Jembrana Disease Virus Tat Can Regulate Human Immunodeficiency Virus (HIV) Long Terminal Repeat-Directed Gene Expression and Can Substitute for HIV Tat in Viral Replication

HEXIN CHEN,[1] JUN HE,[1] STEVEN FONG,[2] GRAHAM WILCOX,[3] AND CHARLES WOOD[1]*

School of Biological Sciences, University of Nebraska—Lincoln, Lincoln, Nebraska 68588[1]; Division of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, WA 6150, Australia[3]; and Division of Toxicology, U.S. Army Medical Research Institute for Infectious Diseases, Fort Detrick, Frederick, Maryland 21702-5011[2]

Jembrana disease virus (JDV) is a bovine lentivirus genetically similar to bovine immunodeficiency virus; it causes an acute and sometimes fatal disease in infected animals. This virus carries a very potent Tat that can strongly activate not only its own long terminal repeat (LTR) but also the human immunodeficiency virus (HIV) LTR. In contrast, HIV Tat cannot reciprocally activate the JDV LTR (H. Chen, G. E. Wilcox, G. Kertayadnya, and C. Wood, J. Virol. 73:658–666, 1999). This indicates that in transactivation JDV Tat may utilize a mechanism similar to but not the same as that of the HIV Tat. To further study the similarity of JDV and HIV tat in transactivation, we first tested the responses of a series of HIV LTR mutants to the JDV Tat. Cross-transactivation of HIV LTR by JDV Tat was impaired by mutations that disrupted the HIV type 1 transactivation response element (TAR) RNA stem-loop structure. Our results demonstrated that JDV Tat, like HIV Tat, transactivated the HIV LTR at least partially in a TAR-dependent manner. However, the sequence in the loop region of TAR was not as critical for the function of JDV Tat as it was for HIV Tat. The competitive inhibition of Tat-induced transactivation by the truncated JDV or HIV Tat, which consisted only of the activation domain, suggested that similar cellular factors were involved in both JDV and HIV Tat-induced transactivation. Based on the one-round transfection assay with HIV tat mutant proviruses, the cotransfected JDV tat plasmid can functionally complement the HIV tat defect. To further characterize the effect of JDV Tat on HIV, a stable chimeric HIV carrying the JDV tat gene was generated. This chimeric HIV replicated in a T-cell line, C8166, and in peripheral blood mononuclear cells, which suggested that JDV Tat can functionally substitute for HIV Tat. Further characterization of this chimeric virus will help to elucidate how JDV Tat functions and to explain the differences between HIV and JDV Tat transactivation.

Lentiviruses are a group of retroviruses usually associated with slowly developing diseases with a number of different clinical manifestations of infection in different virus-host systems (13, 14). For example, human immunodeficiency virus (HIV) causes immunodeficiency and ultimately leads to the death of infected patients several years after infection (14). In contrast, a recently characterized bovine lentivirus, Jembrana disease virus (JDV), causes an acute disease in infected animals after a short incubation period (7, 60, 61). It is probable that an understanding of the differences in molecular mechanisms involved in the pathogenesis of different lentiviruses will provide important insights into methods of controlling lentivirus-induced diseases such as AIDS.

The genomes of all lentiviruses contain three major structural genes, *gag*, *pol*, and *env*, as well as several accessory and regulatory genes flanked by the two long terminal repeats (LTRs) (14). In HIV, at least six accessory and regulatory genes, *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu*, have been identified previously (14). Studies of these accessory genes have suggested that most of them are involved in viral replication and pathogenesis (19, 33, 39, 42, 44, 46, 57). The HIV *tat* gene has been extensively studied and is an essential determinant of viral replication and the pathogenesis of infection (3, 4, 12, 16, 18, 31, 58, 62). Tat is a potent transactivator for HIV gene expression (3, 4) and has also been shown to modulate the expression of cellular genes, such as those for major histocompatibility complex class I (30), tumor necrosis factor alpha (8, 48), interleukin-2 (IL-2) (59), IL-6 (53), and several extracellular matrix proteins (54). The Tat protein is released from HIV type 1 (HIV-1)-infected cells and can be detected in sera of HIV-1-infected individuals (24). Extracellular Tat may be involved in suppression of the host immune response and cellular disorders associated with AIDS pathology (2, 23). Tat can synergize with cellular basic fibroblast growth factor and may be involved in the induction of Kaposi's sarcoma lesions (6, 22, 23). In addition, Tat may participate in the induction of apoptosis in lymphocytes and contribute to the depletion of the $CD4^+$ T cells in AIDS patients (40, 43, 58). The role of Tat in HIV pathogenesis may not be limited to its transactivation function (31). Tat may also play a role in viral replication. Indeed, Tat has recently been shown to be required for efficient HIV-1 reverse transcription (27, 28, 29). Thus, there is ample evidence to suggest that the HIV Tat is a pleiotropic protein, but how Tat is involved in HIV pathogenesis is still an enigma. Further understanding of the molecular basis for how Tat plays a role in viral replication and viral pathogenesis would be of fundamental importance to our understanding of lentiviruses.

JDV is a recently identified lentivirus (9, 10, 61) related to bovine immunodeficiency virus (BIV), but in contrast to BIV, it causes an acute disease atypical of most lentivirus infections (7, 60, 61). JDV infection of cattle can lead to death of the animal within 1 to 2 weeks (60, 61). The virus replicates to

* Corresponding author. Mailing address: School of Biological Sciences, University of Nebraska—Lincoln, E319 Beadle Center, P.O. Box 880666, Lincoln, NE 68588-0666. Phone: (402) 472-4550. Fax: (402) 472-8722. E-mail: cwood1@unl.edu.
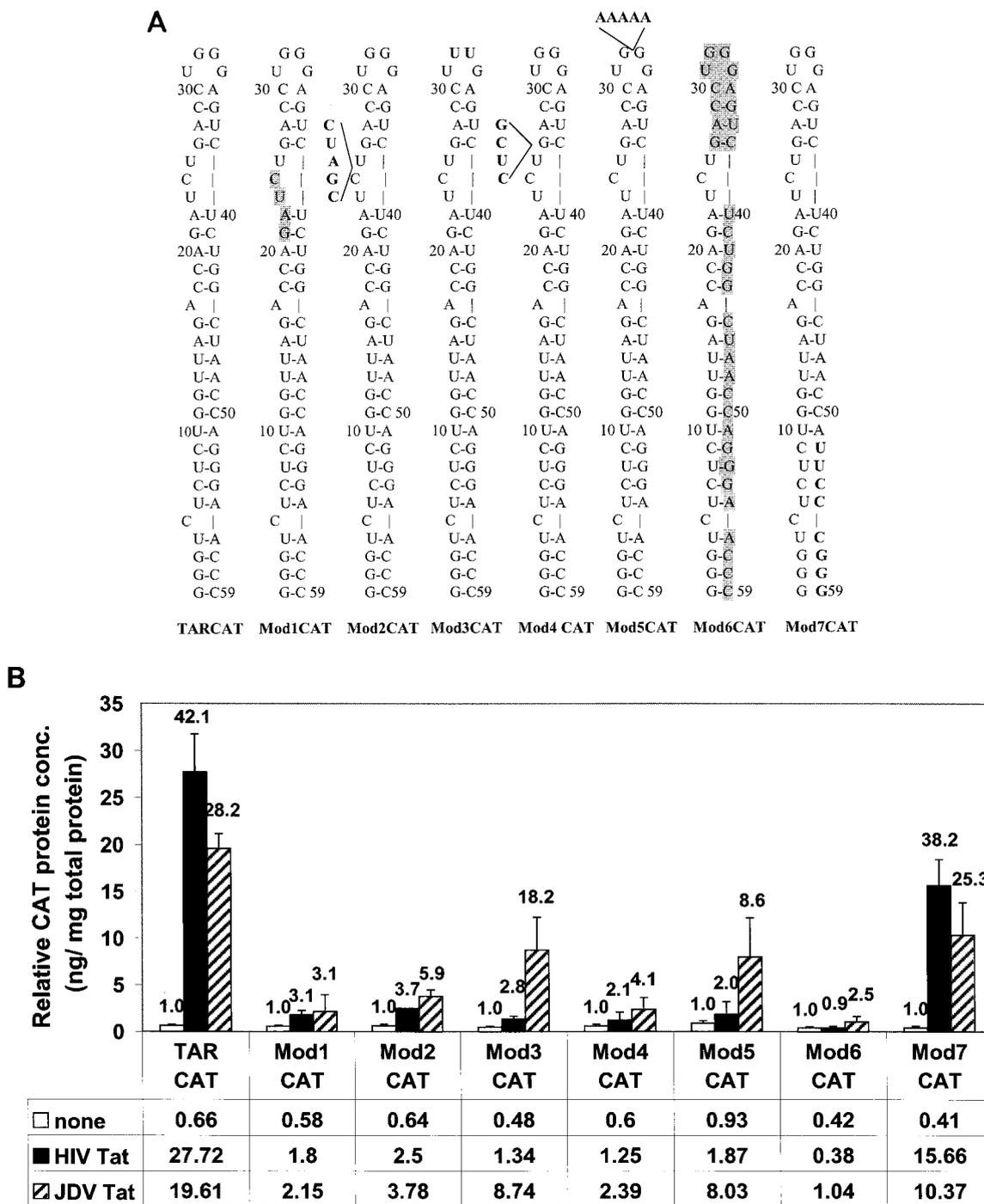
FIG. 1. Effects of HIV TAR mutations on the transactivation of HIV LTR by both JDV Tat and HIV Tat. (A) Schematic presentation of the HIV LTR and mutant TAR constructs which were described in detail previously (25). TARCAT represents the wild-type LTR. All other modifications are as follows: Mod1CAT, 4-base deletion (GAUC) in the bulge region; Mod2CAT, 5-base insertion (CGAUC) in the bulge region; Mod3CAT, 2-base substitution (GG mutated to UU) in the loop region; Mod4CAT, 4-base insertion (CUCG) between the bulge and the upper stem-loop region; Mod5CAT, 5-base insertion (AAAAA) in the loop region; Mod6CAT, 35-base deletion, including the bulge and the downstream sequences; Mod7CAT, 8-base substitution (UUCCCGGG) in the lower stem-loop region. The shaded letters represent the deleted residues, and the boldface letters represent the substituted residues. (B) A 0.05-μg amount of LTR mutants was cotransfected with 0.5 μg of either pUC-Jtat or pUC-Htat. At 48 h posttransfection, cell lysates were collected and subjected to bicinchoninic acid assay. By using the same amount of total cellular protein, the expressed CAT protein in the transfected cell lysate was measured with the CAT ELISA kit as described in Materials and Methods. The relative CAT concentrations (conc.) shown in the figure represent the results from at least three independent experiments. The number above each bar represents fold activation for each LTR, and the transactivation fold was obtained by dividing the amount of CAT protein in the presence of Tat protein by the amount of CAT in the absence of Tat (11).
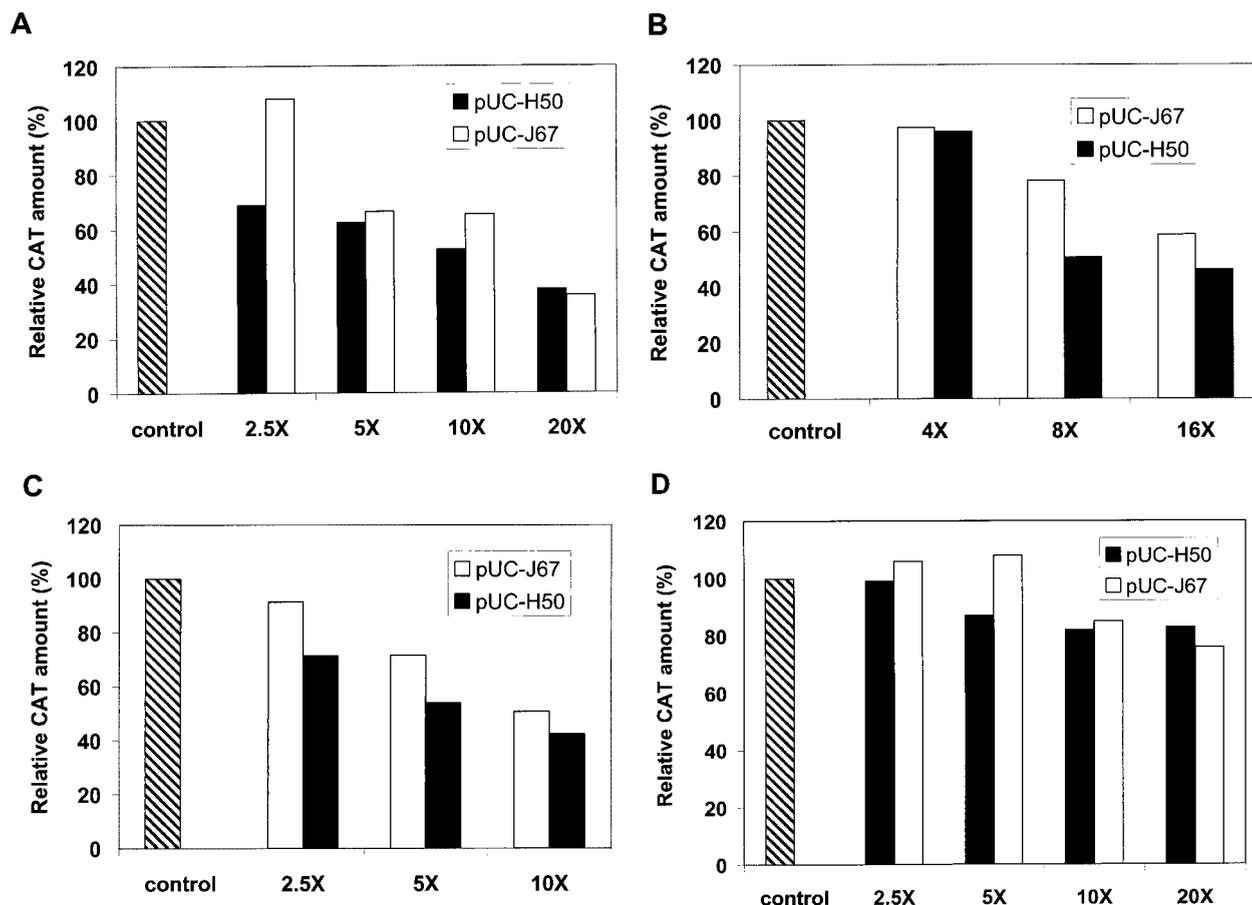
FIG. 2. Competitive inhibition of Tat-induced transactivation by the truncated Tat proteins. (A) CV-1 cells were cotransfected with 0.05 μg of pHIV-CAT, 0.1 μg of HIV *tat* plasmid pUC-Htat, and the indicated amount of the competitors (pUC-J67 expresses the truncated JDV Tat protein and pUC-H50 expresses the truncated HIV Tat protein). The indicated values represent the percentages of the amount of CAT in the presence of competitor relative to that in the absence of competitor (defined as 100%). (B) CV-1 cells were cotransfected with 0.05 μg of pHIV-CAT plasmid, 0.25 μg of JDV *tat* plasmid pUC-Jtat, and the indicated amounts of competitors. (C) CV-1 cells were cotransfected with 0.5 μg of pJU3R-CAT plasmid, 0.25 μg of JDV *tat* plasmid pUC-Jtat, and the indicated amounts of competitors. (D) CV-1 cells were cotransfected with 0.15 μg of pHTLV-CAT plasmid, 0.1 μg of pCMV-Tax plasmid, and the indicated amounts of competitors. The total amount of DNA used in each transfection was equalized with a control plasmid. Control in the figure represents no competitor addition.

extremely high titers in infected animals, and since Tat plays an important role in viral replication, we hypothesized that JDV encodes a potent transactivator, Tat, responsible for its robust replication activity in vivo. In previous studies, we have shown that JDV encodes a Tat that can strongly activate not only the JDV LTR but also the LTRs of other lentiviruses, including the HIV LTR (11). Other than BIV Tat, JDV Tat is the only other nonprimate viral Tat to effectively transactivate the HIV LTR (41), which implies that similar mechanisms may be shared between HIV and the bovine lentivirus Tat proteins. It also suggests that bovine lentiviruses may have a close evolutionary relationship with primate lentiviruses, at least in terms of transactivation mechanism, and may be used as a model to further dissect the molecular mechanism of Tat transactivation and its role in the pathogenesis of lentivirus infections.

To further study the similarities and differences between the JDV and the HIV Tat, we investigated the transactivation of the HIV LTR by the JDV Tat. As expected, JDV Tat transactivated HIV LTR at least partially in a TAR-dependent manner, and this may involve similar mechanisms and common cellular factors in the two systems. Moreover, we demonstrated that JDV Tat functionally substituted for the HIV Tat when the JDV *tat* gene was introduced into the HIV genome. Our

study provides additional evidence that the JDV Tat is a potent transactivator and indicates that further characterization of the molecular mechanism involved in transactivation by JDV Tat is warranted.

MATERIALS AND METHODS

Plasmids. Several HIV and JDV LTR promoter constructs were used in this study. A series of HIV LTR mutants with deletions and mutations in the TAR region of the LTR, TARCAT and Mod1CAT to Mod7CAT, were used. The clone TARCAT, used as a positive control, contained the HIV 5′ LTR region (nucleotide [nt] −121 to +82, encompassing the intact TAR sequence) cloned in front of the indicator chloramphenicol acetyltransferase (CAT). Additional mutant HIV LTR promoter constructs were derivatives of TARCAT; these were used previously to test their responsiveness to Tat transactivation (25). A pHIV-CAT clone that contained the intact HIV U3R region was described previously (35). The constructs pHTLV-CAT and pCMV-Tax were a kind gift from Fatah Kashanchi (37). The mutant HIV Tat construct pΔCys1-4 used was described previously (26). The JDV promoter construct, pJU3R-CAT, was constructed as previously described (14); this clone was generated by insertion of the CAT gene into a site 3′ of the U3R region of the JDV LTR.

Various HIV and JDV *tat* expression constructs were generated using the expression plasmid pUC-RSV. The pUC-RSV plasmid was generated by inserting the Rous sarcoma virus LTR and the bovine growth hormone polyadenylation signal sequence from pRc/RSV (Invitrogen, Carlsbad, Calif.) into pUC18. The HIV or the JDV *tat* exon 1 was PCR amplified and inserted into vector pUC-RSV to generate pUC-Htat and pUC-Jtat, both of which encoded the

transactivation-competent Tat peptides. The truncated HIV *tat* (corresponding to amino acids 1 to 50) and the truncated JDV *tat* sequences (corresponding to amino acids 1 to 67) were cloned into pUC-RSV to generate plasmids pUC-H50 and pUC-J67. These two plasmids encoded truncated peptides which had lost their transactivation abilities. Two *tat* mutant proviral clones, pMtat(−) and pMtat30, were obtained from the AIDS Research and References Program National Institute of Allergy and Infectious Diseases [NIAID], National Institutes of Health, Bethesda, Md.): pMtat(−) contains a termination codon (TGA) in place of the ATG initiation codon and is unable to synthesize Tat (49); pMtat30, which carries a Cys→Gly substitution within the cysteine-rich region, produces a missense Tat (50).

The HIV infectious proviral clone pNL43 was obtained from the AIDS Repository (NIAID) (1). This proviral clone was used to generate other modified HIV proviral clones. The *tat* gene mutant pNL-tat$_m$ proviral plasmid was generated by introducing a stop codon into the *tat* gene of pNL43 using a PCR-based mutagenesis method (5). This mutation was verified by *Eco*RI restriction enzyme digestion, since a new *Eco*RI site was generated in the mutated *tat* gene (TGG AAG CAT mutated to TGA ATT CAT). Using this plasmid, an *Nde*I restriction site was introduced into the ATG site of the *nef* gene and was then used to construct another modified proviral clone that carried the JDV *tat* gene. The plasmid pNL-Jtat contained the JDV *tat* gene, in which the *Nde*I/*Xho*I fragment of the *nef* gene of pNL-tat$_m$ was replaced by the JDV *tat* gene exon 1 sequences. In addition, a random stretch of sequence (32 bp) was introduced into the *nef* gene of pNL-tat$_m$ to generate pNL-nef, which was used as the negative control in the various experiments. As a control, the plasmid Tat(−)1ex, which contains the HIV *tat* in place of the *nef* gene, was constructed using a similar strategy (45), and was kindly provided by K. T. Jeang (NIAID).

Transfection, virus stock, and CAT ELISA. CV-1, HeLa, and 293T cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum. As described previously (11), 2×10^5 cells were seeded into 60-mm-diameter dishes 24 h prior to transfection. The cells were transfected with 1 to 2 μg of CsCl-purified plasmid DNA using Lipofectamine (GIBCO BRL, Grand Island, N.Y.). At 48 h after transfection, the cells were harvested and lysed, and the amount of protein in the lysate was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.). For cells transfected with the promoter CAT construct plasmids, the level of CAT expression was determined by a commercial CAT enzyme-linked immunosorbent assay (ELISA) (Boehringer Mannheim, Indianapolis, Ind.) according to the protocols described by the manufacturer.

Stocks of infectious virus were produced by transfection of cells with proviral DNA. The medium from the transfected cells was collected 48 h posttransfection, the cells and cellular debris were removed by centrifugation, and the supernatant was used as a source of infectious virus. The titer of virus was measured by the p24 assay according to protocols described by the manufacturer (NEN Life Sciences Products, Boston, Mass.).

Viral infection and p24 assays. Human peripheral blood mononuclear cells (PBMC) were maintained in RPMI 1640 with 10% fetal bovine serum and IL-2 (2 U/ml) (Pharmacia, Piscataway, N.J.) and stimulated with phytohemagglutinin (at a final concentration of 250 ng/ml) for 1 to 3 days prior to infection. For each infection, 2×10^6 PBMC in a 0.5-ml volume were incubated with virus at 37°C for 2 h. The virus-containing medium was then decanted and replaced with fresh RPMI medium containing IL-2 (2 U/ml). The infected cultures were sampled every 6 to 7 days, and the amount of virus produced was measured by p24 assay.

Sequence analysis. The alignment of Tat amino acid sequences was performed using the Clustal W program in the GCG package (Madison, Wis.). Phylogenetic reconstruction trees were generated using the Evolution program in the GCG package. The Distance program (with Kimura's formula) was used to generate a pairwise matrix of the evolutionary distance of the amino acid sequences. Phylogenetic trees were constructed from the same distance matrices with the Growtree program (neighbor-joining algorithm or unweighted pair group method with averages [UPGMA]).

RESULTS

Transactivation of HIV LTR by JDV Tat is TAR dependent. Our previous study demonstrated that JDV Tat was a potent transactivator which could significantly increase the LTR-directed gene expression of different animal and primate lentiviruses, including HIV (11, 14). We found that the JDV Tat not only stimulated its own LTR significantly but also stimulated HIV LTR-directed CAT expression to high levels equivalent to those obtained with HIV Tat (11). The activation of JDV LTR by JDV Tat was mediated by TAR-like elements located in the LTR, and the loop region in the TAR seemed to be less critical for JDV-mediated transactivation.

To further investigate the activation of HIV LTR by JDV Tat and whether similar mechanisms of transactivation were involved, a series of HIV TAR and LTR mutants were studied. These constructs were generated previously (25) and consisted
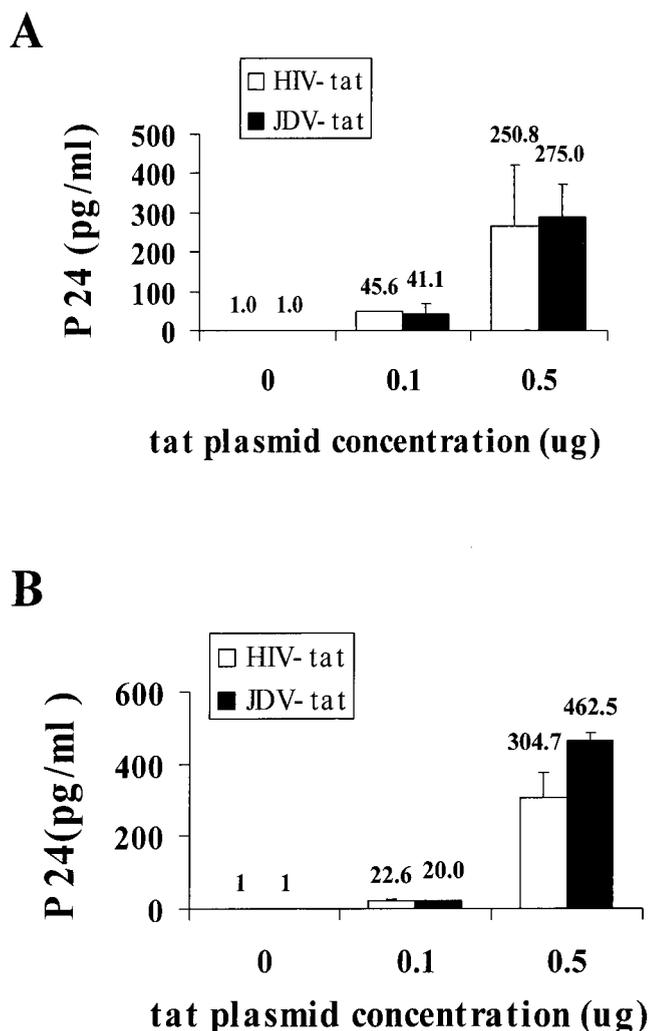

FIG. 3. Complementation of the *tat* mutant HIV proviruses by JDV Tat and HIV Tat. A 0.1-μg amount of *tat* mutant HIV proviral DNA was cotransfected into CV-1 cells with varying concentrations of the JDV and HIV *tat* plasmids, as indicated. The culture supernatant was collected at 72 h posttransfection, and the amount of HIV produced was determined by p24 ELISA. (A) Complementation of the HIV *tat* mutant clone pMtat(−) by JDV Tat and HIV Tat. This mutant carries a termination codon in place of the ATG (methionine) initiator codon in the Tat coding region (49). (B) Complementation of the HIV *tat* mutant clone pMtat30 by JDV and HIV Tat. This mutant has a Cys→Gly substitution within the Tat cysteine cluster and produces a missense Tat (50).

of either deletions or mutations in the stem, the bulge, or the loop region of the HIV TAR. These mutants are summarized in Fig. 1A and were designated TARCAT, Mod1CAT, Mod2CAT, Mod3CAT, Mod4CAT, Mod5CAT, Mod6CAT, and Mod7CAT. Each plasmid construct was cotransfected into CV-1 cells with the JDV *tat* expression plasmid, pUC-Jtat, and tested for its ability to be activated by the JDV Tat (Fig. 1B). TARCAT containing the intact TAR sequence was used as the wild-type LTR. Mod7CAT, which contained the minimum HIV TAR required for HIV Tat transactivation, showed wild-type levels of transactivation in the presence of either JDV or HIV Tat. The responsiveness of the remaining HIV LTR mutants to transactivation by HIV Tat and JDV Tat was severely impaired and varied. The mutants Mod1CAT, Mod2CAT, Mod4CAT, and Mod6CAT, with mutations in the TAR stem and bulge region, were activated by JDV Tat about 60 to 80%
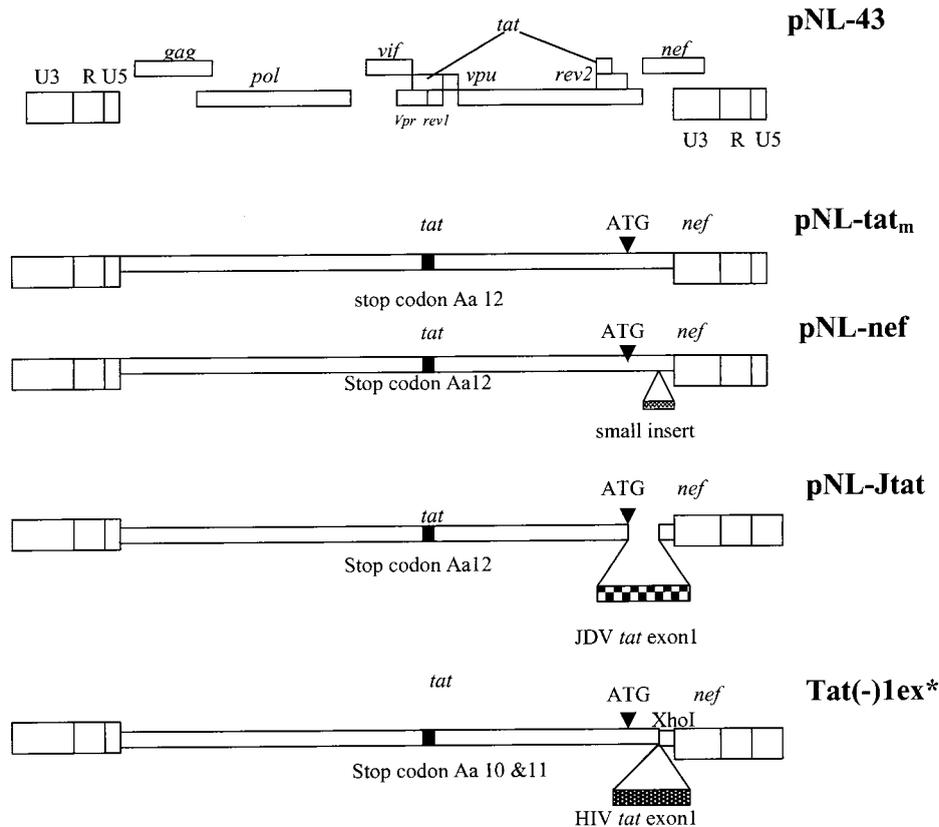
FIG. 4. Schematic representation of the wild-type pNL43 and the various modified virus genomes. The pNL-tat$_m$ was generated by introducing a stop codon at amino acid 12 of the *tat* gene to render it inactive. This clone was then used to construct the other modified HIVs. pNL-Jtat express the JDV Tat exon 1 using the ATG of the *nef* gene. pNL-nef contains a random sequence insertion in the *nef* gene. Tat(−)1ex was used and described previously (45).

less than was the wild-type HIV LTR. However, we consistently observed that these mutant promoters still responded better to the JDV Tat than to the HIV Tat, even though the activities were much lower than that with the wild-type LTR. The Mod3CAT mutant, which had a 2-base substitution in the loop region, retained most of its responsiveness to JDV Tat, but not to HIV Tat. Mod5CAT, which had a 5-base insertion in the loop region, was impaired in its ability to be activated although it has a relatively higher basal level of promoter activity. The data demonstrated that the JDV Tat transactivated the HIV LTR at least partially in a TAR-dependent manner, and sequences in the loop region of HIV LTR seemed to be less important. The data further support our earlier results that showed that activation of the JDV LTR by JDV Tat did not involve the loop sequence of the TAR region (11).

JDV and HIV Tat activation of the LTR can be competitively inhibited by coexpression of the HIV or JDV Tat activation domain. HIV Tat has been shown to transactivate the LTR by recruiting the cyclin T-CDK9 complex to the TAR region which then phosphorylates the C-terminal domain of RNA polymerase II (15, 32, 34, 47, 56). In comparing the JDV Tat sequence with HIV Tat, we found that the JDV Tat contained a very conserved cysteine-rich domain and a core domain, which are both involved in the HIV Tat binding of cellular factors. Therefore, it is likely that JDV Tat transactivates HIV LTR by a similar mechanism and might involve similar cellular factors.

We constructed two truncated *tat* plasmids, pUC-J67, which encoded a JDV Tat peptide, JTat67 (amino acids 1 to 67), and

pUC-H50, which encoded an HIV Tat activation domain, HTat50 (amino acids 1 to 50). We first examined the ability of the expressed truncated Tat peptide JTat67 and HTat50 to inhibit the transactivation function of HIV Tat and the effect of intact JDV Tat on the HIV LTR (Fig. 2A and B). These truncated proteins lost their abilities to transactivate HIV LTR and JDV LTR and did not inhibit the basal level of LTR expression (data not shown). We then cotransfected CV-1 cells with the target plasmid pHIV-CAT, effector plasmid pUC-Htat or pUC-Jtat, and varying concentrations of the competitor plasmid pUC-H50 or pUC-J67 (Fig. 2A and B); the total amount of DNA in each transfection was normalized by co-transfection of the parent vector pUC18. As shown in Fig. 2A and B, JTat67 and HTat50 efficiently inhibited transactivation of the HIV LTR by both HIV Tat and JDV Tat, in a dose-dependent manner. A 20-fold excess of the competitor plasmid pUC-J67 or pUC-H50 inhibited HIV Tat activation of the HIV LTR by about 65% (Fig. 2A), whereas a 10-fold excess of pUC-J67 inhibited HIV Tat transactivation of the HIV LTR by about 40% and a 10-fold excess of pUC-H50 resulted in a 50% decrease in activity (Fig. 2A). However, when only a 2.5-fold excess of competitor was added, the JDV peptide JTat67 was ineffective while the HIV peptide inhibited activation about 30%. The inhibition of HIV LTR activation by JDV Tat was similar (Fig. 2B): a 16-fold excess of either pUC-J67 or pUC-H50 caused significant inhibition of HIV LTR activation, 40% inhibition with pUC-J67 and 50% inhibition with pUC-H50; lower concentrations of pUC-H50 seemed to cause greater inhibition (Fig. 2B).

We next examined the effect of the truncated Tat proteins on transactivation of the JDV LTR by JDV Tat (Fig. 2C). Cotransfection of CV-1 cells with pJU3R-CAT, pUC-Jtat, and varying concentrations of competitor plasmids (either pUC-H50 or pUC-J67) inhibited JDV Tat transactivation to levels similar to those observed with the HIV LTR. A 10-fold excess of either of the Tat inhibitors HTat50 and JTat67 inhibited activation by about 50%. At lower concentrations of the inhibitors, the HIV Tat truncated protein also seemed to be more effective than the JDV truncated Tat protein. These results demonstrated that both truncated JDV and HIV Tat may inhibit transactivation by the intact Tat, possibly by competing for common cellular factors used by both JDV and HIV Tat.

To confirm that the inhibition by the JDV and HIV Tat activation domains was specific to Tat-mediated activation, we also examined the effects of these activation domains on the activation of human T-cell leukemia virus (HTLV) LTR by its cognate transactivator Tax. The presence of either HIV or JDV Tat transactivation domain did not dramatically affect the activation of the HTLV LTR by Tax. However, a slight inhibition by a fivefold or more excess of HIV Tat transactivation domain expression plasmid pUC-H50 and a 10-fold or more excess of JDV Tat transactivation domain expression plasmid pUC-J67 was observed. These results suggested that the inhibition effects of the JDV and HIV Tat activation domain were Tat specific but may also have involved some nonspecific transcription factors (Fig. 2D).

JDV Tat can complement HIV *tat*(−) provirus expression. Tat is a potent and essential transcriptional transactivator of HIV LTR, and HIV-1 proviruses mutated in *tat* are nonviable (18, 49). However, this defect can be complemented by coexpression of the HIV Tat in *trans*. To determine whether JDV Tat can also complement the HIV *tat*(−) provirus expression in *trans*, two *tat* mutant HIV proviral plasmids, pMtat(−) and pMtat30, were cotransfected into CV-1 cells with a JDV Tat expression plasmid. Both *tat* mutants were demonstrated previously to be defective due to the mutations at the initiation codon of the Tat protein or at the cysteine-rich transactivation domain (49, 50). At 48 h posttransfection, supernatant was collected and assayed for the expression of HIV by p24 analysis. It was determined that transfection of the two *tat* mutant cDNA plasmids alone into CV-1 cells did not produce detectable levels of HIV as measured by p24 assays. However, in the presence of either the JDV *tat* or the HIV *tat* plasmid, HIV p24 was detected (Fig. 3). An increase in the amount of the JDV or HIV Tat plasmid transfected resulted in higher levels of HIV production. Our results demonstrated that both *tat* HIV mutants were complemented equally well by the presence of JDV or HIV Tat. This complementation assay demonstrated that the JDV Tat could functionally complement the defect in the *tat* gene in the HIV provirus to enable the replication of HIV in the proviral DNA-transfected cell.

Construction and characterization of chimeric HIV carrying the JDV *tat* gene. To further investigate whether JDV *tat* could functionally substitute for the HIV *tat* gene, we constructed a chimeric proviral clone by inserting the JDV *tat* gene into HIV proviral backbone, pNL43 (Fig. 4). To avoid affecting the function of other genes which overlap with the *tat* gene, we introduced a stop codon into the *tat* region of the HIV proviral plasmid pNL43 to inactivate *tat*, and an *Eco*RI site was generated in the mutated *tat* region. The resulting plasmid, pNL-tat$_m$, with a *tat* mutation, otherwise isogenic to pNL43, was wild type in all other genes. A chimeric pNL-Jtat virus was also constructed, wherein a small fragment of *nef* was replaced with cDNA coding for JDV Tat exon 1 but still using the native *nef* ATG translation initiation site. The pNL43 *nef* could be effec-

TABLE 1. p24 assay of supernatant from chimeric proviral DNA-transfected cells[a]

Plasmid(s)	Level of p24 in cell line:		
	HeLa (pg/ml)	293T (ng/ml)	CV-1 (pg/ml)
pNL43	636.5	21.8	>1,000.0
pNL43 + pUC-Jtat	>600.0	16.3	1,063.9
pNL43 + pUC-Htat	625.3	19.9	1,064.8
pNL43 + pΔCys1-4	ND	20.6	ND
pNL-tat$_m$	5.8	1.3	267.8
pNL-tat$_m$ + pUC-Jtat	>600.0	15.8	>1,000.0
pNL-tat$_m$ + pUC-Htat	>600.0	20.6	1,105.6
pNL-tat$_m$ + pΔCys1-4	ND	0.7	ND
pNL-nef	1.2	0.6	77.4
pNL-nef + pUC-Jtat	177.7	18.2	1,005.6
pNL-nef + pUC-Htat	638.2	15.7	885.2
pNL-nef + pΔCys1-4	ND	0.05	ND
pNL-Jtat	605.7	12.6	1,122.1
pNL-Jtat + pUC-Jtat	>600.0	11.3	1,132.1
pNL-Jtat + pUC-Htat	645.7	13.3	1,137.8
pNL-Jtat + pΔCys1-4	ND	8.5	ND
Tat(−)1ex	650.2	13.4	1,058.6
Tat(−)1ex + pUC-Jtat	>600.0	8.9	1,160.4
Tat(−)1ex + pUC-Htat	>600.0	8.4	>1,000.0
Tat(−)1ex + pΔCys1-4	ND	3.4	ND

[a] One microgram of proviral DNA was transfected into either HeLa, 293T, or CV-1 cells with or without *tat* plasmids. At 48 h posttransfection, the supernatants were collected and assayed for p24 production. ND, not determined.

tively replaced since such *nef* mutations do not significantly affect the in vitro replication and infectivity of the mutant HIV in T-cell lines (45). As a positive control, a chimeric virus, Tat(−)1ex, containing the HIV exon 1 *tat*, was constructed using a similar strategy (45). A pNL-nef plasmid, in which random sequences were inserted into the *nef* region of pNL-tat$_m$ to further inactivate *nef*, was used as a negative control.

We first characterized the ability of these modified proviral clones to replicate and generate viruses in transfected cells. When 1 μg of the above plasmids was transfected into nonpermissive cells such as CV-1, HeLa, and 293T cells, the *tat* mutant proviral clones pNL-tat$_m$ and pNL-nef did not produce detectable levels of HIV in HeLa cells and produced much lower levels of HIV in 293T and CV-1 cells than did wild-type virus pNL43 (Table 1). In contrast, Tat(−)1ex and pNL-Jtat, which contain the HIV *tat* and JDV *tat*, respectively, produced wild-type levels of expression of HIV in all the tested cell lines.

To rule out the possibility that the failure to express virus in cells transfected with pNL-tat$_m$ or pNL-nef was due to other unexpected defects besides the *tat* mutation in the genome, we cotransfected these cells with *tat* mutant proviral DNAs and either JDV or HIV Tat-expressing plasmids. For both pNL-tat$_m$ and pNL-nef, in the presence of HIV or JDV *tat*, the replication of the viruses in the transfected cells was restored and high levels of p24 were detected in the culture supernatant. Cotransfection of Tat-expressing plasmids with Tat(−)1ex, pNL-Jtat, or pNL43 proviral DNA only slightly increased or did not change the level of HIV expression. Cotransfection of a mutant HIV Tat-expressing plasmid, pΔCys1-4, which carries four Cys→Ser amino acid substitutions in the Cys-rich region and has lost its transactivation ability, failed to rescue the *tat*-defective virus (Table 1).
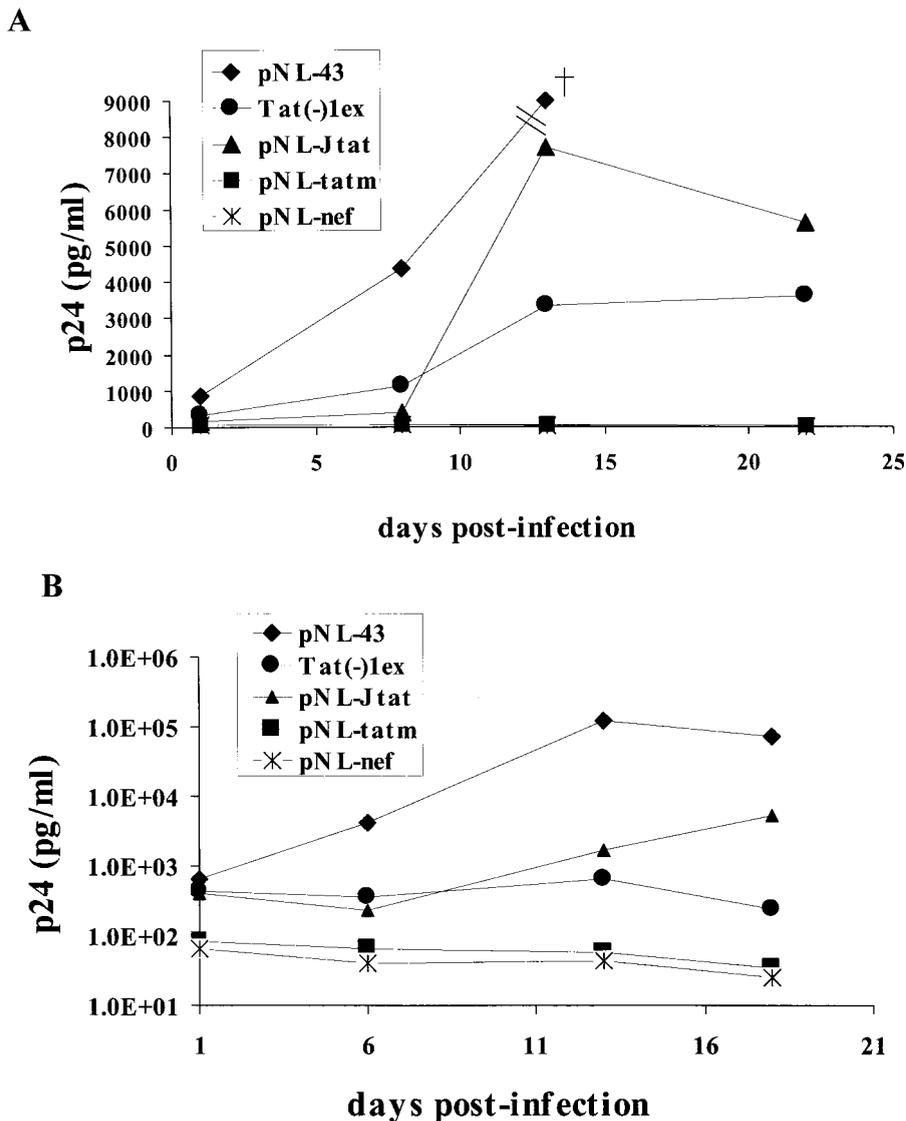
FIG. 5. Growth and replication kinetics of the various recombinant HIV-1 viruses in the C8166 T-cell line and PBMC. (A) Different viral stocks generated by transfection of 293T cells were used to infect the C8166 T-cell line. The 293T cells were transfected with the recombinant proviral DNA using Lipofectamine. At 48 h posttransfection, supernatants were collected, and the p24 levels were determined. C8166 cells (2×10^5) were infected by 10 ng of the different viruses as determined by the p24 assay. The amount of virus produced was monitored at the indicated time points by p24 analysis. The cross in the figure represents a p24 concentration of 103 ng/ml, which is beyond the y-axis scale. (B) PBMC (2×10^6) were infected with 10 ng of virus as determined by the p24 assay, and the amount of virus produced was monitored at the indicated time points as described above.

To further determine whether the presence of JDV *tat* in the chimeric virus could fully complement the HIV *tat* functions and enable the virus to productively infect CD4$^+$ T cells, the viruses generated by transfection were used to infect both primary PBMC and a T-cell line, C8166 (45). The amount of virus produced from the transfected 293T cells was determined by p24 antigen levels, a similar amount of virus from each transfection experiment was then used to infect both fresh PBMC and C8166 cells, and the infected cells were monitored for virus growth by p24 assay at various time points after infection (Fig. 5A). The control chimeric virus Tat(−)1ex, containing the HIV *tat* gene, replicated in the T-cell line as demonstrated previously (45). The NL-Jtat virus, containing JDV *tat*, also replicated efficiently in this T-cell line, and substantial amounts of p24 were detected 14 days after infection. Almost twice as much p24 was detected in NL-Jtat-infected cells as in

those infected by the control Tat(−)1ex virus. Our results thus showed that the chimeric virus with the HIV *tat* replaced by the JDV *tat* was fully competent in its ability to infect and replicate in C8166 T cells. As expected, the negative control viruses, NL-tat$_m$ and NL-nef, did not replicate.

To further characterize the chimeric viruses, their ability to infect and replicate in primary T cells was tested. It was previously shown that the HIV chimera with the HIV *nef* gene replaced by HIV *tat* was unable to replicate in PBMC (45), suggesting that either the HIV *tat* exon 1 alone or the destruction of the HIV *nef* affected the ability of HIV to replicate in primary cells. Indeed, when we tested our various chimeric viruses in PBMC (Fig. 5B), the Tat(−)1ex virus containing the HIV *tat* exon 1 did not replicate in PBMC even though it replicated in C8166 cells, similar to results obtained previously with this virus (45). In contrast, the chimeric NL-Jtat which

A

B

JDV *tat* insert
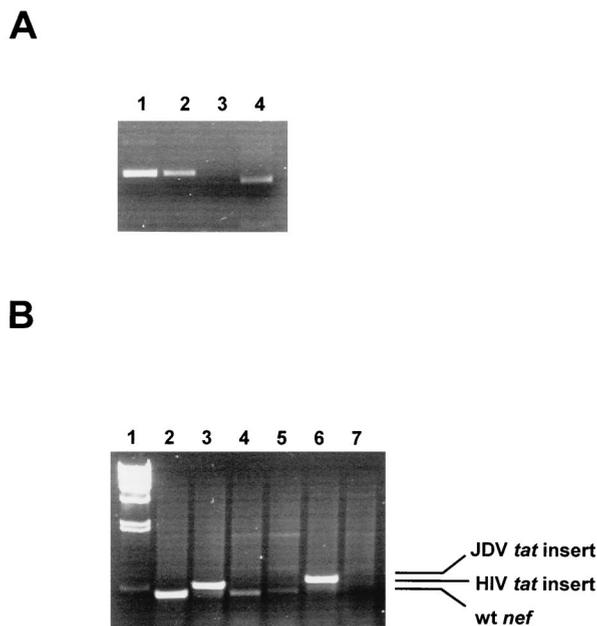HIV *tat* insert
wt *nef*

FIG. 6. PCR analysis of the viruses generated from the infected PBMC cultures. The figure shows PCR amplification of *tat* and *nef* gene sequences from the infected PBMC cellular DNA. At 18 days after infection, PBMC cellular DNA was extracted from cells infected with different viruses, and PCR amplification with the appropriate primers was performed. (A) Verification of *tat* mutation in the recombinant virus by *Eco*RI digestion. PCR products of the *tat* region amplified from infected PBMC DNA were purified from 1% agarose gels and then cut with *Eco*RI. To avoid amplification of the inserted *tat* sequences in the recombinant virus, the primers NL-tat1 (nt 5762 to 5781, 5′ GTT TAT CCA TTT CAG AAT TG 3′) and NL-tat2 (nt 6428 to 6409, 5′ CCA AAC ATT ATG TAC CTC TG 3′) used were located outside the *tat* sequences (nt 5830 to 6044) in the NL43 genome (GenBank accession no. M19921). Lane 1, NL43 virus *tat* DNA; lane 2, Tat(−)1ex virus *tat* DNA; lane 3, NL-tat$_m$ virus *tat* DNA; lane 4, NL-Jtat virus *tat* DNA. (B) PCR amplification of *nef* sequences from the infected PBMC DNA to verify the presence of the *tat* insert in the *nef* region of the recombinant virus genome. PCR was performed with primers NL-nef1 (nt 8447 to 8465, 5′ TCC ATT CGA TTA GTG AAC G3′) and NL-nef2 (nt 8904 to 8886, 5′ CTA CTT GTG ATT GCT CCA T3′). Lane 1, lambda/*Hin*dIII; lane 2, NL43; lane 3, Tat(−)1ex; lane 4, NL-tat$_m$; lane 5, NL-nef, lane 6, NL-Jtat; lane 7, mock-infected control. wt, wild type.

contained the JDV *tat* infected and replicated effectively in the primary PBMC, even though the levels seemed to be lower than those of the wild-type virus NL43. It produced lower levels of virus expression initially, but by 14 days the levels seemed to have increased and by 18 days were much higher. Our results suggested that the presence of JDV exon 1 *tat* complemented both HIV *tat* and *nef* to render the virus fully replication competent in both PBMC and T-cell lines. As expected, both *tat* mutant virus NL-tat$_m$ and NL-nef failed to replicate in PBMC.

To rule out the possibility that infection of PBMC and C8166 cells by chimeric virus NL-Jtat was due to the back mutation of the HIV *tat* mutation to wild type, the HIV *tat* gene of the infected cells was analyzed by PCR and restriction analysis of the infected PBMC viral DNA (Fig. 6). Since the mutated *tat* gene was engineered to have an *Eco*RI site, the presence of the restriction site would indicate persistence of the mutation. Indeed, the amplified *tat* gene product from the chimeric virus NL-Jtat-infected cellular DNA, but not the wild-type virus-infected cellular DNA, was cut completely by *Eco*RI to generate a smaller *tat* gene fragment of the expected size (Fig. 6A, compare lanes 1 and 4). This result suggested that the mutation in the *tat* gene for NL-Jtat persisted and that rever-

sion to the wild type was not responsible for the ability of this virus to replicate in PBMC. To further confirm that the JDV and HIV *tat* gene insertion in the *nef* region had not been deleted, the *nef* region of the viruses from the infected PBMC was analyzed by PCR. As was expected, the PCR-amplified fragments from NL-Jtat and Tat(−)1ex viruses were relatively larger than the wild-type *nef* fragment, indicating that the *tat* insert was retained (Fig. 6B).

Phylogenetic analysis of lentivirus Tat proteins. The cross-transactivation by JDV Tat of the HIV LTR suggested a close evolutionary relationship between JDV and other primate lentiviruses. To analyze the evolutionary genetic relationship between JDV and other lentiviruses, a neighbor-joining phylogenetic tree based on the published sequences of lentiviral Tat peptides which are competent in transactivation was constructed (3, 11, 17, 20, 21, 36, 41, 51, 52, 55) (Fig. 7A). With the constructed tree, two major subgroups of Tat were formed (primate versus nonprimate lentivirus Tat proteins). Within the primate lentivirus subgroup, the simian immunodeficiency virus Tat was more closely related to HIV-2 Tat than to HIV-1 Tat. Within the nonprimate lentivirus Tat group, the JDV and BIV Tat proteins were closely related phylogenetically. Interestingly, the two bovine lentivirus Tat proteins were more closely related to the primate lentivirus Tat proteins than they were to the nonprimate lentivirus Tat proteins. Another phylogenetic tree created by using UPGMA also showed a similar phylogenetic relationship, and although no distinctive nonprimate lentiviral Tat subgroup was formed with this method of analysis (Fig. 7B), JDV and BIV Tat proteins were within a subgroup which mainly contained the primate lentivirus Tat proteins.

DISCUSSION

A comparison of the amino acid sequences of the JDV and HIV Tat showed a striking homology in the cysteine-rich and the core activation domain, but very little similarity in the RNA binding basic domain (11). Indeed, competitors containing the HIV-1 and JDV Tat activation domain could inhibit the HIV and JDV Tat transactivation of the HIV LTR or the JDV Tat transactivation of the JDV LTR. Since both truncated proteins did not contain the basic RNA binding domain that is responsible for the TAR binding, the inhibition was unlikely to be due to competition for binding to TAR and more likely was due to the effects of the transactivating domain itself. These observations support the notion that the JDV and HIV Tat activation domains may involve a similar transactivation mechanism and both may involve interaction with cellular cofactors. The cross-inhibition of Tat functions may be due to the sequestering of the cellular factors involved.

In this study, we have demonstrated that the transactivation effect of JDV Tat on HIV LTR is at least partially TAR dependent. Mutations of TAR in the HIV LTR, except for mutations in the loop region as represented by mutant Mod3CAT, resulted in significant loss of responsiveness to JDV Tat transactivation. However, differences in the Tat of JDV and HIV were noted. First, as the HIV LTR mutant Mod3CAT was responsive to transactivation by JDV Tat but not to that by HIV Tat, this suggested that a sequence in the loop region of HIV TAR was not critical for transactivation by JDV Tat but was essential for transactivation by HIV Tat. Second, most HIV LTR mutants retained some responsiveness to the JDV Tat even though they were not activated by HIV Tat. These differences could be due to the JDV Tat transactivating the HIV LTR using other mechanisms in addition to the TAR-dependent pathway, or the JDV Tat-TAR interaction
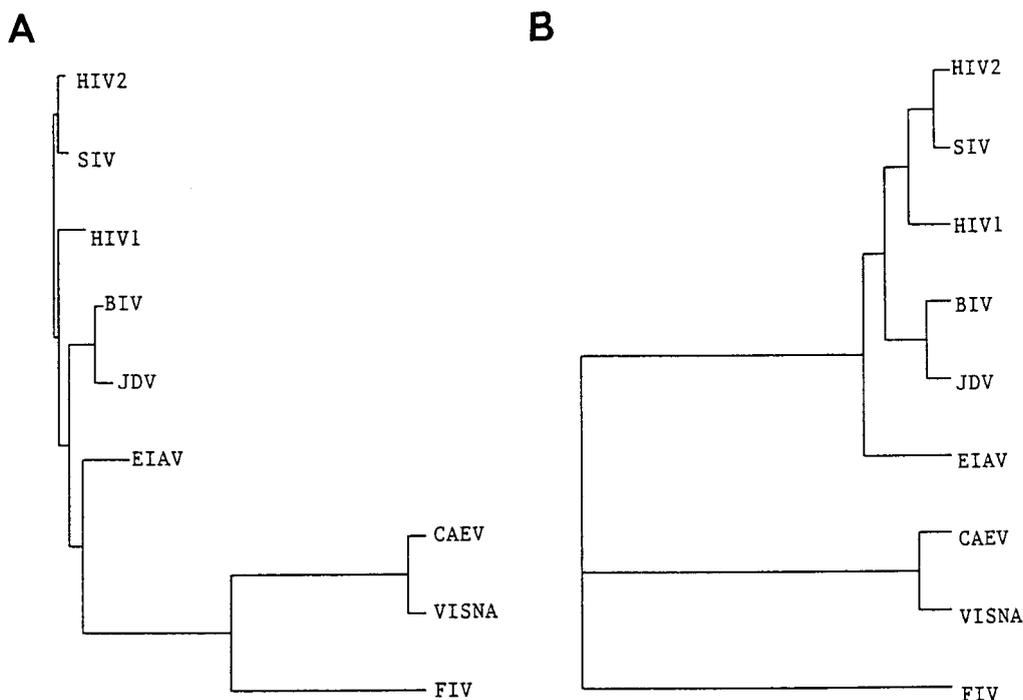
FIG. 7. Phylogenetic analysis of the various lentivirus Tat proteins. The Tat amino acid sequences were deduced from the *tat* nucleotide sequences: JDV genome (GenBank accession no. U21603), BIV (GenBank accession no. M32690), HIV-1 (GenBank locus hivhxb2cg), HIV-2 (GenBank accession no. M38432), simian immunodeficiency virus (SIV) (GenBank accession no. M19499), equine infectious anemia virus (EIAV) (GenBank accession no. M30138), caprine arthritis-encephalitis virus (CAEV) (GenBank accession no. M34092), feline immunodeficiency virus (FIV) (GenBank accession no. M36968), and visna virus *tat* sequence (accession no. J04359). The trees were reconstructed using two separate programs as described in Materials and Methods. Phylogenetic analysis used the Evolution program in GCG. (A) Analysis using the neighbor-joining algorithm; (B) analysis using the UPGMA algorithm.

may be different from the HIV Tat-TAR interaction. We favor the latter explanation, since our results suggest that the loop sequence in the TAR is not critical for JDV transactivation, which is different from that by HIV. Studies of HIV have shown that the loop region is the binding site for cyclin T, and the binding of cyclin T to the loop can significantly stabilize the HIV Tat-TAR interaction (56). Recent data have convincingly demonstrated that the binding of the HIV Tat activation domain to the cyclin T1 subunit of the human TAK/P-TEFb transcription elongation complex is a critical first step in TAR RNA recognition and Tat-mediated transactivation (32). It is possible that JDV Tat can directly interact with the TAR elements independent of cyclin T complex binding, and thus the mutations of the cyclin target in the loop region would not affect the transactivation function of the JDV Tat. However, this does not exclude the possibility that the cyclin T complex is still involved in the JDV Tat-induced transactivation of the HIV LTR. Further studies are necessary in order to decipher the role of cyclin T in JDV Tat transactivation.

In addition to a transactivation function, Tat is essential for viral replication and pathogenesis (12, 18, 45, 62). HIV Tat was reported to activate quiescent T lymphocytes and induce apoptosis (38, 40) and may contribute to the selective depletion of CD4^{+} lymphocytes in vivo. Tat is also vital for viral replication and infection; mutations in *tat* led to viruses that were defective (3, 4). Relocation of *tat* in the HIV genome or the substitution of HIV *tat* for the *nef* gene still led to viruses that were defective; they were able to replicate in T-cell lines but not in PBMC (45). Substitution for the HIV *tat* by alternative transactivators, such as HTLV Tax and Vp16, which were cloned into HIV *tat*(−) has resulted in viruses that replicate very poorly in PBMC and other cell types (45). Our chimeric virus that used

JDV *tat* exon 1 is therefore unique, as JDV *tat* exon 1 was able to substitute for HIV *tat* and enable successful replication in both a T-cell line and PBMC, even though resulting virus replication was less than that with the wild-type virus. Our results indicated that JDV Tat can functionally replace HIV Tat and suggest that JDV Tat should play a role in viral replication and pathogenesis of infection similar to that of HIV Tat in HIV. In addition, the JDV Tat could rescue a mutant HIV when it was mutated not only in the *tat* but also in the *nef* gene, suggesting that JDV Tat can substitute for both HIV Tat and Nef to enable the virus to replicate effectively in vitro. However, it is possible that this HIV-JDV chimeric virus may be nonpathogenic and attenuated in vivo. Further characterization of this virus and its pathogenicity will be important not only for elucidation of the functions of Tat but also for vaccine development if the HIV-JDV chimeric virus is found to be attenuated.

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