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A Novel CDK9-Associated C-Type Cyclin Interacts Directly with HIV-1 Tat and Mediates Its High-Affinity, Loop-Specific Binding to TAR RNA

Ping Wei,* Mitchell E. Garber,*‡ Shi-Min Fang,* Wolfgang H. Fischer,† and Katherine A. Jones*§
*Regulatory Biology Laboratory
†Peptide Biology Laboratory
The Salk Institute for Biological Studies
La Jolla, California 92037-1099
‡Graduate Program in Biomedical Sciences
University of California, San Diego
La Jolla, California 92093

Summary

The HIV-1 Tat protein regulates transcription elongation through binding to the viral TAR RNA stem-loop structure. We have isolated a novel 87 kDa cyclin C–related protein (cyclin T) that interacts specifically with the transactivation domain of Tat. Cyclin T is a partner for CDK9, an RNAPII transcription elongation factor. Remarkably, the interaction of Tat with cyclin T strongly enhances the affinity and specificity of the Tat:TAR RNA interaction, and confers a requirement for sequences in the loop of TAR that are not recognized by Tat alone. Moreover, overexpression of human cyclin T rescues Tat activity in nonpermissive rodent cells. We propose that Tat directs cyclin T–CDK9 to RNAPII through cooperative binding to TAR RNA.

Introduction

The human immunodeficiency virus (HIV) encodes a nuclear transcriptional activator, Tat, which acts to enhance the processivity of RNA polymerase II (RNAPII) complexes that would otherwise terminate transcription prematurely at random locations downstream of the viral RNA start site. The mechanism of Tat transactivation is unique in that the *cis*-acting transactivation response element (TAR) is a stable RNA stem-loop structure that forms at the 5′ end of nascent viral transcripts. Transcriptional activation by Tat through TAR requires proper folding of the RNA as well as specific bases in the bulge and apical loop of the TAR RNA hairpin structure (for review, see Cullen, 1993; Jones and Peterlin, 1994).

The interaction of Tat with TAR RNA is mediated through an arginine-rich motif (ARM) that is characteristic of a family of sequence-specific RNA-binding proteins (reviewed by Gait and Karn, 1993). However, several lines of evidence suggest that the ARM of Tat is not an independent domain. First, the transactivation domain of Tat cannot be substituted by the activation domains of other transcription factors, such as the herpesvirus VP16 protein, even though the VP16 activation domain is capable of activating transcription when recruited to RNA through a different RNA-binding domain (Tiley et al., 1992; Ghosh et al., 1993). Second, the full-length Tat-1 protein, but not a mutant Tat protein lacking the transactivation domain, is able to target a heterologous protein to TAR RNA in vivo (Luo et al., 1993). Third, Tat transactivation through TAR is blocked by amino acid insertions that separate the activation domain from the ARM (Luo and Peterlin, 1993), and the isolated ARM does not function as a dominant negative inhibitor of the wild-type Tat protein (Madore and Cullen, 1993). In addition, residues in the core of the transactivation domain enhance the affinity and specificity of the Tat:TAR interaction in vitro (Churcher et al., 1993). Taken together, these studies suggest that the transactivation domain is required, directly or indirectly, for efficient binding of Tat to TAR RNA in vivo.

In vitro, Tat binds to the bulge region of TAR RNA but does not recognize sequences in the loop of the RNA hairpin that are essential for transactivation. Moreover, exogenous Tat cannot overcome the specific inhibition to Tat transactivation that is observed when cells or cell extracts are treated with synthetic TAR (decoy) RNAs. Consequently, it has been suggested that Tat must first interact with a host cell factor in order to recognize TAR RNA with high affinity and in a sequence-appropriate manner (reviewed by Cullen 1993; Jones and Peterlin, 1994). Additional evidence in support of a cofactor for Tat comes from the observation that murine and Chinese hamster ovary (CHO) cell lines do not support efficient Tat activation through TAR RNA, and studies with chimeric Tat proteins have attributed the defect in rodent cells to a problem of TAR RNA recognition. Complementation studies using human:CHO hybrid cell clones revealed that a factor encoded on human chromosome 12 (Chr. 12) enhances Tat activity 5- to 10-fold in rodent cells and confers a requirement for sequences in the loop of TAR RNA (Hart et al., 1993; Alonso et al., 1994).

By contrast with the ARM, the N-terminal half of Tat (aa 1–48) functions autonomously as a transcriptional activation domain in the context of a chimeric DNA- or RNA-binding protein and can act on its own to inhibit (or "squelch") the activity of the wild-type Tat protein (reviewed by Cullen, 1993; Jones and Peterlin, 1994). Tat acts through TAR to control an early step in transcription elongation that is sensitive to inhibition by protein kinase inhibitors and requires the carboxyl-terminal domain (CTD) of the large subunit of RNA polymerase II (reviewed by Jones, 1997). The transactivation domain of Tat interacts strongly with a nuclear **T**at-**a**ssociated **k**inase, called TAK (Herrmann and Rice, 1993; 1995), which was recently shown to be identical to the kinase subunit of P-TEFb (Mancebo et al., 1997; Zhu et al., 1997), a **p**ositive-acting **t**ranscription **e**longation **f**actor complex that is required for elongation at many genes (Marshall and Price, 1992, 1995). Purified TAK/P-TEFb can hyperphosphorylate the RNAPII CTD (Marshall et al., 1996; Yang et al. 1996). Biochemical analysis of purified TAK/P-TEFb identified the 42 kDa catalytic subunit as a CDC2-related kinase, PITALRE (hereafter called CDK9), and CDK9 inhibitors as well as a dominant negative CDK9 mutant were found to block Tat transactivation

§To whom correspondence should be addressed.

in vivo and in vitro, indicating that CDK9 kinase activity is critical for Tat activity (Mancebo et al., 1997; Yang et al. 1997; Zhu et al., 1997). Taken together, these studies provide strong evidence that the CDK9-containing TAK/P-TEFb complex is an important host cell transcriptional coactivator for Tat.

We have recently found that the addition of Tat to nuclear extracts induces the binding of CDK9-containing TAK/P-TEFb complexes to TAR RNA (M. E. G., P. W., and K. A. J., unpublished data). Moreover, we noted that the nuclear Tat-TAK/P-TEFb complexes did not associate with loop mutant TAR RNA, indicating that the interaction of Tat with TAK/P-TEFb might alter its RNA-binding specificity. In this paper, we report the cloning of a novel 87 kDa cyclin C–related protein, designated cyclin T, which is a constituent of the Tat-associated TAK/P-TEFb complex in HeLa nuclear extracts. Interestingly, we find that purified recombinant cyclin T interacts specifically with Tat and dramatically enhances its affinity for TAR RNA. Most importantly, the binding of recombinant cyclin T–Tat complexes to TAR RNA was found to require sequences in the loop of TAR RNA that are not recognized by free Tat alone. These results suggest that Tat can recruit cyclin T–CDK9 complexes to RNAPII through cooperative binding to nascent TAR RNA. Based on these findings, we propose that cyclin T is the TAR RNA-binding cofactor for Tat.

Results

Identification of a Nuclear 87 kDa Protein Associated with HIV-1 Tat in the TAK/P-TEFb Complex

To further characterize the proteins in the TAK/P-TEFb complex that interact with the transactivation domain of Tat, GST-Tat-1 (aa 1–48)–coupled beads were incubated with a crude HeLa nuclear extract, and the nuclear proteins associated with the transactivation domain of HIV-1 Tat were analyzed by SDS-PAGE and silver-staining. As shown in Figure 1A (right panel), an 87 kDa protein (p87) was found to bind avidly to the wild-type HIV-1 Tat protein and not to mutant GST-Tat (aa 1–48) proteins (P18IS, C22G, K41A) containing substitutions that inactivate Tat transactivation in vivo (Herrmann and Rice, 1993, 1995). Both p87 and the 42 kDa CDK9 subunit of the TAK/P-TEFb complex were phosphorylated when the GST-Tat-1 (aa 1–48) beads were incubated with [γ-^{32}P]ATP and analyzed for TAK activity (Figure 1A, left panel). As expected, CDK9 was also detected only in association with the wild-type Tat protein. Interestingly, the 87 kDa protein was recovered in apparent stoichiometric excess to the CDK9 protein (Figure 1A, right panel), suggesting that Tat might interact most tightly with the p87 subunit of the TAK/P-TEFb complex.

The GST-Tat-1 (aa 1–48) protein used in these experiments contained a functional transactivation domain, as demonstrated by its ability to block ("squelch") transcriptional activation by wild-type Tat-1 in vitro when incubated with nuclear transcription extracts at levels 1.5- to 15-fold higher than the wild-type Tat-1 protein (Figure 1B). Comparable inhibition of Tat transactivation was observed with a mutant GST-Tat-2 (aa 1–84) protein

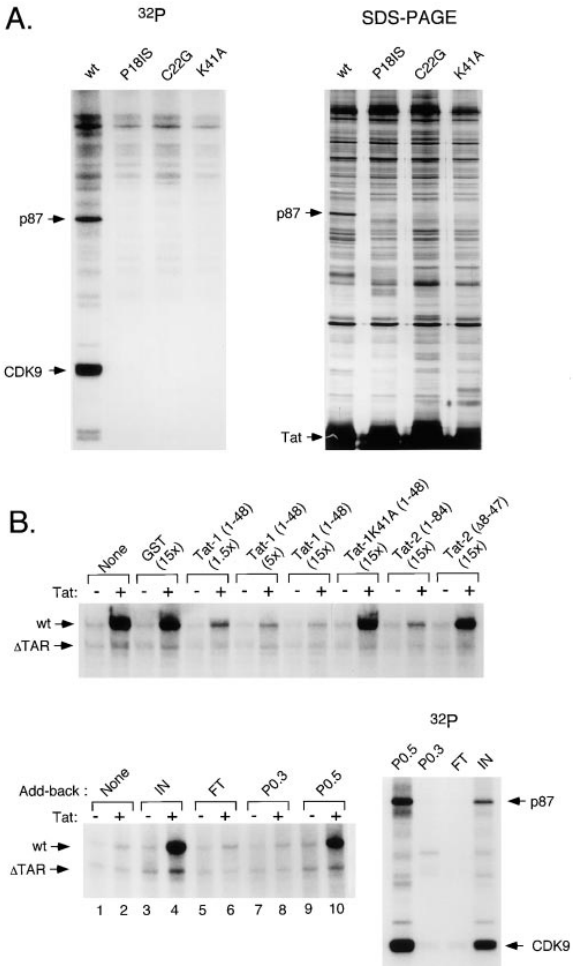

Figure 1. Identification of an 87 kDa HeLa Nuclear Protein (p87) that Associates Specifically with the Transactivation Domain of HIV-1 Tat

(A) HeLa nuclear proteins associated with GST-Tat-1 (aa 1–48)-coupled beads were isolated and analyzed for kinase activity (left panel) or by SDS-PAGE (right panel). Approximately 15 μg of each GST-Tat protein was incubated with 1 mg of HeLa nuclear extract; one-third of the reaction was analyzed for TAK activity (left panel), and the remainder was examined by silver-staining (right panel). Wild-type (wt) and mutant GST-Tat-1 (aa 1–48) proteins are indicated above each lane. A 63 kDa protein seen exclusively in the wild-type GST-Tat fraction is a contaminant originating from the Tat preparation.

(B) Top panel: analysis of wild-type GST-Tat-1 and transdominant GST-Tat-1 (aa 1–48) proteins by run-off transcription experiments. Transdominant GST-Tat proteins, indicated above each lane, were preincubated with HeLa nuclear extract prior to transcription. Arrows indicate the location of run-off transcripts from the wild-type (wt) pHIV-2/CAT or the control pHIV-2ΔTAR/CAT (ΔTAR) template. Bottom panel, left: analysis of the ability of nuclear extract or phosphocellulose column fractions to restore transactivation to reactions that were inhibited by incubation with GST-Tat-1 (aa 1–48). Reactions were supplemented with either HeLa nuclear extract (IN, 60 μg; lanes 3 and 4), P0.1 MKCl (FT; lanes 5 and 6), P0.3 MKCl (lanes 7 and 8), or P0.5 MKCl (lanes 9 and 10) phosphocellulose column fractions. Bottom panel, right: Tat-associated kinase activity in the nuclear extract (IN) or column fractions.

that contains an intact transactivation domain but lacks the ARM. The GST-Tat-1 (aa 1–48) and GST-Tat-2 (aa 1–84) proteins did not block basal transcription in the

absence of Tat, nor did they affect transcription from HIV templates that lack TAR (ΔTAR). The ability of these truncated Tat proteins to inhibit Tat transactivation in vitro was destroyed by mutations affecting key residues within the transactivation domain (Tat-1 K41A, Tat-1 C22G, Tat-1 P18IS, Tat-2 Δ8–47) (Figure 1B; other data not shown). Inhibition of Tat transactivation by Tat-1 (aa 1–48) could not be overcome by the addition of exogeneous wild-type Tat protein, but was restored by nuclear extract (Figure 1B, compare lanes 1 and 2 with lanes 3 and 4). Fractionation of the HeLa nuclear extract by phosphocellulose column chromatography revealed that the Tat-associated p87 and CDK9 proteins eluted principally in the P0.5MKCl fraction, as determined by SDS-PAGE and silver-staining for the p87 protein (data not shown), and by the in vitro kinase assay (Figure 1B, lower right panel). The P0.5MKCl fraction was as effective as nuclear extract in its ability to restore Tat-activated transcription to reactions that had been inhibited by incubation with GST-Tat-1 (aa 1–48) (Figure 1B, lanes 9 and 10), whereas the other column fractions were inactive. Collectively, these data suggest that p87 is a component of the TAK/P-TEFb complex that binds tightly to the transactivation domain of HIV-1 Tat.

Molecular Cloning of p87 (Cyclin T)

To better characterize the 87 kDa protein, approximately 110 pmol of p87 was isolated from 40 mg of crude HeLa nuclear extract using a scaled-up version of the GST-Tat-1 (aa 1–48) affinity selection protocol used in Figure 1A (see Experimental Procedures). The p87 protein was excised from a preparative SDS-PAGE and subjected to tryptic digestion, and nine different tryptic peptide fragments were purified by HPLC and subjected to microsequence analysis. The derived amino acid sequence established that p87 was a novel protein, and a search of the GenBank database revealed an expressed sequence tag clone (EST yd48c03; I.M.A.G.E. ID111460) that encoded several of the p87-derived peptides. A RACE RCR protocol was used to extend the cDNA clone to the 3′ end. A human Jurkat (T cell) cDNA library was then screened with radiolabeled probes derived from the EST cDNA and 3′RACE PCR products, and a 7.2 kB cDNA clone encompassing the entire (726 amino acid) open reading frame (ORF), as well as 5′- and 3′-untranslated leader sequences, was isolated and sequenced. Inspection of the amino acid sequence encoded by the cDNA (Figure 2A) revealed the presence of all nine peptides obtained from the native p87 protein (P1–P9; Figure 2B). In vitro translation of the cDNA clone confirmed that the intact ORF encodes a protein of 87 kDa (data not shown), indicating that the open reading frame is complete.

Database searches with the predicted protein sequence (hereafter called cyclin T, for **T**at) identified an amino-terminal cyclin box motif that is closely related (39% identity within the most conserved 25 aa region of the cyclin box) to human cyclin C. A longer 250 aa region encompassing the entire duplicated cyclin fold is highly conserved amongst C-type cyclins from various organisms (alignment not shown). Cyclin T is most closely related to the essential *S. pombe* C-type cyclin,

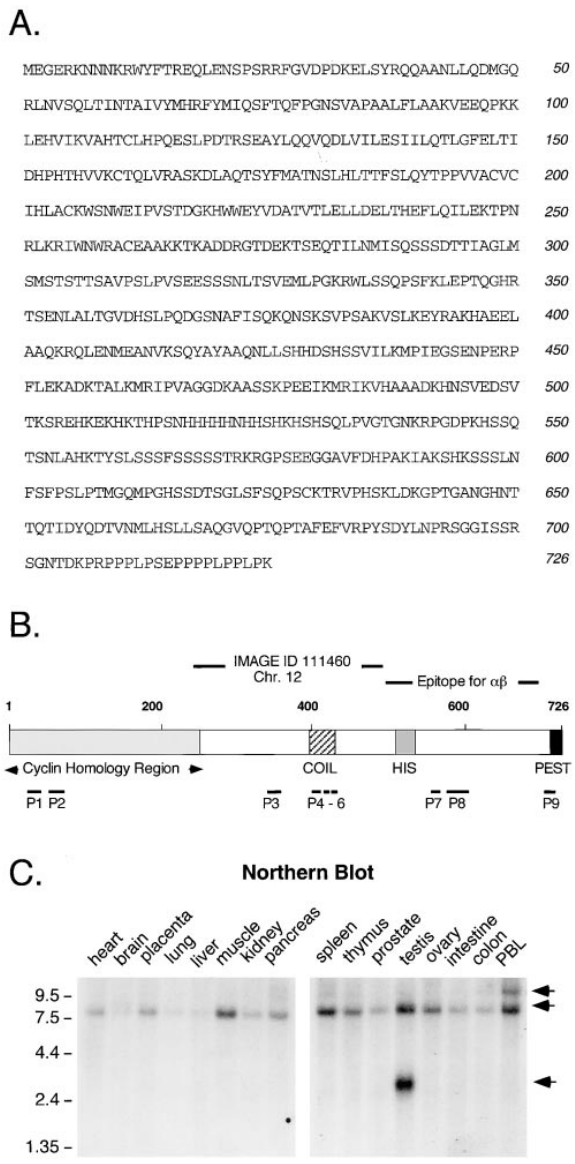

Figure 2. Analysis of the Sequence and Predicted Domain Structure of the Human Cyclin T (p87) Protein

(A) Amino acid sequence of human cyclin T encoded by the 2.3 kb cDNA.

(B) Predicted domain structure of human cyclin T. The locations of nine peptides (P1–9) derived from microsequencing of purified HeLa p87 are indicated below the diagram.

(C) Northern blot analysis of cyclin T mRNA in various human tissues (Clontech), using a probe corresponding to the cyclin homology domain (aa 1–176).

Pch1(+), which was isolated through its ability to interact with CDC2 (Furnari et al., 1997). In addition to the N-terminal cyclin domain, the cyclin T protein contains a putative coiled-coil motif (aa 379–430), a His-rich motif (aa 506–530) and a carboxy-terminal PEST sequence (aa 709–726; Figure 2B). C-terminal PEST sequences are commonly found in G1 cyclins and serve to regulate protein turnover by the cellular ubiquitination and proteolysis pathways (Rechsteiner and Rogers, 1996). Cyclin T is encoded by a single gene, and its genomic location

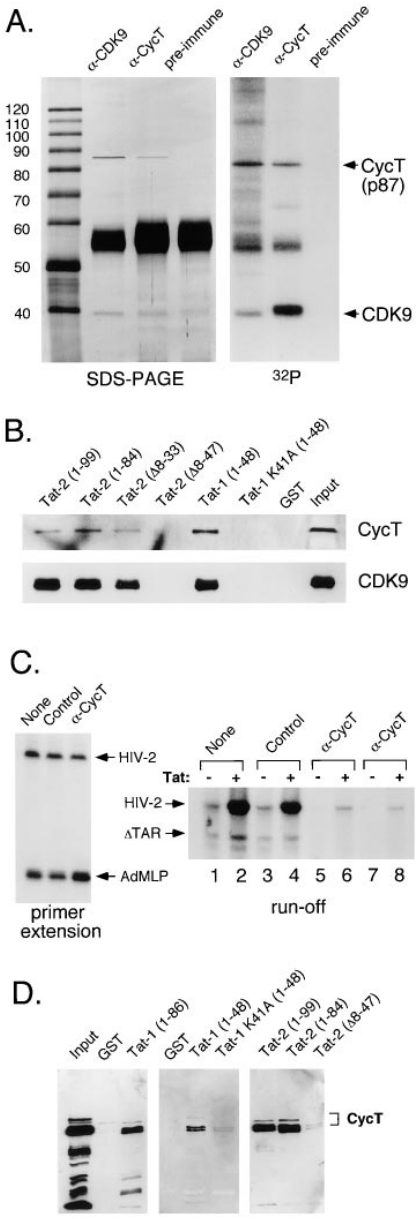

Figure 3. Cyclin T Is a Partner for CDK9 (PITALRE) and Is Required for Transcription Elongation In Vitro

(A) Cyclin T associates with CDK9 in nuclear extracts. Immunoprecipitation reactions with HeLa nuclear extract were carried out using antisera specific either to cyclin T or CDK9 (PITALRE), or with preimmune serum. Proteins were visualized by staining with silver (left panel) or by incubation of the beads with [^{32}P]ATP to analyze phosphorylated proteins (right panel). The identification of cyclin T and CDK9 was confirmed by Western blot (data not shown).

(B) Analysis of the binding of nuclear cyclin T and CDK9 to wild-type and activation domain mutant HIV-1 and HIV-2 Tat proteins. HeLa nuclear extract was incubated with various GST-Tat proteins coupled to beads as described in Figure 1A, and cyclin T and CDK9 proteins were examined by Western blot.

(C) Cyclin T is required for HIV-1 transcription elongation and Tat transactivation in vitro. RNA initiation from the HIV-2 and adenovirus major late promoter was analyzed by primer extension, and RNA elongation and Tat transactivation was measured using a run-off transcription assay. HeLa nuclear extracts were depleted with control (GST-specific) antiserum (lanes 3 and 4), or with antiserum specific to cyclin T (lanes 5–8), as indicated above each lane.

has been mapped from sequence-tagged sites (STS G25423; G28091) to a position located 295.9 cR (centirays; 1 cR = 270 kb) from the top of human chromosome 12.

Northern blot analysis with a probe derived from the region of the cyclin box (aa 1–176) revealed that the major cyclin T transcript (8 kb) is widely expressed in human tissues (Figure 2C). A larger transcript (~9.5 kb) was also detected in peripheral blood lymphocytes (PBLs), and relatively high levels of a shorter transcript (3.0 kb) were detected exclusively in the testis. These same transcripts were also detected with a radiolabeled probe derived from the C-terminal region of the cyclin T gene, and, in addition, the C-terminal probe detected an abundant 3.5 kb transcript in the ovary, which did not hybridize to a probe from the cyclin box (data not shown). These results indicate that cyclin T mRNA is expressed widely in adult human tissues and may be spliced differentially in PBLs as well as in cells derived from the human germ line.

Cyclin T Interacts with CDK9 in Nuclear Extracts and Is Required for RNAPII Transcription Elongation In Vitro

To confirm that the cyclin T cDNA encodes the 87 kDa Tat-associated protein that we originally identified in HeLa nuclear extracts, a region near the carboxy-terminus of cyclin T was expressed in bacteria and used to generate rabbit polyclonal antiserum. Both the native 87 kDa cyclin T protein and CDK9 were detected by immunoprecipitation using the anti-cyclin T antibodies, and the same proteins were also detected with anti-CDK9 (PITALRE) antiserum (Figure 3A). The identity of the 87 kDa and 42 kDa proteins as cyclin T and CDK9, respectively, was established by Western blot analysis of the immunoprecipitated reactions (data not shown). Neither cyclin T nor CDK9 were detected in control immunoprecipitation reactions with preimmune serum (Figure 3A). Western blot analysis was used to confirm the presence of native cyclin T and CDK9 proteins in TAK/P-TEFb fractions derived by GST-Tat affinity selection from HeLa nuclear extract (Figure 3B). We conclude that cyclin T, like CDK9, associates specifically with the wild-type Tat-1 and Tat-2 proteins and not with mutant Tat proteins altered within the transactivation domain.

Previous studies have shown that P-TEFb is critical for DRB-sensitive RNAPII transcription elongation at many promoters (Marshall and Price, 1995; Marshall et al., 1996; Zhu et al., 1997), and immunodepletion of CDK9 from HeLa nuclear extracts has been shown to inhibit RNAPII elongation as well as Tat transactivation (Mancebo et al., 1997; Zhu et al., 1997). To test the role of the cyclin T–CDK9 complex in transcription, HeLa nuclear extracts were depleted of endogeneous cyclin T by repeated incubation with anti-cyclin T antibody-coupled

(D) Analysis of the ability of recombinant cyclin T to bind directly to Tat. Different HIV-1 and HIV-2 GST-Tat proteins were coupled to beads and incubated with GST-cleaved cyclin T, as indicated above each lane. The binding of cyclin T to the different GST-Tat proteins was visualized by Western blot.

beads. Western blot analysis indicated that this procedure resulted in the loss of approximately 80% of the endogeneous HeLa cyclin T protein as well as at least 50% of the endogeneous CDK9 (data not shown). Analysis of the transcriptional activity of the cyclin T–depleted extracts revealed that the loss of cyclin T had only a modest effect on transcription initiation from the HIV-1 promoter and no effect on initiation from other promoters (HIV-2, alpha-globin, and AdMLP) as determined by primer extension (Figure 3C; other data not shown). By contrast, immunodepletion of cyclin T caused a dramatic reduction in the synthesis of longer run-off transcripts from the wild-type and TAR-deleted HIV promoter templates, and also greatly reduced TAR-dependent transactivation by Tat (Figure 3C). Transcription elongation was unaffected in extracts treated with control (GST) antiserum. From these results, we conclude that cyclin T is required, directly or indirectly, for elongation of transcription by RNAPII.

Recombinant Cyclin T Interacts Directly with the Transcriptional Activation Domain of Tat

Because p87 was most prominent among the nuclear proteins that bound to the Tat transactivation domain in extract (see Figure 1A), it was important to determine whether the recombinant p87 protein could interact directly with Tat. Recombinant cyclin T protein was expressed in bacteria and purified as a GST–cyclin T fusion protein, and the GST domain was removed by cleavage with thrombin. Purified cyclin T was then incubated with wild-type or mutant GST-Tat-coupled beads and the affinity column eluates were analyzed for binding of cyclin T by Western blotting. As shown in Figure 3D, cyclin T bound to wild-type GST-Tat-1 and GST-Tat-2 proteins, as well as to truncated Tat proteins that contain an intact transactivation domain. By contrast, cyclin T did not associate with either GST or mutant Tat-1 (K41A) or Tat-2 (Tat-2 Δ8–47) proteins. Moreover, we also found that GST-cleaved Tat-2 protein bound in an activation domain–dependent manner to GST–cyclin T–coupled beads (data not shown), and we conclude that cyclin T binds directly to the transactivation domains of the HIV-1 and HIV-2 Tat proteins in vitro.

The Interaction of Cyclin T with Tat Dramatically Enhances Its Affinity for TAR RNA and Alters the Specificity of the Tat:TAR Interaction

If the cyclin T subunit of the TAK/P-TEFb complex is indeed the direct target for Tat in the cell, we reasoned that the interaction between Tat and cyclin T might have a dramatic effect on binding to TAR RNA. Binding of Tat to TAR RNA in vitro does not correlate well with the sequence requirements for TAR in Tat transactivation, and in particular, Tat binding is only modestly affected by mutations in the loop of the RNA that play a critical role in Tat transactivation. We recently found that HIV-1 and HIV-2 Tat proteins bind with much higher affinity to HIV-2 TAR (TAR-2) than to HIV-1 TAR (TAR-1) RNA in vitro, despite the fact that both TARs function nearly equivalently in transactivation, and that binding of free Tat to TAR-2 is significantly reduced by mutations in the duplicated loop sequences of TAR-2 RNA. Moreover, the affinity of free Tat for the TAR-2 loop mutant RNA is significantly higher than that observed for wild-type TAR-1 RNA in vitro (M. E. G., P. W., and K. A. J, unpublished data). Therefore, the interaction of Tat with a cellular RNA-binding cofactor is predicted to enhance selectively the affinity of Tat for wild-type TAR-1 RNA, as well as block the residual nonspecific binding of Tat to the bulge region on TAR-1 and TAR-2 loop mutant RNAs.

To determine whether the interaction of Tat with cyclin T alters its TAR RNA recognition properties, gel mobility shift experiments were carried out with recombinant Tat and cyclin T proteins and wild-type and loop mutant HIV-1 and HIV-2 TAR RNAs. The full-length HIV-1 Tat bound weakly to wild-type TAR-1 RNA (Figure 4A, lane 5), and a four-base substitution of residues in the loop of TAR-1 reduced the binding of Tat by approximately 3-fold (Figure 4A, lane 6). Interestingly, the binding of HIV-1 Tat to TAR-1 RNA was dramatically enhanced in the presence of cyclin T (compare lanes 5 and 7), and the cyclin T–Tat complex did not bind to loop mutant TAR-1 RNA (lane 8). Cyclin T had no significant effect on the binding of mutant Tat proteins that lack a functional transactivation domain (C22G, P18IS; lanes 9–16). Cyclin T also strongly enhanced the binding of Tat to a minimal TAR RNA element (+17 to +43) and mutations in either the loop or bulge of the TAR RNA destroyed the interaction (Figure 4A, compare lane 23 with lanes 24 and 25). We conclude that the binding of Tat-1 to cyclin T strongly enhances its affinity for TAR RNA, and that the formation the cyclin T–Tat:TAR complex requires both the integrity of the Tat transactivation domain as well as sequences in the loop and bulge of the TAR RNA hairpin. Moreover, purified cyclin T displayed no affinity for TAR RNA in the absence of Tat (Figure 4A, lanes 3 and 4), indicating that the ability of cyclin T to associate with TAR depends entirely upon its ability to bind cooperatively with Tat to the RNA.

Tat binds with intrinsically higher affinity to TAR-2 RNA than TAR-1 RNA and recognizes two or possibly three sites on the duplicated TAR-2 hairpin structure. Addition of cyclin T to the wild-type Tat-2 protein yielded a new complex containing both the cyclin and Tat-2, and the cyclin T–Tat-2 complex did not bind to a double loop mutant TAR-2 RNA (Figure 4B, compare lanes 7 and 8). Cyclin T did not bind TAR-2 RNA in the absence of Tat and did not affect the binding of a mutant Tat-2 protein lacking the transactivation domain (Tat-2 Δ8–47). Because Tat has a higher affinity for TAR-2 RNA than for TAR-1 RNA, the extent of cooperativity observed upon addition of cyclin T was less dramatic than that observed for Tat-1 on TAR-1 RNA; however, the binding of cyclin T and Tat-2 is cooperative at limiting concentrations of Tat-2 (data not shown), and cyclin T can be shown to enhance the binding of Tat-2 to TAR in RNase footprint protection experiments, which are more sensitive to the formation of stable complexes (see below). We conclude that the direct binding of Tat to the cyclin T subunit of TAK/P-TEFb strongly influences both the affinity and specificity of TAR RNA recognition.

If cyclin T is the TAR RNA-binding cofactor for Tat, one prediction is that exogeneous synthetic wild-type TAR decoy RNAs, as well as transdominant mutant Tat proteins, should compete effectively for the formation

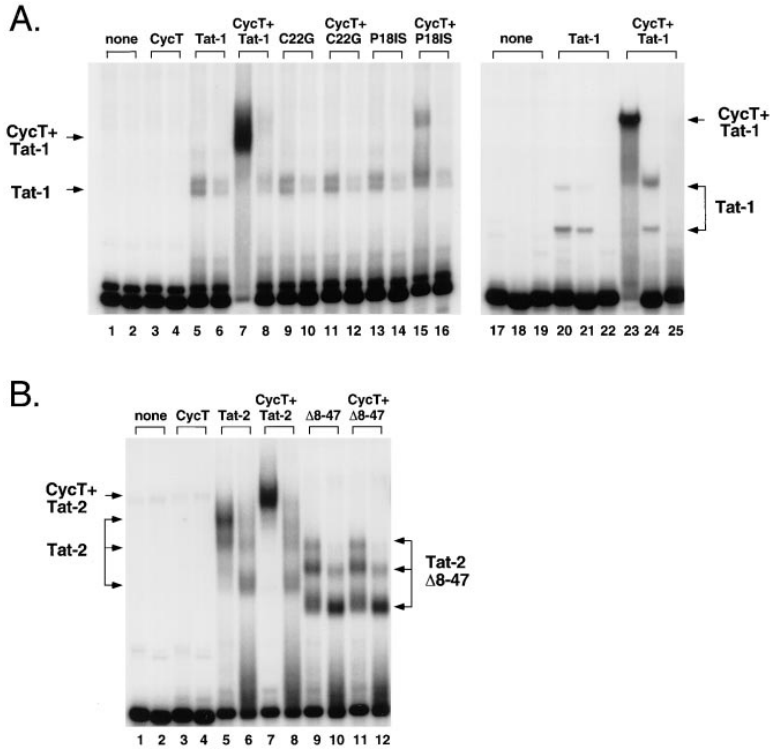

Figure 4. Analysis of the Binding of Tat, Cyclin T, and Cyclin T–Tat Complexes to TAR RNA in Gel Mobility Shift Experiments

(A) Binding of cyclin T and Tat-1 proteins to TAR-1 RNA. Left panel: binding to wild-type (odd-numbered lanes) or loop mutant (even-numbered lanes) TAR-1 RNAs (+1 to +80). Where indicated, reactions contained 400 ng of wild-type or activation domain mutant GST-Tat-1 (aa 1–86) proteins and 750 ng of GST–cyclin T. Right panel: analysis of binding of Tat-1 and cyclin T to minimal (+17 to +43) TAR-1 RNAs. Where indicated, reactions contained 125 ng GST-cleaved cyclin T and 100 ng GST-cleaved Tat-1 (aa 1–86). TAR-1 RNA probes (5 ng) were either wild-type (lanes 17, 20, and 23), loop mutant (+29/+34, substituting all six loop residues; lanes 18, 21, and 24), or bulge point mutant (U22A; lanes 19, 22, and 25).

(B) Binding of cyclin T and Tat-2 proteins to wild-type (odd-numbered lanes) or double loop mutant (even-numbered lanes) TAR-2 RNA. Where indicated, reactions contained 400 ng of wild-type GST-Tat-2 (aa 1–99) or 400 ng of activation domain mutant GST-Tat-2Δ8–47, and 750 ng of GST–cyclin T.

of the cyclin T–Tat:TAR ternary complex in vitro. As shown in Figure 5A, binding of the cyclin T–Tat-1 complex to TAR-1 RNA was effectively competed by an excess of wild-type TAR-1 or TAR-2 RNA and was not inhibited by equivalent amounts of TAR loop mutant RNAs. The RNA-binding specificity of the cyclin T–Tat:TAR complex correlates precisely with the ability of these TAR decoy RNAs to block Tat transactivation in vitro (M. E. G., P. W., and K. A. J., unpublished data). In addition, preincubation of the cyclin T protein with an excess of the transdominant negative mutant Tat-1 (aa 1–48) protein was sufficient to block the formation of the cyclin T–Tat:TAR ternary complex (Figure 5B). By contrast, the weak binding of free Tat to TAR-1 RNA was not inhibited by GST-Tat-1 (aa 1–48) (compare lanes 6 and 7 with lane 4), and comparable levels of the mutant GST-Tat K41A (aa 1–48) protein did not interfere with the formation of the cyclin T–Tat:TAR complex (compare lanes 8 and 9 with lane 5). We conclude that the binding of the transdominant mutant Tat-1 (aa 1–48) protein to cyclin T is sufficient to prevent formation of the ternary complex with TAR RNA. The experiment shown in Figure 5B also demonstrates that the ARM of Tat is critical for formation of the cyclin T–Tat:TAR complex, because the transdominant GST-Tat-1 (aa 1–48) protein displayed no affinity for TAR RNA, either alone or in the presence of cyclin T (Figure 5B, lanes 2 and 3).

The Cyclin T–Tat Complex Protects Sequences in the Loop and Upper Stem of TAR RNA in RNase Footprint Experiments

The binding of the cyclin T–Tat complex to TAR RNA was further assessed by RNase footprint protection experiments. Radiolabeled TAR-1 RNA was incubated with either purified Tat-1, cyclin T, or the cyclin T–Tat-1 complex and subjected to partial digestion with different ribonucleases (RNase T1, cobra venom RNase, RNase A). As shown in Figure 6A, binding of the cyclin T–Tat-1 complex resulted in a specific protection of sequences in the upper stem and loop of the TAR-1 RNA hairpin (cf. lanes 5, 9, and 13). No specific binding to TAR-1 RNA was observed with either cyclin T or Tat-1 alone. These data suggest that the cyclin greatly enhances the stability and affinity of the Tat-1:TAR-1 interaction, and suggest that the binding of Tat-1 and cyclin T to TAR-1 RNA is cooperative. The binding of free Tat-2 protein to TAR-2 RNA resulted in the formation of a nuclease-hypersensitive site at position G57 and a weak protection of sequences in the bulge and loop of the 5′-hairpin stem of TAR-2, whereas free cyclin T did not interact with TAR-2 RNA. By contrast, the cyclin T–Tat-2 complex bound avidly to sequences in the loop and stem of both the 5′- and 3′-TAR-2 hairpin structures (Figure 6B, lanes 5, 9, and 13). The cyclin T–Tat-2 complex bound specifically to TAR under these conditions and did not protect RNA sequences outside or between the two TAR-2 hairpin structures. Thus, cyclin T also enhances the affinity and stability of the Tat-2:TAR-2 interaction in vitro.

Overexpression of Human Cyclin T Enhances Tat Transactivation in Rodent (NIH3T3, CHO) Cell Lines

The observation that cyclin T is encoded on chromosome 12 and is required for high-affinity binding of Tat to TAR RNA raises the possibility that differences between the murine and human homologs of cyclin T may be responsible for the partial defect in Tat activity that

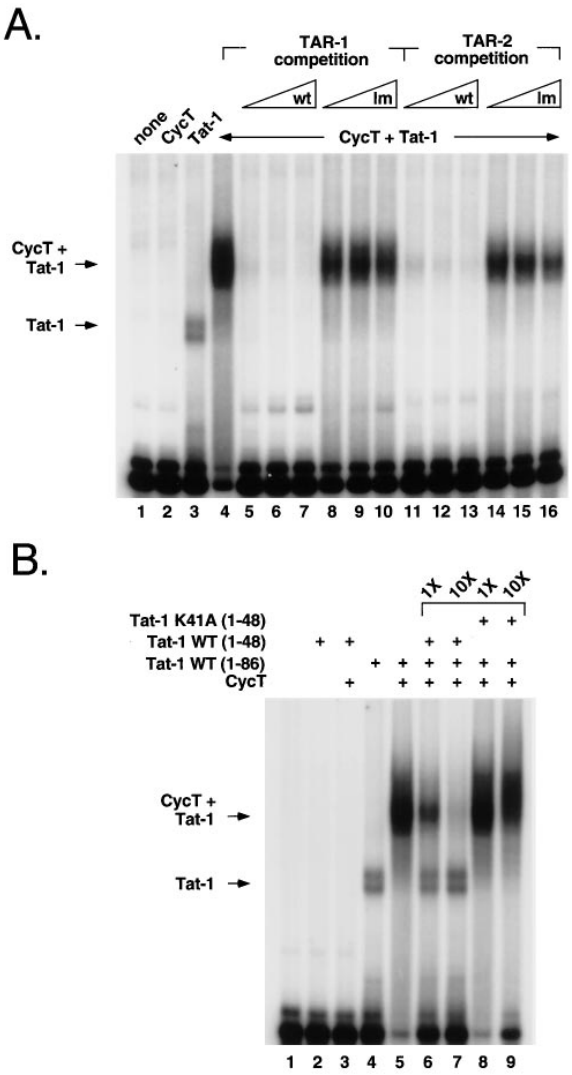

Figure 5. Analysis of the Specificity of the Cyclin T–Tat-1:TAR-1 Complex

(A) Competition of the complex with wild-type and loop mutant TAR RNAs. Where indicated, reactions contained 400 ng of GST-Tat-1 (aa 1–86), 750 ng of GST-cleaved cyclin T, and 5 ng labeled wild-type TAR-1 RNA. Reactions either lacked competitor RNA (lanes 1–4), or contained competitor TAR RNA at a 100-fold (lanes 5, 8, 11, and 14), 200-fold (lanes 6, 9, 12, and 15) or 400-fold (lanes 7, 10, 13, and 16) molar excess to the radiolabeled RNA.

(B) Analysis of the ability of GST-Tat-1 (1–48) to block the formation of the cyclin T–Tat-1:TAR RNA complex. Reactions contained recombinant cyclin T and Tat proteins at the following levels: 750 ng of GST–cyclin T (lanes 3 and 5–9), 400 ng of wild-type GST-Tat-1 (aa 1–86) (lanes 4–9), 400 ng GST-Tat-1 (aa 1–48) (lane 6), 4 μg GST-Tat-1 (aa1–48) (lanes 2, 3, and 7), 400 ng GST-Tat-1 K41A (aa 1–48) (lane 8), or 4 μg GST-Tat-1 K41A (aa 1–48) (lane 9).

has been characterized in murine (rodent) cell lines. To assess this possibility, we carried out transient transfection experiments to examine the effect of overexpression of human cyclin T protein on Tat activity in rodent cell lines. Interestingly, overexpression of a form of the human cyclin T protein lacking the C-terminal PEST sequence (pCMV-HACycT) was found to enhance HIV-1 Tat transactivation by as much as 18-fold in murine NIH3T3 and CHO (chinese hamster ovary) cell lines (Figure 7A). Basal HIV-1 transcription was unaffected by cyclin T in these experiments, although a modest increase in basal expression was observed at higher levels of cyclin T (data not shown), consistent with the proposed general role of TAK/P-TEFb in transcription elongation. These findings suggest that the human cyclin T can act in combination with murine CDK9 to rescue Tat activity in rodent cells. Overexpression of the human CDK9 subunit had no significant effect on Tat transactivation in these experiments (data not shown), indicating that the kinase is not limiting for Tat activity in rodent cells. These data suggest that cyclin T is limiting for Tat transactivation in murine cells and may be responsible for the previously-characterized species restriction to Tat activity. Collectively, the data presented in this paper suggest that binding of Tat to the cyclin T subunit of TAK/P-TEFb, as well as the subsequent cooperative binding of the complex to TAR RNA, plays a central role in transcriptional activation (Figure 7B). Details of this model are discussed below.

Discussion

Tat Interacts Directly with a Nuclear Cyclin that Is Associated with CDK9 in a Transcription Elongation Complex

In the model shown in Figure 7B, Tat interacts through its activation domain (AD) with the cyclin T subunit of a preexisting TAK/P-TEFb complex. We speculate that the interaction of Tat with cyclin T alters the conformation of Tat to enhance greatly the affinity and specificity of the Tat:TAR interaction. Although cyclin T has no affinity for RNA on its own, it may nevertheless also provide important contacts for TAR RNA when bound in the complex together with Tat. Importantly, the in vitro RNA-binding properties of the cyclin T–Tat:TAR complex correlate precisely with the known sequence requirements for TAR-dependent Tat transactivation. In particular, the formation of this complex requires both the activation domain and the ARM of Tat as well as the key sequences in the loop and bulge of the TAR RNA hairpin. Although it has been suggested that the transcriptional coactivator for Tat would be distinct from its TAR RNA-binding cofactor, our results indicate that a single molecule (cyclin T) subserves both functions and that cyclin T provides a direct link between Tat, TAR, and the TAK/P-TEFb elongation factor complex.

The RNA-binding experiments shown here were carried out with Tat and free cyclin T, whereas cyclin T is normally bound to CDK9 in the cell (Figure 3). We have recently found that Tat can also interact with a recombinant cyclin T–CDK9 complex and direct its specific binding to TAR RNA (M. E. G., P. W., and K. A. J., unpublished data). However, CDK9 had no affect on the affinity or RNA-binding specificity of the complex beyond that reported here for cyclin T alone, and CDK9 did not influence the binding of Tat to TAR in the absence of the cyclin. Thus, the HIV-1 TAR RNA element appears to have evolved to recognize Tat when bound to cyclin T in the TAK/P-TEFb complex, rather than free Tat alone. Cooperative binding to TAR RNA might be an important

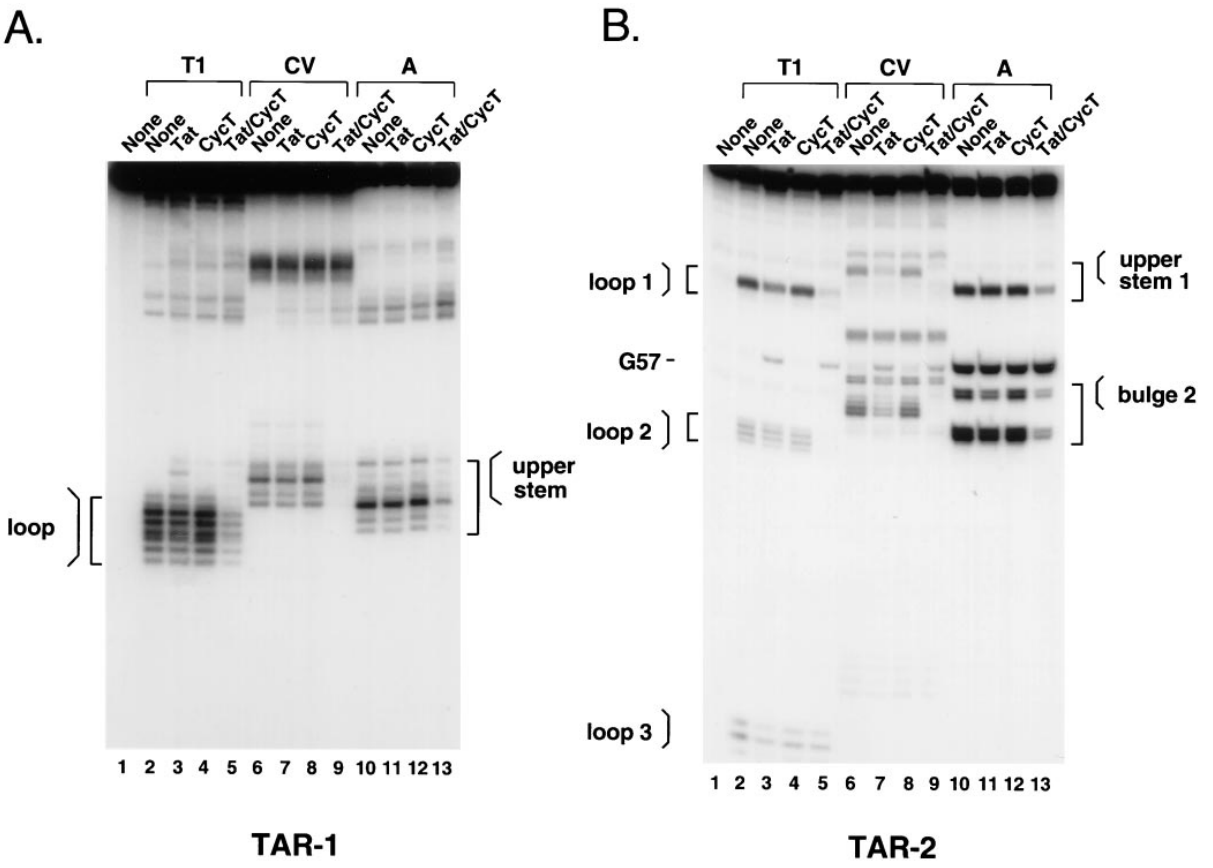

Figure 6. RNase Footprint Analysis of the Binding of Cyclin T and Tat to HIV-1 TAR (TAR-1) AND HIV-2 TAR (TAR-2) RNAs

(A) RNase footprint protection analysis of the binding of cyclin T, Tat, and cyclin T–Tat complex to TAR-1 RNA. Where indicated, the 3′ end-labeled TAR-1 probe was incubated with 400 ng GST-Tat-1 (aa 1–86) and 750 ng GST-cleaved cyclin T. Reactions were treated with single-strand specific RNase T1 (lanes 2–5), double-strand specific RNase CV (lanes 6–9), or single-strand specific RNase A (lanes 10–13). Brackets highlight regions of RNase protection over the loop and upper stem of the TAR-1 stem-loop structure.

(B) RNase footprint analysis of the binding of GST-cleaved cyclin T and GST-Tat-2 (aa 1–99) proteins to TAR-2 RNA. Protein amounts were the same as for (A).

mechanism to ensure that TAR molecules are bound only by Tat proteins that have previously associated with cyclin T–CDK9 complexes in the cell.

It is interesting to note that the activation domain of Tat-1 (aa 1–48) functions as a potent dominant negative inhibitor of the wild-type Tat protein without affecting basal HIV-1 transcription or general elongation, even though cyclin T and the TAK/P-TEFb complex are required for basal transcription from the HIV-1 promoter (Figure 3C; Mancebo et al., 1997; Zhu et al., 1997). These results suggest that Tat binds to a region of cyclin T that is not important for general transcription elongation by the cyclin T–CDK9 complex. Thus, the region of the cyclin that is responsible for binding to Tat (and TAR) may constitute an effective target for the development of new inhibitory compounds that block Tat activity without affecting the general transcription elongation properties of the TAK/P-TEFb complex.

Cyclin T as the TAR RNA-Binding Cofactor for Tat

Truncation of the N-terminal activation domain of Tat greatly enhances its affinity for TAR RNA and reduces the specificity of the Tat:TAR interaction (M. E. G., P. W., and K. A. J., unpublished data), which suggests that the free Tat protein may be configured poorly for binding to RNA in the absence of cyclin T. Consequently, the interaction of Tat with cyclin T may be necessary to alter the structure of Tat for high-affinity binding to TAR RNA. The cooperative binding of Tat and cyclin T to TAR RNA provides a molecular explanation for the long-standing observation that targeting and transactivation through TAR RNA always requires the transactivation domain of Tat, and that this region cannot be substituted by the activation domains of other transcription factors.

The TAR RNA recognition mechanism suggested here may resemble that proposed for other heteromeric RNA-binding protein complexes. For example, it has been shown that the mammalian U2 snRNP proteins, U2A′ and U2B′′, must first interact with each other in order to bind with high-affinity and specificity to U2 snRNA (Scherly et al., 1990a, 1990b). Interestingly, neither protein can recognize the specific U2 snRNA recognition element on its own, although U2B′′ binds RNA in a relatively nonspecific manner through its RNA recognition motif (RRM; Scherly et al., 1990a) and U2A′ interacts weakly with double-stranded RNA (Boelens et al., 1991).

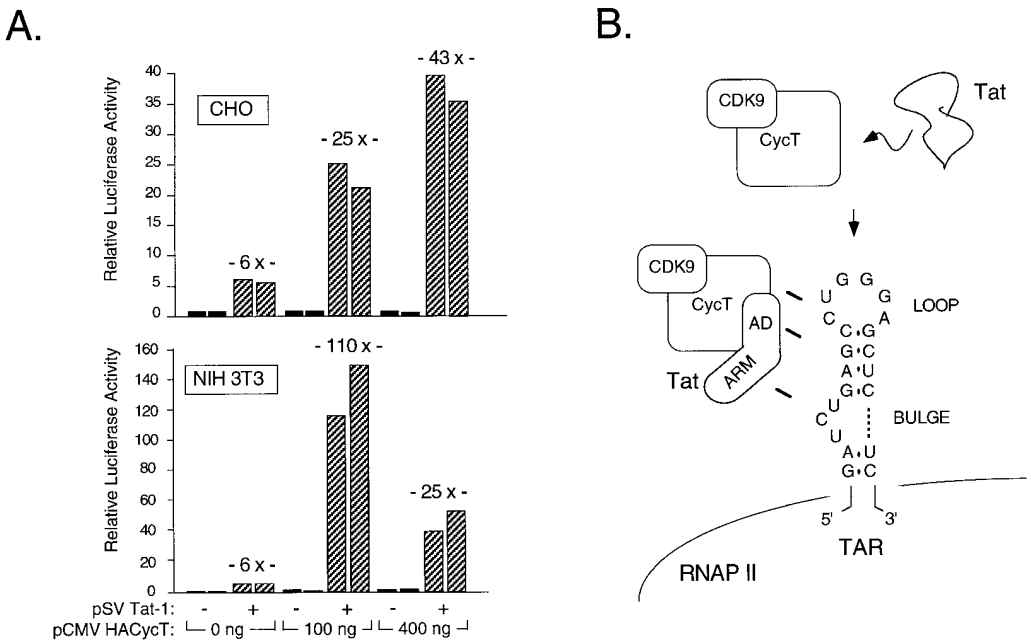

Figure 7. Overexpression of Human Cyclin T Enhances Tat Activity in Murine Cells

(A) Human cyclin T stimulates Tat transactivation in rodent cell lines. NIH3T3 and CHO cells were transfected with 100 ng pHIV-1/LUC, 10 ng pSV-TatZX (−Tat), or pSV-Tat (+Tat), 10 ng of pRL-CMV (control plasmid), and different amounts of pCMV-HACycT, as indicated in the figure. Shown are duplicate transfections from the same experiment. The firefly luciferase activity produced from pHIV-1/LUC was normalized to the Renilla luciferase activity from the pRL-CMV plasmid (Promega). Cells were transfected with Lipofectamine (GIBCO-BRL) and luciferase activity was measured 48 hr posttransfection.

(B) Biochemical view of the interaction of Tat with the cyclin T subunit of the CDK9-containing TAK/P-TEFb complex, and the subsequent cooperative binding of Tat and TAK/P-TEFb to TAR RNA. See text for details.

Formation of the U2A′:U2B′′ complex is thought to alter the conformation of U2B′′ in a manner that greatly enhances its specific binding to the U2 snRNA element and also reduces its nonspecific binding to U1 snRNA.

Tat belongs to a different class of RNA-binding proteins and interacts with RNA through an arginine-rich motif (ARM), and its partner is a cyclin that lacks RNA recognition motifs and displays no intrinsic affinity for RNA. Nevertheless, we envision that the interaction of Tat with cyclin T induces a conformational change in Tat that significantly enhances its affinity and specificity for TAR-1 RNA, and may also inhibit the nonspecific binding of Tat to the bulge region of loop mutant RNAs. The ARM is essential for binding of the cyclin T–Tat complex to TAR and provides important contacts within the bulge of TAR RNA. It is possible that one or more of the conserved aromatic residues in the hydrophobic core of the transactivation domain of Tat might provide important stacking interactions with bases in loop of the TAR hairpin, as has been observed for other RNA-binding proteins that recognize stem-loop structures (Oubridge et al., 1994; for review, see Moras and Poterszman, 1995). Finally, cyclin T may also directly contact TAR RNA within the complex (Figure 7B).

What Is the Role of TAR RNA?

The TAK/P-TEFb elongation factor complex is limiting in extracts, and it has not been established how this complex is recruited to promoters, although the *Drosophila* TAK/P-TEFb is not present in RNAPII complexes prior to transcription (Marshall and Price, 1992, 1995). Tat may function to bypass the normal recruitment mechanism for TAK/P-TEFb by interacting directly with cyclin T and promoting the association of the TAK/P-TEFb complex with RNAPII. Thus, cooperative binding of Tat-TAK/P-TEFb complex to TAR RNA might serve to recruit the elongation factor to the HIV-1 promoter. Because it has been reported that Tat can associate with the RNAPII preinitiation complex (Garcia-Martinez et al., 1997) or the RNAPII holoenzyme (Cujec et al., 1997), it is possible that binding to TAR serves a different function, for example to enhance CDK9 kinase activity or alter the substrate specificity of the kinase. Biochemical studies with immobilized DNA templates have shown that Tat associates with elongating RNAPII only on templates that contain the wild-type TAR element, which is consistent with a role for TAR in recruitment (Keen et al., 1996, 1997). At later times in elongation, Tat binds to the RNAPII complex through protein:protein interactions, and not through its ability to bind nascent TAR RNA (Keen et al., 1997). Consequently, it will be important to determine whether the cyclin T–CDK9 complex associates with Tat in the RNAPII elongation complex, or whether it acts only transiently through TAR RNA, and whether TAK/P-TEFb plays any role in the transfer of Tat from TAR to RNAPII.

Once recruited to the RNAPII transcription complex, CDK9 may activate transcription through its demonstrated ability to hyperphosphorylate the RNAPII CTD, or it may function to phosphorylate other proteins that

regulate elongation in a CTD-dependent manner. Reconstituted transcription systems support efficient elongation of RNAPII transcription in the absence of TAK/P-TEFb (Maldonado and Reinberg, 1995; Shilatifard et al., 1997), however transcription in such systems is not dependent upon the CTD and does not support Tat activation through TAR (Mancebo et al., 1997). Reconstitution of Tat activation in a reconstituted system was shown to require TAK/P-TEFb and a negative inhibitor (Mancebo et al., 1997), which suggests that TAK/P-TEFb may ultimately counteract a negative regulator of transcription elongation. Tat activity is also likely to require other positively acting elongation factors (Zhou and Sharp, 1995, 1996).

The role of the cyclin T–CDK9 complex in Tat-mediated transactivation is novel, although other transcription factors have been shown previously interact with cyclin–CDK complexes as direct substrates for phosphorylation. In an interesting variation on this theme, it was recently shown that a direct interaction between the human estrogen receptor (hER) and cyclin D1 enhances binding of hER to DNA and induces ERE-dependent transcription in the absence of ligand, thereby enabling estrogen-independent growth of breast tumor cells that overexpress cyclin D1 (Zwijsen et al., 1997).

Regulation of TAK/P-TEFb (CTD Kinase) Activity

Cyclin T is a novel human C-type cyclin, most closely related to the essential *S. pombe* Pch1(+) cyclin, which is a partner for CDC2-related proteins (Furnari et al., 1997). In yeast, the closest kinase to CDK9 is CTDK1, a CTD kinase that has been implicated in the control of transcription elongation, although CTDK1 does not mediate the DRB-sensitive step in transcription elongation in yeast extracts (Sterner et al., 1995; Lee and Greenleaf, 1997). Other well-characterized cyclin C–related proteins are also nuclear and have been implicated in transcriptional control, including the cyclin H subunit of TFIIH (Fisher and Morgan, 1994; Fisher et al., 1995) and the yeast SRB10/11 cyclin-kinase pair associated with RNAPII (Liao et al., 1995). Unlike other cyclins, which are differentially regulated throughout the cell cycle, the levels of the C-type cyclins generally do not vary during cell division (Li et al., 1996). However, the C-type cyclins could represent important targets for environmental signaling pathways in the cell. For example, the UME3p (SRB11) cyclin, which has been implicated in transcriptional repression of selected genes, is down-regulated by proteolysis during heat shock (Cooper et al., 1997). Tat-associated CDK9 activity increases upon activation of peripheral blood lymphocytes or differentiation of promonocytic cell lines (Yang et al., 1997), which are conditions that induce viral transcription strongly. Consequently it will be interesting to learn whether the levels of cyclin T protein or mRNA are increased upon T cell activation, and whether the interaction of Tat with cyclin T enhances the stability of the cyclin or TAK/P-TEFb activity. Beyond these specific implications for Tat activity, cyclin T could be regulated under the many varied cellular conditions that have been shown to enhance overall levels of RNAPII CTD hyperphosphorylation, including serum stimulation (Dubois et al., 1994) and the onset of major zygotic gene activation during mammalian development (Bellier et al., 1997).

Species-Specific Regulation of Tat Activity

Interestingly, cyclin T fits several of the criteria that have been established previously for the species-restricted host cell cofactor for Tat (Hart et al., 1993; Alonso et al., 1994). In particular, cyclin T confers upon Tat the ability to bind to TAR RNA in a loop-dependent manner and is encoded on human chromosome 12. Moreover, we find that overexpression of human cyclin T in NIH3T3 and CHO cells significantly enhances Tat activity in vivo (Figure 7A). The identification of proteins that can restore Tat function in rodent cells is an important goal for the development of transgenic mice that can be infected with HIV-1, which would provide a useful system to study the impact of viral infection on the immune system. In addition, it will be important to determine whether the enhancement in Tat activation caused by overexpression of human cyclin T in rodent cells is sufficient to permit virus replication and to assess directly whether the murine cyclin T protein is defective in its ability to interact with Tat or TAR RNA. Further studies characterizing cyclin T should help to elaborate the mechanism of Tat transactivation and may also suggest new approaches to selectively block Tat activity in infected cells.

Experimental Procedures

DNA Constructs

Plasmid pGST-Tat-1 K41A (aa 1–48) was subcloned into the BamHI and SmaI sites of pGEX-2T (Pharmacia), all other GST-Tat vector constructs were obtained from Dr. A. P. Rice (Baylor Univ.). The full-length human cyclin T cDNA was subcloned into the NcoI and HindIII site of pGEX-KG to generate pGST-CycT. Exogenous TAR RNAs for RNA-binding and in vitro competition experiments were prepared from plasmids pH96 WT and pH96 30/33 (+1 to +80) (Sheline et al., 1991). Plasmids pTAR2 WT and pTAR2 LM contain a single copy of the wild-type or double loop mutant of TAR-2 RNA (+1 to +123), respectively, and were subcloned into the HindIII and BamHI sites of pSP64 polyA vector (Promega). pCMV-HA-CycT encodes the cyclin T gene lacking the PEST sequence (aa 1–708) as a XbaI-BamHI fragment in pCGN.

Purification of p87 (CycT) from HeLa Nuclear Extract

Protein affinity selection with GST-Tat-1 (aa 1–48) and in vitro kinase assays were as described by Herrmann and Rice (1993), with a modified washing in buffer D (20 mM Tris-HCl [pH 8.0], 0.2 mM EDTA, 150 mM KCl, 0.5% NP-40, 0.05% SDS, 20% glycerol, 2 mM dithiothreitol, and 0.2 mM PMSF). These reactions were scaled up 80-fold, and 1 μg (110 pmol) of human cyclin T protein was isolated on a preparative SDS-PAGE (6%), transferred to a PVDF membrane (MSI), and stained with amido black. A detailed protocol is available from the authors upon request. HPLC-purified tryptic fragments of p87 were subjected to peptide sequencing using the Perkin Elmer ABI 470 and Procise 494 systems.

Cloning of Cyclin T cDNA

The I.M.A.G.E. Consortium Clone (ID 111460) containing an 0.9 kb cDNA fragment of the gene (aa 236–481) encoding human cyclin T was obtained from the American Type Culture Collection. The cDNA insert was sequenced on both strands and used to design primers for 3′ RACE PCR. The 0.8 kb 3′ RACE PCR products (aa 482–726) were subcloned in the EcoRI site of pGEM-7Z(f) and sequenced. The full-length cyclin T gene was obtained by screening one million phage from a λZAPII Jurkat cDNA library (Waterman et al., 1991) using probes from the 0.9 kb cDNA and the 0.8 kb 3′ RACE PCR

product. Probes for screening were labeled by random priming to a specific activity of 1×10^9 dpm/μg. Positive plaques were purified by rescreening, and the clone with the longest insert (7.2 kb) was subjected to automatic DNA sequencing.

Protein–Protein Interaction Experiments

Reactions containing 4 μg of the wild-type or mutant GST-Tat proteins were incubated with 1 μg of thrombin-cleaved cyclin T protein in 500 μl binding buffer (20 mM HEPES [pH 7.9], 0.5% NP-40, 1% Triton X-100, 0.7% beta-mercaptoethanol, 0.1% BSA) containing 200 mM KCl, at 4°C for 4 hr. Reactions were then incubated for 30 min with 10 μl of glutathione beads and washed three times with binding buffer containing 1 M KCl.

Preparation and Use of Polyclonal Antibodies

For the production of polyclonal antibodies specific to cyclin T, a His-p86II (aa 483–701) antigen was produced as a hexahistidine fusion protein using the pET-28a(+) expression plasmid (Novagen), and the antigen was used to raise polyclonal antiserum (Pocono Rabbit Farm and Laboratory, Inc.). For immunoprecipitation reactions, aliquots of 50 μl (750 μg) of HeLa nuclear extract were diluted 1:4 with IP buffer (20 mM Tris-HCl [pH 7.9], 0.5% NP-40, 1% Triton X-100, 5 mM DTT) containing 150 mM KCl, and the reactions were incubated with either 1 μg of anti-CDK9 IgG (PITALRE-CT, Santa Cruz Biotechnology), or with 1 μl of preimmune or anti-cyclin T antisera, at 4°C for 4 hr. The reactions were mixed with 10 μl of Protein A–Sepharose, incubated at 4°C for 1 hr, and washed five times with 1 ml aliquots of IP buffer containing 1 M KCl. Transcription extacts were depleted of cyclin T using His-tagged p86 affinity-purified polyclonal antiserum.

Preparation of TAR RNA and RNA-Binding Experiments

TAR-1 RNAs were synthesized using T7 RNA polymerase from Hind III-digested pH96 WT and pH96 30/33, and TAR-2 RNAs were transcribed with SP6 RNA polymerase after linearization of pTAR2WT or pTAR2LM with BamHI (Sheline et al., 1991). Large-scale TAR synthesis was performed in a 0.4 ml final reaction volume containing RNA synthesis buffer (40 mM Tris-HCl [pH 8.0], 2 mM spermidine, 20 mM DTT, 6 mM $MgCl_2$), 0.5 mM of each of the ribonucleoside triphosphates (rNTPs), 20 pmol linear DNA template, 0.8 U/μl T7 or Sp6 RNA polymerase (Ambion), and 100 U RNasin (USB). Reactions were incubated at 37°C for 2 hr. For synthesis of high specific-activity TAR RNAs for use in gel mobility shift experiments, 80 μl reactions were prepared with 1 pmol of linear DNA templates in RNA synthesis buffer containing 20 mM rUTP, ^{32}P-UTP (30 μCi, 800 Ci/mmol, 20 μCi/μl, Amersham), and 0.5 mM each of rATP, rGTP, and rCTP. Reactions were incubated at 37°C for 1 hr, and the DNA template was treated with 2 U DNase I (Promega) per μg DNA, extracted with a phenol:chloroform mixture, and precipitated with ethanol. The RNA pellet was dissolved in 0.1 M NaCl and applied to a G-50 spin column (Boehringer) prior to use.

TAR RNAs used for the RNase footprint experiment were 3′ end-labeled with T4 RNA ligase and [5′-^{32}P]pCp as described previously (Churcher et al., 1993). Reactions (20 μl) contained 50 mM Tris-HCl (pH 8.0), 3 mM DTT, 10 mM $MgCl_2$, 25 mM NaCl, 50 mM ATP, 200 pmol TAR RNA, 65 μCi [^{32}P]pCp (3000 Ci/mmol, Andotek), 40 U T4 RNA ligase (NEB), 80 U RNasin (USB), 25 μg/ml BSA, and 10% DMSO. Following incubation overnight at 4°C, the RNA was extracted, precipitated, and passed through a G-50 spin column, and typically contained 2.5×10^4 cpm/pmol RNA. End-labeled TAR probes were purified on a 6% denaturing polyacrylamide gel and eluted prior to use.

Gel mobility shift reactions (16 μl final reaction volume) were carried out in binding buffer (30 mM Tris-HCl [pH 8.0], 12% glycerol, 70 mM KCl, 1.3 mM DTT, 0.01% NP-40, 5.5 mM $MgCl_2$) and contained 5 ng of labeled TAR RNA as well as 850 ng d(I-C) and 500 ng r(I-C) as competitor RNAs. Reactions were incubated for 10 min at 30°C, and RNA-binding complexes were separated on a pre-run 4% Tris-Glycine gel (7 watts, 2.5 hr at 4°C). RNase footprint reactions (16 μl) were carried out with 2.5 ng of 3 end-labeled TAR RNA under the conditions listed above with 1 μg tRNA added as additional competitor. RNA-binding complexes were equilibrated for 10 min at 30°C and treated with 1 μl of either RNase T1 (0.3 U, Boehringer), RNase CV (0.035 U, Pharmacia), or RNase A (0.6 ng, Boehringer), on ice for 15 min. Reactions were terminated with 85 μl of stop buffer (100 mM Tris-HCl [pH 8.0], 1% Sarkosyl, 100 mM NaCl, 10 mM EDTA, and 25 μg/ml tRNA), prior to analysis on 12% denaturing polyacrylamide gels.

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References

Alonso, A., Cujec, T., and Peterlin, B. (1994). Effects of human chromosome 12 on interactions between Tat and TAR of human immunodeficiency virus type 1. J. Virol. *66*, 6505–6513.

Bellier, S., Chastant, S., Adenot, P., Vincent, M., Renard, J., and Bensaude, O. (1997). Nuclear translocation and carboxyl-terminal domain phosphorylation of RNA polymerase II delineate the two phases of zygotic gene activation in mammalian embryos. EMBO J. *16*, 6250–6262.

Boelens, W., Scherly, D., Beijer, R., Jansen, E., Dathan, N., Mattaj, I., and van Venrooij, W. (1991). A weak interaction between the U2A′ protein and the U2 snRNA helps to stablilize their complex with the U2B′′ protein. Nucleic Acids Res. *19*, 455–460.

Churcher, M., Lamont, C., Hamy, F., Dingwall, C., Green, S., Lowe, A., Butler, P., Gait, M., and Karn, J. (1993). High affinity binding of TAR RNA by the human immunodeficiency virus type-1 tat protein requires base-pairs in the RNA stem and amino acid residues flanking the basic region. J. Mol. Biol. *230*, 90–110.

Cooper, K., Mallory, M., Smith, J., and Strich, R. (1997). Stress and developmental regulation of the yeast C-type cyclin Ume3p (Srb11p/Ssn8p). EMBO J. *16*, 4665–4675.

Cujec, T., Cho, H., Maldonado, E., Meyer, J., Reinberg, D., and Peterlin, B. (1997). The human immunodeficiency virus transactivator Tat interacts with the RNA polymerase II holoenzyme. Mol. Cell. Biol. *17*, 1817–1823.

Cullen, B. (1993). Does HIV-1 Tat induce a change in viral initiation rights? Cell *73*, 417–420.

Dubois, M., Nguyen, V., Dahmus, M., Pages, G., Pouyssegur, J., and Bensaude, O. (1994). Enhanced phosphorylation of the C-terminal domain of RNA polymerase II upon serum stimulation of quiescent cells: possible involvement of MAP kinases. EMBO J. *13*, 4787–4797.

Fisher, R., and Morgan, D. (1994). A novel cyclin associates with MO15/CDK7 to form the CDK-activating kinase. Cell *78*, 713–724.

Fisher, R., Jin, P., Chamberlin, H., and Morgan, D. (1995). Alternative mechanisms of CAK assembly require an assembly factor or an activating kinase. Cell *83*, 47–57.

Furnari, B., Russell, P., and Leatherwood, J. (1997). Pch1(+), a second essential C-type cyclin gene in Schizosaccharomyces pombe. J. Biol. Chem. *272*, 12100–12106.

Gait, M.J., and Karn, J. (1993). RNA recognition by the human immunodeficiency virus Tat and Rev proteins. Trends Biochem. Sci. *18*, 255–259.

Garcia-Martinez, L., Ivanov, D., and Gaynor, R. (1997). Association of Tat with purified HIV-1 and HIV-2 transcription preinitiation complexes. J. Biol. Chem. *272*, 6951–6958.

Ghosh, S., Selby, M., and Peterlin, B. (1993). Synergism between Tat and VP16 in trans-activation of HIV-1 LTR. J. Mol. Biol. *234*, 610–619.

Hart, C., Galphin, J., Westhafer, M., and Schochetman, G. (1993).

TAR loop-dependent human immunodeficiency virus trans activation requires factors encoded on human chromosome 12. J. Virol. *67*, 5020–5024.

Herrmann, C., and Rice, A. (1993). Specific interaction of the human immunodeficiency virus Tat proteins with a cellular protein kinase. Virol. *197*, 601–608.

Herrmann, C., and Rice, A. (1995). Lentivirus Tat proteins specifically associate with a cellular protein kinase, TAK, that hyperphosphorylates the carboxyl-terminal domain of the large subunit of RNA polymerase II: candidate for a Tat cofactor. J. Virol. *69*, 1612–1620.

Jones, K.A. (1997). Taking a new TAK on Tat transactivation. Genes Dev. *11*, 2593–2599.

Jones, K.A., and Peterlin, B.M. (1994). Control of RNA initiation and elongation at the HIV-1 promoter. Annu. Rev. Biochem. *63*, 717–743.

Keen, N., Gait, M., and Karn, J. (1996). Human immunodeficiency virus type-1 Tat is an integral component of the activated transcription-elongation complex. Proc. Natl. Acad. Sci. USA *93*, 2505–2510.

Keen, N., Churcher, M., and Karn, J. (1997). Transfer of Tat and release of TAR RNA during the activation of the human immunodeficiency virus type-1 transcription elongation complex. EMBO J. *16*, 5260–5272.

Lee, J., and Greenleaf, A. (1997). Modulation of RNA polymerase II elongation efficiency by C-terminal heptapeptide repeat domain kinase I. J. Biol. Chem. *272*, 10990–10993.

Li, H., Lahti, J., and Kidd, V. (1996). Alternatively spliced cyclin C mRNA is widely expressed, cell cycle regulated, and encodes a truncated protein. Oncogene *13*, 705–712.

Liao, S.-M., Zhang, J., Jeffery, D., Koleske, A., Thompson, C., Chao, D., Viljoen, M., and Vuuren, H., and Young, R. (1995). A kinase-cyclin pair in the RNA polymerase II holoenzyme. Nature *374*, 193–196.

Luo, Y., and Peterlin, B. (1993). Juxtaposition between activation and basic domains of human immunodeficiency virus type 1 Tat is required for optimal interactions between Tat and TAR. J. Virol. *67*, 3441–3445.

Luo, Y., Madore, S., Parslow, T., Cullen, B., and Peterlin, B. (1993). Functional analysis of interactions between Tat and the trans-activation response element of human immunodeficiency virus type 1 in cells. J. Virol. *67*, 5617–5622.

Madore, S., and Cullen, B. (1993). Genetic analysis of the cofactor requirement for human immunodeficiency virus type 1 Tat function. J. Virol. *67*, 3703–3711.

Maldonado, E., and Reinberg, D. (1995). News on initiation and elongation of transcription by RNA polymerase II. Curr. Opin. Cell. Biol. *7*, 352–361.

Mancebo, H., Lee, G., Flygare, J., Tomassini, J., Luu, P., Zhu, Y., Blau, C., Hazuda, D., Price, D., and Flores, O. (1997). P-TEFb kinase is required for HIV Tat transcriptional activation in vivo and in vitro. Genes Dev. *11*, 2633–2644.

Marshall, N., and Price, D. (1992). Control of formation of two distinct classes of RNA polymerase II elongation complexes. Mol. Cell. Biol. *12*, 2078–2090.

Marshall, N., and Price, D. (1995). Purification of P-TEFb, a transcription factor required for the transition into productive elongation. J. Biol. Chem. *270*, 12335–12338.

Marshall, N., Peng, J., Xie, Z., and Price, D. (1996). Control of RNA polymerase II elongation potential by a novel carboxyl-terminal domain kinase. J. Biol. Chem. *271*, 27176–27183.

Moras, D., and Poterszman, A. (1995). Diverse modes of recognition. Curr. Biol. *5*, 249–251.

Oubridge, C., Ito, N., Evans, P., Teo, C.-H., and Nagai, K. (1994). Crystal structure at 1.92Å resolution of the RNA-binding domain of the U1A spliceosomal protein complexed with an RNA hairpin. Nature *372*, 432–438.

Rechsteiner, M., and Rogers, S. (1996). PEST sequences and regulation by proteolysis. Trends Biochem. Sci. *21*, 267–271.

Scherly, D., Boelens, W., Dathan, N., van Venrooij, W., and Mattaj, I. (1990a). Major determinants of the specificity of interaction between small nuclear ribonucleoproteins U1A and U2B′′ and their cognate RNAs. Nature *345*, 502–506.

Scherly, D., Dathan, N., Boelens, W., van Venrooij, W., and Mattaj, I. (1990b). The U2B′′ RNP motif as a site of protein–protein interaction. EMBO J. *9*, 3675–3681.

Sheline, C.T., Milocco, L.M., and Jones, K.A. (1991). Two distinct nuclear transcription factors recognize loop and bulge residues of the HIV-1 TAR RNA hairpin. Genes Dev. *5*, 2508–2520.

Shilatifard, A., Conaway, J., and Conaway, R. (1997). Mechanism and regulation of transcriptional elongation and termination by RNA polymerase II. Curr. Opin. Genet. Dev. *7*, 199–204.

Sterner, D., Lee, J., Hardin, S., and Greenleaf, A. (1995). The yeast carboxy-terminal repeat domain kinase CTDK-I is a divergent cyclin-cyclin-dependent kinase complex. Mol. Cell. Biol. *15*, 5716–5724.

Tiley, L.S., Madore, S.J., Malim, M.H., and Cullen, B.R. (1992). The VP16 transcription activation domain is functional when targeted to a promoter-proximal RNA sequence. Genes Dev. *6*, 2077–2087.

Waterman, M.L., Fischer, W.H., and Jones, K.A. (1991). A thymus-specific member of the HMG protein family regulates the human T cell receptor C alpha enhancer. Genes Dev. *5*, 656–669.

Yang, X., Herrmann, C., and Rice, A. (1996). The human immunodeficiency virus Tat proteins specifically associate with TAK in vivo and require the carboxyl-terminal domain of RNA polymerase II for function. J. Virol. *70*, 4576–4584.

Yang, X., Gold, M.O., Tang, D.N., Lewis, D.E., Aguilar-Cordova, E., Rice, A.P., and Herrmann, C.H. (1997). TAK, an HIV Tat-associated kinase, is a member of the cyclin-dependent family of protein kinases and is induced by activation of peripheral blood lymphocytes and differentiation of promonocytic cell lines. Proc. Natl. Acad. Sci. USA *94*, 12331–12336.

Zhou, Q., and Sharp, P. (1995). Novel mechanism and factor for regulation by HIV-1 Tat. EMBO J. *14*, 321–328.

Zhou, Q., and Sharp, P. (1996). Tat-SF1: cofactor for stimulation of transcriptional elongation by HIV-1 Tat. Science *274*, 605–610.

Zhu, Y., Pe'ery, T., Peng, J., Ramanathan, Y., Marshall, N., Marshall, T., Amendt, B., Mathews, M., and Price, D. (1997). Transcription elongation factor P-TEFb is required for HIV-1 Tat transactivation in vitro. Genes Dev. *11*, 2622–2632.

Zwijsen, R., Wientjens, E., Klompmaker, R., van der Sman, J., Bernards, R., and Michalides, R. (1997). CDK-independent activation of the estrogen receptor by cyclin D1. Cell *88*, 405–415.

GenBank Accession Number

The accession number for the human *CycT* gene reported in this paper is AF045161.

Note Added in Proof

The cDNA for human cyclin T has been independently isolated by D. H. Price and colleagues from studies of the cyclin partners for CDK9 (D. H. Price, personal communication).