HIV-1 Tat-associated RNA polymerase C-terminal domain kinase, CDK2, phosphorylates CDK7 and stimulates Tat-mediated transcription

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HIV-1 gene expression is regulated by a viral transactivator protein (Tat) which induces transcriptional elongation of HIV-1 long tandem repeat (LTR). This induction requires hyperphosphorylation of the C-terminal domain (CTD) repeats of RNA polymerase II (Pol II). To achieve CTD hyperphosphorylation, Tat stimulates CTD kinases associated with general transcription factors of the promoter complex, specifically TFIIH-associated CDK7 and positive transcription factor b-associated CDK9 (cyclin-dependent kinase 9). Other studies indicate that Tat may bind an additional CTD kinase that regulates the target-specific phosphorylation of RNA Pol II CTD. We previously reported that Tat-associated T-cell-derived kinase (TTK), purified from human primary T-cells, stimulates Tat-dependent transcription of HIV-1 LTR *in vivo* [Nekhai, Shukla, Fernandez, Kumar and Lamb (2000) *Virology* **266**, 246–256]. In the work presented here, we characterized the components of TTK by biochemical frac-

tionation and the function of TTK in transcription assays *in vitro*. TTK uniquely co-purified with CDK2 and not with either CDK9 or CDK7. Tat induced the TTK-associated CDK2 kinase to phosphorylate CTD, specifically at Ser-2 residues. The TTK fraction restored Tat-mediated transcription activation of HIV-1 LTR in a HeLa nuclear extract immunodepleted of CDK9, but not in the HeLa nuclear extract double-depleted of CDK9 and CDK7. Direct microinjection of the TTK fraction augmented Tat transactivation of HIV-1 LTR in human primary HS68 fibroblasts. The results argue that TTK-associated CDK2 may function to maintain target-specific phosphorylation of RNA Pol II that is essential for Tat transactivation of HIV-1 promoter. They are also consistent with the observed cell-cycle-specific induction of viral gene transactivation.

Key words: microinjection, protein microsequencing.

INTRODUCTION

Expression of the HIV-1 proviral genome requires host-cell transcription factors, and is uniquely dependent upon the virally encoded transactivator Tat protein [1–4]. HIV-1 Tat stimulates the formation of full-length transcripts from the HIV-1 promoter [5–7]. Tat interacts with transactivation response (TAR) RNA, a hairpin-loop structure at the 5' end of all nascent viral transcripts [8]. A number of genetic and biochemical studies indicate that Tat-mediated transactivation requires co-operative interaction between Tat and host-cell cofactors [1–4] to promote elongation of transcription [9]. Because elongation of transcription can be blocked by the nucleoside analogue 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) [10,11], it has been proposed that it is DRB-sensitive cellular kinases that phosphorylate the RNA polymerase II (Pol II) C-terminal domain (CTD) heptapeptide repeats during transcription elongation. HIV-1 Tat associates with a kinase (Tat-associated kinase) that hyperphosphorylates RNA Pol II CTD [12] and is a human homologue of positive transcription elongation factor b (P-TEFb) [13,14]. The catalytic subunit of Tat-associated kinase corresponds to the earlier-described nuclear cyclin-dependent kinase, PITALRE [15], and has been renamed CDK9 (cyclin-dependent kinase 9). P-TEFb was characterized previously to regulate transcription elongation and to be a CTD kinase [16].

We have reported that full-length Tat associates with a CTD kinase complex isolated from mitotically stimulated human primary T-lymphocytes (Tat-associated T-cell-derived kinase, or TTK) [17]. Direct microinjections into human primary fibroblasts of TTK, purified from human primary T-cells, stimulated Tat-dependent expression of a reporter *lacZ* gene placed under the control of HIV-1 long tandem repeat (LTR) [18]. The expression of LacZ in this system was cell-cycle-dependent (primarily in the G₁ phase) and required CTD repeats [18]. Furthermore, overexpression of specific CDK inhibitors, p16 and p27, blocked Tat transactivation [18]. It was tempting to suggest that TTK contained CDK9, but we did not find CDK9 by immunoblotting assay [18]. Moreover, TTK was biochemically distinct from CDK9, because TTK phosphorylated CDK7 and promoted association of CDK7 and cyclin H [18]. Taken together, our data indicate that a CDK-like kinase distinct from CDK9 may be involved in Tat-mediated transactivation.

In the present study, we resolved the components of TTK by biochemical fractionations, immunoblotting and ion-trap mass-spectrometric peptide sequencing. We found that TTK is a multimeric complex containing CDK2, nuclear phosphatase 1 and other proteins. Specifically, TTK hyperphosphorylated Ser-2 residues within the RNA Pol II CTD repeats in the presence of Tat. Furthermore, purified TTK phosphorylated CDK7 and promoted association of CDK7 and cyclin H. Direct micro-

Abbreviations used: TAR, transactivation response; CTD, C-terminal domain; CTDA, hypophosphorylated CTD; CTDO, hyperphosphorylated CTD; Pol II, polymerase II; TTK, Tat-associated T-cell-derived kinase; GST, glutathione S-transferase; MPF, M-phase-promoting factor; DTT, dithiothreitol; SP, sulphonylpropyl; CAK, CDK-activating kinase; NIPP1, nuclear inhibitor of protein phosphatase 1; MAT1, ménage-a-trois 1; LTR, long tandem repeat; CDK, cyclin-dependent kinase; DRB, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole; Tat, a viral transactivator protein.

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injection of TTK into primary fibroblasts stimulated Tat transactivation. In cell-free transcription assays, TTK restored Tat-mediated transcription activation of HIV-1 LTR in nuclear extracts immunodepleted of CDK9, but not in the extracts depleted of both CDK9 and CDK7. The data suggest that the phosphorylation of CTD Ser-2 residues by the Tat-associated TTK fraction may function to sustain the hyperphosphorylated state of RNA Pol II during transactivation of the HIV-1 promoter.

MATERIALS AND METHODS

Jurkat T-cells were purchased from the National Cell Culture Center (Cellex Biosciences, Minneapolis, MN, U.S.A.). DEAE-Sepharose (Fast-Flow), sulphopropyl-(SP-)Sepharose (Fast-Flow), Hi-Trap heparin columns and [γ - 32 P]ATP (6000 Ci/mmol) were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). Econo-Pac CHT-II cartridge (ceramic hydroxyapatite) was from Bio-Rad (Hercules, CA, U.S.A.). Glutathione-agarose was from Sigma (Atlanta, GA, U.S.A.). Glutathione S-transferase (GST)-CTD was expressed in *Escherichia coli* and purified as described in [19]. The Tat expression plasmids GST-Tat 86, GST-Tat 72, GST-Tat 72 C22G, GST-Tat 48 and GST-Tat 48 C22G were obtained from the National Institutes of Health AIDS repository, expressed in *Escherichia coli*. Tat protein was purified on glutathione-agarose beads as described in [17]. CDK7, CDK7T176A, cyclin H and ménage-à-trois 1 (MAT1) expression vectors were provided kindly by Dr Marcel Doreé (CNRS, Montpellier, France).

The reporter plasmid pJK2 (courtesy of Dr Michael Emmerman, Fred Hutchinson Cancer Institute, Seattle, WA, U.S.A.) expressed nuclear localization signal-tagged β -galactosidase under the control of HIV-1 LTR [20]. A control reporter plasmid expressed tagged β -galactosidase under the control of the β -actin promoter (courtesy of Dr J. M. Blanchard, Institut de génétique moléculaire, CNRS, Montpellier, France). Bacterially expressed HIV-1 Tat protein was used at 1 mg/ml in 50 mM β -mercaptoethanol. A coupled transcription/translation system based on rabbit reticulocyte lysate was purchased from Ambion (Austin, TX, U.S.A.). M-phase-promoting factor (MPF) was kindly provided by Dr James Maller (University of Denver, Denver, CO, U.S.A.). Polyclonal antibodies to CDK7, the motif PSTAIRE, casein kinase 2, purified rabbit IgG and Protein A/G-agarose were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Polyclonal antibodies to CDK9 (PITALRE) were purchased from Biodesign Company (Saco, ME, U.S.A.). Antibodies for the p62 subunit of TFIIF were a gift from Dr Hua Xiao (Rockefeller University, NY, U.S.A.). Monoclonal antibodies to the unphosphorylated form of CTD (8WG16), phosphorylated on Ser-2 (H5) and Ser-5 (H14), were from BabCo (Richmond, CA, U.S.A.).

CTD kinase assays

Fractions eluted from each chromatography column (described below) were assayed for total CTD kinase activity as well as Tat-associated CTD kinase activity.

Total CTD kinase assay

For this, 2 μ l of a fraction (about 1/1000 of the total amount) was incubated in a 20 μ l reaction volume with 50 μ M ATP, 1 μ Ci of [γ - 32 P]ATP and 100 ng of GST-CTD in kinase buffer containing 50 mM Hepes (pH 7.9), 10 mM MgCl₂, 6 mM EGTA and 2.5 mM dithiothreitol (DTT), for 10 min at room temperature.

Tat-associated CTD kinase assay

Again, 2 μ l of a fraction (about 1/1000 of the total amount) was incubated with 10 μ g of GST-Tat 72 immobilized on glutathione-agarose beads for 1 h at 4 °C. The beads were washed with the buffer B containing 20 mM Hepes (pH 7.9), 250 mM NaCl, 1 % Nonidet P-40, 5 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF and 10 μ g/ml aprotinin, followed by washing with the kinase buffer, described above. Tat-associated CTD kinase activity was assayed by incubating the kinase-bound beads with 100 ng of GST-CTD in kinase buffer containing 50 μ M ATP and 10 μ Ci of [γ - 32 P]ATP for 10 min at room temperature. Phosphorylated GST-CTD was resolved on SDS/PAGE (10 % gels) and subjected to autoradiography and quantification with PhosphorImager Storm 860 (Molecular Dynamics).

Purification of CTD kinases

Extract from T-cells was prepared and fractionated by (NH₄)₂SO₄ precipitation as described previously [21]. Briefly, 100 litres of Jurkat T-cell culture at concentration of 5×10^5 cells/ml were centrifuged, washed and Dounce-homogenized in buffer A (50 mM Hepes, pH 7.9, 5 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 10 μ g/ml aprotinin and 10 % glycerol) supplemented with 0.1 % Nonidet P-40. (NH₄)₂SO₄ was added to 10 % saturation to extract nuclei. After centrifugation, the supernatant, containing approx. 10 g of protein, was fractionated further with (NH₄)₂SO₄ added to 20 %, 40 % and 80 % saturation. The presence of total Tat-associated CTD kinase activity was analysed and is described in the Results section. The 40 %-(NH₄)₂SO₄ fraction (about 3.5 g of protein) was found to contain the major part of total and Tat-associated CTD kinase activity. This fraction was diluted with buffer A until the conductivity was equivalent to 50 mM KCl and then loaded on to a DEAE-Sepharose column (about 500 mg of protein/50 ml column). The column was eluted with a linear gradient of KCl (0.1–1 M) in buffer A. Fractions were assayed for total and Tat-associated CTD kinase activity as described above. Two peaks, containing Tat-associated CTD kinase activity, designated as TTK and TTK' (see the Results section), were collected, diluted with buffer A until conductivity was equivalent to 50 mM KCl and loaded on to a 10 ml SP-Sepharose column, which was eluted with a linear gradient of KCl (0.1–1 M) in buffer A. Tat-associated CTD kinase activity of TTK and TTK' retained on SP-Sepharose was further fractionated on Hi-Trap heparin columns (1 ml; three in series). Fractions were analysed for total and Tat-associated CTD kinase activity. The peaks of Tat-associated CTD hyperphosphorylating activity were pooled, diluted 10-fold with 0.1 M sodium phosphate buffer (pH 6.8) and loaded on to an Econo-Pac CHT-II cartridge (ceramic hydroxyapatite; 5 ml). The column was eluted with a linear gradient of sodium phosphate (pH 6.8; 0.1–1 M) containing 5 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 10 μ g/ml aprotinin and 10 % glycerol. Fractions were collected and analysed for the Tat-associated CTD-kinase activity as described above, as well as by immunoblotting. Fractions containing TTK were resolved on SDS/PAGE (12 % gels; 20 cm $\times$ 20 cm), stained with colloidal Coomassie Brilliant Blue (ICN) and subjected to protein microsequencing.

Preparation of CTD kinase for microinjection of HS68 cells

Fractions from the hydroxyapatite column, containing either TTK or TTK' peaks of CTD hyperphosphorylation activity (approx. 100 μ g of protein), were pooled, diluted 10-fold with buffer A and incubated with GST-Tat 72 immobilized on agarose beads (1 ml of 50 % slurry), for 1 h at 4 °C. The beads were

Figure 1 Purification of TTK

(A) DEAE-Sepharose column-chromatographic elution profile of CTD kinase activity of TTK. Jurkat T-cell extract 40%-(NH₄)₂SO₄ cut was applied to a DEAE-Sepharose column. Fractions were analysed for total (– Tat) and Tat-associated (+ Tat) CTD kinase activities as described in the Materials and methods section. For total CTD kinase assay, a portion of each fraction was incubated with [γ -³²P]ATP and recombinant GST-CTD in the kinase buffer. For Tat-associated CTD kinase assay, a portion of each fraction was bound to GST-Tat 72 immobilized on glutathione–agarose beads and then incubated with [γ -³²P]ATP and recombinant GST-CTD. Phosphorylated GST-CTD was resolved on SDS/10%-(w/v)-PAGE. Two peaks of CTD kinase activity, designated TTK and TTK', are underlined. I, input; numbers correspond to the fraction numbers. Positions of CTDo and CTDa are shown. The Figure is an autoradiogram. (B) TTK fraction is distinct from TFIH-associated kinase. Fractions from DEAE-Sepharose were analysed by Western blotting with antibodies against CDK7, CDK9, TFIH p62 and PSTAIRE. (C) SP-Sepharose column chromatography separates TTK fraction from CDK9. TTK fractions from DEAE-Sepharose (fractions 34–36) were resolved on SP-Sepharose. Column fractions were assayed for Tat-associated CTD kinase activity, as described in (A) and the Materials and methods. An autoradiogram of phosphorylated CTD resolved on 10% SDS/PAGE gel is shown in the kinase assay panel. Column fractions were analysed by Western blotting with antibodies against PSTAIRE, CDK9 and CDK7. F, flow-through. (D) TTK contains CDK2. TTK activity resided in the 40%-(NH₄)₂SO₄ cut, DEAE-Sepharose (fractions 34–36), SP-Sepharose (fractions 28–30), heparin–agarose (Hep.; fractions 20–22) and hydroxyapatite (Hydrox.; fractions 32–34). These fractions were resolved by SDS/10%-(w/v)-PAGE, and immunoblotted with antibodies against CDK7, PSTAIRE, CDK2 and CDK9.

packed into a 1 ml column, washed with buffer B, followed by buffer A, and eluted with buffer A containing 0.5 M KCl. Eluates were concentrated and desalted on Centricon-10 spin columns (Millipore, Tempe, AZ, U.S.A.), assayed for CTD kinase activity and used for microinjections.

Microinjection and immunostaining

HS68 cells (human foreskin fibroblasts) were cultured on coverslips (in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum) until 30–50% confluency. HS68 cells were injected with the reporter plasmid pJK2 alone or in combination with other expression vectors. The amount of microinjected proteins and plasmids was adjusted to approx. 8–10 molecules of each component/nucleus, based on the microinjection volume of 10^{–15} ml and known molecular masses. The molecular mass of TTK was estimated at about 300 000 kDa by size-exclusion chromatography on Superdex 200 10/30 (Pharmacia). The reporter plasmid DNA, with or without the TTK, was mixed with rabbit IgGs as a marker just before injection into HS68 cell nuclei. The injected cells were fixed 2–3 h post injection with 3.7% formaldehyde (in PBS) for 5 min at room temperature. Fixed cells were permeabilized (for 20 s) with acetone at

–20 °C and immunostained with anti- β -galactosidase monoclonal antibody, followed by FITC-conjugated anti-mouse IgG. Rabbit IgG injection marker was stained with biotinylated anti-rabbit IgG and secondarily with Texas Red-conjugated streptavidin. The efficiency of the Tat response was monitored by counting the fraction of Texas Red-stained cells that were also stained with FITC-conjugated β -galactosidase antibody.

In vitro transcription

The HIV-1 LTR template (nt –110 to +168) was created by PCR using CD12 expression plasmid as a template [7]. The template was incubated with HeLa nuclear extract or the extract immunodepleted of CDK9 as described in [22]. Transcription reactions (50 μ l) contained 50 μ l of the indicated nuclear extract, 1 μ g of HIV-1 LTR template and 0.2 μ g of purified Tat protein in buffer C (20 mM Hepes, pH 7.9, 50 mM KCl, 6.25 mM MgCl₂, 0.5 mM EDTA, 2 mM DTT, 10 μ M ZnSO₄, 10 mM creatine phosphate, 100 μ g/ml creatine kinase and 8.5% glycerol). After 45 min of incubation at 30 °C, RNA transcripts were purified by phenol extraction and resolved on 6% sequencing acrylamide gel containing 7 M urea.

NanoLC ion-trap MS and peptide sequencing

TTK-containing fractions purified on hydroxyapatite column were concentrated on Centricon-10 spin columns, resuspended in SDS-loading buffer and resolved by SDS/PAGE (12% gel). Protein bands visible after colloidal Coomassie Brilliant Blue staining and corresponding to the peak of CTD-hyperphosphorylating activity were subjected to in-gel reduction, carboxamidomethylation and tryptic digestion (Promega, Madison, WI, U.S.A.). Multiple peptide sequences were determined in a single run by microcapillary reverse-phase chromatography coupled directly to a Finnigan LCQ quadrupole ion-trap mass spectrometer equipped with a custom nanoelectrospray source. The column was packed in-house with 5 cm of C_{18} support into a New Objective one-piece 75 μ m (inner diameter) column terminating in a 15 μ m tip. Flow rate was 190 nl/min. The ion trap was programmed to acquire successive sets of three scan modes consisting of full-scan MS over alternating ranges of 395–800 m/z and 800–1300 m/z , followed by two data-dependent scans on the most abundant ion in those full scans. These data-dependent scans allowed the automatic acquisition of a high-resolution (zoom) scan to determine charge state and exact mass, and tandem MS spectra for peptide sequence information. Tandem MS spectra were acquired with a relative collision energy of 30%, an isolation width of 2.5 Da and recurring ions dynamically excluded. Interpretation of the resulting MS/MS spectra of the peptides was facilitated by programs developed in the Harvard Microchemistry Facility (Cambridge, MA, U.S.A.) and by database correlation with the algorithm SyQuest [23,24].

Assembly of CDK-activating kinase (CAK)

Reconstitution of CAK from components transcribed/translated *in vitro* was carried out as described previously [25]. Briefly, wild-type *Xenopus* CDK7, control non-phosphorylatable mutant CDK7 T176A, cyclin H and MAT1 cDNAs were transcribed/translated in a coupled rabbit reticulocyte system according to the manufacturer's recommendations (Ambion). Phosphorylation of CDK7 was performed by incubating 4 μ l of 35 S-labelled CDK7-programmed reticulocyte lysate, and 2 μ l of 35 S-labelled cyclin H-programmed reticulocyte lysate with an indicated kinase, which included MPF, or purified CTD kinase fractions. The incubation was carried out in a 15 μ l volume with 0.2 mM ATP, 9.9 mM $MgCl_2$ and 6.7 μ M microcystin for 30 min at 30 °C. MAT1-programmed lysate was added as indicated. Non-phosphorylatable CDK7T176A was used as a negative control. After the incubation, 5 μ l of anti-CDK7 polyclonal antiserum and 50 μ l of Protein A-agarose (50% slurry) in TNN buffer (50 mM Tris/HCl, pH 8.0/150 mM NaCl/0.5% Nonidet P-40) were added and the volume was adjusted to 100 μ l by TNN buffer. Immunoprecipitation with anti-CDK7 antibody was carried out for 2 h at 4 °C. The beads were washed three times with 0.5 ml of TNN buffer and once with 50 mM Tris/HCl (pH 8.0), and the proteins were resolved by SDS/PAGE (12% gel). The gel was treated with Amplify solution (Amersham Pharmacia Biotech), dried and exposed to X-ray film with an intensifying screen at -70 °C.

Immunodepletion of PSTAIRE kinase

Reconstitution of CAK was performed as described above. The mixture of wild-type CDK7 (10 μ l of 35 S-labelled CDK7-programmed reticulocyte lysate), cyclin H (7.5 μ l of 35 S-labelled cyclin H-programmed reticulocyte lysate) and 2.5 μ l of 100 μ M microcystin was preincubated for 30 min at 4 °C with 10 μ l of Protein A/G-agarose (Santa Cruz Biotechnology). About 10 μ g

of TTK was preincubated with the indicated amount of either anti-PSTAIRE antibodies or purified rabbit IgG at 4 °C for 30 min in 18 μ l of kinase buffer, and then added to the reticulocyte lysate preincubated with Protein A/G as described above. Incubation was continued for 1 h at 4 °C with occasional shaking. Protein A/G-agarose was removed by brief centrifugation. An aliquot of 26 μ l from each sample was supplemented with 6 μ l of mixture containing 1 mM ATP and 50 mM $MgCl_2$ and incubated for 1 h at 30 °C. After the incubation, CDK7 was immunoprecipitated and resolved on SDS/PAGE as described above.

RESULTS

Purification of Tat-associated CTD kinase

Whole-cell lysate from Jurkat T-cells was prepared and subjected to $(NH_4)_2SO_4$ fractionation as described in the Materials and methods section. $(NH_4)_2SO_4$ cuts of 10, 20, 40 and 80% were analysed for total and Tat-associated CTD kinase activities. CTD phosphorylated at a low level (hypophosphorylated CTD or CTDa) migrated on SDS/PAGE with the same mobility as

Figure 2 Composition of TTK

(A) Fractionation on an Econo-Pac CHT-II (hydroxyapatite) column. TTK activity corresponding to fractions 28–30 of SP-Sepharose fractionation, and further resolved on a Hi-Trap heparin column, was fractionated on a hydroxyapatite column as described in the Materials and methods section. Fractions were assayed for Tat-associated CTD kinase activity. (B) Econo-Pac CHT-II (hydroxyapatite) column fractions corresponding to (A) resolved by SDS/10%-PAGE and stained with colloidal Coomassie Brilliant Blue. Positions of the protein bands which co-migrated with Tat-associated CTD kinase activity and which were subjected to sequencing by ion-trap MS are indicated.

Figure 3 HIV-1 Tat stimulates hyperphosphorylation of Ser-2

(A) Association of TTK with GST-Tat stimulates CTD hyperphosphorylation. Lane 1, control reaction, where TTK (SP-Sepharose fractions 28–30) was incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and recombinant GST-CTD in the absence of Tat. Lanes 2–5, TTK was bound to GST fusion proteins of Tat 86, Tat 72, Tat 48 and Tat 48 C22G, and then incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and recombinant GST-CTD. Phosphorylated GST-CTD was resolved by SDS/10% PAGE. Positions of CTD α and CTD β are shown. The Figure is an autoradiogram. (B) Purified Tat stimulates TTK to hyperphosphorylate CTD on Ser-2. Tat was expressed in bacteria and purified on HPLC as described in the Materials and methods section. Purified Tat was added to TTK (hydroxyapatite column, fractions 32–34). Enhanced chemifluorescence (ECF) immunoblotting analysis of CTD phosphorylated with or without Tat with antibodies against non-phosphorylated CTD (8WG16), or CTD phosphorylated on Ser-2 (H5) or Ser-5 (H14) is shown. The portion of the same membrane exposed to a PhosphorImager screen is shown in the kinase assay panel. (C) Purified Tat stimulates CDK2/cyclin E to hyperphosphorylate CTD on Ser-2. Purified Tat was added to recombinant CDK2/cyclin E (overexpressed in baculovirus [17]). ECF immunoblotting analysis of phosphorylated CTD was performed as described in (B). (D) Tat stimulates CDK7/cyclin H to hyperphosphorylate CTD on Ser-2 and Ser-5. Tat was added to CDK7/cyclin H purified in this study (flow-through fraction of SP-Sepharose purified further on heparin–agarose). ECF immunoblotting analysis of phosphorylated CTD was performed as described in (B).

non-phosphorylated CTD. In contrast, hyperphosphorylated CTD (CTD α) migrated on SDS/PAGE with a high degree of retardation, probably because of the SDS repelling effect. Tat-associated CTD kinase activity fractionated in the 40%-(NH_4) $_2\text{SO}_4$ cut showed little or no CTD α hyperphosphorylation activity (Figure 1A, lane I), in agreement with our earlier observation [17]. Subsequent fractionation of the 40% (NH_4) $_2\text{SO}_4$ cut on DEAE-Sepharose resulted in the separation of total CTD α hypophosphorylating activity into two peaks (Figure 1A, upper panel, – Tat). Surprisingly, when the DEAE fractions were applied to GST-Tat 72 to analyse Tat-associated CTD kinase activity, CTD α hyperphosphorylation was observed (Figure 1A, lower panel, + Tat, fractions 34–38). Fractions 34–38 corresponding to this hypophosphorylating Tat-associated CTD kinase activity were designated TTK. The second peak of Tat-associated CTD α hypophosphorylation activity was designated TTK'. Immunoblotting of the DEAE-Sepharose fractions showed that TTK activity co-elutes with CDK7, CDK9 and a kinase recognized antigenically by the anti-PSTAIR antibody (Figure 1B). Further resolution on an SP-Sepharose column showed that the Tat-associated CTD kinase activity (TTK) co-migrating with PSTAIR kinase was clearly resolved from the CDK7- and CDK9-containing fractions (Figure 1C). TTK, upon fractionation on Hi-Trap heparin (Figure 1D) and Econo-Pac CHT-II columns (Figure 1D), co-eluted with the PSTAIR kinase. The PSTAIR-like kinase was identified as CDK2 by immunoblotting (Figure 1D). Neither CDK7 nor CDK9 was detected in the later stages of TTK purification (Figure 1D).

The hydroxyapatite fraction of TTK contained protein bands of 32, 42, 60, 80 and 150 kDa (Figure 2B, fraction 32). Protein bands of 32, 60, 80 and 150 kDa, visible after colloidal Coomassie

Brilliant Blue staining (Figure 2B, bands marked in fraction 32), were subjected to tryptic digestion and nanoelectrospray MS (described in the Materials and methods section). Two peptides derived from the 32 kDa protein had sequence homology to a nuclear inhibitor of protein phosphatase 1 (NIPPI1) [26]. The amino acid sequences of 60, 80 and 150 kDa proteins matched to unnamed proteins coded by open reading frames of human genes. Their relevance to the regulation of Tat transactivation is under investigation.

Tat stimulates TTK to hyperphosphorylate CTD on Ser-2 of heptad repeats

We next asked whether Tat stimulated the TTK-associated kinase activity and which specific domain of Tat is required for retaining the kinase activity. TTK (Figure 2A, hydroxyapatite column fraction 32) was applied to the following GST fusion constructs: GST-Tat 86, GST-Tat 72, GST-Tat 48 and GST-Tat 48 C22G. In the absence of Tat, no hyperphosphorylation of CTD was observed (Figure 3A). Binding to Tat 86 and Tat 72 retained the CTD kinase activity and stimulated CTD α hyperphosphorylation (Figure 3A). Binding to the activation domain of Tat (GST-Tat 48) on the other hand retained the kinase activity which hypophosphorylated CTD α (Figure 3A). The N-terminal domain of the transcriptionally inactive mutant of Tat (GST-Tat 48 C22G) did not associate with the kinase (Figure 3A). To exclude the possibility that the effect of Tat is due to the interaction of GST portions of GST-Tat 72 and GST-CTD, Tat protein was purified as described in the Materials and methods section and added to the kinase reaction. No CTD α hyperphosphorylation was observed in the absence of Tat, whereas the level

Figure 5 Induction of Tat response by TTK *in vivo*

HS68 cells were injected with HIV-1 LTR-LacZ (pJK2) (A) or β -actin-LacZ (B) expression vectors with or without Tat, Tat plus TTK or TTK'. After 3 h of incubation to allow expression of β -galactosidase, cells were fixed and immunostained as described in the Materials and methods section. The percentage of injected nuclei expressing β -galactosidase is shown.

precipitation of cyclin H with wild-type CDK7, but not with the CDK7 T176A mutant (Figure 4A, compare lanes 9 and 10). Therefore, phosphorylation of Thr-176 by MPF promoted binding of CDK7 to cyclin H. Alternatively, CDK7–cyclin H complex assembly factor, MAT1, which has been shown to stimulate CAK independently of CDK7 phosphorylation [27], stimulated binding of cyclin H to either CDK7 or CDK7 T176A (Figure 4A, compare lanes 5 and 6). TTK promoted binding of cyclin H to wild-type CDK7, but not to the CDK7 T176A mutant (Figure 4A, compare lanes 11 and 12). Therefore, TTK probably promotes CDK7–cyclin H complex formation by phosphorylating Thr-176. As a negative control, the TTK' fraction (Figure 1A), purified on SP-Sepharose, heparin-agarose and hydroxyapatite columns, did not promote cyclin H binding to CDK7 (Figure 4A, lanes 13 and 14).

Since TTK is a multicomponent complex, we could not exclude the possibility that a kinase other than CDK2/PSTAIRE is responsible for phosphorylation of CDK7. To test this possibility, immunodepletion of TTK was performed as described in the Materials and methods section. Depletion of TTK with anti-PSTAIRE antibody reduced the ability of the kinase to promote CDK7–cyclin H complex formation (Figure 4B, lanes 3–5). In contrast, immunodepletion with preimmune serum did not affect the ability of TTK to promote CAK formation (Figure 4B, lanes 6–8).

Figure 6 TTK restores Tat-dependent transcription

(A) TTK restores Tat-dependent HIV-1 LTR transcription in CDK9-depleted HeLa nuclear extract. *In vitro* transcription reactions contained HIV-1 LTR template and HeLa nuclear extract not depleted (lanes 1 and 7) or depleted (lanes 2–6 and 8–12) of CDK9. Tat was added where indicated, during assembly of the pre-initiation complex. The reaction was supplemented with nucleotides, [32 P]UTP and increasing amounts of TTK, as indicated, and incubated for 45 min at 30 °C. Transcription reactions were separated on 10% PAGE containing 7 M urea. Lanes 1 and 7, non-depleted extract without and with Tat. Lane 2, transcription in the depleted extract. Lanes 3–5, TTK added to the depleted extract. Lane 6, CDK9/cyclin T added to the depleted extract. Lane 8, Tat added to the depleted extract. Lanes 9–11, Tat and TTK added to the depleted extract. Lane 12, Tat and CDK9/cyclin T added to the depleted extract. (B) CDK7/cyclin H does not restore Tat-dependent HIV-1 LTR transcription in CDK9-depleted HeLa nuclear extract. CDK7/cyclin H complex was purified from Jurkat T-cells and added to the transcription reaction in CDK9-depleted HeLa nuclear extract. Lanes were as for (A). (C) TTK does not restore Tat-dependent HIV-1 LTR transcription in HeLa nuclear extract depleted of CDK9 and CDK7. Lanes 1 and 7, non-depleted extract without and with Tat. Lane 2, transcription in the depleted extract. To the depleted extract the following was added: lanes 3–5, TTK; lane 6, CDK9/cyclin T and purified TFIIH; lane 7, CDK9/cyclin T; lane 8, Tat and CDK9/cyclin T; lane 9, Tat; lanes 10–12, Tat and TTK; lane 13, Tat and TFIIH; lane 14, Tat and CDK9/cyclin T.

TTK augments Tat-mediated transactivation *in vivo*

To assess the role of TTK in Tat transactivation *in vivo*, we microinjected TTK, or, as an experimental control, TTK' fractions, into HS68 cells (human foreskin fibroblasts), utilizing the indicator plasmid HIV-1 LTR-LacZ (pJK2 [20]) or the control plasmid β -actin-LacZ [18]. The injected cells were marked with rabbit IgG and immunostained with Texas Red-conjugated anti-rabbit IgG and the β -galactosidase was stained with FITC-conjugated anti- β -galactosidase monoclonal antibodies. The score of β -galactosidase-positive microinjected cells is shown in Figure 5. Note that neither TTK nor TTK' alone induced the HIV-1 LTR-LacZ reporter in the absence of Tat, suggesting that the Tat-associated kinase fraction functions to augment Tat-mediated transactivation (Figure 5A). Inclusion of recombinant Tat protein induced LTR-LacZ expression. In the absence of TTK, 14 out of 31 injected cells (45%) showed a response to Tat. Inclusion of TTK along with Tat augmented the Tat response to 80% (20 out of 25 injected cells, Figure 5A). In contrast with TTK, inclusion of the TTK' fraction along with Tat protein showed no measurable induction of Tat response (Figure 5A). Thus it appears that TTK and not the TTK' fraction is capable

of inducing Tat transactivation *in vivo*. The induction appears to be specific, since a non-specific reporter construct, under the control of β -actin promoter, is neither induced by Tat nor stimulated by TTK or TTK' (Figure 5B).

TTK stimulates Tat-dependent transcription elongation *in vitro*

We utilized an *in vitro* transcription assay with the HIV-1 LTR template and HeLa nuclear extract to determine the effect of purified TTK on Tat-dependent transcription. Since CDK9 present in the HeLa nuclear extract makes it highly active in basal and Tat-dependent transcription, the nuclear extract was depleted of CDK9 as described in [22] (Figure 6A, compare lanes 1 and 7 with lanes 2 and 8). Addition of TTK restored Tat-dependent transactivation in CDK9-depleted nuclear extract (Figure 6A, lanes 9–11). In control experiments, addition of the CDK7-containing fraction did not restore Tat transactivation (Figure 6B, lanes 9–11). Furthermore, Tat transactivation was not restored by TTK in extracts that were double-depleted of CDK9 and CDK7 (Figure 6C, lanes 9–11).

DISCUSSION

In this report we analysed the components of a Tat-associated CTD kinase to determine its specific role in Tat transactivation of HIV-1 LTR. We have previously reported that HIV-1 Tat associates with a CTD kinase (TTK) in human primary T-cells [17,18]. The TTK fraction associated with full-length Tat, and was shown to contain CDK7. The TTK fraction phosphorylated CTD as well as it stimulated CAK activity [17]. Upon further fractionations on DEAE-Sepharose, SP-Sepharose, heparin-agarose and hydroxyapatite columns, the TTK fraction resolved from CDK7; however, it did retain the ability to induce CAK [18]. Moreover, the purified TTK induced transcription from HIV-1 LTR in assays *in vivo* [18]. In the present study we characterized the components of purified TTK. At the initial stages of fractionation, CDK2, CDK7 and CDK9 co-eluted with the Tat-associated CTD kinase activity. After further purification on SP-Sepharose, heparin-agarose and hydroxyapatite columns, only CDK2 co-eluted with the TTK activity, and not CDK7 or CDK9. In agreement with our earlier results, TTK stimulated CDK7–cyclin H complex formation. TTK phosphorylated Thr-176 of *Xenopus* CDK7 and induced CDK7–cyclin H interaction. Moreover, immunodepletion of the CDK2 component of TTK by anti-PSTAIRE antibodies greatly reduced the ability of TTK to phosphorylate CDK7. Therefore, it appears that the CDK2 component of the TTK fraction is responsible for CAK activation.

With respect to the RNA Pol II CTD kinase activity, the TTK fraction showed only CTD hypophosphorylation activity at the initial stages of purification [17]. This hypophosphorylation activity resolved into two peaks (TTK and TTK') on DEAE-Sepharose. Surprisingly, after binding to Tat, the TTK and not the TTK' fraction hyperphosphorylated CTD. An interesting aspect of the CTD hyperphosphorylation activity is that binding of the kinase to GST-Tat has a stimulatory effect on CTD hyperphosphorylation. When TTK was bound to the N-terminal domain of Tat only CTD hypophosphorylation was observed in contrast with CTD hyperphosphorylation by full-length Tat-bound TTK. We also demonstrated that purified soluble Tat greatly stimulated TTK to hyperphosphorylate CTD. Curiously, this hyperphosphorylation occurs specifically on Ser-2 residues of CTD, in contrast with phosphorylation of CTD by CDK7, which phosphorylates CTD on Ser-2 and Ser-5 (Figure 3D), or CDK9, which phosphorylates CTD on Ser-5 in response to

Tat [22,28]. Tat also had a similar stimulatory effect on purified CDK2/cyclin E, which hyperphosphorylated CTD on Ser-2 in the presence of Tat (Figure 3C). In our preliminary study, we determined that amino acids 42–72 of Tat stimulated CTD hyperphosphorylation (S. Nekhai and F. Kashanchi, unpublished work). It is likely therefore that both the N-terminal domain of Tat and the core domain of Tat are required to bind TTK and to stimulate Ser-2 target-specific hyperphosphorylation of CTD.

To analyse the biological relevance of the TTK fraction to Tat transactivation we performed direct microinjections of TTK into human primary fibroblasts. In these experiments, TTK augmented Tat-mediated transactivation *in vivo*. In contrast, TTK' did not induce Tat-mediated transactivation. Although the level of induction we observed was a modest 2-fold, this effect was observed in a short induction time (3 h post-injection). Therefore, the net effect of TTK on the regulation of HIV-1 expression might be significantly higher. Co-transfection of HEK-293 cells with CMV-Tat and HIV-1 LTR-LacZ expression vectors showed only a 3-fold increase in the number of β -galactosidase-positive cells, as compared with the control HIV-1 LTR-LacZ transfection (results not shown). Strikingly, when β -galactosidase enzymic activity was assayed, the effect of Tat increased to 33-fold (results not shown). Therefore, the procedure to score for β -galactosidase-positive cells may underestimate the effect of TTK. To confirm that TTK indeed stimulates Tat-dependent transcription elongation *in vitro*, we utilized an *in vitro* transcription assay with an HIV LTR template. The cell-free transcription reaction was immunodepleted of CDK9 to make a nuclear extract deficient in Tat transactivation. Addition of TTK partially restored Tat-dependent transactivation in CDK9-depleted nuclear extract. In control experiments, addition of CDK7-containing kinase fraction did not restore Tat transactivation in the CDK9-depleted extract. Further, addition of TTK to CDK9 and CDK7 double-depleted nuclear extract did not restore Tat transactivation. The results indicate that TTK may require CDK7 for its function in CDK9-depleted transcription activation. Recently, CDK9 has been shown to hyperphosphorylate Ser-5 in the presence of Tat [22,29]. In the absence of Tat only Ser-2 phosphorylation was observed [22].

Sequencing of the TTK components revealed the presence of a regulatory subunit of a nuclear phosphatase, NIPPI1 [26]. The role of NIPPI1 in HIV-1 transcription is currently under investigation in our laboratory. In addition, our unpublished observations indicate that protein phosphatase 1 can dephosphorylate Ser-2 of CTD [29a]. Therefore, CDK2 and nuclear protein phosphatase 1 may represent a novel kinase–phosphatase complex that reversibly phosphorylates CTD on Ser-2. The roles of other protein components of the TTK complex are yet to be determined.

The specific role of Ser-2 phosphorylation in HIV-1 transcription is yet to be clarified. The biochemical distinction between TTK and CDK9 indicates that Tat might utilize more than one pathway to hyperphosphorylate RNA Pol II CTD. CDK9 alone does not interact with Tat [13]. The CDK9/cyclin T complex interacts with Tat to a greater extent in the presence of TAR RNA [30,31]. Also, CDK9 autophosphorylation stimulates binding of CDK9/cyclin T to TAR RNA in the presence of Tat [29]. Therefore it is possible that prior to the activation of CDK9, Tat may stimulate an additional kinase, such as CDK2/TTK, to provide Ser-2 phosphorylation of CTD. The role of CDK2 in the stimulation of Tat-mediated transcription is intriguing, due to the fact that HIV-1 transcription is regulated during the cell cycle [18,32]. It has been demonstrated that transcription from HIV-1 LTR is Tat-dependent at the G₁ phase and less so at G₂/M phase of the cell cycle [32]. We observed the

highest Tat response of HIV-1 LTR transcription in the cells progressing from the G₀ to the G₁ phase [18]. Moreover, overexpression of a CDK inhibitor p16 blocks Tat transactivation [18]. Overexpression of dominant-negative mutants of CDK2 partially blocked Tat transactivation [18]. Recently, increased activity of CDK2/cyclin E was reported in HIV-1-infected quiescent peripheral CD4 lymphocytes [33]. Therefore, it is possible that HIV-1 Tat may utilize this abundant CDK2 activity to stimulate transcription.

The presented detailed analysis of the components of TTK suggests a mechanism whereby a positive-feedback mechanism helps to maintain a hyperphosphorylated state of RNA Pol II CTD repeats (CTDo). The results envision a means to sustain CTDo whereby the PSTAIRE kinase in the TTK fraction phosphorylates the CDK7 component to reconstitute active CAK. Secondly, Tat may activate CDK2 to promote Ser-2 phosphorylation, in addition to Ser-5 phosphorylation provided by CDK9. Yet to be resolved is the role of other TTK components in Tat transactivation *in vivo*, which may include their specific role in tethering the active CAK components to HIV-1 Tat.

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REFERENCES

- Cullen, B. R. (1998) HIV-1 auxiliary proteins: making connections in a dying cell. *Cell* **93**, 685–692.
- Jeang, K. T. (1998) Tat, Tat-associated kinase, and transcription. *J. Biomed. Sci.* **5**, 24–27.
- Taube, R., Fujinaga, K., Wimmer, J., Barboric, M. and Peterlin, M. B. (1999) Tat transactivation: a model for the regulation of eukaryotic transcriptional elongation. *Virology* **264**, 245–253.
- Karn, J. (1999) Tackling Tat. *J. Mol. Biol.* **293**, 235–254.
- Adams, M., Sharmeen, L., Kimpton, J., Romeo, J. M., Garcia, J. V., Peterlin, M. B., Groudine, M. and Emerman, M. (1994) Cellular latency in human immunodeficiency virus-infected individuals with high CD4 levels can be detected by the presence of promoter-proximal transcripts. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 3862–3866.
- Antoni, B. A., Stein, S. B. and Rabson, A. B. (1994) Regulation of human immunodeficiency virus infection: implications for pathogenesis. *Adv. Virus Res.* **43**, 53–145.
- Boris-Lawrie, K. A., Brady, J. N. and Kumar, A. (1992) Sequences within the R region of the long terminal repeat activate basal transcription from the HIV-1 promoter. *Gene Express.* **2**, 215–230.
- Feng, S. and Holland, E. C. (1988) HIV-1 tat trans-activation requires the loop sequence within TAR. *Nature (London)* **334**, 165–167.
- Laspi, M. F., Rice, A. P. and Mathews, M. B. (1989) HIV-1 Tat protein increases transcriptional initiation and stabilizes elongation. *Cell* **59**, 283–292.
- Chodosh, L. A., Fire, A., Samuels, M. and Sharp, P. A. (1989) 5,6-Dichloro-1-beta-D-ribofuranosylbenzimidazole inhibits transcription elongation by RNA polymerase II *in vitro*. *J. Biol. Chem.* **264**, 2250–2257.
- Yankulov, K., Yamashita, K., Roy, R., Egly, J.-M. and Bentley, D. L. (1995) The transcriptional elongation inhibitor 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole inhibits transcription factor IIH-associated protein kinase. *J. Biol. Chem.* **270**, 23922–23925.
- Herrmann, C. H. and Rice, A. P. (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.
- Zhu, Y., Pe'ery, T., Peng, J., Ramanathan, Y., Marshall, N., Amendt, B., Mathews, M. B. and Price, D. H. (1997) Transcription elongation factor P-TEFb is required for HIV-1 tat transactivation *in vitro*. *Genes Dev.* **11**, 2622–2632.
- 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. U.S.A.* **94**, 12331–12336.
- Grana, X., DeLuca, A., Sang, N., Fu, X., Claudio, P. P., Rosenblatt, J., Morgan, D. O. and Giordano, A. (1994) PITALRE, a nuclear CDC2-related protein kinase that phosphorylates the retinoblastoma protein *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 3834–3838.
- Marshall, N. F. and Price, D. H. (1995) Purification of p-TEFb, a transcription factor required for the transition into productive elongation. *J. Biol. Chem.* **270**, 12335–12338.
- Nekhai, S., Shukla, R. R. and Kumar, A. (1997) A human primary T-lymphocyte-derived human immunodeficiency virus type 1 Tat-associated kinase phosphorylates the C-terminal domain of RNA polymerase II and induces CAK activity. *J. Virol.* **71**, 7436–7441.
- Nekhai, S., Shukla, R. R., Fernandez, A., Kumar, A. and Lamb, N. J. C. (2000) Cell cycle-dependent stimulation of HIV-1 promoter by Tat-associated CAK activator. *Virology* **266**, 246–256.
- Peterson, S. R., Dvir, A., Anderson, C. W. and Dynan, W. S. (1992) DNA binding provides a signal for phosphorylation of the RNA polymerase II heptapeptide repeats. *Genes Dev.* **6**, 426–438.
- Kimpton, J. and Emerman, M. (1992) Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated beta-galactosidase gene. *J. Virol.* **66**, 2232–2239.
- Cisek, L. J. and Corden, J. L. (1991) Purification of protein kinases that phosphorylate the repetitive carboxyl-terminal domain of eukaryotic RNA polymerase II. *Methods Enzymol.* **200**, 301–325.
- Zhou, M., Halanski, M. A., Radonovich, M. F., Kashanchi, F., Peng, J., Price, D. H. and Brady, J. N. (2000) Tat modifies the activity of CDK9 to phosphorylate serine 5 of the RNA polymerase II carboxyl-terminal domain during human immunodeficiency virus type 1 transcription. *Mol. Cell. Biol.* **20**, 5077–5086.
- Eng, J. K., McCormick, A. L. and Yates, III, J. R. (1996) Mining genomes with MS. *Anal. Chem.* **68**, 534A–540A.
- Chittum, H. S., Lane, W. S., Carlson, B. A., Roller, P. P., Lung, F. D., Lee, B. J. and Hatfield, D. L. (1998) Rabbit beta-globin is extended beyond its UGA stop codon by multiple suppressions and translational reading gaps. *Biochemistry* **37**, 10866–10870.
- Martinez, A. M., Afshar, M., Martin, F., Cavadore, J. C., Labbe, J. C. and Doree, M. (1997) Dual phosphorylation of the T-loop in cdk7: its role in controlling cyclin H binding and CAK activity. *EMBO J.* **16**, 343–354.
- Vulsteke, V., Beullens, M., Waelkens, E., Stalmans, W. and Bollen, M. (1997) Properties and phosphorylation sites of baculovirus-expressed nuclear inhibitor of protein phosphatase-1 (NIPP-1). *J. Biol. Chem.* **272**, 32972–32978.
- Fisher, R. P., Jin, P., Chamberlin, H. M. and Morgan, D. O. (1995) Alternative mechanisms of CAK assembly require an assembly factor or an activating kinase. *Cell* **83**, 47–57.
- Ivanov, D., Kwak, Y. T., Nee, E., Guo, J., Garcia-Martinez, L. F. and Gaynor, R. B. (1999) Cyclin T1 domains involved in complex formation with Tat and TAR RNA are critical for tat-activation. *J. Mol. Biol.* **288**, 41–56.
- Garber, M. E., Mayall, T. P., Suess, E. M., Meisenhelder, J., Thompson, N. E. and Jones, K. A. (2000) CDK9 autophosphorylation regulates high-affinity binding of the human immunodeficiency virus type 1 tat-P-TEFb complex to TAR RNA. *Mol. Cell. Biol.* **20**, 6958–6969.
- Bharucha, D. C., Zhou, M., Nekhai, S., Brady, J. N., Shukla, R. R. and Kumar, A. (2002) Protein phosphatase from human T cells augments Tat transactivation of the immunodeficiency virus type 1 long terminal repeat. *Virology*, in the press.
- Wei, P., Garber, M. E., Fang, S. M., Fischer, W. H. and Jones, K. A. (1998) A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. *Cell* **92**, 451–462.
- Garber, M. E., Wei, P., Kewal-Ramani, V. N., Mayall, T. P., Herrmann, C. H., Rice, A. P., Littman, D. R. and Jones, K. A. (1998) The interaction between HIV-1 Tat and human cyclin T1 requires zinc and a critical cysteine residue that is not conserved in the murine CycT1 protein. *Genes Dev.* **12**, 3512–3527.
- Kashanchi, F., Agbottah, E. T., Pise-Masison, C. A., Mahieux, R., Duvall, J., Kumar, A. and Brady, J. N. (2000) Cell cycle-regulated transcription by the human immunodeficiency virus type 1 Tat transactivator. *J. Virol.* **74**, 652–660.
- Clark, E., Santiago, F., Deng, L., Chong, S., de La Fuente, C., Wang, L., Fu, P., Stein, D., Denny, T., Lanka, V. et al. (2000) Loss of G(1)/S checkpoint in human immunodeficiency virus type 1-infected cells is associated with a lack of cyclin-dependent kinase inhibitor p21/Waf1. *J. Virol.* **74**, 5040–5052.