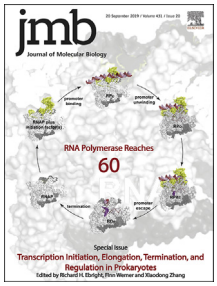
Review



Mechanisms of Transcriptional Pausing in Bacteria

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Abstract

Pausing by RNA polymerase (RNAP) during transcription regulates gene expression in all domains of life. In this review, we recap the history of transcriptional pausing discovery, summarize advances in our understanding of the underlying causes of pausing since then, and describe new insights into the pausing mechanisms and pause modulation by transcription factors gained from structural and biochemical experiments. The accumulated evidence to date suggests that upon encountering a pause signal in the nucleic-acid sequence being transcribed, RNAP rearranges into an elemental, catalytically inactive conformer unable to load NTP substrate. The conformation, and as a consequence lifetime, of an elemental paused RNAP is modulated by backtracking, nascent RNA structure, binding of transcription regulators, or a combination of these mechanisms. We conclude the review by outlining open questions and directions for future research in the field of transcriptional pausing.

Introduction

In 1973, Maizels [1] observed that *Escherichia coli* RNA polymerase (RNAP) transiently paused at discrete sites during transcription of the lactose operon *in vitro*. This was indicated by the accumulation and disappearance of RNA transcripts of discrete intermediate lengths over the course of the transcription reaction. That same year, Dahlberg and Blattner [2] reported a similar phenomenon, this time during RNA synthesis on bacteriophage λ DNA. These two seminal studies marked the start of an entire field of research on transcriptional pausing (Fig. 1), the breadth of which increased immensely over the past 4.5 decades to encompass not only basic research into the fundamental nature of a paused RNAP, but also its modulation by transcription factors across all domains of life, genome-wide sequence dependence of pausing, and the ever-growing body of important roles transcriptional pausing plays in fine-tuning gene expression.

The initially observed *in vitro* pausing of bacterial RNAP during transcription was confirmed on a variety of DNA templates in the 1970s and early 1980s, suggesting that pausing is ubiquitous and generating some insight into its underlying causes. In many cases, these pauses took place immediately following synthesis of an RNA hairpin structure: *E. coli trp* (tryptophan operon) leader and terminator [3–5]; *E. coli thr* (threonine operon) leader [6]; some sites in the *E. coli rrnB* (one of the seven ribosomal RNA operons in *E. coli*) leader [7]; some sites in bacteriophage λ t_{R1} terminator, located downstream of the structural gene for the lytic repressor cro [8]; and some sites in SV40 virus DNA F_1 region [9]. At other sites, however, RNAP paused following synthesis of an RNA apparently lacking a hairpin structure: the initially reported *E. coli lacZ* [1,10,11]

and bacteriophage λ 6S RNA [2]; phage T7 early DNA region [12]; other sites in the *E. coli rrnB* leader [7]; and other sites in the SV40 DNA F_1 region [9]. Thus, although not all *in vitro* pauses observed in early studies correlated with a predicted secondary structure in the nascent RNA, formation of a stable RNA hairpin did appear to signal RNAP to pause.

The phenomenon of transcriptional pausing was first demonstrated *in vivo* in 1981 by the Chambon laboratory in hen erythrocytes, where a transcription run-on technique (Box 1) revealed accumulation of eukaryotic RNAPII, or Pol II (we will use "RNAPII" throughout this review to avoid confusion with DNA polymerase II) at the 5′-end of the β-globin gene—an indication of RNAPII pausing proximally to the gene promoter [13]. The next milestone in the field arrived with the advent of methods to map RNAP binding to specific DNA locations *in vivo* (later termed chromatin immunoprecipitation, or ChIP; Box 1), first successfully applied by Gilmour and Lis [14] in 1984 to map bacterial RNAP binding sites across several genes in *E. coli* and *Salmonella*. Using ChIP in eukaryotes, transcription start site-proximal accumulation of RNAPII was demonstrated on a handful of additional eukaryotic genes in mid-1980s, in *Drosophila* and mammalian cells [15–17]. Importantly, by complementing ChIP with nuclear run-on experiments, the Lis laboratory showed these accumulated RNAPII molecules to be transcriptionally engaged but paused, as opposed to simply being tightly associated with the promoter [16]. In the mid-2000s, approaches that coupled ChIP with either microarray hybridization (ChIP-chip) or with high-throughput sequencing (ChIP-seq) revealed that RNAP pausing *in vivo* is not restricted to the promoter regions of specific genes but occurs globally at 5′ and 3′ ends of genes [18–22]. More recently, techniques mapping RNAP position at nucleotide resolution, including native elongating transcript sequencing (NET-seq, Box 1) and precision nuclear run-on and sequencing (PRO-seq, Box 1), established that transcriptional pauses are universal: they take place throughout the gene body across the genomes of different organisms, ranging from bacteria to human cells, and occur on average every 20–100 base pairs (bp) of DNA transcribed [23–26]. Altogether, these studies suggested that pausing is a general feature of transcription and is not limited to specific genes or specific locations within the genes.

Sequencing- and microarray-based approaches to detect transcriptional pauses *in vivo* have been complemented by imaging real-time transcription in live cells. In 2007, the Singer laboratory measured mammalian RNAPII transcription kinetics in living cells by using a combination of RNAPII fluorescent fusion and fluorescent phage MS2-labeled mRNA transcripts, generated from an engineered *lacO* gene array. Here, computational modeling of fluorescence recovery after photobleaching (FRAP) of tagged α-amanitin-resistant RNAPII revealed at least three kinetically different populations of RNAPII, representing enzyme engaged in transcription initiation, elongation, and pausing [27]. By implementing a version of this approach a decade later, this time with endogenously expressed mammalian GFP-RNAPII, Steurer *et al*. [28] assigned the paused kinetic population to the promoter-proximally paused RNAPII, with calculated residence time of only 42 s—in sharp contrast to much longer 20 min RNAPII spends bound to the chromatin. These findings suggested a rapid turnover of RNAPII at promoters due to termination of promoter-proximally paused RNAPII, which could contribute to regulation of gene expression by, for instance, keeping promoters of active genes free of nucleosomes.

As the tools to map and image RNAP pauses *in vivo* became more and more sophisticated, so did the tools to observe transcriptional pausing in purified *in vitro* systems—beyond the traditional workhorse, gel electrophoresis. Optical traps, single-molecule Förster resonance energy transfer (smFRET) microscopy, and fluorescence co-localization microscopy approaches allowed detection and monitoring of single molecules of RNAP in action [29–31]. Optical trapping studies in early 2000s, for instance, revealed that individual bacterial RNAP molecules pause every 100–200 bp of transcribed DNA—a number confirmed by *in vivo* RNAP mapping methods years later—for brief durations of 1–6 s on average at saturating 1 mM NTPs (Fig. 1) [32,33]. In addition, smFRET studies reported in 2005 and Co-localization Single-Molecule Spectroscopy (CoSMoS) studies reported in 2016 demonstrated at the single-molecule level that σ^{70}, which is historically thought of solely as a transcription initiation protein that aids in promoter recognition, can remain bound to a fraction of elongating RNAPs *in vitro* well past initiation [34,35], confirming an observation made in ensemble measurements earlier [36,37]. This retained σ factor then appears to modulate the behavior of RNAP during productive RNA synthesis, for example, by inducing pauses at promoter-like sequences and by blocking binding of competing transcription regulators to RNAP [34,38,39], although the question of σ retention *versus* re-binding *in vivo* remains open [40]. By monitoring initial transcription, that is, synthesis of the early short RNAs by RNAP, in real time with smFRET, the Kapanidis laboratory captured a long pause (~20 s) during the transition from a 6-nucleotide (nt) to a 7-nt RNA transcript at a *lac* promoter [41]. At around the time of the Kapanidis report, the Weiss group observed the "initiation pause" in their smFRET and single-molecule magnetic tweezers experiments [42]. The initiation pause may serve as a decision point between promoter escape to productive elongation and aborting RNA

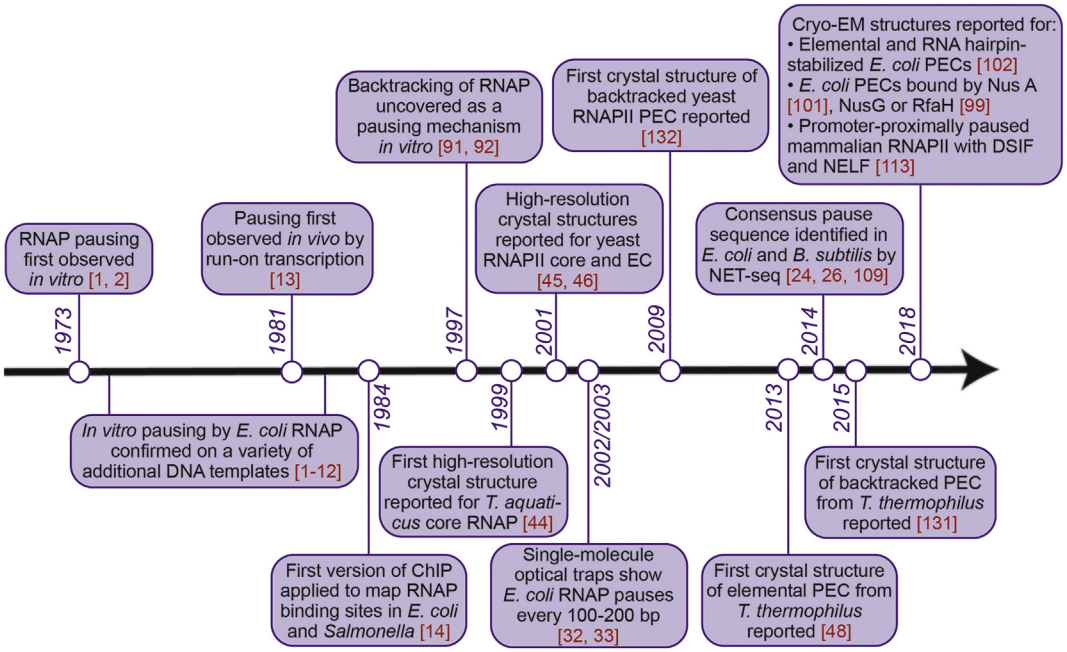


Fig. 1. Milestones in the field of transcriptional pausing research, with emphasis on pausing by bacterial RNAP.

synthesis, and is largely controlled by a specific region of the bacterial initiation factor σ^{70} [41,43]. Here, single-molecule studies revealed features of transcriptional pausing often hidden in ensemble measurements.

Along with the improvements in genomic analyses and single-molecule assays described above, structural studies of RNAPs both in prokaryotes and eukaryotes has progressed apace since 1999, and the new structural information greatly aided the design and interpretation of the biochemical assays. The first high-resolution crystal structure of a multi-subunit cellular RNAP, of a thermostable RNAP from *Thermus aquaticus* (*T. aquaticus*) [44] (Fig. 2A) provided a long-awaited structural framework to interpret decades of biochemical and genetic results. A *Saccharomyces cerevisiae* RNAPII crystal structure followed in 2001, confirming high structural conservation of RNAP from prokaryotes to eukaryotes [45,46] (Fig. 2B). Many crystal structures containing thermophilic bacterial RNAPs from *T. aquaticus* or *T. thermophilus* followed, including structures of an elongation complex (EC) and an elemental paused complex (ePEC) [47,48]. More than a decade passed until *E. coli* RNAP structures were determined in 2013 by three different groups independently [49–51]. During this time of great advancement in our structural understanding of the bacterial transcription cycle, x-ray crystallography was the only structural tool available, limiting structural studies to transcription complexes that could be crystallized and sometimes complicating structural analyses due to the confounding effects of crystal packing forces. Fortunately, thanks to the recent advances in cryo-electron microscopy (cryo-EM), structure determination of challenging targets, including RNAPs, at atomic resolution without crystallization became possible [52]. As a result, dozens of structures of transcription complexes without the influence of crystal packing forces have been reported in the last few years and deepened our understanding of transcription, including transcriptional pausing control, which we cover in the Mechanisms of Pausing by RNAPs and Regulation of RNAP Pausing by Transcription Factors sections.

Pausing of RNAP during transcription is broadly involved in regulating gene expression in both prokaryotes and eukaryotes. Generally speaking, transcriptional pauses define windows of time and space for co-transcriptional regulatory events to occur. In some cases, pausing of RNAP at specific positions on the DNA template provides a time window to allow the interaction of the transcribing complex with small molecules, regulatory proteins, or RNAs [53–55]. In other cases, pauses make transcribing complexes susceptible to termination, thus prematurely terminating RNA synthesis [56,57] and decreasing RNA abundance in the cell—unless regulatory proteins stabilize these complexes for continued transcription ("antitermination"). The body of specific examples of the established and emerging roles transcriptional pauses play to regulate gene expression continues to grow. In eukaryotes, promoter-proximally paused RNAPII physically blocks nucleosome re-assembly ("nucleosome occlusion") to keep promoters open and accessible to activator and transcription factor binding; allows rapid or

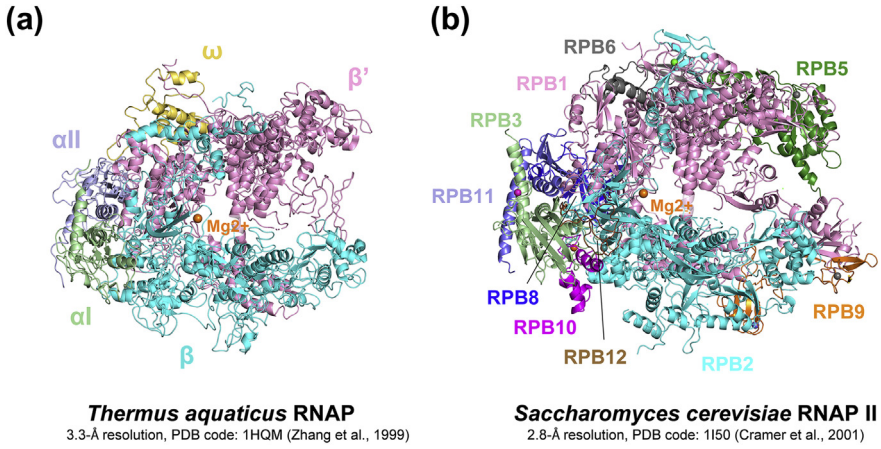


Fig. 2. First high-resolution structures of RNAP. (A) First high-resolution structure of a bacterial RNAP, *T. aquaticus* RNAP core at 3.3 Å resolution [44]. (B) First high-resolution structure of a eukaryotic RNAP, *S. cerevisiae* RNAP II core at 2.8 Å resolution [45]. All subunits are labeled on the figure, and catalytic magnesium is drawn as an orange sphere in the center of the structures.

synchronous gene activation; and couples elongation and co-transcriptional RNA processing (5′-capping, 3′-end processing, splicing), such that nascent RNA is protected from degradation and efficiently matures into a functional mRNA [58,59]. In bacteria, transcriptional pauses control co-transcriptional folding of nascent RNA into its biologically functional forms, such as catalytic RNAs or alternative structures of riboswitch or attenuator RNAs [60–64]. Paused bacterial RNAP gives the translating ribosome time to catch up and release RNAP from the pause, thereby synchronizing transcription with translation [65,66]. Transcriptional pauses at the intrinsic (factor-independent) termination sites provide time for a terminator hairpin to form [67]. Pausing also serves as a key first step in Rho-dependent termination of transcription by stalling RNAP at terminators long enough for Rho protein to bind nascent RNA and dissociate it from the transcribing complex [68]. Finally, RNAP pausing precedes excision of misincorporated nucleotides, thus playing an important role in proofreading and maintaining fidelity of transcription [69–71].

While paused RNAP regulates co-transcriptional events like RNA folding and regulator binding, these events and regulators in turn modulate pausing behavior of RNAP [53,60,72,73]. Pausing of RNAP *in vivo* is fine-tuned by a plethora of cellular regulators, a few of which are discussed in this review. Although the knowledge of intricacies of pausing regulation *in vivo* might make it an attractive target for applied research, before this complex regulation can be fully exploited, we must first understand the basic molecular mechanisms by which RNAP enters and escapes a transient pause state, alone and in the presence of regulatory proteins. Accordingly, this review focuses on the accumulated biochemical and structural insights into the structural and mechanistic basis of transcription pausing.

Mechanisms of Pausing by RNAPs

Types of transcriptional pauses

RNA synthesis during productive elongation takes place inside an EC, composed of RNAP and the nucleic acids (DNA and nascent RNA). Transcript extension within the EC occurs one nucleotide at a time through a repeating sequence of steps termed the nucleotide addition cycle (NAC) (Fig. 3). The NAC comprises (i) nucleic-acid translocation from pre- to post-translocated register, which vacates the binding site for the incoming nucleoside triphosphate (NTP) and aligns the next template DNA base for base-pairing; (ii) NTP binding; (iii) folding of the RNAP active site-proximal trigger loop (TL) motif into the trigger helices (TH); and (iv) formation of the phosphodiester bond [79]. Unfolding of the TH and release of pyrophosphate accompany the translocation step to prepare active site for the next nucleotide addition cycle. RNAPs from many gram-negative bacterial lineages, including *E. coli*, contain a large amino-acid sequence insertion (sequence insertion 3, or SI3) in the middle of their TL [80], which has to move during TL-TH transition, thereby affecting RNAP function during elongation, pausing, and proofreading hydrolysis [81–83]. Transcriptional pauses interrupt the NAC at one or more of the above steps in a paused elongation complex (PEC). Pauses characterized to date represent off-pathway events, meaning that they branch off of the main nucleotide addition pathway and kinetically compete

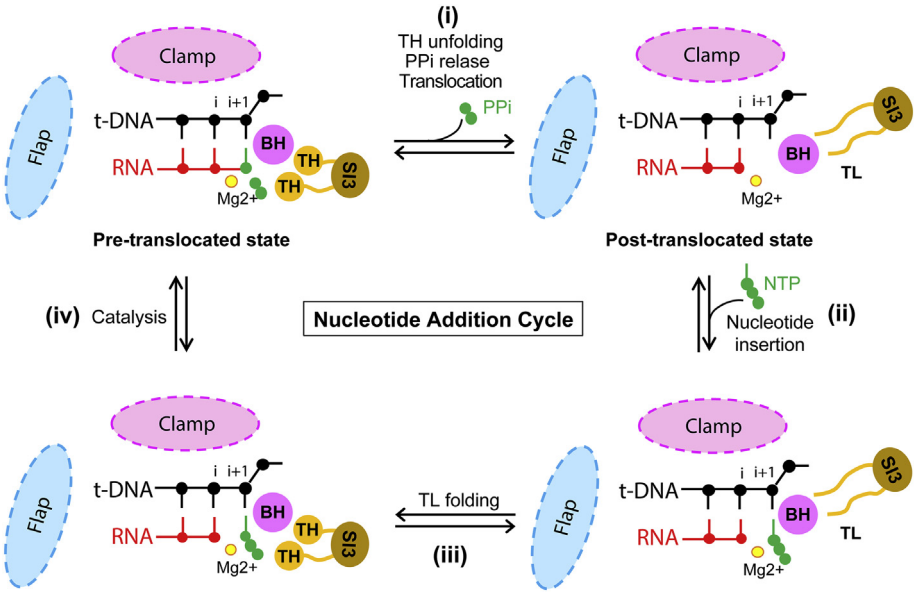


Fig. 3. Schematic diagram of nucleotide addition cycle. An active-site view of the nucleotide addition cycle by RNAP. A part of RNA–DNA hybrid in the proximity of the catalytic magnesium is drawn with the neighboring protein components, BH, TL, and SI3 connected to the TL. Clamp domain of RNAP forms the main channel that accommodates the RNA–DNA hybrid, and the flap domain is located at the upstream end of RNA–DNA hybrid. The locations of clamp and flap domains are marked by colored ovals, and their sizes are not to scale. Each round of nucletide addition occurs in the following steps: (i) nucleic-acid translocation from pre- to post-translocated register, which vacates the binding site for the incoming nucleoside triphosphate (NTP) and aligns the next template DNA base for base-pairing with NTP; (ii) NTP binding; (iii) TL folding into the trigger helices (TH); and (iv) formation of the phosphodiester bond. Unfolding of the TH and release of pyrophosphate accompany the translocation step (i).

with it [12,84] (Fig. 4). A corollary of this definition of pausing is that formation of the offline pause state involves a change in the structure of the EC that disrupts the NAC. Thus, a key feature of an off-pathway pause is that only a fraction of the ECs transcribing through the site enter the pause state, with the rest of the complexes moving past the site without pausing. An on-pathway, so-called pre-translocated pause has also been proposed to result from slow translocation from pre- to post-translocated register following nucleotide addition [85,86]. The phenomenon resulting from translocation bias toward the pre-translocated state, however, is best defined as a slow nucleotide addition step rather than a "pre-translocated pause" because the EC is not structurally rearranged during this slow step, and the pre- and post-translocated registers likely still equilibrate rapidly relative to the rate of nucleotide addition (see accompanying review of the mechanism of translocation by Belogurov and Artsimovitch [182]). Such slow nucleotide addition steps may promote pausing at a site by allowing time for formation of an off-pathway pause state [87,88]. Regardless of the detailed mechanism, pauses are caused by sequence-specific interactions of RNAP with DNA and nascent RNA rather than by stochastic fluctuations in the structure of EC independent of nucleic-acid sequence.

Transcriptional pauses are believed to initiate with an isomerization of the EC into an off-pathway, transient (lifetime of a few seconds), catalytically inactive elemental pause state, producing an elemental paused elongation complex (ePEC) [61,84,88] (Fig. 4). The ePEC can then further re-arrange into long-lived pause states by backtracking (i.e., a backward movement of RNAP that disengages the 3' RNA end from the active site and extrudes it into the secondary channel); by the formation of RNA secondary structures, such as hairpins, in the RNA exit channel that modify ePEC conformation [60]; by the action of transcription regulators, or a combination of several of these mechanisms. Backtracking appears to be the most common mechanism of stabilizing the initial elemental pause [89–92] and is favored when the RNA–DNA hybrid is destabilized by a UA-rich RNA–DNA hybrid or by nucleotide misincorporation into the transcript [71,93]. The two well-characterized pause examples in bacteria are accompanied by backtracking: the *ops* (*o*peron *p*olarity *s*uppressor) pause, which occurs in the early transcribed region of *E. coli* operons that encode or affect biosynthesis of extracytoplasmic macromolecules (e.g., hemolysin) [89]; and promoter-proximal pauses caused by σ^{70} factor failing to disengage from the transcription complex after initiation, thus allowing the σ^{70}-

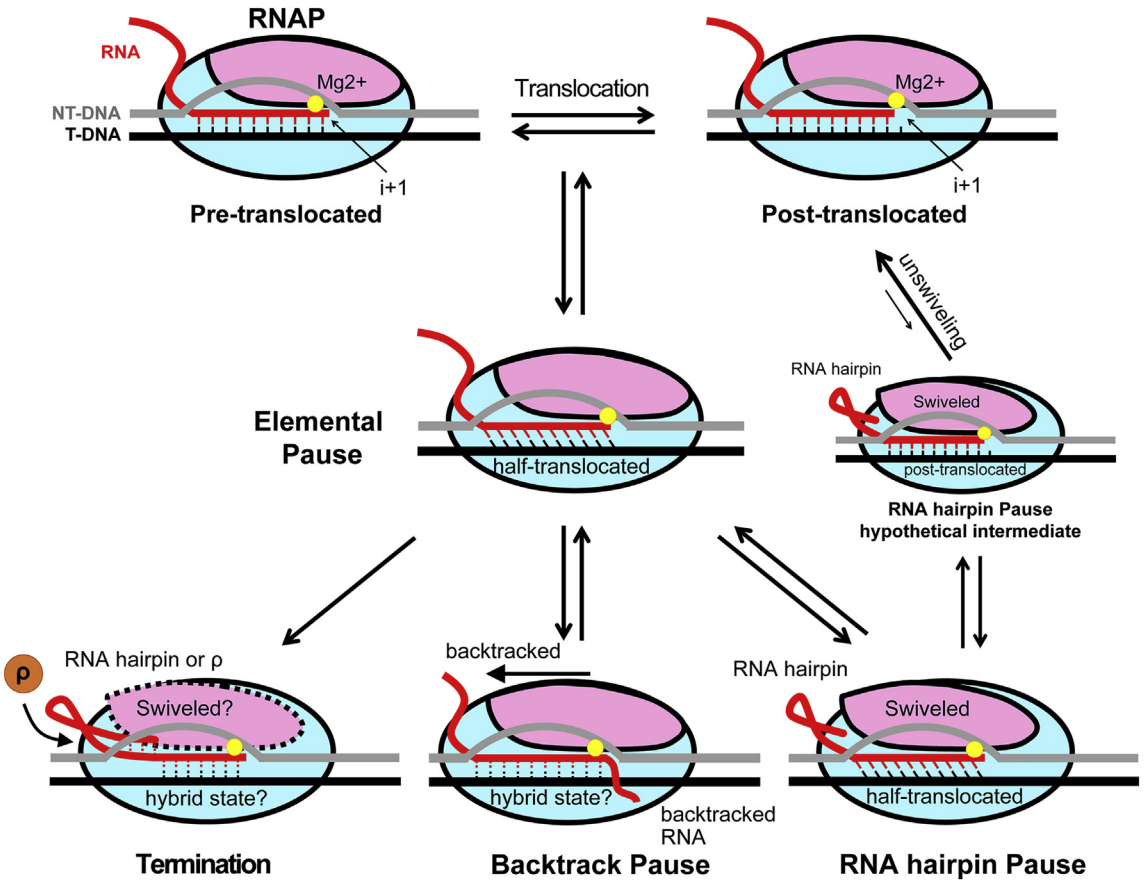


Fig. 4. Schematic diagram of transcriptional pauses. In the on-pathway process of nucleotide addition, RNAP translocates from pre-translocated to post-translocated register to vacate the $i+1$ site for the next nucleotide to bind as shown in the top part of Fig. 3. During this translocation, RNAP can isomerize into an off-pathway conformation that blocks catalysis for a few seconds in response to the nucleic acid sequence interacting with the enzyme. This isomerized state of an EC is termed the "elemental pause." Elemental paused EC samples multiple RNA–DNA hybrid registers, including the half-translocated register (see the main text). Elemental paused EC can further isomerize into longer-lived paused states, such as backtrack and RNA hairpin-stabilized pauses, or precede transcription termination. In an RNA hairpin-stabilized paused EC, RNA hairpin formation in the RNA exit channel induces conformational change of RNAP (swiveling), while RNA–DNA hybrid remains half-translocated. To escape a hairpin-stabilized pause, the minor population of swiveled RNAP with post-translocated RNA–DNA hybrid may be unswiveled, returning to the on-pathway. In a backtrack paused EC, RNAP moves backward on the nucleic acids, extruding the 3′-end of RNA into the secondary channel. Hybrid conformation here is drawn with dotted lines because both canonical pre-translocated and the half-translocated hybrid conformations have been observed in backtracked ECs. During intrinsic termination, RNA hairpin in a paused EC invades RNA–DNA hybrid, while Rho-dependent termination is likely preceded by an elemental pause. The conformation of RNA–DNA hybrid in the termination process is unknown.

associated ECs pause at −10-like promoter sequences downstream of the promoter [94–98]. Backtracking at the *ops* assists loading of the transcription factor RfaH onto the EC [53,99].

Another means of stabilizing the elemental pause is *via* formation of an RNA hairpin in the RNA exit channel of an ePEC. Formation of an RNA duplex in the RNA exit channel of an ePEC 11–12 nt upstream from a paused RNA 3′ nt prolongs the initial elemental pause ~10-fold [100]. An RNA hairpin-stabilized pause can be prolonged even further, up to 100-fold, by the transcription factor NusA [100,101], as described in later sections of this review. The spacing between the RNA duplex in the RNA exit channel of RNAP and 3′ end of the transcript determines the interactions of the duplex with the flap domain of RNAP [102,103]. Because of the specific spacing requirement between the 3′ nt of the elemental paused RNA and the upstream RNA duplex, hairpin-stabilized pauses likely occur infrequently, although genome-wide mapping of such pauses in any organism remains an important experimental challenge due to the complexities of predicting RNA structures. Hairpin-stabilized pauses are prevalent in attenuation control of enterobacterial amino-acid biosynthetic operons (e.g., the *his* pause

in the leader region of histidine biosynthetic operon), where they synchronize transcription of the attenuation control regions with translation of the leader peptide-coding regions. A hairpin-stabilized pause governs folding of a regulatory leader RNA in *mgtA* operon, which encodes an Mg^{2+} transporter in enterobacteria, into one of two mutually exclusive conformations, one of which serves as a substrate for Rho-dependent transcription termination at high intracellular Mg^{2+} [104,105]. Finally, RNA hairpin formation serves as a precursor to transcription termination, a platform for recruitment of anti-termination proteins (e.g., λN), and plays a role in reiterative transcription, or transcript slippage [60].

Sequence specificity of transcriptional pausing

Multiple lines of evidence over the years suggested that RNAP pausing is programmed by specific elements in the nucleic-acid sequence. *In vitro* work on the *his* pause in *E. coli* showed that certain base substitutions in the template DNA increased pausing, whereas others decreased it, and altering the 3′-terminal nucleotide at the pause site changed not only pause strength but also the location of the pause on the transcribed template [106]. Biochemical experiments with *E. coli* RNAP pausing in the early transcribed region of the bacteriophage T7 D111 deletion variant also pointed to the importance of the nature of the incoming NTP [107]. Single-molecule studies on *E. coli* RNAP transcribing an *rpoB* gene demonstrated a statistically significant variation in pausing as a function of template position [33]. A burning question became: is there a common nucleic acid sequence shared between different genes that drives RNAP to pause?

The efforts to identify the consensus sequence that causes transcriptional pausing date almost four decades back. In 1981, based on the analyses of several *E. coli* RNAP pause sites in the early transcribed region of bacteriophage T7 and its D111 deletion mutant mentioned above, Aivazashvili and colleagues [108] suggested that the rate of nucleotide addition was influenced by the identity of the nucleobase at the 3′ end of the RNA transcript, the identity of the next NTP to be incorporated into the paused RNA, and on the nucleic-acid sequence context in the immediate proximity of these two RNA elements. Sequencing of nascent RNAs from bacteria by NET-seq by several laboratories in 2014 and 2015, followed by sequence alignments of the observed pause sites, finally revealed a 16-nt consensus elemental pause sequence conserved among different bacterial RNAPs [24,26,109] (Fig. 5). The consensus pause signal is multipartite, as discussed in a later section of this review, where elements that have most pronounced effect on pausing propensity are the G_{-10} in the nascent RNA of the upstream fork junction and $Y_{-1}G_{+1}$, where -1 refers to the 3′ end of nascent RNA $+1$ represents the incoming NTP [24,84], although it is not known whether the sequence specificity comes from template DNA, non-template DNA, nascent RNA, or a combination of thereof. In addition to these sequence elements independently identified by Larson *et al*. [24], Vvedenskaya *et al*. [26], and Imashimizu *et al*. [109], the consensus determined by Larson *et al*. also contains a G_{-11} in the non-template DNA. The difference could potentially stem from the difference in PEC trapping methods between the studies, where relatively slow cooling of cells by Vvedenskaya *et al*. and Imashimizu *et al*. (*versus* flash-freezing in liquid nitrogen by Larson *et al*.) may have captured only the strongest contributors to pausing. The work by Imashimizu *et al*. further showed that PECs positioned on the consensus nucleic-acid sequence sample multiple translocation registers including post-, pre-translocated and 1-nt backtracked hybrid conformations [109].

In *E. coli*, the consensus pause sequence not only accounted for the known regulatory pause sites, such as attenuator pauses in *thr*, *leu* and *his* leader regions, but also allowed identification of ~20,000 additional previously undocumented *in vivo* sites [24]. In the case of expressed genes, these transcriptional pauses were enriched at translation start sites and occurred within the first 100 nt, in line with transcriptional pausing playing an important role in synchronizing transcription and translation in bacteria.

The elemental pause

As backtrack- and RNA hairpin-stabilized pauses were studied in more detail, a transient intermediate that connects these off-pathway long-lived pauses to the on-pathway efficient elongation was proposed and its existence supported by single-molecule optical assays and cross-linking experiments [33,89,110,111]. Newman *et al*. [33] found that 95% of RNAP pausing on the *rpoB* template exhibited transient pausing a few seconds long while the remaining 5% pausing lasted much longer, in excess of 25 s, probably aided by RNAP backtracking or nascent RNA hairpin formation. The major transient pausing was observed throughout the *rpoB* DNA template and was not affected by assisting *versus* opposing applied force, suggesting that this frequent pausing was rate-limited by neither backtracking nor hyper-translocation [33,110]. Based on realization that these non-backtracked pauses appeared to also precede the hairpin-stabilized and possibly the backtrack-stabilized paused states, this pause was named "elemental" [61].

Initial structural studies of elemental paused transcription complexes used x-ray crystallography [48]. Three separate crystal structures of *Thermus*

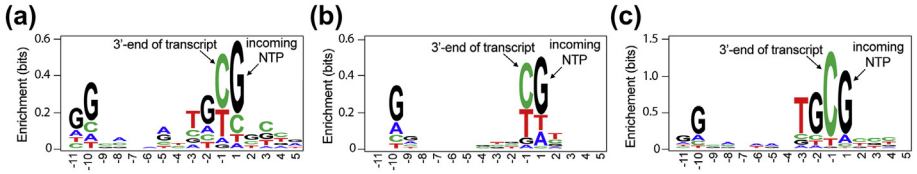


Fig. 5. Consensus elemental pause sequence. (A) Consensus sequence logo obtained by using NET-seq from *B. subtilis* and *E. coli*. Cells were harvested by filtration and flash-frozen in liquid nitrogen before RNA isolation. Native elongating transcripts were harvested by immunoprecipitating Flag-tagged RNAP, and the transcripts were convered to DNA for sequencing [24]. (B) Consensus sequence obtained by NET-seq in *E. coli* under conditions similar to those in panel A, except that the cells were not flash-frozen in liquid nitrogen but instead cooled on dry ice [26]. (C) Consensus sequence logo obtained by RNET-seq (read-length-specific NET-seq) [109]. Contrast to the experiments performed in above panels B and C, only the RNA region buried in RNAP was isolated and analyzed.

RNAP assembled on a minimal nucleic acid scaffold comprising the *E. coli his* pause sequence but without the RNA pause hairpin (so an elemental pause scaffold) were determined: one crystal form with *T. aquaticus* RNAP at 7.8 Å resolution, and two with *T. thermophilus* RNAP at 4.5 and 3.6 Å resolution. All three structures shared two common features, an open clamp and a kinked bridge helix (BH) that blocked substrate NTP binding. The higher resolution structure (3.6 Å) revealed the kinked BH trapped the template DNA base from entering the active site; thus, the complex was in a state of partial translocation. This active-site conformation resembled that of the active site structure in the α-amanitin-stalled *S. cerevisiae* RNAPII EC [112]. In addition, the RNA exit channel was widened, suggesting that an RNA hairpin could form within the RNA exit channel.

Recently, however, an ePEC structure was determined by single-particle cryo-EM suggesting different features for an elemental pause [102]. The cryo-EM sample was prepared with *E. coli* RNAP on a full nucleic acid scaffold comprising the *E. coli his* pause sequence including the RNA pause hairpin (see below). Thanks to the ability to separate distinct conformational states through cryo-EM analysis, a minor population (~12%) of the particles missing the RNA pause hairpin was detected (it is not known if the RNA hairpin was degraded or simply unformed in these particles). RNA hairpin-less ECs in the cryo-EM preparation led to a 5.5-Å electron density map with a 4.2-Å local resolution around the active site and showed the following preliminary features of ePEC. First, the clamp was closed in contrast to the crystal structures. Second, the active site exhibited a half-translocated state in which the RNA was post-translocated while the template DNA was pre-translocated. In this conformation, the NTP substrate-binding site was empty, but the template DNA base for the substrate was still base-paired with its non-template DNA partner, thereby inhibiting binding of the incoming NTP, consistent with the result that elemental pause escape was not dependent on the NTP concentration [84]. This half (or partial)-translocated state may be a common feature of paused transcription complexes, as it was also observed in an RNA hairpin-stabilized paused state, backtracked RNAPII and in promoter-proximally paused RNAPII structures [101,102,113,114] as discussed in a later section.

On the basis of the reported consensus sequence (see the Sequence specificity of transcriptional pausing section above) and structures of the ePEC discussed in this section, Saba *et al*. [84] recently performed a battery of biochemical assays to probe the molecular mechanism of elemental pause. The authors showed that the elemental pause signal is multipartite, similar to an RNA hairpin-stabilized pause [115]. The elemental pause dwell time was affected by mutations in the upstream fork junction, downstream fork, RNA–DNA hybrid, and downstream DNA duplex, implying that the elemental pause mechanism is complex, requiring orchestration of multiple interactions between RNAP and nucleic acids. In addition, the authors showed that the ePEC samples the 1-nt backtracked state in addition to the pre-translocated [24,26] and half-translocated [102] states, in agreement with a previous report [109], although backtracking did not limit the rate of pause escape on the consensus pause sequence. Instead, based on the results of the fluorescence-based translocation assays with the fluorescent guanine analog 6-methylisoxanthopterin (6-MI) at either the upstream fork of RNA–DNA hybrid or downstream DNA duplex of the transcription bubble, the authors proposed that progressing past the half-translocated state rate-limits escape from the pause, although the route by which this barrier is overcome remains to be determined. Interestingly, the rate of RNA–DNA hybrid translocation at the upstream fork was similar in non-paused and elemental paused ECs. This study triggers additional questions on the details of the elemental pause mechanism. For example, how the hybrid translocation at the upstream fork is uncoupled with downstream duplex translocation, and what interactions between RNAP and

consensus DNA or RNA sequence induce the elemental pause.

In summary, from the biochemical and structural studies of the elemental pause, we can conclude the following:

1) The elemental pause signal is multipartite: sequences at the upstream fork, hybrid, downstream fork and downstream duplex DNA affect pause duration [24,26,84,110]. Among the elements of the sequence, the upstream-fork G at -10 and the downstream-fork YG at -1 and $+1$ are most crucial for the pause, although the effect from other nucleic-acid scaffold areas might have averaged out in the analysis of biochemical pause-escape data.
2) An ePEC samples multiple translocation registers during its lifetime, including the half-translocated, pre-translocated, and 1-nt backtracked states. Post-translocated state cannot exist in an elemental paused state because ePEC does not bind NTP substrate while post-translocated state has an empty active site for the substrate binding. An ePEC ensemble likely consists mostly of pre-translocated and half-translocated populations, whereas a backtracked state exists only transiently, since backtracking did not affect pause life times although it was observed indirectly *via* GreA/B cleavage [84]. The equilibrium between pre- and half-translocated states might be determined by the DNA sequence.
3) Complete downstream DNA translocation to form the fully post-translocated state is the rate-limiting step for elemental pause escape. Nevertheless, as the elemental pause is caused by multiple signals spanning the upstream fork to the downstream DNA duplex, a more careful explanation of how multiple signals orchestrate a block to downstream DNA translocation is needed [84]. Determination of higher-resolution ePEC structures by cryo-EM, avoiding crystal packing issues, will deepen our understanding of the molecular mechanism of elemental pausing in transcription.

RNA hairpin-stabilized pausing

Since the discovery of RNA hairpin-stabilized pausing, studies have been directed toward uncovering the mechanistic basis for exactly how an RNA hairpin prolongs the lifetime of a transcriptional pause. In an early hypothesis, the RNAP was viewed as a rigid body that could not accommodate an RNA hairpin within the RNA exit channel [116]. Consequently, the folding of the RNA hairpin might "pull" the RNA transcript out of the RNA exit channel in the upstream direction, thus altering the nucleic-acid structure in the active site and inhibiting addition of the next nucleotide. This model was refuted by the finding that a 1-nt insertion between the RNA hairpin and RNA–DNA hybrid increased pause duration, instead of releasing the tension of RNA pulling and shortening the pause as predicted by the model [72].

Another general hypothesis for an RNA hairpin-stabilized pausing mechanism is an allosteric model in which global conformational changes in RNAP induced by RNA hairpin formation in the RNA exit channel modulate catalysis by altering the active-site architecture allosterically. Note that the RNA exit channel where the RNA hairpin forms is more than 50 Å away from the RNAP active site Mg^{2+}. To probe the active-site architecture at an RNA hairpin-stabilized pause, Toulokhonov *et al*. [72] performed cross-linking experiments between RNAP and RNA, where photosensitive cross-linkable nucleotide analogs were placed at -11 position of RNA and the 3′-end of RNA. These cross-linking experiments revealed that RNA duplex formation in the RNA exit channel did not change the cross-linking pattern at -11 position of RNA at the entrance of the exit channel but altered the crosslinking ratio between β and β′ subunits at the 3′-end of RNA near the active site, supporting the allosteric model. A nucleotide cross-linkable analog introduced into the loop region of RNA hairpin cross-linked to β flap-tip helix region of RNAP indicating the interaction between RNA hairpin and β flap-tip [103]. Interestingly, an RNAP mutant lacking the entire flap tip or flap-tip helix abolished or decreased the RNA hairpin-stabilized pausing on the *his* pause sequence without altering RNA hairpin formation or RNA–DNA hybrid register [103]. Furthermore, the pause suppressing RNAP mutants, β′ F773 V and β T563I, where mutations are located adjacent to the active site, exhibited slower RNA duplex formation in the exit channel, supporting the idea that the exit channel and active site are energetically linked [100].

In accordance with the crucial role of the TL in the NAC, TL dynamics has been shown to affect pausing as well [81,117–121]. Deletion of the TL reduced the fold-change in catalysis rates both in the paused and non-paused states, but diminished the effect of hairpin formation on pause duration [120]. A cysteine-pair reporter assay revealed that in the RNA hairpin paused RNAP TL movement is restricted. Thus, even if a hairpin-stabilized PEC samples the post-translocated register, which can bind NTP, the TL probably does not fold properly in response to NTP binding in the active site [119]. Complete deletion or small deletions in SI3 abrogate the RNA pause hairpin effect without affecting the elemental pause [81,83,122].

Aided by the recent technical advances in cryo-EM, structures of *his* pause elongation complexes

containing *his* RNA pause hairpin (*his*PEC) with and without NusA were independently determined by two groups [101,102]. The most significant structural features of the *his*PEC were consistent in both structures regardless of the presence of NusA. The interesting features observed in these structures are as follows (Fig. 6):

1) The RNA hairpin stem resided within the RNA exit channel, while the loop is exposed to the solvent and not resolved in the cryo-EM structure (Fig. 6A, B). The inner wall of the RNA exit channel is lined with positively charged amino acid residues, presumably assisting formation of an RNA hairpin within the channel.
2) Contrary to the expectation from crystallographic work, the RNAP clamp was closed, but not in the position seen in a non-pause EC. Instead, a set of RNAP domains including the clamp, jaw, shelf, and SI3 domains (mostly in β′ subunit) was rotated ("swiveled") by about 3° around an axis almost overlapping the BH (Fig. 6A, C). Swiveling explains the results of the cysteine-pair reporter assay with *his*PEC mentioned above: the swiveled clamp also favors the disulfide bond formation designed to promote open clamp conformation in the

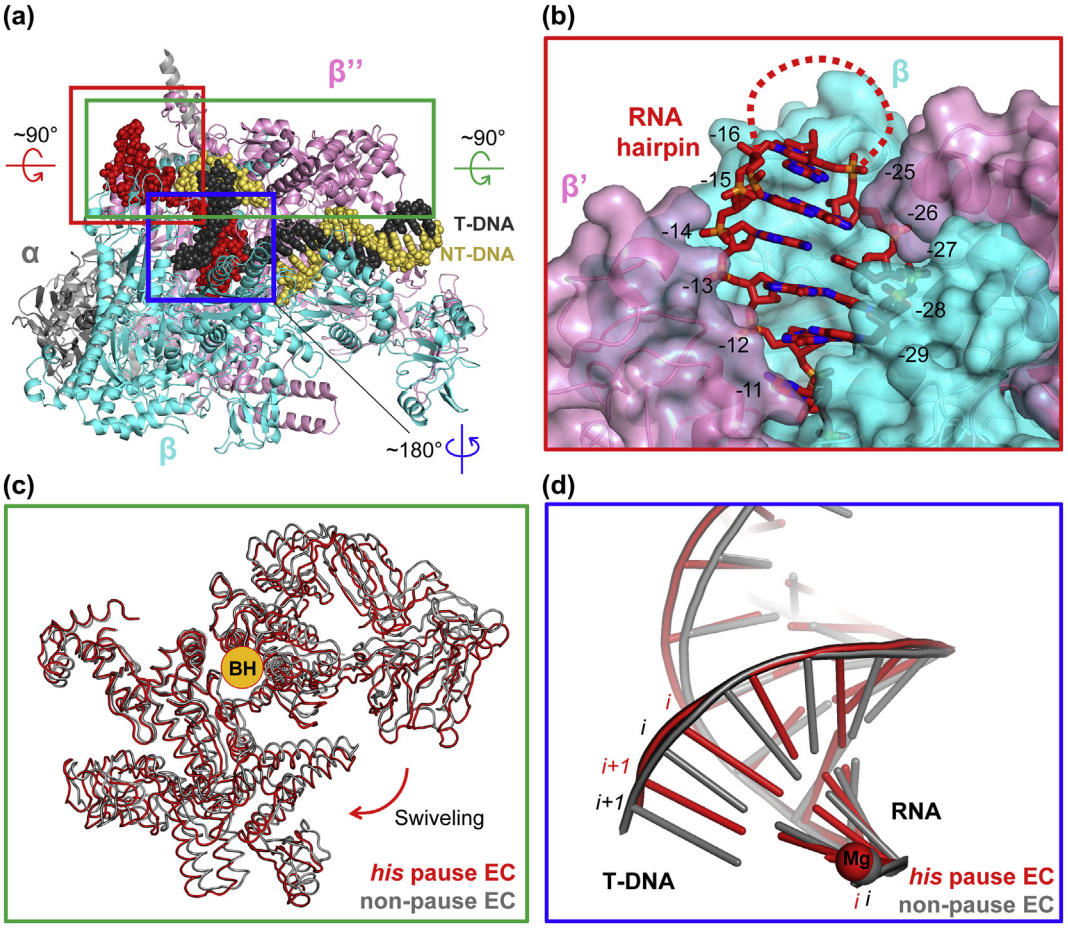


Fig. 6. Structural features of an RNA hairpin-stabilized pause elongation complex (*his*PEC). (A) The overall structure of *E. coli his*PEC. Specific areas are boxed, rotated, and zoomed for detailed description. (B) Red boxed region from panel A rotated by 90° and magnified to show RNA exit channel with *his* pause RNA hairpin stem duplex in it. RNA hairpin stem duplex formed fully and resided in the RNA exit channel while the loop region of the hairpin was disordered (marked by a dotted line). The opening of the channel widened compared to that of non-paused EC. The residues in RNA are numbered by their positions relative to the active site, where the RNA 3′-end nucleotide is at −1 position. The −11 RNA base connects RNA−DNA hybrid and RNA hairpin. (C) Green boxed region from panel A rotated by 90° and zoomed in. Comparison of Cα traces of swivel modules from *his*PEC and non-pause EC (PDB code: 6ALF). Compared to the swivel module of a non-pause EC, swivel module in *his*PEC rotated about 3° around the axis overlapping BH. (D) Blue boxed region from panel A rotated by 180° and zoomed in. Only nucleic acid structures were drawn for clarity. Non-pause EC exhibits a post-translocated hybrid while *his*PEC has a half-translocated hybrid, harboring DNA base in $i+1$ site base-paired with RNA base in *i* site.

assay. The swiveling movement inhibits nucleotide addition because the swiveled SI3 would clash with the β lobe upon TL folding, which is a crucial step for catalysis, explaining the pause-reducing effect from SI3 deletion [81,83,122].

3) The RNA–DNA hybrid was observed in a half-translocated state, as described for the ePEC in The elemental pause section (Fig. 6D). This is consistent with the idea that the RNA hairpin pause derives from the ePEC.

Based on the accumulated evidence so far, we envision the following model for the formation of an RNA hairpin-stabilized pause. First, RNAP pauses at a pause site *via* the elemental pause mechanism, providing a time window for RNA hairpin formation in the RNA exit channel. At this stage, the RNA–DNA hybrid assumes a half-translocated conformation, at least in a subpopulation of the complexes. Second, RNA hairpin formation allosterically stabilizes the swiveled conformer, thereby relocating SI3. This relocation inhibits proper folding of the TL for the NAC and blocks nucleotide addition for minutes. Unfortunately, the TL was disordered in both cryo-EM structures of hairpin-stabilized PECs and flap-tip helix was invisible in *his*PEC without NusA, complicating understanding of the roles of TL and flap-tip in RNA hairpin-stabilized PEC. This may suggest that TL, flap-tip, or both stabilize RNA-hairpin pause in a way that cryo-EM cannot reveal due to the dynamic conformational change, or play roles during the initial stages of entry into an RNA hairpin-stabilized pause rather than stabilizing the final paused state. Escape from a hairpin-stabilized pause requires both the reversal of swiveling and a shift of RNA–DNA hybrid to a post-translocated state. It is possible that a minor population, unresolved by single-particle cryo-EM, of the swiveled hairpin-stabilized PECs occupies the post-translocated register, which is capable of binding NTP but not catalysis due to inhibited TL folding (Box 2).

Parallels between bacterial RNAP and eukaryotic RNAPII pausing

Transcriptional pausing in eukaryotes plays critical roles in maintenance of nucleosome-free regions at promoters, activation of gene expression, RNA splicing, polyadenylation, and cellular differentiation and development [58,59,123,124]. In particular, promoter-proximal pausing by eukaryotic RNAPII is a rate-determining step for transcription elongation and serves as a checkpoint for transcript and RNAPII modification [74]. Promoter-proximal pausing has not been observed in yeast but is widespread in other eukaryotes, such as *Drosophila* and humans [19,125–128]. The recently reported cryo-EM structure of a promoter-proximally paused *Sus scrofa* (pig) RNAPII contains *Homo sapiens* DSIF (*D*RB *s*ensitivity-*i*nducing *f*actor) and NELF (*n*egative *e*longation *f*actor) [113]. DSIF is a heterodimer of Spt4 and Spt5. Spt5 is an ortholog of bacterial transcription elongation factor NusG, and the NusG family of regulators is the only transcription factor family conserved in all domains of life [129]. NELF contains four subunits, NELF-A, -B, -C/D, and -E. In the structure, NELF binds to the RNAPII funnel region, which is equivalent to the secondary channel in prokaryotic RNAP, bridges the core and shelf modules, and contacts the TL. Thus, NELF restrains mobility of part of RNAPII required for pause release. In addition, NELF prevents binding of the anti-pausing transcription elongation factor IIS (TFIIS) by occupying its binding site [130]. Interestingly, the RNA–DNA hybrid in the promoter-proximal paused RNAPII structure is half-translocated, superimposable with the elemental and RNA hairpin-stabilized PECs of *E. coli* RNAP near the active site, suggesting that the half-translocated conformation of the hybrid might be a universal mechanism for interrupting RNAP catalysis. In addition, Spt5 binds to the same site of RNAPII as NusG binds on *E. coli* RNAP [99].

A half-translocated RNA–DNA hybrid has also been observed in a structure of paused *S. cerevisiae* RNAPII EC stabilized by backtracking. Structures of backtracked PECs from multiple species and with various length of backtracked RNA have been reported [114,131,132], but only the *S. cerevisiae* RNAPII complex with a 9-mer backtracked RNA exhibited the half-translocated RNA–DNA hybrid, also called a "tilted" hybrid [114]. Why only this particular backtracked complex has a tilted hybrid is unclear, but neither the length of RNA nor the species origin of RNAP would explain this observation since other backtracked complexes that either contain RNAP from the same species or have backtracked RNAs of various lengths did not exhibit the half-translocated hybrid [132]. The nucleic-acid sequence used to reconstitute backtracked RNAP complexes, the way of assembling the backtracked complexes or the different crystal packing forces experiences by each complex might have influenced the observed hybrid conformation.

A half-translocated RNA–DNA hybrid was also observed in the structures of initial transcribing complex (ITC) of *S. cerevisiae* RNAPII [133,134], where the initial RNA–DNA hybrids shorter than 8-bp presented as half-translocated. Interestingly, addition of NTP substrate to a half-translocated ITC changed its register to post-translocated, with NTP bound in the $i+1$ site, suggesting that the half-translocated hybrid might compensate for instability of a short hybrid to help transcription with short RNA. This might indicate that the half-translocated state is not only a catalytically inactive paused

intermediate in elongation but also an initiation intermediate that tolerates short RNA–DNA hybrids.

The structures described above demonstrate that RNAPII exhibits the half-translocated hybrid, just as prokaryotic RNAPs, and all observed RNAPII half-translocated hybrids are either in a paused state or in an initially transcribing state, which are not on-line elongating states. Combined with the prokaryotic RNAP structures, this finding suggests that the half-translocated, or tilted, hybrid structure could be a universal conformation accompanying the paused state.

Structural features of backtrack pauses in prokaryotes and eukaryotes

The first backtracked RNAP complex structure was solved with *S. cerevisiae* RNAPII in 2009 by x-ray crystallography [132]. In this study, multiple backtracked complex structures were determined by using RNA transcripts containing mismatched nucleotides at the RNA 3′-end or by using a template DNA strand bearing DNA damage downstream of the 3′-end of the RNA. The overall crystal structures of the RNAPII backtracked complexes were similar regardless of the cause of backtracking or backtracked RNA lengths. In the structures, the first backtracked nucleotide (3′ to the $i+1$ site) was bound in a pocket created by the BH, TL, and other RNAPII residues, termed the "P" site based on its potential role in proofreading. The rest of the backtracked RNA appeared highly mobile and had no clear electron density in the secondary channel.

Another *S. cerevisiae* RNAPII backtracked complex structure containing a 15-mer polyC RNA revealed better electron density for the backtracked RNA, showing that it makes interactions with the conserved residues lining the inside of the secondary channel and suggesting that backtracked RNA might have a preferred conformation [114]. A TFIIS-bound backtracked complex structure showed that TFIIS rearranges the backtracked RNA location within the secondary channel to perform RNA cleavage, explaining why TFIIS-catalyzed cleavage of long backtracked RNAs is slower than that of short backtracked RNAs. As commented in the previous section, the RNA–DNA hybrid in the backtracked RNAPII was tilted about ~25° similarly to the half-translocated state observed in *E. coli his*PEC, although this altered hybrid conformation was not observed in the previously reported *S. cerevisiae* RNAPII backtracked complex [132].

Backtracked complex structures were also determined with bacterial RNAPs. Sekine *et al*. [131] solved crystal structures of *T. thermus* RNAP backtracked complex with and without GreA/Gfh1 chimeric protein. Consistent with the earlier reported backtracked RNAPII structures [114,132], first backtracked nucleotide was located in the P site and the clamp was closed in *T. thermus* RNAP backtracked complex. GreA/Gfh1 chimeric protein binding to backtracked *T. thermus* RNAP induced "ratcheting" opening the clamp and rotating the shelf module, whereas TFIIS binding to *S. cerevisiae* RNAPII did not change the clamp position.

In summary, the crystal structures of backtracked ECs from eukaryotes and prokaryotes showed few conformational changes in RNAP compared to non-paused EC, whereas the hybrid conformation varied in different complexes. The first backtracked RNA base was located in the "P site" at the end of the secondary channel, and the remainder of the backtracked RNA was extruded into the secondary channel. To avoid the possibility of having crystal-packing biased structures, obtaining cryo-EM structures of backtracked ECs is necessary.

Regulation of RNAP Pausing by Transcription Factors

To tune gene expression, every step of the transcription cycle is modulated by a multiplicity of transcription factors, and the regulation of transcriptional pausing during elongation is no exception. In this section, we describe bacterial transcription factors known to enhance or attenuate RNAP pausing and summarize what is known about them mechanistically.

Stabilization of RNA hairpin-dependent pausing by NusA

NusA (*N u*tilization *s*ubstance protein *A*) is a multifunctional transcription elongation factor that is universally conserved among eubacteria, and plays diverse roles in a context-dependent manner [136]. In this section, our discussion will be limited to *E. coli* NusA because the function of NusA in other species is not yet known clearly. NusA was first discovered (and named, *N-u*tilizing *s*ubstance) as a factor required for λ phage protein N-mediated antitermination, along with other factors such as NusG, B, and E [137]. NusA was also shown to be necessary for the antitermination of ribosomal RNA synthesis [138] and for the efficient expression of endogenous genes like β-galactosidase [139]. In contrast to its antitermination activity in the context of λN antitermination complex, NusA also enhances intrinsic termination [140] and extends the lifetime of pauses at sites where pausing is prolonged by nascent RNA structures [89,141]. In addition, NusA facilitates RNA folding, transcription-translation coupling, and DNA repair, probably partially by modulating transcriptional pausing [63,142,143]. Out of the many roles of NusA, here we will focus on its RNA hairpin-stabilized pause enhancing activity [89].

Suitable to its multiple functionalities, NusA contains multiple domains: NTD (N-terminal domain), S1, KH1 (K-homology), KH2, AR1 (acidic-rich), and AR2 [144,145]. NusA-NTD is necessary and sufficient to enhance pausing of RNAP [146]. S1, KH1, and KH2 bind RNA [147,148] and the two AR domains modulate binding affinity of NusA to RNAP [146]. In particular, AR2 auto-inhibits NusA by blocking RNA binding to it in the absence of RNAP, and this auto-inhibition is relieved in the presence of RNAP by AR2 binding to the C-terminal domain of the RNAP α subunit [149]. The contributions of NusA to stimulation of intrinsic termination are partially from its pause-enhancing activity, but not exclusively dependent on it [146].

Recently, two independent structures of NusA-bound *E. coli* RNAP ECs—the *his*PEC and the λN anti-termination complex—were reported in contrasting biological contexts [101,150].

The 3.6-Å cryo-EM structure of a NusA-bound *his*PEC structure revealed four main interactions of NusA to the TEC to stabilize the RNA hairpin pause (Fig. 7A). First, NusA-NTD was bound to the FTH of RNAP, as predicted from previous biochemical and NMR studies [72,151,152]. The observation that deletion of the FTH abolished the pause-enhancing effect of the NusA-NTD also emphasizes the role of this interaction in hairpin pause stabilization [72,146]. Second, NusA-AR2 was bound to the α1-CTD of RNAP. This interaction relieves the auto-inhibition of NusA, as proposed from gel-shift assays and NMR study [146,153]. Third, NusA-NTD was bound to the α2-CTD. Deletion of the α-CTDs eliminated the pause-enhancing activity of the NusA-NTD, implying that the interaction between NusA-NTD and α2-CTD is required for this activity. This interaction likely increases the binding affinity of NusA-NTD to RNAP rather than playing a functional role since the α2-CTD is highly mobile without NusA and does not have a defined interaction with other RNAP subunits [101]. Fourth, the NusA-KH domains were bound to the RNAP ω subunit. A KH deletion did not abolish pause-enhancing activity of NusA, but higher concentrations of NusA were required for maximum activity, indicating that the KH domain contributes to NusA affinity for the EC [146]. The interaction between the KH domains and the RNAP ω subunit explains the positive effect of the KH domains on the binding affinity of NusA to EC.

In the 3.7-Å cryo-EM structure of the λN anti-termination complex, NusA exhibited a largely distinct conformation compared to NusA in the *his*PEC–NusA complex due to extensive interaction with λN (Fig. 7B) [150]. The λN anti-termination complex comprises NusA, NusB, NusE, and NusG in addition to λN and the RNAP EC. In the structure, λN remodeled the Nus factors to inhibit their pause stabilizing or termination-promoting functions, as well as penetrated the EC to stabilize an active RNAP conformation. Superimposition of the *his*PEC–NusA complex with the λN anti-

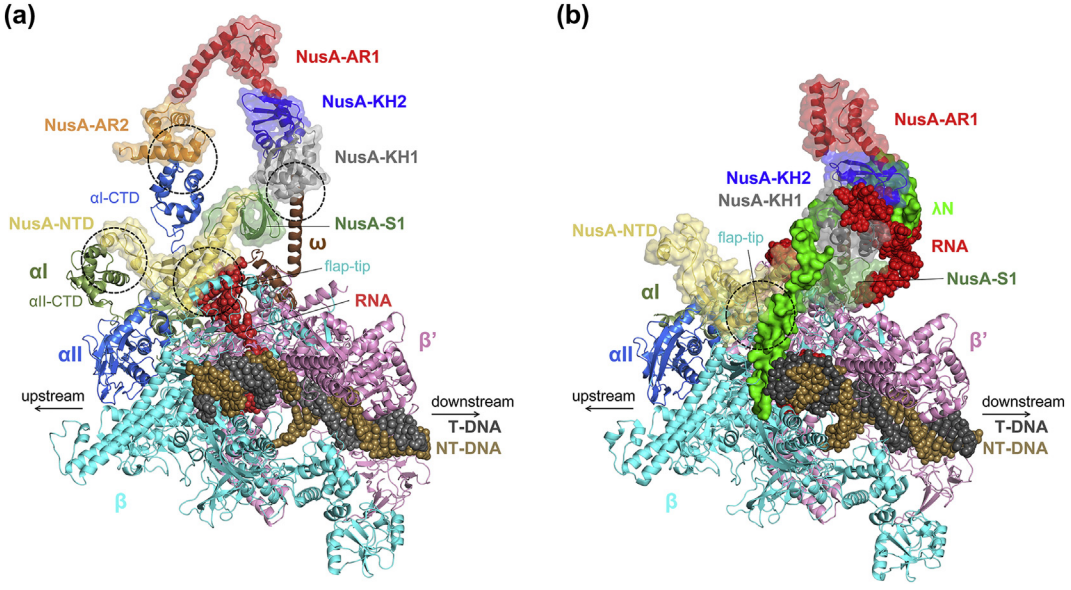


Fig. 7. Comparison of NusA-bound *his*PEC and λN anti-termination complex. Protein region is drawn in cartoon format, and nucleic acids are drawn as spheres. NusA is marked by a transparent surface, and each domain of NusA is colored differently. (A) *his*PEC–NusA complex (PDB code: 6FLQ). The main contact points between RNAP and NusA are marked with dotted circles. (B) λN anti-termination complex (PDB code: 6GOV) drawn from the same vantage point as in panel A. λN is drawn as a non-transparent green surface. The main contact point between RNAP and NusA is marked with a dotted circle. NusA-AR2 was disordered. NusB and NusE are not shown for clarity.

termination complex revealed that the angle between the Cα of D344 at the end of the KH2 domains from both structures and the Cα of NusA D103, a residue that contact with RNAP at the same position in both structures, is ~ 40°. Contrary to the *his*PEC-bound NusA that relocated the β flap-tip to stabilize the RNA hairpin and enhance pausing allosterically, NusA in the λN anti-termination complex was displaced from the RNA exit channel and so was prevented from stabilizing a pause RNA hairpin. The β flap-tip was also moved away with NusA-NTD from the RNA exit channel. The RNAP α-CTDs were not visualized in the λN anti-termination complex, suggesting that the αCTD−NusA interactions were broken during λN-mediated repositioning of NusA.

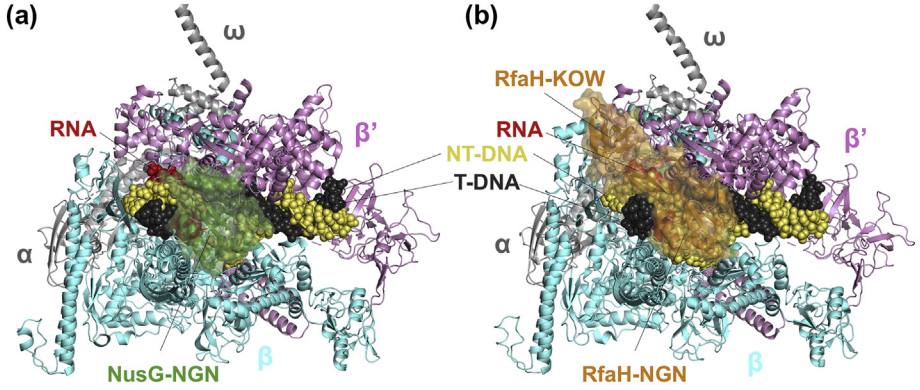
In summary, the two structures of NusA-bound ECs demonstrated the following:

1) NusA promotes the RNA hairpin-stabilized pause by situating the RNAP flap-tip to stabilize the RNA hairpin or to enhance the allosteric effect of the RNA hairpin.
2) λN eliminates the pause-enhancing activity of NusA by relocating NusA and altering the interactions between NusA and RNAP.

Pause suppression by NusG and RfaH

Although pausing aids timely recruitment of transcription regulators, guides nascent RNA folding, opposes promoter occlusion by nucleosomes, and permits termination, excessive pausing can lead to premature transcription termination or genome instability [59]. NusG and RfaH both are members of the NusG/Spt5 family, and their most prominent function is to suppress pausing in order to prevent these deleterious effects. Both NusG and RfaH consist of an N-terminal domain NGN (*Nus*G-like *N*-terminal domain) and a C-terminal domain KOW (named after its discoverers, Kyrpides, Ouzounis, Woese [154]). NusG reduces backtrack pauses, whereas RfaH inhibits both backtrack and RNA hairpin-stabilized pauses [155]. Other than their anti-pausing properties, NusG and RfaH have a few differences. RfaH, whose RNAP-binding surface is autoinhibited by the CTD, can be loaded onto RNAP only at a specific sequence *ops* exposed in the non-template DNA of a PEC that can displace the CTD. In contrast, *E. coli* NusG does not exhibit sequence specificity [89], although *Bacillus subtilis* and *Mycobacterium tuberculosis* NusGs exhibit some sequence-specific stimulation of pausing or termination [73,156]. Both NusG and RfaH KOW domains are known to bind to ribosomal proteins such as S10 [157,158], whereas NusG KOW interacts with termination factor Rho to favor its action at suboptimal termination sequences [159,160].

Cryo-EM structures of NusG- and RfaH-bound ECs in combination with biochemical assays revealed the anti-pausing mechanism of NusG and RfaH in the work by Kang *et al*. [99] (Fig. 8). Both NusG and RfaH bind to the same site on RNAP and contact the β protrusion, β lobe domain, and β′ clamp helices. Although RNAP structures in NusG- and RfaH-bound ECs were similar to that of a non-paused EC, the angle between upstream and downstream duplex DNAs decreased upon binding of NusG and RfaH, implying NusG and RfaH



***E.coli* EC-NusG complex**
(PDB: 6C6U)

***E.coli* EC-RfaH complex**
(PDB: 6C6S)

Fig. 8. NusG- and RfaH-bound EC structures. RNAP subunits and nucleic acids are labeled. (A) *E. coli* NusG (colored in green) binds between β and β′ subunits, contacting β protrusion, β lobe, and β′ clamp helices. NusG KOW domain was disordered in the cryo-EM structure. (B) *E. coli* RfaH (colored in orange) binds to the same site as NusG in panel A. In addition, RfaH recognizes a short-hairpin formed by *ops* sequence in the non-template DNA (not visible in the figure) and RfaH KOW domain binds flap tip of RNAP, covering upstream duplex DNA near the transcriptpion bubble.

Box 1
Methods to map paused RNA polymerase (RNAP) in vivo.

Refer to Refs. [58,59,74] for more detailed description of the methods, including limitations of each, and relevant literature resources.

Transcription run-on technique determines in vivo location of an actively transcribing RNAP on a gene from the analysis of nascent RNA product of that gene. In a transcription run-on experiment, cells are lysed and transcription is halted. Transcription by RNAPs that are still bound to DNA and nascent RNA is then briefly re-started in vitro in the presence of radiolabeled or modified NTPs. The amount of labeled RNA produced at a given location of a gene reports on the abundance of RNAP at that location and thus serves as a measure of RNAP occupancy at the genes of interest. Global run-on sequencing (GRO-seq) [75] using brominated NTPs or 4-thioUTP, and its nucleotide-resolution variation, precision nuclear run-on and sequencing (PRO-seq) [76] using chain-terminating biotinylated NTPs, are genome-wide transcription run-on methods, in which labeled RNA is purified, converted into a DNA sequencing library, and sequenced.

Chromatin immunoprecipitation (ChIP) determines in vivo location of RNAP on genes from the analysis of DNA crosslinked to RNAP and immunoprecipitated with an RNAP-specific antibody. In a ChIP experiment, RNAP is reversibly cross-linked to the chromatin DNA, typically with formaldehyde, although in the seminal versions of this approach, intact cells were irradiated with UV light to crosslink DNA to the protein irreversibly [14]. Protein−DNA complexes are then fragmented and solubilized, and DNA cross-linked to RNAP is purified by immunoprecipitation, followed by reversal of the crosslinks. The relative amount of DNA captured this way is quantified by microarray hydridization (ChIP-chip), high-throughput sequencing (ChIP-seq), or quantitative PCR (ChIP-qPCR). Versions of ChIP-seq that introduce exonuclease digestion step to eliminate DNA not directly bound to RNAP (ChIP-exo and ChIP-nexus) achieve higher resolution and sensitivity.

Native elongating transcript sequencing (NET-seq) determines both the genomic location and abundance of RNAP from the analysis of 3′-ends of nascent RNA transcripts. Nascent RNA associated with the extremely stable RNAP-RNA−DNA transcription complex is purified by either immunoprecipitation or cell fractionation. The 3′-ends of these purified RNAs are converted into a DNA library, which is sequenced to reveal the identities and abundance of each 3′-end. Mapping the sequencing reads to the genome being analyzed reports DNA strand-specific genomic position and the RNAP active site at single-nucleotide resolution. NET-seq has been given other names (e.g., mNET-seq, NET-prism) when other pull-down targets are used [26,77,78], but it would lessen confusion to use NET-seq (without modifiers) for all methods that sequence nascent transcripts 3′-ends from the RNAP active site.

rearrange the upstream duplex DNA. Based on psoralen cross-linking at the upstream DNA duplex near transcription bubble fork, the authors suggested that NusG and RfaH inhibit backtracking by stabilizing upstream duplex DNA, which must be melted during backtracking [161]. In addition, biochemical assays revealed that RfaH could inhibit RNA hairpin-stabilized pausing by tightly binding to EC and preventing swiveling caused by RNA hairpin formation. In contrast, NusG showed much lower affinity to the EC explaining why NusG could not block swiveling.

Roles of the NusG-family transcription factors in prokaryotes: RfaH and others

RfaH is a paralog of NusG and a poster child showing how NusG family transcription factors can be utilized for the expression of specific genes. RfaH was first discovered as a component in lipopolysaccharide synthesis [162]. It was first thought to be an enzyme but was later shown to be a positive regulator of *rfa* operon [163]. Afterward, the list of gene operons regulated by RfaH, or RfaH regulons, has increased to include *tra* (F factor synthesis), *hly* (hemolysin synthesis), and *kps* (exopolysaccharide synthesis) [164]. As mentioned above, RfaH needs to be recruited to the *ops* site exposed on the non-template single-stranded DNA in the EC [53,165]. Deletion of either RfaH or the *ops* sequence abrogated full expression of the genes in the operon, showing a consistent pattern of moderate transcription decrease in early genes and of almost complete abolition of transcription of the distal genes [164,166−170].

A number of biochemical experiments, combined with multiple structural studies [99,158,171],

Box 2
The motions of biomolecular complexes differ from those of macroscopic machines

It is important to remember that structural states (i.e., conformations) of macromolecules are in constant thermal motion such that all available conformations are stochastically sampled with varying probabilities. For example, ECs and PECs sample different states, with the relative stabilities of the array of states determining the longevity of events such as pausing. In a paused complex, a non-paused state may be sampled transiently but not readily lead to pause escape because, for example, NTP substrate fails to bind while the state exists. It is tempting to describe functional states of biomolecular machines, like RNAP, in terms of reminiscent of discrete, long-lived states of a macroscopic machine that cycle in precisely timed intervals, but the consequences of thermal activity create a much different reality at the molecular scale. Side chains and domains of RNAP are in constant movement relative to one another driven by thermal energy (e.g., see simulations in Ref. [135]). As a consequence, ECs fluctuate to transiently attain PEC-like conformations (and vice versa), although the alternative state may be occupied for only a small fraction of the time. Structures of ECs or PECs, whether crystal structures or cryo-electron microscopy structures, should not be interpreted as snapshots of a rigid machine, but as the most probable representations of a dynamic distribution of structures.

demonstrated that RfaH assists the full expression of long operons that contain the *ops* site in their leader sequence by inhibiting both backtracking and RNA hairpin pause. The polarity-suppressing activity of RfaH is likely common in NusG-like proteins from diverse bacterial species. *Myxococcus xanthus taA* is a NusG-like protein and located first in the type I polyketide synthase (PKS) gene cluster for the antibiotic TA (Myxovirescin) [172]. Deletion of *taA* does not affect the normal growth and development of the cells but abrogates antibiotic production. *Bacteroides fragilis* NCTC9343 synthesizes eight distinct capsular polysaccharides (PS) from separate biosynthetic loci, and each locus contains a NusG-like proteins, named UpxY, where x varies from a to h. Deletion of UpxY abolished transcription of the operon, whereas swapping a short NGN peptide between UpxYs altered the specificity of UpxYs toward the gene cluster. The swapped peptide was located at the homologous region to the *ops* recognition site in RfaH, implying that UpxY might recognize a specific sequence in the operon through this region in order to activate transcription in a similar way to RfaH [99]. *Bacillus amyloliquefaciens* LoaP (Long operon-associated Protein) is another example of a NusG-like protein that

Box 3
Open questions in transcriptional pausing research

- What is the mechanistic basis of sequence specificity of transcriptional pausing; i.e., why does RNA polymerase pause more frequently on some sequences and not others?
- How general is the bacterial consensus pause sequence?
- Does the consensus pause sequence change in response to environmental changes or in different species?
- What could adjust the pause efficiency and duration other than the DNA sequence?
- What is the structural basis of hairpin-stabilized pausing in bacterial RNAPs lacking SI3?
- How can we apply the understanding of pausing mechanisms to practical purposes, e.g., bacterial engineering for natural product production or clinical diagnostic and therapeutic application?

regulates transcription elongation of a biosynthetic gene cluster [173]. In contrast to taA and UpxY that control one operon, LoaP controls two type I PKS gene clusters, difficidin and macrolactin synthetic clusters. Systematic NusG-like protein sequence alignment in the study also revealed that most LoaP homologs in NusG-like proteins are located adjacent to large gene clusters, implying universal usage of NusG-like proteins as polarity-suppressing biosynthesis regulators.

Concluding Remarks

Since its inception in the early 1970s, the field of transcriptional pausing research has evolved and expanded. Our understanding of pausing mechanisms has been deepened, largely thanks to advances in experimental methods: ensemble and single-molecule *in vitro* approaches, improved structural biology methods, and *in vivo* genome-wide sequencing and imaging techniques. Nevertheless, open questions still remain (Box 3), making this active field of research an exciting avenue for the future.

Although we now know the sequence elements associated with paused RNAP and that these sequences cause pausing by inducing the half-translocated state, at least in some bacteria, we do not fully understand how interactions of these pause sequences with RNAP interfere with translocation. One future direction that could shed light on this issue is determination of high-resolution structures of ePECs formed by nucleotide addition. Because of the transient nature of ePECs, it seems likely that complexes that arrive at the pause by NTP incorporation, as opposed to direct reconstitution at the pause site, would better represent physiologically relevant species. These structural outputs could then be used as a starting point for theoretical work. Computational analyses of the PECs in different nucleic-acid contexts in turn could complement structural efforts to address the mechanistic basis for the sequence specificity of transcriptional pausing.

The consensus pause sequence (Fig. 5) described in this review was determined by sequencing nascent RNA from *E. coli* and *B. subtilis* grown in a rich medium [24,26]. Although purified RNAPs from several additional organisms (*Rhodobacter sphaeroides*, *Mycobacteria bovis*, *Thermus thermophiles*, and mammalian RNAPII from *Bos taurus*) responded to the consensus pause sequence *in vitro* [24], it seems important to verify that the consensus holds across prokaryotes and eukaryotes by NET-seq, or determine if other pause sequences exist. Another interesting open question is whether changing growth conditions will alter the consensus pause sequence. For example, the small-molecule alarmone (p)ppGpp, which rapidly accumulates in bacterial cells exposed to environmental or nutritional stress (so-called stringent response), enhances *in vitro* pausing of *E. coli* RNAP during elongation on genes under stringent control [174,175]. Furthermore, different solutes (e.g., chaotropic *versus* kosmotropic) have been demonstrated to differentially affect RNAP conformation and both its interactions with nucleic acids and its pausing behavior *in vitro* [115]. Increased pressure has also been shown to influence elongation behavior of RNAP *in vitro* [176,177]. Finally, altering pH changes transcriptional pausing both *in vitro* and *in vivo* [178,179]. Thus, it would be interesting to see if subjecting bacteria to stress or changing osmolarity, pressure, or pH within the cells alters the consensus pause sequence *in vivo*. These effects may be of particular importance in industrial applications of synthetic biology where growth conditions may be suboptimal or highly variable in addition to insights into fundamental mechanisms that may be gained by defining the effects.

From the structures of RNA hairpin-stabilized PECs from *E. coli*, it is clear that the SI3 domain of RNAP plays a key role in inhibiting catalysis at the pause site by not allowing TL folding as a consequence of swiveling. Many bacterial RNAPs, however, lack the SI3 domain yet are capable of sensing RNA structure as a pause signal, raising the question of whether these bacteria employ a different mechanism for hairpin-stabilized pausing. For example, *B. subtilis* RNAP, which does not contain SI3, stalled at a site that has a hairpin structure similar to *his* pause hairpin but shifted away from the RNA 3′-end by 1 nt [180]. Determination of PEC structures from these organisms will shed light on this question and inform us on how conserved the allosteric mode of hairpin action is.

Heterologous expression of biosynthetic gene clusters for natural product synthesis is often unsuccessful for poorly understood reasons. One possible reason for these failures could be our inability to predict pausing in a given organism to avoid deleterious pauses or programming the EC to override them (e.g., using regulators like RfaH). If pauses could be predicted accurately, in order to remove negative effects from the synthetic sequence (analogously to codon optimization for recombinant protein expression) or optimally maintain RNA folding and transcription–translation coupling, then genetic design will be more powerful. Furthermore, our growing understanding of NusG-family factors modulating transcription of biosynthetic gene clusters in different bacteria is likely to boost our ability to generate natural products heterologously. Another area that will benefit from the ability to predict, and control, transcriptional pauses is bacterial engineering for clinical diagnostic and therapeutic applications [181].

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Abbreviations used:
RNAP, RNA polymerase; RNAPII, eukaryotic Pol II RNA polymerase; EC, elongation complex; PEC, paused elongation complex; ePEC, elemental paused elongation complex; bp, base pair; nt, nucleotide; NTP, nucleoside triphosphate; NAC, nucleotide addition cycle; TL, trigger loop; BH, bridge helix; FT, flap tip; FTH, flap-tip helix; SI3, sequence insertion 3; CTD, carboxy-terminal domain; NTD, amino-terminal domain; NGN, NusG-like N-terminal domain; NET-seq, native elongating transcript sequencing; CPX, cysteine-pair cross-linking.

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