Backtracked and paused transcription initiation intermediate of *Escherichia coli* RNA polymerase


Initiation is a highly regulated, rate-limiting step in transcription. We used a series of approaches to examine the kinetics of RNA polymerase (RNAP) transcription initiation in greater detail. Quenched kinetics assays, in combination with gel-based assays, showed that RNAP exit kinetics from complexes stalled at later stages of initiation (e.g., from a 7-base transcript) were markedly slower than from earlier stages (e.g., from a 2- or 4-base transcript). In addition, the RNAP–GreA endonuclease accelerated transcription kinetics from otherwise delayed initiation states. Further examination with magnetic tweezer transcription experiments showed that RNAP adopted a long-lived backtracked state during initiation and that the paused–backtracked initiation intermediate was populated abundantly at physiologically relevant nucleoside triphosphate (NTP) concentrations. The paused intermediate population was further increased when the NTP concentration was decreased and/or when an imbalance in NTP concentration was introduced (situations that mimic stress). Our results confirm the existence of a previously hypothesized paused and backtracked RNAP initiation intermediate and suggest it is biologically relevant; furthermore, such intermediates could be exploited for therapeutic purposes and may reflect a conserved state among paused, initiating eukaryotic RNA polymerase II enzymes.

Transcription initiation by RNA polymerase (RNAP) is a highly regulated rate-limiting step in many genes and involves numerous intermediate states that remain incompletely understood. Here, we report the characterization of a previously hypothesized slow initiation pathway involving RNAP backtracking and pausing. This backtracked and paused state is observed when all nucleoside triphosphates (NTPs) are present at physiologically relevant concentrations, but becomes more prevalent with unbalanced NTP levels, which may occur in vivo under conditions of metabolic stress. Pausing and backtracking in initiation may play an important role in regulating RNAP transcription. Moreover, similar RNA backtracked states may contribute to promoter-proximal pausing among eukaryotic RNA polymerase II enzymes.


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between these states (16–18). In particular, the strong σ70–PRS interactions (absent in elongation) (19–21) and the interactions (steric and electrostatic) of the acidic tip of the σ70 region 3.2 (σR3.2, which blocks the RNA exit channel) and the 5′ end of the nascent transcript (22–25) contribute most to the slow rate of initiation. Nascent RNAs of up to 9 nt in length are stabilized by hybridization to the melted template DNA in the RNA:DNA hybrid (18, 26–28). Although RNAP can accommodate an RNA of up to 14 nt long (i.e., 9 nt in RNA:DNA hybrid + 5 nt in RNA exit channel) (29), the transition into elongation, whereby the RNA exit channel blockage by σR3.2 is relieved, begins when RNA reaches the length of 11 nt (16, 30–32). After removal of the exit channel blockage, RNAP undergoes a global conformational change, σ70 loses its grip on the PRS and RNAP processively and rapidly elongates until it reaches the termination signal (23).

In what ways can RNAP overcome the barrier between initiation and elongation? Previous experimental data suggested that in AI, RNAP scrunches downstream DNA into its active site (3, 4). Because σ70 holds the −10 and −35 DNA elements tightly, the dsDNA upstream to the transcription bubble stays as a duplex, whereas the 10 DNA bases in the noncoding part of the transcription bubble (bases −10 to −1 in both strands) become compressed. This results in a strain buildup into what is known as the “stressed intermediate” complex (5, 33). Additionally, the longer the initially transcribed nascent RNA is, the closer its negatively charged 5′ end will be to the acidic tip of σR3.2 (19, 24). Simultaneously, a longer nascent transcript makes more stable RNA:DNA hybrid, which, in turn, helps counteract strain from the compressed DNA bubble and electrostatic repulsion from the acidic tip of σR3.2. In AI, transcripts are held in RNAP until reaching a certain length, above which the strain is relieved either through RNA backtranslocation and its abortive release through the secondary channel (14, 34–36) or by pushing σR3.2 and unblocking the RNA exit channel (22–25).

The electrostatic repulsion between the acidic tip of σR3.2 and the negatively charged 5′ end of the nascent transcript should decrease with each backtranslocation step (i.e., the distance between the negatively-charged groups increases). Nascent transcripts >4 nt in length could be stabilized in a backtracked state resulting in initiation pausing without immediate abortive release. Similar situations in elongation stabilize the nascent transcript in the pretranslocated state (37–41), eventually causing the nascent RNA 3′ end to disengage from the active site, backtrack into the secondary channel and pause transcription. The backtracked RNA can be cleaved either by the intrinsic endonucleolytic activity of RNAP (very inefficient at physiological pH) or by the action of extrinsic transcript cleavage factors GreA and GreB (TFIIS in eukaryotic RNA polymerase II system (42)). This cleavage leads to realignment of the newly formed RNA 3′-terminus with the active site and reactivation of transcription (pause release) (43–45). Due to these parallels, we wanted to examine if backtracking and pausing occur in transcription initiation by RNAP as well.

Previously it has been shown that stalling of transcription initiation occurs in strong promoters (8, 46). In addition, it has been shown that GreA and GreB induce RNA cleavages in AI, reduce abortive cycling and stimulate RNAP transition to elongation in vitro and in vivo at certain promoters (5, 25, 47–55). Linking these

![Fig. 1. Single-round transcription quenched kinetics assay.](https://www.pnas.org/doi/fig/10.1073/pnas.1605038113)

(A) Representative promoter sequence used here (lacCONS promoter) to show how by changing the initially transcribed sequence (ITS; cyan), different NTP-starved states can be generated (RP_{R,2}, RP_{R,EC4,6,9}, RP_{EC,1}). Other regions of the promoter include the promoter recognition sequence (PRS; pink) and the elongation sequence (yellow), including a probe target complementary sequence (red). All promoters measured are described in Fig. 2. (B) Schematic of RNAP runoff transcription starting from a particular NTP-starved state (incubation with a partial set of NTPs for _t-in trance_). Upon supplementing all NTPs, transcription kinetics start and transcripts are quantified via hybridization to a SDNA FRET probe for different incubation times (_t-in trance_). (C) Example of quenched kinetics data generated from quantification of runoff transcripts. The example follows one repetition of the kinetics exiting from RP_{R,2}. (D) As an example for kinetic curve extraction, average runoff kinetics from various RP_{R,2} are shown. The data points are averages of three repeats and the error bars are the SDs about these averages. The data are represented as points and the solid line represents the best-fit result to the model described in Methods. The best-fit values of the model parameters are shown in Table 2.
biochemical studies to the aforementioned endonucleolytic activity that the GreA–RNAP complex has in elongation, it was hypothesized that RNAP backtracking may occur in initiation and that GreA factors, as in elongation, act upon the nascent RNA bases that enter into the secondary channel of RNAP through backtracking. To directly address this question and to study the mechanism of transcription initiation in greater detail, we developed a solution-based, single-round quenched kinetics transcription assay that measures the kinetics of runoff transcript production. This assay was initially used to assess the kinetics of exit out of NTP-starved RPITC states, using an *E. coli* transcription system reconstituted from native RNAP core enzyme and σ70. Such NTP-starvation experiments provided us with reliable means to interrogate RNAP transcription from specific initiation states and offered mechanistic insights that were further validated in the follow-up experiments with minimal or no starvation. In addition to quenched kinetics assays of runoff RNA, we performed gel-based in vitro transcription assays that focused on kinetics of abortive products. Finally, we carried out single-molecule magnetic tweezers experiments to monitor changes in transcription bubble size during transcription initiation by RNAP. Altogether, we report here the detection and direct observation of RNAP in a backtracked, paused state during early stages of transcription initiation. Although previously hypothesized, our results establish the backtracked, paused state during early stages of transcription initiation. This observation could have potential applications for molecular therapeutics and mechanistic implications for mammalian RNA polymerase II enzymes.

**Results and Discussion**

**Single-Round Transcription Quenched Kinetics Assay.** We developed a single-round quenched kinetics assay (Fig. 1) to probe the kinetics of transcription by directly counting the number of transcripts produced over time. Using this assay, we initially examined the kinetics of *E. coli* RNAP transcription from distinct RPITC states generated via NTP starvation (Fig. 1A and B). The assay was based on quantification of single runoff transcripts by hybridization with a doubly labeled ssDNA probe (Fig. 1B). The number of hybridized probes (and hence the number of transcripts) was accurately determined using microsecond alternating-laser excitation (μsALEX)-based fluorescence-aided molecule sorting (ALEX-FAMS) (56, 57) (Fig. 1C). ALEX-FAMS is a method based on single-molecule Förster resonance energy transfer (smFRET) (58). A significant advantage of smFRET and ALEX-FAMS is the ability to identify distinct populations in a model-free manner, simply by counting single-molecule events of one sort (with a given FRET efficiency population) and comparing their number to single-molecule events of another sort (representing a distinct FRET efficiency population) (56, 57, 59, 60) (Fig. 1C). Hence, FAMS is a suitable method for the quantification of runoff transcripts at picomolar probe concentrations.

Our experimental design circumvented potential drawbacks of the quenched kinetics assay. Because we used low concentrations of RNAP and promoter DNA, the probability for RNAP reassembly/reinitiation was low, whereas single-round transcription reaction conditions were achieved kinetically and thermodynamically (61, 62) (details in Supporting Information, Rationale of the Single-Round Character of the Quenched Kinetics Transcription Assay). Furthermore, we designed the DNA template so that the probe hybridization sequence would anneal at the end of the fully elongated RNA (Fig. L4); in this way, probe hybridization would not interfere with transcription initiation but instead report on successful promoter escape. Also, because transcription initiation in many genes is much slower than elongation (i.e., initiation is typically rate limiting) (63), the synthesis of relatively short, yet full-length RNA products (39-base and 41-base transcripts for the promoters studied here) reflected the rate of transcription initiation.

![Fig. 2. Quenched kinetics transcription results identify an initiation-related stalled state. Shown are runoff kinetics from various NTP-starved states. Kinetics starting from late initiation states (e.g., RPITC≥4 blue) are slower than from an earlier initiation state (e.g., RPITC=2 black). The data are represented as points and solid lines represent best-fit results to the model described in Methods. The best-fit values of the model parameters are shown in Table S1.](https://doi.org/10.1073/pnas.0619065105)
Slower Transcription Initiation Kinetics from Select NTP-Starved States. Because promoter escape is the rate-limiting step in initiation, we anticipated that RNAP transcription kinetics starting from different RP states (“exit kinetics”) would be similar. However, we found that exit kinetics from the RP_{ITC≤4}, RP_{ITC≤6}, or RP_{ITC≤7} states were slower than from RP_{ITC=2} state (Fig. 2). In fact, whereas exit kinetics from RP_{ITC=2} were similar to those of RNAP already in the elongation state (RD_{E=11}), the exit kinetics from RP_{ITC≤7} were at least 3.5 times slower (Table S1). These results suggested the existence of a previously hypothesized paused state in RNAP transcription initiation (RP_{ITC≤7}; see Fig. 5). Importantly, this state was transient and overall RNAP activity remained unchanged, given that all “delayed” RNAP complexes (RP_{ITC≤4}, RP_{ITC≤6}, or RP_{ITC≤7}) eventually transitioned to elongation (Fig. 2).

Delayed Initiation Kinetics Are Associated with Backtracking. It is well established that elongating RNAP can backtrack and pause (44, 45). In such circumstances, the nascent RNA 3’ end backtranslocates into the secondary channel, where it can undergo endonucleolytic cleavage by the GreA–RNAP complex (25, 44, 48, 66). To test whether delayed exit kinetics for RP_{ITC≤7} were associated with backtracking during initiation, we assessed the effect of GreA, using our single-round quenched kinetics assay. As shown in Fig. 3 A and B, the addition of GreA at physiologically relevant concentration of 1 μM accelerated the exit kinetics from RP_{ITC≤7} relative to the exit kinetics from RP_{ITC≤2} (~50% recovery from the RP_{ITC≤7} stalled state; Methods and Table S1). These results were consistent with GreA-dependent release of RNAP from a backtracked and paused state in elongation (44, 45, 47).

To further test the effect of GreA during RNAP transcription initiation, we performed in vitro transcription assays in which 32P-labeled abortive transcripts were quantified following polyacrylamide gel electrophoresis (PAGE). This enabled identification of various abortive transcripts (band assignment in Fig. S8 and accompanying legend) and thus provided a means to determine whether GreA catalyzed the cleavage of short transcripts during transcription initiation. As shown in Fig. 3 C and D, the
production of the 7-base abortive RNA transcript was significantly suppressed in the presence of GreA. Because GreA stimulates cleavage only of “backtracked” RNA (i.e., when the RNA 3’ end is inserted into the secondary channel), these data, combined with our single-round kinetics data, confirmed that RNAP backtracks during transcription initiation. Because GreA predominantly induces cleavage of 2–3 bases from the RNA 3’-terminus (44, 45, 50–52), the lack of the 7-base abortive transcript is most likely due to GreA-induced shortening (compare Fig. 3 C and D) to a 5-mer, suggesting that RNAP in NTP-starved RP\textsubscript{ITC\textsubscript{C}57} backtracks by 1 base.

**RNAP Backtracking and Pausing in the Presence of All NTPs.** The data summarized in Figs. 2 and 3 studied pausing and backtracking in RNAP transcription initiation under conditions in which one or more NTPs were absent. Whereas the complete absence of select NTPs is highly unlikely in vivo, changes in pools of NTP levels may occur in bacteria in several conditions (e.g., metabolic stress, stages in cell growth; discussions in refs. 67–69). Accordingly, we examined RNAP transcription with the quenched kinetics assay with all NTPs present but under NTP concentration imbalance (UTP and GTP >> ATP and CTP at the lacCONS promoter; UTP and ATP >> CTP and GTP at the T5N25 promoter). According to the DNA sequences of these promoters (Fig. S1), these conditions were expected to temporarily populate delayed RP\textsubscript{ITC} states (RP\textsubscript{ITC\textsubscript{C}57} in lacCONS and RP\textsubscript{ITC\textsubscript{C}58} in T5N25, respectively). Consistent with the results described above, we observed a delay in exit kinetics from the RP\textsubscript{ITC\textsubscript{C}2} state under conditions of NTP imbalance (Fig. S9, orange) compared with equimolar conditions (Fig. S9, black) at each of the two different tested promoter templates. In addition, the observed delay was smaller in magnitude than the delay achieved upon exit from an NTP-starved RP\textsubscript{ITC} state (Fig. S9, blue). This result implies the kinetic delay inversely correlates with the abundance of the NTPs required for promoter clearance.

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The single-round quenched transcription kinetics assay and transcription assays with 3P-labeled UTP are ensemble experiments and hence cannot reliably detect infrequent or transient events. Under conditions of NTP starvation, abortive transcripts are identifiable by the gel-based assay (Fig. 3B). However, in the presence of all NTPs we did not obtain radiolabeled bands with sufficient intensity for the quantification of abortive transcripts, due to a limited number of abortive transcripts produced under single-round transcription conditions (Fig. S8B). The single-round transcription kinetics starting from RPITC=2 may already be influenced by pausing and backtracking; however, in the quenched kinetics assay RPITC=2 serves as a reference point.

To detect the full initiation behavior on the lacCONS promoter in the presence of equimolar NTPs (100 μM each) with high sensitivity, we implemented magnetic tweezers experiments with positively supercoiled promoter templates (Fig. 4A). This assay allowed us to track individual RNAP complexes over time and simultaneously detect and identify distinct RPITC states, based upon well-established changes in DNA extension (4). In the absence of GreA, we observed short- and long-lived RPITC states (Fig. 4 B, D, F, and H). The lifetimes of RPITC states (n = 216) are summarized in a histogram fitted with a double exponential, in which ~60% of events were short-lived (τ = 340 ± 60 s, SEM) and ~40% were long-lived (τ = 4,600 ± 2,700 s, SEM; Fig. 4H, blue; fractions based on quadrant analysis as discussed in Fig. 3 legend). Correlating these lifetimes with DNA bubble sizes (representing distinct RPITC states; Fig. 4 A, D, and F and accompanying legend) revealed that short-lived RPITC states are characterized by a Gaussian distribution of transcription bubble sizes with a mean apparent unwinding of 15.9 ± 0.4 bp (SEM) and a SD of 3.6 ± 0.4 bp, whereas long-lived events display two distinct states of apparent unwinding: a 20% fraction with mean apparent unwinding of only 9.9 ± 0.3 bp (SEM) and a SD of 0.7 ± 0.3 bp (SEM) and an 80% fraction with mean apparent unwinding of 14.8 ± 0.4 bp (SEM) and a SD of 2.3 ± 0.4 bp (SEM).

These data suggested that, in addition to the well-characterized RPITC state (Fig. 4A), a large fraction of RNAP complexes entered a distinct, long-lived state characterized by a smaller transcription bubble (denoted RPITC* in Fig. 4A-C). We hypothesized that this long-lived initiation intermediate represented a backtracked RNAP that was characterized also in our quenched kinetics and gel-based transcription assays. If correct, the addition of GreA would be expected to markedly reduce the number of these long-lived events. In agreement, RPITC states (n = 209), observed in the presence of GreA, became uniformly short-lived (τ = 350 ± 30 s, SEM) and displayed a bubble size distribution similar to that observed for the short-lived RPITC states in the absence of GreA [16.2 ± 0.2 bp (SEM); SD 2 ± 0.2 bp (SEM); Fig. 4 C, E, G, and H]). The results shown in Fig. 4 followed transcription rounds of single RNAP molecules on immobilized DNA molecules carrying the lacCONS promoter sequence. These experiments were performed in the presence of the initiating dinucleotide used in the quenched kinetics assay (Figs. 1D, 2, and 3 and Figs. S3, S4, S8, and S9). ApA. Additional magnetic tweezer transcription experiments using the lacCONS promoter with all NTPs in the absence of ApA yielded similar trends to those in the presence of it (example of a trajectory in Fig. S10). Experiments conducted on a different promoter template (TSN25) at 100 μM each NTP also showed the same trends, which were furthermore independent of the presence (Fig. S11) or absence (Fig. S12) of the initiating dinucleotide of TSN25 promoter, ApA. Specifically, we observed both short-lived RPITC states lasting tens of seconds and long-lived RPITC* states lasting thousands of seconds, for which the RPITC state systematically displays a smaller mean unwinding amplitude with narrower distribution than the RPITC state. As observed for the lacCONS promoter, addition of GreA once again abrogates the minimally unwound, long-lived RPITC* state in favor of short-lived RPITC states with extensive unwinding and leading to highly efficient promoter escape.

Finally, we performed magnetic tweezer transcription experiments on the lacCONS promoter and at higher NTP concentrations (1 mM each NTP; no initiating dinucleotide), which resemble the concentrations of NTPs under normal physiological conditions. The results obtained in the absence of GreA (Fig. S13) continue to show long-lived RPITC* events that resemble the ones measured under lower NTP concentrations. The transcription initiation complexes formed at the lacCONS promoter in these conditions continue to display double-exponential lifetimes before bona fide escape and regular processive elongation: 84%
with a fast phase of only 91 ± 15 s (SEM) and 16% with a slow phase remaining in the thousands of seconds (Fig. S13).

In summary, the presence of GreA abrogates RP<sub>TTC</sub> states characterized by long lifetimes and smaller transcription bubbles in favor of RP<sub>TTC</sub> states with short lifetimes and larger transcription bubbles, leading to highly efficient promoter escape (Figs. S11 and S12 D and E, quadrants, and S13C, quadrants). Hence, we conclude that GreA catalyzes the cleavage of nascent RNA transcript in backtracked complexes. The cleavage allows scrunching to resume so that the transcription bubble may reach its maximal size, efficiently driving the transition to a productive elongation. It also implies that the long-lived paused state is either caused by or stabilized through backtracking. These data further support the existence of a long-lived initiation state, in which the transcription bubble is smaller than its size in the presence of GreA, most likely due to backtracking. Altogether, the results provide evidence for the existence of the previously hypothesized paused and backtracked initiation state.

Conclusions

Our results support the existence of a previously hypothesized, yet uncharacterized, state in which RNAP backtracks and pauses during transcription initiation. We identified this paused—backtracked initiation intermediate under in vitro conditions with NTP concentrations that resemble physiological conditions (∼100 μM to 1 mM). Moreover, GreA and NTP availability appear to play key roles in regulating the flux in or out of this state. Based upon these results, we propose a modified transcription initiation model (Fig. 5). The model proposes that transcription initiation is rate limiting not only due to multiple abortive cycles that can occur before transitioning into elongation (5, 31, 63, 70), but also due to the existence of a backtracked, paused state. Thus, we hypothesize the presence of two initiation pathways. If nascent RNA backtracks into the secondary channel, either RNAP can swiftly release it as an abortive product, if the hybrid is short enough (14, 35, 36), or the backtracked complex can be stabilized. The prolonged association of the backtracked transcript may lead to intrinsic (or GreA-induced) RNA cleavage by RNAP. The backtracked-paused complex may also slowly release the nascent RNA through further backtracking. The model depicted in Fig. 5 adds an additional “slow” pathway (red arrows and beige rectangle background) to the conventional abortive initiation model.

Although the paused—backtracked initiation intermediate was observed frequently even at high NTP concentration, its effect on transcription and promoter escape was most pronounced under limiting NTP concentrations. These findings could have direct implications for in vivo conditions. First, the intracellular concentrations of NTPs can vary by severalfold, even during normal bacterial growth from midlog to early stationary phase (68). Additionally, nutritional limitation (e.g., carbon sources) can lead to a significant decrease of intracellular NTPs that directly affect the efficiency of transcription initiation and start-site selection at promoters of ribosomal and pyrimidine biosynthetic operons [reviewed by Turnbough (69)]. Moreover, decreases in nucleotide pools become even more dramatic during metabolic stress induced by antibiotics, oxidative stress, etc. (67). Therefore, under certain growth and environmental conditions, NTP concentrations may become limiting and thus are likely to affect abortive synthesis, early transcription, pausing, and promoter escape. Second, a number of in vitro studies have shown that the rate-limiting steps in transcription initiation include promoter DNA recognition/binding, open complex formation, abortive RNA synthesis/release, and promoter escape, all of which are targets for regulation (53). In vivo analysis of the chromosomal distribution of bacterial RNAPs by ChiP-chip, ChiP-seq, and DNA footprinting demonstrated that even under normal growth conditions (without NTP deprivation), a substantial fraction of RNAP accumulated at promoters of both transcriptionally active and inactive regions, engaged in either “poised” or open promoter complexes or in initial transcribing and paused complexes (43, 71–74). These results indicate that abortive initiation and promoter escape are the major rate-limiting steps in vivo.

Mechanisms of transcription by cellular RNA polymerases are broadly conserved (75). For example, scrunching (3, 4, 76) and trigger loop function during catalysis (37, 77–80) are similar, as are some of the mechanistic roles of e70 and TFIIIB (1, 81). It will be important to determine whether similar backtracked states are adopted during transcription initiation by eukaryotic RNA polymerases. Mammalian RNA polymerase II (Pol II) pauses during early stages of transcription, and this represents a common regulatory intermediate (82). Potentially, a mechanistic intermediate of paused mammalian Pol II enzymes may involve RNA backtracking; such backtracked intermediates may help explain why TFIIIS, a eukaryotic convergently evolved analog of GreA, has been linked to transcription initiation and assemblies with Pol II at the promoter (42, 83, 84). Finally, we emphasize that, because RNAP transcription initiation is rate limiting at many genes and highly regulated in vivo, this previously hypothesized yet uncharacterized backtracked-paused RNAP state may lead to potential new strategies for molecular therapeutics and to the development of novel antibiotics.

Methods

Transcription Quenched Kinetics Assay.

Preparation of a stable RP<sub>o</sub>, RP<sub>TTC</sub>, RP<sub>E</sub> solution is prepared with 3 μL E. coli RNAP holoenzyme (NEB, M0551S; 1.6 μM), 10 μL 2x transcription buffer [80 mM Hepes KOH, 100 mM KCl, 20 mM MgCl<sub>2</sub>, 2 mM dithirotetox (DTT), 2 mM 2-mercaptoethanol-HCl (MEC), 200 μg/mL BSA, pH 7], 1 μL of 1 μM lacCONS (65) or TSN25 promoter (5) (sequence in Fig. 1A and Fig. 51), and 6 μL of water. RP<sub>o</sub> is then incubated in solution at 37 °C for 30 min. To remove unreacted and nonspecifically bound RNAP, 1 μL of 100 mM Heparin-Sepharose CL-6B beads (GE Healthcare) is added to RP<sub>p</sub> solution together with 10 μL of prewarmed 1x transcription buffer [Heparin challenge (3, 65)]. The mixture is incubated for 1 min at 37 °C and centrifuged for at least 45 s at 6,000 rpm. A total of 20 μL of the supernatant containing RP<sub>p</sub> is transferred into a new tube with initiating dinucleotide, for sDNA-chemistry. Each kinetic curve was measured in two or three separate repeats to show reproducibility. The model depicted in Fig. 5 adds an additional “slow” pathway (red arrows and beige rectangle background) to the conventional abortive initiation model.

To produce runoff transcripts, high-purity ribonucleotide triphosphates (NTPs) (GE Healthcare) were used in all transcription reactions at 100 μM each. To obtain a specific initiation or elongation state, only a partial set of NTPs was used. The choice of the partial set of NTPs depended on the sequence of ITSs being used (Fig. 1B). To exit from the initiation/elongation NTP-starved state the reaction mixture was complemented with all four NTPs. The nontemplate strands of all promoter DNAs have the sequence of 20 da (20 consecutive A), the complementary sequence of ssDNA FRET probe (20 dt), at the end (Fig. 1A and Fig. 51 for probe target sequence). The ssDNA FRET probe is doubly labeled with a pair of fluorophores suitable for smFRET: a donor, tetramethylrhodamine, at the 5′ end (5′ TAMRA modification) and an acceptor, Alexa Fluor 647, at the 3′ end (3′ Alexa Fluor 647 modification) [ordered from IDT (65)].

For kinetics, the reaction mixture is incubated with the partial set of NTPs for a constant duration of 40 min at 37 °C. All four NTPs are then added to the reaction mixture and incubated for various times, t<sub>sec</sub>, at 37 °C. The reaction is quenched by addition of 0.5 M GndHCl. Subsequently, a ssDNA FRET probe is added to the quenched reaction mixture and incubated for 20 min at room temperature to detect runoff RNA transcripts produced during t<sub>sec</sub> (see Fig. 56 for quantification kinetics in 0.5M GndHCl). The quenched-probed reaction mixtures were then used for qALEX measurements. An example of the quenched kinetic assay FRET results is shown in Fig. 1C and D.

Each kinetic curve was measured in two or three separate repeats to show reproducibility (Fig. 57). The concentration of the ssDNA FRET probe in each
experimental repeat was designed so that the steady-state level of a kinetic curve would be 0.3 ± 0.05. To make all repeats of kinetic curves reach a steady-state level of exactly 0.9 and not close to it, these repeats had to be rescaled (as shown in Fig. S14 and a detailed explanation in Supporting Information, RNAP-DNA Complex Concentration Adjustment and Rescaling for Transcription Kinetics Assays).

Each time point in the quenched kinetics assay is measured for duration of 10–15 min, using a setup described in Panzeri et al. (85), using Perkin-Elmer SPADs and 532-nm and 638-nm CW lasers operating at powers of 170 μW and 80 μW, respectively.

For transcription kinetics experiments with GreA, 1 μM GreA is added to transcription complexes in NTP-starved initiation or elongation states and incubated for 15 min before adding all four NTPs to initiate transcription reactions for 4 h.

Each kinetic measurement was performed at least in duplicates, using different preparations obtained on different days (Fig. S7). For each batch, we made sure of the following:

i) The FRET probe in the presence of transcription complexes without NTPs yielded a high-FRET population with no or a negligible amount of low-FRET population (no NTP, negative control).

ii) After 20 min incubation of RP1-ΔC2 with all four NTPs (steady state for RP1-ΔC2; Figs. 1D, 2, and 3A and B and Figs. S3, S4A, S7, S9, and S14), the fraction of hybridized probe reaches 90 ± 5% (positive control). This control is performed daily on the same batch used to prepare NTP-starved RNAP states.

iii) With an elongation incubation time (typically several hours) after quenching the reaction, the measurement yielded the same hybridized fraction as measured without the extra incubation (quenching does work).

The result of the negative control (no NTP) serve as the “t = 0” time point. The positive control shows that the prepared transcription complexes are active and produce a lower amount of transcripts than the detection limit of the assay (overall amount of transcripts is lower than the amount of ssDNA FRET probe) at very long times, “t = ∞.”

All NTP-starved states were prepared from RP1-ΔC2 stock solution. Because the other experimental conditions (concentrations, temperature, etc.) are identical, any changes in activity that may be caused solely due to the starvation of NTPs will show a change in the hybridized fraction in long time points of the kinetic trace. Such comparisons were routinely performed and have never shown a difference in the long time point baseline between the kinetics from RP1-ΔC2 and from NTP-starved states (within 5% error). Therefore, we conclude that our experimental conditions (e.g., NTP starvation) did not alter the transcription activity but only the kinetics. In this regard, the same quantity of the stock solution could be used for kinetics assays for NTP-starved states as the one used for the above-mentioned positive control.

μsALEX-smFRET data were analyzed as described in Supporting Information, μsALEX-smFRET Analysis for the Quantification of Transcription Kinetics.

Transcription Assays Visualizing Abrupt Product Formation Using Urea-Denaturing PAGE Analysis of [3H]-Radiolabeled RNA Products. Abrupt transcription assays were run using the lacCDS promoter, having its probe target 20A sequence replaced by the WT lacUV5 sequence (results from +20 to +39 [Fig. S8]). Three units of RNAP holoenzyme (NEB; M0551S) was mixed with 50 nM promoter DNA in 1x transcription buffer in a final volume of 20 μL. The reaction was then incubated at 37 °C for 20 min to form RP1, followed by addition of 1 μL of 100 mM Heparin-Sepharose beads and 10 μL of transcription buffer. The mixture was incubated for ~1 min and centrifuged, and 20 μL of the supernatant was removed and added to 10 μL prewarmed transcription buffer. After incubating an additional 10 min, A,E was added at a final concentration of 1.3 mM and incubated for 30 min to form the RP1-ΔC2. The RP1-ΔC2 was then diluted to 400 μL with transcription buffer containing SUPERaseIN (AM2696; Thermo Fisher Scientific) to final concentrations of 1.7 nM template, 112 μM A,E, and 0.3 units/μL SUPERaseIN. This solution was stored at room temperature and used as a stock for each time course.

For time-course experiments, 90 μL of the stock solution was briefly incubated to bring it to 37 °C. To analyze the production kinetics of abortive products from RP1-ΔC2, stock solution was mixed with 10 μL of 200 μM UTP+GTP mixture supplemented with ~10 μCi [α-32P]UTP. At each time point, 10 μL aliquot was then removed and mixed with an equal volume of formamide gel loading buffer. To analyze abortive product formation from RNAP that was not stalled, the UTP+GTP mixture was replaced by a complete set of NTPs. In experiments looking at the effects of GreA on abortive product formation, additional 15-min incubation at 37 °C was performed before the addition of NTPs, either in the presence or in the absence of 1 μM GreA. The stopped reaction aliquots were held at −20 °C until running the urea-denaturing PAGE.

Samples were heated for 3 min at 90 °C and loaded on a 23% (wt/vol, 19:1 acrylamide-bis-acrylamide), 0.4-mm-thick urea-denaturing polyacrylamide gel. The gel usually ran for 5–6 h at 1,500 V in 1× TBE with an additional 0.3 M sodium acetate in the bottom well. The gels were then removed, dried, and exposed on a phosphor-storage screen about 2 d. Screens were visualized using a Typhoon PhosphorImager.

Magnetic Trapping Assay. Single-molecule experiments. For the single-molecule experiments we used the DNA constructs described in Supporting Information, DNA Constructs for the Magnetic Trapping Assay.

Functionalyzed 2.2-kbp DNA molecules were first attached to 1-μm-diameter streptavidin-coated magnetic beads (MyOne Strepavidin C1; Life Technologies) and then tethered to a modified glass capillary surface coated with anti-digoxigenin (Roche) (86). Experiments were carried out on a homemade magnetic tweezer microscope to extend and supercoil the DNA, running the events using the Picowisf software suite to track and analyze the position of the magnetic bead. This position marks the free end and thus the extension of the functionalized DNA. Data were analyzed using custom routines in the Xvin software suite. Experiments were carried out in standard buffer at 34 °C, using 100 μM RNAP saturated with n70 (prepared as in ref. 87) and 100 μM A,E (for experiments on lacCDS promoter) (we used 100 μM A,U for experiments on TNS25 promoter) and 100 μM each of ATP, UTP, GTP, and CTP. When added, GreA is at 1 μM.

μsALEX-smFRET data were analyzed as described in Supporting Information, μsALEX-smFRET Analysis for the Quantification of Transcription Kinetics.

Transformation of apparent DNA unwinding during scrunching and backtracking and its relation to the length of the transcribed RNA as seen in the quenched kinetics assay. In the supercoiling transcription assay where plenometric supercoils are present (+4 positive supercoils throughout), the extension changes of the DNA construct report on the number of supercoils. Specifically, the DNA typically contracts by ~55 nm for every additional supercoil when extended at low force (F = 0.3 pN) as in these experiments. DNA unwinding by RNAP is sensitively reported via its effect on overall DNA supercoiling: Conservation of linking number means that topological unwinding of 10.5 bp results in an ~55-nm decrease in DNA extension.

During initial transcription and scrunching, an “N=2” rule has been observed (88), relating the length of the RNA and the extent of DNA unwinding in the bubble. Because an RNA 2-mer can be formed in RPo without a need for additional DNA unwinding and scrunching, an RNA N-mer can be formed by additional unwinding and scrunching of N-2 bases. However, during backtracking, this linear relationship is lost, although the simplest hypothesis is that backtracking by 1 bp dehybridizes 1 bp of the RNA-DNA hybrid and reduces the bubble size by the same amount. Thus, in this work the single-molecule assay can report only on the apparent bubble size, which results from the final backtracked state. This measurement provides information complementary to that obtained from the quenched kinetics assay, which instead provides insight into the relation between accessible RNA lengths and likelihood of entry into a state that is not competent for promoter escape.

Separation of correlative lifetime/amplitude data into quadrants. We separate short- and long-lifetime initiation events by setting a boundary corresponding to the short mean lifetime plus 1 SD (as it is a single-exponential distribution this essentially means two times the short lifetime). We separate small and large bubbles by setting a boundary corresponding to the mean unwinding amplitude plus 1 SD. Furthermore, the length of an RNA 2-mer, 3-mer, or long lived and displaying small or large bubble sizes.

Illustrations. All illustrations of RNAP transcription initiation and elongation states have been prepared in Adobe Illustrator CC 2015.
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