*Proc. Natl. Acad. Sci. USA*
Vol. 96, pp. 1285–1290, February 1999
Biochemistry

# Interactions between human cyclin T, Tat, and the transactivation response element (TAR) are disrupted by a cysteine to tyrosine substitution found in mouse cyclin T

KOH FUJINAGA*, RAN TAUBE*, JÖRG WIMMER*, THOMAS P. CUJEC, AND B. MATIJA PETERLIN†

Howard Hughes Medical Institute, Departments of Medicine, Microbiology, and Immunology, University of California, San Francisco, CA 94143-0703

*Edited by Malcolm A. Martin, National Institutes of Health, Bethesda, MD, and approved December 21, 1998 (received for review October 29, 1998)*

**ABSTRACT** **The transcriptional transactivator Tat from HIV binds to the transactivation response element (TAR) RNA to increase rates of elongation of viral transcription. Human cyclin T supports these interactions between Tat and TAR. In this study, we report the sequence of mouse cyclin T and identify the residues from positions 1 to 281 in human cyclin T that bind to Tat and TAR. Mouse cyclin T binds to Tat weakly and is unable to facilitate interactions between Tat and TAR. Reciprocal exchanges of the cysteine and tyrosine at position 261 in human and mouse cyclin T proteins also render human cyclin T inactive and mouse cyclin T active. These findings reveal the molecular basis for the restriction of Tat transactivation in rodent cells.**

HIV encodes the transcriptional transactivator Tat that is essential for viral replication (1, 2). Tat binds to the transactivation response element (TAR) RNA, which forms a stem-loop downstream from the site of initiation of transcription in the 5′ long terminal repeat (3, 4). *In vitro* binding studies revealed that Tat interacts with the 5′ bulge from positions +23 to +25 in TAR. However, the central loop from positions +31 to +36 in TAR also is required for Tat transactivation *in vivo* (3, 4). Thus, a cellular protein that binds to Tat and the central loop in TAR is required for optimal effects of Tat in cells. Additional evidence in support of the loop-binding protein comes from rodent cells, such as the Chinese hamster ovary (CHO) cells, that do not support efficient Tat transactivation. Complementation studies using CHO:human somatic cell hybrids revealed that a protein encoded on the human chromosome 12 provides this function for the central loop in TAR (5–7).

These initial observations gained strong support with the identification of human cyclin T, which binds to the activation domain of Tat and the central loop in TAR (8). Cyclin T, as well as cyclin T2a and T2b proteins, forms a complex with the cyclin-dependent kinase 9 (CDK9), which is called the positive transcription elongation factor b (P-TEFb) (9). The positive transcription elongation factor b hyperphosphorylates the C-terminal domain of RNA polymerase II, which is required for Tat transactivation (10–15). Not only did human cyclin T increase the binding of Tat to TAR *in vitro*, but its introduction into rodent cells led to higher levels of Tat transactivation (8). Moreover, cyclin T was mapped to the human chromosome 12. It is the only C-type cyclin that mediates interactions between Tat and TAR and supports virus expression and particle production in CHO cells (J.W. and B.M.P., unpublished data).

Although human cyclin T is the loop-binding cofactor of Tat, it is unclear which region of the protein mediates interactions between Tat and TAR. Additionally, the rodent cyclin T, which does not support Tat transactivation, should reveal residues required for these interactions. To address these issues, we first cloned mouse cyclin T and compared its amino acid sequence to that of human cyclin T. Second, we demonstrated that N-terminal 281 amino acids of human cyclin T support interactions between Tat and TAR and are sufficient for Tat transactivation in cells. The substitution of the cysteine at position 261 to the tyrosine found in mouse cyclin T abolished these interactions *in vitro* and failed to increase Tat transactivation *in vivo*. Finally, the reciprocal exchange rendered mouse cyclin T fully functional for effects of Tat in cells.

## MATERIALS AND METHODS

**Cloning of Mouse Cyclin T (mT).** Mouse cDNA was synthesized from total RNA extracted from NIH 3T3 cells by reverse transcription using random hexamer primers. PCR reactions were performed on this cDNA by using degenerate primers (5′NNTCTAGAACCATGGARGGNGARAGRAARAAYAAYAAYAAR, 5′NNGGATCCNTYAYTTNGGNAGNGGNGGNAGNGGNGGNGGNGG), which were designed from the sequence of the termini of human cyclin T (8, 9). The resulting 2.2-kilobase cDNA fragment was cloned into the PCR2.1 TOPO vector (Invitrogen), resulting in the plasmid pmT724, which was subjected to dideoxynucleotide DNA sequencing.

**Plasmid Constructions.** The plasmid reporter pHIVSCAT and the plasmid effector pcDNA3-Tat have been described elsewhere (16). To construct plasmids coding for glutathione *S*-transferase (GST)-fusion proteins, the indicated fragments were amplified by PCR from phT726 (9) or pmT724 (see above) by using appropriate primers with *Bam*HI and *Eco*RI linkers. Amplified fragments were inserted into *Bam*HI and *Eco*RI sites of pGEX-2TK vector (Amersham Pharmacia). The plasmids phT726K265M, phT372A269P, phT281K277N, phT281C261Y, and pmT724Y261C were made by introducing amino acid substitutions or stop codons into phT726 or pmT724 by using the Transformer Site-Directed Mutagenesis Kit (CLONTECH). The hemagglutinin tag was added to some of these cyclin T proteins. phT227 was constructed by removing the *Xcm*I fragment from phT726. All mutations were confirmed by DNA sequencing. Sequences of oligonucleotide primers are available on request.

**Protein Purification.** Hybrid GST-human cyclin T proteins were expressed in DH5$\alpha$ strain of *Escherichia coli* and were purified by using glutathione-Sepharose beads (Amersham

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

PNAS is available online at www.pnas.org.

This paper was submitted directly (Track II) to the *Proceedings* office.
Abbreviations: TAR, transactivation response element; CHO, Chinese hamster ovary; GST, glutathione *S*-transferase.
*K.F., R.T., and J.W. contributed equally to this work.
Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF113951).
†To whom reprint requests should be addressed at: University of California, San Francisco, Box 0703, San Francisco, CA 94143-0703. e-mail: matija@itsa.ucsf.edu.

Pharmacia) with a modified lysis buffer (buffer A) containing 50 mM Hepes (pH 7.8), 100 mM KCl, 1% Triton X-100, 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 2 mg/ml lysozyme (17). Expressed proteins were eluted from glutathione-Sepharose beads by using 10 mM glutathione and were subjected to dialysis against buffer B containing 30 mM Tris·HCl (pH 8.0), 70 mM KCl, and 1 mM DTT. The purity of eluted proteins was examined by Coomassie blue staining of SDS/PAGE. Their concentrations were determined by a protein assay kit (Bio-Rad).

***In Vitro* Binding Assays.** Binding assays between different hybrid GST-cyclin T proteins and Tat were performed as follows: 5 $\mu$g of each chimera were incubated with $^{35}$S-labeled Tat expressed in the coupled transcription and translation system from the rabbit reticulocyte lysate (T7 TNT system, Promega) in 300 $\mu$l of buffer C (20 mM Hepes, pH 7.9/1% Triton X-100/0.5% Nonidet P-40/0.3% BSA/2 mM DTT/200 mM KCl/0.5 mM $ZnCl_2$/0.5% SDS) at 4°C for 3 hr. After the binding, 20 $\mu$l of glutathione Sepharose beads were added to the reaction and were incubated for an additional 30 min. Beads were washed extensively three times with buffer C containing 1M KCl. Bound proteins were eluted with equal volume of 2× SDS-loading buffer, were resolved by 12% SDS/PAGE, and were analyzed by autoradiography.

**Transient Transfections and CAT Assays.** CHO cells were cotransfected with pHIVSCAT (0.1 $\mu$g) and pcDNA3-Tat (0.1 $\mu$g) in the presence or absence of phT or pmT plasmids (1 $\mu$g) with Lipofectamine as recommended by the manufacturer (GIBCO/BRL). All transfections were balanced to a total 1.2 $\mu$g of DNA with pcDNA3. Forty-eight hours after the trans-

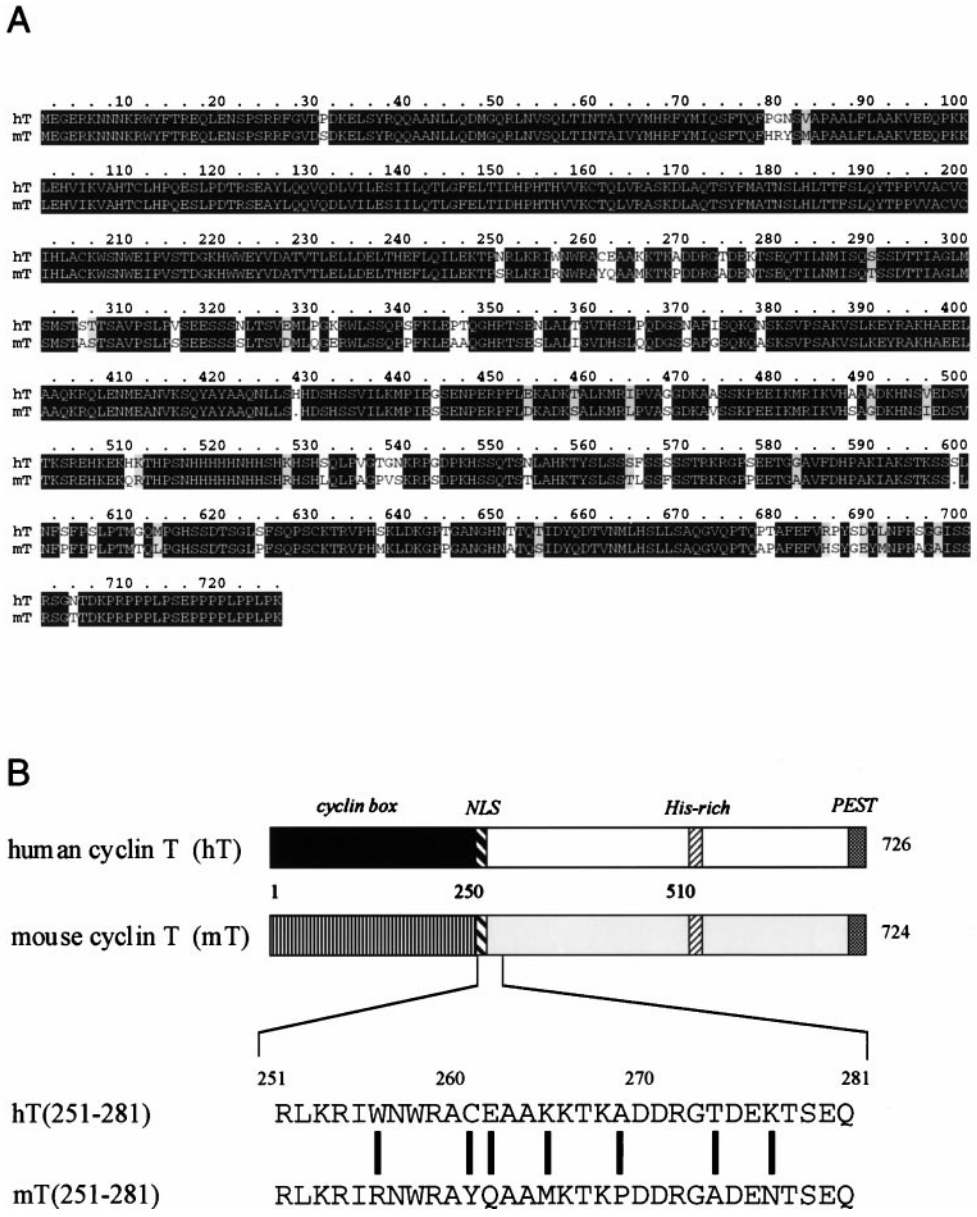


FIG. 1. Comparison of human and mouse cyclin T proteins. (*A*) Alignment of sequences from the human and mouse cyclin T proteins. Identical and similar amino acids are depicted on black and gray backgrounds, respectively. These two cyclins share 90% sequence identity. (*B*) Schematic representation of cyclin T proteins. Of note are duplicated cyclin boxes from positions 1 to 250, the putative nuclear localization signal (NLS) from positions 251 to 255, the histidine-rich region (His-rich) from positions 506 to 530, and the C-terminal PEST sequence from positions 709 to 726. The shading between these cyclins is slightly different for easier reference in Figs. 2 and 3. Sequences flanking cyclin boxes from positions 251 to 281 are presented below the schematic representation. Seven amino acids differ between the human and mouse cyclin T. Of these, the cysteine, lysine, alanine, and lysine at positions 261, 265, 269, and 277 in human cyclin T were changed individually to tyrosine, methionine, proline, and asparagine from mouse cyclin T, respectively (see below).

fection, cells were lysed in buffer D (250 mM Tris·HCl, pH 7.5/0.1% Triton X-100), and CAT activities were measured by a liquid scintillation assay as described (16). Western blotting was performed on some of these lysates with the antihemagglutinin antibody (12CA5)(18).

**Electrophoretic Mobility-Shift Assays.** $^{32}$P-labeled TAR and TAR lacking the 5′ bulge sequence (Δbulge) or the central loop (Δloop) were prepared by *in vitro* transcription of linearized plasmid templates as described (16). TAR RNA was incubated with 0.2 μg of cyclin T expressed in *E. coli* (Fig. 3) or 5 μl of cyclin T synthesized in the coupled transcription and translation system from the reticulocyte lysate (Fig. 5) in the absence or presence of 80 ng of purified Tat (18) in buffer E [30 mM Tris·HCl, pH8.0/70 mM KCl/0.01% Nonidet P-40/5.5 mM $MgCl_2$/1 mM DTT/13% glycerol/53 μg/ml poly(dI-)poly(dC)/31 μg/ml poly(I)poly(C)] for 10 min at 30°C. A 100-fold excess of cold competitor RNA was used as indicated. RNA–protein complexes were separated by a 6% nondenaturing polyacrylamide gel (4 watts, 3 hr at 4°C). Gels were dried and analyzed by autoradiography.

## RESULTS

**The Isolation of Mouse Cyclin T.** Previous studies demonstrated a block to Tat transactivation in rodent cells, which could be rescued by introducing the human chromosome 12 (6, 7) or human cyclin T (8) into these cells. Thus, specific changes between the human and mouse cyclin T proteins should determine their roles in Tat transactivation. To this end, we cloned mouse cyclin T by using PCR, degenerate oligonucleotides, and sequences from expressed sequence tags and compared its sequence to that of human cyclin T. As presented in Fig. 1*A*, these proteins share 90% sequence identity, which is highest in duplicated cyclin boxes from positions 1 to 250 (8). Identical amino acids are depicted within the black background. Changed residues are presented in gray and white backgrounds (Fig. 1*A*). This comparison helped us to identify which residues in human cyclin T could mediate interactions between Tat and TAR (Fig. 1*B* and see below). Highlighted are residues just C-terminal to cyclin boxes, where seven amino acids differ between the human and mouse cyclin T proteins (Fig. 1*B*).

**Tat Binds to Residues from Positions 1 to 300 in Human Cyclin T.** To determine whether Tat binds to human cyclin T, binding assays between these proteins were performed *in vitro* (Fig. 2). The hybrid GST-human cyclin T protein obtained from *E. coli* was incubated with $^{35}$S-labeled Tat, which was expressed by using the coupled transcription and translation system from the rabbit reticulocyte lysate. We compared the binding of human cyclin T to Tat and two mutant Tat proteins,

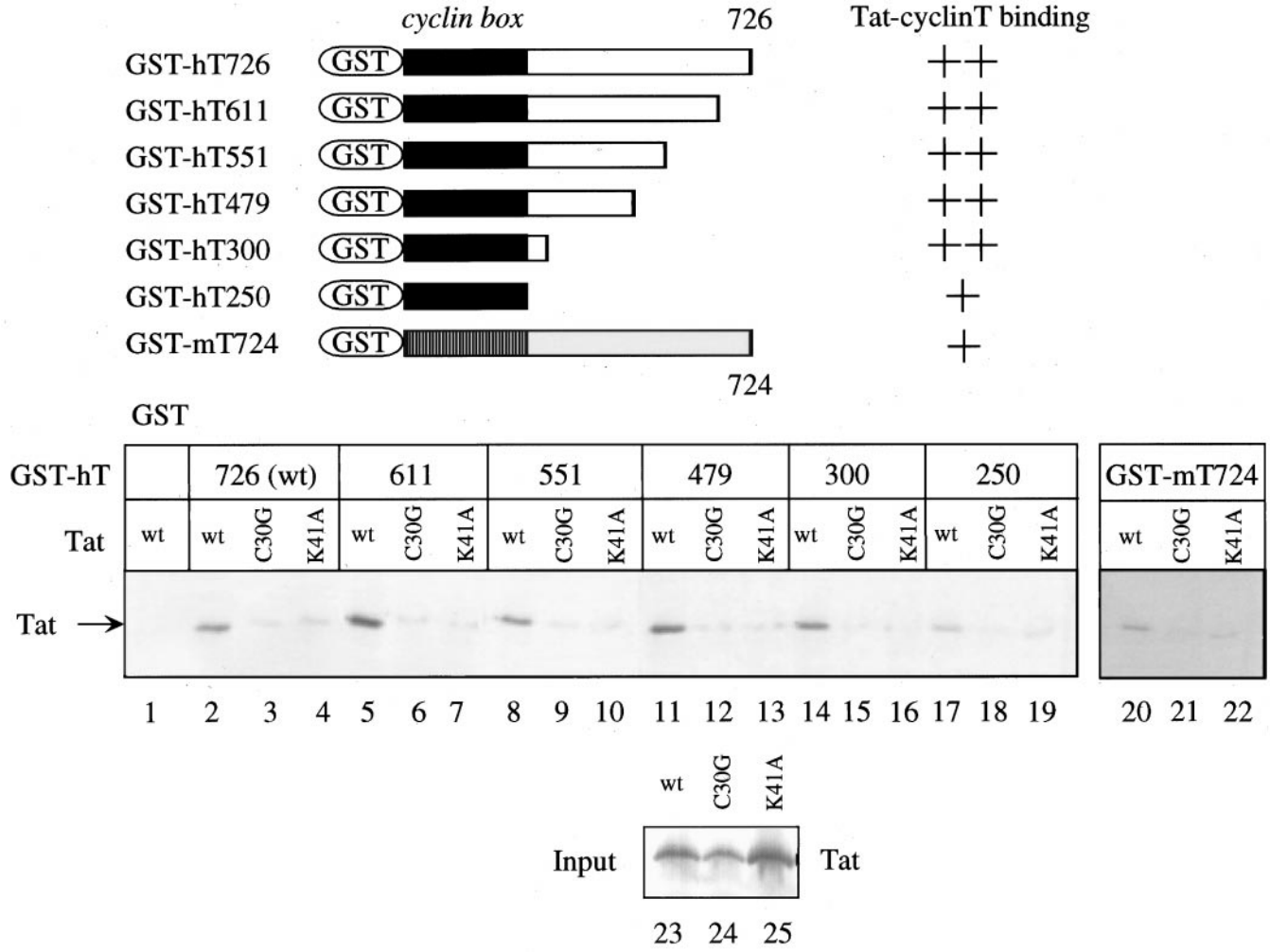


FIG. 2. N-terminal 300 residues in human cyclin T mediate its specific binding to Tat, and mouse cyclin T binds weakly to Tat. Wild-type and mutant $^{35}$S-labeled Tat proteins were incubated with GST and hybrid GST-cyclin T proteins and were selected on glutathione-Sepharose beads. Bound proteins were revealed by SDS/PAGE followed by autoradiography. Besides full length human [GST-hT726, wild-type (wt)] and mouse cyclin T proteins (GST-mT724), C-terminal truncations of human cyclin T to positions 611 (GST-hT611), 551 (GST-hT551), 479 (GST-hT479), 300 (GST-hT300), and 250 (GST-hT250) also were examined. These truncations and the summary of the binding data are depicted on the top. Mutant Tat proteins contained a substitution of the cysteine to glycine at position 30 (TatC30G) and of the lysine to alanine at position 41 (TatK41A) in the activation domain of Tat, which render Tat inactive. The input of Tat, mutant Tat proteins, and chimeras was equal in all reactions (lanes 23 to 25).

where the cysteine at position 30 (C30G) and the lysine at position 41 (K41A) in the activation domain of Tat were changed to glycine and alanine, respectively. Complexes containing cyclin T and Tat were isolated by adding glutathione-Sepharose beads to the reaction, followed by extensive washing under stringent conditions (1M KCl). Retained proteins were visualized by autoradiography.

As presented in Fig. 2, human cyclin T (hT726) binds efficiently to Tat (lane 2) but not mutant Tat proteins (lanes 3 and 4), confirming the specificity of the interaction between human cyclin T and Tat. Because Tat does not function well in rodent cells (6, 7), we also examined the binding of Tat to mouse cyclin T (mT724) (Fig. 2, lanes 20–22). We found that mT724 binds specifically to Tat but not mutant Tat proteins. However, the binding of mouse cyclin T to Tat was ≈4-fold weaker (Fig. 2, compare lanes 2 and 20).

To define sequences in human cyclin T that interact with Tat, further binding studies were performed by using a series of truncated hybrid GST-human cyclin T proteins (Fig. 2, lanes 5–19). The specific binding of human cyclin T to Tat was detected with all C-terminal truncations up to position 300 (Fig. 2, lanes 5, 8, 11, and 14). No diminution of binding occurred when 426 C-terminal residues were removed from human cyclin T. However, a further truncation of 50 amino acids to position 250 reduced the binding of human cyclin T to Tat to levels observed with mouse cyclin T (Fig. 2, compare lanes 17–20). No detectable binding was observed with GST alone (Fig. 2, lane 1). Additionally, no specific binding occurred between Tat and residues from positions 300 to 726 in human cyclin T (data not presented). These findings indicate that cyclin boxes and adjacent 50 amino acids are responsible for the binding of human cyclin T to the activation domain of Tat and that mouse cyclin T might lack critical residues in this region.

**Interactions Between Tat and TAR Require Residues from Positions 1 to 300 in Human Cyclin T.** Human cyclin T increases the binding of Tat to TAR *in vitro* (8, 19). To map the region in human cyclin T that is responsible for these interactions, we performed electrophoretic mobility-shift assays using purified GST-cyclin T fusion proteins, Tat, and $^{32}$P-labeled wild-type or mutant TAR transcripts. At first, full length human and mouse cyclin T proteins were tested. As presented in Fig. 3*A*, only human cyclin T (GST-hT726), Tat, and TAR led to a retarded band (lane 2). This binding required the 5′ bulge (Fig. 3*A*, lanes 3 and 4) and the central loop (Fig. 3*A*, lanes 5 and 6) in TAR and Tat (Fig. 3*A*, lane 1). In sharp contrast, no bands were observed with mouse cyclin T (mT724), Tat, and TAR (Fig. 3*A*, lanes 7 and 8), indicating that mouse cyclin T does not support interactions between Tat and TAR. These results are consistent with a previous report that human cyclin T and Tat bind specifically to TAR (8). Using our conditions, Tat alone did not bind to TAR (Fig. 3*A*, lanes 2, 4, and 6). Additionally, no fortuitous binding was observed between mouse cyclin T, Tat, and the mutant TAR (data not presented).

Having established the specificity of binding between human cyclin T, Tat, and TAR, we next performed an electrophoretic mobility-shift assay with truncated hybrid GST-human cyclin T proteins. As presented in Fig. 3*B*, Tat and hybrid GST-human cyclin T proteins containing its N-terminal 479 [lane 2 (GST-hT479)] and 300 [lane 4 (GST-hT300)] amino acids bound to TAR. Similar results were obtained with longer versions of our chimeras (GST-hT551 and GST-hT611) (data not presented). In sharp contrast, Tat and human cyclin T containing only N-terminal 250 amino acids (GST-hT250) failed to bind to TAR (Fig. 3*B*, lanes 5 and 6). We conclude that residues from positions 1 to 300 in human cyclin T are required for its binding to Tat and TAR.

**Reciprocal Exchanges of the Cysteine and Tyrosine at Position 261 in Human and Mouse Cyclin T Proteins Render Human Cyclin T Inactive and Mouse Cyclin T Active for Tat Transactivation.** These results led us to focus on residues from positions 251 to 300 in human cyclin T (Fig. 1 *A* and *B*). Comparing these sequences between human and mouse cyclin T proteins revealed identical amino acids from positions 278 to 300. Between positions 251 and 278, only seven changed amino

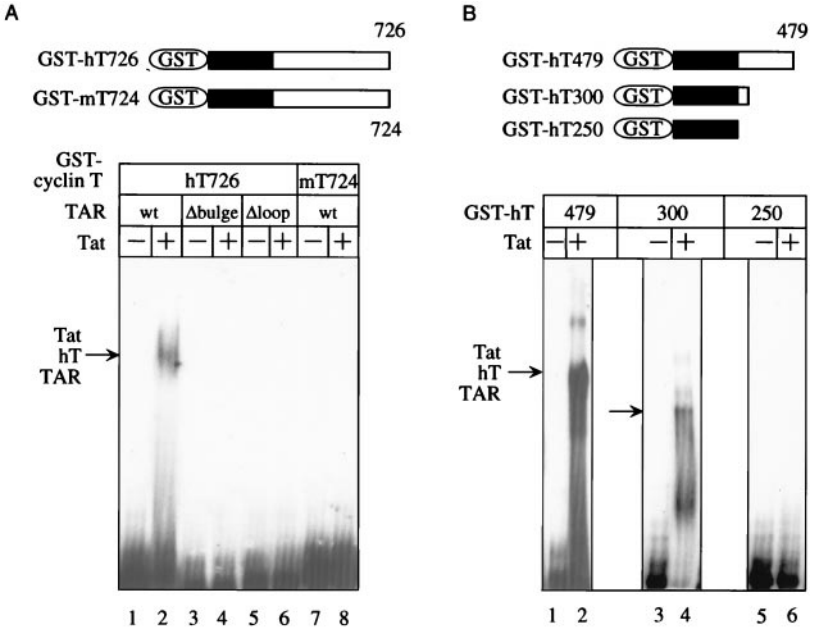


FIG. 3. Analysis of interactions between cyclin T proteins, Tat and TAR. (*A*) Mouse cyclin T does not support interactions between Tat and TAR *in vitro*. The hybrid GST-human cyclin T (hT726) and hybrid GST-mouse cyclin T (mT724) proteins were incubated with $^{32}$P-labeled TAR [lanes 1, 2, 7, and 8, wild-type (wt)], TAR lacking the 5′ bulge (lanes 3 and 4, Δbulge), or TAR lacking the central loop (lanes 5 and 6, Δloop). Tat was added to reactions in lanes 2, 4, 6, and 8. RNA–protein complexes were resolved by a 6% nondenaturing polyacrylamide gel. (*B*) N-terminal 300 residues in human cyclin T support interactions between Tat and TAR *in vitro*. Human cyclin T proteins, which were truncated from the C terminus to position 479, 300, and 250 (hT479, hT300, and hT250), were incubated with TAR. Tat was added to the reaction in lanes 2, 4, and 6. RNA–protein complexes are indicated by arrows. A schematic representation of cyclin T proteins used in these reactions is given on the top.

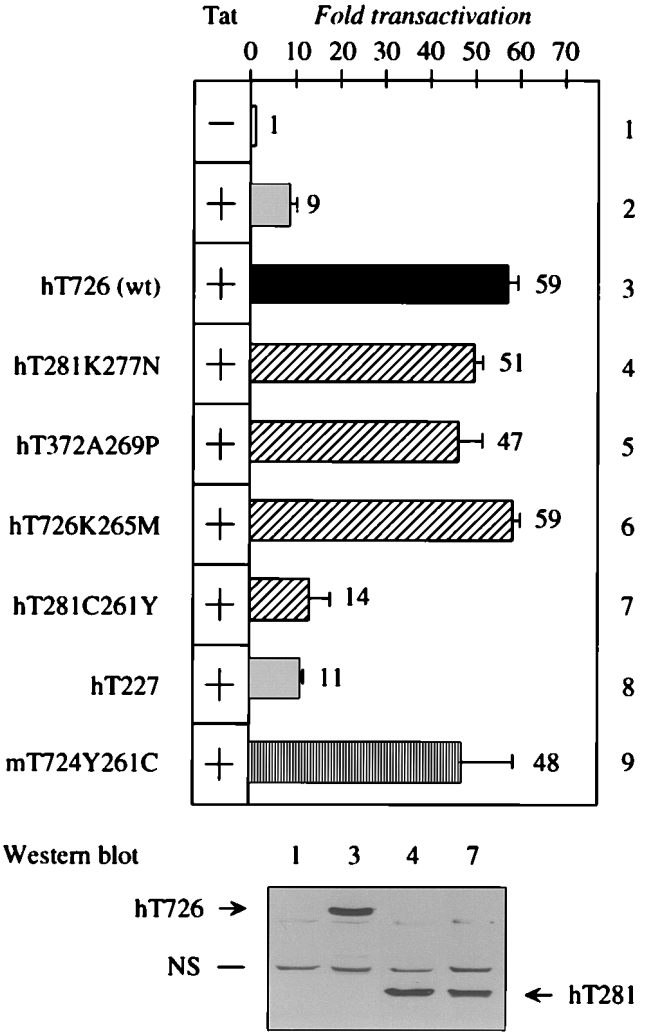


FIG. 4. N-terminal 281 residues in human cyclin T support Tat transactivation. Reciprocal substitutions of the cysteine and tyrosine at position 261 inactivate human cyclin T and activate mouse cyclin T, respectively, in CHO cells. The HIV-1 long terminal repeat was expressed alone [pHIVSCAT (lane 1)] or together with Tat [pcDNA3Tat (lane 2)]. To Tat were added human cyclin T [hT726 (lane 3)], truncated human cyclin T containing the substitution of lysine to asparagine at position 277 [hT281K277N (lane 4)], truncated human cyclin T containing the substitution of the alanine to proline at position 269 [hT372A269P (lane 5)], human cyclin T containing the substitution of the lysine to methionine at position 265 [hT726K265M (lane 6)], truncated human cyclin T containing the substitution of the cysteine to tyrosine at position 261 [hT281C261Y (lane 7)], truncated human cyclin T to position 227 [hT227 (lane 8)], and mouse cyclin T containing the substitution of the tyrosine at position 261 to cysteine [mT724Y261C (lane 9)]. Solid and striped bars denote wild-type or truncated and mutant cyclin T proteins, respectively. The value of the CAT activity of pHIVSCAT alone was set to 1. Standard errors of the mean from three independent transfections are presented. Western blotting revealed that levels of expression of the full length and truncated human cyclin T proteins (hT281K277N and hT281C261Y) were similar. Numbers above the lanes correspond to lanes from coexpression assays. NS, nonspecific bands.

acids were detected. Of these, the most drastic substitutions occurred at positions 261, 265, 269, and 277 (Fig. 1*B*). We introduced single amino acids from mouse cyclin T into human cyclin T and tested them for Tat transactivation in CHO cells and for specific binding to Tat and TAR *in vitro*. Because N-terminal 300 residues from human cyclin T were sufficient for its interactions with Tat and TAR, we also introduced stop codons into these cyclin T proteins.

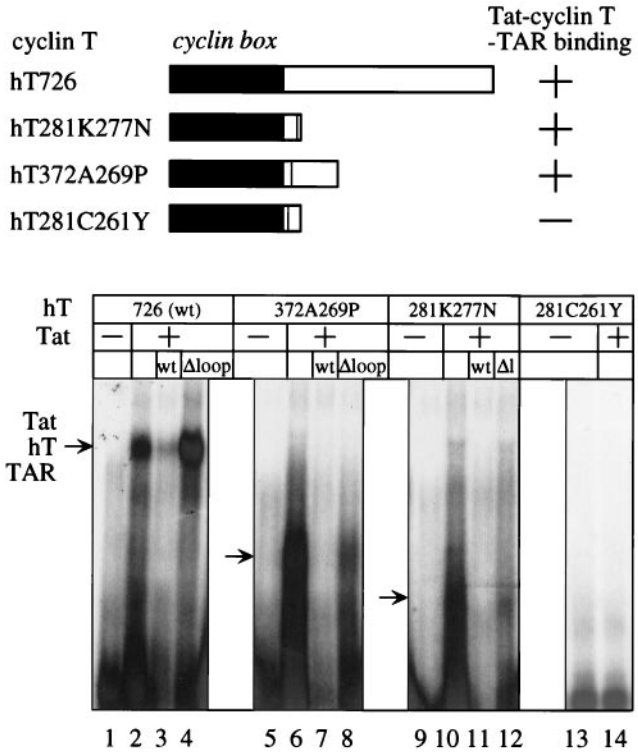


FIG. 5. The substitution of the cysteine (human) to tyrosine (mouse) at position 261 in human cyclin T abolishes its interaction with Tat and TAR *in vitro*. Wild-type (hT726) and mutant cyclin T proteins (hT372A269P, hT281K277N, and hT281C261Y) were incubated with $^{32}$P-labeled TAR in the absence and presence of Tat. RNA–protein complexes were separated by 6% nondenaturing polyacrylamide gel as in Fig. 3. The specificity of the binding was examined by a competition with excess amounts of unlabeled TAR [wild-type (wt) (lanes 3, 7, and 11)] or TAR lacking the 5′ loop [Δloop (lanes 4, 8, and 12)]. RNA–protein complexes are indicated by arrows. The schematic representation of cyclin T proteins and summary of binding results are given on the top.

As presented in Fig. 4, wild-type and truncated human cyclin T proteins increased Tat transactivation in CHO cells equally. Tat increased the expression from the HIV-1 long terminal repeat only 9-fold in these cells (Fig. 4, lane 2). The addition of human cyclin T at a ratio of 10:1 to Tat increased effects of Tat by a factor of six to 59-fold [Fig. 4, lane 3 (hT726)]. A similar increase in the activity of Tat was observed with human cyclin T containing only the N-terminal 281 amino acids [Fig. 4, lane 4 (hT281K277N)]. This human cyclin T also contained the substitution of the lysine to asparagine at position 277 [Figs. 1*B* and 4, lane 4 (hT281K277N)]. Additional point mutations of the alanine to proline at position 269 and the lysine to methionine at position 265 had no effect [Fig. 4, lanes 5 and 6 (hT372A269P and hT726K265M, respectively)]. Thus, these residues are not responsible for the restriction of mouse cyclin T. However, the next mutation, of the cysteine to tyrosine at position 261, abrogated the effect of human cyclin T in CHO cells [Fig. 4, lane 7 (hT281C261Y)]. Indeed, this mutation rendered human cyclin T as inactive as the deletion of the entire adjacent C-terminal sequence and part of cyclin boxes [Fig. 4, lane 8 (hT227)]. In sharp contrast, when the tyrosine at position 261 was mutated to cysteine, mouse cyclin T supported Tat transactivation at similar levels to human cyclin T [Fig. 4, lane 9 (mT724Y261C)]. Levels of expression of full length and truncated cyclin T proteins (hT281K277N and hT281C261Y) were equivalent (Fig. 4, Western blot). We conclude that sequences adjacent to cyclin boxes play a critical role in interactions between human cyclin T, Tat, and TAR. Of these, the cysteine at position 261 in human cyclin T is a key residue. Because this mutation restored the function of mouse

cyclin T, it is solely responsible for the restriction to Tat transactivation in rodent cells.

**A Cysteine to Tyrosine Substitution at Position 261 in Human Cyclin T Abolishes its Interactions with Tat and TAR *in Vitro*.** To examine whether this failure of mutant human cyclin T (hT281C261Y) to rescue Tat transactivation in cells could be correlated with its inability to support interactions between Tat and TAR *in vitro*, we performed an electrophoretic mobility-shift assay (Fig. 5). Wild-type and mutant human cyclin T proteins were synthesized in the coupled *in vitro* transcription and translation system from the rabbit reticulocyte lysate and were combined with Tat and $^{32}$P-labeled TAR transcripts. As presented in Fig. 5, wild-type [lanes 2 and 4 (hT726)] and several mutant human cyclin T proteins [lanes 6 and 8 (hT372A269P) and lanes 10 and 12 (hT281K277N)] resulted in retarded bands. Their binding required Tat (Fig. 5, lanes 1, 5, and 9) and the central loop in TAR (data not presented). Of importance, wild-type but not mutated unlabeled TAR transcripts competed for this binding (Fig. 5, lanes 3, 4, 7, 8, 11, and 12). In sharp contrast, human cyclin T containing the cysteine to tyrosine substitution at position 261 failed to support interactions between Tat and TAR (Fig. 5, lane 14), which is consistent with the result of the functional assay (Fig. 4). From our transient expression assays and *in vitro* binding data, we conclude that the cysteine to tyrosine substitution at position 261 is responsible for the restriction in Tat transactivation in rodent cells and for the lack of interactions between mouse cyclin T, Tat and TAR.

## DISCUSSION

In this study, we mapped residues in human cyclin T that bind to Tat and support its interactions with Tat and TAR. Cyclin boxes and adjacent 31 residues to position 281 are required for optimal interactions between human cyclin T, Tat, and TAR *in vitro* and for Tat transactivation *in vivo*. Moreover, the cloning of mouse cyclin T revealed seven amino acid changes outside of cyclin boxes between the mouse and human cyclin T proteins. One of them, the cysteine to tyrosine substitution at position 261 in mouse cyclin T, was found responsible for low levels of Tat transactivation in CHO cells and for its inability to support interactions between Tat and TAR *in vitro*.

Sequences between human and mouse cyclin T proteins are 90% identical. From positions 1 to 250, which contain 10 potential $\alpha$ helices in an arrangement reminiscent of two tandemly repeated cyclin boxes, there are only six changed amino acids (Fig. 1*A*). Because these sequences most likely interact with cyclin-dependent kinase 9, they should be highly conserved between species. However, adjacent 31 amino acids contain seven substitutions, of which four appeared most deleterious (Fig. 1*B*). These were tested in our functional and binding assays. Three others [tryptophan to arginine at position 256, glutamic acid to glutamine at position 262, and threonine to alanine at position 274 (Fig. 1*B*)] created a less acidic environment for interactions between mouse cyclin T and TAR and were unlikely to block the formation of the RNA–protein complex. Also, the striking effect of the substitution of the cysteine to tyrosine at position 261 in human cyclin T, and the rescue of mouse cyclin T with the reciprocal substitution, met all of our structural and functional criteria for mouse cyclin T. Finally, C-terminal 445 residues in human cyclin T were completely dispensable for Tat transactivation and for its interactions with Tat and TAR.

Although mouse cyclin T does not support efficient Tat transactivation in rodent cells, its overexpression did not decrease effects of Tat in human cells (data not presented). Likewise, a peptide containing just the 30 amino acids centered on the cysteine at position 261 was unable to support interactions between Tat and TAR or to block Tat transactivation and viral replication (data not presented). Thus, N-terminal sequences in human cyclin T are required for its binding to Tat and will have to be included in the creation of a dominant negative cyclin T protein.

This study explains low levels of Tat transactivation in rodent cells (6, 7). A single point mutation in mouse cyclin T is responsible for this phenotype. Because interactions with TAR were compromised the most, the RNA-tethering defect in CHO cells (6, 7) now has found a solid molecular foundation. Cyclin T proteins also recruit cyclin-dependent kinase 9, which hyperphosphorylates the C-terminal domain of RNA polymerase II and possibly other targets (16, 20). By functioning in RNA-binding and coactivation, human cyclin T thus reconciles previous notions of a single cofactor for Tat (21, 22). Finally, our study suggests minimal components that will be required for detailed structural analyses of this RNA–protein complex. They should yield a tantalizing picture of how these proteins and RNA are assembled and how their interactions can be inhibited.

We thank Michael Armanini for expert secretarial assistance, Kathy Jones and David Price for plasmids and antibodies, Jefferson Chan for technical advice, and members of the laboratory for help with some experiments and critical comments on the manuscript. This work was supported by the Howard Hughes Medical Institute and a grant from the Japanese Foundation for AIDS Prevention.

1. Dayton, A. I., Sodroski, J. G., Rosen, C. A., Goh, W. C. & Haseltine, W. A. (1986) *Cell* **44,** 941–947.
2. Fisher, A. G., Feinberg, M. B., Josephs, S. F., Harper, M. E., Marselle, L. M., Reyes, G., Gonda, M. A., Aldovini, A., Debouk, C., Gallo, R. C., *et al.* (1986) *Nature (London)* **320,** 367–371.
3. Frankel, A. D. & Young, J. A. (1998) *Annu. Rev. Biochem.* **67,** 1–25.
4. Jones, K. A. & Peterlin, B. M. (1994) *Annu. Rev. Biochem.* **63,** 717–743.
5. Hart, C. E., Ou, C. Y., Galphin, J. C., Moore, J., Bacheler, L. T., Wasmuth, J. J., Petteway, S., Jr. & Schochetman, G. (1989) *Science* **246,** 488–491.
6. Alonso, A., Derse, D. & Peterlin, B. M. (1992) *J. Virol.* **66,** 4617–4621.
7. Alonso, A., Cujec, T. P. & Peterlin, B. M. (1994) *J. Virol.* **68,** 6505–6513.
8. Wei, P., Garber, M. E., Fang, S. M., Fischer, W. H. & Jones, K. A. (1998) *Cell* **92,** 451–462.
9. Peng, J., Zhu, Y., Milton, J. T. & Price, D. H. (1998) *Genes Dev.* **12,** 755–762.
10. Marshall, N. F., Peng, J., Xie, Z. & Price, D. H. (1996) *J. Biol. Chem.* **271,** 27176–27183.
11. Manchebo, H., Lee, G., Flygare, J., Tomassini, J., Luu, P., Zhu, Y., Blau, C., Hazuda, D., Price, D. & Flores, O. (1997) *Genes Dev.* **11,** 2633–2644.
12. Zhu, Y., Pe'ery, T., Peng, J., Ramanathan, Y., Marshall. N., Marshall, B., Mathews, M. & Price, D. (1997) *Genes Dev.* **11,** 2622–2632.
13. Okamoto, H., Sheline, C. T., Corden, J., Jones, K. A. & Peterlin, B. M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 11575–11579.
14. Chun, R. F. & Jeang, K. T. (1996) *J. Biol. Chem.* **271,** 27888–27894.
15. Yang, X., Gold, M. O., Tang, D. N., Lewis, D. E., Aguilar-Cordova, E., Rice, A. P. & Herrmann, C. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 12331–12336.
16. Fujinaga, K., Cujec, T. P., Peng, J., Garriga, J., Price, D. H., Grana, X. & Peterlin, B. M. (1998) *J. Virol.* **72,** 7154–7159.
17. Herrmann, C. & Rice A (1993) *Virology* **197,** 601–608.
18. Cujec, T. P., Okamoto, H., Fujinaga, K., Meyer, J., Chamberlin, H., Morgan, D. & Peterlin, B. M. (1997) *Genes Dev.* **11,** 1–12.
19. Zhou, Q., Chen, D., Pierstorff, E. & Luo, K. (1998) *EMBO J.* **17,** 3681–3691.
20. Jones, K. A. (1997) *Genes Dev.* **11,** 2593–2599.
21. Carroll, R., Peterlin, B. M. & Derse, D. (1992) *J. Virol.* **66,** 2000–2007.
22. Madore, S. J. & Cullen, B. R. (1993) *J. Virol.* **67,** 3703–3711.