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Identification of Lentivirus Tat Functional Domains through Generation of Equine Infectious Anemia Virus/Human Immunodeficiency Virus Type 1 *tat* Gene Chimeras

RICHARD CARROLL,[1] LUIS MARTARANO,[2] AND DAVID DERSE[1]*

Laboratory of Viral Carcinogenesis[1] and Biological Carcinogenesis and Development Program, PRI/DynCorp,[2] NCI-Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201

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The structural regions that comprise the functional domains of lentivirus Tat proteins were examined. Chimeric *tat* genes and chimeric viral promoters were constructed between the distantly related human immunodeficiency virus type 1 (HIV-1) and equine infectious anemia virus (EIAV). These exchange experiments revealed that the EIAV Tat-responsive element recognition domain is formed by two distinct structural regions. Activation domains of both HIV-1 and EIAV Tat contain a conserved core element, but at least HIV-1 Tat requires the presence of additional structural regions. The interchangeable nature of Tat activation domains suggests that these domains act through a common or ubiquitous cellular transcription factor.

The evolutionarily divergent lentiviruses, equine infectious anemia virus (EIAV) and human immunodeficiency virus type 1 (HIV-1), both encode *tat* genes, whose products stimulate in *trans* long terminal repeat (LTR)-directed gene expression (1, 9, 40, 59). HIV-1 Tat acts primarily to increase the steady-state concentration of viral mRNA (6, 23, 38, 45, 64). The Tat-responsive element (TAR), located at the 5′ end of all viral transcripts, is required for HIV-1 Tat-mediated *trans* activation (17, 23, 29, 46). TAR RNA is capable of assuming a stem-loop conformation (38, 41), and both the sequence of the loop and secondary structure within the stem, including a pyrimidine bulge, are important for *trans* activation (2, 12, 16, 48, 52). EIAV Tat-mediated activation of gene expression is also dependent on sequences in the R region of the LTR (10, 56). These sequences are predicted to form an RNA secondary structure analogous to that of HIV-1 TAR, and mutations which disrupt the secondary structure of the stem or alter the sequence of the loop abolish *trans* activation (4). However, EIAV TAR is predicted to have a much shorter stem than HIV-1 TAR, and it lacks the pyrimidine bulge found in HIV-1 TAR (4).

It has been suggested that the binding of HIV-1 Tat to TAR RNA positions Tat for subsequent interactions with the transcription apparatus (3, 16, 30, 34, 60). This dual-interaction mechanism is reminiscent of other *trans*-acting proteins that contain two functional domains; one recognizes a nucleic acid target sequence, while the other activates gene expression through interactions with the transcription complex. Functional domains of these *trans*-acting proteins have been identified in domain swap experiments whereby the exchange of structural regions confers properties of one *trans* activator upon another (reviewed in references 37 and 43).

Alignment of the amino acid sequences of lentiviral *tat* gene products suggests the existence of five structural motifs: the amino-terminal, cysteine-rich, core, basic, and carboxy-terminal regions (9). Mutagenesis experiments indicate that amino acid residues in the amino-terminal (13, 33, 49), cysteine-rich (13, 15, 33, 49, 50), core (21, 33), and basic (11, 15, 24, 33, 49, 58, 61) regions are required for HIV-1 Tat activity, while the carboxy terminus appears to be dispensable (38, 51).

The common arrangement of structural motifs in lentiviral Tat proteins suggested that these proteins may be composed of discrete, interchangeable functional domains. We constructed chimeric Tat proteins by exchanging structural regions between EIAV and HIV-1 Tat in order to identify functional domains and to explore the interactions between Tat and *cis*-acting promoter elements. The contribution of promoter elements to Tat-mediated *trans* activation was further studied by creating chimeric promoters containing the TAR region of one virus fused to the upstream region of the other virus. We have identified the principal components of the TAR recognition and activation domains of the Tat proteins. Furthermore, specificity in promoter *trans* activation is determined by the TAR element in the promoter and the TAR recognition domain of Tat. The U3 regions of the promoters and the activation domains of Tat were interchangeable, suggesting that Tat activates gene expression through some ubiquitous component of the cellular transcription machinery.

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 (9). pRS-Etat-M contains a cDNA copy of the EIAV *tat* gene, joined to a synthetic initiation codon, cloned into pRSPA (9). pRS-Htat was constructed by inserting the 343-bp *Sal*I-to-*Sau*96-1 fragment of pHXB2, which contains the first HIV-1 *tat* coding exon (55), into the pRSPA polylinker.

pUX-CAT contains the bacterial chloramphenicol acetyltransferase (CAT) gene and simian virus 40 polyadenylation signals cloned into pUC18 (26). 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 (9).

* Corresponding author.

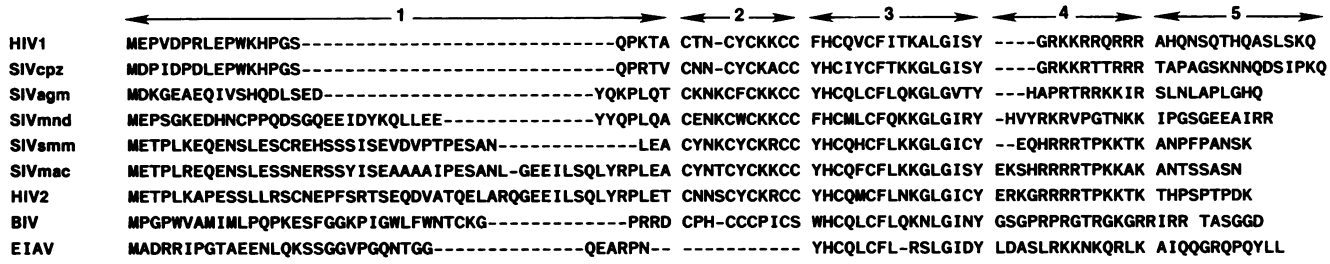

FIG. 1. Amino acid sequences of lentiviral Tat proteins. Dashes represent gaps introduced to facilitate sequence alignment. Tat structural regions are indicated and numbered as follows: region 1 is the amino terminus, region 2 is the cysteine-rich region (absent from EIAV Tat), region 3 is the core region, region 4 is the basic region, and region 5 is the carboxy terminus. Amino acid sequences are shown for the first coding exons of *tat* genes of HIV-1 (1), simian immunodeficiency virus SIV_{cpz} (28), SIV_{agm} (14), SIV_{mnd} (62), SIV_{smm} (27), SIV_{mac} (5), HIV-2 (22), and bovine immunodeficiency-like virus (BIV) (18). The amino acid sequence for EIAV Tat assumes that translation was initiated at a non-AUG codon (9, 40).

Oligonucleotide-directed mutagenesis. Oligonucleotide-directed mutagenesis was performed essentially as described previously (32, 36). The following oligonucleotides (Operon) were used to introduce restriction enzyme sites into pRS-Etat-M and pRS-Htat. The underlined regions refer to the base changes introduced by the mutagenesis procedure. The mutants are shown in Fig. 2A.

5′-TGTTATGAAACACAGCTGGCAATGAAAG-3′ was used to introduce a *Pvu*II site into pRS-Htat between amino acids 35 and 36.

5′-CCGCTTCTTCCTTACGTAGGAGATGCCTAA-3′ was used to introduce a *Sna*BI site between amino acids 47 and 48 of pRS-Htat.

5′-CTAGGAATTGATTACGTAGATGCTTCATTA-3′ was used to introduce a *Sna*BI site between amino acids 49 and 50 of pRS-Etat-M.

5′-AACAAAGACTGAGAGCTCTCCAACAAGGAAG-3′ was used to introduce an *Sst*I site between amino acids 63 and 64 of pRS-Etat-M.

tat gene mutants were identified by restriction enzyme digestion, and the nucleotide sequence was verified by dideoxy sequencing using Sequenase (United States Biochemicals).

Construction of chimeric *tat* genes. The mutated derivatives of pRS-Etat-M and pRS-Htat were digested at restriction enzyme sites introduced by mutagenesis as well as preexisting sites. The fragments were gel purified and ligated into the pRSPA polylinker. Chimeric *tat* genes were sequenced in their entirety.

$pH_{2,3}E_{4,5}$tat was constructed by digesting pRS-Htat with *Rsa*I and *Hin*dIII, and oligonucleotide linkers containing one-half of an *Nco*I site (5′-CATGGCATGC-3′ and 5′-GCATGC-3′) were ligated to the 130-bp fragment of the HIV *tat* gene. This intermediate was digested with *Sna*BI, and the resulting 54-bp fragment was inserted into *Nco*I-*Sna*BI-digested $pH_{1,2,3}E_{4,5}$tat to generate $pH_{2,3}E_{4,5}$tat.

pEΔ3tat was created by digesting pRS-Etat-M with *Apa*I, which cuts downstream of the EIAV *tat* coding sequences, followed by digestion with exonuclease III and mung bean nuclease. An *Xba*I nonsense linker (5′-CTAGTCTAGACTAG-3′; New England BioLabs) was ligated to the deleted *tat* gene sequence. The extent of 3′-end deletions was determined by dideoxy nucleotide sequencing.

Construction of chimeric viral promoters. Recombinant LTRs were constructed by separate polymerase chain reaction amplification of the U3 and R+U5 regions. The EIAV and HIV-1 LTRs were cloned in Bluescript KS+ plasmids. 5′ primers used to amplify the R+U5 regions were complementary to the sequence at the RNA start site and had a short tail to generate a *Sma*I site for subsequent cloning. The sequencing primer KS (Stratagene) served as the 3′ polymerase chain reaction primer and is complementary to plasmid sequences downstream of the LTR U5 region. The oligonucleotide primers were as follows: EIAV, 5′-ATCCCCGGGCACTCAGATTCTGCG-3′; and HIV-1, 5′-ATCCCCGGGTCTCTCTGGTTAGAC-3′. Amplifications were performed in a Bellco thermocycler; the amplified products were digested with *Sma*I and *Hin*dIII and cloned into Bluescript KS+ plasmid.

The oligonucleotide primers used to amplify the U3 regions were the pUC-M13 reverse-sequencing primer (complementary to plasmid sequences upstream of the U3 region) and U3 region-specific primers: EIAV, 5′-AATTGTCAGAATACAAGCACT-3′; and HIV-1, 5′-AGTACAGGCAAAAAGCAGCTG-3′. The U3-region polymerase chain reaction products were digested with *Xba*I, which cuts upstream of U3, and ligated to the R-region plasmids that had been cut with *Sma*I and *Xba*I, thus fusing the blunt ends of the R and U3 regions. The chimeric LTRs were then excised and transferred to pUX-CAT plasmids and verified by nucleotide sequence analysis.

All plasmids were banded twice by cesium chloride density gradient centrifugation. Two independent clones of each plasmid were assayed.

Transfections and CAT assays. D17 cells were maintained in Dulbecco modified Eagle's medium containing 10% fetal calf serum. The day before transfection, D17 cells were seeded at 3×10^5 cells per well in 3-cm-diameter six-well dishes. Cells were transfected with 0.1 μg of Tat expression plasmid and 3.0 μg of CAT reporter construct by the calcium phosphate method (20). Six hours after transfection, the cells were washed in phosphate-buffered saline and refed with fresh medium. Cells extracts were prepared 48 h after transfection (9), and CAT assays were performed by the solvent partition method (39). Basal levels of CAT activity were determined by transfection of the appropriate reporter plasmid and pRSPA. Each reported data point represents the mean of at least three independent transfections.

RESULTS

Activity of wild-type *tat* gene products and construction of *tat* gene chimeras. The predicted amino acid sequences of lentiviral *tat* gene products are shown in Fig. 1. The areas of amino acid similarity between Tat proteins define the structural regions. Although EIAV Tat clearly has structural

TABLE 1. *trans* activation of EIAV and HIV LTRs by *tat* expression plasmids[a]

CAT plasmid	*tat* expression plasmid	CAT activity (10^3 cpm/h)	Fold *trans* activation
pEI-CAT	pRSPA	0.48	NA
	pRS-Etat-M	9.96	20.8
	pRS-Htat	0.48	1.0
pHI-CAT	pRSPA	1.73	NA
	pRS-Htat	19.63	11.3
	pRS-Etat-M	2.04	1.2

[a] D17 cells were transfected with 3 μg of the indicated CAT reporter construct and 0.1 μg of *tat* expression vector. pRS-Etat-M expresses the EIAV *tat* gene, and pRS-Htat expresses the HIV-1 *tat* gene. In pHI-CAT, CAT activity is directed by the HIV-1 LTR; in pEI-CAT, CAT activity is directed by the EIAV LTR. pRSPA contains no *tat* coding sequences and was used to determine the basal level of CAT activity. Fold *trans* activation is expressed as the ratio of Tat-induced to basal activity. NA, not applicable.

regions characteristic of this group, it is the most distantly related member; it lacks the cysteine-rich region and has several unique differences in the core sequences.

The activity of wild-type *tat* gene products was assayed by transfection of D17 cells with *tat* expression plasmids in combination with either pEI-CAT or pHI-CAT, in which expression of the bacterial CAT gene was directed by the EIAV LTR or the HIV-1 LTR, respectively (Table 1). D17, a canine osteosarcoma cell line, is permissive for both EIAV and HIV-1 Tat activity (9). pRS-Etat-M, which expresses the wild-type EIAV *tat* gene, caused an approximately 20-fold increase in the level of pEI-CAT activity but did not stimulate pHI-CAT expression. pRS-Htat, which expresses the wild-type HIV-1 *tat* gene, stimulated pHI-CAT activity approximately 11-fold but did not increase pEI-CAT activity. The relatively low level of *trans* activation of pHI-CAT was due to the high basal activity of this promoter, which may have resulted from either the abundance of cell-specific transcription factors or the absence of the HIV-1 LTR negative regulatory element, which suppresses basal activity (46). This experiment demonstrated that despite their structural similarities, both HIV-1 Tat and EIAV Tat *trans* activated only their cognate promoters, as previously reported (9).

To determine which Tat structural regions comprise functional domains, we introduced restriction enzyme sites into EIAV and HIV-1 *tat* coding sequences, enabling the coding sequences to be cleaved at structural region junctions (Fig. 2A). Introduction of restriction sites caused several amino acid changes (Fig. 2A), but none of the introduced changes altered Tat activity (data not shown). Chimeric *tat* genes were generated by digesting the modified EIAV and HIV-1 *tat* genes and ligating the appropriate fragments into the expression vector pRSPA (Fig. 2B).

Identification of the EIAV Tat promoter recognition domain. $pH_{1,2}E_{3,4,5}$tat contained the HIV-1 Tat amino-terminal and cysteine-rich regions and the EIAV Tat core, basic, and carboxy-terminal regions. It stimulated pEI-CAT expression as efficiently as pRS-Etat-M, while it had a negligible effect on pHI-CAT activity (Fig. 3). This experiment demonstrated that *trans* activation of the EIAV LTR by EIAV Tat did not require the EIAV Tat amino-terminal region, in agreement with previous results (9). Furthermore, it indicated that the HIV-1 Tat amino-terminal and cysteine-rich regions did not disrupt EIAV Tat activity.

$pH_{1,2,3}E_{4,5}$tat contained the HIV-1 Tat amino-terminal, cysteine-rich, and core regions and the EIAV Tat basic and carboxy-terminal regions. It stimulated pEI-CAT activity to approximately 80% of the wild-type EIAV Tat value, while it stimulated pHI-CAT activity to approximately 25% of the wild-type HIV-1 Tat level, which was only 2.5-fold over background. This experiment demonstrated that the EIAV Tat basic and carboxy-terminal regions conferred EIAV LTR-specific *trans* activation when combined with either the EIAV Tat core region or the amino-terminal, cysteine-rich, and core regions of HIV-1 Tat. Thus, the EIAV Tat basic and carboxy-terminal regions are likely to form a promoter recognition domain.

The roles of the EIAV Tat basic and carboxy-terminal regions in promoter recognition were further assayed by

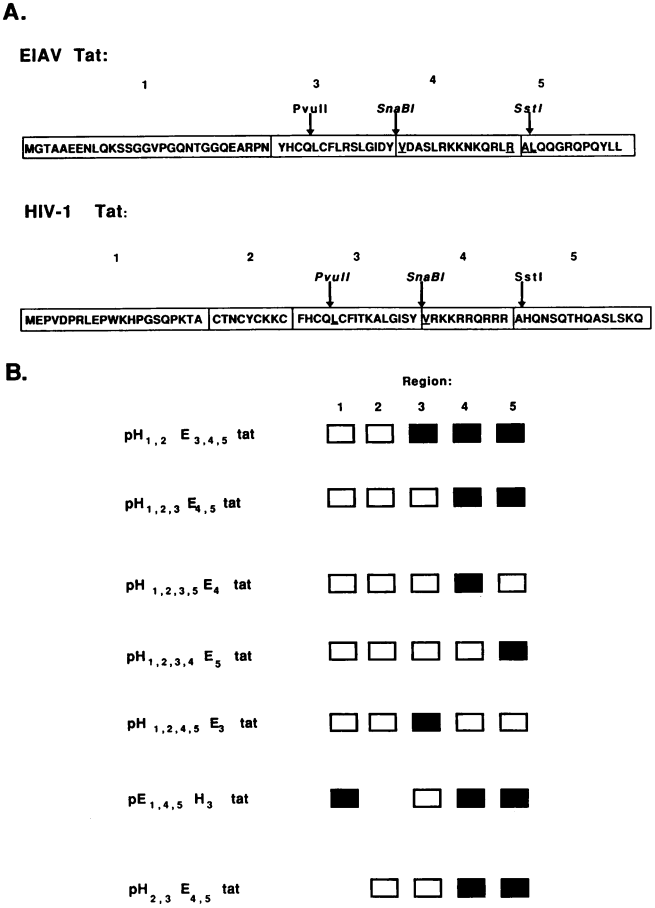

FIG. 2. Oligonucleotide-directed mutagenesis and construction of EIAV/HIV-1 *tat* gene chimeras. (A) Restriction enzyme sites were introduced into the coding sequences of EIAV and HIV-1 *tat* genes by oligonucleotide-directed mutagenesis as described in Materials and Methods. The amino acid sequences of EIAV Tat and HIV-1 Tat are presented, and the locations of pertinent restriction sites are indicated. Restriction sites introduced by mutagenesis are indicated by italics. Amino acid residues altered by the mutagenesis procedure are underlined. The previously described Tat structural regions are boxed. (B) EIAV and HIV-1 *tat* genes were cleaved at introduced or preexisting restriction enzyme sites, and the fragments were combined to generate chimeric *tat* genes. Expression vectors were constructed by inserting the chimeras into the polylinker of the expression vector pRSPA (see Materials and Methods). Black blocks represent structural regions derived from EIAV Tat; white blocks represent structural regions derived from HIV-1 Tat.

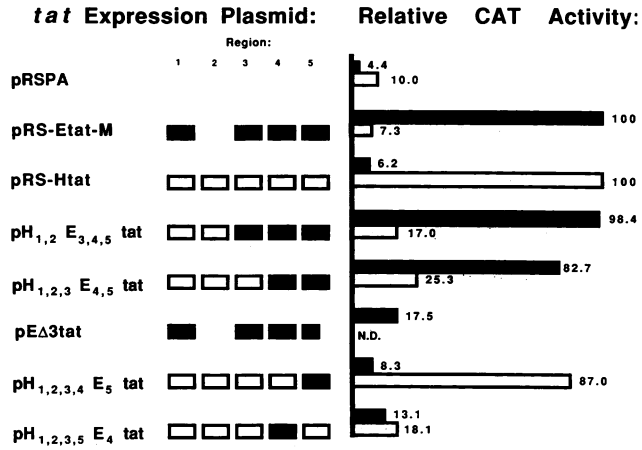

FIG. 3. Identification of the EIAV Tat promoter recognition domain. D17 cells were transfected with LTR-CAT reporter constructs and chimeric *tat* expression plasmids. The structures of both wild-type and chimeric *tat* gene products are shown; black boxes represent structural regions derived from EIAV tat, and white boxes represent structural regions derived from HIV-1 Tat. pEΔ3tat was generated by removing the three carboxy-terminal amino acids from EIAV Tat (see Materials and Methods). CAT activity is expressed relative to the value obtained when wild-type *tat* expression plasmids (pRS-Etat-M and pRS-Htat) were transfected with their cognate promoters (pEI-CAT and pHI-CAT, respectively). pRSPA contains no *tat* coding sequences and was used to determine basal levels of CAT activity. Activity of the HIV-1 LTR-CAT construct is indicated by white bars; activity of the EIAV LTR-CAT construct is indicated by black bars. Relative CAT activity is also indicated numerically. N.D., not done.

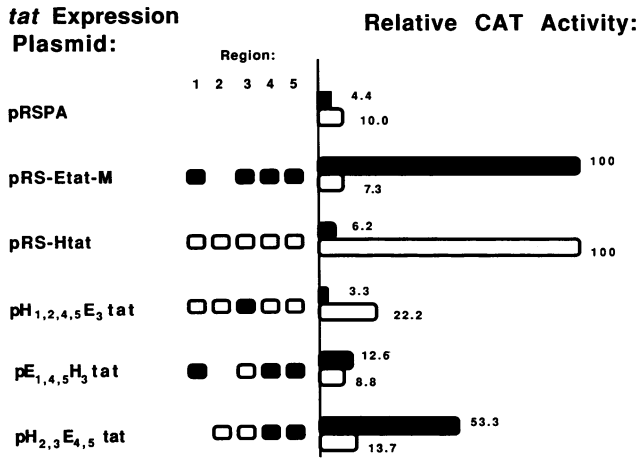

FIG. 4. Identification of EIAV and HIV-1 Tat activation domains. D17 cells were transfected with LTR-CAT reporter constructs and chimeric *tat* expression plasmids. Black boxes represent structural regions derived from EIAV tat; white boxes represent structural regions derived from HIV-1 Tat. CAT activity is expressed relative to the value obtained when wild-type *tat* expression plasmids (pRS-Etat-M and pRS-Htat) were transfected with their cognate promoters (pEI-CAT and pHI-CAT, respectively). Relative activity is expressed numerically and indicated by white bars for HIV-1 LTR-CAT activity and black bars for EIAV LTR-CAT activity.

construction of EIAV Tat deletion mutants as well as *tat* gene chimeras in which the basic and carboxy-terminal regions of EIAV Tat were individually inserted into an HIV-1 Tat backbone. The results are shown in Fig. 3. pEΔ3tat, created by removing the three carboxy-terminal amino acid residues from EIAV Tat, stimulated pEI-CAT activity to less than 20% of the level of pRS-Etat-M, emphasizing the importance of the carboxy-terminal region for EIAV Tat activity. $pH_{1,2,3,4}E_5$tat, in which the carboxy terminus of HIV-1 Tat was replaced with the EIAV Tat carboxy terminus, only slightly stimulated pEI-CAT activity, while it stimulated pHI-CAT activity to 87% of the level of pRS-Htat. However, HIV-1 Tat mutants lacking amino acid sequences downstream of the basic region can still *trans* activate the HIV-1 LTR (38, 51), so it is not surprising that $pH_{1,2,3,4}E_5$tat specifically recognized the HIV promoter. $pH_{1,2,3,5}E_4$tat, made by replacing the HIV-1 Tat basic region with the EIAV Tat basic region, weakly stimulated both pEI-CAT activity and pHI-CAT activity. This experiment indicated that neither the basic nor the carboxy-terminal region of EIAV Tat individually conferred EIAV promoter-specific *trans* activation, suggesting that formation of the EIAV Tat promoter recognition domain required both structural regions.

Identification of EIAV and HIV-1 Tat activation domains. The EIAV Tat core region, while not part of the promoter recognition domain, is essential for EIAV Tat activity (9), implying that it forms an additional functional domain. The EIAV Tat core region could be replaced with little loss of activity by the HIV-1 amino-terminal, cysteine-rich, and core regions ($pH_{1,2,3}E_{4,5}$tat; Fig. 3), suggesting that these regions formed an analogous HIV-1 Tat functional domain. By analogy with other *trans*-acting factors, we refer to this as the putative Tat activation domain. As a first step toward defining the sequences which comprised the EIAV and HIV-1 Tat activation domains, we tested the ability of Tat core regions to function in a heterologous context (Fig. 4). $pE_{1,4,5}H_3$tat was produced by replacing the EIAV Tat core region with the HIV-1 Tat core region, while $pH_{1,2,4,5}E_3$tat was constructed by substituting the EIAV Tat core region for the HIV-1 core region. Neither chimera stimulated significant levels of either EIAV or HIV-1 LTR-directed CAT activity (Fig. 4), demonstrating that despite their high degree of amino acid similarity, the core regions were not interchangeable.

To investigate the contributions of HIV-1 Tat structural regions to the HIV-1 Tat activation domain, we constructed $pH_{2,3}E_{4,5}$tat, which contained the HIV-1 Tat cysteine-rich and core regions and the EIAV Tat basic and carboxy-terminal regions. $pH_{2,3}E_{4,5}$tat stimulated pEI-CAT expression to approximately 50% of the wild-type EIAV Tat level, while it only slightly stimulated pHI-CAT activity (Fig. 4). This experiment demonstrated that the HIV-1 Tat cysteine-rich and core regions formed the minimal essential HIV-1 Tat activation domain. Addition of the HIV-1 Tat amino-terminal region to this construct (yielding $pH_{1,2,3}E_{4,5}$tat; Fig. 3) resulted in a further increase in EIAV LTR-directed CAT activity, suggesting that sequences within the HIV-1 Tat amino-terminal region served to augment the strength of the activation domain.

Construction and activity of chimeric LTRs. While both EIAV and HIV-1 LTRs contain downstream TARs, the U3 regions of the two promoters bear little similarity (Fig. 5A). Inspection of the EIAV U3 region reveals that it lacks the consensus binding sites for SP-1 and NF-κB implicated as important for HIV-1 Tat activity (17). To identify LTR elements that interacted directly or indirectly with EIAV or HIV-1 Tat, we constructed chimeric LTR-CAT reporter

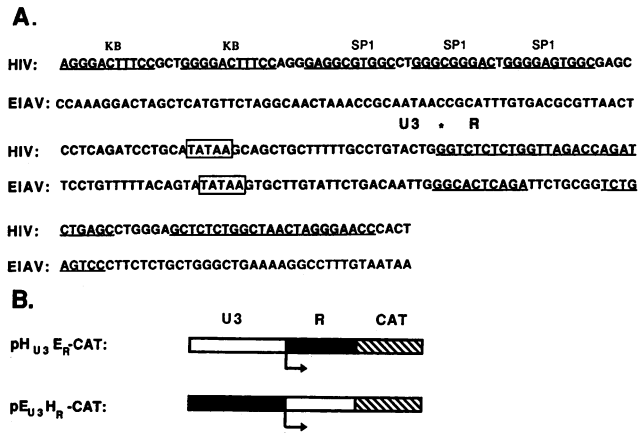

FIG. 5. Construction of EIAV/HIV-1 promoter chimeras. (A) Sequences of the EIAV and HIV-1 LTRs. The transcription start site is indicated by an asterisk. On the HIV-1 LTR, the consensus binding sites for NF-κB and SP-1 are indicated. For both LTRs, the TATA boxes and TAR elements (underlined) are indicated. (B) Chimeric promoter-CAT constructs, made as described in Materials and Methods. Regions derived from the EIAV promoter are in black; sequences derived from the HIV-1 promoter are in white. CAT sequences are represented by diagonal lines. The transcription start site is indicated by an arrow.

plasmids. $pH_{U3}E_R$-CAT contained the HIV-1 LTR U3 region and the EIAV LTR R region, while $pE_{U3}H_R$-CAT contained the EIAV U3 region and the HIV R region. Fusions were made at the RNA start sites (Fig. 5B).

The basal and Tat-induced activities of the chimeric promoters are presented in Table 2. pRS-Etat-M strongly stimulated $pH_{U3}E_R$-CAT activity, while pRS-Htat had no effect on this promoter. Because of its higher basal activity, the fold activation of $pH_{U3}E_R$-CAT by pRS-Etat-M was lower than that for pEI-CAT, although the chimeric promoter reached higher absolute levels of CAT activity. The reciprocal chimeric LTR-CAT construct, $pE_{U3}H_R$-CAT, was strongly activated by pRS-Htat but unaffected by pRS-Etat-M. This demonstrated that both Tat proteins specifically interacted with their cognate R regions and furthermore that both were capable of *trans* activating promoters containing heterologous U3 regions, as has been previously reported (38, 42, 57).

$pH_{1,2,3}E_{4,5}$tat contained the EIAV Tat promoter recognition domain, and it specifically stimulated EIAV LTR-directed CAT activity to high levels (Fig. 2). It also stimulated high levels of $pH_{U3}E_R$-CAT activity (75% of the wild-type EIAV Tat value), but it failed to stimulate $pE_{U3}H_R$-CAT-directed CAT activity significantly. These data indicated that the EIAV Tat basic and carboxy-terminal regions formed a functional domain which specifically recognized the TAR region of the EIAV LTR.

We were unable to identify the corresponding HIV-1 TAR recognition domain (data not shown). It has been demonstrated that the HIV-1 Tat basic region binds TAR RNA in vitro (47, 63), so the basic region, like the EIAV Tat basic region, is likely to form part of the TAR recognition domain. It is likely that the functional TAR recognition domain of HIV-1 Tat is formed through interactions between the basic region and another, as yet unidentified, structural region.

TABLE 2. Activity of EIAV/HIV-1 promoter chimeras

CAT plasmid (U3, R)[a]	CAT activity (10^3 cpm/h)[b]			
	pRSPA	pRS-Htat	pRS-Etat-M	$pH_{1,2,3}E_{4,5}$tat
pHI-CAT	1.7	19.6	2.0	1.7
(HIV, HIV)	(NA)	(11.3)	(1.2)	(1.0)
pEI-CAT	0.5	0.5	10.0	8.3
(EIAV, EIAV)	(NA)	(1.0)	(20.0)	(16.6)
$pE_{U3}H_R$-CAT	0.2	7.3	0.2	0.5
(EIAV, HIV)	(NA)	(36.5)	(1.0)	(2.2)
$pH_{U3}E_R$-CAT	2.3	2.5	24.0	18.3
(HIV, EIAV)	(NA)	(1.1)	(10.4)	(7.8)

[a] D17 cells were transfected with LTR-CAT reporter constructs and the indicated *tat* expression plasmids. The composition of each LTR-CAT construct is indicated in parentheses below the construct name.

[b] Fold *trans* activation for the given *trans* activators is indicated in parentheses. NA, not applicable.

DISCUSSION

The common requirement for downstream promoter elements indicates that EIAV Tat and HIV-1 Tat belong to a novel class of *trans* activators. Although EIAV and HIV-1 Tat have different polarities with respect to the roles of their amino- and carboxy-terminal regions, and neither Tat stimulates expression of genes linked to the heterologous promoter, they share conserved core and basic regions. Alignment of their amino acid sequences suggests the existence of discrete regions, perhaps separately acting in nucleic acid target recognition and transcription activation. These properties make EIAV Tat and HIV-1 Tat ideal candidates for domain swap experiments, which have proven useful in identifying functional domains in other classes of *trans* activators (37, 43). Exchange of structural regions between EIAV and HIV-1 Tat has permitted the identification of lentiviral Tat functional domains. We have found that EIAV and HIV-1 Tat functional domains are formed through the combined actions of highly conserved structural elements and elements that are disparate with respect to both amino acid sequence and location within the protein. This likely represents molecular adaptations of a common process to the conditions imposed by different virus replication strategies in different hosts.

TAR recognition domain. Fragments of HIV-1 Tat containing the basic region bind to the stem and pyrimidine bulge of HIV-1 TAR in vitro, but this binding is not sufficient for *trans* activation (47, 63). The nucleotide sequence of the HIV-1 TAR loop is critical for *trans* activation (2, 12), and the loop sequence itself may serve as a target for cellular RNA-binding proteins (19, 35), yet HIV-1 Tat binds with apparently unaltered affinity to TAR mutants from which the loop has been removed (47). These data imply that a complex interaction, with a Tat-TAR component and a TAR-cellular protein component, is required for *trans* activation.

The smaller size of the EIAV TAR stem and the lack of a pyrimidine bulge (4) suggest that the process of EIAV TAR recognition may differ significantly from TAR recognition in HIV-1. The EIAV TAR recognition domain was formed by the EIAV Tat basic and carboxy-terminal regions. Since neither region, when inserted singly into an HIV-1 Tat backbone, conferred EIAV TAR recognition, it appears that both regions are required for formation of this domain. By analogy with HIV-1 Tat, it is possible that the EIAV Tat basic region binds TAR RNA, and the EIAV Tat carboxy-terminal region either interacts with a cellular TAR RNA-binding protein or serves to stabilize the binding between EIAV Tat and TAR.

Activation domains of EIAV and HIV-1 Tat. While required for activity (9), the EIAV Tat core region is not required for formation of the TAR recognition domain, and we therefore propose that the EIAV core region comprises a second EIAV Tat functional domain, the activation domain. However, since insertion of the EIAV core region into an HIV-1 Tat backbone yielded an inactive Tat protein, we cannot exclude the possibility that additional sequences are required to form a viable EIAV Tat activation domain.

The high degree of amino acid similarity between the core regions of EIAV and HIV-1 Tat suggested that the HIV-1 Tat core region comprised part of the HIV-1 Tat activation domain, as previously suggested (21, 53). However, when the HIV-1 Tat core region was fused to the EIAV TAR recognition domain, the resulting chimeric Tat protein was inactive. Addition of the HIV-1 cysteine-rich domain to this construct resulted in a marked increase in Tat activity, and addition of both HIV-1 amino-terminal and cysteine-rich domains yielded a construct with nearly wild-type EIAV Tat activity. Therefore, we propose that the HIV-1 Tat core and cysteine-rich regions form the essential HIV-1 Tat activation domain, and the amino-terminal region or portions thereof serve to increase the activity of this domain.

Implications of Tat structure for *trans* activation. HIV-1 Tat acts to stimulate gene expression primarily by increasing the rate of HIV-1 LTR-directed transcription (reviewed in reference 7). However, the mechanism by which Tat accomplishes this remains unknown. Tat has been reported to work at both initiation (34) and postinitiation (31, 34, 52) stages of transcription. We constructed chimeric promoters in part to determine whether Tat activation domains required interactions with specific upstream or downstream factors. We found that there were no specific sequence requirements upstream of the TATA box for *trans* activation, as has been previously reported for HIV-1 (38, 42) and EIAV (57).

The combination of relaxed upstream sequence requirements and highly specific downstream (TAR) sequence requirements suggests that transcription in the presence of Tat may proceed in two stages: early events, which are Tat independent, involve the recruitment of transcription factors and RNA polymerase II, resulting in the formation of an initiation complex. The rate at which these complexes are formed is dictated by sequence motifs in the U3 region. The strict orientation and position dependence of TAR (23, 38, 52) place Tat in a unique position to determine the fate of transcription complexes. While under certain circumstances the TAR element can be replaced by viral (60) or bacterial (53) RNA elements, Tat must still be situated near the 5′ end of the nascent transcript. Such positioning would facilitate interactions between the activation domain of Tat and components of the cellular transcription machinery. The observation that the structurally dissimilar activation domains of EIAV Tat and HIV-1 Tat can substitute for one another suggests that their common target is a ubiquitous cellular factor. Possible targets for the Tat activation domain in this area of the promoter include RNA polymerase II, RNA polymerase ancillary transcription factors, and TAR DNA-binding proteins (30, 52, 65).

We have identified functional domains critical for Tat activity in two widely divergent lentiviruses. We have determined that EIAV Tat and HIV-1 Tat display a fundamentally similar architecture, with the amino-terminal halves serving as activation domains, while the carboxy-terminal halves serve as TAR recognition domains. Further elucidation of the mechanism of Tat-mediated stimulation of viral gene expression awaits the identification of the cellular proteins with which Tat interacts.

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