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# Functional Domains of Tat Required for Efficient Human Immunodeficiency Virus Type 1 Reverse Transcription†

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**Tat expression is required for efficient human immunodeficiency virus type 1 (HIV-1) reverse transcription. In the present study, we generated a series of 293 cell lines that contained a provirus with a *tat* gene deletion (Δ*tat*). Cell lines that contained Δ*tat* and stably transfected vectors containing either wild-type *tat* or a number of *tat* mutants were obtained so that the abilities of these *tat* genes to stimulate HIV-1 gene expression and reverse transcription could be compared. *tat* genes with mutations in the amino terminus did not stimulate either viral gene expression or HIV-1 reverse transcription. In contrast, *tat* mutants in the activation, core, and basic domains of Tat did not stimulate HIV-1 gene expression but markedly stimulated HIV-1 reverse transcription. No differences in the levels of virion genomic RNA or $tRNA_3^{Lys}$ were seen in the HIV-1 Δ*tat* viruses complemented with either mutant or wild-type *tat*. Finally, overexpression of the Tat-associated kinases CDK7 and CDK9, which are involved in Tat activation of HIV-1 transcription, was not able to complement the reverse transcription defects associated with the lack of a functional *tat* gene. These results indicate that the mechanism by which *tat* modulates HIV-1 reverse transcription is distinct from its ability to activate HIV-1 gene expression.**

Reverse transcription is the process by which retroviruses synthesize a double-stranded DNA provirus from their positive-strand RNA genomes (4, 71). Studies involving the analysis of human immunodeficiency virus type 1 (HIV-1) reverse transcription have demonstrated that this process is subject to complex regulation by both viral and cellular factors. For example, the virally encoded heterodimeric reverse transcriptase (RT) p51/p66 (6) and nucleocapsid protein (NCp7) (51) interact with a cellular $tRNA_3^{Lys}$, which is preferentially imported into virion particles (32), by complementary base pairing with a region of HIV-1 genomic RNA known as the primer-binding site (64). Interactions between the cellular $tRNA_3^{Lys}$, RT (5, 63), and NCp7 (52, 54, 57) may help influence the specific reverse transcription initiation complex. Other RNA structures, including TAR RNA (31, 35) and an A-rich loop outside the primer-binding site within U5 (40, 42, 44), have also been reported to be necessary and may promote a structurally favored initiation complex required for efficient reverse transcription.

In addition to those in the genes for RT and NCp7, mutations in other HIV-1 structural, regulatory, and accessory genes have resulted in viruses that are defective for reverse transcription. These include the *nef* (1, 67) and *vif* (75) genes, which have been shown to affect HIV-1 reverse transcription by influencing virus particle formation. HIV-1 matrix protein (11, 74) and Vpr (37) may influence reverse transcription efficiency by directing nuclear import of the reverse transcription complex, in addition to having effects on early steps in the life cycle prior to reverse transcription (47). HIV-1 integrase (56) and the viral transactivator Tat (34) are also required for efficient HIV-1 reverse transcription. Cellular proteins, including cyclophilin A (23, 72), DNA topoisomerase I (66), and ERK2 (45), which are specifically incorporated into HIV-1 virions, have also been suggested to play either a direct or indirect role in the process of reverse transcription.

The HIV-1 transactivator Tat is required for efficient viral replication by stimulating HIV-1 transcriptional activation. Tat activation requires a double-stranded RNA structure known as TAR, extending from the transcription initiation site to position +57. Tat directly interacts with at least two cellular kinases, CDK7 (14, 28) and CDK9 (55, 76), to stimulate hyperphosphorylation of the C-terminal domain of RNA polymerase II and increase the processivity of the elongating transcription complexes. Both the activation and basic domains of Tat are required for this function. Specifically, Tat binds through an interaction between its basic domain and the bulge region of its effector molecule TAR RNA (18, 21). The activation domain interacts with the cellular kinases and may direct them into the transcription complexes that are assembling on the HIV-1 promoter and therefore bypass the normal recruitment mechanisms (76).

Although mutations in the *tat* gene reduce viral replication several thousandfold (16, 22), heterologous viral transactivators, which restore HIV-1 gene expression, only partially offset the severe defects in viral replication and cytopathicity (39). This result suggested that Tat might function in steps of the viral life cycle other than increasing transcription. Previously, we have demonstrated that HIV-1 virions with *tat* gene deletion (Δ*tat*) produce levels of negative-strand strong-stop DNA

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at least 10-fold lower than those wild-type HIV-1 (34). Also, HIV-1 proviruses that lack *tat* can be complemented by the expression of a functional *tat* gene in the cell lines producing the mutant HIV-1. This defect in reverse transcription was also seen in endogenous reverse transcription assays. Thus, HIV-1 Tat is required at early stages of reverse transcription, although its exact role in this process has not been determined.

Tat may be involved in the initiation of reverse transcription prior to the subsequent switch to elongation (42, 43). The kinetics of this process have been studied (41, 65) and found to be close to the overall rate of DNA synthesis for other polymerases, with initiation being the rate-limiting step (50). It should be mentioned that reverse transcription can occur in the absence of a functional *tat* gene but that the accumulation of proviral DNA intermediates is greatly reduced (34). These results suggest that in the absence of Tat, an optimal reverse transcription complex is not formed. It is possible that Tat may be directly involved in these early steps and/or that Tat may interact with a cellular factor(s) during initiation to enhance the processivity of HIV-1 RT. Finally, Tat could also function during viral assembly by either recruiting a cellular factor or modifying an existing viral protein.

To better define the role of Tat in reverse transcription, we studied a panel of *tat* mutants to define domains that are required to support efficient HIV-1 reverse transcription. In addition, we wished to identify *tat* mutants that could stimulate reverse transcription but not viral gene expression. We performed both single-cycle infection and natural endogenous reverse transcription (NERT) assays with viruses produced from 293 cells expressing HIV-1 with a *tat* gene deletion and expressed a panel of *tat* mutants both stably and transiently. Our results suggest that the mechanism by which Tat stimulates HIV-1 reverse transcription can be separated from its role in activating HIV-1 gene expression.

## MATERIALS AND METHODS

**Plasmids and constructs.** The amino acids in Tat at positions 3, 5, and 9 were mutated to glycine, and lysine 41 was mutated to alanine, by site-directed mutagenesis by the QuikChange method (Stratagene, Inc.). The wild-type and mutated *tat* genes have been previously described (27, 73). The *tat* genes were ligated into pBK-RSV (Stratagene, Inc.) or pDex (27) and verified by sequencing. Plasmids expressing either CDK7, CDK9, or Cdc5 were the generous gift of León F. Garcia-Martínez. Plasmid pCH110, which expressed the β-galactosidase (β-Gal) gene, was obtained from Amersham Pharmacia Biotech. The positive control $tRNA_3^{Lys}$ plasmid was the generous gift of J. Pata, Yale University.

**Transfections and CAT assays.** HeLa cells were transfected with an HIV-1 long terminal repeat (LTR)-chloramphenicol acetyltransferase (CAT) reporter plasmid, a eucaryotic expression plasmid driven by Rous sarcoma virus (RSV) promoter and containing either the wild-type or mutant *tat* gene, a simian virus 40 β-Gal control plasmid. For each transfection, HeLa cells were grown to 30 to 50% confluence and transfected by using the Lipofectamine transfection protocol (Life Technologies) with 2 μg of each of the eucaryotic expression plasmids containing the *tat* genes, 3 μg of HIV-1 LTR-CAT reporter plasmid and 2 μg of pCH110. At 48 h posttransfection, the cells were washed with phosphate-buffered saline (PBS), resuspended in 500 μl of 0.25 M Tris-HCl (pH 7.8), and lysed by repeated freezing and thawing. The protein content of cell lysates was measured by using the Bio-Rad protein assay. β-Gal activity was determined by using a chlorophenol red galactopyranoside assay with standardized β-Gal concentrations. CAT protein levels were determined with extracts standardized for transfection efficiency according to β-Gal activity by using the Roche Diagnostics CAT enzyme-linked immunosorbent assay (ELISA) kit.

**Cell lines, viruses, and infections.** The isolation and characterization of the 293 cell lines (30) producing HIV-1 Δ*tat* and wild-type HIV-1, designated Δ*tat* and wild type, respectively, have been previously described (33, 34). The Δ*tat* cell line was grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 5% newborn calf serum, 2% fetal calf serum, 1% penicillin-streptomycin, 1% GlutaMax (Life Technologies), and 0.25 μg of puromycin (Sigma) per ml. HIV-1 Δ*tat* cells were transfected by using Lipofectamine (Life Technologies) with either the parental vector pBK-RSV or the same plasmid containing a wild-type or mutated *tat* gene. Cells were serially diluted at 48 h posttransfection and cultured in complete IMDM with the addition of 1 mg of G418 per ml. Next, 36 to 48 individual foci were randomly selected and clones were expanded in 24-well plates. Cell lines were assessed for growth characteristics, cell morphology, and HIV-1 production. Tat expression was determined by RT-PCR as described below. Three individual cell lines were chosen from each stably transfected 293 Δ*tat* cell line.

Peripheral blood mononuclear cells (PBMCs) were obtained from HIV-1-seronegative donors and isolated on a Ficoll-Plaque (Amersham Pharmacia Biotech) gradient as previously described (33). PBMCs were activated in RPMI 1640 medium supplemented with 20% fetal bovine serum, 1% GlutaMax, 1% penicillin-streptomycin, and 1% KaryoMAX phytohemagglutinin (M form) (Life Technologies) for 72 h. The PBMCs were maintained in complete RPMI 1640 medium containing 10 U of interleukin-2 (Roche Diagnostics) per ml and lacking phytohemagglutinin.

Virus stocks were produced and assayed as previously described (34). Briefly, each 293 Δ*tat* cell line containing either a wild-type or mutant *tat* gene was grown in 100-mm-diameter tissue culture dishes in complete IMDM supplemented with 1 mg of G418 per ml and 0.25 μg of puromycin per ml. The supernatant was removed when the cells were 50% confluent, replaced with complete IMDM lacking both puromycin and G418, and cultured for 18 h at 37°C with 5% $CO_2$. The medium was removed, filtered through a 0.45-μm-pore-size PES membrane, and stored in 10-ml aliquots at −80°C. Each virus stock was assayed for HIV-1 p24 antigen (Ag) by ELISA (NEN Life Science Products) and for RT activity by the RT Detect Assay (Roche Diagnostics).

Cell-free supernatant containing 90 mU of RT activity was adjusted to 45 ml with cell-conditioned culture medium and supplemented with 10 mM $MgCl_2$ and 300 U of DNase I (Worthington Biochemical). The viral supernatants were incubated at 37°C for 30 min, after which a 15-ml aliquot of each was heat-inactivated at 60°C for 20 min. Each DNase I-treated viral supernatant was then incubated with $2 \times 10^7$ activated PBMCs for 2 h. The infected PBMCs were washed three times with culture medium to remove residual virus, and low-molecular-weight nucleic acids were isolated from half of the cells by the Hirt lysis method (38). The remaining infected cells, as well as cells infected with heat-inactivated virus, were harvested after an additional 22 h in culture.

To measure virus replication kinetics, $10^7$ PBMCs were infected with 30 ml of HIV-1 supernatant containing 60 mU of total RT activity. The residual virus was removed by washing the cells with complete RPMI 1640 medium, and the infected cells were cultured in 10 ml of complete RPMI 1640 medium supplemented with 10 U of interleukin-2 per ml (infection day 0). The infected cells were passaged every 3 to 4 days for a total of 21 days and supplemented once weekly with newly activated PBMCs at a 1:1 ratio. Cells were removed by centrifugation, and the culture supernatant was assayed for p24 Ag by ELISA.

**NERT assay.** Virus stocks were prepared from 293 cells expressing wild-type HIV-1, HIV-1 Δ*tat*, or HIV-1 Δ*tat* complemented with either wild-type or mutant *tat* genes. These stocks were assayed for total RT activity on a synthetic template according to the directions of the manufacturer (Roche Diagnostics, Inc.). For each NERT assay, virus stock containing 0.75 mU of RT activity was supplemented with 10 mM $MgCl_2$ and incubated for 30 min at 37°C with 100 U of DNase I in a final volume of 200 μl of IMDM. Enzymatic activity was terminated in half of the DNase I-treated virus stock by the addition of 150 μl of stop solution (10 mM Tris-HCl [pH 7.4], 10 mM EDTA, 20 μg of sheared salmon sperm DNA per ml, and 50 μg of proteinase K per ml) followed by incubation at 37°C for 10 min and then boiling for 10 min. The remaining 100 μl was supplemented with 50 μM deoxynucleoside triphosphates (dNTPs) and incubated at 37°C for 90 min before the activity was stopped as described above. The stopped reaction mixtures were centrifuged briefly in a microcentrifuge at 14,000 × *g*, and 10 μl of each was assayed for negative-strand strong-stop DNA by 34 cycles of PCR as described except for the addition of 3.5 mM $MgCl_2$ to compensate for EDTA present in the stop mix.

**PCR and RT-PCR analysis.** Analysis of low-molecular weight nucleic acids by PCR was as previously described (36, 38, 78). All HIV-1-specific oligonucleotides are denoted numerically by using the HIV-1 transcription start site as +1 (genomic RNA). Briefly, an oligonucleotide (5′-ATGCAGCGCAAGTAGGT) complementary to the sense strand of the mitochondrial cytochrome *c*-oxidase II (Cyt-OxyII) gene was end labeled to a specific activity of greater than $10^8$ cpm/μg by using T4 polynucleotide kinase (New England BioLabs) and [γ-$^{32}$P]ATP (>7,000 Ci/mmol) (ICN). Hirt lysates were serially diluted in fivefold increments and assayed for Cyt-OxyII levels by 20 cycles of PCR (65°C for 2 min and 93°C for 1 min) with 25 ng of the $^{32}$P-labeled oligonucleotide, 50 ng of an unlabeled oligonucleotide (5′-GGAAAATGATTATGAGGGCGTG) complementary to the antisense strand, 1.5 mM $MgCl_2$, 1× reaction buffer as supplied, and 0.25 U of Platinum *Taq* DNA polymerase (Life Technologies). The PCR products were resolved on 9% polyacrylamide gels, and the dried gels were visualized and analyzed with a Molecular Dynamics PhosphorImager. All samples were assayed within the linear range of the PCR. The Hirt lysates, which were normalized for equivalent Cyt-OxyII levels, were assayed by PCR for HIV-1 reverse transcription products corresponding to negative-strand strong-stop DNA by using $^{32}$P-labeled oligonucleotides complementary to sequences between +96 and +118 (5′-CAAGTAGTGTGTGCCCGTCTGTT, sense) and +182 and +158 (5′-CT GCTAGAGATTTTCCACACTGAC, antisense). Full-length HIV-1 DNA was detected by using 25 ng of $^{32}$P-labeled +96/+118 HIV-1 oligonucleotide and 50 ng of an oligonucleotide complementary to HIV-1 sequences located downstream from the primer-binding site between +242 and +219 (5′-CCTGCGTC GAGAGAGCTCCTCTGG, antisense). PCR products were resolved by 9%

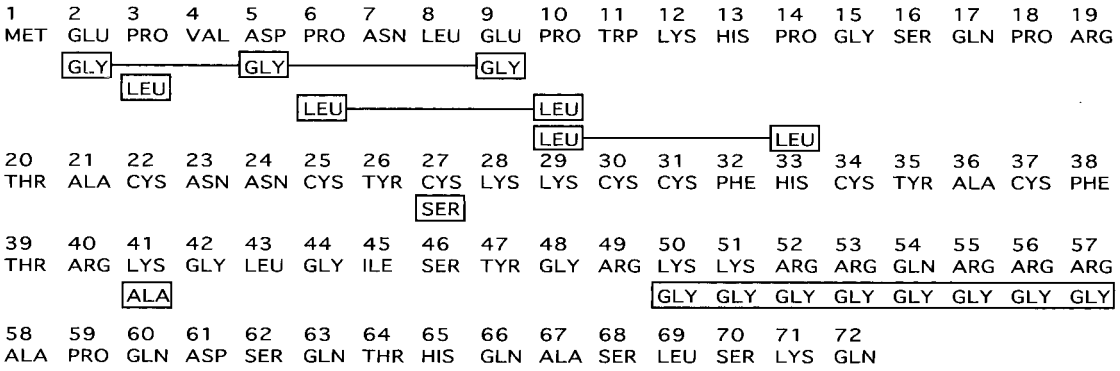


FIG. 1. Schematic of the first exon of the HIV-1 Tat protein. The amino acid changes are shown boxed below the native amino acid sequence. Multiple mutations are indicated by solid lines between boxed amino acids. The mutations in the *tat* gene product which were constructed are [E2G, D5G, E9G], P3L, P[6, 10]L, P[10, 14]L, C27S, K41A, and K/R[50-57]G.

polyacrylamide gel electrophoresis. The gels were dried and analyzed on a Molecular Dynamics PhosphorImager.

RT-PCR to determine the amount of *tat* RNA produced by each of the 293 cell lines was performed on total RNA isolated from 293 stable cell lines by using TriPure reagent (Roche Diagnostics). For each reaction, 10 μg of total RNA, 0.5 μg of an antisense oligonucleotide complementary to β-actin mRNA (BA3, 5′-GGCGTACAGGGACAGCACA), and 0.5 μg of an antisense oligonucleotide complementary to pBK-RSV-directed *tat* mRNA (M13 forward, 5′-GTTTTCC CAGTCACGAC) were heated at 75°C for 15 min and placed on ice. cDNA synthesis reaction mixtures containing the reaction buffer provided, 10 mM dithiothreitol, 2 mM dNTPs, 20 U of RnaseOut (Life Technologies), and 200 U of Moloney murine leukemia virus (M-MLV) RT (Life Technologies) were incubated at 37°C for 1 h. Each reaction mixture was serially diluted in fivefold increments and assayed by PCR with 100 ng of BA3 and 100 ng of a β-actin sense primer, BA4 (5′-GGCGTACAGGGACAGCACA). PCR was performed for 25 cycles at 53, 72, and 94°C for 1 min at each temperature, and the DNA products were resolved on a 1.5% agarose gel. The cDNA reaction mixtures were normalized to β-actin mRNA levels and then assayed for pBK-RSV *tat* cDNA by PCR with a nested *tat* primer, TA3 (5′-AGATCTATACACTCGCACGCC, antisense), and a primer complementary to vector sequences (5′-AGCGGATAA CAATTTCACACAGGA, sense) for 35 cycles at 50, 72, and 94°C for 1 min at each temperature. The products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized on a UV transilluminator.

The positive control $tRNA_3^{Lys}$ plasmid was linearized with the restriction enzyme *Nsi*I. In vitro-synthesized RNA was obtained by using T7 RNA polymerase, treated with RQ-DNase I, and gel purified. Also, an HIV-1 DNA fragment that contained sequences from −22 to +517, and a deletion of sequences from +80 to +151, was ligated into pGem4z (Promega). This was linearized with *Eco*RI, and in vitro-transcribed RNA was made by using T7 AmpliScribe reagents (Epicentre Technologies), treated with RQ-DNase I, and gel purified.

To detect $tRNA_3^{Lys}$ incorporation into virions, DNase I-treated virus stocks of either the wild-type, Δ*tat*, or Δ*tat* complemented viruses were subjected to centrifugation at 22,000 × *g* for 90 min and resuspended in 1× PBS–1% bovine serum albumin (BSA) (PBS-BSA). The viral suspensions were assayed for p24 Ag and RT activity. Exactly 100 ng of p24 Ag of each virus was extracted by using TRIzol reagent (Life Technologies) according to the manufacturer's recommendations. Nucleic acids were precipitated overnight (−20°C) and recovered by centrifugation at 15,000 × *g* at 4°C for 60 min. A visible pellet was washed with 70% ethanol and centrifuged as before, and the pellet was resuspended in 30 μl of TE (10 mM Tris-HCl [pH 7.8], 0.1 mM EDTA). Total HeLa cell RNA (7.5 μg) and an in vitro-transcribed $tRNA_3^{Lys}$ molecule (0.5 μg) were used as positive controls. Duplicate reactions with 5 μl of each viral RNA and the positive control were set up and included 20 U of RNasin (Promega), 0.5 μl of dimethyl sulfoxide, 50 ng of either a $tRNA_3^{Lys}$-specific antisense oligonucleotide (5′-TGGCGC CCGAACAGGGACTTGA) or an HIV-1-specific antisense oligonucleotide (5′-CCTGCGTCGAGAGAGCTCCTCTGG), and 3 μl of $H_2O$. These reaction mixtures were heated to 75°C for 10 min and placed on ice. In vitro reverse transcription reactions were performed in the presence and absence of avian myeloblastosis (AMV) RT (Promega) with buffers provided by the manufacturer plus 0.2 mM dNTPs at 42°C for 1 h followed by 72°C for 5 min. The reverse transcription reactions were amplified by PCR with primer pairs specific for $tRNA_3^{Lys}$ (5′-ATAGCTCAGTCGGTAGAGCAT [sense] and 5′-GCCGAACA GGGACTTGAT [antisense]) and HIV-1 genomic RNA (5′-CAAGTAGTGTG TGCCCGTCTGTT [sense] and 5′-CGAGAGAGCTCCTCTGGTTCTAC [antisense]). The PCR products were separated on a 9% polyacrylamide gel matrix in 1× Tris-borate-EDTA, dried, and quantitated on a Molecular Dynamics PhosphorImager.

Similarly, filtered viral supernatants containing wild-type, Δ*tat*, or complemented Δ*tat* viruses were treated with 300 U of DNase I, and the virus particles were pelleted through 20% sucrose at 75,000 × *g* for 2 h. The pellets were suspended in 0.5 ml of PBS-BSA. The viral suspensions were assayed for p24 Ag. Supernatant containing 60 ng of p24 Ag was treated with TriPure reagent (Roche Diagnostics), 0.5 pg of in vitro-synthesized HIV-1 RNA was added, and total virion RNA was isolated according to the manufacturer's recommendations. Total viral RNA was annealed to an oligonucleotide (5′-GACTGCGAATCGT TCTAG-3′, antisense) complementary to sequences in the *gag* open reading frame at 75°C for 10 min and placed on ice, and cDNA was made by using the supplied buffers, 0.2 mM dNTPs, and M-MLV RT (Life Technologies) at 37°C for 60 min. Each cDNA reaction was assayed by PCR for the internal control (IC) cDNA (reverse transcribed from IC RNA) by using a $^{32}$P-labeled oligonucleotide specific for pGem4z sequences (5′-GGGAGACAAGCTTGCATGCC TG, sense) and an unlabeled HIV-1-specific oligonucleotide (5′-GCAGTGGG TTCCCTAGTTAGC, antisense) for 25 cycles at 93°C for 1 min and 65°C for 2 min. The normalized cDNA reaction mixtures were serially diluted and assayed for HIV-1 DNA by using HIV-1-specific primers ($^{32}$P-labeled +96/+118 and unlabeled +182/+158) for 30 cycles with the same cycling parameters. The PCR DNA products were separated on a 9% polyacrylamide gel, dried, and visualized and quantitated on a PhosphorImager (Molecular Dynamics).

## RESULTS

**Isolation of clonal 293 cell lines containing *tat* deletion HIV-1 and stably transfected wild-type or mutant *tat* genes.** We previously demonstrated that *tat* was required for efficient HIV-1 reverse transcription (34), in addition to its well-characterized role in activating HIV-1 gene expression (reviewed in references 29 and 46). Transient transfection of a wild-type *tat* gene into cells containing an integrated HIV-1 provirus with a *tat* gene deletion produced virus that was fully competent for reverse transcription upon infection of PBMCs. Several *tat* mutants which were defective in activating HIV-1 gene expression were also unable to complement HIV-1 reverse transcription, while *tat* genes that stimulated high levels of HIV-1 gene expression correlated with efficient reverse transcription. Thus, it was important to address whether we could identify *tat* mutants that were defective in transactivation yet were able to stimulate HIV-1 reverse transcription.

To determine whether we could separate these functions of *tat*, we used a panel of mutated *tat* genes that were defective for the activation of viral gene expression (Fig. 1). These included mutants coding for substitutions of three acidic residues in the amino terminus ([E2G, D5G, E9G]), a mutation of proline residue 3 to leucine (P3L), a mutation of proline residues 6 and 10 or 10 and 14 to leucine residues (P[6, 10]L or P[10, 14]L), a mutation of cysteine residue 27 to serine (C27S), a mutation of a lysine residue 41 to alanine (K41A), and replacement of basic amino acids extending from positions 50 to 57 by glycine (K/R[50-57]G). The *tat* genes containing each

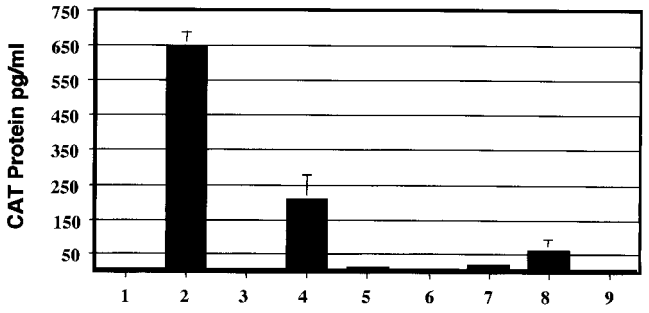


FIG. 2. Activation of HIV-1 gene expression by Tat. HeLa cells were co-transfected with the reporter constructs HIV-1 LTR-CAT and pCH110 (β-Gal) together with plasmids (pDex) that expressed either the β-globin gene (bar 1), the wild type *tat* gene (bar 2), or the mutated *tat* genes corresponding to [E2G, D5G, E9G] (bar 3), P3L (bar 4), P[6, 10]L (bar 5), P[10, 14]L (bar 6), C27S (bar 7), K41A (bar 8), and K/R[50-57]G (bar 9). The cells were harvested at 48 h posttransfection, and equal amounts of protein were normalized to β-Gal activity and assayed for CAT protein by ELISA. The transfections were performed three times with the standard deviations indicated.

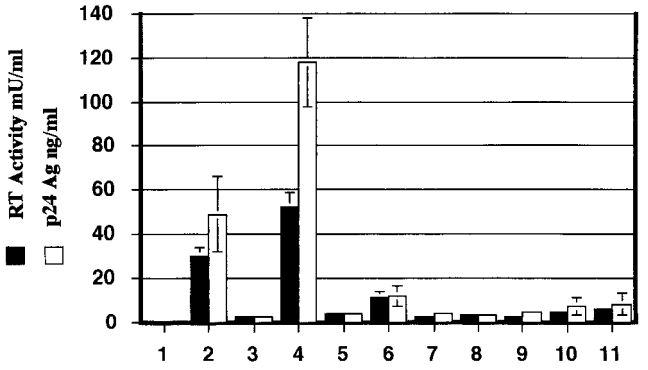


FIG. 4. Analysis of HIV-1 gene expression from 293 cells. Culture supernatants were obtained from either 293 cells (bars 1), 293 cells stably infected with HIV-1 wild-type virus (bars 2), 293 cells infected with an HIV-1 Δ*tat* virus (bars 3), or the Δ*tat* cell line stably transfected with pBK-RSV containing the wild-type *tat* gene (bars 4) or the mutated *tat* genes corresponding to [E2G, D5G, E9G] (bars 5), P3L (bars 6), P[6, 10]L (bars 7), P[10, 14]L (bars 8), C27S (bars 9), K41A (bars 10), and K/R[50-57]G (bars 11). The amounts of p24 Ag and reverse transcriptase activity in each virus stock were determined as described in Materials and Methods. The data from four to six independent virus stocks were averaged and the standard deviation for each assay indicated.

of these mutations or a rabbit β-globin gene were inserted downstream of the RSV promoter and transfected into HeLa cells together with HIV-1 LTR-CAT and simian virus 40–β-Gal reporter constructs. At 48 h posttransfection, CAT production was measured by an ELISA with extracts normalized for β-Gal activity. HeLa cells transfected with the HIV-1 LTR-CAT reporter alone (Fig. 2, bar 1) demonstrated low levels of CAT protein, while wild-type *tat* increased the level of CAT protein to 650 pg/ml (Fig. 2, bar 2). The mutation P3L resulted in a threefold decrease in *tat* stimulation (Fig. 2, bar 4), while the mutation K41A resulted in a 10-fold reduction in *tat* stimulation of CAT levels (Fig. 2, bar 8). The levels of CAT protein produced in the presence of the remaining mutants were either at the threshold of detection for the assay (Fig. 2, bars 5 and 7) or below the level of detection (Fig. 2, bars 3, 6, and 9). Similar results were seen in three independent experiments.

**Characterization of 293 cell lines containing *tat* mutants.** Expression vectors containing each of the different *tat* genes were transfected into 293 cells containing the HIV-1 Δ*tat* provirus. Stable cell lines containing both HIV-1 Δ*tat* and the *tat* expression vectors were obtained by G418 selection as previously described (33). The 293 cell lines were then assayed for p24 Ag levels by ELISA and for plasmid-derived *tat* mRNA by RT-PCR analysis. Western blot analysis of Tat protein from extracts prepared from stably transfected cell lines indicated that Tat was produced at levels of less than 10 ng per sample (32a). No cDNA product for *tat* was observed with RNA obtained from parental 293 cells or 293 cells containing the Δ*tat* provirus or wild-type virus (Fig. 3A, lanes 1 to 3). In contrast, similar levels of plasmid-derived *tat* mRNA were present in 293 cells containing the HIV-1 Δ*tat* provirus and either wild-type *tat* (Fig. 3A, lane 4) or each of the *tat* mutants (Fig. 3A, lanes 5 to 11). PCR analysis of β-actin cDNA levels indicated that the amounts of RNA in all cDNA synthesis reaction mixtures were similar (Fig. 3B, lanes 1 to 11). No cDNA products were detected in RNA samples produced in the absence of added M-MLV RT (Fig. 3C and D). Following PCR analysis, the *tat* cDNA product was isolated and sequenced. In each case, the *tat* genes contained the expected nucleotide changes (data not shown). Chromosomal DNA obtained from each 293 cell line was also subjected to PCR to obtain HIV-1 proviral DNA and to confirm by sequencing that each cell line contained the HIV-1 with a deleted *tat* gene (data not shown).

Viral supernatant was obtained from each cell line and assayed for RT activity (Fig. 4). The 293 cells containing the wild-type HIV-1 produced RT and p24 Ag levels of 30 mU/ml and 50 ng/ml, respectively (Fig. 4, bars 2), while the 293 cells

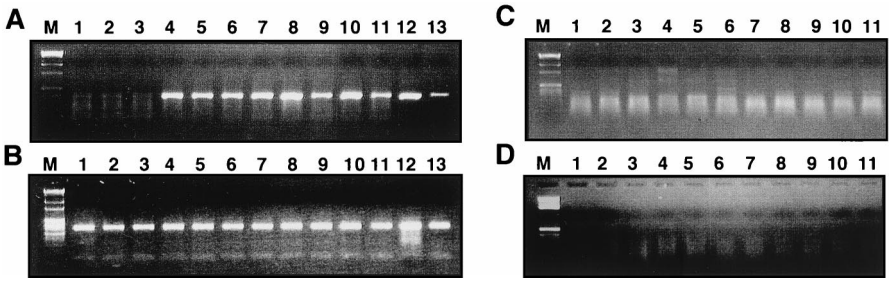


FIG. 3. RT-PCR analysis of wild-type and mutant *tat* genes. Total RNA was obtained from uninfected 293 cells (lanes 1), 293 cells stably transfected with HIV-1 wild-type (lanes 2) or HIV-1 Δ*tat* (lanes 3), and 293 cells containing both HIV-1 Δ*tat* and wild type *tat* (lanes 4) or the mutated *tat* genes corresponding to [E2G, D5G, E9G], P3L, P[6, 10]L, P[10, 14]L, C27S, K41A, and K/R[50-57]G (lanes 5 to 11, respectively). Primers specific for plasmid-derived *tat* mRNA or cellular β-actin mRNA were annealed to RNA obtained from each of the 293 cell lines, and a reverse transcription reaction was performed in the presence (A and B) or absence (C and D) of M-MLV RT. PCR was performed on each cDNA reaction mixture to detect either the *tat* (A and C) or β-actin (B and D) gene. PCR products were resolved on a 1.5% agarose gel. Molecular mass markers are shown for each gel (lanes M). PCRs with a plasmid containing the *tat* gene (panel A, lanes 12 and 13) (equivalent to 0.1 and 0.5 pg) or serially diluted β-globin cDNA (panel B, lanes 12 and 13) are shown.

TABLE 1. Replication kinetics of wild-type and Δ*tat* HIV-1[a]

| Day | p24 Ag (pg/ml) with the following virus: | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Wild type | Δ*tat* | *tat* wild type | [E2G, D5G, E9G] | P3L | P[6, 10]L | P[10, 14]L | C27S | K41A | K/R[50-57]G | Mock |
| 0 | 5 | 15 | 20 | 30 | 20 | 15 | 20 | 20 | 20 | 15 | 0 |
| 3 | 150 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | 750 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 | 3,598 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 14 | 48,763 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 17 | 24,851 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 21 | 36,891 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a] Activated PBMCs were infected with virus stocks containing 60 mU of RT activity. The cells were washed and cultured as described in Materials and Methods, and a sample of culture supernatant was removed and assayed for p24 Ag (day 0) by ELISA. The cells were split 1:2 twice weekly, and an aliquot of the supernatant was assayed for p24 Ag. All infections were supplemented with uninfected, activated PBMCs weekly. Each infection was repeated twice with independent virus stocks.

harboring the Δ*tat* provirus had 1 mU of RT per ml and 1.2 ng of p24 Ag per ml (Fig. 4, bars 3). Stable transfection of wild-type *tat* into 293 cells containing HIV-1 Δ*tat* increased expression of RT and p24 Ag levels 50- and 100-fold, respectively (Fig. 4, bars 4). Clonal 293 cell lines containing the different *tat* genes produced levels of RT and p24 Ag that were slightly greater than those of the parental 293 cell line containing HIV-1 Δ*tat*. The *tat* mutants [E2G, D5G, E9G], P[6, 10]L, P[10, 14]L, and C27S resulted in RT and p24 Ag levels that were 1.5- to 3-fold greater than those of the parental HIV-1 Δ*tat* cell line (Fig. 4, bars 5, 7, 8, and 9). The proline mutant P3L increased HIV-1 gene expression approximately 11-fold, while the *tat* mutants K41A and K/R[50-57]G increased RT and p24 Ag 4- and 6-fold, respectively (Fig. 4, bars 6, 10, and 11). This data represents assays performed on four to six independent virus stocks from each 293 cell line. The increase in the amount of HIV-1 produced in the presence of each *tat* gene correlated with the abilities of these different genes to transactivate the HIV-1 LTR in transient assays of *tat* activity (Fig. 2).

Next, we assayed the replication of the HIV-1 Δ*tat* viruses produced in the 293 cell lines containing the different *tat* mutants. Activated PBMCs were infected with cell-free virus containing equivalent amounts of RT activity for wild-type HIV-1, HIV-1 Δ*tat*, or HIV-1 Δ*tat* complemented with either the wild-type or each of the mutated *tat* genes. The virus was removed from the infected cells at 5 h postinfection, and the PBMCs were cultured and monitored for p24 Ag production for 3 weeks. Small quantities of p24 Ag were present in the PBMCs due to residual virus remaining from the initial infection of HIV-1 Δ*tat* produced in the presence of the different *tat* mutants. However, only wild-type HIV-1 produced in the 293 cell lines was able to efficiently replicate in PBMCs. No p24 Ag was detected in any of the other cultures (the limit of detection was 10 pg of p24 Ag per ml) after 21 days postinfection (Table 1). These results indicate that no detectable recombination had occurred between the stably transfected *tat* genes and the HIV-1 provirus.

**The amino terminus of Tat is critical for modulating HIV-1 reverse transcription.** By using similar quantities of HIV-1 virion-associated RT activity, activated PBMCs were infected with either wild-type HIV-1, HIV-1 Δ*tat*, or HIV-1 Δ*tat* produced from 293 cell lines stably expressing wild-type *tat* or each of the mutated *tat* genes. Nucleic acids were isolated by Hirt lysis at 2 h (Fig. 5A) and 24 h (Fig. 5B and C) postinfection of PBMCs. PCR analysis of the reverse transcription products corresponding to negative-strand strong-stop HIV-1 DNA (Fig. 5A and B) or full-length HIV-1 DNA (Fig. 5C) was performed. HIV-1 Δ*tat* was very defective for reverse transcription, resulting in a 10- to 30-fold reduction in the levels of both negative-strand strong-stop DNA and full-length cDNA (Fig. 5B and C, lanes 3) compared to wild-type HIV-1 (Fig. 5B and C, lanes 2). The reverse transcription defect in HIV-1 Δ*tat* was fully restored by complementation of the 293 cell lines producing this virus with wild-type *tat* (Fig. 5B and C, lanes 4) or a *tat* mutant causing a mutation at proline residue 3 (Fig. 5B and C, lanes 6). Other mutants with mutations in the amino terminus of Tat ([E2G, D5G, E9G], P[6, 10]L, and P[10, 14]L) did not increase either negative-strand strong-stop DNA synthesis (Fig. 5B, lanes 5, 7, and 8) or full-length DNA synthesis (Fig. 5C, lanes 5, 7, and 8).

In contrast to the inability of the majority of the amino-

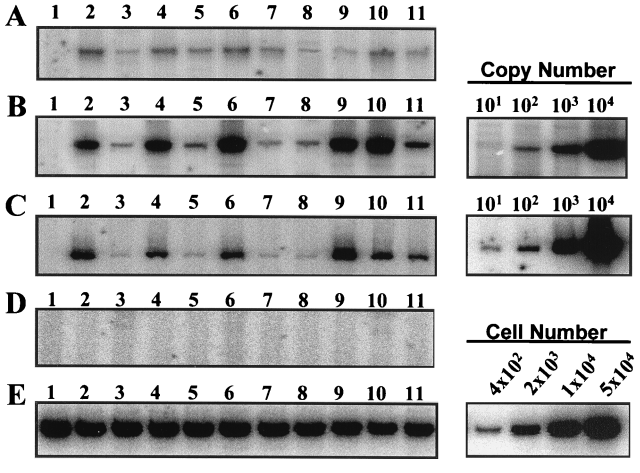


FIG. 5. Reverse transcription of HIV-1 lacking *tat*. Activated PBMCs were infected for 2 h with culture supernatant from transfected 293 cells containing 90 mU of RT activity for either wild-type HIV-1 (lanes 2), Δ*tat* virus (lanes 3), or virus produced from 293 HIV-1 Δ*tat* cells stably transfected with an RSV expression vector containing the wild-type *tat* gene (lanes 4) or the mutated *tat* genes corresponding to [E2G, D5G, E9G], P3L, P[6, 10]L, P[10, 14]L, C27S, K41A, and K/R[50-57]G (panels A to C and E, lanes 5 to 11, respectively), and mock supernatant (panels A to C and E, lanes 1). PBMCs were infected with aliquots of the same viruses that were heat inactivated at 60°C (D). At 2 h postinfection, residual virus was removed and Hirt lysates were prepared from half of the infected cells, while the remaining PBMCs were cultured for 24 h before Hirt lysates were prepared. The recovered nucleic acids were assayed for HIV-1 negative-strand strong-stop DNA in 2-h (A) and 24-h (B) lysates and for full-length DNA in 24-h lysates (C) by quantitative PCR. PCR analysis of the Cyt-OxyII content in Hirt lysates was used to standardize the DNA recovery (E). All PCRs were performed within the linear range of the assay as determined by assays of HIV-1 DNA copy number (10, $10^2$, $10^3$, and $10^4$) or cell number ($4 \times 10^2$, $2 \times 10^3$, $1 \times 10^4$, and $5 \times 10^4$). This analysis is representative of PCRs performed for four separate infections with independently prepared virus stocks.

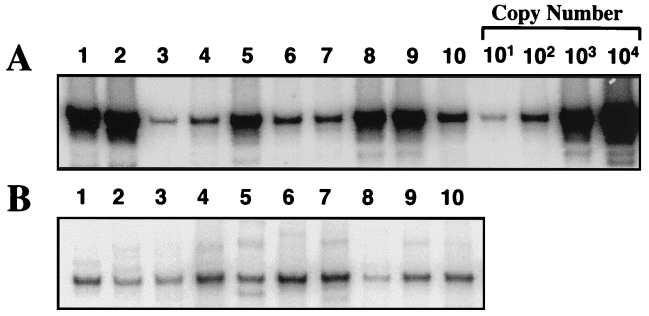


FIG. 6. NERT assay for HIV-1 wild-type and *tat* mutant viruses. Virus stocks for wild-type virus (lanes 1), Δ*tat* virus *trans*-complemented with wild-type *tat* (lanes 2), Δ*tat* virus (lanes 3), or Δ*tat* virus produced in the presence of *tat* mutants [E2G, D5G, E9G], P3L, P[6, 10]L, P[10, 14]L, C27S, K41A, and K/R[50-57]G (lanes 4 to 10, respectively) were analyzed for endogenous reverse transcription. Culture supernatant (200 μl) containing approximately 0.75 mU of RT activity was treated with 100 U of DNase I. Half of each reaction mixture was added to 150 μl of stop solution, incubated at 37°C for 10 min, and then boiled for 10 min (B). The remaining half of each reaction mixture was supplemented with 50 μM dNTPs and incubated at 37°C for 90 minutes before the reaction was terminated as described above. (A) PCR to detect HIV-1 negative-strand strong-stop DNA was performed on NERT reaction mixtures as described in Materials and Methods. All PCRs were performed within the linear range of the assay as determined by assays of HIV-1 DNA copy number (10, $10^2$, $10^3$, and $10^4$).

terminal Tat mutants to stimulate gene expression (Fig. 2, bars 3, 4, and 6, and 4, bars 5, 7, and 8) or complement HIV-1 reverse transcription, mutants containing replacements of cysteine residue 27 or lysine residue 41 were able to restore HIV-1 reverse transcription to levels seen with wild-type *tat* (Fig. 5B and C, lanes 9 and 10). A Tat mutant which has glycine substituted for basic amino acids 50 to 57, which was defective in Tat transcriptional activation, resulted in a four- to eightfold increase in the synthesis of negative-strand strong-stop DNA. These results were consistent for four to six independent virus stocks and suggest that the ability of *tat* to efficiently initiate HIV-1 reverse transcription is largely dependent on an intact Tat amino terminus. There is an additional requirement for the basic domain of Tat to fully complement HIV-1 reverse transcription. Surprisingly, amino acid residues within the Tat cysteine and core domains that are necessary for *tat*-mediated activation of HIV-1 gene expression are not required for *tat* stimulation of HIV-1 reverse transcription. We observed similar patterns of reverse transcription complementation in PBMCs infected with virus stocks produced by transient expression of these genes into 293 Δ*tat* cells, although there was some variability in the overall degree of complementation (data not shown).

**Role of Tat in endogenous HIV-1 reverse transcription.** NERT assays were performed as described previously (34). HIV-1 supernatants containing equal amounts of RT were incubated with 50 μM dNTPs in the presence of DNase I, and each of the viruses was then assayed by PCR for the synthesis of negative-strand strong-stop DNA. Both wild-type HIV-1 and HIV-1 Δ*tat* complemented with wild-type *tat* resulted in 30- to 60-fold more negative-strand strong-stop DNA than seen with HIV-1 Δ*tat* virus alone (Fig. 6, lanes 1 to 3). HIV-1 produced in the presence of amino-terminal mutations of Tat (Fig. 6, lanes 4, 6, and 7) synthesized only three- to fivefold more negative-strand strong-stop DNA than HIV-1 Δ*tat* virus. In contrast, Tat mutants with mutations of proline 3 (Fig. 6, lanes 5), cysteine 27 (Fig. 6, lanes 8), or lysine 41 (Fig. 6, lanes 10) resulted in 20- to 35-fold more negative-strand strong-stop DNA than HIV-1 Δ*tat*. The Tat basic mutant, K/R[50-57]G, (Fig. 6, lanes 10) resulted in approximately 15-fold more negative-strand strong-stop DNA than HIV-1 Δ*tat* (Fig. 6, lanes 3). PCR analysis of molecular standards indicated that all reactions were performed within the linear range of the assay. These NERT assays coupled with our in vivo data indicate a critical role for the amino terminus of Tat in the efficient initiation of HIV-1 reverse transcription. Surprisingly, this effect is not dependent upon cysteine residue 27 or lysine residue 41, both of which are important for Tat-mediated transactivation of HIV-1 transcription.

**Tat-associated kinases CDK7 and CDK9 do not complement reverse transcription defects associated with HIV-1 Δ*tat* virions.** It has been demonstrated that the HIV-1 Tat protein specifically interacts with and activates cyclin-dependent kinases (15, 28, 55, 76, 79) to phosphorylate the C-terminal domain of RNA polymerase II and increase HIV-1 gene expression. Several mutants with mutations in the Tat activation domain, which interacts with cellular kinases to stimulate HIV-1 transcription, were unable to complement the reverse transcription defect in HIV-1 Δ*tat* virions. Thus, it is possible that Tat may interact with a cellular kinase to stimulate reverse transcription. Therefore, we assayed the ability of overexpression of Tat-associated kinases CDK7 and CDK9 to complement the reverse transcription defects seen with the HIV-1 Δ*tat* virions. The 293 cell lines expressing HIV-1 Δ*tat* or wild-type HIV-1 were transiently transfected with expression vectors containing either wild-type cdk7, cdk9, or a control cdc5. Tat does not require cdc5 to activate HIV-1 gene expression. Cell-free supernatants were obtained from 293 cells producing HIV-1 Δ*tat* in the presence or absence of each of these kinases or wild-type *tat*. Equal amounts of 293 viral supernatants were used to infect PBMCs. Hirt lysates were processed after 24 h and assayed for negative-strand strong-stop DNA synthesis. Neither the Tat-associated kinases nor the unrelated cdc5 were able to complement the reverse transcription defects (Fig. 7, lanes 3 to 5) as compared to the results with wild-type *tat* (Fig. 7, lanes 1). There was no change in the amount of negative-strand strong-stop DNA synthesized in PBMCs infected with wild-type HIV-1 produced in the presence or absence of these constructs (Fig. 7, lanes 6 to 10).

**Virion genomic RNA levels are not altered in Δ*tat* viruses.** To determine whether the defects in reverse transcription were due to alterations in HIV-1 RNA encapsidation, we performed RT-PCR on RNA obtained from partially purified virions. We had previously used a first-strand cDNA primer that recognized sequences between the primer-binding site and 5′ splice donor site, and we saw no differences in encapsidated RNA (34). In this analysis, we used a first-strand primer directed at sequences located downstream of the Gag initiating methionine (Fig. 8C). Wild-type, Δ*tat*, or complemented Δ*tat* viruses were pelleted through 20% sucrose, suspended in PBS-BSA, and assayed for p24 Ag and RT. Total virion RNA, along with 0.5 pg of an exogenously added IC RNA, was isolated from equivalent amounts of each virus. cDNA was synthesized from the isolated viral RNA and assayed by using PCR primers that could discriminate between HIV-1 cDNA and IC cDNA (Fig. 8C). Each cDNA reaction mixture was serially diluted in fivefold increments and subjected to 30 cycles of PCR as described in Materials and Methods. Equivalent amounts of HIV-1 cDNA were detected for Δ*tat* virus (Fig. 8A, lanes 1 to 3), Δ*tat* virus complemented with [E2G, D5G, E9G] (Fig. 8A, lanes 4 to 6), Δ*tat* virus complemented with wild-type *tat* (Fig. 8A, lanes 7 to 9), and wild-type virus (Fig. 8A, lanes 10 to 12). No products were observed with mock cDNA (Fig. 8A, lanes 13 to 16) or in reactions performed without M-MLV RT (data not shown). Our analysis showed that other complemented Δ*tat* viruses also had wild-type levels of genomic RNA (data not

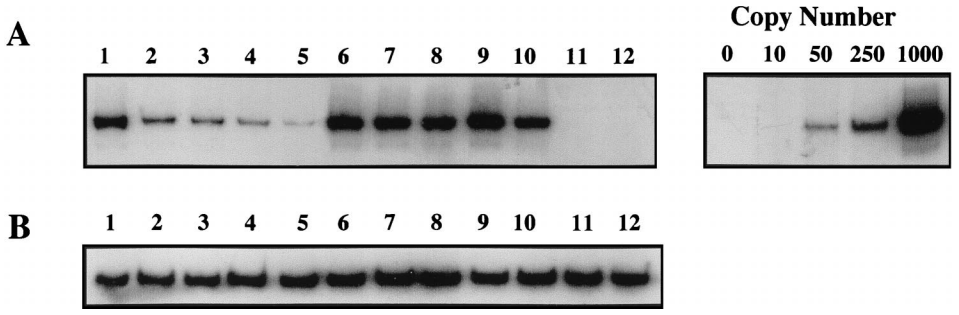


FIG. 7. Cyclin-dependent kinases do not complement reverse transcription defects associated with Δ*tat* viruses. (A) Viral supernatants from 293 cells producing Δ*tat* virus (lanes 1 to 5) or wild-type HIV-1 (lanes 6 to 10) following transfection of wild-type *tat* (lanes 1 and 6), an empty RSV expression vector (lanes 2 and 7), a wild-type cdk7 expression vector (lanes 3 and 8), a wild-type cdk9 expression vector (lanes 4 and 9), a wild-type cdc5 expression vector (lanes 5 and 10), mock supernatant (lane 11), or heat-inactivated wild-type HIV-1 (lane 12) were used to infect $5 \times 10^6$ activated PBMCs. At 2 h postinfection, residual virus was removed by washing, and Hirt lysates were prepared at 24 h postinfection. The recovered nucleic acids were assayed for HIV-1 negative-strand strong-stop DNA. (B) Quantitative PCR analysis of Cyt-OxyII content in Hirt lysates was used to standardize the DNA recovery. All PCRs were performed within the linear range as determined by assays of HIV-1 DNA copy number (0, 10, 50, 250, and 1,000). This analysis is representative of PCRs performed for three separate HIV-1 infections with independently prepared virus stocks.

shown). Finally, PCR analysis of the same cDNA reactions for IC cDNA in either the presence (Fig. 8B, odd-numbered lanes) or absence (Fig. 8B, even-numbered lanes) of M-MLV RT showed that both the RNA recoveries and cDNA synthesis efficiencies were similar in all reactions (Fig. 8B, lanes 1, 3, 5, 7, and 9). Molecular standards showed that the reactions were performed within the linear range of the assays (Fig. 8A, lanes 16 to 20, and B, lanes 11 to 14). These experiments are in agreement with our previous study and indicate that *tat* does not effect genomic RNA packaging.

**$tRNA_3^{Lys}$ is equally incorporated into wild-type, Δ*tat*, and Δ*tat* complemented viruses.** To determine whether the defect

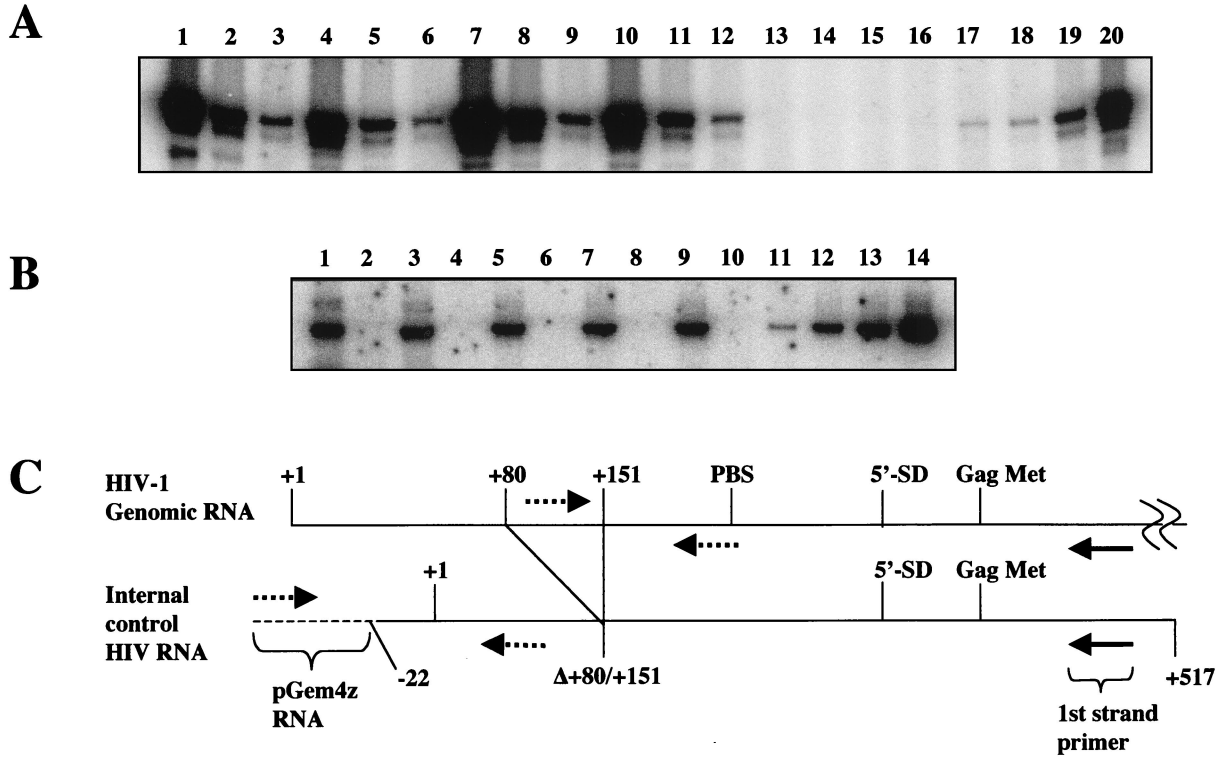


FIG. 8. Analysis of genomic RNA packaging. (A) Supernatants containing wild-type virus (lanes 10 to 12), Δ*tat* virus (lanes 1 to 3), or Δ*tat* virus complemented with wild-type *tat* (lanes 7 to 9) or [E2G, D5G, E9G] (lanes 4 to 6) or mock complemented (lanes 13 to 16) were pelleted through 20% sucrose and suspended in PBS-BSA buffer. An IC RNA was added to purified virus that contained 100 ng of p24 Ag, and both RNAs were copurified. cDNA reactions were performed in either the presence or absence of M-MLV with a first-strand primer that annealed to sequences located downstream from the Gag initiating methionine shown in panel C. The cDNA was serially diluted in fivefold increments and assayed by PCR for HIV-1 DNA with primers indicated in panel C. PCRs were performed on HIV-1 DNA present at 0, $10^1$, $10^2$, $10^3$, and $10^4$ copies (lanes 16 to 20). (B) The RNA recovery and cDNA synthesis were similar for each cDNA reaction corresponding to Δ*tat* (lanes 1 and 2), Δ*tat* plus [E2G, D5G, E9G] (lanes 3 and 4), Δ*tat* plus wild-type *tat* (lanes 5 and 6), wild-type virus (lanes 7 and 8), and mock virus (lanes 9 and 10). IC RNA was reverse transcribed in either the presence (lanes 1, 3, 5, 7, and 9) or absence (lanes 2, 4, 6, 8, and 10) of M-MLV and detected by PCR with the primers shown in panel C (dotted lines). IC plasmid DNA standards present at 20, 100, 300, and 1,000 copies are shown (lanes 11 to 14). (C) Model showing HIV-1 RNA and IC RNA. An internal deletion from +80 to +151 in IC RNA allows detection of IC cDNA from HIV-1 cDNA by PCR with the indicated primers. Solid arrow, first-strand cDNA primer; dotted arrows, PCR primers; dotted line, pGem4Z RNA; solid line, HIV-1 RNA.

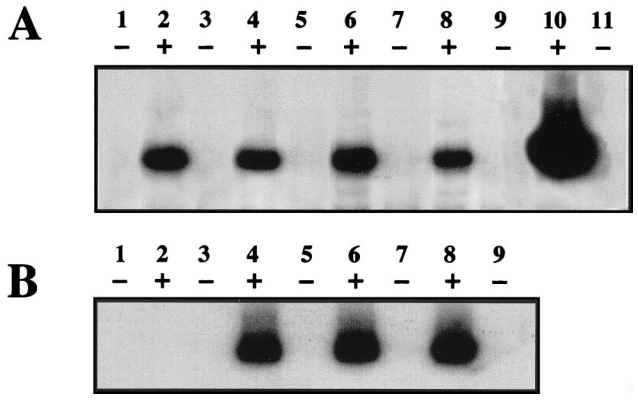


FIG. 9. Analysis of $tRNA_3^{Lys}$ packaging in wild-type and *tat* mutant viruses. RNA was extracted from pelleted virus that contained 100 ng of p24 Ag, and cDNA was synthesized in the presence (+) or absence (−) of AMV RT primed with an antisense oligonucleotide that hybridized to either the 3′-terminal 18 nucleotides of the $tRNA_3^{Lys}$ molecule (A) or HIV-1 sequences extending from +242 to +219 (B). (A) $tRNA_3^{Lys}$ cDNA was detected by PCR with primers that hybridize to internal $tRNA_3^{Lys}$ sequences. Total HeLa cell RNA (lanes 1 and 2) or wild-type HIV-1 (lanes 3 and 4), Δ*tat* virus (lanes 5 and 6), and Δ*tat* virus produced following transfection with a wild-type *tat* expression vector (lanes 7 and 8) contain similar amounts of $tRNA_3^{Lys}$. An in vitro-transcribed $tRNA_3^{Lys}$ molecule was added as a positive control for the reactions (lanes 10 and 11). A PCR-negative control is shown in lane 9. (B) As a control for virus load, HIV-1 cDNA was detected by PCR with a nested antisense primer corresponding to HIV-1 sequences +236 to +214 and a sense primer corresponding to +96 to +118 for HeLa cells (lanes 1 and 2), wild-type HIV-1 (lanes 3 and 4), Δ*tat* virus (lanes 5 and 6), or Δ*tat* virus produced following transfection with a wild-type *tat* expression vector (lanes 7 and 8). A negative PCR control is shown in lane 9.

in HIV-1 reverse transcription in the absence of *tat* was due to a reduction in the packaging of $tRNA_3^{Lys}$, RT-PCR analysis was performed with total RNA isolated from equal amounts of HIV-1 wild-type, Δ*tat*, and Δ*tat* virions produced in the presence of a wild-type *tat* gene. First-strand synthesis was performed with a primer specific for the 3′ tail of the $tRNA_3^{Lys}$ molecule in the presence (Fig. 9, even-numbered lanes) or absence (Fig. 9, odd-numbered lanes) of AMV RT. PCR analysis was then performed with a primer pair specific for the $tRNA_3^{Lys}$ molecule, one primer of which was $^{32}P$ labeled, as described in Materials and Methods. There was no difference in the relative amounts of tRNA for either wild-type or Δ*tat* virions produced in either the presence or absence of a wild-type *tat* gene (Fig. 9A, lanes 3 to 8). Total HeLa cell RNA and an in vitro-synthesized $tRNA_3^{Lys}$ molecule were used as positive controls for the RT-PCRs (Fig. 9A, lanes 1, 2, 10, and 11). As a control for viral RNA recovery, PCR analysis was performed for full-length HIV-1 RNA with the same RNA samples (Fig. 9B) and primers that detect HIV-1 genomic RNA. These results suggest that *tat* does not play a role in the packaging of the $tRNA_3^{Lys}$ primer into HIV-1 virions and support our previous observations that there are no gross biochemical abnormalities in virions produced in the absence of *tat*.

## DISCUSSION

Previously, we demonstrated that *tat* plays a role in early steps in the HIV-1 life cycle, specifically in the process of reverse transcription (34). In the studies outlined here, we employed a panel of *tat* mutants that included activation domain substitution mutants, basic domain substitution mutants, and a variety of point mutants to determine whether we could identify *tat* mutants that could complement reverse transcription but not viral gene expression. We assayed these *tat* mutants for their effects on both viral gene expression and ability to complement reverse transcription defects associated with HIV-1 Δ*tat*. Several *tat* mutants in the amino terminus of Tat were unable to support HIV-1 gene expression or reverse transcription. In contrast, several mutants causing mutations in the activation, core, and the basic domains of Tat, which were defective for viral transcription, were able to complement the reverse transcription defects associated with Δ*tat* virions. Thus, complementation of reverse transcription defects in Δ*tat* virus by exogenously added wild-type *tat* is not simply the result of *tat*-modulated increases in HIV-1 gene expression. No differences in the p24 Ag/RT ratios or the amounts of genomic RNA or $tRNA_3^{Lys}$ packaged into virions in the presence or absence of a functional *tat* gene were observed, indicating that these virions were biochemically similar (32).

Since the Tat basic domain substitution mutant and the TAR RNA bulge mutant (35) exhibited little or no defects in the synthesis of negative-strand strong-stop DNA, it is likely that the defects in reverse transcription seen with Δ*tat* virus (34) are due to a process that can be separated from Tat binding to TAR RNA. The fact that overexpression of the cyclin-dependent kinases CDK9 and CDK7, which are believed to be essential for Tat-mediated transcriptional activation (14, 15, 28, 55, 76), had no effect on the process of reverse transcription further serves to distinguish the role of Tat in reverse transcription from its role in transcription. However, these experiments do not rule out the possibility that Tat and TAR RNA might interact with additional viral and/or cellular factors and form a distinct reverse transcription initiation complex.

It is possible that Tat may interact with other cellular kinases to stimulate efficient reverse transcription. Tat has been reported to activate components of signal transduction pathways, including mitogen-activated protein kinases (MAPKs) (7, 13, 26, 48, 58, 59), and it may be involved in regulating signal transduction pathways leading to apoptosis (8, 53, 77). Tat may also act as a cellular growth factor (2, 3, 17, 49, 60, 62), such as in its involvement in the development of Kaposi's sarcoma (19, 20, 61, 68). Although quiescent T cells can be infected by HIV-1 and reverse transcription can be initiated, full-length proviral DNA cannot be detected and integration does not take place (70, 78). The block in HIV-1 replication in quiescent cells has been reported to involve decreased translocation of the reverse transcription complex and/or the preintegration complex (10) which is regulated by phosphorylation (9, 24). Viral proteins associated with the reverse transcription complex include the heterodimeric RT, integrase, nucleocapsid, Vpr, and matrix protein (11). Studies suggest that phosphorylation of matrix protein on tyrosine (24, 25) by an as-yet-unidentified kinase or on serine residues by a cellular serine/threonine kinase identified as ERK2/MAPK (9, 45) is required to dissociate myristoylated matrix protein from the cell membrane and direct its nuclear import. The latter kinase is induced upon T-cell activation and is specifically incorporated into HIV-1 virions (12, 45). Thus, there is a precedent for alterations in the cellular signal transduction pathways for modulating viral replication.

The effect of Tat on reverse transcription can be distinguished from that found in the studies discussed above, because Tat acts at an earlier step in reverse transcription, perhaps during virion assembly or initiation of reverse transcription. In any event, the defects are present within the virion particles themselves. Like the effects of Tat mutants, mutations in either *vif* (69) or *nef* (1, 67) also likely result in reverse transcription defects by different mechanisms. The fact that we cannot identify Tat as a virion-associated protein lends support to the idea that it has an indirect effect on the effi-

ciency of reverse transcription. Because reverse transcription occurs in the absence of Tat, albeit with greatly reduced efficiency, Tat may be able to stimulate this process in a manner parallel to viral transcription. While RT itself has not been demonstrated to be a target of cellular kinases, it may be possible that it can be modified to become a more processive enzyme and that Tat may function to recruit or activate a kinase involved in this process. Future experiments will aim to identify precisely at what point and with what viral and cellular factors Tat functions in the process of HIV-1 reverse transcription.

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