Tat-associated Kinase (P-TEFb): a Component of Transcription Preinitiation and Elongation Complexes*

Yueh-Hsin Ping and Tariq M. Rana‡

From the Department of Pharmacology, Robert Wood Johnson Medical School, and Molecular Biosciences Graduate Program at Rutgers University, Piscataway, New Jersey 08854

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† Recipient of a Research Career Development Award from the National Institutes of Health. To whom correspondence should be addressed: Dept. of Pharmacology, Robert Wood Johnson Medical School, and Molecular Biosciences Graduate Program at Rutgers University, 675 Hoes Ln., Piscataway, NJ 08854 Tel.: 732-235-4082; Fax: 732-235-3235; E-mail: rana@umdnj.edu.

The abbreviations used are: pol II, polymerase II; HIV-1, human immunodeficiency virus, type 1; CTD, carboxyl-terminal domain; LTR, long terminal repeat; TAR, trans-activation responsive region; PIC, preinitiation complex; TEC, transcription elongation complex; PAGE, polyacrylamide gel electrophoresis.

Human immunodeficiency virus, type 1 (HIV-1) Tat protein activates transcription from the HIV-1 long terminal repeat. Tat interacts with TFIIH and Tat-associated kinase (a transcription elongation factor P-TEFb) and requires the carboxyl-terminal domain of the largest subunit of RNA polymerase II (pol II) for transcriptional activation. We developed a stepwise RNA pol II walking approach and used Western blotting to determine the role of TFIIH and P-TEFb in HIV-1 transcription elongation. Our results demonstrate the new findings that P-TEFb is a component of the preinitiation complex and travels with the elongating RNA pol II, whereas TFIIH is released from the elongation complexes before the trans-activation responsive region RNA is synthesized. Our results suggest that TFIIH and P-TEFb are involved in the clearance of promoter-proximal pausing of RNA pol II on the HIV-1 long terminal repeat at different stages.

Transcription elongation in eukaryotic genes is a complex process that involves a number of regulatory factors. It is becoming increasingly clear that the elongation stage of RNA pol II is a major regulatory process of gene expression (1–6). After successful initiation of RNA synthesis, RNA pol II can pause, get arrested, pass through terminator sequences, or terminate transcription. Release of RNA pol II from stalled complexes is a rate-limiting step in transcription of inducible eukaryotic genes (3, 4, 7). In the absence of inducer protein, RNA pol II elongation complexes pause 20–60 nucleotides downstream of the promoter. Promoter-proximal pausing is released by DNA- or RNA-binding activators that recruit or stimulate positive-acting transcription elongation factors. General transcription factors such as TFIIH play a key role in promoter clearance and the promoter-proximal release of RNA pol II (8, 9).

Human immunodeficiency virus, type 1 (HIV-1) encodes a small regulatory protein, Tat, which is required for efficient transcription of viral genes. Tat enhances the processivity of RNA pol II elongation complexes that initiate in the HIV LTR region. Tat activates transcription by binding to a highly structured RNA element, TAR RNA, that is located at the 5′ end of nascent viral transcripts (for review see Refs. 10 and 11). Tat functions through TAR RNA to control an early step in transcription elongation that is sensitive to protein kinase inhibitors and requires the carboxyl-terminal domain (CTD) of the largest subunit of RNA pol II (12). The Tat protein contains two important functional regions: an arginine-rich region that is required for binding to TAR RNA and an activation domain that mediates the interactions with cellular machinery (11). Tat has been reported to interact with a number of cellular proteins associated with transcription, including TFIIH (13), SP1 (14), TAFII-55 (15), TFIIH (16–18), and RNA pol II (19).

Recent studies indicate that Tat transactivation function is mediated by a nuclear Tat-associated kinase, TAK (12, 20, 21). The transactivation domain of Tat interacts with TAR (22, 23), which was recently shown to be identical to the kinase subunit of P-TEFb, a positive-acting transcription elongation factor (24, 25). P-TEFb is required for transcription elongation at many genes (26, 27) and is proposed to facilitate the transition from abortive to productive elongation by phosphorylating the CTD of the largest subunit of RNA pol II (28). Experimental evidence has established that the CTD is required for Tat transactivation (29–31). It is possible that P-TEFb kinase activities are also involved in regulation of other cellular proteins. P-TEFb is composed of at least two subunits: the catalytic subunit cyclic-dependent kinase CDK9 (previously named PITALRE) and the regulatory subunit cyclin T1 (24, 32, 33). Complexes containing CDK9 and cyclin T1-related proteins, cyclin T2a or cyclin T2b, are also active for P-TEFb activity (34). It has been demonstrated that cyclin T1 interacts directly with the activation domain of Tat and mediates its high affinity and specific binding to TAR RNA (33). Tat-cyclin T1 interaction is distinctive in nature because other Tat-binding proteins identified so far have not been shown to enhance Tat affinity and specificity for TAR RNA. Therefore, cyclin T1 interaction with Tat could be responsible for P-TEFb recruitment to the RNA pol II elongation complexes stalled after TAR RNA.

In this paper, we provide the evidence that P-TEFb is a component of the preinitiation complex and travels with the RNA pol II elongation complex as the nascent RNA is synthesized. As outlined in Fig. 1, we have used an experimental strategy to track RNA pol II in stepwise transcription and to stall elongation complexes at specific sites. The stalled transcription complexes were purified by first magnetic beads and then by releasing the template DNA by cleaving it with restriction enzymes. The protein composition in the released complexes was then measured by Western blotting. Our results demonstrate that P-TEFb is a component of the preinitiation complex and travels with the elongating RNA pol II, whereas TFIIH is released from the elongation complexes before the TAR RNA is synthesized. Our results suggest that two cellular
amide, 7M urea gels, and the protein compositions of the transcription RNA transcripts are analyzed by electrophoresis on 15% polyacrylamide.

...XbaI (1) and to stall elongation complexes at specific sites (right). A target sequence for triple helical DNA is inserted into the DNA template. Plasmid pWT2 is linearized with restriction enzymes (HindIII or AccI). A psoralen- and biotin-containing oligonucleotide is used to form triplex DNA, and UV irradiation covalently cross-links psoralen to the template. Psoralen cross-linked template is immobilized on streptavidin-conjugated magnetic beads, and noncross-linked DNA is washed away with buffer. For stepwise RNA pol II walking, DNA templates containing psoralen cross-links upstream of the promoter are used (left). To stall elongation complexes at positions +185 and +529, psoralen cross-links at downstream sequences are used (right). The RNA transcripts are analyzed by electrophoresis on 15% polyacrylamide, 7M urea gels, and the protein compositions of the transcription RNA transcripts are analyzed by electrophoresis on 15% polyacrylamide. Length of the RNA transcripts was confirmed by molecular weight markers. Numbers on the top show various positions where TECs were stalled.

FIG. 1. A, experimental design to walk RNA pol II in stepwise transcription (left) and to stall elongation complexes at specific sites (right). A target sequence for triple helical DNA is inserted into the DNA template. Plasmid pWT2 is linearized with restriction enzymes (HindIII or AccI). A psoralen- and biotin-containing oligonucleotide is used to form triplex DNA, and UV irradiation covalently cross-links psoralen to the template. Psoralen cross-linked template is immobilized on streptavidin-conjugated magnetic beads, and noncross-linked DNA is washed away with buffer. For stepwise RNA pol II walking, DNA templates containing psoralen cross-links upstream of the promoter are used (left). To stall elongation complexes at positions +185 and +529, psoralen cross-links at downstream sequences are used (right). The RNA transcripts are analyzed by electrophoresis on 15% polyacrylamide, 7M urea gels, and the protein compositions of the transcription complexes are analyzed by Western blotting. B, DNA sequence of the early transcribed region (+1 to +71) in the HIV-1 promoter.

kinases, TFIIH and P-TEFb, are involved in the clearance of promoter-proximal pausing of RNA pol II on the HIV-1 LTR at different stages.

EXPERIMENTAL PROCEDURES

Template DNAs—The test plasmids (pWT2 and pPT529) used in this study were derived from the p10SLT plasmid, which contains HIV-1 5'-LTR (35). Plasmid pWT2 was constructed by inserting a synthesized DNA fragment containing a triplex target sequence (5'-AAA AGA AAA GGG GGG-3') between the HindIII and NalI sites of plasmid p10SLT. Plasmid pPT529 contains a triplex target sequence at +529 between XhoI and XhoI sites of p10SLT.

Oligonucleotide Synthesis and Purification—Oligodeoxyribonucleotides were synthesized on an automated DNA/RNA synthesizer (ABI 392). Psoralen was conjugated to the 5' end of the triplex probes (see "Experimental Procedures"). RNA transcripts were labeled with [α-32P]CTP during transcription. Transcription elongation complexes stalled at various positions were isolated and the RNA transcripts were analyzed on 15% polyacrylamide, 7M urea gels. Length of the RNA transcripts was confirmed by molecular weight markers. Numbers on the top show various positions where TECs were stalled.

Fig. 1) by using phosphoramide derivatives of psoralen. Biotin-modified oligonucleotides were prepared on biotin containing support. Cytosine was replaced by 5-methyl-cytosine. All the oligonucleotides were deprotected in ammonium hydroxide at 55 °C for 6 h and purified on 20% polyacrylamide, 7M urea gels.

Triplex Formation and Cross-linking—Linearized DNA (final concentration, 0.1 μM) was incubated with excess psoralen-oligonucleotide probe (250 ng) in a buffer containing 10 mM Tris-HCl (pH 6.5), 50 mM NaCl, 10 mM MgCl₂, and 0.5 mM spermine for 30 min at 37 °C and then cooled down slowly to room temperature. The mixture was irradiated with UV (360 nm) in a Photochemical Reactor for 8 min. For magnetic bead binding, template DNA was separated from unreacted psoralen-oligonucleotide probe on 1.2% agarose gels.

Immobilization of DNA on Magnetic Beads—Template DNA (0.5 μg) cross-linked to a psoralen probe modified with biotin was bound to 25 μl of streptavidin-coated magnetic bead (250 μg, Dynal Inc.) by incubating DNA and beads in TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) containing 1 M NaCl at room temperature overnight on a shaker.

Stepwise Walking of RNA pol II—HeLa cell nuclear extracts were prepared according to published procedures (36, 37) with minor modifications (38). Preinitiation complexes (PICs) were formed by incubating the immobilized DNA templates (200 ng) in a volume of 25 μl containing 12 μl of nuclear extract, 6 μM MgCl₂, and 0.5 μg of poly(dA·dT) for 15 min at 30 °C. PICs were washed with 25 μl of washing buffer A (20 mM HEPES, pH 7.9, 100 mM KCl, 20% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 6 mM MgCl₂) to remove unbound proteins. The PICs were walked to position U14 by incubation with 12.5 mM phosphocreatine, 20 μM CTP, GTP, and UTP, 20 μM dATP, and 10 μCi of [α-32P]CTP (25 Ci/mmol, ICN; final concentration, 160 μM) for 5 min at room temperature. Transcription elongation complexes (TECs) stalled at U14 were washed with 25 μl of washing buffer B (wash buffer A containing 0.05% Nonidet P-40 and 0.015% Sarkosyl) and twice with washing buffer C (wash buffer A containing 0.05% Nonidet P-40). The TECs stalled at U14 were walked stepwise along the DNA by repeated incubation with different sets of three NTPs. Unincorporated NTPs were removed by washing the immobilized complexes with buffer C. To isolate RNA products from stalled TECs, 175 μl of stop solution (0.3 μM Tris-HCl, pH 7.4, 0.3 mM sodium acetate, 0.5% SDS, 2 mM EDTA) was added. The mixture was extracted with 200 μl of phenol-chloroform-isooamyl alcohol (50:48:2) and then with chloroform (200 μl). RNA transcripts were precipitated with ethanol and analyzed on 15% polyacrylamide, 7M urea gels.

Western Blotting—For the isolation of ternary complexes, the PICs...
FIG. 3. Isolation of preinitiation (A) and elongation complexes (B). PICs and stalled TECs were formed on DNA templates immobilized on streptavidin beads, and the DNA was cleaved with restriction enzymes to isolate the RNA pol II complexes. To isolate PICs, DNA was cleaved at BglI restriction site. For isolation of TECs, RNA pol II complexes were walked to different sites and then cleaved with BglII. Complexes were visualized by using antibodies against RNA pol II CTD, CDK9, and TFIIH (lanes 2, 4, and 3). Lanes 1, 2, 5, and 3 contain 3, 10, and 15 standard transcription reactions, respectively. Lane 1, 5% of the nuclear extract present in the transcription reaction. Lane 2, PICs formed in the presence of sarkosyl.

FIG. 4. A strategy to isolate a homogeneous population of RNA pol II elongation complexes stalled at positions +185 and +529. DNA containing a downstream triplex target site was immobilized on the magnetic beads (Fig. 1, right), and cell-free transcription experiments were performed. TECs stalled at the DNA damage site were separated by magnet. Restriction digestion was performed to remove the promoter sequences. B, transcript analysis for the elongation complexes stalled at DNA damage sites (+185 and +529). DNA templates containing psoralen cross-links at +185 (lane 2) and +529 (lane 3) were used in transcription reactions, and the RNA transcripts were analyzed on denaturing gels. Lane 1 is a 50-base pair DNA marker. C, CDK9 travels with the elongation complexes. Transcription complexes stalled at +185 and +529 were isolated and analyzed on SDS-PAGE. CDK9 was detected by Western blot analysis (lanes 2 and 3). Lanes 2 and 3 contain three and five standard transcription reactions, respectively. Lane 1, 5% of the nuclear extract present in the transcription reaction.

RESULTS

Stepwise Transcription—To determine the protein composition of transcription elongation complexes at different stages, it is important to isolate homogeneous populations of RNA pol II ternary complexes stalled at specific sites. We used triplex DNA structure and psoralen photochemistry to immobilize DNA templates and stall elongation complexes (38). The experimental strategy for site-specific psoralen cross-links in the DNA template containing HIV-1 promoter to isolate the stalled RNA pol II elongation complexes is outlined in Fig. 1. This method involves the following five steps: 1) insertion of a target sequence for triple helix formation at a predetermined position in the DNA template; 2) restriction digest with an enzyme to yield the triplex site upstream or downstream of the promoter sequences; 3) synthesis of a third strand for triplex formation containing a psoralen at its 5' end and a biotin at the 3' end; 4) triplex formation between the DNA template and the third strand followed by nearly ultraviolet irradiation (360 nm); and 5) immobilization of the cross-linked DNA template on streptavidin-conjugated magnetic beads and in vitro transcription. Immobilized DNA templates with beads upstream of the promoter were used for stepwise walking of RNA pol II, and DNA templates with beads downstream of the promoter were used to stall elongation complexes chased with all four nucleotides.

Preinitiation complexes were formed on immobilized DNA tem-
plates, and elongation was initiated by adding dATP, UTP, CTP, and GTP. These elongation complexes were starved for one of the NTPs and then stalled at U14. Further initiation was inhibited by sarkosyl wash as described under “Experimental Procedures.” Stepwise walking of the TECs stalled at U14 was accomplished by repeated incubation with different sets of three NTPs (39). A typical gel of our RNA pol II walking experiments is shown in Fig. 2. TECs stalled at A-22, C-30, G-36, U-46, A-51, and C-61 are shown. Viability of the stalled complexes was confirmed by adding all four NTPs, which produced run-off products of expected lengths indicating that 100% of the complexes were transcriptionally active (data not shown).

Isolation of Preinitiation and Elongation Complexes—We formed preinitiation and stalled transcription elongation complexes on DNA templates immobilized on streptavidin beads, and the DNA was cleaved with restriction enzymes to isolate the RNA pol II complexes. The strategy for these experiments is outlined in Fig. 3 (A and B). To isolate PICs, DNA was cleaved at the BspE1 restriction site. For isolation of TECs, RNA pol II complexes were stalled at different sites and then cleaved with BglII. RNA contents were analyzed on 7 M urea, 15% polyacrylamide gels (as shown in Fig. 2), and proteins were separated on denaturing SDS gels.

Analysis of Proteins in Preinitiation and Elongation Complexes—Recently, it has been reported that P-TEFb is involved in Tat transactivation (12, 24, 25). A number of studies also suggest that TFIIH kinase complex plays an important role in Tat activation (16–18). We planned to investigate the role of TFIIH and P-TEFb in transcription using HeLa nuclear extracts and DNA templates containing HIV-1 promoter. We used immobilized templates and isolated RNA pol II preinitiation and elongation complexes stalled at different sites during transcription as described above. Complexes were released by cleaving DNA templates with restriction enzymes and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membrane, and various proteins were detected by Western blotting with antibodies raised against TBP, two subunits of TFIIH (p89 or ERCC3 and p62), RNA pol II, and a subunit of P-TEFb (CDK9). Affinity purified antibodies against TFIIH and TBP were purchased from Santa Cruz Biotechnology. RNA pol II and CDK9 antibodies were kindly provided by Drs. Michael Dahmus (University of California, Davis) and David Price (University of Iowa, Iowa City), respectively. Results of this analysis are shown in Fig. 3C. P-TEFb is a component of RNA pol II preinitiation complex and remains attached to the elongation complexes stalled at C-30 and C-71. On the other hand, TFIIH is detected in the PICs and not in the TECs stalled at C-30 and C71. To confirm that sarkosyl treatment of TECs did not remove TFIIH and other transcription factors, we isolated TECs stalled at A-15 and washed with sarkosyl containing buffer and analyzed for protein contents as described above. Western analysis showed that TFIIH was present in TECs stalled at A-15 (data not shown). TBP analysis was performed as a control experiment showing that TBP was present in the PICs and not in the stalled elongation complexes (data not shown).

Our findings about the assembly and release of TBP and TFIIH are in agreement with previous studies by Zawel et al. (40) who used a defined reconstituted transcription system containing adenovirus 2 major late promoter. It is interesting to note that using nuclear extracts and DNA templates containing HIV-1 promoter sequences did not change the properties of TFIIH. To normalize the amount of transcription complexes at each step, we detected RNA pol II by immunoblotting in the PICs and the TECs. It is now generally accepted from previous studies that the PIC contains nonphosphorylated CTD of RNA pol II (IIa) and TECs contain phosphorylated CTD of RNA pol II (IIo) (41). RNA pol II in elongation complexes is IIo; however, we did not separate IIa and IIo bands in this gel because we were detecting RNA pol II as an internal standard and wanted to detect CDK9 in the same gel and did not run the gel for longer times to resolve proteins with high molecular weights. We were able to separate IIa and IIo when gels were run for longer times (38). No proteins were detected when the PIC formation was inhibited by the addition of sarkosyl (lane 2 in Fig. 3C).

Western blotting of the nuclear extract was performed as a control experiment for the identification of the correct proteins and to confirm that these proteins are not modified or degraded in our stalled complexes. It is important to note that these experiments are not quantitative; therefore, the relative stoichiometry of P-TEFb in elongation complexes cannot be determined from these results. The intensity of various bands represents the immunoreactivity of the specific antibodies and does not correspond to the amount of proteins present in RNA pol II complexes. For example, TFIIH bands are more intense than CDK9 and RNA pol II (Fig. 3C, lane 3) and do not necessarily represent the stoichiometry of these proteins. During stepwise walking of RNA polymerase II elongation complexes, each wash to remove unincorporated NTPs results in a loss of TECs. To ensure that a sufficient amount of proteins was present in TECs stalled at longer sequences, 10 and 15 standard transcription reactions were used to isolate TECs stalled at C-30 and C-71, respectively. PICs contained three standard transcription reactions. Accordingly, more CDK9 and RNA pol II were detected in TECs stalled at C-30 and C-71 compared with the PICs (Fig. 3C, lanes 3–5). These results establish that P-TEFb is a part of preinitiation and elongation assembly of RNA pol II, whereas TFIIH is released from the elongation complex before full-length TAR RNA is transcribed.

P-TEFb Is a Component of the Transcription Elongation Complex—The above experiments show that P-TEFb associates with preinitiation transcription complexes and remains attached to the elongation complexes stalled at C-71. To deter-
mine whether P-TEFb is released from the TECs after the synthesis of full-length TAR RNA or it associates with the RNA pol II during elongation, we stalled elongation complexes at different stages of the transcription cycle (12). How might P-TEFb interact with TAR RNA and become part of the elongation complexes unless TAR RNA is synthesized? TFIIH is released, whereas Tat remains in the elongation complex after the transcription of a functional TAR RNA structure. Future studies to define the Tat-interacting regions of TFIIH and P-TEFb complexes and to determine various stages of the CTD phosphorylation will reveal mechanistic details of Tat function.

DISCUSSION

We have utilized a stepwise transcription approach and Western blotting to determine the role of TFIIH and P-TEFb in transcription elongation in HIV-1 promoter. Our results provide new insights into the mechanism of Tat transactivation.

Several lines of evidence suggest that Tat activation requires the CTD of RNA pol II (16, 29–31). CTD contains a tandemly reiterated peptide sequence (YSPTSPS) that is differentially phosphorylated during the transcription cycle (for review see Refs. 41 and 42). Mammalian cells contain two forms of RNA pol II, phosphorylated (Ii0) and nonphosphorylated (Iiα), that differ in the extent of phosphorylation within the carboxyterminal domain of their largest subunit (41). The nonphosphorylated form of RNA pol II preferentially associates with the preinitiation complex, whereas RNA pol II derived from isolated ternary complexes is highly phosphorylated (40, 43). Phosphorylation of CTD is suggested to be critical for the release of preinitiation complexes from the promoter and could disrupt interactions between the CTD and other mediator proteins and transcription factors, such as TBP (40, 44). Tat stimulates hyperphosphorylation of the CTD in a transcription dependent manner in vitro (16, 17). These findings suggest that Tat is involved in CTD kinase steps to form processive elongation complexes.

Interaction of Tat with two cellular kinase complexes, TFIIH (16–18) and P-TEFb (22–24), have been reported, and it has been proposed that Tat may interact with these kinase complexes at different stages of the transcription cycle (12). How does Tat interact with these two kinase complexes and control the processivity of the RNA pol II elongation? On the basis of previous findings and our results, we propose a model for transcriptional activation by Tat (Fig. 5). Tat binds to the preinitiation complex and interacts with TFIIH; however, its interaction with other proteins present in the initiation complex cannot be ruled out (13–15, 19). It has been reported earlier that Tat associates with purified transcription preinitiation complexes (45). P-TEFb is also a component of the PIC, but it may not be involved in Tat binding at this point. TFIIH plays a critical role in transcription initiation and promoter clearance (8, 46) and is bound to nonphosphorylated RNA pol II holenzyme. TFIIH alone or with Tat phosphorylates the CTD and assists in promoter clearance. The TFIIH complex dissociates from the RNA pol II 30–50 nucleotides after initiation and is not part of the elongation complex (40). In line with previous findings, we also observed that TFIIH was released from the elongation complexes when 46-nucleotide-long RNA was transcribed. TFIIH is released, whereas Tat remains in the elongation complex by interactions with cyclin T1 subunit of P-TEFb. After the transcription of a functional TAR RNA structure, Tat binds to TAR and repositions P-TEFb kinase in the vicinity of the CTD of RNA pol II. Hyperphosphorylation of the CTD is accomplished by P-TEFb kinase, and processive elongation complexes are formed. We propose that a functional Tat-P-TEFb interaction requires TAR RNA because Tat does not become part of the elongation complexes unless TAR RNA is synthesized (47). It is also possible that more than one molecule of Tat participate in a single round of transactivation and additional Tat is recruited to the elongation complex after TAR is transcribed. Future studies to define the Tat-interacting regions of TFIIH and P-TEFb complexes and to determine various stages of the CTD phosphorylation will reveal mechanistic details of Tat function.

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