Visna Virus Tat Protein: a Potent Transcription Factor with both Activator and Suppressor Domains

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Visna virus is a pathogenic lentivirus of sheep that is distantly related to the primate lentiviruses, including human immunodeficiency virus type 1. The visna virus genome encodes a small regulatory protein, Tat, which is necessary for efficient viral replication and enhanced viral transcription. To investigate the mechanism of action of the visna Tat protein and to localize the protein domain(s) responsible for transcriptional activation, chimeric proteins containing visna virus Tat sequences fused to the DNA binding domain of the yeast transactivation factor GAL4 (residues 1 to 147) were made. The GAL4-Tat fusion proteins were transfected into cells and tested for the ability to activate the adenovirus E1b promoter via upstream GAL4 DNA binding sites. Full-length GAL4-Tat fusion proteins were weak transactivators in this system, giving only a two- to fourfold increase in transcription in several cell types, including HeLa and sheep choroid plexus cells. In contrast, fusion of the N-terminal region of the Tat protein to GAL4 revealed a potent activation domain. Amino acids 13 to 38 appeared to be the most critical for activation. No other region of the protein showed any activation in the GAL4 system. This N-terminal region of the visna virus Tat protein has a large number of acidic and hydrophobic residues, suggesting that Tat has an acidic activation domain common to many transcriptional transactivators. Mutations in hydrophobic and bulky aromatic residues dramatically reduced the activity of the chimeric protein. Competition experiments suggest that mechanism of the visna virus Tat activation domain may closely resemble that of the herpesvirus activator VP16 and human immunodeficiency virus Tat, a related lentivirus activator, since both significantly reduce the level of visna virus Tat activation. Finally, a domain between residues 39 and 53 was identified in the Tat protein that, in the GAL4 system, negatively regulates activation by Tat.

Lentiviruses are a subfamily of nononcogenic retroviruses that cause chronic, multiorgan diseases characterized by long incubation periods and slowly progressive clinical courses. Visna virus causes interstitial pneumonitis and a progressive inflammatory disease of the central nervous system (54). One important biologic property common to all lentiviruses is a tropism for cells of the monocyte/macrophage lineage during natural infection. (21). Infected monocytes, however, do not exhibit significant levels of viral RNA or protein synthesis until these cells mature into macrophages (20, 21, 54). This maturation process can be stimulated by treating monocytes with agents such as phorbol esters or cytokines and is related to the activation of specific cellular transcription factors (1, 2, 8, 11, 16, 37, 60).

Gene expression directed by the visna virus long terminal repeat (LTR) is also induced upon activation of monocytes to macrophages (17, 67). Studies done in this laboratory have identified sequences in the U3 region of the visna virus LTR important for transcriptional activity. These include a TATA box at -20 and a recognition site for the AP-4 transcription factor at -60 (53). In addition, there are multiple AP-1 sites in the viral LTR, and the one most proximal to the TATA box mediates viral activation by phorbol ester in macrophages (17). This AP-1 site has a sequence identical to the AP-1 consensus and appears to be critical for efficient transcription (39, 61, 62). Previous studies have shown that the 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 (66).

In addition to cellular factors, virus-encoded proteins are also involved in the regulation of visna gene expression (12). The visna virus genome, as well as other lentivirus genomes, encodes *trans*-acting proteins that regulate viral gene expression. The visna virus *tat* gene encodes a 10-kDa protein that greatly increases gene expression directed by the visna virus promoter in the 5' LTR (28). Interestingly, recent studies 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* promoter (19a, 19b).

The mechanism of transcriptional transactivation by visna virus Tat is not well understood. Unlike human immunodeficiency virus (HIV) Tat, visna virus does not have a TAR-like structure in the R region of the LTR (5, 14, 19a, 39; reviewed in references 50 and 56). It has also been demonstrated that HIV Tat can transactivate through TAR-independent mechanisms as well by binding to TFIID (27, 34). Also, mechanisms involving interaction via the TAR element or indirect interaction with the transcription initiation machinery via the cellular factor Sp1 have been demonstrated (31, 33, 68). It has been reported that other viral transactivators, which do not bind to DNA directly, activate transcription by binding to cellular factors which in turn bind to upstream promoter elements. The herpesvirus VP16 protein binds to the Oct-1 transcription factor and most recently has been shown to bind to the promoter factors TFIIB, TFIID, and TFIIF (23, 24, 44, 64). The adenovirus E1A protein has recently been shown to bind to the TATA-binding protein (TBP) of TFIID (4, 30, 40, 45).

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TABLE 1. Oligonucleotides used

Type	Sequence (initial amino acid of Tat activation domain)
5' primer ^a	5' GCGCGGAGATCTATGGAAGAAGTACCAAGAAG 3' (1) 5' GCGCGAGATCTGTAGAAGCAGAGGGAGTATTT 3' (13) 5' GCGCCAGATCTTTTCAATTTTATGAAGACTGG 3' (19) 5' GCCGCAGATCTTGGGAATGTTGGGACTATGTA 3' (25) 5' GCGCCAGATCTAGAGTAATCTGACGAAAGACT 3' (34) 5' GCGCCAGATCTGCACAGATTGGATATGGAA 3' (60)
3' primer ^a	5' GCGCCTCTAGATTATCAGTCCCAACATTCCCA 3' (29) 5' GCCGCTCTAGATTATTAGTCAGATACTCTTTGACTTACATA 3' (38) 5' GCGCCAGATCTTTATCACCATCGTTGTAGTCTTTCGT 3' (43) 5' GCCGCGTCTAGATTATCATCTAAGCTGATTATTAGTAAGCAT 3' (53) 5' GCGCCAGATCTTTATCACCACATCCATATCTGTGCTTC 3' (62) 5' TGTACTTCTAGATTATCACAGTTCAACATTCTT 3' (94)
Mutated primer ^b	
5'	5' GCGCGAGATCTGGAGAAGCAGAGGGAGGATTT 3' (13) 5' GCGCAGATCTGTAGAAGCAGAGGGAGTATCTCAATCTTATGAA 3' (13)
3'	5' GCCGCTCTAGATTATTCGTCAGATCCTCTTTGACTTCCATA 3' (38) 5' GCGCGTCTAGATTATCTAAGCTGATTATTAGTATGCATAGCTTGCCA 3' (53)
Oligonucleotide for cloning of Tat into pSVL ^a	5' CGCCGCTCGAGACCTGCCATGGAACCAGTCGA 3' CCGCCGGATCCGGATCCTTACTATTCTTAGG

^a Underlined regions indicate the first codon of Tat for each construct.

^b Underlined regions indicate the first codon of Tat for each construct and single bases changed for point mutations.

Transactivators contain at least two essential domains; one directs the protein to its target promoter through direct DNA binding or protein-protein interactions, and a second facilitates events that increase initiation of RNA transcription presumably through direct contact with other cellular factors at the promoter (57, 58). Previous studies in our laboratory have not detected direct binding of visna virus Tat to the visna virus LTR or to nascent RNA transcripts (19b). Therefore, the visna virus Tat protein may be involved in an indirect activation mechanism, possibly by recruiting or modulating cellular factors involved in the initiation of transcription.

To further study the mechanism of visna virus Tat transactivation, we identified the Tat activation domain and essential amino acid residues within it by using a GAL4-Tat chimera system. Results showed that Tat has a single, potent acidic activation domain. Tat has a pattern of bulky aromatic and hydrophobic residues which are important for its activity. Although, the activation domain is constitutively active when linked to GAL4, its activity is influenced by intramolecular mechanisms in the context of full-length Tat. A leucine-rich region adjacent to the N-terminal activation domain (residues 39 to 53) was found to dramatically reduce Tat transactivation. Further, competition experiments using the visna virus Tat activation domain with VP16, an acidic activator, and the HIV Tat, an unclassified activator with an acidic region, suggest that although the targeting regions of these activators may be different, all three may act through a common set of recruiting factors, resulting in activated transcription.

MATERIALS AND METHODS

Plasmid constructions. The effector plasmid pGH250 and the target plasmids E1b-CAT, (GAL4)₁E1b-CAT, and (GAL4)₅E1b-CAT were obtained from Gary Hayward and previously described by Hardwick et al. (26). Constructs containing chimeric genes of GAL4-Tat were generated by cloning *Bgl*II (5' end)- and *Xba*I (3' end)-digested PCR products of Tat into the *Bgl*II and *Xba*I sites of the GAL4 DNA binding domain in GH250. Sequences of the oligonucleotides synthesized for the generation of these constructs are shown in Table 1. Mutated primers (Table 1) were used to generate single

amino acid changes shown in Fig. 4 and 7.

The SV2VP16 construct contains the entire VP16 open reading frame driven by the simian virus 40 (SV40) promoter as described previously (26). The HIV Tat Designer Gene construct was obtained from the NIAID AIDS Research and Reference Reagent Program (Ogden BioServices Corp.). The HIV-1 Tat gene was subcloned into the pSVL expression vector by using PCR to generate an *Xho*I site (5') and a *Bam*HI site (3'). Sequences of the oligonucleotides designed for cloning of Tat into the pSVL expression vector are shown in Table 1. All new constructs were sequenced to verify reading frames and mutations.

Transfections. HeLa and sheep choroid plexus 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 calcium phosphate method as previously described (26). Briefly, HeLa cells were seeded into six-well cluster dishes at a density of 4 × 10⁵ cells per well 24 h prior to transfection. The cells were given fresh medium 4 h before transfection. The transfection mix contained 1 µg of target and 2 µg of effector plasmid, 125 µl of 2× HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid)-buffered saline (274 mM NaCl, 10 mM KCl, 1.4 mM Na₂HPO₄, 12 mM glucose, 42 mM HEPES [pH 7.04]), and H₂O to a final volume of 238 µl. While the transfection cocktail was bubbled, 12 µl of 2.5 M CaCl₂ was added. Samples were incubated at room temperature for 20 min to allow a precipitate to form. The mix was then added dropwise to the culture medium in each well. The cells were glycerol shocked 18 to 20 h posttransfection with 15% glycerol in phosphate-buffered saline (PBS). The medium was replaced, and cells were incubated for an additional 24 h.

CAT assays. Chloramphenicol acetyltransferase (CAT) assays were performed as previously described (26). Briefly, transfected cells were washed two times with PBS and scraped into 1 ml of PBS. Cells were pelleted and resuspended in 100 µl of 0.25 M Tris hydrochloride (pH 7.8). Cells were disrupted by sonication, and debris was removed by centrifugation. 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

FIG. 1. GAL4-Tat chimeric proteins. (a) A plasmid expressing full-length Tat or its derivatives was fused to the DNA binding domain of GAL4 to generate chimeric proteins. The target plasmid contained the CAT gene with the minimal adenovirus E1b promoter and five upstream GAL4 DNA binding sites. (b) The amino acid sequence of a visna virus Tat clone described previously (12). The domains of the Tat protein fused to GAL4 and used in transfection experiments are outlined.

100 μ g) was mixed with a cocktail containing 70 μ l of 1 M Tris (pH 7.8), 27 μ l of H₂O, 2 μ l of 40 mM acetyl coenzyme A, and 1 μ l of [¹⁴C]chloramphenicol (0.1 μ Ci, 60 mCi/mmol). The mix was incubated at 37°C for 90 min. 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 μ l of ice-cold ethyl acetate and spotted onto a thin-layer chromatography plate. The acetylated forms were separated by a chromatography in a chloroform-methanol solvent (95:05 [vol/vol]). 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. Fold activation was calculated by the ratio of the percent acetylation of each sample to that of the control effector GAL4(1-147) (the GAL4 DNA binding domain, extending from amino acids 1 to 147) transfected with the indicated CAT reporter plasmid, which was arbitrarily assigned a value of 1. In all experiments, the standard error calculated from the percent acetylation ranged from 0.1 to 20.

Immunofluorescence. Cells were transfected as described above and fixed at 24 or 48 h as follows: after cells were washed twice with PBS, 3.7% paraformaldehyde was added for 20 min at 25°C. Cells were permeabilized with 0.1% Nonidet NP-40 for 10 min at 25°C and washed twice with PBS. A monoclonal anti-GAL4 antibody, which was the gift of P. Chambon (71), was used at a dilution of 1:200 in 1 $\times$ PBS containing 2% chicken serum and incubated at 25°C for 1 h. The cells were washed three times with 1 $\times$ PBS. Secondary antibodies were diluted 1:1,000 in PBS-2% chick serum and incubated for 1 h at 25°C.

RESULTS

Mapping of the activation domain of Tat. Previous studies from this laboratory have shown that visna virus Tat activates transcription from the viral LTR as well as from heterologous promoters containing AP-1 sites (19a, 19b). Transactivator proteins usually contain at least one activation domain which

interacts with the transcription initiation complex. Transactivators also contain targeting domains which serve to localize the protein to its site of action. For some activators, the protein itself binds to DNA sequences, usually upstream of the initiation site. Other transcriptional activators act more like bridging factors and exert their effect by interacting directly with other DNA-binding proteins. The activation and targeting domains of most proteins are separable and can be transferred to heterologous proteins. Since visna virus Tat does not appear to bind DNA directly, we used the well-characterized yeast transactivator GAL4 DNA binding domain (amino acids 1 to 147) as the DNA targeting element. A series of fusion proteins was constructed to define the region(s) of the Tat protein necessary for transactivation (Fig. 1). Chimeric genes were generated by substituting portions of the *tat* gene for the activation domain of GAL4. Visna virus Tat is a small protein of 94 amino acids. Therefore, as a first step in defining regions important for transactivation, the Tat protein was divided into three regions of approximately 35 to 40 amino acids each (Fig. 1b). The entire Tat gene as well as the coding regions for these three domains were cloned in frame and downstream of the GAL4 DNA binding domain [GAL4(1-147)]. These fusion constructs were transfected into HeLa cells and tested for the ability to activate transcription of a cotransfected vector containing the CAT gene driven by a minimal adenovirus E1b TATA element with five upstream GAL4 DNA binding sites.

All constructs used in these experiments were tested in an immunofluorescence assay with anti-GAL4 antiserum (71) to ensure that they yielded a protein product that was translocated to the nucleus. All constructs were estimated to produce similar levels of protein by the immunofluorescence assay (data not shown). In all experiments, fold activation was calculated on the basis of the percent acetylation induced by GAL4(1-147) alone. Fold activation for this vector in each experiment was assigned a value of 1 (see Materials and Methods). The full-length visna virus Tat protein was a weak transactivator in the GAL4 system, with only a fourfold increase in activity over the GAL4 control (Fig. 2a, lane 2). It cannot be ruled out that the full-length Tat protein may have artificially low activity in the GAL4 system; however, previous studies in this laboratory have shown that the full-length

FIG. 2. Localization of the activation domain of Tat. (a) HeLa cells were cotransfected with GAL4-Tat effector constructs containing either full-length Tat or the domains depicted in Fig. 1b, with a target construct, (GAL4)₅E1b-CAT. Dilutions of cell lysates were made to maintain a fold activation within the linear range of the assay. Fold activations reflect the increase in activity over that obtained with GAL4(1-147), which has been assigned a value of 1. Calculation of fold activation is described in Materials and Methods. In experiments in which the percent acetylation of a sample was less than that of the control, the sample was assigned a value of 1 as well. (b) HeLa cells were transfected with effector plasmids as in panel a. The target plasmid used in these experiments is E1b-CAT, containing no GAL4 binding sites. (c) Sheep choroid plexus cells were transfected with effector constructs as in panel a with the target plasmid (GAL4)₅E1b-CAT.

protein itself is not an especially strong transactivator (12). The N-terminal portion of the protein (domain I) contained a potent activation domain with a 594-fold increase in activity over the control (Fig. 2a, lane 3). Initial experiments showed that constructs containing the activation domain had activity that was out of the linear range of the CAT assay. Therefore, in subsequent experiments, lysates were diluted either 1:10 or 1:50 to give results within the linear range (dilutions are indicated in Fig. 2). The activity of the N terminus was shown to be due to a specific interaction between the chimeric proteins and the GAL4 binding sites, as a construct containing no GAL4 binding sites was not responsive to the GAL4-Tat fusion proteins (Fig. 2b). Domains II and III of Tat do not have activity, suggesting that the Tat protein contains a single activation domain (Fig. 2a, lanes 4 and 5). To ensure that a possible activation domain was not interrupted in the first series of constructs, a vector containing amino acids 34 to 94 [Tat(34-94)] was tested. As shown in Fig. 3, this construct had no activity.

To determine if the Tat chimeric protein was functional in a natural host cell, transfections were done in sheep choroid plexus cells, a primary cell type which can be productively infected by visna virus. Results in Fig. 2c show that the full-length Tat protein had essentially no activity, while the N-terminal portion of the protein contained the activation domain and had a 28-fold increase over GAL4 alone. The fold activation in these cells is notably lower than in HeLa cells. This difference is thought to be due to a lower efficiency of expression resulting from the difficulty in transfecting this primary cell type. These results indicate that the activation domain of Tat does not appear to be cell type specific, although this does not rule out the possibility that it is a stronger activator in certain cell types, depending on the cellular factors important for its activity.

Determination of the boundaries of the activation domain.

To define a C-terminal boundary for the activation domain, a series of deletions was made in domain I from amino acid 43. Interestingly, the activity of the Tat protein increased 60% when amino acids 39 to 43 were removed [Tat(13-38); Fig. 3]. Further deletion of amino acids 30 to 38 [compare Tat(13-38) with Tat(13-29)] decreased activity almost threefold. Therefore, it appears that residues between 30 and 38 are important for transactivation.

To localize the amino acids that comprise the minimal activation domain, N-terminal deletions were also made in domain I (Fig. 3). Removal of the first 12 amino acids of Tat increased its ability to transactivate almost 2-fold (1,226-fold versus 613-fold). Further N-terminal deletions in Tat demonstrated that residues between amino acids 13 and 19 were critical for transactivation, as the Tat(19-38) construct had a fourfold decrease in activity (Fig. 3). Finally, the Tat(25-38) construct had no activity, indicating that critical residues and/or structure had been lost by deletion of amino acids 19 to 25. The results in Fig. 3 clearly demonstrate that Tat has a potent activation domain between residues 13 and 38. The minimal activation domain lies between amino acids 19 and 29. It is difficult to discern from these experiments whether critical residues had been deleted in Tat(25-38) or whether the protein was simply reduced to a nonfunctional size. However, mutational analysis described below supports the former theory, that critical residues had been lost.

Hydrophobic and aromatic residues are important for the acidic activation domain of Tat. Examination of the N-terminal amino acid sequence of Tat revealed a characteristic acidic domain, common to many other viral transcriptional transactivators (26, 46, 65). Recent reports have suggested, however, that it is not the acidic residues which are critical for function (10). Rather, it appears that hydrophobic and bulky aromatic

FIG. 3. Identification of the activation domain of Tat. HeLa cells were transfected with effector plasmids as indicated by inclusive amino acid numbers indicated to the left of each construct. The target construct was (GAL4)₅E1b-CAT. Fold activations are the averages of at least three CAT assays, reflecting the increase in percent acetylation over that of the GAL4(1-147) effector plasmid alone.

residues may be more important in defining the activation domain. Cress and Triezenberg (10) reported that in the case of VP16, bulky hydrophobic residues in the VP16 acidic activation domain are critical for activator function. This pattern of important hydrophobic residues was not limited to acidic activators and was also found in the glutamine-rich activator Sp1 (10). Figure 4 is an alignment of several known viral transactivators with conserved hydrophobic and aromatic residues in a characteristic pattern such that when mutated, the activity of the domain is diminished (10, 26, 46). Hydrophobic residues of visna virus Tat can be aligned at least two different ways with VP16 and Rta (Fig. 4). Point mutations made in this region are shown in Fig. 5. When Val-13 and Val-18 were changed to glycine residues, there was a twofold drop in activation. When constructs containing mutations at Val-31 and Val-35 were tested, there was an eightfold drop in activation, indicating that these residues are clearly important for activity in this system. When a construct containing mutations in all four valines in the hydrophobic pattern were tested, the activity resembled that of the construct with Val-31 and Val-35, showing that there was no additive effect with these mutations. We cannot determine from these studies if the amino acid substitutions disrupted direct protein-protein contacts or overall structure.

It has previously been shown for the herpesvirus transactivator VP16 that Phe-442 in the activation domain is critical for function (63). Tat has two Phe residues in the region from amino acids 19 to 25 of the activation domain. Mutation of both of these residues resulted in the most dramatic loss of activity compared with other mutations in the valine residues (Fig. 5). This finding indicates that in visna virus Tat, the bulky

aromatic residues are critical for the function of the activation domain. However, it is not yet clear if their spatial arrangement is critical.

Competition of Tat activation by VP16 and HIV Tat. The data thus far have shown that the activation domain of visna virus Tat shares some important characteristics with VP16. First, both Tat and VP16 have a high content of acidic and hydrophobic residues in their activation domains. Second, Tat has hydrophobic residues in a characteristic pattern, similar to VP16, which may play a role in the secondary structure of these activators. Finally, Phe residues within the activation domains of both proteins are critical for activity. To determine if these two activators utilize similar cellular factors, competition assays were performed to determine if an SV40-driven VP16 construct containing the entire coding region could inhibit the activity of a GAL4-Tat(13-38) chimera. An SV40 HIV-Tat construct containing the entire protein was also used in these experiments as a control for a nonacidic activator. The results in Fig. 6 show that VP16 did, in fact, inhibit the activity of GAL4-Tat(13-38) and caused a more than five- to sixfold decrease in activation. Somewhat surprisingly, HIV Tat was also effective as a competitor, although this effect is probably due to the TAR-independent activation capacity of HIV Tat, since there is no TAR element present in this system. pSV₂Neo was used as a control for squelching in these experiments and did not compete with visna virus Tat.

Identification of an inhibitory domain. Initial experiments (Fig. 2) with GAL4-Tat fusion proteins showed that the full-length protein is a relatively poor transactivator, although Tat does contain a potent activation domain, in the GAL4 system. Results of these initial experiments suggested that

Herpes

VP16	C (457)	H	L	D	G	E	D	V	A	M	A	H	A	D	A	L	D	D	F	D	L	D	M	L		
	N (427)	F	T	P	H	D	S	A	P	Y		G	A	L	D	M	A	D	F	E	F	E	Q	M	F	
Rta	HVS (a)	S	H	E	N	Q	T	L	E	I	D	N	N	V	D	I	E	D	L	G	L	S	F	P	M	S
	EBV (2b)	L	N	L	D	S	P	L	T	P	E	L	N	E	I	L	D	T	F	-	L	N	D	E	C	L
	HVS (b)	N	V	D	I	E	D	L	G	L	S	F	P	M	S	L	Q	D	F	-	L	N	D	E		
	EBV (2c)	F	L	N	D	E	C	L	L	H	A	M	H	I	S	T	G	L	S	I	-	F	D	T	S	L
na	Tat (aa 3)	E	V	P	R	R	Q	P	G	G	L	V	E	A	E	G	V	F	Q	F	Y	E	D	W	E	
	(aa 12)	L	V	E	A	E	G	V	F	Q	F	Y	E	D	W	E	C	W	D	Y	V	S	Q	R	V	S

FIG. 4. Comparison of amino acid sequences of viral activation domains. Hydrophobic residues (boxed) of several herpesvirus activators and visna virus Tat are aligned. Alignment of viral and cellular sequences has been previously described (10, 26, 46). Notations in parentheses indicate beginning amino acid (aa) of the Tat activation domain.

domains in the protein may have been repressing the activity of the activation domain. To determine which regions of the protein were responsible for this repression, deletions were made in the C-terminal portion of the Tat protein. Results in Fig. 7 show that deletion of amino acids 63 to 94 from GAL4-Tat did not increase activity, and further deletion to residue to 53 [Tat(13-53)] also did not increase activation. It was also noted that removal of residues 39 to 43 from the N-terminal constructs shown in Fig. 2 resulted in a 1.5-fold increase in activity. These results suggested that the region of repression was located between residues 39 and 53. This region of the Tat protein contains a high content of leucine residues that are conserved not only between visna virus strains but also in the Tat protein of caprine arthritis encephalitis virus, a closely related lentivirus. These leucines do not form a typical leucine zipper (38), characteristic of many transactivators in the bZIP family. However, it has been recently reported that leucine residues (not necessarily forming a leucine zipper) may be involved in protein-protein interactions in other transcriptional activators (15).

To determine if the leucines in this region were, in fact, responsible for the inhibitory activity of the Tat(13-53) construct, point mutations were made in Leu-44 and Leu-47 to

change them to His and Gln, respectively. Mutation of the two leucines in this domain partially restores the activity of the Tat protein to 57-fold, as opposed to the 3-fold activity of the Tat(13-53) construct. The possibility exists that this region of the protein is responsible for binding a cellular factor which, when bound, represses the activity of Tat. Previous reports have suggested that this is the case with the inhibitory domain of *c-jun* (3). This is consistent with the observation that activity of the activation domain of Tat, by itself, is constitutive. Alternatively, this region could cause intramolecular interactions that repressed Tat activity.

DISCUSSION

Visna Tat is a regulatory protein necessary for efficient viral replication and transcription from the viral LTR. The identification of Tat as a transactivator has been previously described (12). Through the use of the yeast GAL4 fusion system, we have identified the activation domain of the Tat protein as well as residues important for its activity. Results in this study revealed that the activation domain of Tat is located in the N-terminal 38 amino acids of the protein, with a minimal region of activity between residues 19 and 29. Mutational

FIG. 5. Mutational analysis of the visna Tat activation domain. HeLa cells were transfected with effector plasmids as indicated by inclusive amino acid numbers indicated to the left of each construct. The target construct used was (GAL4)₅E1b-CAT. Point mutations were made in the Tat activation domain as described in Materials and Methods and are indicated to the right of each construct. The amino acid sequence of visna virus Tat from residues 13 to 38 is shown above the Tat(13-38) construct. Fold activations are the averages of at least three CAT assays, reflecting the increase in percent acetylation over that of the GAL4(1-147) effector plasmid alone.

FIG. 6. Competition experiment with visna virus Tat and VP16 or HIV Tat. Increasing concentrations of the indicated competitors (SV40-driven VP16, HIV Tat, or *neo* gene) were cotransfected with 2 μ g of GAL4-Tat(13-38) and 1 μ g of the target plasmid (GAL4)₅E1b-CAT. Fold activations were calculated as described in Materials and Methods.

analysis of the activation domain showed that Phe residues at 19 and 21 are important for activity. It also appears that other hydrophobic residues may be important in the activation domain, as mutation of valine residues at 13, 18, 31, and 35 reduces the activity of Tat by severalfold. It should be noted

that there is a potential phosphorylation site in the region of Val-35 (Ser-36). Thus, mutation of Val-35 may have disrupted a consensus site for phosphorylation. It is possible that phosphorylation within the acidic activation domain of Tat enhances its activity by adding to the net negative charge of the region. Computer-generated analysis of the amino acid sequence of visna virus Tat predicts a helical structure in the region of the minimal activation domain, although the helix it is not amphipathic. It is still unclear if there is a secondary structure that is characteristic of acidic activating regions. Recent reports have suggested both amphipathic α -helical structures and β -pleated sheets as possible structures for acidic domains (10, 25, 41).

Activation domains have been demonstrated to function as independent molecular components of transcription factors. These domains are not typified by a single consensus sequence but fall into several categories. For example, there are acidic domains in GAL4 (48), GCN4 (29), and VP16 (65), glutamine-rich stretches in Sp1 (9) and Oct-2/OTF2 (22), proline-rich sequences in CTF/NF-1 (52), serine/threonine-rich regions in Pit-1/GHF-1 (69) and Erg-1 (18), and a combination of acidic and proline-rich domain in Jun and Fos (1, 6). Experiments done thus far indicate that Tat is an acidic activator. This is the first report of the identification and characterization of an acidic activation domain in the Tat protein of lentiviruses. Although studies on HIV Tat with the GAL4 system have been reported, it appears that Sp1, or at least an Sp1 binding site, is required for activity, even when the N-terminal activation

FIG. 7. Identification of the inhibitory domain of Tat. HeLa cells were transfected with effector plasmids as indicated by inclusive amino acid numbers indicated to the left of each construct. Point mutations were made in the Tat inhibitory domain as described in Materials and Methods and are indicated to the right of each construct. The target construct used was (GAL4)₅E1b-CAT. Fold activations are the averages of at least three CAT assays, reflecting the increase in percent acetylation over that of the GAL4(1-147) effector plasmid alone.

domain of HIV Tat was used (68). Furthermore, it appears that while HIV-1 Tat has a negatively charged region in its N terminus, this region is not considered to be the complete activation domain (47).

It has been shown that acidic activators, although nonrigid in their sequence requirements, may have an alignment of hydrophobic residues and a net negative charge which allows them to interact with basal transcription factors (24). It has recently been reported that acidic activators bind to TBP through an interaction between positively charged lysine residues in TBP and the negatively charged domain of the activator (24). In this study, the activity of visna virus Tat was compared with that of another acidic activator, VP16, and a nonacidic activator, HIV Tat. A competition assay indicated that native VP16 acted as a competitive inhibitor of GAL4-Tat. HIV-1 Tat also competed in these experiments. This result is intriguing because competition by HIV Tat in the absence of TAR would presumably be at the level of the initiation complex, possibly through an interaction with TFIID. Since both VP16 and HIV Tat are known to bind factors within the initiation complex itself, perhaps the level of competition for both activators is at the initiation complex and not targeting elements.

Interestingly, Tat is similar to VP16 in its pattern of hydrophobic residues and its essential Phe residues (Fig. 3 and 4, and reference 63). These results are consistent with the idea that different classes of activators interact with different transcription factors. The small size of the visna virus Tat acidic activation domain and the results of our mutagenesis experiments suggest that the visna virus Tat activation domain is not composed of separate functional subdomains, although this possibility has not been formally tested. In contrast, the VP16 (25a) and Rta (70) acidic activation domains are composed of at least two distinct functional subdomains.

Reports from our laboratory and other laboratories have shown that Tat is a transcriptional transactivator (12, 55). In most cases, however, the level of transactivation has been considered relatively low (10- to 40-fold), in part because of a high basal level of transcription of the viral LTR (12). Therefore, it was not particularly surprising that activity of the full-length Tat was low in the GAL4 system (fourfold; Fig. 2a, lane 2). It cannot be ruled out that the full-length Tat protein may have artificially low activity in the GAL4 system; however, this result is consistent with previous studies in this laboratory using the wild-type, full-length Tat as described above. It was, however, surprising that within Tat, there was a small, potent activation domain that had nearly 150 times the activity of the full-length protein. It appeared from analysis of GAL4 fusion proteins containing the entire Tat protein that other regions in the protein may have been repressing the activation domain. The region between residues 39 and 53 was found to contain an inhibitory domain. Furthermore, mutations of conserved leucine residues in this region restore partial function to a construct containing residues 13 to 53 (57-fold; Fig. 7). A fusion construct with this region deleted [Tat(1-38/60-94)] gave a significant increase in activity over the full-length protein (40-fold) but did not regain the full activity of the activation domain itself (data not shown). This finding suggests that there may be another inhibitory domain at the C terminus of the protein or that residues 39 to 53 are only part of the inhibitory region. It is possible that there is an intramolecular interaction that regulates the activation domain. Such interactions are known to exist in the case of the serum response factor, which has a modular inhibitory domain specific only to its activation domain (32). Another alternative is that an intermolecular interaction occurs with a cellular protein to inhibit transcription. The repression domain in Tat does not, however, resem-

ble other reported inhibitory domains such as the alanine-rich Kruppel protein of *Drosophila melanogaster* (42) or the immediate-early transcription factor Erg-1 (18), which has been reported to contain a proline-directed phosphorylation site within its sequence. The fact that this leucine-rich region is conserved among visna viruses and caprine arthritis encephalitis virus suggests that it is important for the function of the Tat protein in the viral life cycle.

Since the observed inhibitory effects have all been detected in the context of GAL4-Tat constructs, it will be important to examine the role of these domains with Tat alone. It is reasonable to assume that Tat is regulated in the cell, although it is not known what effect constitutive activity of the activation domain would have in the cell. It is intriguing to hypothesize that unregulated Tat might lead to deleterious effects on the cell which would ultimately limit the level of virus replication. Interestingly, none of the lentiviruses are known to have transforming potential. In its unregulated form, the visna virus Tat protein might have the potential to alter cell growth properties.

Other lentivirus activators that have been identified are HIV-1 Tat, equine infectious anemia virus Tat, Bel-1 of human foamy virus, and Taf of simian foamy virus type 1 (5, 13, 14, 36, 39, 51). With the exception of HIV-1 Tat, little is known about the mechanism of action of these activators. Much of the work that has been done has centered on the identification of the Tat-responsive elements in the lentivirus LTRs. In the case of HIV-1 and equine infectious anemia virus, the responsive element is the TAR region, which forms a stem-loop structure located approximately 40 bases downstream of the transcription initiation site (5, 13, 14). Experiments have shown that TAR is recognized as RNA rather than DNA and that HIV-1 Tat does, in fact, bind to the nascent mRNA (14). It remains controversial whether the Tat protein binds TAR to recruit basal transcription factors and thus promote the formation of new initiation complexes, as is the function of most transactivators, or to aid in the processivity of the polymerase, allowing full-length transcripts to be made (5, 13, 14, 31, 34, 35, 49, 68).

Indeed, HIV-1 Tat is unique among viral and cellular transactivators, which have been shown to work through a variety of strategies involved in binding to DNA consensus sites proximal to the TATA site and interacting with basal transcription factors to increase both the rate and amount of polymerase II initiation. TAR-like structures have not been identified in visna viruses or in caprine arthritis encephalitis virus. We propose that visna virus Tat, unlike HIV-1 Tat, acts more like other viral activators. These transactivator proteins, such as VP16 and E1A, have been shown to bind cellular factors which in turn bind to their DNA consensus sites. Recent studies have shown, however, that VP16 can bind to factors within the initiation complex itself, such as TBP and TFIIB (4, 23, 24, 44). Recent studies by Ransone et al. (59) have suggested that Jun and Fos bind to TBP as well as their target AP-1 consensus site (59). In the case of the herpesvirus Zta protein, it has been hypothesized that this activator may have originated from a cellular AP-1 family DNA-binding protein (43). Its responsive element (ZRE) contains an AP-1 site, and Zta can bind as a dimer to this site (7). Studies have shown that instead of the characteristic leucine zipper found in bZIP proteins, Zta has a region of repeated hydrophobic residues that can support its dimerization (15). Interestingly, this domain can substitute for the leucine zipper of c-Fos (15).

Studies from our laboratory have not detected direct binding of visna virus Tat to DNA. Rather, Tat may act more like adenovirus E1A or herpesvirus VP16 in its requirement for

cellular factors for activity. We have previously shown that the responsive element in the visna virus LTR is an AP-1 site and that c-Fos and c-Jun are capable of binding this site in the visna virus LTR (66). Furthermore, Neuve et al. recently reported that visna virus Tat is not an efficient transactivator in F9 teratocarcinoma cells, which are known to lack significant levels of c-Jun and c-Fos (55). This possible requirement for Jun and Fos proteins could provide a level of cell type specificity for the activity of visna virus Tat. It is not known what the endogenous levels of Jun and Fos homologs are in the natural host cells of visna virus, the sheep monocyte/macrophage. It is possible that the activation of the sheep monocyte to a macrophage, which is necessary for visna virus activation, is also required to relieve repression by the inhibitory domain of Tat. This could occur by activation or upregulation of cellular factors, possibly Jun and Fos, required to bind to Tat and cause a conformational change to allow exposure of the activation domain. Clearly, the mechanism of activation is complex and has some redundancies built in to fulfill the requirements of cell specificity and distinct signal transduction pathways utilized within the cell under different conditions.

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