A Highly Purified RNA Polymerase II Elongation Control System*

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The balance of activity between both positive and negative factors achieves accurate control of many cellular processes. Accumulating evidence indicates that such a process regulates the control of transcription elongation (1). It has been proposed that shortly after initiation, negative transcription elongation factors act upon RNA polymerase II to cause production of short transcripts (2). With the action of P-TEFb the polymerase enters productive elongation and transcription is no longer influenced by the negative factors (3, 4). After this transition, the polymerase is acted upon by general elongation factors such as S-II, TFIIF, ELL, and elongin to generate long transcripts (5–7).

The key step in the elongation control process, the transition from abortive elongation to productive elongation, requires the positive elongation factor P-TEFb (1, 3, 4). P-TEFb was originally purified from Drosophila nuclear extracts, as a factor required for reconstitution of 5,6-dichloro-1-(3-n-ribofuranosylbenzimidazole (DRB) sensitivity in vitro transcription assays (5). Active human P-TEFb consists of a heterodimer of cyclin-dependent kinase 9 (Cdk9) and either cyclin T1, cyclin T2, or cyclin K (8, 9). The elongation properties of P-TEFb are dependent on its kinase activity, and both the kinase and elongation activities are sensitive to the nucleotide analog DRB (3, 4, 8, 10–12), a kinase inhibitor known for its ability to inhibit transcription elongation (13). P-TEFb is also strongly inhibited by flavopiridol, a drug currently in clinical trials as an anti-cancer treatment that might also be useful as an anti-HIV therapy (14). Depletion of P-TEFb from HeLa nuclear extract (HNE) greatly reduces the ability of RNA polymerase II to produce full-length transcripts and eliminates the DRB sensitivity of that extract (8). The addition of purified P-TEFb to HNE depleted of Cdk9 restores the ability of RNA polymerase II to generate full-length transcripts and restores DRB sensitivity (8, 15).

Another factor required for DRB sensitivity, DRB sensitivity-inducing factor (DSIF), was purified based on its ability to reconstitute DRB sensitivity in a partially purified transcription system (16). DSIF is composed of the 14-kDa Spt4 subunit (p14) and the 120-kDa Spt5 subunit (p160) (17). Spt4 possesses a putative zinc finger domain and probably interacts with Spt5 through mainly hydrophobic interactions (18). Spt5 has a highly acidic N-terminal region, four copies of the KOW repeat, and two sets of repeats in the C-terminal region (CTR1 and CTR2). RNA polymerase II has been shown to directly interact with Spt5 through a region encompassing the KOW repeats (18, 19). KOW repeats are also found in the Escherichia coli elongation control protein NusG (20). NusG binds the prokaryotic RNA polymerase and is required for certain specific termination and anti-termination activities (21, 22). Saturating amounts of NusG also enhance the elongation rate of the E. coli RNA polymerase by ∼20% (23). Genetic and biochemical studies of the yeast, fruit fly, and zebrafish homologs of Spt5 have also linked these proteins to the regulation of transcription elongation (24–26). Depletion of DSIF from HNE greatly reduces the DRB sensitivity of that extract without significantly affecting the ability of RNA polymerase II to generate long transcripts (16). Depletion of P-TEFb from a HNE eliminates DRB sensitivity and greatly reduces the ability of RNA polymerase II to generate long transcripts (8). Immunodepletion of both DSIF and P-TEFb from an extract restores the ability of RNA polymerase II to make long transcripts while eliminating the DRB sensitivity of that extract (15). A dose-dependent add-back of purified DSIF and P-TEFb reconstitutes DRB-dependent elongation control (15). This finding suggests that P-TEFb functions to reverse the negative activity associated with DRB sensitivity and that this negative activity is dependent on the presence of DSIF. The expression of a dominant negative form of DSIF in HeLa cells increased the expression level of each of four reporter genes severalfold (18), suggesting that DSIF is involved in general repression of transcription.

The search for factors required for the reconstitution of DRB sensitivity in a partially purified transcription assay led to the discovery of a third factor, negative elongation factor (NELF) (27). NELF consists of four subunits. The smallest of these, NELF-E, was cloned and found to contain repeats of the dipen-
tide Arg-Asp (RD repeats), an RNA recognition motif, and a leucine zipper at the C-terminal end (27). Immunodepletion of NELF-E from an HNE significantly reduced the DRB sensitivity of that extract, while an add-back of purified NELF restored the DRB sensitivity to original levels. Using a deoxyctosine-tailed template assay, the effect of combining DSIF and NELF together with purified RNA polymerase II was tested (27). Under these conditions, transcription was nearly completely inhibited. When DSIF and NELF were added to polymerases that had been paused on a tagged template, further elongation was also significantly reduced (27).

To further study the role of DSIF, NELF, and P-TEFb in elongation control, an assay using isolated RNA polymerase II elongation complexes was utilized. With this system, elongation control mediated by P-TEFb was reproduced in both a crude system, incorporating a HeLa nuclear extract and in a highly purified system, using only purified factors. We found that DSIF and NELF work cooperatively to decrease the elongation rate of the polymerase by increasing the time it spends at pause sites. The original elongation rate of the polymerase was restored by the addition of P-TEFb to the assay. We also show that NELF functionally competes with TFIIF in the presence of DSIF.

EXPERIMENTAL PROCEDURES

Materials—Generation of pET constructs for the expression of both human Spt4 and Spt5 with an N-terminal His tag is described elsewhere (16). Bacterial expression and purification of P-TEFb (Cdk9 and cyclin T2a) are described by Peng et al. (8). Anti-FLAG M2-agarose affinity gel (A-1205) and FLAG peptide (F-3290) were obtained from Sigma. Recombinant human TFIIF was purified as described in Peng et al. (28).

Expression and Purification of DSIF—E. coli DE3 cells transformed with plasmids expressing Spt4 and Spt5 were grown at 37 °C in Luria medium containing 100 μg/ml ampicillin. Because the expression of Spt5 had a negative effect on the growth of the cells, ampicillin selection was maintained by washing the cells in fresh medium several times during growth. When a liter of culture reached an absorbance of 0.5 absorbance units, it was induced with 400 μM isopropyl-1-thio-β-D-galactopyranoside. After a 3-h induction, cells were washed with PBS, resuspended in lysis buffer (PBS, 1% Triton X-100, 200 μM EDTA, 1 mM DTT, 0.1% of a saturated solution of PMSF in isopropyl alcohol), and sonicated. The lysate was spun at 50,000 × g for 40 min, the supernatant was removed, and the pellet was resuspended in urea buffer (25 mM HEPES, 100 μM EDTA, 100 mM KCl, 6 μM urea). The urea-extracted pellet was spun at 15,000 × g for 30 min. 3.2 mM CaCl2 was added to complex the EDTA immediately before loading onto a 4-ml Ni2-

column equilibrated with 200 mM imidazole. Purified Spt5 was combined with an approximate 1:1 complexation with HNE or purified factors in the presence of 20 mM HEPES, 60 mM KCl, 7 mM MgCl2, and 50 μM DRB for 10 min at room temperature (28). Transcription was initiated during a 30-s pulse with the addition of ATP, GTP, and UTP to 500 μM and 5 μM of [α-32P]CTP for each final elongation reaction. Elongation was halted by the addition of EDTA to 25 mM. Complexes associated with the immobilized template were stringently washed three times with HKS (20 mM HEPES, 1 mM KCl, and 1% Sarkosyl), two times with HKB (20 mM HEPES, 60 mM KCl, and 200 μg/ml bovine serum albumin), and resuspended in HKB.

Transcription Extension—Extension of transcripts in early elongation complexes was carried out in 19-μl reactions by first mixing the isolated complexes with HNE or purified factors in the presence of 20 mM HEPES, 60 mM KCl, 200 μg/ml bovine serum albumin, and 1.1 units/μl RNAsin (Promega). After 3–5 min, elongation was resumed by the simultaneous addition of NTPs to 500 μM and MgCl2 to 7 mM. Reactions were allowed to elongate for the indicated amounts of time at room temperature and were stopped by the addition of 200 μl of Sarkosyl Stop Solution (100 mM Tris, 100 mM NaCl, 10 mM EDTA, 1% Sarkosyl, 200 μg/ml RNA). RNA preparation and analysis on 6% denaturing gel was described previously (29).

RESULTS

To further our understanding of elongation control by P-TEFb, we developed an in vitro transcription system in which factors could be added back to isolated early elongation complexes. The attachment of the template to magnetic beads through a biotin-streptavidin linkage allows for the isolation of the early elongation complexes. The template is incubated with HNE to form preinitiation complexes on the CMV immediate early promoter. Upon the addition of nucleotides, including limiting [α-32P]CTP, RNA polymerase II initiates and generates transcripts predominately less than 25 nt in length. The elongating polymerases are halted and stripped of associated factors by repeatedly washing with buffer containing 1 mM KCl and 1% Sarkosyl and resuspended in transcription buffer without nucleotides. The addition of nuclear extract to such complexes has been demonstrated to reconstitute P-TEFb-dependent elongation control (28). Here we use isolated elongation complexes to determine whether highly purified DSIF, NELF, and P-TEFb could reconstitute elongation control in a similar manner.

Readhead of Nuclear Extract to Isolated Elongation Complexes Reconstitutes Elongation Control—We first examined the kinetics of RNA polymerase II elongation in the presence of a nuclear extract added back to early elongation complexes in the absence or presence of DRB as diagrammed in Fig. 1A. Previous work, which is briefly described in the Introduction,
**Fig. 1.** Reconstitution of DRB sensitivity with an HNE. A, diagram of the procedure used to isolate elongation complexes. B, the effect of HNE on isolated elongation complexes. HNE was added to high salt and detergent-washed early elongation complexes either with or without 50 μM DRB. The 0-s time points represent the isolated early elongation complexes before further transcription. In the remaining reactions, NTPs were added and elongation was allowed to proceed for the indicated times.

Strongly indicates that DRB sensitivity observed in reconstituted transcription assays is due exclusively to the inhibition of P-TEFb (1, 4, 8, 12). After add-back of HNE, the early elongation complexes were chased for 0, 20, 30, 50, 80, 130, 210, or 340 s (Fig. 1B). At the first few time points, little difference was observed in the elongation potential of the transcription complexes with or without DRB present. At longer time points, DRB reduced the elongation potential of a large fraction of the polymerases. This kinetic delay to the function of P-TEFb suggests that the kinase only functions on complexes that have either traveled a certain distance down the template or that P-TEFb requires a certain period of time to function. In the presence of DRB, complexes continued elongation, but at a much slower rate. The kinetics of elongation and the timing of P-TEFb function seen here match what was found previously using a system in which initiation and elongation were not separated by isolation of the early elongation complexes (2). The results presented here demonstrate that the add-back assay faithfully reconstitutes appropriate kinetic aspects of elongation control.

**Expression and Purification of DSIF and NELF**—The two subunits of DSIF, Spt4 and Spt5, were both expressed in E. coli and purified as described under “Experimental Procedures” and summarized in Fig. 2A. Cells transformed with the Spt5 pET vector reproducibly caused slow growth, suggesting that expression of human Spt5 is toxic to E. coli. The resulting loss of the expression plasmid was minimized by maintaining ampicillin selection with several fresh medium changes during growth. This allowed a significant increase in the final level of expressed protein. The majority of both the Spt4 and Spt5 proteins produced in E. coli was insoluble, but both proteins were solubilized in the presence of 6 M urea. Each subunit was individually purified over a nickel column in the presence of 0.1% Nonidet P-40 throughout purification and 80°C. We tested the activity of the two NELF fractions, 31 and 32, that differ in NELF-C/D composition but found no differences in activity in our add-back transcription assay (data not shown).

**DSIF and NELF Cause Production of Short Transcripts**—In an attempt to reconstitute the negative elongation activity observed with HNE, the effect of adding purified DSIF and purified NELF to isolated early elongation complexes was tested (Fig. 4). Transcripts in the isolated complexes were elongated for 5 min in the presence of 0, 6, 13, 25, 50, or 100 ng of DSIF and 5 ng of NELF or with 0, 0.6, 1.3, 2.5, 5, or 10 ng of NELF and 50 ng of DSIF. At the concentrations used in this assay, DSIF or NELF added individually had no observable effects on the elongation rate. When both of these factors were added together, a negative effect was observed on approximately half of the polymerases (Fig. 4). We were unable to find conditions in which all polymerases were affected by DSIF and NELF (see “Discussion”). Using saturating concentrations of DSIF and NELF, polymerases that were affected by these factors elongated at one-third the rate of the unaffected polymerases. A maximal reduction in the rate of DSIF and

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NELF affected polymerases occurred when ~50 ng of DSIF and 5 ng of NELF were added (Fig. 4 and data not shown). Increasing the concentrations of DSIF or NELF above these values did not affect the percentage of polymerases that were affected by these factors. When DSIF was added at a 10-fold higher concentration than the highest concentration tested in Fig. 4, all polymerases increased their rate of elongation by ~20% (data not shown). This stimulatory activity was independent of NELF. Concentrations of NELF up to 15-fold higher than those used here had no effect on the elongating polymerase in the absence of DSIF. Because independent purifications resulted in DSIF preparations that had specific activities that differed by up to a factor of 5, it is likely that only a fraction of the DSIF in any of these samples was active. In contrast, separate purifications of NELF had very similar specific activities (data not shown).

**P-TEFb Reverses the Negative Effect of DSIF and NELF**—To determine whether the negative activity of DSIF and NELF could be reversed, increasing amounts of P-TEFb (0, 25, 50, 100, or 200 ng) were added back to isolated elongation complexes in reactions containing 50 ng of DSIF and 5 ng of NELF. As the level of P-TEFb increased, the ability of the polymerase to generate longer transcripts was restored (Fig. 5). The transcripts generated with the highest level of P-TEFb were on average slightly shorter than those seen in the absence of any added factors. This was not changed by the addition of even higher levels of P-TEFb (data not shown). This would be expected if the highly purified system exhibited a kinetic delay of P-TEFb action like that seen in reactions containing nuclear extract (see Fig. 1). In the absence of DSIF and NELF, P-TEFb did not have any effect on the elongation rate of the polymerase (Fig. 5). When DRB was added to a reaction with DSIF, NELF, and P-TEFb, the reversal was blocked, indicating that the kinase activity of P-TEFb is required (Fig. 5).

Since the sequence of the NELF-E subunit suggests that it may bind RNA (27), we examined the effect of transcript length on the activity of DSIF, NELF, and P-TEFb. Transcripts from isolated early elongation complexes (~15–25 nt) were extended to ~70 nucleotides in the absence of any additional proteins. Purified factors were then added, and elongation was examined. No difference in the activities of these three proteins was found, suggesting that the factors work independent of transcript length (data not shown).

**DSIF and NELF Increase the Time the Polymerase Spends at Pause Sites**—To examine the kinetics of action of DSIF, NELF, and P-TEFb, three time courses were performed. The elongation reactions contained 1) no added factors, 2) DSIF and NELF, or 3) DSIF, NELF, and P-TEFb. Transcripts from each time point were analyzed on a denaturing gel, and the autoradiograph of that gel is shown in Fig. 6. To aid in visualizing the results, the autoradiograph was analyzed using Scion Image (Scion Corp.). Plot profiles of each lane were generated and arranged so that a comparison of the three different reactions at each time point could be made (Fig. 7). In the first reaction

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**Fig. 2. Purification of DSIF.** A, diagram of DSIF purification. B, purification of DSIF on Mono Q. Both subunits of DSIF (Spt4 and Spt5) were individually expressed in E. coli and purified under denaturing conditions. After renaturing the subunits together, DSIF was chromatographed on Mono Q. Details can be found under “Experimental Procedures.” Fractions (numbered) from a KCl gradient elution were analyzed by SDS-PAGE followed by silver staining. M, 10-kDa ladder; OP, output; P, pellet or insoluble subunits after renaturation; FT, flow-through.

**Fig. 3. Purification of NELF.** A, diagram of NELF purification. B, purification of NELF from Mono Q. Affinity-purified NELF (OP) obtained from expression of a FLAG-tagged subunit in HeLa cells was chromatographed on Mono Q. Individual NELF subunits are labeled NELF-A through NELF-E. Details can be found under “Experimental Procedures.” Fractions (numbered) from a KCl gradient elution were analyzed by SDS-PAGE followed by silver staining. M, 10-kDa ladder; OP, output; FT, flow-through.
with RNA polymerase II alone, examination of the transcripts isolated from each time point indicated that polymerases moved relatively synchronously down the template, pausing at the typical, heterogeneous array of sites (Fig. 6). In the reaction with DSIF and NELF, the pattern of transcripts indicated that half of the polymerases carried out elongation in a manner indistinguishable from that of RNA polymerase II alone and half were dramatically slowed (Figs. 6 and 7). The plot profile of the longest time point with DSIF and NELF present shows a population of transcripts that are nearly 80 nt long and a population of transcripts between 200 and 300 nt with very few transcripts between each of these regions. These results are not compatible with a model in which DSIF and NELF act on all polymerases to varying degrees. The pause sites seen with and without DSIF and NELF are essentially the same, suggesting that DSIF and NELF slow the elongation rate by increasing the time the polymerase spends at the pause sites. The action of DSIF and NELF was very rapid, as indicated by the fact that within the first 20 s of elongation, DSIF and NELF had already decreased the length of approximately half of the transcripts. At early time points, the presence of P-TEFb in a reaction with NELF and DSIF had little or no effect (Figs. 6 and 7, 20- and 40-s points). However, at 80, 160, and 320 s, 90% of the transcripts shorter than 45, 60, and 110 nucleotides, respectively, were reduced by the addition of P-TEFb to elongation reactions containing DSIF and NELF (Figs. 6 and 7). Overall, the kinetics of action of DSIF and NELF and the subsequent action of P-TEFb are similar to what was observed with HNE (see Fig. 1). In both the highly purified system and the crude HNE add-back, there was a rapid negative effect followed by an approximately 1-min delay before the action of P-TEFb produced a noticeable effect.

**TFIIF Functionally Competes with DSIF and NELF**—Because DSIF and NELF seem to function by increasing the time the polymerase spends at pause sites and TFIIF functions by decreasing the time the polymerase spends at pause sites (30), the effect of adding TFIIF together with DSIF and NELF was explored. Due to the 10-fold increase in elongation rate in the presence of TFIIF, 1-min elongation assays were utilized. A titration of TFIIF (0, 2, 6, 20, or 60 ng) was used to demonstrate this positive effect. As the TFIIF concentration was increased, the length of the transcripts generated in 1 min dramatically increased (Fig. 8). When 50 ng of DSIF and 5 ng of NELF were added to a low concentration of TFIIF (2 ng), the stimulatory effect associated with TFIIF and the repressive effect associated with DSIF and NELF were both observed (Fig. 8). As the concentration of TFIIF was increased in the presence of DSIF and NELF, the shorter transcripts associated with DSIF and NELF function were no longer present (Fig. 8). At high concen-
trations, TFIIF greatly reduced or eliminated the effect of the two negative factors, but at intermediate concentrations both effects were seen, suggesting a functional competition between the positive and negative factors.

To further examine the competition of TFIIF with DSIF and NELF and to determine which of the two proteins might be responsible for this concentration-dependent competition, each was titrated upward with the other held constant into reactions containing TFIIF. A dose-dependent appearance of shorter transcripts was only observed when NELF was increased. The effect of increasing NELF and DSIF in parallel or individually can be visualized best in the plot profiles found in Fig. 9B. Note especially the effect on transcripts between 50 and 150 nt in length. From the experiments in Figs. 8 and 9, we conclude that in the presence of DSIF, TFIIF functionally competes with NELF in a concentration-dependent manner.

**DISCUSSION**

The results obtained with the highly purified system described here are dictated by the composition of the elongation complexes used and the factor preparations added back. To avoid the presence of factors that might influence elongation, early elongation complexes were repeatedly washed with 1 M KCl and 1% Sarkosyl. No transcription factors are known to be resistant to such stringent washing. The properties of the isolated complexes reflected the lack of all known factors that affect elongation including, TFIIF (see Fig. 8), S-II (data not shown), termination factor 2 (28), DSIF (see Fig. 4), NELF (see Fig. 4), and P-TEFb (see Fig. 4). It is possible that the washed elongation complexes lost specific RNA polymerase II subunits as discussed below. It is also possible that unknown factors have become tightly associated with the polymerase during initiation; however, this is not likely, since there are no examples of the incorporation of salt and detergent-resistant factors into prokaryotic or eukaryotic elongation complexes. The subunits of DSIF and TFIIF were produced in *E. coli*, denatured, purified, reconstituted, and then repurified. Therefore, these highly purified recombinant factors are not likely to contain contaminating activities or to be functionally modified. It is possible that a fraction of DSIF is nonfunctional as discussed below. NELF and P-TEFb were purified from human and insect
cells, respectively, using affinity chromatography in the presence of high salt to eliminate associated factors, followed by high resolution ion exchange chromatography. Silver-stained gel analysis indicated the absence of significant amounts of other proteins in the preparations of DSIF, TFIIF, NELF, and P-TEFb. There was heterogeneity in the C/D subunit of NELF, as described under "Results"; however, this heterogeneity was functionally insignificant. In summary, our highly purified preparations of early elongation complexes and elongation factors allow us to reasonably conclude that the identified factors are responsible for the effects seen on the isolated elongation complexes.

Our results indicate that a number of the features of elongation control can be reproduced with the combination of highly purified components described above. The most characteristic feature of elongation control is the P-TEFb-dependent reversal of the effect of negative factors. Using a system in which factors were added back to isolated elongation complexes, the negative effect of DSIF and NELF was abrogated by P-TEFb. It is interesting that DSIF, NELF, or P-TEFb alone did not have any effect on the elongation properties of RNA polymerase II. The only combination of two factors that had any effect was the one composed of DSIF and NELF. Together, DSIF and NELF slowed the rate of elongation of RNA polymerase II. The only effect of P-TEFb was to eliminate the negative effect of DSIF and NELF, and this reversal required the kinase activity of the factor. A second feature of elongation control seen in vitro using whole nuclear extract is the rapid function of negative factors followed by a kinetic delay in the function of P-TEFb on early elongation complexes. Importantly, the kinetics of both the action of the negative factors and P-TEFb are similar in the crude system and in the highly purified system. The negative factors begin to exert their influence very quickly (within 20 s). In both types of assays, there was a kinetic delay of about 1 min before P-TEFb reversed the effect of the negative factors. The delay did not depend on transcript length, because identical results were obtained when the starting elongation complexes had RNA that was 50–75 nt instead of less than 25 nt in length (data not shown).

Our results support the idea that there is a minimum length of time required for P-TEFb function, which is independent of transcript length. P-TEFb is known to heavily phosphorylate both the CTD of RNA polymerase II and the carboxyl-terminal domain of Spt5 at multiple sites (4, 19, 31, 32). It is possible that the delay is due to an accumulation of phosphorylated residues on one or both of these subunits.

In an attempt to determine which P-TEFb phosphorylation event is responsible for the reversal of the DSIF and NELF effect, DSIF, NELF, and/or the early elongation complexes were prephosphorylated independently or with all three components together. P-TEFb phosphorylation was subsequently inhibited by DRB addition, the remaining unphosphorylated factors were added to the reactions, and the early elongation complexes were then allowed to transcribe. Prephosphorylation of any or all of the factors together had no effect on the negative activity of DSIF and NELF. This suggests that active transcription or a conformational change in the polymerase or a conformational change of the factors associated with the elongation complex is required for the reversal by P-TEFb.

Another system was recently reported that demonstrates a requirement for P-TEFb, DSIF, and NELF (33) in controlling the appearance of run-off transcripts. From those studies, it was concluded that P-TEFb (not TFIIH) reversed the negative effect of DSIF and NELF. The system coupled initiation and elongation and used the appearance of run-off transcripts as a read out. Besides factors needed for initiation, another factor, FACT, was needed to see an effect of P-TEFb. FACT has been demonstrated to exert a positive effect on elongation through chromatin templates (34). It is not clear what role FACT plays in a system devoid of chromatin or why FACT was required for the function of P-TEFb. It is likely that the differences in the systems used could explain the dissimilarities in the results. In the coupled initiation and elongation assay, effects of the factors might be on either step of transcription. For example, FACT or P-TEFb might affect the formation of initiation complexes in the presence of NELF and DSIF. Differences between the two systems also could reside in the factor preparations.

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used. In the studies presented here, P-TEFb composed of Cdk9 and cyclin T2a was used, and in the other study P-TEFb containing cyclin T1 was used. We have found that P-TEFb containing cyclin T1 does not as effectively reverse the negative effect of DSIF and NELF in the highly purified assay (data not shown). Further analysis is needed to resolve the differences seen between the two studies, but both indicate that DSIF and NELF have a negative effect that can be reversed by P-TEFb.

Kinetic studies presented here indicated that DSIF and NELF work cooperatively to increase the time the polymerase spends at pause sites; however, in all of our assays, about 50% of the polymerases were unaffected by the addition of the negative factors. This fraction was not affected by the addition of amounts of DSIF and NELF greater than that required to achieve a saturation of their effect on elongation. Explanations for this observation include possible modification states of DSIF, NELF, and/or RNA polymerase II, contaminating inactive forms of DSIF and NELF, or differences in the subunit composition of the polymerases. We are currently addressing these possibilities. Since nearly all of the polymerases in the isolated elongation complexes come under the influence of negative factors when whole nuclear extract is added back, whatever is missing in our highly purified system can be complemented by the extract. The missing component could be a weakly bound RNA polymerase II subunit, another unidentified factor(s), or an enzymatic activity that modifies DSIF, NELF, P-TEFb, or the polymerase.

It is clear that elongation by RNA polymerase II can be influenced by the combinatorial action of a number of factors (1, 6, 35-37). The highly purified elongation control system described here allows the effect of elongation factors to be examined in the presence of any combination of other factors. As a step toward a more complete system, we examined the influence of TFIIF on the effect of NELF and DSIF. We found that the ability of TFIIF to increase the elongation rate was dominant over the negative effect of DSIF and NELF when those two factors were held at a specific concentration. However, the addition of more NELF, but not more DSIF, was able to overcome the effect of TFIIF. This functional competition between NELF and TFIIF could be due to a physical competition for an interaction site on the polymerase or to the ability of the factors to influence the conformation of the polymerase from different sites. TFIIF functions by decreasing the time RNA polymerase II spends at pause sites, and one model proposes that the factor facilitates a conformational change from the paused to the elongation competent state (30). The combination of NELF and DSIF could have exactly the opposite effect, by stabilizing the paused conformation. TFIIF also inhibits the action of the ATP-dependent RNA polymerase II termination factor 2 (28). A requirement of the paused conformation for efficient termination by termination factor 2 is consistent with all results obtained so far (38–41). When added into the highly purified elongation control system described here, termination factor 2 causes termination of all polymerases irrespective of the presence of NELF, DSIF, or P-TEFb (39–41) (data not shown). Further studies are needed to examine the delicate balance between the positive effects of elongation factors such as TFIIF and S-II and the negative factors, DSIF, NELF, and factor 2.

One aspect of the function of both P-TEFb and the negative factors that warrants more attention is how they relate to the elongation complex. Concentrations of P-TEFb required for the reversal of the DSIF and NELF in our assays are ~100-fold higher than the estimated concentration of the early elongation complexes. The requirement for this high concentration may be a result of missing activators and/or recruiting factors. Recruitment of P-TEFb to the polymerase may increase the local concentration and stimulate transcription selectively. Direct recruitment of P-TEFb by the coactivator CITA has been shown to play a role in controlling MHC class II gene expression (42), and Drosophila P-TEFb is recruited to the hsp70 promoter by an unknown mechanism in response to heat shock (43). Both immunofluorescence and chromatin immunoprecipitation assays have also demonstrated that Drosophila Spt5 is also recruited to the heat shock promoter in response to heat shock (44, 45). Recently, it was found that the androgen receptor interacted with P-TEFb and that this interaction enhanced elongation from a promoter under the control of the androgen receptor (46). Additional evidence that activation of elongation is a result of selective P-TEFb recruitment is observed in the well studied area of Tat transactivation of the HIV long terminal repeat (47). The HIV Tat protein recruits P-TEFb to the TAR element present on nascent HIV transcripts, and this allows the production of full-length HIV transcripts (10, 48). The highly purified system described here should be useful in elucidating the differences in activation of transcription by recruitment of P-TEFb through DNA and RNA elements.

Analysis of the data published thus far suggests that DSIF, NELF, and P-TEFb function together to regulate transcription elongation, but the reason this mechanism exists is not yet clear. One possibility is that the number of full-length transcripts produced could be controlled by the selective action of P-TEFb on elongation complexes. This regulation could be accomplished as described above by the recruitment of P-TEFb to particular genes. A second possibility not necessarily exclusive of the first is that the process could facilitate the exchange of factors used during initiation with those used during elongation. It is likely that some of the latter factors include capping enzymes, splicing factors, nucleosome remodeling factors, and other general elongation factors (5, 6, 49–52). DSIF and NELF may slow the polymerase to provide time for it to load the machinery needed to efficiently transcribe the full-length gene and process the primary transcript into a mature mRNA (53). Consistent with this last idea, recent studies have found that DRB treatment of HeLa cells induces the appearance of intron-containing transcripts (54) and that the large subunit of DSIF (Spt5) can interact with the capping enzyme (55). Further understanding of this multifactor process will require better in vitro systems that allow efficient transcription through chromatin templates and coupling of transcription with RNA processing.

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