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Inhibition of Human Immunodeficiency Virus Type 1 Tat Activity by Coexpression of Heterologous *trans* Activators

RICHARD CARROLL,[1] B. MATIJA PETERLIN,[2] AND DAVID DERSE[1]*

Laboratory of Viral Carcinogenesis, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201,[1] and Howard Hughes Medical Institute and Departments of Medicine, Microbiology, and Immunology, University of California, San Francisco, California 94143-0724[2]

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We examined the mechanism of Tat-mediated *trans* activation through competition experiments employing Tat proteins of human immunodeficiency virus type 1 (HIV-1) and equine infectious anemia virus (EIAV). EIAV Tat, as well as chimeric EIAV/HIV-1 Tat proteins, inhibited HIV-1 Tat-mediated *trans* activation in a cell-type-dependent fashion. Furthermore, these proteins inhibited *trans* activation by Tat-bacteriophage R17 coat protein chimeras. Inhibition resulted from competition between activation domains of effectors and competitors for a limiting cellular cofactor. The context in which competitor activation domains were expressed contributed to the extent of inhibition. In transfected cells, EIAV Tat and all chimeric competitors were located primarily in the cytoplasm, whereas HIV-1 Tat was primarily located in the nucleus. These data are consistent with a model for *trans* activation in which the activation domain of Tat associates with and conveys a cellular factor to the transcription complex via the *trans*-acting-responsive element (TAR).

The evolutionarily divergent lentiviruses equine infectious anemia virus (EIAV) and human immunodeficiency virus type 1 (HIV-1) both contain *tat* genes whose protein products stimulate viral long terminal repeat (LTR)-directed gene expression in *trans* (1, 10, 26, 38). EIAV and HIV-1 Tat proteins belong to a novel class of *trans* activators that require an RNA stem-loop structure, called the *trans*-acting-responsive element (TAR), for function. TAR is located at the 5′ end of all viral transcripts (5, 14, 23, 31, 36). In the absence of HIV-1 Tat, transcription from the HIV-1 promoter yields primarily truncated mRNA species, while in the presence of Tat, full-length HIV-1 transcripts predominate, suggesting that Tat increases the processivity and prevents premature termination of RNA polymerase II (7, 17, 20, 34, 41).

Tat functional domains have been identified through the exchange of EIAV and HIV-1 Tat structural regions, as well as by the fusion of HIV-1 and EIAV Tat to heterologous RNA-binding proteins (4, 8, 35, 39). These studies demonstrated that Tat is composed of a TAR recognition domain, which associates with TAR RNA, and an activation domain, which could either modify RNA polymerase II directly or convey a cellular factor to the transcription complex.

Despite their different structures and sequences, HIV-1 and EIAV Tat activation domains can be exchanged, yielding chimeric *trans* activators whose promoter specificity is dictated by their respective TAR recognition domains (4). The interchangeable nature of Tat activation domains suggests that they interact with a common cellular factor. Therefore, a *trans* activator that contains a functional activation domain yet is unable to *trans* activate the HIV-1 LTR (a pseudoeffector) should compete with native HIV-1 Tat for this factor, resulting in reduced HIV-1 Tat activity. Since EIAV Tat is unable to *trans* activate the HIV-1 LTR (10), it fulfills these criteria. Additionally, we constructed chimeric Tat proteins by fusing HIV-1 or EIAV Tat activation domains to either the EIAV TAR recognition domain or to the bacteriophage R17 coat protein, a prokaryotic RNA-binding protein which recognizes a stem-loop structure analogous to TAR (30).

To assess the ability of these competitors to inhibit HIV-1 Tat activity and to define the mechanism by which Tat stimulates lentivirus gene expression, we coexpressed HIV-1 Tat and potential competitors and measured the ensuing HIV-1 LTR-directed gene expression. We found that *trans* activation was inhibited by the overexpression of competitor activation domains, that the extent of inhibition varied between cell types, and that regions outside the activation domain (i.e., the TAR recognition domain) also influenced the extent of inhibition. These data suggest that inhibition of Tat activity results from competition between activation domains for a limiting cellular factor and are compatible with a model for *trans* activation in which Tat, instead of directly affecting the transcription complex, associates with and conveys a cellular cofactor to the transcription complex.

MATERIALS AND METHODS

Plasmid construction. pRSPA was constructed by inserting the Rous sarcoma virus LTR and simian virus 40 polyadenylation signals into the distal sites of the Bluescript KS+ (Stratagene) polylinker (10). pRS-ETat contains a cDNA copy of the EIAV *tat* gene, joined to a synthetic initiation codon, cloned into pRSPA (10). pRS-HTat contains the first HIV-1 *tat* coding exon of pHXB2 (37) cloned into pRSPA.

pUX-CAT contains the bacterial chloramphenicol acetyltransferase (CAT) gene and simian virus 40 polyadenylation signals cloned into pUC18 (16). pUXCAT-RSV contains the Rous sarcoma virus LTR cloned into pUX-CAT (10). pHI-CAT was created by inserting a 197-bp *Taq*I-to-*Hin*dIII fragment of the HIV-1 LTR from pHXB2 into pUX-CAT. pEI-CAT was constructed by inserting the EIAV 5′ LTR into pUX-CAT (10). pHIVSR-CAT was constructed by inserting the operator of bacteriophage R17 in place of the HIV-1 TAR sequence (35).

The construction of the chimeric *tat* genes used in this

* Corresponding author.

report has been described previously (4). Briefly, restriction sites were introduced into EIAV and HIV-1 *tat* coding sequences by oligonucleotide-directed mutagenesis (18), enabling the coding sequences to be cleaved at the junctions of structural regions shown in Fig. 1A. The cleaved fragments were recombined to create *tat* gene chimeras. pRS-ETat-3d was constructed by exonuclease III and mung bean nuclease digestion of pRS-ETat followed by ligation of a stop codon-containing linker (New England Biolabs) (4). pRS-HTat-CP and pRS-ETat-CP were constructed by ligating the amino-terminal halves of HIV-1 and EIAV Tat, respectively, to amino acids 2 to 129 of the bacteriophage R17 coat protein (8). pRS-HTat(1-47X) was generated by inserting a stop codon after amino acid 47 of pRS-HTat.

Transfections and CAT assays. D17 and HeLa cells were maintained in Dulbecco's modified Eagle's medium containing 10 and 5% fetal calf serum, respectively. The day before transfection, D17 cells were seeded at 3×10^5 cells per well and HeLa cells were seeded at 5×10^5 cells per well in 3-cm-diameter six-well dishes. Cells were transfected with 3.0 μg of CAT reporter construct, 0.1 μg of *tat* expression plasmid, and either 1.0 μg of competitor expression plasmid or 1.0 μg of pRSPA (to maintain equivalent DNA levels) by the calcium phosphate procedure (11). Cell extracts were prepared 48 h after transfection (10), and CAT assays were performed by the solvent partition method (25). Basal levels of CAT activity were determined by transfection of the appropriate reporter plasmid and pRSPA. Absolute levels of [^{14}C]acetyl coenzyme A incorporation occasionally varied between experiments. In these cases, raw data were normalized to the activity of the constitutive reporter plasmid pUXCAT-RSV. Where indicated, relative CAT activity was calculated by comparing *trans*-activation levels in the presence of competitor with those obtained in the absence of competitor. Each datum point represents the mean of at least three independent transfections, and in all cases, relative activity values varied by less than 20% from the mean value shown.

Immunoprecipitations and cell fractionations. D17 cells were transfected with 5 μg of the appropriate expression plasmid, and 48 h later, they were labelled for 3 h with 400 μCi of [^{35}S]cysteine (NEN) per ml. The cells were lysed in Nonidet P-40 (NP-40) lysis buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid]-KOH [pH 7.9], 140 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% NP-40, 10% glycerol) and centrifuged at 1,000 × *g*. The supernatant (cytoplasmic fraction) was decanted and cleared by centrifugation. The pellet, which contained the crude nuclear fraction, was washed three times in NP-40 lysis buffer. Loosely associated nuclear proteins were extracted on ice in high-salt lysis buffer (50 mM HEPES-KOH [pH 8.0], 500 mM NaCl, 1.0% NP-40, 100 μg of phenylmethylsulfonyl fluoride per ml). Tightly associated nuclear proteins were extracted by resuspending the pellet in RIPA buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) and passing the suspension through a 26-gauge needle. Two micrograms of RNase A was added, and the suspension was incubated at 37°C for 20 min and cleared. All fractions were diluted with an equal volume of NP-40 lysis buffer and incubated with rabbit antisera prepared against peptides containing EIAV Tat amino acid residues 34 to 63 or HIV-1 Tat residues 1 to 67 (see Fig. 1A for location of these residues). The immune complexes were precipitated by addition of protein A-Sepharose CL4B (Pharmacia) and washed three times in wash buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, 0.25% gelatin, 100 μg of phenylmethylsulfonyl fluoride per ml). The precipitates were boiled in 1× SDS sample buffer, and resolved by SDS-polyacrylamide gel electrophoresis (PAGE) on 5 to 15% polyacrylamide gels (19).

RESULTS

HIV-1 Tat activity is inhibited by coexpression of a related *trans* activator. The amino acid sequences of EIAV and HIV-1 Tat proteins are shown in Fig. 1A. The sequences can be subdivided into structural regions, and the formation of functional domains from structural regions has been described previously (4, 8, 35). Both proteins contain a conserved central core region, region 3, which in EIAV Tat forms the activation domain (Fig. 1B). The minimal HIV-1 Tat activation domain requires the cysteine-rich region (region 2) in addition to the core region. The HIV-1 Tat amino-terminal region (region 1) augments the strength of this domain. Both Tat proteins also contain a stretch of basic amino acids (region 4). The EIAV Tat basic region, along with the carboxy terminus (region 5), forms the EIAV TAR recognition domain, while the HIV-1 Tat basic region is sufficient for TAR RNA binding in vitro (6, 32, 42).

Both EIAV and HIV-1 Tat proteins are active in D17 cells, a canine osteosarcoma cell line, but EIAV Tat is only weakly active in HeLa cells (10). Furthermore, despite their structural similarities, neither *tat* gene product stimulates expression of reporter genes linked to the heterologous promoter (4). We reasoned, therefore, that EIAV Tat might inhibit *trans* activation of the HIV-1 LTR by HIV-1 Tat. D17 cells were cotransfected with effector plasmid pRS-HTat (which encodes HIV-1 Tat), the target plasmid pHI-CAT (which contains the HIV-1 LTR fused to the bacterial CAT gene), and competitor plasmids (encoding *trans* activators which do not recognize HIV-1 TAR). The results are shown in Fig. 2. Cotransfection of pRS-HTat and a 10-fold excess of the EIAV Tat expression plasmid pRS-ETat resulted in a 60% decrease in pHI-CAT activity. Repetition of this experiment in HeLa cells yielded different results. Expression of a 10-fold excess of EIAV Tat barely affected the HIV-1 Tat-mediated increase in pHI-CAT activity (Fig. 2). In the converse experiment, cotransfection of D17 cells with pEI-CAT, pRS-ETat, and a 10-fold excess of pRS-HTat resulted in a 35% decrease in pEI-CAT activity (3a). Since the EIAV promoter functions poorly in HeLa cells (3a), we examined only the inhibition of HIV-1 Tat activity further. These experiments demonstrate that HIV-1 Tat-mediated *trans* activation can be decreased, albeit in a cell-type-specific fashion, by a functionally analogous *trans*-activator protein.

Cell-type-specific competition between Tat activation domains. The strong amino acid sequence similarity (Fig. 1A) and the functional interchangeability (4) of EIAV and HIV-1 Tat activation domains suggested that these elements compete for a common cellular factor. To test this hypothesis, we constructed chimeric competitors in which EIAV and HIV-1 Tat activation domains were fused to the EIAV TAR recognition domain (Fig. 1B) and tested their ability to inhibit the HIV-1 Tat-mediated *trans* activation of pHI-CAT (Fig. 2). pRS-$H_{123}E_{45}$Tat contains the HIV-1 activation domain joined to the EIAV TAR recognition domain. pRS-$H_{12}E_{345}$Tat contains the activation and TAR recognition domains of EIAV Tat joined to the HIV-1 Tat cysteine-rich and amino-terminal regions. Neither construct *trans* activates the HIV-1 LTR (4). Cotransfection of pRS-HTat and a 10-fold excess of pRS-$H_{123}E_{45}$Tat resulted in a 60% decrease

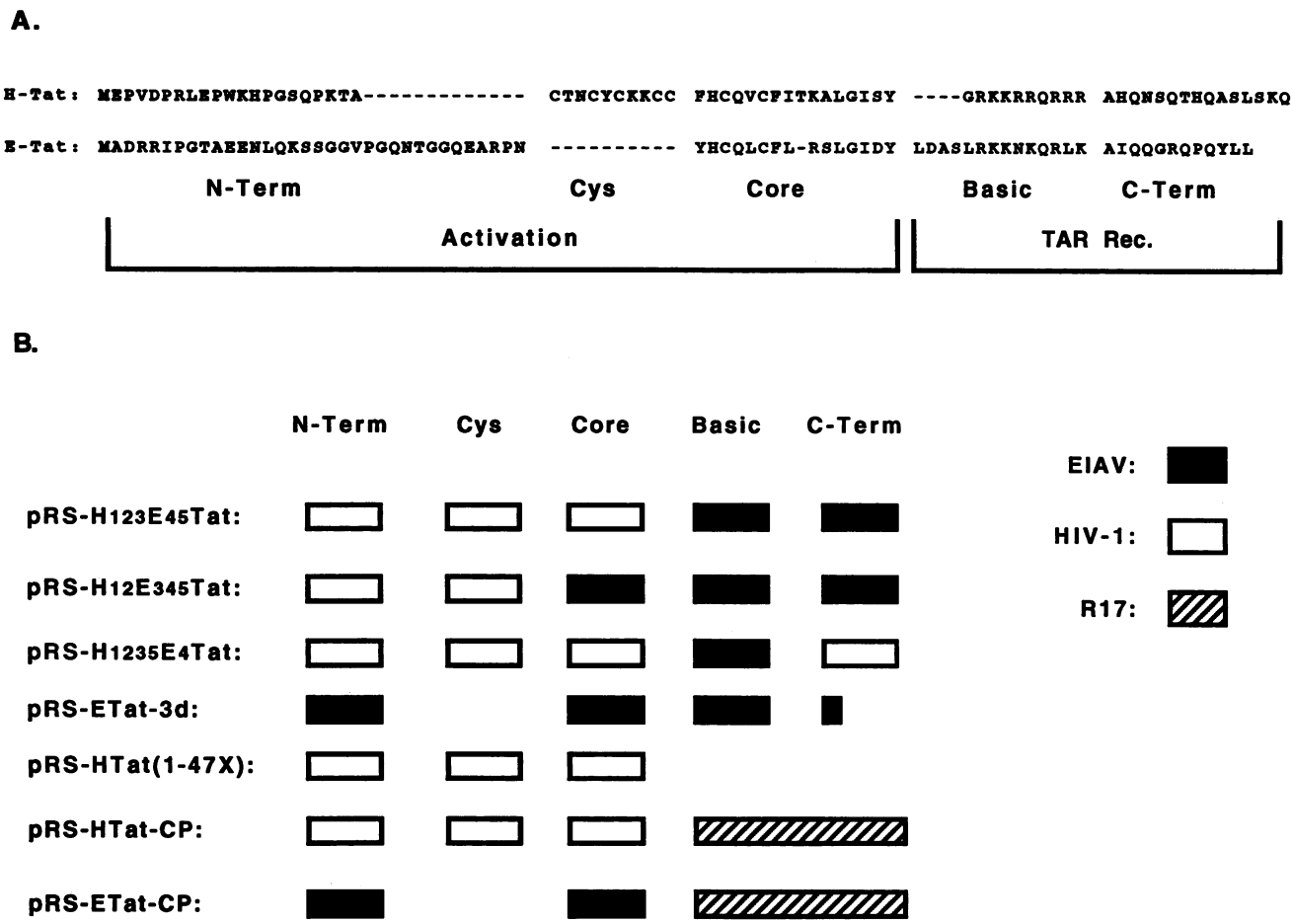

FIG. 1. Amino acid sequences and functional domains of wild-type and chimeric *tat* gene products. (A) Amino acid sequences of EIAV Tat (E-Tat) (10, 26) and the first coding exon of HIV-1 Tat (H-Tat) (1). The dashes were introduced to facilitate sequence alignment. The structural regions and functional domains (4, 8) are indicated. Rec., recognition domain. (B) Structures of chimeric *trans* activators. Restriction enzyme sites were introduced into EIAV and HIV-1 *tat* coding sequences at the junctions of structural regions. Chimeras were generated by combining digested fragments (4). Black rectangles represent EIAV Tat structural regions, while white rectangles represent HIV-1 Tat structural regions. The three carboxy-terminal amino acids were removed from EIAV Tat to generate pRS-ETat-3d. A stop codon was inserted immediately after the HIV-1 Tat core region to generate pRS-HTat(1-47X). pRS-HTat-CP and pRS-ETat-CP were constructed by fusing the HIV-1 Tat amino-terminal, cysteine-rich, and core regions or the EIAV Tat amino-terminal and core regions, respectively, to amino acids 2 to 129 of the bacteriophage R17 coat protein (8, 35). Coat protein sequences are represented by hatched rectangles.

in pHI-CAT activity in both D17 and HeLa cells. In contrast, whereas coexpression of pRS-HTat and a 10-fold excess of pRS-$H_{12}E_{345}$Tat resulted in a 50% drop in pHI-CAT activity in D17 cells, no inhibition was observed in HeLa cells. pRS-$H_{123}E_{45}$Tat and pRS-$H_{12}E_{345}$Tat differ only in the source of their core regions, indicating that the core region is the primary determinant of their differential ability to inhibit HIV-1 Tat activity in HeLa cells. Since in EIAV Tat, the core region forms the activation domain, while in HIV-1 Tat, it is a critical component of the activation domain (4), these experiments suggest that the inhibition of HIV-1 Tat activity results from competition between competitor and effector activation domains for a limiting cellular cofactor. These data further suggest that the affinities of the EIAV and HIV-1 Tat activation domains for this cofactor vary in different cell types and that the inability of competitors containing the EIAV activation domain (pRS-$H_{12}E_{345}$Tat as well as wild-type EIAV Tat) to inhibit HIV-1 Tat activity in HeLa cells results from the low affinity of the EIAV activation domain for the HeLa cell factor.

To examine the relative affinities of Tat activation domains for the cellular cofactor further, we fused the EIAV and HIV-1 Tat activation domains to the bacteriophage R17 coat protein, generating pRS-ETat-CP and pRS-HTat-CP, respectively (Fig. 1B). These fusion proteins *trans* activate chimeric LTRs containing the R17 operator sequence in place of the TAR element, but they do not *trans* activate wild-type lentivirus LTRs. Additionally, wild-type Tat proteins do not *trans* activate R17 operator-containing promoters (8, 35). pRS-ETat-CP and pRS-HTat-CP were cotransfected with pHIVSR-CAT, which contains the HIV-1 U3 region and the R17 coat protein operator in place of the HIV-1 TAR element (35). In D17 cells, both effectors increased pHIVSR-CAT expression approximately ninefold (Fig. 3). However, in HeLa cells, pRS-ETat-CP increased pHIVSR-CAT expression only 3-fold, while pRS-HTat-CP stimulated pHIVSR-CAT activity approximately 30-fold. The cell type preferences exhibited by the activation domains in these *trans*-activation experiments paralleled their

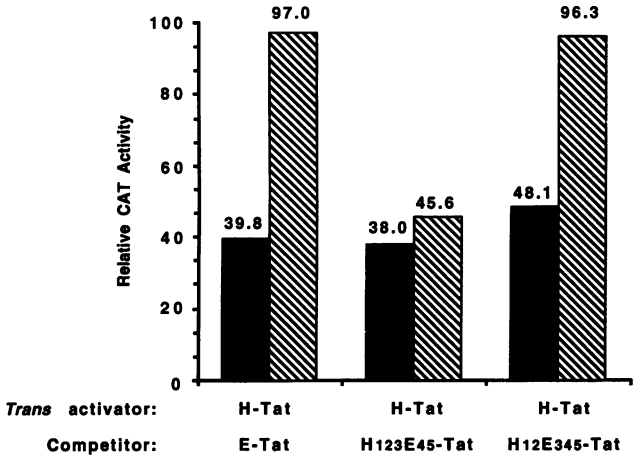

FIG. 2. Cell type-specific competition between Tat activation domains. D17 cells (solid columns) and HeLa cells (hatched columns) were cotransfected with the HIV-1 LTR-CAT reporter plasmid pHI-CAT, the HIV-1 *tat* expression plasmid pRS-HTat, and a 10-fold excess of the indicated competitor (see Fig. 1B for structures of competitors). CAT activity was measured and is expressed as a percentage of the value obtained in matching control experiments in which pHI-CAT and pRS-HTat were transfected without competitor plasmid.

relative strengths as inhibitors, suggesting that the putative cofactors in HeLa and D17 cells differ.

Influence of the TAR recognition domain on activation domain affinity. To determine whether the TAR recognition domain plays a role in this competition, we tested the ability of chimeric competitors with defective or absent TAR recognition domains to inhibit the activity of HIV-1 Tat. We also tested the ability of mutant and wild-type *trans* activators to inhibit the activity of hybrid HIV-1 Tat-R17 coat proteins.

pRS-HTat(1-47X), pRS-HTat-CP, and pRS-$H_{1235}E_4$Tat all contain an intact HIV-1 Tat activation domain, either expressed alone, fused to the R17 coat protein, or fused to a defective EIAV TAR recognition domain, respectively (Fig. 1B). Additionally, pRS-ETat-3d contains an intact EIAV Tat activation domain and a defective EIAV TAR recognition domain (Fig. 1B). pRS-$H_{1235}E_4$Tat and pRS-ETat-3d *trans* activate the EIAV LTR weakly, and pRS-$H_{1235}E_4$Tat also weakly *trans* activates the HIV-1 LTR (4). pRS-HTat-CP and pRS-HTat(1-47X) do not *trans* activate the EIAV or the HIV-1 LTR (3a, 10). While a 10-fold excess of pRS-$H_{1235}E_4$Tat weakly inhibited HIV-1 Tat activity (approximately 20% inhibition in D17 cells and 25% inhibition in HeLa cells), the other competitors did not inhibit HIV-1 Tat activity (Fig. 4). Thus, the activation domains of these competitors, with nonfunctional TAR recognition domains, are poor inhibitors of HIV-1 Tat. This could be due to the influence of the carboxy-terminal half of Tat on the proper folding and stable secondary structure of the protein.

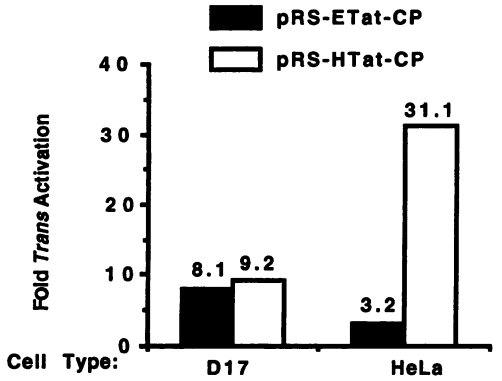

FIG. 3. Activity of Tat-coat protein fusion constructs. D17 and HeLa cells were transfected with 3 μg of the LTR-CAT reporter construct pHIVSR-CAT and 0.1 μg of pRS-ETat-CP or pRS-HTat-CP. pHIVSR-CAT contains the HIV-1 U3 region, with the TAR element replaced by the bacteriophage R17 operator, and the CAT gene (35). pRS-HTat-CP and pRS-ETat-CP contain the HIV-1 Tat amino-terminal, cysteine-rich, and core regions and the EIAV Tat amino-terminal and core regions, respectively, fused to the bacteriophage R17 coat protein (Fig. 1B). Fold *trans* activation is expressed as the ratio of *trans* activator-induced to basal CAT activity. Fold *trans* activation values are indicated above each bar.

The experiments discussed above suggest that pRS-HTat-CP, as well as other competitors with altered TAR recognition domains, has a lower relative affinity for the cellular cofactor. Thus, pRS-HTat-CP, when used as an effector, might be easily inhibited by any or all *trans* activators. In fact, overexpression of HIV-1 Tat strongly inhibited pRS-HTat-CP activity in HeLa cells, as reported previously (22), and in D17 cells (Fig. 4). Additionally, EIAV Tat, which failed to inhibit HIV-1 Tat activity in HeLa cells, strongly inhibited pRS-HTat-CP activity in HeLa and D17 cells. Surprisingly, pRS-HTat(1-47X), which lacks any RNA recognition motif, strongly inhibited pRS-HTat-CP activity in both cell types (Fig. 4). These data strengthen and support the idea that optimal binding of Tat with the cellular cofactor requires the properly folded protein.

Subcellular localization of effectors and inhibitors. The basic region of HIV-1 Tat, in addition to binding TAR RNA (6, 9, 32, 42), contains a nucleolar localization signal which targets Tat to its correct subcellular location (15, 33, 40). Recently, *trans*-dominant inhibitors of HIV-1 Tat containing truncated or altered basic regions were described (22, 27). These mutants, which *trans* activated the HIV-1 LTR poorly, failed to localize to the nucleus but did not prevent the nucleolar localization of wild-type HIV-1 Tat (22). It should be noted that several of our competitors are also functional *trans* activators. Therefore, we wished to determine whether the ability of these competitors to inhibit HIV-1 Tat activity (or *trans* activate other LTRs) was a function of their subcellular localization. To this end, D17 cells were transfected with *tat* expression constructs and fractionated into cytoplasmic and nuclear extracts. The nuclear extract was first gently extracted with high-salt buffer to remove loosely associated proteins and then vigorously disrupted to extract tightly associated proteins. Immunoprecipitation analysis was performed on both cytoplasmic and nuclear fractions (Fig. 5).

HIV-1 Tat localized mainly in the tightly associated nuclear fraction, with smaller proportions found in the cytoplasmic and loosely associated nuclear fractions. The anti-HIV-1 Tat antisera recognized a doublet, with only the higher-molecular-weight band found in the tightly associated nuclear fraction. It is likely that this doublet results from posttranslational modification of amino acids in the carboxy-terminal region, since it was detected only in chimeric proteins containing the HIV-1 Tat carboxy terminus (for example, see pRS-$H_{1235}E_4$Tat in Fig. 5).

In contrast to HIV-1 Tat, EIAV Tat was located predominantly in the cytoplasm. Only a small portion of EIAV Tat

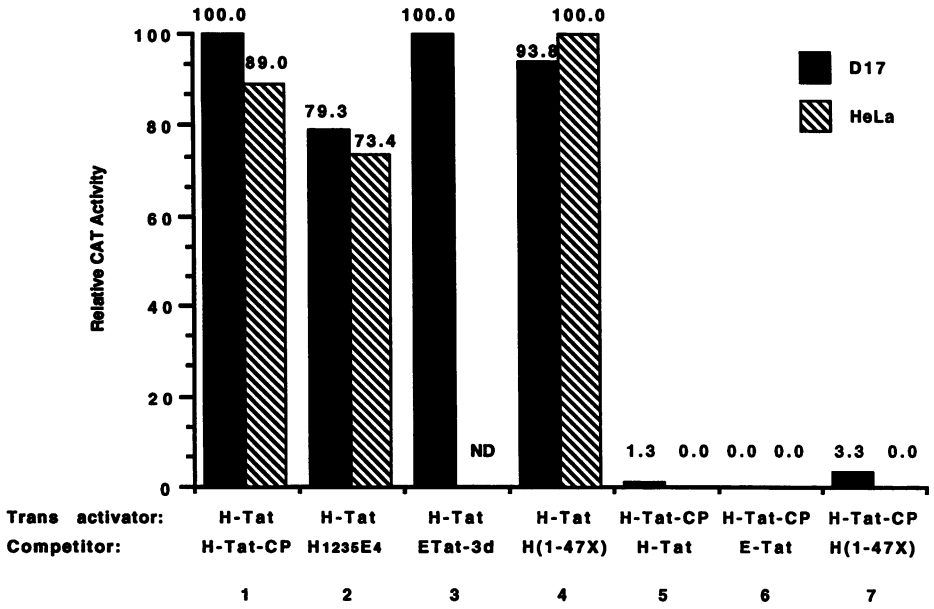

FIG. 4. Role of the TAR recognition domain in competition. D17 cells and HeLa cells were cotransfected with either the HIV-1 LTR-CAT reporter plasmid pHI-CAT and the HIV-1 *tat* expression plasmid pRS-HTat (lanes 1 to 4) or the R17 operator-containing plasmid pHIVSR-CAT and the Tat-coat protein fusion construct pRS-HTat-CP (lanes 5 to 7). A 10-fold excess of the indicated competitor was also transfected. CAT activity was measured and is expressed as a percentage of the activity obtained in matching control experiments in which pHI-CAT and pRS-HTat (lanes 1 to 4) or pHIVSR-CAT and pRS-HTat-CP (lanes 5 to 7) were transfected without competitor plasmid. ND, not done.

was detected in the loosely associated nuclear fraction, while barely detectable levels were observed in the tightly associated nuclear fraction. The chimeric construct pRS-$H_{123}E_{45}$Tat, which effectively inhibited HIV-1 Tat activity in both HeLa and D17 cells, had a similar distribution.

The HIV-1 Tat-coat protein fusion construct pRS-HTat-CP, which lacks the HIV-1 basic region, was found predominantly in the cytoplasmic fraction, but significant quantities were detected in both the loosely and tightly bound nuclear fractions. The two Tat proteins with defective EIAV TAR recognition domains, pRS-$H_{1235}E_4$Tat and pRS-ETat-3d, had different distributions. An appreciable portion of pRS-$H_{1235}E_4$Tat, like wild-type HIV-1 Tat, was found in the tightly associated nuclear fraction, while pRS-ETat-3d, like wild-type EIAV Tat, was located mainly in the cytoplasm.

Some proteins found almost exclusively in the cytoplasm, including wild-type EIAV Tat and pRS-$H_{123}E_{45}$Tat, functioned as potent *trans* activators of the EIAV LTR (4) and effective inhibitors of HIV-1 Tat activity. Other proteins which localized more efficiently to the nucleus, such as pRS-HTat-CP and pRS-$H_{1235}E_4$Tat, functioned poorly as inhibitors and *trans* activators. Thus, no correlation between the subcellular localization of pseudoeffectors and their ability to inhibit HIV-1 Tat activity was observed, and furthermore, the relative distribution of effectors within cells did not correlate with their ability to *trans* activate other LTRs. These data raise the possibility that the cellular cofactor that interacts with Tat is located in the cytoplasm.

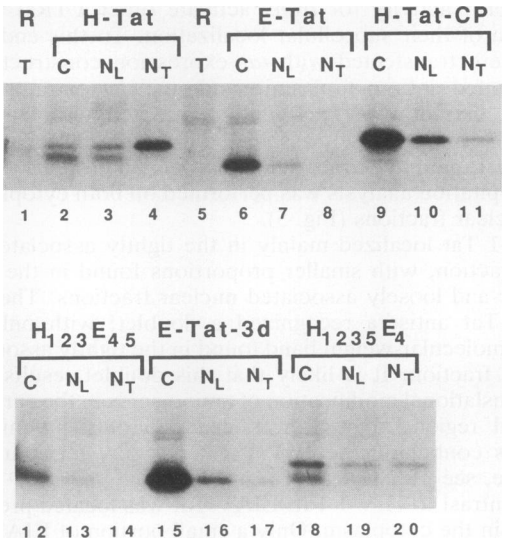

FIG. 5. Subcellular localization of wild-type and mutant Tat proteins. D17 cells were transfected with the indicated *tat* expression plasmids. The [^{35}S]cysteine-labelled proteins were precipitated with anti-HIV-1 Tat antisera (lanes 1 to 4, 9 to 14, and 18 to 20) or anti-EIAV Tat antisera (lanes 5 to 8 and 15 to 17). Precipitated samples were resolved by SDS-PAGE on a 5 to 15% polyacrylamide gel. R refers to cells transfected with pRSPA. T, total cell lysate; C, cytoplasmic fraction; N_L, loosely associated nuclear fraction; N_T, tightly associated nuclear fraction.

DISCUSSION

The key regulatory role of the HIV-1 *tat* gene product in the viral life cycle makes it an attractive target for antiviral therapy. Inhibition of HIV-1 Tat activity by multimerized TAR elements (12, 21), fused TAR-bacteriophage MS2 operator sequences (3), and *trans*-dominant HIV-1 Tat mutants

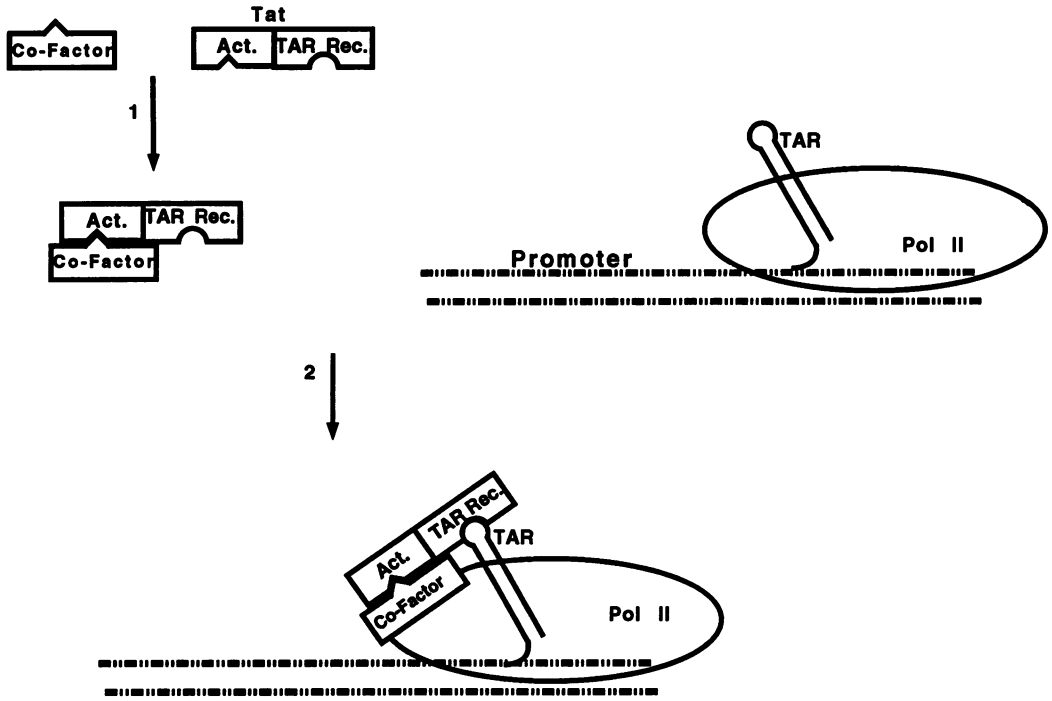

FIG. 6. Tat-mediated *trans* activation. This model depicts possible stages in the process of Tat-mediated *trans* activation. The first step involves an interaction between the activation domain (Act.) of Tat and a cellular cofactor. In the second step, Tat conveys the cofactor to RNA polymerase (Pol II) via the tethering interaction between the TAR element and the TAR recognition domain (Rec.) of Tat.

(13, 22, 27) has been reported. In this article, we have shown that the Tat protein of the distantly related lentivirus EIAV, as well as the products of EIAV/HIV-1 *tat* gene chimeras, also inhibits HIV-1 Tat activity.

The striking amino acid similarity between the core regions and therefore the activation domains of EIAV and HIV-1 Tat (Fig. 1A) suggested a common mechanism of *trans* activation. In fact, competitors containing the HIV-1 Tat activation domain inhibit HIV-1 Tat in both D17 and HeLa cells, while competitors containing the EIAV activation domain inhibit HIV-1 Tat in D17 cells only. These observations strongly suggest that both EIAV and HIV-1 Tat activation domains interact with a cellular cofactor required for *trans* activation and that inhibition of HIV-1 Tat activity occurs via a squelching mechanism (28) in which competitor activation domains compete for and sequester this cofactor. The inhibition profile parallels the activity of the hybrid HIV-1 and EIAV Tat-coat proteins in these two cell lines, suggesting that the strength of inhibition is determined by the affinities of their respective activation domains for the D17 or the HeLa cell factor. Furthermore, the predominantly cytoplasmic localization of EIAV Tat and several chimeric *trans* activators suggests that the association between this factor and the Tat activation domain may occur in the cytoplasm prior to nuclear migration and Tat-TAR interaction. Our results argue against the possibility that Tat itself modifies the transcription complex and are consistent with the model shown in Fig. 6, in which Tat acts by conveying the cofactor to the transcription complex, possibly through the tethering function of TAR (35, 39). In light of this proposed mechanism, the activation domain of Tat might more accurately be termed an effector domain.

Our observations indicate that an activation domain is necessary but not sufficient for inhibition; competitors lacking a functional EIAV TAR recognition domain inhibited HIV-1 Tat activity weakly or not at all. It is conceivable that the TAR recognition domain contributes to the overall folding of Tat and that disruption of this domain can affect the adjacent activation domain. Although modular, the activation and RNA-binding domains have evolved as a single functional unit. It may not be surprising that residues in one domain contribute to the association of proteins with the other domain, either through direct interactions with the protein or through indirect contributions to the secondary structure. This assertion is supported by the observation that mutations in the core region of HIV-1 Tat, in addition to impairing activity, perturb the highly compact, protease-resistant structure of the protein (29). This implies that the activation domain function depends on the overall folding of Tat. While it is also possible that the TAR recognition domain of competitors binds heterologous TARs or competes for TAR-binding proteins, our results and those of others (22) argue against this model.

Recent reports indicate that HIV-1 Tat mutants with truncated basic regions (and therefore defective TAR recognition domains) still inhibit HIV-1 Tat activity (22, 27). It may be that the requirement for an intact TAR recognition domain is more important for EIAV Tat and that the EIAV TAR recognition domain may be differently structured than that of HIV-1 Tat. Furthermore, defective EIAV TAR recognition domains, such as those present in pRS-$H_{1235}E_4$Tat and pRS-ETat-3d, may destabilize the overall folding of this protein and hence sterically impair activation domain function.

Studies of competition between heterologous *trans* activators permit the in vivo analysis of discrete events which make up the process of Tat-mediated *trans* activation. The use of functional *trans* activators as competitors provides a

powerful tool for the detailed study of interactions between Tat and cellular targets. Through the use of this assay, evidence for the existence of a cellular cofactor that associates with the Tat activation domain and functions as a positive effector of *trans* activation has been obtained. Cell-type-specific differences in Tat activity have previously been described (2), and a Tat-associated HeLa cell protein which functions as a negative modulator of Tat activity has been reported (24), but the identity of the putative positive cofactor described in this report remains unknown. Its presence in both human and canine cells suggests that it is ubiquitous, and its identification and characterization will undoubtedly reveal further details of Tat-mediated *trans* activation.

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