIV-1 proviral genome with a 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 particles after transfection with Tat expressing clones, stimulation with cytokines, such as TNF-$\alpha$, or general inducers, such as sodium butyrate. In order to test whether the coactivator p300 plays a critical role in the activation of integrated HIV-1, we transfected the HLM-1 cells with either the wild-type or the mutant Tat in the presence or absence of p300. Supernatants were collected at days 3, 7, and 10 for p24 gag ELISA. The results of such an experiment are shown in Fig. 5A where ectopic expression of p300 along with wild-type Tat activated the viral production by three- to fourfold. However, the lysine 41 mutant of Tat either alone or in combination with p300 did not activate the latent virus. Similar results were seen with increased titrations of lysine 41 mutant plasmid that were carried out to 90 days (data not shown). Taken together these data indicate that the Tat 41 lysine residue is important for the activation of integrated HIV-1 promoter activity.

## DISCUSSION

Virus infection or activation of infected cells results in a dramatic hyperacetylation of histones H3 and H4, which have been shown to localize to specific cellular promoters (Parekh and Maniatis, 1999). By inference, results from a number of laboratories in the past few years have shown a dramatic increase in viral transcription when HIV-1-infected cells or cells carrying stably integrated HIV-1 LTR were treated with histone deacetylase inhibitors, such as *n*-butyrate (Laughlin *et al.,* 1993, 1995), TSA, or trapoxin (Van Lint *et al.,* 1996). This is due mainly to the fact that stably integrated HIV-1 provirus is packaged into chromatin (Mirkovitch, 1997; El Kharroubi *et al.,* 1998), and two nucleosomes (called nuc-0 and nuc-1) have been shown to be deposited onto the viral DNA promoter at precise locations with respect to regulatory elements (Verdin, 1991; Verdin *et al.,* 1993). It has been shown that the binding of transcription factors p50 and Sp1 leads to a reconfiguration of the chromatin structure in the proximal promoter region (Pazin *et al.,* 1996) and that the LTR nucleosomal arrays containing Sp1 and NF-$\kappa$B1 produce regions of enhanced DNase I sensitivity, specifically at the HIV-1 nucleosome (Steger and Workman, 1997). Therefore, a combination of Sp1 and multiple distal enhancer binding proteins was required to derepress the HIV-1 transcription in chromatin reconstitution experiments (Sheridan *et al.,* 1995).

Tat is also acetylated by CBP/p300 and P/CAF (Kiernan *et al.,* 1999; Ott *et al.,* 1999), which increases the affinity for the CDK9/P-TEFb CTD kinase complex. We have previously shown that Tat binds to CBP/p300 in the minimal HAT domain (aa 1253–1790) and the interaction results in a change of conformation on CBP/p300 (Deng *et al.,* in press). Our current results indicate that the Tat–p300 interaction increases the HAT activity of p300 on histone H4-associated nucleosomal DNA and not free histones. Nucleosomal histone H4 was acetylated to varying degrees on lysines 8, 12, and 16, which correlates well with the increased transcriptional activity. This is in contrast to p300's specificity, where it can acetylate preferentially at Lys 5 and Lys 8 (Sobel *et al.,* 1994, 1995; Schiltz *et al.,* 1999). Therefore, Tat's ability to modulate p300 HAT activity and increase its acetylation profile may explain the increase in transcription observed in our *in vitro* run-off assay. A clear correlation between H4 acetylation and increased transcription has been demonstrated on a number of promoters, including the Hsp26 gene and activator protein MOF (Vettese-Dadey *et al.,* 1996; Imhof *et al.,* 1997; Akhtar and Becker, 2000). This is also consistent with previously published reports where in the absence of transcription (Marzio *et al.,* 1998), HAT-associated complexes showed an increase in the

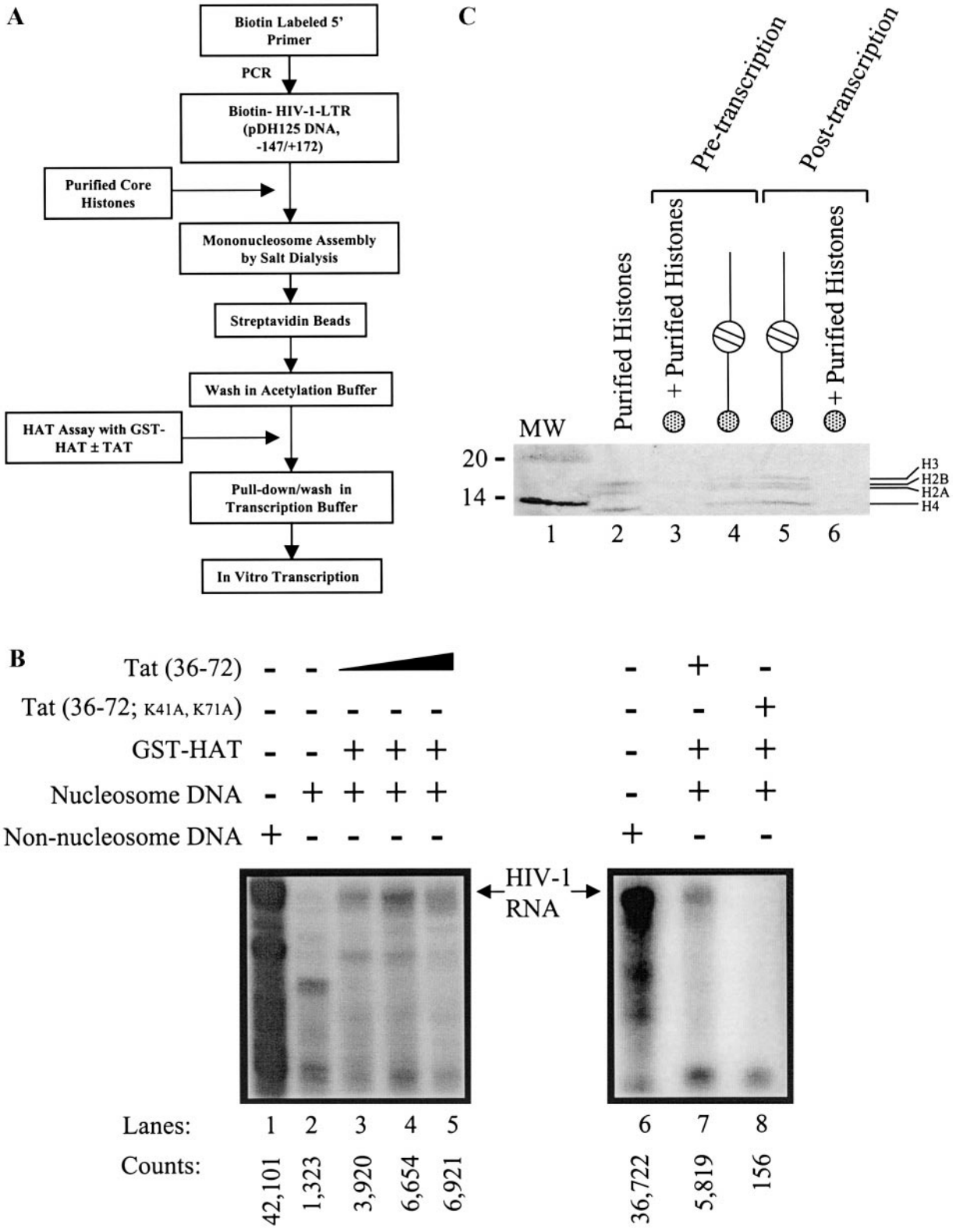



FIG. 4. Histone acetylation increases the transcription of HIV-1 chromatin template *in vitro*. (A) The diagram depicts the experimental procedure for obtaining biotin-labeled HIV-1 LTR, chromatin assembly, and pull-down with streptavidin beads followed by an *in vitro* transcription reaction. (B) Immobilized chromatin HIV-1 LTR templates (1 $\mu$g) were acetylated in the presence of GST–HAT (lane 3–5, 7, and 8) or wild-type Tat (36–72) (lane 3, 100 ng; lane 4, 200 ng; lane 5, 400 ng). Lanes 1 and 6 represent 1 $\mu$g of immobilized histone free naked DNA used in an *in vitro* transcription reaction. Lane 7 represents addition of wild-type Tat (36–72) (400 ng) and lane 8 represents the addition of mutant Tat (36–72) (400 ng) to the reaction. Immobilized templates were then washed in transcription buffer and tested for transcription *in vitro* using HeLa nuclear extracts. Run-off of $^{32}$P-labeled transcripts (172 bases) was separated on a 6% urea/PAGE and exposed to a PhosphorImager cassette. Counts (cpm) from the RNA bands are shown

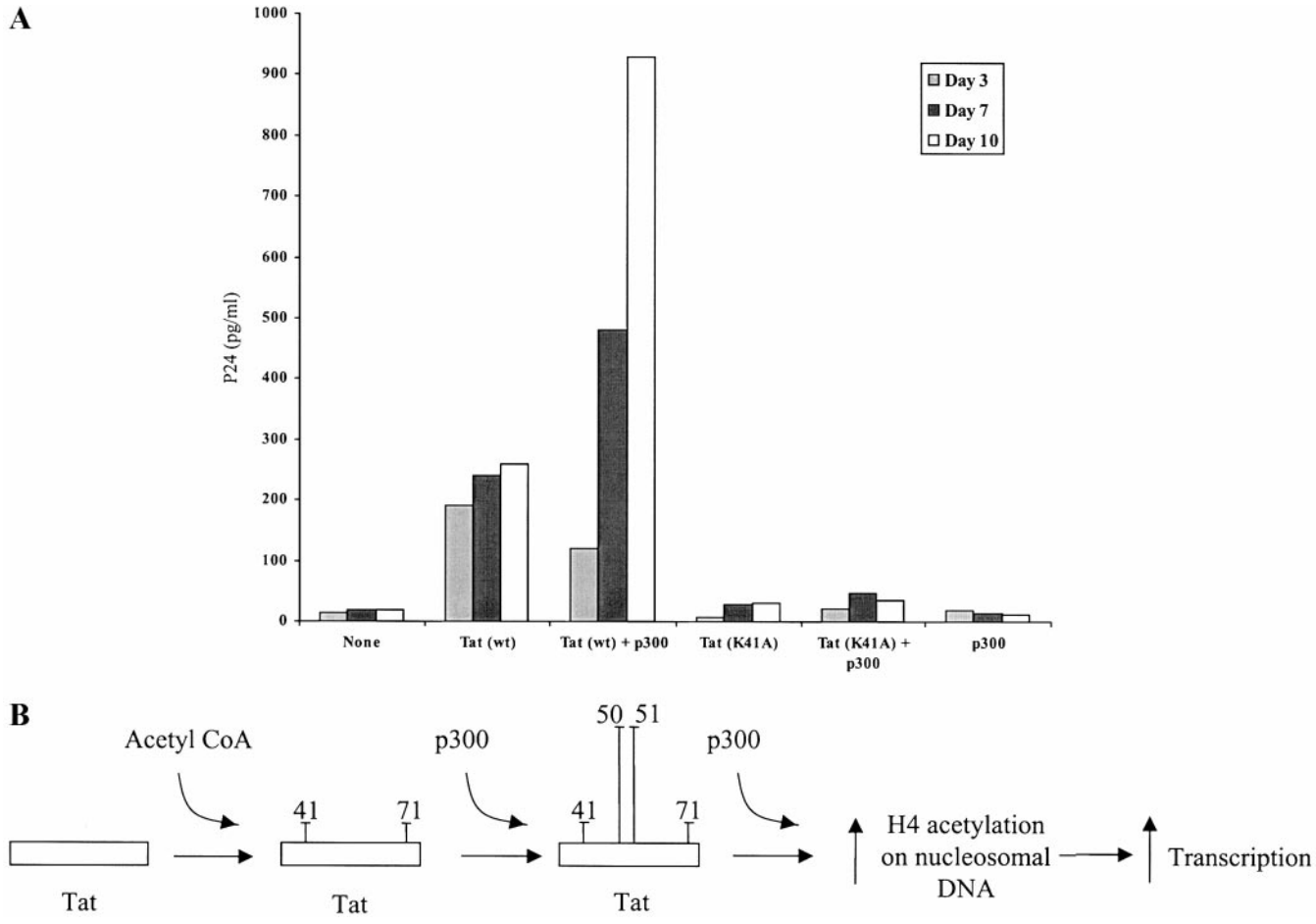



FIG. 5. Effect of wild-type and mutant Tat on HIV-1 proviral genome. (A) HLM-1 cells were transfected either alone or with wild-type or mutant Tat (50 ng) and CMV-p300 (6 $\mu$g) by the $CaPO_4$ precipitation method. The p24 gag antigen ELISA was performed from supernatants obtained at days 3, 7, and 10 after transfection. All experiments were performed twice in HLM-1 cells. (B) Proposed model of how Tat acetylation and p300 HAT activity enhancement increases transcription on an HIV-1 nucleosomal DNA. Autoacetylation of Tat at positions 41 and 71 allows for the efficient recruitment of p300, which can acetylate Tat at positions 50 and 51. This complex may allow a change of conformation on p300 to increase its acetylation activity on nucleosomal histone H4, which in turn enhances transcription of the HIV-1 promoter.

cleavage efficiency of restriction endonucleases targeted to the HIV-1 chromatin template, suggesting that histone acetylation alone leads to nucleosome remodeling (Steger *et al.,* 1998; Li *et al.,* 1999). Finally, in an attempt to find other nonhistone transcription factors that could serve as a substrate for Tat and p300 HAT complex, we utilized all the known transcription factors affecting HIV-1 transcription *in vitro* and have found that the human TATA binding protein (TBP) can serve as a substrate for acetylation *in vitro* (L. Deng and F. Kashanchi, unpublished results). The increased acetylation on TBP increased its DNA binding to the HIV-1 TATA box. It remains to be seen whether TBP acetylation either alone or in the context of SL1, TFIID, or TFIIIB could increase the rate of cellular Pol I, II, and III transcription or simple reinitiation of HIV-1- or TAR-independent promoters normally affected by Tat.

Unexpectedly, we also observed that Tat could autoacetylate itself, which was specific to the wild-type and 36–72 peptide but not the double-lysine mutant or 42–70 peptide, indicating that lysine residues 41 and 71 were acceptor sites for autoacetylation. Peptide 42–70 could

at the bottom. (C) The presence of intact nucleosomes in pre- and posttranscription reaction. Reactions were carried out at 5× for each sample. Following pull-down in transcription buffer, samples were prepared for *in vitro* transcription (see the last two parts in A). Lane 2 represents 1/10 of the original free histones used for the assembly reaction. Lanes 3 and 5 serve as controls where DNA was not added to the original reaction; however, samples contained the addition of purified core histones prior to assembly. Lanes 4 and 5 represent samples from pre- and posttranscription reactions, respectively. Samples from lanes 3–6 were finally washed twice in buffer D, plus 300 mM KCl and 0.1% NP-40, resuspended in 100 $\mu$l of 1 M KCl buffer, incubated at 37°C for 4 h (to remove core histones from DNA), TCA precipitated the supernatant, dried, denatured, run on a 4–20% SDS/PAGE, and stained with Coomassie blue.

not enhance the HAT activity of p300 to acetylate nucleosomal H4. Therefore, the presence of acetylated lysine 41 and 71 accounts for increased p300 HAT activity. Lysine 41 mutation has been shown by us and others to completely inactivate Tat function *in vivo* and it remains to be seen whether lysine 71 also has similar importance for Tat inactivation *in vivo.* It is important to note that in our assays Tat autoacetylation was not 100% efficient. We attribute the inefficiency of acetylation to various *in vitro* preparations of Tat. For instance, *Escherichia coli*-made or machine-synthesized Tat, fractionated on a denatured HPLC column, lyophilized, and renatured prior to use, is at best 2–10% active, as judged by activation of the HIV-1 promoter in an *in vitro* transcription assays. However, we have recently observed a higher acetylation efficiency (30–40%) when Tat was made as a soluble protein produced from Baculovirus-infected cells (L. Deng, unpublished results). Future MALDI-TOF experiments will determine whether autoacetylation requires Tat dimers (absent in denatured preps) or other post-translational modifications for optimal enzymatic activity.

It is important to note that our current study does not adequately address whether Tat autoacetylation occurs *in vivo.* However, we have performed a number of experiments to address this issue and to this end have found ambiguous and therefore unreportable results. For instance, we find labeling of cells with any low or high beta emitter, for the purpose of *in vivo* labeling of proteins, to cause an artificial cell cycle blockage. This is true when labels are added prior to or subsequent to transfection experiments. Specifically, low emitters block cells at G2/M and high emitters block cells at the G1/S phase of the cell cycle. Therefore, in our view, results obtained from the *in vivo* labeling of Tat and its reported acetylation site at various positions *in vivo* are ambiguous. To circumvent this problem, we have utilized Tat immunoprecipitations in the presence of deacetylase inhibitors from either transfected (HA tagged antibody) or infected (polyclonal anti-Tat antibody) cells, followed by separation on SDS/PAGE, trypsin digestion, and acquisition of peptide mass fingerprints, using MALDI-TOF. Again, we find inconsistent results depending on which cell types we use. For instance, K28, 41, and 50 are all acetylated at early G1 (to varying degrees) in CEM and HL-60 cells, but not in HeLa or U937 cells. However, we observe efficient acetylation of K28, 41, 50, 51, and 71 in late G1/S in CEM cells and efficient acetylation in HeLa and U937 cells in G2 cells. To add to the complexity of *in vivo* acetylation sites, in HIV-1-infected latent cells (ACH2, 8E5, U1, and OM10.1) Tat shows variable levels of acetylation on K28 and 71 and no acetylation at some of the other reported *in vivo* sites. Therefore, this level of compounding complexity on *in vivo* acetylation sites requires further and careful analysis with methods that truly score for *in vivo* Tat acetylation sites. Future experiments utilizing elutriated cell cycle fractions, and infection with various HIV-1 clades in lymphocytes, will determine the exact sites of Tat acetylation *in vivo.*

The effect of acetylation on transcription elongation has also been demonstrated. Wittschieben *et al.* (1999) showed that histone modification may be directly coupled to transcription elongation by RNA polymerase II. *In vitro,* purified RNA polymerase II could transcribe naked DNA without substantial impediments, but its ability to transcribe a chromatin template efficiently is severely compromised. However, *in vivo,* the actively transcribing complex contains not only RNA polymerase II, but also a multisubunit complex, termed "elongator," which associates with the hyperphosphorylated C-terminal domain of the largest polymerase subunit (Otero *et al.,* 1999), and the piggybacking of chromatin modification activities by helicases in general and RNA polymerase II in particular is needed for transcription and other activities such as mRNA capping, polyadenylation, and pre-mRNA splicing (Otero *et al.,* 1999). Therefore, in the case of HIV-1, it is conceivable that cyclin T/cdk9 phosphorylation of RNA Pol II CTD may aid in piggybacking of the Tat and HAT complexes, which may in turn primarily acetylate histones and play a significant role in transcriptional elongation on repressed chromatin DNA.

Finally, it has been reported that the HAT activity of CBP/p300 is stimulated by interaction with E1A or phosphorylation by cdk2/cyclin E complex (Ait-Si-Ali *et al.,* 1998). Along the same lines, we can envision a cdk9/cyclin T complex that binds to substrates other than CTD to modulate their activity. For instance, phosphorylation of the CBP/p300 complex by cdk9/cyclin T could increase its intrinsic HAT activity. Alternatively, cdk9/cyclin T complex could phosphorylate histone tails, which may serve as better substrates for HAT enzymes (L. Deng, unpublished results). Future experiments will define whether substrates other than CTD could contribute to Tat's multifunctional role in the HIV-1 life cycle.

## MATERIALS AND METHODS

### Antibodies

Antibodies were purchased from Upstate Biotechnology (UBI): anti-acetyl histone H4, ChIPs grade, polyclonal (Catalog No. 06-866); anti-acetyl-histone H4 (Lys 5), polyclonal (Catalog No. 06-866); anti-acetyl-histone H4 (Lys 8), (Catalog No. 06-0760); anti-acetyl-histone H4 (Lys 12), (Catalog No. 06-761); and anti-acetyl-histone H4 (Lys 16), (Catalog No. 06-762). Anti-acetyl histone H4, ChIP grade, is a polyclonal antiserum from rabbit. It recognizes acetylated histone H4 and may cross-react with other acetylated proteins, such as H2B. All antibodies recognizing histone H4-acetylated forms at positions 5, 8, 12, and 16 were IgG affinity-purified.

### Immunoprecipitation

The nucleosomal arrays were assembled on pDH125 plasmid DNA with a histone:DNA ratio of 1:1.5 by dialysis. The nucleosome histones were acetylated by p300 (HAT) in the presence or in the absence of Tat. The acetylated histones ($^{14}$C-labeled) were immunoprecipitated by polyclonal anti-acetyl histone H4, ChIPs grade, anti-acetyl-histone H4 (Lys 5), anti-acetyl-histone H4 (Lys 8), anti-acetyl-histone H4 (Lys 12), and anti-acetyl-histone H4 (Lys 16) antibodies. All antibodies were equalized on SDS/PAGE by staining for similar amounts of heavy and light chain prior to the IP reaction. Samples (IPs) were allowed to rotate overnight at 4°C and the next day protein A+G beads (30% slurry, 100 $\mu$l) were added for an additional 2 h. The IP beads were then washed with TNE 150 buffer (150 mM NaCl; 100 mM Tris–HCl, pH 7.5, 1 mM EDTA, and 0.1% NP-40) at least four times. The acetylated histones H4 ($^{14}$C-labeled) were separated in a 4–20% SDS/PAGE (Invitrogen, Inc.), dried, and exposed to a PhosphorImager cassette.

### Peptide synthesis

The Tat peptides (36–72) 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), and Ser and Tyr (But). 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. A similar approach was used to synthesize the Tat (1–72) protein with an efficiency of 2–3% yield for the wild-type and 1.5% yield for the mutated protein. The purity of the peptides and proteins was further confirmed by analytical reverse-phase HPLC, capillary zone electrophoresis, and MALDI-TOF mass spectrometry. The amounts of each peptide were determined by Bio-Rad protein assay as well as by running small aliquots on 4–20% or 15% SDS/PAGE followed by silver staining (Silver Stain Plus, Bio-Rad).

### Biotinylated HIV-1 LTR DNA

The HIV-1 LTR DNA from −147 to +172 was amplified by PCR from the pDH125 plasmid DNA (Dual T- and M-tropic HIV-1 genome, the generous gift of D. Cho and M. Martin, NIH/NIAID) with the biotinylated 5′ primer, biotin-5′ACT TTTCCGGGGAGGCGCGATC3′, and the 3′ primer, 5′GCCACTGCTAGAGATTTCCACACTG3′, oligonucleotides providing every PCR product to contain a biotin moiety at the 5′ termini. The sequences of the HIV-1 LTR DNA were confirmed by the dideoxy sequencing method.

### Nucleosome reconstitution by salt sequential dilution

Core histones were purified from HeLa cells by the method of Simon and Felsenfeld (1979). The biotinylated mononucleosomes were prepared by mixing the biotinylated HIV-1 LTR DNA and purified core histones by salt sequential dilution from 1 to 0.1 M NaCl (Imblazano, 1998; Stein and Mitchell, 1988). Ten to 20 $\mu$g of DNA of HIV-1 LTR PCR products from plasmid pDH125 was mixed with 5 $\mu$l of 5 M NaCl, and 2 $\mu$l of 10× reconstitution buffer (0.15 M Tris–HCl, pH 7.5; 1 mM DTT; 2 mM EDTA) by pipetting up and down repeatedly. Next 30–40 $\mu$g of core histones was added in a total volume of 50 $\mu$l adjusting the volume by adding dd $H_2O$. Samples were then gently flicked in the tube and incubated at 37°C for 20 min. Sequential dilution was carried out by adding 10 $\mu$l of 1× reconstitution buffer every 10 min, for 3 h at 37°C. At each time point, samples were mixed by gentle up and down pipetting. An aliquot was run on agarose gel to ensure proper assembly prior to each experiment.

### Histone acetyltransferase assay

HAT assays were performed in a total of 30- to 60-$\mu$l reactions at 37°C for 60 min in a buffer containing 1 $\mu$l of purified GST–p300 HAT (1 mg/ml), 200–400 ng of substrate proteins or peptides, and 1 $\mu$g 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 $\mu$l of [$^{14}$C]acetyl-Coenzyme A (65 mCi/mmol/ICN). Proteins and peptides were resolved on a 4–20% or 15% SDS/PAGE (Novex, Inc.). Gels were dried and exposed to a PhosphorImager cassette and bands were quantified using Molecular Dynamics software. Both HAT inhibitors, Lys-CoA and H3-CoA, were a generous gift from P. A. Cole (Johns Hopkins University).

### MNase digestion of the nucleosomal and naked DNA

Aliquots of the assembled pDH125 chromatin DNA (100 $\mu$l, ~5 $\mu$gDNA) were added to 100 $\mu$l of prewarmed (37°C) digestion buffer (50 mM NaCl, 10 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.15% NP-40, and 25 units of MNase, USB Catalog No. 70196Y) incubated for 15, 45, and 65 min at 37°C. The reactions were terminated by the addition of 10 $\mu$l of 0.5 M EDTA and incubated for an additional 15 min at 37°C. Following termination, the DNA was phenol/chloroform extracted, precipitated, and resuspended in 10 $\mu$l of TE buffer. As a control, aliquots of naked plasmid pDH125 DNA were also digested under the same conditions as described above. The digested DNAs were precipitated, run on a 1% agarose gel, and stained with ethidium bromide.

### *In vitro* transcription on chromatin templates

The biotinylated DNA of HIV-1 LTR (from −147 to +172) was assembled with core histones into mononu-

cleosomal chromatin. Biotinylated chromatin templates were pulled down with streptavidin–agarose beads after HAT assay with or without acetyl-CoA and Tat. The chromatin beads were washed with transcription buffer (20 mM HEPES (pH 7.9), 100 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 17% glycerol, and 1 mM dithiothreitol) twice and preincubated at 30°C for 30 min, followed by the addition of 3 $\mu$l [$^{32}$P]UTP (Amersham Pharmacia Biotech, 400 Ci/mmol) and 20 $\mu$l HeLa nuclear cell extract (14 $\mu$g/$\mu$l), 3 $\mu$l of 20% PEG (6000), 3 $\mu$l of 50 mM $MgCl_2$, 3 $\mu$l of 1 mM dithiothreitol, 1.5 $\mu$l of 50 mM ATP/CTP/GTP, and 18 $\mu$l of transcription buffer. $^{32}$P-labeled RNA products were purified and separated on a 6% denaturing acrylamide urea gel (Invitrogen, Inc.).

### Mass spectrometry

The unacetylated or autoacetylated peptides were digested with proline-specific endopeptidase (Seikagaku Corp.). The digested or undigested peptides were desalted using $C_{18}$ ZipTips (Millipore) according to the manufacturer's procedure. A 1-$\mu$l aliquot of sample was taken for peptide mass mapping on a PerSeptive Biosystem DEPRO MALDI-TOF Mass Spectrometer using $\alpha$-cyano-4-hydroxycinnamic acid as the matrix. Analysis was performed in the linear delayed-extraction mode, with external calibration. The analysis of acetylation mass was performed through the ProFound Web site located at Rockefeller University (http://prowl.rockefeller.edu).

### Transfection and HIV-1 detection of HLM-1 cells

HLM-1 cells (AIDS Research and Reference Reagent Program, Catalog No. 2029) were derived from HeLa-T4+ cells integrated with one copy of 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. HLM-1 cells are negative for virus particle production, but can be induced to express one cycle of infectious HIV-1 particles after transfection with Tat cDNA or mitogens such as TNF-$\alpha$ or sodium butyrate. HLM1 cells were grown in DMEM containing 10% FBS, 100 $\mu$g/ml of G418, plus 1% streptomycin, penicillin, and 1% L-glutamine (Gibco BRL). Cells were grown to 75% confluency prior to the transfection or passages.

HLM-1 cells were transfected with the plasmid DNAs including Tat, mutant Tat lys41 (generous gift of D. Singer at NIH/NCI), and p300 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 by using standard ELISA kit (Abbott) using the supernatants of transfected cells at days 3, 7, and 10.

### Protein transfection

Lymphocyte (CEM (12D7)) cells were grown to the mid-log phase of growth and processed for protein electroporation as described previously (Kashanchi *et al.,* 1992), with only minor modification, where cells were electroporated at 230 V with HIV-1 LTR CAT DNA (3 $\mu$g) along with Tat wild-type (1.0 $\mu$g) or mutant (KGLG deletion) protein, and plated in 10 ml of complete RPMI 1640 medium for 18 h prior to harvest.

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