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Transcriptional Pausing as a Mediator of Bacterial Gene Regulation

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Abstract

Cellular life depends on transcription of DNA by RNA polymerase to express genetic information. RNA polymerase has evolved not just to read information from DNA and write it to RNA but also to sense and process information from the cellular and extracellular environments. Much of this information processing occurs during transcript elongation, when transcriptional pausing enables regulatory decisions. Transcriptional pauses halt RNA polymerase in response to DNA and RNA sequences and structures at locations and times that help coordinate interactions with small molecules and transcription factors important for regulation. Four classes of transcriptional pause signals are now evident after decades of study: elemental pauses, backtrack pauses, hairpin-stabilized pauses, and regulator-stabilized pauses. In this review, I describe current understanding of the molecular mechanisms of these four classes of pause signals, remaining questions about how RNA polymerase responds to pause signals, and the many exciting directions now open to understand pausing and the regulation of transcript elongation on a genome-wide scale.

Contents

INTRODUCTION

RNA polymerase (RNAP) is the central enzyme of gene expression and is responsible for converting the stored information in DNA into active form as an RNA transcript. Thus, understanding how RNAP is regulated has been a central focus of microbiology since bacterial RNAP was first purified, in 1961 (20). Upon development of assays that could visualize specific RNA products, it became obvious that RNA synthesis by RNAP is discontinuous (97). RNAP adds nucleotides to the growing transcript rapidly at most DNA positions (in 10–50 ms) but takes much longer (seconds to minutes) at other positions. These slow steps are called pauses. It was also immediately clear that pausing is sequence dependent. RNAP dwells reproducibly at certain DNA positions (i.e., all transcribing RNAPs recognize the same subset of DNA locations), causing buildup and eventual decay of intermediate-length RNAs during synchronous transcription. Pausing is not a universal property of polymerase enzymes but rather is an evolved feature of the multi-subunit RNAPs responsible for cellular transcription (e.g., DNA polymerases do not exhibit extensive sequence-dependent pausing). Once pausing was discovered, the key questions became, "What is the function of these pauses?" and "What mechanisms explain pausing by multi-subunit RNAPs?"

In the broadest context, transcriptional pauses are timing signals in the information processing system of life (**Figure 1**). Although we usually think of microbial life in terms of chemistry (79) and chemistry explains molecular mechanism, life itself depends on constant processing of information by cells (41)—both stored information read from DNA and information sensed from cellular and extracellular environments via molecular interactions. RNAP functions at the center of cellular information processing as both the retriever of information from DNA and the receiver of information from regulators. In this sense, RNAP is the cell's CPU (central processing unit) and can be viewed as a type of Turing machine, the classic conceptualization of a universal computer (103).

To process information, RNAP must respond to it within windows of time and location along DNA during which a decision to continue transcription is relevant. For example, if RNAP

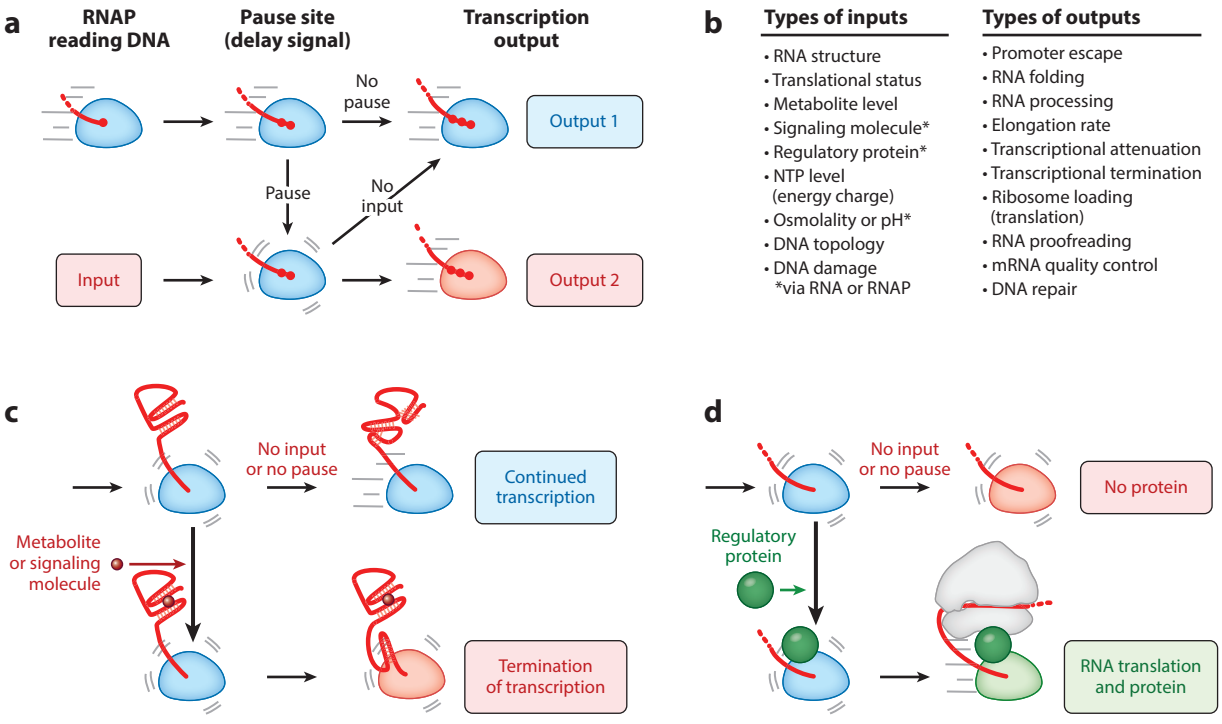

Figure 1

Transcriptional pausing as a timing signal for cellular information processing by RNA polymerase (RNAP). (*a*) A pausing delay enables input to output processing. (*b*) Types of inputs and outputs processed by RNAP at pause sites. (*c*) Example of RNA folding as an output. (*d*) Example of protein synthesis (translation) as output.

proceeds past an AUG start codon and a ribosome loads onto the nascent RNA, it may be too late to alter the protein output. Although key decisions occur by controlling RNAP initiation at promoters, evolution has expanded the information processing capacity of cells by equipping the elongating RNAP with the ability to respond to regulatory inputs, including inputs sensed via nascent RNA structures. Fundamentally, transcriptional pauses punctuate RNA synthesis to provide the time and location windows necessary for these regulatory decisions. Overriding a pause event, conversely, may cause RNA synthesis to proceed too far for regulatory input to be useful. For instance, if transcription continues past a 5′ untranslated region (UTR) and into the body of a gene, ribosome loading onto the nascent RNA may then commit the cell to expression of the gene.

A transcriptional pause is thus analogous to a timing loop in a computer program that instructs the processor to wait until an instruction is received or to move on after an interval if no instruction is received. In the analog world of biological information processing, the precise duration of the delay is specified stochastically within a probability distribution of possible dwell times at pause sites. The inputs potentially received at pauses are diverse and can be sensed directly by RNAP or indirectly via the nascent RNA (**Figure 1*b***). The outputs are also diverse. For example, known pauses can (*a*) allow time for a metabolite or signaling molecule to bind to a riboswitch aptamer and control a termination decision in a 5′ UTR, such as flavin mononucleotide (FMN) binding to the *Bacillus subtilis ribD* leader RNA (148, 153) (**Figure 1*c***) or (*b*) allow time for a regulatory protein to load onto RNAP to enable subsequent ribosome recruitment and translation of the RNA into a protein product, like the *Escherichia coli* RfaH protein (3, 6) (**Figure 1*d***). Although a

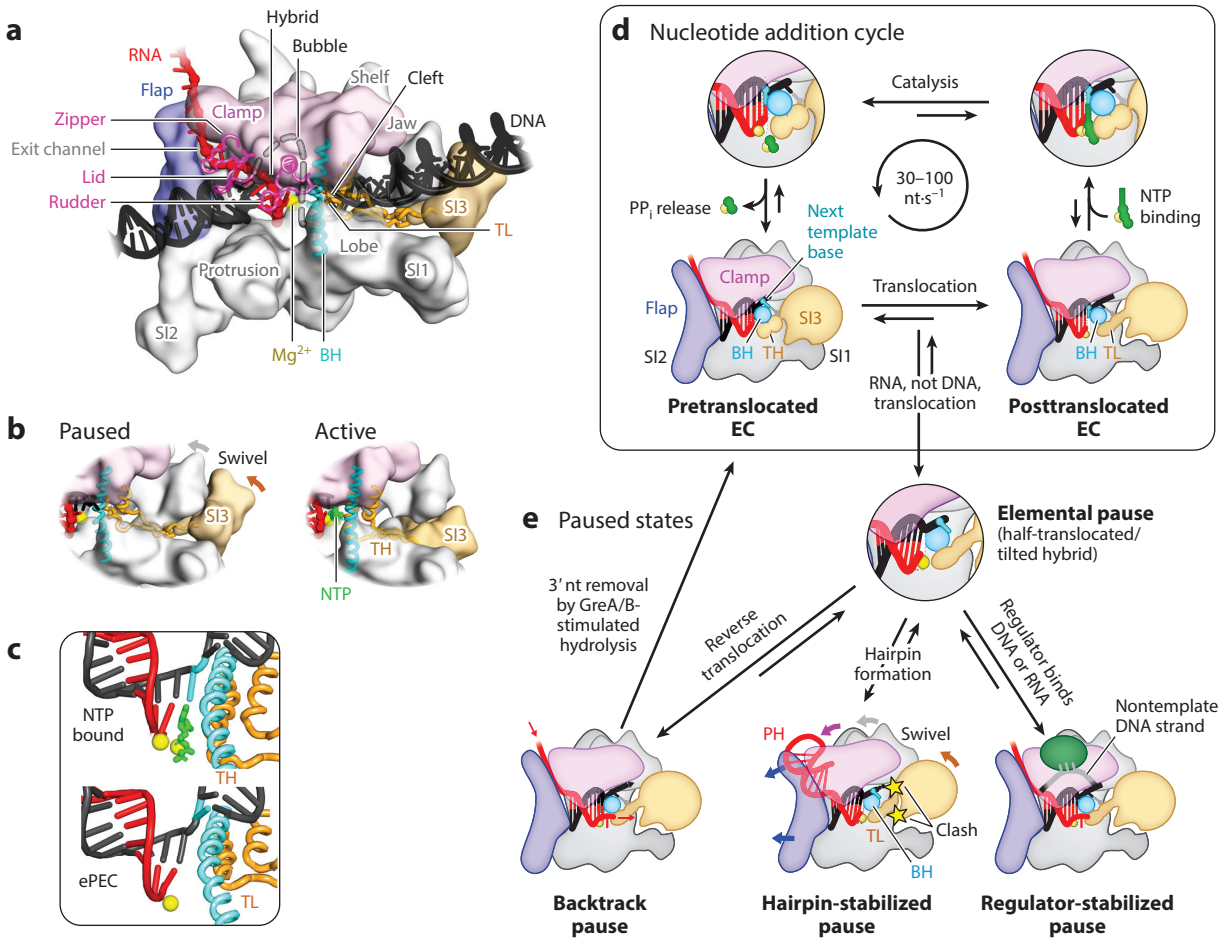

Figure 2

Four classes of transcriptional pause signals. (*a*) Structure of the *Escherichia coli* EC (71). Nontemplate DNA in the transcription bubble, shown as gray segments, is disordered in most structures. (*b*) SI3 locations in swiveled and active ECs (1, 52, 68). (*c*) Structures of the NTP-bound and elemental paused forms of the EC (1, 52, 68). (*d*) The nucleotide addition cycle. Mg^{2+} (*yellow circle*) is associated with NTP (*green*) during binding and PP_i during release. (*e*) Four types of paused ECs. Abbreviations: BH, bridge helix; EC, elongation complex; ePEC, elemental paused EC; PH, pause hairpin; PP_i, inorganic pyrophosphate; SI1, sequence insertion 1; TH, trigger helix; TL, trigger loop.

detailed description of pausing within a formalism of biological information processing would be illuminating, my intention here is only to suggest why pausing evolved in cellular RNAPs and to focus principally on its molecular mechanisms and roles in gene regulation.

STRUCTURAL FEATURES OF RNAP RELEVANT TO TRANSCRIPTIONAL PAUSING

Several features of RNAP are key to understanding mechanisms of transcriptional pausing. First, RNAP makes extensive contacts to DNA and RNA in a deep cleft formed by its two large subunits (**Figure 2*a***). Duplex DNA (~20 bp) bound in the cleft is separated 1 bp in front of the active site, with the template strand forming a 9- to 10-bp hybrid with product RNA held deep in the cleft

before reannealing passively with the nontemplate strand outside the cleft. In the ~12-bp melted transcription bubble, the nontemplate DNA stays on the outside surface of RNAP. A complex set of mobile domains, modules, and loops that include the jaw, lobe, protrusion, shelf, clamp, rudder, bridge helix, fork loop 2, switches, lid, zipper, and flap contact DNA and RNA and mediate their movements. In every round of nucleotide addition, a polymorphous trigger loop–trigger helices (TL–TH) module alternates between a random coil (TL) to admit NTP and a helical hairpin (TH) to contact NTP and stabilize the transition state (**Figure 2*b,c***). Most bacterial RNAPs also contain lineage-specific surface modules that mediate regulation; *E. coli* RNAP contains three called sequence insertions (SI1, SI2, and SI3). SI3, inserted in the TL, plays a key role in pausing.

RNAP Conformational Fluctuations and Pausing

The elongation complex (EC) remains flexible during transcription. Thermal energy causes small movements of the domains, modules, and loops (34, 141, 158). These flexible RNAP movements are necessary for transcriptional activity. Increasing the thermodynamic activity of water in a pressure cell grinds RNAP to a complete but reversible halt (40), presumably because exposure of a hydrophobic surface during RNAP movements becomes highly disfavored.

The ability of RNAP to fluctuate among multiple conformations helps explain a fundamental feature of transcriptional pausing: Paused states arise in kinetic competition with bypass of pause sites by EC rearrangement into a paused EC (PEC) conformation (73). Thus, pauses are characterized by both a dwell time distribution (i.e., half-life) and an efficiency of pausing, defined as the fraction of ECs passing a given template position that enter the paused state (89). In some cases, multiple PEC conformations form and generate multiphasic dwell time distributions. Although on-pathway pausing resulting from slow translocation of DNA rather than rearrangement to off-pathway states has been proposed (7, 14), as a practical matter, on-pathway pausing cannot be distinguished experimentally from highly efficient EC-to-PEC isomerization. Since isomerization demonstrably competes with elongation at most pause sites (42, 60, 73, 76, 89, 135), it is least confusing to simply define pausing as isomerization to an off-pathway state.

It is also crucial to appreciate that structural states of ECs and PECs are in constant thermal motion on a nanosecond to microsecond time scale at biologically relevant temperatures (131). All accessible states, even those less thermodynamically stable, are sampled stochastically (i.e., less-stable states are sampled less often and for shorter durations). Active ECs and inactive PECs exist as a fluctuating collection of different states. Thus, PECs may sample active states transiently but not escape if they fluctuate back to an inactive state before NTP can bind and react. Structural models shown here and elsewhere represent the most probable or average state among a dynamic distribution of structures. From an information processing standpoint, this means that regulatory decisions mediated by RNAP are probabilistic rather than absolute at a single-molecule level.

The Nucleotide Addition Cycle

Pauses disrupt the nucleotide addition cycle (NAC), which involves four main steps (**Figure 2*d***): (*a*) translocation of the DNA and RNA chains through RNAP, from the pretranslocated conformation generated by the previous round of nucleotide addition to the posttranslocated conformation in which a new template DNA base is loaded into the active site; (*b*) binding of cognate Mg^{2+}·NTP into the active site by rapid sampling among the four NTPs diffusing through the RNAP secondary channel and closure of the active site by TL-to-TH isomerization after the cognate NTP binds; (*c*) catalysis of nucleotide addition that generates pyrophosphate in a reversible phosphoryl transfer reaction involving S_N2 attack of the RNA 3′ oxygen on the NTP α-phosphorus via a

trigonal bipyramidal transition state stabilized by the TH; and (*d*) isomerization of the TH back to the TL with release of pyrophosphate from RNAP. Although these steps of the NAC are clear, important questions remain, including how RNAP side-chain contacts to DNA and RNA change to accomplish the steps and which domain, module, and loop movements accompany each step (11). At pause sites, the NAC is interrupted by effects of RNA and DNA sequence on RNAP conformation and on the energetics of base-pairing.

FOUR CLASSES OF TRANSCRIPTIONAL PAUSE SIGNALS

Four distinct pause conformations can be described with supporting structural and biochemical data: (*a*) the elemental PEC, (*b*) the backtrack PEC, (*c*) the hairpin-stabilized PEC, and (*d*) the regulator-stabilized PEC (**Figure 2*e***).

The Elemental Pause Signal

The elemental pause was first hypothesized based on the observation that elimination of the pause hairpin (PH) from the hairpin-stabilized *his* pause signal, which coordinates translation of a leader peptide–coding region with transcription of the *E. coli his* operon attenuator, leaves a residual, shorter pause that depends on the RNA and DNA sequences in contact with RNAP (22, 24, 87). The name elemental pause was proposed in 2006 (87), prior to which the terms ubiquitous, basal, and unactivated were used to describe sequence-dependent pauses for which neither hairpin stabilization nor backtracking was evident (5, 22, 110). The existence of a non-backtracked elemental pause state was initially debated based on different interpretations of the effect of applied force on pausing (33, 44, 60) but was unambiguously resolved by mechanistic analyses (42, 127) and direct imaging of elemental PECs (ePECs) (68, 147).

The elemental pause signal is multipartite. Formation and longevity of the elemental pause depend on multipartite interactions of distinct parts of the RNA–DNA scaffold in contact with RNAP (**Figure 3**). The multipartite nature of pause signals was first established for the hairpin-stabilized *his* pause over two decades ago (22, 24). Similar multipartite components for the elemental pause became clear from their sequence signatures in precisely defined pause locations in the *E. coli* genome by native elongating transcript sequencing (NET-seq) (90) (see the sidebar titled What Is NET-seq?). The same sequence signatures were evident in vitro from single-molecule analyses at single–base pair resolution (90). The similarity of the in vivo and in vitro signatures established that pausing in live bacteria is controlled principally by RNAP–nucleic acid interactions and base-pairing energetics rather than by regulators present in cells but not in vitro. Each pause signal component is validated by pause-weakening substitutions in the conserved sequences (90, 127) (**Figure 3*a*,*b***). The consensus elemental pause signal stimulates pausing by diverse bacterial RNAPs and by mammalian RNAPII, suggesting that it affects fundamental RNAP–nucleic acid interactions conserved during evolution. Attempts to define consensus eukaryotic pause sequences using NET-seq were unsuccessful until recent improvements in analysis algorithms enabled success and verified the similarities of eukaryotic pause sequences to the bacterial elemental pause sequence (43). Minor deviations in the consensus are seen in *Bacillus subtilis* (90, 153), suggesting that some changes in RNAP–nucleic acid interactions important for pausing have occurred during bacterial evolution. However, pausing by only a few bacterial RNAPs has been studied. Given the remarkable differences uncovered recently in mechanisms of initiation in different bacterial lineages (27, 58, 142), examination of pausing in diverse bacteria is likely to provide new insights into evolution of these interactions.

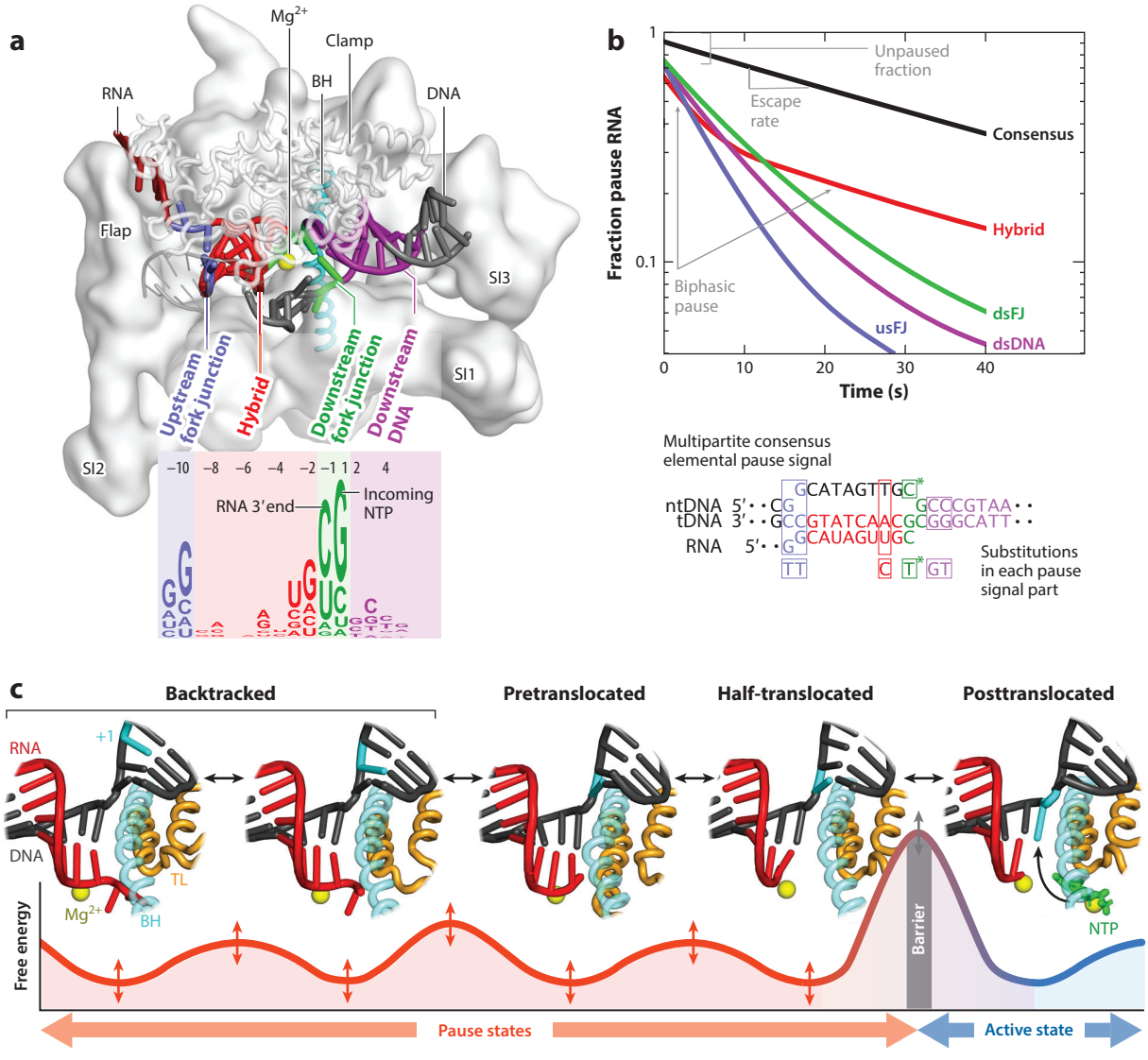

Figure 3

The elemental pause signal. (*a*) Structure of the ePEC showing four components of the elemental pause signal. (*b*) Pause dwell times for consensus and mutant elemental pause signals. Note the biphasic pause created by a hybrid substitution. Substitutions are shown for only the nontemplate (*top*) strand but are present in all strands, as indicated by boxes, except for the dsFJ change (*asterisk*), which is present only in the nontemplate DNA strand (90, 127). (*c*) Translocational interconversions in the ePEC. States are modeled from currently available structures (1, 52, 68, 92, 128). The energy diagram is hypothetical. The free energy of states and the barriers between them, and thus their occupancies and contributions to pause dwell time, will vary depending on the pause sequence (*vertical arrows*). Abbreviations: BH, bridge helix; dsDNA, downstream DNA; dsFJ, downstream fork junction; ePEC, elemental paused elongation complex; ntDNA, nontemplate DNA; SI1, sequence insertion 1; tDNA, template DNA; TL, trigger loop; usFJ, upstream fork junction.

WHAT IS NET-seq?

Native elongating transcript sequencing (NET-seq) determines the 3′ ends of nascent RNAs and thus the locations of transcribing RNAPs and pause sites in vivo based on 3′ end abundances found by high-throughput sequencing of RNAs recovered from ECs that are affinity captured after cryo-lysis of flash-frozen cells (30, 90). When combined with nucleases that trim RNA to the RNAP upstream edge, a modified NET-seq method (rNET-seq) also can detect backtracking by paused ECs (64, 153). Other methods exist to map EC and PEC locations in vivo (see 69), but NET-seq has proven most suitable for bacterial transcription studies.

Elemental pause signal components stimulate pausing additively. The four components of the elemental pause signal are (*a*) duplex DNA (~8 bp) in contact with RNAP in the downstream DNA cleft (contacting principally the clamp, lobe, bridge helix, and fork loop 2); (*b*) the nucleotides forming the downstream fork junction, which correspond to the RNA 3′ nucleotide and incoming NTP (C–G or, more broadly, pyrimidine–purine in the consensus sequence); (*c*) the RNA–DNA hybrid; and (*d*) the upstream fork junction, which includes the first RNA nucleotide upstream from the hybrid (G in the consensus) (64, 90, 127) (**Figure 3*a***). The downstream fork junction–active site sequence is the most highly conserved component and is easily detected in NET-seq experiments that capture primarily stronger pauses (64, 144). However, this pause signal component alone does not trigger pausing; changing the other components to sequences found least at pauses while retaining the consensus active site C–G completely eliminates pausing (90). Substitutions in each component individually decrease pausing (**Figure 3*b***). Combining these substitutions additively decreases pausing further (127). These additive effects are most easily explained if each component affects the same rate-limiting step in the pause mechanism. Although the hybrid and downstream DNA contributions are harder to detect by NET-seq (64, 144), all four components are validated by multiple studies (13, 14, 22, 90, 127, 135). Exclusion of weaker pauses may explain differences in pause sequence signatures (69). Not every pause needs all four components. Further, the possibility of multiple hybrid and downstream pause motifs cannot be excluded. Improvements in bioinformatic analyses of high-accuracy NET-seq analyses may continue to improve our understanding of elemental pause signals.

Structure of the ePEC. Cryo–electron microscopy (cryo-EM) provided the key insight into the structure of the ePEC by resolving a half-translocated state of the RNA–DNA scaffold in *his* ePECs lacking a PH (52, 68) (**Figure 3**). The nascent RNA in the *his* ePEC is translocated, meaning that the RNA 3′ end has shifted to the product subsite of the active site, whereas the DNA template strand remains in the pretranslocated register. As a consequence, the RNA–DNA base pairs are tilted ~15° relative to an active EC, and the position into which NTPs must bind lacks a free template base to recognize an incoming NTP. Thus, the NAC is blocked in a half-translocated state, with the template base needed for the next round of nucleotide addition remaining paired to the nontemplate strand on the active-site distal side of the bridge helix. Consistent with elemental pausing being a universal regulatory feature of RNAP (90), this same tilted hybrid state also occurs in mammalian RNAPII PECs (143). It is unclear whether translocation always occurs in two steps (RNA first, then DNA) or the half-translocated state forms only at pauses.

Contribution of each elemental pause signal component to pausing. The additive contributions of each pause signal component suggest that each may contribute to a global RNAP conformational change that inhibits the completion of translocation. Indeed, a modest swiveling of a

WHAT IS BACKTRACKING?

Backtracking refers to reverse translocation of RNA and DNA through RNAP. During transcription, DNA translocates into RNAP from the downstream direction, melts at the downstream fork junction, and translocates away from RNAP after reannealing at the upstream fork junction. The RNA–DNA hybrid translocates away from the active site. RNA exits out a side channel after the hybrid separates at the upstream junction. During backtracking, these translocation steps all occur in the reverse direction, resulting in the 3′ RNA separating from DNA and occupying the NTP-entry pore. Pausing, nucleotide misincorporation, DNA-bound obstacles, DNA supercoiling, and a weak RNA–DNA hybrid all can favor backtracking. RNAP is sometimes said to backtrack on the DNA. However, both forward and reverse translocation require ~36° per base pair rotation and are best viewed as DNA movements relative to a stationary RNAP; in cells, RNAP attached to nascent RNA bound by ribosomes cannot easily rotate.

RNAP can cleave backtracked RNA hydrolytically in its active site. Cleavage is greatly stimulated by Gre factors, which stabilize binding of Mg^{2+}II, increase transcription fidelity, and rescue RNAP arrested after backtracking. The ATP-dependent translocase Mfd can counteract backtracking by RNAP but also can push RNAP off DNA at damage sites.

swivel module (described below for hairpin-stabilized pauses) occurs in the ePEC (52, 127). This change may alter contacts made by the clamp, rudder, and switches to DNA in the hybrid, which are more extensive than contacts to the RNA, enough to inhibit DNA translocation (52). Additionally, a key Arg residue in fork loop 2 may inhibit release of the +1 template base from the downstream base pair (127). Strong +1 base-pairing may inherently inhibit translocation, whereas interaction of +1 nontemplate G with a core recognition element pocket after translocation reduces pausing (144). However, G–C base-pairing alone does not explain how the downstream fork junction affects pausing. The *B. subtilis* consensus pause sequence features +1A or +1T in place of +1G (90, 153). Finally, lobe–clamp contacts to downstream DNA and lid–clamp contacts to −10,−11 Gs also may disfavor DNA translocation (64, 90).

Translocational interconversions in the ePEC. The half-translocated intermediate is just one of several translocational states formed by a consensus ePEC (127) (**Figure 3*c***). Backtracked and pretranslocated states are detectable by transcript cleavage and pyrophosphorolysis reactions, respectively (see the sidebar titled What Is Backtracking?). In the consensus ePEC, these states equilibrate so quickly that inducing rapid transcript cleavage with GreA or GreB does not reduce the pause dwell time (127). Rather, the strong kinetic barrier for escape of a consensus ePEC is the shift from the half- to the posttranslocated state. This absence of kinetically significant backtracking is unlikely to be true for all ePECs; for many pause sequences additional states may become kinetically significant (e.g., biphasic pausing when the hybrid is altered; **Figure 3*b***). Elemental and backtrack pausing may occur on a continuum rather than as discrete mechanisms (**Figure 3*c***). This model of rapidly interconverting ePEC states resembles descriptions of pausing for yeast RNAPII (33, 63) and provides opportunities for pause modulation by regulators that favor or disfavor backtracking (e.g., *E. coli* NusA or NusG, respectively) (8, 59, 140).

Backtrack Pause Signals

Although backtracking is not kinetically significant for some elemental pauses, it appears to be the prevalent mode of pausing by eukaryotic RNAPII and could prove to be more dominant in other bacterial lineages and in archaea. Backtracking can arise from initially formed ePECs

(42, 127) but also may be triggered when RNAP encounters a barrier to translocation like a protein bound to DNA (39, 106), by misincorporation of a mismatched 3′ RNA nucleotide (136), by DNA damage (67), or when transcribing RNAP encounters a DNA end. Once RNAP halts on DNA in response to one of these triggers, the extent of backtracking is determined by the relative stability of the RNA–DNA hybrids in the forward versus backtracked registers. An unstable hybrid in the register occupied by RNAP will favor backtracking if backtracking results in a more stable hybrid (112, 113). This model of backtrack pausing has been verified directly by genome-scale analysis of pauses in *E. coli*, *B. subtilis*, yeast, and mammalian cells (64, 101, 153). Backtrack pausing triggered by DNA damage or nucleotide misincorporation is thus the first step in transcription-coupled DNA repair, when backtracked RNAP is either displaced by Mfd or driven back further by UvrD to uncover the damage for repair, as well as in transcriptional error correction, when backtracking enables GreA/B–TFIIS-stimulated removal of 3′ error–containing RNA (107).

Backtracking requires that single-stranded RNA be available to move into the RNA exit channel. Thus, nascent RNA structures can block backtracking (156) and antisense DNAs or RNAs that create nascent structures can be used to assess the contribution of backtracking to pausing (4). Extensive backtracking leads to EC arrest, from which it is important to rescue or remove RNAP (e.g., by GreB-stimulated transcript cleavage or by Mfd) to avoid collisions with replication that cause double-stranded breaks in DNA (132). Thus, backtrack pausing plays central roles both in maintaining information in DNA and in accurately copying information to RNA.

Hairpin-Stabilized Pause Signals

Hairpin-stabilized pauses were first discovered in the leader regions of gammaproteobacterial amino acid biosynthesis operons regulated by attenuation (23, 151). These pauses synchronize transcription of the attenuator with translation of a leader peptide–coding region because a translating ribosome melts or blocks formation of the pause RNA hairpin. Most pauses in *E. coli* appear to be elemental or backtrack pauses rather than hairpin-stabilized pauses (90), but hairpin-stabilized pauses are nonetheless widespread among diverse 5′-proximal regulatory regions (26, 157), can involve pseudoknots rather than simple stem-loop structures (149), and also occur in *B. subtilis* (152, 153). The difficulty of predicting complex and alternative nascent RNA structures accurately has prevented a genome-scale accounting of hairpin-stabilized pausing. Solving this experimental challenge is a key need in the field.

The cryo-EM structure of the *his* PEC revealed that hairpin stabilization of swiveling by the clamp, shelf, jaw, SI3, and β′C, which rotate as a unit, explains the mystery of how hairpin stimulation of pausing in *E. coli* depends on the 188–amino acid insertion in the TL called SI3 (52, 68) (**Figure 2**). PHs form 11 or 12 nucleotides from the nascent RNA 3′ end. Nascent RNA structures leaving >12 or <11 3′ nucleotides do not stimulate pausing but can inhibit backtracking and, when leaving 7–8 3′ nucleotides, destabilize an EC (e.g., at intrinsic terminators) (22, 137). Deleting *E. coli* SI3 ≥50 Å away from the PH, or even altering SI3 structure, greatly reduces PH stimulation of pausing (31, 150). PH stabilization of swiveling explains this long-distance effect. Swiveling shifts the interface between SI3 and the core RNAP so that the SI3 movement required for TH formation creates steric clash (52, 68) (**Figure 2**).

Swiveling is also seen in backtracked PECs (1) and in a hairpin-containing *Thermus thermophilus* EC, which lacks SI3 (128). Thus, PHs that stimulate pausing in *B. subtilis* RNAP (152, 153), which also lacks SI3, might do so simply by stabilizing the swiveled state.

Key questions now include whether and how the PH-remodeled RNA exit channel, which contacts PH phosphates via conserved basic residues (68), aids PH formation and nascent RNA

folding. Formation of biologically active RNA often requires cotranscriptional folding, but the interplay between pausing and RNA structure formation remains incompletely understood (157).

Regulator-Stabilized Pause Signals

Regulators known to aid transcriptional pausing include NusA, the initiation factor σ^{70}, RfaH in *E. coli*, and NusG in bacterial lineages that include *B. subtilis*, *T. thermophilus*, and *Mycobacterium tuberculosis*. More pause regulators may await discovery as diverse bacterial lineages become more fully explored. Two mechanisms of regulator-stabilized pausing can be described.

NusA-stimulated pausing. The NusA type of pause stimulation depends on interactions with the nascent RNA that may aid PH formation, stabilize PHs, or aid backtracking (52, 53, 57, 102, 159). NusA contains four RNA-binding domains [N-terminal domain (NTD), S1, KH1, and KH2]. Although the hairpin loop potentially contacts the S1 domain, only the NTD (when supplied at high concentration) is required for the full effect of NusA on the *his* pause (53). The other NusA domains increase NusA affinity for the PEC. Key current questions include which NusA interactions are required to promote backtracking, whether additional NusA–RNA interactions play roles in modulating more complex RNA structures (149) or in aiding terminator hairpin formation (53), and the potentially broader role of NusA in promoting cotranscriptional folding of nascent RNAs (157).

σ^{70}-RfaH-NusG-stimulated pausing. The σ^{70}-RfaH-NusG class of pause enhancement depends on interactions of the regulator with the nontemplate DNA strand. NusG, the only elongation regulator found in all domains of life, binds ECs via its conserved NGN (NusG NTD) and ribosomes, ρ, or other regulators via its C-terminal domain. Only one type of housekeeping NusG ortholog (found in firmicutes, mycobacteria, and other lineages; hereafter referred to as *B. subtilis*–like NusG) exhibits pause enhancement via sequence-specific binding of the NGN to the nontemplate strand. *E. coli*–like NusG orthologs suppress pausing and backtracking upon binding to ECs and lack affinity for nontemplate strand bases. However, many bacteria encode one or more NusG paralogs that bind non–template strand bases and stimulate pausing (e.g., *E. coli* RfaH). Mutational analysis of *B. subtilis* NusG reveals an Asn/His-Thr motif in the NGN that aids non–template strand T recognition and may distinguish the two types of housekeeping NusG orthologs (99, 153–155). RfaH recognizes a non–template strand DNA hairpin (70), whereas σ^{70} stimulates EC pausing by binding to a −10-like sequence similar to the promoter Pribnow box (117). Pause stimulation by σ^{70}, RfaH, and NusG is strikingly similar, making σ^{70} an excellent paradigm to understand how regulators in this class work.

The regulator-induced pausing cycle. The pioneering analysis of σ^{70}-stabilized pausing by Jeff Roberts and coworkers provides paradigmatic insight into this class of pause regulators. Its discovery in the early 1990s came as a surprise (125); like many regulatory paradigms, this insight arose from studies of gene regulation in the coliphage λ. To allow time for binding of antiterminator Q to RNAP, which enables λ late gene expression, RNAP pauses at +16,17 downstream from the $\lambda P_{R'}$ promoter. Elemental pauses at +16,17 become long-lived when σ^{70} relocates from $\lambda P_{R'}$ contacts to a −10-like element exposed in the nontemplate strand of the PEC (13, 124, 125, 135) (**Figure 4**). Once bound, σ^{70} traps the nontemplate strand while nucleotide addition proceeds, causing the DNA strands to scrunch rather than reanneal as needed for continued transcription. This scrunching resembles an expansion of the transcription bubble that also occurs prior to promoter escape in the open complexes (123). Once bound, σ^{70} captures RNAP in a regulator-induced

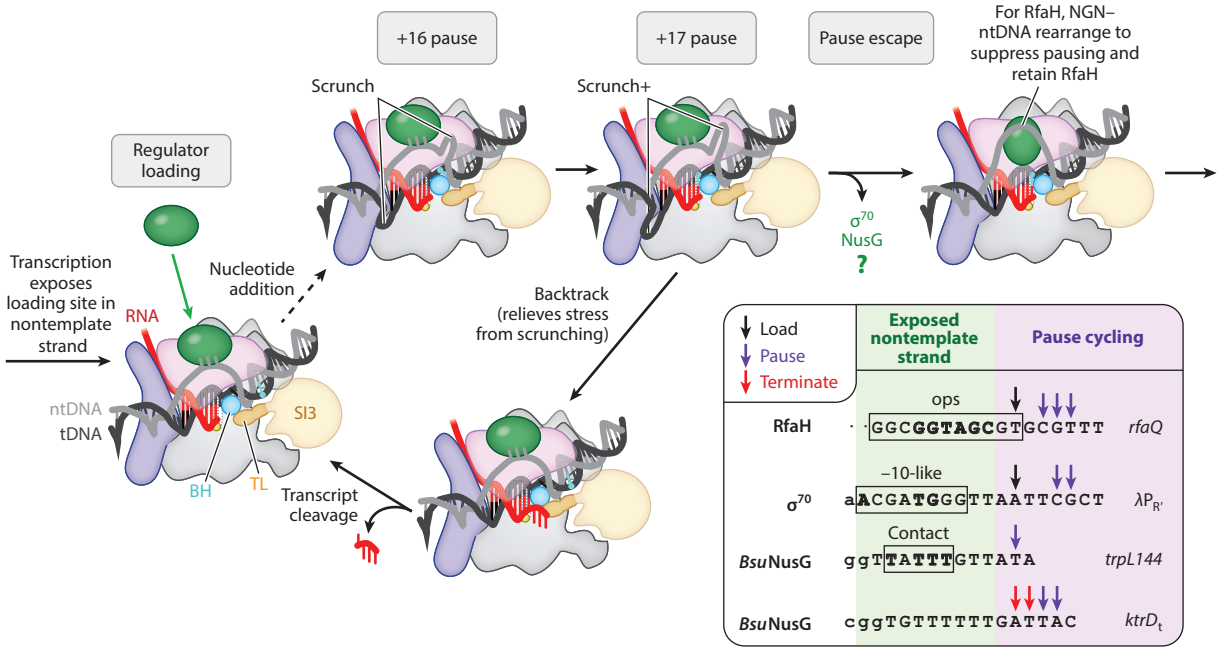

Figure 4

The regulator-induced pausing cycle. The cycle is illustrated for the σ^{70} pause in the λ late operon (117). Analogous sequences that mediate RfaH, σ^{70}, and *Bacillus subtilis* NusG-stabilized pausing are shown. (*Inset*) Conserved bases recognized by regulator are in boldface. The site of regulator loading is green. The region or potential region within which pause cycling occurs is purple. Abbreviations: BH, bridge helix; NGN, NusG N-terminal domain; ntDNA, nontemplate DNA; SI3, sequence insertion 3; tDNA, template DNA; TL, trigger loop.

pausing cycle (135) (**Figure 4**). The scrunched PEC is destabilized by increased DNA melting and possibly by steric strain from the scrunched DNA strands. This strain is relieved either by release of the σ^{70} contacts and escape from the pausing cycle or by backtracking of the PEC to prolong the pause and shrink the DNA bubble (116), which can be detected by Gre-induced transcript cleavage (100, 134). This pausing cycle involving PEC capture via nontemplate DNA binding, scrunching, backtracking, and transcript cleavage may be a common mechanism for the σ^{70}-RfaH-NusG class of pause stimulators. It also may operate at pauses associated with physical barriers, topological barriers, or DNA ends.

σ^{70}-Stimulated pausing via the pausing cycle occurs after many *E. coli* promoters, including lac_P (56, 111), and extends to at least some alternative sigma factors (119, 130); its extent remains incompletely defined (117, 119). Although σ^{70} can transfer from promoter contacts to PEC contacts (124), σ^{70} also can be retained persistently by a fraction of ECs (9, 35, 54, 55, 72, 104) or can rebind ECs and stimulate pausing far downstream from promoters (45, 105).

Both RfaH and *B. subtilis*–like NusG induce pausing via nontemplate strand binding mechanisms that resemble the σ^{70}-induced pause cycle, although with interesting variations. RfaH enhances pausing at its recognition sequence (*ops*), but once the EC escapes *ops*, RfaH remains stably bound in a pause-suppression mode that inhibits RNAP swiveling (70) and may guide the nontemplate DNA, aid translocation, and inhibit backtracking (109). The stably associated RfaH aids ribosome recruitment to nascent RNA and the resulting ribosome–RfaH–EC complexes suppress termination and aid translation in long operons that encode

lipopolysaccharide-synthesizing enzymes (17). RfaH loads on unscrunched ePECs paused at U11 of *ops* (GGC**GGUAGC**G$\underline{U}_{11}$GC$_{13}$GUUU; underline, *ops* consensus; bold, DNA hairpin bound by RfaH) (109). RfaH suppresses the U11 pause but dramatically enhances pausing at C13 and to lesser extents at G14 and U15, consistent with entry in a σ^{70}-like pausing cycle (6, 12). Pause escape appears to be triggered by rearrangement of the nontemplate strand, possibly triggered by stress from scrunching, into a path over the surface of the RfaH NGN that could explain processive RfaH retention and exclusion of σ^{70} and NusG.

B. subtilis NusG also may function similarly to σ^{70}-stimulated pausing (154). *B. subtilis* NusG enhances pauses that allow time for RNA folding or interactions with ligands in vivo (152). TnTTT in the exposed nontemplate strand interacts with the NusG NGN (153, 155). Pausing at *trpL* U144, which provides time for binding of the Trp RNA-binding attenuation protein (TRAP) (2, 152), occurs in the same window observed for σ^{70}- and RfaH-stimulated pausing (**Figure 4**), but a PH that increases pausing in concert with NusA may inhibit extension and backtracking prior to pause escape. Interestingly, NusG-stimulated pausing occurs at many *B. subtilis* intrinsic terminators just after the mapped termination points (e.g., *ktrD*$_t$; **Figure 4**). It is tempting to speculate that cycles of scrunching, backtracking, and cleavage might help explain NusG action at these terminators. Whether *B. subtilis* NusG causes pause cycling is unknown, but it is intriguing that the transcript cleavage factor GreA, which can facilitate escape from σ^{70} pause cycles (100), reduces promoter-proximal pausing in *B. subtilis* (83).

The remarkable similarity of regulator-induced pausing by σ^{70}, RfaH, and *B. subtilis*–like NusG raises several interesting questions. Conversion of RfaH into a processively bound form upon pause escape is well established, but the mechanism of RfaH retention remains unclear. Simple topological trapping of RfaH by the nontemplate strand is an attractive hypothesis. Might the nontemplate DNA topologically trap σ^{70} or NusG in some situations (e.g., to explain σ^{70} retention)? Does retained σ^{70} suppress pausing and NusG binding like RfaH? How widespread among diverse bacteria is the involvement of sigma factors in pausing and elongation? Might some conformation of *B. subtilis*–like NusGs suppress pausing similarly to RfaH? Finally, NusG–RfaH orthologs and paralogs are ubiquitous in diverse bacterial lineages controlling a variety of functions (19, 25, 46, 47, 115). Do all these regulators function like NusG or RfaH, or do additional modes of pausing-based regulation remain to be uncovered?

Pausing by single-subunit RNAPs. Although pausing by single-subunit RNAPs has been described, available data suggest these single-subunit RNAPs evolved different pausing behaviors and sequence dependencies than multisubunit RNAPs. For example, T7 RNAP recognizes a specific DNA sequence directly in addition to pausing on U-tracts (94); Qß RNA-dependent RNAP pauses when it encounters template RNA structures (85); and backtrack pausing by SARS-CoV-2 RNAP has been proposed to mediate template switching (98). However, more detailed and extensive studies are needed to understand these mechanisms.

GENOME-SCALE REGULATORS OF PAUSING

An in-depth description of the diverse roles pausing plays in cellular information processing is beyond the scope of this review. Many that involve pausing at specific locations are covered well in other recent reviews (10, 69, 101, 138, 154, 157). However, pausing also supports cellular information processing via global inputs to RNAP that affect its responses to broad sets of pause sites or even pausing genome wide. We know less about the global regulation of pausing, despite its importance. To encourage future studies to fill this knowledge gap, I conclude this review by describing what is known and what we need to know about the global regulation of pausing in

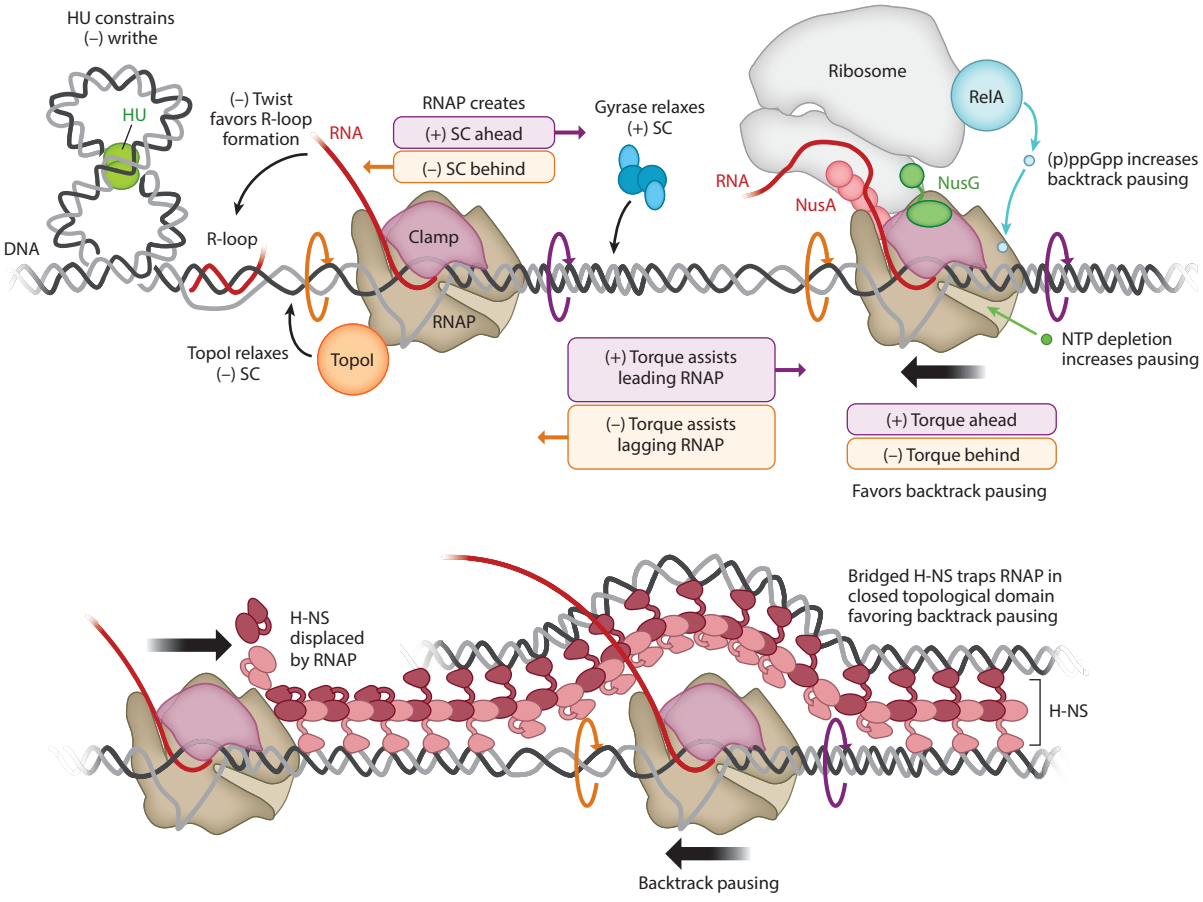

Figure 5

Global regulators of transcriptional pausing. DNA topology (supercoiling), R-loop formation, translational coupling modulated by NusA and NusG, small-molecule regulators [e.g., (p)ppGpp], and bacterial chromatin proteins like H-NS and HU all modulate transcriptional pausing in an interconnected network of interactions that regulates transcription of chromosomal DNA. RNAP both generates and responds to changes in DNA supercoiling. Plus signs and minus signs indicate positive and negative supercoiling. Abbreviations: RNAP, RNA polymerase; SC, supercoiling; TopoI, DNA topoisomerase I.

bacteria, with specific attention to small-molecule regulators of pausing, the interplay of pausing and DNA topology, and the effects of bacterial chromatin proteins on pausing (**Figure 5**).

Small-Molecule Regulators of Pausing

Although most cellular regulators of transcriptional pausing are macromolecules (proteins or RNAs), small molecules also interact with RNAP and modulate transcriptional pausing. These small molecules can interact either in the active site to affect catalysis or at other locations to affect RNAP structure allosterically.

NTP concentration. The most obvious small-molecule regulators of pausing are the substrates for transcription, NTPs. Although enzyme substrates are not typically considered regulators, NTP levels directly connect RNAP pausing to cell physiology because pause durations depend on NTP

levels that vary as a function of cellular state (154). NTP-sensing pauses are most clearly illustrated by the trailblazing work of Charles Turnbough on the *E. coli pyrBI* attenuator (36, 138, 139). Here, pauses at sites of UTP addition delay RNAP to allow time for ribosome loading on a leader peptide–coding region when UTP levels are low. Translation by the ribosome then suppresses intrinsic termination at the *pyrBI* attenuator, enabling transcription of the genes encoding the pyrimidine biosynthetic enzyme aspartate transcarbamoylase. More broadly, changes in NTP levels will alter dwell times at all pause sites, slowing transcription when NTP levels are low. For example, less pausing when NTP levels are high will increase rates of RNA production where translational capacity is greater even without direct coupling of ribosomes and RNAP.

(p)ppGpp regulation of pausing. In some bacteria (e.g., *E. coli*), pausing by RNAP is tied more directly to translation by the signaling molecule (p)ppGpp (**Figure 5**). (p)ppGpp is synthesized by the ribosome-binding enzyme RelA when the ribosome A site is empty (16) and binds *E. coli* RNAP at two sites, termed site 1 and site 2 (126). Site 1 is at the interface of the ω and β′ subunits, whereas site 2 is formed when the dissociable regulator DksA binds in the RNAP secondary channel. (p)ppGpp regulation of transcription initiation is largely worked out (48). Although less studied, it has long been known that (p)ppGpp also functions to stimulate pausing by RNAP in the absence of DksA (i.e., via site 1) (73, 75). (p)ppGpp-stimulated pause sites are found throughout genes (82), but the underlying mechanism is incompletely understood. (p)ppGpp has been shown to stimulate backtracking (67). In contrast, the hairpin-stabilized *his*PEC, in which backtracking is blocked by the PH, is unaffected by (p)ppGpp (R. Landick, unpublished observation). (p)ppGpp appears to stabilize partial clamp opening in σ^{70} holoenzyme (38); if also true for ECs, clamp loosening could explain increased backtracking. Global stimulation of pausing by (p)ppGpp appears to be a central circuit in regulation of cell physiology in *E. coli*–like bacteria where the aggregate effect of (p)ppGpp on pausing can significantly reduce transcription rate both in vitro and in vivo (18, 133) and is proposed to couple the rates of chain elongation by ribosomes and RNAP without requiring direct ribosome–RNAP interactions (160).

Cellular osmolality and water activity. (p)ppGpp may be only one example of a broader impact of small molecules on pausing by RNAP. For example, some osmolytes may alter pausing nonspecifically by affecting the relative stability of particular RNAP conformations (e.g., swiveling and TL folding) either electrostatically or via changes in the thermodynamic activity of water (21, 40). These types of effects have been characterized for initiation (78), including in vivo (49, 91), but they remain to be studied systematically for elongation and pausing. In addition to effects on RNAP, effects on pausing via effects on nascent RNA folding also are possible. Both changes in pH and changes in Mg^{2+} concentration are known to modulate pausing through effects on nascent RNA structures (61, 108). Bacterial cells respond to changes in their environments with large changes in intracellular solute composition (122), but the impacts of these changes on RNAP structure, activity, and pausing are largely unexplored areas in need of research.

Transcription–Translation Coupling

Ribosomes translating nascent RNA may affect pausing by forming complexes with RNAP termed expressomes, either directly through a tight coupling interface or more loosely though interfaces mediated by NusA, NusG, or both (77, 114, 145, 146) (**Figure 5**). These ribosome–RNAP interactions explain how ribosomes suppress hairpin-stabilized pausing by inhibiting or melting PHs (88) but also raise many questions. Some nascent RNA structures may be able to form in loosely coupled expressomes (28, 145). It is currently unclear which expressome conformations are present

during which stages of transcript elongation and pausing in vivo. The presence of pause regulators NusA and NusG at the ribosome–RNAP interface creates the potential for complex effects on pausing depending on expressome conformation; the plasticity of NusA and NusG is evident from their different configurations in antitermination complexes (62). The extent to which expressomes versus (p)ppGpp coordinates transcription and translation in *E. coli*–like bacteria (121, 160) and whether pausing within genes is important in *B. subtilis*–like bacteria that appear to use neither expressome-like nor (p)ppGpp-mediated coupling (66) remain unclear. These questions about coupling should drive high-impact research for the next decade or longer.

DNA Topology

Another key but poorly researched area of cellular transcriptional regulation is the connection between DNA topology and pausing. Transcription is inextricably linked to changes in DNA topology because neither RNAP nor DNA freely rotates within cells, even though one or the other must rotate 360° for every ~10 base pairs of DNA threaded through RNAP. The inability to rotate causes accumulation of positive DNA supercoiling in front of the EC and negative supercoiling behind it, which is known as the twin-supercoiled domain model of transcription (93) (**Figure 5**). The accumulated supercoiling is manifest as changes in either DNA twist, which generates a torsional force along the DNA helix, or DNA writhe, which relieves the torsional stress. In topologically closed DNA, the sum of twist and writhe is constant. These topological effects of transcription have been validated both in vitro (95) and in vivo (74). They explain how multiple ECs cooperate without requiring physical interactions of RNAP (74). Changes in DNA topology can either increase or decrease pausing by RNAP (81). Increases in negative and positive torsion upstream and downstream of RNAP, respectively, generate a force that favors backtracking of PECs (**Figure 5**); correspondingly GreA, which helps ECs recover from backtrack pauses, increases EC resistance to stalling in response to torsional force (96).

In cells, the accumulation of DNA supercoiling from transcription is relieved by topoisomerases. In *E. coli*, DNA gyrase relieves positive supercoiling in front of ECs and topoisomerase I relieves negative supercoiling behind ECs. Intriguingly, *E. coli* topoisomerase I appears to bind directly to RNAP (29). Combined, they maintain a modest negative supercoiling of the *E. coli* genome on average, but gene-by-gene supercoiling levels remain ill-defined. Differences in psoralen binding suggest that supercoiling varies considerably across the genome (86), but higher-resolution assays are needed to define supercoiling levels and effects on pausing in individual genes. GapR from *Caulobacter crescentus*, which binds positive twist in DNA (51), offers one attractive approach.

An interplay among negative supercoiling, pausing, and formation of nascent RNA structures also will impact R-loop formation (**Figure 5**). R-loops, in turn, impact both transcription and DNA replication (37, 84). Effects on pausing of R-loops upstream or downstream of PECs require study. New genome-scale methods are needed to define relationships among supercoiling, pausing, R-loop formation, and gene regulation.

Bacterial Chromatin, DNA Topology, and Pausing

In bacteria, transcriptional pausing functions not on the naked DNA templates used for nearly all mechanistic studies to date but instead on complex nucleoprotein complexes colloquially referred to as bacterial chromatin (129). Bacterial chromatin is complex; its protein components are diverse and generate more heterogeneous structures than nucleosomes in eukaryotic chromatin (32). Both the structures of bacterial chromatin and the ways they affect pausing require

more study. Two points deserve highlighting. First, bacterial chromatin is not necessarily an impediment to transcription. The most abundant chromatin protein in rapidly growing *E. coli*, HU, colocalizes with RNAP on actively transcribed genes (120). HU, which is also the most widely conserved bacterial chromatin protein, constrains negative supercoiling as writhe (50). Thus, HU may reduce both backtrack pausing and R-loop formation by relieving negative torsion upstream of ECs (**Figure 5**). Another abundant and widely distributed chromatin protein, Dps, binds all DNA in stationary phase but does not inhibit transcription (65). Thus, some bacterial chromatin proteins may turn out to aid transcription, but their direct effects on transcriptional pausing remain to be elucidated.

Second, the best-characterized bacterial chromatin protein involved in transcriptional silencing, H-NS in *E. coli*–like bacteria, can form different types of nucleoprotein filaments with different effects on transcriptional pausing (15, 80). Linear H-NS filaments, in which one H-NS polymer binds one DNA duplex, inhibit promoter binding but do not pose barriers to transcript elongation in vitro (**Figure 5**). In contrast, bridged H-NS filaments, in which one H-NS polymer binds two DNA duplex segments, trap ECs in a topologically closed domain that stimulates pausing at sites where backtracking is possible while having little if any effect at elemental pause sites where backtracking does not occur (80). Thus, switching between linear and bridged H-NS conformations may govern H-NS stimulation of pausing and ρ-dependent termination, which is crucial to silencing of laterally transferred DNA (118). The distributions of linear versus bridged H-NS filaments in vivo and the abilities of other regulators of pausing and topology to act on topologically trapped PECs are unknown and offer fertile ground for future study.

CONCLUSION

The study of transcriptional pausing has advanced from the stage of discovery and the search for function and mechanism to one of understanding how pausing mediates cellular information processing in different ways at an organismal scale. Much remains to be discovered both at the mechanistic level of signal processing within RNAP and at the global level of how regulators mediate information flow to and from PECs. Emerging approaches like time-resolved cryo-EM and massively parallel single-molecule and single-cell analyses may provide key tools for these discoveries. Human ingenuity and collaboration will provide the driving force.

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