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Promoter proximal pausing and the control of gene expression

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Summary

The advent of methods for mapping the location of specific proteins across genomes is substantially enlightening our understanding of gene regulation. One recent discovery is that Pol II is concentrated at the 5' end of thousands of genes in mammalian and Drosophila cells. Prior to this, much research had focused on understanding how sequence-specific, DNA-binding proteins orchestrate the actions of regulators of chromatin structure and the general transcriptional machinery to control transcription initiation. The concentration of Pol II at the 5' ends of genes indicates that key steps regulating transcription occur after Pol II has associated with a gene's promoter.

Introduction

Nuclear run-on assays and in vivo crosslinking analyses first revealed that Pol II was concentrated at the 5' end of a handful of genes including *hsp70* in Drosophila, and *c-myc* and *c-fos* in mammals [1,2]. Notably, crosslinking detected the Pol II but the nuclear run-on analysis established that the Pol II was transcriptionally engaged [3,4]. High-resolution analyses of the nuclear run-on products of the *hsp70* and *c-fos* genes revealed that Pol II pauses in the region 20 to 40 nucleotides downstream from the transcription start site. Permanganate genomic footprinting, which maps the location of transcription bubbles associated with elongation complexes in living cells, corroborated the results of the run-on assays.

Biochemical experiments primarily in the laboratories of Handa and Price have provided a framework for understanding the behavior of Pol II in the promoter proximal region [5]. Both groups were investigating the mechanism by which a nucleoside analog called DRB inhibits transcription elongation. By fractionating nuclear extracts and assaying for DRB sensitive transcription, Handa's laboratory discovered two proteins called DSIF and NELF that inhibit elongation [6,7] and Price's laboratory discovered a protein called P-TEFb that promotes elongation [8] (Fig. 1). In nuclear extracts, DSIF and NELF collaborate to inhibit transcription elongation after Pol II transcribes approximately 30 nucleotides [7]. Recent results show that the distance between the start site and the paused Pol II could be dictated by length of transcript needed to cause the association of DSIF with the elongation complex [9,10]. P-TEFb is a kinase that alleviates inhibition by NELF and DSIF. In a defined

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transcription system, optimal reversal of inhibition by NELF and DSIF is achieved by phosphorylating an intact elongation complex containing Pol II, DSIF, and NELF [11,12].

Much attention has been directed at understanding how P-TEFb's activity is regulated at genes as this is thought to influence the reactivation of the paused Pol II as well as contribute to the processivity of the Pol II and to transcription-coupled RNA processing. Multiple mechanisms appear to exist for recruitment of P-TEFb to genes including direct association with DNA binding proteins such as Myc and NF-κB [13,14], indirect association with acetylated histones via Brd4 [15], and association with the mediator [16](Fig. 1). While P-TEFb appears to trigger the dissociation of NELF from the elongation complex, it is unclear to what extent factors control the initial association of NELF or DSIF with the elongation complex. The finding that NELF associates with the estrogen receptor and other nuclear receptors, and that this association functions to attenuate the level of induction of target genes indicates that NELF may be actively recruited by DNA binding proteins to the elongation complex [17,18].

Functions of promoter associated Pol II

Given that NELF and DSIF inhibit transcription elongation in vitro, one might have anticipated that promoter proximal pausing represses gene expression and, indeed, this appears to be true for many genes. Several estrogen-regulated genes were found to be upregulated upon RNAi-mediated depletion of NELF [19] and *junB* and *c-fos* behaved similarly [20]. However, genome-wide expression analysis revealed that more genes decrease than increase expression in NELF-depleted cells suggesting that pausing plays a positive role in gene expression [t1][21,22]. In accordance with this conclusion, almost half of the genes expressed above the 90th percentile level in Drosophila are associated with NELF [23].

One way in which pausing appears to positively influence gene expression is by maintaining the accessibility of the promoter region. Genome-wide mapping of nucleosomes in Drosophila revealed that the first nucleosome downstream from the transcription start is often located adjacent to paused Pol II and that the core promoter is nucleosome-free [24]. Depletion of NELF from Drosophila cells resulted in the appearance of a nucleosome over the promoters of the *TepII* and *Tl* genes, which also exhibited reduced expression upon depletion of NELF [21]. In the case of a human breast cancer cell line, the depletion of NELF resulted in the loss of histone modifications typically associated with active transcription [22]. Thus, the presence of paused Pol II could function to prevent formation of repressive chromatin both by excluding nucleosomes from the promoter region and by maintaining patterns of histone modification that are conducive to activation.

It has been proposed that pausing of Pol II in the promoter proximal region serves as a checkpoint for coupling transcription and mRNA processing, and for delaying the elongation complex until modifications occur that increase the processivity of Pol II [25]. These hypotheses clearly require more rigorous testing in vivo. DSIF and serine 5 phosphorylation of the CTD of Pol II stimulate mRNA capping but there is no in vivo data demonstrating that disruption of pausing results in aberrant capping [20]. NELF has been shown to be involved in 3' end processing of the nonpolyadenylated histone RNAs [26] and to influence splicing [18], but it is unclear if these activities are connected to promoter proximal pausing. It is also unclear whether promoter proximal pausing is necessary for establishing the transcriptional competency of Pol II in vivo. To address these issues, one needs to disrupt pausing and monitor the behavior of Pol II during activation.

Promoter proximal pausing allows the process of recruiting Pol II to a promoter to be uncoupled from transcription of a gene, thus allowing genes to be potentiated for rapid

induction in response to external stimuli. The heat shock genes are excellent examples since these genes are fully induced within minutes of a heat shock [27]. Paused Pol II is also detected on genes that respond rapidly to LPS and estrogen [15,19,28]. However, the connection between pausing and rapid induction remains correlative and has not been directly tested by demonstrating that disruption of pausing slows the induction rate of a gene. For example, the depletion of NELF in the case of estrogen responsive genes results in precocious expression in the absence of hormone [19], and the kinetics of *junB* induction in response to IL6 remained unchanged following depletion of NELF and loss of paused Pol II [20].

Several recent studies have linked pausing to developmental processes. Knockout of a NELF subunit, which would presumably impair promoter proximal pausing, results in embryonic lethality in mice and Drosophila [29,30]. Many developmental control genes in Drosophila have paused Pol II at their promoters [31,32]. An analysis of the spatial and temporal pattern of transcription of specific genes in the Drosophila embryo revealed that genes with paused Pol II are synchronously induced in fields of nuclei [33]. In contrast, those genes without paused Pol II tend to be induced with significantly less synchrony. The Drosophila embryo is a special case because the time in development at which these analyses were done was at the late syncitial blastoderm stage, just as cellularization was beginning. This situation does not exist in developing mammals. In addition, it remains to be demonstrated that perturbing pausing affects the synchrony of expression.

An intriguing activity to be linked to the paused Pol II is enhancer blocking activity [34,35]. Levine and colleagues noted that the genes flanking the bithorax and antennapedia complexes of Drosophila have paused Pol II while the genes within the loci do not. They hypothesized that the paused Pol II might insulate the genes within the developmental loci from the effects of enhancers in flanking regions of the chromosome. When the paused Pol II was positioned between an enhancer and a target promoter, it blocked enhancer activity. Although pausing was not directly measured on the transgenes, it was inferred from the finding that embryos heterozygous for NELF or DSIF mutations impaired insulator activity. Intriguingly, the NELF mutation also impaired the ability of the *Fab7* and *Fab8* insulator elements to block enhancer action. Since paused Pol II is not known to be associated with the *Fab7* and *Fab8* insulators, it was proposed that these insulator elements might associate with paused Pol II to regulate the targeting of enhancers. Consistent with such a model is the recent finding that an insulator-associated protein called BEAF is commonly located near paused Pol II [36]. Perhaps paused Pol II can function in establishing higher order structure of the chromatin fiber. It will be interesting to see if perturbing pausing alters patterns of chromosome looping and the targeting of enhancer function for endogenous loci.

The state of Pol II at the 5’ ends of genes in Drosophila and mammalian cells

Chromatin immunoprecipitation (ChIP) analyses of individual genes and genome-wide clearly establish that Pol II tends to concentrate at the 5’ ends of genes. However, ChIP is unable to assess the transcriptional status of the Pol II, so one cannot know definitively from these analyses alone whether the Pol II is in a preinitiated or transcriptionally engaged state. Currently, nuclear run-on and permanganate genomic footprinting are the only techniques that directly monitor transcriptionally engaged Pol II. Lis and colleagues recently developed a nuclear run-on technique that queries the distribution of transcriptionally engaged Pol II throughout the genome [37]. This technique, called GRO-seq, incorporates bromouridine into nuclear run-on products. The bromouridinated RNA products are isolated and analyzed by massively parallel sequencing. Not only does the method identify transcriptionally engaged Pol II, which is a prerequisite for run-on, but it also reveals the direction of

transcription. Transcriptionally engaged Pol II is detected in the promoter proximal region of thousands of genes in human cells. Unexpectedly, many of these genes also have Pol II transcribing in the opposite direction, upstream from the gene's start site. The significance of this antisense transcription is not known.

Other methods for assessing the behavior of Pol II also provide evidence that the Pol II concentrated in the promoter proximal region is transcriptionally engaged. Sequencing of 5' capped RNAs in the size range from 25 to 120 nucleotides, and sequencing of short uncapped RNAs indicate that Pol II is at some point transcriptionally engaged in the promoter proximal region [38,39]. Importantly, neither of these analyses distinguishes whether these RNAs are nascent transcripts associated with engaged Pol II or products of premature termination or RNA degradation. For example, for a collection of promoters that have similar levels of cross-linked Pol II in a ChIP-seq analysis, the corresponding amounts of 5' capped RNAs can range at least 10-fold, raising the possibility that transcripts and Pol II are dissociating from the DNA (38). The small uncapped RNAs known as tiny RNAs or tiRNAs are intriguing and could be hinting at a possible fate of some of the Pol II. It was proposed that tiRNAs might be the products of TFIIS-induced cleavage of backtracked Pol II [40]. However, these tiRNAs are typically 18 nucleotides in size, and there is no data indicating that TFIIS-induced cleavage occurs at 18 nucleotide intervals. Instead, 18 nucleotides is the length of RNA associated with an elongation complex that is protected from ribonucleolytic attack [41]. Hence, it is possible that these tiRNAs are the products of nucleolytic attack of the nascent transcripts in vivo. This would suggest that some paused Pol II might abort from the template rather than resuming transcription. [t2]

The phosphorylation state of the carboxyl-terminal domain of the largest subunit of Pol II has been used to infer the transcriptional status of Pol II. Serine 5 phosphorylation of the CTD is an indication that TFIIH has acted on the Pol II and that Pol II has initiated transcription [25]. Hence, the absence of serine 5 phosphorylation has been taken as an indication that Pol II is in a preinitiated state. By this criterion, several investigators have identified cases where Pol II is deemed to be in a preinitiation complex [19,42,43]. Byun et al. [43] have proposed that Pol II in a preinitiation complex bookmarks genes like *c-fos* for rapid induction. In contrast, others have shown by permanganate footprinting and nuclear run-on that the Pol II associated with *c-fos* is in a transcriptionally engaged, but paused state [20]. The recent finding that serine 5 phosphorylation is not required for Pol II to initiate transcription in yeast serves as a precaution against relying solely on this marker to assess the state of Pol II [44[t3]]. The inconsistencies over the status of Pol II at the *c-fos* promoter serve to highlight a weakness in our current methods for evaluating the status of the Pol II. No one method allows quantification of the proportions of Pol II in the preinitiated and the paused states.

Conclusion

The discovery that Pol II is concentrated at the 5' ends of many genes in metazoans has directed much interest towards understanding the control of Pol II after it initiates transcription. Promoter proximal pausing mediated by NELF and DSIF represents as early stage in the control of transcription elongation, and cells appear to use this pausing in different ways to either positively or negatively regulate gene expression. The wide-spread application of chromatin immunoprecipitation has made it relative simple to determine if Pol II is concentrated at the promoter of a particular gene but nuclear run-on and permanganate footprinting assays are required to assess whether these Pol II are pausing in the promoter proximal regions of genes. In lieu of this information, it remains possible that regulation is targeting the behavior of the Pol II in a preinitiation complex, or the rate of elongation and

processivity of the Pol II as it advances beyond the promoter proximal region and into the body of the gene.

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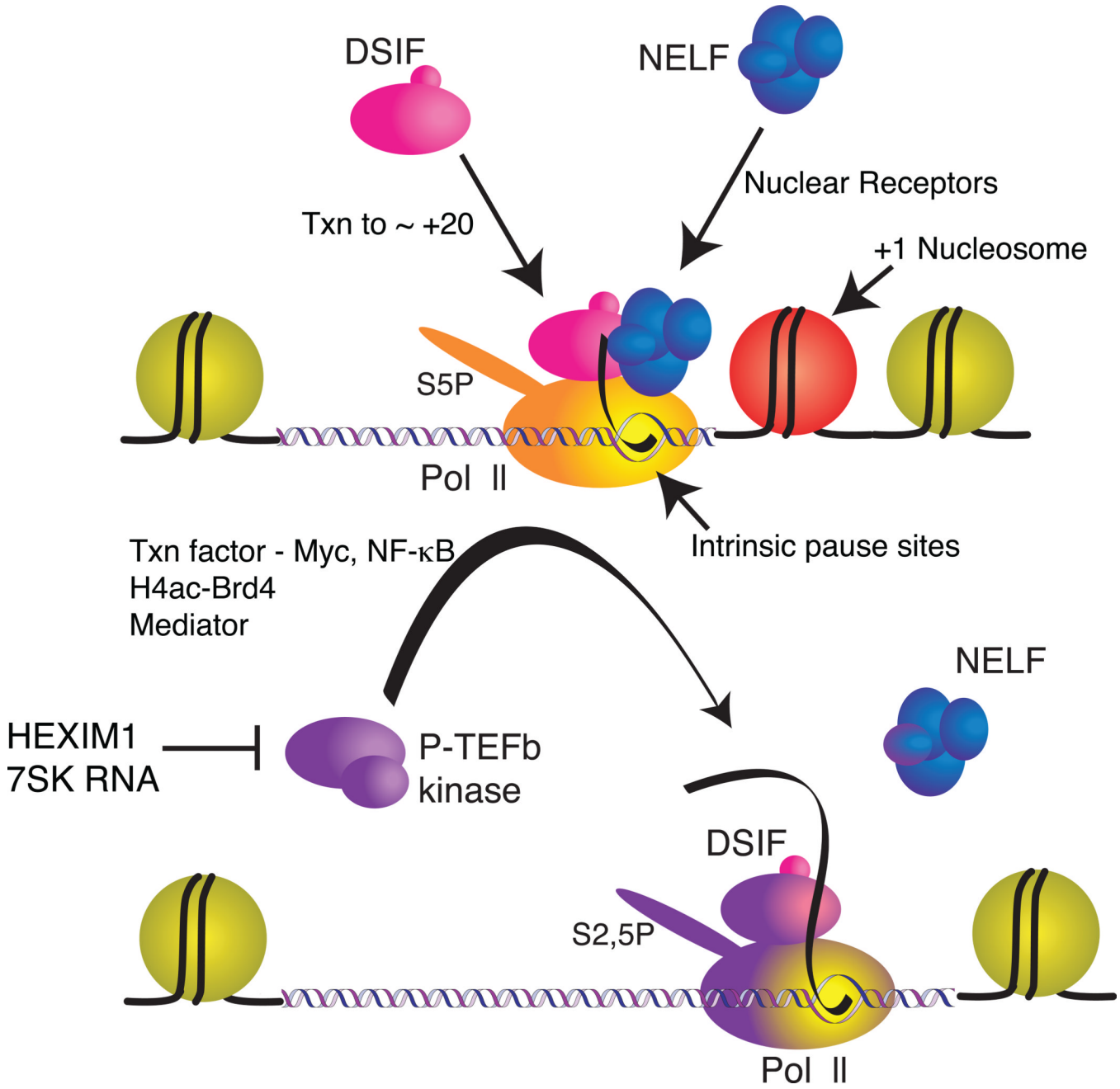

Figure 1.

Interactions impacting promoter proximal pausing. DSIF and NELF provide the basis for promoter proximal pausing [45]. NELF's association with the elongation complex is dependent on DSIF, and DSIF's association requires the Pol II transcribe approximately 20 nucleotides [9,10]. NELF's recruitment to particular promoters can also be facilitated by sequence-specific, DNA-binding proteins such as nuclear hormone receptors [17,18]. Nucleic acid sequences affecting the stability of the RNA-DNA heteroduplex in the transcription bubble may also influence where Pol II pauses [38]. The +1 nucleosome may also contribute to the pause [24]. P-TEFb is a kinase that phosphorylates DSIF, NELF, and the CTD of Pol II, and appears to reactivate the paused Pol II [46]. P-TEFb activity is repressed by the 7SK RNA/HEXIM1 complex in mammals[t4] [47]. P-TEFb's recruitment to

a gene may occur through association with DNA binding proteins such as c-Myc [13] and NF-κB [14], association with Brd4 that is in turn associated with acetylated tails of histone H4 [15], or mediator [16].