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A transdominant *tat* mutant that inhibits tat-induced gene expression from the human immunodeficiency virus long terminal repeat

LORI PEARSON*, JOSEPH GARCIA*, FOON WU*, NIDIA MODESTI*, JAY NELSON†, AND RICHARD GAYNOR*‡

Division of Hematology–Oncology, Department of Medicine, University of California–Los Angeles School of Medicine, Los Angeles, CA 90024; ‡Wadsworth Veterans Hospital, Los Angeles, CA 90024; and †Scripps Institute, La Jolla, CA 92037

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ABSTRACT **Regulation of human immunodeficiency virus (HIV) gene expression is dependent on specific regulatory regions in the long terminal repeat. These regions include the enhancer, SP1, "TATA," and trans-activating (TAR) regions. In addition, viral regulatory proteins such as tat and rev are important in regulating HIV gene expression. The mechanism of tat activation remains the subject of investigation, but effects at both transcriptional and posttranscriptional levels seem likely. Previous mutagenesis of the tat protein revealed that the amino terminus, the cysteine-rich domain, and the basic domain were all required for complete tat activation. Mutants of other viral trans-acting regulatory proteins, including E1A, tax, and VM65, have been identified that were capable of antagonizing the activity of their corresponding wild-type proteins. We wished to determine whether mutants of the tat protein could be identified that exhibited a similar phenotype. One mutant (Δ*tat*) that truncated the basic domain of tat resulted in a transdominant phenotype inhibiting tat-induced gene expression of the HIV long terminal repeat but not other viral promoters. This mutant exhibited its maximal phenotype in cotransfection experiments when present in an 8- to 30-fold molar excess over the wild-type *tat* gene. Trans-activation of the HIV long terminal repeat by Δtat was very defective at the DNA concentrations used in these experiments. RNase protection analysis indicated that this mutant decreased tat-induced steady-state mRNA levels of the HIV long terminal repeat. Second-site mutations of the Δ*tat* gene in either the amino terminus or cysteine region eliminated the transdominant phenotype. In contrast to tat, which was localized predominantly to the nucleolus, Δtat was present in both the nucleus and cytoplasm, suggesting that it may inhibit tat function by preventing nucleolar localization. Transdominant mutants of *tat* may have a role in potentially inhibiting HIV gene expression.**

Regulation of gene expression of the human immunodeficiency virus (HIV), the causative agent of AIDS (1–3), is dependent on specific regulatory regions in the long terminal repeat (LTR). These regions include multiple enhancer elements, the SP1, the "TATA," and the trans-activating region (TAR) (4–10). In addition, viral proteins such as tat (11–14) and rev (15, 16) are important in regulating viral gene expression. However, the mechanism by which these viral proteins activate HIV gene expression remains open to question.

tat is an 86-amino acid nuclear protein that is required for efficient viral gene expression (17). The *tat* gene is capable of activating the HIV LTR fused to reporter genes such as chloramphenicol acetyltransferase (CAT) from 40- to 300-fold (11–14). This increase in gene expression is accompanied by increases in steady-state RNA levels (18, 19), though effects on translation have also been reported (20, 21). tat has been demonstrated to increase transcription in nuclear run-on experiments with the HIV LTR (22). Other studies using microinjection *Xenopus* oocytes demonstrated that tat was able to increase gene expression of the HIV LTR mRNA when both were injected into the nucleus, suggesting that tat acted at posttranscriptional levels (23). The relative contribution of tat at both the transcriptional and posttranscriptional levels remains the subject of intense investigation.

The TAR extending from positions −17 to +80 in the HIV LTR is required for tat activation (7, 8, 11, 24–27). This element forms a stable stem–loop RNA structure. The maintenance of this stem–loop structure is required for tat activation (7, 8, 24, 25, 27). Both cellular proteins and tat itself bind to TAR RNA (28, 29). The significance of this binding has not yet been determined. In addition, TAR DNA also serves as binding sites for cellular proteins such as untranslated binding protein (UBP) 1 and 2, which may play a role in tat activation (8, 9, 30).

Mutagenesis of the tat protein indicated that multiple domains, including the amino terminus, the cysteine-rich region, and the basic domain, are required for complete tat activation (31–34). Both zinc and cadmium bind to the cysteine-rich moiety, and this binding is associated with metal-linked dimers (35). Mutation of the cysteine moieties results in HIV mutants that are severely defective in gene expression and growth (34). The basic domain is required for nuclear (32, 33) and likely nucleolar localization of tat (17), though it may also play a role in either DNA or RNA binding. The role of the amino terminus for tat function is not known.

Mutants of a number of other viral trans-activator proteins such as E1A (36), tax (37), and VM65 (38) have been identified that inhibit the promoter activation of their wild-type proteins. These so-called transdominant mutants may play a role in creating cell lines "immune" to subsequent viral infection (38, 39). To attempt to create such cell lines for HIV, we have studied a series of *tat* mutants for potential transdominant phenotypes. This work characterizes a transdominant *tat* mutant, Δ*tat*, which severely inhibits tat activation of the HIV LTR over a large concentration range.

MATERIALS AND METHODS

Mutagenesis. A *Hin*cII–*Ssp* I fragment (nucleotides 5791–6061 of the Arv 2 genome) containing the second exon of *tat* (first 72 amino acids) was cloned into the *Hin*cII site of pUC19. A partial *Hin*dIII–*Bam*HI fragment containing the *tat* gene was then cloned into the M13mp18 vector for subsequent mutagenesis. Oligonucleotides (21-mers) made to the coding strand of *tat* and containing the mutations indicated in Fig. 1 were synthesized for use in site-directed mutagenesis of the *tat* gene. Mutagenized plasmids were transformed into

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Abbreviations: HIV, human immunodeficiency virus; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; TAR, trans-activating region; RSV, Rous sarcoma virus.

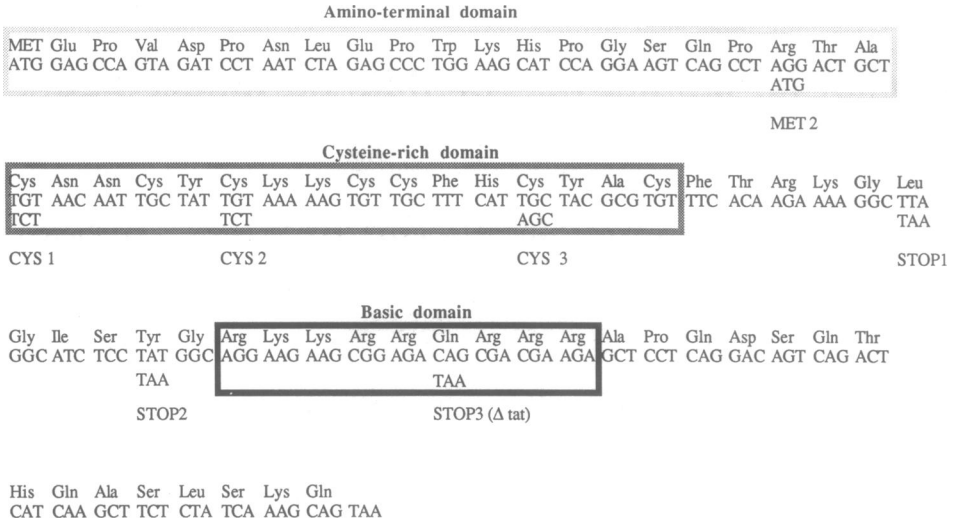

FIG. 1. HIV tat amino acid sequence and functional domains. The first 72 amino acids in the HIV tat protein and the substitutions introduced by oligonucleotide-directed mutangenesis are indicated.

JM103 and plaques were picked for subsequent sequence analysis. All clones were sequenced to verify the appropriate changes. Replicative form DNA was prepared from positive clones and fragments were gel-isolated that contained the mutated *tat* genes. These fragments were used to replace the wild-type *tat* gene in an expression plasmid containing the Rous sarcoma virus (RSV) promoter sequence and simian virus 40 splice acceptor and polyadenylylation sequences (32).

Transfections and CAT Assays. By using the calcium phosphate transfection protocol, RSV–*tat* expression plasmids or a control plasmid RSV–β-globin were transfected into HeLa cells in 100-mm plates (50–70% confluent at time of transfection) along with 3 μg of an HIV LTR–CAT construct containing nucleotides 287–476 of the Arv 2 genome (positions −177 to +83 from the start of transcription) fused to the CAT gene. For experiments using both *tat* and mutant *tat* expression vectors, 0.1 μg of wild-type *tat* and 0.2 μg, 0.8 μg, or 3.0 μg of the mutant *tat* expression vector was added along with 3 μg of HIV LTR–CAT construct. Total expression vector DNA concentration was adjusted to 3 μg with a construct containing the RSV promoter cloned into pUC19. Transfections were glycerol-shocked 4 hr after transfection, harvested 44 hr later, and CAT activity was determined as described (40).

RNA Analysis. HeLa cells in five plates were transfected. Each plate received 3 μg of HIV LTR–CAT, 1 μg of *tat*, and 8 μg of RSV–β-globin, RSV–*tat*–Δ*cys*, or RSV–*tat*. Cytoplasmic RNA was prepared 48 hr after transfection as described and used in RNase protection analysis (41).

RNase Protection Analysis. A 240-base-pair (bp) *Hin*dIII–*Pvu* II fragment of the CAT gene from RSV–CAT was cloned into pGEM-2 for RNase protection experiments. A 210-bp *Eco*RI–*Xba* I fragment from RSV–*tat* was also cloned into pGEM-2. RNA probes were transcribed with either bacteriophage T7 or SP6 RNA polymerase in the pGEM polylinker. The [α-^{32}P]GTP-labeled RNA probes (250,000 cpm) and 50 μg of HeLa RNA were hybridized and digested with T2 RNase (41). T2 RNase-resistant products were analyzed on an 8% polyacrylamide gel containing 8 M urea followed by autoradiography.

Immunofluorescence. HeLa cells were plated at 1×10^5 cells per 60-mm dish 24 hr prior to transfection. Either 10 μg of RSV–*tat* or RSV–Δ*tat* was transfected by calcium phosphate precipitation with glycerol shock 4 hr after transfection. At 48 hr after transfection, the cells were washed with isotonic phosphate-buffered saline (PBS) and fixed as described (33). Nontransfected cells were also treated similarly and served as controls. Both preimmune rabbit serum and tat antibody prepared against a peptide corresponding to amino acids 1–17 of the tat protein were used for staining. Serum was diluted 1:300, added to the transfected cells, and incubated at room temperature overnight. Cells were washed and incubated in the dark with a 1:100 dilution of rhodamine isothiocyanate-conjugated goat anti-rabbit IgG second antibody. Finally, cover slips were mounted, and cells were viewed with a light fluorescent microscope.

RESULTS

A *tat* Mutant with a Truncation in the Basic Domain Antagonizes Wild-Type tat Activation. Several domains of the tat protein have been shown to be required for activation (31–34). These include the amino terminus, the cysteine-rich domain containing four repeats of Cys-Xaa-Xaa-Cys, and the basic domain, which contains eight basic amino acids (lysine and arginine). A schematic of mutants used in this study is shown in Fig. 1. Each of these mutants was severely defective in activation of the HIV LTR compared to wild-type tat as reported (31). Mutants in each of these domains were tested in cotransfection assays with the wild-type *tat* gene in HeLa cells to determine if any of these mutants antagonized tat activation of an HIV LTR–CAT construct. Each *tat* mutant was transfected into HeLa cells in an 8-fold molar excess over that of the wild-type *tat* gene along with an HIV LTR–CAT plasmid. CAT assays demonstrated that one mutant with a truncation in the basic domain referred to as stop3 or Δ*tat* decreased tat activation of the HIV LTR by a factor of 10–15 in three independent experiments (Fig. 2*A*). Other mutants had no detectable effect on the level of tat activation.

To determine if second-site mutations introduced into the Δ*tat* construct altered the transdominant phenotype, additional mutations were placed into Δ*tat* and then assayed for their effects on antagonism of tat activation of the HIV LTR–CAT construct. Mutations of each of the three cysteine residues, individually or combined, or a mutation in the amino terminus were inserted into the Δ*tat* construct. As shown in Fig. 2*B*, lane 1, the Δ*tat* mutation inhibited tat activation of the HIV LTR when present in an 8-fold molar excess over *tat*. However, the introduction of other second-site mutations eliminated this antagonism of tat activation (Fig. 2*B*, lanes 2–5). The level of the HIV LTR–CAT construct in the presence of *tat* is also shown (Fig. 2*B*, lane 6).

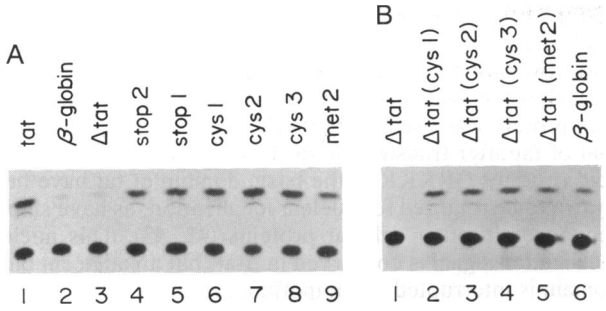

FIG. 2. Role of *tat* mutants on wild-type tat activation of the HIV LTR. (*A*) CAT assays from transfections of the HIV LTR–CAT construct with wild-type *tat* (lane 1), β-globin (lane 2), or wild-type *tat* and an 8-fold molar excess of stop3 (Δ*tat*) (lane 3), stop2 (lane 4), stop1 (lane 5), cys1 (lane 6), cys2 (lane 7), cys3 (lane 8), or met2 (lane 9) are shown. (*B*) CAT assays were performed after transfection of an HIV LTR–CAT construct and *tat* onto HeLa cells in the presence of an 8-fold excess of Δ*tat* (lane 1), Δ*tat* (cys1) (lane 2), Δ*tat* (cys2) (lane 3), Δ*tat* (cys3) (lane 4), Δ*tat* (met2) (lane 5), or β-globin (lane 6).

This suggests that the conformation of the Δtat protein was critical for the maintenance of the transdominant phenotype.

Transdominant Repression of tat Function. Titration of wild-type *tat* gene with either Δ*tat* or another mutant, Δ*cys*, which eliminated the three critical cysteine residues in the *tat* gene (illustrated in Fig. 1), was performed to determine the range over which these mutants antagonized wild-type tat activation. As shown in Fig. 3*A*, lanes 4–6, wild-type tat activation of the HIV LTR–CAT remained constant when titrated with a 2-, 8-, or 30-fold excess of a β-globin control. A similar lack of effect on tat activation was seen with a 2-, 8-, or 30-fold excess of the Δ*cys* mutant (Fig. 3*A*, lanes 7–9). However, transfection of either a 2-, 8-, or 30-fold excess of Δ*tat* resulted in marked antagonism of tat activation of the HIV LTR (Fig. 3*A*, lanes 1–3). The HIV LTR–CAT plasmid in the absence of *tat* is also shown (Fig. 3*A*, lanes 10–12). This antagonism by Δ*tat* when present in an 8- or 30-fold molar excess over wild-type *tat* resulted in severe decreases in CAT activity in four independent experiments.

The Δ*tat* mutant was markedly defective (20- to 40-fold in three experiments) in activating the HIV LTR–CAT plasmid as compared to wild-type *tat* (Fig. 3*B*). However, at higher DNA concentrations (5–10 μg), low-level activation of the HIV LTR was noted (data not shown). Δ*tat* did not decrease gene expression from the RSV–CAT plasmid (Fig. 3*C*) or other viral promoters tested. Thus, Δ*tat* was very defective for activation of the HIV LTR and it appeared to specifically antagonize tat activation of the HIV LTR.

Transdominant Inhibition of tat-Induced Steady-State RNA Levels. To determine whether Δtat inhibition resulted in a decrease in tat-induced steady-state RNA levels of the HIV LTR, RNase protection was performed on RNA prepared after transfection of HIV LTR–CAT and wild-type *tat*, β-globin, or wild-type *tat* plus an 8-fold excess of either Δ*tat* or Δ*cys*. Transfections were normalized for the levels of luciferase activity by inclusion of a thymidine kinase–luciferase construct in each transfection mixture (data not shown). As shown in Fig. 4*A*, the presence of Δ*tat* and *tat* resulted in a marked decrease in the 150-bp RNase protected CAT mRNA band as compared to the presence of Δ*cys* and *tat* (Fig. 4, lanes 1 and 2). The level of HIV LTR–CAT mRNA in the presence of either β-globin or *tat* alone are also indicated (Fig. 4, lanes 3 and 4). The intensity of the 142-bp RNase-protected tat mRNA band was roughly equivalent in the presence of *tat* and either Δ*tat* or Δ*cys* (Fig. 4, lanes 1 and 2). These results indicated that Δ*tat* decreased the amount of tat-induced steady-state mRNA from the HIV LTR.

Nucleolar Localization of Δtat Is Altered. The tat protein has been shown by immunofluorescence to be localized to the nucleus with the majority of the protein present in the nucleolus (17, 33). To compare the cellular localization of tat and Δtat, these constructs were individually transfected into HeLa cells and localized by immunofluorescence with tat antibody. As shown in Fig. 5*B*, tat was primarily localized to the nucleolus. However, Δtat was localized to both the nucleus and the cytoplasm with little detectable accumulation in the nucleolus (Fig. 5*D*). This pattern of staining was not seen with preimmune serum, indicating the specificity of the tat antibody (data not shown). Thus, a potential mechanism of Δtat antagonism of tat activation may be inhibition of tat localization to the nucleolus.

DISCUSSION

The results presented herein demonstrate that a truncation of the *tat* gene, Δ*tat*, was capable of antagonizing wild-type tat activation of the HIV LTR. This antagonism was maximal when Δ*tat* was present in an 8- to 30-fold molar excess over the wild-type *tat* gene in transfection experiments. This effect was due to an inhibition of the tat-induced steady-state RNA levels from the HIV LTR–CAT construct. Second-site mutations of the Δ*tat* construct resulted in the loss of the transdominant phenotype. This inhibition was specific for the HIV LTR and not for other promoters. The mechanism of this transdominant phenotype is not known. However, the requirement for the structural integrity of the Δtat protein and the previous demonstration of the presence of metal-linked dimers of tat would be consistent with a model that tat and Δtat may form heterodimers with a defective activation phenotype. Potential inhibition in tat localization to the nucleolus or defective interaction with HIV LTR DNA, RNA, or cellular transcription factors remain other possible explanations for this transdominant phenotype.

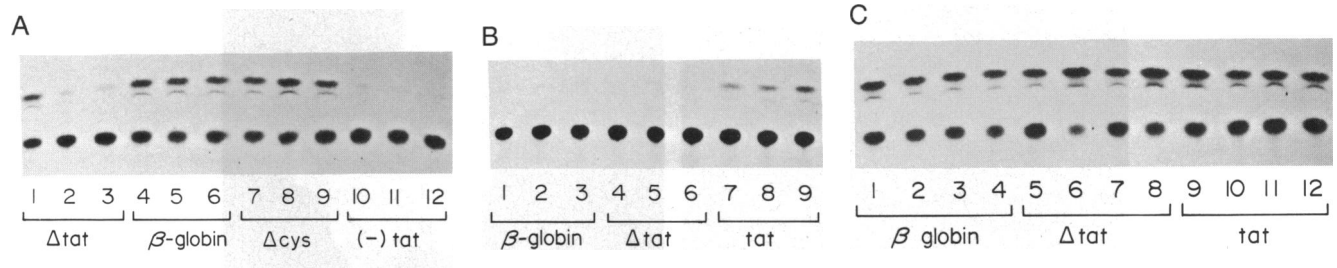

FIG. 3. Titration of Δ*tat* and the wild-type *tat*. CAT assays were performed from transfections of the HIV LTR–CAT construct and wild-type *tat* (0.1 μg) with a 2-fold (lanes 1, 4, and 7), 8-fold (lanes 2, 5, and 8), or 30-fold (lanes 3, 6, and 9) molar excess of Δ*tat* (lanes 1–3), β-globin (lanes 4–6), or Δ*cys* (lanes 7–9). Lanes 10–12 contain equivalent amounts of β-globin as in lanes 4–6 but without *tat*. (*B*) The HIV LTR–CAT plasmid in the presence of β-globin (lanes 1–3), Δ*tat* (lanes 4–6), or *tat* (lanes 7–9) was assayed for CAT activity. Lanes: 1, 4, and 7, 0.1 μg; 2, 5, and 7, 0.8 μg; and 3, 6, and 9, 3 μg. (*C*) The RSV–CAT plasmid in the presence of β-globin (lanes 1–4), Δ*tat* (lanes 5–8), or *tat* (lanes 9–12) was assayed for CAT activity. Lanes: 1, 5, and 9, 0.1 μg of these plasmids; 2, 6, and 10, 0.2 μg; 3, 7, and 11, 0.8 μg; and 4, 8, and 12, 3.0 μg.

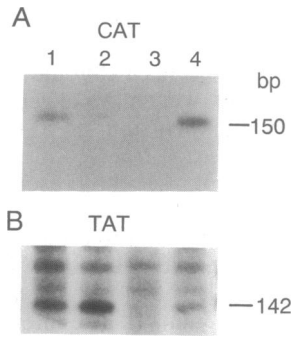

FIG. 4. Δtat decreases steady-state levels of HIV LTR–CAT mRNA. The HIV LTR–CAT construct was cotransfected with 1 μg of *tat* and 8 μg of Δ*cys* (lane 1), 1 μg of *tat* and 8 μg of Δ*tat* (lane 2), 9 μg of β-globin (lane 3), or 1 μg of tat and 8 μg of β-globin (lane 4). RNase protection was performed with either a 240-bp CAT-specific probe (*A*) or a 210-bp tat-specific probe (*B*). The positions of the 150-bp CAT-specific band and the 142-bp tat-specific band are indicated.

A number of previous *tat* mutants in the basic domain have been constructed (32, 33). These studies indicate that point mutations in the basic domain do not result in large decreases in tat activation. However, mutations that either truncated the *tat* gene in the basic domain or substituted multiple amino acids in this domain result in marked decreases in tat activation. Immunofluorescence experiments have indicated that the basic domain functioned in the nuclear and likely the nucleolar localization of tat (17, 32, 33), though this domain may also play a role in the *in vivo* stability of the tat protein (32). However, our immunofluorescence studies indicate that Δtat protein was present in roughly equivalent quantities as that of tat after transfection of these constructs. The amino acid residues GRKKR in the basic domain of tat have been shown to be required for nuclear localization, as have similar sequences in other cellular proteins (42, 43). This nuclear localization signal is conserved in Δtat, but an adjacent basic domain is interrupted. Immunofluorescence studies indicate that Δtat is localized to both the nucleus and the cytoplasm, though there appeared to be defects in nucleolar localization. Thus Δtat may inhibit tat nucleolar localization and thus prevent its presence in a nuclear compartment required for subsequent activation of HIV gene expression.

Transdominant mutations of other viral trans-activator proteins have been characterized. These include mutations of the E1A (36), tax (37), and VM65 (38) proteins. Each of these mutant proteins resulted in an inhibition of activation of the wild-type proteins for their specific viral promoters when present in a 5- to 20-fold excess over the wild-type protein. The mechanism of inhibition for these other viral trans-activator proteins is not known. A transdominant mutation of the herpesvirus transactivator, VM65, which eliminated large portions of the acidic domain, was used to construct stable cell lines that were resistant to infection with herpes simplex virus (38). This suggests that such transdominant constructs

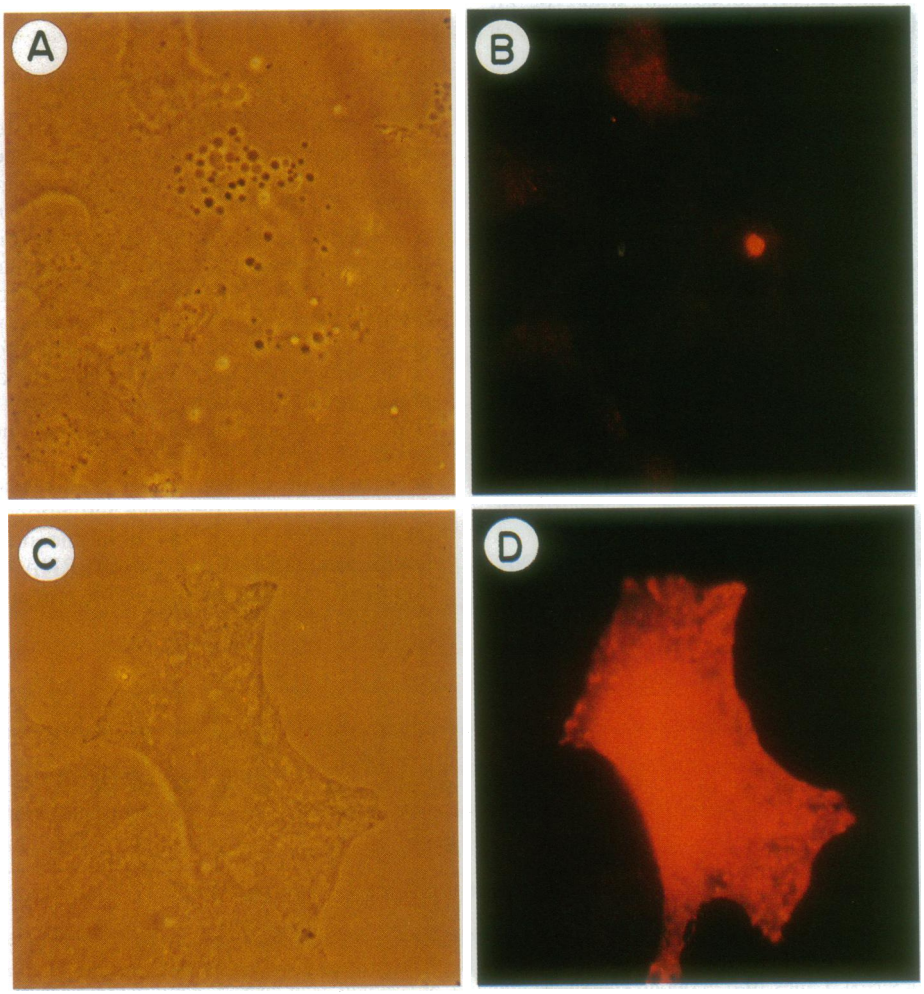

FIG. 5. Indirect immunofluorescence staining of HeLa cells expressing either *tat* or Δ*tat*. HeLa cells were transfected with either RSV–*tat* (*A* and *B*) or RSV–Δ*tat* (*C* and *D*). Phase-contrast (*A* and *C*) and fluorescence (*B* and *D*) photomicrographs are shown. (×790.)

can be used to create cell lines that are "immune" to viral infection (39).

Transdominant mutations have been described for other HIV proteins including rev (44) and gag (45). These mutant proteins were capable of antagonizing the growth of HIV. Previous studies of the tat protein using chemically synthesized peptides have been reported (46–48). One group reported that a 50-amino acid tat peptide between amino acids 37 and 86, which eliminated the cysteine-rich domain, had wild-type trans-activation properties (46). Furthermore, peptides containing amino acids 37–62 and substitutions between amino acids 40 and 47 were reported to have transdominant phenotypes (47). However, another study using similar peptides failed to confirm either the activation by these tat peptides or their transdominant phenotype (48). Our mutagenesis studies suggest that both an intact amino terminus and cysteine-rich domain were required for the transdominant phenotype.

tat protein may enter cells after being placed into tissue culture medium and thereupon be capable of transactivation of the HIV LTR (49). The mechanism of this entry is unknown. It is possible that transdominant mutant tat proteins could be used to inhibit HIV gene expression in infected cells. At present, we have been unable to inhibit HIV gene expression with bacterial-synthesized Δtat added to the tissue culture medium. This may be due to poor cellular uptake of bacterial-synthesized Δtat as compared to tat and decreased nuclear localization. However, other transdominant tat proteins could potentially inhibit HIV gene expression. A study of Δtat and other transdominant mutations and a determination of their mechanisms of inhibition will be important in understanding tat function and may lead to the development of reagents of potential therapeutic importance.

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