DSIF and NELF Interact with RNA Polymerase II Elongation Complex and HIV-1 Tat Stimulates P-TEFb-mediated Phosphorylation of RNA Polymerase II and DSIF during Transcription Elongation*

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Control of transcription elongation requires a complex interplay between the recently discovered positive transcription elongation factor b (P-TEFb) and negative transcription elongation factors, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) sensitivity inducing factors (DSIF) and the negative elongation factor (NELF). Activation of HIV-1 gene expression is regulated by a nascent RNA structure, termed TAR RNA, in concert with HIV-1 Tat protein and these positive and negative elongation factors. We have used a stepwise RNA pol II walking approach and Western blotting to determine the dynamics of interactions between HIV-1 Tat, DSIF/NELF, and the transcription complexes actively engaged in elongation. In addition, we developed an in vitro kinase assay to determine the phosphorylation status of proteins during elongation stages. Our results demonstrate that DSIF/NELF associates with RNA pol II complexes during early transcription elongation and travels with elongation complexes as the nascent RNA is synthesized. Our results also show that HIV-1 Tat protein stimulated DSIF and RNA pol II phosphorylation by P-TEFb during elongation. These findings reveal a molecular mechanism for the negative and positive regulation of transcriptional elongation at the HIV-1 promoter.

Transcription in eukaryotic cells is a complex process and involves three major steps including initiation, elongation, and termination. Although it was thought originally that regulation occurs primarily at the level of initiation, it is now recognized that the elongation step of transcription is a critical target for regulation of gene expression (1–3). Density analysis of RNA pol II in cells led to the identification of a number of genes including c-myc (4), c-fms (5), hsp70 (6), and HIV (7), which are potentially regulated at the elongation stage of transcription. Shortly after initiation, RNA pol II faces a barrier of negative transcription elongation factors and enters abortive elongation. The action of positive transcription elongation factors (P-TEF) lowers the barrier of negative transcription elongation factors and helps RNA pol II to escape from this transition phase which could lead to premature termination of transcription (for an excellent review, see Ref. 8). A positive elongation factor, P-TEFb, allows the transition into productive elongation producing longer mRNA transcripts (8).

Proteins involved in positive and negative regulation of elongation were discovered during studies aimed at understanding the mechanism of transcription inhibition by a nucleoside analog, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). DRB was discovered as an inhibitor of hnRNA and mRNA synthesis in HeLa cells (9, 10). DRB inhibits the production of full-length RNA and increases the amount of short transcripts from a variety of genes, suggesting that RNA pol II elongation was affected (10). In addition, DRB has no effect on promoter-independent RNA pol II transcription and on transcription reconstituted by purified general transcription factors and RNA pol II (11, 12). These studies suggested that there are cellular proteins other than the RNA pol II and general transcription factors that confer DRB sensitivity on elongation.

Recent studies using DRB as a transcription inhibitor led to the discovery of positive and negative elongation factors. P-TEFb was originally identified as an activity that released RNA pol II from an elongation pause in a DRB-sensitive manner (13, 14) and is proposed to facilitate the transition from abortive to productive elongation by phosphorylating the CTD of the largest subunit of RNA pol II (15). P-TEFb is composed of two subunits: the catalytic subunit cyclic-dependent kinase CDK9 (previously named PITALRE) and the regulatory subunit cyclin T1 (16–18). Complexes containing CDK9 and cyclin T1-related proteins, cyclin T2a or cyclin T2b, are also active for P-TEFb activity (19). Two negative transcription elongation factors, DSIF (DRB sensitivity-inducing factor) and NELF (negative elongation factor), have recently been identified and characterized (12, 20). DSIF is composed of two subunits, p160 and p14, which are homologs of the Saccharomyces cerevisiae transcription factors Spt5 and Spt4, respectively (12, 21). NELF is composed of five polypeptides, named as NELF-A to -E, and contains a subunit identical to RD, which is a putative RNA-binding protein of unknown function. DSIF and NELF function cooperatively and strongly repress RNA pol II elongation (20).

One elegant example of transcription elongation control is the mechanism of HIV-1 gene expression (for recent reviews,
see Refs. 22–26). 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 long terminal repeat (LTR) region. Tat activates transcription by binding to a highly structured RNA element, TAR RNA, which is located at the 5′-end of nascent viral transcripts (27). Tat functions through TAR RNA to control an early step in transcription elongation that is sensitive to protein kinase inhibitors and requires the C-terminal domain (CTD) of the large subunit of RNA pol II (22). Mutational analysis of HIV-1 Tat protein has identified two important functional domains: an arginine-rich region that is required for binding to TAR RNA, and an activation domain that mediates the interactions with cellular machinery (28, 29). Recent studies showed that Tat transactivation function is mediated by a nuclear Tat-associated kinase, TAK (22–26). The transactivation domain of Tat interacts with TAK (30, 31), which was recently shown to be identical to the kinase subunit of P-TEFb (17, 32). Tat interacts with cyclin T1 subunit of P-TEFb and recruits the kinase complex to TAR RNA. Recruitment of P-TEFb to TAR has been proposed to be both necessary and sufficient for activation of transcription elongation from the HIV-1 long terminal repeat promoter (33).

P-TEFb is a component of preinitiation transcription complexes and functions by phosphorylating the CTD of the largest subunit of RNA pol II during elongation steps (34–36). The CTD of the largest subunit of RNA pol II contains tandem repeats of the consensus sequence (YSPTPPS) that is differentially phosphorylated during the transcription cycle and phosphorylation of the CTD is critical for transcription regulation (37). Cellular kinases and phosphatases may contribute in transcription regulation based on their ability to alter the phosphorylation status of the RNA pol II CTD (37). For example, TFIIH phosphorylates the CTD of RNA pol II and assists in promoter clearance (38, 39).

It is not well understood when DSIF and NELF are recruited to the active elongation complexes and how they function. We were intrigued by the studies reporting that Spt5, one of the subunits of DSIF, is involved in Tat-mediated transactivation and functions as a positive elongation factor in HIV-1 LTR promoter in the presence of Tat (40, 41). Since DSIF was identified as a negative elongation factor, these results suggested that there is a critical transition step which converts Spt5 from a negative to a positive elongation factor during Tat transactivation. We reasoned that DSIF could be a substrate for P-TEFb phosphorylation and the phosphorylation status of Spt5 was a key element to determine the Spt5 function in transcription.

In this paper, we used a stepwise transcription approach to isolate homogeneous populations of active RNA pol II elongation complexes to study the interaction between negative elongation factors and RNA pol II transcription complex. In addition, we carried out kinase experiments during elongation to analyze the function of Spt5 in HIV-1 Tat transactivation. Our results demonstrate that DSIF and NELF associate with RNA pol II complexes during early transcription elongation and travel with elongation complexes as the nascent RNA is synthesized. Our results also show that HIV-1 Tat protein stimulated Spt5 and RNA pol II phosphorylation by P-TEFb. We propose a model for the regulation of elongation in HIV-1 LTR by Tat and Spt5.

Experimental Procedures

Tat Protein Purification—Recombinant HIV-1 Tat protein expressed in *Escherichia coli* as a glutathione S-transferase fusion protein was purified by glutathione-Sepharose affinity chromatography according to previously described procedures (42). HIV-1 glutathione S-transferase-Tat expression vectors were obtained from AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health, which were made available by Dr. Andrew Rice.

DNA Templates—The DNA templates used for preparing transcriptional ternary complexes were generated by polymerase chain reaction. Polymerase chain reaction was carried out by using plasmid pI0SLT containing HIV-1 LTR promoter as a template (43), and three primers based on pI0SLT plasmid sequence (5′-ACCCAGTGAAGAGCAGGAG, 5′-CACACTGACTAAAGAGGCT, and 5′-CACCATTATGCCTCCCGCT). The first primer contained a biotin at the 5′-end which was used to immobilize DNA to magnetic beads. Polymerase chain reaction products were purified on 1.0% agarose gels.

**Immobilization of DNA on Magnetic Beads**—DNA templates (0.25 μg) with biotin at 5′-end was bound to 25 μl of streptavidin-coated magnetic beads (Dynal Inc.) by incubating DNA and beads in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) containing 1 mM NaCl at room temperature overnight on a shaker.

**Stepwise Walking of RNA Pol II**—HeLa cell nuclear extracts were prepared according to published procedures (44, 45). Stepwise transcription were performed as described previously (35). Preinitiation complexes (PICs) were assembled by incubating the immobilized DNA templates (200 ng) in a volume of 25 μl containing 12 μl of nuclear extract, 6 mM MgCl₂ and 0.5 μg of poly(dA-dT) for 15 min at 30 °C. For RNA pol II ligation experiments at the PIC formation step, nuclear complexes were first incubated with 10 units of casein kinase II and 10 μCi of [γ-32P]ATP for 15 min at 30 °C. Then immobilized DNA templates and poly(dA-dT) were added into the mixture with 20 μM DRB, and incubated for an additional 15 min at 30 °C. RNA pol II complexes at the PIC stage were formed by adding 200 μM dATP during incubation. To remove unbound materials, PICs were washed with 25 μl of washing buffer A (20 mM HEPES, pH 7.9, 100 mM KCl, 2% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 6 mM MgCl₂). PICs were washed stepwise along the DNA templates (0.25 μg) with biotin at 5′-end to 25 μl of wash buffer A (20 mM HEPES, pH 7.9, 100 mM KCl, 2% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 6 mM MgCl₂). PICs were washed with 25 μl of wash buffer B (wash buffer A containing 0.05% Nonidet P-40 and 0.015% Sarkosyl) and twice with wash buffer C (wash buffer A containing 0.05% Nonidet P-40). The TECS were washed stepwise along the DNA templates by incubating with different combinations of NTPs. For all experiments that required additional cellular factors supplied by nuclear extract, TECS were incubated with nuclear extract at room temperature for 10 min and washed three times with wash buffer C. For chase experiments, incubated TECS were incubated with 4 NTP mixtures and incubated at room temperature for 5 min.

**Western Blotting**—For the isolation of ternary complexes on immobilized DNA template, the PICs and TECS at different steps were cleaved by restriction enzymes at 30 °C. The solution phase containing ternary complexes was mixed with 1 × SDS loading buffer and heated at 100 °C for 5 min. Released proteins were fractionated by SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Bio-Rad) to detect protein compositions. DSIF and NELF antibodies were kindly provided by Dr. Hiroshi Handa (Tokyo Institute of Technology, Japan). P-TEFb and RNA pol II antibodies were kind gifts from Dr. David H. Price (University of Iowa, Iowa City) and Dr. Michael Dahmus (University of California, Davis), respectively. The anti-[H]a biotin mouse antibodies were purchased from Roche Molecular Biochemicals. Protein contents were visualized with either ECL system (Amersham Pharmacia Biotech) or BM chemiluminescence Blotting Kit (Roche Molecular Biochemicals). The blots were exposed to x-ray film for various times (between 15 s and 10 min).

**Dye and Immunoprecipitation Assays**—TECS stalled at A-22 were incubated with additional nuclear extract in the absence or presence of 100 ng of HA-Tat at room temperature for 10 min. After removing the unbound proteins, TECS were chased by adding 10 μCi of [γ-32P]ATP, 20 μM CTP, GTP, UTP, and 2 μM ATP, in the absence or presence of 20 μM DRB. To reduce nonspecific phosphorylation by other kinases such as DNA-dependent protein kinase, 50 μM inhibitor LY294002 (Sigma) was included in the chase reactions. TECS were washed three times with 25 μl of wash buffer C to remove unincorporated radioisotope and then cleaved by restricted ternary complex to isolate the ternary complexes. The isolated TECS were incubated with Protein G-Sepharose beads (Amersham Pharmacia Biotech), which were pre-bound with Spt5 antibodies (Transduction Laboratories) in RIPA buffer (20 mM Tris-HCl, pH 8.0, 0.5% Nonidet P-40, 1% Tritone X-100, and 150 mM KCl), at 4 °C for overnight. The beads were washed with 300 μl of RIPA buffer containing 0.015% Sarkosyl three times and once with 300 μl of RIPA buffer.
RESULTS

DSIF and NELF Are Not Components of Preinitiation Complex—To determine whether DSIF and NELF are present in PICs, we prepared RNA pol II complexes on immobilized DNA templates containing HIV-1 LTR. Experimental design is shown in Fig. 1A. We prepared PICs containing RNA pol IIA and RNA pol IIO forms as described under “Experimental Procedures.” To visualize the formation of RNA pol IIA and RNA pol IIO forms in the presence of dATP, the largest subunit of RNA pol II was labeled by phosphorylation of the most C terminus residue (position 1928) by incubation with casein kinase II in the presence of [γ-32P]ATP and this phosphorylation of RNA pol IIA did not alter the electrophoretic mobility of the enzyme (46, 47). Kinase activity of TFIIH at this step was inhibited by the addition of DRB. PICs containing labeled RNA pol IIA were washed to remove excess radioisotope, DRB, and unbound proteins. RNA pol IIA was observed in the absence of dATP (Fig. 1B, lane 3) which was converted to IIO form in the presence of dATP (lane 4). These results show that PICs containing RNA pol IIA and IIO can be isolated and both forms of PICs are stable on immobilized DNA template.

Next, we analyzed the composition of PICs by Western blotting. PICs containing RNA pol IIA and IIO forms were formed as described above and released from immobilized DNA templates by cleaving with restriction enzyme and subjected to SDS-PAGE. Protein contents were transferred to polyvinylidene difluoride membrane, and detected by Western blotting with various antibodies raised against RNA pol II, a subunit of DSIF (Spt5), a subunit of NELF (NELF-E), and a subunit of P-TEFb (CDK9). Antibodies against RNA pol II were kindly provided by Dr. Michael Dahmus (University of California, Davis). DSIF and NELF antibodies were a kind gift from Dr. Hiroshi Handa (Tokyo Institute of Technology, Japan). P-TEFb antibodies were a kind gift from Dr. David H. Price (University of Iowa, Iowa City). Results of this analysis are shown in Fig. 1C. Regardless of the phosphorylation states of RNA pol II, DSIF and NELF are clearly not present in the PICs (Fig. 1C, lanes 2 and 3). However, P-TEFb was detected in PICs containing RNA pol IIA and IIO forms (Fig. 1C). P-TEFb as a component of PIC has been previously reported (35), and these results show that the phosphorylation status of RNA pol II did not affect the P-TEFb-PIC association. In addition, these results also indicate that there is no nonspecific interaction between DSIF, NELF, and DNA templates. To normalize the amount of transcription complexes, RNA pol II was detected as an internal standard and RNA pol IIA was converted to pol IIO form in the presence of dATP (Fig. 1B, lane 2) 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 (45). 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 nuclear extracts. These results indicate that DSIF and NELF do not interact with the RNA pol IIA and IIO forms in the PICs.

DSIF and NELF Associate with RNA Pol II Transcription Complexes during Elongation—Since DSIF and NELF are not components of the PICs (Fig. 1C), it is likely that these factors are recruited to the transcription complexes during the elongation stage. To test this hypothesis and determine the stage of elongation where DSIF and NELF interact with the transcription complex, we isolated homogeneous populations of TECs by a stepwise transcription approach (35). Preinitiation complexes were formed on immobilized DNA templates and elongation was initiated by adding dATP, UTP, CTP, and GTP. These elongation complexes were starved for ATP and therefore stalled at U-14. Further initiation was inhibited by Sarkosyl wash as described under “Experimental Procedures.” Stepwise walking of the TECs stalled at U-14 was accomplished by repeated incubation with different sets of 3 NTPs. The viability of the stalled complexes was confirmed by adding all 4 NTPs, which produced runoff products of expected lengths indicating that 100% of the complexes were transcriptionally active (data not shown).

We isolated TECs at A-22 and C-61 positions which display different sequence and structure of RNA transcripts (Fig. 2).
TECs were incubated with HeLa nuclear extracts to provide with DSIF and NELF since these factors were not present in the PICs. After removing the unbound proteins from nuclear extracts, TECs were released by cleaving the DNA template with 

\[ Pvu \] II, which is located downstream of TATA box sequence and this restriction digest avoids further initiation events. Protein contents were separated by SDS-PAGE and detected by Western blotting using RNA pol II, DSIF, and NELF antibodies. As shown in Fig. 2, DSIF and NELF can be detected in TECs after incubation with the nuclear extracts (lanes 2 and 4). As expected, there was no DSIF and NELF present in TECs without the addition of nuclear extract (Fig. 2B, lanes 1 and 3). Since there was no detectable binding of DSIF and NELF to the DNA templates or PICs (Fig. 1C), the associations of DSIF and NELF with TECs represent that these factors specifically interact with the elongation machinery. In agreement with our previous studies, P-TEFb was present in the elongation complexes. It is important to note that these experiments are not quantitative, therefore, the relative stoichiometry of P-TEFb, DSIF, NELF, and RNA pol II 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 the elongation complexes. For example, DSIF bands are more intense than CDK9 and RNA pol II (Fig. 2B) and do not necessarily represent the stoichiometry of these proteins. Our results showing the coexistence of DSIF and NELF in TECs are in agreement with previous findings indicating that NELF acts cooperatively with DSIF (20). Taken together, these results demonstrate that DSIF and NELF associate with RNA pol II complexes during the elongation stage and form a functional DSIF-NELF complex.

**DSIF and NELF Association with RNA Pol II Elongation Complexes Does Not Require RNA**—NELF is composed of five polypeptides, the smallest of which is identical to RD, a putative RNA-binding protein of unknown function (20). RD contains a tract of alternating Arg-Asp residues (RD motif). Since NELF interacts with elongation complexes (Fig. 2), it is possible that it recognizes RNA and this RNA binding activity of NELF recruits DSIF-NELF to the elongation complex. To address this question, we treated TECs with RNase A to remove the RNA moiety extruded outside of RNA pol II complexes and examined the binding of DSIF-NELF to these elongation complexes.

Before testing the RNA dependence of DSIF/NELF-TEC interactions, it was necessary to demonstrate that RNase A can hydrolyze RNA exposed out of the TECs and these TECs containing short nascent transcripts are competent to carry out transcription elongation. The experimental outline to remove nascent RNA and to test elongation is shown in Fig. 3. TECs stalled at A-22 were prepared by stepwise transcription reactions. The isolated TECs (A-22') were treated with RNase A to digest the extruded 5'-end of the RNA. After removing the enzyme, TECs were further walked to G-26. RNA transcripts were isolated from RNase A-treated TECs, A22', was ~7 nucleotides shorter than a 22-nucleotide transcript (Fig. 3B, lane 2). The protected 15-nucleotide RNA represents the RNA which is part of the RNA-DNA hybrid as well as single-stranded RNA inaccessible to the RNase A. The addition of cold ATP-free nucleotide mixture was able to move TECs from A-22 to G-26 (Fig. 3B, lane 3). These results demonstrate that nascent RNA exposed from the TECs can be removed by RNase A digestion and RNase A-treated TECs are competent to carry out further transcription elongation.

We next analyzed the DSIF/NELF interaction with TECs that were treated with RNase A (Fig. 3C). TECs were stalled at A-22 and RNA was digested with RNase A. After incubating the TECs with nuclear extracts and removing the unbound proteins, the TECs were released from the beads by PvuII cleavage. Proteins were separated by SDS-PAGE and detected by immunoblotting as described above. As shown in Fig. 3C, RNase A treatment did not affect the DSIF/NELF interaction with TECs (lanes 2 and 3). There was no DSIF/NELF detected in TECs which were not incubated with nuclear extracts (lane 1). Based on these results, we conclude that DSIF and NELF associate with elongation complexes and this interaction is RNA-independent.

**DSIF/NELF Travels with RNA Pol II Complexes through the Process of Transcription Elongation**—We next determined whether DSIF/NELF is released from TECs or it remains attached with TECs during elongation (Fig. 4). TECs stalled at A-22 were incubated with or without nuclear extract, washed extensively with buffers to remove unbound proteins, and chased by the addition of NTTPs mixture for short periods of time. After TECs were released by restriction enzyme digestion, protein contents of TECs were separated on SDS-PAGE.
on 15% polyacrylamide, 7M urea gels. Isolated TECs were further walked to G-26. RNA transcripts were -end of the RNA. After removing the RNase A to digest the extruded 5′9 nucleotides. The isolated TECs (A22) were treated with A22, RNA and to test elongation. TECs were formed at A-22 position by stepwise transcription. The isolated TECs (A22) were treated with RNase A to digest the extruded 5′ nucleotides. The isolated TECs (A22) were treated with RNase A to digest the extruded 5′ nucleotides. The isolated TECs (A22) were treated with RNase A to digest the extruded 5′ nucleotides. The isolated TECs (A22) were treated with RNase A to digest the extruded 5′ nucleotides.

FIG. 3. DSIF and NELF association with elongation complex does not require RNA. A, experimental outline to remove nascent RNA and to test elongation. TECs were formed at A-22 position by stepwise transcription. The isolated TECs (A22) were treated with RNase A to digest the extruded 5′-end of the RNA. After removing the enzyme, TECs were further walked to G-26. RNA transcripts were isolated by phenol extraction and ethanol precipitation, and analyzed on 15% polyacrylamide, 7M urea gels. B, RNA transcripts isolated from different TECs are shown: radiolabeled RNA transcript from TECs at A-22 position before RNase A treatment (lane 1), after RNase A digestion (lane 2), and TECs walked to G-26 after RNase A digestion (lane 3). C, the TECs were stalled at A-22 position by stepwise transcription and RNA was digested by RNase A treatment. TECs were incubated with nuclear extract and unbound proteins were removed by extensive washing with transcription buffer. TECs were isolated by PvuII cleavage and analyzed on 10% SDS-PAGE and proteins were detected by immunoblotting using antibodies against RNA pol II CTD, DSIF, and NELF. TECs stalled at A-22 were isolated without and with nuclear extract incubation (lanes 1 and 2). TECs stalled at A-22 were treated with RNase A prior to nuclear extract incubation (lane 3).

and detected by immunoblotting. RNA transcript analysis showed that the TECs stalled at A-22 could be chased to G-646 after incubation with NTPs for short periods of time. DSIF and NELF are present in TECs stalled at A-22 only when complexes are incubated with nuclear extracts (Figs. 2 and 3). DSIF and NELF were also detected in TECs stalled at G-646 (Fig. 4B, lane 2). Since there is no further addition of cellular factors during chase from A-22 to G-646, these results indicate that DSIF/NELF remains attached to the TECs during the elongation process.

HIV-1 Tat Does Not Interfere with the Association of DSIF/ NELF with RNA Pol II Elongation Complexes—HIV-1 Tat protein stimulates elongation through the positive effects of P-TEFb. It is quite reasonable to postulate that Tat could dislodge negative elongation factors such as DSIF-NELF complex from the elongating polymerase. To determine the effect of Tat on the interaction of DSIF/NELF with RNA pol II elongation complexes in HIV LTR promoter, we prepared TECs stalled at the C-61 position which would expose a functional TAR RNA structure for Tat binding. TECs stalled at C-61 were incubated with HIV-1 Tat containing HA-tag in the presence and absence of HeLa nuclear extracts. After removing the unbound proteins, TECs were isolated by PvuII restriction digest and analyzed by immunoblotting using antibodies against RNA pol II CTD, DSIF, and HA. FIG. 5 shows that Tat can bind to TECs in the absence and presence of nuclear extracts (lanes 2 and 4) and DSIF also interacts with the TECs in the presence of Tat (lanes 3 and 4). RNase A digestion of TECs stalled at C-61 in the presence of Tat did not release Tat protein from the elongation complexes indicating that Tat binds TAR RNA and then transfers to the elongation machinery (48). DSIF was identified as one of the negative transcription elongation factors by Handa and co-workers (12, 49) and P-TEFb could alleviate the negative effects of DSIF. Our previous studies have shown that P-TEFb is a component of the preinitiation and elongation complexes (35). Therefore, the presence of Tat, P-TEFb, and DSIF/NELF in the elongation complexes suggests that Tat could be involved in regulating the function of negative elongation factors through activation of P-TEFb (see below).

HIV-1 Tat Stimulates P-TEFb-mediated Phosphorylation of RNA Pol II and Spt5—Spt4 and Spt5 inhibit DRB-mediated transcription elongation (12, 49). Recent studies also showed that Spt5 is involved in Tat transactivation which is a positive elongation activity (40, 41). Since our results indicate that DSIF, P-TEFb, and Tat are present in elongation complexes, we proposed two hypotheses. 1 In addition to CTD of RNA pol

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II, Spt5 is the other cellular target for P-TEFb. 2) The phosphorylation status of Spt5 may play a key role in DRB-mediated repression and Tat-dependent transactivation. To address these questions, we set up a protein phosphorylation experiment during elongation (Fig. 6). TECs stalled at A-22 were isolated, incubated with nuclear extracts in the absence or presence of HIV-1 Tat protein, washed with buffers to remove unbound proteins, and chased to G-646 by the addition of nonradioactive NTPs and [γ-32P]ATP. DRB (20 μM) was included during elongation reactions to specifically inhibit Tat-dependent P-TEFb activity in HIV-1 LTR promoter (17). TECs were isolated by BglII cleavage, immunoprecipitated with Spt5, analyzed on 7.5% SDS-PAGE, and visualized by autoradiography. Protein contents were normalized by immunoblotting using antibodies against RNA pol II and Spt5 (as described in Figs. 2–5). Therefore, the different intensity of radiolabeled proteins in Fig. 6A represents the degree of phosphorylation of these proteins.

Fig. 6A shows that RNA pol II and Spt5 were phosphorylated during elongation from position A-22 to G-646 (lane 1). Tat stimulated phosphorylation of RNA pol II and Spt5 (compare lanes 1 and 3). DRB inhibited phosphorylation of these proteins and Tat was unable to overcome this effect (lanes 2 and 4). Enhanced phosphorylation of RNA pol II by Tat and DRB effect on phosphorylation are in agreement with previous reports showing that DRB inhibits the kinase activity of CDK9 which is responsible for RNA pol II hyperphosphorylation by Tat (17, 32, 36). Tat-mediated phosphorylation of RNA pol II showed that Tat bound to elongation complexes was functional. One interesting observation from these experiments was that Spt5 phosphorylation was also enhanced 2 ± 0.1-fold by Tat during elongation and inhibited by DRB (lanes 3 and 4). These results show that Tat stimulates Spt5 phosphorylation which is inhibited by DRB and P-TEFb activity suggesting that Spt5 is a target of P-TEFb phosphorylation and the status of Spt5 phosphorylation determines the function of Spt5 in transcription elongation.

DISCUSSION

We have utilized a stepwise transcription approach and Western blotting to demonstrate the interaction of negative elongation factors, DSIF and NELF, with active RNA pol II transcription machinery during various stages of transcription. Our results also show that DSIF/NELF associates with the elongation complex through protein-protein interactions and does not depend upon the nascent RNA sequence. In addition, we demonstrate that Tat does not dislodge negative elongation factors from the RNA pol II complex and stimulates RNA pol II and Spt5 phosphorylation. Our results provide new insights into the mechanisms of transcription elongation and the role of Tat protein in the regulation of HIV-1 gene expression.

Recent studies have uncovered that shortly after postinitiation, RNA pol II comes under the control of negative and positive elongation factors (for a recent review, see Ref. 8). DSIF and NELF are two negative elongation factors which are able to impede RNA pol II elongation (12, 20). The kinase activity of P-TEFb may be required to overcome their negative effects (49). DSIF interacts with RNA pol II and may directly modulate its elongation activity (49). Since P-TEFb phosphorylates the CTD of the largest subunit of RNA pol II, it was suggested that CTD phosphorylation by P-TEFb somehow alleviates the negative effect of DSIF (49). Our results show that the Spt5 subunit of DSIF is phosphorylated by P-TEFb in the presence of Tat suggesting a mechanism for overcoming the negative effect of DSIF by P-TEFb.

Spt4 and Spt5 are present in a variety of species from yeast to humans (12, 21, 40, 50). The C-terminal domain of human Spt5 (hSpt5) is rich in Ser, Pro, and Tyr and contains two C-terminal repeat motifs, CTR1 and CTR2 (51). The consensus sequence of CTR2 is similar to the CTD of the largest subunit of RNA pol II. CTR1 and CTR2 contain multiple Ser and Thr residues and may provide phosphorylation sites for cellular kinases. In vivo phosphorylation of hSpt5 during mitosis has recently been reported (51). Recent studies showed that Spt4 and Spt5 function during early transcription elongation process which is regulated by P-TEFb (12, 49). Immunodepletion of DSIF and P-TEFb could restore transcription to normal levels, and addition of recombinant DSIF was able to repress transcription in a dose-dependent manner (49). In addition, in the presence of P-TEFb, DSIF had no effect without DRB (49). These results indicated that DSIF in the absence of P-TEFb plays a role of negative regulator in transcription (49). Ivanov
et al. (52) reported that the CTR1 domain of Spt5 is important in transcription elongation in the presence of DRB or the HIV-1 Tat protein. In vitro kinase assays using Spt5 as a substrate showed that the addition of CDK9 resulted in phosphorylation of Spt5 (52, 53). Spt5 and TFIIF are phosphorylated by P-TEFB, however, no functional significance of the phosphorylation of these factors has been observed.3

How do these negative and positive transcription regulator proteins work together to control the processivity of RNA pol II that is enhanced by viral Tat protein? On the basis of our results, combined with previous reports, we propose a model for the regulation of transcription elongation by Tat (Fig. 7). RNA pol II containing nonphosphorylated CTD of the largest subunit (IIA) assembles on the HIV LTR promoter to form a preinitiation complex. TFIHH binds to nonphosphorylated RNA pol II and plays a critical role in transcription initiation and promoter clearance (38, 54–56). TFIHH phosphorylates the CTD of the largest subunit of RNA pol II and assists in promoter clearance. The TFIHH complex dissociates from TECs 30 to 50 nucleotides after initiation and is not part of the elongation complexes (35, 57). P-TEFB, composed of CDK9 and cyclin T1, is a component of PICs, however, it may not be an active kinase at this stage (14, 35). After promoter clearance, DSIF and NELF associate with transcription complex during early elongation stage. Under standard physiological conditions and non-HIV-1 LTR promoters, Spt5 is phosphorylated by CDK9 once DSIF/NELF associate with early elongation complex and this phosphorylation of Spt5 may sufficiently support regular transcription elongation. In the presence of DRB, the kinase activity of CDK9 is inhibited and Spt5 cannot be phosphorylated by P-TEFB. The unphosphorylated form of Spt5 acts as a negative regulator and causes inhibition of RNA pol II elongation. In contrast to cellular promoters, transcription from HIV-1 LTR promoter is not efficient and CDK9 is activated by Tat protein. In the absence of Tat, elongation complexes originated at HIV-1 promoter meet DSIF and NELF and CDK9 is unable to efficiently phosphorylate Spt5 and as a result elongation is not processive. After the transcription of a functional TAR RNA structure, Tat binds to TAR and repositions P-TEFB in the vicinity of the CTD of RNA pol II and Spt5. Hyperphosphorylation of the CTD is carried out by P-TEFB after the formation of Tat:TAR-P-TEFB complexes (23). In addition to CTD phosphorylation, Tat also enhances the phosphorylation of Spt5 mediated by P-TEFB and phosphorylated form of Spt5 turns DSIF into a positive regulator of transcription elongation. It is still unclear how P-TEFB gets activated. It is quite possible that autophosphorylation of P-TEFB or phosphorylation by a cellular kinase changes P-TEFB conformation and functional properties during transcription stages. During elongation on HIV-1 LTR, assembly of the P-TEFB:Tat:TAR ternary complex could be critical for activation of CDK9 kinase function.

It is interesting that after joining the elongation complexes at early stages, negative elongation factors travel with the transcription machinery. There are three possible explanations for postinitiation interaction of DSIF/NELF with the RNA pol II complexes: (a) departure of initiation factors from the complex allows more accessible surfaces for protein-protein interaction, (b) a conformational change in the structure of RNA pol II occurs that is recognized by DSIF/NELF, (c) DSIF/NELF are recruited through interaction with other proteins such as Tat-SF1 (41, 58) and this interaction is only possible during elongation. These results provide a number of intriguing possibilities related to the function of negative regulators of elongation. 1) DSIF associates with the early elongation complex to achieve a kinetic delay in elongation so that RNA processing machinery can load onto the RNA pol II complex. 2) Negative elongation factors in conjunction with P-TEFB could be involved in altering the phosphorylation status of the RNA pol II CTD which is important in recruitment of RNA capping and polyadenylation factors (59, 60). It has been reported that phosphorylation of specific residues in the CTD has a differential effect on recruitment and activation of the capping enzyme (61). 3) These factors could directly play a role in RNA processing. For exam-

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3 D. Price, personal communications.
ple, Spt5 stimulates mRNA capping (62). 4) DSIF can be converted into a transcription repressor during elongation by dephosphorylation of Spt5. Future studies on P-TEFb and DSIF/NELF would provide exciting insights into the mechanisms of gene expression.

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