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Genetic Analysis of the Cofactor Requirement for Human Immunodeficiency Virus Type 1 Tat Function

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The Tat protein of human immunodeficiency virus type 1 is a potent transcriptional *trans* activator of the viral long terminal repeat promoter element. Tat function requires the direct interaction of Tat with a *cis*-acting viral RNA target sequence termed the *trans*-activation response (TAR) element and has also been proposed to require at least one cellular cofactor. We have used a genetic approach to attempt to experimentally define the role of the cellular cofactor in Tat function and TAR binding. Our data suggest that neither Tat nor the cellular cofactor binds to TAR alone in vivo and indicate, instead, that the interaction of Tat with its cellular cofactor is a prerequisite for TAR binding. The known species tropism of lentivirus Tat proteins appears to arise from the fact that not only Tat but also the cellular cofactor can markedly influence the RNA sequence specificity of the resultant protein complex. These data also suggest that the Tat cofactor is likely a cellular transcription factor that has been highly conserved during vertebrate evolution. We hypothesize that the primary function of Tat is to redirect this cellular factor to a novel viral RNA target site and to thereby induce activation of viral gene expression.

Replication of the pathogenic retrovirus human immunodeficiency virus type 1 (HIV-1) is critically dependent on the functional expression of the viral nuclear regulatory proteins Tat and Rev (reviewed in reference 12). The Tat protein is a transcriptional *trans* activator of the HIV-1 long terminal repeat (LTR) promoter element, while Rev acts posttranscriptionally to induce the cytoplasmic expression of mRNAs that encode the viral structural proteins. The mechanisms of action of Tat and Rev, while therefore clearly distinct, are nevertheless each dependent on their direct interaction with *cis*-acting viral RNA target sites termed, respectively, the *trans*-activation response (TAR) element (14, 40, 49) and the Rev response element (RRE) (29, 51).

Although many DNA and RNA tumor viruses encode sequence-specific transcriptional *trans* activators, the HIV-1 Tat protein is highly unusual in acting via an RNA, rather than a DNA, target sequence. Indeed, similar RNA sequence-specific regulatory proteins have as yet been observed only in the other primate immunodeficiency viruses and in a subset of the more distantly related ungulate lentiviruses, including equine infectious anemia virus (EIAV) and bovine immunodeficiency virus (12, 13, 27). While the precise mechanism of action of these lentivirus Tat proteins has remained uncertain, activation of viral RNA expression at both the level of transcription initiation and the level of elongation has been proposed (15, 19, 23, 24, 26, 31, 44).

Many eukaryotic transcription factors display a modular domain structure featuring discrete protein sequences involved, respectively, in conferring nucleic acid sequence specificity and in activating transcription from the bound template (32, 37). Similarly, mutational analysis has led to the definition of two domains within the 86-amino-acid Tat protein that appear to fulfill comparable functions (Fig. 1). The arginine-rich basic domain of Tat not only acts as a nuclear localization signal (41) but also is both necessary and fully sufficient for specific binding to TAR in vitro (6, 40, 49). The core motif of Tat is highly conserved among all lentiviral Tat proteins, while the cysteine motif is conserved in all primate lentiviruses (8). Mutation of either of these two motifs can inactivate HIV-1 Tat function in vivo without affecting the ability of the protein to bind TAR in vitro (17, 22, 41, 47). These sequences have therefore been proposed to form a cellular cofactor binding domain (8, 9, 13, 42, 47). While less well conserved sequences located N terminal to these Tat motifs are essential for full biological activity in vivo, the more C-terminal sequences, including the second coding exon of Tat, appear dispensable for efficient *trans* activation of the HIV-1 LTR (8, 13, 17, 25, 39, 44, 47) (Fig. 1).

The *cis*-acting RNA target site for HIV-1 Tat is a 59-nucleotide (nt) RNA stem-loop structure located immediately proximal to the start site for viral mRNA transcription (Fig. 2) (4, 16). Mutations that disrupt the helical structure of the 27-nt apical region of TAR, or that affect the pyrimidine-rich 3-nt bulge, prevent TAR function in vivo and also inhibit the in vitro interaction of Tat with TAR (3, 14, 40). In contrast, mutation of the 6-nt terminal loop of TAR has no detectable affect on the in vitro Tat-TAR interaction yet effectively blocks TAR function in vivo (14, 16). It has therefore been proposed that *trans* activation of the HIV-1 LTR by Tat might require the specific interaction of not only Tat but also a cellular cofactor(s) with TAR (40). It has further been suggested that a specific interaction of this TAR-binding cellular cofactor(s) with Tat might well be critical to the formation of this hypothetical ternary complex (46). Although several cellular proteins that bind to TAR or Tat have been reported, the identification of a cellular cofactor required for Tat function and the definition of the role of this cofactor in the mechanism of action of Tat have so far remained elusive (30, 34, 50).

In this study, we have used a range of genetic approaches to address this latter question. Our data support the hypothesis that the primary role of Tat is to recruit a cellular transcription factor to the HIV-1 LTR TAR element. These

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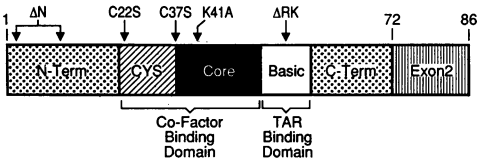

FIG. 1. Domain organization of the Tat protein of HIV-1. The functional domain organization of the 86-amino-acid Tat protein presented is based on reports from a number of laboratories, including, particularly, the work of Kuppuswamy et al. (25) and Derse and coworkers (8, 13). The core motif, marked by the consensus amino acid sequence N′-$\frac{K}{R}$XLGIXY-C′, is conserved in all lentivirus Tat proteins. In contrast, five of the seven cysteine residues that constitute the essential Cys motif of HIV-1 Tat are lacking in the eTat protein (8).

data further suggest that neither Tat nor the cellular cofactor is capable of interacting with TAR on its own in vivo.

MATERIALS AND METHODS

Construction of molecular clones. We have previously described the cytomegalovirus immediate-early promoter-based expression vector pBC12/CMV as well as derivatives containing cDNA forms of the HIV-1 *tat* gene (pcTat) and *rev* gene (pcRev) (48). Expression plasmids containing the mutant ΔN, C22S, C37S, and K41A forms of HIV-1 Tat were derived from pcTat as previously described (41, 47). The ΔRK Tat mutant contains an extensive substitution mutation within the Tat basic motif that changes Tat amino acids 50 to 56 from N′-KKRRQRR-C′ to N′-YVQILLY-C′. The ΔRK mutation was generated in the pcTat context by using the polymerase chain reaction (33) with overlapping oligonucleotide primers encoding the appropriate amino acid changes. The expression plasmid pcTat/Rev encodes a fusion protein consisting of the full-length HIV-1 Tat protein attached to the N terminus of the full-length HIV-1 Rev protein (48). Plasmids encoding mutant forms of Tat in this Tat-Rev fusion context were derived as previously described (48). Reporter plasmids containing the *cat* indicator gene under the control of the wild-type HIV-1 LTR (pTAR/CAT) or under the control of an HIV-1 LTR containing the stem-loop IIB (SLIIB) domain of the RRE in place of TAR (pSLIIB/CAT) have been described previously, as has the Rev function reporter plasmid pDM128/CMV (21, 28, 48).

All EIAV expression plasmids were derived from the pMA-1 proviral clone (1). A full-length cDNA copy of the EIAV *tat* gene was derived by the polymerase chain reaction, using oligonucleotide primers that permitted isolation of both coding exons of EIAV *tat*. The 5′ primer used to isolate the first exon substituted an *Nco*I site and a consensus translation initiation codon (5′-CCAUGG-3′) in place of the leucine codon that normally serves as the initiation codon for EIAV Tat (eTat) (8). The 3′ primer inserted an *Eag*I site at the end of the first exon. Oligonucleotide primers used to isolate the second eTat exon inserted an *Eag*I site at the beginning of this exon and introduced a unique *Xho*I site immediately 3′ to the Tat translation termination codon. A cDNA generated by ligation of these two genomic EIAV *tat* gene fragments at the introduced *Eag*I site encodes the correct eTat amino acid sequence, although the codon usage for arginine 32 (AGA→CGG) and proline 33 (CCC→CCG) is modified. The resultant *Nco*I-to-*Xho*I fragment, containing the full-length EIAV *tat* gene, was inserted into the pBC12/CMV expression plasmid to generate peTat. Similarly, pcRev/eTat contains this full-length eTat cDNA fused to the C terminus of the HIV-1 Rev protein. The pR43G derivative of peTat contains a mutation of arginine 43 to glycine that was introduced into the EIAV *tat* gene by the polymerase chain reaction. This critical arginine residue is located at the equivalent position in the Tat core consensus sequence to lysine 41 of HIV-1 Tat (Fig. 1) (8). The pEIAV/CAT plasmid contains EIAV LTR sequences, extending from −212 to +40 relative to the cap site, substituted in place of the HIV-1 LTR of pTAR/CAT. The pEIAV/CAT plasmid therefore contains the entire EIAV TAR element (10).

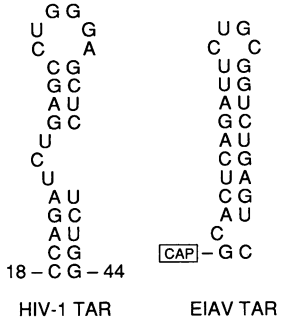

FIG. 2. Comparison of the HIV-1 and EIAV TAR elements. The primary sequence and proposed secondary structure of the critical 27-nt apical region of the 59-nt HIV-1 TAR element are compared with those of the complete 25-nt EIAV TAR element.

Cell culture and DNA transfection. The cell lines COS, HeLa, L, and QC1-3 were maintained as previously described (28). HeLa and L-cell cultures were transfected by the calcium phosphate procedure, using a total of 5.1 μg of DNA per 35-mm-diameter plate. COS and QC1-3 cells were transfected by using DEAE-dextran and chloroquine with a total of 500 ng of DNA per 35-mm-diameter plate (11). For assays of *trans* dominance, HeLa cells were transfected with 2 μg of the relevant chloramphenicol acetyltransferase (CAT) reporter plasmid, 0.1 μg of the effector plasmid, 2.0 μg of the competitor plasmid, and 1 μg of salmon sperm carrier DNA. At ~48 h after transfection, cultures were harvested and relative levels of CAT enzyme activity were determined by the diffusion method (35). All values reported were obtained during the linear phase of this kinetic assay for CAT activity and have been adjusted for any minor variability in the level of total protein in each extract, as determined by the method of Bradford (5). Expression of the various Tat mutants in transfected COS cells was monitored by radiolabeling with [^{35}S]cysteine followed by immunoprecipitation with a rabbit polyclonal anti-Tat antiserum as previously described (11, 47, 48).

RESULTS

Many eukaryotic transcription factors contain discrete functional domains that confer nucleic acid sequence specificity or transcription activation potential (32, 37). It has therefore proven possible to construct functional chimeric *trans* activators consisting of the nucleic acid binding domain of one protein attached to the effector domain of a second protein (37). Similarly, we and others have shown that fusion proteins consisting of Tat linked to a heterologous RNA binding domain can activate gene expression from an HIV-1 LTR in which TAR has been replaced by the appropriate RNA target (42, 43, 48). In particular, it has been shown that a Tat-Rev fusion protein can efficiently activate

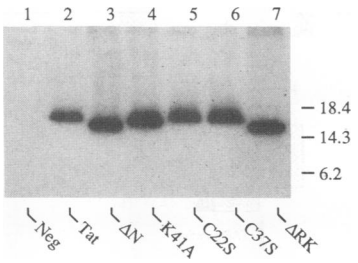

FIG. 3. Immunoprecipitation analysis of mutant HIV-1 Tat proteins. Expression plasmids encoding the indicated Tat proteins were transfected into COS cells as previously described (11, 47). At 48 h after transfection, cells were labeled with [^{35}S]cysteine (11), and the resultant extract was subjected to immunoprecipitation with a rabbit polyclonal anti-Tat antiserum (11, 47). Precipitated proteins were resolved by electrophoresis and visualized by autoradiography. The wild-type Tat protein is predicted to migrate at ~16 kDa (47). Positions of protein molecular weight markers are indicated at the right in kilodaltons. Neg, negative control.

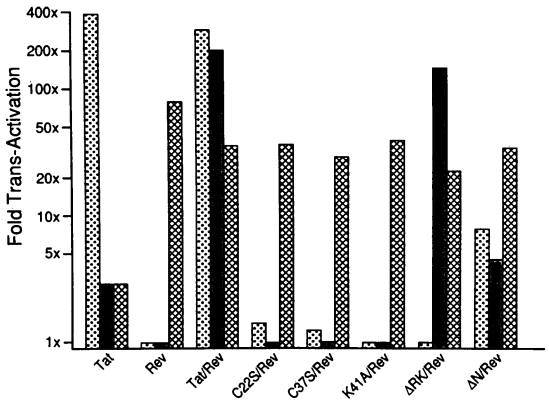

FIG. 4. Biological activity of wild-type and mutant Tat-Rev fusion proteins. The relative abilities of the indicated proteins to *trans* activate the HIV-1 LTR via the wild-type TAR element present in the indicator plasmid pTAR/CAT (dotted bars) or via the RRE-derived RNA target sequence present in the indicator plasmid pSLIIB/CAT (grey bars) are compared. Also shown are the abilities of these same proteins to posttranscriptionally regulate *cat* gene expression by interacting with the RRE target present in the indicator construct pDM128/CMV (cross-hatched bars). The parental expression plasmid pBC12/CMV served as a negative control. These data are representative of a series of transfection experiments performed with the human cell line HeLa.

gene expression from an HIV-1 LTR in which critical TAR sequences have been replaced by the RRE SLIIB primary Rev binding site (48).

In the context of such a Tat-Rev fusion, Rev serves as an autonomous nucleic acid binding domain while Tat contributes the effector domain. Mutations introduced into Tat that specifically inactivate the Tat RNA binding domain, while highly deleterious in the context of the wild-type protein, should therefore have no effect on the activity of the chimeric protein. However, mutations that inactivate the effector domain of Tat should be equally inhibitory in both assay systems.

To examine this issue, we introduced five distinct, well-characterized mutations into the Tat-Rev fusion protein context (Fig. 1) (25, 41, 47). In the ΔN mutation, Tat amino acids 2 to 9 inclusively have been deleted. The cysteine 22-to-serine (C22S) mutation is in the Tat cysteine motif, the cysteine 37-to-serine (C37S) mutation is at the border of the Tat core motif, and the lysine 41-to-alanine (K41A) mutation lies within the core motif. A fourth missense mutation, termed ΔRK, introduces six missense mutations into the Tat basic domain (50-KKRRQRR-56 changed to 50-YVQILLY-56). The ΔN deletion mutant of Tat has been reported to retain a low level of Tat activity (47), while the four Tat missense mutations have been reported to be entirely inactive (25, 41, 47). With the exception of the ΔRK mutant, which lacks a functional nuclear localization signal, these Tat mutants are predicted to show the nuclear/nucleolar subcellular localization characteristic of Tat (25, 41, 47). To confirm that these mutations also do not significantly effect the level of synthesis and expression of Tat, we determined the level of expression of these mutant proteins in transfected tissue culture cells by using immunoprecipitation analysis (11, 47, 48). As predicted from earlier work (25, 41, 47), these mutations were shown to have no detectable effect on the level of Tat protein expression (Fig. 3).

As expected, analysis of the biological activity of these fusion proteins in transfected HeLa cells demonstrated that all four Tat missense mutations abrogated the ability of the Tat-Rev fusion protein to activate expression of an indicator gene linked to the wild-type HIV-1 LTR, while the ΔN/Rev deletion mutant retained a minimal level of activity (Fig. 4). Similarly, the three mutations introduced into the cysteine and core motifs of Tat also abolished the ability of the Tat-Rev fusion protein to activate HIV-1 LTR-dependent gene expression via the RRE SLIIB RNA target, while the ΔN/Rev mutant displayed a barely detectable level of Tat function (Fig. 4). In marked contrast, the ΔRK/Rev mutant remained fully active on this heterologous RNA target site.

The pDM128/CMV indicator construct contains the *cat* indicator gene within an RRE-containing intron and is unable to express significant levels of cytoplasmic *cat* mRNA in the absence of HIV-1 Rev function (21, 28). Both Rev and the Tat-Rev fusion protein efficiently activate CAT expression from the pDM128/CMV construct upon cotransfection into HeLa cells (Fig. 4). Similarly, all five mutant Tat-Rev fusion proteins retained the ability to induce *cat* gene expression from this Rev indicator construct (Fig. 4). We therefore conclude that all of these fusion proteins are fully capable of functionally interacting with the RRE element present on the DM128/CMV-encoded *cat* transcript. The inability of the C22S, C37S, and K41A and ΔN fusion proteins to effectively activate HIV-1 LTR gene expression via the RRE-derived SLIIB RNA target therefore does not result from instability or from an inability to bind the RRE.

The Tat RNA binding domain is not autonomous in vivo. The data presented in Fig. 4 are consistent with the hypothesis, enunciated perhaps most clearly by Derse and coworkers (8, 13), that the Tat cysteine and core motifs form the minimal Tat activation domain while the N-terminal sequences of Tat contribute strongly to full effector domain function. Importantly, these data also clearly show that this activation domain remains active in the presence of a defective TAR RNA binding motif, i.e., is fully autonomous. However, they do not address the question of whether the Tat RNA binding domain is also independent of effector domain function in vivo. To address this issue, we examined whether any of these Tat mutants could act as competitive inhibitors of either Tat or Tat-Rev. The rationale for these experiments derives from the observation in several systems that an inactive but stable *trans* activator that retains a

TABLE 1. Analysis of *trans* inhibition of Tat function by various Tat and Rev derivatives

Challenge plasmid	Mean relative residual CAT activity ± SD[a]	
	Tat on TAR	Tat-Rev on SLIIB
pcTat	1.10 ± 0.12	0.17 ± 0.06
pcRev	0.75 ± 0.26	0.24 ± 0.08
pΔN	0.63 ± 0.18	0.53 ± 0.05
pC22S	0.97 ± 0.06	1.07 ± 0.15
pC37S	0.70 ± 0.06	1.02 ± 0.14
pK41A	1.09 ± 0.34	0.98 ± 0.24
pΔRK	0.63 ± 0.07	0.27 ± 0.07

[a] The relative abilities of an ~20-fold excess of the indicated proteins to block *trans* activation of the wild-type pTAT/CAT reporter plasmid by Tat or of the pSLIIB/CAT reporter plasmid by Tat-Rev were compared by transfection into HeLa cells. The data are derived from three distinct experiments and are given relative to the value for a culture in which the parental pBC12/CMV vector was used as a negative control, which was arbitrarily set at 1.00.

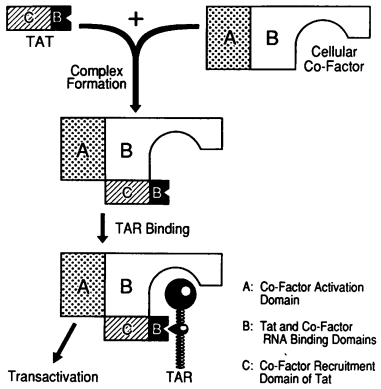

FIG. 5. Model for the temporal assembly of the HIV-1 Tat-TAR activation complex. It is proposed that the interaction of the viral Tat protein with a cellular cofactor represents the first step in the mechanism of action of Tat. This protein-protein interaction is predicted to be dependent on the integrity of the core, cysteine, and, to a lesser extent, N-terminal domains of HIV-1 Tat. This model predicts that neither Tat nor the cellular cofactor is able to bind TAR alone in vivo. The second step is the interaction of the Tat-cofactor complex with TAR to form an active ternary complex. This is dependent both on the specific interaction of Tat with the TAR pyrimidine bulge and on the specific interaction of the cellular cofactor with the TAR loop. It is further hypothesized that the cellular cofactor is directly responsible for the *trans* activation of HIV-1 LTR-dependent gene expression that results from this protein-RNA interaction. Although Carroll et al. (9) have also proposed a model in which Tat conveys a cellular cofactor to the TAR element, the presented model differs in that the cellular cofactor is predicted to be essential for TAR binding by Tat.

functional nucleic acid binding motif but lacks an effector domain will compete with the wild-type protein for binding to the target sequence and, hence, inhibit *trans* activation (20, 37). As the efficiency of this competition should simply reflect the ratio of wild-type to mutant protein, such a *trans*-dominant negative phenotype should be a defining characteristic of stable, inactive regulatory proteins that retain full nucleic acid binding potential. A second class of *trans*-dominant negative mutants is observed when the defective regulatory protein lacks the ability to bind the nucleic acid target but retains the ability to bind to and sequester a limiting cellular cofactor, a phenomenon generally known as squelching (37).

To ascertain whether any of these mutant proteins could display a dominant negative phenotype, we examined the ability of Tat to activate the wild-type HIV-1 LTR or of Tat-Rev to activate the pSLIIB/CAT indicator construct in the presence of a 20-fold molar excess of the relevant mutant protein expression plasmid. As shown in Table 1, several proteins were able to significantly inhibit Tat-Rev function on pSLIIB/CAT. In particular, the wild-type Tat protein inhibited Tat-Rev function by ~83%, while Rev inhibited Tat-Rev activity by ~76%. These controls in fact serve as examples of the two distinct classes of *trans*-dominant inhibitors predicted above. The Rev protein is incapable of interacting with the Tat cofactor but can inhibit Tat-Rev function by competing for binding to the RRE-derived SLIIB RNA target. Tat, on the other hand, is unable to bind to the SLIIB RNA target yet is capable of interacting with the cellular Tat cofactor. Inhibition of Tat-Rev function by Tat must therefore result from cofactor sequestration or squelching. As predicted by this hypothesis, the three Tat mutants C22S, C37S, and K41A, which lack a functional effector domain, have also lost the ability to inhibit Tat-Rev function via SLIIB. In contrast, the ΔRK mutant of Tat, which lacks a functional RNA binding motif but retains the effector domain (Fig. 1), also retains the ability to inhibit Tat-Rev function via the SLIIB target (Table 1). The somewhat reduced effectiveness of this inhibition, compared with the inhibition of Tat itself, may reflect the inappropriate subcellular localization of the ΔRK mutant. The ΔN mutant of Tat, which retains a low but detectable level of Tat function (47), also retained a low but apparently significant capacity to inhibit Tat-Rev function on SLIIB in *trans*.

In contrast to the observations with Tat-Rev on SLIIB, none of the various proteins tested were able to effectively inhibit Tat function via the wild-type TAR element (Table 1). It is therefore apparent that the C22S, C37S, and K41A proteins, despite an apparently intact TAR binding motif, are nevertheless incapable of competing with Tat for binding to TAR. Of interest, the ΔRK mutant also exhibited a significantly reduced ability to squelch *trans* activation of the wild-type HIV-1 LTR by Tat compared with its ability to inhibit Tat-Rev function via the SLIIB RNA target. While this may suggest that the interaction of Tat with its cellular cofactor is stabilized by binding to the viral TAR element, the data presented in Table 1 also clearly demonstrate that the interaction of Tat with a limiting cellular cofactor can readily occur in the absence of binding to TAR. In addition, these observations argue that mutant Tat proteins that are unable to bind this cellular cofactor are also unable to effectively compete for binding to TAR regardless of whether they retain a fully intact basic domain. The simplest interpretation of these data is that the interaction of Tat with a cellular cofactor precedes, and is a prerequisite for, binding to TAR in vivo. A model for the in vivo Tat-TAR interaction that incorporates this prediction is presented in Fig. 5.

Tat species specificity results from inefficient binding to TAR. Several groups have demonstrated that Tat is only minimally active in murine cells and have further shown that this low activity can be complemented in hybrid mouse cells containing human chromosome 12 (2, 18, 36). Indeed, these data provide one of the strongest arguments for the importance of a human cellular cofactor in the mechanism of action of HIV-1 Tat. If the model for the temporal assembly of the Tat activation complex proposed in Fig. 5 is accurate, we can then hypothesize either that mouse cells do not

TABLE 2. Species specificity of *trans* activation by lentivirus Tat proteins

Reporter plasmid	Effector plasmid	Fold *trans* activation[a]		
		HeLa	L	QC1-3
Part A				
pTAR/CAT	pcTat	257	9	7
	pcTat/Rev	117	2	1
	peTat	1	1	1
pSLIIB/CAT	pcTat	3	2	3
	pcTat/Rev	110	35	146
Part B				
pEIAV/CAT	peTat	11	15	6
	pcRev/eTat	3	7	8
	pcTat	2	1	2
pSLIIB/CAT	peTat	1	1	1
	pcRev/eTat	103	21	19

[a] The data compare the relative abilities of the listed effector plasmids to *trans* activate CAT expression directed by the three indicated reporter constructs after transfection into cells of human (HeLa), mouse (L), or quail (QC1-3) origin and represent the averages of three transfection experiments.

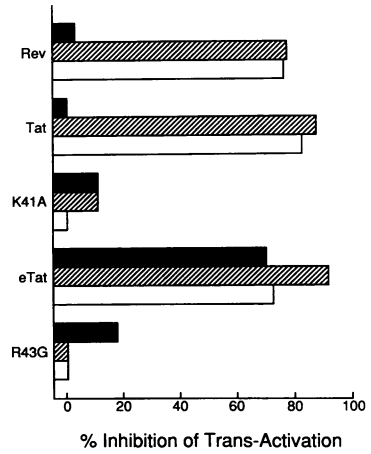

FIG. 6. Competition between the Tat proteins of HIV-1 and EIAV for the same cellular cofactor. The abilities of an excess of the indicated proteins to block *trans* activation of the pTAR/CAT indicator construct by Tat (solid bars) or of the pSLIIB/CAT indicator construct by HIV-1 Tat-Rev (hatched bars) or Rev-eTat (open bars) are compared. These data are representative of three separate transfection experiments in HeLa cells.

provide a cellular cofactor that is capable of interacting with Tat or that such a complex indeed forms but is then unable to interact with TAR. If the former is correct, then it is predicted that Tat-Rev function via SLIIB should also be inefficient in mouse cells. Conversely, if TAR binding is the rate-limiting step, then Tat-Rev should be fully active. It should be noted, however, that these two possibilities are not mutually exclusive.

To examine this issue, we introduced appropriate HIV-1-based indicator and effector plasmids into human HeLa cells, into mouse L cells, and into the quail cell line QC1-3. As shown in Table 2, part A, these data reproduce the previous observation that neither Tat nor Tat-Rev can effectively *trans* activate the wild-type HIV-1 LTR in cells derived from these other species. In contrast, Tat-Rev was observed to be a very effective *trans* activator of the HIV-1 LTR via the introduced SLIIB RNA target in both murine and avian cells. We therefore conclude that it is the interaction of Tat with TAR, rather than the recruitment by Tat of an appropriate cellular cofactor, that is inefficient in these nonhuman cell lines.

Although RNA sequence-specific *trans* activation of transcription was first described for HIV-1, comparable regulatory proteins have subsequently been described in other primate lentiviruses and in the ungulate lentiviruses EIAV and bovine immunodeficiency virus (8, 13, 27). The eTat protein has been extensively studied by Derse and coworkers and has a number of interesting properties. EIAV TAR, like HIV-1 TAR, has been shown to form an RNA stem-loop structure (10). However, EIAV TAR lacks both the bulge and the loop sequences that are known to be critical for HIV-1 TAR function (Fig. 2). Further, while eTat contains sequences that are similar to the HIV-1 Tat basic and core motifs, it lacks any equivalent of the equally critical cysteine motif (Fig. 1) (8). Finally, although eTat functions effectively in cells of equine or canine origin, it is only minimally active in human cells and cannot functionally interact with the HIV-1 TAR element (Table 2, part A) (8).

To examine whether the mechanistic basis for the species tropism of the eTat protein was similar to that seen with HIV-1 Tat, we derived effector plasmids that encoded either an eTat cDNA (peTat) or a fusion protein of Rev and eTat (pcRev/eTat). In addition, we constructed an indicator plasmid, termed pEIAV/CAT, that placed the *cat* gene under the control of EIAV LTR sequences previously shown to be fully responsive to eTat (10). Analysis of these constructs in transfected HeLa cells confirmed the modest activity of eTat in human cells (Table 2, part B). However, the Rev-eTat fusion protein proved as effective as the HIV-1 Tat-Rev fusion protein in activating the HIV-1 LTR via the SLIIB RNA target. Enhanced activity on the SLIIB RNA target was also observed with the Rev-eTat fusion protein in both mouse and quail cells. We therefore conclude that the species tropism of eTat again primarily reflects species-specific differences in the efficiency of the interaction between eTat and EIAV TAR rather than differences in eTat activation potential.

HIV-1 Tat and eTat interact with the same cellular cofactor. The markedly different RNA sequence specificities and primary sequences of the Tat proteins of HIV-1 and EIAV, when combined with their dissimilar species tropism, could be viewed as inconsistent with the hypothesis that these distantly related lentivirus regulatory proteins interact with the same cellular cofactor. However, the observation that these proteins can each *trans* activate the HIV-1 LTR by >100-fold when targeted to the introduced SLIIB target by fusion to Rev (Table 2) could also indicate similar mechanisms of action.

To address whether HIV-1 Tat and eTat indeed interact with the same cofactor in human cells, we examined whether Tat and eTat could inhibit the activity of Tat on HIV-1 TAR, Tat/Rev on SLIIB, or Rev/eTat on SLIIB (Fig. 6) in transfected HeLa cells. Specificity controls were provided by the effector domain-minus K41A mutant of Tat and the similar R43G mutant of eTat. As shown in Fig. 6, both Tat and eTat, but not the K41A or R43G derivative, were able to very effectively inhibit the activity of either Tat-Rev and Rev-eTat on the SLIIB target. Remarkably, eTat, which is not active on the HIV-1 TAR element (Table 2, part A), also proved able to inhibit HIV-1 Tat function via the wild-type HIV-1 TAR element by ~70% under these assay conditions. These data therefore support the hypothesis that HIV-1 Tat

and eTat interact with, and compete for, the same cellular cofactor in transfected HeLa cells.

DISCUSSION

The experiments reported in this article were designed to address the importance of cellular cofactor(s) in the mechanism of action of Tat and to shed light on how the molecular interaction between Tat, TAR, and this cofactor(s) can lead to *trans* activation of the HIV-1 LTR. As the identity of the cellular cofactor(s) for Tat remains unknown, we have been forced to use an entirely genetic approach to these questions. Although the full verification of the experimental conclusions derived from this work must therefore await the future biochemical dissection of the mechanism of action of Tat, these data nevertheless provide clear evidence in favor of the model for Tat function presented in Fig. 5. In particular, these data support the following hypotheses.

(i) The interaction of Tat with an essential cellular cofactor is independent of TAR binding. This hypothesis is supported by two observations. First, the Tat-Rev fusion protein is fully functional when targeted to the heterologous SLIIB RNA target sequence, and this activity is not affected by mutation of the Tat basic domain (Fig. 4). Second, both Tat and a Tat mutant (ΔRK) lacking a functional basic domain are able to effectively squelch the activity of Tat-Rev via the SLIIB target sequence (Table 1). In contrast, the activity of Tat-Rev on the SLIIB RNA target and the ability of Tat to inhibit this activity are both dependent on the integrity of the Tat cysteine and core motifs and, to a lesser extent, the N-terminal domain. These observations confirm and extend previously published results obtained by using Tat-MS2 coat protein fusions and an HIV-1 LTR construct containing the MS2 operator RNA in place of TAR (9, 42) or by using a GAL4-Tat fusion protein targeted to a GAL4 DNA binding site introduced into the HIV-1 LTR U3 region (44). Overall, these data demonstrate that the arginine-rich RNA binding domain of Tat plays at most a minor role in mediating the interaction of Tat with a cellular cofactor(s) and further suggest that the Tat cofactor binding domain is coincident with the first ~48 amino acids of Tat, including, in particular, the Tat core and cysteine motifs (8, 13, 44).

(ii) The interaction of Tat with a cellular cofactor is a prerequisite for TAR binding in vivo. Several groups have demonstrated that the Tat basic domain is not only necessary but also fully sufficient for TAR binding in vitro (6, 40, 49). In particular, neither the Tat core motif nor the Tat cysteine motif appears to play any detectable role in mediating this interaction in vitro (22). Tat mutants lacking a functional cysteine or core motif, such as C22S, C37S, and K41A, should therefore compete effectively with wild-type Tat for TAR binding in vivo. However, none of these three inactive mutants proved capable of exerting any inhibitory effect on Tat in transfected cells (Table 1). In contrast, Rev, which could be viewed as a Tat-Rev derivative lacking a functional effector domain, was able to markedly inhibit Tat-Rev function via SLIIB by competing for SLIIB RNA binding. It should be emphasized that the C22S, C37S, and K41A Tat proteins are all fully stable in vivo and also display an apparently wild-type subcellular localization (Fig. 3) (25, 41, 47). The simplest interpretation of this result is therefore that the cofactor binding domain of Tat plays a critical role in mediating TAR binding in vivo.

(iii) Tat may directly interact with only a single cellular cofactor. As shown in Fig. 1, Tat appears to contain at least three motifs that are essential for in vivo function. The basic domain's sole purpose lies in mediating the interaction of Tat with TAR, while both the cysteine and core motifs are required for Tat effector function. The question then becomes whether these motifs bind one or more cellular cofactors. Clearly, genetic experiments such as those described in this report cannot distinguish between binding of a single polypeptide, binding of a preformed multiprotein complex, or the highly cooperative binding of two or more cofactors to Tat. However, these data do not appear consistent with the hypothesis that Tat binds two or more cellular cofactors independently. For example, if the core and cysteine motifs each bound a distinct cellular cofactor that was independently required for activation, then one would predict that mutation of one motif would leave the other free to bind, and hence sequester, the other cellular cofactor. However, none of these point mutants proved able to exert such a squelching phenotype (Table 1). Further, if a distinct sequence located within either the Tat core or cysteine motif was involved in effector function while a second was involved in recruiting a cofactor required for TAR binding, we would then predict that mutation of the former would give a protein that could compete for TAR binding, while mutation of the latter would give a protein that was active when fused to Rev, i.e., the phenotype seen with ΔRK. However, all core and cysteine motif mutants proved inactive in the Rev fusion context. The simplest hypothesis to explain these observations is therefore that the N-terminal ~48 amino acids of Tat, including, in particular, the cysteine and core motifs, likely bind a single cellular cofactor.

(iv) Tat does not contain a transcription activation domain per se. Several classes of transcription activation domains have been defined. Of these, the most intensively studied is clearly the acidic activation motif first defined in the yeast protein GAL4 and seen in a particularly active form in the herpes simplex virus *trans* activator VP16 (32, 37). Rappaport et al. (39) have proposed that the Tat protein contains a comparable acidic activation motif toward the protein N terminus. However, the observation that point mutations within the Tat core and cysteine motifs block *trans* activation by the Tat-Rev fusion protein demonstrate that the Tat N terminus does not contain an autonomous activation motif. In fact, the observation that the same mutations that block Tat *trans* activation in the Tat-Rev context also block squelching by Tat strongly suggests that Tat does not contain a transcription activation motif per se but instead recruits a cellular cofactor that contains sequences able to fulfill this function; i.e., it is the cofactor that is recruited to TAR by Tat that is either directly or indirectly responsible for *trans* activation of HIV-1 LTR-dependent gene expression.

(v) **The species tropism of Tat reflects differences in the efficiency of the Tat-TAR interaction in vivo.** The Tat protein of HIV-1 is highly active in human cells but displays modest activity in cells of rodent or avian origin. Similarly, the distantly related eTat protein is active in equine and canine cells but only minimally active in human cells (8). In the case of HIV-1 Tat, it has been demonstrated that a cellular factor encoded on human chromosome 12 markedly enhances *trans* activation of the HIV-1 LTR in rodent cells (18, 36), thus leading to the suggestion that an equivalent cofactor might be lacking in cells of murine origin. Here, we demonstrate that the species tropism of HIV-1 and eTat can be essentially overcome if Tat is targeted to the heterologous SLIIB RNA target site by fusion to Rev (Table 1). In particular, *trans* activation of the HIV-1 LTR by Tat-Rev was ~18-fold more efficient on the SLIIB RNA target in mouse cells and ~100-fold more efficient in avian cells. Similarly, the Rev-

eTat fusion proved far more active on the SLIIB RNA target than on the homologous EIAV TAR element when tested in human cells. It is therefore apparent that species tropism does not result from the inability of HIV-1 or eTat to interact with the appropriate cellular cofactor. It is, rather, the efficiency of the subsequent interaction of the Tat-cofactor complex with TAR that primarily determines the level of *trans* activation in cells of the species in question. We note that comparable data demonstrating efficient *trans* activation of the HIV-1 LTR in mouse cells by a Tat-MS2 coat protein fusion have recently been presented by Alonso et al. (2).

(vi) Lentivirus Tat proteins interact with the same cellular cofactor. Among lentivirus Tat proteins, that of EIAV is the most dissimilar to that of HIV-1. The eTat protein is unique among Tat proteins in lacking a cysteine motif and is also unusual in that it shows no ability to *trans* activate the HIV-1 LTR (8, 13). Remarkably, however, the eTat protein proved as effective as HIV-1 Tat in *trans* activating the HIV-1 LTR when both were targeted to the SLIIB RNA sequence by fusion to Rev (Table 1). The equivalent, ~100-fold *trans* activation seen with these two distinct Tat fusion proteins suggested that they might be recruiting the same transcription factor to the SLIIB RNA target. To address this issue, we examined whether these proteins could inhibit SLIIB RNA-dependent activation of the HIV-1 LTR by the Tat-Rev or Rev-eTat fusion protein. Remarkably, both Tat and eTat proved able to efficiently inhibit *trans* activation by both fusion proteins. Indeed, eTat proved able to exert a significant inhibitory effect on wild-type Tat function via the HIV-1 TAR element. This inhibition was entirely dependent on the integrity of the conserved effector domain core motif of these two Tat proteins. These data therefore indicate that HIV-1 Tat and eTat compete with each other for the same limiting cellular cofactor in transfected human cells. Prior to completion of this work, Carroll et al. (9) also reported that EIAV Tat could inhibit the activity of a HIV-1 Tat-MS2 coat protein hybrid, but not of HIV-1 Tat itself, in transformed HeLa cells. Although these recent data therefore differ at least in part from the results presented in this report, Carroll et al. (9) nevertheless also proposed that HIV-1 Tat and eTat interact with the same cellular cofactor prior to binding their homologous TAR element (Fig. 5).

(vii) The cellular cofactor modulates the RNA sequence specificity of the Tat-cofactor complex. The TAR elements of the lentiviruses HIV-1 and EIAV are quite distinct (Fig. 2). In particular, the EIAV TAR element clearly lacks any equivalent of the pyrimidine-rich bulge and conserved hexanucleotide loop that are critical for HIV-1 TAR function in vivo (10). It is, therefore, not surprising that HIV-1 Tat is inactive on the EIAV TAR element and vice versa (Table 2). However, the evidence presented above clearly indicates that the cellular cofactors recruited by Tat and eTat are the same, at least in human cells. We have argued that Tat interacts with a single cofactor and that this cofactor affects the efficiency of the Tat-TAR interaction. This view implies that the same cellular cofactor must be involved in mediating the interaction of both HIV-1 Tat and eTat with their respective, highly divergent, TAR elements. It is, therefore, not surprising that these interactions are not equivalently efficient in cells from different species (Table 2). The human cofactor is, according to this hypothesis, highly effective at mediating the Tat-TAR interaction of the human virus HIV-1 but is far less competent to mediate the Tat-TAR interaction of the equine virus EIAV. The high activity of eTat in equine and canine cells (8, 13) implies that these species express an analogous protein that can effectively mediate this latter binding event. The equivalent protein expressed by mouse and quail cells, in contrast, is not proficient at mediating either of these Tat-TAR interactions but could, presumably, be effective on yet a third RNA sequence.

Of interest, the observation that the HIV-1 and EIAV Tat proteins can work effectively in a range of species, including nonmammalian cells, when targeted to a heterologous RNA binding domain (Table 2) indicates that the Tat cofactor has been conserved since the evolutionary divergence of mammals and birds and has also retained the structural features that allow it to interact with Tat. However, it also appears that the RNA sequence specificity of this cofactor has evolved significantly over time, thus generating the species tropisms that are now characteristic of the various lentivirus Tat proteins.

A model for the temporal assembly of a ternary complex on TAR. In this report, we have presented a genetic analysis of the role of the cellular cofactor in the mechanism of action of Tat. These data, in combination with recent observations from other groups (2, 9, 42, 46), have suggested a specific model for the genesis of the Tat transcription activation complex (Fig. 5). The primary, and most novel, feature of this model is that neither Tat nor the cellular Tat cofactor binds TAR alone in vivo. Instead, we propose that these proteins must first form a protein complex. This complex then binds to TAR with a sequence specificity that is determined both by Tat and by the cellular cofactor (Fig. 5). This hypothesis is therefore inconsistent with the contention, based entirely on in vitro analysis, that Tat alone can bind to TAR in vivo.

Although the binding of TAR by the Tat basic motif in vitro occurs with high affinity, it displays a relatively low level of specificity. Thus, Tat proteins in which the basic motif has been substituted by other natural or artificial stretches of basic amino acids have been reported to retain full in vivo activity (6, 45), while the ability of the basic domain of Tat to selectively bind TAR appears to be specified by a single arginine residue within the basic motif (7). Indeed, the amino acid arginine itself seems to retain much of the same RNA specificity (38). It has therefore seemed improbable that the sequence information present in the basic motif of Tat could alone be sufficient to direct the specific binding of Tat to TAR under in vivo conditions. The hypothesis that a cellular cofactor might be involved in mediating the Tat-TAR interaction was first suggested by the observation that the ability of a TAR RNA decoy to sequester the Tat protein in vivo, and hence inhibit HIV-1 LTR *trans* activation, was critically dependent on the integrity of terminal loop RNA sequences that have no effect on Tat binding in vitro (46). As noted above, the data presented in this report strongly support the proposal that the interaction of Tat with a cellular cofactor is, in fact, an essential prerequisite for TAR binding in vivo.

The TAR element cannot bind an essential cellular cofactor distinct from the one that binds to Tat, because it would then be impossible to functionally substitute a heterologous RNA target sequence for TAR. Given, then, that the cellular cofactors that bind Tat and TAR are one and the same, what is the evidence that the cofactor does not bind TAR alone? The most compelling evidence for this hypothesis derives from the observation that the interaction of TAR with a cellular cofactor is necessary but not sufficient to direct Tat to TAR. The evidence for necessity includes the observation that TAR sequences that are not directly involved in binding to Tat are nevertheless required for TAR function in vivo (14, 40) as well as the finding that the species origin of the Tat

cofactor markedly affects the level of *trans* activation obtained with a particular TAR element (Table 2). The evidence that the TAR-cofactor interaction is not sufficient to recruit Tat to TAR derives from the finding that the Tat basic domain is critical for TAR binding, but not effector function, in vivo (Fig. 4) (42, 44) and that complexes of the same cellular Tat cofactor with either HIV-1 Tat or eTat display entirely different TAR RNA sequence specificities in vivo (Table 2). Indeed, the data presented in this report are fully consistent with the hypothesis, first suggested by Carroll et al. (9), that the entire purpose of Tat is simply to recruit the apparently ubiquitous cellular transcription factor referred to here as the Tat cofactor to a novel, viral RNA target sequence and to thereby activate viral LTR-specific gene expression. Tat could therefore be viewed more as a *trans* specifier of a preexisting cellular transcription factor than as a transcriptional *trans* activator in its own right.

An important prediction of the model presented in Fig. 5 is that the cellular Tat cofactor is unlikely to bind TAR with significant affinity in the absence of Tat. Efforts to define the Tat cofactor on the basis of its ability to bind TAR in vitro may therefore be doomed to failure. Instead, our data would suggest that it is the affinity of the cofactor for Tat itself that should provide the best biochemical or genetic tool for the identification of this interesting cellular regulatory protein.

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