ANNUAL REVIEWS

Annual Review of Biochemistry

Transcription in Living Cells: Molecular Mechanisms of Bursting

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Annu. Rev. Biochem. 2020. 89:189–212

First published as a Review in Advance on March 24, 2020

The *Annual Review of Biochemistry* is online at biochem.annualreviews.org

https://doi.org/10.1146/annurev-biochem-011520-105250

Keywords

transcription, single-molecule, single-cell, gene regulation, bursting

Abstract

Transcription in several organisms from certain bacteria to humans has been observed to be stochastic in nature: toggling between active and inactive states. Periods of active nascent RNA synthesis known as bursts represent individual gene activation events in which multiple polymerases are initiated. Therefore, bursting is the single locus illustration of both gene activation and repression. Although transcriptional bursting was originally observed decades ago, only recently have technological advances enabled the field to begin elucidating gene regulation at the single-locus level. In this review, we focus on how biochemical, genomic, and single-cell data describe the regulatory steps of transcriptional bursts.

Contents

INTRODUCTION

Over the past 50 years, a variety of gene regulatory mechanisms have been defined in both unicellular and multicellular organisms. Gene expression is a result of many steps, from chromosome organization to nucleosome remodeling, transcriptional initiation, elongation, termination, and RNA processing. These steps must be coordinately regulated in a timely manner, especially during development, when some cell stages last minutes. In development and adulthood, these systems respond to environmental stimuli and are important for stress responses, differentiation, and homeostasis. While genomic assays have illustrated the orchestrated process of gene activation for thousands of genes, single-cell studies have quietly enhanced our view of complexity and dynamics. Early on, methods employing electron microscopy through Miller spreads showed that genes toggle between active periods of nascent RNA synthesis and periods devoid of RNA (1, 2). These transcription units or transcriptional bursts illustrate the random nature of the molecular and biochemical process of transcription.

Bursts represent the culmination of multiple regulatory processes, and their behavior can be divided into three main regulatory steps. First, how frequently one observes bursts, or burst frequency, is representative of steps that occur prior and up to transcriptional initiation, including activator binding, enhancer looping, chromatin and nucleosome remodeling, preinitiation complex (PIC) formation, and initiation. Second, once bursts are initiated, the promoter is in an open state, and thus multiple polymerases can be loaded during a burst. Indeed, bursts of up to 200 polymerases have been reported in eukaryotes (3). Therefore, upon initiation of a burst, promoters must be maintained in an open state for multiple polymerases to be recruited during this transcriptionally permissive state. Last, the burst is of a limited size and duration, which indicates that the promoter switches from an active to an inactive state. Therefore, by understanding how bursting features such as burst frequency and size are modulated in response to environmental, chemical, and genetic stimuli, we can gain a more detailed view into the molecular mechanisms of how transcription is regulated at the single-locus level. To understand the biochemistry of bursting is to understand how transcription works in the nucleus.

In this review, we focus on what makes genes burst and how biochemical and single-molecule literature describe our current knowledge of bursting. We discuss the role of transcription factors, chromatin, nucleosomes, enhancers, and chromosome topology in bursting. While the stochastic and variable nature of transcriptional bursting can lead to variability at the level of mRNA (4, 5), this review does not focus on these aspects. We also highlight the importance of and increasing trend toward understanding gene regulation at the single-cell level.

OBSERVING STOCHASTIC GENE EXPRESSION

Over the past decade, numerous advances in microscopy have enabled the real-time imaging of endogenous biomolecules. The development of imaging methods such as highly inclined and laminated optical sheet and light sheet microscopy (6, 7) has made it significantly easier to visualize single protein molecules such as transcription factors in living cells. These landmark achievements are in part due to the development and usage of HaloTag and SNAP-tag protein tagging (**Figure 1**) and organic dyes (8–10). Recently, it has been possible to image multiple single proteins by fusing HaloTag and SNAP-tag protein tags to a protein of interest. HaloTag and SNAP-tag specific ligands are conjugated with organic dyes (Janelia Fluor 549 and 646). They are incubated with the cells and passively enter the cell nucleus. When these liganded dyes are bound by their cognate protein tag, they emit several-fold more light and thus achieve an excellent signal-to-noise ratio (10). This method has been used successfully by several groups to quantify the residence times of transcription factors, CCCTC-binding factor (CTCF), and cohesin (9, 11–13).

Observing the real-time behavior of single protein molecules of RNA polymerase, transcription factors, and cofactors gives us the events that lead to activation as well as those that do not. Therefore, the ability to characterize RNA synthesis and expression is key to determining how gene activity is functionally modulated. It is possible to observe nascent transcription in live cells using the RNA stem-loop and cognate protein imaging systems MS2 and PP7 (14–16) (**Figure 1**), as well as RNA aptamer–dye-based methods such as Mango (17, 18), riboswitches (19), and programmable RNA binding (20, 21). Nearly all our knowledge of transcriptional bursting in living cells comes from the MS2 imaging method, which is based on the strong interaction between the MS2 coat protein, which is fused to green fluorescent protein (GFP), and an RNA stem-loop (**Figure 1**). Repeats of this stem-loop are inserted into a gene of interest using clustered regularly interspaced short palindromic repeat–CRISPR associated protein 9 (CRISPR-Cas9)-directed homologous repair (22). Additionally, MS2-GFP is stably integrated into the genome to provide a constant supply of coat protein. Upon transcriptional activation of the gene, the MS2 stem-loops are transcribed and are bound by multiple MS2-GFP proteins. Activation is then visualized using standard widefield microscopy and appears as a bright, punctate, diffraction-limited spot. Upon termination of the transcript, the RNA diffuses away, and the transcription site is no longer observed. This method can be used as a proxy for active and inactive periods of nascent RNA synthesis or transcriptional bursts; however, it is important to consider that this measurement is the transcript dwell time and includes time needed for transcript elongation and termination.

While these live-cell methods elucidate the real-time dynamics of transcriptional activation, it is less feasible to use them to characterize the steady-state RNA output across thousands of cells. Single-molecule fluorescence in situ hybridization (smFISH) enables the quantification of RNA in thousands of fixed single cells (**Figure 1**). Originally designed to look at levels of ribosomal RNA in oocytes of *Xenopus* chromosomes (23), single-molecule visualization is achieved through the use of 40–50 small, approximately 20-base-pair probes labeled by single fluorophores. When a probe-set binds a specific transcript, the enrichment of multiple probes to a single transcript manifests as a bright, punctate spot in fixed cells. This methodology enables the characterization

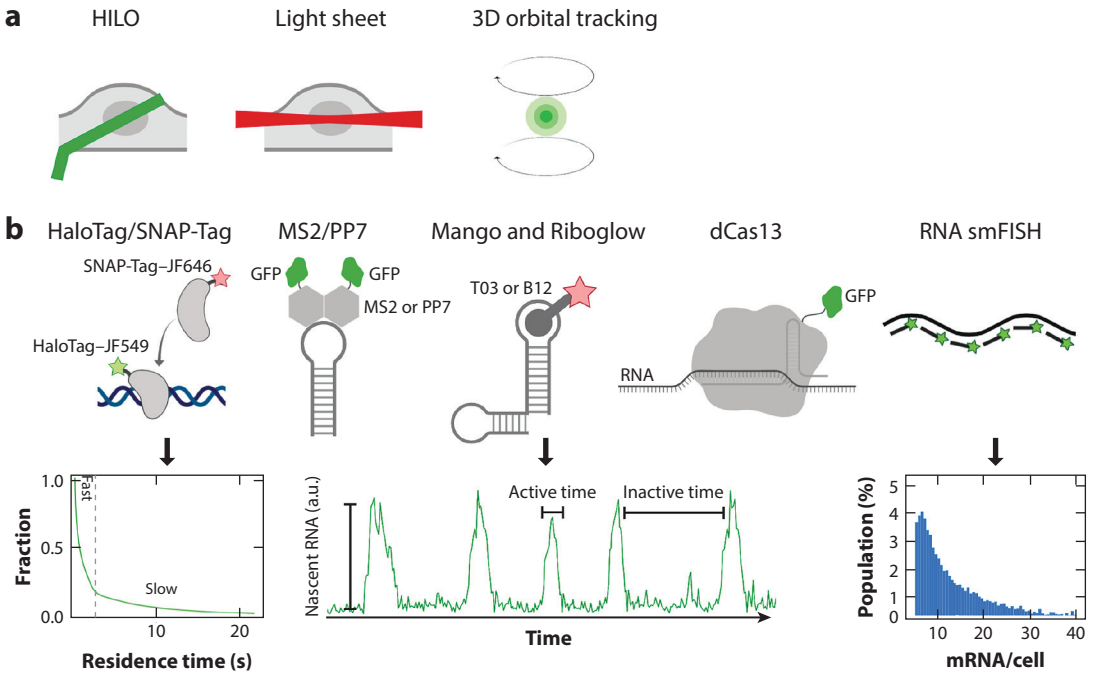

Figure 1

Advances in imaging methods for investigating stochastic transcription. (*a*) Several imaging modalities have increased the signal-to-noise ratio and enabled longer timescale imaging of single biomolecules. Both highly inclined and laminated optical sheet (HILO) and light sheet microscopy excite a thin section of cells, effectively reducing the amount of out-of-focus light. Three-dimensional (3D) orbital tracking enables long-term single molecule tracking by rotating the laser focus above and below the molecule of interest, thus reducing the bleaching rate. (*b*) HaloTag and SNAP-tag protein tags are fused to a protein of interest, and when cells are incubated with the liganded dye, they are bound by the respective tags and can emit several-fold more light relative to unbound dye. Popular dyes used for this imaging method are Janelia Fluor 549 and 646 (JF549 and JF646). MS2 and PP7 imaging rely on the high-affinity association of the MS2 or PP7 coat protein, which is fused to green fluorescent protein (GFP) and its cognate RNA stem-loop. Other methods such as Mango and Riboglow rely on RNA structure to bind dye-conjugated molecules such as T03 and vitamin B12. Catalytically inactive Cas13 (dCas13) uses a guide RNA to find and bind complementary RNA. Lastly, RNA single-molecule fluorescence in situ hybridization (smFISH) uses multiple labeled probes to image single RNAs in fixed cells. Each of these methods can be used to extract different protein and transcription parameters in living and fixed cells. Figure adapted from image created with BioRender.com.

of the single-cell distribution of an RNA in a fixed-cell population using discrete RNA counts. The subsequent RNA distributions can then be characterized as having a normal or broad distribution and can suggest different modes of regulation using modeling. Taken together, these technologies allow us to observe several parts of the dynamic biochemical process of transcription that are otherwise averaged out in population or snapshot methods. By characterizing how DNA-binding protein residence times change or how gene activity or expression is modulated in response to stimuli, we can obtain a unique and dynamic perspective of gene regulation.

WHAT CAUSES A GENE TO BURST?

Proximal Regulation

Transcriptional activation is rate limited by the binding of sequence-specific factors to DNA proximal to the gene transcription start site, followed by the assembly of the RNA polymerase II (Pol II)

PIC and initiation and elongation of the nascent transcript. Here, we specifically discuss the role of regulatory regions surrounding the core promoter where the PIC assembles, along with general transcription factors and other transcriptional activators. Distal regulation by enhancers is discussed in the section titled Distal Regulation. In the simplest model, transcriptional bursts are initiated by the binding of transcription factors to *cis*-acting sequences within promoters. Direct evidence for this model comes from yeast. Mutagenesis of approximately 20 yeast promoters indicated that burst frequency (i.e., the propensity to fire) was the primary quantity that changed with mutation (24). Similarly, exposing these promoter proximal sites by introducing nucleosome disfavoring sequences resulted in a higher frequency of active states (25). Moreover, by tuning the size of the nucleosome disfavoring region, the rate of transcriptional bursting could be controlled to an even greater extent than by changing transcription factor binding sites (26). These data were also indirectly supported by a large-scale measurement of the dose response of thousands of synthetic promoters, which demonstrated that gene output was determined by transcription factor concentration (27). At the single-molecule level, indirect evidence comes from the observation that the search times of the Mbp1 transcription factor, as inferred from diffusion measurements done with fluorescence correlation spectroscopy, were similar to the burst frequencies of a reporter gene (16). Finally, in a recent live-cell single-molecule study that used orbital scanning microscopy to directly visualize the transcription factor Gal4 activating the target gene *GAL10*, Donovan and colleagues (28) directly showed that Gal4 binding occurs immediately before the start of the transcriptional burst. These data overwhelmingly support an activator-centric view of bursting in yeast, in which the frequency of bursting is rate limited by the successful binding events to cognate binding sites in the region proximal to the transcription start site.

Although this model is appealing in yeast, there are several reasons it might not immediately apply to metazoans. For example, the human genome is larger, and *cis*-regulatory elements can be located farther from the genes they regulate. Moreover, unlike yeast promoters, metazoan promoters in general are less uniformly accessible (29). Nevertheless, related results about the importance of the promoter proximal region have been reported in mammalian cells, even though transcription factors must search through a massive array of off-target binding sites (30). As in yeast, increasing transcription factor levels would allow these activators to sample promoters more frequently, and thus bursting could be more likely to occur. This modulation has been observed for the *FOS* gene, for which transcription factor levels are positively correlated with the frequency of *FOS* bursts, as measured through the number of active transcription sites per cell by smFISH (31) and for a glucocorticoid-responsive gene, as determined through MS2 imaging of RNA in living cells (32). Transcription factor levels also exhibit dynamic behavior manifested as pulsatile and oscillatory expression levels (33, 34). This feature would allow regulatory elements to tune the probability of activation by integrating multiple dynamic signals through sampling rate. Moreover, the *cis*-acting sequence composition of individual promoters, including the number and affinity of DNA regulatory elements, can also contribute to burst frequency (35). Systematic perturbation of promoter proximal *cis*-acting sequences was shown by smFISH to regulate burst frequency, size, or both features of a major histocompatibility complex class I gene in primary B cells (36). For example, mutation of the initiator element of the promoter did indeed change burst frequency, but mutation of the binding site for the specificity protein 1 transcription factor affected burst size. Finally, changes in promoter sequence also occur naturally and result in diversification of bursting dynamics. For example, *Dictyostelium* has more than 30 actin genes, 17 of which code for the same protein and are expressed at comparable levels. Yet the bursting dynamics are highly variable, and this variability is conferred by the promoter region (37).

These results indicate that different aspects of bursting are modulated by transcription factors and coactivators that are recruited to promoters located proximal to the transcription start site.

Transcription factors can bind to a range of degenerate consensus sequences that can positively or negatively affect the binding affinity. If transcription factors spend more time bound at a specific promoter, burst frequency should be positively correlated with binding affinity. This increased transcription factor dwell time could increase the probability of forming a PIC and subsequent rounds of transcription.

Distal Regulation

Enhancers are crucial for transcriptional activation of genes in many processes, including development, circadian rhythms, and nuclear receptor activity (38–41), and can be located megabases away from their target genes (42). The general mechanism of enhancer function is still unclear. Enhancers contain *cis*-acting sequences that can recruit transcription factors and chromatin remodelers (43). Enhancers can both activate and repress transcription (33), and two major models of enhancer-mediated activation have been proposed. In the first, since enhancers often contain many transcription factor binding sites that lead to the recruitment of cofactors and Pol II, it is posited that enhancers that are in close proximity to their target gene could increase the local concentration of the above factors (**Figure 2**). This local increase in transcription factor levels could increase the promoter sampling rate and thus increase the probability of initiating a

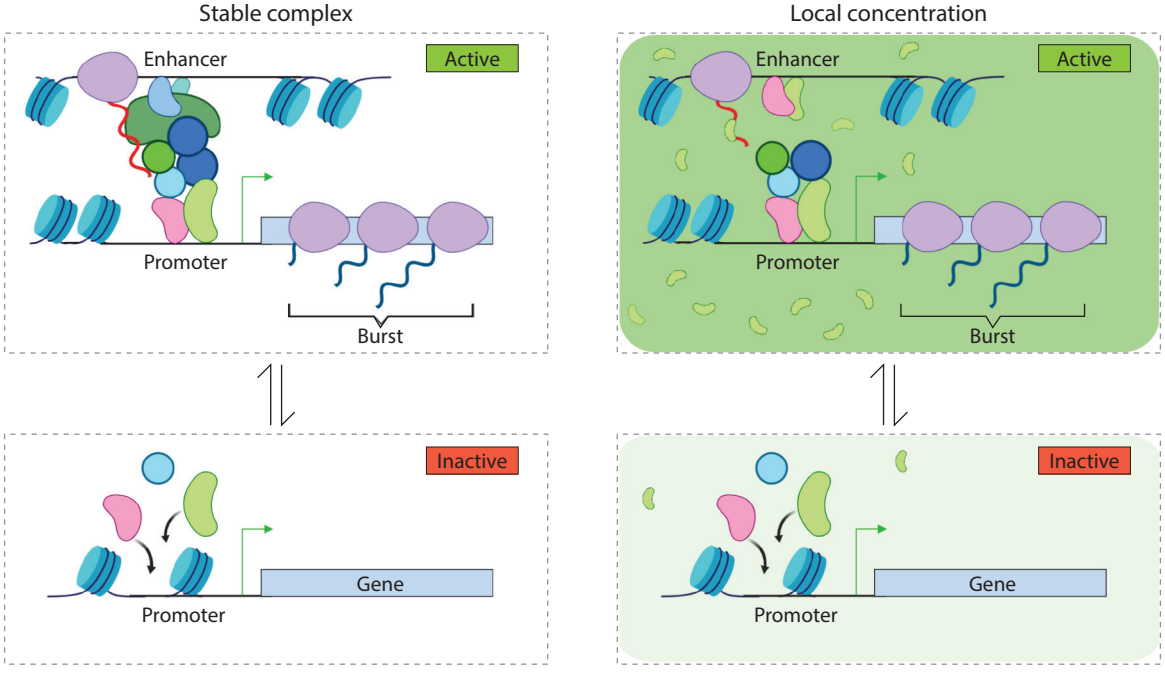

Figure 2

Simple model of transcriptional bursting. Genes can toggle between active (*top*) and inactive states (*bottom*). Two models of enhancer-mediated activation have been proposed. During transcriptional activation, genes can form stable complexes with distal enhancers (*left*). Factors that modulate stability of this complex can lead to loading of multiple polymerases and are known as transcriptional bursts. Inactive states are periods during which the gene is being sampled by transcription factors to reposition nucleosomes or actively repressed. No RNA is produced during these periods. Additionally, enhancers in close proximity to a gene promoter might increase the local concentration of factors needed for activation (*right*). This increase in local concentration increases the probability of forming a preinitiation complex at the promoter. Figure adapted from image created with BioRender.com.

transcriptional burst (44, 45). In the second model, transient chromosome looping between enhancers and promoters could allow deposition of Mediator, transcription factors, or even chromatin remodelers on the target gene promoter. This transient looping could lead to the formation of a stable complex that recruits machinery needed to activate target genes (46). How stability of this enhancer promoter complex contributes to bursting dynamics is unknown. However, if stability is necessary for transcriptional initiation, prolonged stability could lead to re-initiation events, which increase the burst size (**Figure 2**). Interestingly, enhancer reporter constructs have been shown to simultaneously activate two genes, such that the bursts are coordinated in time (47). These data argue that enhancers function through proximity rather than acting as stable complexes. However, these data do not preclude the possibility that multiple complexes are formed at the same locus or that these complexes turn over on faster timescales than the resolution of the experiment.

Enhancers have been shown to increase the probability of transcriptional bursts at a gene locus (48–50). Increases in probability of transcription can be interpreted as an increase in the fraction of cells that are activated in response to stimuli. With live-cell RNA imaging, this modulation can be observed at a single locus as more frequently occurring transcriptional bursts. Using MS2 labeling of the estrogen-responsive *TFF1* gene, we showed that CRISPR deleting a proximal enhancer located 10 kb away slightly reduced the number of RNA produced per transcriptional burst but substantially lowered the frequency of activation (51). These data indicate that enhancers can affect both burst frequency and duration. Yet the fact that the *TFF1* gene continued to transcribe in an estrogen-dependent manner indicates the use of other *cis*-regulatory sequences. One possible interpretation is that alternative or redundant enhancers form less-stable contacts with the promoter, resulting in decreased duration of active periods. Furthermore, the decreased stability of the alternate enhancer–promoter complex might lead to fewer productive activation events and hence lower observed bursting frequency. Another possibility, which is not mutually exclusive, is that the alternate enhancer is simply less likely to form contacts with the promoter. Whether these effects are mediated by the stability of *trans*-activators or loops in chromatin is unknown. However, given that promoters are often regulated by multiple enhancers (52), it will be important to investigate how enhancers coordinate gene bursting and how the intrinsic activity of the enhancer affects individual bursts.

Chromosome proximity assays have shown that upon induction, enhancers and promoters are spatially close to each other, leading to the hypothesis that proximity is necessary for transcriptional activation (53, 54). Using live-cell MS2–PP7 imaging of RNA coupled with DNA imaging through insertion of exogenous sequences, Chen and colleagues (50) showed that enhancer–promoter proximity of the even skipped locus during *Drosophila* development is crucial for transcriptional activation of individual loci. Moreover, Bartman and colleagues (49) used a zinc-finger fusion protein that bound both the locus control region and the β-globin promoter region. This protein increased looping between the enhancer and the promoter and subsequently increased the frequency of transcriptional bursting for the β-globin locus in mammalian cells. However, it is unclear whether proximity is a general requirement, as enhancer–promoter proximity is not required for transcriptional bursting of *SOX2* (55). Compounding this issue has been the difficulty in determining how frequently enhancers contact promoters as a function of time, how long they maintain proximity, and how these features contribute to the activation strength and variability of individual bursts (56).

In addition, enhancers often exhibit uni- and bidirectional transcription, which raises questions about the functional role of enhancer RNAs (eRNAs) in transcriptional bursting (41, 57, 58). Several studies have investigated the importance of eRNAs on *cis* gene activation by either knockdown of the eRNA (59–62) or insertion of a polyadenylation site immediately after the enhancer

transcription site (63, 64). These studies show that lowering eRNA levels leads to a reduction in target gene activation. So what is the mechanistic role of eRNAs in gene activation? If eRNAs help stabilize enhancer complexes (59, 61), their presence may be correlated with larger burst sizes. The eRNA could also be necessary for removal of the pausing factors negative elongation factor and DRB sensitivity-inducing factor from each Pol II within a burst, allowing Pol II to enter elongation (65). However, only a few eRNAs, including the distal regulatory region (DRR) eRNA transcribed from the *MYOD1* enhancer, have shown the ability to *trans*-activate another gene when either tethered to a reporter gene or overexpressed off a plasmid (60, 66–68). In support of these studies, eRNAs have been chromatin immunoprecipitated at loci different than their *cis* targeted gene (59). For example, the *trans*-activating eRNA transcribed from a *MYOD1* enhancer called the DRR specifically targets and activates the myogenin locus, even though it is transcribed from a different chromosome (68). Surprisingly, 60% of DRR eRNAs were colocalized with myogenin transcriptional bursts. Moreover, a significant fraction of myogenin transcriptional bursts (55%) exhibited colocalized DRR eRNA, suggesting that this DRR eRNA is predictive of myogenin transcription. Interestingly, the DRR eRNA has a short half-life of 30 min, longer than other measured eRNA half-lives (7.5 min) (65, 69). The ability of an eRNA to function in *trans* as a signaling molecule would depend on its half-life and the time needed to find its target. Given that most eRNAs are unstable, it seems unlikely that many eRNAs function in *trans*.

Post-Inititation Regulation

Genome-wide assays have shown that Pol II signal is enriched 50–100 base pairs proximally from the transcription start site, suggesting that Pol II pauses shortly after initiation (70, 71). The transition from the paused to elongating phase of transcription is a regulated step accompanied by phosphorylation of the C-terminal domain on serine 2 (72). Given the large number of proteins involved in transcriptional initiation, proximal polymerase pausing was proposed as a mechanism to keep polymerases stably engaged for rapid induction of heat-shock responsive genes (71, 73). However, a recent study has illustrated that polymerase pausing may be more dynamic than the prevailing view suggests. Fluorescence recovery after photobleaching of GFP-RPB1 showed multiple populations of Pol II with different rates of fluorescence recovery. After performing this technique in cells treated with drugs that inhibit different stages of transcription and modeling, Steurer and colleagues (74) inferred the length of time Pol II spends in various stages and the fraction of polymerase that makes the transition from initiation to pausing to elongation. Mathematical modeling of these measurements allowed them to infer a polymerase pause time of approximately 40 s (74). They also calculated that only 10% of paused polymerases enter productive elongation, in agreement with other live-cell estimates of polymerase initiation and release carried out on gene arrays (75). These studies indicate that polymerase pausing is a highly dynamic process, and in response to environmental stimuli, genes modulate this rate-limiting step from pause release into elongation. These data contradict bulk cell studies that used triptolide to inhibit polymerase initiation to measure Pol II pausing half-lives. In these studies, promoter Pol II pausing half-lives are on the order of 2.5–20 min and are longer than enhancer Pol II pausing half-lives (76, 77). Given that enhancer transcription is prevalent in metazoans, it is possible that single-molecule measurements are skewed toward enhancer Pol II pausing. Until recently, the contributions of paused polymerase to transcriptional bursting have been difficult to tease apart from initiation and polymerase recruitment. By integrating smFISH with bulk cell assays, a recent study determined that polymerase pause release is a key regulatory feature of transcriptional bursts that is modulated in response to biological stimuli (78). Thus, pausing allows cells to mount a rapid transcriptional response simply by modulating how many polymerases are released during each transcriptional burst rather than modulating polymerase recruitment.

Topological Regulation

The structure of the DNA helix is intimately connected to proximal and distal regulation and also to post-initiation control. The ability of *trans*-acting factors to find their cognate binding sites—be they located proximal or distal to transcription start sites—depends on the accessibility of DNA, which is in part determined by the extent of supercoiling (79). Likewise, DNA supercoiling is a structural byproduct of RNA transcription observed in both prokaryotes and eukaryotes with the potential to impact transcription dynamics. The prevailing model is that positive and negative supercoiling are created in front of and behind polymerase, respectively, as it reads through the gene locus (80). Therefore, supercoiling could limit the duration of transcriptional bursts by acting as a structural constraint: Each polymerase that enters into productive elongation during a burst contributes to the cumulative total of supercoiling. Ultimately, these structural constraints would lead to a negative effect on transcriptional initiation, ending the burst and potentially leading to a refractory period. Indeed, bulk cell assays have shown that supercoiling negatively affects transcription of both ribosomal RNAs synthesized by RNA polymerase I and polyadenylated RNA transcripts synthesized by Pol II (81–83).

Relieving this structural constraint must therefore be a crucial regulatory event for single bursts. Type I and type II topoisomerase enzymes can relieve this stress induced by supercoiling. Using an elegant, real-time, single-molecule assay to visualize transcription reconstituted with T7 or *Escherichia coli* RNA polymerases, Chong et al. (84) observed that positive supercoiling accumulates and inhibits transcription initiation and elongation on a 12-kb reporter template. This inhibition was relieved in a concentration-dependent manner by the type II topoisomerase DNA gyrase. Moreover, in intact cells, bursting of the *lac* operon was dependent on the expression level of DNA gyrase and the rates at which the enzyme bound the locus. Insertion of a strong gyrase site next to the *lac* operon enhanced bursting of this locus, suggesting that both gyrase concentration and binding rates influence transcriptional bursting. Thus, both in vitro and in bacterial cells, transcriptional bursting was dependent on the activity and concentration of type II topoisomerase DNA gyrase.

There are also examples of this requirement in eukaryotes. For example, topoisomerase type II and components of the DNA damage repair pathway are recruited to glucocorticoid and estrogen receptor gene targets. These factors are necessary for the estrogen-mediated activation of *TFF1*, and double-stranded breaks are observed at the *TFF1* promoter as well as glucocorticoid receptor gene targets (85, 86). These double-stranded DNA breaks are transient and site-specific consequences of DNA topoisomerase type II activity at the promoter. Furthermore, live-cell imaging of RNA synthesis of an HIV-1 reporter coupled with mathematical modeling led to a model of polymerase spacing dependent on DNA torsional stress (87). Specifically, the authors proposed that supercoiling generated by each elongating polymerase is an active mechanism to maintain a certain distance between polymerases in a burst. The prediction from these studies is that if supercoiling is a barrier to initiation and elongation, we would expect to see burst sizes generally conserved among most organisms. Overall, the role of DNA topology in transcriptional bursting has not been systematically addressed in eukaryotes.

WHAT SETS THE DURATION OF A BURST?

Simply put, a transcriptional burst describes correlations between active polymerases or nascent RNA: One transcribing polymerase is likely to lead to another. The null hypothesis—the non-bursting gene—is a limiting case in which each transcribing polymerase is independent. If the polymerases are independent, and the probability of initiation is uniform in time, the distribution of mRNA in the cell will follow a Poisson distribution (88). The property of bursts most easily

characterized by a number of experimental approaches is the burst size, which is the number of transcribing polymerases over which correlations occur. Burst sizes have been reported from 1 to >200 (3). A more difficult quantity to measure is the burst duration, which is the timescale over which these multiple initiation events occur. Burst durations—where they have been measured with techniques such as live-cell imaging, single RNA counting in fixed cells, and single-cell RNA sequencing (scRNA-seq)—have been reported from seconds to minutes to hours (51, 89, 90). From a regulation perspective, the scope and modulation of burst size has implications for how a cell responds to a stimulus and how such a response might lead to variability in the downstream gene products (91). Burst duration, by contrast, contains more mechanistic information and provides direct clues to the molecular mechanisms that determine the burst.

Recent work suggests that burst sizes are smaller and more uniform than previously hypothesized. In yeast, live-cell imaging of nascent RNA using MS2 and PP7 determined that the *GAL10* gene has a burst duration of approximately 70 s and a burst size of about 2 RNA (92). In mouse cells, simultaneous imaging of Pol II and β-actin nascent RNA produced from the endogenous locus demonstrated clustering of polymerases at active genes with a duration of 5–30 s (93). In human cells, live-cell imaging of nascent RNA produced from the endogenous *TFF1* locus also showed small burst sizes (about 2 RNA) with short active periods (approximately 30 s). Critically for that gene, the off periods showed large variation (51). With high time-resolution imaging, it was possible to resolve individual bursts that might have appeared as a single large burst under a different labeling or imaging strategy (**Figure 3**). Recent genome-wide scRNA-seq measurements coupled with modeling of variation reported median burst sizes of approximately 3 RNA (94). These results are in contrast to earlier measurements, which reported extensive burst size modulation or large bursts of transcriptional activity. Ultimately, experiments that rely heavily on computational models or inference of transcriptional bursting from protein time series have more caveats than direct live-cell imaging of nascent RNA, and these latter methods have yet to identify genes that show large transcriptional bursts.

Based on this evidence, we suggest that burst size and duration are thermodynamic properties of transcription, related to the stability of activators, the PIC, or the nucleosome, for example. Support for this view comes from in vitro reconstitution experiments that visualized transcription at

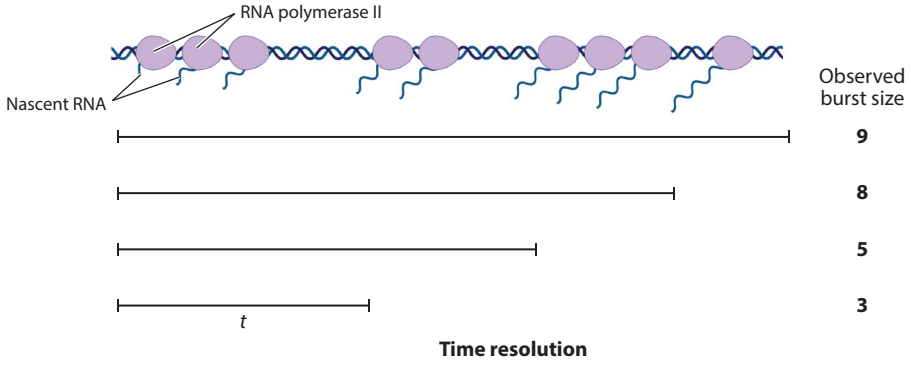

Figure 3

Limits of live-cell methods to determine burst size. Quantification of burst sizes is limited by the time resolution of the method used to observe transcriptional bursts. For example, fluorescent proteins that have been used to infer burst dynamics have longer maturation times than the burst duration as observed by methods that directly visualize nascent transcription. Thus, several bursts could be summed to give larger burst sizes. Figure adapted from image created with BioRender.com.

the single-molecule level through combined imaging of polymerase, general transcription factors, and nascent RNA. Revyakin, Zhang, and colleagues (95–97) observed that transcription factors such as transcription factor IIB (TFIIB) showed a residence time of a few seconds, which also corresponded to the period over which polymerases initiated. In this same experimental setup, they also observed multiple rounds of transcriptional initiation, from 2 to >10 events, which is close to the burst sizes observed by live-cell imaging in vivo. Similarly, Senecal and colleagues (31) showed that increased promoter affinity by transcription factors leads to increased burst size of the *FOS* gene. Finally, in the only study that simultaneously measured transcription factor dynamics and nascent RNA production, Donovan et al. (28) tracked single Gal4p activators interacting with the active *GAL10* locus in yeast. They observed a dwell time of approximately 30 s at the active locus, and changes in the activator dwell time through mutation to the *cis*-acting upstream activating sequence directly manifested as changes in the burst duration. This measurement relies on a new technology applied to in vivo transcription: orbital tracking microscopy. The microscope setup consists of a laser beam that orbits the actively transcribing gene, thereby allowing active positioning of the nascent RNA in the center of the orbit (98). Simultaneously, proteins that interact with the active locus can be observed passing through the orbit of the laser. The technique enables observation of a large dynamic range, encompassing both the on and off rates of protein on DNA that occur on the order of seconds and the bursting timescales that occur over minutes to hours. Together, these studies demonstrate a causative role for activator dwell time in determining burst duration.

Overall, the similarity in burst sizes across genes and organisms, coupled with the emerging body of work suggesting that frequency modulation is the more common regulatory mechanism (47, 49–51, 89, 99), argues against burst size as a regulatory knob in transcription. Burst sizes can be changed, but it is often through perturbation rather than regulation. For example, chemical inhibition of the chromatin reader tripartite motif-containing 24 (TRIM24) changes the burst size of the estrogen-regulated *TFF1* gene. Similar results have been obtained by perturbing lysine deacetylases (see the section titled What Is the Role of Chromatin in Bursting?). Moreover, core promoter sequences seem to confer different burst sizes (88), and systematic mutagenesis of *cis*-acting promoter elements for a reporter gene change burst size, as described above (35, 36).

The lack of evidence for large cooperative bursts of transcription involving many polymerases initiating over an extended period of time suggests a mechanism closer to transcription reinitiation (100). Transcription reinitiation or recycling of components of the PIC was described in vitro by several labs (101–103), and the comparison to bursting was suggested early on in single-molecule studies. However, limitations in cellular single-molecule measurements and potential artifacts in in vitro experiments precluded making a convincing quantitative comparison. This comparison should be revisited for two examples for which there are in vitro and in vivo live-cell data. Kraus & Kadonaga (104) measured transcription rates from a chromatinized estrogen-responsive promoter in vitro. They carried out studies under conditions that favor either a single round of transcription or transcription reinitiation and concluded that ligand-activated estrogen receptor promotes transcription initiation and reinitiation, with the latter being the larger effect. In fact, in the presence of estradiol, there was a 5-fold increase in the number of rounds of transcription, which is within a factor of 2–3 of the burst size measured for the endogenous estrogen-responsive gene *TFF1*. Thus, an important component of the ligand-activated response in solution is reinitiation. Similarly, for an HIV-1 viral template, allowing reinitiation results in an approximately 10-fold increase in the rounds of transcription (105), which is remarkably similar to the burst size measured in living cells (approximately 19 RNA) (87). The latter study reported the existence of regularly spaced polymerase convoys, which are functionally similar to bursts. These convoys depend on Mediator, as evidenced by the observation that a knockdown of *MED11* reduced the size of the convoy by a

factor of 2. Notably, Mediator has also been shown to be part of a reinitiation intermediate, along with other general transcription factors (106). Thus, two transcriptional paradigms—the estrogen response and an HIV-1 reporter—show intriguing concordance between in vivo and in vitro studies. Future studies that rely on simultaneous imaging of protein and nascent RNA will be essential in solidifying this connection.

WHAT IS THE ROLE OF CHROMATIN IN BURSTING?

The nucleosome presents an obstacle that must be negotiated by *trans*-regulatory factors and RNA polymerase, but the precise role of the nucleosome in modulating transcriptional bursting remains enigmatic. In part, the difficulty in defining the contributions of nucleosomes to bursting arises from the technical challenge of labeling and imaging specific nucleosomes on specific genes during the act of transcription in the nucleus. In contrast to transcription factors and nascent RNA, which can be visualized with reasonable contrast against nonspecific events, the nucleosome is refractory to these approaches: Even if the gene of interest is identified, most of the signal from an optical focal volume will arise from nucleosomes on regions of the genome that are likely not transcribing. For example, a rough approximation of the nuclear volume in a diploid human cell is 0.25 pL, containing 6 billion bases, which, when divided by the approximate focal volume of 0.1 fL, gives 2,500 voxels, each containing 2.4 million bases and 16,000 nucleosomes, assuming isotropic distribution. Superresolution approaches (107, 108) will improve the contrast, but it remains to be seen whether a 25-fold improvement in optical resolution in all three dimensions is experimentally feasible in living cells. Thus, most of our information about the role of nucleosome dynamics in transcriptional bursting comes from bulk perturbation of histones followed by single-cell readouts, sequencing approaches adapted to give kinetic information, or single-molecule imaging of nonspecific dynamics in the nucleus.

The most well-studied aspect of this question is the turnover of histones on DNA, which is related to the stability of the nucleosome. Early microscopy studies relying on fluorescence photobleaching recovery of GFP-labeled histones revealed that histones could rapidly turn over on DNA on timescales of minutes (109, 110). In recent years, pulse-chase methods followed by chromatin isolation and sequencing have provided a genome-wide view of histone dynamics with single-gene specificity. Pioneering studies from the Henikoff (111) and Rando (112) labs measured mean histone lifetimes of approximately 1 h in flies and yeast. Importantly, peak turnover occurred just downstream of the transcriptional start site of active genes (111). Both studies relied on biochemical isolation of histones synthesized during a defined period of time, either by incorporation of a methione surrogate azidohomoalanine during translation (111) or by transcriptional activation of an epitope-labeled histone protein (112). Likewise, the histone variant H3.3 is incorporated into chromatin independently of DNA replication, allowing it to be a complementary record of where histone turnover is occurring during transcription (113). Enhancers that are important for differentiation show high turnover of H3.3 (114), and H3.3 becomes deposited in active chromatin during gene activation (115). Strikingly, the turnover of H3.3 at promoters is directly proportional to expression levels, and there is an overall correlation between gene activity and the deposition of this histone variant. The histone turnover time of H3.3 in mammalian cells—approximately 2 h at promoters and enhancers—is similar to the time between transcriptional bursts in estrogen-responsive genes (51, 89).

Histones are also posttranslationally modified, but the causative role of these modifications in transcription is subject to debate (116). The best-studied modifications are acetylation and methylation of the histone tails, and these modifications correlate with changes in nucleosome stability (117). Perturbation of the lysine acetyl modifications has been shown to modify bursting

dynamics—for example, in the way that inhibiting lysine deacetylases with trichostatin A results in changes to the bursting dynamics from a prolactin reporter construct (90). Likewise, studies on the *Bmal1* gene demonstrated that acetylation-mediated control of transcriptional burst frequency was important in timing of circadian expression (118). Here, the authors specifically increased acetylation of *Bmal1* by targeting p300 to the locus with dCas9 and observed a concomitant increase in burst frequency. Histone acetylation has also been shown to modulate burst size and frequency in mouse neurons (119), and burst size was shown to be controlled by acetylation for the estrogen-responsive *GREB1* gene (89). Similarly, for the estrogen-responsive *TFF1* gene, knockdowns of the chromatin reader TRIM24, which binds H3K23ac and H3K4me3, resulted in changes in burst duration, but burst frequency remained unchanged (51). However, these data do not specify whether histones are themselves the mechanistic intermediate of lysine deacetylase inhibitors (120), considering that many enzymes are regulated by acetylation (121).

The half-life of an acetyl modification on a histone is measured in seconds or minutes (122), but histone methylation turns over with slower kinetics. This longer timescale of histone methylation changes likely corresponds to transcription dynamics on much longer timescales than transcriptional bursting. In a recent study, components of different repressive complexes including DNA methyltransferase 3B, embryonic ectoderm development (part of the polycomb repressive complex 2 that methylates histone H3 at lysine 27), Krüppel-associated box (a zinc-finger transcription factor involved in chromatin regulation through H3K9Me3), and histone deacetylase 4 (a deacetylase whose substrates include histones 3 and 4) were recruited to a fluorescent reporter gene to determine repression and derepression kinetics (123). The reporter gene consisted of an H2B-citrine transcription unit adjacent to Tet operator sequences in the promoter region, all of which were flanked by insulators and integrated on a human artificial chromosome. Recruitment of repressive complexes occurred through the Tet repressor, and the experimental readout was the disappearance of fluorescence signal with respect to a control fluorescence reporter. While histone deacetylase 4 recruitment induced reversible repression that lasted 5 days, embryonic ectoderm development and Krüppel-associated box recruitment exhibited 2- to 3-week repression. Related mechanisms could also lead to inherited bursting dynamics that were methylation dependent (124). Here, the authors followed single *Dictyostelium* cells through cell division and observed similar bursting frequencies of genes between mother and daughter cells compared to randomly chosen cells in the same field. This inheritance required the methylation of H3K4, which is associated with gene activation. In both cases—repression and activation—methylation seems to play a general role in the activity of the gene but not the timing of individual bursts. Strong repressive mechanisms can completely inhibit transcriptional bursts, likely through chromatin compaction, thereby inhibiting transcription factor accessibility to enhancer and promoter regions. For such long repressive timescales, one might expect periods of monoallelic expression in which bursts would be observed only for one allele. This phenomenon was recently observed for the estrogen response and in T cell differentiation (125). In both instances, multiday repressive periods were observed for individual alleles. Similarly, Dey and coworkers (126) used random integration of a lentiviral fluorescent protein reporter to show that genomic locations displaying high expression noise were more associated with repressed chromatin. The clones that exhibited the highest variability had more inaccessible chromatin, as measured by DNase I sensitivity. The authors concluded that such regions of the genome permitted transcription only in rare bursts interspersed with long off periods, leading to high variability in gene expression. In summary, although histone turnover bears a strong resemblance to transcriptional bursting dynamics, it is presently unclear what role, if any, modifications of those histones directly play in the dynamics of bursting.

Single-molecule studies in vivo and in vitro provide a more nuanced view of the role of nucleosomes in transcription initiation and hence bursting. Although it is obvious how nucleosomes

might hinder access of sequence-specific transcription factors to their cognate sites and hence alter on rates, nucleosomes also play a profound role in modulating the off rate of transcription factors. Single-molecule measurements in vitro demonstrated that Gal4 and LexA show 1,000-fold accelerated off rates when binding to a site near a positioned nucleosome. Luo et al. (127) postulate a competition model in which the nucleosome essentially drives off the transcription factor from DNA. This same dwell time (approximately 15 s) was observed for Gal4 in vivo using single-molecule imaging, and the dwell time correlates well with the duration of transcriptional bursts from the *GAL10* gene. Moreover, changing the dwell time of Gal4 through mutagenesis of the upstream activating sequence changed the burst duration accordingly (28). In addition, the authors could visualize stable binding of Gal4 immediately before a burst, indicating that binding of the activator was the rate-limiting step. These data suggest that nucleosome remodeling per se may not be the rate-limiting step in activation, but nucleosome dynamics near the binding site may play a defining role in limiting the duration of the burst. An integrated mathematical model that attempts to predict the average lifetime of the nucleosome-free TATA box in front of the *GAL10* promoter arrives at a value of approximately 12 s (128), which is close to the measured Gal4 dwell time from live-cell measurements (28, 128). Finally, a complementary technique used to directly visualize the nucleosome occupancy in an ensemble of purified DNA templates also inferred a quantitative relationship between the bursting behavior of the *PHO5* gene and the number of nucleosome-free templates (129). In summary, although much attention has been given to the role the nucleosome plays in reducing access, the role it plays in terminating bursts may be more important.

From these data, it is possible to construct a plausible generic model for the role of the nucleosome in bursting. Successful transcription factor binding is the rate-limiting step but instigates remodeling or removal of critical nucleosomes. This nucleosome turnover is empirically observed as the histone lifetime on DNA but is a downstream consequence of transcription factor binding. Sequence-specific transcription factors recruit the general transcription machinery (e.g., Mediator, TFIID, A, B, F, H, E, Pol II) through a well-characterized sequence of events, ultimately leading to the productive synthesis of nascent RNA. However, this productive state is short lived (lasting approximately 15–30 s), initiating only a few RNA polymerases, and is actively removed by ATP-driven processes such as nucleosome remodeling. In this model, each and every burst is accompanied by nucleosome remodeling or removal and occurs during the brief period when the DNA near the transcription start site is free of nucleosomes.

WHAT IS THE CONNECTION BETWEEN BURSTING AND ARCHITECTURE?

Whole-genome measurements of chromosome conformation capture or proximity (Hi-C) suggest that chromosomes can be organized into distinct structures (130, 131). This organization partitions the genome into compartments and topologically associated domains (TADs) that are presumed to have structural and transcriptional roles (132). Compartments correspond to active and inactive regions of chromosomes and are thought to form through attractions between heterochromatic regions (133); TADs are proposed to arise through loop extrusion (134). The location of genes and enhancers within TADs can also have regulatory functions (43, 135). Enhancers are posited to interact more frequently with genes within a TAD than with regions in adjacent TADs (131, 135–139). However, single-cell imaging studies using DNA FISH show that chromosome interactions captured by Hi-C measurements correspond to infrequent instances of colocalization and are only modestly increased within TADs (140). In other words, chromosomal regions within a TAD are only approximately 2-fold more likely to interact than chromosomal regions in

adjacent TADs. Also, acute depletion of TADs results in only subtle changes to gene expression (141).

So what happens to TADs during gene induction? We applied Shannon entropy as a measure of uncertainty to estimate enhancer and genomic contact specificity. Applying this metric to 3-enzyme Hi-C data from different estradiol concentrations, we observed that upon induction, enhancers form more specific contacts but rarely reach the level of a single, well-defined loop (51). Single-cell imaging studies carried out with DNA FISH tiling approaches have also shown that chromosomes can exhibit different conformations (107, 140, 142–144). For example, Bintu and colleagues (107) were able to map chromosome structures in single cells at approximately 30-kb genomic resolution and approximately 50-nm spatial resolution through iterative probe hybridization, imaging, and centroid localization. Using these data, the authors showed that different chromosome configurations exist in the cell population. Moreover, they could recapitulate Hi-C maps obtained from pooled cells by summing across single chromosome interaction frequencies. If enhancers form specific contacts that are constrained within transiently forming TADs, one would expect that individual genes interact with different sets of enhancers over time. These sets of enhancers could impart different bursting dynamics or, alternatively, could lead to long periods of inactivity if no compatible enhancers are located within the TAD. Therefore, the stability of TADs could contribute to how frequently genes burst and subsequently increase or decrease expression noise. CTCF and cohesin are important for establishing certain TADs, and their disruption can lead to a merger between two adjacent TADs. Moreover, ablation of TAD boundaries can lead to enhancer hijacking, aberrant gene activation by inappropriate enhancers due to the merging of adjacent TADs (145, 146). Similarly, CTCF sites can stabilize enhancer and promoter interactions, and upon deletion of a CTCF site near an enhancer, one observes an increase in expression variability in single cells and fewer enhancer–promoter interactions (147). Yet the mean expression level is hardly changed, arguing for counterbalancing effects on burst size and burst frequency. Understanding TAD dynamics and heterogeneity in relation to gene activity is an active area of research.

Genes have also been found to be clustered within the nucleus in what are termed transcription hubs or transcription factories. In one model, genes are recruited into regions of high Pol II density to facilitate transcription initiation by centralizing factors involved in activation (148, 149). This centralization effectively increases the concentration of factors locally and thus increases rates of transcription. Fanucchi and colleagues (150) observed coactivation of clustered genes in this manner. However, analyses of the dynamics of fluorescently labeled Pol II molecules by super-resolution microscopy show that Pol II clusters are transient, occurring for 5 s before dispersing (151). While this model is intriguing, it remains to be seen how multiple polymerases could be coordinated if genes are reeled in, as has been suggested for transcription factories (152).

WHAT DO GENOME-WIDE MEASUREMENTS REVEAL ABOUT BURSTING?

The idea of stochastic gene expression is many decades old (153). The first rigorous mathematical treatment connecting transcription dynamics to stochastic gene expression came later, resulting in the introduction of the prevailing telegraph model of bursting (154). In this model, the gene can be in one of two states: The on state is permissive for transcription, and RNA can be synthesized as long as the gene is in this state; the off state is the quiescent period during which no RNA is produced. The notion of bursting as a useful description for understanding gene expression heterogeneity came next, followed by the direct visualization of transcriptional bursting in living cells (155, 156). Two observations of this progression stand out. First, there has long been a close

coupling between theory and measurement in this field. Indeed, there is not sufficient space in this review to cover the large and growing body of work dedicated to developing quantitative models of gene regulation based on these phenomena. Second, measurements of bursting have always been tangled up with the causes and consequences of expression variability. In recent years, the explosion in scRNA-seq, coupled with other genome-wide approaches, has led to efforts to derive general principles of transcription. Here, we discuss how these approaches attempt to infer dynamic quantities from static measurements (mostly sequencing), using mathematical models as the connecting thread.

One approach is to use single-cell imaging approaches for a few genes or transcripts as a scale bar to interpret genome-wide data. Bartman et al. (78) used a comparison between nascent RNA measurements by smFISH and Pol II occupancy as measured by chromatin immunoprecipitation sequencing to estimate how bursting phenomena might relate to specific aspects of the transcription cycle, such as recruitment, initiation, pause release, and termination. They determined that polymerase recruitment, as measured by chromatin immunoprecipitation, occurs during the burst but that pause release is a critical determinant of the nascent RNA levels measured by smFISH. Indeed, during erythroid differentiation, the properties of bursting that changed were the burst frequency and the downstream pause release. Moreover, they concluded that their results were not consistent with the prevailing two-state model of transcription. These results stand in contrast to a model (for example) in which polymerase is recruited uniformly but then only released from pausing during a burst (78). One of the first studies to apply the conceptual underpinning of bursting to scRNA-seq data concluded that there is only a mild correlation between expression levels and burst size, and that pausing is weakly associated with bursting (157). Larsson et al. (94) used the initial two-state telegraph model developed by Peccoud & Ycart (154) to infer bursting from the distribution of mRNA across cells as measured by scRNA-seq. They provided evidence that burst size largely depends on promoter elements and burst frequency is the regulatory variable for most genes in response to stimulus or during differentiation (94). The strength of these approaches is the ability to leverage genome-wide downstream mRNA variation with specific sequences to infer the relationship between bursting and regulation. The weakness is that it is impossible to infer kinetic parameters from steady-state data without using at least one rate as an input, as has been pointed out previously (158).

One important caveat to these approaches is the assumption of a certain underlying statistical model of transcription. Interestingly, the underlying statistical model for differential RNA-seq analysis of bulk measurements using the differential gene expression analysis packages DESeq2 or edgeR is the negative binomial distribution (159–161), which arises as a natural limiting case of the original bursting model (162). Simply put, the negative binomial distribution allows for variance that is greater than the expectation from the Poisson distribution in which the variance and the mean are equal. This excess variance in RNA-seq could come from experimental error but is also expected, given the underlying mechanisms of transcriptional bursting. At this point, the two-state telegraph model (154) is the de facto description for mRNA expression, despite the fact that multiple live-cell studies indicate this model does not quantitatively describe bursting dynamics for all genes (51, 163).

CONCLUSION AND OUTLOOK

The thesis of this review is that breaking down the transcription cycle into the initiation, duration, and termination of a burst provides insight into the mechanisms of transcription and processes of gene regulation in the nucleus. These concepts also have particular synergy with the fast-growing field of single-cell transcriptomics. In fact, the union of concepts in stochastic gene expression

with experiments in single-cell sequencing presents an opportunity. Understanding the biophysical behavior of transcription in the mammalian nucleus, for example, may aid in the development of better clustering algorithms and more robust thresholds for detecting subpopulations of cells. Knowledge of the general dynamic principles of gene regulation will be essential in determining which subsets of cells are stable biological states and which are simply dynamic fluctuations. Ultimately, gene dynamics are impossible to infer from fixed snapshots in time. However, the scope of single-cell imaging is always growing, and the sensitivity of scRNA-seq is always improving, leading toward a convergence of these two approaches.

Much has been learned in recent years about the role of activators and enhancers in controlling bursting, but relatively little is known about how higher levels of organization in human cells, such as nucleosome organization, chromosome conformation, topological domains, or compartmentalization, impact transcription dynamics. Human transcription is sporadic—bursts are often separated by hours of inactivity—which may be due in part to these emergent properties of the nucleus. A major challenge in the coming years will be to experimentally observe chromosome structure with nanometer spatial resolution and subsecond time resolution while simultaneously monitoring functional readouts such as transcriptional activation. Such approaches will also be essential for resolving the role of liquid-liquid phase separation in transcriptional activation. These measurements, coupled with gene editing and high-throughput imaging, will enable investigators to generate a comprehensive view of transcriptional regulation at the single-gene level.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

This work was supported by the Intramural Research Program of the National Institutes of Health. We thank Tineke Lenstra, Murali Palangat, and the reviewing editor for critical reading of the manuscript.

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