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Regulation of Human Immunodeficiency Virus Type 1 Gene Expression by Clade-Specific Tat Proteins

Yan Desfosses,[1] Mayra Solis,[1] Qiang Sun,[1] Nathalie Grandvaux,[1] Carine Van Lint,[2] Arsene Burny,[2] Anne Gatignol,[1] Mark A. Wainberg,[1] Rongtuan Lin,[1] and John Hiscott[1]*

McGill AIDS Center, Lady Davis Institute, Jewish General Hospital, Departments of Microbiology & Immunology and Medicine, McGill University, Montréal, Canada H3T 1E2,[1] and Université Libre de Bruxelles, Institut de Biologie et de Medecine Moleculaires, Laboratoire de Virologie Moleculaire, 6041 Gosselies, Belgium[2]

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The major group of human immunodeficiency virus type 1 (HIV-1) strains that comprise the current global pandemic have diversified during their worldwide spread into at least 10 distinct subtypes, or clades. Subtype C predominates in sub-Saharan Africa and is responsible for the majority of worldwide HIV-1 infections, subtype B predominates in North America and Europe, and subtype E is prevalent in Southeast Asia. Significant amino acid variations have been observed among the clade-specific Tat proteins. For the present study, we examined clade-specific interactions between Tat, transactivation-responsive (TAR) element, and P-TEFb proteins and how these interactions may modulate the efficiency of HIV-1 transcription. Clade-specific Tat proteins significantly modified viral gene expression. Tat proteins derived from HIV-1 clades C and E were strong transactivators of long terminal repeat (LTR) activity; Tat E also had a longer half-life than the other Tat proteins and interacted more efficiently with the stem-loop TAR element. Chimeric Tat proteins harboring the Tat E activation domain were strong transactivators of LTR expression. While Tat B, C, and E were able to rescue a Tat-defective HIV-1 proviral clone, Tat E was significantly more efficient at rescue than Tat C, possibly due to the relative stability of the Tat protein. Swapping the activation domains of Tat B, C, and E identified the cyclin T1 association domain as a critical determinant of the transactivation efficiency and of Tat-defective HIV-1 provirus rescue.

Based on the genomic sequences of the *gag* and *env* genes of human immunodeficiency virus type 1 (HIV-1), at least 10 distinct subtypes and circulating recombinant forms (A to D, A/E, A/G, F, H, J, and K) have been defined throughout the world (40, 41, 51, 63, 64). Analyses of the current worldwide distribution of HIV-1 subtypes have concluded that HIV A/E and HIV C are the most prevalent HIV-1 subtypes and are responsible for the vast majority of global HIV-1 infections (8, 49). The relationship between the virus subtype, biological properties, and pathogenicity is almost completely unknown, in part because virus replication studies have been performed almost exclusively with subtype B viruses (31).

HIV-1 utilizes a combination of viral regulatory proteins and host signaling pathways to mediate its own transcriptional regulation (25, 27). In the absence of Tat, cellular transcription factors such as NF-κB are required to achieve HIV transcription at the long terminal repeat (LTR) region (56, 73). However, the configurations of the HIV-1 subtype LTR elements are highly divergent and therefore have a significant impact on the HIV transcription efficiency (31, 48, 52, 69, 70, 71).

Productive HIV-1 replication is absolutely dependent upon the 14-kDa viral Tat protein (reviewed in references 7, 23, 29, 30, 32, 36, and 66). Tat acts through the binding of its RNA binding domain to a 59-bp stem-loop RNA structure, called the transactivation-responsive (TAR) element, located at the 5′ end of all nascent HIV-1 transcripts, in order to achieve a high level of gene transactivation (3, 11, 21, 59, 62). Moreover, the Tat function requires its association with Tat-associated kinases (TAKs) and Tat-associated histone acetyltransferases (TAHs). TAKs are responsible for RNA polymerase II (RNA Pol II) C-terminal domain (CTD) phosphorylation (10, 13, 26, 39, 45, 74, 76, 80, 81), which triggers polymerase departure, promoter clearance, and rapid reinitiation as well as efficient elongation (14, 78). Among the described TAK kinases, cyclin-dependent kinase 9 (CDK9) binds its cyclin partner, cyclin T1, to form the positive transcription elongation factor b (P-TEFb) complex (24, 47, 55, 67, 75, 81). The interaction between Tat and cyclin T1 facilitates the binding of Tat to the TAR element (72), thus initiating the formation of an elongation-competent transcription complex. Thereafter, CDK9 promotes RNA Pol II CTD phosphorylation and the Tat–P-TEFb complex dissociates from the TAR element (34, 37), which triggers transcription from the LTR. In addition to Tat, the p65/RelA component of NF-κB also uses P-TEFb to stimulate transcriptional elongation by RNA Pol II (2). Importantly, this may in part explain the enigma of how the first rounds of HIV transcription occur prior to the synthesis of Tat: NF-κB binds to the HIV LTR and recruits P-TEFb, which phosphorylates the CTD of RNA Pol II. This idea is supported by the observation that cells stimulated with tumor necrosis factor alpha support HIV replication in a TAR-independent and NF-κB-dependent manner (1). However, the HIV LTR was shown to be responsive to

* Corresponding author. Mailing address: Lady Davis Institute for Medical Research, Jewish General Hospital, McGill University, 3755 Cote Ste. Catherine, Montreal, Quebec, Canada H3T1E2. Phone: (514) 340-8222, ext. 5265. Fax: (514) 340-7576. E-mail: john.hiscott@mcgill.ca.

TABLE 1. Primers used for this study

Primer name	Sequence (5′-3′)
Tat B mutants	
5′Tat B T23N	CCTAGGACGCCTTGTAATAATTGCTATTGTAAA
3′Tat B T23N	TTTACAATAGCAATTATTACAAGGCGTCCTAGG
5′Tat B T23S	CCTAGGACGCCTTGTAGCAATTGCTATTGTAAA
3′Tat B T23S	TTTACAATAGCAATTGCTACAAGGCGTCCTAGG
Tat C mutants	
5′Tat C N23T	CCTAAAACTGCTTGTACCAATTGCTATTGTAAA
3′Tat C N23T	TTTACAATAGCAATTGGTACAAGCAGTTTTAGG
5′Tat C N23S	CCTAAAACTGCTTGTAGCAATTGCTATTGTAAA
3′Tat C N23S	TTTACAATAGCAATTGCTACAAGCAGTTTTAGG
Tat E mutants	
5′Tat E S23T	CCTACAACTGCTTGTACCAAGTGTTACTGTAAA
3′Tat E S23T	TTTACAGTAACACTTGGTACAAGCAGTTGTAGG
5′Tat E S23N	CCTACAACTGCTTGTAATAAGTGTTACTGTAAA
3′Tat E S23N	TTTACAGTAACACTTATTACAAGCAGTTGTAGG

P-TEFb when NF-κB motifs were mutated and Sp-1 motifs were left intact. The reverse situation showed no LTR responsiveness to P-TEFb. Consequently, Sp-1 may be the major player in the absence of Tat during HIV transcription (77). Thus, cellular transcription factors such as NF-κB and/or Sp-1 may substitute for the lack of elongation effects in the absence of Tat, but controversy still remains regarding their importance.

Tat-associated acetyltransferases (TAH) include the transcriptional coactivator p300/CBP, the p300/CBP-associated protein (P/CAF), and hGCN5 (12, 15, 38, 54). The recruitment of TAHs to the LTR by Tat leads to histone acetylation and the subsequent activation of chromatin-associated HIV-1 proviruses (reviewed in references 57 and 68). Moreover, Tat is also directly modified by acetylation (reviewed in references 7, 9, 57, and 79). p300/CBP and hGCN5 acetylate Tat in the TAR RNA binding domain at lysine (K) 50, and to a lesser extent at K51, whereas P/CAF acetylates K28 in the activation domain (12, 15, 38, 54). Acetylation at K28 promotes the association between Tat and P-TEFb (38) and acetylation at K50 induces the dissociation of Tat from the TAR RNA during early transcriptional elongation (6, 16, 34, 38, 50), thereby enhancing transcriptional activity.

Just as the different HIV-1 subtypes possess distinct enhancer organizations, structurally distinct Tat proteins have likewise been identified from various virus clades (48, 49). For the present study, we analyzed how clade-specific Tat proteins modulate the efficiency of HIV-1 LTR-specific gene expression. Our results indicate that variations in Tat proteins significantly modify HIV-1 transcriptional activation through alterations in the transactivation efficiency, protein half-life, and association with the TAR element of the LTR.

MATERIALS AND METHODS

Cell culture. Jurkat T cells were cultured in RPMI 1640 medium with L-glutamine (Wisent) supplemented with 10% fetal bovine serum (Wisent), 100 mM HEPES, 1 mM sodium pyruvate, 80 mM glucose, and antibiotics. Cos7 cells and HEK 293 cells were cultured in Dulbecco's modified Eagle's medium with L-glutamine (Wisent) supplemented with 10% fetal bovine serum and antibiotics.

Plasmids and cloning. pcDNA3.1/Zeo-Tat B, C, and E constructs and selected HIV-1 B LTR-pβ-gal constructs were obtained from M. Montano, Harvard AIDS Institute, and subcloned as previously described into the pGL3 basic vector (LTR-pGL3 construct) (65). The selected HIV-1 A-G LTR-luciferase constructs were obtained from B. Berkhout, University of Amsterdam, Amsterdam, The Netherlands. Clade-specific Tat proteins represent consensus sequences derived from an analysis of >20 different strains from HIV^+ individuals for each clade (49). LTR elements were derived from HIV-1 strains isolated from patients with non-subtype B HIV-1 infections (31). The selected clade-specific LTRs and Tat proteins represent sequences closest to the consensus sequences found in the Los Alamos database (http://www.hiv.lanl.gov/content/hiv-db/mainpage.html). Expression plasmids encoding Flag-tagged Tat B, C, and E proteins from a pcDNA3.1/Zeo-Flag backbone were generated by PCR amplification of the cDNAs, using specific primers containing forward EcoRI sites and reverse BamHI sites, as follows: Tat B Forward, 5′-CCGGAATTCAATGGAGCCAG TAGATCCTAGA-3′; Tat B Reverse, 5′-CGCGGATCCTTACTGCTTTGATA AAAAAAC-3′; Tat C Forward, 5′-CCGGAATTCAATGGAGCCAGTAGAT CCTAAC-3′; Tat C Reverse, 5′-CGCGGATCCTTACTGCTTTGATACAAGA TT-3′; Tat E Forward, 5′-CCGGAATTCAATGGAGCCGGTAGATCCTAAC-3′; and Tat E Reverse, 5′-CGCGGATCCTTACTGCTCTGGTATAGGATT-3′. The respective cDNAs were inserted into a pcDNA3.1/Zeo vector carrying a Flag epitope sequence cut with EcoRI sites and BamHI restriction enzymes. Expression constructs encoding different lysine-to-alanine (K→A) point mutants were generated by overlap PCRs using pcDNA3.1/Zeo-Flag-Tat B, C, and E cDNAs as templates. The pcDNA3.1/Zeo-Flag chimeric constructs Tat BC, EC, BE, and EB were generated as follows. First, fragments were amplified by PCR using the following primers containing a forward EcoRI site and a reverse BamHI site: for the first 144 bp of the N-terminal fragments, 5′-GAAGATCT TCATGGACTACAAAGACGATGAC-3′ and 5′-CTTCTTCCTGCCATAGG A-3′; and for the C-terminal fragments, 5′-CCGCTCGAGCGGCCGGGATCC TTACTGCT-3′ and 5′-TCCTATGGCAGGAAGAAG-3′. The resulting PCR products were used to perform overlap PCR. Finally, the recombinant PCR products were subcloned into pcDNA3.1/Zeo-Flag cut with EcoRI and BamHI. For the generation of pGEX-4T-2-Tat B, C, and E constructs, plasmid-specific primers containing BamHI restriction sites and a hemagglutinin (HA) tag sequence (reverse primer) were used to amplify Tat cDNAs by PCR. BamHI-restricted PCR fragments were cloned into the unique BamHI site of the pGEX-4T-2 plasmid, placing the Tat sequence in frame with the glutathione *S*-transferase (GST) sequence at the N terminus and with the HA epitope at the C terminus. pGEM3Zf-T7-TAR was a generous gift from C. Van Lint (Université Libre de Bruxelles). The HIV-1 Tat-defective provirus was a generous gift from M. A. Wainberg (McGill University, Montreal, Canada). pDRASCAT and pCMV-CIITA were generous gifts from B. M. Peterlin (University of California at San Francisco).

Expression constructs encoding different point mutations at position 23 were generated by overlap PCRs using pcDNA3.1/Zeo-Flag-Tat B, C, and E cDNAs as templates and the primers listed in Table 1.

In a second round of PCR, final PCR products were amplified by using the following primers designed for each end of the *tat* gene: 5′ Tat B, 5′-CCGGA ATTCCATGGAGCCAGTAGATCC-3′; 3′ Tat B, 5′-CGGGATCCCGTTACT GCTTTGATAAAAA-3′; 5′ Tat C, same as 5′ Tat B; 3′ Tat C, 5′-CGGGATC CCGTTACTGCTTTGATACAAG-3′; 5′ Tat E, 5′-CCGGAATTCCATGGAG

CCGGTAGATCC-3′; and 3′ Tat E, 5′-CGGGATCCCGTTACTGCTCTGGTATAGG-3′.

Transfections and reporter assays. For luciferase assays, Jurkat T cells (10^6 cells per transfection) were seeded at 10^6 cells/ml at least 1 h prior to transfection in six-well plates. HIV-1 LTR B-pGL3 constructs and pcDNA3.1/Zeo-Flag-Tat C or E (K→A) or the pcDNA3.1/Zeo-Flag chimeric construct Tat BC, EC, BE, or EB (50 ng) was cotransfected with 100 ng of pRLTK (encoding *Renilla* luciferase as an internal control) by use of the FUGENE 6 transfection reagent (Roche) as indicated by the manufacturer. In some experiments, the HIV-1 LTR B luciferase construct and various amounts of the pcDNA3.1/Zeo-Flag-Tat B, C, or E construct (2, 10, 50, and 500 ng) were cotransfected with 100 ng of the pRLTK HIV-1 LTR-luciferase construct (80 ng), and the pcDNA3.1/Zeo-Flag-Tat B, C, or E construct (50 ng) was cotransfected with 100 ng of pRLTK by use of the FUGENE 6 transfection reagent (Roche). At 48 h posttransfection, the cells were washed with phosphate-buffered saline (PBS) and lysed in passive lysis buffer. Luciferase activities were measured with a dual-luciferase reporter assay system (Promega).

For chloramphenicol acetyltransferase (CAT) assays, Cos7 cells were seeded 16 h prior to transfection in 6-cm dishes. The pDRASCAT (250 ng) and pEGFP (250 ng) constructs were cotransfected along with pCMV-CIITA (250 ng) and the pcDNA3.1/Zeo-Flag-Tat B, C, E, BC, EC, BE, or EB (250 ng or 1.25 μg) construct by use of the Lipofectamine 2000 transfection reagent (Invitrogen) as indicated by the manufacturer. At 24 h posttransfection, the cells were washed with PBS and harvested by using scrape buffer (40 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA). The cells were then lysed in 100 μl of 0.25 M Tris-HCl, pH 7.8, buffer by three rounds of freezing-thawing and centrifuged for 10 min at 13,000 rpm. A volume of 50 μl of each extract was used to perform the CAT assay. First, extracts were heated at 60°C to inactivate deacetylases. Each extract was then mixed with a reaction mixture containing 0.1 M Tris-HCl, pH 7.8, 1 mM chloramphenicol, and 0.1 μCi of ^{3}H-labeled acetyl-coenzyme A. The reaction mixtures were then overlaid with 4 ml of immiscible water in 7-ml glass scintillation vials. The reaction mixtures were finally incubated at 37°C and the ^{3}H counts per minute (cpm) were measured with a scintillation counter. Measurements were taken up to 3 h postincubation.

RNA EMSA. For RNA electrophoretic mobility shift assays (EMSAs), TAR RNA was synthesized by T7 RNA polymerase from the HindIII-digested pGEM3Zf-T7-TAR plasmid (38) by an in vitro transcription reaction (Ambion) containing [α-^{32}P]UTP (Amersham). The reaction mixtures were incubated at 37°C for 3 h, and the DNA template was digested with 2 U of DNase I. The TAR RNA was purified by phenol-chloroform-isoamyl alcohol extraction (GibcoBRL) and precipitated with ethanol. Prior to use, the RNA pellet was dissolved in 0.1 M NaCl and applied to a G-50 Quick Spin column (Roche). GST-Tat B, C, E/pGEX-4T-2-HA and His-human cyclin T1 (hcyclin T1)/pET15b plasmids were transformed into BL21 (DE3)/pLysS competent bacteria by electroporation. Protein expression was induced by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to exponentially growing bacteria at 37°C. The purification of GST and His-tagged recombinant proteins was performed by using glutathione-Sepharose and nickel resin, respectively, as indicated by the manufacturer's protocols (Amersham Bioscience and Novagene, respectively). Purified proteins were dialyzed against a buffer containing 30 mM Tris-HCl (pH 8.0), 70 mM KCl, and 1 mM dithiothreitol at 4°C. The purity of fusion proteins was determined by Coomassie blue staining of sodium dodecyl sulfate (SDS)-polyacrylamide gels. The protein concentrations were determined by the Bradford assay (Bio-Rad). Tat, hcyclin T1, and TAR interaction analyses were performed as described previously (72). Briefly, 5 ng ^{32}P-labeled TAR RNA was mixed with various amounts of GST-Tat and with His-hcyclin T1 and incubated for 10 min at 30°C. RNA-protein complexes were separated in nondenaturing 6% Tris-glycine polyacrylamide gels in 1× Tris-glycine buffer run at 8 W at 4°C. The gels were dried and then analyzed with a Typhoon image system (Amersham Bioscience) and quantified with ImageQuant software.

Rescue of a Tat-defective HIV-1 provirus. The Tat-defective HIV-1 provirus has a mutation in the initiation codon of the Tat gene. Jurkat T cells (10^6 cells) were seeded in six-well plates at least 1 h prior to transfection. The pcDNA3.1/Zeo-Flag Tat B, C, or E expression plasmid or a plasmid expressing a chimeric Tat protein (BC, EC, BE, or EB) (800 ng) was cotransfected with 800 ng of a plasmid encoding the Tat-defective HIV-1 provirus by use of the FUGENE 6 transfection reagent (Roche) as indicated by the manufacturer. After 24 h, the cells were transferred to 75-cm^2 flasks containing 20 ml of medium. Aliquots of the supernatants (500 μl) were harvested at 24-h intervals posttransfection, and p24 antigen levels were measured by an enzyme-linked immunosorbent assay (ELISA) (Vironostika HIV-1 antigen kit; Biomerieux).

^{35}S-pulse-chase metabolic labeling. For analyses of the half-lives of the different subtype-specific Tat proteins, HEK 293 cells were transfected with 10 μg (Tat B and C) or 5 μg (Tat E) of a pcDNA3.1/Zeo-Flag Tat construct in 60-mm dishes by the calcium phosphate coprecipitation method. At 24 h posttransfection, the cells were pulse labeled for 1 h with 200 μCi/ml of [^{35}S]methionine and [^{35}S]cysteine (Tran^{35}S-Label; ICN) in Dulbecco's modified Eagle's medium without methionine and cysteine (ICN) supplemented with 10% dialyzed serum (GIBCO) and then chased for the indicated times with medium containing an excess of methionine and cysteine (150 mg/liter). The cells were then washed in ice-cold PBS and stored at −80°C before lysis. The cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml pepstatin, and 5 μg/ml aprotinin), and whole-cell extracts (WCE) (400 μg) were immunoprecipitated as follows. WCE were incubated for 3 h at 4°C with protein G-Sepharose beads that had been preadsorbed to 1 μg of anti-Flag antibody. Immunocomplexes were washed four times with lysis buffer, and the proteins were eluted by heating at 95°C for 5 min in denaturing sample buffer and then analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 15% acrylamide gels. ^{35}S-labeled Tat proteins were detected by exposition to autoradiographic films, and quantification was accomplished by densitometric analysis using NIH Image software.

Immunoblot analysis. Cos7 cells were washed once with PBS, trypsinized, and lysed in lysis buffer. WCE (50 μg) were resolved by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad). The membranes were blocked in PBS containing 0.1% Tween 20 and 5% nonfat dry milk for 2 h at room temperature and then incubated with anti-Flag M2 (Sigma) (1 μg/ml), anti-α-actin (Chemicon), anti-green fluorescent protein (anti-GFP) (Santa Cruz Biotechnology), or anti-class II transactivator (anti-CIITA) (Santa Cruz Biotechnology) in blocking solution. After five washes in PBS containing 0.1% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G in blocking solution. Immunoreactive proteins were visualized by enhanced chemiluminescence.

RESULTS

Transactivation of LTRs A to G by clade B-, C-, and E-specific Tat proteins. Previous studies demonstrated that Tat E had the highest transactivation potential, whereas Tat B had the lowest transactivation potential, when assayed with the clade B, C, and E LTRs (65). These studies were extended to include each of the HIV-1 clade LTRs, A to G, and the results were consistent with the previous data. All Tat proteins were capable of stimulating the different LTRs; however, Tat E was the strongest transactivator of LTR activity, regardless of the origin of the HIV LTR (Fig. 1B). The combination of Tat E with LTRs D and F displayed the highest level of induction, whereas LTRs E and G demonstrated the weakest Tat response. Also, a dose-dependent LTR B transactivation by clade-specific Tat proteins confirmed that Tat E was the strongest transactivator and Tat B was the weakest transactivator, regardless of the amount of plasmids transfected (data not shown).

Transactivation of HIV-1 clade B LTR by Tat (K→A) point mutants. The possibility that clade-specific differences in the transactivation capacities of Tat proteins may be related to the acetylation of lysine (K) residues in the transactivation or RNA binding domain was next investigated. Indeed, previous studies revealed that K28 and K50 in the Tat B protein are important for interactions with the histone acetyltransferase adapters CBP and p300 (15, 38). Thus, several point mutations were generated in different lysine residues of the Tat C and E proteins (Fig. 2A). The mutation of other lysine residues, i.e., K19, K41, K50, and K51 to alanine in the Tat C protein, demonstrated that not only did the K28A and K41A mutations have a dramatic inhibitory effect on transactivation, but K50A and K51A also reduced transactivation, although to a lesser extent (Fig. 2B). In the context of the Tat E protein, multiple

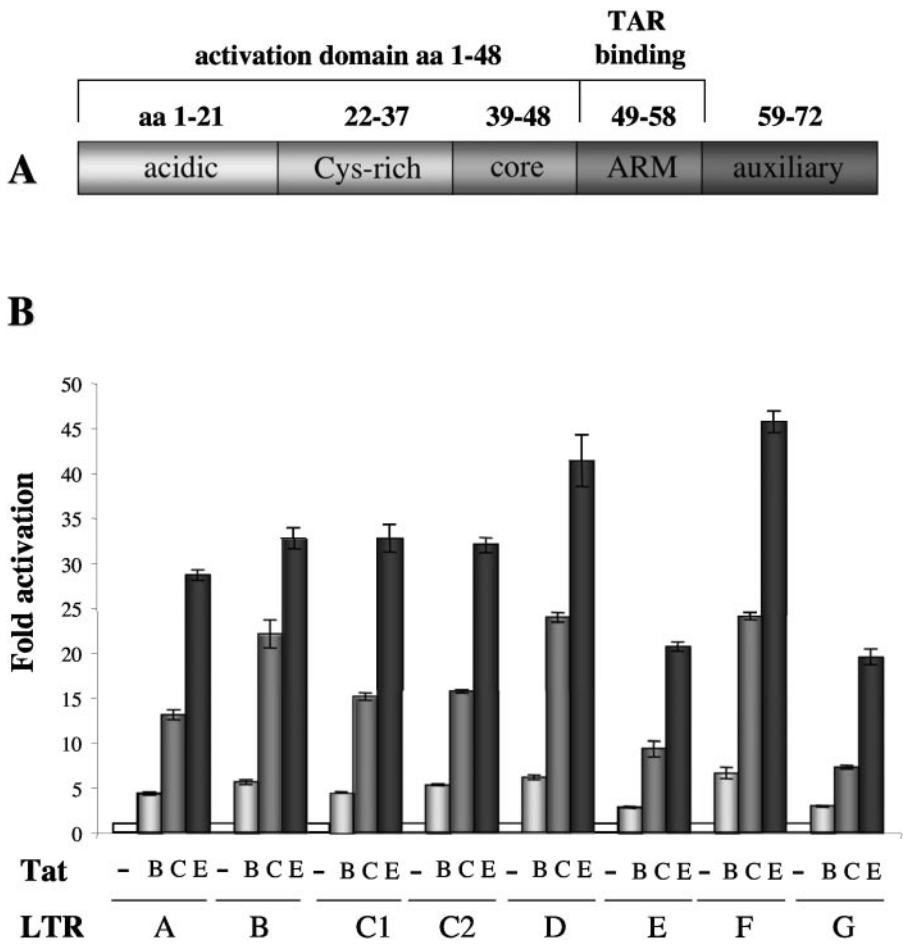

FIG. 1. Transactivation of clade-specific LTRs A to G by clade B-, C-, and E-specific Tat proteins. (A) Schematic representation of the functional domains of the Tat protein. (B) Jurkat T cells were transfected with luciferase reporter constructs (80 ng) containing HIV-1 clade-specific LTR elements in the absence or presence of expression plasmids encoding Tat B, C, or E (50 ng). Relative luciferase activities (expressed as fold activation) were determined as the ratios of luciferase activities between Tat/LTR-cotransfected cells and LTR-transfected cells. The results were normalized by using *Renilla* luciferase. The basal activity of each LTR was set to onefold activation.

lysine residues were mutated, but only the K41A mutation interfered significantly with Tat E transactivation; K28A, K40A, and K50A mutations produced a twofold inhibitory effect on transactivation, whereas other lysine residues had a modest impact on the transactivation efficiency (Fig. 2C). These results demonstrate that K28 is also an important residue in Tat C and Tat E, and they extend previous experiments demonstrating that the acetylation of K28 by CBP/p300 is a critical modification of Tat B. Furthermore, the mutation of lysine 41 to alanine in all Tat proteins completely abrogated transactivation by inhibiting the capacity of Tat to associate with cyclin T1. Although both Tat C and Tat E contain novel lysine substitutions within amino acids 1 to 72 of exon 1, these lysine residues do not appear to play a significant role in the regulation of Tat transactivation.

Determination of the half-lives of HIV-1 Tat proteins. In view of the amino acid variation of the clade-specific Tat proteins, it is possible that protein stability contributes to the differential transactivation of Tat proteins. Flag-tagged Tat B-, C-, and E-expressing constructs were transiently transfected into HEK 293 cells and pulse labeled for 1 h with [^{35}S]Met/Cys, followed by an unlabeled chase for different times (Fig. 3A). Quantification of the [^{35}S]Met/Cys pulse-chase results demonstrated that both Tat B and Tat C had a half-life of ~3 h (Fig. 3B). However, Tat E was significantly more stable, with an extended half-life of >6 h (Fig. 3B), suggesting that the prolonged half-life of Tat E may contribute to its increased transactivation capacity.

Tat-TAR EMSA analysis with different clade-specific B, C, and E Tat proteins. To further analyze the biochemical properties of the clade-specific Tat proteins, we performed a Tat-TAR EMSA analysis using the TAR element from a clade B HIV-1 LTR as a probe, together with increasing amounts of purified GST-Tat fusion proteins (Fig. 4A). The Tat-TAR RNA EMSA analysis demonstrated that in the absence of cyclin T1, all of the clade-specific Tat proteins bound to TAR RNA as both monomers and dimers in a concentration-dependent manner (Fig. 4A). Quantification of the Tat-TAR complexes composed of the Tat C (Fig. 4A, lanes 8 to 14) and Tat E (Fig. 4A, lanes 15 to 21) proteins revealed that Tat C and E bound to the TAR element with a three- to fourfold higher affinity than that of Tat B (Fig. 4A, lanes 1 to 7, and B). The

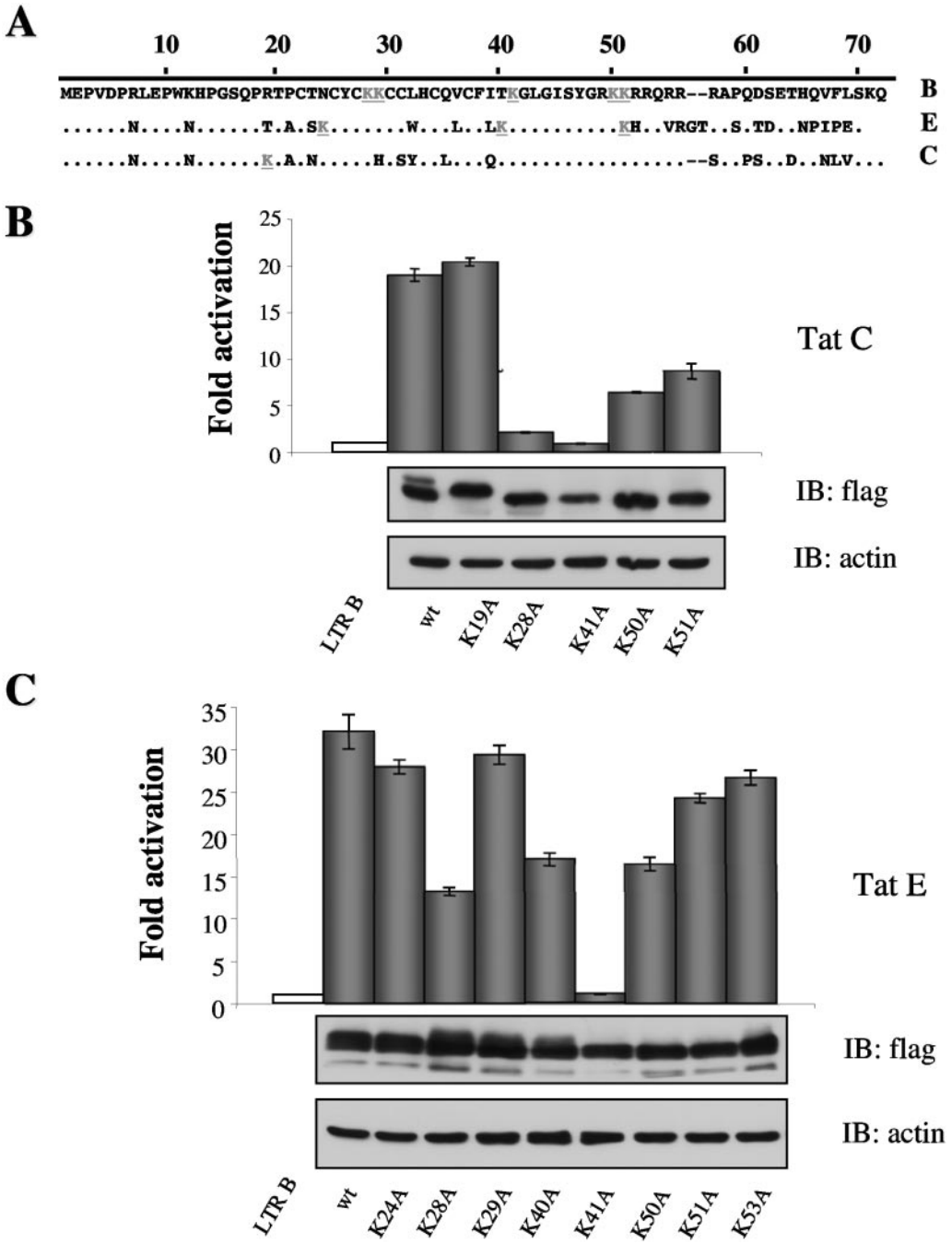

FIG. 2. Transactivation of HIV-1 clade B LTR by Tat (K→A) mutant proteins. (A) Primary sequence information for Tat B, C, and E proteins. Different lysine (K)-to-alanine (A) point mutants of Tat C and E were generated as described in Materials and Methods. (B) For luciferase assays, Jurkat T cells were transiently transfected with the LTR B-pGL3 reporter construct (80 ng) in the absence or presence of an expression plasmid encoding the Flag-tagged Tat C or E mutant protein (50 ng). Relative luciferase activities were determined as described in the legend to Fig. 1. For an analysis of protein expression, Cos7 cells were transfected with 5 μg of a plasmid encoding the Flag-tagged Tat C or E mutant protein. WCE (50 μg) were resolved by SDS-PAGE and immunoblotted (IB) with anti-Flag and anti-actin antibodies.

addition of purified cyclin T1 to the reactions did not augment the binding of Tat C and Tat E and essentially confirmed the results of the Tat-TAR binding assay (Fig. 4A and B), although it was possible to detect the formation of a Tat/hcyclin T1/TAR complex with increasing amounts of added cyclin T1 (data not shown). These data suggest that the Tat C and E proteins possess increased transactivation potentials, in part through an increased affinity for binding to the TAR element.

Transactivation of HIV-1 clade B LTR by chimeras of clade-specific Tat proteins. Next, to evaluate the differential transactivation potentials and the affinities of various clade-specific Tat proteins for cyclin T1, we generated chimeric Tat proteins

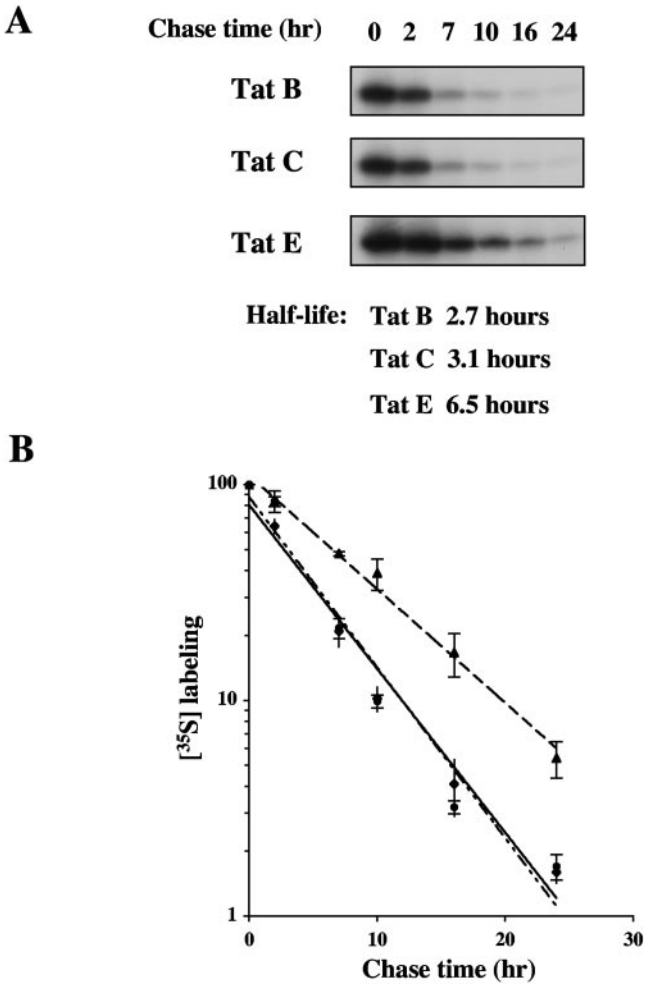

FIG. 3. Determination of half-lives of HIV-1 Tat proteins. (A) HEK293 cells were transiently transfected with Flag-tagged expression constructs (5 or 10 μg) encoding Tat B, C, or E. At 24 h posttransfection, the cells were pulse labeled with [^{35}S]methionine and [^{35}S]cysteine for 1 h and then chased with unlabeled medium for the indicated times. WCE were then subjected to immunoprecipitation with an anti-Flag antibody. Immunocomplexes were resolved by SDS-PAGE and analyzed by autoradiography. (B) The incorporated radioactivity was quantified, plotted in a semilogarithmic graph representing the means of triplicates ± standard errors, and subjected to linear regression. Tat B (circles), Tat C (diamonds), and Tat E (triangles) are represented.

by swapping the clade-specific N-terminal activation domains responsible for cyclin T1 binding. Replacement of the Tat B and C N-terminal activation domains with the Tat E cyclin T1-binding activation domain resulted in enhanced transactivation capacities of both the Tat EB and EC proteins in Jurkat T cells compared to the respective Tat B and C wild-type proteins (Fig. 5). Conversely, the transactivation potentials of both the Tat BC and BE proteins were dramatically decreased compared to the activities of the wild-type Tat C and E proteins, respectively. Thus, variations in the N-terminal activation domain sequences among clade-specific Tat proteins appear to be crucial determinants in conferring different binding affinities for the cofactor cyclin T1. The stabilities of the Tat E, BE, and EB proteins were also evaluated by pulse-chase analysis; no significant differences were observed between the wild-type and chimeric proteins (data not shown), indicating that a specific domain of Tat E does not appear to be responsible for the increased stability of the protein.

Based on the recent studies of Reza et al. (60), the possibility that clade-specific differences in the transactivation capacities of Tat proteins may be related to the residue located at position 23 in the activation domain was next investigated. Thus, the residue at position 23 of a particular subtype Tat protein was mutated to the residues found in the other subtype Tat proteins, using the primers listed in Table 1 for first-round amplification. Proteins harboring mutations of the threonine, asparagine, and serine residues found in Tat B, C, and E, respectively, showed the same activities as the respective wild-type proteins (data not shown). Therefore, the residue at position 23 is not critical for determining subtype-specific differences between Tat proteins in terms of LTR transactivation.

Rescue of a Tat-defective HIV-1 proviral genome by various Tat proteins. To determine whether the increased transactivation potentials of the Tat C and E proteins could also impact de novo HIV-1 replication, we transfected a Tat-negative proviral cDNA clone which was defective for Tat expression into Jurkat T cells in combination with expression plasmids encoding the clade-specific Flag-tagged Tat proteins or Flag-tagged chimeric Tat proteins. At different times posttransfection, culture supernatants were analyzed for rescue of the p24 capsid antigen by the use of ELISA (Fig. 6). The rescue of the Tat-defective HIV-1 proviral genome replication was achieved by the Tat B, C, and E proteins along with the chimeric Tat proteins. Tat E was clearly the most efficient protein in the rescue assay, Tat C had an intermediate rescue efficiency, and Tat B was relatively inefficient compared to the Tat C and E proteins (Fig. 6A). For chimeric Tat proteins, those harboring the Tat E activation domain showed efficiencies in the rescue assay as high as that of the wild-type Tat E protein (Fig. 6B and C). Inversely, chimeric Tat proteins harboring the Tat B activation domain clearly showed low efficiencies of HIV particle production (Fig. 6B and C). These results demonstrate significant differences between the clade-specific Tat proteins in terms of the ability to rescue a Tat-defective proviral genome. In particular, the clade E Tat protein, which possessed the highest activity in terms of LTR transactivation, was the most efficient at rescuing the replication of the HIV-1 Tat-defective proviral genome.

Competition between CIITA and Tat proteins for P-TEFb binding. To determine the affinities of clade-specific and chimeric Tat proteins for P-TEFb, we cotransfected the class II transactivator (CIITA), a transcription factor that utilizes P-TEFb to initiate the transcription of major histocompatibility complex class II (MHC II) molecules (Fig. 7A), along with different Tat proteins. While Tat B decreased the HLA-DR promoter activity about 30 to 40%, Tat E strongly inhibited CIITA transactivation (70 to 80%), resulting in a low HLA-DR promoter activity (Fig. 7B). This pattern was observed independent of the amount of plasmid transfected. When the chimeric Tat proteins were used in the same competition analysis, chimeric proteins harboring the Tat E activation domain were as efficient at inhibiting CIITA's function as the wild-type Tat E protein. Conversely, chimeric Tat proteins harboring the Tat B activation domain decreased the transactivation of HLA-DR

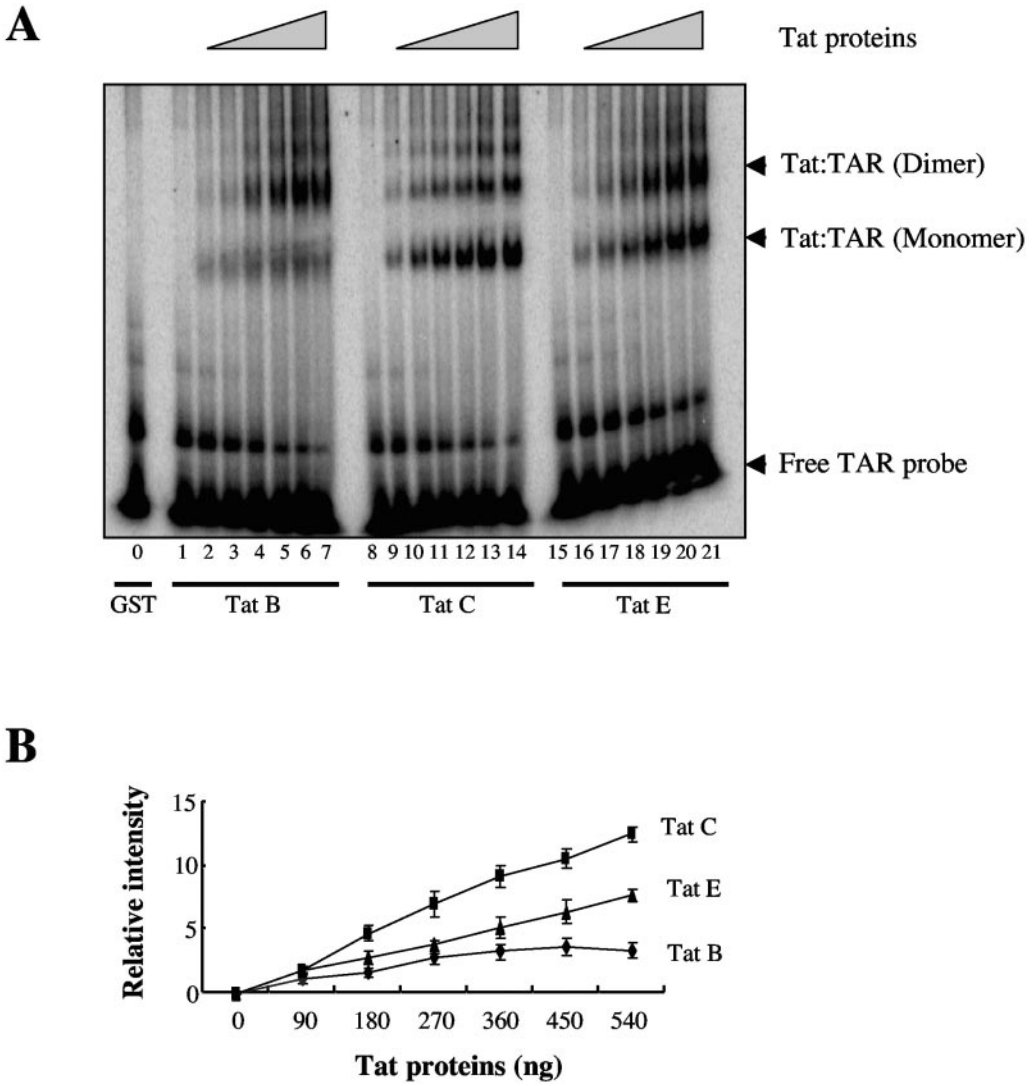

FIG. 4. Tat-TAR EMSA analysis with clade-specific Tat B, C, and E proteins. (A) Increasing amounts of purified GST-Tat B, GST-Tat C, and GST-Tat E fusion proteins (90, 180, 270, 360, 450, and 540 ng), corresponding to lanes 2 to 7, 9 to 14, and 16 to 21, respectively, were resolved with 5 ng of ^{32}P-labeled TAR element from HIV-1 clade B. (B) The Tat-TAR monomer band intensities in panel A were quantified by using ImageQuant 5.2, and the relative band intensities were plotted versus the amounts of Tat protein (ng). Tat B (diamonds), Tat C (squares), and Tat E (triangles) are represented.

promoter activity to the same level as that by the wild-type Tat B protein (Fig. 7C). These results demonstrate that the clade E Tat protein more effectively inhibited CIITA function than did Tat B and imply that Tat E has a stronger ability to sequester P-TEFb than does Tat B.

DISCUSSION

HIV-1 can be classified into at least 10 different subtypes, or clades, with distinct worldwide distributions and patterns of disease transmission and pathogenesis (40, 41, 51, 63, 64). Just as the genomic sequences of the *gag* and *env* genes have mutated extensively during the evolution of the virus, the HIV-1 Tat transactivator proteins from different consensus clade viruses have also diversified and possess different transactivation potentials in assays using conventional reporter gene readouts (48, 49). The objective of the present study was to evaluate clade-specific Tat proteins and the mechanisms by which structurally distinct Tat proteins may modulate HIV-1 LTR gene expression. Our results demonstrate that the Tat proteins derived from HIV-1 clades C and E—the most prevalent HIV subtypes in the world—display dramatically distinct patterns of gene activation. Both Tat C and E had stronger transactivation potentials than the consensus clade B Tat protein, and both displayed higher affinities for the TAR RNA element in a Tat-TAR EMSA analysis. Furthermore, Tat E possessed a demonstrably longer half-life than that of either clade B or clade C Tat. While both Tat C and E were able to rescue a Tat-defective HIV-1 proviral clone, Tat E was significantly more efficient at rescue than Tat C, possibly due to the relative stability of the Tat protein. Swapping the activation domains of Tat B, C, and E identified the cyclin T1 association domain as

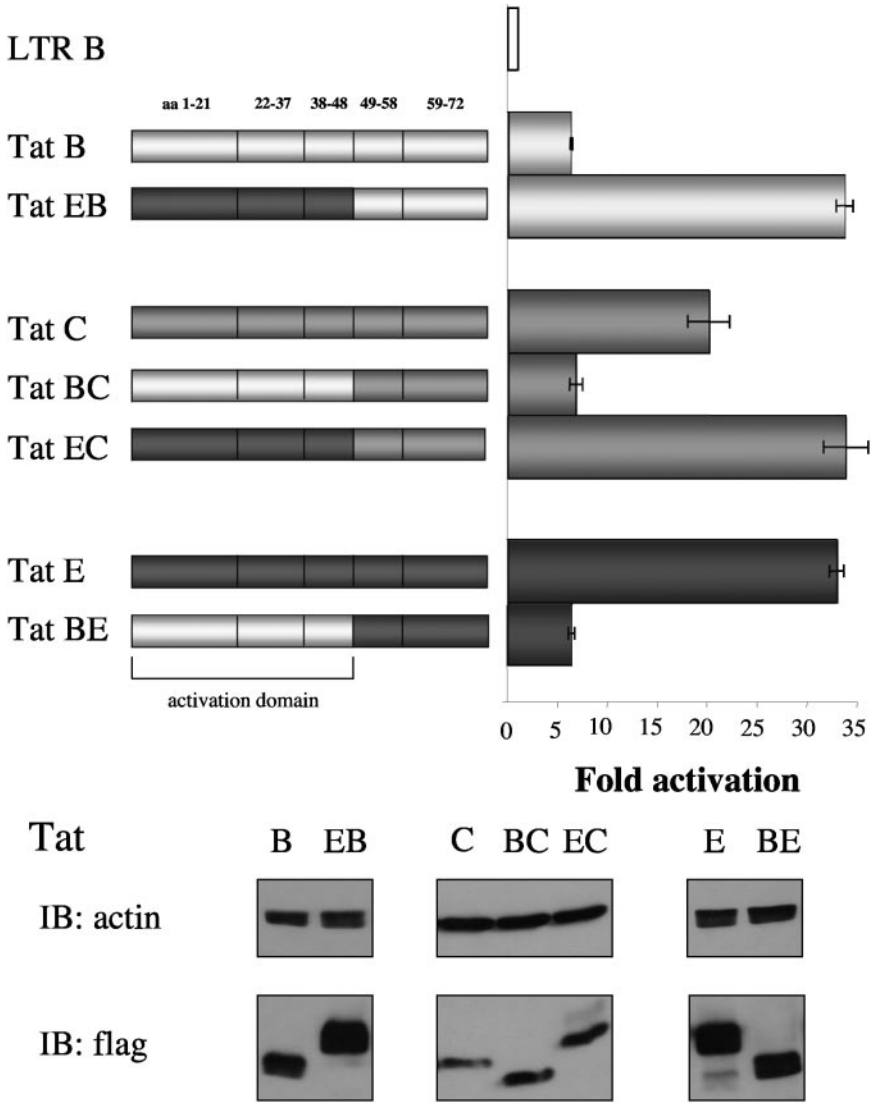

FIG. 5. Transactivation of HIV-1 clade B LTR by recombinant Tat proteins. For luciferase assays, Jurkat T cells were transiently transfected with the LTR B-pGL3 reporter construct (80 ng) in the absence or presence of an expression plasmid encoding Flag-tagged Tat BC, EC, BE, or EB (50 ng). Relative luciferase activities were determined as described in the legend to Fig. 1. For an analysis of protein expression, Cos7 cells were transfected with 5 μg of a plasmid encoding the Flag-tagged Tat B, C, E, BC, EC, BE, or EB protein. WCE (50 μg) were resolved by SDS-PAGE and immunoblotted by using anti-Flag and anti-actin antibodies.

a critical determinant of transactivation efficiency and of Tat-defective HIV-1 provirus rescue.

The transactivation potentials of the clade-specific Tat B, C, and E proteins were next examined by using the LTR elements from HIV-1 clades A through G. Independent of the LTR origin, Tat E was the strongest transactivator of LTR gene expression, which corroborates previous studies comparing Tat proteins in the context of the LTR elements B, C, and E (65). Independently, Kurosu et al. demonstrated that the subtype C Tat protein exhibited the highest transcriptional activity among Tat B, C, and E (44). Such differences may be due to the divergence in the primary Tat amino acid sequence; it should be noted that the sequences used for the present study are closest to the consensus sequences found in the Los Alamos database (http://www.hiv.lanl.gov/content/hiv-db/mainpage.html).

An affinity for the cellular cofactor P-TEFb is an important determinant of the Tat transactivation potentials observed with clade C and E Tat proteins compared to that observed with clade B Tat. The activation domain of Tat, encompassing the first 48 amino acids, is required for P-TEFb binding (30, 33, 58, 81) and promotes HIV-1 transcription by phosphorylating the RNA Pol II CTD (39, 74, 80, 81). The effect of this region on Tat transactivation was investigated by swapping different activation domains between the clade-specific Tat proteins. Chimeric proteins containing the Tat E activation domain were as efficient as the wild-type Tat E protein in LTR transactivation and Tat-defective HIV provirus rescue. In contrast, the Tat B activation domain decreased the transactivation potentials of both the Tat C and E proteins. These results are consistent with a recent study using a Tat protein containing a single

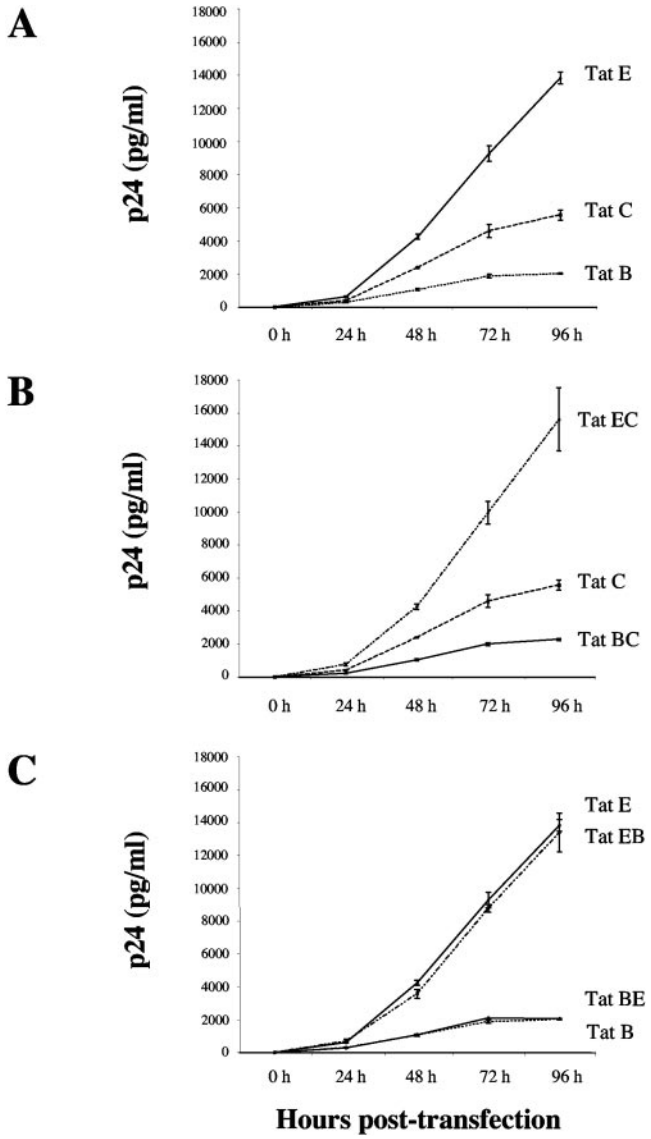

FIG. 6. Rescue of Tat-defective HIV-1 clone by various Tat proteins. (A) Jurkat T cells were cotransfected with a plasmid encoding the Tat B, C, or E protein (800 ng) along with a plasmid encoding a Tat-defective proviral genome (800 ng). (B) Jurkat T cells were cotransfected with a plasmid encoding the Tat C, BC, or EC protein along with a plasmid encoding a Tat-defective proviral genome (800 ng). (C) Jurkat T cells were cotransfected with a plasmid encoding the Tat B, E, BE, or EB protein along with a plasmid encoding a Tat-defective proviral genome (800 ng). Aliquots of supernatants were harvested at the indicated times posttransfection (24 to 96 h), and p24 antigen levels (pg/ml) were measured by ELISA. The data plotted in the graph represent the means of triplicates ± standard errors. In panel A, Tat B (diamonds), Tat C (squares), and Tat E (triangles) are represented; in panel B, Tat C (squares), Tat BC (diamonds), and Tat EC (triangles) are represented; in panel C, Tat B (diamonds), Tat BE (circles), Tat EB (crosses), and Tat E (triangles) are represented.

threonine-to-asparagine substitution (position 23) within the cysteine-rich region that led to an increased Tat/P-TEFb interaction and increased the Tat transactivation potential (60). Interestingly, Tat B contains a threonine at position 23, while Tat C contains an asparagine at this position; this natural substitution in Tat C may provide a replicative advantage to subtype C HIV-1 strains. However, our studies demonstrated that mutations of Tat B, C, and E at position 23 to either a threonine, an asparagine, or a serine residue did not show significant differences in transactivation in Jurkat T cells compared with the respective wild-type Tat proteins. This observation may be dependent on the different primary sequences found in the cysteine-rich domains of different Tat proteins within a particular subtype. In fact, the cysteine-rich region of Tat is crucial for its activity (43, 61) and is also involved in Zn^{2+} cation binding (19, 28). Human cyclin T1 requires Zn^{2+} to bind Tat, since a mutation of cysteine 261 to tyrosine (found in murine cyclin T1) disrupts the Tat/P-TEFb interaction (20, 22). Furthermore, this specific region of Tat is the most variable of the regions forming the activation domain among the clade-specific Tat proteins. Consequently, the overall architecture of the cysteine-rich region may determine the affinity for P-TEFb. Therefore, primary amino acid sequence variations among the different clade-specific Tat activation domains seem to account for the different transactivation potentials observed with the clade-specific Tat proteins.

In order to confirm the role of P-TEFb in the differences seen between clade-specific Tat proteins in terms of LTR transactivation, we performed a competition assay between CIITA and Tat. CIITA is a transcription factor known to initiate the expression of MHC II and exerts its function primarily by binding general transcription factors (17, 46), histone acetyltransferases (18, 42), and the positive transcription elongation factor b complex. Previous studies have shown that CIITA and Tat compete for P-TEFb binding (35, 53). Competition between CIITA and clade-specific Tat proteins revealed that Tat E competed to a higher extent with CIITA to bind P-TEFb than did Tat B. The results obtained with the chimeric proteins harboring the Tat B and E activation domains also showed similar patterns to those observed with the wild-type Tat B and E proteins, respectively. Therefore, the activation domains of the various Tat proteins have an important impact on the recruitment of and binding to P-TEFb.

The observation that clade E Tat displays more stability (6 h) than that of Tat B and C (3 h) may also contribute to the differences seen in transactivation by the Tat B, C, and E proteins. The improved stability of Tat E relative to that of Tat B and C could improve the transactivation of different enhancer elements, independent of their origins. The combination of a high affinity for TAR, a high affinity for cyclin T1, and an increased stability of Tat E is consistent with the efficient rescue of the Tat-defective HIV-1 provirus compared to that with the Tat B and C proteins. The greater stability of the subtype Tat E protein may be dependent on its ubiquitination pattern. Recently, Bres et al. demonstrated a nonproteolytic role for Tat-mediated ubiquitination (5). In fact, Tat ubiquitination by the proto-oncoprotein Hdm2 did not target Tat for degradation. Since the subtype Tat E protein contains more lysine residues, which are ubiquitin acceptor residues, Tat E ubiquitination could occur more readily and contribute to the longer half-life of Tat E than that of Tat B and C. Unique lysine residues such as K40 within the primary sequence of Tat may act as ubiquitin acceptor residues.

The posttranslational modification of Tat by acetylation is crucial for Tat function (7, 9, 57, 79). Lysine residues of Tat are

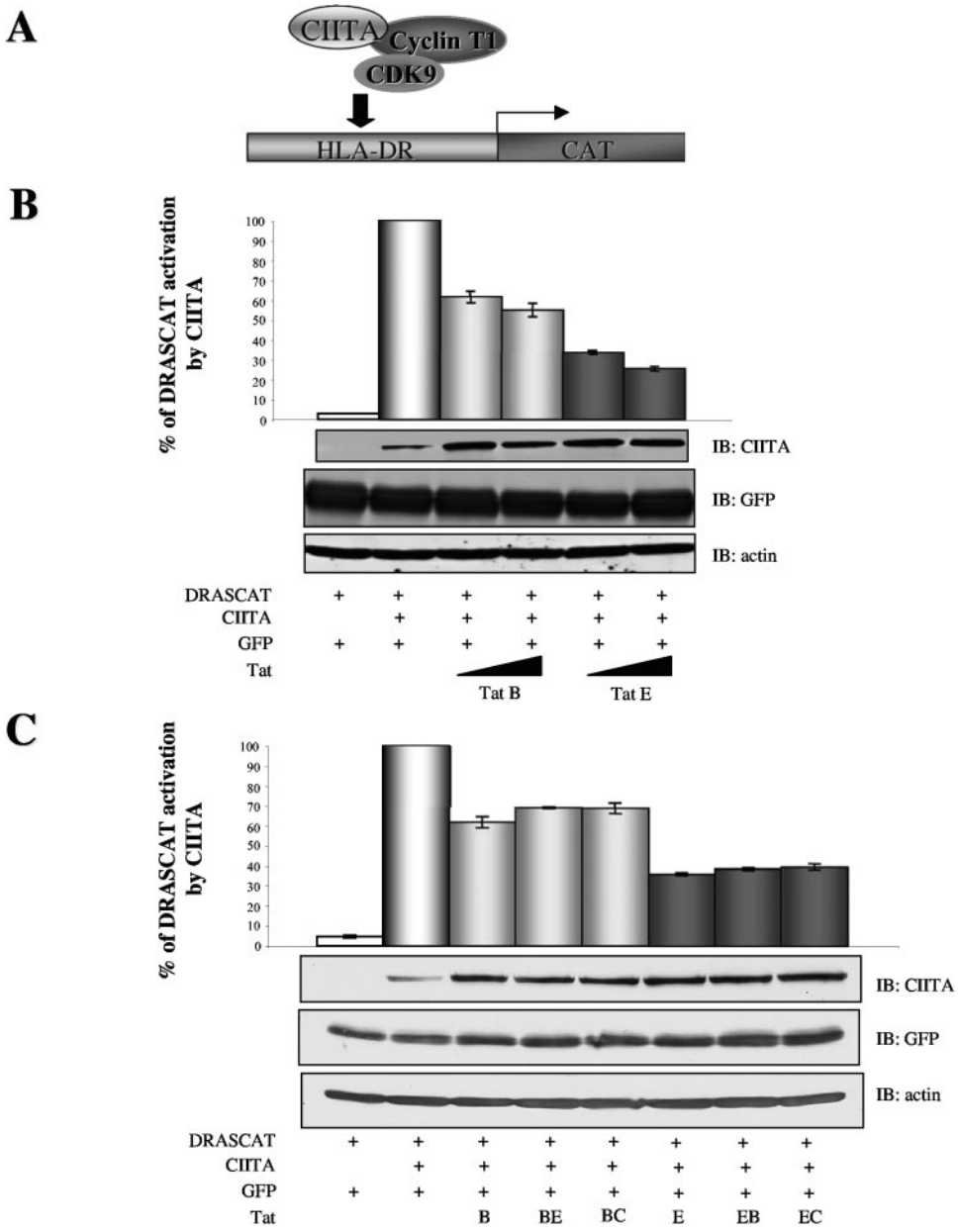

FIG. 7. Competition between CIITA and Tat proteins for P-TEFb binding. (A) Schematic representation of MHC II promoter (DRA) activation by the transcription factor CIITA. (B) Cos7 cells were cotransfected with the pDRASCAT reporter construct (250 ng), a plasmid encoding EGFP (250 ng), in the absence or presence of pCMV-CIITA (250 ng), and a plasmid encoding the Tat B or E protein (250 ng or 1.25 μg). CAT activity (cpm of ^{3}H) was measured with a scintillation counter, and the results are plotted as percentages of the DRASCAT activity in the presence of CIITA. (C) Cos7 cells were cotransfected with the pDRASCAT reporter construct (250 ng), a plasmid encoding EGFP (250 ng), in the absence or presence of pCMV-CIITA (250 ng), and a plasmid encoding the Tat B, BE, BC, E, EB, or EC protein (250 ng). CAT activity (cpm of ^{3}H) was measured with a scintillation counter, and the results are plotted as percentages of the DRASCAT activity in the presence of CIITA. For an analysis of protein expression, equal volumes of the extracts (45 μl) were resolved by SDS-PAGE and immunoblotted (IB) by using anti-CIITA, anti-GFP, and anti-actin antibodies.

acetylated by different histone acetyltransferases: P/CAF acetylates K28 (38), whereas p300/CBP and hGCN5 are required for the acetylation of K50 and K51, respectively (12, 15, 38, 54). While P/CAF-dependent acetylation at K28 enhances the Tat transactivation potential by coordinating its interaction with cyclin T1 and the TAR RNA element (38), acetylation by p300/CBP at K50 triggers a disruption of the Tat–TAR–P-TEFb complex (6, 34, 50). Our transactivation studies using Jurkat T cells with Tat proteins containing different lysine-to-alanine point mutations demonstrated that K28A and K50A mutations also decreased the Tat C and E protein function, as described previously in the context of Tat B (4, 38). Another lysine residue in the cysteine-rich domain of Tat, namely, K41, is essential for Tat function, since the mutation of this residue

in Tat C and E led to a complete abrogation of Tat function, as also described previously for Tat B (43, 61). This conserved residue is absolutely required for interactions with cyclin T1 (4, 72) and may be important for interactions with other proteins such as P/CAF (4). The unique K40 residue of Tat E may also contribute to the interaction with cyclin T1 and to the higher affinity of Tat E for cyclin T1 than that of Tat C. As suggested before, this specific residue may also serve as a ubiquitin acceptor residue. Finally, we also found that a K51A mutation had an effect on Tat C function: K51 acts as an acetyl acceptor residue (12) but also plays a role in the Tat-P/CAF interaction (16). The fact that the K51A mutation is less important for Tat E function may be explained by the presence of an extra lysine residue at position 53 that may compensate for the K51 mutation. Thus, the Tat C and E proteins conserve the K28 and K50 residues, which are targets for the histone acetyltransferases, and both Tat proteins also conserve K41, which is crucial for the cyclin T1 association. Other lysine residues in Tat C and E appear to play a minor role in Tat function. In conclusion, the present studies provide evidence that Tat protein stability and affinity for the TAR element contribute to the enhanced transcriptional function of Tat C and E. Ongoing studies will investigate the relationship between Tat function and HIV replication in primary T lymphocytes and dendritic cells.

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