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# Recruitment of cyclin T1/P-TEFb to an HIV type 1 long terminal repeat promoter proximal RNA target is both necessary and sufficient for full activation of transcription

PAUL D. BIENIASZ, THERESE A. GRDINA, HAL P. BOGERD, AND BRYAN R. CULLEN*

Howard Hughes Medical Institute and Department of Genetics, Box 3025, Duke University Medical Center, Durham, NC 27710

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**ABSTRACT** **Transcriptional activation of the HIV type 1 (HIV-1) long terminal repeat (LTR) promoter element by the viral Tat protein is an essential step in the HIV-1 life cycle. Tat function is mediated by the TAR RNA target element encoded within the LTR and is known to require the recruitment of a complex consisting of Tat and the cyclin T1 (CycT1) component of positive transcription elongation factor b (P-TEFb) to TAR. Here, we demonstrate that both TAR and Tat become entirely dispensable for activation of the HIV-1 LTR promoter when CycT1/P-TEFb is artificially recruited to a heterologous promoter proximal RNA target. The level of activation observed was indistinguishable from the level induced by Tat and was neither inhibited nor increased when Tat was expressed in trans. Activation by artificially recruited CycT1 depended on the ability to bind the CDK9 component of P-TEFb. In contrast, although binding to both Tat and TAR was essential for the ability of CycT1 to act as a Tat cofactor, these interactions became dispensable when CycT1 was directly recruited to the LTR. Importantly, activation of the LTR both by Tat and by directly recruited CycT1 was found to be at the level of transcription elongation. Together, these data demonstrate that recruitment of CycT1/P-TEFb to the HIV-1 LTR is fully sufficient to activate this promoter element and imply that the sole role of the Tat/TAR axis in viral transcription is to permit the recruitment of CycT1/P-TEFb.**

The Tat protein encoded by HIV type 1 (HIV-1) is a potent activator of the viral long terminal repeat (LTR) promoter and is required for virus replication (reviewed by refs. 1, 2). Properties that distinguish Tat from other transcription factors include that it acts predominantly at the level of transcription elongation (3–6), rather than initiation, and that the target sequence for Tat is an RNA stem–loop structure, termed TAR, located immediately 3′ to the LTR transcription start site. The human cyclin T1 (hCycT1) component of positive transcription elongation factor b (P-TEFb) rescues Tat function in otherwise nonpermissive murine cells and binds to TAR cooperatively with Tat (7–10). Recruitment of hCycT1/P-TEFb to TAR may enhance transcription elongation by allowing the CDK9 component of P-TEFb to phosphorylate the carboxyl-terminal domain (CTD) of initiated RNA polymerase II (PolII) complexes (11–18), a modification thought to be required for efficient elongation by PolII (19). Although hCycT1/P-TEFb is therefore clearly an essential Tat cofactor, it has remained uncertain whether hCycT1/P-TEFb recruitment to the LTR is the only activity of Tat or, instead, one of two or more critical functions.

In addition to hCycT1, a number of other potential Tat interacting proteins and/or cofactors have been proposed including MSS1, HT2A, CA150, TFIID, Tat-SF1, TIP30, and PolII itself (20–26), and other groups have reported interactions between Tat and the coactivator proteins p300 and CREB-binding protein (CBP) (27–29) or between Tat and the transcription factor TFIIH (6, 18, 30, 31). Recruitment of p300 or CBP, two similar proteins that possess histone acetyltransferase activity, could enhance HIV-1 LTR transcription by increasing the level of acetylation of histone molecules bound to this promoter element. In other systems, histone hyperacetylation has been shown to be characteristic of transcriptionally active chromatin (32). The reported interaction of Tat with TFIIH (6, 18, 30, 31), which contains a CTD kinase activity, has led to the proposal that Tat might activate the HIV-1 LTR by the sequential TAR-independent recruitment of TFIIH to LTR-bound PolII molecules, followed by the subsequent TAR-dependent recruitment of hCycT1/P-TEFb (2, 33).

Here, we demonstrate that recruitment of hCycT1/P-TEFb to the HIV-1 LTR, by using a heterologous RNA-binding domain, is in fact fully sufficient to induce efficient transcription elongation. Coexpression of Tat neither enhanced nor inhibited this effect, thus arguing against a second TAR-independent Tat function. Although the ability to bind to Tat, to TAR, and to the essential P-TEFb component CDK9 was found to be critical for hCycT1 to support transcriptional activation via TAR, only CDK9 binding was required for activation when hCycT1 was recruited to a heterologous RNA target. Together, these data indicate that the sole role of Tat during transcriptional activation of the HIV-1 LTR is to promote appropriate recruitment of hCycT1/P-TEFb to this viral promoter element.

## MATERIALS AND METHODS

**Plasmid Construction.** The mammalian reporter and effector plasmids pHIV/TAR/CAT, pHIV/SLIIB/CAT, pBC12/CMV/lacZ, pcTat, and pTat-Rev have been described (34), as have the cyclin T1 (CycT1) expression plasmids pBC12/CMV/hCycT1 and pBC12/CMV/mCycT1 (8). pBC12/CMV-based plasmids encoding cyclin/Rev fusion proteins were generated from pTat-Rev by substitution of the relevant cyclin in place of Tat such that the cyclin was separated from the Rev sequence by a three-glycine spacer.

**Mutagenesis of hCycT1.** Missense mutations in the hCycT1 cDNA were constructed by recombinant PCR. In mutant hCycT1(X5), residues Cys 261 and Glu 262 are both replaced by alanine. To generate hCycT1 mutants that are defective for CDK9 binding, codons for residues Lys-93 and Glu-96, which by analogy with other cyclins are predicted to contact CDK9

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Abbreviations: HIV-1, HIV type 1; CTD, carboxyl-terminal domain; CycT1, cyclin T1; hCycT1, human cyclin T1; LTR, long terminal repeat; PolII, RNA polymerase type II; P-TEFb, positive transcription elongation factor b; PV UTR, poliovirus untranslated region; SLIIB, stem–loop IIB; Dist, distal RNA.

*To whom reprint requests should be addressed. e-mail: culle002@mc.duke.edu.

(35), were randomized and the resultant mutants screened by using the yeast two-hybrid assay for hCycT1 variants that could interact with HIV-1 Tat and TAR but not with CDK9. One such mutant, in which Lys-93 and Glu-96 are replaced with Leu and Lys, respectively, was designated hCycT1(K26) and used in subsequent experiments. Mutant hCycT1 proteins were transferred to pBC12/CMV for expression of unfused proteins and into pTat-Rev for expression as Rev fusions in mammalian cells.

**Mammalian Cell Transfection Assays.** Human 293T cells and murine LmTK cells were transfected by using calcium phosphate coprecipitation and DEAE dextran, respectively. For experiments that determined the ability of CycT1, CycH, or Tat, expressed either as wild-type proteins or fused to Rev, to activate the HIV-1 LTR, 200 ng of pHIV/TAR/CAT or pHIV/SLIIB/CAT was cotransfected with 500 ng of the activator expression plasmid. For experiments that measured the effect of Tat on reporter gene expression in the presence of CycT1, CycT1 mutants, or CycT1-Rev, 500 ng of the CycT1 expression plasmid and 25 ng (human cells) or 100 ng (murine cells) of pcTat or a control plasmid were used. At 48 hr after transfection, cells were harvested and CAT activities determined (8). Alternatively, total RNA was extracted by the guanidium isothiocyanate method and analyzed by ribonuclease protection. For Western blot analysis, 293T cells (35 mm culture) were transfected with 2 $\mu$g of the relevant Rev fusion protein expression plasmid. Cell lysates were prepared at $\approx$48 hr after transfection and analyzed as previously described (8) by using a rabbit polyclonal anti-Rev antiserum (34).

**Ribonuclease Protection Assay.** The promoter proximal poliovirus untranslated region (PV UTR)-derived probe was synthesized as previously described (36). Sequences from the 3′ end of the *cat* gene (132 nucleotides) were amplified by PCR and cloned into pCR2.1 (Invitrogen). The distal RNA (Dist) probe was synthesized by linearizing this construct with *Hin*dIII and transcription with T7 polymerase.

Total RNA (20 $\mu$g) extracted from transfected 293T cells was hybridized with $2 \times 10^5$ cpm of each probe, digested with RNase T1 (PV UTR probe) or an RNase T1/RNase A mixture (Dist probe) and precipitated by using the HybSpeed RPA kit (Ambion, Austin, TX). Protected fragments were visualized by autoradiography after gel electrophoresis.

**Protein/Protein and Protein/RNA Interaction Assays in Yeast.** Two-hybrid protein/protein interaction assays and three-hybrid protein/RNA interaction assays were performed as described previously (8, 37). Briefly, to measure the ability of hCycT1 mutants to bind CDK9 or Tat, yeast Y190 cells were transformed with plasmids expressing GAL4-CDK9 or GAL4-Tat fusion proteins together with a VP16-hCycT1 expression plasmid. To determine the ability of Tat/CycT1 complexes to bind TAR, yeast L40-coat cells were transformed with plasmids expressing a hybrid MS2/TAR RNA, wild-type HIV-1 Tat, and a VP16-hCycT1 fusion protein.

## RESULTS

Activation of the HIV-1 LTR promoter by Tat normally depends on the recruitment of Tat and of the hCycT1/P-TEFb cofactor to the promoter proximal TAR RNA target. However, efficient activation of an HIV-1 LTR in which the TAR element has been replaced by a heterologous RNA target sequence can instead be mediated by a fusion protein consisting of Tat linked to the relevant RNA-binding protein (34, 38–40). For example, a fusion protein consisting of Tat fused to HIV-1 Rev can efficiently activate an HIV-1 LTR in which the TAR element has been replaced with the stem–loop IIB (SLIIB) RNA-binding site for Rev. This result is reproduced in Fig. 1 *a* and *b*, which show that a Tat-Rev fusion protein effectively and equivalently activates both the wild-type HIV-1 LTR present in the pHIV/TAR/CAT indicator plasmid and the SLIIB$^+$ TAR-HIV-1 LTR present in pHIV/SLIIB/CAT, in transfected 293T cells. In contrast, although Tat can activate the wild-type HIV-1 LTR, it fails to act on the LTR bearing SLIIB in place of TAR.

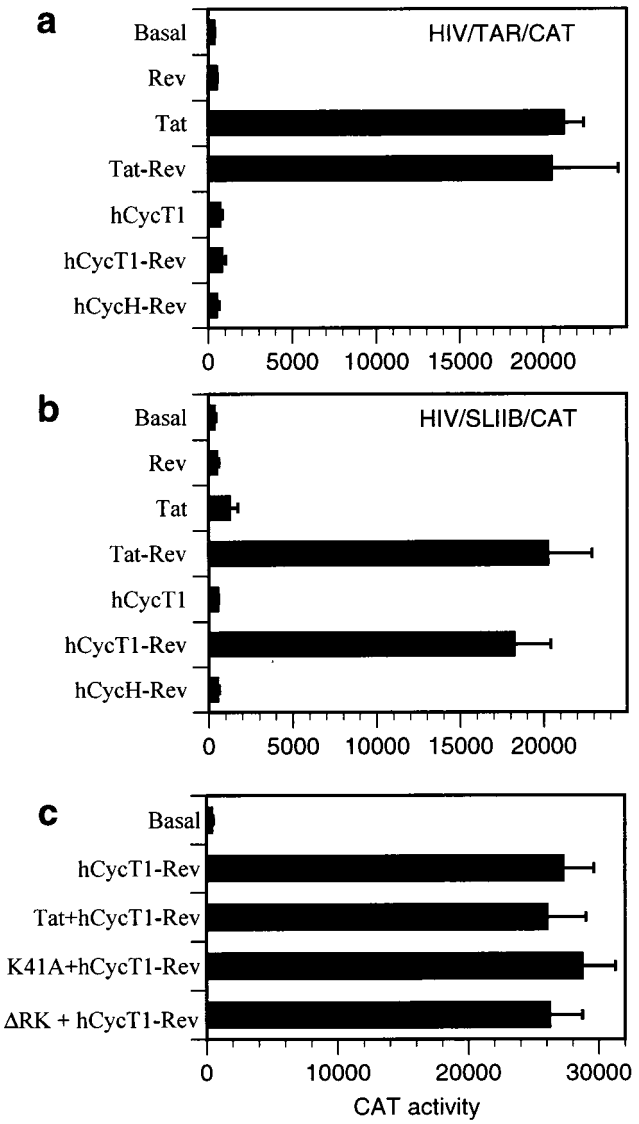


FIG. 1. Recruitment of CycT1/P-TEFb activates the HIV-1 LTR promoter independently of Tat and TAR. 293T cells were transfected with HIV-1 LTR-based reporter plasmids containing the TAR (*a*) or SLIIB (*b*) RNA target. Transactivation by the indicated effectors, expressed either as wild-type unfused proteins or fused to Rev, was determined by measuring CAT levels 48 hr after transfection. In *c*, cells were transfected with the pHIV/SLIIB/CAT reporter plasmid, along with plasmids expressing hCycT1-Rev and the indicated wild-type or mutant Tat expression plasmid. In each case, results represent the mean ± standard deviation of three transfections.

**Efficient Activation of the HIV-1 LTR in the Absence of Tat or TAR.** The activity of the Tat-Rev fusion protein on the pHIV/SLIIB/CAT indicator construct presumably reflects the recruitment of a functional Tat cofactor complex to SLIIB. Importantly, Tat is known to efficiently bind to hCycT1/P-TEFb in the absence of TAR (7–10), and mutations in Tat that block hCycT1 binding also block Tat-Rev function via both TAR and SLIIB (8, 39). As Rev can apparently effectively recruit not only Tat but also all other factors required for transactivation to the SLIIB RNA target, we next asked whether fusion of the hCycT1 component of P-TEFb to Rev would also be sufficient to activate the HIV-1 LTR promoter via SLIIB. As shown in Fig. 1*b*, the hCycT1-Rev fusion protein

indeed proved to be as active as Tat-Rev when tested on the pHIV/SLIIB/CAT indicator plasmid. In contrast, and as expected, the hCycT1-Rev fusion differed from Tat-Rev in being unable to activate via the TAR element (Fig. 1*a*). A similar fusion protein consisting of human cyclin H (hCycH), the cyclin component of TFIIH, fused to Rev failed to activate either indicator construct (Fig. 1 *a* and *b*) even though hCycT1-Rev and hCycH-Rev were expressed at comparable levels, as shown both by Western analysis and by equivalent levels of Rev function (data not shown). We therefore conclude that the Tat independent recruitment of hCycT1 to the heterologous SLIIB RNA target can activate gene expression driven by the HIV-1 LTR as effectively as the normal Tat dependent recruitment of hCycT1 to TAR.

**Expression of Tat Does Not Affect Activation by the hCycT1-Rev Chimera.** It could be argued that recruitment of the hCycT1-Rev chimera to the HIV-1 LTR reproduces only the TAR-dependent activity of Tat, i.e., reflects only a part of the biological activity of Tat. This hypothesis would predict that coexpression of Tat would also permit any TAR-independent Tat function to occur and thereby would result in a synergistic activation of the HIV-1 LTR promoter. One argument against this proposal is the observation that the level of activation of the pHIV/SLIIB/CAT indicator construct by the hCycT1-Rev chimera is comparable to the level seen with the Tat-Rev fusion on either pHIV/SLIIB/CAT or pHIV/TAR/CAT, which in turn is similar to the activity seen with wild-type Tat on the latter construct (Fig. 1 *a* and *b*). However, to test this hypothesis more rigorously, we coexpressed hCycT1-Rev in 293T cells along with saturating levels of either the wild-type Tat protein or of previously described (8, 34) Tat mutants bearing a defective hCycT1-binding domain (K41A) or a defective RNA-binding motif (ΔRK). As shown in Fig. 1*c*, none of these Tat proteins had any significant effect on the observed level of activation of the pHIV/SLIIB/CAT indicator plasmid by the hCycT1-Rev chimera.

Mouse cells are not permissive for HIV-1 Tat function because, although the murine form of CycT1 (mCycT1) is able to bind to Tat, the resultant Tat/mCycT1 complex is not effectively recruited to TAR (8–10). However, Tat function in murine cells can be rescued by expression of hCycT1. To test whether the hCycT1 component of the hCycT1-Rev chimera retained the ability to interact with Tat and to be recruited to TAR, we coexpressed Tat and hCycT1-Rev in murine cells. As shown in Fig. 2*a*, hCycT1-Rev indeed rescued HIV-1 Tat function, thus showing that the lack of an effect of Tat on hCycT1-Rev function reported in Fig. 1*c* was not because of an inability to bind to hCycT1-Rev. As shown in Fig. 2*b*, the hCycT1-Rev chimera was, in contrast to Tat, fully active in murine cells when tested with the pHIV/SLIIB/CAT indicator plasmid. Again, expression of Tat failed to enhance hCycT1-Rev function significantly (Fig. 2*b*). In fact, activation of the HIV LTR in murine cells by Tat/hCycT1-Rev complexes was equivalent whether they were recruited via a Tat/TAR or a Rev/SLIIB interaction.

**Activation of the HIV-1 LTR by hCycT1-Rev Requires CDK9 Activity.** It has been demonstrated that the ability of hCycT1 to support Tat function depends on a direct interaction between hCycT1 and both Tat and TAR (8–10). It is further predicted, although this has not yet been shown, that this activity would also require binding of CDK9 to hCycT1. If activation of the HIV-1 LTR via direct recruitment of hCycT1/P-TEFb occurs via the same general mechanism, then binding to both Tat and TAR should become irrelevant, whereas CDK9 recruitment should remain critical.

To test this hypothesis, we derived two mutants of hCycT1 that specifically affect either CDK9 binding or Tat binding. As shown in Fig. 3*a*, the K26 mutant of hCycT1 has lost the ability to interact with CDK9 but displays wild-type levels of binding to both Tat (measured by two-hybrid assay) or TAR (measured by three-hybrid assay). In contrast, the X5 mutant of hCycT1 displays only a vestigial level of binding to Tat and does not detectably interact with TAR, yet retains the ability to bind CDK9. We have previously reported that mCycT1 can bind to both CDK9 and HIV-1 Tat effectively *in vivo* but fails to interact with TAR (8).

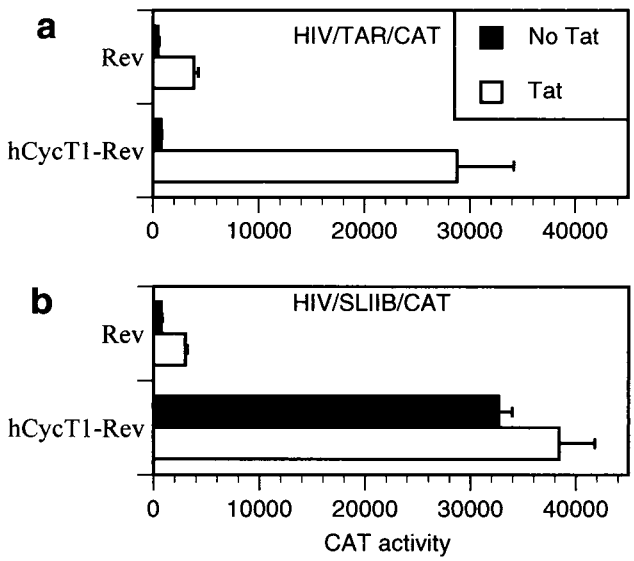


FIG. 2. hCycT1-Rev forms a complex with Tat that activates the HIV-1 LTR equivalently via TAR or SLIIB. Murine LmTK-cells were transfected with HIV-1 LTR-based reporter plasmids containing TAR (*a*) or SLIIB (*b*) RNA target elements, as in Fig. 1, and plasmids expressing Rev or hCycT1-Rev, along with the indicated Tat expression plasmid. The depicted results reflect mean CAT levels determined 48 hr after transfection ± the standard deviation of three transfection experiments.

As shown in Fig. 3*b*, the X5 and K26 mutants of hCycT1, as well as mCycT1, all differ from wild-type hCycT1 in being unable to rescue activation of the wild-type HIV-1 LTR by Tat in murine cells. In contrast, both mCycT1 and the X5 mutant of hCycT1 are fully able to activate the pHIV/SLIIB/CAT indicator plasmid when recruited to the LTR by fusion to Rev (Fig. 3*c*). The K26 mutant of hCycT1 is, however, entirely inactive even though hCycT1(K26)-Rev and hCycT1-Rev are expressed at comparable levels in transfected cells, as measured by Western blot analysis (Fig. 3*d*), and all four CycT1-Rev fusion proteins exhibit readily detectable and comparable levels of Rev activity (data not shown). These data therefore demonstrate that activation of the HIV-1 LTR by hCycT1 requires an hCycT1/CDK9 interaction, regardless of whether recruitment of hCycT1 is mediated by Rev and SLIIB or by Tat and TAR. However, the ability of hCycT1 to bind Tat and TAR is important only in the latter case.

**Activation by hCycT1-Rev Occurs at the Level of Elongation.** Tat is unusual among transcription factors in that it acts primarily, or even exclusively, at the level of transcription elongation (3–6, 36). Thus, in the absence of Tat, transcription from the HIV-1 LTR promoter initiates efficiently but declines rapidly with increasing distance from the cap site. If the hCycT1-Rev fusion protein is in fact acting via the same mechanism as Tat, then a similar increase in processivity should be induced.

To test this hypothesis, we used a previously described assay (36) that relies on the observation that stable RNA secondary structures present in prematurely terminated transcripts are resistant to degradation *in vivo*. Several groups have used measurement of the level of such stable RNA stem–loop structures as a means of determining the level of processive vs. nonprocessive transcription (36, 41–43). The pHIV/SLIIB/CAT indicator construct contains the PV UTR inserted be-

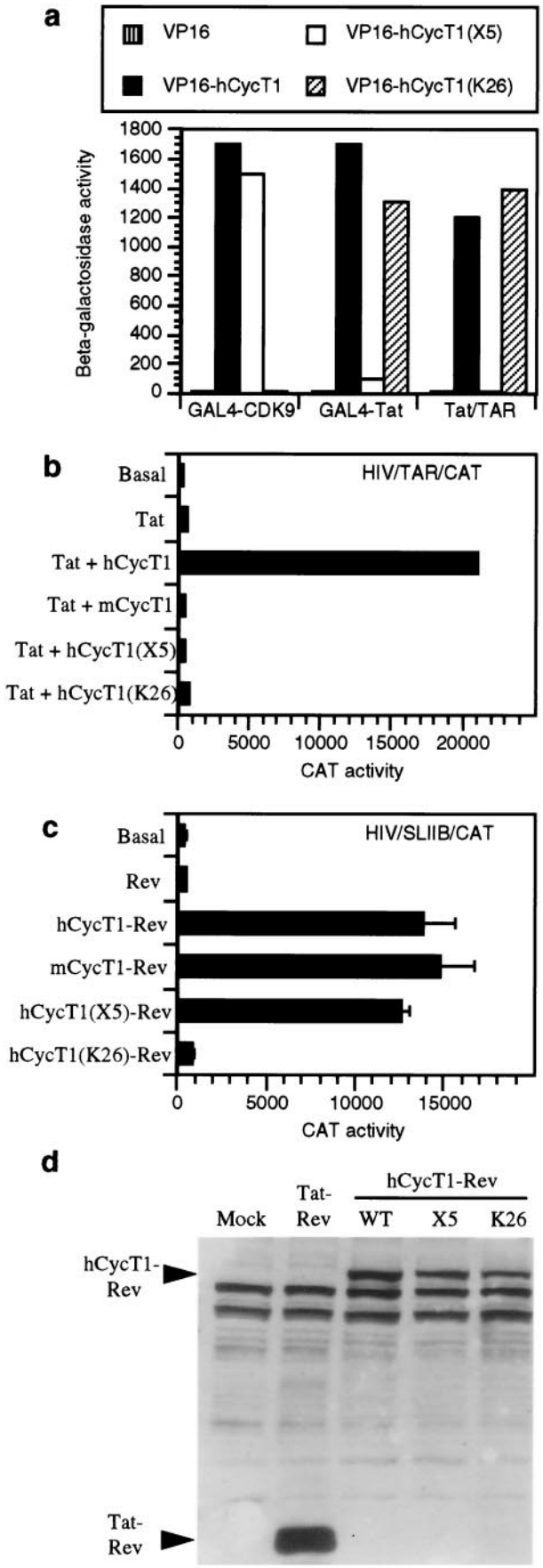


FIG. 3. Tat and TAR binding by hCycT1 is dispensable for activation when P-TEFb is artificially recruited to the HIV-1 LTR. (*a*) Analysis of hCycT1 interactions with CDK9, Tat, and TAR in yeast cells. Interactions between VP16-hCycT1 fusion proteins and GAL4-CDK9 or GAL4-Tat were measured by two-hybrid assay. Alternatively, the ability of hCycT1/Tat complexes to interact with HIV-1 TAR was determined by yeast three-hybrid assay. Values indicate the level of β-galactosidase activity in yeast cells after growth on appropriate selective media. (*b*) The ability of CycT1 to interact with Tat, TAR, and CDK9 is required to support Tat/TAR function. Murine LmTK-cells were transfected with the pHIV/TAR/CAT reporter plasmid along with plasmids expressing Tat and wild-type or mutant hCycT1. (*c*) CDK9 but not Tat/TAR-binding activity is required for hCycT1-Rev/SLIIB-mediated activation of the HIV-1 LTR. 293T cells were transfected with the pHIV/SLIIB/CAT reporter along with plasmids expressing the indicated hCycT1-Rev fusion proteins. Values are the mean ± standard deviation of CAT levels determined 48 hr after transfection from three transfections. (*d*) Relative levels of expression of the indicated Rev fusion proteins in transfected 293T cells were determined by Western blot analysis by using a polyclonal anti-Rev antiserum.

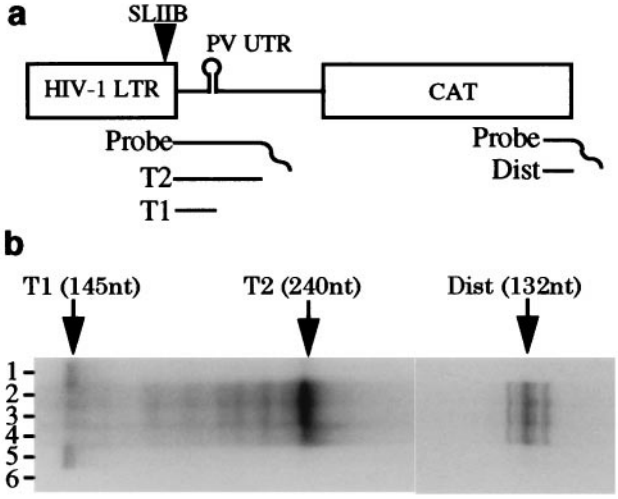


FIG. 4. Recruitment of hCycT1 to the HIV-1 LTR promoter enhances transcriptional processivity. (*a*) Schematic representation of the pHIV/SLIIB/CAT reporter plasmid indicating the positions of the PV UTR and Dist probes used to measure the levels of promoter proximal (T1), intermediate (T2), and distal (Dist) RNA. (*b*) Ribonuclease protection assay performed by using total RNA extracted from 293T cells transfected with pHIV/SLIIB/CAT and a plasmid expressing Rev (lane 1), Tat-Rev (lane 2), hCycT1-Rev (lane 3), hCycT1(X5)-Rev (lane 4), or hCycT1(K26)-Rev (lane 5). A mock protection assay (lane 6) used RNA from untransfected 293T cells.

tween the HIV-1 LTR and the *cat* indicator gene. The PV UTR is highly structured and, in particular, encodes an RNA cloverleaf structure near its 5′ end that was previously shown to resist degradation *in vivo* when present in prematurely terminated transcripts (36).

The strategy used in this experiment is shown in Fig. 4. Two RNA probes were used, a promoter proximal probe that would detect both prematurely terminated transcripts that were degraded, by 3′ exonuclease activity, to the base of the PV UTR-derived cloverleaf structure (fragment T1) as well as intermediate length, mostly nonterminated transcripts (fragment T2). A Dist probe, directed against the 3′ end of the *cat* indicator gene, detects only full-length mRNAs. As shown in Fig. 4, the pHIV/SLIIB/CAT indicator construct normally gives rise to readily detectable levels of the T1 RNA fragment after transfection into 293T cells together with a Rev expression plasmid, which here serves as a negative control, yet no T2 or Dist signal is observed. Coexpression of Tat-Rev, of hCycT1-Rev, or of the hCycT1(X5)-Rev mutant greatly enhances the expression of RNAs able to rescue the T2 or Dist probe fragment but has little effect on the promoter-proximal T1 signal. In contrast, the CycT1(K25)-Rev mutant, which

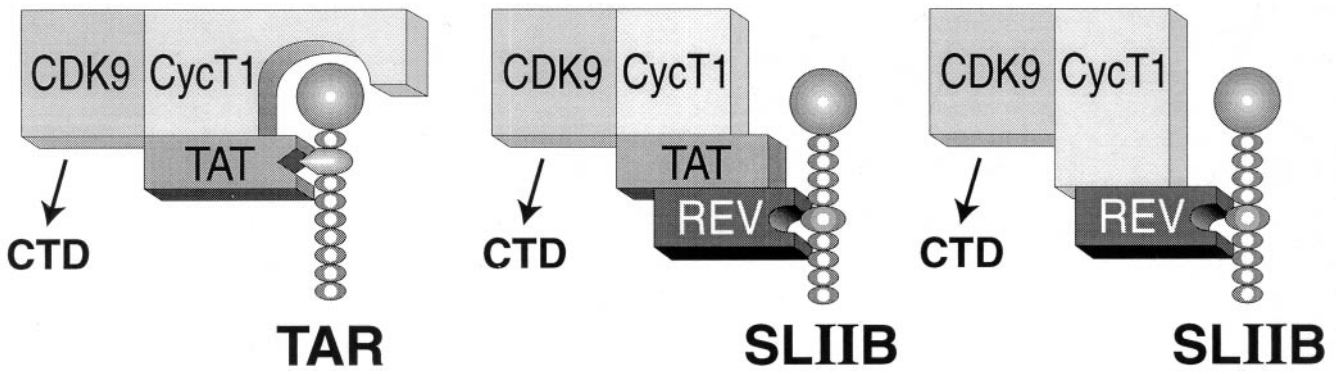


FIG. 5. Schematic representation of three modes of recruitment of CycT1/P-TEFb to the HIV-1 LTR promoter. As shown in this manuscript, each mode produces an equivalent level of activation of this promoter (see text for detailed discussion).

lacks the ability to recruit CDK9, has no effect on the efficiency of transcription elongation and displays a phenotype identical to the negative control. Together, these data demonstrate that the hCycT1-Rev chimera activates HIV-1 LTR-dependent transcription by enhancing transcriptional processivity in a manner that requires CDK9 recruitment and is indistinguishable from that seen with Tat.

## DISCUSSION

Transcriptional transactivation of the wild-type HIV-1 LTR by the viral Tat protein requires the recruitment of a complex consisting of Tat and the hCycT1 component of P-TEFb to the viral TAR element (Fig. 5). Binding of the hCycT1/Tat heterodimer to TAR is highly cooperative (7–10) and involves a direct and specific interaction between Tat and a bulge-loop in TAR and, most probably, a second direct interaction between hCycT1 and the terminal loop of TAR (Fig. 5). hCycT1 is in turn bound by the CDK9 component of P-TEFb (7, 44), and it is believed to be the phosphorylation of the PolII CTD by CDK9 (11–18) that is primarily responsible for the enhanced elongation that results from this RNA sequence-dependent recruitment event (Fig. 5).

As previously shown by several groups, activation of the HIV-1 LTR by Tat can also be mediated by a heterologous RNA target substituted in place of TAR, as long as Tat is expressed as a fusion to the relevant RNA-binding protein (34, 38–40). Thus, an HIV-1 LTR bearing the SLIIB RNA stem–loop structure in place of TAR is effectively activated by a Tat-Rev fusion protein but not by Tat (Fig. 1). Because formation of the Tat/hCycT1 complex appears to be a prerequisite for TAR binding by either protein *in vivo* (7–10), and because mutations of Tat that block the hCycT1/Tat interaction also block activation by Tat-Rev (8, 39), it is likely that activation of an HIV-1 LTR bearing the SLIIB RNA target by the Tat-Rev fusion protein also involves recruitment of the hCycT1/P-TEFb protein complex to the LTR (Fig. 5).

Here, we demonstrate that this heterologous RNA tethering strategy can be taken one step further, i.e., that Tat becomes entirely dispensable if CycT1, and hence presumably P-TEFb, is recruited to the HIV-1 LTR by fusion to a heterologous RNA-binding protein, in this case Rev (Fig. 5). The level of activation of the HIV-1 LTR induced by an hCycT1-Rev fusion protein targeted to SLIIB is closely comparable to the level of activation observed on recruitment of Tat to TAR or of the Tat-Rev fusion protein to SLIIB (Fig. 1). It is therefore apparent that recruitment of hCycT1/P-TEFb to a promoter proximal RNA target is both necessary and sufficient for full transcriptional activation of the HIV-1 LTR and that any of the three recruitment strategies depicted in Fig. 5 is equally effective in achieving this aim. Therefore, the sole purpose of both TAR and Tat, at least as relates to activation of transcription from the HIV-1 LTR, is to mediate the appropriate recruitment of hCycT1/P-TEFb. In other words, Tat is simply a molecular adaptor protein whose role is to confer a new viral RNA target specificity on a preexisting host-cell transcription factor.

Several lines of evidence argue that activation of the HIV-1 LTR by hCycT1-Rev occurs by the same mechanism used by Tat. Thus, the activation of HIV-1 LTR-driven gene expression observed on recruitment of hCycT1-Rev to the SLIIB RNA target resulted from enhanced transcription elongation (Fig. 4) and was indistinguishable from the effect induced by recruitment of Tat-Rev to SLIIB (Fig. 4) or reported previously for Tat (36). Secondly, activation by hCycT1-Rev, like activation by Tat, depended on recruitment of CDK9 to the LTR promoter. Both activities were therefore blocked by the K26 missense mutation in hCycT1 that prevents CDK9 binding (Fig. 3). However, and as expected, the ability of hCycT1 to bind to Tat and to TAR was required only to support Tat function and became irrelevant when hCycT1 was directly recruited to the LTR (Fig. 3).

We also tested whether expression of Tat in *trans* would enhance the activation of the HIV-1 LTR by hCycT1-Rev, as would be predicted if Tat played a second TAR-independent role in mediating this response. In fact, expression of saturating levels of Tat in either human cells (Fig. 1*c*) or murine cells (Fig. 2*b*) did not affect the ability of hCycT1-Rev to activate the HIV-1 LTR promoter, even though the hCycT1 component of the hCycT1-Rev fusion protein retained the ability to interact with both Tat and TAR, as shown by its ability to rescue Tat function in murine cells (Fig. 2*a*). Therefore, binding to Tat neither enhances nor inhibits the ability of hCycT1-Rev to activate the HIV-1 LTR, and it is therefore very unlikely that a Tat-induced conformational change in hCycT1 plays a role in the activation of this viral promoter.

The hCycT1-Rev fusion protein is not the only heterologous fusion protein that has been shown to enhance HIV-1 LTR-driven gene expression when recruited to a promoter proximal RNA target, although this property is clearly rare (34, 45–48). The first fusion protein shown to exert this effect consisted of the VP16 transcription activation domain linked to Rev (34). Although the VP16 activation domain associates with a kinase that can phosphorylate the PolII CTD (49) and can enhance transcription elongation (6, 36), the primary effect of VP16 on transcription is clearly at the level of initiation (6, 36), and VP16 does not bind to hCycT1 under conditions where an hCycT1/Tat interaction can be readily detected (8). Also, activation of the HIV-1 LTR by the VP16-Rev fusion protein differs from hCycT1-Rev-mediated activation in being significantly lower than is seen with Tat-Rev (45). Similarly, whereas direct recruitment of either the CDK8 or the CDK9 kinase to the SLIIB RNA target can also activate the HIV-1 LTR promoter (46–48), this activation was reported to be less than

10% of the level seen with Tat-Rev (46, 48). This is an important difference, because it is precisely the fact that hCycT1-Rev is just as effective as either Tat-Rev or Tat in activating HIV-1 LTR driven gene expression (Fig. 1) that argues most convincingly for the hypothesis that hCycT1/P-TEFb recruitment is the only relevant biological activity of Tat in mediating this activation.

In conclusion, we have presented data showing that the role of Tat in the transcriptional activation of the HIV-1 LTR is restricted to acting as a molecular adaptor that permits recruitment of hCycT1/P-TEFb to the LTR. These observations suggest that the interactions between Tat and a variety of other cellular proteins that have been previously described (20–26) are either indirect or represent binding events that have no functional relevance to transcriptional activation by Tat. It is, however, important to note that these data do not preclude an additional role for Tat in other aspects of the viral life cycle, such as reverse transcription or regulation of apoptosis, and it remains possible that other cellular proteins may interact with Tat and play a role in mediating these distinct processes. A complete analysis of the molecular contacts that facilitate the recruitment of hCycT1 and Tat to TAR, preferably by using an x-ray crystallographic approach, would represent a key next step toward the future design of agents that can specifically block this critical interaction and, hence, the replication of HIV-1.


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