TFIIFH Inhibits CDK9 Phosphorylation during Human Immunodeficiency Virus Type 1 Transcription*

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Tat stimulates human immunodeficiency virus, type 1 (HIV-1), transcription elongation by recruitment of the human transcription elongation factor P-TEFb, consisting of CDK9 and cyclin T1, to the TAR RNA structure. It has been demonstrated further that CDK9 phosphorylation is required for high affinity binding of Tat/P-TEFb to the TAR RNA structure and that the state of P-TEFb phosphorylation may regulate Tat transactivation. We now demonstrate that CDK9 phosphorylation is uniquely regulated in the HIV-1 preinitiation and elongation complexes. The presence of TFIIFH in the HIV-1 preinitiation complex inhibits CDK9 phosphorylation. As TFIIFH is released from the elongation complex between +14 and +36, CDK9 phosphorylation is observed. In contrast to the activity in the “soluble” complex, phosphorylation of CDK9 is increased by the presence of Tat in the transcription complexes. Consistent with these observations, we have demonstrated that purified TFIIFH directly inhibits CDK9 autophosphorylation. By using recombinant TFIIFH subcomplexes, our results suggest that the XBP subunit of TFIIFH is responsible for this inhibition of CDK9 phosphorylation. Interestingly, our results further suggest that the phosphorylated form of CDK9 is the active kinase for RNA polymerase II carboxyl-terminal domain phosphorylation.

Human immunodeficiency virus type 1 (HIV-1) encodes a transactivator protein, Tat, that stimulates transcription elongation through interaction with the transactivation response (TAR) RNA structure located at the 5′ end of nascent transcripts (1–4). In view of the fact that hyperphosphorylation of the carboxy-terminal domain (CTD) of the large subunit of RNA polymerase II (RNAP II) correlates with the formation of processive elongation complexes (5) and that Tat transactivation requires the CTD of RNAP II (6–9), it has been proposed that a critical step in Tat transactivation is mediated through a cellular kinase(s) (3, 10). Two CDK-cyclin pairs, present in two distinct transcription factor complexes, have been implicated as Tat cofactors that could phosphorylate the RNAP II CTD (3, 11). Positive transcription elongation factor (P-TEFb) (12, 13) or Tat-associated kinase (14–16), composed of CDK9 and cyclin T1 (CycT1) (14, 17–22), regulates Tat transactivation at an early step in transcription elongation. CDK9 is a Cdc2-related kinase termed PITALRE (22–25) that phosphorylates the RNAP II CTD and promotes transcriptional elongation from many promoters in vitro (5, 17, 22). Several lines of evidence suggest that P-TEFb is important for Tat transactivation. First, depletion of P-TEFb from HeLa nuclear extracts renders the extract unable to carry out Tat transactivation (17, 19, 21). Second, dominant-negative mutants of CDK9 or kinase inhibitors that preferentially block CDK9 inhibit Tat transactivation and HIV-1 replication in vivo (14, 17, 22, 26). Third, CycT1 is responsible for the species-specific restrictions to HIV-1 Tat transactivation in vivo. Tat transactivation is abolished in mouse cells because HIV-1 Tat is unable to bind cooperatively with murine CycT1 to TAR RNA structure (27–32). Tat transactivation could be restored by expression of human CycT1 in mouse cells or a murine CycT1 protein containing a point mutation (Y261C) in the Tat-TAR recognition motif (28, 31). Species-specific differences in the cyclin partners for CDK9 also underlie the failure of the equine infectious anemia virus Tat to recognize HIV-1 TAR RNA structure in human cells (33, 34). Although multiple cyclin partners for CDK9 have been identified (18, 35), Tat acts only with CycT1-CDK9 (36–38).

The function of the Tat-P-TEFb complex is mediated through the high affinity, loop-specific binding of the Tat-P-TEFb complex to the TAR RNA structure. The formation of the tripartite complex between Tat, CycT1, and TAR depends on the 5′ bulge and central loop in TAR (3, 11, 19, 28, 30–32, 39). Biochemical studies indicate that Tat binds to CycT1 and forms a zinc-dependent complex with residues in the Tat-TAR recognition motif of CycT1 (28, 31, 36, 37, 39). Recent studies have reported that autophosphorylation of CDK9 regulates this high affinity binding of the Tat-P-TEFb complex to TAR RNA structure (40, 41). Genetic studies have shown that TAR can be functionally replaced by heterologous RNA structures. The subsequent recruitment of Tat to these RNA targets by fusion of Tat to an RNA binding domain can clearly fully activate HIV-1 LTR-dependent transcription (42, 43). Furthermore, chimeric CycT1 or
CDK9 proteins can also activate transcription if tethered directly to nascent RNA (14, 33, 37). The results suggest that the primary role of Tat is to recruit P-TEFb to the TAR RNA structure.

The second CTD kinase that has been implicated in Tat transactivation is TFIIH, a general transcription factor that contains nine polypeptides (ERCC3/XPB, ERCC2/XPD, p62, p52, p44, CDK7 (MO15), cyclin H, MAT1, and p34) (44, 45), and possesses CTD kinase activity (46, 47). The kinase activity of TFIIH resides in the cyclin-dependent kinase 7 (CDK7) subunit (48–51). In association with cyclin H and MAT1, CDK7 forms the CDK-activating kinase (CAK) complex that phosphorylates CDKs involved in the regulation of the cell cycle (52–56). The association of CAK with core TFIIH switches its substrate specificity from CDKs to the CTD of RNAP II (57, 58). Interestingly, the yeast homologue of CDK7, Kin28, is found only in a complex with TFIIH and is devoid of CAK activity (59). Although it has been reported that Tat enhances CDK7 kinase activity (8, 60, 61), the role of TFIIH in Tat transactivation is controversial (62).

Several lines of evidence indicate that CDK7 (TFIIH) and CDK9 (P-TEFb) associate with the HIV-1 preinitiation complex (63–66). Furthermore, our previous published results (63) also demonstrated that CDK7 phosphorylates RNAP II CTD at serine 5 and CDK9 phosphorylates CTD at serine 2 during HIV-1 transcription. Thus, it is important to understand the relationship between CDK7 (TFIIH) and CDK9 (P-TEFb) during Tat transactivation. The recent reports (40, 41) have demonstrated that CDK9 autoprophosphorylation is important for binding to TAR RNA structure. The results presented in this study indicate that TFIIH plays a significant role in regulating CDK9 phosphorylation and thus Tat-P-TEFb binding to the TAR RNA structure. TFIIH apparently inhibits CDK9 phosphorylation until it is released from the transcription complex between +14 and +36. Once TFIIH is released, CDK9 phosphorylation occurs, allowing the Tat-P-TEFb complex to bind to the newly synthesized TAR RNA structure and facilitate transcription elongation. The orchestrated release of TFIIH and induction of Tat-P-TEFb binding to the TAR RNA structure almost certainly contributes to the normal efficiency of HIV-1 transcription in infected cells.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Anti-TBP and anti-TFIIH monoclonal antibodies were bought from Promega. Anti-TFIIIEe, anti-RAP74, anti-p62 (a subunit of TFIIH), anti-CDK7, and anti-Spi antibodies are products of Santa Cruz Biotechnology. Anti-CDK9 antibody was purchased from Biodesign Co. Anti-CTD of RNAP II monoclonal antibodies SWG16, H5 (phosphoserine 2) and H14 (phosphoserine 5), and anti-Tat monoclonal antibody are products of Babco.

**Biotinylation of Template DNAs**—HIV-1LTR templates (nucleotides −110 to +168) were amplified by PCR with the forward primer 5′-biotinylated TAT GGA TTT ACA AGG GAC TTT C-3′ and the reverse primer 5′-GAT CCG ATT ACA GAG TTC TTT C-3′. The primers were synthesized and biotinylated by Lofstrand Laboratories.

**Purification of Transcription Factors**—The production of recombinant P-TEFb proteins was carried out as described by Peng et al. (18). TFIIH and TFID were purified from HeLa extracts (67, 68). The production of recombinant subcomplexes of TFIIH was carried out as described by Tirole et al. (69).

**Immunodepletion of CDK7 from HeLa Nuclear Extract**—HeLa nuclear extract (100 µl) in 0.8 M KCl buffer D (20 mM HEPES (pH 7.9), 15% glycerol, 800 mM KCl, 10 mM MgCl₂, 0.2 mM EDTA (pH 8.0), 0.1% Nonidet P-40, and 1 mM DTT) was incubated with 20 µl of protein A-Sepharose beads to which anti-CDK7 had been pre-bound (10 µg of IgG). Antigen-antibody complexes were removed by centrifugation. After repeating the procedure twice, depleted nuclear extracts were dialyzed against 0.1 M KCl buffer D and assayed by Western blot analysis.

**Purification of Preinitiation Complexes (PICs)**—Association reaction mixtures (30 µl) contained 15 µl of HeLa nuclear extract, 1.0 µg of biotinylated templates, and 1.0 µg of poly[dI-dC]) in the absence or presence of Tat. The in vitro transcription (IVT) buffer contained 50 mM KCl, 6.25 mM MgCl₂, 20 mM HEPES (pH 7.9), 2 mM DTT, 0.5 mM EDTA (pH 8.0), 10 mM ZnSO₄, 10 mM creatine phosphate, 100 µg/ml creatine kinase, and 8.5% glycerol (1× IVT buffer). After a 30-min incubation at 30 °C, streptavidin-coated magnetic beads (Dynabeads, Dynal) pre-bound in binding buffer (20 mM HEPES (pH 7.9), 80 mM KCl, 10 mM MgCl₂, 2 mM DTT, 10 µM ZnSO₄, 100 µg/ml bovine serum albumin, 0.05% Nonidet P-40, and 10% glycerol) were then added to the reaction, and the mixtures were further incubated for 30 min at 30 °C. The immobilized templates were then harvested using a magnetic stand, and the PICs were washed extensively with 1× IVT buffer. In vitro transcription and Western blot analysis could be performed using the purified PICs assembled on the immobilized templates.

**Western Blot Analysis of the Purified PICs**—The purified PICs assembled on the immobilized templates were heated for 10 min at 100 °C in SDS loading buffer. The released proteins were fractionated by electrophoresis on 4–20% SDS-polyacrylamide gels and then transblotted onto polyvinylidene fluoride membranes (Millipore). The protein components of PICs were analyzed with specific antibodies as indicated above.

**In Vitro Transcription with the Purified PICs**—In vitro transcription reactions (100 µl) were set up by resuspending the purified PICs in 100 µl of 1× IVT buffer, 50 µM ATP, 50 µM CTP, 50 µM GTP, 20 µCi of [α-³²P]UTP, and 10 units of RNase (Promega). The transcription reactions were allowed to take place for 60 min at 30 °C. The radiolabeled transcripts were fractionated by electrophoresis on 6% denaturing polyacrylamide gels and detected by PhosphoImager.

**Kinase Reactions in the Purified PICs and Immunoprecipitation of the Phosphorylated Proteins**—Kinase reactions were performed by mixing the purified PICs with 20 µCi of [γ-³²P]ATP in 100 µl of 1× IVT buffer. After an incubation of 10 min at 30 °C, the PICs were separated from supernatants and washed extensively. 500 µl of RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) was then added into the tubes containing PICs immobilized by streptavidin-coated beads, and the mixtures were incubated for 120 min at 4 °C with rocking. The supernatants were saved, and phosphorylated proteins were immunoprecipitated by specific antibodies.

**Preparation of Transcription Elongation Complexes**—The purified PICs were incubated with ATP for 10 min and then washed extensively with 1× IVT buffer. The PICs were walked to position U-14 by incubation with 50 µM DATP, CTP, GTP, and UTP for 10 min at 30 °C and then washed extensively with 1× IVT buffer. The transcriptional elongation complexes (TECs) stalled at U-14 were walked stepwise along the DNA by repeated incubation with different sets of three NTPs, and then washed extensively with 1× IVT buffer to remove the uncoupled NTPs. To detect phosphorylation of proteins during moves, phosphorylated proteins were labeled with [γ-³²P]PIATP during stepwise moves. To analyze the proteins components of TECs stalled, the TECs were washed with cold NTPs, and TECs stalled at different positions were then analyzed by Western blot.

**CDK9 Autophosphorylation Assays**—CDK9 autophosphorylation assays were performed by mixing 50 ng of P-TEFb, 10 µM ATP, and 20 µCi of [γ-³²P]ATP in the absence or presence of Tat and incubating for 60 min at 23 °C. The total reaction volume was 20 µl, and the final conditions were 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 5 mM MnCl₂, 4 mM MgCl₂, and 10 µM ZnSO₄. Phosphorylated CDK9 was then immunoprecipitated with anti-CDK9 antibody and fractionated by electrophoresis on 10% SDS-polyacrylamide gels.

**CTD Kinase Assay**—CTD kinase assays were performed by mixing 100 ng of GST-CTD, 50 ng of P-TEFb, 200 µM ATP and incubating for 60 min at 23 °C. The total reaction volume was 20 µl, and the final conditions were 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 5 mM MnCl₂, 4 mM MgCl₂, and 10 µM ZnSO₄. Others proteins were added as indicated in the figure legends.

**RESULTS**

**CDK7 and CDK9 Associate with HIV-1 PICs**—We recently developed an immobilized template assay to analyze Tat transactivation (63, 70). Briefly, HIV-1 LTR promoter templates were 5′-end labeled with biotin at position −110 and incubated with HeLa nuclear extracts. PICs were subsequently purified with streptavidin-coated magnetic beads and analyzed by Western blots (Fig. 1A). Nonspecific DNA (poly[dI-dC]) was included during the incubation of templates with HeLa nuclear extract to minimize nonspecific binding of protein to the DNA.
TFIIH Inhibits P-TEFb

**FIG. 1.** Western blot analyses of HIV-1 PICs and in vitro transcription with the purified PICs. A. Western blot analyses of the HIV-1 PICs. PICs were assembled by incubating biotinylated wild-type (WT) HIV-1 LTR templates (lanes 3 and 4) or biotinylated TATA box mutant (Mut) HIV-1 LTR templates (lanes 1 and 2) with HeLa nuclear extract in the absence or presence of Tat. The protein components of PICs were analyzed with Western blots. Antibodies used in the Western blot analyses are indicated on the left, and the corresponding proteins are shown on the right. Input is indicated as In, B, in vitro transcription with the purified HIV-1 PICs. PICs were assembled by incubating biotinylated HIV-1 LTR templates with HeLa nuclear extract in the absence or presence of Tat, and the amount of Tat was added into the reactions as indicated. In vitro transcription reactions were then performed by incubating the purified PICs with nucleotides, and transcripts were labeled with [α-32P]UTP. The labeled RNA products were separated on a 6% denaturing polyacrylamide gel and detected by autoradiography.

Template. Parallel binding reactions were also carried out with a biotinylated TATA box mutant template of the HIV-1 LTR as a negative control for nonspecific binding of protein to templates. Finally, the magnetic beads used to purify the templates were extensively blocked with bovine serum albumin to minimize background binding of proteins to the beads, thus interfering with Western blot analysis.

The results shown in Fig. 1A demonstrate several important points. First, consistent with previous reports (63, 65), Tat is a component of the HIV-1 PICs (9th panel). Second, RNAP II and general transcription factors including TFIIID, TFIIIB, TFIIIE, TFIIF, and TFIIH associate with HIV-1 PICs (lanes 3 and 4). Tat does not affect the level of association of RNAP II and general transcription factors with PICs (lanes 3 and 4). Third, both CDK7 and CDK9, the two kinases responsible for RNAP II CTD phosphorylation during HIV-1 transcription, are present in the HIV-1 PICs (7th and 8th panels). Interestingly, the amount of either kinase is equal in the absence or presence of Tat with the wild-type HIV-1 LTR template (lanes 3 and 4). The appearance of proteins bound to the templates is specific. Parallel assays performed with a TATA box mutant HIV-1 LTR, which is transcriptionally inactive, failed to precipitate RNAP II and general transcription factors (lanes 1 and 2). Importantly, the presence of the TATA box mutation did not affect Sp-1 binding to the template (Fig. 1A, 10th panel).

In vitro transcription was performed using the purified HIV-1 PICs (Fig. 1B). In the absence of Tat, a low level of basal HIV-1 transcription was observed (lane 1). The addition of increasing amounts of Tat protein to the preincubation mix significantly increased transcription from the HIV-1 promoter (lanes 2–5). Optimum Tat transactivation was observed when ~100 ng of Tat was added to the reaction (Fig. 1B, lane 4). Several control experiments were performed to demonstrate the specificity of Tat transactivation. First, we utilized a template that contains a base substitution in the TAR RNA bulge. This mutation knocks out the ability of Tat to bind TAR RNA, inhibiting Tat transactivation in vitro and in vivo (71). The TAR RNA mutation inhibited the ability of Tat to transactivate the template, although the mutation did not significantly affect the level of basal transcription (data not shown) (63, 70). To demonstrate further the specificity of the Tat transactivation, we utilized Tat mutants with single amino acid substitutions at lysine 41 or cysteine 22. Consistent with previous results (70), the mutants failed to activate transcription (data not shown).

TFIIH Inhibits Tat-induced CDK9 Phosphorylation in HIV-1 PICs—It has been demonstrated recently (40, 41) that CDK9 autophosphorylation increases the interaction of P-TEFb with the HIV-1 TAR RNA structure. In an independent study, it was demonstrated that Tat modifies the activity/substrate specificity of the CDK9 kinase on the RNAP II CTD (63). To investigate whether Tat regulates CDK9 phosphorylation in the HIV-1 transcription complexes, mock-depleted or CDK7 (TFIIH)-depleted extracts were prepared for in vitro assays (Fig. 2A). The results presented in Fig. 2A demonstrate that the immunodepletion was specific. CDK7 was specifically not detected in the extract immunodepleted with anti-CDK7 antibody (top panel). In contrast, Western blot analysis with control anti-CDK9, anti-TBP, and anti-CTD antibodies showed no difference in the level of these proteins between the control and immunodepleted extracts (2nd to 4th panels). HIV-1 LTR templates were incubated with immunodepleted extracts, and the PICs were then purified with streptavidin-coated magnetic beads. Kinase reactions were performed with the purified PICs, and the phosphorylated proteins were labeled with [γ-32P]ATP. Phosphorylated CDK9 was immunoprecipitated with anti-CDK9 antibody and analyzed by SDS gel electrophoresis. The results shown in Fig. 2B demonstrate two significant points. First, the level of CDK9 phosphorylation in the PICs is low in the presence of CDK7 (lanes 1 and 3). This kinase activity is not affected by Tat. Remarkably, when the extract was depleted of CDK7 (TFIIH), an increase in CDK9 phosphorylation was observed in the presence of Tat (lanes 2 and 4). These results suggest that Tat facilitates CDK9 phosphorylation when TFIIH is not present in the PICs.

Tat-induced CDK9 Phosphorylation during HIV-1 Transcription Takes Place after TFIIH Is Released from Transcription Complexes—The above results suggest that CDK7 (TFIIH) inhibits Tat-induced CDK9 phosphorylation during HIV-1 transcription. To test this hypothesis further, the purified PICs were preincubated with ATP for 10 min and then walked to position U-14 by incubation with 50 μM dATP, CTP, GTP, and UTP for 10 min at 30 °C. The elongation complexes (TECs) stalled at U-14 were walked stepwise along the DNA by repeated incubation with different sets of three NTPs and then washed extensively. Immunoblot analysis of the transcription complexes indicates that CDK7 (TFIIH) associates with HIV-1 PICs and is released from the transcription complex between +14 and +36 (Fig. 3A, top panel). CDK9, in contrast, stays...
associated with the elongation complex through +36 (middle panel).

In light of the results obtained above, we tested to see whether CDK9 phosphorylation activity was recovered when TFIIH is released from HIV-1 transcription complexes. To detect CDK9 phosphorylation during transcription, the complexes were incubated with \([\gamma\text{-}^{32}\text{P}]\)ATP during the stepwise elongation steps, and phosphorylated CDK9 was immunoprecipitated with specific antibody. The results are shown in Fig. 3B. CDK9 phosphorylation was uniquely observed after the elongation complexes moved from nucleotide +14 to +36 in the presence of Tat (lane 6). Thus, at the same time that TFIIH was shown to exit from the transcription complex, phosphorylation of CDK9 was observed. Western blot analysis of CDK9 in the PIC and elongation complexes supports the above result. Tat-induced CDK9 phosphorylation resulted in a retarded migration of the CDK9 protein in the SDS gel (Fig. 3A), and the corresponding proteins are shown on the right. Input is indicated as In. B, Tat induced CDK9 phosphorylation during the HIV-1 transcription after TFIIH was released from transcription complexes. Phosphorylated proteins were labeled with \([\gamma\text{-}^{32}\text{P}]\)ATP during stepwise moves, and phosphorylated CDK9 was immunoprecipitated with anti-CDK9 antibody.

addition of okadaic acid did not affect the level of CDK9 phosphorylation (data not shown).

Purified TFIIH Inhibits CDK9 Autophosphorylation in in Vitro Kinase Assay—To determine whether TFIIH directly inhibits CDK9 autophosphorylation, in vitro kinase assays were performed with purified TFIIH (68) and P-TEFb (18). The kinase reactions were performed by incubating 50 ng of P-TEFb, 10 µM ATP, and 20 µCi of \(\gamma\text{-}^{32}\text{P}\)ATP in the absence or presence of purified Tat and TFIIH. CDK9 was then immunoprecipitated with anti-CDK9 antibody and fractionated by electrophoresis on 4–20% SDS-polyacrylamide gels. The results of the experiment demonstrated that purified TFIIH inhibited CDK9 autophosphorylation (Fig. 4A, compare lanes 5 and 6 with 1 and 2). As a control, P-TEFb was incubated with the purified multisubunit basal transcription factor TFIIH. In contrast to the results obtained with TFIIH, TFIIID did not inhibit P-TEFb phosphorylation (Fig. 4A, lanes 3 and 4).

To eliminate the possibility that the decrease in \(^{32}\text{P}\)-labeled CDK9 (Fig. 4A, lanes 1 and 2) resulted from phosphatase contamination, TFIIH was added after the kinase assay was completed and a further incubation was performed. To inhibit any further kinase activity during the further incubation, 5 mM EDTA was added to the reaction mixture. No decrease in the level of CDK9 phosphorylation was observed (Fig. 4A, lanes 7 and 8). These results suggest that the decrease in \(^{32}\text{P}\)-labeled CDK9 (lanes 1 and 2) was not the result of phosphatase activity, but rather that TFIIH inhibits CDK9 autophosphorylation in the in vitro kinase assays.

![Figure 2](http://www.jbc.org/)

**Fig. 2.** TFIIH inhibits Tat-induced CDK9 phosphorylation in HIV-1 PICs. A, Western blot analyses of immunodepleted extracts. Antibodies used in the Western blot analyses were indicated on the left, and the corresponding proteins were shown on the right. B, TFIIH inhibited Tat-induced CDK9 phosphorylation in HIV-1 PICs. HIV-1 LTR templates were incubated with mock-depleted extract (odd lanes) or CDK7-depleted extract (even lanes), and PICs were then purified with streptavidin-coated magnetic beads. Kinase reactions were performed with the purified PICs, and phosphorylated proteins were labeled with \([\gamma\text{-}^{32}\text{P}]\)ATP. Phosphorylated CDK9 was immunoprecipitated with anti-CDK9 antibody.

![Figure 3](http://www.jbc.org/)

**Fig. 3.** Tat-induced CDK9 phosphorylation during HIV-1 transcription takes place after TFIIH is released from transcription complexes. The purified PICs were incubated with ATP for 10 min at 30 °C and then washed extensively with 1× IVT buffer. PICs were walked to position U-14 by incubation with 50 µM dATP, CTP, GTP, and UTP for 10 min at 30 °C and then washed extensively with 1× IVT buffer. The TECs stalled at U-14 were walked stepwise along the DNA by repeated incubation with different sets of three NTPs and then washed extensively with 1× IVT buffer to remove the unincorporated NTPs. A, Kinetic analyses of TFIIH and P-TEFb during the HIV-1 transcription. Western blot analyses of HIV-1 transcription complexes stalled at different positions were performed with anti-CDK7, anti-CDK9, and anti-Tat antibodies. Antibodies used in the Western blot analyses are indicated on the left, and the corresponding proteins are shown on the right. Input is indicated as In. B, Tat induced CDK9 phosphorylation during the HIV-1 transcription after TFIIH was released from transcription complexes. Phosphorylated proteins were labeled with \([\gamma\text{-}^{32}\text{P}]\)ATP during stepwise moves, and phosphorylated CDK9 was immunoprecipitated with anti-CDK9 antibody.
It is also obvious that there is a fundamental difference in the ability of Tat to stimulate CDK9 phosphorylation in a “soluble” assay and in HIV-1 transcription complexes (compare Fig. 4A with Figs. 2B and 3B). In a soluble assay containing Tat and CDK9, Tat does not increase the level of CDK9 phosphorylation (Fig. 4A, lanes 5 and 6). This result is true whether the CDK9 kinase assay was performed as an end point assay (Fig. 4A) or as a kinetic analysis in which the activity of CDK9 autophosphorylation was assayed at various times during the reactions (Fig. 4B). These results are consistent with the recent report of Garber et al. (40) who also found no stimulation of CDK9 autophosphorylation by the HIV-1 Tat protein using a soluble kinase assay with purified P-TEFb and Tat. In contrast, Tat does appear to stimulate CDK9 phosphorylation when the two factors are part of the transcription complexes (Figs. 2B and 3B). Although we cannot rule out the possibility that there is not a distinct kinase responsible for CDK9 phosphorylation in the transcription complexes, the fact that both the transcription complexes and soluble complex are inhibited by TFIIH argues that this is not the case. In addition, both kinase activities are sensitive to low concentrations of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (data not shown). Given the ability of Tat to modify P-TEFb kinase activity on the CTD, it will be of interest to map the domain of XBP that is responsible for inhibiting P-TEFb kinase activity. These studies will require incorporation of Tat mutants into the IIH5 complex because the XBP subunit is not stable alone.

**Inhibition of CDK9 Autophosphorylation by TFIIH Decreases the Level of CTD Phosphorylation at Serine 2**—Another substrate for the CDK9 kinase is the RNA II CTD. It was of interest, therefore, to determine whether TFIIH inhibits phosphorylation of this substrate. CTD kinase assays were performed with purified TFIIH and P-TEFb. To detect the CTD kinase activity of CDK9 specifically, the CTD phosphorylation was analyzed by Western blot analysis with anti-CTD monoclonal antibodies H5 (phosphoserine 2) and H14 (phosphoserine 5).

When the Western blot analysis was performed with anti-CTD antibody H5 (phosphoserine 2), a partial inhibition of CTD phosphorylation at serine 2 was observed (Fig. 6A, top panel, lanes 2 and 3). This result could indicate that serine 2 phosphorylation catalyzed by CDK9 was more resistant to TFIIH inhibition. Alternatively, the results might suggest that CDK9-dependent phosphorylation of the CTD at serine 2 involves multiple steps, some of which are sensitive to TFIIH inhibition. Remarkably, when the P-TEFb was preincubated with ATP to allow autophosphorylation of CDK9, TFIIH did not inhibit CTD phosphorylation at serine 2 (Fig. 6A, top panel, lanes 5 and 6).

When the Western blot analysis was performed with anti-CTD antibody H14 (phosphoserine 5), two conclusions could be reached. First, TFIIH specifically phosphorylates the CTD at serine 5 (Fig. 6A, bottom panel, lanes 1 and 4). Second, P-TEFb does not phosphorylate serine 5 of CTD (lanes 2 and 5); furthermore, P-TEFb has no inhibitory effect on CTD kinase activity of TFIIH (Fig. 6A, lanes J, 3, 4, and 6).

Our results are consistent with a model in which the CTD kinase activity of CDK9 is dependent upon “activation” of CDK9 through an autophosphorylation step (Fig. 6B). We interpret these results to indicate that TFIIH inhibits the CDK9 autophosphorylation but does not directly inhibit CTD phosphorylation catalyzed by activated CDK9. Given that CDK9 kinase activity appears to be differentially regulated in soluble...
The total reaction volume was 20 µl, and the final conditions were 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 5 mM MnCl₂, 4 mM MgCl₂, and 10 mM ZnSO₄. To eliminate the possibility that the decrease in CTD phosphorylation resulted from the direct inhibition of the CTD kinase activity of CDK9 by TFIIH, TFIIH was added, and CTD kinase assays were performed after a preincubation of P-TEFb with ATP was done (see *lanes 4–6*). Phosphorylated GST-CTD was separated on 8% SDS-polyacrylamide gels and then transblotted onto polyvinylidene fluoride membranes (Millipore). Western blot analyses were performed with anti-CTD monoclonal antibodies H5 (phosphoserine 2) and H14 (phosphoserine 5). Input is indicated as In. A, a model for CDK9 autophosphorylation and its CTD kinase activity.

complexes compared with transcription complexes, this model will require verification with RNAP II contained in a functional elongation complex. Although we have shown previously (63) that the CTD substrate specificities of CDK7 and CDK9 are identical in soluble and transcription complexes, the requirement for CDK9 autophosphorylation to activate CTD kinase activity in the transcription complex awaits further investigation.

DISCUSSION

Several lines of investigation support the conclusion that P-TEFb plays a key role in Tat transactivation. First, depletion of P-TEFb from nuclear extracts blocks Tat transactivation (17, 19, 21). Second, dominant-negative mutants of CDK9 or CDK9 kinase inhibitors inhibit Tat transactivation (14, 17, 22, 26). Finally, the species-specific restriction of HIV-1 Tat transactivation has been closely linked to the CycT1 subunit of P-TEFb (27–32). More recent studies (40, 41) have demonstrated that autophosphorylation of the CDK9 subunit of P-TEFb increases the binding of Tat-P-TEFb to the TAR RNA structure, a step that is critical for Tat transactivation. The results presented in this study indicate that TFIIH regulates CDK9 phosphorylation. TFIIH apparently inhibits CDK9 phosphorylation until it is released from the transcription complex between +14 and +36. Once TFIIH is released, CDK9 phosphorylation occurs, allowing the P-TEFb and Tat to bind to the newly synthesized TAR RNA structure and facilitate transcription elongation. The orchestrated release of TFIIH and induction of Tat-P-TEFb binding to the TAR RNA structure almost certainly contributes to the efficiency of HIV-1 transcription in infected cells. Several reports (64, 73–75) have suggested that CDK9 is present, but inactive, in HIV-1 preinitiation complexes. Our data provide evidence that the inactive state of CDK9 in the HIV-1 PICs may be due to the presence of TFIIH.

In a very elegant analysis of the fate of transcription factors during the transition from initiation to elongation, Zawel et al. (76) have demonstrated that TFIIID remains promoter-bound, whereas TFIIH, TFIIIE, TFIIF, and TFIIJ are released rapidly. TFIIH release occurs after the complex reaches +30 to +50. Interestingly, Hahn and co-workers (77) have recently reported that TFIIH is not released from transcription complexes in the presence of the mediator complex. Consistent with the report from Ping and Rana (64), our analyses of HIV-1 transcription complexes indicate that TFIIH is associated with HIV-1 preinitiation complex but is released from elongation complexes during HIV-1 transcription. The release of TFIIH would be accompanied by CDK9 autophosphorylation, allowing TAR RNA binding (40, 41), posing the complex for the transition from nonprocessive to processive transcription elongation.

It is interesting to speculate that the functional interaction between TFIIH and P-TEFb plays an important role in an efficient transition from initiation and promoter clearance to elongation. TFIIH is a multifunctional transcription factor that plays a critical role not only in transcription initiation, where it catalyzes an ATP-dependent formation of the open complex, but also in promoter escape, where it suppresses arrest of early RNA elongation intermediates (78, 79). The results presented here suggest the CDK9 phosphorylation is required for the RNAP II CTD phosphorylation. By inhibiting CDK9 phosphorylation, TFIIH may ensure that the transition from initiation to elongation proceeds in an efficient and programmed manner. Once TFIIH is released from the transcription complex, CDK9 phosphorylation enables P-TEFb to increase transcription elongation through phosphorylation of the RNAP II CTD.

Our previous results (63) demonstrate that the RNAP II containing an unphosphorylated CTD is recruited into the HIV-1 PIC and phosphorylated by P-TEFb and TFIIH during HIV-1 transcription. Moreover, Tat modifies the CTD kinase activity of CDK9 during HIV-1 transcription. The results presented in this study indicate that TFIIH regulates CDK9 phosphorylation. By inhibiting CDK9 phosphorylation, TFIIH may ensure that the transition from initiation to elongation proceeds in an efficient programmed manner. It is interesting that Dahmus and co-workers (80, 81) have recently reported that Tat regulates CTD phosphatase activity. In view of the fact that each cycle of transcription appears to be associated with the reversible phosphorylation of RNAP II CTD (5, 82), the dephosphorylation of RNAP II by CTD phosphatase (80, 81, 83–90) may play an important role in Tat transactivation.
The potential involvement of protein phosphatases in changes in P-TEFb phosphorylation has been addressed by two experiments. First, the phosphatase inhibitor okadaic acid was shown not to affect the level of CDK9 phosphorylation. Because some protein phosphatases are resistant to okadaic acid, this result does not rule out the possibility that specific classes of phosphatases are involved in the regulation of CDK-9 phosphorylation. Second, the experiment to look at release of 32P from labeled CDK9 was carried out in the presence of 5 mM EDTA. Therefore, the results do not indicate that specific protein phosphatases are involved in the regulation of CDK-9 phosphorylation.

Several substrates have now been identified for CDK9, including CDK9 itself (15, 40, 41), the RNAPII CT (12, 15, 40, 43, 72, 75), and more recently the SPT5 subunit of 5,6-dichloro-1-β-d-ribofuranosylbenzimidazole sensitivity-inducing factor (40, 72, 75). Phosphorylation of the RNAPII CT plays a critical role in transcription elongation. CDK9 phosphorylation appears to regulate two important functions of CDK9. The recent reports (40, 41) have shown that CDK9 phosphorylation increases TAR RNA binding. The results presented in this study suggest that CDK9 autophosphorylation activates the CTD kinase function of CDK9. Our previous result (63) and the results presented here demonstrate that CDK9 phosphorylates the full-length CT at serine 2. It has recently been reported that CDK9 phosphorylates serine 5 in a CT peptide (three repeats) kinase assay (103). Preliminary results suggest that the apparent contradiction between these results may be due to the difference of substrates. Considering the recent reports (40, 72, 75), it will be of interest to determine whether TFIIH inhibits P-TEFb phosphorylation of the SPT5 subunit of 5,6-dichloro-1-β-d-ribofuranosylbenzimidazole sensitivity-inducing factor which inhibits promoter proximal elongation by RNAPII (104, 105). These analyses will provide important insight into Tat transactivation and the programmed regulation of HIV-1 transcription.

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