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Acetylated Tat Regulates Human Immunodeficiency Virus Type 1 Splicing through Its Interaction with the Splicing Regulator p32

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The human immunodeficiency virus type 1 (HIV-1) potent transactivator Tat protein mediates pleiotropic effects on various cell functions. Posttranslational modification of Tat affects its activity during viral transcription. Tat binds to TAR and subsequently becomes acetylated on lysine residues by histone acetyltransferases. Novel protein-protein interaction domains on acetylated Tat are then established, which are necessary for both sustained transcriptional activation of the HIV-1 promoter and viral transcription elongation. In this study, we investigated the identity of proteins that preferentially bound acetylated Tat. Using a proteomic approach, we identified a number of proteins that preferentially bound AcTat, among which p32, a cofactor of splicing factor ASF/SF-2, was identified. We found that p32 was recruited to the HIV-1 genome, suggesting a mechanism by which acetylation of Tat may inhibit HIV-1 splicing needed for the production of full-length transcripts. Using Tat from different clades, harboring a different number of acetylation sites, as well as Tat mutated at lysine residues, we demonstrated that Tat acetylation affected splicing in vivo. Finally, using confocal microscopy, we found that p32 and Tat colocalize in vivo in HIV-1-infected cells.

Human immunodeficiency virus type 1 (HIV-1), a member of the lentivirus family of retroviruses, is the etiologic agent of AIDS. The virus enters the cells by binding the (Env) glycoprotein to the CD4 receptor along with the chemokine receptor CXCR4 or CCR5 (25, 100). The viral RNA is then reverse transcribed into proviral DNA in the cytoplasm and integrated into the host genome (63). Once integrated, the activity of the viral promoter located in the 5′ long terminal repeat (LTR) is regulated by several factors, including the local chromatin structure at the site of integration (37, 49, 70), epigenetic mechanisms affecting the 5′ LTR (29), activation-dependent host transcription factors (22, 52), and the presence of the viral transactivator Tat (27, 33).

Tat, a 14-kDa viral protein, acts as a potent transactivator of viral genes and is essential for viral replication. There is evidence that Tat is involved in transcription initiation through binding general transcription factors including TATA box-binding protein, TAFII250, TFIIB, and TFIIH (6, 39–41, 91, 92, 95, 106), as well as through stimulation of preintegration complex assembly (67). Consistent with previously published data, it has recently been shown that, like typical activators, Tat dramatically stimulates transcriptional and preinitiation complex formation by directly recruiting TATA box-binding protein (72). However, the current favored model supports a predominant role of Tat in transcription elongation by binding to TAR (transactivation-responsive region), a structured RNA element located at the 5′ end of all HIV-1 transcripts (53). Tat binding to TAR requires cyclin T1, a component of positive transcription elongation factor b (pTEF-b), which subsequently recruits Cdk9 to the HIV-1 promoter (5, 23, 105). Cdk9 and other Tat-activated kinases hyperphosphorylate the C-terminal domain of RNA polymerase II (Pol II), increasing its processivity (15, 105, 107). In addition, Tat transactivates viral gene expression by recruiting chromatin remodeling complexes and histone-modifying enzymes (3, 18, 42, 53, 54, 66, 70). Histone acetyltransferases (HATs), including p300 and hGCN5, not only acetylate histones but also acetylate Tat at lysine positions 50 and 51 in the arginine-rich motif (17, 18, 42, 66). Acetylation of lysine 50 of Tat by p300 promotes the dissociation of Tat from TAR RNA during early transcription elongation and recruits p300/CBP-associated factor (P/CAF) to the elongating RNA Pol II (20, 62, 70). Subsequently, P/CAF acetylates Tat at lysine 28 (42) enhancing its binding to p-TEFb (7). The current model suggests that acetylation of Tat regulates two discrete and functionally critical steps in transcription, binding to an RNA Pol II C-terminal domain kinase and release of Tat from TAR RNA (38, 65).

Constitutive and alternative mRNA splicing occurs in HIV-1 to remove upstream Gag and Pol coding sequences from the Env mRNA and to generate the essential regulatory proteins. Splicing of HIV-1 leads to the production of three classes of RNA: unspliced (9 kb), including Gag and Pol; singly spliced (4 kb), including Env, Vpu, Vif, and Vpr; and doubly spliced (2 kb), including Tat, Rev, and Nef (Fig. 1). The pattern of splic-

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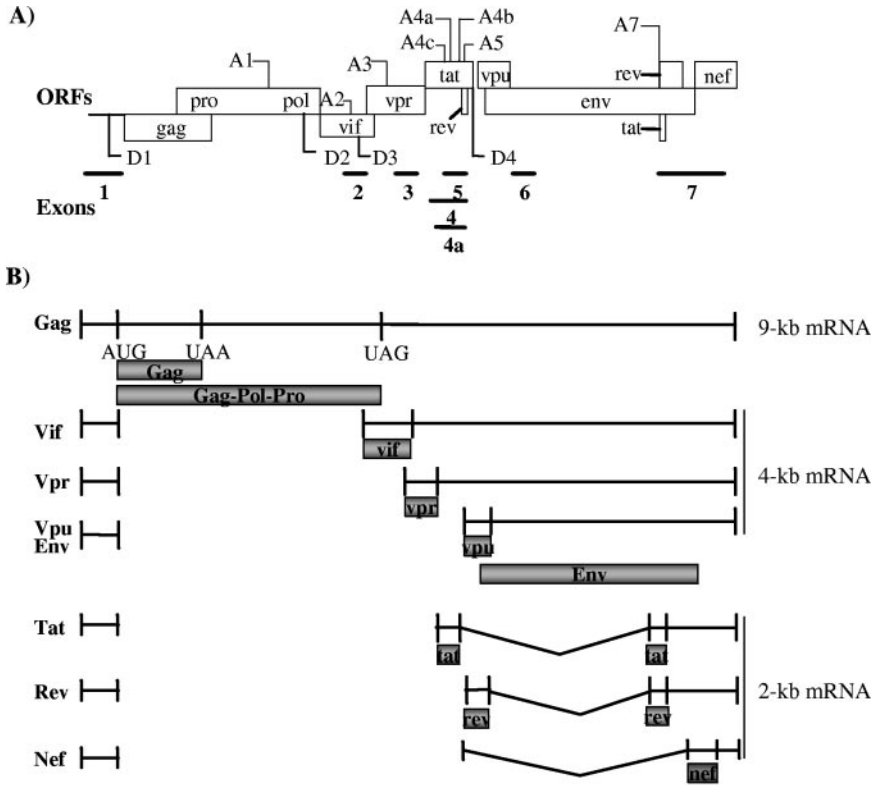


FIG. 1. Genomic structure of HIV-1. (A) Structure of HIV-1 proviral genome and location of the HIV-1 exons and splice donor (D) and splice acceptor (A) sites. (B) The structure of the alternatively spliced mRNAs (lines) and open reading frames (ORFs) (filled boxes).

ing in HIV-1-infected cells varies according to the phase of HIV-1 infection. While subgenomic species (doubly and singly spliced) are abundant during early stages of infection, genomic transcripts (unspliced) appear at the later stage of infection (43, 44, 57). In models of proviral latency in lymphoid and monocytic cells as well as nonlymphoid cells, stimulation with sodium butyrate, phorbol esters, or tumor necrosis factor alpha leads to a shift in the RNA splicing pattern from multiply spliced low-molecular-weight messages to full-length genomic species, which are needed for the production of viral structural proteins (Gag) and serve as genomic RNA for new virions (44, 48, 58, 69). The splicing of the HIV-1 RNA is complex because of the presence of four 5′ splice donor sites D1 to D4 and eight 3′ splice acceptor sites (A_1, A_2, A_3, $A_{4a,b,c}$, A_5, and A_7) (71, 73, 83) (Fig. 1). A comprehensive study examining the steady-state levels of viral mRNA during viral infection determined the utilization of different splice sites for the production of the 2-kb and 4-kb transcripts. The A4 and A7 sites were essential for the production of Tat, while A4 (a, b, or c) and A7 were needed to produce Rev. Nef and Env required A5, and Vpr required A2 (71).

Although most of the available evidence hints to an indirect regulation of splicing by Tat through up-regulation of genes involved in splicing (including splicing factors arginine/serine-rich 7 and 9) and mRNA processing (11, 46, 50, 101), we have recently found that Tat regulates splicing through recruitment of the ASF/SF-2 inhibitor p32. Using a proteomic approach to study the binding partners of acetylated Tat, we found that the acetylated form of Tat preferentially binds to p32, an inhibitor of splicing factor ASF/SF-2. We also found that p32 is recruited to the HIV-1 promoter, suggesting a mechanism by which acetylation of Tat preferentially binds p32 and thereby inhibits the HIV-1 splicing needed for the production of full-length transcripts. Using Tat from different clades, harboring different numbers of acetylation sites, as well as Tat mutated at lysine residues, we directly demonstrated that Tat acetylation affected splicing of the HIV-1 genome. Finally, using confocal microscopy, we demonstrated that Tat colocalized with p32 in vivo.

MATERIALS AND METHODS

Plasmids. Plasmids encoding wild-type, chimeric, and mutant Tat proteins from clades B, C, and E were described previously (75). These constructs were cloned into a modified version of the pcDNA3.1/Zeo vector, which contains a Flag-encoding sequence at the 5′ end of multiple cloning sites. The proviral plasmids pNLCAT (32) and pNL-Luc (89) were obtained from the NIH AIDS Reagents program (www.aidsreagent.org). We generated the LTR-Target 3 minigene by PCR amplification of the HIV-1 LTR from nucleotide (nt) −156 to +73, containing the *cis*-regulatory elements NF-$\kappa B^{II,I}$ and $SP1^{III,\ II,I}$, the TATA box, and TAR using the primers 5′-CCGCTCGAGCGAGAGCTGCATCCGG AGTACTTCAA-3′ (forward) and 5′-CCGGAATTCTGAGGCTTAAGCAGT GGGTTCCCTA-3′ (reverse), having the XhoI and EcoRI recognition sequences, respectively (underlined). The LTR was subcloned into the XhoI and EcoRI sites of a reporter minigene (Target 3) spanning the human cytomegalovirus (HCMV) UL37 exon 3 donor, the complete intron 3, and the exon 4 acceptor and downstream exonic sequences called Target 3 and was driven by the HCMV major immediate-early (IE) promoter (87). Polyadenylation of the reporter minigene is directed by the HIV-1 polyadenylation (PA) signal that was generated by PCR amplification of HIV-1 DNA using primers containing BamHI and XbaI sites (underlined) for cloning (forward, 5′-CGCGGATCCG GAACCCACTGCTTAAGCCTCAATAAAGC-3′; reverse, 5′-CTAGTCTAGA

GATTTTCCACACTGACTAAAAGGGTCTGAGG-3′). The complete minigene with the LTR and PA was cloned into the mammalian expression vector Bluescript II (SK+) and sequenced to verify that sequences were not mutated during amplification and cloning. The glutathione *S*-transferase (GST)–p32 (pGEX-2TK-p32) vector was kindly provided by J. Kjems (University of Aarhus, Aarhus C, Denmark) (88), and the pGEM-Tat wild-type and K41A mutant vectors for the TNT system as well as GST-Tat were previously described (40). Double mutant pcTat (K50, 51A) was derived from pcTat (31, 41) as previously described (17). PGN-ASF/Sf-2 was a kind gift from J. L. Manely (93), and pcDNA-p32 was kindly provided by B. M. Peterlin (104).

Cell culture and transfections. HeLa and 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin-streptomycin. HeLa and 293T cells (2.5×10^6) cultured on 60-mm plates and grown overnight were transfected using metafectene (Biontex, Munich, Germany). HeLa cells were transfected with 12 μg of pNL-Luc or pNL-CAT in combination with murine leukemia virus (MLV)-Env, and 293T cells were transfected with 3 μg of minigene plasmids alone or with 6 μg of pcTat or pcTat (K50, 51A) vector. HLM-1 cells are HeLa ($CD4^+$) cells transduced with the Tat-defective mutant pMTat derived from pHXBgpt (NIH AIDS Reagent Catalog). They were grown under the same conditions described above with addition of G418 (100 μg/ml) for selection. HLM-1 cells were transfected with 1 μg of Flag-Tat B, C, E, chimeric Tat, or Tat mutants by nucleofection according to the manufacturer's protocol (Amexa, Cologne, Germany). OM10.1 cells are derived from HL-60 promyelocyte cells, which survived an acute infection (NIH AIDS Reagent Catalog) and contain a single copy of integrated wild-type (WT) HIV-1. OM10.1 cells were grown in RPMI supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin-streptomycin (Quality Biological).

Streptavidin bead pull-down assay. Tat peptides (amino acids [aa] 36 to 53 and 42 to 52) were synthesized with a biotin tag on a PAL-polyethylene glycol-polystyrene resin by continuous flow solid-phase synthesis on a Perspective Biosystems Pioneer synthesizer (Framingham, MA) using HBTU-activated 9-fluorenylmethoxy carboxyl amino acids and were synthetically acetylated at positions 41/50/51 or 50/51, respectively (18). Synthesized Tat peptides (aa 36 to 53 and 42 to 54), labeled with biotin at the N terminus and with or without an acetyl group at lysines 50 and 51, were used in the pull-down assays (described in reference 17). CEM whole-cell extracts were prepared as previously described (77) and were incubated with biotin-labeled Tat peptides in TNE_{50} buffer (100 mM Tris-HCl, pH 7.5; 50 mM NaCl; 1 mM EDTA; 0.1% NP-40) at 4°C overnight, in the presence or absence of competing peptides (Tat Ac K50 and K51, Tat Ac K50, and Tat Ac K51). Streptavidin beads (Boehringer Mannheim) were added to the mixture and incubated for 2 h at 4°C. The beads were washed twice with TNE_{600} or TNE_{2000} (100 mM Tris-HCl, pH 7.5; 600 or 2,000 mM NaCl; 1 mM EDTA; 0.1% NP-40), once with TNE_{150}, and once with TNE_{50}. Bound proteins were separated on 4 to 20% sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE), and subjected to mass spectrometry or Western blotting with antibodies against p32 (kindly provided by W. C. Russell, University of St. Andrews, United Kingdom), HSP70 (Santa Cruz Biotech), ASF/SF-2 (Zymed Laboratories, Inc.), and HDAC1 (Santa Cruz).

MALDI-TOF and database analysis. Following streptavidin bead pull down, proteins were separated by SDS-PAGE, excised from a Coomassie blue-stained gel, and trypsin digested (97). Sample solutions were further desalted and concentrated with C_{18} ZipTips (Millipore). Samples were mixed with the same volume of a matrix solution (α-cyano-4-hydroxycynnamic acid in 50% acetonitrile–0.1% [vol/vol] trifluoroacetic acid). Two microliters of the mixtures was applied to the sample plate and introduced into the mass spectrometer after drying. Mass spectra were recorded in the reflectron mode of a matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometer (Axima CFR Plus; Kratos).

Proteins were identified using the peptide mass fingerprinting analysis software Prospector, Mascot (Matrix Science), and Profound and the NCBInr and SwissProt databases. In general, all spots were searched with methionine oxidation and no limitation for pI. The best match for each spot was considered with higher coverage rate, more matched peptides, and higher score, with a limitation on the taxonomic category to *Homo sapiens*. One missed cleavage by trypsin and a mass tolerance of 1 Da were considered for most of the proteins.

TNT-binding assays. pGEM-Tat (40) was used to produce ^{35}S-labeled Tat using the TNT coupled reticulocyte lysate system (Promega) according to the manufacturer's protocol. To confirm synthesis, proteins were separated by SDS-PAGE on a 4 to 20% Tris-glycine polyacrylamide gel. The gels were dried and exposed to a PhosphorImager cassette. Binding assays included GST-p32 constructs (0.5 μg) or GST alone (concentration determined by Coomassie blue staining) and 5 μl of TNT reaction mixtures and were carried out at 4°C overnight. The next day, glutathione-Sepharose beads were added and incubated at 4°C for 2 h. Complexes were then washed twice with TNE_{600} plus 1.0% NP-40, once with TNE_{150} plus 0.1% NP-40, and once with TNE_{50} plus 0.1% NP-40. Complexes were separated on a 4 to 20% Tris-glycine gel by SDS-PAGE. The gel was dried and exposed to a PhosphorImager cassette.

ChIP of HIV-1 latently infected cells. Chromatin immunoprecipitation (ChIP) was performed as described previously (16). Five to 10 million OM10.1 cells were incubated for 2 h with or without trichostatin A (TSA; 450 nM). After a 24-h induction, cells were cross-linked (1% formaldehyde, 10 min at 37°C) and samples were sonicated to reduce DNA fragments to 200 to 800 nt for ChIP assays. Transcription complexes were immunoprecipitated with 5 μg antibodies including Tab 172 (control antibody [Ab]) that can recognize the human T-cell leukemia virus type 1 viral protein Tax (NIH AIDS Reagent program), p32 (kindly provided by W. C. Russel), AcTat (immunoglobulin G [IgG]-purified antibody raised in our lab against Tat acetylated at positions 50, 51), cdk-9 (Biodesign International, Maine), ASF/SF-2 (Zymed Laboratories, California) and RNA Pol II (C-21; Santa Cruz). Specific DNA sequences in the immunoprecipitates were detected by PCR by using primers specific for the HIV-1 LTR (forward, 5′-AAGGGCTAATTCACTCCCAA-3′; reverse, 5′-TCTAACACTTCTCTCTC CGGG-3′) and for actin (forward, 5′-GTGACACAGCATCACTAAGG-3′; reverse, 5′-ACAGCACCGTGTTGGCGT-3′).

Luciferase and CAT assays. HeLa cells were infected with MLV-pseudotyped pNL-CAT or pNL-Luc and treated with 450 nM TSA 24 h later. After 48 h, cells were lysed and chloramphenicol acetyltransferase (CAT) and luciferase activities were determined. Luciferase was measured using the Luciferase assay system (Promega). For the CAT assay, a standard reaction was performed by adding the cofactor acetyl coenzyme A to a microcentrifuge tube containing cell extract and radiolabeled (^{14}C) chloramphenicol in a final volume of 50 μl and incubating the mixture at 37°C for 1 h. The reaction mixture was then extracted with ethyl acetate and separated by thin-later chromatography on silica gel plates (Baker-flex silica gel thin-later chromatography plates) in a chloroform-methanol (19:1) solvent. The resolved reaction products were then detected by exposing the plate to a PhosphorImager cassette.

RPA. The probe for RNase protection assays (RPA) is expressed from pBS/HIV978-340 containing the major 5′ splice donor sequence of HIV-1 which was cut by HindIII (4). One microgram of purified DNA was used to generate the RNA probe in the presence of [^{32}P]UTP using the T7 MAXIscript kit (Ambion, Austin, TX), which was then gel purified. RNA (30 μg) extracted from HLM-1 cells using RNA-Bee according to the manufacturer's directions (TEL-TEST, Texas) was hybridized with 5×10^5 cpm probe using the RPA III kit (Ambion). The hybridized RNA was digested with RNase T1 at a 1:100 dilution. Protected fragments were resolved on a 6% denaturing Tris-borate-EDTA-urea acrylamide gel and visualized by autoradiography.

RNA isolation and RT-PCR. Total cellular RNA was isolated using RNA-Bee (TEL-TEST, Texas). One microgram of total RNA was treated with RQ1 RNase free-DNase (Promega) and reverse transcribed using oligo(dT) primers (Invitrogen) and SuperScript II reverse transcriptase (RT; Invitrogen). Control reactions containing no RT were assayed in parallel. cDNAs were amplified by PCR using Biolase DNA polymerase (Bioline, London, United Kingdom) with the primers indicated in each figure legend. PCR products were detected by ethidium bromide staining following electrophoresis on nondenaturing 12% polyacrylamide gels. A DNA marker (100-bp ladder; Promega) served as a molecular size standard. Gel photographs were digitized using SynGene.

Immunofluorescent staining. Cells were grown on coverslips and then fixed for 20 min with 4% paraformaldehyde (Fisher Scientific) at room temperature. Next, cells were washed with phosphate-buffered saline without Mg^{2+} and Ca^{2+} (Quality Biological) at 4°C. The primary antibody, in 10% bovine serum albumin, was added and incubated for 1 h, in the dark, at 37°C. Primary antibodies used were rabbit polyclonal anti-p32 (Ab-1, 1:1,000 dilution) and mouse monoclonal Flag antibody (1:200 dilution; Santa Cruz Biotechnologies). Coverslips were then washed three times with 5 ml of phosphate-buffered saline without Mg^{2+} and Ca^{2+}. Secondary antibodies in 10% bovine serum albumin were added, and the slides were again incubated for 1 h, in the dark, at 37°C. Secondary antibodies used were Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200) and Alexa Fluor 568-conjugated goat anti-mouse IgG (1:50) from Molecular Probes/Invitrogen. The previous washes were repeated. Coverslips were then incubated at room temperature for 20 min with 2 μM TOTO-3, a dimeric cyanine nucleic acid stain from Molecular Probes, for nuclear staining. Coverslips were washed briefly with H_2O, and the excess liquid was removed. Coverslips were mounted onto slides with Prolong anti-fade (Molecular Probes), which also prevents photobleaching. After drying, the slides were sealed with clear nail polish.

Confocal laser scanning microscopy. Slides were viewed with a Bio-Rad MRC1024 confocal laser scanning microscope (Center for Microscopy and Im-

age Analysis, George Washington University) using the 60× oil objective and Bio-Rad LaserSharp software, version 5.2. Triple excitation at 488, 568, and 647 nm was used. Optical sections were taken using z-dimensions between 0.5 to 1.0 μm. Pictures were produced using Adobe Photoshop 5.0 and Bio-Rad plug-ins.

RESULTS

Tat acetylation alters its binding partners. Lysine acetylation is an important posttranslational modification that regulates the function of many proteins (99). Tat expressed from clade B viruses, the most studied HIV-1 subtype, harbors 12 lysine residues in its sequence, including one triple lysine motif at positions 88/89/90 in the C terminus and two double lysines at positions 28/29 in the cysteine-rich region and at position 50/51 in the arginine-rich motif. Some of the HIV-1 Tat lysine residues have been shown to be acetylated by different HATs (K^{50} and K^{28}) (24, 96) or by auto-acetylation (K^{41} and K^{71}) (18). These modifications influence the binding partners of AcTat. Acetylated Tat was shown to interact with proteins having a bromodomain including P/CAF and SWI/SNF (62, 70) and to increase transcription initiation and elongation on chromatinized templates (17, 18).

To determine the binding partners of acetylated Tat, we used a proteomic approach to identify cellular factors that differentially interacted with unmodified or acetylated Tat. We initially chose Tat peptides (aa 36 to 53) containing the core region as well as the basic domain because of the importance of these two regions in regulating viral gene expression (10, 90). Unmodified or acetylated biotin-tagged Tat peptides were used to pull down protein complexes from human T-cell extracts (CEM cells). After a series of high-salt/detergent washes, bound complexes were separated by SDS-PAGE. Bands that specifically interacted with acetylated Tat were excised from the gel, and in-gel digestion with trypsin was performed overnight to produce tryptic peptides for each protein for further identification by mass spectrometry. A few cellular proteins were found to differentially bind unmodified versus acetylated Tat (Fig. 2A). Although the high-salt wash condition (2 M NaCl) did not show significant differences between unmodified Tat and AcTat (lanes 4 and 5), the less-stringent wash conditions (0.6 M NaCl) revealed unique bands binding to AcTat (lanes 2 and 3; bands 1, 6, 7, and 8). Bands 4 and 5 bound to both forms of Tat but had a higher affinity for AcTat. They also acted as positive controls for the database searches, since bands 2 and 3 may have similar identities to bands 4 and 5, respectively. Beads alone incubated with CEM cell extracts were used as a negative control (lane 6). Using MALDI-TOF mass spectrometry, we identified the selected proteins (Table 1) as DNA polymerase delta catalytic subunit p125 (band 1), members of the HSP70 family (bands 2 to 5), tubulin beta-5 (band 6), the splicing regulator p32 (band 7), and ribosomal protein L24, a component of the 60S subunit (band 8). All of these proteins were found using three independent search engines: Mascot, Prospector, and Profound. Some of the identified proteins have been shown to play a role in HIV-1 viral replication. GRF78 associates with and facilitates folding of viral proteins, namely the large surface protein of hepatitis B virus (14) and the HIV-1 Env protein (21). On the other hand, HSP70 is involved in the folding/stabilization of Cdk9 and the production of the mature Cdk9/cyclin T1 p-TEFb complex

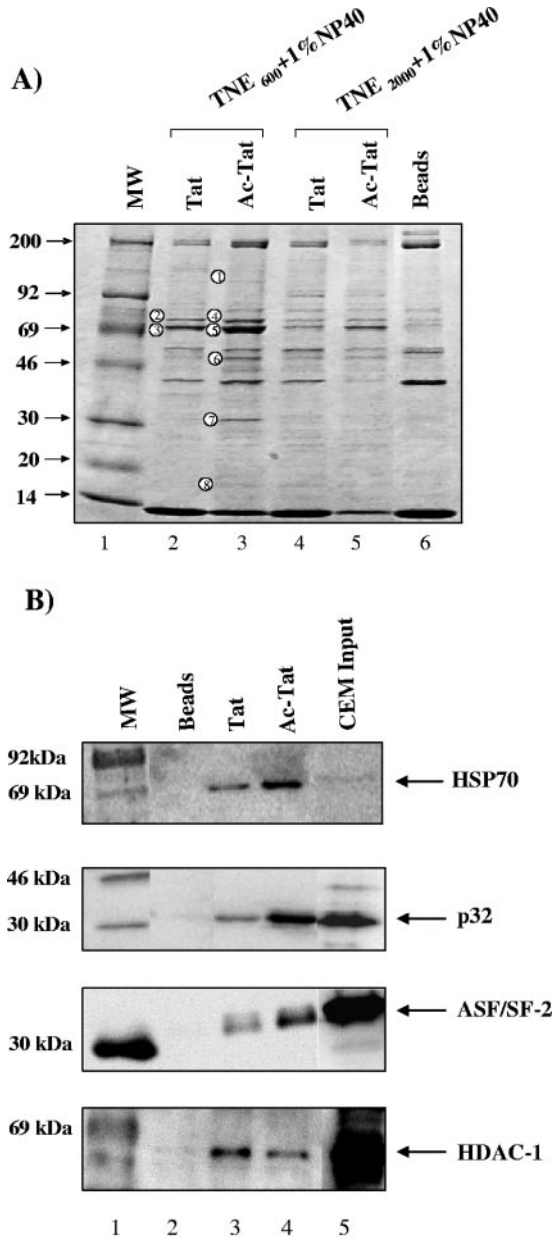


FIG. 2. Identification of cellular Tat-binding proteins. (A) Uninfected CEM extracts (2 mg) were incubated with 100 μg of biotinylated unmodified or acetylated Tat (AcTat) peptides (aa 36 to 54) for 2 h at 4°C, pulled down with streptavidin beads, and washed with TNE_{600} or TNE_{2000} plus 1% NP-40 (0.6 M and 2 M NaCl, respectively). As a negative control, whole-cell lysates were incubated with streptavidin beads alone (Beads). Bound proteins were separated on a 4 to 20% Tris-glycine gel and stained with Coomassie blue. Eight bands were chosen for mass spectrometric analysis (numbered circles). (B) Western analysis of Tat-interacting proteins. Samples were prepared as for panel A and washed with TNE_{600} plus 1% NP-40. Bound proteins were separated on a 4 to 20% Tris-glycine gel, transferred overnight to an Immobilon P membrane, and Western blotted with antibodies against p32, HSP-70, ASF/SF-2, and HDAC1. MW, DNA marker.

(64). Tubulin beta protein is the major constituent of microtubules. Interestingly, Tat has been shown to bind tubulin/microtubules through a 4-aa subdomain of its conserved core region, leading to the alteration of microtubule dynamics and

TABLE 1. Protein partners of AcTat[a]

Sample no.	Accession no.	Molecular mass (kDa)	Gene identification from 3 databases
1	P28340	123.52	DNA polymerase delta catalytic subunit
2	NP_005338.1	72.33	Heat shock 70-kDa protein 5 (glucose-regulated protein, 78 kDa [GRP78])
3	NP_006588.1	70.88	Heat shock 70-kDa protein 8
4	NP_005338.1	72.33	Heat shock 70-kDa protein 5 (glucose-regulated protein, 78 kDa [GRP78])
5	NP_006588.1	70.88	Heat shock 70-kDa protein 8
6	NP_006078	49.67	Tubulin beta 5 chain
7	NP_001203.1	31.34	P32, complement component 1, splicing factor SF-2-associated protein
8	NP_000977.1	17.78	Ribosomal protein L24

[a] Cellular proteins that differentially bound to amino acids 36 to 54 of acetylated Tat. Unique proteins were digested in gel with trypsin, and peptides were eluted, desalted, concentrated with a C_{18} Zip-tip, and analyzed by MALDI-TOF mass spectrometry as described in reference 97. Proteins were identified using the peptide mass fingerprinting analysis software Prospector, Mascot (Matrix Science), and Profound, and the NCBInr and SwissProt databases.

activation of a mitochondrion-dependent apoptotic pathway (13). p32, the ASF/SF2-associated protein, was of particular interest to us, since it suggests a direct role of Tat in RNA splicing, an area that has not been extensively explored. p32 has been reported to regulate splicing by inhibiting the activity of splicing factor 2 (ASF/SF-2) and to inhibit excessive splicing of the HIV-1 RNA (68). Since we were interested in the role of Tat acetylation in the context of viral gene expression and regulation, we decided to focus on this particular protein and its functional significance in HIV-1 splicing. As a first step, we confirmed the preferential interaction of acetylated Tat peptides (aa 36 to 53) with p32 by Western blot analysis (Fig. 2B). We performed the same experiment as previously described using biotin-labeled Tat (unmodified or acetylated) and CEM extracts and Western blotted with an antibody against p32. Figure 2B clearly shows that Tat acetylated at positions 41, 50, and 51 preferentially interacted with p32 (compare lanes 2 and 3). We also performed Western blotting using antibodies against HSP70 and confirmed the results obtained by mass spectrometry (Fig. 2B). Since p32 is an ASF/SF-2-associated protein, we determined whether ASF/SF-2 also bound preferentially to AcTat by Western blotting. Results in Fig. 2B show that the splicing factor ASF/SF-2 preferentially bound to AcTat, either directly or indirectly, through interaction with p32. To show that acetylation does not increase binding of Tat to cellular factors nonspecifically, we showed that Tat acetylation decreases binding to histone deacetylase 1 (HDAC1) compared to unmodified Tat. HDAC1 has been shown to bind Tat-interactive protein, 60 kDa (Tip60) (26), and to compete with Tat over special AT-rich sequence binding protein 1 binding (47). Based on these results, we suggest that acetylation of Tat allows the recruitment of factors involved in splicing.

Tat acetylated at positions 50 and 51 increases Tat binding to the ASF/SF2-associated splicing regulator p32. In the previous experiment, we used biotin-labeled synthesized Tat (aa 36 to 53) that was either unmodified or acetylated at positions 41, 50, and 51. To narrow down the lysine residues whose acetylation was important for p32 binding, we used additional synthesized Tat peptides (aa 42 to 54) that were unmodified or acetylated at lysine residues 50 and 51. We subsequently performed a similar experiment, as shown in Fig. 2. The results in Fig. 3A show that both Tat (aa 36 to 53) acetylated at lysine 41, 50, and 51 and Tat (aa 42 to 54) acetylated at positions 50 and 51 preferentially bound to p32 compared to the unmodified form of Tat (compare lanes 3 to 6). While the acetylated form of Tat (aa 36 to 53) showed a fivefold increase in binding to p32 compared to unmodified Tat (aa 36 to 53) (compare lanes 3 and 4), unmodified Tat (aa 42 to 54) did not show any interaction with p32, while the acetylated form of Tat did, suggesting that acetylation of Tat at positions 50 and 51 is important for p32 binding. To further support that lysine residues 50 and 51 are important for p32 interaction, we used nonbiotinylated Tat peptides (aa 42 to 54) that are acetylated at positions 50 and/or 51 to compete with p32 binding to biotin-AcTat (aa 36 to 53) (Fig. 3B). We showed that the synthetic Tat peptide (aa 42 to 54) acetylated at positions 50 and 51 competed out p32 binding to biotin-AcTat (aa 36 to 53) almost completely (Fig. 3B, lane 4). While p32 binds almost 4 times more to the AcTat compared to the unmodified Tat in the absence of the competing peptides, p32 binding to AcTat drops after addition of the competing peptide Ac Tat 50,51 to levels similar to unmodified Tat (1.3-fold higher than binding to unmodified Tat). Peptides Tat Ac K50 and Tat Ac K51 compete out p32 partially and drop p32 binding to AcTat (aa 36 to 53) by around 40% and 30%, respectively (2- and 2.5-fold higher than binding of p32 to unmodified Tat) (Fig. 3B, lanes 5 and 6).

In the previous set of experiments, we used Tat peptides spanning the core and the basic regions of Tat. To determine whether acetylation of full-length Tat demonstrates the same preferential binding to p32, we incubated in vitro-transcribed and -translated full-length Tat with GST-p32 in the presence or absence of CBP/p300, a HAT domain that has previously been demonstrated to acetylate Tat at positions 50 and 51 (42, 66). Associated complexes were isolated, washed in high-salt/detergent buffer, and separated by SDS-PAGE. GST alone was used as a negative control. As shown in Fig. 3C, we observed a 10-fold-greater binding of p32 with full-length ^{35}S-Tat in the presence of GST-HAT (CBP/p300) (compare lanes 2 and 3). ^{35}S-Tat mutated at lysines 50 and 51 showed a decrease of p32 binding compared to wild-type ^{35}S-Tat in the presence of GST-HAT (compare lanes 3 and 6). The specificity of this preferential binding of p32 to acetylated Tat was confirmed by the addition of curcumin, a specific inhibitor of the p300/CREB-binding protein (CBP) HAT activity (2). In the presence of curcumin, binding of wild-type ^{35}S-Tat to p32 decreased to basal levels (compare lanes 3 to 4), demonstrating that acetylation of Tat at positions 50 and 51 was responsible for its preferential binding to p32.

To directly study the role of lysine 41 acetylation on p32 binding, we used a T7-driven vector expressing a full-length Tat with a K41A mutation and performed an in vitro binding assay by incubating ^{35}S-Tat K41A with GST-p32 as described for Fig. 3C. Results of such an experiment show that mutation at lysine 41 did not affect p32 binding to Tat in the presence of HAT (Fig. 3D, lanes 3 and 5), as quantified using the Image Quant software, while interaction of p32 with Tat (K50,51A) was dramatically affected compared to wild-type Tat (Fig. 3C, lanes 3 and 6). We conclude from these results that p32 inter-

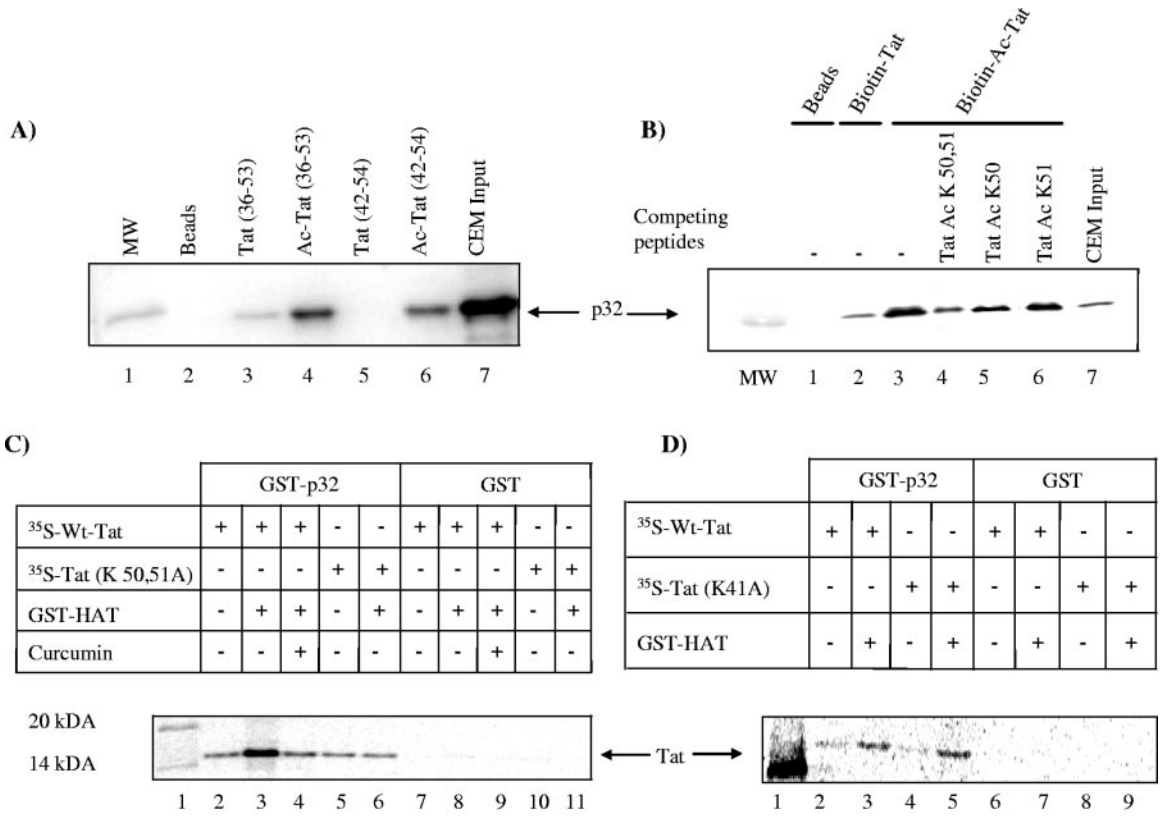


FIG. 3. p32 preferentially interacts with acetylated Tat in vitro. (A) Biotin-labeled Tat (aa 36 to 53) unmodified (lane 3) or acetylated at positions 41, 50, and 51 (lane 4) and biotin-labeled Tat (aa 42 to 54) unmodified (lane 5) or acetylated at lysine 50 and 51 (lane 6) were incubated with CEM cell extracts. Bound proteins were separated on 4 to 20% Tris-glycine gels, transferred overnight to an Immobilon P membrane, and Western blotted with antibodies against p32. (B) Three competing peptides (aa 42 to 54) were added to the biotin-labeled AcTat pull down: Tat Ac K50 and 51, Tat Ac K50, or Tat Ac K51, and the same experiment described for panel A was performed. (C, D) TNT-synthesized ^{32}P-labeled full-length WT Tat, mutant Tat (K50, 51A) (C), and Tat (K41A) (D) were incubated with acetyl coenzyme A in the presence (+) or absence (−) of GST-p300 (HAT domain) for 2 h and then incubated with GST-p32 or GST overnight. In panel C, curcumin (100 μM) was added to inhibit HAT activity. Bound complexes were washed with TNE_{600} plus 1% NP-40, and proteins were separated on a 4 to 20% Tris-glycine polyacrylamide gel, dried, and exposed to a PhosphorImager cassette. MW, DNA marker.

acts directly with full-length AcTat and that acetylation at lysines 50 and 51 is needed for this preferential binding.

Treatment with the deacetylase inhibitor TSA increases recruitment of p32 to the HIV-1 promoter. After studying the interaction between acetylated Tat and p32 in vitro, we attempted to demonstrate that this interaction occurs in the context of viral gene expression in vivo. Therefore, we used ChIP to study the presence of AcTat, p32, and ASF/SF-2 on the HIV-1 promoter before or after virus activation. OM10.1 cells, a promyelocytic cell line that is latently infected with full-length wild-type HIV-1, was grown to mid-log phase and treated with TSA. TSA is a deacetylase inhibitor that increases acetylation of not only histones but also of Tat and synergizes with Tat in transcriptional activation of the HIV-1 LTR (42). Results shown in Fig. 4A demonstrated that TSA induction of acetylation resulted in an increase of p32 (compare lanes 3 and 4) and AcTat (compare lanes 7 and 8) occupancy on the HIV-1 LTR. ASF/SF-2 was present in small amounts on the HIV-1 promoter, but it did not vary after TSA treatment (Fig. 4A, lanes 9 and 10). As a control, we showed that RNA Pol II and cdk-9 also increase on the promoter after treatment, since virus activation induces the recruitment of both RNA Pol II and cdk9 to the HIV-1 promoter (lanes 5, 6, 11, and 12). p32, RNA Pol II, ASF/SF-2, and cdk-9 are cellular factors that are involved in regulating cellular genes. To show specificity of their presence on the HIV-1 LTR, we performed PCR using actin primers as a control gene (Fig. 4A, panel 2). Since TSA increases the overall transcription in the cells, RNA Pol II is expected to increase on cellular genes, including actin, as shown in Fig. 4A (panel 2, lanes 5 and 6). Although cdk-9 levels increased on the HIV-1 LTR after treatment with TSA (Fig. 4A), it showed no binding to the actin gene before or after induction with TSA. However, p32 levels decreased on the actin gene after induction. We speculate that p32 during viral activation is functional in the context of gene expression of the virus at the expense of regulating cellular genes such as actin. ASF/SF-2 levels on the actin gene remained similar before or after induction. As for the viral protein Tat, the increase of AcTat at the level of HIV-1 LTR is specific since it decreased on the HIV-1 Env after TSA treatment (data not shown). Tab172, the negative-control antibody that recognizes the human T-cell leukemia virus type 1 viral protein Tax, did not show any binding to the HIV-1 promoter or to the actin gene. These results further indicate that increase of p32, RNA Pol II, AcTat, and cdk-9 after virus activation is LTR specific.

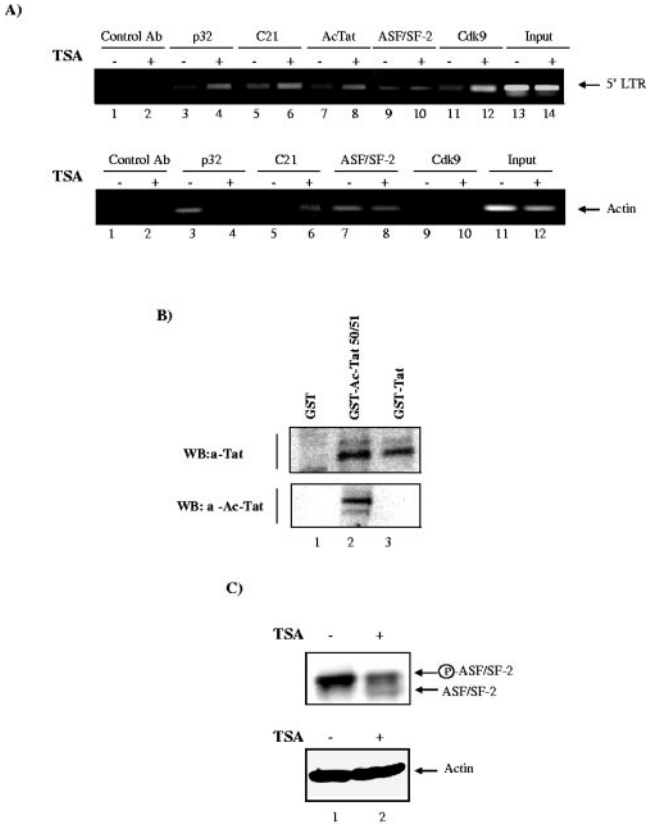


FIG. 4. TSA treatment increases the presence of p32 and acetylated Tat at the HIV-1 promoter. (A) OM10.1 cells were treated with (+) or without (−) 450 nM TSA to induce Tat acetylation followed by ChIP assays. Equal amounts of treated and untreated cells were harvested after 24 h, cross-linked, and lysed using an SDS-containing buffer. The cross-linked chromatin was sonicated, and transcription complexes were immunoprecipitated with 5 μg of antibodies to anti-Tab172 (Control Ab), anti-Pol II (C-21), anti-p32, cdk-9, ASF/SF-2, or anti-AcTat 50/51 (AcTat), and recovered DNA was used for PCR amplification of the HIV-1 LTR to determine the occupancy of the immunoprecipitated protein on the HIV-1 promoter (upper panel) and on the actin gene (lower panel). (B) Specificity of the AcTat antibody. One microgram of either GST (lane 1), GST-Tat acetylated with p300 HAT domain (lane 2; as described in legend to Fig. 3), or unmodified GST-Tat (lane 3) were separated by 4 to 20% SDS-PAGE, transferred, and Western blotted (WB) with anti-Tat (α-Tat) antibody (NIH AIDS reagent) or anti-AcTat (α-AcTat) antibody (rabbit polyclonal antibody raised against the Tat peptide 36 to 70 acetylated at positions 50 and 51). (C) Western blot for ASF/SF-2 and actin (as a loading control) of OM10.1 cell extracts before and after treatment with TSA. The circled P indicates phosphorylation.

The specificity of the AcTat antibody was tested and is shown in Fig. 4B. While Tat polyclonal antibody recognized both GST-Tat acetylated in vitro (lane 2) and unmodified GST-Tat (lane 3), the AcTat antibody only recognized GST-Tat acetylated in vitro (lower panel), indicating the specificity of this antibody to the acetylated form of Tat. GST was used as a negative control in both cases.

Since the increase in p32 levels did not correlate with an increase with ASF/SF-2 levels on the HIV-1 promoter, we performed a Western blot to determine the effect of TSA treatment on ASF/SF-2 protein levels. Interestingly, virus activation by TSA induced a decrease in the overall level of ASF/SF-2 and a shift in phosphorylation state from a predominantly hyperphosphorylated form before treatment to the appearance of a hypophosphorylated form of ASF/SF-2 after treatment (Fig. 4C). This observation is consistent with previous studies that demonstrated virus-induced dephosphorylation of serine-arginine-rich (SR) proteins in several viral systems. Namely, vaccinia and adenoviruses induced a partial dephosphorylation of SR proteins, leading to their inactivation (35), and HSV-1 infection reduced phosphorylation of SR proteins and impaired their ability to function in splicesome assembly (78). We speculate that the production of the hypophosphorylated form of ASF/SF-2 is due to p32, since p32 was shown to inhibit phosphorylation of ASF/SF-2 and thus inhibit its interaction with RNA (65).

Acetylation regulates HIV-1 splicing. The splicing pattern in the different HIV-expressing cells is highly conserved, suggesting that splicing regulation is critical for efficient virus replication (28, 71, 85). SR proteins, including ASF/SF-2, regulate splicing in HIV-1 and maintain a balance between unspliced versus spliced viral RNAs (36, 76). p32 inhibits ASF/SF2 function as both a splicing enhancer and splicing repressor protein by preventing ASF/SF2 phosphorylation and, subsequently, its stable interaction with RNA (68). For this reason, we propose that AcTat interaction with p32 affects HIV-1 splicing by regulating ASF/SF-2 activity. To show that Tat acetylation regulated splicing, we used two HIV-1 reporter viruses, pNL-CAT and pNL-Luc, with CAT and Luc expressed from singly spliced and doubly spliced transcripts, respectively (Fig. 5A). Both of those plasmids express Tat. These two reporter viruses pseudotyped with the MLV Env glycoprotein were used to infect HeLa cells, and both CAT and Luc activities were measured in the presence or absence of TSA. Figure 5B demonstrated that treatment with TSA for 24 h decreased luciferase activity and increased CAT activity, indicating that acetylation induced a shift in the pattern of splicing from multiply spliced to singly spliced mRNA. To check whether the levels of cellular proteins were also affected by TSA treatment, we performed a Western blot for two cellular proteins, actin and cyclin E. No change in the actin protein levels has been observed after TSA treatment (Fig. 3C). Cyclin E is expressed as a full-length 48-kDa protein and has 3 isoforms, E_L, E_T, and E_S, expressed as 50-, 45-, and 43-kDa proteins, respectively. Figure 3C shows that neither cyclin E nor its isoforms are affected by TSA treatment. These results show that the variation in the HIV-1 protein level after TSA treatment as shown by CAT and Luc is specific.

Tat acetylation affects splicing in vivo. To show that the shift in splicing was mediated by Tat acetylation, we used an RPA using a probe derived from pBS/HIV(78-340) containing the major 5′ splice donor site of all HIV-1 transcripts which distinguishes between spliced (4 and 2 kb) and unspliced (9 kb) transcripts (104). In vitro-synthesized probe was mixed with total RNA from HLM-1 cells (HIV-1^+/Tat^-), a HeLa-derived cell line containing an integrated full-length HIV-1 provirus with a defective Tat, transfected with either WT pcTat or a mutant pcTat (K50A, K51A). HLM-1 cells with no DNA transfected was used as a negative control. Figure 6A shows that transfection of HLM-1 cells (HIV-1^+/Tat^-) with wild-type pcTat leads to the production of more unspliced than spliced transcripts (lane 3), while the double lysine mutant pcTat

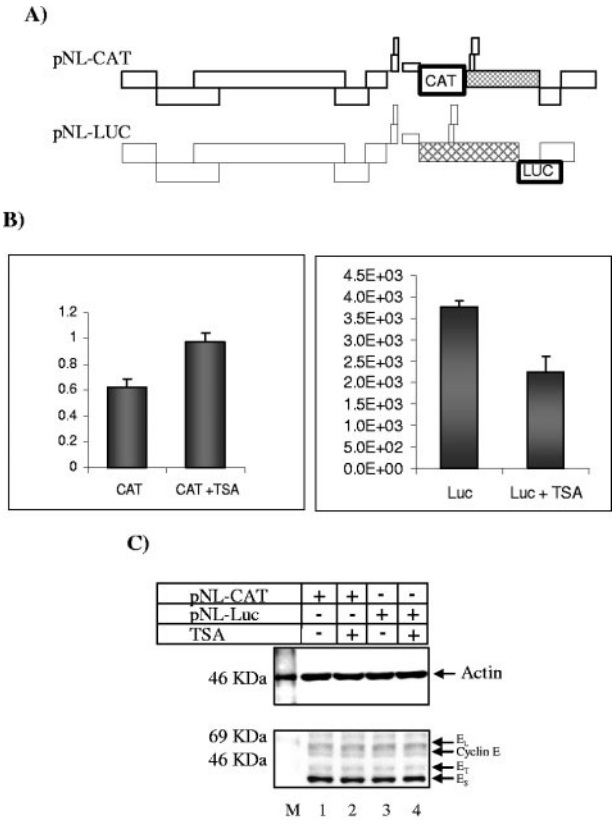


FIG. 5. Effect of TSA on HIV-1 singly and doubly spliced mRNA. (A) The proviral plasmid pNL-CAT contains the *CAT* reporter gene cloned into the *env* open reading frame (ORF) (32), which is expressed from singly spliced (4-kb) viral transcripts. The proviral plasmid pNL-Luc contains the firefly luciferase gene cloned into the *nef* ORF (89), and luciferase is expressed from the doubly spliced (2-kb) viral transcripts. (B) HeLa cells were infected with MLV-pseudotyped pNL-CAT or pNL-Luc and treated with 450 nM TSA 24 h later. After 48 h, cells were lysed and CAT and luciferase activities were determined. The average of results from three independent experiments is shown. (C) Western blot for actin (Santa Cruz) and cyclin E (Santa Cruz) on cells transfected with pNL-CAT and pNL-Luc treated or untreated with 450 nM TSA. +, present; −, absent.

(K50A, K51A) produced more spliced then unspliced transcripts (lane 4). We calculated the ratios of unspliced to spliced mRNA (US/S) in HLM-1 cells transfected with WT Tat and mutant Tat (K50A, K51A) to take into account the difference in the overall mRNA produced due to differences in transactivation. WT Tat induced a higher ratio of unspliced to spliced transcripts compared to the Tat double mutant (Fig. 6C, panel 1), indicating that acetylation of Tat at positions 50 and 51 affected the pattern of splicing, favoring the production of unspliced mRNA. In addition, Tat is known to synergize with TSA in transcription activation. To prove that the shift in the pattern of splicing in Fig. 6A, lanes 3 and 4, correlated with the effect of Tat on splicing and not merely on transactivation, we treated cells with TSA to activate transcription and studied the synergistic effect of TSA with Tat on the pattern of splicing. The results show that spliced and unspliced mRNAs were not significantly affected by TSA treatment in cells transfected with WT Tat. However, only spliced products increased when cells were transfected with the Tat double mutant and treated with TSA (Fig. 6A, lanes 5 and 6). Figure 6C demonstrates that WT Tat induced a higher ratio of unspliced to spliced transcripts than the double mutant Tat in the presence or absence of TSA (panels 1 and 2).

Since we showed in Fig. 2B that ASF/SF-2 binds to AcTat, either directly or indirectly, through interaction with p32, we studied whether overexpression of ASF/SF-2 affects Tat-mediated splicing. We performed RPA in HLM-1 (HIV-1^+/Tat^-) transfected with Tat alone or with Tat and ASF/SF-2 and showed that overexpression of ASF/SF-2 partially reverts the effect of Tat on HIV-1 splicing by decreasing the overall production of transcripts and decreasing the ratio of unspliced to spliced from 1 to 0.7. Transfection of HLM-1 with ASF/SF-2 alone did not lead to the production of any transcript. However, overexpression of p32 as expected increased the ratio of unspliced to spliced by 60% (from 1 to 1.6) compared to Tat alone (Fig. 6B).

To further support the hypothesis that Tat acetylation induced a shift of RNA transcripts from spliced to unspliced, we explored sequence variations between the subtype-specific Tat proteins. Figure 7A shows a sequence comparison of Tat amino acid residues 1 to 72 from clades B, C, and E. Although lysines 28 and 50, which are acetylated by P/CAF and p300, respectively, are conserved among these different subtypes, additional lysine residues exist on Tat from clades E and C that may be targets of acetylation (75). Based on this observation, we studied the effect of Tat from these different clades on the pattern of splicing. HLM-1 (HIV-1^+/Tat^-) cells were transfected with Tat B, C, or E, and the splicing pattern was determined using the RPA method described above. We observed that Tat B and C induced an increase in the amount of both spliced and unspliced mRNA products compared to untransfected HLM-1 cells (Fig. 7B). As expected, Tat E had the highest effect on the production of unspliced mRNA (Fig. 7B). This increase in unspliced mRNA may be due to an increase in the overall production of transcripts, since Tat E has a higher transactivation potential than Tat B or C (75). The unspliced-to-spliced ratio was calculated to take into account the overall difference in the amount of transcripts produced and was normalized to the Tat B ratio, which was set at 1, demonstrating that Tat E increased the unspliced-to-spliced ratio compared to Tat B or C. Although Tat C has one additional lysine at position 19, our results show that this lysine does not have an effect on splicing (Fig. 7B, lane 3). To narrow down the region of Tat E that has the highest effect on the shift in the splicing pattern, we used chimeric Tat plasmids (Tat BE, EB, and EC) expressing protein in which the first 48 amino acids that correspond to the activation domain have been swapped for the sequence of another Tat. Tat BE did not produce any spliced nor unspliced mRNA (data not shown), consistent with the observation that Tat BE dramatically loses its transactivation potential (19). However, Tat EB and Tat EC, which retain their transactivation potential, can induce a shift from spliced to unspliced mRNA but to a lesser extent than WT Tat E (Fig. 7D), implying that the C terminus of Tat E is important for regulation of splicing and that this effect is independent of the activation domain.

Desfosses et al. published an article showing that the transactivation capacity of Tat is related to the acetylation of lysine residues (19). Using a Tat E construct and its lysine-to-alanine

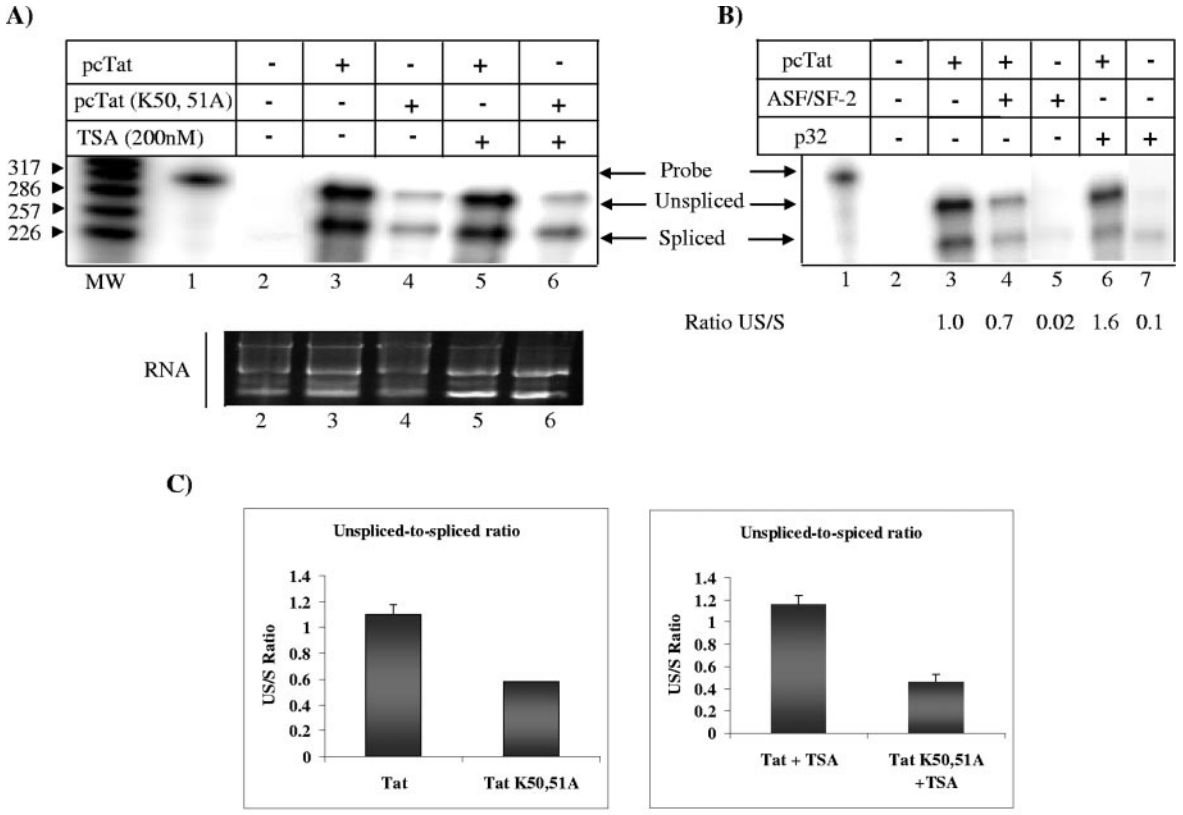
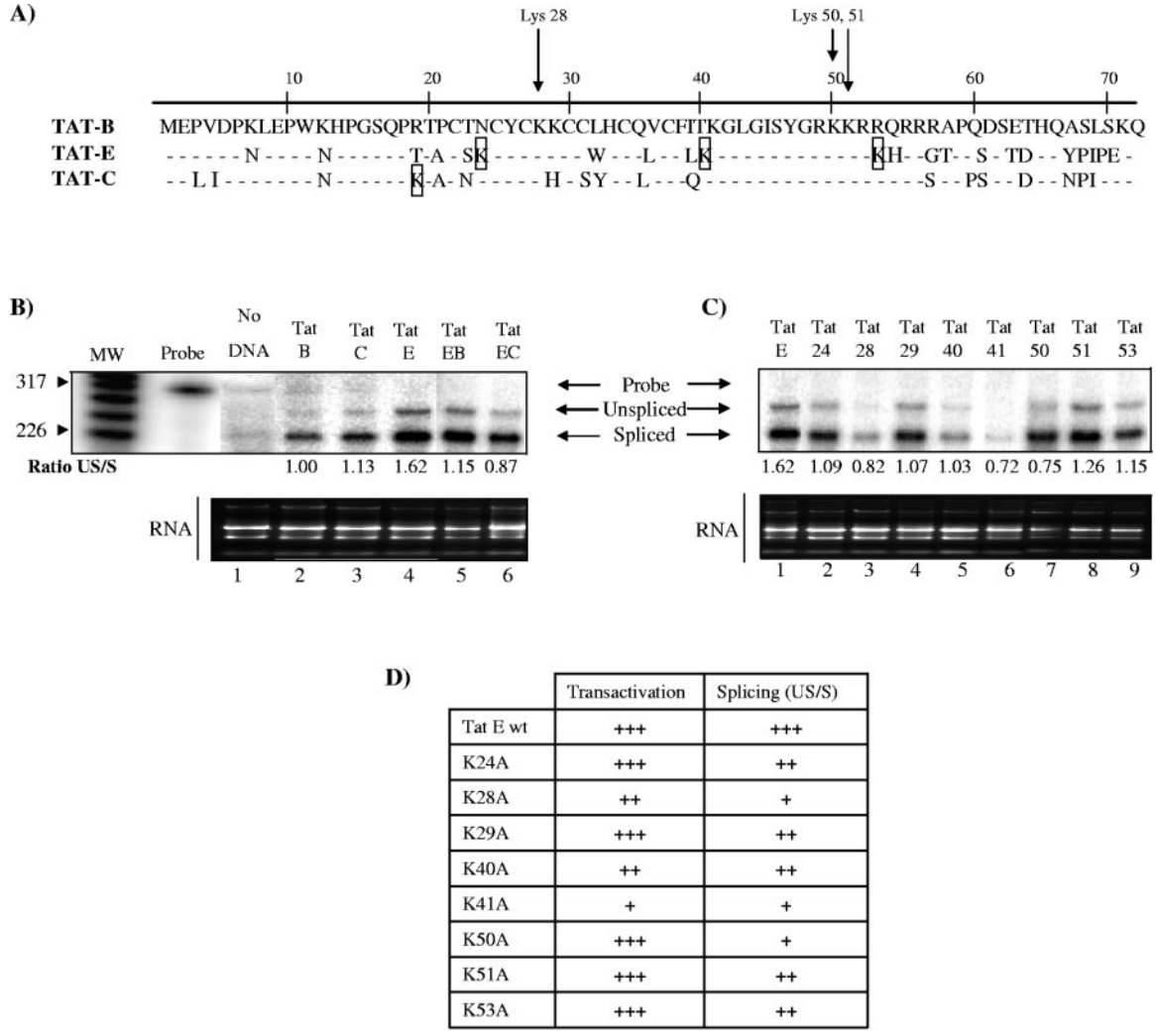


FIG. 6. Effect of WT-Tat and mutant Tat on the pattern of HIV-1 splicing. (A) Total RNA was extracted from HLM-1 (HIV-1^+/Tat^-) cells transfected with WT Tat or mutant (K50A, K51A) Tat and analyzed by RPA after 48 h. Upper panel: RPA. Lanes 3 and 4, untreated; lanes 5 and 6, treatment with 200 nM TSA for 4 h. The free probe (312 nucleotides) and two protected fragments from unspliced (262 nucleotides) and spliced (213 nucleotides) transcripts are indicated by arrows. MW, RNA marker. Lower panel: RNA from the different samples was run on 1% agarose gel and stained with ethidium bromide. (B) RNA from HLM-1 transfected with pcTat alone, pcTat with ASF/SF-2, or pcTat with p32 was extracted and analyzed by RPA 48 h after transfection. The US/S ratio is calculated and normalized to the ratio of pcTat which is set as 1. (C) Ratios of unspliced to spliced mRNA in panel A are shown as bars and correspond to the averages of results from three independent transfections. +, present; −, absent.

point mutants, they showed that while K41A interfered significantly with Tat E transactivation, K28A, K40A, and K50A produced a twofold inhibitory effect on transactivation, whereas the other lysine residues had a modest impact on transactivation efficiency. Based on our hypothesis that the interaction between acetylated Tat (lysines 50 and 51) and p32 inhibits splicing and on the fact that Tat E has an additional lysine at positions 24, 29, 40, and 53, which may be a potential site for acetylation, we tested whether mutation of those lysines in Tat E would revert the levels of unspliced transcripts to levels similar to that produced by Tat B and C. To this end, we performed RPA in HLM-1 cells transfected with Tat E mutated at lysines 24, 28, 29, 40, 41, and 53 in addition to mutants at positions 50 and 51 (Fig. 7C) and calculated both the transactivation efficiency and the effect on splicing of each of the mutants. The results were summarized in Fig. 7D. Transactivation efficiency was calculated as the sum of both unspliced and spliced transcripts, while the effect on splicing was measured as the ratio of unspliced over spliced transcripts. Our results were consistent with those of Desfosses et al. in that Tat E K41A abrogated transactivation almost completely, K28A and K40A around 50%, while all the others did not have a significant effect on transactivation (19). The only difference in the two results is in Tat K50A, and this can be explained by the fact that our analysis was in the context of the whole virus, while the study done by Desfosses et al. was in the context of the HIV-1 LTR. We determined the effect of the different lysine mutants on splicing by calculating the ratio of unspliced over spliced transcripts. While mutants K24A, K29A, K40A, K51A, and K53A had a moderate effect on splicing (around 20 to 30% decrease), K28, K41, and K50 affect splicing significantly (decrease unspliced-to spliced [US/S] ratio around 50% or more). However, since the production of transcript overall is abrogated or greatly decreased in the K28 and K41 mutants, K50 is the only mutation that affects splicing significantly without greatly affecting transactivation. These results show on one hand that lysine 50 has the greater effect on splicing inhibition and on the other that the additional lysines in Tat E, including lysines 24, 29, 40, and 53, affect splicing moderately. Collectively, these experiments demonstrated that while Tat B and C were able to produce both spliced and unspliced products, Tat E induced a higher production of unspliced mRNA. Mutations of the different lysine residues of Tat E and especially lysine 50 affected the splicing pattern and led to a decrease in the unspliced to spliced ratio compared to wild type Tat E.



D)

	Transactivation	Splicing (US/S)
Tat E wt	+++	+++
K24A	+++	++
K28A	++	+
K29A	+++	++
K40A	++	++
K41A	+	+
K50A	+++	+
K51A	+++	++
K53A	+++	++

FIG. 7. Effect of Tat from different clades and Tat acetylation mutants on the pattern of HIV-1 splicing. (A) Sequence variation in subtype-specific Tat proteins. Differences in amino acid residues (1 to 72 aa) in Tat B, C, and E are shown. Lys 28, 50, and 51, which are targets for acetylation by P/CAF and p300, are conserved (indicated by arrows). Additional lysine residues of Tat E and C that may be targets of acetylation are boxed. (B and C) Total RNA was extracted from HLM-1 cells transfected with plasmids encoding Tat from clades B, C, and E, and chimeric Tat BE and EC (B) or Tat E and Tat E mutants (Tat E K 24A, K28A, K29A, K40A, K41A, K50A, K51A, and K53A) (C) and analyzed by RPA after 48 h as described for Fig. 6 (upper panel). RNA from the different samples was run on a 1% agarose gel (lower panel). Ratios of US/S transcripts normalized according to the Tat B ratio (set at 1) are indicated at the bottom. (D) Table representing the transactivation efficiency and the effect on splicing of all the Tat E lysine mutants. Transactivation efficiency was calculated as the sum of unspliced and spliced transcript intensity as measured by Image Quant. +++, no significant effect on transactivation; ++, modest decrease in transactivation; +, complete disruption of transactivation. The effect on splicing was measured as the US/S ratio. US/S ratios were classified as follows: high ratio, +++; moderate ratio, ++; low ratio, +.

AcTat regulates splicing of an LTR-driven minigene. As outlined in Fig. 1, splicing of the HIV-1 genomic pre-RNA is a complex process leading to the formation of at least 30 differentially spliced RNAs (71). To minimize the complexity in studying the role of Tat in viral splicing, we developed an expression system referred to as LTR-Target 3, in which the HIV-1 LTR drives the expression of the HCMV UL37 gene spanning exon 3 splice donor, the complete intron 3, and the exon 4 splice acceptor (Fig. 8A). The vector was constructed by subcloning PCR-amplified HIV-1 LTR, UL37 (exon 3, intron 3, and exon 4), and HIV-1 PA into a BlueScript expression vector. Target 3 is the vector from which the UL37 exon 3, intron 3, and exon 4 were derived. It is driven by the HCMV major IE promoter, and its polyadenylation is directed by the simian virus 40 early PA signal (87). Splicing of LTR-Target and Target 3 can be studied by RT-PCR using primers 182 and 183 indicated in Fig. 8A, and transcription of the UL37 gene yields two species of RNA: an unspliced cDNA of 202 nt and a spliced transcript of 99 nt. To study the effect of Tat on splicing of the UL37 reporter gene, we transfected 293T cells with LTR-Target 3 with or without pcTat. 293T cells transfected with empty vector were used as a negative control. 293T cells transfected with Target 3 produced unspliced HCMV transcripts of 202 bp (Fig. 8B, lane 2). This construct was used

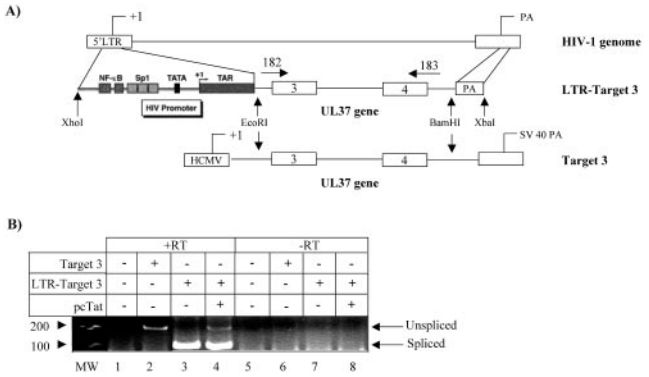


FIG. 8. Effect of Tat acetylation on the splicing of the LTR-Target 3 reporter minigene. (A) The HIV-1 LTR was cloned from HIV-1 (pLAI strain) and inserted in place of the HCMV IE promoter of the Target 3 reporter minigene (Target 3) and is referred to as LTR-Target 3. The polyadenylation signal from HIV-1 (pLAI) was cloned downstream of Target 3. The localization of the forward (182) and reverse (183) primers used in the RT-PCR assay are indicated (87). (B) 293T cells were cotransfected with no DNA (lanes 1 and 5), Target 3 (lanes 2 and 6), or LTR-Target 3 with (+) (lanes 4 and 8) or without (−) (lanes 3 and 7) pcTat. Total RNA was isolated, reverse transcribed, and PCR amplified using primers 182 and 183 to detect spliced and unspliced messages. PCR products were separated by electrophoresis through a 1% agarose gel and stained with ethidium bromide. Control reactions did not contain RT (−) (lanes 5 to 8). Arrows indicate the positions of unspliced (202 bp) and spliced (99 bp) cDNA products. MW, DNA marker.

as a positive control, since it reproduces the alternative splicing of HCMV UL37 pre-mRNA accurately and efficiently (87). However, transfection with LTR-Target 3 produced spliced transcripts in the absence of Tat (lane 3). Transfection of LTR-Target 3 with pcTat produced both spliced (99 bp) cDNA as well as unspliced (202 bp) transcripts (lane 4). Since spliced and unspliced transcripts were produced in the presence of Tat, while only spliced transcripts were produced in the absence of Tat, these results demonstrate that Tat inhibits splicing and increases the production of unspliced products. Control reactions containing no RT were also PCR amplified, but no cDNA products were observed. These results confirm that Tat regulates splicing and favors the production of unspliced mRNAs.

Tat and p32 colocalize in vivo. Since we showed that p32 affects Tat-mediated HIV-1 splicing, we thought it was critical to confirm that p32 and Tat interact in vivo. To this end, HLM-1 cells either transfected with Flag-Tat or nontransfected were fixed and fluorescently stained for both p32 and Flag proteins after treatment with TSA for 2 h (Fig. 9). TOTO-3, a dimeric cyanine nucleic acid stain from Molecular Probes, was utilized as a nuclear stain. p32 was ubiquitously present mostly in the cytoplasm and the perinuclear region in cells that were either untreated or treated with TSA (Fig. 9A and B, panels a and d), with the majority of p32 being cytoplasmic. This is consistent with previous reports on p32 localization (8, 12, 84). Interestingly, in cells transfected with Flag-Tat, p32 becomes mostly nuclear (Fig. 9C, panel g), consistent with previous studies that show p32 being shuttled to the nucleus by viral proteins (30, 55). As for Tat, it shows a nuclear distribution and a higher concentration in the nucleolus (Fig. 9C, panel h). This is also consistent with previous studies describing nucleolar distribution of Tat (59, 82, 86). When red and green channels are merged, colocalization between p32 and Tat can be observed. Examples of colocalization have been indicated in panel i. According to our results, we believe that in the presence of Tat, p32 is shuttled from the cytoplasm to the nucleus and that p32 and Tat colocalization occurs predominantly in the nucleolus.

DISCUSSION

In our previous work, we reported that Tat acetylation enhanced transcription of an integrated HIV-1 genome and enhanced binding to core histones (17). In addition, other reports have shown that Tat acetylation by p300 at lysine 50 is a necessary step in Tat-mediated transactivation (38, 42). Since most studies have focused on the interacting partners of Tat regardless of its posttranslational modifications, we decided to study the cellular factors that preferentially interacted with the acetylated form of Tat. We now report the identification of several proteins that preferentially interacted with AcTat, including DNA polymerase delta, heat shock proteins, tubulin, ribosomal proteins, and the splicing regulator p32. p32 was of particular interest to us, since it suggested a direct role of Tat in RNA splicing. p32 is synthesized as a 282-amino-acid-long proprotein (34 kDa) that is posttranscriptionally processed in human cells by removal of the N-terminal 73 amino acids to form the 209-amino-acid-long mature protein (32 kDa). Zheng et al. has shown that amino acids 1 to 57 (of the mature form) are responsible for the splicing activity of human p32 and that the mutation G35D disrupts its splicing function (104).

Our results demonstrate that p32 preferentially interacted with Tat peptides 36 to 53 that were acetylated at lysines 41, 50, and 51 (Fig. 3A). We mapped the lysine residues that were important for the preferential interaction with p32 to lysines 50 and 51, by showing that p32 bound to Tat peptides 42 to 54 only if lysines 50 and 51 were acetylated (Fig. 3A). In addition, p32 binding to full-length Tat was increased upon acetylation of lysines 50 and 51 with the HAT domain of p300 and decreased when a specific inhibitor of p300 was added (Fig. 3B). Mutations at lysine residues 50 and 51 decreased p32 binding to levels comparable to nonacetylated Tat, while mutation at lysine position 41 did not affect p32 binding (Fig. 3C and D). Furthermore, we showed that p32 interacted with Tat acetylated at lysine residues 50 and 51 in the basic region. The dependence of this interaction on Tat acetylation is further supported by Yu et al. (102), who detected p32 interaction with truncated Tat peptides at the activation and core regions but not the basic region, since they did not use the acetylated form of Tat in their system. These authors defined amino acids 244 to 255 of p32 as required for Tat binding and showed that specific binding also occurred in vivo (102). Since p32 was first isolated as a protein tightly associated with SF2/ASF during its purification from HeLa cells (45), we demonstrated that AcTat bound more strongly to ASF/SF-2 than to unmodified Tat, either directly or indirectly, through binding to p32. ASF/SF-2 is an essential SR protein that plays a role in multiple steps of spliceosome assembly. It facilitates entry of U2AF65 into the branch point, contacts the 5′ splice site and promotes U6 snRNA base pairing (79, 80). p32 was shown to regulate ASF/

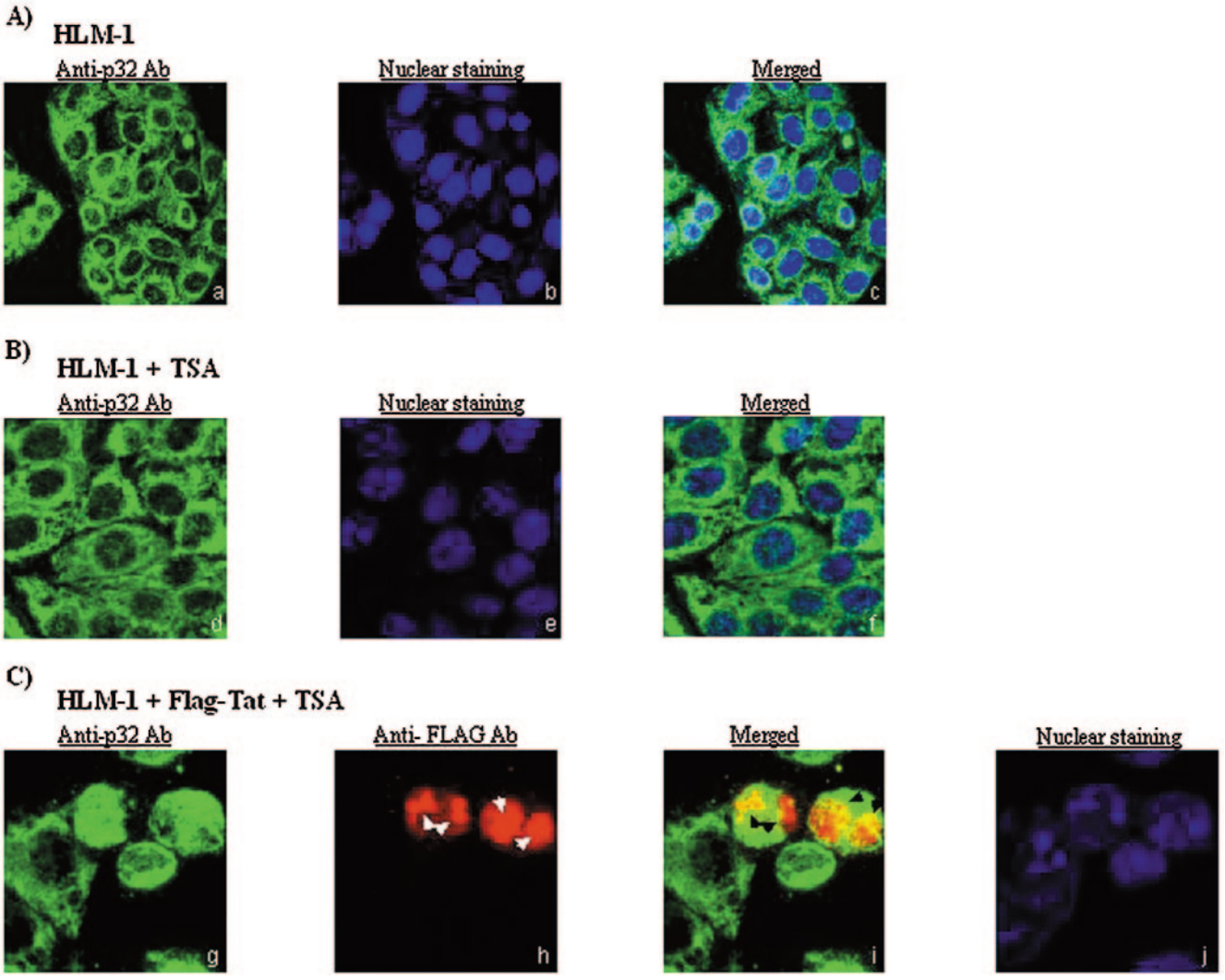


FIG. 9. Colocalization of Tat and p32 in HIV-1-infected cells. Confocal optical sections (z = 1.0 μm) are shown in all panels. Localization of p32 in HLM-1 untreated cells (A) or cells treated with 450 nM TSA for 2 h (B). p32 staining as detected by rabbit polyclonal anti-p32 primary antibody and Alexa Fluor 488 secondary antibody is shown in panels a and d. Nuclear staining detected utilizing TOTO-3, a dimeric cyanine nucleic acid stain, is shown in panels b and e. Merged images of p32 and nuclear staining are shown in panels c and f. (C) HLM-1 cells transfected with Flag-Tat and treated with 450 nM TSA for 2 h. p32 staining as detected by rabbit polyclonal anti-p32 primary antibody and Alexa Fluor 488 secondary antibody is shown in panel g. Tat staining detected by mouse monoclonal anti-Flag primary antibody and Alexa Fluor 568 secondary antibody is shown in panel h. White arrows indicate nucleolar localization of Tat. A merged image of p32 and Tat staining is shown in panel i. Black arrows indicate points where colocalization is occurring, shown as yellow coloring when the two images are merged. Nuclear staining detected utilizing TOTO-3, a dimeric cyanine nucleic acid stain, is shown in panel j.

SF-2 activity by preventing its phosphorylation needed for RNA binding and subsequent spliceosomal assembly (68).

To determine the functional significance of the interaction between AcTat and p32 in vivo, we investigated whether AcTat recruited p32 and ASF/SF-2 to the HIV-1 promoter. TSA is a specific inhibitor of the deacetylase activity of class I and II HDACs (reviewed in reference 61). TSA increases acetylation of not only histones but also of Tat and synergizes with Tat in transcriptional activation of the HIV-1 LTR (42). In vivo treatment with TSA induced the recruitment of p32 to the HIV-1 promoter along with AcTat, suggesting a role of p32 in the regulation of HIV-1 gene expression. However, the levels of ASF/SF-2 on the HIV-1 LTR remained similar before and after treatment with TSA.

Since the increase in p32 levels did not correlate with an increase with ASF/SF-2 levels on the HIV-1 promoter, we performed a Western blot to determine the effect of TSA treatment on ASF/SF-2 protein levels. Interestingly, virus activation by TSA induced a decrease in the level of ASF/SF-2 and a shift in phosphorylation state from a predominantly hyperphosphorylated form before treatment to the appearance of a hypophosphorylated form of ASF/SF-2 after treatment (Fig. 4C). Virus-induced dephosphorylation has previously been shown in several viral systems, including vaccinia and adenoviruses, whereby the virus induces a partial dephosphorylation of SR proteins leading to their inactivation (35), and in HSV-1 infection, whereby phosphorylation of SR proteins is reduced impairing their ability to function in splicesome assembly (78).

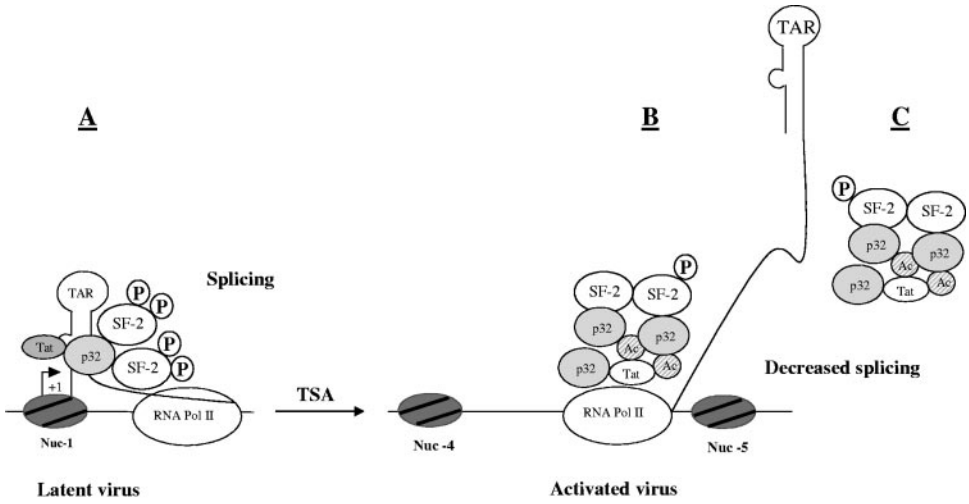


FIG. 10. Proposed model for AcTat regulation of HIV-1 splicing. (A) Low amounts of Tat, p32, and hyperphysphorylated ASF/SF-2 are bound to the HIV-1 LTR before virus activation. (B) Upon treatment with TSA, Tat becomes acetylated and recruits more p32 to the LTR, which in turn acts on ASF/SF-2 and decreases its phosphorylation, impairing its binding to RNA and subsequently leading to a decrease in HIV-1 splicing. (C) AcTat/p32/SF-2 form a complex and affect splicing, probably sequestering ASF/SF-2 away from the HIV-1 mRNA, which may occur in the absence of HIV-1 LTR DNA.

Subsequent studies have shown that p32 interacts with many cellular and viral proteins, including protein kinase Cμ (74), lamin B receptor (81), herpes simplex virus IE63 (9), adenovirus polypeptide V (55), Epstein-Barr virus EBNA I (94), HIV Rev (51, 88), and HIV Tat (102). The association between p32 and HIV Rev is particularly interesting, since it suggests a possible connection between p32 and regulation of splicing. In addition, p32 involvement in splicing inhibition in murine cells helps reverse the posttranscriptional block of HIV-1 expression by inhibiting excessive splicing of the HIV-1 RNA (104). Our data indicating that AcTat interacts with p32 suggest the involvement of Tat acetylation in HIV-1 splicing.

In this report we provide evidence that HIV-1 splicing is regulated by Tat acetylation. While treatment with TSA decreased the production of doubly spliced messages, it increased the production of the singly spliced mRNA. We also demonstrate that this effect on splicing was mediated by acetylated Tat, since WT Tat B produced more unspliced than spliced transcripts, while Tat mutated at lysines 50 and 51 produced more spliced than unspliced transcripts. In addition, overexpression of ASF/SF-2 reverts the effect of Tat and decreases the US/S ratio, while overexpression of p32 increases it, demonstrating that the Tat effect on splicing is p32 and ASF/SF-2 dependent. Using Tat from various clades, harboring different numbers of lysine residues, we showed that Tat E induced a higher production of unspliced mRNA. Mutations of the different lysine residues of Tat E affected the splicing pattern and led to a decrease in the unspliced-to-spliced ratio compared to wild-type Tat E, suggesting that the presence of the additional lysines in Tat E was responsible for the higher shift from spliced to unspliced messages compared to the other clades. We also showed that AcTat induced the production of unspliced mRNA of a reporter minigene driven by the LTR promoter, indicating that the observed effect on splicing inhibition was related to AcTat expression.

Although most of the available evidence hints to an indirect regulation of splicing by Tat through up-regulation of genes involved in splicing (including splicing factors arginine/serine-rich 7 and 9) and mRNA processing (11, 46, 50, 101), our data suggest a direct role of AcTat in splicing regulation. We demonstrated that, on one hand, p32 and ASF/SF-2 preferentially bind to AcTat rather than to unmodified Tat (Fig. 2B) and, on the other hand, that activation of the virus with TSA induces the recruitment of AcTat and p32 to the HIV-1 promoter, while the levels of ASF/SF-2 remained similar before and after treatment. According to these results, we propose a model in which AcTat/p32/SF-2 form a complex and affect splicing probably for sequestering ASF/SF-2 away from the mRNA and this happens without the actual presence of the complex on the HIV-1 LTR. Alternatively, low amounts of Tat, p32, and hyperphosphorylated ASF/SF-2 are bound to the HIV-1 LTR before virus activation. Upon treatment with TSA, Tat becomes acetylated and recruits more p32 to the LTR, which in turn, acts on ASF/SF-2 and decreases its phosphorylation, impairing its binding to RNA and subsequently leading to a decrease in HIV-1 splicing (Fig. 10).

ASF/SF-2 is an essential SR protein that controls cellular spliceosome assembly and splicing of viral mRNA during viral infection. This has been observed in both adenovirus and papillomavirus infections where ASF/SF-2 regulates the early-to-late shift in viral mRNA splicing and expression (56, 60). In the context of HCMV infection, both hypo- and hyperphosphorylated forms of ASF/SF-2 are induced within IE and early times of infection and are altered in different cell types (1). Recently, it has been shown that HIV-2 Gag down-regulates splicing by binding to PRP4, serine/threonine kinase, sequestering it away from ASF/SF-2, and preventing its phosphorylation needed for its splicing activity. In addition, p32 was shown to inhibit ASF/SF2 function as both a splicing enhancer and splicing repressor protein by preventing ASF/SF2 phosphorylation and, subse-

quently, its stable interaction with RNA. In addition, doubly spliced transcripts Tat, Rev, and Nef were shown to be regulated by ASF/SF-2. The two silencer elements (ESS3 and ISS) and two enhancer elements (ESE2 and ESE3/[GAA]3) were identified at the splice acceptor site A7, which is involved in the production of Tat and Rev. While hnRNP A1 binds ISS and ESS3 and is involved in the inhibitory process, ASF/SF2 activates site A7 utilization.

Our results fit into a model where unspliced mRNA needs to be synthesized for the production of infectious virions. In fact, during early HIV infection, small, multiply spliced RNAs encoding Tat and Rev, among others, are generated. When a threshold of Rev is reached, singly and unspliced mRNA is transported from the nucleus to the cytoplasm via interaction of Rev with a highly structured element (Rev-responsive element) that is present in all unspliced and singly spliced HIV transcripts (34, 103). As Rev increases the steady-state level of those mRNAs in the cytoplasm, Gag, Pol, and Env are efficiently synthesized along with the accumulation of full-length transcripts for packaging. In this situation, Rev is acting as a negative feedback factor, limiting its own production through inhibiting the export of spliced messages. We believe that while Rev mediates the export of singly and unspliced mRNA, AcTat is responsible for the splicing regulation by bringing splicing regulators such as p32 into the complex.

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