The Leucine Domain of the Visna Virus Tat Protein Mediates Targeting to an AP-1 Site in the Viral Long Terminal Repeat

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The visna virus Tat protein is a strong transcriptional activator and is necessary for efficient viral replication. The Tat protein regulates transcription through an AP-1 site proximal to the TATA box within the viral long terminal repeat (LTR). Previous studies from our laboratory using Tat-Gal4 chimeric proteins showed that Tat has a potent acidic activation domain. Furthermore, a region adjacent to the Tat activation domain contains a highly conserved leucine-rich domain which, in the context of the full-length protein, suppressed the activity of the activation domain. To further elucidate the role of this region, four leucine residues within this region of Tat were mutated. In transient-transfection assays using visna virus LTR-CAT as a reporter construct, the activity of this leucine mutant was dramatically reduced. Additionally, domain-swapping experiments using the N-terminal activation domain of VP16 showed that the leucine-rich domain of Tat confers AP-1 responsiveness to the chimeric VP16-Tat protein. A chimeric VP16-Tat construct containing the leucine mutations showed no increased AP-1 responsiveness in comparison with that of the VP16 activation domain alone. Furthermore, in competition experiments, a Gal4-Tat protein containing only the leucine region of Tat (amino acids 34 to 62) was able to inhibit by competition the activity of full-length Tat. These studies strongly suggest that this leucine-rich domain is responsible for targeting the Tat protein to AP-1 sites in the viral LTR. In addition, examination of the amino acid sequence of this region of Tat revealed a highly helical secondary structure and a pattern of residues similar to that in the leucine zippers in the bZIP family of DNA-binding proteins. This has important implications for the interaction of Tat with cellular proteins, specifically Fos and Jun, that contain bZIP domains.

Visna virus belongs to the lentivirus subfamily of nononcogenic retroviruses that cause chronic, multiorgan diseases characterized by long incubation periods and slowly progressive clinical courses. Visna virus-infected sheep develop interstitial pneumonitis and a progressive inflammatory disease of the central nervous system (15, 28). It has previously been shown by our laboratory that visna virus, like other lentiviruses, infects cells of the monocyte-macrophage lineage (16). Gene expression directed by the visna virus long terminal repeat (LTR) is induced upon activation of monocytes to macrophages (13, 34). Within the U3 region of the visna virus LTR, there are multiple binding sites for the AP-1 cellular transcription factor. Deletion mutagenesis experiments have shown that the AP-1 site most proximal to the TATA box mediates viral activation by phorbol esters in macrophages (13, 18). It has also been shown that the cellular Jun and Fos proteins bind to this AP-1 site in the visna virus LTR, which leads to the subsequent activation of visna virus gene expression as monocytes mature into macrophages (1, 2, 33).

Like human immunodeficiency virus (HIV) and equine infectious anemia virus, the visna viral genome encodes a *trans*-acting protein that regulates viral gene expression. The visna virus *tat* gene encodes a 10-kDa protein that greatly increases gene expression directed by the visna viral promoter in the 5' LTR (9, 18). Studies from this and other laboratories have shown that the visna virus Tat protein acts through AP-1 sites in the viral LTR as well as in heterologous promoters containing AP-1 sites, including the *c-fos* and *c-jun* promoters (14, 29).

The mechanism of transcriptional transactivation by visna virus Tat, however, is not well understood. Unlike HIV and equine infectious anemia virus, visna virus does not have a TAR-like structure in the R region of the LTR, which in HIV and equine infectious anemia virus serves to tether Tat proteins to nascent RNA transcripts (4, 10, 11, 25, 30). This interaction is thought to bring the Tat protein into proximity with the pol II initiation complex, where it can then associate with members of this complex (21, 37). Visna virus Tat protein has not been found to bind either RNA or DNA directly, making identification of a targeting domain difficult. In a previous study, we defined the activation domain of the Tat protein in the N-terminal 38 residues, using Gal4-Tat chimeric proteins. Interestingly, we also found that Tat contains an adjacent regulatory domain that, in the context of the full-length protein, serves to regulate the activation domain in the Gal4 system (6). In this study, we confirm the activation potential of the Tat protein outside of the Gal4 system and provide evidence for the location of the targeting domain of the Tat protein that directs it to the AP-1 site in the viral LTR.

MATERIALS AND METHODS

Plasmid constructions. Constructs containing chimeric genes of Gal4-Tat have been previously described (6). The effector plasmid, pGH250, and the target plasmids E1b-CAT, (GAL4)₃E1b-CAT and (GAL4)₅E1b-CAT were obtained from Gary Hayward and were previously described by Hardwick et al. (17). Tat derivatives of the GH250 construct have been described elsewhere (6). The full-length CMV-Tat construct was made by subcloning the *tat* gene into *Hind*III and *Xba*I sites of the cytomegalovirus (CMV) link vector, which has been previously described (40). Mutagenesis of the *tat* gene was done with complimentary PCR oligonucleotides that contained the region of the gene with the desired mutations. Two rounds of PCR using the primer sets listed in Table 1 were used to construct TatΔF5', TatΔL, and TatΔC3'. The PCR products were digested with *Hind*III and *Xba*I and cloned into *Hind*III and *Xba*I sites in the CMV link vector. The VP16-Tat chimeric constructs containing the activation domain of

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VP16, fused to the Tat gene beginning at amino acid 38, were made by using PCR primers listed in Table 1 and cloned into the CMV link as described above. The Gal4(5)-CAT and Gal4(5)AP-1-CAT vectors were generated by making complementary oligonucleotides containing an AP-1 site flanked by *Xho*I sites (Table 1) and subcloned into the *Xho*I site in the Elb and Elb₃ vectors. All new constructs were sequenced to verify reading frames and mutations.

Transfections. HeLa and sheep choroid plexus (SCP) cells were maintained in modified Eagle medium supplemented with 10% fetal bovine serum and incubated at 5% CO₂ at 37°C. Cells were transfected by the Lipofectamine method and used according to the manufacturer's instructions (Life Technologies). Briefly, HeLa cells or SCP cells were plated into six-well cluster dishes at a density of 2×10^5 or 1×10^5 cells per well 24 h prior to transfection. Effector (0.8 µg) and target DNA (1 µg) were mixed with an Opti-Mem solution of 5 µl of Lipofectamine-sample. The DNA-lipofectamine sample was allowed to incubate for 30 min at room temperature and was added to the rinsed cell monolayer. The mix was left on the cells for 6 h and replaced with fresh medium for 36 h. Cells were harvested for chloramphenicol acetyltransferase (CAT) assays or fixed for immunofluorescence as described below.

CAT assays. CAT assays were performed as previously described (6). Briefly, transfected cells were washed two times with phosphate-buffered saline (PBS) and scraped into 1 ml of PBS. The cells were pelleted and resuspended in 100 µl of 0.25 M Tris hydrochloride (pH 7.8). The cells were disrupted by sonication, and debris was removed by centrifugation. The lysates were then treated at 65°C for 10 min to inactivate CAT inhibitors. The lysates were centrifuged, and the resulting supernatant was used in the CAT assay. Each sample (50 to 100 µg) was mixed with a cocktail containing 70 µl of 1 M Tris (pH 7.8), 27 µl of H₂O, 2 µl of 40 mM acetyl coenzyme A, and 1 µl of [¹⁴C]chloramphenicol (0.1 mCi; 60 mCi/mmol). The mix was incubated at 37°C for 90 min (HeLa) or 3 h (SCP). The chloramphenicol and its acetylated forms were extracted by adding 1 ml of ice-cold ethyl acetate, vortexing briefly, and centrifuging for 30 s. The organic phase was transferred to a fresh tube and dried down in a vacuum. The samples were resuspended in 20 µl of ice-cold ethyl acetate and spotted onto a thin-layer chromatography plate. The acetylated forms were separated by chromatography in a chloroform-methanol solvent (95:5 [vol/vol]). The plates were exposed to XAR film, and radioactivity was counted by liquid scintillation. The percent acetylation was calculated by the ratio of the acetylated forms of chloramphenicol to the total radioactivity in each sample. In all figures, percent acetylation is shown with the background activity of the control subtracted out. Background values for Gal4 (residues 1 to 147) [Gal4(1-147)] or CMV-βGal ranged from >1% acetylation to 5%. Values less than 1% were set at 1. Fold activation was calculated as the ratio of the percent acetylation of each sample to that of the control of the effector Gal4(1-147) or CMV-βGal transfected with the indicated CAT reporter plasmid. All experiments were done in at least duplicate and were repeated at least three times. Samples were determined to be within the linear range of the assay if their acetylation was less than 50%. Samples whose acetylation was greater than 50% were diluted accordingly. In all experiments, the standard error calculated from the percent acetylation ranged from 0.1 to 15.

Western blots (immunoblots) and immunofluorescence. Cells were transfected as described and lysed with RIPA buffer (1% Zwittergent, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.5 M NaCl, 1 mM sodium EDTA, 25 mM Tris HCl (pH 7.6), and 1 mM phenylmethylsulfonyl fluoride) 72 h after transfection. The protein content in the lysates was quantitated with the Bio-Rad protein assay, and 250 µg of total protein was used for each sample. The Tat protein was immunoprecipitated with polyclonal antibody made to the carboxy-terminal Tat peptide (1:50 dilution) (6). The immune precipitates were

collected on protein A-Sepharose beads, and the proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The Tat protein was detected on the Western blots with the same antibody (1:200 dilution) and developed by enhanced chemiluminescence (Amersham).

Cells were transfected as described previously and fixed at 24 or 48 h by washing twice with PBS and then adding 3.7% paraformaldehyde for 20 min at 25°C. Cells were permeabilized with 1% Nonidet P-40 for 10 min at 25°C and washed twice with PBS. A polyclonal Tat peptide antibody was used at a dilution of 1:50 in 1× PBS containing 2% chicken serum and incubated at 4°C overnight. The cells were washed three times with 1× PBS. Secondary antibodies were diluted 1:1,000 in PBS-2% chick serum and incubated for 1 h at 25°C.

RESULTS

Mapping of targeting domain of Tat. A recent report from our laboratory showed that, in the Gal4 system, the Tat protein has a potent N-terminal activation domain (6). Interestingly, the full-length Gal4-Tat protein had minimal activity on a promoter containing five Gal4 binding sites. Our results led us to the conclusion that Tat has a suppressor region adjacent to the activation domain. Further analysis of this region has prompted us to propose an alternative explanation for this lack of activity in the full-length construct. A new CAT reporter construct in which an AP-1 site was inserted just upstream of the Gal4 binding sites was made. This reporter construct was tested in cotransfection assays with Gal4-Tat and its derivatives, and its activity was compared with that of the original reporter construct containing only Gal4 binding sites (Fig. 1). Activation by full-length Gal4-Tat protein on the Gal4 promoter with the AP-1 site Gal4(5)AP-1-CAT was 14-fold greater than that on the Gal4(5)-CAT. The addition of an AP-1 site in the Gal4(5)-CAT promoter also increased the activation by the Gal4-Tat activation domain construct by more than twofold and increased that of the Gal4-Tat(34-62) construct fourfold. Increased activation in the promoter construct containing the AP-1 site was specific for the Tat protein, since the Gal4 protein alone or Gal4-Tat containing the C-terminal domain of Tat(60-94) showed a minimal increase in activity when the AP-1 site was present in the Gal4(5)-CAT promoter. These results indicate that the activity of the Tat protein is maximal when an AP-1 site is present in the promoter. The lack of activation observed for full-length Tat in the Gal4 system could be due to the protein being targeted to cellular promoters that contain AP-1 sites rather than to the Gal4(5)-CAT promoter. Interestingly, the full-length Gal4-Tat construct had the most activity on a reporter construct that

TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence (initial amino acid of Tat)
5' primer	5' TGTGAAGCTTCTTCCACCATGGAAGAAGTACCAAGAAG 3' (1) ^a
	5' TACGCAAAGCTTCCACCATGAGAGTAATCTGACGAAGACT 3' (34) ^a
	5' CGCCGAAGCTTCCACCATGCTGTGACGGCCCCCCCCG 3' (VP16) ^b
3' primer	5' TGTACTTCTAGATTATCACAGTTCACATTTCTT 3' (94) ^a
	5' AGCCATCGTTGTAGTCTTTC TCCCGGCCCCGGGA 3' (VP16-Tat38) ^b
Mutated complementary primers	5' (39) AGACAACAACGATGGCAAGCTATGCATACTAATAATCAGCATAGA 3' (53) ^c
	3' (53) TCTGTTGTTGCTACCGTTCGATACGTATGATTATTAGTCGTATCT 5' (39) ^c
	5' (74) AGAAATGGTGGTGGCAGGCTAGGTAACCCC 3' (83) ^c
	3' (83) TCTTTACCAACCGTCCGATCCATTGGGG 5' (74) ^c
AP-1 complementary primers with <i>Xho</i> I ends	5' GCGCGCTCGAGTGTAGCAGCTGATGCTTGAGTCATAACCCTCGAGCGGC 3' ^d
	3' CGCGGAGCTCACATCGTCGACTACGA ACTCAGGTAT TGGAGTCTCTCCG 5' ^d

^a Underlined regions indicate the first (5') or last (3') codon of Tat for each construct.

^b Regions in boldface italic type indicate VP16 sequences.

^c Underlined regions indicate single base changes for point mutations.

^d Underlined regions indicate AP-1 sequences.

FIG. 1. (A) Tat activation of the Gal4-CAT promoter construct with an AP-1 site added. HeLa cells were transfected with the effector plasmids indicated by inclusive amino acid numbers below each sample. The reporter constructs used were Gal4(5)-CAT, which has five Gal4 binding sites, and Gal4(5)AP-1-CAT, which has one AP-1 site inserted just upstream of the five Gal4 binding sites. For Gal4-Tat(1-38), the lysate was diluted 1:25 to bring the percent conversion into the linear range. The values for dilutions are given in the text. (B) Tat activation of the Gal4-CAT promoter with an AP-1 site added. (Graphic representation of Fig. 1A.) HeLa cells were transfected with the effector plasmids indicated by inclusive amino acid numbers below the graph. For Gal4-Tat(1-38), the lysate was diluted 1:25 to bring the percent conversion into the linear range. The values for dilutions are given in the text. Percent acetylation is calculated from the average of at least three CAT assays. The value for the negative control of Gal4 alone was less than 1% (not shown). Shaded bars, Gal4(5)-CAT; hatched bars, Gal4(5)AP-1-CAT. Error bars, standard errors.

contained no Gal4 binding sites but had the upstream AP-1 site (data not shown).

Mutational analysis of full-length Tat. To analyze wild-type and mutant Tat protein activity on the visna viral LTR, cotransfection assays, using a CAT reporter construct that has previously been shown in our laboratory to be the most responsive to Tat activation, were performed. This construct contains the U3 region of the LTR starting at bp 67 and includes the proximal AP-1 site and the TATA element. All constructs used in these experiments were analyzed for expression by Western blotting as well as in an immunofluorescence assay with an anti-Tat polyclonal rabbit antibody generated against a C-terminal Tat peptide (6). All constructs were esti-

mated to produce similar levels of protein in both of these assays, and all Tat proteins were translocated to the nucleus (data not shown). In all experiments, fold activation was calculated on the basis of percent acetylation induced by CMV- β Gal, which was used as a control. Fold activation for this vector was assigned a value of 1 (see Materials and Methods).

The wild-type Tat protein activated the viral LTR 40-fold in SCP cells. This is a primary sheep cell that can be infected with visna virus in culture. The activity of the Tat protein was somewhat less in HeLa cells (13-fold), possibly because of a lower affinity for human transcription factors. Previous results in the Gal4 system showed that mutations at Phe-19 and -21 in the activation domain decreased the level of activity by more than 100-fold. To determine if these residues were important in the context of full-length Tat, the same Phe-19 and -21 mutations were made, as shown in Fig. 2, and tested with -67LTR-CAT in cotransfection assays. While loss of the Phe residues caused a fivefold decrease in activity in HeLa cells (Fig. 3, Tat5' Δ F), the effect was much less dramatic in SCP cells, demonstrating only a minor decrease in activity. This difference could be due to the fact that in SCP cells, Tat's activity is optimized, and the mutation of the Phe residues may be compensated for by other hydrophobic residues that have been shown to be important in this region (6).

Previously in the Gal4 system, mutation of the leucine residues within the regulatory region was shown to relieve some of the repression observed for the full-length Gal4-Tat protein. To determine if these leucine residues were important in the full-length Tat, they were mutated to histidine or glutamine residues (Fig. 2). Figure 3 shows that loss of these leucines causes an almost-total loss of Tat activity in both HeLa and SCP cells. This suggests that this region is important for targeting the Tat protein to the viral promoter. Although it cannot be ruled out that these mutations alter the secondary structure of the protein, the experiments described below do not support this notion.

To investigate the function of the cysteine residues at the carboxy terminus of the Tat protein, the three conserved cysteine residues were changed to glycine residues. In SCP cells, loss of these cysteine residues caused a threefold drop in activity, while in HeLa cells, a fourfold drop occurred. This region of Tat may be involved in intra- or intermolecular interactions, and the loss of activity observed could be due to the lack of proper protein-protein association.

Targeting the VP16 activation domain to the viral AP-1 site. Transcription activator proteins are modular in that their domains can be separated and remain functional (7, 31, 32). Often one domain can be swapped for another between two activator proteins. To further examine how the visna virus Tat protein was targeted to AP-1 sites, a chimeric protein containing the herpesvirus VP16 protein N-terminal activation domain (residues 1 to 47) and the visna virus Tat amino acids 38 to 94 was tested with the -67LTR-CAT (Fig. 2). The chimeric protein had twice the activity on the visna virus -67LTR-CAT promoter as the protein containing the VP16 activation domain alone (Fig. 4). Expression of amino acids 38 to 94 of Tat showed no activity on this promoter (data not shown). The VP16(1-47) activation domain alone or the chimeric protein containing Tat amino acids 38 to 94 had no activity on an Oct-1 containing the CAT reporter construct, indicating that these proteins were not targeted to a VP16-responsive promoter (data not shown). Mutation of the leucines in the leucine-rich domain of the VP16-Tat chimeric protein (as shown in Fig. 2) caused the activity to drop to a level near that of the VP16 activation domain alone (Fig. 5), indicating that the protein had lost its ability to target the AP-1 site.

Tat Constructs

VP16/Tat Chimeric Constructs

FIG. 2. Tat protein and VP16-Tat chimeric protein expressing constructs used in transfection assays. All wild-type Tat and VP16-Tat constructs were made in vectors driven by the CMV promoter. Mutations are indicated for each construct by asterisks. The corresponding amino acid change caused by the point mutation is indicated to the right of each bar. The VP16-Tat chimeric proteins were made by fusion of the genes encoding the first 47 amino acids of VP16, representing its N-terminal activation domain, to the portion of Tat encoding amino acids 38 to 94.

To further examine if this leucine region of Tat targets the protein to the AP-1 site, a competition experiment was performed by expression of the leucine-rich region [Gal4-Tat(34-62)] together with wild-type Tat (CMV-Tat). Figure 5 shows that increasing amounts of the competitor Gal4-Tat(34-62) construct competes with full-length Tat, decreasing its activity threefold over the range used in this competition. These results suggest that this leucine-rich region of the Tat protein binds to cellular factors, such as Jun and Fos, and competes with the full-length Tat protein for binding to the AP-1 site in the -67LTR-CAT promoter.

DISCUSSION

The visna virus Tat protein, like other lentiviral Tat proteins, regulates transcription of the viral LTR. The Tat-responsive region of the viral LTR has been mapped to an AP-1 site proximal to the TATA box in the U3 region (14, 18). In the present study, mutagenesis experiments, as well as domain-swapping and competition assays, have revealed a targeting domain within the Tat protein. In a previous study, we showed that while Tat has a potent activation domain, a full-length Gal4-Tat construct had little activity (6). In the present study, we reexamined this finding by adding an AP-1 site to the Gal4-CAT construct. When an AP-1 site was inserted just

upstream of a CAT reporter construct containing five Gal4 binding sites, the activity of a full-length Gal4-Tat fusion construct increased 14-fold. The addition of the AP-1 site also increased the activity (fourfold) of a construct containing the leucine-rich region of Tat, Gal4-Tat(34-62). This provided evidence that this region was important in targeting Tat protein to AP-1 sites. An increase in activity was also observed for the Gal4-Tat(1-38) construct; this could be due to increased binding of the activation domain at AP-1 sites by interactions with Jun or Fos. Binding of the Tat protein to Jun and Fos has been observed in vitro with GST-Tat fusion constructs. These studies are similar to those done with the human T-cell leukemia virus type 1 (HTLV-1) Tax protein, and the addition of the 21-bp repeats from the HTLV-1 promoter to a construct containing only Gal4 binding sites greatly increased the activity of Gal4-Tax (8). Similar results were observed for HIV-Tat in the Gal4 system when an SP-1 site was added to the Gal4 promoter construct (20, 36). These results indicate that flanking sequences are important for the ability of these proteins to function as transactivators.

Cotransfection experiments using Tat in the context of its own LTR revealed that, as in the Gal4 system, mutation of Phe-19 and -21 reduced Tat's activity fivefold in HeLa cells (Fig. 3). Interestingly, the activity of this mutant construct was

FIG. 3. Mutational analysis of wild-type Tat protein. SCP cells or HeLa cells (as indicated) were transfected with the effector plasmids indicated. The target construct was -67LTR-CAT . Fold activation is calculated from the average of at least three CAT assays. The value for the negative control of CMV- βGal was subtracted out of each value to give the percent acetylation and fold activation. Error bars, standard errors.

only slightly reduced in SCP cells, a cell type that is highly permissive for visna virus infection. These results suggest that in a natural host cell these Phe residues are less critical for the activity of this domain and that other hydrophobic residues may either compensate for the Phe residues or be sufficient for activation.

Further mutational analysis of Tat revealed another important region of the protein. The C-terminal portion of Tat, while not highly conserved overall, has three conserved cysteine residues. When these residues were changed to glycines, there was a fourfold loss of activity in HeLa cells and a threefold loss of activity in SCP cells (Fig. 3). The cysteine-rich region of HIV-Tat has been shown to be critical for activity and has also been included in the mapping of the activation domain. Furthermore, studies have shown that this domain binds to zinc (12). As yet, we have no evidence to suggest that the C-terminal portion of Tat is a metal-binding domain. Alternatively, this region may be important for protein dimerization of Tat. Tat can bind to other proteins *in vitro* and may possibly form weak homodimers under certain conditions (27). Yet, another potential function for this region is involvement in redox regulation with Jun and Fos in the infected cell. It has been shown that the DNA binding of the Fos-Jun heterodimer is modulated by reduction-oxidation of a single conserved cysteine residue in the DNA-binding domains of these two proteins (1, 44). Xanthoudakis and Curran went on to identify the Ref-1 protein as the mediator of this redox reaction (43). Clearly further studies are needed to understand the function of this region in the visna virus Tat protein.

The leucine-rich region of the Tat protein (residues 38 to 62) is highly conserved not only among different strains of visna virus but also between visna virus and caprine arthritis encephalitis virus. When four of the leucines within this region were

mutagenized, Tat lost its ability to activate the viral LTR-CAT construct containing an AP-1 site in HeLa and SCP cells (Fig. 3). Moreover, a chimeric construct containing the N-terminal activation domain of VP16 fused to Tat amino acids 38 to 94 conferred AP-1 responsiveness to the VP16 activation domain (Fig. 4). When the leucines in this region were mutated (Fig. 3), the activity dropped to the level of the VP16 activation domain alone. Finally, competition experiments revealed that the expression of increasing amounts of Gal4-Tat(34-62) reduced the activity of full-length CMV-Tat (Fig. 5). The Gal4(1-147) construct alone was used to eliminate the possibility of promoter competition. Initially, we were surprised to find that Gal4(1-147) alone also competed with Tat, albeit to a much lesser extent than did Gal4-Tat(34-62). A recent study by Sollerbrant et al., however, showed that the DNA-binding domains of Gal4 and Jun can interact through a zinc finger and bZIP binding motifs (35). We believe that our results are consistent with this study, in that the Gal4 construct used as a control lowered the level of activity and may be competing for Jun binding in these cells (Fig. 5).

Evidence presented thus far indicates that the leucine-rich domain of Tat is responsible for targeting the protein to AP-1 sites. The possible mechanism for this interaction with the AP-1 complex is unclear. Previous studies from our laboratory have shown that the Tat protein does not bind to DNA or RNA in mobility shift assays (5). This would suggest that Tat's targeting mechanism might not be like that of HIV-Tat, which is tethered to RNA by its TAR region (4, 11, 25, 30). Rather, visna virus Tat may be more like other viral activators such as VP16, HTLV-Tax, and the adenovirus E1A protein (3, 24, 39, 42). These potent activators have been shown to bind to cellular factors, which in turn bind to their respective DNA consensus sites. Their activity may be a combination of regulation

FIG. 4. Swapping of activation domains of the Tat protein and VP16 protein to identify promoter targeting region. SCP or HeLa cells (as indicated) were transfected with the effector plasmids indicated. All effector plasmids encoded the first 47 amino acids of VP16 and Tat residues 38 to 94. Mutations of the VP16-Tat construct are shown in Fig. 2. The target construct was -67LTR-CAT. Fold activation is calculated as the average of at least three CAT assays. The value for the negative control of CMV-βGal was subtracted out of each value to give the percent acetylation and fold activation. Error bars, standard errors.

not only at the initiation complex through binding to TBP and TFIIB but also at upstream promoter sites by binding to cellular factors and modulating or enhancing their activity.

In our laboratory we have observed that the Tat protein binds to both Jun and Fos (27). Further, it appears that the leucine-rich domain of Tat (residues 34 to 62) is important for

binding to Fos (27). This leucine-rich region of Tat appears to have a helical structure similar to the leucine zipper region of bZIP proteins. It is intriguing that the binding of Tat to Fos may alter the specificity of heterodimer formation with Jun or other members of the bZIP family of proteins. It is possible that this altered binding pattern is responsible for the complex's preferential binding the AP-1 site in the visna virus LTR. It is now known that interactions between different members of the bZIP family can alter their DNA-binding specificities and that sequences that flank the DNA-binding site are important in determining this specificity (19, 22, 23, 26, 38, 41, 42). Future experiments will test these hypotheses to determine if Tat is functioning in this manner.

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FIG. 5. Competition experiment with CMV-Tat and Gal4-Tat(34-62). HeLa or SCP cells were transfected with 1 mg of CMV-Tat and increasing amounts of Gal4-Tat(34-62) (diamonds) or Gal4 alone (squares) as indicated. The target construct was -67LTR-CAT. Fold activation is calculated as the average of at least three CAT assays. The value for the negative control of CMV-βGal was subtracted out of each value to give the percent acetylation and fold activation. Error bars, standard errors.

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