

Inhibition of Human Immunodeficiency Virus Type 1 and Type 2 Tat Function by Transdominant Tat Protein Localized to both the Nucleus and Cytoplasm

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We introduced various mutations into the activation and RNA binding domains of human immunodeficiency virus type 1 (HIV-1) Tat in order to develop a novel and potent transdominant Tat protein and to characterize its mechanism of action. The different mutant Tat proteins were characterized for their abilities to activate the HIV LTR and inhibit the function of wild-type Tat in *trans*. A Tat protein containing a deletion of the basic domain (Tat Δ 49-57) localized exclusively to the cytoplasm of transfected human cells was nonfunctional and inhibited both HIV-1 and HIV-2 Tat function in a transdominant manner. Tat proteins containing mutations in the cysteine-rich and core domains were nonfunctional but failed to inhibit Tat function in *trans*. When Tat nuclear or nucleolar localization signals were fused to the carboxy terminus of Tat Δ 49-57, the chimeric proteins localized to the nucleus or nucleolus, respectively, and remained capable of acting in a transdominant manner. Introduction of secondary mutations in the cysteine-rich and core domains of the various transdominant Tat proteins completely eliminated their abilities to act in a transdominant fashion. Our data best support a mechanism in which these transdominant Tat proteins squelch a cellular factor or factors that interact with the Tat activation domain and are required for Tat to function.

The human immunodeficiency virus type 1 (HIV-1) and HIV-2 Tat proteins (Tat-1 and Tat-2) transactivate HIV gene expression at the transcriptional level by interacting with elements of the promoter region in the viral long terminal repeat (LTR) (reviewed in references 13 and 34). A functional Tat protein is required for high levels of HIV gene expression and viral replication (6). Tat function requires the binding of Tat to an RNA element present in the viral LTR known as the transactivation response element (TAR) (for a review, see reference 76). Both transcriptional elongation and initiation of transcription have been shown to be affected by Tat (13, 31, 37, 46, 52). Tat is believed to function by interacting with and directing components of the transcriptional machinery to the vicinity of the HIV promoter (11, 12, 50). Cellular transcription factors such as NF- κ B and Sp1 that interact with DNA elements present in the viral LTR have been reported to cooperate and synergize with transcription facilitated by Tat-TAR interaction (32, 35, 36, 44, 51). The detailed mechanism by which Tat activates transcription and the roles played by various cellular factors that associate with Tat and TAR remain to be elucidated.

Extensive mutagenesis and functional studies suggest that Tat-1 and Tat-2 function in similar manners. Although the overall homology between Tat-1 and Tat-2 is just 40%, they have nearly 80% homology in the highly conserved activation and basic domains (4, 5, 24). The activation domain is thought to interact with cellular factors, possibly transcription factors, while Tat is bound to TAR RNA (49, 50, 71). This domain has been further subdivided into cysteine-rich and core domains which are highly conserved between the two Tat proteins (15, 19, 41, 58, 59). The remainder of the amino-terminal domain, which is larger for Tat-2, shows little homology between Tat-1

and Tat-2, although mutations throughout this region significantly reduce or eliminate the ability of Tat to function. Both Tat-1 and Tat-2 also contain a positively charged basic domain that functions as a nuclear localization signal (NLS) and mediates the binding of Tat to TAR RNA *in vitro* (28, 65, 66). However, interaction of Tat with TAR in cells appears to also require residues throughout the entire amino terminus of Tat (48). Recently, it has been suggested that interaction of Tat and a cellular cofactor may be required for interaction of Tat with TAR *in vivo* (50).

Because of the importance of Tat in the life cycle of the virus, numerous strategies to interfere with Tat function have been suggested as potential antiviral therapies. One approach involves the use of transdominant mutant Tat proteins that inhibit Tat function when present in excess over Tat. Transdominant mutations in both the HIV Tat and Rev transactivators have been studied extensively in order to better understand the mechanism of action of these proteins. The best-characterized class of transdominant Tat proteins contain mutations within the basic domain (15, 53, 55). Tat peptides and proteins containing mutations in either the core or the cysteine-rich domain have also been reported to act in a transdominant fashion (7, 9, 23). However, little is known about the mechanism of action of transdominant Tat proteins. Three models have been proposed to explain the mechanism of action of the different transdominant Tat proteins. One mechanism envisions the competition of the transdominant and wild-type proteins for binding to TAR RNA (7, 9). Second, the propensity of Tat to multimerize, at least *in vitro* (16, 17), has led to the suggestion that certain transdominant Tat proteins may form mixed complexes with wild-type Tat that serve to trap wild-type Tat in the cytoplasm, where it cannot function (53). Third, since the interaction of Tat with various cellular factors has been proposed to be required for function, transdominant Tat has been suggested to function by sequestering a critical cellular factor required for Tat to function, a mecha-

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nism known as squelching (12, 50, 53). Transdominant proteins that function in this manner have been previously described for the yeast Gal4, herpesvirus VP16, and adenovirus E1a transactivators (29, 56, 73, 75).

To better understand the mechanism of action of transdominant Tat protein, we constructed and characterized several different Tat proteins that contained mutations in the cysteine-rich, core, and basic domains. The abilities of these mutants to activate HIV gene expression as well as to inhibit Tat function were investigated by using cell lines that contained integrated LTR-reporter constructs. We found that deletion of the basic domain between residues 49 and 57 (Tat Δ 49-57) resulted in an inactive Tat protein that acted as a transdominant inhibitor of wild-type Tat function. This protein localized exclusively to the cytoplasm of transfected cells and inhibited both Tat-1 and Tat-2 function in concert with both the HIV-1 and HIV-2 LTRs. We added either an NLS or a nucleolar localization signal (NOLS) to examine the ability of the transdominant protein to function in different cellular compartments. We also introduced secondary mutations in the cysteine-rich and core domains to examine whether mutations in domains shown to be critical for interaction with cellular factors affected the function of transdominant Tat. Our results support a model in which transdominant Tat acts by sequestering, or squelching, a cellular factor(s) that interacts with the activation domain of Tat.

MATERIALS AND METHODS

Plasmid constructions. pTat-1 was created by PCR amplification of the full-length (86-amino-acid) Tat-1 cDNA (HXB2 isolate), using pOTStat-4 (3) as the template. Since all primary isolates of HIV-1 encode a Tat protein at least 101 amino acids long, this shorter two-exon version is likely to be a laboratory artifact (54). The Tat-1 cDNA was amplified by using oligomers which contained *Eco*RI and *Nco*I sites at the 5' end and *Xba*I and *Hind*III sites at the 3' end. The resulting product was digested with *Eco*RI and *Hind*III and ligated to pCDN (2) digested with the same enzymes. The same strategy was used to subclone the full-length Tat-2 cDNA (ROD isolate, 130 amino acids; a kind gift from R. Gaynor) into pCDN to create pTat-2. The Tat-1 constructs pTatKL/A and pTat Δ 49-57 were constructed by using splicing-by-overlap-extension PCR (30). The same 5' and 3' outside oligomers were used together with internal oligomers that contained the desired mutations. For pTatKL/A, the lysine at position 41 and the leucine at position 43 were each changed to alanine (K41,L43-to-A mutation). pTat Δ 49-57 contains a deletion of residues 49 through 57. pTatC/S was constructed by PCR amplification of the Tat cDNA from pOTStatC27,30,31/S, which was constructed by oligonucleotide synthesis of the entire Tat cDNA (30a). pTat Δ /TNLS was constructed by PCR using pTat Δ 49-57 as the template together with the original 5' oligomer and a 3' oligomer containing the coding sequence for the C-terminal eight amino acids of Tat followed by the Tat NLS as previously described (66), a stop codon (TAG), and an *Xba*I site. The NLS (LRKRRRQRRV [65]) was designed so that the leucine codon replaced the original Tat stop codon. The resulting PCR product was cleaved with *Eco*RI and *Xba*I and ligated to pTat-1 digested with the same enzymes. pTat Δ /TNLS/C/S and pTat Δ /C/S were created by exchanging the *Eco*RI-*Bsu*36I fragment from pTatC/S for the same fragment from either pTat Δ 49-57 or pTat Δ /TNLS. pTat Δ /KL/A and pTat Δ /TNLS/KL/A were created by introducing the K41,L43-to-A mutation by PCR as described above into pTat Δ 49-57 and pTat Δ /TNLS, respectively. pTat Δ /NOLS was created by using the same strategy used to construct pTat Δ /TNLS, in which the NOLS sequence encoded the amino acids LRKRRRQRRRAHQN found in the Tat protein of several HIV-1 strains (61). pTat Δ /NOLS/KL/A was constructed by using PCR to introduce the K41,L43-to-A mutation into pTat Δ /NOLS. All inserts were sequenced by using the Taq Dideoxy Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's instructions.

Transfections, Western blot (immunoblot) analysis, and CAT assays. HeLa cells and cell lines were maintained in Dulbecco's modified Eagle medium containing 10% fetal calf serum, 200 μ g of G418 per ml, and 1% penicillin-streptomycin-amphotericin B (Fungizone) (Gibco-BRL). The construction of a HeLa cell line, referred to as A5, containing an integrated HIV-1 LTR (nucleotides -465 to +80) driving expression of chloramphenicol acetyltransferase (CAT) was described previously (74). A HeLa cell line containing the HIV-2 ROD LTR (nucleotides -254 to +156) driving expression of CAT, referred to as O3, was constructed as described for A5 (74). HeLa cells and cell lines were transfected by the calcium phosphate method (22). Cells were harvested between 60 and 65 h after transfection, and CAT activity was determined as previously described (21). COS cells were transfected by the DEAE-dextran method (25).

For Western blot analysis, cells were harvested 65 h after transfection, and proteins from cell lysates were separated on a sodium dodecyl sulfate (SDS)-18% polyacrylamide gel. Tat proteins were detected by using monoclonal antibody (MAb) 11, directed against amino acids 5 to 22 of Tat (10). The blots were developed by enhanced chemiluminescence (ECL kit; Amersham) according to the manufacturer's instructions.

Indirect immunofluorescence. COS cells were transfected in 35-mm-diameter wells by the DEAE-dextran method. At 65 h posttransfection, cells were washed three times in phosphate-buffered saline (PBS) and fixed in the wells with ice-cold acetone-methanol (1:1). Cells were incubated for 1 h at 37°C with either Tat MAb 9 (directed against the RGD amino acid sequence, amino acids 78 to 80) or MAb 11 (directed against an epitope between amino acids 5 and 22) diluted 1:100 in PBS (10). The two antibodies gave equivalent results for all proteins tested. Cells were washed again and incubated for 1 h at 37°C with goat anti-mouse immunoglobulin G-fluorescein isothiocyanate conjugate (Sigma) diluted 1:100. Cells were washed extensively with PBS and examined by phase-contrast and fluorescence microscopy.

RESULTS

Construction and characterization of a potent transdominant Tat protein. Several mutant Tat proteins have been reported to act as transdominant repressors of wild-type Tat function. Tat proteins containing mutations in the basic domain have been shown to act in a transdominant manner (15, 53, 55); however, most of these mutants retain the ability to partially transactivate the HIV-1 or HIV-2 LTR and localize diffusely throughout the cell (53, 55), complicating analysis of their mechanisms of action. Tat peptides containing mutations in the core domain also have been reported to act in a transdominant manner (23); however, full-length Tat proteins containing the same mutations do not appear to inhibit Tat function (50, 71). Mutations in the cysteine-rich domain of Tat have been reported to act in a transdominant fashion by some groups (7, 9) but not by others (50). To better understand the mechanism of action of transdominant Tat, we sought to clearly define the domains of Tat that, when altered, result in transdominant proteins and to construct a transdominant Tat protein that was completely inactive and exhibited a well-defined cellular localization.

The amino acid sequence of full-length HIV-1 Tat protein and its functional domains (19, 28, 41, 57, 58) are shown in Fig. 1A, and the constructs used in this study are shown in Fig. 1B. We first introduced mutations into the cysteine-rich, core, and basic domains of Tat. To evaluate the ability of the mutant Tat proteins to activate HIV gene expression and to inhibit wild-type Tat function *in trans*, we used cell lines containing an integrated HIV-1 LTR driving expression of CAT, for the following reasons. Previous studies of transdominant Tat proteins have generally been done by transient cotransfection of an LTR-driven reporter gene together with one-exon forms of Tat-1 or Tat-2. However, it has been shown that the action of Tat at the level of transcription initiation or elongation may be related to the integration state of the LTR (31). Furthermore, the second exon of Tat, which has little effect on transactivation of an unintegrated LTR, appears to significantly enhance transactivation of an integrated LTR (31). As shown in Fig. 2A, transfection of a HeLa cell line containing an integrated HIV-1 LTR with pTat-1, which expresses full-length Tat-1, resulted in a dose-dependent, 20- to 40-fold increase in CAT expression. In contrast, transfection of this cell line with Tat constructs containing mutations in either the cysteine-rich (TatC/S), core (TatKL/A), or basic (Tat Δ 49-57) domain resulted in little or no increase in CAT expression even when 5- to 50-fold-higher amounts of plasmid DNA were transfected (Fig. 2A). We obtained similar results in assays using a cell line containing an integrated HIV-2 LTR-CAT reporter cassette (data not shown). The wild-type and mutant Tat proteins were efficiently expressed in transiently transfected COS cells (Fig.

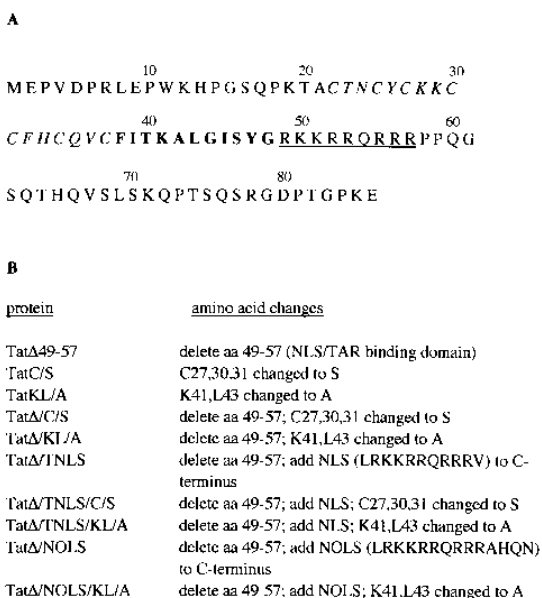


FIG. 1. Sequence of HIV-1 Tat protein and locations of Tat mutants. (A) Primary amino acid sequence of HIV-1 Tat. The functional domains as identified by Kuppuswamy et al. (41) are shown. The cysteine-rich domain is italicized, the core domain is indicated in boldface, and the basic domain is underlined. (B) Specific amino acid (aa) changes, features, and nomenclature of Tat mutants used in this study.

3, lanes 2 to 5), suggesting that this result was not due to dramatically different levels of expression. To determine whether the mutant Tat proteins inhibited wild-type Tat function in *trans*, HIV-1 LTR-containing cells were transfected with a constant amount of pTat-1 and increasing amounts of either pTat Δ 49-57, pTatKL/A, or pTatC/S. As a control, a separate transfection in which cells were transfected with pTat-1 and increasing amounts of parent vector, pCDN, was performed. The CAT activity present in cells cotransfected with pTat-1 and pTatKL/A, pTat Δ 49-57, or pTatC/S was normalized to that present in cells cotransfected with pTat-1 and the same amounts of pCDN. Cotransfection of pTatKL/A or pTatC/S had little or no effect on wild-type Tat function (Fig. 2B). In contrast, Tat function was inhibited in a dose-dependent manner by cotransfection of pTat Δ 49-57. A 5-fold excess of pTat Δ 49-57 inhibited Tat function by approximately 50%, and a 10-fold excess inhibited Tat function by nearly 80%. The level of inhibition of wild-type Tat function by Tat Δ 49-57 is equal to or exceeds that of other previously characterized transdominant Tat proteins analyzed in cells that were transiently cotransfected with both Tat and LTR-reporter constructs (15, 53).

We next compared the abilities of Tat Δ 49-57 to inhibit the function of full-length Tat-1 and Tat-2 in concert with both the HIV-1 and HIV-2 LTRs. In the first experiment, a HeLa cell line containing an integrated HIV-1 LTR-CAT reporter cassette was cotransfected with a constant amount of either pTat-1 or pTat-2 and increasing amounts of pTat Δ 49-57. The total amount of DNA in each transfection was normalized by cotransfection of the parent vector, pCDN. As a control, cells were cotransfected with a constant amount of pTat-1 and increasing amounts of pTatKL/A, which has no effect on Tat function (Fig. 2A). As shown in Fig. 4, Tat Δ 49-57 inhibited both Tat-2 (lanes 2 to 5) and Tat-1 (lanes 10 to 13) transactivation of the HIV-1 LTR in a dose-dependent manner. A 10- to 20-fold excess of pTat Δ 49-57 inhibited Tat function between 50 and 70%,

and a 50-fold excess inhibited Tat function between 75 and 85%. As expected, Tat-1 and Tat-2 function was unaffected by cotransfection of pTatKL/A (lanes 6 to 9 and 14 to 17). The lower level of inhibition in this experiment compared with that in Fig. 2B is likely to result from promoter competition and reduced transfection efficiency due to cotransfection of parent vector DNA. To determine the ability of Tat Δ 49-57 to inhibit Tat-1 and Tat-2 transactivation of the HIV-2 LTR, this experiment was repeated with cells containing an integrated HIV-2 LTR driving expression of CAT. As shown in Fig. 5, both Tat-1 transactivation and Tat-2 transactivation of the HIV-2 LTR were inhibited in a dose-dependent manner by Tat Δ 49-57. As expected, cotransfection of pTatKL/A had no effect on the ability of Tat-1 and Tat-2 to function. Our results demonstrate that Tat Δ 49-57 acts in a transdominant manner to inhibit both Tat-1 and Tat-2 function in concert with both the HIV-1 and HIV-2 LTRs.

Tat Δ 49-57 inhibits Tat function when localized to both the nucleus and cytoplasm. It was previously shown that a trans-

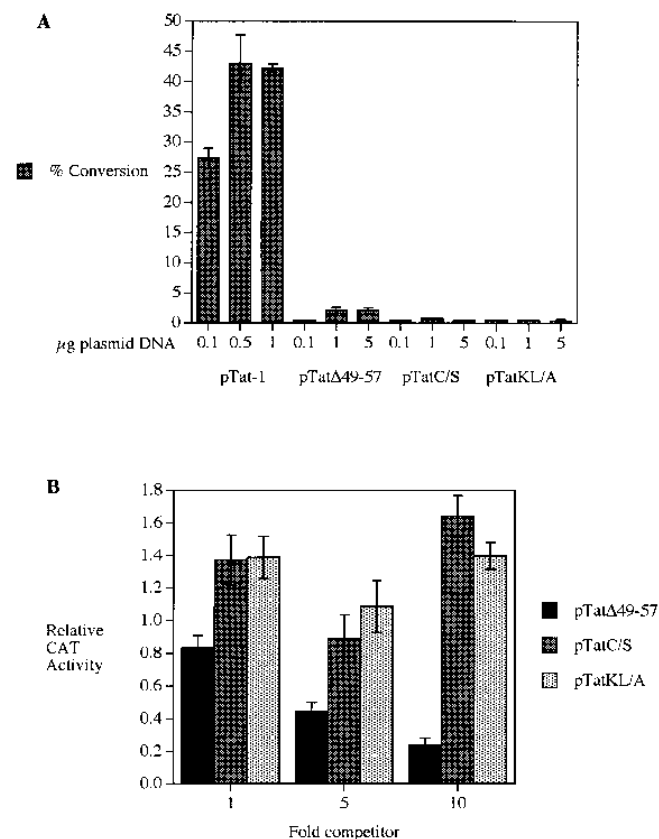


FIG. 2. Activities of wild-type and mutant Tat proteins on the HIV-1 LTR and inhibition of Tat function in *trans*. (A) HeLa cells containing an integrated HIV-1 LTR-CAT reporter gene were transfected with the indicated amounts of pTat-1, pTat Δ 49-57, pTatKL/A, or pTatC/S by the calcium phosphate method. Cells were harvested 65 h after transfection, and CAT activity was determined. The bars represent CAT activity present after subtraction of background activity from untransfected cells (1.1% in this experiment). (B) HeLa cells containing the integrated HIV-1 LTR-CAT cassette were transfected with 50 ng of pTat-1 and 50, 250, or 500 ng of pTat Δ 49-57, pTatC/S, pTatKL/A, or pCDN by the calcium phosphate method. Cells were harvested 65 h after transfection, and CAT activity was determined. The relative CAT activity was calculated by dividing the activity present in cells cotransfected with pTat-1 and pTat Δ 49-57, pTatC/S, or pTatKL/A by the activity in cells cotransfected with pTat-1 and pCDN. Mean values and average errors from duplicate transfections is shown for both experiments.

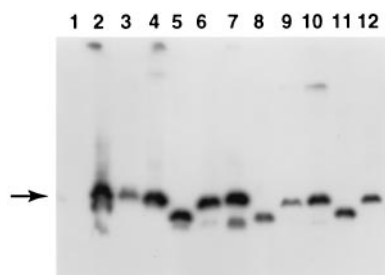


FIG. 3. Expression of Tat and mutant Tat proteins in COS cells. COS-1 cells in 100-mm-diameter dishes were transiently transfected with 5 μ g of plasmid DNA by the DEAE-dextran method. Cells were harvested 65 h after transfection, and proteins from cell extracts were separated on an SDS-18% polyacrylamide gel. The gel was subjected to immunoblot analysis, and Tat proteins were detected with a MAb directed against residues 5 to 22 of Tat. Proteins were visualized by the enhanced chemiluminescence method, using a horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G antibody. Cells were transfected as follows: lane 1, no DNA; lane 2, pTat-1; lane 3, pTatC/S; lane 4, pTatKL/A; lane 5, pTat Δ 49-57; lane 6, pTat Δ /TNLS; lane 7, pTat Δ /NOLS; lane 8, pTat Δ /KL/A; lane 9, pTat Δ /TNLS/KL/A; lane 10, pTat Δ /NOLS/KL/A; lane 11, pTat Δ /C/S; lane 12, pTat Δ /TNLS/C/S.

dominant Tat protein in which amino acids 52 to 57 within the basic domain were replaced with glycine residues was impaired in its ability to selectively localize to the nucleus and instead was localized throughout the nucleus and cytoplasm (53). We determined the subcellular distribution of both wild-type Tat and Tat Δ 49-57 in transfected cells by indirect immunofluorescence. Wild-type Tat-1 localized selectively to the nucleus, with a predominant accumulation in the nucleoli (Fig. 6B). In contrast, Tat Δ 49-57 localized exclusively to the cytoplasm (Fig. 6C). In an attempt to manipulate the subcellular localization of Tat Δ 49-57, we constructed two chimeric proteins, Tat Δ /TNLS and Tat Δ /NOLS. Tat Δ /TNLS contains the sequence LRKKR RQRRRV, which has previously been shown to direct a cytoplasmic protein, β -galactosidase, to the nucleus (66), at the C terminus of Tat Δ 49-57; Tat Δ /NOLS contains the sequence LRKKRRQRRRAHQN, which has been reported to direct β -galactosidase to the nucleolus (62), at the C terminus. Both Tat Δ /TNLS and Tat Δ /NOLS were strongly expressed in transfected cells (Fig. 3, lanes 6 and 7). As expected, the addition of the positively charged NLS and NOLS sequences to Tat Δ 49-57 resulted in proteins that migrated near the same apparent molecular weight as wild-type Tat. As shown in Fig. 6D and E, Tat Δ /TNLS localized to the nucleus. Interestingly, Tat Δ /TNLS did not appear to localize to the nucleoli as efficiently as wild-type Tat. In some cells, the nucleoli were excluded and only the nucleus was stained (Fig. 6E). In contrast, Tat Δ /NOLS localized exclusively to the nucleoli (Fig. 6F) and appeared to have the same subcellular distribution as wild-type Tat (compare Fig. 6B and F). Thus, addition of the sequence AHQN specifies nucleolar localization of Tat, in agreement with results previously reported by Siomi et al. (62). Neither Tat Δ /TNLS nor Tat Δ /NOLS transactivated the HIV-1 LTR (data not shown), demonstrating that the presence of the NLS/TAR binding domain out of its normal context was unable to restore Tat activity but could function as an NLS.

We then determined whether either Tat Δ /TNLS or Tat Δ /NOLS remained capable of inhibiting Tat function *in trans*. To this end, cells containing the HIV-1 LTR were transfected with a constant amount of pTat-1 and a 1-, 10-, or 50-fold excess of pTat Δ 49-57, pTat Δ /TNLS, or pTat Δ /NOLS, and the total amount of transfected DNA was normalized by cotransfection of pCDN. The CAT activity present in cells cotransfected with pTat-1 and pTat Δ 49-57, pTat Δ /TNLS, or pTat Δ /NOLS was

normalized to that present in cells transfected with pTat-1 and pTatKL/A. As shown in Fig. 7, cotransfection of pTat Δ /TNLS or pTat Δ /NOLS inhibited the function of the wild type in a transdominant manner. The inhibition of Tat function by Tat Δ /TNLS was less efficient than that by Tat Δ 49-57 when a lower amount (i.e., 10-fold excess) was transfected but was equally efficient when a 50-fold excess was transfected. Tat Δ /NOLS inhibited Tat function as efficiently as Tat Δ 49-57 at all amounts. Therefore, Tat Δ 49-57 appears to be capable of inhibiting Tat function when localized to either the nucleus or the cytoplasm. These results clearly suggest that cytoplasmic localization of Tat Δ 49-57 is not required to inhibit Tat function and that Tat Δ 49-57 does not appear to function by sequestering wild-type Tat in the cytoplasm.

Mutations in the activation domain of transdominant Tat proteins eliminate their abilities to act in a transdominant manner. It has been proposed that Tat functions by interacting with a cellular factor(s) bound to the Tat activation domain (11, 12, 14, 50, 71). Thus, transdominant Tat has been suggested to function by squelching a limiting cellular cofactor that interacts with Tat and is required for Tat function (12, 15, 50, 53). We therefore reasoned that mutations in the activation domain of Tat Δ 49-57 might impair its ability to act in a transdominant manner, since Tat Δ 49-57 would no longer interact with the Tat cofactor. To test this hypothesis, we introduced

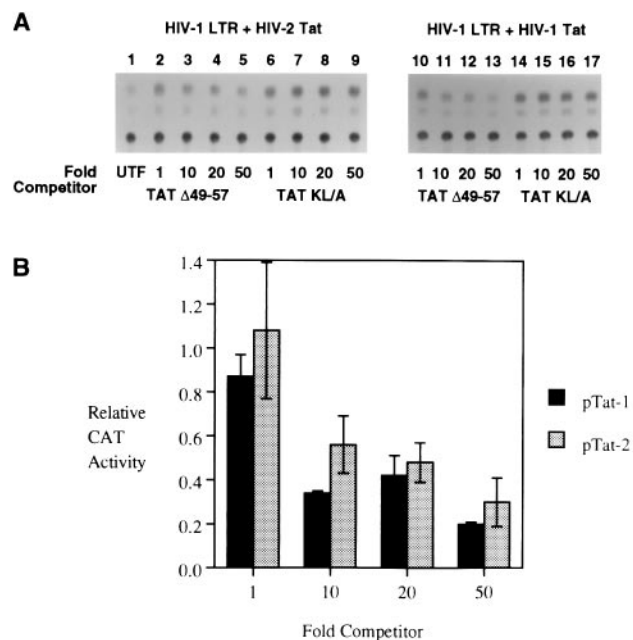


FIG. 4. Tat Δ 49-57 inhibition of both Tat-1 and Tat-2 transactivation of the HIV-1 LTR. (A) Autoradiograms of CAT assay. HeLa cells containing the integrated HIV-1 LTR-CAT cassette were transfected with 50 ng of pTat-2 (lanes 2 to 9) or pTat-1 (lanes 10 to 17) and the indicated fold excess of either pTat Δ 49-57 (lanes 2 to 5 and 10 to 13) or pTatKL/A (lanes 6 to 9 and 14 to 17) by the calcium phosphate method. The total amount of DNA in each transfection was normalized to 2,550 ng with pCDN. Cells were harvested 65 h after transfection, and CAT activity was determined. (B) The autoradiograms in panel A were quantitated with a Betagen 603 Betascope. The relative CAT activity was calculated by dividing the activity in cells cotransfected with pTat-1 or pTat-2 and pTat Δ 49-57 by that present in cells cotransfected with pTat-1 or pTat-2 and the same amounts of pTatKL/A, after subtraction of the background activity from untransfected cells (UTF; A, lane 1). The higher activity present in untransfected cells resulted from using five times the protein for the assay in order to visualize the weaker activity of HIV-2 Tat on the HIV-1 LTR. The CAT activity present in untransfected cells in the assay for HIV-1 Tat (A, right panel) is similar to that in lanes 1 and 10 in Fig. 5A. Mean values and average errors from duplicate transfections are shown.

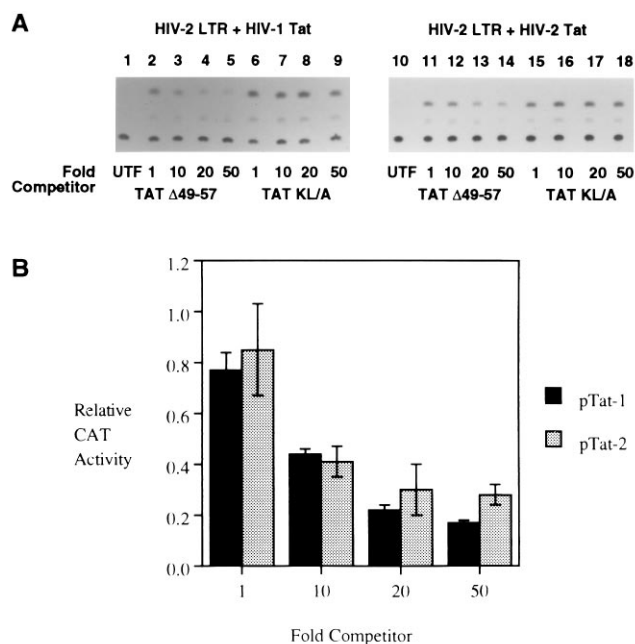


FIG. 5. Tat Δ 49-57 inhibition of both Tat-1 and Tat-2 transactivation of the HIV-2 LTR. HeLa cells containing the integrated HIV-2 LTR-CAT cassette were transfected with 50 ng of pTat-1 (lanes 2 to 9) or pTat-2 (lanes 11 to 18) and the indicated fold excess of either pTat Δ 49-57 (lanes 2 to 5 and 11 to 14) or pTatKL/A (lanes 6 to 9 and 15 to 18) by the calcium phosphate method. The total amount of DNA in each transfection was normalized to 2,550 ng with pCDN. Cells were harvested 65 h after transfection, and CAT activity was determined. (A) Autoradiograms of CAT assay. (B) The autoradiograms in panel A were quantitated with a Betagen 603 Betascope. The relative CAT activity was calculated as described in the legend to Fig. 4 after subtraction of background activity from untransfected cells (UTF; A, lanes 1 and 10). Mean values and average errors from duplicate transfections are shown.

the K41,L43-to-A core domain mutations into pTat Δ 49-57, pTat Δ /TNLS, and pTat Δ /NOLS. These constructs are referred to as pTat Δ /KL/A, pTat Δ /TNLS/KL/A and pTat Δ /NOLS/KL/A, respectively. We also introduced the mutations of cysteines 27, 30, and 31 to serine in the cysteine-rich domain into pTat Δ 49-57 and pTat Δ /TNLS. These constructs are referred to as pTat Δ /C/S and pTat Δ /TNLS/C/S. All of these constructs were efficiently expressed in transiently transfected COS cells (Fig. 3, lanes 8 to 12). To determine if these mutations affected the ability of Tat Δ 49-57, Tat Δ /TNLS, or Tat Δ /NOLS to inhibit Tat function, a constant amount of pTat-1 and 1-, 10-, and 50-fold excesses of the different constructs were cotransfected into the HIV-1 LTR-CAT cell line. The total amount of transfected DNA was normalized by cotransfection with pCDN. Three separate experiments were performed, one for the Tat Δ 49-57 series of constructs (Fig. 8A), one for the Tat Δ /TNLS series (Fig. 8B), and one for the Tat Δ /NOLS series (Fig. 8C). As described above, the CAT activity was normalized to that present in cells cotransfected with pTatKL/A. As expected, cotransfection of pTat Δ 49-57 inhibited Tat function in a dose-dependent manner in each experiment (Fig. 8). In contrast, cotransfection of pTat Δ /KL/A and pTat Δ /C/S had no effect on Tat function, even after cotransfection of a 50-fold excess of plasmid DNA (Fig. 8A). Similarly, mutation of the core and cysteine-rich domains of Tat Δ /TNLS completely eliminated its ability to inhibit Tat function in *trans* (Fig. 8B). Mutation of the core domain of Tat Δ /NOLS also completely abrogated its ability to function in a transdominant manner (Fig. 8C). Thus, mutations within the Tat activation domain

completely eliminated the ability of Tat Δ 49-57 to act in a transdominant manner, regardless of subcellular localization, suggesting that these domains may interact with a cellular Tat cofactor that is squelched by transdominant Tat. Our results suggest that the integrity of both the core and cysteine-rich domains of Tat is critical for the function of both wild-type Tat and transdominant Tat proteins.

DISCUSSION

In this report, we characterized the abilities of several mutant Tat proteins to act as transdominant repressors of Tat function in cell lines containing an integrated HIV-1 or HIV-2 LTR in conjunction with full-length Tat-1 or Tat-2 protein. This assay system was chosen for several reasons. First, Tat-activated transcription in HeLa cells that contain an integrated HIV-1 LTR has been shown to closely resemble transcriptional activation of latently HIV-infected U1 and Ach2 cell lines (31). Second, the second exon of Tat has been shown to significantly increase Tat activity on an integrated LTR but has little or no effect on the ability of Tat to function in concert with an unintegrated LTR (31, 41, 57). Third, in the absence of Tat, prematurely truncated transcripts resulting from abortive elon-

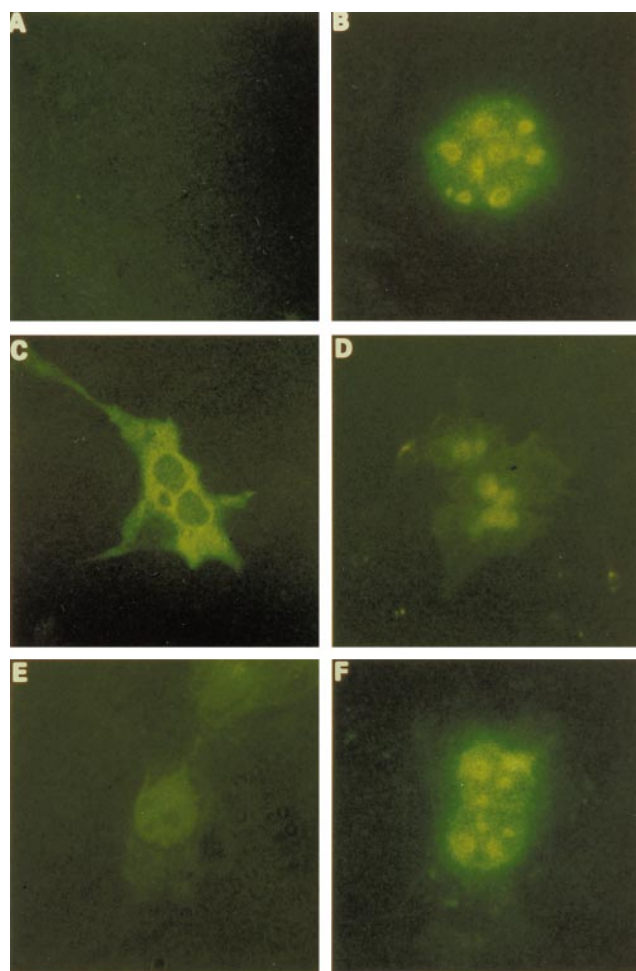


FIG. 6. Subcellular localization of Tat and transdominant Tat proteins. COS cells in 35-mm-diameter wells were transfected with no DNA (A), pTat-1 (B), pTat Δ 49-57 (C), pTat Δ /TNLS (D and E), or pTat Δ /NOLS (F). Cells were analyzed by indirect immunofluorescence 65 h after transfection as described in Materials and Methods.

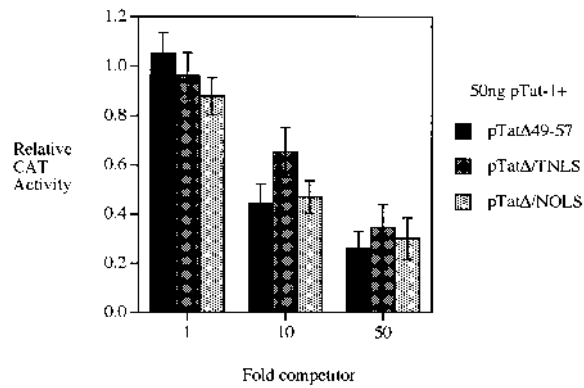


FIG. 7. Effect of subcellular localization on the ability of Tat Δ 49-57 to inhibit Tat function *in trans*. HeLa cells containing the integrated HIV-1 LTR-CAT cassette were transfected by the calcium phosphate method with 50 ng of pTat-1 and the indicated fold excess of pTat Δ 49-57, pTatKL/A, pTat Δ /TNLS, or pTat Δ /NOLS. The total amount of DNA transfected was normalized to 2,550 ng with pCDN. The cells were harvested 65 h after transfection, and CAT activity was determined. Relative CAT activity was calculated by dividing the activity present in cells cotransfected with pTat-1 and pTat Δ 49-57, pTat Δ /TNLS, or pTat Δ /NOLS by that in cells cotransfected with pTat-1 and the same amounts of pTatKL/A. Mean values and average errors from duplicate transfections are shown.

gation are not present in cells containing an integrated LTR but are abundant in cells that contain an unintegrated LTR (31, 37, 39, 46, 52). Thus, Tat may act on different transcriptional processes depending on the integration state of the reporter gene and its ability to replicate (31). Using our system, we found that mutations in the conserved cysteine-rich and core domains of Tat resulted in inactive proteins that failed to inhibit wild-type Tat function *in trans*. In contrast, deletion of the basic domain of Tat resulted in a protein that was not functional but acted as a transdominant inhibitor of both Tat-1 and Tat-2 function in concert with both the HIV-1 and HIV-2 LTRs.

Transdominant Tat proteins have been proposed to function by competing with Tat for binding to TAR, by forming inactive mixed multimers with Tat, or by squelching a cellular cofactor required for Tat function (7, 9, 23, 50, 53, 55). Many of the transdominant Tat proteins characterized in previous studies partially activated the HIV-1 LTR and localized to both the cytoplasm and nucleus (53). In contrast, Tat Δ 49-57 localized exclusively to the cytoplasm and was completely nonfunctional on both the HIV-1 and HIV-2 LTRs. Since Tat Δ 49-57 localized to the cytoplasm and lacks the TAR RNA binding domain, it is unlikely to inhibit Tat function by competing for binding to TAR RNA. Our data, taken together with those of previous studies, suggest that formation of mixed complexes between wild-type and transdominant Tat also fails to account for the behavior of transdominant Tat. Indeed, transdominant Tat proteins that contain an altered basic domain do not appear to affect the ability of wild-type Tat to localize to the nucleus (53, 54a), and analysis of Tat expressed in transfected cells suggests that it is monomeric (60). The observation that Tat Δ 49-57, which localizes to the cytoplasm, and Tat Δ /TNLS and Tat Δ /NOLS, which localize to the nucleus and nucleolus, respectively, inhibit Tat function provides further evidence that Tat Δ 49-57 does not act by sequestering wild-type Tat in the cytoplasm. Furthermore, our results suggest that it is disruption of the ability of the basic domain to bind to TAR, rather than act as an NLS, that is primarily responsible for the transdominant phenotype.

Tat has been proposed to transactivate HIV gene expression

by interacting with components of the cellular transcription machinery while bound to TAR (11, 12, 50, 61, 71, 72). Therefore, it has been proposed that transdominant Tat may act by competing with, or squelching, a cellular cofactor(s) that interacts with the activation domain of wild-type Tat (12, 50). This domain is able to act independently of Tat-TAR interaction, since the amino-terminal 48 amino acids of Tat are functional when directed to the promoter by fusion with Gal4, VP16, or Rev RNA or DNA binding domains (20, 63, 64, 72). Further, mutations in the core and cysteine-rich domains similar to those described here abrogate transactivation by these fusion proteins (64). If transdominant Tat acts by squelching a

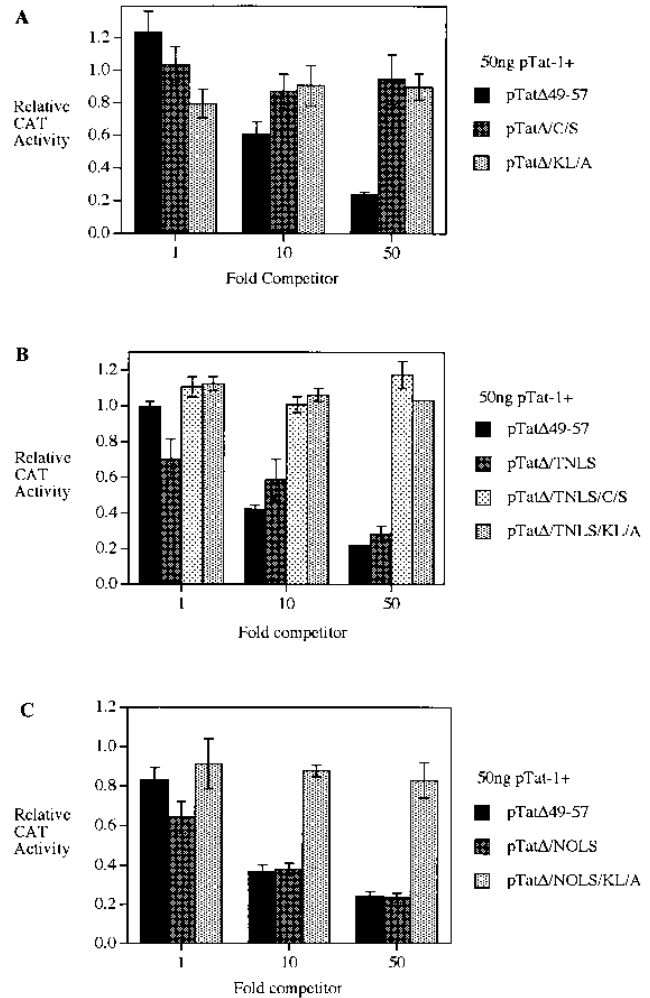


FIG. 8. Effects of mutations in the cysteine-rich and core domains on the ability of Tat Δ 49-57 to inhibit Tat function. (A) HeLa cells containing the integrated HIV-1 LTR-CAT cassette were transfected by the calcium phosphate method with 50 ng of pTat-1 and the indicated fold excess of pTat Δ 49-57, pTatKL/A, pTat Δ /C/S, or pTat Δ /KL/A. The total amount of DNA transfected was normalized to 2,550 ng with pCDN. The cells were harvested 65 h after transfection, and CAT activity was determined. Relative CAT activity was determined by dividing the activity present in cells cotransfected with pTat-1 and pTat Δ 49-57, pTat Δ /C/S, or pTat Δ /KL/A by that present in cells cotransfected with pTat-1 and pTatKL/A. Mean values and average errors from duplicate transfections are shown. (B) The experiment represented in panel A was repeated with pTat Δ /TNLS, pTat Δ /TNLS/C/S, or pTat Δ /TNLS/KL/A cotransfected in excess. (C) The experiment represented in panel A was repeated with pTat Δ /NOLS or pTat Δ /NOLS/KL/A cotransfected in excess. Relative CAT activities were calculated as described for panel A. Transfections were performed in duplicate, and pTat Δ 49-57 and pTatKL/A series were included for each experiment.

cellular factor bound to the activation domain, mutations in the activation domain would be predicted to disrupt this interaction and reduce or eliminate the ability of transdominant Tat protein to inhibit Tat function. Consistent with this hypothesis, we found that mutations in the cysteine-rich or core domains of Tat Δ 49-57, Tat Δ /TNLS, and Tat Δ /NOLS eliminated the abilities of these proteins to act in a transdominant manner. Carroll et al. previously showed that inhibition of HIV-1 Tat function by the functionally equivalent equine infectious anemia virus Tat protein, which localizes to the cytoplasm, was dependent on an intact equine infectious anemia virus Tat core domain, which is highly homologous to the Tat core domain (12). Madore and Cullen reported that a chimeric Tat-Rev protein directed to the HIV promoter by interaction of the Rev moiety with the Rev-responsive element inserted in place of TAR effectively mimicked Tat function in a TAR-independent manner (50). Interestingly, wild-type Tat inhibited the function of this chimeric Tat-Rev protein in *trans*. This was proposed to occur because wild-type Tat was unable to bind to the Rev-responsive element but competed for and sequestered the Tat cofactor(s). The ability of wild-type Tat to inhibit Tat-Rev function required an intact Tat activation domain but was unaffected by mutation of the basic domain (18, 50). In this context, Tat Δ /TNLS and Tat Δ /NOLS may function in a manner similar to inhibition of Tat-Rev function by wild-type Tat, since Tat Δ /TNLS and Tat Δ /NOLS localize to the nucleus or nucleolus and would interact with the Tat cofactor(s) but not with TAR. Mutation of the activation domain would thus prevent transdominant Tat from interacting with the Tat cofactor.

Several cellular proteins including TFIIID, Tat-associated protein, and Sp1 have been shown to interact with Tat through the core domain (32, 38, 47). Recently, a cellular factor containing a zinc finger domain and homology to several oncogenes and transcription factors that interacts with Tat through both the core and cysteine-rich domains was identified (18). It is likely that Tat interacts with several different cellular factors and that sequestering may entail competition for one or more of these factors. Since *de novo* protein synthesis has been shown to be dispensable for Tat function (33), such a cofactor would be envisioned to be stably present in the cell. Taken together with the observation that Tat Δ 49-57 is transdominant, this finding suggests that Tat and transdominant Tat may interact with a cofactor shortly after its synthesis in the cytoplasm. It has previously been suggested that Tat may interact with a cofactor in the cytoplasm prior to migration to the nucleus and binding to TAR (12). Tat Δ /TNLS and Tat Δ /NOLS may sequester by interacting with the Tat cofactor in the cytoplasm prior to migration to the nucleus. Sequestering by transdominant Tat localized to the nucleus may also occur through a mechanism whereby Tat Δ /TNLS and Tat Δ /NOLS would compete for the nuclear pool of cofactor that enters the nucleus alone or in conjunction with wild-type Tat. However, Tat Δ /TNLS and Tat Δ /NOLS would not be able to direct the factor to the HIV promoter, since neither protein can bind TAR. Madore and Cullen have proposed that interaction of Tat with a cofactor may be a prerequisite for the binding of Tat to TAR in vivo (50). Thus, it is conceivable that interaction of transdominant Tat with a cellular cofactor may prevent the binding of wild-type Tat to TAR. A detailed biochemical analysis of Tat-TAR and Tat-cofactor interactions in the presence of transdominant Tat will be required to fully understand the mechanism of action of transdominant Tat proteins.

There has been considerable interest in the use of transdominant HIV proteins in gene therapy approaches to treat AIDS. A detailed understanding of the mechanism of action of transdominant Tat proteins is critical to this approach. If trans-

dominant Tat interferes with a cellular cofactor that is required for normal cellular function, it is possible that overexpression of transdominant Tat proteins would be toxic to the cell. It has been suggested that Tat-specific transcription factor(s) that are not required for basal transcription are present in cells (50, 68). The identification and targeting of these factors may circumvent this problem. The experiments presented in this report and by others suggest that the use of transdominant Tat protein alone as a gene therapy approach is not likely to be effective since large amounts of transdominant Tat are required to achieve partial inhibition of Tat function (1, 42). Furthermore, HIV transcription can be activated by Tat in a TAR-independent manner in certain cell types (8, 26, 27). For example, HIV transcription can be activated by Tat in astrocytes in the absence of TAR (69, 70), and we have confirmed that Tat Δ 49-57 can activate TAR-independent transcription in astrocytes, whereas mutations in the activation domain display a recessive negative phenotype (40). Thus, inhibition of HIV gene expression at the transcriptional level is likely to require multiple approaches. These include overexpression of TAR-containing transcripts to act as a sink for Tat or Tat-cofactor complexes and simultaneous inhibition of Tat and Rev by transdominant proteins or antisense RNAs (1, 42, 43, 45, 67). We are currently attempting to evaluate and compare the effects of these transdominant Tat proteins on viral replication, as well as their abilities to inhibit Tat function in different TAR-independent models of transactivation. These studies should yield additional information on the mechanism of action of Tat and the potential usefulness of transdominant Tat proteins in antiviral therapy.

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