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Counterregulation of Chromatin Deacetylation and Histone Deacetylase Occupancy at the Integrated Promoter of Human Immunodeficiency Virus Type 1 (HIV-1) by the HIV-1 Repressor YY1 and HIV-1 Activator Tat

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Repression of human immunodeficiency virus type 1 (HIV-1) transcription may contribute to the establishment or maintenance of proviral quiescence in infected CD4$^+$ cells. The host factors YY1 and LSF cooperatively recruit histone deacetylase 1 (HDAC1) to the HIV-1 long terminal repeat (LTR) and inhibit transcription. We demonstrate here regulation of occupancy of HDAC1 at a positioned nucleosome (nuc 1) near the transcription start site of integrated LTR. We find that expression of YY1 increases occupancy by HDAC1, decreases acetylation at nuc 1, and downregulates LTR expression. HDAC1 recruitment and histone hypoacetylation were also seen when Tat activation was inhibited by the overexpression of YY1. A YY1 mutant without an HDAC1 interaction domain and incompetent to inhibit LTR activation fails to recruit HDAC1 to LTR or decrease nuc 1 acetylation. Further, expression of a dominant-negative mutant of LSF (dnLSF), which inhibits LSF occupancy and LTR repression, results in acetylation and decreased HDAC1 occupancy at nuc 1. Conversely, exposure of cells to the histone deacetylase inhibitor trichostatin A or activation of LTR expression by HIV-1 Tat results in the displacement of HDAC1 from nuc 1, in association with increased acetylation of histone H4. Recruitment of HDAC1 to the LTR nuc 1 can counteract Tat activation and repress LTR expression. Significantly, when repression is overcome, LTR activation is associated with decreased HDAC1 occupancy. Since the persistence of integrated HIV-1 genomes despite potent suppression of viral replication is a major obstacle for current antiretroviral therapy, strategies to selectively disrupt the quiescence of chromosomal provirus may play a role in the future treatment of AIDS.

Biochemical and epigenetic studies have revealed that nucleosomes at eukaryotic promoters function as dynamic units in transcriptional regulation (4, 5, 8, 18, 21, 38, 45). The nucleosome core contains a central histone octamer consisting of four histone dimers, H2A, H2B, H3, and H4 (30). Posttranscriptional modifications of the tails (31, 42) of histone proteins, such as acetylation (19, 48, 50), methylation (43), and ubiquination and phosphorylation (9, 11), modulate the conformation of a nucleosome. Nucleosome structure is thought to influence the accessibility of transcription factors to the promoter and the formation of the transcription initiation complex. Hyperacetylation of core histones is correlated with transcription activation (7, 20, 35) while hypoacetylation is correlated with repression (27, 32). Acetylation is reversed by histone deacetylases (HDACs), a family of enzymes that removes acetyl groups from the tails of acetylated core histones (14, 22, 49). Although the mechanism by which HDACs regulate nucleosome structure is not clear, several transcriptional repressors associate with HDACs. Interestingly, DNA-binding domains of HDACs have not yet been identified. Targeted recruitment of HDACs to a specific promoter by sequence specific DNA-binding factors is a favored model to explain the selective silencing of individual eukaryotic genes (23, 24, 33, 36, 37).

Human immunodeficiency virus type 1 (HIV-1) is an intracellular parasite dependent on the host cellular metabolism to complete its life cycle. Once integrated into the host genome, the 5′ long terminal repeat (LTR) of HIV-1 serves as the promoter regulating viral gene expression and replication. HIV-1 can establish a quiescent, latent state within resting CD4$^+$ T cells (16). Mechanisms that allow the establishment or maintenance of proviral quiescence have not yet been elucidated. While recruitment of selected host transcription activators and viral activator Tat to the LTR allows powerful activation of HIV transcription and viral replication, restriction of LTR expression by host repressors may allow activated, infected lymphocytes to return to the nonproductive resting state and establish viral quiescence.

A positioned nucleosome (nuc 1) spanning the region from +1 to +155 with respect to the transcription start site of HIV-1 LTR has been mapped in DNase I protection studies (52, 53). Experiments examining the accessibility of integrated HIV-1 LTR to restriction endonucleases at this region suggest that disruption of this nucleosome accompanies transcriptional activation of integrated LTR by the viral factor Tat (13, 40) or the HDAC inhibitors trichostatin A (TSA) and trapoxin (51).

Our previous studies have identified two ubiquitous transcription factors, YY1 and LSF, that cooperate in the repres-

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sion of HIV-1 LTR and viral production (12, 34, 44). LSF binds to a sequence located at the region from −10 to +27 of the HIV-1 LTR and recruits YY1 to LTR via a specific interaction with the zinc-finger domain of YY1. YY1 and LSF repress the LTR via recruitment of HDAC1. These three cellular factors copurify in a complex binding the LTR RCS site, and repression of HIV LTR expression requires both LSF capable of binding the LTR RCS site and YY1 capable of recruitment of HDAC1 (12). Further, mutations within the RCS ablate the inhibitory effect of YY1 (44), TSA blocks YY1-mediated repression (12), and sequence-specific polyamides antagonizing LSF binding to LTR allow HIV expression in vivo (J. J. Coull et al., unpublished data). Deacetylation of histones may remodel nuc 1 at LTR, resulting in transcriptional repression of HIV-1.

We have analyzed in vivo factor occupancy at the nuc 1 region of the integrated LTR by using a chromatin immunoprecipitation (ChIP) assay. We demonstrate for the first time that ChIP assays detect HDAC1 at the nuc 1 site of the LTR and loss of HDAC1 after TSA treatment in vivo. We then examined the occupancy of HDAC1 and the acetylation state of nuc 1 simultaneously and found an inverse correlation between LTR expression and both HDAC1 occupancy and nuc 1 hypoacetylation. Further, we found that the HIV-1 activator Tat decreased HDAC1 occupancy, while the LTR host repressor YY1 could overcome this effect, decreasing H4 acetylation at nuc 1 and increasing HDAC1 occupancy.

MATERIALS AND METHODS

Cell culture and TSA treatment. The HeLa-CD4-LTR-CAT cell line (10) was maintained in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS). Cells between passages 2 and 10 were grown in 0.5% FBS overnight to synchronize them at G_0/G_1. Then, 5×10^5 cells were fed with 10% FBS, and 400 nM TSA was added. Cells were washed with phosphate-buffered saline (PBS), and cellular extracts were prepared after 2 h of TSA treatment and prior to extensive cell cycling.

Transfection. 2×10^6 cells were seeded in 100-mm culture dishes and grown overnight. Expression plasmids driven by the cytomegalovirus (CMV) immediate-early promoter, CMV-empty vector, CMV-green fluorescent protein (GFP) (Clontech, Palo Alto, Calif.), CMV-YY1 (46), and CMV-Tat were purified by using an Endofree plasmid kit (Qiagen, Valencia, Calif.) and quantified by using a spectrophotometer. Cells were cotransfected by using Lipofectamine 2000 reagent according to the manufacturer's instructions (Life Technologies). A total of 5 μg of DNA, including 1 μg of CMV-GFP, was mixed with 20 μl of Lipofectamine 2000 reagent, added to cells in serum-free medium, and incubated for 4 h before DMEM with 10% FBS was added.

FACS analyses. Transfected cells were grown in DMEM for 18 to 24 h before harvest for fluorescence-activated cell sorting (FACS) analysis. Cells were raised in 10% FBS, gated for high GFP expression, and sorted into GFP-negative or GFP-positive populations.

ChIP assays. After being sorted by FACS, at least 2×10^5 GFP-expressing cells were used for each ChIP assay. Cells were fixed in 1% formaldehyde at 37°C for 8 min. After being cross-linked, cells were rinsed twice with PBS. Then, 100 μl of lysis buffer (Upstate Biotechnology, Lake Placid, N.Y.), together with 5 μl of protease inhibitor cocktail (Sigma, St. Louis, Mo.), was added to the cell pellets, followed by incubation at 4°C for 10 min. Lysates were resuspended with 1 ml of dilution buffer (Upstate Biotechnology) in a 15-ml conical tube, subjected to sonication for five 30-s pulses with 15-s pauses in a microtip ultrasonicator, and transferred to a 1.5-ml microcentrifuge tube. Soluble chromatin was collected as supernatant after a 10-min centrifugation at 13,000 rpm and 4°C. Appropriate chromatin fragmentation (300 to 1,000 bp) was confirmed by agarose gel electrophoresis. Next, 50 μg of soluble chromatin was incubated on a rotating platform with 4 μl of anti-acetyl-histone H4 (Upstate Biotechnology), anti-HDAC1 (Upstate Biotechnology), anti-LSF (gift of M. Sheffery and S. Swendenmann), or rabbit preimmune immunoglobulin G (IgG) serum (Sigma), as appropriate, overnight at 4°C. Immunoprecipitates were incubated with 40 μl of salmon sperm DNA–protein A-agarose beads (Upstate Biotechnology) for 1 h at 4°C. Agarose beads were recovered by centrifugation and washed sequentially for 5 min with 1 ml of each of the following five buffers (Upstate Biotechnology): ChIP dilution buffer, low-salt wash buffer, high-salt wash buffer, LiCl wash buffer, and Tris-EDTA buffer. Immunoprecipitated DNA was eluted with 500 μl of elution buffer (1% sodium dodecyl sulfate [SDS], 0.1 M $NaHCO_3$). Reversal of DNA cross-linking DNA was performed by incubating 50 μg of soluble chromatin fraction with 19 μg of proteinase K (PCR grade; Boehringer, Mannheim, Germany) at 56°C for 1 h. DNA was extracted in phenol-chloroform-isoamyl alcohol, precipitated in ethanol, washed, and resuspended in 50 μl of water.

Quantitative duplex PCR assay was performed to analyze the amount of DNA precipitated by specified antibodies in proportion to input DNA. Two pairs of primers were used: LTR-109F (5′-TAC AAG GGA CTT TCC GCT GG-3′) and LTR+82R (5′-AGC TTT ATT GAG GCT TAA GC-3′) for the HIV-1 LTR promoter and P-β-actin-F (5′-TGC ACT GTG CGG CGA AGC-3′) and P-β-actin-R (5′-TCG AGC CAT AAA AGG CAA-3′) for the β-actin promoter. A total of 28 to 34 cycles of PCR were carried out with 2 to 10 μl of precipitated DNA in a 25-μl PCR containing 12.5 μl of master mix (Qiagen) and 12.5 pmol of each primer. Serial twofold dilutions of input DNA (prior to immunoprecipitation) were also subjected to PCR to ensure linear amplification in each experiment. Then, a 10-μl portion of the PCR products was resolved by 8% polyacrylamide gel electrophoresis and visualized by ethidium bromide staining. PCR products were quantified by using an AlphaImager 2000 (Alpha Innotech Corporation). Relative fold changes of PCR products were calculated by using the amounts of PCR products obtained from standard serial dilutions of input DNA.

Western blots of nuclear proteins. A total of 5×10^6 cells were harvested, washed with PBS, and lysed in 250 μl of buffer A (10 mM HEPES, pH 8.0; 10 mM KCl; 1.5 mM $MgCl_2$; 0.5% NP-40) with 50 μl of protease inhibitor cocktail (Sigma) for 1 min at 4°C. Nuclei were pelleted by centrifugation at 5,500 rpm at 4°C for 3 min. Buffer B (20 mM HEPES, pH 8.0; 420 mM NaCl; 25% glycerol; 0.2 mM EDTA) with 10 μl of protease inhibitor cocktail was used to resuspend the nuclei. After a 15-min incubation on ice, nuclear extracts were obtained as supernatant by centrifugation at 10,000 rpm at 4°C for 10 min. Then, 50 μg of nuclear extracts was subjected to SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. HDAC1 proteins were detected by rabbit anti-HDAC1 (Upstate Biotechnology) by using an ECL kit (Amersham, Piscataway, N.J.).

RNA preparation and reverse transcription-PCR (RT-PCR) analysis. Total RNA was isolated from 2×10^5 HeLa CD4 cells by using the TRIazol reagent (Life Technologies, Rockville, Md.) according to the manufacturer's instructions. Next, 5 μg of total RNA was treated with 20 U of RNase-free DNase I (Boehringer Mannheim) in 20 μl of a buffer containing 10 mM $MgCl_2$ and 20 mM Tris-HCl (pH 7.5) at 37°C for 30 min. DNase I was inactivated by heating at 100°C for 10 min. Half of the DNase I-treated RNA was transcribed into cDNA with 125 ng of random hexamers (Promega, Madison, Wis.) by using an Omniscript RT Kit (Qiagen) according to the supplier's protocols. The β-actin gene was first amplified from 1/20 of the cDNA to ensure equal input of mRNA in PCRs with the following primer set: β-actin-F (5′-GTC GAC AAC GGC TCC GGC-3′) and β-actin-R (5′ GGT GTG GTG CCA GAT TTT CT-3′). Two pairs of primers—the pair of CAT 65F (5′-TTG AGG CAT TTC AGT CAG TTG C-3′) and CAT 323R (5′-TCA CTC CAG AGC GAT GAA AAC G-3′) and the pair of HDAC1-754F (5′-ACG GGA TTG ATG ACG AGT CC-3′) and HDAC1-855R (5′-GGT CTT ACA GTG TGG CTC AG)—were designed based on the coding sequence of the CAT and HDAC1 genes, respectively. Duplex PCRs were performed with primer sets for CAT and HDAC1 or β-actin and HDAC1. PCRs were carried out with a *Taq* PCR Master Mix Kit (Qiagen). The PCR program consisted of 2 min of denaturation at 94°C, 34 cycles of amplification, and 7 min of final elongation. Each cycle included 20 s of denaturation at 94°C, 15 s of annealing at 55°C, and 15 s of elongation at 72°C.

CAT assays. Cell extracts were prepared from transfected cells by using 5× reporter lysis buffer (Promega). Approximately 2×10^5 cells were lysed with 100 μl of lysis buffer and subjected to three freeze-thaw treatments with dry ice and a 37°C water bath. Input extracts for chloramphenicol acetyltransferase (CAT) assays were normalized by equal amounts of protein as measured by Bradford assay (Bio-Rad). CAT assays were carried out as described previously (29).

RESULTS

Hyperacetylation of histone H4 at nuc 1 of HIV-1 LTR correlates with transcriptional activation of LTR by the HDAC inhibitor TSA. The HDAC inhibitor TSA has been shown to activate viral expression from provirus in HIV-1-infected cell

lines (51). A reversible inhibitor of HDACs (15), TSA causes global acetylation of the genome and increases the accessibility of restriction endonuclease sites at the nuc 1 region of the LTR. Although DNase I protection assays indicate that disruption of nuc 1 is accompanied by transcriptional activation of LTR by TSA, direct evidence showing changes of histone acetylation at nuc 1 is not provided by these studies.

We performed ChIP assays in a HeLa cell line containing a single integrated copy of an LTR-CAT reporter gene to document changes in histone H4 acetylation at nuc 1 in vivo. Using an antibody-directed against acetylated histone H4, we precipitated DNA fragments associated with acetylated histone H4 from LTR-CAT cell extracts. The precipitated DNA was quantified by duplex PCR with primers spanning the nuc 1 region of LTR and the constitutive host β-actin promoter. Amplification of serial dilutions of DNA demonstrated that a twofold increase in density units of PCR product represents as much as a fourfold increase in target DNA (Fig. 1A). Therefore, we consider any change of density units that is >2-fold to be significant. However, it is important to point out that the semiquantitative nature of these assays only allows us to make qualitative statements as to increased or decreased occupancy and not quantitative comparisons between assays. As shown in Fig. 1B, a 2-h exposure of cells to TSA results in a significant increase in acetylated histone H4 at nuc 1 but an insignificant increase in acetylated histone H4 at the constitutive β-actin promoter.

We carried out quantitative RT-PCR analysis of the CAT transcripts from cell extracts used for ChIP assays to correlate hyperacetylation at nuc 1 with the expression level of LTR. A significant increase of LTR expression was detected in cells (Fig. 1C) treated with TSA.

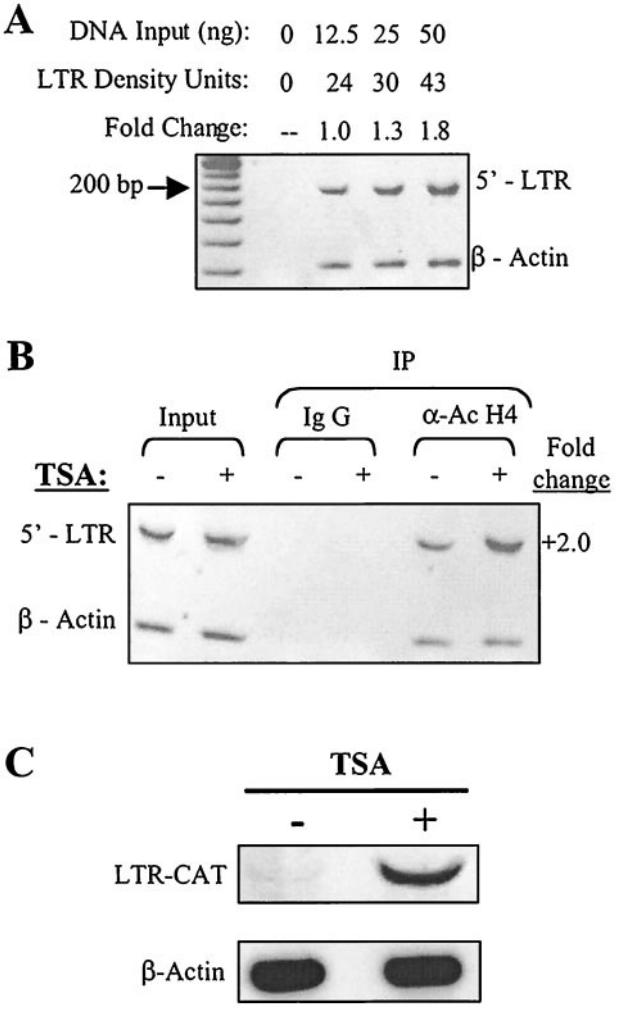

FIG. 1. The HDAC inhibitor TSA increases acetylation of histone H4 at the nuc 1 region of HIV-1 LTR and activates the integrated LTR. A HeLa cell line with an integrated copy of a LTR-CAT reporter gene was grown in 0.5% serum overnight, refed with complete medium, and stimulated with TSA for 2 h. Cell extracts were subjected to ChIP assays with antibody directed against acetylated histone H4. Total RNA was isolated from a portion of cells and analyzed by RT-PCR. (A) Semiquantitative duplex PCR was performed to amplify both LTR and β-actin promoter from genomic DNA extracted from HeLa LTR CAT cells. Serial dilutions of input were subjected to PCR amplification in parallel with each experiment. The relative intensity of the PCR product bands correlated with the amount of input DNA. These results were used to validate the fold change in quantification. (B) Duplex PCR amplifications of whole-cell extract (Input), nonspecific IgG immunoprecipitate (IgG), and α-acetylated histone H4 immunoprecipitate (IP) were carried out to show changes of acetylated histone H4 at nuc 1 and β-actin promoter after TSA treatment. The abundance of DNAs associated with acetylated histone H4 (IP) is shown as PCR products. (C) RT-PCR assay compared CAT transcripts after TSA treatment. β-Actin transcripts were amplified to ensure the equivalent input of cDNA.

HDAC1 is localized at the nuc 1 region of HIV-1 LTR. Our previous studies have shown that HDAC1 is recruited to the LTR of HIV-1 by the host factor YY1 and is present in a complex containing YY1 and LSF that binds to the LTR (12). To document the presence of HDAC1 at the LTR in vivo, we performed ChIP assays with antibody directed against HDAC1 (2). Fixation of whole cells with formaldehyde induces cross-linking of protein-DNA and protein-protein complexes in vivo (28). Sonication of cell extracts and immunoprecipitation with antibody against HDAC1 allow recovery of protein-DNA complexes that include HDAC1. As shown in Fig. 2A, duplex PCR analysis of this immunoprecipitate with primers flanking the nuc 1 region of LTR and β-actin promoter demonstrates HDAC1 occupancy at nuc 1 but no detectable occupancy of HDAC1 at the β-actin promoter in vivo. No PCR product is detected with extracts subjected to immunoprecipitation with nonspecific antiserum. These data directly confirm previous in vitro studies identifying HDAC1 within a factor complex binding at the LTR of HIV-1.

Cells were treated with TSA for only 2 h, to avoid significant cell cycle effects. Parallel ChIP assays performed with anti-acetylated histone H4 or anti-HDAC1 antibodies repeatedly demonstrate that TSA treatment decreases the ability to detect HDAC1 by ChIP at the nuc 1 region of the LTR; no change is seen in the presence of nonspecific IgG (Fig. 2B). To address the possibility that a decrease of HDAC1 at the LTR is caused by overall reduced expression of HDAC1 induced by TSA, we performed RT-PCR and Western blot analysis of HDAC1 expression. We found no detectable change of HDAC1 RNA expression level (Fig. 2C, lower panel) or nuclear protein level (Fig. 2C, upper panel) with TSA treatment. The loss of the ability to detect HDAC1 at nuc 1 is therefore not due to gross changes in HDAC1 gene or protein expression induced by TSA. TSA treatment, nuc 1 acetylation, and increased LTR

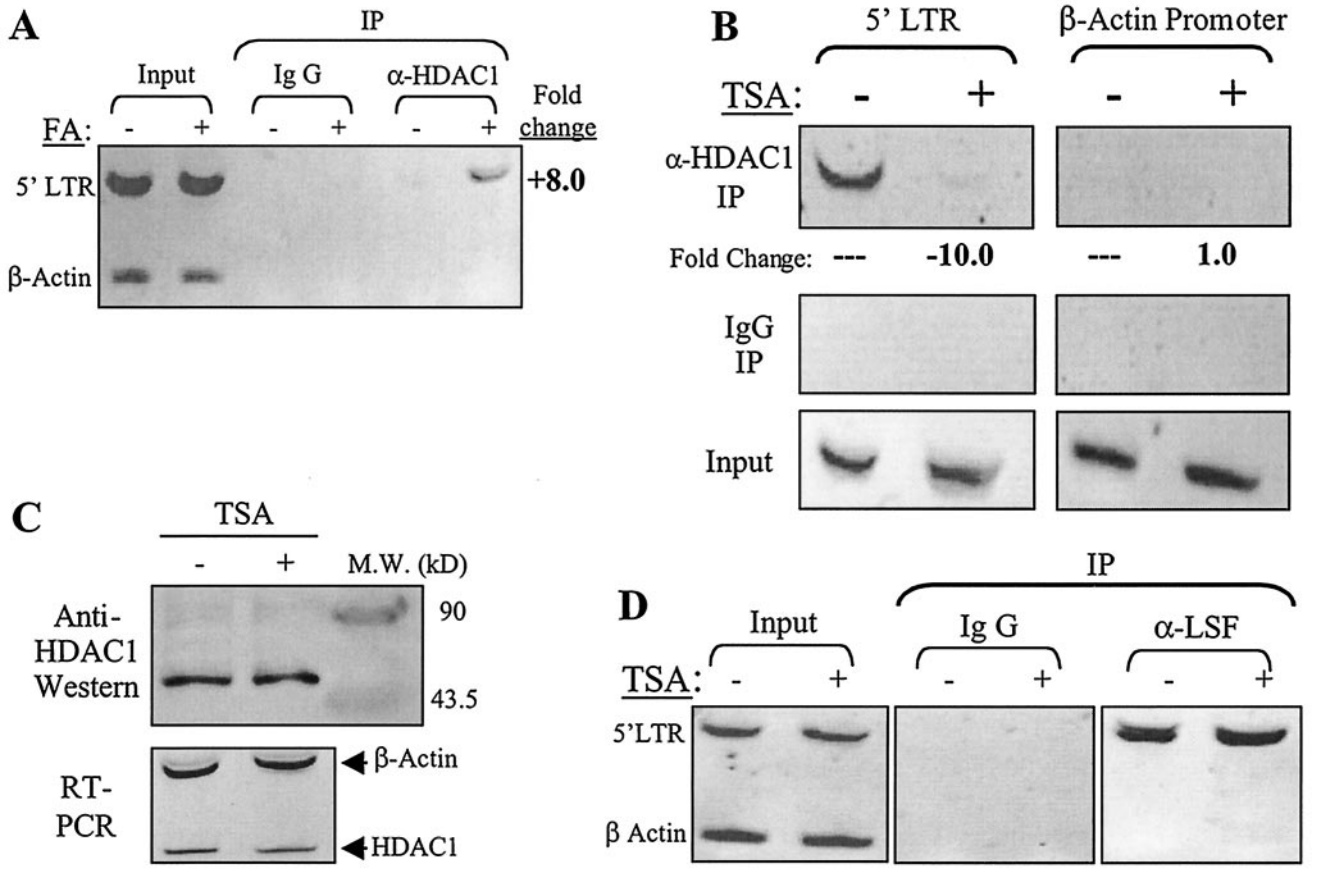

FIG. 2. HDAC1 is localized at the nuc 1 region of HIV-1 LTR in vivo. (A) A ChIP assay was performed with antibody directed against HDAC1. Formaldehyde cross-linking retained HDAC1 at the LTR. HDAC1 occupancy was not detected at the constitutive β-actin promoter. (B) ChIP assays with antibodies against HDAC1 detected the displacement of HDAC1 occupancy at nuc 1 after LTR activation by TSA. (C). Transient TSA treatment did not alter the expression of HDAC1 as shown by Western blot and RT-PCR. (D) TSA does not decrease HDAC1 occupancy by decreasing LSF occupancy.

expression are likely associated with decreased occupancy by HDAC1 at the nuc 1 region of LTR. We cannot completely exclude other possibilities, however, such as masking of HDAC by an induced protein or displacement of HDAC1 from nuc 1 beyond the 4 Å distance cross-linked by formaldehyde. Decreased HDAC1 occupancy at the LTR upon TSA treatment appears to be a direct effect of TSA on the interaction of HDAC1 with YY1 or of YY1 with LSF, since ChIP assays performed with anti-LSF antibody showed that TSA treatment did not affect LSF occupancy at the LTR (Fig. 2D).

Transcriptional activation of LTR by Tat correlates with disassociation of HDAC1 from nuc 1 and hyperacetylation of histone H4 at nuc 1. The changes observed in HDAC1 occupancy and histone H4 acetylation at nuc 1 suggest a mechanistic link between these events. The HIV Tat protein has been shown to be a powerful activator of integrated LTR expression. It has been suggested that Tat can interact with histone acetyltransferases (26, 39, 51). We hypothesized that similar counterregulatory changes in HDAC1 occupancy and acetylated histone H4 quantity might occur upon LTR activation by viral factor Tat.

We cotransfected GFP and Tat expression plasmids into the HeLa LTR-CAT cell line and separated cells into GFP-positive and -negative populations by FACS. CAT assays performed before FACS showed that cotransfection with Tat strongly activated the integrated LTR-CAT gene (Fig. 3A), while transfection of GFP alone did not change CAT activity. To verify that Tat is delivered to GFP-positive cells by cotransfection, CAT activities of GFP-expressing and -nonexpressing cells were measured. Significant CAT activity induced by Tat was only detected in GFP-expressing cells (Fig. 3B). When cell extracts from GFP-expressing cells were subjected to ChIP assays with antibody against HDAC1 and acetylated histone H4, decreased occupancy by HDAC1 and increased acetylation at the nuc 1 region of the LTR were repeatedly observed (Fig. 3C). The PCR product of acetylated histone H4 ChIP increased from 1.4- to 4.0-fold (median, 2.4; mean, 2.4 ± 0.4) upon Tat expression. LSF occupancy did not change at the LTR after Tat activation (Fig. 3D).

Binding of LSF to LTR is required for HDAC1 recruitment and deacetylation of nuc 1. There is no evidence that HDAC1 binds directly to the nuc 1 region of LTR promoter. LSF has been shown to bind to the LTR and recruit YY1. YY1 then recruits HDAC1, mediating repression (12, 34, 44). LSF binds to DNA as a tetramer (47). The cellular pool of LSF capable of binding DNA can be depleted by the overexpression of dnLSF, which is unable to bind DNA but competent to multimerize with functional LSF (54). The overexpression of

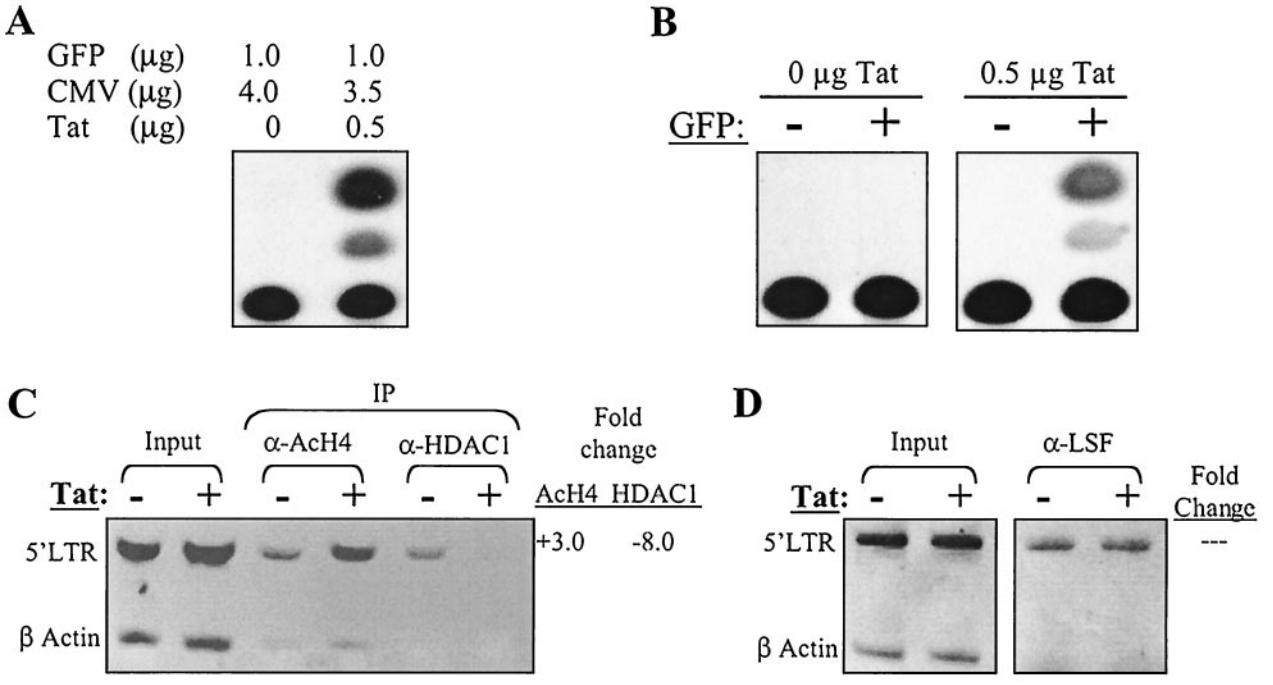

FIG. 3. Changes of histone H4 acetylation and HDAC1 occupancy at nuc 1 correlate with Tat activation of LTR. HeLa LTR-CAT cells were cotransfected with GFP and empty CMV vector or with GFP and CMV-Tat plasmids. After 24 h, transfected cells were separated into GFP-positive and -negative populations by FACS. Extracts were prepared for CAT and ChIP assays with antibodies against acetylated histone H4, HDAC1, or LSF. (A) Tat-activated integrated LTR-CAT in HeLa reporter line as shown by CAT assay. (B) A CAT assay showed cotransfection exclusively delivered Tat plasmids into GFP-positive cells. (C) ChIP assays showed increased acetylation of histone H4 and decreased HDAC1 occupancy at nuc 1 of LTR upon Tat activation in GFP-positive cells. (D) Overexpression of Tat did not affect LSF occupancy at nuc 1.

dnLSF has been shown to relieve YY1-mediated repression of Tat-activated LTR expression (12, 44). Overexpression of dnLSF should therefore decrease HDAC occupancy and increase the levels of acetylated histone H4, presumably by blocking binding of functional LSF to LTR. To test this hypothesis, cotransfection with wild type and dnLSF mutant were performed as before. Since ChIP assays are not quantitative, it is important to note that the results displayed inform as to qualitative changes in factor occupancy and do not measure the absolute presence or absence of a factor. ChIP assays repeatedly demonstrated that the overexpression of dnLSF was sufficient to decrease LSF occupancy at the LTR (Fig. 4A) and resulted in decreased HDAC1 occupancy and increased nuc 1 acetylation (Fig. 4B). No product is seen in parallel ChIP assays of the β-actin promoter (data not shown). LSF occupancy at the LTR is therefore associated with HDAC1 recruitment and nuc 1 remodeling. Further, overexpression of LSF does not appear to increase LSF occupancy above basal levels (Fig. 4). This is consistent with previous observations that LTR expression is little affected by overexpression of LSF alone (12, 44).

Transcriptional repression of LTR by YY1 correlates with HDAC1 recruitment and hypoacetylation of histone H4 at the nuc 1 region of LTR. Current models of eukaryotic gene regulation suggest that an equilibrium exists between initiation and repression of transcription at a gene promoter. Since disassociation of HDAC1 and hyperacetylation of histone H4 at nuc 1 accompany LTR activation by TSA or Tat, repression of LTR expression might correlate with increased HDAC1 occupancy and deacetylation of histone H4 at nuc 1. We compared ChIP assays with HDAC1 and acetylated histone H4 antibodies in LTR-CAT cells transfected with GFP alone and cotransfected with GFP and YY1. Repeated and reproducible increases in HDAC1 occupancy and decreased acetylation of histone H4 at LTR were detected in cells transfected with YY1 (Fig. 5A). Overexpression of YY1 does not change LSF occupancy at LTR (Fig. 5B).

Interaction of YY1 and HDAC1 in vivo is required for repression of Tat by YY1. YY1 mapping studies have indicated that YY1 interacts with HDAC1 through its glycine/alanine domain (1), and this domain is known to be required for repression of the LTR by YY1 (12). We therefore tested the ability of a YY1 mutant lacking this HDAC1 interaction domain to increase HDAC1 occupancy and decrease histone H4 acetylation. We compared CAT activity in a HeLa LTR-CAT line cotransfected with GFP alone, GFP plus Tat, GFP plus wild-type YY1 and Tat, and GFP plus mutant YY1 and Tat. ChIP assays from a portion of cells used in CAT assays allowed a comparison of the levels of LTR expression as measured by CAT activity with HDAC1 occupancy and histone H4 acetylation at nuc 1.

Overexpression of wild-type YY1 inhibits Tat activation and results in increased HDAC1 occupancy and decreased acetylation of histone H4 at nuc 1 (Fig. 6, lane B compared with lane C). As we have previously described, a glycine/alanine-rich domain mutant of YY1 incapable of HDAC1 interaction was unable to repress LTR activation by Tat (Fig. 6, lane D). Increased HDAC1 occupancy and decreased acetylated his-

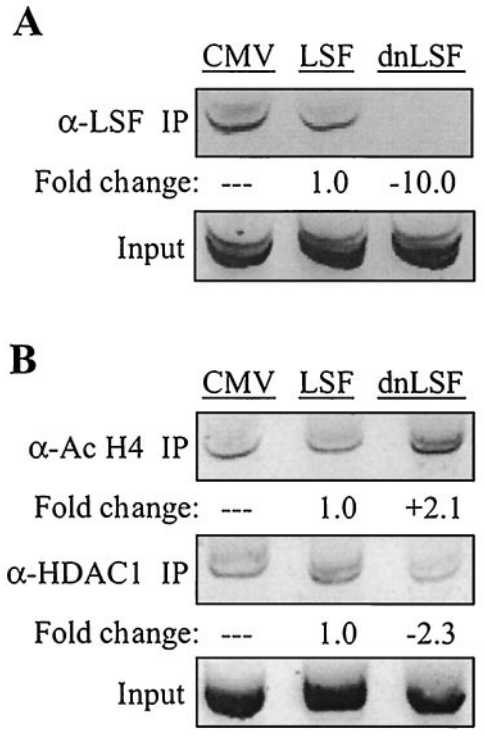

FIG. 4. Binding of LSF to LTR correlates with HDAC1 recruitment and hypoacetylation at the nuc 1 region of LTR. HeLa LTR-CAT cells cotransfected with GFP plus empty CMV vector, GFP plus CMV-LSF, or GFP plus CMV-dnLSF were sorted by FACS and ChIP assays performed with antibodies against acetylated histone H4 or HDAC1. (A) Overexpression of dnLSF decreased LSF occupancy at nuc 1. (B) Overexpression of dnLSF increased acetylation of histone H4 (upper panel) and decreased HDAC1 occupancy (middle panel) at nuc 1.

tone H4 at nuc 1 was not observed in cells transfected with YY1 mutant (Fig. 6, lane C compared with lane D). That the acetylation of histone H4 in the presence of Tat and mutant YY1 did not increase (Fig. 6, lane B compared with lane D) could be due to incomplete competition of the transfected YY1 mutant with endogenous wild-type YY1. In this setting, basal acetylation is sufficient to allow Tat activation. However, it is clear that recruitment of HDAC1 to the LTR is required for repression of LTR expression by YY1, and primarily mediated by the glycine/alanine-rich domain of YY1.

DISCUSSION

HIV-1 depends on the host transcription machinery to complete its life cycle. The level of transcription from HIV-1 genome is an important factor in determining the rate of viral replication. Binding of cellular transcription factors and host activators to the LTR results in the assembly of a transcriptional complex which, once modified by the viral factor Tat, can rapidly undergo efficient transcriptional elongation and reinitiation (3, 17, 25). The accessibility of the LTR in the natural context of chromatin to such host factors may therefore represent a critical mechanism by which HIV-1 transcription is regulated.

Host factors that modulate chromatin structure at the LTR have been implicated in the transcriptional regulation of HIV-1 (13, 40). Our study is the first to directly demonstrate that HIV LTR nuc 1 deacetylation is induced by YY1. We have shown that expression of the viral activator Tat is associated with histone acetylation at nuc 1 and downregulation of HDAC1 occupancy. Conversely, recruitment of HDAC1 to nuc 1 by YY1 results in the hypoacetylation of nuc 1 and LTR repression. Parallel reporter assays of LTR expression and real-time promoter occupancy illustrate that hyperacetylation of nuc 1 is associated with LTR activation and, conversely, that hypoacetylation of nuc 1 is associated with the repression of LTR. These findings strongly suggest that chromatin structure at nuc 1 plays a significant role in regulating HIV-1 expression.

We utilized in vivo ChIP assays to document changes of histone H4 acetylation and factor occupancy at nuc 1. Since the cross-linking distance of formaldehyde is 4 Å, the possibility exists that some changes observed represent changes in localization rather than the absolute removal of a factor. However, in agreement with findings from DNase I protection and restriction endonuclease accessibility studies (13, 51, 53), ChIP assays verify that transcriptional activation of LTR by TSA is accompanied by hyperacetylation of H4 histones at nuc 1. Surprisingly, the occupancy of HDAC1 at nuc 1 region of LTR measured in vivo by formaldehyde cross-linking declined significantly upon TSA activation of LTR. A reversible inhibitor

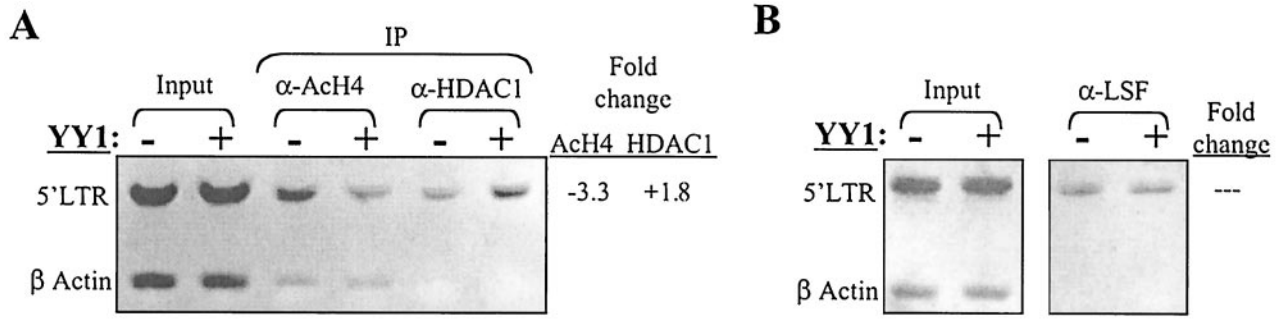

FIG. 5. YY1 recruits HDAC1 and inhibits histone H4 acetylation at the nuc 1 region of LTR. HeLa LTR-CAT cells were cotransfected with GFP plus empty CMV vector or with GFP plus CMV-YY1 and then sorted by FACS and ChIP assays performed with antibodies against acetylated histone H4, HDAC1, and LSF. (A) YY1 increased HDAC1 occupancy and decreased acetylation of histone H4 at nuc 1. (B) Overexpression of YY1 did not change LSF occupancy at nuc 1.

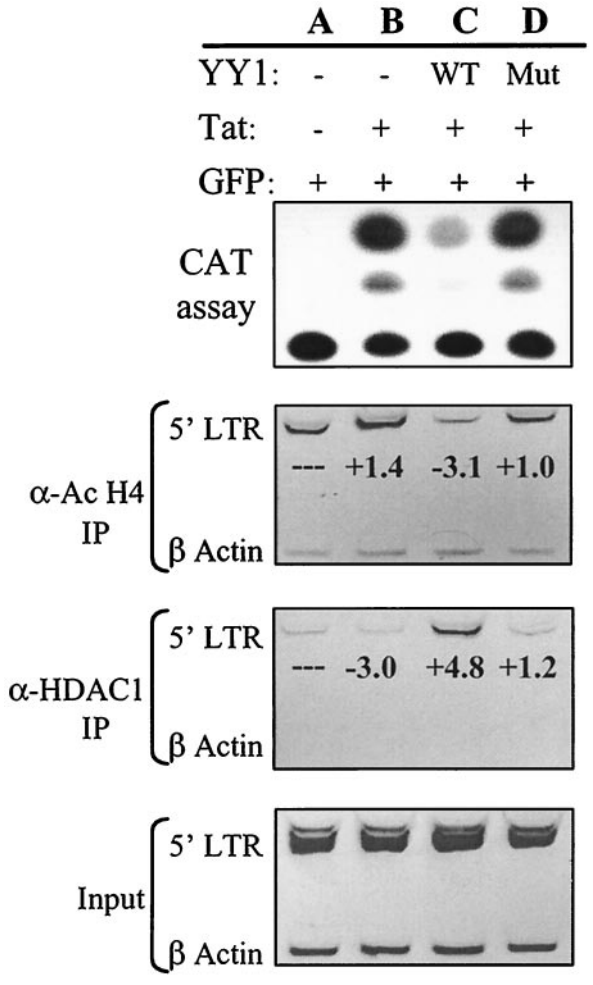

FIG. 6. HDAC1 recruitment by YY1 is required for the repression of LTR. Cell extracts were prepared from GFP-positive cells cotransfected with empty CMV vector, Tat, Tat plus wild-type YY1, and Tat plus mutant YY1 lacking the HDAC1 interaction domain. CAT assays (upper panel) were performed to correlate the level of LTR expression with the acetylation of histone H4 and HDAC1 occupancy (middle panel) at nuc 1, as measured by ChIP assays. A YY1 mutant with a deletion of HDAC1 interaction domain was unable to repress the LTR, increase HDAC1 occupancy, or significantly decrease H4 acetylation at nuc 1.

of HDAC1, TSA is thought to bind HDAC1 and causes conformational change of the enzyme at its active site (15). This conformational change could directly result in the disassociation of HDAC1 from nuc 1 within chromatin about the HIV-1 LTR.

The *trans*-activator Tat has been shown to interact with histone acetyltransferases (26, 39). In this study, we have shown that Tat activation is associated with histone acetylation at nuc 1. Further, we have identified an inverse relationship between acetylation of histone H4 and HDAC1 occupancy upon activation at LTR by Tat or repression by YY1. This correlation is also observed when the formation of YY1-LSF repression complex at LTR is blocked by dnLSF. Disassociation of HDAC1 from nuc 1 of LTR may therefore allow unrestrained histone acetyltransferase activity, resulting in local hyperacetylation of histone H4. These observations not only confirm that the transcriptional activation is associated with remodeling of nuc 1 by acetylation but suggest the possibility that histone acetyltransferases or other factors recruited to LTR by Tat may interact with HDAC1, YY1, or both and may regulate their functions or interaction. While both TSA and Tat activation result in the loss of the repression and decreased detection of HDAC1 in ChIP assay, further study is required to define the mechanism(s) by which TSA and Tat decrease HDAC1 occupancy. Possibilities include an effect on HDAC1, YY1, or both.

Our data suggest that HDAC1 plays an important role at the LTR. Our previous studies (12, 34, 44) used a HDAC1 antibody that cross-reacted with HDAC3 but with no other HDAC family members. The molecular weight of HDAC detected in the YY1-LSF complex at the LTR was consistent only with the presence of HDAC1 (12). However, a role for other HDAC family members in the regulation of LTR chromatin structure is a topic for future study.

Regulation of transcription factors by acetylation has been reported (48). HDAC1 could be modified by acetylation, resulting in dissociation from the LTR. Another attractive mechanism to explain the apparent displacement of HDAC1 upon LTR activation is the modulation of factors that recruit HDAC1 to the LTR promoter, such as YY1 and LSF. Our previous studies have shown that transcription factors YY1 and LSF cooperate to inhibit HIV-1 transcription in both integrated LTR cell lines and primary CD4 lymphocytes. We now demonstrate that HDAC1 is recruited to nuc 1 by the glycine/alanine-rich domain (YY1 154-199) of YY1 in vivo. Interaction of histone acetyltransferases, such as P300 and PCAF, and HDACs has been shown to regulate the DNA binding activity of YY1 (55). HDAC1 serves in an autoregulatory capacity, regulating YY1 activity within the context of the LTR.

Histone acetyltransferases may also play an important role in the regulation of LTR. Tat recruits p300 and PCAF to LTR (26, 39). Tat mutants unable to bind histone acetyltransferases fail to activate LTR. The host coactivators Sp1 and NF-κB have been reported to interact with histone acetyltransferases (41). Sp1 has been reported to functionally interact with HDACs (6) as well. Influenced by the local chromatin environment and the level of viral activator Tat, cellular activators, and repressors, the integrated HIV-1 promoter is likely to be remodeled to allow activation or repression. The counterregulation of chromatin acetylation and HDAC1 occupancy could integrate a complex network of countervailing host and viral signals. Taken together, our findings tightly link deacetylation of nuc 1 to the repression of LTR expression.

The persistent reservoir of HIV infection, established in a subpopulation of resting $CD4^+$ T lymphocytes that have escaped viral and immune cytolysis, may be unaffected despite years of continuous antiretroviral therapy. Our study confirms a correlation of hyperacetylation accompanying the disruption of nuc 1 with the transcriptional activation of the LTR and demonstrates for the first time that the deacetylation of nuc 1 correlates with the transcriptional repression of the LTR. These findings reveal novel counterregulatory interactions between HDAC1 occupancy and chromatin acetylation at the integrated promoter of HIV-1 LTR and suggest that specific modulation of chromatin architecture may allow the establish-

ment of persistent chromosomal provirus. A detailed molecular understanding of the biochemical mechanisms which establish and maintain the quiescent state of integrated HIV proviral genomes may allow the design of future therapeutics to directly attack this reservoir of HIV-1 infection.

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