The SWI/SNF Chromatin-remodeling Complex Is a Cofactor for Tat Transactivation of the HIV Promoter*

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Tat is a critical viral transactivator essential for human immunodeficiency virus (HIV) gene expression. Activation involves binding to an RNA stem-loop structure and recruitment of the positive transcription elongation factor b. Tat also induces the remodeling of a single nucleosome in the HIV promoter. However, the mechanism of this remodeling has remained unclear. Knockdown of INI-1 and BRG-1, two components of the SWI/SNF chromatin-remodeling complex, suppressed Tat-mediated transactivation. Cells lacking INI-1 (G401 and MON) or BRG-1 (C33A) exhibited defective transactivation by Tat that was restored upon INI-1 and BRG-1 expression, respectively. Tat was co-immunoprecipitated with several SWI/SNF subunits, including INI-1, BRG-1, and β-actin. The SWI/SNF complex interacted with the integrated HIV promoter in a Tat-dependent manner. We also found that INI-1 and BRG-1 synergized with the p300 acetyltransferase to activate the HIV promoter. This synergism depended on the acetyltransferase activity of p300 and on Tat Lys60 and Lys61. In conclusion, Tat-mediated activation of the HIV promoter requires the SWI/SNF complex in synergy with the coactivator p300.

Infection by human immunodeficiency virus (HIV) sets in motion a complex series of actions that result in the efficient transcription of the viral genome. Once the virus is integrated into the host genome, nucleosomes are deposited at specific positions within the HIV promoter region (1). A large nucleosome-free region is present between nucleosome (nuc)-0 and nuc-1 and contains binding sites for transcription factors such as NF-κB and Sp1 and other basal transcription factors. Transcription is initiated within this nucleosome-free region. Transcriptional activation of the HIV promoter is associated with the remodeling of nuc-1, which is positioned immediately downstream of the transcription start site (1).

In the early phase of HIV infection, cellular transcription factors activate transcription from the HIV promoter. However, the basal HIV promoter shows a striking elongation defect, resulting in the accumulation of short transcripts corresponding to the first ~50 transcribed nucleotides. This elongation defect is presumed to occur because of deficient loading of the transcription elongation complex pTEFb at the HIV promoter. We propose that the presence of nuc-1 (immediately downstream of the transcription start site) accentuates the elongation defect of the polymerase complex assembled at the HIV promoter.

However, the elongation defect of RNA polymerase II assembled at the HIV promoter is not absolute, and basal transcription leads to the accumulation of the viral Tat protein, a potent transactivator. Tat binds to TAR (an RNA stem-loop in the nascent viral RNA) and recruits pTEFb (which contains CDK9 and cyclin T1). The recruitment of pTEFb leads to phosphorylation of the C-terminal domain of RNA polymerase II and increased transcriptional elongation of the HIV promoter. Efficient transcription elongation of the HIV genome in response to Tat leads to more Tat synthesis and generates a Tat-dependent positive feedback loop (2).

Tat expression also leads to the remodeling of nuc-1 (1, 3). This remodeling is thought to remove an obstacle to RNA polymerase II elongation. Both Tat activities (pTEFb recruitment and nuc-1 remodeling) are thought to synergize in enhancing the ability of RNA polymerase II to elongate. The molecular mechanism of this Tat-induced nucleosome-remodeling event has remained unclear.

Chromatin-modifying complexes are classified into two main groups. The first contains factors that mediate covalent modifications of histones. The N-terminal tails of histone proteins are subject to extensive post-transcriptional modifications, including acetylation, phosphorylation, and methylation. The interaction of Tat with a number of histone acetyltransferase complexes such as p300/CPB, p300/CPB-associated factor (pCAF), and human GCN5 and their relevance to Tat-mediated activation of the HIV promoter have been established (4–11). The complexes acetylare the N-terminal tails of histones of nucleosomes at the HIV promoter, inducing destabilization of histone-DNA contacts and thus facilitating transcrip-
SWI/SNF Remodeling Complex in Tat Activation of HIV LTR

EXPERIMENTAL PROCEDURES

Plasmids and Retroviral Vectors—The HIV LTR-luciferase reporter construct (pEV229) (6); the cytomegalovirus (CMV)-driven expression vectors for N-terminally FLAG-tagged wild-type Tat (pEV280) and mutant Tat(K50R/K51R) (pEV538) (6); and SV40-driven C-terminally FLAG-tagged INI-1 (21), CMV-driven hemagglutinin-tagged BRG-1 (22), CMV-driven p300 (6), and Δp300 catalytic mutant (6) expression constructs have been described. To construct the retroviral vector LTR-enhanced green fluorescent protein (EGFP)-internal ribosome entry site (IRES)-luciferase-LTR (pEV677), the SalI fragment containing IRES-luciferase of pEV676 was inserted into the Sall-restricted retroviral vector pRRL-EGFP (pEV658). Glutathione S-transferase (GST)-fused BRG-1 deletion constructs have been described previously (23).

Cell Lines—G401 and C33A cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 5% penicillin/streptomycin. MON cells, Jurkat T cells, and Jurkat T cell clones A2 (24, 25), containing integrated latent LTR-green fluorescent protein (GFP) and LTR-Tat-IRES-GFP, respectively, were transfected with the retroviral vector LTR-EGFP-IRES-luciferase (pEV677). Forty-eight hours after infection, the cells were transfected with CMV-Tat to stimulate GFP expression in cells containing the integrated LTR-luciferase-GFP reporter. After 24 h, GFP-positive cells were sorted by flow cytometry and maintained in culture until they became GFP-negative upon dilution of the CMV-Tat expression vector. G401 and MON cells containing integrated LTR-luciferase-GFP were then used in transient transfection experiments.

HCT116 cell lines expressing doxycycline-inducible short hairpin RNA against the INI-1 gene were generated essentially as described (26). Briefly, a monoclonal Tet repressor-expressing HCT116 cell line (HCT116TR) was first generated in accordance with the manufacturer’s instructions with pCDNA3.1 (Invitrogen) and blasticidin selection. HCT116TR cells were then cotransfected with four pTER-INI-1 (for Tet-inducible RNA interference against INI-1) vectors generated by cloning gene-specific oligonucleotides against the INI-1 gene into pTER (26). Zeocin-resistant doxycycline-inducible INI-1 knockdown HCT116 cell lines were then tested for their ability to knock down INI-1 by Western blotting.

Antibodies, Co-immunoprecipitation, and Protein-Protein Interactions—The specific antibodies used for immunoprecipitations were anti-YY1, anti-BRM, anti-BRG-1, anti-p300, anti-14-3-3, anti-PKD-1, anti-cyclin T1, and anti-CDK9 (Santa Cruz Biotechnology, Inc.); anti-INI-1 (anti-SMARCB1) (Abcam); and anti-acetyllysine (Cell Signaling Technology). For immunoprecipitations, Jurkat A2 cells were treated with 10 nM phorbol 12-myristate 13-acetate (PMA) for 12–16 h to produce FLAG-Tat. Cells were lysed in immunoprecipitation buffer (25 mM Hepes (pH 7.9), 150 mM KCl, 1 mM EDTA, 5 mM MgCl2, 5% glycerol, 1% Nonidet P-40, 0.5 mM dithiothreitol, 1 μM trichostatin A (TSA), 1 mM nicotinamide, and protease inhibitor mixture (Sigma)) for 20 min on ice and passed twice through a 26-gauge needle. Lysates were centrifuged, and 2 mg of whole cell protein lysate was incubated overnight with 20 μl of acetyllysine (Sigma) for 20 min on ice and passed twice through a 26-gauge needle. Lysates were centrifuged, and 2 mg of whole cell protein lysate was incubated overnight with 20 μl of acetyllysine (Sigma) for 20 min on ice and passed twice through a 26-gauge needle. Lysates were centrifuged, and 2 mg of whole cell protein lysate was incubated overnight with 20 μl of acetyllysine (Sigma) for 20 min on ice and passed twice through a 26-gauge needle. Lysates were centrifuged, and 2 mg of whole cell protein lysate was incubated overnight with 20 μl of acetyllysine (Sigma) for 20 min on ice and passed twice through a 26-gauge needle. Lysates were centrifuged, and 2 mg of whole cell protein lysate was incubated overnight with 20 μl of acetyllysine (Sigma) for 20 min on ice and passed twice through a 26-gauge needle. Lysates were centrifuged, and 2 mg of whole cell protein lysate was incubated overnight with 20 μl of acetyllysine (Sigma) for 20 min on ice and passed twice through a 26-gauge needle. Lysates were centrifuged, and 2 mg of whole cell protein lysate was incubated overnight with 20 μl of acetyllysine (Sigma) for 20 min on ice and passed twice through a 26-gauge needle. Lysates were centrifuged, and 2 mg of whole cell protein lysate was incubated overnight with 20 μl of acetyllysine (Sigma) for 20 min on ice and passed twice through a 26-gauge needle.
with 1 \mu M TSA and 5 mM nicotinamide for 6 h. Cells were then harvested and lysed in phosphate lysis buffer (PLB; phosphate-buffered saline, 2 mM EDTA, 1% Triton X-100, 0.5 mM dithiothreitol, 1 \mu M TSA, 5 mM nicotinamide, and protease inhibitor mixture). Lysates were centrifuged, and 2 mg of protein lysate was incubated overnight with 20 \mu l of M2-agarose beads at 4 °C on a rotator. After extensive washing with PLB, the beads were resuspended in SDS loading buffer containing 2-mercaptoethanol and separated on an SDS-polyacrylamide gel. Coprecipitated proteins were identified by immunoblotting with the indicated antibodies.

For GST pulldown experiments, GST fusion proteins were expressed in bacteria and purified on glutathione beads. GST-BRG-1 deletion peptides immobilized on beads were then incubated with synthetic acetylated or unacetylated biotinylated Tat in PLB for 2 h at 4 °C on a rotator. The beads were washed extensively with PLB and wash buffer (25 mM Hapes (pH 7.9), 400 mM KCl, 1 mM EDTA, 5 mM MgCl2, 5% glycerol, 1% Nonidet P-40, 0.5 mM dithiothreitol, 1 \mu M TSA, 1 mM nicotinamide, and protease inhibitor mixture) before addition of SDS loading buffer and electrophoresis. Acetylated and unacetylated biotinylated Tat proteins were detected by Western blotting using horseradish peroxidase-conjugated anti-streptavidin antibody.

**Transient Transfection and Luciferase Assays**—G401, C33A, MON, and doxycycline-inducible INI-1 knockdown HCT116 cells were seeded at a density of 5 \times 10^4 cells/35-mm plate and transfected the next day with FuGENE 6 reagent (Roche Applied Science) according to the manufacturer’s instructions. Typically, transient transfections were carried out with 200 ng of LTR-luciferase reporter plasmid and expression vectors CMV-FLAG-Tat (5–30 ng), Rous sarcoma virus-FLAG-INI-1 (50–500 ng), CMV-BRG-1 (50 ng), CMV-p300 (50 ng), and CMV-\Delta p300 (50 ng) as indicated. The total DNA amount was adjusted using the corresponding empty vectors. Transfection efficiencies were >50% for all assays. Transfection efficiency was normalized using the cotransfected Renilla luciferase activity as an internal control. The cells were lysed after 24 h using luciferase lysis buffer (Promega Corp.), and luciferase activities were measured using the Dual-Luciferase reporter assay system (Promega Corp.), and luciferase activities were measured using the Dual-Luciferase reporter assay system (Promega Corp.).

**RNA Interference**—Pre-designed Dharmacon siRNA pools targeting transcripts of the human SNF5 (INI-1) and BRG-1 genes, as well as a control siRNA pool, were used to knock down the respective genes in Jurkat A72 cells. siRNA was delivered into Jurkat cell clones by nucleofection (AMAXA). siRNA (2 \mu g) was used to nucleofect 5 million cells; and 24, 48, and 72 h after nucleofection, protein levels were examined by Western blot analysis.

**Chromatin Immunoprecipitation**—Jurkat cell clones A72 and A2 were fixed by adding formaldehyde to a final concentration of 1% for 15 min at room temperature. Glycine was added to a final concentration of 125 mM to stop the cross-linking. Nuclei were isolated by incubation in hypotonic buffer (25 mM Hapes (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.1% Nonidet P-40, 1 mM dithiothreitol, and protease inhibitor mixture) for 10 min, followed by homogenization 15 times in a Dounce homogenizer. Nuclei were lysed in sonication buffer (50 mM Hapes (pH 7.9), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 0.1% sodium deoxycholate, 0.5% SDS, and protease inhibitor mixture) and sonicated to obtain DNA fragments of 500–1500 bp. Lysates were centrifuged at 14,000 rpm for 10 min at 4 °C and precleared by incubation with protein A-agarose beads for 2 h at 4 °C. Immunoprecipitations were conducted by incubating 5 \mu g of the indicated antibodies with 800 \mu l of the sheared cross-linked chromatin overnight at 4 °C. Approximately 15 million cells were used per immunoprecipitation. Protein A-agarose beads were blocked by incubation overnight with 1 mg/ml bovine serum albumin and 0.1 mg/ml salmon sperm DNA at 4 °C. Immunoprecipitates were bound to blocked protein A-agarose beads for 2 h at 4 °C and washed twice with each buffer A (20 mM Tris (pH 8.1), 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, and protease inhibitor mixture), buffer B (50 mM Hapes (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and protease inhibitor mixture), and buffer C (20 mM Tris (pH 8.0), 250 mM LiCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and protease inhibitor mixture). Immunoprecipitated complexes were eluted in 1% SDS, 0.1 M NaHCO3, and 500 mg/ml protease K and incubated for 2 h on a shaker at 37 °C and overnight at 65 °C for reversion of cross-links. DNA was extracted twice with phenol/chloroform and once with chloroform/isoamyl alcohol, ethanol-precipitated, and resuspended in 100 \mu l of H2O by shaking at 37 °C. Input and immunoprecipitated DNAs (10 \mu l) were subjected to 33 PCR cycles with primers 996 (5’-TTGCCCTGTACTGGGTCTCTCTTCG-3’) and 997 (5’-TGCCTTTTAAAGCTCCGTGTTCG-3’).

**RESULTS**

**RNA Interference-mediated Depletion of INI-1 and BRG-1 Compromises Tat Transactivation**—To examine whether INI-1-containing chromatin-remodeling complexes are involved in the transcriptional activation of the HIV promoter, we used a cell line containing a doxycycline-inducible short hairpin RNA against the INI-1 gene. Cells were transfected with the HIV population. Cells were further gated using forward scatter versus FL1 to differentiate between GFP-positive and GFP-negative cells.
INI-1 and BRG-1 are cofactors for Tat-mediated transactivation of the HIV promoter. A, depletion of INI-1 by RNA interference suppresses Tat activation of the HIV LTR. The HCT116 cell line expressing doxycycline-inducible short hairpin RNA against INI-1 was treated with doxycycline 48 h before cotransfection with the HIV LTR-luciferase reporter construct in the presence or absence of the Tat expression vector. Depletion of INI-1 was confirmed by Western blotting using a specific antibody for INI-1. B, INI-1 enhances Tat activation of the LTR in transient transfection experiments in the INI-1-deficient G401 cell line. Cells were cotransfected with the LTR-luciferase reporter construct and expression vectors for FLAG-Tat and INI-1 as indicated. INI-1 and Tat expression levels were visualized by Western blotting. C, depletion of INI-1 or BRG-1 in Jurkat cells by siRNA knockdown. Shown are the results from Western blot analysis of BRG-1 and INI-1 protein levels 48 or 72 h after transfection of control (Cont.), BRG-1, and INI-1 siRNA oligonucleotides in Jurkat cells. Depletion of BRG-1 or INI-1 did not result in destabilization or degradation of the other subunit. D, LTR activation by Tat is reduced in response to BRG-1 or INI-1 depletion. Jurkat cells containing an integrated HIV promoter driving expression of the GFP protein (A72 cells) were treated with BRG-1 or INI-1 siRNA for 48 h. These cells were nucleofected with either an empty vector or Tat expression vectors. Cells were analyzed by flow cytometry 20 h after nucleofection of the Tat expression vector. Transfection results shown in A, B, and D represent the mean ± S.D. of three independent experiments conducted in triplicate.

FIGURE 1. INI-1 and BRG-1 are cofactors for Tat-mediated transactivation of the HIV promoter. A, depletion of INI-1 by RNA interference suppresses Tat activation of the HIV LTR. The HCT116 cell line expressing doxycycline-inducible short hairpin RNA against INI-1 was treated with doxycycline 48 h before cotransfection with the HIV LTR-luciferase reporter construct in the presence or absence of the Tat expression vector. Depletion of INI-1 was confirmed by Western blotting using a specific antibody for INI-1. B, INI-1 enhances Tat activation of the LTR in transient transfection experiments in the INI-1-deficient G401 cell line. Cells were cotransfected with the LTR-luciferase reporter construct and expression vectors for FLAG-Tat and INI-1 as indicated. INI-1 and Tat expression levels were visualized by Western blotting. C, depletion of INI-1 or BRG-1 in Jurkat cells by siRNA knockdown. Shown are the results from Western blot analysis of BRG-1 and INI-1 protein levels 48 or 72 h after transfection of control (Cont.), BRG-1, and INI-1 siRNA oligonucleotides in Jurkat cells. Depletion of BRG-1 or INI-1 did not result in destabilization or degradation of the other subunit. D, LTR activation by Tat is reduced in response to BRG-1 or INI-1 depletion. Jurkat cells containing an integrated HIV promoter driving expression of the GFP protein (A72 cells) were treated with BRG-1 or INI-1 siRNA for 48 h. These cells were nucleofected with either an empty vector or Tat expression vectors. Cells were analyzed by flow cytometry 20 h after nucleofection of the Tat expression vector. Transfection results shown in A, B, and D represent the mean ± S.D. of three independent experiments conducted in triplicate.

The LTR-luciferase reporter construct in the presence or absence of Tat. Induction of the short hairpin RNA against INI-1 by doxycycline led to nearly complete suppression of INI-1 expression (Fig. 1A). Under these conditions, Tat-mediated transactivation of the HIV promoter was compromised, whereas basal HIV promoter activity was unchanged (Fig. 1A).

To confirm this result, we used INI-1-deficient G401 cells. These cells were transfected with the LTR-luciferase reporter vector in the presence or absence of expression vectors for Tat and INI-1. In the absence of Tat, INI-1 had no effect on basal HIV promoter activity (Fig. 1B). Expression of INI-1 enhanced the HIV LTR activation mediated by Tat (Fig. 1B). Expression of both Tat and INI-1 was confirmed by Western blotting (Fig. 1B). These results indicate that INI-1 is necessary for optimal Tat activation of the HIV promoter.

Next, we studied the role of INI-1 and BRG-1 in HIV promoter activity in the Jurkat A72 T cell line. This clonal cell line was generated by infection of Jurkat cells with viral particles containing an HIV retroviral vector lacking the tat gene. In this vector, the HIV promoter drives expression of GFP (25). siRNAs specific for INI-1 or BRG-1 were transfected by nucleofection, resulting in transfection of ~80% of the cells (data not shown) and leading to the specific depletion of either INI-1 or BRG-1 (Fig. 1C). Notably, depletion of INI-1 did not affect the expression of BRG-1, and conversely, depletion of BRG-1 did not deplete INI-1 (Fig. 1C). Cells were transfected with an expression vector for Tat or the control empty vector, and Tat-mediated transactivation was assessed by measuring GFP expression by flow cytometry. Depletion of either INI-1 or BRG-1 suppressed Tat-mediated transactivation of the HIV promoter as indicated by a decrease in the percentage of GFP-positive cells (Fig. 1D). Similar results were also obtained when the mean fluorescence intensity was measured (siRNA control, mean fluorescence intensity = 92.9; siRNA for BRG-1, mean fluorescence intensity = 92.9; and siRNA for INI-1, mean fluorescence intensity = 58.6), indicating that depletion of INI-1 and BRG-1 decreases Tat-mediated transcriptional activation of the LTR. To ensure that the observed decrease in transactivation was not due to lowered activity of the CMV promoter and therefore lower Tat expression, we transfected Jurkat A72 cells lacking either INI-1 or BRG-1 with a CMV-luciferase reporter vector. Luciferase values in cells with or without INI-1 and BRG-1 were similar (data not shown). Together, these results demonstrate that Tat activation of the LTR depends on INI-1 and BRG-1. In addition, the reduced transactivation activity of Tat in the absence of BRG-1 suggests that INI-1 plays a role in the transactivation as a subunit of the BRG-1 complex.

Tat Interacts with Subunits of the SWI/SNF Chromatin-remodeling Complex—Our results above showed that Tat requires INI-1 and BRG-1 for transcriptional activation. To determine whether Tat interacts with SWI/SNF components in cells, we used Jurkat A2 cells containing a latently integrated LTR-Tat-IRES-GFP virus (24). This cell line expresses detectable levels of FLAG-tagged Tat protein under the control of the HIV promoter only after stimulation of these cells with PMA (Fig. 2A, upper panel). We immunoprecipitated Tat from Jurkat A2 cells extracted after PMA stimulation and probed for association of Tat with endogenous components of SWI/SNF. The immunoprecipitations showed that Tat specifically associated with the core components of SWI/SNF: INI-1, BRG-1, and β-actin (Fig. 2A). Interestingly, Tat co-immunoprecipitated with BRG-1 but not BRM (Fig. 2A), indicating that Tat interacts specifically with the SWI/SNF complex containing BRG-1 as its catalytic subunit. We used the unrelated proteins 14-3-3 and
PKD-1 as controls, and they did not co-immunoprecipitate with Tat.

We also examined the interaction between Tat and SWI/SNF by immunoprecipitating the endogenous INI-1 or BRG-1 complexes using antibodies specific for each protein. Western blot analysis of the immunoprecipitated proteins confirmed that BRG-1 co-immunoprecipitated with INI-1 and vice versa (Fig. 2B, upper panel). Immunoprecipitated complexes bound to protein A-Sepharose beads were incubated with in vitro translated 35S-labeled Tat, washed, and analyzed by autoradiography after SDS-PAGE. In vitro 35S-labeled Tat efficiently bound to both the BRG-1 and INI-1 complexes, but not to control or PKD-1-coated beads (Fig. 2B, lower panel).

INI-1 Synergizes with Tat and the Transcriptional Coactivator p300—Previous studies showed that p300 is a cofactor in the Tat-dependent activation of the HIV LTR (4–6, 9). To test whether INI-1 and p300 act synergistically, we transfected the INI-1-negative G401 and MON cells with the HIV LTR-luciferase reporter plasmid with or without expression vectors for Tat, INI-1, p300, and a mutant p300 protein with a defective histone acetyltransferase domain (p300ΔHAT) (Fig. 3A). In the absence of Tat, INI-1, p300, and p300ΔHAT did not significantly affect basal reporter activity. Expression of Tat alone mediated relatively weak (~10-fold) activation of transcription in both the G401 and MON cell lines. Expression of INI-1 or p300 individually with Tat present at limiting concentrations resulted in a slight increase in transcription compared with Tat alone. Strikingly, coconcurrent expression of INI-1, p300, and Tat strongly activated the HIV LTR to 450-fold in G401 cells and to 100-fold in MON cells (Fig. 3A). The synergistic activation of the HIV promoter by Tat, INI-1, and p300 was abolished when p300ΔHAT was used (Fig. 3A). The cooperative effects on transcription were specific for the HIV promoter, as the effect was not observed with the CMV promoter (Fig. 3A).

During the HIV life cycle, activation of the HIV LTR takes place after integration of the HIV genome in the host cell genome. Therefore, we examined the effect of INI-1 on the regulation of the integrated HIV promoter. We generated viral particles containing the retroviral vector LTR-EGFP-RES-luciferase-LTR (Fig. 3B) and infected the INI-1-deficient G401 and MON cell lines. Polyclonal cell lines containing an integrated LTR-luciferase reporter were obtained and transiently transfected with the expression vectors for Tat, INI-1, p300, and p300ΔHAT. In this system, the same synergy was noted between INI-1, p300, and Tat (Fig. 3B). This synergy was also dependent on the histone acetyltransferase domain of p300 (Fig. 3B). In conclusion, these results reveal a striking cooperation between the ATP-dependent chromatin-remodeling subunit INI-1 and the acetyltransferase p300 during Tat-directed transcription of the HIV LTR.

Tat Lys50 and Lys51 Are Necessary for the Synergy between Tat, p300, and SWI/SNF—The p300 transcriptional coactivator acetylates Tat at Lys50 (6, 11, 12). This acetylation mediates the dissociation of Tat from TAR (11, 12, 27). Because our results indicated that the synergy between INI-1 and p300 in Tat activation of the LTR depends on the acetyltransferase activity of p300, we tested the role of Tat Lys50 in SWI/SNF recruitment and LTR activation. Although Lys50 is the primary target of acetylation in Tat, its mutation to alanine leads to the secondary acetylation of Lys51 (6). Introduction of both mutations is therefore necessary to abrogate acetylation. Accordingly, we tested the ability of a mutant Tat protein in which both Lys50 and Lys51 were substituted with arginine (Tat(K50R/K51R)) to recruit SWI/SNF. Notably, the synergistic activation by p300 and INI-1 was abolished in both G401 and MON cells when coexpressed with Tat(K50R/K51R) (Fig. 4A).

We also used the BRG-1-deficient C33A cell line to examine the synergism between Tat, BRG-1, and p300 (Fig. 4B). The cells exhibited low LTR activation by Tat that was increased upon BRG-1 expression (Fig. 4B). Comcomitant expression of BRG-1, p300, and Tat strongly activated the LTR to 70-fold. This synergism was abolished in the presence of the p300 catalytic mutant (p300ΔHAT) as well as the Tat(K50R/K51R) mutant (Fig. 4B).

These results raised the possibility that the interaction between SWI/SNF and Tat is disrupted if Tat cannot be acetylated. To determine whether the interaction of Tat with SWI/SNF is modulated by acetylation, wild-type Tat or the Tat(K50R/K51R) mutant was cotransfected in 293T cells with or without p300. To prevent deacetylation of Tat, cells were further treated with nicotinamide, an inhibitor of class III histone deacetylases (28), and trichostatin A, an inhibitor of class I and II histone deacetylases (Fig. 4C). We found that Tat association with BRG-1 increased in the presence of p300 as shown by co-immunoprecipitation of BRG-1 with Tat (Fig. 4C). The same treatment markedly increased Tat acetylation (Fig. 4C). We also examined the effect of Tat acetylation on its interaction with subunits of the pTEFβ complex. A concomitant decrease in the interaction of acetylated Tat with cyclin T1 and CDK9.
was observed (Fig. 4C). Notably, the Tat(K50R/K51R) mutant did not display increased affinity for BRG-1 in response to p300. In agreement with these data, the affinity of the Tat(K50R/K51R) mutant for pTEFb subunits was not decreased in response to p300 (Fig. 4C). Mutation of Tat residues Lys50 and Lys51 to arginine decreased Tat acetylation significantly, but not completely, consistent with the existence of other Tat acetylation sites (29).

A likely candidate subunit in the SWI/SNF complex to directly interact with acetylated Tat is BRG-1. BRG-1 contains a C-terminal bromodomain, a recognition motif for acetylated lysine-containing proteins. Acetylated Tat has been shown to specifically interact with another transcriptional coactivator, PCAF, via its bromodomain (7). According to structure-based sequence alignment of bromodomains, the BRG-1 bromodomain shares the highest sequence homology with the PCAF bromodomain, including conserved key amino acid residues important for Tat binding (7). To test this model, we used GST fusion proteins made to overlap 300-amino acid stretches of the BRG-1 protein (Fig. 4D, upper panel) (23). The BRG-1 bromodomain is located within the C-terminal domain of BRG-1 (amino acids 1400–1700). Fusion proteins were immobilized on glutathione beads and tested for binding to synthetic biotinylated Tat protein acetylated at Lys50 or to unacetylated Tat protein (27). The BRG-1 bromodomain specifically bound acetylated Tat (Fig. 4D). This binding was critically dependent on Tat acetylation at Lys50, as unacetylated Tat did not bind to the BRG-1 bromodomain-containing fragment. An additional weaker interaction was also observed between BRG-1 amino acids 400–700 and acetylated Tat. Together, these results are consistent with the model that acetylated Tat recruits the SWI/SNF complex to the HIV LTR via BRG-1.

**FIGURE 3.** INI-1, p300, and Tat cooperate to activate the HIV promoter. A, transient transfection experiments were performed in the INI-1-deficient cell lines G401 and MON. LTR-luciferase and CMV-luciferase reporter plasmids were cotransfected in the presence of various combinations of expression vectors for Tat, INI-1, p300, and the p300HAT catalytic mutant as indicated. B, the structure of the retroviral vector packaged into vesicular stomatitis virus G-pseudotyped viral particles and used to infect G401 and MON cells is shown. This infection led to integration of a chromatinized LTR-EGFP-IRES-luciferase reporter vector. C, INI-1-deficient G401 and MON cell lines containing an integrated LTR-luciferase reporter were transfected with various combinations of expression vectors for Tat, INI-1, p300, and the p300HAT catalytic mutant in the indicated combinations. HIV promoter activity was assessed by measuring luciferase activity in cell lysates. The results are expressed as fold activation over the basal activity of the HIV promoter. The results shown in A and C represent the mean ± S.D. of three independent experiments conducted each in triplicate.

**Tat Mediates the Recruitment of the SWI/SNF Complex to the HIV Promoter in Vivo**—To demonstrate that Tat mediates the recruitment of SWI/SNF to the HIV promoter in vivo, we performed chromatin immunoprecipitation assays. We stimulated the Jurkat cell line A2 containing a latently integrated LTR-Tat-IRES-GFP virus (24) with PMA. PMA stimulation of the A2 cells resulted in GFP expression in 86% of the cells (Fig. 5A). Tat was expressed to detectable levels in response to PMA stimulation at 30 min and peaked at 4 h post-stimulation (Fig. 5B). Chromatin was prepared from cells at 0, 0.5, 4, and 8 h post-stimulation and subjected to chromatin immunoprecipitation with antibodies specific for BRG-1, p300, and YY1. PCR analysis of the immunoprecipitated material with primers specific for the HIV promoter indicated that BRG-1 and p300, while initially absent from the LTR, were specifically recruited to the HIV promoter in response to PMA stimulation (Fig. 5B).
In contrast and in agreement with published observations (30), the transcriptional repressor YY1 was bound to the HIV promoter under basal conditions and was displaced in response to PMA (Fig. 5B). These results suggest that the SWI/SNF complex is recruited to the LTR in response to Tat. However, in this experiment, we could not exclude the possibility that recruitment of SWI/SNF to the LTR occurs indirectly via other LTR activators in response to PMA.

To demonstrate that SWI/SNF recruitment occurs directly via Tat and in a Tat-dependent manner, we used Jurkat A72 cells containing an integrated LTR-GFP virus that lacks Tat (24) in chromatin immunoprecipitation experiments (Fig. 5, C and D). We transfected the Jurkat A72 cells with an expression vector for Tat or the control empty vector and observed that 46% of the cells expressed GFP 16 h after Tat transfection (Fig. 5C). Tat was expressed 4.5 h post-transfection (Fig. 5D). Chromatin was prepared from cells 4.5 h after introduction of the Tat expression vector and subjected to chromatin immunoprecipitation with antibodies specific for BRG-1 and YY1. PCR analysis of the immunoprecipitated material with primers specific for the HIV promoter indicated that BRG-1 was specifically recruited to the HIV promoter, whereas YY1 was displaced in response to Tat expression (Fig. 5D). These results demonstrate that the SWI/SNF complex is specifically recruited to the LTR by Tat in vivo.

DISCUSSION

We have shown that the SWI/SNF chromatin-remodeling complex is a cofactor for Tat activation of the HIV promoter. Knockdown of INI-1 and BRG-1, two critical components of mammalian chromatin-remodeling complexes, suppresses Tat-mediated transactivation. Similarly, cells without INI-1 or BRG-1 exhibit defective transactivation by Tat that can be rescued by INI-1 or BRG-1 expression. Tat specifically interacts with several SWI/SNF subunits, INI-1, BRG-1, and β-actin. Similarly, SWI/SNF interacts with the integrated HIV promoter in a Tat-dependent manner. In addition, we found that INI-1 and BRG-1 act synergistically with the p300 acetyltransferase to activate the HIV promoter. This synergism is critically dependent on the histone acetyltransferase activity of p300 and on Tat Lys30 and Lys51.

Studies in several experimental systems have revealed that enzymes that post-translationally modify chromatin proteins and chromatin-remodeling complexes are recruited in a stepwise fashion to specific promoters (31–35). The combinatorial assembly of transcription factors and these chromatin-modifying proteins mediates a precise transcriptional response. However, chromatin-modulating factors do not appear in a set order at all genes. For example, the human SWI/SNF complex is recruited at the interferon-β promoter after the histone acetyltransferases CBP and PCAF, whereas the yeast SWI/SNF chromatin-remodeling complex is recruited first and is required for the subsequent recruitment of the SAGA histone acetyltransferase complex at the yeast HO promoter (31, 32).

We observed that Tat, p300, and SWI/SNF synergistically activate the HIV promoter. This synergy depends on the acetyltransferase activity of p300 and on Tat Lys30 and Lys51. We (6) and others (11) have reported that p300 acetylates Tat at Lys30, a modification that plays a significant role in Tat transcriptional

FIGURE 4. Activation of the HIV promoter by SWI/SNF, p300, and Tat is dependent on Tat Lys30 and Lys51. A, LTR-luciferase reporter plasmids were cotransfected in the presence of various combinations of expression vectors for INI-1, p300, the p300ΔHAT catalytic mutant, Tat, and the acetylation mutant Tat(K50R/K51R) (K50,51R) in G401 (left panel) or MON (right panel) cells. Tat was co-immunoprecipitated with pTEFb and SWI/SNF is modulated by Tat acetylation. Tat (wild-type or K50R/K51R) was immunoprecipitated (IP) using anti-FLAG antibody, and the immunoprecipitated material was analyzed by Western blotting using antibodies specific for BRG-1, cyclin T1, and CDK9. Tat acetylation levels were assessed using anti-acetylysine antibody. All proteins were expressed at similar levels under the different experimental conditions (Input). B, the BRG-1 bromodomain binds preferentially to Tat acetylated at Lys50. GST fusions to the N terminus of the BRG-1 bromodomain were expressed in bacteria. BRG-1 deletions were purified and immobilized on glutathione beads (upper panel) and incubated with acetylated (Ac-Tat) or unacetylated biotinylated Tat. Beads were washed; bound proteins were separated by SDS-PAGE; and bound Tat was visualized using horseradish peroxidase-conjugated streptavidin.

In contrast and in agreement with published observations (30), the transcriptional repressor YY1 was bound to the HIV promoter under basal conditions and was displaced in response to PMA (Fig. 5B). These results suggest that the SWI/SNF complex is recruited to the LTR in response to Tat. However, in this experiment, we could not exclude the possibility that recruitment of SWI/SNF to the LTR occurs indirectly via other LTR activators in response to PMA.

To demonstrate that SWI/SNF recruitment occurs directly via Tat and in a Tat-dependent manner, we used Jurkat A72 cells containing an integrated LTR-GFP virus that lacks Tat (24) in chromatin immunoprecipitation experiments (Fig. 5, C and D). We transfected the Jurkat A72 cells with an expression vector for Tat or the control empty vector and observed that 46% of the cells expressed GFP 16 h after Tat transfection (Fig. 5C). Tat was expressed 4.5 h post-transfection (Fig. 5D). Chromatin was prepared from cells 4.5 h after introduction of the...
activity in the HIV promoter. We have proposed previously that Tat acetylation serves as a molecular switch that coordinates the recruitment of different cofactors to the HIV promoter (7, 8, 27, 36). Early in the transcription cycle, unacetylated Tat binds to the RNA element TAR and recruits pTEFb, including CDK9 and cyclin T1, to the HIV promoter. Tat bound to the HIV promoter becomes acetylated by p300, leading to the dissociation of the ternary complex between Tat, TAR, and pTEFb.

The results presented here are consistent with the model that acetylated Tat facilitates the recruitment of the SWI/SNF complex to the HIV promoter, leading to nuc-1 remodeling. Acetylated Tat preferentially interacts with another histone acetyltransferase (PCAF) via its bromodomain (7, 8, 37). We cannot presently determine whether acetylated Tat recruits PCAF and SWI/SNF sequentially, in a mutually exclusive manner, or simultaneously. The orthologs of these two protein complexes in Saccharomyces cerevisiae (GCN5 and SWI/SNF) cooperate in the transcriptional activation of several promoters (38–42). Evidence has been presented that the bromodomain of GCN5 stabilizes the SWI/SNF complex in an artificial promoter and is required for nucleosome remodeling and transcriptional activation (43). This observation indicates that, in some cases, both GCN5 and SWI/SNF may bind together to a given promoter (43). Because Tat can also interact with PCAF, it is possible that PCAF-mediated hyperacetylation of the HIV promoter further stabilizes SWI/SNF binding to the HIV promoter by creating a hyperacetylated chromatin environment. Indeed, PCAF targets histones for acetylation in the HIV promoter (44).

Our observations represent the first example of recruitment of a chromatin-remodeling complex to a promoter via an RNA-binding protein. The HIV Tat protein represents a unique transcriptional activator targeted downstream of the transcription start site via its interaction with the TAR RNA element. No rationale has emerged thus far to explain why Tat has evolved as an RNA-binding transactivator instead of a more classical DNA-binding protein. It is intriguing that nuc-1, the nucleosome that is remodeled by SWI/SNF in a Tat-dependent manner, is located immediately downstream of the transcription start site. Our observations that Tat contributes to the recruitment of a chromatin-remodeling complex could provide a rationale for the need for Tat to function via RNA instead of DNA. SWI/SNF bound to TAR via Tat would be positioned immediately at the site of nuc-1 and could explain the selective remodeling of nuc-1 by Tat. We cannot exclude the possibility that SWI/SNF also interacts with Tat when the latter is bound to the elongating polymerase (45). However, such a model is inconsistent with the observations that a single nucleosome (nuc-1) is remodeled at the level of the HIV promoter and that remodeling of nuc-1 is insensitive to α-amanitin, a specific inhibitor of RNA polymerase II (1).

Recruitment of the SWI/SNF complex to the HIV promoter in response to Tat is accompanied by removal of the YY1 transcriptional repressor. Previous reports have documented that YY1 is recruited to the HIV promoter at the transcription start site via its specific interaction with LSF (30, 46). YY1 specifically recruits HDAC-1 and is likely to contribute to histone hypoacetylation at the level of nuc-1 in the absence of Tat (30). It is not entirely clear how Tat leads to the displacement of YY1, but nuc-1 remodeling could lead to a change in the affinity of the DNA for YY1 and its dissociation from the HIV promoter. Alternatively, Tat may mediate the displacement of YY1 from the HIV promoter independently of nuc-1 remodeling. Irrespective of the mechanism, removal of YY1 from the HIV promoter and the resulting loss of HDAC-1 could further contribute to the hyperacetylation of the HIV promoter mediated by PCAF. Such a mechanism could contribute to the hyperacetylation of the HIV promoter that has been observed in response to Tat expression (4).

The identification of the SWI/SNF chromatin-remodeling complex as a Tat cofactor provides a mechanism for the longstanding observation that Tat leads to the selective remodeling
of nuc-1. The recruitment of SWI/SNF via Tat and RNA represents a novel mechanism for the recruitment of a chromatin-remodeling complex to a promoter. Further study of this process will contribute to providing an integrated understanding of HIV transcriptional regulation in the context of chromatin.

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