HIV-1 Tat Interaction with RNA Polymerase II C-terminal Domain (CTD) and a Dynamic Association with CDK2 Induce CTD Phosphorylation and Transcription from HIV-1 Promoter*

Longwen Deng‡, Tatyana Ammosova§, Anne Pumfery‡, Fatah Kashanchi‡, and Sergei Nekhai‡§

From the ‡Department of Biochemistry & Molecular Biology, George Washington University Medical Center, Washington, D. C. 20037, and the §Center for Sickle Cell Disease and Department of Biochemistry, Howard University, Washington, D. C. 20059

HIV-1 Tat is a viral protein that interacts with transcriptional elongation (3), which is regulated by phosphorylation of the largest subunit of RNA polymerase II (RNAPII). In this process Tat enhances phosphorylation of the C-terminal domain (CTD) of RNAPII by activating cell cycle-dependent kinases (CDKs) associated with general transcription factors of the promoter complex, specifically CDK7 and CDK9. Here, we provide further evidence that CDK2 is involved in Tat-mediated CTD phosphorylation and in HIV-1 transcription in vitro. Tat-mediated CTD phosphorylation by CDK2 required cysteine 22 in the activation domain of Tat and amino acids 42–72 of Tat. CDK2 phosphorylated Tat itself, apparently by forming dynamic contacts with amino acids 15–24 and 36–49 of Tat. Also, amino acids 24–36 and 45–72 of Tat interacted with CTD. CDK2 associated with RNAPII and was found in elongation complexes assembled on HIV-1 long-terminal repeat template. Recombinant CDK2/cyclin E stimulated Tat-dependent HIV-1 transcription in reconstituted transcription assay. Immunodepletion of CDK2/cyclin E in HeLa nuclear extract blocked Tat-dependent transcription. We suggest that CDK2 is part of a transcription complex that is required for Tat-dependent transcription and that interaction of Tat with CTD and a dynamic association of Tat with CDK2/cyclin E stimulated CTD phosphorylation by CDK2.

* This work was supported by National Institutes of Health (NIH) Grants AI44357 and AI43894, an Alexandria and Alexander Sinheimer Foundation grant, and a grant from George Washington University (to F. K.) and by NHLBI, NIH Research Grant UH1 HL03679 and The Office of Research on Minority Health (to T. A. and S. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Center for Sickle Cell Disease, Howard University, 2121 Georgia Ave., NW, Washington, D. C. 20059. Tel.: 202-885-4845; Fax: 202-884-7861; E-mail: anekhai@howard.edu.

¶ The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; TAR, transactivation response; RNAPII, RNA polymerase II; CTD, C-terminal domain; CDK, cell cycle-dependent kinase; TTK, Tat-associated T-cell-derived kinase; GST, glutathione S-transferase; DTT, dithiothreitol; LTR, long terminal repeat; CTDa, hypophosphorylated form of CTD; CTDa, hyperphosphorylated form of CTD; ARM, arginine-rich motif.

\[1\] The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; TAR, transactivation response; RNAPII, RNA polymerase II; CTD, C-terminal domain; CDK, cell cycle-dependent kinase; TTK, Tat-associated T-cell-derived kinase; GST, glutathione S-transferase; DTT, dithiothreitol; LTR, long terminal repeat; CTDa, hypophosphorylated form of CTD; CTDa, hyperphosphorylated form of CTD; ARM, arginine-rich motif.

\[2\] HIV-1 Tat is a viral protein that interacts with transcriptional elongation (3), which is regulated by phosphorylation of the largest subunit of RNA polymerase II (RNAPII). In this process Tat enhances phosphorylation of the C-terminal domain (CTD) of RNAPII by activating cell cycle-dependent kinases (CDKs) associated with general transcription factors of the promoter complex, specifically CDK7 and CDK9. We reported a Tat-associated T-cell-derived kinase, which contained CDK2. Here, we provide further evidence that CDK2 is involved in Tat-mediated CTD phosphorylation and in HIV-1 transcription in vitro. Tat-mediated CTD phosphorylation by CDK2 required cysteine 22 in the activation domain of Tat and amino acids 42–72 of Tat. CDK2 phosphorylated Tat itself, apparently by forming dynamic contacts with amino acids 15–24 and 36–49 of Tat. Also, amino acids 24–36 and 45–72 of Tat interacted with CTD. CDK2 associated with RNAPII and was found in elongation complexes assembled on HIV-1 long-terminal repeat template. Recombinant CDK2/cyclin E stimulated Tat-dependent HIV-1 transcription in reconstituted transcription assay. Immunodepletion of CDK2/cyclin E in HeLa nuclear extract blocked Tat-dependent transcription. We suggest that CDK2 is part of a transcription complex that is required for Tat-dependent transcription and that interaction of Tat with CTD and a dynamic association of Tat with CDK2/cyclin E stimulated CTD phosphorylation by CDK2.

\[3\] Although the evidence for the role of CDK9/cyclin T1 in Tat-mediated HIV-1 transcription is overwhelming, our recent data suggest that there may be an additional CTD kinase involved in the Tat response. We have purified a Tat-associated T-cell-derived kinase (TTK) that phosphorylates CTD (9–11). TTK stimulates Tat transactivation in vitro (11) and in vivo (10, 11). TTK contains CDK2, which phosphorylates CDK7 (11).

In the work presented here, we analyze the effect of Tat on CTD phosphorylation by CDK2/cyclin E and the function of CDK2 in transcription assays of HIV-1 promoter in vitro. Our finding demonstrated that interaction of Tat with CTD and a dynamic interaction with CDK2/cyclin E stimulated CTD phosphorylation by CDK2. Also we showed that CDK2 was part of transcription complex and was required for Tat-dependent transcription in vitro.

EXPERIMENTAL PROCEDURES

Materials—Recombinant CDK2/cyclin E expressed and purified as described previously (12) was a gift from Dr. M. Beulken (Catholic University of Leuven, Belgium). Recombinant CDK9/cyclin T1 purified as described previously (13) was a gift of Dr. D. Price (Iowa State University). Glutathione S-transferase (GST)-fused CTD was expressed in E. coli and purified on Aquepore RP-300 column (Applied Biosystems, Foster City, CA) by reverse-phase chromatography as described (15). Mutant Tat C22G fused to GST was expressed in E. coli, purified on glutathione-agarose beads, cleaved off the GST moiety with thrombin, recovered from the beads in 7 M guanidine-HCl and purified on Aquepore RP-300 column (Applied Biosystems, Foster City, CA). Peptides representing partial sequence of Tat were chemically synthesized by Peptide Technologies (Gaithersburg, MD). Yeast RNA polymerase II was purified as described (16) and was a gift of Dr. Vladimir Tchernaenko (Henry Ford Health System, Detroit, MI). CDT peptides were provided by Dr. S. Trigun and Dr. M. Morange (Ecole Normale Superieure, Unite de Genetique Moléculaire, France).
Antibodies—Anti-CDK9 (Biosign, Saco, ME), anti-CDK2 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-RNAPII (BabCo, Richmond, CA) were purchased, aliquoted, and kept at −70 °C until later use. Antibodies against the α-subunit of the translation initiation factor eIF-2 were generated as described (17).

Cyclin E phosphorylates serines 2 and 5 of CTD heptapeptide repeat

CDK2/cyclin E was immunoprecipitated and incubated with 1 μg of wild type or A5, A9, A12, or A7/A14 mutant CDK2 peptide. Labeled peptides were resolved on a 20% SDS-PAGE and analyzed on a PhosphorImager. The PhosphorImager counts from two independent experiments are shown in millions. Roscovitine was added at 9.6 μM. Data are means ± S.D.

| CTD peptide | CDK2/cyclin E | CDK2/cyclin E + roscovitine |
|-------------|--------------|----------------------------|
| Hepta-2: SPTAPSYSPYTPSY | 6.80 ± 0.20 | 0.09 ± 0.32 |
| A5: SPTAPSYSPYTPSY | 0.90 ± 07 | 0.06 ± 0.21 |
| A9: SPTAPSYSPYTPSY | 1.20 ± 0.16 | 0.04 ± 0.33 |
| A12: SPTAPSYSPYTPSY | 5.30 ± 0.10 | 0.09 ± 0.22 |
| A7/A14: SPTAPSYSPYTPSY | 4.70 ± 0.38 | 0.05 ± 0.38 |

In Vitro Transcription Assay—The immobilized biotin HIV-1 LTR DNA (0.3 or 3 μg/reaction) was bound to streptavidin-Sepharose beads (high performance, Amersham Biosciences) and used for in vitro transcription, which was performed with DNA and a mixture of basal factors, including HeLa RNAPII (100 ng), e-TFIIID (epitope-tagged, 100 ng of TBP and TAF proteins), rTFIIA (40 ng of three recombinant subunits), rTFIIB (1 μg, 20 ng), rTFIIF (50 ng of both subunits), TFIIH (75 ng from HeLa cells), RHA (50 ng, gift of Dr. C. G. Lee), and p300 (100 ng). In addition, 2.5 mM ATP/CTP/GTP mix and 30 μM of [32P]UTP (400 Ci/mmol, Amersham Biosciences) were added. Also Tat (1 μg) and either CDK9/cyclin T1 (150 ng, gift of Dr. D. Price) or CDK2/cyclin E (200 ng) was added as indicated. The final volume (30 μl) was adjusted with transcription buffer (20 mM HEPES (pH 7.9), 100 mM KCl, 12.5 mM MgCl2, 1 mM EDTA, 17% glycerol, and 1 mM DTT). 32P-Labeled RNA products were purified and separated on a 6% denaturing acrylamide urea gel, dried, and visualized using PhosphorImager software (Amersham Biosciences).

RESULTS

CDK2/Cyclin E Phosphorylates Ser-2 and Ser-5 of the CTD Heptapeptide Repeat—We have recently demonstrated that CDK2/cyclin E phosphorylates CDT (11). CDK2 binds to a (ST/I)PKRX/K consensus phosphorylation site (19). The CTD heptapeptide (YPSTPS) contains two SF repeats that could potentially be recognized by CDK2/cyclin E. To analyze the phosphorylation sites, we used synthetic peptides containing two CTD repeats (hepta-2). We also used mutated CTD peptides containing a Ser-5 mutation in the first hepta repeat (A5), a Ser-2 mutation in the second hepta repeat (A12) or two Ser-7 mutations in the first and second hepta repeats (A7/A14) (20). Mutations in Ser-5 of the first hepta repeat (A5) or Ser-2 in the second repeat (A12) or two Ser-7 mutations in the first and second hepta peptides (A7/A14) (20).
HIV-1 Tat Associates with CTD and CDK2

A) HIV-1 Tat stimulates CDK2/cyclin E to phosphorylate RNAPII CTD. A, Tat stimulated GTS-CTD phosphorylation in a dose-dependent manner. Recombinant GST-CTD was incubated with CDK2/cyclin E in the presence of 100, 200, and 400 ng of wild type Tat (lanes 2–4) or Tat C22G (lanes 5–7). Addition of Tat resulted in the appearance of hyperphosphorylated form of CTD, indicated as CTDa, and hypophosphorylated form of CTD is indicated as CTDo. Lane 1, control without Tat. B, Tat stimulated RNAPII CTD phosphorylation in a dose-dependent manner. Yeast RNAPII was incubated with CDK2/cyclin E in the presence of 100, 200, and 400 ng of Tat (lanes 6–8). Tat stimulates RNAPII phosphorylation in a dose-dependent manner. Phosphorylated RNAPII CTD, marked as RNAPII, Lane 5, control without Tat. Lanes 1–4, reactions without CDK2/cyclin E and with 0, 100, 200, and 400 ng of Tat. Kinase reactions were resolved on 10% (A) and 5% (B) SDS-PAGE and analyzed on a PhosphorImager. Quantitation of the phosphorylated CTD is shown in arbitrary units, proportional to the PhosphorImager units. 

B) Amino acids 42–70 of Tat stimulate CTD phosphorylation. CTD was incubated with CDK2/cyclin E in the presence of 100, 200, and 400 ng of full-length Tat (lanes 2–4), Tat-(1–49) (lanes 5–7), Tat-(42–72) (lanes 8–10), Tat-(42–54) (lanes 11–13), Tat-(49–60) (lanes 14–16), and Tat-(56–70) (lanes 17–19). Full-length Tat and Tat-(42–72) stimulated CTD hyperphosphorylation, indicated as CTDa, and CTD hypophosphorylation, indicated as CTDo, by CDK2/cyclin E. Lane 1, control reaction without Tat. Kinase reactions were resolved on 10% SDS-PAGE and analyzed on a PhosphorImager. Quantitation of phosphorylated CTD is shown in arbitrary units, which are proportional to the PhosphorImager units.
concentration-dependent manner (Fig. 3A, lanes 2–4). To analyze which domain of Tat may form dynamic contacts with CDK2/cyclin E during phosphorylation reaction, a competition assay was performed with the excess of Tat peptides, which spanned different domains of Tat. The peptide containing amino acids 11–24 of Tat (Tat-(11–24)) completely blocked Tat phosphorylation (Fig. 3B, lane 3). In contrast, the peptide containing amino acids 6–14 of Tat (Tat-(6–14)) was not inhibitory. Therefore amino acids 15–24 are critical for the CDK2/cyclin E-mediated phosphorylation of Tat. Neither Tat-(49–60) nor Tat-(56–70) interfered with Tat phosphorylation (Fig. 3B, lane 4 and 5). We observed that Tat-(36–72) inhibited Tat phosphorylation (Fig. 3B, lane 6). Therefore, we concluded that amino acids 36–49 of Tat also dynamically interact with CDK2/cyclin E during phosphorylation reaction. Taken together, our observations demonstrate that CDK2/cyclin E dynamically interacts with the N-terminal domain of Tat.

Tat Binds to CTD—It has been suggested that Tat binds to RNAPII CTD through the RNA-binding arginine-rich motif of Tat, based on the observation that TAR RNA inhibited Tat-mediated CTD phosphorylation by CDK9/cyclin T1 (24). Therefore it was of interest to directly investigate binding of Tat to CTD. For this purpose, we utilized a modified gel shift assay that was previously developed to study the binding of TAR RNA to short peptides derived from double-stranded RNA-activated kinase (17). In the modified assay, we analyzed retardation of phosphorylated CTD as a function of Tat binding. CTD migrated on 4% polyacrylamide gel as a diffuse band (Fig. 4A, lane 1). Addition of full-length Tat resulted in a dose-dependent shift of CTD (Fig. 4A, lanes 2–4). Mutation in the N-terminal domain of Tat (C22G) reduced the efficiency of the shift (Fig. 4A, lanes 5–7). Truncation of first N-terminal 36 or 42 amino acids significantly reduced the ability of mutated Tat to bind to CTD (Fig. 4B, lanes 3 and 4). Further truncated Tat-(56–70) or isolated RNA-binding domain of Tat-(49–60) did not bind to CTD (Fig. 4B, lanes 5 and 6). However, the isolated activation domain of Tat, Tat-(1–44) bound to CTD (Fig. 4C, lane 5), although less efficiently than full-length Tat (Fig. 4C, lane 2). Because the N-terminal peptides, Tat-(6–14) and Tat-(11–24), did not bind CTD (Fig. 4C, lanes 3 and 4), then amino acids 24–44 may contain a CTD binding site in addition to the one or more sites located in the amino acids 42–72.

Therefore, Tat interacts with CTD via two binding sites located within amino acids 24–72.

CDK2 Associates with RNAPII in HeLa Nuclear Extract—To assess whether CDK2 is part of a transcription complex we analyzed whether CDK2 is associated with complexes that contain RNAPII. Following fractionation of HeLa nuclear extract on Superose 6 size exclusion column, CDK2 was found to be present in the high molecular weight fractions that also contained RNAPII (Fig. 5A, fractions 15–19). To assess whether CDK2 and RNA II were part of the same macromolecular complex, we analyzed whether CDK2 is associated with complexes that contain RNAPII. Following fractionation of HeLa nuclear extract on Superose 6 size exclusion column, CDK2 was found to be present in the high molecular weight fractions that also contained RNAPII (Fig. 5A, fractions 15–19). To assess whether CDK2 and RNA II were part of the same macromolecular complex, we analyzed whether CDK2 is associated with complexes that contain RNAPII. Following fractionation of HeLa nuclear extract on Superose 6 size exclusion column, CDK2 was found to be present in the high molecular weight fractions that also contained RNAPII (Fig. 5A, fractions 15–19). To assess whether CDK2 and RNA II were part of the same macromolecular complex, we analyzed whether CDK2 is associated with complexes that contain RNAPII. Following fractionation of HeLa nuclear extract on Superose 6 size exclusion column, CDK2 was found to be present in the high molecular weight fractions that also contained RNAPII (Fig. 5A, fractions 15–19). To assess whether CDK2 and RNA II were part of the same macromolecular complex.
of the transcriptional apparatus. Also Tat was added to the reaction. In the absence of UTP, RNAPII is paused on the fifth nucleotide of TAR RNA. Analysis of proteins associated with this paused RNAPII complex showed that both CDK2 was present in the paused complex (Fig. 6, lanes 2 and 4). These observations suggest that CDK2 is present in early elongation complex.

**CDK2/Cyclin E Is Required for Tat-mediated Transcription from HIV-1 Promoter**—The requirement of CDK2 in HIV-1 transcription was tested in a reconstituted transcription reaction. The reaction was assembled with biotinylated HIV-1 LTR template immobilized on streptavidin-Sepharose beads and with a mixture of basal factors, including RNAPII, e-TFIID (TBP and TAF proteins), rTFIIB, rTFIIB, hTFIIH, hRHA, and rp300. Under these conditions, we observed very little activated transcription with basal factors (Fig. 7, lane 1). Strikingly, addition of recombinant CDK2/cyclin E stimulated activated transcription to about 20-fold (Fig. 7, lane 2). Addition of CDK9/cyclin T1 stimulated activated transcription to about 9-fold (Fig. 7, lane 3). Next we analyzed whether CDK2/ cyclin E present in HeLa nuclear extract was essential for Tat-dependent transcription on immobilized template. Addition of Tat to HeLa nuclear extract stimulated transcription 65-fold (Fig. 7, compare lane 5 with lane 4). Immunodepletion of CDK2/cyclin E from the HeLa nuclear extract completely abolished the Tat-mediated activation of transcription (Fig. 7, lane 6). Addition of recombinant CDK2/cyclin E to the immunodepleted nuclear extract restored the Tat-activated transcription in a concentration-dependent manner (Fig. 7, lanes 7–9). Therefore, collectively, these results suggest that the CDK2/cyclin E complex is necessary for Tat-activated transcription in vitro.

**DISCUSSION**

This study describes a potentially novel mechanism by which HIV-1 Tat stimulates CTD phosphorylation by CDK2/cyclin E. More specifically, we have found that full-length Tat binds to
CTD and is also dynamically associated with CDK2/cyclin E, which greatly stimulated recombinant CDK2/cyclin E to phosphorylate CTD. We found that CDK2 associates with RNAPII and is part of transcription complex assembled on the HIV-1 LTR template. Finally, we have shown that CDK2 was required for Tat-dependent transcription.

Recent studies provided substantial evidence that a general transcription elongation factor-b-associated CDK9/cyclin T1 plays a key role in the Tat-mediated activation of HIV-1 transcription (reviewed in Refs. 1 and 2). Tat associates with the bulge of TAR RNA and also binds to cyclin T1, a cyclin partner of CDK9, which in turn interacts with the loop of TAR RNA (6, 22). This allows CDK9/cyclin T1 to be recruited by Tat to the HIV-1 promoter and stimulate transcription elongation by RNAPII (7). Although the evidence for the role of CDK9/cyclin T1 in Tat-mediated HIV-1 transcription is overwhelming, the collective data from our laboratories suggest that there may be an additional CTD kinase involved in the Tat response.

We have reported that Tat associates with a CTD kinase isolated from human primary T-lymphocytes (TTK) (9). Microinjections of TTK into human primary fibroblasts stimulated Tat-dependent expression of a reporter LacZ gene placed under the control of HIV-1 LTR (10). It was tempting to suggest that TTK contained CDK9, but we did not find CDK9 by immunoblotting assays (10). Moreover, biochemically TTK was distinct from CDK9, because TTK phosphorylated CDK7 and promoted association of CDK7 and cyclin H (10).

Analysis of TTK by biochemical fractionation showed that TTK uniquely copurified with CDK2 and not with CDK9 or CDK7 (11). Tat induced the TTK and CDK2 to phosphorylate CTD, specifically at serine 2 residues (11), which was in contrast to the reported phosphorylation of Ser-5 by CDK9 in the presence of Tat (8). TTK restored Tat-mediated transcription activation of HIV-1 LTR in an HeLa nuclear extract immunodepleted of CDK9 but not in the HeLa nuclear extract double-depleted of CDK9 and CDK7 (11). Therefore CDK2 was likely to be involved in the regulation of Tat-mediated transcription.

Another line of evidence, which points to CDK2, comes from the analysis of the cell cycle regulation of HIV-1 transcription. We have observed that Tat-mediated transcription is regulated during the cell cycle (10, 26). We have demonstrated that transcription from HIV-1 LTR is Tat-dependent at the G1 and less so at G2/M phase of the cell cycle (26). We observed highest Tat response of HIV-1 LTR transcription in the cells progressing from G0 to G1 phase (10). Overexpression of specific CDK inhibitors, p16 and p27, blocked Tat transactivation (10). Overexpression of dominant negative mutants of CDK2 and CDK4 but not CDK1 blocked Tat transactivation (10). These data indicate that Tat-dependent transcription may in part be regulated by CDK2, which activity is highest at the G1 phase. Finally, we have observed that the activity of CDK2/cyclin E is increased in HIV-1-infected quiescent peripheral CD4 lymphocytes (27). Collectively, our published data (9–11, 26, 27) points to a possibility that Tat-dependent transcription may be regulated by CDK2.

We report here that Tat stimulated CDK2 to phosphorylate CTD in a concentration-dependent manner. Little phosphorylation was observed in the absence of Tat. Analysis of the Tat regions responsible for stimulation of the kinase showed that two regions of Tat were required for efficient stimulation of CTD hypo- and hyperphosphorylation: cysteine 22 and the peptide, containing amino acids 42–72 of Tat. Mutation in the cysteine 22 decreased the efficiency of Tat stimulation, implying that the N terminus of Tat is important for stimulation. On the other hand, analysis of Tat truncation mutants, lacking N terminus amino acids showed that Tat-(42–72) partially stimulated CTD phosphorylation by CDK2/cyclin E. The full-length Tat showed 2-fold higher level of CTDa and CTDo phosphorylation at 2 μM concentration, compared to CTD phosphorylation at 6 μM Tat-(42–72) (Fig. 2B). Therefore Tat-(42–72) is ~10-times weaker activator than the full-length Tat. Therefore, it is likely that there is a binding site located in the N-terminal 42 amino acids of Tat, in addition to the one or more binding sites located within amino acids 42–72. These binding sites may interact with CTD and/or CDK2/cyclin E to stimulate CTD phosphorylation. Our results are in agreement with recent demonstration that full-length Tat, but not the isolated N-terminal domain of Tat, stimulated CTD hyperphosphorylation by CDK9 (24).

To study if CDK2/cyclin E dynamically interacted with Tat we analyzed phosphorylation of Tat by CDK2/cyclin E. Tat was phosphorylated by CDK2/cyclin E in a concentration-dependent manner. Competition analysis showed that amino acids 15–24 and 36–49 of Tat interacted with CDK2/cyclin E. The amino acids 15–24 of Tat contain a 16SQPK19 sequence, which resembles a putative CDK phosphorylation site (S/T)P (K/R) (19) and therefore may serve as a CDK2 phosphorylation site. The amino acids 36–49 of Tat contain a 41KAL43 sequence, which resembles a cyclin binding motif (Cy or RxL). This motif binds to a hydrophobic groove on the surface of the cyclin E and allows potential CDK2 phosphorylation site to be in proximity to CDK2 active center (28). The distance between the CDK2 phosphorylation site and the cyclin E binding site has been determined to be at least a 12 amino acids long to allow CDK2/cyclin E binding (28). In the case of Tat, the distance between SQPK sequence and KAL sequence is 21 amino acids, which is sufficient to allow simultaneous binding of CDK2 and cyclin E to Tat. Whether these motifs interact directly with CDK2/cyclin E still remains to be established. Also of interest would be to identify what position of Tat is phosphorylated by CDK2/cyclin E. Finally, it would be of interest to find out a role of Tat phosphorylation in HIV-1 transcription.

The formation of Tat-TAR RNA-CDK9/cyclin T1 complex has been demonstrated in a gel-shift assay (6, 24). In contrast, we did not detect binding of CDK2/cyclin E to Tat-TAR RNA complex in a gel-shift assay (data not shown). Therefore, the find-
ing, that CDK2 phosphorylates Tat, but does not form a stable complex with Tat-TAR RNA suggests that CDK2-Tat interaction is dynamic. Our data point to the possibility that this dynamic interaction between Tat and CDK2/cyclin E may target CDK2 to CTD. Accordingly, we demonstrated that Tat interacted with CTD in gel-shift assay through the interactions within the amino acids 24–72. We observed increase of both hypo- and hyperphosphorylation of CTD (Figs. 1 and 2). For mouse CTD phosphorylated by cdc2, the cdc2 mobility shift takes place when at least 15 phosphates are incorporated into 52 heptapeptide repeats (29). However, in contrast to CTD phosphorylation by cdc2, which resulted in a gradual shift of CTD (29), we observed both hypo- and hyperphosphorylated forms of CTD at the same time. This indicates that phosphorylation of CTD occurs in two different ways; in stochastic fashion, which generates CTDa, and in processive fashion, which results in multiple phosphorylations of the same CTD molecule. Accumulation of CTDa indicates that a single round phosphorylation is a more frequent event than multiphosphorylation. Because we did not observe intermediately phosphorylated CTD, CTDa is likely to be a result of a cooperative phosphorylation reaction, which means that consecutive phosphorylation of already phosphorylated CTD is more efficient than the first phosphorylation event. We present a hypothetical model of the Tat-mediated CTD phosphorylation by CDK2/cyclin E (Fig. 8). Tat interacts with CTD through the arginine-rich motif (ARM, amino acids 49–60) that may interact with the phosphorylated CTD residues and through the amino acids 24–36 (not shown in the figure). We speculate that the activation domain of Tat dynamically interacts with CDK2 through the proposed binding of SPQK sequence and with cyclin E through the cyclin-binding KAL sequence. Upon Tat phosphorylation by CDK2, the SPQK motif dissociates from CDK2 and the kinase is redirected to the SP sequence on CTD due to the continuing interaction of Tat with cyclin E and CTD. A single round of phosphorylation reaction will generate hypophosphorylated CTD, and the amount of the CTDa will be increased with the increase of concentration of Tat, which will bring CDK2 in proximity of CTD. We speculate that, after a single round of CTD phosphorylation by CDK2, the CDK2-cyclin E-Tat complex may slide along the CTD. Ionic interactions between ARM and CTD may permit a quick movement along the CTD, similar to the described movement of lae repressor toward lae operator on DNA when the repressor is intermittently associated with DNA through ionic contacts (30). As a result, CDK2 may phosphorylate CTD at multiple sites, generating hyperphosphorylated form of CTD. It has been shown that the CTD heptapeptide is primarily phosphorylated on Ser-2 and Ser-5 during transcription (4). In the present paper we showed that CDK2 phosphorylates both Ser-2 and Ser-5 of CTD heptapeptide repeat when only two repeats were used as a substrate. On full-length CTD, Tat induced primarily hyperphosphorylation of Ser-2 by CDK2/cyclin E (11). Interestingly, Tat stimulated CDK9 to phosphorylate Ser-5 in vitro transcription assays (8, 24). It indicates that there is a principal distinction in the mechanisms of CTD recognition by CDK2 and CDK9 in the presence of Tat. Also, it may indicate that the kinases are activated at different stages of transcription. In yeast, phosphorylation of Ser-5 during preinitiation transcription allows association of capping factors with CTD (31–33). Then Ser-5 is dephosphorylated by an as yet unknown phosphatase that allows dissociation of capping factors and then Ser-2 is phosphorylated by CDK9 (34). The pattern of Ser-2 and Ser-5 phosphorylation in higher eukaryotes during HIV-1 transcription remains to be established as well as the role of Tat in this process. We observed that CDK2 was associated with RNAPII. Fractionation of HeLa nuclear extract on the size-exclusion column showed that a portion of CDK2 coeluted with RNAPII. In addition, CDK2 associated with RNAPII on an affinity column with immobilized anti-RNAPII CTD antibodies. Therefore a portion of CDK2 is likely to be associated with the transcription complex. Surprisingly, we did not observe CDK2 in the preinitiation complex assembled on HIV-1 LTR template. Instead we found CDK2 present in the early elongation complex. This finding indicates that CDK2 may be recruited to the HIV-1 promoter after the preinitiation. Because CDK2 phosphorylates Ser-2 in the presence of Tat (11), then finding of CDK2 in the elongation complex is consistent with the observation that Ser-2 phosphorylation occurs at elongation and not at the initiation of transcription (34). We investigated the requirement of CDK2/cyclin E for HIV-1 transcription by analyzing the effect of CDK2 in reconstituted transcription assay, which was devoid of CDK9/cyclin T1. In this system, addition of either CDK2/cyclin E or CDK9/cyclin T1 stimulated Tat-dependent transcription. In HeLa nuclear extract, immunodepleted of CDK2/cyclin E dramatically decreased Tat-dependent transcription. Addition of CDK2/cyclin E to the depleted extract fully restored Tat-dependent transcription. The results indicate that CDK2/cyclin E is required for Tat-dependent transcription in vitro. Because CDK2/cyclin E did not bind to the Tat-TAR RNA complex (data not shown), therefore, CDK2 may only partially reconstitute transcription activation on the HIV-1 promoter. The role of CDK2 in stimulation of Tat-mediated transcription is intriguing due to the discussed regulation of HIV-1 transcription during the cell cycle (10, 26). It is also possible that HIV-1 Tat may utilize CDK2 activity, elevated during HIV-1 infection (27), to induce transcription. This study describes a potentially novel mechanism by which HIV-1 Tat stimulates CTD phosphorylation by CDK2/cyclin E. Uncovering an alternative pathway for Tat-mediated hyperphosphorylation of RNAPII CTD may provide additional valuable targets for anti-HIV-1 therapeutics.

Acknowledgments—We thank Dr. Emma Lees (DNAX, Palo Alto, CA) for the cyclin E and CDK2 viruses; Dr. M. Beuillon (Catholic University, Leuven, Belgium) for CDK2/cyclin E; Dr. L. Meijer (CNRS, France) for recombinant CDK9/cyclin T1 stimulated Tat-dependent transcription. In HeLa nuclear extract, immunodepleted of CDK2/cyclin E dramatically decreased Tat-dependent transcription. Addition of CDK2/cyclin E to the depleted extract fully restored Tat-dependent transcription. The results indicate that CDK2/cyclin E is required for Tat-dependent transcription in vitro. Because CDK2/cyclin E did not bind to the Tat-TAR RNA complex (data not shown), therefore, CDK2 may only partially reconstitute transcription activation on the HIV-1 promoter.

This role of CDK2 in stimulation of Tat-mediated transcription is intriguing due to the discussed regulation of HIV-1 transcription during the cell cycle (10, 26). It is also possible that HIV-1 Tat may utilize CDK2 activity, elevated during HIV-1 infection (27), to induce transcription.

This study describes a potentially novel mechanism by which HIV-1 Tat stimulates CTD phosphorylation by CDK2/cyclin E. Uncovering an alternative pathway for Tat-mediated hyperphosphorylation of RNAPII CTD may provide additional valuable targets for anti-HIV-1 therapeutics.

REFERENCES
1. Karn, J. (1999) J. Mol. Biol. 283, 235–254
2. Taube, R., Fujinaga, K., Wimmer, J., Barberic, M., and Peterlin, M. B. (1999) Virology 264, 245–253
3. Lapgia, M. F., Rice, A. P., and Mathews, M. B. (1999) Cell 99, 283–292
4. Dahmus, M. E. (1996) J. Biol. Chem. 271, 19009–19102
5. Majello, B., and Napolitano, G. (2001) Front. Biosci. 6, 1358–1368
6. Wei, P., Garber, M. E., Fang, S. M., Fischer, W. H., and Jones, K. A. (1998) Cell 92, 451–462
7. Bieniasz, P. D., Gréna, T. A., Bogerd, H. P., and Cullen, B. R. (1998) EMBO J. 17, 7066–7068
8. Zhou, M., Halanski, M. F., Radonovich, M. F., Kaushan, F., Peng, J., Price, D. H., and Brady, J. (2000) Nat. Neurosci. 3, 849–855
9. Peterson, S. R., Dvir, A., Anderson, C. W., and Dynan, W. S. (1992) Genes Dev. 6, 755–762
15. Allet, B., Payton, M., Mattaliano, R. J., Gronenborn, A. M., Clore, G. M., Wingfield, P. T. (1988) Gene 65, 259–268
16. Huet, J., Manaud, N., Dieci, G., Peyroche, G., Conesa, C., Lefevre, O., Ruet, A., Riva, M., Sentenac, A. (1996) Methods Enzymol. 273, 249–267
17. Nekhai, S., Bottaro, D. P., Woldehawariat, G., Spellerberg, A., and Petryshyn, R. A. (2000) Peptides 21, 1449–1456
18. Cujec, T. P., Okamoto, H., Fujinaga, K., Meyer, J., Chamberlin, H., Morgan, D. O., and Peterlin, B. M. (1997) Genes Dev. 11, 2645–2657
19. Morgan, D. O. (1997) Annu. Rev. Cell Dev. Biol. 13, 261–291
20. Trigon, S., Serizawa, H., Conaway, J. W., Conaway, R. C., Jackson, S. P., and Morange, M. (1998) J. Biol. Chem. 273, 6769–6775
21. Wang, D., de la Fuente, C., Deng, L., Wang, L., Zilberman, I., Eadie, C., Healey, M., Stein, D., Denny, T., Harrison, L. E., Meijer, L., and Kashanchi, F. (2001) J. Virol. 75, 7266–7279
22. Parada, C. A., and Roeder, R. G. (1996) Nature (London) 384, 375–378
23. Garcia-Martinez, I. F., Mavankal, G., Neve, J. M., Lane, W. S., Ivanov, D., and Gaynor, R. B. (1997) EMBO J. 16, 2836–2850
24. Garber, M. E., Mayall, T. P., Suess, E. M., Meisenhelder, J., Thompson, N. E., and Jones, K. A. (2000) Mol. Cell. Biol. 20, 6958–6969
25. Herrmann, C. H., and Rice, A. P. (1995) J. Virol. 69, 1612–1620
26. Kashanchi, F., Agbottah, E. T., Pise-Masison, C. A., Mahieux, R., Duvall, J., Kumar, A., and Brady, J. N. (2000) J. Virol. 74, 652–660
27. Clark, E., Santiago, F., Deng, L., Chong, S. Y., De La Fuente, C., Wang, L., Fu, P., Stein, D., Denny, T., Lanka, V., Mozafari, F., Okamoto, T., and Kashanchi, F. J. (2000) J. Virol. 74, 5049–5052
28. David, Y., Takeda, D. Y., James, A., Wohlschlegel, J. A., and Dutta, A. (2001) J. Biol. Chem. 276, 1993–1997
29. Zhang, J., and Corden, J. L. (1991) J. Biol. Chem. 266, 2297–2302
30. Lohman, T. M., DeHaseth, P. L., and Record, M. T. (1978) Biophys. Chem. 8, 281–294
31. McCracken, S., Fong, N., Yankulov, K., Ballantyne, S., Pan, G., Greenblatt, J., Patterson, S. D., Wickens, M., and Bentley, D. L. (1997) Nature 383, 357–361
32. Komarnitsky, P., Cho, E. J., and Buratowski, S. (2000) Genes Dev. 14, 2432–2440
33. Schroder, S. C., Schwer, B., Shuman, S., and Bentley, D. (2000) Genes Dev. 14, 2435–2440
34. Cho, E. J., Kobor, M. S., Kim, M., Greenblatt, J., and Buratowski, S. (2001) Genes Dev. 15, 3319–3329
HIV-1 Tat Interaction with RNA Polymerase II C-terminal Domain (CTD) and a Dynamic Association with CDK2 Induce CTD Phosphorylation and Transcription from HIV-1 Promoter

Longwen Deng, Tatyana Ammosova, Anne Pumfery, Fatah Kashanchi and Sergei Nekhai

J. Biol. Chem. 2002, 277:33922-33929.
doi: 10.1074/jbc.M111349200 originally published online July 11, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M111349200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 19 of which can be accessed free at http://www.jbc.org/content/277/37/33922.full.html#ref-list-1