Properties of RNA Polymerase II Elongation Complexes Before and After the P-TEFb-mediated Transition into Productive Elongation*

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The positive transcription elongation factor, P-TEFb, controls the fraction of initiated RNA polymerase II molecules that enter into the productive mode of elongation necessary to generate mRNAs. To better understand the mechanism of this transition into productive elongation we optimized a defined in vitro transcription system and compared results obtained with it to those obtained with a crude system. We found that controlling the function of TFIIF is a key aspect of RNA polymerase II elongation control. Before P-TEFb function, early elongation complexes under the control of negative factors are completely unresponsive to the robust elongation stimulatory activity of TFIIF. P-TEFb-mediated phosphorylation events, targeting the elongation complex containing DSIF and NELF, reverse the negative effect of DSIF and NELF and simultaneously facilitate the action of TFIIF. We also found that productive elongation complexes are completely resistant to negative elongation factors. Our data suggest that an additional factor(s) is involved in establishing the unique resistance activities of the elongation complexes before and after P-TEFb function. Furthermore, we provide evidence for the existence of another positive activity required for efficient function of P-TEFb. A model of the mechanism of P-TEFb-mediated elongation control is proposed in which P-TEFb induces the transition into productive elongation by changing the accessibility of elongation factors to elongation complexes. Our results have uncovered important properties of elongation complexes that allow a more complete understanding of how P-TEFb controls the elongation phases of transcription by RNA polymerase II.

The elongation stage of eukaryotic RNA polymerase II (RNAPII) transcription is not only essential for generating full-length mRNA but also is a critical target for the regulation of gene expression (1, 2). An elongation control process was initially uncovered during studies of the transcription inhibitory mechanism of the ATP analog DRB (3, 4). DRB treatment blocks RNAPII transcription specifically at an early step in elongation without inhibiting the enzymatic activity of purified RNAPII itself (4, 5). Further studies uncovered a new class of elongation factors responsible for this DRB sensitivity. Two negative elongation factors, the DRB sensitivity-inducing factor (DSIF) and the negative elongation factor (NELF) cause transcription pausing by physically associating with RNAPII (6–8). Positive transcription elongation factor b (P-TEFb), a cyclin-dependent kinase that can be inhibited by DRB, counteracts the negative effects of DSIF and NELF and allows RNAPII to enter productive elongation (9–14).

Early studies identified many genes that were regulated at the stage of transcription elongation, including hsp70 (15), c-myc (16, 17), and the HIV-LTR (18), and later a common regulatory mechanism utilized by these genes was uncovered, which was the recruitment of P-TEFb to the transcription machinery (12, 19, 20). Indeed, P-TEFb is the key regulator that causes RNAPII to overcome the rate-limiting step during the early stage of elongation. P-TEFb is an essential cellular coactivator for the viral transactivator Tat in stimulating transcription from HIV-LTR (12). Accumulating evidence indicates that control of gene expression by P-TEFb plays an important role in cellular activation, proliferation, and differentiation (2). It has been shown that many causes of cardiac hypertrophy converge at the critical step of up-regulation of P-TEFb activity (21, 22). High P-TEFb activity may also play a role in maintenance of the cancer state, evidenced by the fact that one potential anti-cancer drug, flavopiridol, has been found to act as a potent P-TEFb inhibitor (23).

Although required for the productive elongation of many genes, P-TEFb by itself has been shown to have no direct effect on RNAPII elongation rate in vitro, emphasizing that P-TEFb functions as a regulator of other elongation factors (11). P-TEFb can phosphorylate several proteins during elongation. It is responsible for hyperphosphorylation of the serine 2 positions of heptapeptide repeats in the C-terminal domain (CTD) of the large subunit of RNAPII (10). Similarly, the CTD-like heptapeptide repeats in the C-terminal region of the Spt5 subunit of DSIF can also be phosphorylated by P-TEFb, which is crucial for the positive elongation activity of DSIF (24). In addition, phosphorylation of one of the NELF subunits, NELFe, has been correlated with dissociation of NELF from elongation complexes that contain nascent RNA with double-stranded regions (25). It remains unclear if phosphorylation of all or only
a subset of these P-TEFb substrates is directly responsible and sufficient for the regulation of elongation. Indeed, P-TEFb-mediated phosphorylations on RNAPII-CTD and DSIF are known to be involved in pre-mRNA processing (26–29). Moreover, exactly how P-TEFb-mediated phosphorylations cause functional changes in elongation complexes is not well understood. Other known elongation factors such as TFIIF (30), TFIIIS (31), and elongin (32) affect elongation, but their role in the elongation control process is unclear (33).

DSIF has been implicated as both a positive and negative elongation factor (6). In humans it is composed of two subunits (160 and 14 kDa) that are homologs of yeast proteins Spt5 and Spt4 (6). Its transcription repression activity requires NELF and can be alleviated by P-TEFb (7, 14). Therefore, it has been proposed that P-TEFb causes the transition of RNAPII into productive elongation mode by reversing the negative effect of DSIF and NELF (14, 34). Recent studies suggested that phosphorylation of DSIF by P-TEFb can switch it into a positive elongation factor (24). DSIF has been shown to have a stimulatory effect on elongation only in reactions containing nuclear extract and limiting concentrations of ribonucleoside triphosphates (6), but it has no effects in the absence of other proteins (11). The exact role of DSIF and other factors in stimulating the rate of transcription after P-TEFb function and the mechanisms utilized to transduce the signal from P-TEFb are not known.

Most of the factors affecting RNAPII elongation were identified through utilizing functional assays that provided a means of purifying factors that had observable effects on the native elongation properties of RNAPII (35). Previously, we developed an in vitro transcription system using an immobilized DNA template to study elongation control of RNAPII (11, 35). Early elongation complexes containing pulse-labeled nascent RNAs were isolated and allowed to further extend transcripts in the presence of elongation factors that were added back. When purified DSIF and NELF along with recombinant P-TEFb were tested, we confirmed that the two negative factors, when present together, were able to slow the elongation of RNAPII and this effect could be eliminated by P-TEFb (11). Our data also demonstrated that the general transcription factor, TFIIF, could dramatically stimulate the elongation rate and could functionally compete with DSIF and NELF in controlling RNAPII elongation (11).

In this study, we employed an in vitro transcription system optimized to further dissect the functional mechanisms of P-TEFb-mediated elongation control. We found that P-TEFb-directed phosphorylation events can be fulfilled without active transcription, making it possible to more thoroughly study the functional targets of P-TEFb. During the course of our studies, we realized that the properties of elongation complexes are completely resistant to known positive factors before P-TEFb function and completely resistant to negative factors after P-TEFb function. In addition, our data strongly suggest that P-TEFb not only reverses the negative effect of DSIF and NELF, but also facilitates the function of TFIIF as the major positive elongation factor in productive elongation.

**EXPERIMENTAL PROCEDURES**

**Materials**—HeLa nuclear extract (HNE) was prepared as described by Adamson *et al.* (35). Bacterially expressed human DSIF was purified as described by Renner *et al.* (11). NELF was affinity-purified from HeLa S3 cells stably transfected with FLAG-tagged NELFe as described by Renner *et al.* (11). P-TEFb containing Cdk9 and cyclin T2a was expressed in baculovirus-infected insect cells and purified as described by Peng *et al.* (36). Recombinant human TFIIF was purified as described in Peng *et al.* (37).

**Isolation of Early Elongation Complexes (EECs)**—An immobilized DNA template was generated as previously described which contained the full cytomegalovirus promoter driving the production of a 548 nt run-off transcript (11). The protocol used to generate early elongation complexes was also as previously described (11, 35) except for some modifications. For each individual transcription reaction, 8 μl of preincubation mixture containing 200 ng of template (−0.5 pmol) and 1 μl of HNE was incubated with 20 mM HEPES, 60 mM KCl, 7 mM MgCl₂, 10 units of RNaseOUT™ (Invitrogen), and 1 μM flavopiridol for 10 min at room temperature. Transcription was initiated upon the addition of physiological concentrations of ATP, GTP, UTP (500 μM), and 5 μCi of [α-32P]CTP. After 30 s of pulse, elongation was halted with the addition of EDTA to 20 mM, and the resultant EECs contained labeled nascent RNA predominantly less than 25 nt in length. Complexes associated with the immobilized templates were stringently washed three times with high salt EEC isolation buffer (20 mM HEPES and 1.6 M KCl) followed by two washes with low salt EEC isolation buffer (20 mM HEPES, 60 mM KCl, and 200 μg/ml bovine serum albumin), and resuspended in low salt EEC isolation buffer. The isolated EECs used in each experiment were generated in one large reaction, isolated, and aliquoted into individual elongation reactions.

** Transcript Extension (Chase)**—Extension of transcripts from isolated EECs was carried out in 18-μl reactions by first mixing the isolated complexes with either HNE or purified factors in transcription buffer containing 20 mM HEPES, 60 mM KCl, 200 μg/ml bovine serum albumin, 3 mM MgCl₂, and 10 units of RNaseOUT™. Elongation was then resumed upon the addition of NTPs to 500 μM and allowed to proceed for the indicated amounts of time at room temperature. Except for specific indications, we carried out the elongation reactions for 7 min. The reactions were stopped by the addition of 200 μl of Stop Solution (100 mM Tris, 100 mM NaCl, 10 mM EDTA, 1% Sarkosyl, 200 μg/ml *Torula* yeast RNA (Sigma)). RNA preparation and analyses on denaturing gels were described previously (38). Autoradiography of the dried gels provided images of the results, and quantitation was accomplished with a Packard InstantImager (PerkinElmer Life Sciences).

** Prephosphorylation Reactions**—Recombinant P-TEFb and the indicated combinations of components, including preterminated EECs (see pretermination reactions below), DSIF, NELF, and HNE, were assembled in 9-μl reactions (half size of the final transcription elongation reactions) in the same transcript extension conditions as described above excluding NTPs. The prephosphorylation reactions were started upon the addi-
tion of ATP to 500 μM and were allowed to proceed for 5 min at room temperature, and then P-TEFb activity was inhibited by the addition of flavopiridol to 1 μM. When only a subset of the components was subjected to prephosphorylation, the indicated unphosphorylated components were supplemented after the termination of P-TEFb-directed prephosphorylation. Each of the final transcription reactions was 18 μl with the transcript extension conditions kept the same as above. Elongation was allowed to proceed for indicated times upon the addition of CTP, UTP, and GTP to 500 μM.

**Pre-termination Reactions**—When isolated EECs were incubated with P-TEFb in the presence of 500 μM ATP, some polymerases terminated because of the presence of a trace amount of TTF2 remaining bound to the DNA template. TTF2 is a transcription termination factor and its dsDNA-dependent ATPase activity is required for releasing transcripts from the template (39). In the presence of ATP, TTF2 preferentially binds with ssDNA, and its ATPase activity is thus suppressed (40). To limit the impact of contaminating TTF2 during prephosphorylation reactions and subsequent transcription reactions, we carried out a “pre-termination” reaction to inhibit the continuous activity of TTF2. Isolated EECs were incubated with 500 μM ATP, 3 mM MgCl₂, and 100 ng per reaction of ssDNA (a 50-nt DNA oligo with random sequences, synthesized by IDT) at room temperature for 5 min. During this process, only a small fraction of EECs terminated, and once TTF2 came off from these terminated DNA it bound to ssDNA. The pre-terminated EECs were then washed with high salt and is isolated buffer to remove the terminated polymerases, released RNA, contaminating TTF2 and ssDNA. Eventually, the beads were washed and resuspended with low salt EEC isolation buffer and ready for the prephosphorylation treatment.

**Amounts of HeLa Nuclear Extract or Purified Factors Used in Transcription Reactions**—1 μl per reaction of HNE was used for generating early elongation complexes. The same amount of HNE was also used in indicated add-back assays except for specific indications. The amounts of recombinant DSIF and NELF were optimized to achieve the maximal negative effect on elongation and called “1×,” “1×” of DSIF was 0.45 pmol per reaction and 1× of NELF was 0.03 pmol per reaction. 1× of TFIIF was 0.2 pmol, which is the lowest level that achieved the maximal increase in elongation rate in a 2-min reaction. 1× of P-TEFb was 3.3 pmol. 1× of factors was applied in either prephosphorylation reactions or in add-back assays unless otherwise indicated.

**RESULTS**

A previously developed in vitro transcription system using an immobilized DNA template (11, 35) was further optimized and employed here to explore the details of RNAPII elongation control by P-TEFb. The core of this technique is to determine the influence of a crude extract or purified elongation factors on elongation of nascent transcripts in isolated early elongation complexes (EECs) using add-back assays. A 5′-biotinylated DNA template containing the CMV immediate early promoter is immobilized onto paramagnetic beads through a biotin-streptavidin linkage, which allows for the subsequent isolation of the early elongation complexes. Preinitiation complexes are formed on the promoter by incubation with HNE. Upon the addition of nucleotides including limiting [α-32P]CTP, RNAPII initiates and generates labeled, nascent transcripts that are 15–20 nt in length within 30 s. Polymerases are halted by addition of EDTA and repeatedly washed with very high salt buffer followed by low salt washes and are finally resuspended in transcription buffer without nucleotides. The isolated EECs are then supplemented with either a crude extract or purified elongation factors and allowed to extend nascent transcripts upon addition of nucleotides. The transcripts are extracted and analyzed on a denaturing RNA gel and the effects of the factors on elongation are evaluated from the resultant change in the sizes of the RNAs. For brevity, we shall refer to the transcription assays with a HeLa nuclear extract added back with or without purified factors added back as the “crude system” or “defined system,” respectively. The defined system is named not because every component is known to be 100% pure, but because the components are highly purified and have defined methods of isolation. Although DSIF and NELF have been demonstrated to be very pure (11), it is possible that other proteins besides RNA polymerase II subunits may be present in the isolated elongation complexes (41). A systematic examination of the high salt EEC isolation conditions was carried out, and we found that raising the salt from 1 to 1.6 M (data not shown) and elimination of the Sarkosyl resulted in the isolated EECs that responded more efficiently to DSIF and NELF and other elongation factors such as TFIIF (supplemental Fig. S1). This eliminated a serious limitation in our early attempt to reconstitute elongation control in a defined system in which only about half of the isolated EECs responded to DSIF and NELF (11). We believe that Sarkosyl was not completely removed during the following low salt washes, and this negatively affected the function of the factors added back, presumably, by interfering with their interaction with EECs.

**Reconstitution of P-TEFb-mediated Elongation Control in Vitro**—Using EECs isolated under the new optimized conditions, we first examined the effect of recombinant DSIF and affinity-purified NELF on RNAPII elongation by examining the change in the kinetics of elongation. Isolated EECs were allowed to elongate for indicated times (from 0.5 to 16 min) in the absence or presence of DSIF and NELF (Fig. 1A). With no factors added back, isolated EECs that initially contained transcripts predominantly less than 25 nt in length (Fig. 1A, lane 1) moved slowly down the template at a relatively constant rate, as evidenced by the increase in the average lengths of the accumulated transcripts with time (Fig. 1A, lanes 2–7). The native elongation rate under these experimental conditions was about 25 nt per minute, and no run-off transcripts (548 nt) were detected even after 16 min of elongation. With the addition of DSIF and NELF, a decrease in the length of transcripts was observed at all the time points tested (Fig. 1A, lanes 8–13). Consistent with our previous report (11), DSIF and NELF appeared to significantly increase the rate that RNAPII elongation complexes spent at intrinsic pause sites. Inclusion of DSIF and NELF resulted in the reduction of the elongation rate for almost all polymerases to about 30% of their native rate (Fig. 1A, compare lanes 8–13 to 2–7).
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Next we attempted to reconstitute P-TEFb-mediated elongation control using both a defined system and a crude system. Isolated EECs were allowed to elongate for 7 min with or without additional factors. The nascent transcripts were extended to about 200 nt on average in 7 min in the absence or presence of DSIF and NELF. Isolated EECs were allowed to elongate transcripts for indicated times in the presence or absence of purified DSIF and NELF. The synthesized transcripts were extracted and analyzed on a 6% denaturing RNA gel followed by autoradiography of the dried gel. The effect of purified elongation factors (DSIF, NELF, and P-TEFb) or HNE on isolated EECs were analyzed in add-back assays. The first lane represents the isolated EECs before further elongation. In the remaining reactions, elongation was allowed to proceed for 7 min. (+, presence; 1× of factors was supplemented, and the absolute amounts of proteins used are described under “Experimental Procedures”; −, absence. The final concentration of flavopiridol in the indicated reactions was 1 μM.)

FIGURE 1. Reconstitution of elongation control in vitro. A, kinetics of RNAPII elongation in the absence or presence of DSIF and NELF. Isolated EECs were allowed to elongate transcripts for indicated times in the absence or presence of purified DSIF and NELF. The synthesized transcripts were extracted and analyzed on a 6% denaturing RNA gel followed by autoradiography of the dried gel. B, reconstitution of P-TEFb-mediated elongation control in both a defined system and a crude system. The effect of purified elongation factors (DSIF, NELF, and P-TEFb) or HNE on isolated EECs were analyzed in add-back assays. The first lane represents the isolated EECs before further elongation. In the remaining reactions, elongation was allowed to proceed for 7 min. (+, presence; 1× of factors was supplemented, and the absolute amounts of proteins used are described under “Experimental Procedures”; −, absence. The final concentration of flavopiridol in the indicated reactions was 1 μM.)

Functional Targets of P-TEFb in Elongation Control—Because P-TEFb activity was shown to be required for releasing the effect of negative factors on elongation, we wanted to clarify the responsible functional targets of P-TEFb. We first tested whether P-TEFb function could be reconstituted by prephosphorylation of its potential targets in the defined system. The isolated EECs, DSIF and NELF were phosphorylated altogether through incubation with P-TEFb and ATP. After this prephosphorylation, P-TEFb activity was inactivated with flavopiridol, and elongation was allowed to proceed for 7 min without continued P-TEFb function. As shown in Fig. 2A, prephosphorylation of all components together reversed the negative effect of DSIF and NELF even slightly better than P-TEFb did during elongation (Fig. 2A, compare lanes 3 and 4 to lane 2). Using Western blotting, we found that there was a mobility shift of DSIF upon the prephosphorylation treatment, indicating that DSIF was indeed phosphorylated as expected (see supplemental Fig. S2 and Ref. 54). The possibility of proteolysis of the factors during prephosphorylation treatment was ruled out, as evidenced by the fact that the levels of the full-length factors were not changed (supplemental Fig. S2). Therefore, the observed release of the negative effect was because of the action of P-TEFb. That is, the function of P-TEFb can occur in the absence of active transcription, making it feasible to detect the contribution of phosphorylation of each putative substrate to the ultimate release of the negative effect of DSIF and NELF. The isolated EECs, DSIF, and NELF were prephosphorylated individually or in combination. After the treatment, P-TEFb was inactivated with flavopiridol and the remaining unphosphorylated EECs and/or factors were added before transcripts were allowed to elongate for 7 min. Neither prephosphorylation of EECs (RNAPII-CTD) nor NELF alone had a noticeable influence on the negative effect of DSIF and NELF (Fig. 2A, lanes 5 and 6) while prephosphorylation of EECs and NELF did release some of the negative effect (Fig. 2A, lane 9). Prephosphorylation of DSIF alone was able to significantly reduce the negative effect, and prephosphorylation of EECs together with DSIF further enhanced this reversal (Fig. 2A, lanes 7 and 8). In general, prephosphorylation of any two of
these P-TEFb substrates reversed the negative effect better than prephosphorylation of only one component, but not as efficiently as the treatment of all three did (Fig. 2A, compare lanes 5–10 to lane 4). These results suggest that P-TEFb may target most efficiently a complex containing EECs, DSIF and NELF.

Similar but not identical results were obtained with prephosphorylation experiments using the crude system. The isolated EECs and HNE were prephosphorylated with P-TEFb individually or in combination. After the prephosphorylation, the remaining unphosphorylated component (either EECs or HNE) was added, and elongation was allowed to proceed for 7 min in the presence or absence of flavopiridol. When EECs and HNE were prephosphorylated together, run-off transcripts were produced quickly during the subsequent elongation regardless of the presence of flavopiridol (Fig. 2B, lanes 7 and 8). We confirmed that both RNAPII and DSIF were well phosphorylated during the treatment, and no proteolysis occurred during ATP incubation of HNE on critical factors, such as DSIF and NELF (supplemental Fig. S2). Thus, we concluded that P-TEFb function could be fulfilled during prephosphorylation in the crude system as was found in the defined system. In the crude system, prephosphorylation of EECs alone was also not sufficient to cause the transition into productive elongation (Fig. 2B, lane 4). The results with the crude system differed from those found in the defined system in that prephosphorylation of DSIF or DSIF plus NELF in the defined system caused a significant level of reduction of their negative effect (see Fig. 2A), while prephosphorylation of the HNE that contained DSIF and NELF did not reverse the negative elongation potential at all (Fig. 2B, lane 6). In addition, the combination of separately prephosphorylated HNE and EECs did not cause the transition (Fig. 2B, lane 10). The fact that the prephosphorylation of EECs and HNE have to be carried out in concert to fulfill P-TEFb function emphasizes that the elongation complexes and the associated factors are both required for effective reversal of the negative effects of DSIF and NELF by P-TEFb. This again suggests that the functional target of P-TEFb is really an elongation complex containing DSIF and NELF.

The Productive Elongation Complexes Are Open to Effects of Positive Elongation Factor(s) but Immune to Negative Elongation Factors—Because the P-TEFb-mediated transition into productive elongation could be accomplished during a prephosphorylation reaction, we next wondered whether the productive elongation complexes could be isolated and their properties examined. EECs were prephosphorylated in the presence of HNE to allow P-TEFb to induce the transition as described above, and then the resulting productive elongation complexes (PECs) were re-isolated by either a gentle low salt wash or a high salt wash. The EECs left unphosphorylated or prephosphorylated in the absence of HNE were low salt-washed and then served as negative controls. In case that some factor(s) required for keeping productive elongation potential would be lost during the re-isolation washes, HNE was added back after re-isolation to supplement all factors. The subsequent elongation was allowed to proceed for 7 min in the presence or absence of flavopiridol. The low salt-re-isolated PECs maintained the ability to reach run-off site regardless of the presence of flavopiridol (Fig. 3, lanes 5 and 6) whereas the high salt-treated ones, similar to the controls, produced full-length transcripts only when flavopiridol was absent (Fig. 3, lanes 7–8 and 1–4). This suggests that an additional factor(s), associated with the productive elongation complexes in a salt-sensitive manner, is required to maintain the productive elongation potential, even in the presence of additional negative elongation factors supplied by the HNE added back (Fig. 3, lane 6). Strikingly, when the low salt-re-isolated PECs were allowed to elongate in the absence of any additional factors, they were still able to reach run-off quickly (Fig. 3, lane 13). In contrast, the high salt-re-isolated counterpart elongated similarly to control elongation complexes, which was the native elongation rate of RNAPII (Fig. 3, lanes 9, 11, and 15). These observations demonstrated that the factors required for keeping the high elongation efficiency were fairly stably assembled in PECs and, therefore, the PECs were re-isolatable by high salt buffer. The control elongation complexes and the re-isolated PECs were also analyzed to determine how they were affected by the addition of purified DSIF and NELF. Consistently to what was found in lane 6, the low salt-washed PECs were resistant to the addition of DSIF and NELF. Once being re-isolated by high salt wash, the elongation complexes were resensitized to the negative elonga-
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Isolated EECs were left untreated (none, no P-TEFb added back) or pre-phosphorylated by P-TEFb in the absence (EEC) or presence of HNE (EEC + HNE) as described in the legend to Fig. 2. After prephosphorylation, flavopiridol was added to 1 μM to inhibit P-TEFb activity. Then the complexes were either gently washed and resuspended with low salt EEC isolation buffer (LSW) or washed by high salt EEC-isolation buffer (HSW) followed with wash and resuspension with LSW as indicated (flavopiridol was removed through washes). Then elongation reactions were assembled with the re-isolated elongation complexes and other indicated components and allowed to elongate for 7 min. The amount of factors added and the method of how transcripts were analyzed are described in the legend to Fig. 1.

TFIIF Association with Productive Elongation Complexes—Because the low salt-re-isolated PECs were able to produce long transcripts, and TFIIF is the only elongation factor known to be able to dramatically stimulate the elongation rate of RNAPII, we reasoned that TFIIF might be present in the isolated PECs. First, the kinetics of elongation of the following four kinds of elongation complexes were compared: (i) EECs, (ii) EECs with HNE and P-TEFb added back, (iii) PECs (EECs prephosphorylated by P-TEFb in the presence of HNE), (iv) EECs-TFIIF (EECs washed by low salt after incubation with a saturating amount of purified TFIIF). Elongation was allowed to proceed for 20 s or 1 or 3 min, and the lengths of the transcripts were analyzed. As shown previously, EECs by themselves extended transcripts very slowly at the rate of about 25 nt/min (Fig. 4A, lanes 1–3). When HNE and additional P-TEFb were added, the kinetics of P-TEFb function during elongation was observed (Fig. 4A, lanes 4–6). Immediately after elongation restarted (within 20 s), the elongation rate was even slower than the native rate of the polymerase, indicating that elongation was controlled by the negative factors in HNE (Fig. 4A, lane 4). One minute later, an intermediate state appeared: some polymerases were still in abortive elongation mode and the others had escaped from the negative control and entered productive mode (Fig. 4A, lane 5). A majority of the polymerases reached the run-off site within 3 min of elongation (Fig. 4A, lane 6). This clearly demonstrates a kinetic delay during the switching of polymerases into productive elongation state. Interestingly, when P-TEFb functioned prior to the chase through prephosphorylation treatment, the kinetic delay seen in lane 4 was abrogated. After prephosphorylation, the polymerases could elongate at a very high rate immediately upon the addition of nucleotides and arrive at the 548 nt-run-off site in 1 min (Fig. 4A, lanes 7–9). The elongation rate of PECs was about 30 times higher than the native rate of EECs, and this value is close to the estimated elongation rate in vivo (43), suggesting that our in vitro experimental system can efficiently simulate RNAPII elongation in vivo. Comparing the elongation rates determined from lanes 10–12 to those determined from lanes 1–3, the elongation rate of EECs-TFIIF was about ten times higher than that of EECs, which indicates that TFIIF can associate with stalled EECs and subsequently, dramatically stimulate elongation. This result suggests that TFIIF is likely present in PECs and is the major positive elongation factor. The fact that the elongation rate of PECs was about three times higher than that of EECs-TFIIF (Fig. 4A, compare lanes 7–9 with lanes 10–12) suggests the existence of additional positive elongation factors or a P-TEFb-mediated, improved efficiency of TFIIF function.

A modification of an assay that was previously used to show that the Drosophila homolog of TFIIF was able to move dynamically from one elongation complex to another (30) was employed here to determine if human TFIIF had similar properties. The isolated EECs, EECs-TFIIF (produced as described in Fig. 4A) or a mixture containing identical amounts of the two were allowed to elongate for a short period of time (20 s or 1 min). The patterns of transcripts generated by each set of complexes or the mixture of the two were compared to determine if elongation by the slowly moving polymerases (EECs) could be stimulated by the fast moving polymerases (EECs-TFIIF). As shown in Fig. 4B, the elongation rate of EECs was indeed accelerated dramatically when mixed with EECs-TFIIF such that the pattern of the transcripts generated by the mixture was very similar to that made by EECs-TFIIF alone (Fig. 4B, compare lanes 5 and 6 to lanes 1 and 2 and 3 and 4). These results indicate that human TFIIF, like fly TFIIF, is not stably associated with elongation complexes during transcription, even though it can stably associate with stalled EECs.

Next, this experimental strategy was utilized to determine if the positive factor associated with re-isolated PECs had similar properties to TFIIF. When low salt-re-isolated PECs were mixed with EECs, a reduction of short transcripts and simultaneous increase of long transcripts was seen (Fig. 4B, compare lanes 9 and 10 to lanes 1 and 2 and 7 and 8). To better visualize the data, a profile analysis of the lanes was carried out (Fig. 4C). Total counts for specific regions (from 15 nt to the 548 nt run-off site) of each lane were acquired with a Packard InstantImager, and plot profiles of each lane were generated. The related profiles were overlaid so that a comparison of the three different reactions at each time point could be made. As seen with EECs-TFIIF, PECs were able to stimulate the elongation of EECs, suggesting that the positive factor in the re-isolated PECs...
is TFIIF. However, the factor released by the PECs did not stimulate the EECs as much as TFIIF released from EECs/H18528. This could be caused by an increased stability of TFIIF association with PECs. It is also clear that none of the EECs stimulated by the factor released from PECs were able to elongate as rapidly as PECs, which could be due to the lower concentration of TFIIF or because of the presence of another factor stably associated with PECs that facilitates TFIIF function.

Before P-TEFb Function Elongation Complexes Are Dominantly Controlled by Negative Elongation Factors and Resistant to the Stimulatory Activity of TFIIF—As shown above, TFIIF can accelerate elongation of the isolated EECs very efficiently and also is a potential factor in PECs. Because the generation of full-length transcripts in the extract requires P-TEFb activity (see Fig. 1B), we propose that the strong stimulatory activity of TFIIF should be inhibited before P-TEFb acts or when P-TEFb activity is blocked by flavopiridol. To test this, we attempted to isolate “abortive elongation complexes” (AECs) and determined whether the AECs were resistant to TFIIF. First we wanted to find conditions to generate AECs and analyze their elongation properties after complex isolation. Isolated EECs were allowed to elongate in the presence of HNE and flavopiridol for indicated times, and the kinetics of elongation was compared with the controls (either EECs alone or EECs controlled by DSIF and NELF). Longer time points (up to 10 min) were taken to make sure the transition into productive elongation was really blocked. As shown in Fig. 5, when P-TEFb activity was inhibited, the polymerases elongating in the presence of HNE and flavopiridol for indicated times, and the kinetics of elongation was compared with the controls (either EECs alone or EECs controlled by DSIF and NELF). Longer time points (up to 10 min) were taken to make sure the transition into productive elongation was really blocked. As shown in Fig. 5, when P-TEFb activity was inhibited, the polymerases elongating in the presence of HNE and flavopiridol for indicated times, and the kinetics of elongation was compared with the controls (either EECs alone or EECs controlled by DSIF and NELF). Longer time points (up to 10 min) were taken to make sure the transition into productive elongation was really blocked. As shown in Fig. 5, when P-TEFb activity was inhibited, the polymerases elongating in the presence of HNE and flavopiridol for indicated times, and the kinetics of elongation was compared with the controls (either EECs alone or EECs controlled by DSIF and NELF). Longer time points (up to 10 min) were taken to make sure the transition into productive elongation was really blocked. As shown in Fig. 5, when P-TEFb activity was inhibited, the polymerases elongating in the presence of HNE and flavopiridol for indicated times, and the kinetics of elongation was compared with the controls (either EECs alone or EECs controlled by DSIF and NELF). Longer time points (up to 10 min) were taken to make sure the transition into productive elongation was really blocked. As shown in Fig. 5, when P-TEFb activity was inhibited, the polymerases elongating in the presence of HNE and flavopiridol for indicated times, and the kinetics of elongation was compared with the controls (either EECs alone or EECs controlled by DSIF and NELF). Longer time points (up to 10 min) were taken to make sure the transition into productive elongation was really blocked. As shown in Fig. 5, when P-TEFb activity was inhibited, the polymerases elongating in the presence of HNE and flavopiridol for indicated times, and the kinetics of elongation was compared with the controls (either EECs alone or EECs controlled by DSIF and NELF). Longer time points (up to 10 min) were taken to make sure the transition into productive elongation was really blocked.
some long transcripts were produced, indicating that the negative factors were not stably bound. However, the re-isolated AECs were completely resistant to the addition of TFIIF. Comparing lanes 13 and 14 to lane 12, add-back of a saturating amount of TFIIF to the AECs did not have any elongation stimulatory effect. We also found in another experiment that the EECs under the influence of the negative factors in the extract were still completely resistant to a level of TFIIF that was 500 times higher than that needed to have detectable stimulatory effect on EECs alone (supplemental Fig. S3). Even when TFIIF was allowed to interact with EECs prior to the addition of HNE and flavopiridol, the positive effect of TFIIF did not survive isolation (data not shown). These results confirm that the function of TFIIF in stimulating elongation is inhibited before the P-TEFb-mediated transition occurs, even though TFIIF molecules are present. The basis for this complete resistance is not understood and could be caused by modifications of known factors involved or caused by additional factors that potentiate the known factors.

P-TEFb-mediated Elongation Control Modulates the Antagonism of Positive and Negative Elongation Factors—Because TFIIF could not act on elongation complexes before P-TEFb function in the crude system (see Fig. 5) and our data suggested that TFIIF was the major positive elongation factor acting on PECs (see Fig. 4), we reasoned that P-TEFb-mediated phosphorylation events might facilitate the action of TFIIF. We further tested the influence of P-TEFb function on the competition between DSIF, NELF, and TFIIF observed earlier (11) in the defined system. Isolated EECs incubated with indicated amounts of DSIF and NELF were left untreated or prephosphorylated by P-TEFb. The reactions were supplemented with TFIIF and then transcripts were elongated for 7 min. As found before TFIIF competed with DSIF and NELF in controlling the elongation rate of RNAPII (Fig. 6, compare lane 4 to lanes 2 and 3) and the elongation rate was dependent on the relative concentrations of the positive and negative factors (Fig. 6, lanes 4–6). Strikingly, when DSIF, NELF, and EECs were prephosphorylated by P-TEFb prior to the addition of TFIIF, the polymerases were now dominantly controlled by TFIIF causing the majority of the transcripts to reach the run-off site (Fig. 6, lane 8). In addition, prephosphorylation of DSIF, NELF, and EECs slightly enhanced the stimulatory effect of TFIIF (Fig. 6, compare lane 8 to lane 3). We conclude that a defined system with EECs, DSIF, NELF, P-TEFb, and TFIIF better mimics the positive effect of P-TEFb on elongation seen in the crude system.

Although the new defined system reproduced the positive effect of P-TEFb on TFIIF function, the dramatic resistance features demonstrated for the crude system were not seen. EECs under the influence of DSIF and NELF (Fig. 6, lanes 4–6) were not resistant to TFIIF as was found for EECs in the presence of HNE (Fig. 6, lane 7). Also addition of more DSIF and
NELF or HNE after P-TEFb function had a negative effect on the elongation rate in the defined system (Fig. 6, lanes 9 and 10), but not the crude system (Fig. 6, lanes 11–15). This suggests that there is an activity missing in the defined system, which is required to keep the polymerases controlled dominantly by the negative elongation factors before P-TEFb function and by positive factors after P-TEFb function.

To better uncover the interplay between negative and positive elongation factors in the extract, an experiment was designed that utilized the addition of decreasing amounts of HNE to a constant number of isolated EECs. In the presence of flavopiridol to inhibit P-TEFb function, addition of HNE caused a strong negative effect on elongation at all concentrations except at the lowest concentration when 1/25 of the normal amount of extract (1 μl) was added (Fig. 7, lanes 2–4). At that concentration some polymerases reached run-off, suggesting that only as the effect of the negative factors was being titrated away, the effect of positive factors such as TFIIF could take over. In the absence of HNE, addition of a saturating amount of TFIIF to EECs showed the expected strong positive effect (Fig. 7, lane 5). However, addition of HNE at even fairly low levels abolished the positive effect of TFIIF completely (Fig. 7, compare lanes 6 and 7 to lane 5). Only at the lowest concentration of HNE was TFIIF able to slightly enhance the run-off signal (Fig. 7, compare lane 8 to lane 4). Addition of 4 times more TFIIF had no additional effect (data not shown). We conclude that in the absence of P-TEFb function the negative elongation factors in the crude extract are absolutely dominant over TFIIF. On the other hand, addition of a constant amount of DSIF and NELF to EECs before addition of decreasing amounts of HNE had no effect except that DSIF and NELF were able to inhibit the elongation of EECs that escaped the effect of the 

function (see Fig. 7, lane 4). These results imply that there is a factor(s) required to facilitate P-TEFb function present at a relatively limiting concentration in HNE. As found earlier in Fig. 3, addition of a constant amount of DSIF and NELF to the extract titration had essentially no effect (compare Fig. 7, lanes 24–26 with lanes 15–17) except that polymerases escaping the effect of negative factors in the extract at the lowest extract concentration were negatively controlled by the additional DSIF and NELF.

DISCUSSION

In this study the properties of RNAPII elongation complexes were examined under a variety of conditions which led to several significant findings. Conditions for isolation of elongation complexes were discovered that allowed more efficient function of both positive and negative elongation factors, and this in turn enabled prephosphorylation experiments to yield meaningful results. Importantly, the comparison of results from defined and crude systems exposed a critical role played by TFIIF in elongation control. We also provided direct evidence that before P-TEFb function elongation complexes are completely resistant to positive factors, including TFIIF, and that after the P-TEFb-dependent transition into productive elongation occurs, negative factors are no longer able to have any influence. Finally we provide evidence for the existence of a new factor required for efficient P-TEFb function.

Current Model for Elongation Control—Taking into account previously published studies and our new results, a model for the mechanism of P-TEFb-mediated RNAPII elongation control is proposed (Fig. 8). A key feature of the model is the switch of TFIIF for NELF that is promoted by the phosphorylation of components of the elongation complex by P-TEFb. It has been
hypothesized that NELF and TFIIF interact with a similar domain of RNAPII, but result in different functional outputs (11). In the model unphosphorylated DSIF helps recruit and stabilize the interaction of NELF, which increases the propensity of the polymerase to pause. The elongation complex before P-TEFb function is unable to respond to TFIIF due to the exclusion of the positive factor by NELF. A possible resistance factor (RF?) identified in our study may further stabilize the abortive elongation complex and make it more resistant to TFIIF and other positive factors such as TFIIS (44). Phosphorylation of the elongation complex in the presence of DSIF and NELF causes the dissociation of NELF from the complex and the entrance of TFIIF. At this point the polymerase enters productive elongation because TFIIF reduces the dwell time at intrinsic pause sites along the template. Our results showed that TFIIF is not stably associated with the elongation complex during transcription. The negative factors are unable to act on productive elongation complexes in part caused by a putative resistance factor that may be the same as the one acting during abortive elongation or may be different. Results from chromatin immunoprecipitation assays indicate that DSIF is found throughout coding regions of transcribed genes suggesting that it may remain associated with elongating RNAPII while NELF and also facilitates the function of TFIIF. The elongation complexes are then in productive elongation mode and are resistant to the negative elongation factors. Resistance factors (RF?) may be involved in establishment of the unique resistance activities of elongation complexes. More details are described in the text.

FIGURE 8. A model for P-TEFb-mediated elongation control. Before P-TEFb function, RNAPII elongation is dominantly controlled by the negative elongation factors, such as DSIF and NELF. The elongation complexes are in abortive elongation mode and are resistant to the function of positive elongation factors, such as TFIIF. P-TEFb phosphorylates the Ser\(^2\) positions in RNAPII-CTD, DSIF, and NELF, which leads to the release of the negative effect of DSIF and NELF and also facilitates the function of TFIIF. The elongation complexes are then in productive elongation mode and are resistant to the negative elongation factors. Resistance factors (RF?) may be involved in establishment of the unique resistance activities of elongation complexes. More details are described in the text.

The C-terminal region of the hSpt5 subunit of DSIF contains a conserved repetitive heptapeptide motif that can be efficiently phosphorylated by P-TEFb both in vitro and in vivo (24). A mutant DSIF with the major phosphorylation sites abolished functioned normally with NELF to cause transcription pausing, and the negative effect was released by P-TEFb (24). Instead, the mutant was deficient in allowing efficient positive factor function after P-TEFb action. These findings suggest that elimination of the negative effect and stimulation of positive effect on elongation are separable events and that both events are regulated by P-TEFb. When prephosphorylation of the EECs, DSIF, or NELF were individually carried out here, only prephosphorylation of DSIF had a major effect on reducing the negative effects of DSIF and NELF. But when pairs of components were examined, phosphorylation of any two components had a significant effect and prephosphorylation of all three components had the largest effect. Our results suggest that the most efficient target of P-TEFb is an elongation complex containing DSIF and NELF and that phosphorylation of all components contribute to the reversal of the negative effects of DSIF and NELF. Although others have found that phosphorylation of DSIF contributes only to the action of positive factors after P-TEFb function (14, 24), those results were obtained using a crude system, under significantly different conditions than used here. Hopefully, it will be possible to identify the target(s) of P-TEFb needed for subtle effects of both reversal of negative factor function and subsequent positive factor function. The development of a defined system that more closely mimics the crude system is essential for these studies.

Resistance Activities—Comparison of the properties of elongation complexes in the crude and defined systems provided strong evidence for the existence of a factor(s) that confers resistance of abortive elongation complexes to TFIIF and productive elongation complexes to DSIF and NELF. Using the defined system we demonstrated a competition between NELF and TFIIF function and the resistance activities seen in the crude system may involve a strengthening of this competition. It is not clear if both resistance activities are due to the same factor(s) or if the two activities are separable. It is possible that the resistance is caused by a post-translational modification of one of the known factors or a combination of a modification and an additional factor. We favor the idea that DSIF is locked onto the elongation complex by the resistance factor as shown in the model above and that phosphorylation of DSIF determines if NELF or TFIIF is allowed to function. The resistance factor(s) may also be responsible for the enhanced negative properties of abortive elongation complexes seen in the crude system (see Fig. 4). Using the assays developed here we are attempting to purify the factor(s) responsible for the
resistance activities so that a defined system that more accurately mimics the crude system can be studied.

Functional Interactions during Elongation—Consideration of the levels of factors needed to achieve functional consequences on isolated elongation complexes provides evidence that the affinity of some factors changes during the progression into productive elongation. Using Western blotting we estimated that about 1% of the total RNAPII in HNE became engaged in transcription and was recovered in EECs (supplemental Fig. S4). The absolute amount was about 0.01 pmol per reaction. The amounts of DSIF, NELF, TFIIF, and P-TEFb used in reactions with the defined system were all in excess (0.03 to 3 pmol/reaction) over the polymerase in isolated EECs. The amounts of factors needed to see maximal effects in the defined system was determined empirically, and likely reflect the affinity of the factors for the EECs, as well as the fraction of recombiant proteins that were active. Compared with the amounts needed for optimal function in the defined system, we found that the extract had ~4 times more TFIIF, ~10 times more NELF (considering that not all of the NELFe subunit quantitated was found in the five subunit complex of NELF (48)), similar amounts of DSIF and about 2 times less P-TEFb. It is possible that the amounts of factors present in HNE are different than the amounts needed in the defined system due to the influence of other activities such as the resistance activities and the P-TEFb facilitating activity. The requirement of a molar excess of factors over RNAPII is likely dictated by their respective binding constants. When the stability of factor association with EECs was examined, TFIIF was found to stably associate with stalled elongation complexes, but dynamically redistributed to other elongation complexes during transcription. The negative effect of NELF and DSIF did not survive low salt re-isolation of EECs, so at least one of the factors was not stably bound. The TFIIF resistance activity, reconstituted on EECs by incubation with HNE was stably associated, but the relationship of this activity to NELF and DSIF is not yet clear. Likewise, the NELF and DSIF resistance activity is stably associated with productive elongation complexes. It is likely that the association of each factor is influenced by the presence of other factors, the RNA, and the phosphorylation state of RNAPII–CTD.

Potential P-TEFb Facilitator—The finding that P-TEFb likely targets the elongation complex with DSIF and NELF emphasizes that the recruitment of P-TEFb to the paused elongation machinery is a prerequisite for P-TEFb function. This is consistent with the fact that regulating the recruitment of P-TEFb is utilized as a critical strategy for controlling expression of many genes such as hsp70, c-myc, and HIV-LTR (12, 19, 20). Our data provide evidence for the existence of a P-TEFb facilitator in the extract that is required for efficient action of P-TEFb. This P-TEFb facilitating activity may represent the function of a P-TEFb recruiting factor. Brd4, a mammalian bromodomain protein that binds to acetylated chromatin, was recently reported as a general P-TEFb recruiting factor (49, 50). However, Brd4 stimulated P-TEFb function on a chromatin-free template in vitro (50). More recently, it was shown that the fission yeast cap-methyl transferase, Pcm1, is required for efficient recruitment of P-TEFb to chromatin (51), which fits with a proposal that mRNA capping is coupled with elongation control as a transcription checkpoint to ensure only properly capped transcripts are elongated (52, 53). Therefore, it is possible that the P-TEFb facilitating or recruiting activity uncovered in our assays is due to Brd4 or is connected with mRNA 5’-end processing. The identification of this activity is currently in progress.

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