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RNA Polymerase II Elongation Control

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Abstract

Regulation of the elongation phase of transcription by RNA Polymerase II (Pol II) is utilized extensively to generate the pattern of mRNAs needed to specify cell types and to respond to environmental changes. After Pol II initiates, negative elongation factors cause it to pause in a promoter proximal position. These polymerases are poised to respond to the positive transcription elongation factor, P-TEFb, and then enter productive elongation only under the appropriate set of signals to generate full length properly processed mRNAs. Recent global analyses of Pol II and elongation factors, mechanisms that regulate P-TEFb involving the 7SK snRNP, factors that control both the negative and positive elongation properties of Pol II and the mRNA processing events that are coupled with elongation are discussed.

Keywords

P-TEFb; NELF; DSIF; SEC; 7SK snRNP

1. Introduction

Normal development and cellular responses to environmental challenges are accomplished by dynamic regulation of the expression of tens of thousands of genes in mammals. Transcription is the obvious first step in gene expression and, overall, provides many mechanisms that are essential to achieving the appropriate mix of proteins and RNAs in each cell. Access to promoter regions encoded in the DNA template as well as the body of genes is complicated by the fact that much of each organism's genome is buried in highly repressive nucleosomes. A host of factors are required to deliver RNA Polymerase II (Pol II) to the appropriate promoters, allow initiation, cause the transition into productive elongation, and couple the splicing and polyadenylation machinery that is needed to generate a properly processed mRNA. All of these steps are connected to each other to varying extents and our goal is to review recent findings concerning post-initiation transcriptional events. Results from mammalian systems will be emphasized and findings in yeast and Drosophila will be denoted as such, since there is some species specificity.

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It is now clear that many transcriptional choices are available to fine tune the repertoire of genes expressed. Most are not a matter of yes or no decisions, but rather to what frequency the regulated events are allowed. On some genes, in any particular cell or cell type, there is no initiation by Pol II and the consequences on gene expression are obvious. Recently, a choice was uncovered by looking at Pol II occupation by ChIP-Seq or by sequencing nascent transcripts. From many promoters in yeast (1, 2) and mammals (3, 4), transcription can proceed in either direction (Figure 1), but in general only one direction is allowed to proceed into productive elongation (5). The same techniques have also demonstrated that many metazoan genes experience initiation to a fairly high extent with little correlation to the level of expression achieved. The resulting promoter proximally paused polymerases are a prevalent feature on Drosophila (6, 7) and mammalian (8, 9) chromosomes and accordingly these poised polymerases permit a choice of either terminating or entering productive elongation through the action of the Positive Transcription Elongation Factor, P-TEFb (10) (Figure 1). Finally, there is evidence that all long Pol II transcripts are not necessarily processed into functional mRNAs (11) arguing that the coupled RNA processing events are not guaranteed by default.

ChIP-Seq has provided an unprecedented view of the transcription of metazoan genomes. A number of high quality ChIP-Seq datasets examining the chromosomal distribution of Pol II and elongation factors in mouse embryonic stem (MES) cells were recently published (9). As an example of what was found, a very active gene (RPL11) and a neighboring gene that is much less active (TCEB3) is presented in Figure 2. Information from the MES cell datasets was used to generate a plot showing the relative positions for Pol II before and after a 1 hour Flavopiridol treatment of cells that inhibits P-TEFb function (12). Additionally shown are the distributions of two phosphorylated forms of Pol II that are found in elongation complexes (Ser5 and Ser2 of the C-terminal domain (CTD) of the large subunit) (13). The total signals for each of the four tracks for the two genes were normalized so that the relative distributions across the regions can be viewed. The height of the signals between datasets cannot be compared because each is the end result of a complicated protocol that starts with a potentially variable immunoprecipitation step. However, the relative position of signals within a dataset is informative. To interpret the graphs presented it is important to realize that for a protein located precisely at a specific point on the DNA, the signal detecting that protein will be about 400 bp wide with the center of the peak exactly over the site of binding.

The pattern of Pol II found over the two example genes is different and leads to significant insight into the transcription elongation process. For an active gene like the ribosomal protein L11, there is a peak of Pol II about 50 bp downstream of the transcription start site (TSS) due to promoter proximally paused polymerases (Figure 2A, left). Downstream of the poised polymerase a low but significant signal is found over the body of the gene, as well as a large peak downstream of the poly(A) addition site. Treatment of cells with Flavopiridol for 1 hour eliminated almost all of the downstream signals indicating that productive elongation and the production of mRNAs was blocked by inhibiting P-TEFb. Ser5 phosphorylation tracks with Pol II over the 5’ gene regions, but is greatly reduced in the region downstream of the poly(A) site. Ser2 phosphorylation is found predominately over the polymerases downstream of the poly(A) site. This pattern is typical for all highly

transcribed genes (9). A different pattern is found over the more inactive gene (Figure 2A, right). In this case, only poised polymerases are found before and after flavopiridol treatment of the cells. Ser5, but not Ser2 phosphorylation is found over the poised polymerases. A third type of gene exists that has no polymerase at all (not shown), but most mammalian and many Drosophila genes are similar to the lowly expressed gene (6, 9).

The distribution of Pol II across genes in budding yeast has some similarities, as well as some significant differences. Importantly, there are no prominent poised polymerases on the closely packed yeast genes and this is likely due to the lack of the Negative ELongation Factor, NELF. Overall, the pattern of CTD phosphorylation is similar, with Pol II near the 5' end of genes having more Ser5 phosphorylation and Pol II gaining more Ser2 phosphorylation at the 3' end of genes (14). The lack of poised polymerases does not necessarily mean that elongation is not controlled in a related way in yeast compared to metazoans. Indeed a number of factors are exchanged on and off the polymerase during elongation (15).

2. Promoter Proximally Paused Polymerases

To control any pathway it is first necessary to place a restriction in the flow through that pathway which can then be modulated. As demonstrated by the examples in Figure 2A, in the production of mRNA a major restriction point occurs shortly after Pol II initiates. The polymerase becomes stably engaged by elongating the nascent transcript past about 12 nt and then comes under the control of factors that significantly slow or halt elongation (16). These promoter proximately paused polymerases are the major form of polymerase found bound to metazoan chromosomes and are poised for entry into productive elongation (9, 17). Two factors, the DRB Sensitivity Inducing Factor, DSIF (18), and NELF (19), have been clearly implicated in generating poised polymerases (20, 21)(Figure 2). Published ChIP-Seq data for Pol II, DSIF, and NELF in MES cells (9) were reanalyzed to generate a genome wide average (Figure 2B). A set of 20,000 genes containing all genes except for those with transcription start sites, TSSs, within 1000 bp of another TSS were aligned by their TSSs and for each protein the ChIP-Seq signal at each base pair position from −10,000 to +10,000 bp was summed. To correct for background the average of the lowest 10% of the signals for each base pair was subtracted from all other positions and then the total area under each curve was normalized as in Figure 2A. The predominant peak of Pol II is found at about +50 bp (Figure 2B). Pol II is virtually absent in the −10,000 to −2,000 region, and is found at low but significant levels downstream of the poised polymerase peak (Figure 2B). The distribution of DSIF is very similar to that of the polymerase except that the regions downstream of the poised polymerase are slightly more populated, but NELF is found only over the poised polymerase peak (Figure 2B). The data support the idea that poised polymerases are under the control of DSIF and NELF (Figure 2C).

The positional information described above supports what was learned about the function of DSIF and NELF from earlier biochemical and molecular analyses. In vitro transcription experiments initially provided the tools to identify both the positive and negative factors involved in controlling the elongation phase of transcription (16). A key feature of transcription of virtually any promoter in vitro using a crude nuclear extract is the inhibition

of the generation of long transcripts by the ATP analog 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (16, 22). P-TEFb (23), NELF (19) and DSIF (18) were purified based on their activities during reconstitution of DRB-sensitive transcription using fractionated nuclear extracts. Individually, the three factors have almost no effect on elongation of pure Pol II, but together NELF and DSIF reduce the elongation rate of Pol II significantly, and P-TEFb can reverse the negative effect of the two factors (20, 21). Pol II in a crude nuclear extract in which P-TEFb has been inhibited, elongates significantly slower than Pol II in the presence of DSIF and NELF (21), and the search for the other negative factors is ongoing.

While poised polymerases in the bodies of genes have garnered the most attention, the polymerases synthesizing divergent transcripts also seem to be paused (5). Divergent transcripts arise by initiation in an antisense direction about 200 bp upstream from the TSS driving mRNA production. The location of the bulk of polymerases determined using ChIP-Seq as well as short transcripts from RNA-Seq are about +50 and −250 bp from the orienting TSS (5). This suggests that polymerases encounter a block to elongation in both directions. Supporting this idea NELF and DSIF are found over both regions and knockdown of either factor increased transcription in the divergent direction (5). Although most divergent transcripts are degraded rapidly by the RNA exosome, some can be extended for more than a thousand nucleotides (24). The longer divergent transcripts are sensitive to flavopiridol indicating that they are dependent on P-TEFb just like mRNAs derived from the sense direction (24). ChIP-Seq data demonstrate that even for promoters of highly active genes, polymerases synthesizing the associated divergent transcripts do not experience much productive elongation (9). It will be important to determine what directs P-TEFb to function primarily on poised polymerases transcribing in the sense direction. The purpose of this divergent transcription is not clear, but it could be involved in altering the promoter architecture by helping to maintain a nucleosome free region between the two transcription start sites (sense and antisense) or by generating strong torsional strain on the promoter region caused by the helical tension (negative supercoiling) imparted by polymerases traveling away from each other (5).

3. The positive transcription elongation factor, P-TEFb

By default, negative elongation factors associate with Pol II during initiation leading to the generation of poised polymerases. P-TEFb is required to reverse their effects and allow the polymerases to enter into productive elongation (10, 25). P-TEFb is an essential cyclin-dependent kinase that has been identified in eukaryotes from yeast to humans and is regulated in many standard ways and by an unusual mechanism involving reversible association with a small nuclear ribonucleoprotein (snRNP) complex (10, 26). Because poised polymerases are prevalent across the genomes of metazoans, the function of P-TEFb must be accurately directed at the correct time to the appropriate genes. There are a number of comprehensive reviews (10, 25, 27–35) on the history of discovery and details of P-TEFb and its function and accordingly we will focus on mainly on general mechanisms, recent results, and new directions.

3.1 Structure and Function of P-TEFb

P-TEFb is a nuclear localized, cyclin-dependent kinase that has some properties similar to all cyclin-dependent kinases, others that are similar only to cyclin-dependent kinases involved in transcription, and a few that are totally unique to P-TEFb. A common property to all is the two-subunit arrangement. A Cdk subunit contains the active site and a cyclin subunit is needed to invoke a conformational change that in conjunction with phosphorylation of the T-loop in the Cdk subunit leads to activation of the kinase. Phosphorylation of Thr186 is critical for activation of the catalytic subunit of P-TEFb, Cdk9 (36, 37), although other sites can be phosphorylated with other functional consequences (38, 39). The cyclin subunit in mammals is usually either Cyclin T1 or Cyclin T2 (40), although Cyclin K can also be found associated with Cdk9 (41–43). Similar to other Cdks involved in transcription (Cdk7, 8, 12, 13), neither Cyclin T1, Cyclin T2 or Cdk9 change concentration significantly during the cell cycle (44). X-ray structures are available for P-TEFb containing most of Cdk9 and the amino terminal third of Cyclin T1 (45) and for HIV Tat•P-TEFb complex containing a similarly truncated Cyclin T1 and 86 amino acid Tat (46). It should be noted that the structure of P-TEFb alone contains 3 accidental mutations in Cdk9, one of which likely causes a conformational change in the active site compared to the Tat•P-TEFb structure (Tahir Tahirov, personal communication). The Tat•P-TEFb structure should prove useful in the design of anti-HIV drugs that target the transcription of the virus.

P-TEFb has been demonstrated to phosphorylate a number of proteins. The CTD of the largest subunit of mammalian Pol II comprises 52 tandemly repeated copies of a heptapeptide with the consensus Tyr-Ser-Pro-Thr-Ser-Pro-Ser ($Y_1S_2P_3T_4S_5P_6S_7$). Like Cdk7•Cyclin H, P-TEFb phosphorylates the CTD (47), but while Cdk7 phosphorylates Ser5 of the CTD repeats, P-TEFb can phosphorylate primarily Ser2 (48). As will be described later, CTD phosphorylation is likely to play a role more in the coupling of RNA processing with transcription than in the release of promoter proximally paused polymerases. The role of P-TEFb in CTD phosphorylation has been challenged by the discovery that Cdk12 and Cdk13, two human kinases evolutionarily related to the Ser2 kinase Ctk1 in yeast, can also phosphorylate Ser2 (49, 50). Treatment of cells with the potent P-TEFb inhibitor, Flavopiridol, does lead to loss of Ser2 phosphorylation, but this could be due to loss of Pol II in the 3' regions of genes where Ser2 phosphorylation occurs (Figure 2A) and this could explain why Ser2 phosphorylation was lost in *C. elegans* when Cdk9 or Cyclin T were knocked down (51). For the main function of P-TEFb in releasing poised polymerases, two negative factors, DSIF and NELF are targeted (10). Phosphorylation of the C-terminal region of the larger of the two DSIF subunits, hSpt5, by P-TEFb is required for the transition into productive elongation (52, 53) and the factor then tracks with Pol II throughout the gene and in the region downstream of the Poly(A) site (9). Phosphorylation of one of the four NELF subunits (NELFe) has also been linked with its removal from Pol II during the transition into productive elongation (54). Even though yeast do not have NELF or poised polymerases, the Bur1/2 kinase has been demonstrated to phosphorylate DSIF and seems to be performing a similar function to transition the polymerase into productive elongation (55, 56).

3.2 Regulation of P-TEFb

Because poised polymerases that are waiting for P-TEFb to begin mRNA production are prevalent across genomes, regulating the activity of the kinase is essential for proper gene expression. Most mechanisms used to control protein levels such as expression of alternative forms (40, 57), transcriptional (58), translational (58) and post-translational (27) control, some of which involves miRNAs (59), and regulated turnover of the proteins (60) have been found to be involved in controlling P-TEFb subunits. However, one unique mechanism, reversible association with the 7SK snRNP, plays the major role (Figure 3). In rapidly growing cells such as HeLa cells, up to 90% of P-TEFb is sequestered in an inactive form by an RNA binding protein HEXIM1 or HEXIM2 (HEXIM) that associates with the 7SK snRNP (36, 61–67). The bound Cdk9 has been activated by T-loop phosphorylation (36, 37), but is held in an inactive state by an inhibitory domain of HEXIM that is exposed when it is bound to 7SK RNA (36, 68). The La related protein, LARP7 (69–71), and 7SK methyl phosphate capping enzyme, MePCE (72, 73), are constitutive components of the 7SK snRNP. Together they stabilize the RNA and may be involved in regulation of the release of P-TEFb. Interestingly, the C-terminal domain of LARP7 can bind to the active site of MePCE and inactivate its methyl transferase activity, which prevents the reverse reaction that removes the 7SK cap structure (72).

The biochemical properties of the 7SK snRNP with HEXIM and P-TEFb sequestered are ideal for a role in delivering P-TEFb to genes to be expressed while protecting poised polymerases on many other genes from inappropriate activation. In nuclei treated with mild detergents, the 7SK snRNP is the only snRNP that is readily extracted under low salt conditions (74). This indicates that it is not tightly anchored anywhere in the nucleus although transient interactions of the 7SK snRNP with chromatin, have been found over the HIV LTR (75). Diffusion of the 7SK snRNP would provide a means to supply P-TEFb to any location. Higher salt is needed to extract the7SK-free P-TEFb which is likely associated with the genes undergoing productive elongation at the time of extraction (74). Thus, an important mechanism for achieving selective P-TEFb function is the extraction of P-TEFb from the 7SK snRNP and recent evidence has demonstrated that this is possible. The HIV Tat protein and the P-TEFb binding domain of the cellular bromodomain protein Brd4 can individually directly release P-TEFb from the 7SK snRNP without any energy or covalent modifications of the complex (76–83) (Figure 4). On the HIV LTR, the released P-TEFb is recruited to the nascent transcript held by the poised polymerase (Figure 4). Extraction of P-TEFb by Tat does not require the HIV LTR (77, 79) and may be a special case since HIV has evolved to take over the P-TEFb control process. In contrast, Brd4 is generally found associated with acetylated histone tails on active chromatin and its release of P-TEFb from 7SK snRNP could represent a more normal example of selective extraction at the site of activation (32, 84). Many cellular genes that employ Brd4 to recruit P-TEFb to their promoters are primary response genes, whose expression proceeds through signal-induced transcriptional elongation (85). A large number of transcription factors can bind to P-TEFb, but it is not yet clear if any or all of these can extract P-TEFb (10, 86). Because Tat has been found bound to P-TEFb in the Super Elongation Complex (SEC) that also contain several other transcription factors/co-factors (81, 87), it is likely that the overall recruitment of P-TEFb, especially at the HIV LTR, is a multi-step process. The interactions of SEC with the

Polymerase-Associated Factor complex (PAFc) through the PAF1 subunit and the human Mediator complex through the MED26 component may provide additional means for delivering P-TEFb to cellular genes (88–92). Many details of the recruitment of P-TEFb to specific sites of action are currently unknown and this is an important area of future research.

What happens to P-TEFb after it has completed its function on a specific gene is just as important as its initial recruitment. Because “free” P-TEFb might inappropriately trigger the release of poised polymerases into productive elongation on undesired genes it is critical to re-sequester it in the 7SK snRNP. During the extraction of P-TEFb, 7SK undergoes a conformational change that causes the release of HEXIM (76) (Figure 3). This conformation is likely stabilized by the binding of several hnRNPs (93, 94). For P-TEFb to be picked back up by the 7SK snRNP, HEXIM must first rebind. Because the concentration of HEXIM is about 5 times the concentration of P-TEFb in HeLa cells and half of the 7SK snRNPs do not contain HEXIM or P-TEFb (36, 61, 66), it is likely that entry of HEXIM into the 7SK snRNP is a regulated step. How this is accomplished is not clear. Support for the general model of P-TEFb extraction and critical re-sequestration was recently obtained by observing the movement of 7SK in living cells containing an inducible gene array (95). During activation of the array, no specific recruitment of 7SK was observed, but when the genes were shut down 7SK concentration increased in that region. One speculative model would be that the 7SK snRNP containing hnRNPs might be temporarily recruited to the genes as they are shutting down to transfer the hnRNPs to the mRNA and this might activate 7SK by changing the conformation of the RNA, allowing rebinding of HEXIM and then P-TEFb. Evidence for some of this model was provided by studies indicating the importance of the hnRNPs in 7SK dynamics (94).

4. The productive elongation phase of Pol II transcription

The properties of Pol II after P-TEFb triggers its release from the poised position are dictated by the factors that are now allowed to function. Prior to the transition into productive elongation, NELF and DSIF are primary elongation factors (9). After phosphorylation of DSIF by P-TEFb, NELF is ejected from the elongation complex while DSIF is retained (9). Other elongation factors come into the complex and provide it with the remarkable ability to elongate at a steady ~4 kb/minute through all obstacles for up to 2 million bp (96). The continued kinase activity of P-TEFb is not needed to maintain this rate (97). The identity and exact function of all of these factors is not known, but a number of candidate factors have been described. Some have been identified by their ability to affect Pol II elongation through activity-based biochemical approaches or by assays that detect their presence in elongation complexes in vivo. For example, TFIIS aids in restarting arrested Pol II after it falls into a backtracked state (98) and the initiation factor TFIIF has been shown to stimulate the elongation rate of Pol II in the absence of other factors (99). The Polymerase Associated Factor complex (PAFc) and the Super Elongation Complex (SEC) containing ELL, which like TFIIF stimulates elongation of pure Pol II, have recently gained much attention for their intricate roles in transcription, as well as transcription-coupled pre-mRNA processing. An excellent review of the Pol II elongation factors was

published in 2004 (100), so we will focus more heavily on recent work on the factors implicating them in productive elongation.

4.1 TFIIS and TFIIF

During transcription in vitro Pol II has a strong propensity to pause or enter an arrested state at frequent sites along the template and the first two factors discovered that affect elongation, TFIIS and TFIIF, affect pausing and arrest. The difference between pausing and arrest is that Pol II pausing is transient and if given time will the polymerase will return to the elongation mode, but arrest cannot be overcome with time. If pausing persists, such as when NTPs are removed or a physical roadblock is imposed, the pause gradually decays into arrest (101), which is characterized by the “back-tracking” of Pol II such that the active site within the polymerase is no longer aligned with the 3’ end of the nascent RNA. Irreversibly backtracked Pol II is frequently a target for ubiquitylation/degradation (102). In eukaryotic cells, the transcript cleavage stimulatory factor TFIIS (originally S-II) is necessary to rescue Pol II from this arrested state by allowing Pol II to endonucleolytically cleave the RNA to generate a new 3’ end that is properly aligned with the catalytic site (98). This type of activity is present throughout evolution as evidenced by the presence of two TFIIS like factors, GreA and GreB in E. coli (103).

In cells Pol II likely oscillates between the paused and arrested states especially at promoter-proximal regions where negative factors act restrict the forward momentum of the polymerase (104–106). Thus, TFIIS is expected to play a critical role in promoting the activity of early elongation complexes. Indeed, global analysis of paused genes in Drosophila reveals that Pol II backtracking and TFIIS-induced transcript cleavage are widespread among early elongation complexes (105). Depletion of TFIIS via RNAi from Drosophila cells delayed the induction of heat-shock genes (107), however global gene expression profiles are not significantly affected in TFIIS-depleted Drosophila cells (105). The yeast *ppr2* gene encoding the TFIIS ortholog is not essential, although *ppr2* mutants display sensitivity to 6-azauracil (108), a phenotype that is frequently associated with elongation defects (109).

The second factor discovered that affected elongation by Pol II was TFIIF. Identified by its strong affinity for Pol II (110) and by its requirement for initiation of transcription, it also was found to greatly increase the rate of elongation of Pol II in vitro (99, 111). Although it associates with free Pol II and paused elongation complexes, it does not maintain this tight association during elongation (21, 99). Its positive action during elongation can be ascribed to its ability to bind to paused elongation complexes and subsequently convert them into an elongation competent form. Since elongation rates in vitro are dictated by the dwell time at many pause sites and TFIIF reduces this time, it has a concentration dependent effect on elongation with the maximum rate stimulation of about 20 fold. Although ChIP experiments indicate that TFIIF is mostly found in promoter proximal regions in yeast (112) it has been found downstream including at the 3' end of genes in humans (113).

4.2 ELL1/2/3

ELL1, the founding member of the ELL family that also contains ELL2 and ELL3, was originally purified from rat liver extracts as a factor that promoted Pol II elongation in vitro (114). Employing a mechanism different from that of TFIIS, ELL1 directly increases the catalytic rate of Pol II by suppressing transient pausing of Pol II at multiple sites along the DNA template (114). Prior to this discovery, ELL1 had been identified as a fusion partner of the mixed lineage leukemia protein (MLL) in acute myeloid leukemia (115).

MLL plays an important role in maintaining the appropriate expression of the *Hox* gene loci in hematopoietic cells and is a key regulator of hematopoietic stem cell development and maintenance (88). However, the *MLL* gene is frequently involved in aberrant chromosomal translocations with a large number of other genes to create fusion proteins, leading to the development of acute and aggressive myeloid and lymphoblastic leukemia. Wild-type MLL has histone methyltransferase activity capable of methylating histone H3K4 and this function is lost after the translocation. However, the myriad of DNA- and protein-binding domains present in the MLL N-terminal portion that is retained in the chimeras contribute to leukemogenesis. At the time when little was known about the functions of most of the MLL fusion partners, the discovery of the elongation stimulatory activity of ELL1 provided one of the earliest clues to how a fusion protein may induce aberrant expression of MLL target genes to cause leukemia (see below for further discussions).

Based on their homology to ELL1, ELL2 and ELL3 were identified and shown to have similar elongation stimulatory activity in vitro (116, 117). While ELL2 is expressed in many of the same tissues as ELL1, ELL3 is a testis-specific factor. In Drosophila, there is only one ELL ortholog dELL that is detected at numerous sites of active transcription on polytene chromosomes and co-localizes with Pol II at heat shock gene loci upon heat shock (118). Interestingly, mutations in dELL preferentially affect the expression of large genes (119), implicating a critical role for this factor in facilitating promoter-distal elongation by Pol II, a process that is tightly coupled to mRNA 3’ end formation. Indeed, human ELL2 has been shown to function in this coupling (see below).

4.3 PAFc

Composed of five subunits Paf1, Ctr9, Cdc73, Rtf1, and Leo1, the Pol II-Associated Factor complex (PAFc) is a multi-functional factor that contributes to transcriptional elongation, the generation of transcription-associated histone modifications, and the coupling of elongation with downstream events of mRNA biogenesis. The dependence on several but not all the PAFc subunits for histone H2B monoubiquitylation and H3K4 and H3K79 methylation on active genes has been demonstrated (100). The PAFc-mediated recruitment of Rad6 and the SET enzymes to coding regions is responsible for these modifications. While the modified nucleosomes are expected to influence the Pol II elongation on chromatin templates, PAFc has been shown to employ other modification-independent mechanisms to further control the elongation process. Indeed, PAFc has been demonstrated to promote Pol II elongation on both naked DNA and chromatin templates in the presence of other factors (120–122). Notably, the effect on chromatin is mechanistically different from the established role of PAFc in histone monoubiquitylation and methylation (122).

Like most other elongation factors PAFc does not possess any enzymatic activity, however the purified complex has very little ability to stimulate the elongation rate in the absence of other factors. Instead, it functions as a mediator or adaptor to facilitate other elongation factors to bind and affect Pol II. Recent studies have revealed both genetic and physical interactions between PAFc and a number of elongation factors including DSIF, Tat-SF1, TFIIS and SEC as well as their cooperative interactions with Pol II (81, 120, 122–124). Through these interactions, PAFc and its partners coordinate the various aspects of transcriptional elongation and modulate the elongation complex at multiple stages. In agreement with its broad involvement in elongation control, PAF1c has been detected along the entire length of several actively transcribed genes in yeast (112, 125). A recent ChIP-seq analysis in MES cells further corroborates this view and shows that the PAFc subunit Ctr9 cross-links throughout coding regions of active genes and extends to the 3’ end together with the Ser2-phosphorylated Pol II (9). The peak Ctr9 occupancy is at the 3’ end of genes, reflecting the fact that PAFc also plays a key role in transcription-coupled mRNA 3’ end formation (see below).

4.4 SECs

4.4.1 Compositions of human and Drosophila SECs—Besides being sequestered in the 7SK snRNP and the Brd4-containing complex, P-TEFb is also found in several closely related complexes that are collectively referred to as the Super Elongation Complexes or SECs (81, 87, 126–128). Although only a small fraction of P-TEFb is present in the SECs (88), it is highly active as a CTD kinase (126). Besides P-TEFb, SECs also contain AFF1, AFF4, ELL1, ELL2, ENL, AF9 and probably additional subunits. Not all of them exist in a single complex. For example, the highly homologous ENL and AF9 were recently shown to exist in separate SECs that display similar but non-identical functions (124). It is possible that the homologous ELL1 and ELL2 as well as the similar AFF1 and AFF4 proteins are also present in different but closely related complexes (88). Thus, the number of possible combinations among these homologous subunits could be huge, which can greatly increase the regulatory diversity and gene-control options by a family of SECs.

Unlike mammals, Drosophila has only one dELL, one AFF-like protein encoded by the *lilliputian* (*lilli*) gene, and a single ENL/AF9-like protein called Ear. Together with Drosophila Cdk9 and Cyclin T, they existed in a fly version of the SEC (88). Both Lilli and dELL are well-known transcriptional regulators that exhibit strong genetic interactions with genes that contain paused Pol II and are essential for normal embryonic and eye development (reviewed in (88, 129). As expected from the functional coupling between dELL and P-TEFb within the Drosophila SEC, Cdk9 depletion in larvae reduces the amount of dELL detected on the chromosomes (130) and dELL knockdown decreases Ser2 phosphorylation on the Pol II CTD (131).

4.4.2 SECs and human diseases—Human SECs were initially identified because of their close connection to two important diseases: HIV/AIDS and leukemia. As discussed above, HIV Tat can efficiently capture P-TEFb from the 7SK snRNP, raising the question of whether Tat delivers P-TEFb alone or together with other factors to the HIV LTR to activate viral transcription. To address this question, a tandem affinity-purification strategy was

employed to isolate the complex that contains both Cdk9 and Tat. This has led to the identification of the SEC components ELL2, AFF4, ENL and AF9 as additional subunits of the Tat-P-TEFb complex (87). Through directly isolating proteins associated with Tat, the same set of factors plus a few others (e.g. AFF1, ELL1, and PAFc subunits) are also found to interact with Tat and P-TEFb (81). The bromodomain protein Brd4 is noticeably absent from the list, which is consistent with earlier demonstrations that Brd4 and Tat compete for binding to P-TEFb and that the Brd4–P-TEFb complex is incompatible with Tat-transactivation (82, 83).

The interaction between Tat and SECs serves two purposes: (1) it recruits SECs to the HIV LTR through cooperative bindings to the viral TAR RNA, thereby allowing P-TEFb and ELL, elongation factors of two distinct classes, to act on the same polymerase enzyme to synergistically activate transcription (81, 87); and (2) it enhances the SEC formation through increasing the half-life of ELL2, which otherwise would be a short-lived protein targeted by the 26S proteasome for degradation (87). In HeLa and HEK293 cells, about 40% of total ELL2 are in SECs, whereas a much smaller percentage of ELL1, which is stable in the absence of Tat, is sequestered in these complexes (87).

Not only are SECs targeted and regulated by the HIV-1 Tat protein, they also play an important role in causing “runaway” transcription of genes that are normally subject to stringent control by wild-type MLL in developing lymphocytes. AFF1, AFF4, AF9, ENL and ELL1, the five integral SEC components, are all well-known translocation partners of MLL (132). Their fusions to the N-terminal chromatin-targeting portion of MLL result in the recruitment of SECs and their associated elongation stimulatory activity to the MLL target genes to induce leukemic transformation (126–128).

4.4.3 Targeting SECs to Pol II for general elongation—SECs are not made just for HIV-1 Tat or the MLL fusions. Rather, they are required for general transcriptional elongation of many non-HIV and non-MLL-target genes. In both mouse and human cells, they are recruited to many rapidly induced genes in response to differentiation signals to release paused Pol II for dynamic induction of transcription (89). Interestingly, most of these genes contained paused Pol II in their undifferentiated, un-stimulated state. Even for a gene, Cyp26a1, that displays no detectable paused Pol II at the promoter, SECs are still required for rapid induction although the precise role of SECs and their targets are unknown in this case (89). Nevertheless, these data underscore the general importance of SECs during developmental control of gene transcription.

In the absence of gene-specific recruitment factors such as Tat and MLL, how are SECs recruited to the Pol II elongation complex? One potential mechanism relies on the interaction of the conserved YEATS domain in the N-terminal regions of ENL/AF9 with PAFc and, through PAFc, the elongating Pol II on chromatin templates (124) (Figure 5). This model is consistent with the previous demonstrations of an interaction between PAFc and SECs (81) and also the requirement of PAFc for efficient Ser2 phosphorylation of the Pol II CTD (133). Moreover, it also explains why the YEATS domain is dispensable for leukemogenesis when ENL/AF9 is translocated to MLL (134). Apparently, the interactions with PAFc and DNA by the N-terminal portion of MLL in the fusion proteins (134) (135)

can effectively substitute for the PAFc/chromatin-targeting function of the ENL/AF9 YEATS domain, which is frequently missing in the MLL chimeras. Notably, PAFc may not be the only means to recruit SECs to the elongation complex. A recent proteomic study has identified the human Mediator subunit MED26 as a docking site for SECs and proposed MED26 as a molecular switch for interacting first with TFIID during transcription initiation and then SECs during the transition into early elongation (90). This observation agrees well with the previous data indicating a role for Mediator in controlling events that occur during transcriptional elongation of some genes (136).

5. Transcription elongation coupled events

The Pol II elongation complex does not merely extend a nascent mRNA chain to produce the full-length mRNA precursor. It also serves as a central coordinator to facilitate efficient integration of multiple nuclear processes that collectively control the proper expression of a gene. Recently, using an assay in which elongation of long genes was synchronized by manipulating P-TEFb in vivo, it was found that splicing of exons, even when the intervening intron was several hundred thousand bp in length, occurred within 10 minutes of the time the polymerase passed the downstream exon (96). In many of the genes studied the elongation complex was still in productive elongation moving at about 4 kb/minute as the splicing machinery efficiently removed the intron (96). The coupling of transcription with key nuclear events such as mRNA surveillance and export has been reviewed elsewhere (100, 137). This review will focus on the roles of Pol II and several key elongation factors in modulating mRNA processing and maturation, which consist of the addition of the 7-methylguanylate cap structure to the 5’ end of nascent mRNA, the removal of intronic sequences, and the cleavage of RNA at the 3’ end followed by the addition of a poly(A) tail. Importantly, being co-transcriptional not only allows these events to occur more efficiently than if they do completely independently, it is also a necessity for the controlled expression of many, especially large genes in a cell type- or/and developmental stage-specific fashion. An essential player in the coordination of these processing events is the CTD of Pol II, which is conveniently located next to the pre-mRNA exit channel (138) and functions as a platform for the assembly and actions of the various enzymes and proteins responsible for mRNA maturation (100). Additional post-translational modifications of the CTD consensus sequences (e.g. serine and threonine glycosylation and proline isomerization) have also been reported (139, 140). These modifications, in conjunction with the many degenerate heptads in the CTD, offer tremendous combinatorial possibilities to modulate the interactions as well as the activities of the myriad of RNA processing factors, which in turn influence the many steps during the conversion of a gene into its corresponding mature, translatable mRNA.

5.1 Capping

Capping of the 5’ ends of nascent pre-mRNAs, which occurs when the transcripts reach a length of 25 to 30 nucleotides, is accomplished by the sequential actions of the capping enzyme which contains both RNA triphosphatase and guanylyltransferase activities and the guanine-7-methyltransferase (141) and is influenced by the phosphorylation status of the CTD. mRNAs produced by yeast Pol II mutants lacking an intact CTD are not efficiently capped (142, 143). Yeast guanylyltransferase interacted tightly and specifically with the

CTD phosphorylated on Ser5 (141, 144). Phosphorylation of the CTD on Ser5, but not Ser2, stimulated the guanylyltransferase activity (145, 146). Kin28, the TFIIH-associated kinase responsible for Ser5 phosphorylation in yeast, is required for targeting guanylyltransferase to the Pol II transcription complex (147). The Spt5 subunit of DSIF, which is part of the early elongation complex, also stimulates capping together with the Ser5-phosphorylated CTD (142, 148). However, the functional coupling of the human capping enzyme with the transcription complex in vitro only showed a 3-fold dependence on Ser5 phosphorylation compared to an overall 100,000-fold stimulation by the elongation complex compared to naked RNA (146).

These findings suggest the existence of a “checkpoint” mechanism to control the tight coupling between capping and early elongation. As Pol II containing phospho-Ser5 CTD and bound by DSIF is specifically recognized by NELF, which serves to pause the elongation complex (19, 149), a window of opportunity is created for the CTD and DSIF to recruit and stimulate the capping machinery to cap a nascent transcript. Since the capping enzyme is also capable of relieving NELF-mediated repression of elongation (150), it may signal and cooperate with other enzymes such as P-TEFb to convert a paused elongation complex into a productive one once capping is completed. The second step of capping carried out by the methyltransferase has also been shown to be a control point in Myc-activation of genes (11, 151, 152). Control of the fraction of caps that are methylated regulate the subsequent translation of mRNAs (151).

5.2 Splicing

Like capping, both constitutive and alternative splicing also maintain close crosstalk with mammalian transcription. Furthermore, the Pol II CTD, especially the hyperphosphorylated form is intimately involved in this crosstalk (100). Although the exact mechanism is yet to be defined, it is suspected that the hyperphosphorylated CTD of elongating Pol II acts by recruiting key splicing factors to the elongation complex. For instance, the phospho-Ser2 CTD has been shown to interact with the Ser/Arg-rich (SR) proteins (e.g. SF2/ASF and SC35), Spt6, and snRNP particles (153–157). Providing visual support for this recruitment model, Pol II with the complete, but not a truncated CTD, was found to recruit splicing factors to sites of active transcription in living cells (158). Besides the recruitment of splicing factors, the CTD may also assist the formation of early splicing intermediates, such as the U1 snRNP-containing complex assembled at the 5’ splice site and the U2 snRNP complex at the branch point adenosine (159).

Given the importance of the Ser2-phosphorylated CTD in the coupling between splicing and transcript elongation, it does not come as a surprise that recent studies have revealed a key role for P-TEFb in controlling this process. The release of P-TEFb from the 7SK snRNP as a result of LARP7 or MePCE depletion is shown to promote the inclusion of an alternative exon containing an exonic splicing enhancer (ESE) element and a sub-optimal 3' splice site (154). This is likely achieved through the increased occupancy of P-TEFb at the promoter and exonic region of the gene, which leads to a chain of events including enhanced elongation and CTD phosphorylation on Ser2, elevated associations of the SR proteins SF2/ASF and probably others with the CTD, and the SR protein-mediated assembly of the

so-called cross-exon recognition complex (160) at the alternative exon, and ultimately the inclusion of this exon in the mature mRNA. Besides the P-TEFb-dependent CTD phosphorylation on Ser2, reducing the rate of Pol II elongation by mutation or drugs also results in different splicing patterns in several other genes, suggesting a general role for the Pol II elongation complex in controlling splice-site selection during alternative splicing (161).

The P-TEFb-induced co-transcriptional mRNA splicing is also important for controlling the inducible inflammatory gene expression program in response to Toll-like receptor signaling in macrophages (85). In the absence of stimulation, Pol II at many of the GC-rich primary response genes generates low levels of full-length but unspliced and untranslatable transcripts. Gene induction is accomplished through signal-dependent recruitment of P-TEFb by Brd4, which recognizes the inducibly acquired histone H4 acetylation on lysines 5, 8, and 12. This results in robust CTD phosphorylation on Ser2 and production of high levels of fully spliced mature mRNA transcripts.

Not only does the splicing machinery take advantage of the Pol II elongation complex to efficiently remove introns from pre-mRNAs, splicing factors can also promote elongation, providing yet another “checkpoint control” to ensure that a pre-mRNA is not synthesized unless the machinery for its processing is properly assembled and positioned. Demonstrating a reciprocal crosstalk between SR proteins and P-TEFb, RNAi-mediated depletion of SF2/ASF or SC35 decreases the levels of P-TEFb and phospho-Ser2 CTD at the promoter and exonic regions of some genes (154, 162). Furthermore, SC35 depletion causes Pol II accumulation within the gene body and attenuated transcriptional elongation in a gene-specific manner (162). Another example illustrating the stimulatory effect of the splicing machinery on Pol II elongation concerns the spliceosomal U snRNPs, which stimulates elongation when directed to an intron-free DNA template by elongation factor Tat-SF1 (163). When bound to the U snRNPs, Tat-SF1 can also interact with P-TEFb (163) and probably also DSIF and PAFc (120), all are components of the Pol II elongation complex.

5.3 Cleavage and polyadenylation

The proper processing of human mRNA 3’ end is carried out by coordinated actions of a set of proteins and proceeds in two steps: (1) cleavage of the RNA precursor at a position 10-35 nucleotides downstream of the consensus AAUAAA signal; and (2) addition of 200–300 adenosine nucleotides to the newly generated 3’-OH terminus of the transcript by poly (A) polymerase. Multiple lines of evidence indicate that the Pol II CTD, especially the Ser2-phosphorylated form, promotes transcription-coupled 3’ end formation through recruiting key components of the processing machinery to their target sites. For instance, the Pcf11 subunit of the yeast cleavage/polyadenylation factor IA (CFIA) complex specifically recognizes the phospho-Ser2 CTD (164, 165). Similarly, the binding of human cleavage stimulation factor CstF-64 to Pol II depends on Ser2 phosphorylation in the CTD (166).

Although components of the cleavage/polyadenylation machinery can bind to the phospho-Ser2 CTD in vitro (e.g. see (164, 165), the bindings are likely enhanced in vivo by transcription elongation factors that track along with Pol II, resulting in more efficient recognition and cleavage at poly(A) sites (Figure 6). For example, the multifunctional PAFc

was found to be required for transcription-coupled polyadenylation stimulated by the prototypical transcriptional activator GAL4-VP16 (167). Moreover, the PAFc subunit Cdc73 is essential for proper histone mRNA 3' processing as well as export to the cytoplasm (168). PAFc likely accomplishes these tasks through recruiting key polyadenylation factors such as the cleavage/polyadenylation specificity factor (CPSF), cleavage stimulation factor (CstF), and symplekin, a scaffolding protein for 3' processing, to transcribing Pol II (169, 170). Consistent with a critical role for PAFc in this process, the occupancy of the PAFc subunit Ctr9 peaks at the 3' end of actively transcribed genes (9).

Another elongation factor that also facilitates the association of mRNA 3' processing machinery with elongating Pol II is the SEC component ELL2 (171). It enhances the association of CstF-64 with Pol II across the gene encoding the immunoglobulin heavy-chain complex in plasma cells. As a result, ELL2 accelerates the use of a weak promoter-proximal secretory-specific poly(A) site and enhances exon skipping of a nonconsensus splice signal that is in direct competition with the poly(A) site (171). This finding is consistent with the demonstrations that utilization of alternative polyadenylation sites, which is emerging as a common mechanism for controlling gene expression during development and disease, can be caused by alterations in the levels or activity of polyadenylation factors associated with genes that contain such sites (167). Like ELL2, PAFc has also been implicated in the control of alternative polyadenylation site usage. The usage of the promoter-proximal poly(A) site of *Ints6*, a target gene of the PAFc component Cdc73, is stimulated by Cdc73 in human cells (169).

The ELL2-promoted 3' processing of mRNA correlates with a significant increase in Ser2 phosphorylation in the Pol II CTD (166). Notably, PAFc is also required for this modification (133). It had been difficult to explain these results prior to the recent discovery that ELL2 and P-TEFb are components of SECs, which depend on PAFc to interact with elongating Pol II (87, 91). With all this new information, many seemingly unrelated events that have previously been attributed separately to ELL2 and PAFc such as the promotion of Pol II elongation, phosphorylation of the CTD on Ser2, enhanced recruitment of polyadenylation factors to Pol II containing the phospho-Ser2 CTD, and finally accelerated mRNA 3' formation can now be placed into a complete and coherent picture (Figure 6).

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Summary points

1. RNA polymerase II elongation control is essential for the regulation of gene expression.
2. Promoter proximally paused polymerases under the control of NELF and DSIF are prevalent across metazoan genomes.
3. P-TEFb controls the transition into productive elongation generating mature mRNAs in a process at the 5' end of genes.
4. The 7SK snRNP allows regulated release of active P-TEFb by specific transcription factors.
5. Productive elongation complexes are influenced by DSIF, PAFc, SEC and other factors.
6. RNA processing is functionally coupled with transcription elongation.

Future Issues

1. Are there other negative factors besides DSIF and NELF that are involved in promoter proximal pausing?
2. Does divergent transcription have a functional consequence?
3. What regulates directional preference in productive elongation at divergent promoters?
4. What are the mechanisms for delivery of active P-TEFb to specific genes?
5. How is P-TEFb re-sequestered in the 7SK snRNP?
6. What are all the factors needed for productive elongation, how are they sequentially recruited and then removed at the 3' end of genes?

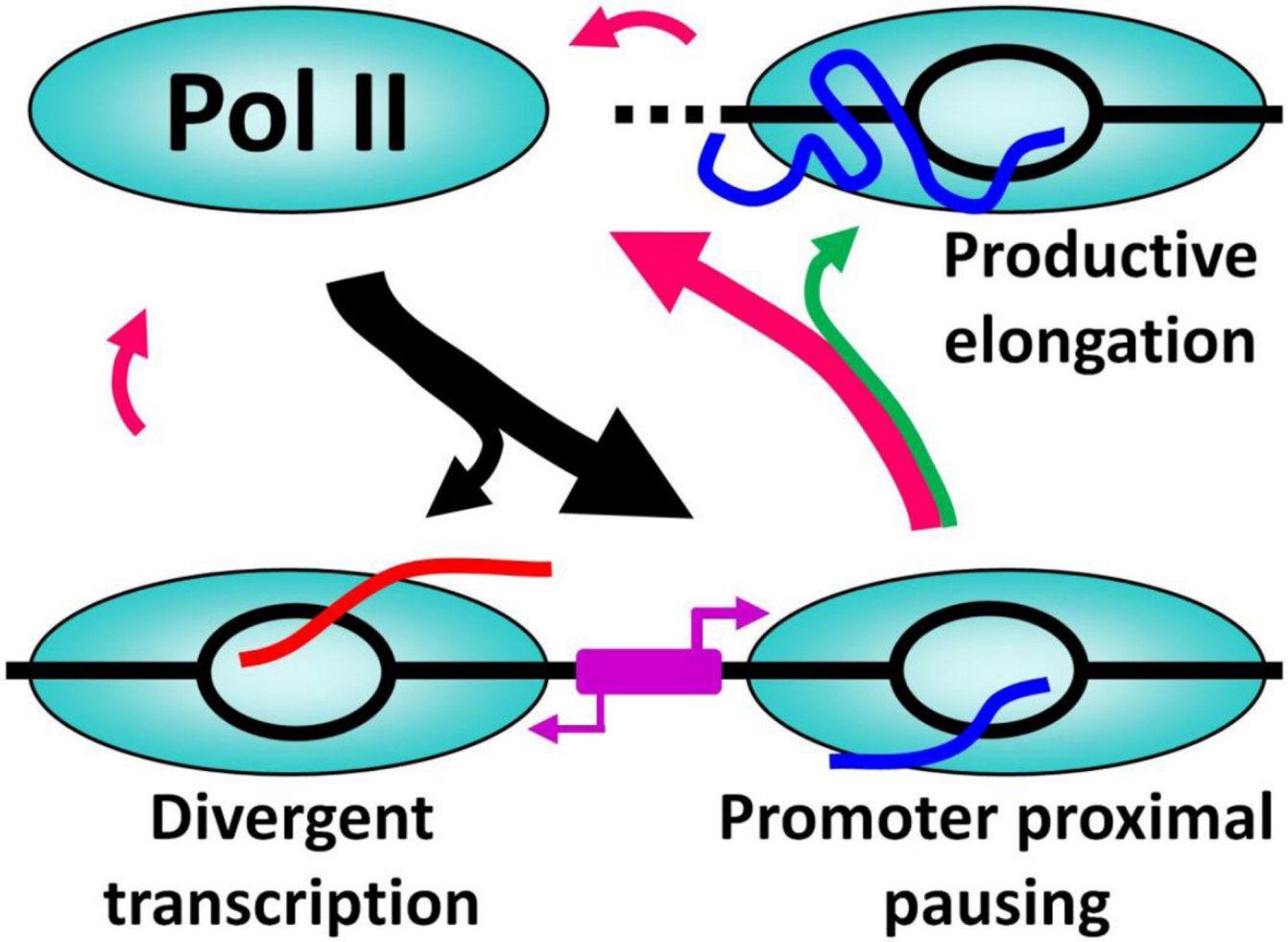

Figure 1. Divergent transcription, promoter proximally paused polymerases, and productive elongation

Many promoters (purple box) allow Pol II to initiate and elongate in both directions using two transcription start sites. Transcription upstream of the gene gives rise to short unstable transcripts and transcription into the gene produces paused polymerases that are poised for entry into productive elongation or termination. The thickness of the arrows is represent relative flow of polymerases; initiation (black), termination (pink), transition into productive elongation (green).

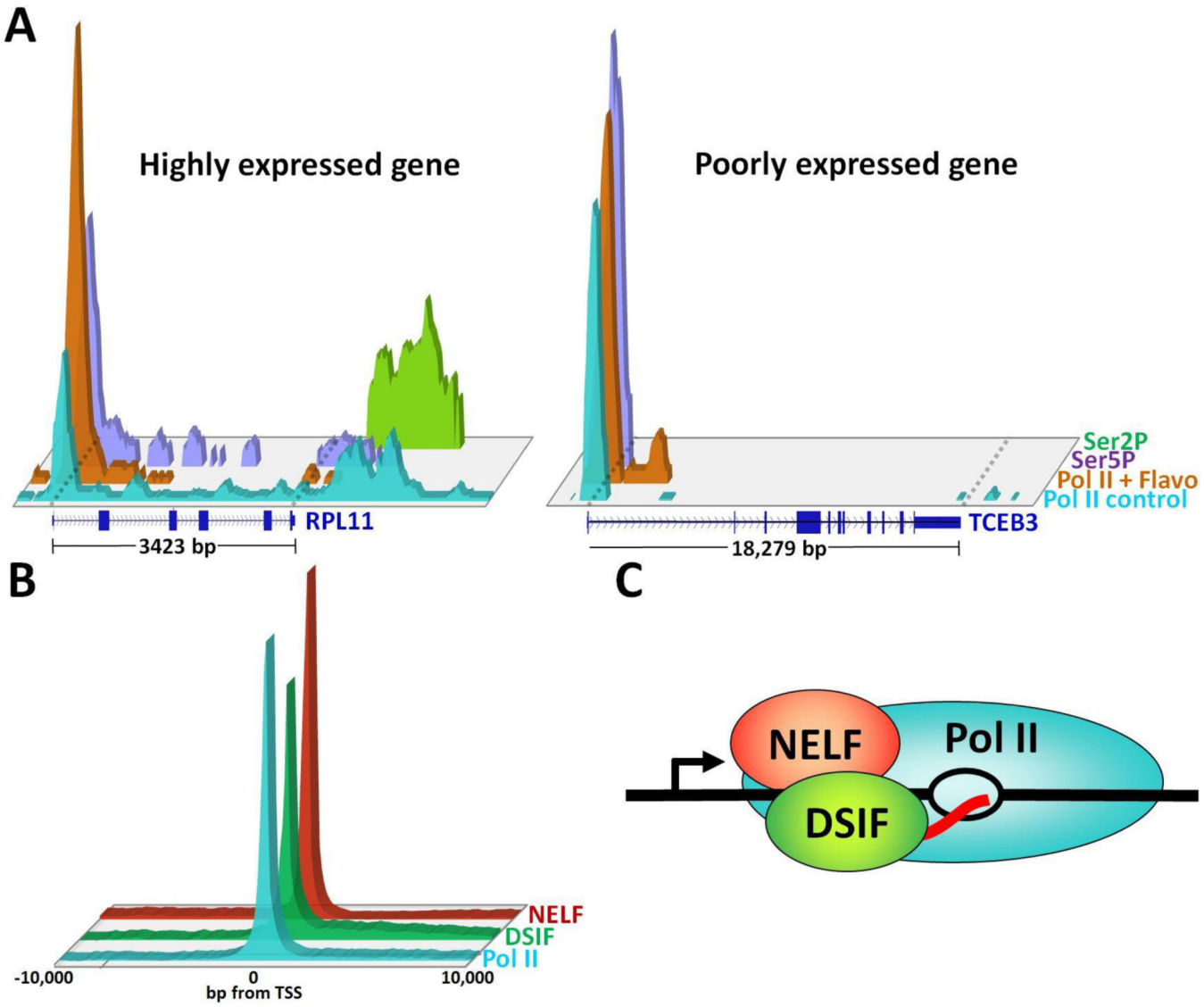

Figure 2. Pol II and elongation factor occupancy from ChIP-Seq

(**A**) MES cell ChIP-Seq datasets from Rahl et al. 2010 (9) were used to generate the views of Pol II from control or Flavopiridol treated cells as well as Ser5 and Ser2 phosphorylation of the CTD of the large subunit of Pol II across two neighboring genes on mouse chromosome 4. The data for each track for each gene was normalized so that the area under all curves was equal (except for the Ser2P for TCEB3 for which there was no data). (**B**) Metagene analysis of MES cell ChIP-Seq data (9) for Pol II, Spt5 subunit of DSIF and NELFe subunit of NELF. The relative distributions of the polymerase and factors were compiled for the region from −10,000 to +10,000 bp around the TSSs for ~20,000 RefSeq genes. In (A) and (B) background signals from the lowest 10% of the 20,000 data points in each distribution were averaged and subtracted from all data points and the resulting curves were normalized so that the total area under each was equal. (**C**) Diagram of the engaged polymerase with nascent transcript (red line) under the influence of DSIF and NELF.

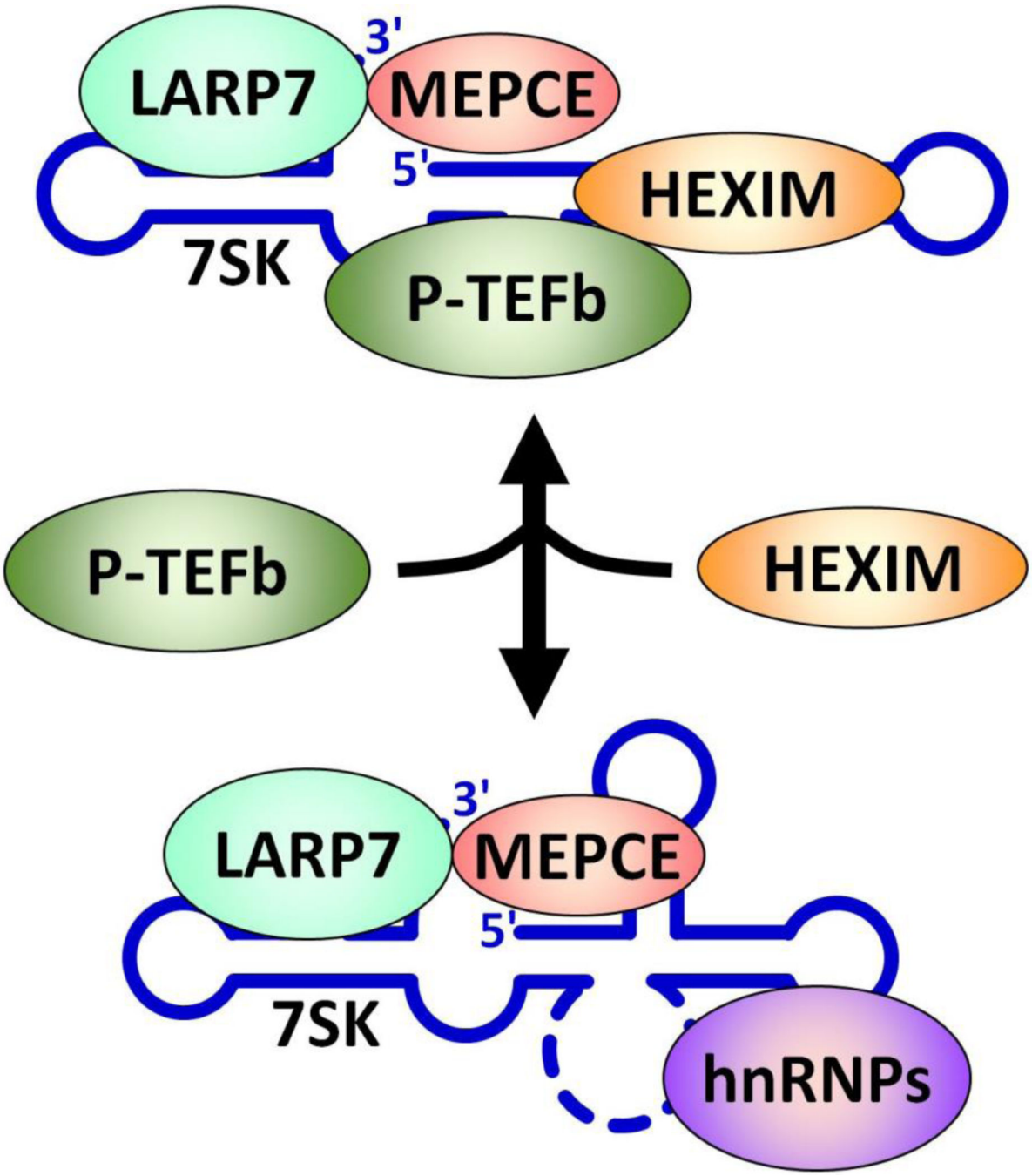

Figure 3. Regulation of P-TEFb by the 7SK snRNP

7SK snRNA is depicted in a cartoon view in which the secondary structures known to be involved in its function are shown. The LA Related Protein, LARP7, is constitutively associated with 7SK and the Methyl Phosphate Capping enzyme, MePCE, which methylates the triphosphate at the 5' end of 7SK is also bound. After binding to the major 5' stem and loop HEXIM1 or HEXIM2 (HEXIM) undergoes a conformational change and binds to and inhibits P-TEFb. When P-TEFb is released from the 7SK snRNP, HEXIM is also released and there is a structural change in 7SK. hnRNPs then replace P-TEFb and HEXIM.

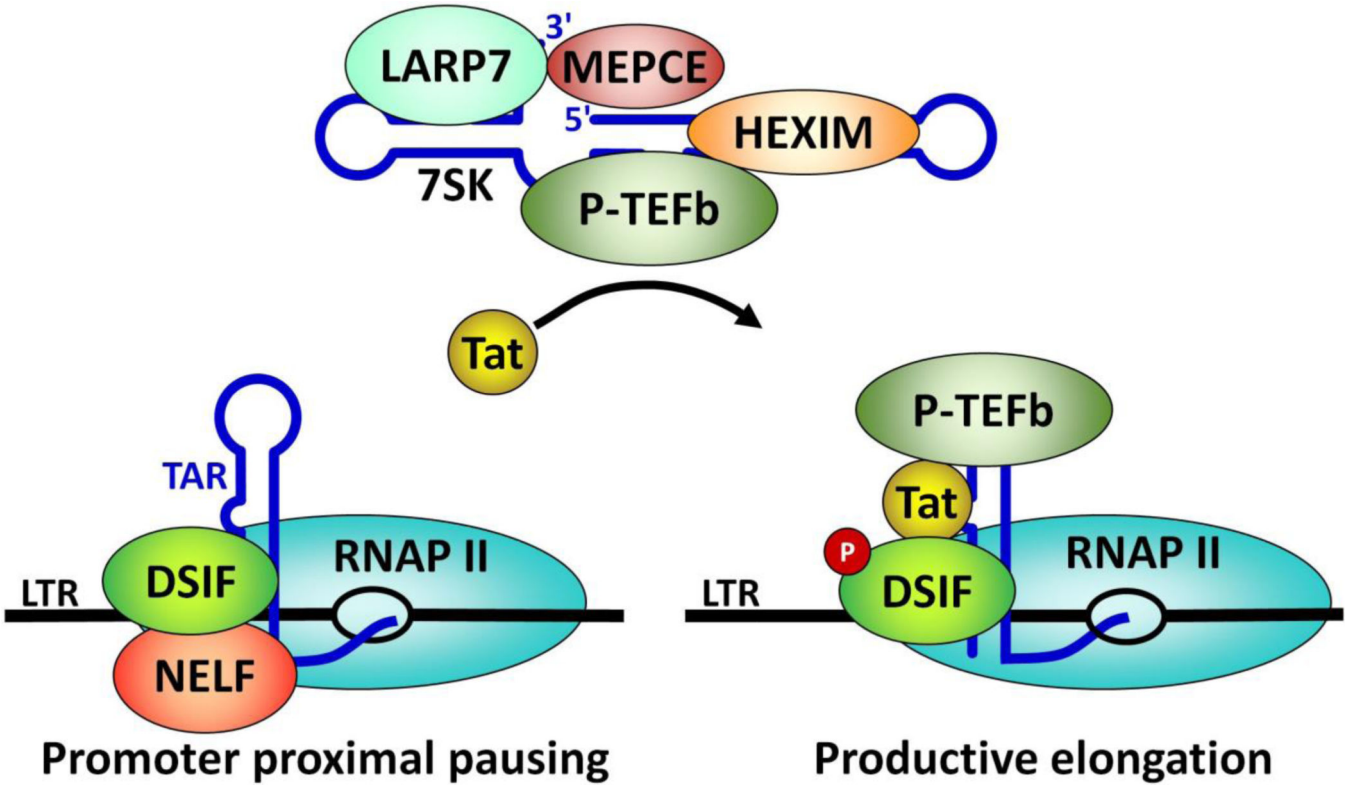

Figure 4. P-TEFb mediated transition into productive elongation

Regulation of HIV transcription is used as an example of how P-TEFb is released from the 7SK snRNP and specifically recruited to its site of action. HIV Tat interacts with P-TEFb in the 7SK snRNP leading to extraction of P-TEFb and the formation of a Tat P-TEFb complex. P-TEFb is recruited to the poised polymerase on the HIV LTR by an interaction with the HIV nascent transcript TAR, where it phosphorylates DSIF (P) as well as NELF and the polymerase (not shown). Also not shown is the SEC which can accompany and potentially help recruit P-TEFb.

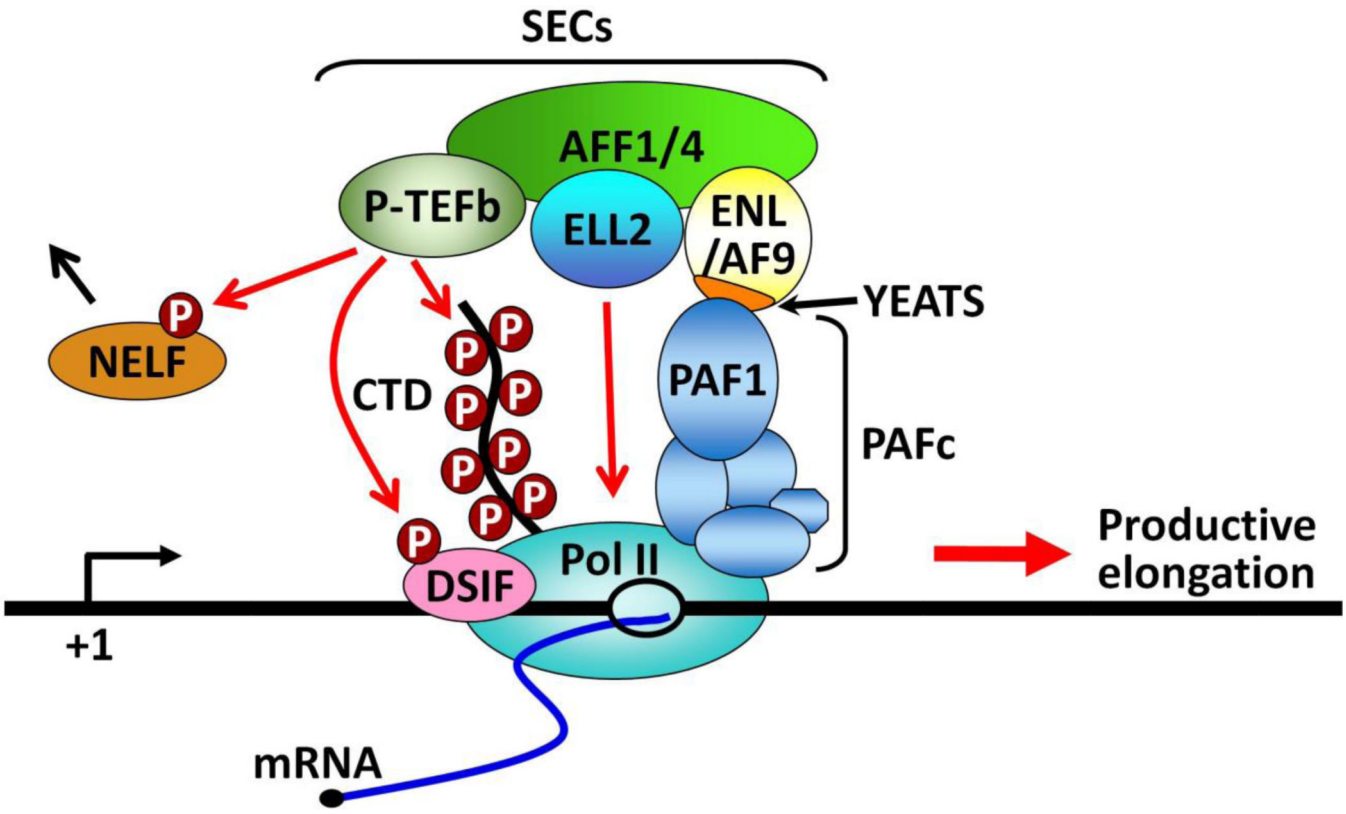

Figure 5. The super elongation complex, SEC

In the absence of sequence-specific recruitment factors such as HIV-1 Tat and the MLL fusions, the SEC complex, which is assembled around the scaffolding protein AFF4, is recruited to the elongating Pol II through the interaction of the YEATS domain of either ENL or AF9 with the PAF1 subunit of PAFc. This allows the SEC to use its P-TEFb and ELL2 functional modules to exert a multitude of effects (e.g. direct stimulation of the Pol II catalytic rate by ELL2, phosphorylation of Ser2 on the Pol II CTD and DSIF by P-TEFb, and phosphorylation and release of NELF by P-TEFb) that result in the synergistic activation of Pol II elongation.

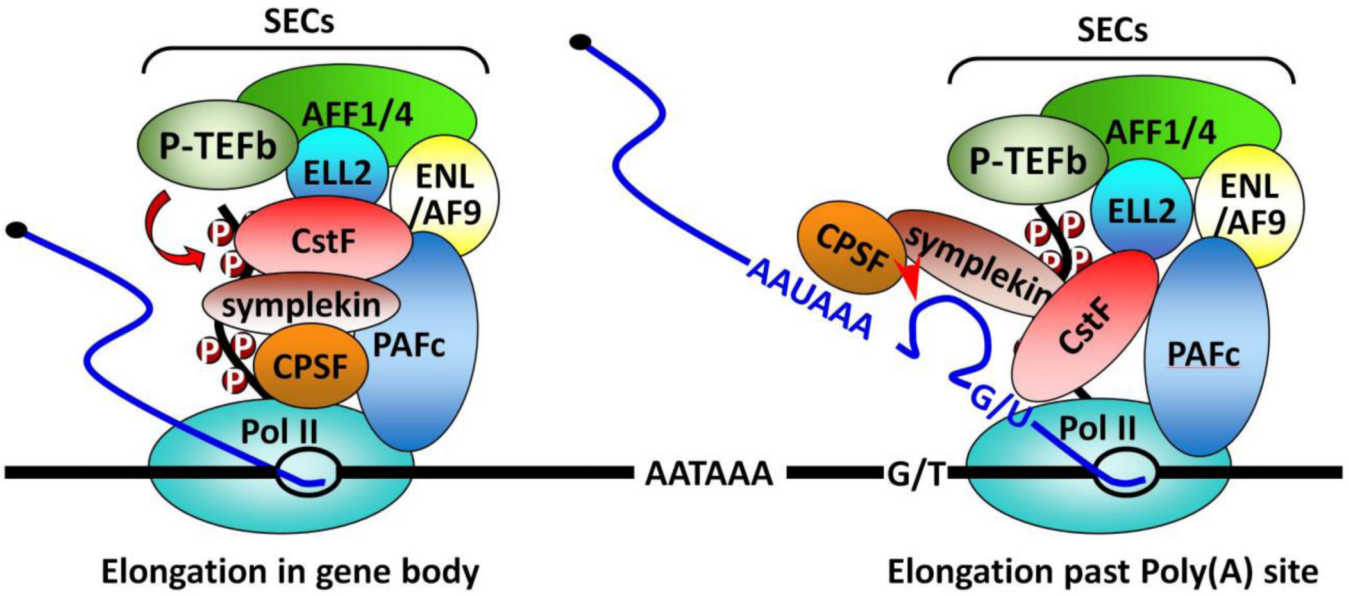

Figure 6. Coupling of 3' end processing with transcription

Polyadenylation factors such as CstF, CPSF and symplekin are recruited to the Pol II elongation complex through the concerted actions of the phospho-Ser2 CTD and transcription elongation factors ELL2 and PAFc, which track along with Pol II during productive elongation. Once the cleavage/polyadenylation signals (AAUAAA followed by a G/U-rich sequence) emerge in the nascent mRNA, they are recognized by the processing machinery to result in efficient polyadenylation.