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Complexity of RNA polymerase II elongation dynamics★

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Abstract

Transcription of protein-coding genes by RNA polymerase II can be regulated at multiple points during the process of RNA synthesis, including initiation, elongation, and termination. *In vivo* data suggests that elongating polymerases exhibit heterogeneity throughout the gene body, suggestive of changes in elongation rate and/or pausing. Here, we review evidence from a variety of different experimental approaches for understanding regulation of transcription elongation. We compare steady-state measurements of nascent RNA density and polymerase occupancy to time-resolved measurements and point out areas of disagreement. Finally, we discuss future avenues of investigation for understanding this critically important step in gene regulation. This article is part of a Special Issue entitled: Chromatin in time and space.

Keywords

Transcription; Elongation; Single-molecule; Splicing; Imaging; Polymerase

1. Introduction

The process of transcription can be broadly divided into three phases namely, initiation, elongation and termination. These steps are mechanistically distinct: 1) initiation consists of promoter recognition, opening of the DNA helix, and possible abortive synthesis of the first few bases of RNA; 2) elongation begins with promoter clearance after synthesis of 8–9 bases; 3) and termination results in the dissociation of the RNA–DNA hybrid and release of the nascent transcript. Historical efforts to understand gene regulation have focused on the biochemical steps of initiation: understanding how, when, and where DNA is unwound to permit RNA polymerase to access the template strand. Although post-initiation gene regulation has been known for decades [1], the prevalence of post-initiation control *in vivo* has come into sharper focus through the use of genome-wide approaches [2]. Thus, the straightforward biochemical distinction of elongation as a uniform enzymatic activity state fails to capture the variety of polymerase behaviors observed in cells. In fact, both the rate of elongation and efficiency of elongation are proposed as regulatory mechanisms in cells, allowing one to ask both “when” and also “if” an initiated polymerase reaches the end of the gene. In this review, we address the first question: is modulation of transcription elongation

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rate a regulatory mechanism *in vivo*? Moreover, for simplicity, we restrict ourselves to transcription elongation which occurs in the body of the gene, far from the 5' end and the observed promoter proximal pausing, which is treated by Adelman and co-authors in this issue.

In recent years, the question of modulation of transcript elongation has been addressed using a variety of techniques, including deep sequencing, chromatin immunoprecipitation, optical force microscopy, and single-molecule fluorescence imaging. The results have been illuminating, and new developments indicate a range of unanticipated behavior for an elongating polymerase. For example, polymerase speeds have been observed from a low of 0.8 kb/min [3] to over 50 kb/min in living cells [4]. Nucleosomes can function as barriers to elongation [5], but the elongation rate can also change the composition of transcribed nucleosomes [6]. These measurements and others reviewed here indicate the field is at an exciting juncture: it is now possible to do *in vivo* enzymology with RNA polymerase for direct comparison to *in vitro* models. In this review, we focus primarily on eukaryotic RNA polymerase II (RNAPII), provide a brief motivation based on *in vitro* work, examine the evidence for changes and/or heterogeneity of elongation *in vivo*, and finally address the implications for gene expression regulation. Our survey of published results suggests that polymerase elongation heterogeneity inferred from steady-state measurements is only partially recapitulated by time-resolved measurements, and we discuss several hypotheses for reconciling these differences.

2. Transcript elongation by RNA polymerase II *in vitro*

All protein coding genes are transcribed by RNAPII, and transcript elongation by RNAPII has been the subject of numerous studies *in vitro* using ensemble and single-molecule assays to understand the biochemical properties, mechanistic details and the regulatory processes that control elongation properties of this enzyme (for a comprehensive review, see [7]). RNAPII incorporates nucleotides to the 3'-end of a growing RNA chain by a Brownian-Ratchet mechanism that allows for forward and backward movement of the polymerase along the DNA template [8–10]. Transcript elongation is often punctuated by reversible transitions of RNAPII from an “on-line” pathway to short lived “off-line” states of temporary inactivity defined as pausing [11] or prolonged and irreversible inactive states called transcriptional arrest [12]. Such transitions and extension times define how long it takes RNAPII to transcribe a gene.

Mammalian RNAPII transcribes naked DNA *in vitro* with an average elongation rate of 300–400 nucleotides a minute. The overall rate of transcript elongation by RNAPII is a function of pause recognition (frequency of pausing), the time spent by the polymerase at the pause (pause duration), and the rate of elongation between pause events (pause free velocity). Several of the general transcription factors such as TFIIF, Elongin and ELL enhance the rate of elongation by RNAPII principally by suppressing pausing [13–16], the primary effect being either on pause duration or enhancing the pause free velocity. There is little evidence to indicate that elongation factors can reduce the frequency of pausing *in vitro*.

Although RNAPII escapes from most pause states where the 3'-end of the transcript has dislodged from the active center of the enzyme, or has traveled backwards (backtracking) along the nucleic acid chain by one or two nucleotides relative to the 3'-end of the transcript [17], extensive backtracking by several nucleotides results in long-lived arrested state. RNAPII can resume elongation from a backtracked arrest state in the presence of the transcription factor TFIIS that induces the intrinsic transcript cleavage activity of RNAPII, cleaving the RNA internally to create a new 3'-end [18–21]. The ability of TFIIS to help RNA polymerase transcribe at high opposing forces is further indication of the active role of TFIIS in rescuing backtracked polymerases [22]. This role is further highlighted by the fact that TFIIF and TFIIS function cooperatively to reduce backtracking by enhancing transcript cleavage and repositioning the polymerase back on the “on-line” pathway [23]. Although TFIIS cooperates with TFIIF to enhance the elongation properties of RNAPII, binding of TFIIS to RNA polymerase is also controlled by the negative elongation factor NELF, which might play a crucial role in NELF induced pausing by RNAPII [24]. In support of this idea, phosphorylation of Ser2 residue of the heptapeptide repeat of the CTD of RNAPII by the cyclin dependent kinase of PTEFb is essential to overcome the inhibitory effect of NELF and promoting elongation [25–27]. Thus, the fine-tuned interplay of both positive and negative elongation factors controls the elongation properties of RNAPII to regulate transcription *in vitro*.

In addition to pausing and arrest, ensemble studies of transcript elongation have also found that nucleosomes reduce the elongation rate by presenting a significant barrier to transcript elongation by RNAPII *in vitro* [28–32]. It is evident from modeling studies combining nucleosomal structure with that of an elongating polymerase that this barrier arises from the strong DNA binding of nucleosomal dyad downstream of the transcription bubble [33]. Once this barrier is crossed, the polymerase can transcribe to the end of the nucleosome with relatively little difficulty. This structural modeling also suggests a model for traversal of the transcription complex through nucleosomes in which the DNA upstream of the polymerase loops back, allowing the deposition of the displaced downstream nucleosome behind the elongating polymerase.

Several protein factors (TFIIS, TFIIF and FACT) mediate elongation of RNAPII through nucleosomes *in vitro* [32,34]. Although the general transcription factors TFIIF and TFIIS individually do not support robust transcription through nucleosomes *in vitro*, they do so in combination; TFIIS suppresses the backtracked pause 45 bp into the nucleosome while TFIIF simultaneously enhances the elongation of the polymerase [32]. FACT functions to destabilize the nucleosome core by removing the H2A/H2B dimer and re-depositing it in the wake of the elongating polymerase after transcribing past the nucleosome core [35]. In this way, elongation factors can function as histone chaperones. The connection between transcript elongation and histone chaperone activity has recently been visualized using atomic force microscopy [6]. By simultaneously observing RNAPII and the nucleosome on a single segment of DNA, the authors use a quantitative kinetic model to argue in favor of the DNA looping model [33] with nucleosomes re-deposited behind the polymerase. Moreover, this study suggests that efficient traversal through a nucleosome and the ability to redeposit the hexosome in its wake might be directly related to the elongation rate of the polymerase, with a slow polymerase better able to cover its tracks.

3. Transcription elongation *in vivo*

Although nucleosomes present a clear elongation barrier *in vitro*, the extent to which chromatin might modify elongation rates *in vivo* is indeterminate. Two approaches have been taken to investigate this topic. First, the position of active RNAPII relative to nucleosomes has been measured for all genes in budding yeast [36]. A genome-wide RNAPII footprinting approach coupled with deep sequencing revealed an increased number of reads near the nucleosome dyad. This increase in read density was interpreted as RNAPII pausing at the nucleosome boundaries. Second, an orthogonal approach attempts to position nucleosomes *in vivo* in order to directly observe the effect on RNAPII elongation. These experiments attempt to establish a direct comparison to *in vitro* results obtained from the high affinity 601 nucleosome positioning sequence [37]. Gracey and coworkers were able to position nucleosomes on the 601 sequence of an episomal vector injected into the mouse and taken up by hepatocytes, but this positioning was only transient [38]. In a related study in mammalian cell lines, this same sequence led to a ~3 fold enrichment of nucleosome occupancy and resulted in an increase in RNAPII density immediately upstream of the sequence [39]. Conversely in yeast, the 601 sequence had no effect on RNAPII distribution on the gene and in fact resulted in a decrease in nucleosome occupancy [40].

One area where RNAPII elongation has attracted considerable attention is in relation to splicing (Oberdoerffer and colleagues, this issue). Changes in velocity or pausing in the body of the gene have been invoked as a mechanism for controlling the fidelity and/or choice of exon inclusion. A mutant polymerase which has a slower elongation rate *in vitro* has been shown to effect alternative splicing *in vivo* in human cell lines [41] and budding yeast [42]. Furthermore, changes in velocity are triggered by UV-induced hyperphosphorylation of the RNAPII CTD, suggesting a regulatory role for RNAPII elongation speeds [43]. Increased RNAPII density on variant exons correlates with a RNAPII CTD phosphorylation state (Ser5-P) which is similar to that of promoter-proximal polymerases [44]. Likewise, alternative splicing of CD45 correlates with an increased density of RNAPII in a CTCF (CCCTC-binding factor)-dependent manner [45]. In yeast, a peak of RNAPII occupancy at the 3' splice site motivated a model that transcriptional pausing at this position may be a checkpoint associated with co-transcriptional splicing [46]. In this same study, the cyclical time-dependent accumulation of RNAPII occupancy suggested bursts of splicing activity. Finally, in a complementary study which directly quantified nascent pre-mRNA distribution across the genome, increased density of transcribing RNAPII was observed at the 3' end, which was attributed to a slower elongation rate of polymerases in that region of the gene [47] as was suggested by earlier single-molecule RNA FISH experiments [48,49]. In summary, the density of RNAPII and nascent RNA appears heterogeneous over the gene body, suggesting kinetic variability in the rate of elongation.

However, these measurements are frequently done under steady-state conditions. Time-resolved measurements done both at the single-cell and population level suggest that elongation rates are remarkably constant over the gene, leading to an apparent contradiction between steady-state and time-resolved approaches. In a comprehensive analysis done by Singh and Padgett which used release from a transcriptional block followed by qRT-PCR,

the speed of RNAPII was measured at 3.8 kb/min [50]. Elongation rates were highly uniform for all genes and over different parts of the gene, regardless of the number of introns. The authors concluded that there were few or no pausing events over hundreds of kilobases of chromatin. Single-cell microscopy studies which directly observe the production of nascent pre-mRNA at an active locus using the MS2 stem loop system [51] (Fig. 1) reached a similar conclusion. Brody et al. created reporter genes with a variable number of introns preceding the stem-loop cassette and determined by fluorescence recovery after photobleaching (FRAP) that elongation kinetics are not modulated by splicing events [52]. Moreover, by measuring the dynamics of both RNAPII and the nascent RNA, the authors were able to tease apart the relative contributions of elongation and post-elongation to the observed kinetics. They observed splicing-dependent changes in the recovery of the MS2-RNA fluorescence but not in the RNAPII fluorescence or in RNAPII distribution as measured by ChIP. These results suggest that the transcripts were retained for processing after the polymerases have departed. In some cases, this processing time lasted ~10 min. A different single-cell study measured retention of the transcript after the poly-adenylation site, linking proof-reading to a step occurring post-elongation [53]. Again, different dynamics were observed for RNAPII and the nascent transcript, indicating that the occupancy of the RNA was not an indicator of active transcription dynamics. These analyses draw attention to the multiple kinetic steps involved in RNA synthesis and to the difficulty in unambiguously assigning an observed change to one particular step such as elongation, or pausing, or termination (Fig. 1B) [54,55].

Recently, several groups have achieved single gene transcription imaging in both mammalian cell lines and yeast [3,4,56]. In yeast, we observed that the dwell time of nascent transcripts was narrowly distributed, implying that the elongation rate was highly uniform over the gene at a rate of 1.2 kb/min (Fig. 1C). Interestingly, the elongation rate increased during G2, but this increase may have been due to global changes in chromatin approaching mitosis. Yunger et al. observed a rate of 0.3–0.8 kb/min in HEK293 cells and were unable to detect pausing in their assay. On the opposite end of the spectrum, polymerase velocities over 50 kb/min were measured on a viral reporter gene, reflecting highly efficient initiation and elongation from a single integration site [4]. Oddly, this same gene exhibited a drastically slower velocity of 1.6 kb/min when integrated as an array. Integration of transgene repeats can cause changes to the chromatin structure [57], so the difference between arrays and single integrations will need to be considered when comparing different reporter systems. However, these measurements raise the possibility that the measured elongation rate is the result of a continuous progression of fits and starts by the polymerase, but that under favorable conditions the polymerase can move at extremely fast speeds down a gene. In that sense, current time-resolved measurements may simply not have the resolution to capture the heterogeneity. Conversely, with the exception of yeast cell cycle variable elongation which occurs on the same gene [56], the extreme range of measured elongation rates may primarily reflect different experimental approaches and analyses. Regulated changes in elongation have yet to be observed using time-resolved measurements. Thus, steady-state and time-resolved measurements are presently at odds, necessitating a systematic comparison of these two approaches.

4. Comparing steady-state and time-resolved measurements

While most *in vitro* measurements are based on direct observations of the kinetics of RNA synthesis using time-resolved measurements, *in vivo* measurements of elongation are based on both steady-state and time-resolved measurements. The distinction between these two approaches is important. Steady-state measurements report the density of some quantity, which is the ratio of kinetic rate constants. Time-resolved measurements report the kinetic rate constants directly. An example is nascent RNA quantification, which is a measure of the density of elongating polymerases on a particular gene (Fig. 2A). Steady-state occupancy of RNAPII is determined by the kinetic balance of the frequency of initiation and the time that RNAPII spends on the gene:

$$m = c\tau \quad (1)$$

where m is the number of polymerases or nascent RNA, c is the initiation rate, and τ is the time the polymerase spends on the gene. At a minimum, the time τ depends on the velocity of the polymerase (v), the distance in bases the polymerase must transcribe (l), and the termination time (T) which includes the time for cleavage, poly-adenylation, and processing:

$$m = c\left(\frac{l}{v} + T\right) \quad (2)$$

Experimentally, by measuring the amount of nascent RNA (m) as a function of position from the 3' end of the gene (l), one can determine the slope (c/v) and the offset (cT) (Fig. 2B).

The critical point in this analysis is that neither quantity (v , T) can be separated from the initiation rate c using this approach. For example, a change in the slope can be due either to a change in velocity or a change in initiation rate. By using a ratiometric measure (for example the ratio of proximal nascent RNA to distal nascent RNA) one can remove the initiation rate, but it is still impossible to separate the effects of elongation and termination. Eliminating the initiation rate and expressing the result in terms of the measured parameters give:

$$vT = \frac{l_1 - l_2}{\frac{m_1}{m_2} - 1} \quad (3)$$

where $l_{1,2}$ is the distance to the end of the gene from the proximal and distal positions; $m_{1,2}$ is the abundance of RNA with the proximal and distal sequence, respectively. Alternatively, to eliminate the initiation rate, one could simply take the ratio of the offset to slope to obtain vT . Again, it is impossible to separate effects on velocity and termination using this steady-state measurement. Intuitively, this result can be understood by considering the case where

the termination rate is very slow compared to the velocity: polymerases pile up at the 3' end, and the ratio $m_1/m_2 \sim 1$.

This analysis points to the inherent difficulty in separating the contributions of initiation, elongation, and termination to the occupancy of polymerases and nascent RNA on a particular gene [54–56]. Moreover, under steady-state conditions, a number of assumptions are necessary to infer changes in elongation rate and/or pausing, and these assumptions may not be valid under all circumstances [58]. For example, a local increase in RNAPII density, say over a particular region of the gene body, can be caused by a change in the initiation rate into the region or by the elongation rate out of the region (Eq. (1)). Therefore, cryptic initiation—initiation at positions other than the annotated transcription start site—would be indistinguishable from pausing. It is becoming clear that most of the eukaryotic genome is transcribed (85% in yeast [59,60], 98% in humans [61]), and it may be that: 1) the resulting RNAs are functional, 2) the process of transcription itself is functional (independent of the synthesized RNAs), or 3) that pervasive transcription is simply spurious and results from RNAPII initiating from nucleosome-depleted DNA [62]. Regardless of the functional role, the existence of pervasive transcription must be considered when inferring elongation changes from steady-state data. Likewise, the emerging ubiquity of anti-sense transcription which often initiates near the terminators of annotated genes urges caution when interpreting RNAPII pausing at the 3' end [2,63].

Complicating the analysis even further is the fact that the same factors which facilitate elongation (and which therefore might be involved in modulating elongation rates) also play an essential role in repressing cryptic initiation. For example, Spt6 plays a critical role in maintaining chromatin structure in yeast, and Spt6 mutants lead to cryptic initiation across the genome [64,65]. In addition, the disruption of nucleosome structure is dependent on active transcription, suggesting that many elongation factors function as histone chaperones which help the removal and replacement of nucleosomes during elongation [66]. Based on several studies in yeast, a general model of chromatin organization by elongating polymerases has emerged [67–71]. RNAPII recruits the histone methyltransferase Set2 through interactions with the phosphorylated RNAPII CTD, resulting in transcription-dependent H3 methylation. The histone deacetylase Rpd3S recognizes methylated histones and deacetylates these histones within transcribed regions, thereby erasing the transcriptional memory of the active gene and restoring the chromatin to a hypoacetylated state that is repressive for transcription. Thus, the coordinated regulation between Rpd3S and Set2 is essential for repressing transcription initiation from intragenic cryptic promoters [68]. Also, because this coordination is in part mediated by the RNAPII CTD, mutations in this region may have pleiotropic effects which alter not only RNAPII elongation but also transcriptional fidelity and the ability to suppress cryptic initiation during elongation.

5. Conclusion and outlook

The complexity of post-initiation steps in transcription provides numerous opportunities for regulation of RNA processing and gene expression [72]. However, the interdependent nature of initiation, elongation, termination, and chromatin modifications makes it difficult to precisely attribute observed changes in RNAPII occupancy or nascent RNA distribution to

one process or the other. Steady-state population measurements utilizing PCR-based approaches have exquisite sensitivity and may be able to detect very small changes in elongation heterogeneity. However, density measurements are always measurements of the relative balance of initiation and elongation, just as an equilibrium constant is the ratio of “on” and “off” rates. On the other hand, kinetic measurements are difficult to make and lower throughput, usually relying on chemical inhibition or genetic modification. Although there is not a definitive consensus from these time-resolved measurements, the preponderance of evidence suggests highly uniform elongation, in contrast to the peaks and variability seen from steady-state measurements. These data then beg the question: what is the minimum length of a detectable pause and what sort of kinetic pause is necessary to be biologically meaningful? In other words, if the cell is regulating elongation, how much modulation is necessary to change the fate of an RNA? Recent work suggests that for a multi-step kinetic process such as splicing, the distribution of splicing time has lower variability, which also means that small changes in elongation (2-fold) might have a large effect on splicing efficiency [73,74]. Detecting such changes *in vivo* and correlating them with a biological outcome will be a significant experimental challenge.

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Fig. 1.

Live cell kinetic measurements of transcription elongation. A) Pre-mRNA can be visualized using the MS2 bacteriophage capsid protein. DNA which encodes for RNA stem loops is inserted into the reporter gene of interest [51]. Once transcribed, the stem loops bind the constitutively-expressed fluorescently-labeled capsid protein. Each RNA stem loop binds a dimer of the capsid protein. The nascent RNA is then visible at the active locus in the living cell. B) Fluorescence recovery after photobleaching (FRAP) of nascent RNA. The fluorescence recovers on a time scale determined by both elongation and termination [55].

The slope of the curve depends on the length of the MS2 array, the length of the gene after the MS2 array, the velocity of the polymerase, and the termination time. The FRAP curves display the recovery dependence for different termination times (T) at a fixed velocity (v). C) Fluctuation analysis on single yeast genes [56]. Using time-lapse imaging coupled with automated tracking enables the determination of the transcription autocorrelation (red circles). The decay depends only on the dwell time of the nascent RNA (τ), and the amplitude is the product of the initiation rate (c) and the dwell time. The original data is from [55,56].

Fig. 2.

Steady-state measurements for determining the relative balance of initiation, elongation, and termination. A) Cartoon of a nascent RNA abundance measurement. The position of a PCR amplicon or FISH probe used to quantify abundance (m) is shown by the red and gray bars. In this instance, $m_1=2$; $m_2=1$. The distance from the position of the polymerase to the end of the gene is shown by $l_{1,2}$. B) Length dependence of nascent RNA abundance. As the distance to the 3' end increases, the time that a nascent RNA is present increases (red line). The slope is the ratio of initiation rate (c) to velocity (v); the offset is the product of the initiation rate (c) and the termination time (T).