Bacterial RNA polymerase can retain σ^{70} throughout transcription

Timothy T. Harden,a,b Christopher D. Wells,c Larry J. Friedman,b Robert Landickd,e, Ann Hochschildf, Jane Kondevg,h,i, and Jeff Gellesg,ih

Department of Physics, Brandeis University, Waltham, MA 02454; bDepartment of Biochemistry, Brandeis University, Waltham, MA 02454; cDepartment of Microbiology and Immunobiology, Harvard Medical School, Boston, MA 02115; dDepartment of Biochemistry, University of Wisconsin, Madison, WI 53706; and eDepartment of Bacteriology, University of Wisconsin, Madison, WI 53706

Edited by Jeffrey W. Roberts, Cornell University, Ithaca, NY, and approved December 15, 2015 (received for review July 15, 2015)

Production of a messenger RNA proceeds through sequential stages of transcription initiation and transcript elongation and termination. During each of these stages, RNA polymerase (RNAP) function is regulated by RNAP-associated protein factors. In bacteria, RNAP-associated α factors are strictly required for promoter recognition and have historically been regarded as dedicated initiation factors. However, the primary α factor in Escherichia coli, σ^{70}, can remain associated with RNAP during the transition from initiation to elongation, influencing events that occur after initiation. Quantitative studies on the extent of σ^{70} retention have been limited to complexes halted during early elongation. Here, we used multiwavelength single-molecule fluorescence-colocalization microscopy to observe the σ^{70}-RNAP complex during initiation from the initiating promoter and throughout the elongation of a long (≈2,000 nt) transcript. Our results provide direct measurements of the fraction of actively transcribing complexes with bound σ^{70} and the kinetics of its release from actively transcribing complexes. σ^{70} release from mature elongation complexes was slow (0.0038 s^{-1}); a substantial subpopulation of elongation complexes retained σ^{70} throughout transcript elongation, and this fraction depended on the sequence of the initially transcribed region. We also show that elongation complexes containing σ^{70} manifest enhanced recognition of a promoter-like pause element positioned hundreds of nucleotides downstream of the promoter. Together, the results provide a quantitative framework for understanding the postinitiation roles of σ^{70} during transcription.

CoSMoS | single-molecule fluorescence | sigma factor | elongation complex | transcription regulation

Although DNA-directed RNA synthesis can be carried out by RNA polymerase (RNAP) alone, it is well established that transcribing RNAPs in the cell have bound accessory proteins that modulate transcription initiation and elongation (1). Elucidating the dynamics of accessory factor binding to and release from the transcription apparatus is essential to achieving a quantitative understanding of the molecular mechanisms that control transcription in cells.

In bacteria, any of a variety of σ subunits can associate with the core RNAP, conferring on the enzyme the ability to bind to distinct subsets of promoter sequences (2). Some σ subunits release from core RNAP immediately upon the initiation of RNA synthesis (3). In contrast, the primary σ factor in Escherichia coli, σ^{70}, may be associated in vivo with a fraction of transcription elongation complexes (TECs) even far downstream of the promoter (4–8). It is unclear whether this downstream association in vivo reflects retention of the initiating σ^{70} subunit or binding of σ^{70} after TEC formation and whether the σ^{70}-TEC association is kinetically stable during transcript elongation (9).

Retention of σ^{70} by early elongation TECs has demonstrated consequences for gene regulation. In particular, the σ^{70}-containing TEC (σ^{70}-TEC) plays an essential role in bacteriophage λ late gene expression (10–12) because bound σ^{70} is required for the recognition of a promoter-like pause element that induces a critical early elongation pause. This early elongation pause, in turn, allows loading of an antitermination factor that enables transcription of the late gene operon (10, 13–15). Similar promoter-proximal pause elements are also associated with many E. coli promoters (16–19), but the function of these elements is yet unknown. Furthermore, σ^{70} interaction sites on core RNAP partially overlap with those of transcription elongation factors such as NusA, NusG, and RfaH (20–23). This and other evidence raises the possibility that σ^{70} retained in TECs sterically occludes the binding of other factors, which in turn could affect processes modulated by these factors, including intrinsic termination, rho-dependent termination, and transcription–translation coupling (10, 14, 15, 23).

σ^{70} retention by TECs early in elongation (<100 bp downstream of the promoter) is well established in vitro (9, 24). Retention can be detected indirectly as pausing that occurs at downstream pause elements that resemble promoter−10 elements (7, 12, 23). In addition, TECs with σ^{70} stably bound have been reported (25), and retention of σ^{70} by TECs stalled at different positions downstream of promoters has been confirmed in bulk (26, 27) and single-molecule (28) studies. The latter data have been interpreted to support models in which σ^{70} is stochastically released after promoter escape, but there are no studies directly characterizing release kinetics on actively elongating TECs.

To understand the postinitiation roles of σ^{70}, it is essential to identify the conditions under which σ^{70} is retained by RNAP after promoter escape and to describe the kinetics of its release from actively elongating TECs. Here, we use multiwavelength light microscopy to directly observe the behavior of individual fluorescently labeled σ^{70} subunits during transcript elongation by Escherichia coli RNA polymerase. We show that σ^{70} can be retained on an RNA polymerase molecule throughout transcription and alters polymerase behavior during transcript elongation.

Significance

In all kingdoms of life, gene transcription is not carried out by RNA polymerase enzymes alone. Instead, the behavior of RNA polymerases during transcription initiation, elongation, and termination is regulated by accessory proteins that bind to the polymerase molecule. Bacterial α proteins are historically thought of as transcription initiation factors primarily involved in promoter recognition. Here, we use light microscopy to directly observe the behavior of individual fluorescently labeled σ^{70} subunits during transcript elongation by Escherichia coli RNA polymerase. We show that σ^{70} can be retained on a RNA polymerase molecule throughout transcription and alters polymerase behavior during transcript elongation.

Author contributions: T.T.H., C.D.W., A.H., J.K., and J.G. designed research; T.T.H. and C.D.W. performed research; T.T.H., C.D.W., L.J.F., and R.L. contributed new reagents/analytic tools; T.T.H. and L.J.F. analyzed data; T.T.H., J.K., and J.G. prepared the original draft and all authors contributed to writing the final manuscript.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. KT326913–KT326916).

1To whom correspondence may be addressed. Email: kondev@brandeis.edu or gelles@brandeis.edu.

This article contains supporting information online at www.pnas.orglookup/suppl/doi:10.1073/pnas.1513899113/-/DCSupplemental.
single-molecule fluorescence techniques to follow in real time the initiation and elongation of transcription complexes from the phage λ P_R promoter. The measurements allow us to directly observe σ^{70} retention on and subsequent departure from transcription complexes, both near to and far (>2,000 nt) downstream of the promoter, and to separately characterize the behavior of TECs and σ^{70}TECs with respect to elongation velocity, intrinsic termination efficiency, and ~10-like pause element recognition.

**Results**

**Direct Detection of σ^{70} on Actively Elongating Transcription Complexes.** To observe the presence of σ^{70} within promoter complexes and TECs, we tethered linear DNA molecules labeled with AlexaFluor 488 (AF488) dye and containing the phage λ P_R promoter to the surface of a glass flow chamber (Fig. 1A). We incubated the surface with a solution containing *E. coli* RNAP holoenzyme containing a σ^{70} subunit labeled on a single cysteine with Cy5 dye. Formation of promoter complexes was visualized as the appearance in total internal reflection fluorescence microscopy (29) of discrete spots of fluorescence that colocalized with the spots from AF488–DNA (Fig. 1B; t = 0). Unbound holoenzyme was then removed from the chamber by extensive washing with buffer. After washing, the DNA-colocalized σ^{70} spots persisted for several minutes or longer, suggesting that they reflect the formation of the kinetically stable open complexes that are expected on this promoter (30).

Once open complexes formed, we initiated transcription at time *t* = 0 by introducing 0.5 mM each of ATP, CTP, GTP, and UTP (NTPs). The solution also contained a Cy3-labeled oligonucleotide probe that was used to detect the nascent transcript by hybridization to a repeated target sequence near the 5′ end of the RNA (Fig. 1A) (3). At 34% of the 576 DNA locations that displayed a spot of Cy5–σ^{70}RNAP fluorescence before NTP addition, we subsequently observed colocalization of a Cy3–probe spot, indicating the formation of a nascent transcript (Fig. 1B). A control experiment without NTPs showed only 4% probe colocalization. The two intrinsic terminators near the downstream end of the template (Fig. 1A) are expected to efficiently induce rapid (<1s) release of the transcript from RNAP (31–33). Consistent with transcript release upon termination, 94% of transcript probe spots seen in the NTPs-containing sample disappeared during the 47 min duration recording; the spots that disappeared (for example, Fig. 1C and Fig. S1, green traces) had a median lifetime of 79 ± 34 s (±SE). The median Cy3–probe spot lifetime was not significantly altered by changes in laser exposure (Fig. S2), indicating that most or all probe colocalizations were not prematurely terminated by photobleaching. During the median duration of the probe spot, an elongation complex is expected to transcribe 1260 ± 540 bp of DNA [at 15.9 ± 0.6 bp/s (34)]. Since the template encodes a 2134- to 2322-nt long RNA, this analysis implies that the transcript is first detected by probe hybridization when the TEC is located 870 ± 540 bp downstream of the promoter, a value consistent within experimental uncertainty with the probe association rate constant, 1.1 × 10^11 s^{-1} M^{-1} (3).

To assess the extent to which σ^{70} was retained during early elongation and beyond, we examined each DNA template location that had a colocalized Cy5–σ^{70} spot at *t* = 0 and determined whether σ^{70} was still present when the Cy3–probe spot was first observed at the same location. On most complexes, the Cy5–σ^{70} spot was lost before transcript was detected (Fig. 1C, Upper). In contrast, 29 ± 3% retained σ^{70} (Fig. 1B; Fig. 1C, Lower; Fig. 1D; Fig. S1; and Fig. S3), consistent with previous literature suggesting that σ^{70} can be retained by early and in some cases also mature TECs (7, 25, 26, 28). This fraction was significantly reduced when we used a transcription template with two point mutations that ablate the promoter-proximal pause element (Fig. 1D and Fig. S4B), consistent with earlier bulk measurements of actively elongating transcription complexes initiated from λ P_R (7).

![Fig. 1. Direct detection of σ^{70} on active TECs.](image)

**In Fig. 1. Direct detection of σ^{70} on active TECs. (A) Transcription template. The template contains the wild-type λ P_R promoter region (blue) with its transcription start site (bent arrow) and promoter proximal pause element (orange), followed by seven tandem repeats of a 21-nt cassette (mave), followed by a portion of the E. coli rpoB coding region (gray) and by two consecutive intrinsic terminators (X). (B) Images (65 × 65 μm) of the same microscope field of view of AF488–DNA (blue), Cy5–σ^{70} (red), and transcript-hybridization probe (green) taken at the specified times. Insets are magnified views of the marked regions. NTPs were introduced at time *t* = 0. The blue arrow marks a DNA spot; red and green arrows mark the same surface location in the other images, with the presence (filled arrows) and absence (open arrows) of a colocalized fluorescence spot indicated. Cartoon show the molecular structures hypothesized to be at the arrow at the three times shown; blue, red, and green stars represent the dye molecules attached to template DNA, σ^{70}, and transcript probe, respectively. (C) Two examples of time records of transcript probe (green) and σ^{70} (red) fluorescence, each colocalized at a DNA spot. (C, Upper) σ^{70} fluorescence disappears before the time interval (shaded) during which transcript probe fluorescence is present. (C, Lower) σ^{70} fluorescence persists throughout transcript probe interval. (D) The fraction (± SEM) of TECs that retain σ^{70} at the time transcript probe is first detected on the TEC. Retention is reduced when the wild-type promoter-proximal transcription pause is disrupted by mutation of the pause sequence. The reported values are corrected for photobleaching (Fig. S3).**

Harden et al.
but inconsistent with previous single-molecule measurements of TECs halted 50 bp downstream of a different promoter (P_{lacUV5}) (28). Our data establish by direct observation on actively elongating transcription complexes that a substantial fraction (29%) can retain bound ς²⁰ hundreds of base pairs downstream of the promoter.

ς²⁰ Can Be Retained During Synthesis of Thousands of Nucleotides of RNA. We next investigated the fate of TEC-associated ς²⁰ molecules from the time when transcript was first detected (with the Cy-3 probe) until transcript was released after the synthesis of >2,000 nt of RNA. In a minority of cases (31%), Cy5-ς²⁰ fluorescence disappeared before loss of Cy3-probe fluorescence. However, the majority of complexes retained Cy5-ς²⁰, releasing it either simultaneously with or subsequent to termination as judged by Cy3-probe disappearance (Fig. 2A). Fluorescence intensity measurements (Fig. S5) were consistent with the idea that the ς²⁰ present had been carried by the TEC to the terminator, rather than remaining behind at the promoter or nonspecifically bound to the slide surface. Thus, even on a long transcription unit, most TECs that retained ς²⁰ until nascent transcript was first detected retained ς²⁰ until termination or longer.

To ask whether ς²⁰ is released stochastically albeit slowly during elongation, we compiled ς²⁰-TEC lifetimes. We separately tabulated the populations that appeared to dissociate during elongation (Fig. 2A, green) and those that persisted at least until termination (Fig. 2A, purple and blue). Joint fitting of these two sets of lifetime data (Methods) showed that the observations were consistent with slow stochastic release of ς²⁰ (Fig. 2B). The apparent ς²⁰ dissociation rate constant k_{app} derived from the fit includes contributions both from dissociation and from photo-bleaching; to determine the true dissociation rate constant, we repeated the experiment at different laser exposures and extrapolated to zero exposure (Fig. S6), yielding k₅ = (3.8 ± 0.8) × 10⁻³ s⁻¹. Fig. 2C summarizes this dissociation process and the fates of the complexes we observe in our experiments. We see that a majority of transcription complexes release ς²⁰ early in the transcription cycle during or shortly after the transition from initiation to elongation, whereas a subset remains stably bound to the TEC. Under our experimental conditions, once a ς²⁰-TEC has transcribed ~870 bp or less, dissociation becomes extremely slow. In fact, the ~100 s required to transcribe an E. coli transcription unit of average length (~1,700 bp; Methods) at 15.9 ± 0.6 bps (34) is less than the characteristic lifetime of the slowly dissociating ς²⁰ (1/k₅ = 260 s). Thus, once early transcription is completed, retained ς²⁰ subunits would usually remain bound to TECs until termination on a transcription unit of typical length.

Effect of Retained ς²⁰ on TEC Function. The foregoing experiments demonstrated that two different types of complexes, canonical TECs and ς²⁰TECs, are synthesizing RNA transcripts in our experiments. Do the functional properties of these two species differ? We first determined the relative elongation rates of canonical TECs and ς²⁰TECs by measuring the transcript probe lifetime as the time difference between the first detection of probe fluorescence (estimated to occur 870 bp downstream of the promoter) and its departure (TECs: Fig. 3A and B, blue; ς²⁰TECs: Fig. 3A and B, purple). The distributions of probe lifetimes show pronounced tails, which is consistent with bulk measurements of transcript elongation kinetics (35) and is presumably attributable, at least in part, to heterogeneity in elongation rates across the populations of TECs and ς²⁰TECs (34). The probe lifetime distribution was indistinguishable within experimental uncertainty for canonical TECs and ς²⁰TECs, indicating that the two types of complexes elongated transcripts at the same rate.

We also examined whether the termination efficiency at an intrinsic transcription terminator is different for canonical TECs and ς²⁰TECs. We prepared a terminator insertion template (Fig. S4D) on which the only TECs detected by probe hybridization were those that had already read through a terminator of moderate strength (λ, ς_R2; termination efficiency 49 ± 4% (36)). We reasoned that if ς²⁰TECs recognized the terminator more (or less) efficiently than canonical TECs, then the fraction of TECs that retain ς²⁰ would be decreased (or increased) on template sequences downstream of the terminator. Instead, we found that the presence of the terminator did not significantly change this fraction either immediately after terminator readthrough or at the time of transcript probe departure (Fig. S7). This finding also indicates that terminator read-through did not detectably stimulate ς²⁰ dissociation from the TEC.

When functionally engaged with the TEC, either because of retention or rebinding, ς²⁰ is expected to be able to mediate recognition of promoter –10-like pause elements within the transcribed sequences (7, 12, 16, 22, 24). We therefore examined whether such a pause element positioned 224 bp downstream of the promoter (Fig. S4C) affected the time required for transcript elongation. This promoter-distal pause element significantly increased the elongation time but did so only for the subpopulation of TECs that retained ς²⁰ (Fig. 3). The statistical significance of this difference (red vs. green distributions in Fig. 3A and B) was confirmed by a
production of the >2,000-nt transcript, a substantial fraction (∼29%) of TECs retained σ70 throughout the early phase of elongation. This fraction depended on the initially transcribed sequence: a −10-like sequence element directing a promotor-proximal pause on the wild-type template increased the fraction of TECs that retain σ70 over that retaining σ70 when this sequence element was mutated. Release of the retained σ70 from actively transcribing σ70TECs is slow enough that most such complexes retain σ70 until termination. TECs and σ70TECs appeared identical with respect to their elongation rates and termination efficiencies at an intrinsic terminator. However, σ70TECs but not TECs could recognize a −10-like pause element >200 bp downstream of the promoter. Although our work examined initiation from only the λ PrK promoter, ChIP-chip data (5, 6) suggest that fractional σ70 retention during elongation might occur on many E. coli transcription units in vivo. However, those data do not distinguish between σ70 retention and σ70 rebinding to TECs [which has been demonstrated to occur in vivo (37)], nor do the data measure the fraction of TECs containing σ70.

Several prior studies that examined the σ70 content of TECs stalled very early in elongation, after the synthesis of ≤50 nt of RNA, concluded that a variable fraction, 20–100%, of TECs retain σ70 (16, 26–28). It is difficult to quantitatively compare those results to ours. In particular, because the earlier studies examined artificially stalled complexes, it was not always clear whether detected σ70 release occurred during promoter escape, during the brief period of active elongation or after stalling (which was in some cases accompanied by RNAS backtracking). Our study bypasses this ambiguity by examining actively elongating complexes in real time. The kinetics of the dissociation of σ70TECs we see after the first phase of elongation are consistent with a low, constant probability of release per unit time (Fig. 2B). Furthermore, the rate of σ70 dissociation from active TECs we measured is more than 10-fold faster than was observed previously for σ70 dissociation from stalled TECs under similar conditions (28). This finding suggests that σ70TECs undergoing elongation enter states more prone to σ70 release than are stalled σ70TECs, highlighting the importance of characterizing actively elongating TECs.

In another previous study (25), stalled TECs with σ70 stably bound were characterized after anti-TEC affinity immobilization; it is unclear whether or not this isolated TEC species corresponds to the σ70TECs in steady-state elongation that we studied. However, those complexes displayed normal elongation rates and termination efficiencies when restarted, similar to the results we obtained observing σ70TECs during steady-state elongation.

The structure of the σ70TEC has not been directly characterized. Nevertheless, we can make some educated guesses about its features (see ref. 9 and references cited therein). In the σ70 holoenzyme structure, σ70 region 3.2 interacts with core RNAS in such a way to obstruct the RNA exit channel; thus, this interaction is likely absent in the σ70TEC, in which the exit channel is occupied with RNA. In addition, an interaction between σ70 region 4 and core RNAS that is required for recognition of the promoter −35 element is likely absent in the σ70TEC, also because of clashes with the nascent RNA. In contrast, the interaction of σ70 region 2 with core RNAS that is seen in the holoenzyme and open complex structures is likely to be present in σ70TECs, because this interaction is presumed necessary for the recognition of the distal σ70-dependent pause element that we observe.

In our experiments, the majority of complexes release σ70 before transcript probe arrival (which occurs after synthesis of ∼870 nt of RNA). Our experiments do not address the question of whether this release occurs during promoter escape or from early TECs. Although we observe that a sequence element near the promoter can alter the amount of early release, we cannot exclude the possibility that other factors (e.g., posttranslational modification of a
fraction of RNAP molecules; see refs. 25 and 34) might also influence the retention of $\sigma^{70}$ before transcript probe arrival.

For TECs free to diffuse in solution, it is well established that the RNA transcript loses its association with the template DNA within seconds of successful termination at intrinsic terminators (32, 33). Less is known about dissociation of RNAP from the template upon termination (38). We see a significant number of retained $\sigma^{70}$ subunits depart from the template simultaneously with RNA dissociation, consistent with RNAP dissociation from DNA at the terminator (Fig. 2A, purple bar). Interestingly, we see that many more [31/(31 + 11) = 74%; Fig. 2A] of the $\sigma^{70}$ subunits that were retained on a TEC up to the point of termination then persist on the template, typically for hundreds of seconds, following probe departure (Fig. S1 A–F). This is an unexpected result. The simplest interpretation of these data is that $\sigma^{70}$-RNAP holozyme often remains associated with template DNA after termination under the conditions of these experiments, raising the possibility that this species might be able to reinitiate on a nearby promoter. An optical trapping study (33) detected efficient rapid release of core RNAP from template DNA upon termination; the apparent discrepancy between that result and our observation of kinetically stable association may arise from the application of force (3 pN or more) to the optical trap in the former experiment.

Does the lengthy $\sigma^{70}$ retention within TECs that we observe in vitro also occur in living cells? In principle, other proteins present in cells (but not present in our experiments in vitro) might bind to $\sigma^{70}$-TECs and accelerate $\sigma^{70}$ release (e.g., by forming a ternary complex with the $\sigma^{70}$-TEC). However, several lines of evidence suggest that transcription complexes in living cells exhibit behaviors that correspond to the phenomena we observe in vitro: (i) genome-wide ChIP-chip experiments (4, 6, 8) detected a low level of $\sigma^{70}$ on many transcription units, and the level did not change systematically beyond the peak at the transcription start site; (ii) TECs initiated from a P$_R$ in vivo exhibited $\sigma^{70}$ ChIP that was suppressed by the same promoter-proximal pause element mutations that we observe suppress $\sigma^{70}$ retention in vitro (7); and (iii) recognition of downstream −10-like pause elements in vivo (7) was ablated by the same mutations, paralleling our observations in vitro that recognition of these pause elements is restricted to $\sigma^{70}$-TECs.

It is currently uncertain why the mutations that abolish the promoter-proximal pause also promote the functionalization of TECs that retain $\sigma^{70}$. One of a number of possible hypotheses is that pausing provides time to form new, stabilizing $\sigma^{70}$-transcription complex interactions that are not present in promoter complexes. Another is that pausing itself is irrelevant to $\sigma^{70}$ retention: it may be that the mutations disrupt a sequence element in the nascent RNA that binds directly to $\sigma^{70}$ and inhibits its dissociation during early elongation.

Taken in the context of previous results, our observations suggest that $\sigma^{70}$ can maintain a kinetically stable association with TECs to the end of a long transcription unit and that such transcription units are thus transcribed by at least two subpopulations of elongation complexes with distinct subunit compositions and functional properties, similar to the well-established examples of the stably bound phage antitermination complexes (10, 12, 14, 24). With the antitermination complexes, the biological role of an augmented TEC is well established, whereas the biological function of $\sigma^{70}$ retention within at least a subset of TECs distal from the start site is not clearly established. Although the presence of $\sigma^{70}$ within TECs induces the recognition of promoter-distal −10-like pause elements, the regulatory role of such pausing remains speculative. Nevertheless, it seems likely that $\sigma^{70}$ retention within TECs has significant regulatory consequences through its effect on the binding of other elongation factors to TECs. In particular, evidence from a variety of sources suggest that NusG and its paralog RfaH compete with $\sigma^{70}$ for binding to TECs (6, 9, 23). These proteins have a variety of biological functions in rho-dependent transcription, transcription-translation coupling, and regulating the expression of horizontally acquired genes (11, 38, 39), and these functions may be suppressed in the subpopulation of TECs with stably bound $\sigma^{70}$. Further research will be required to test for the occurrence of this suppression and to explore its consequences.

The work presented here demonstrates a method for quantitatively characterizing the dynamics of the interaction of a transcription factor with TECs engaged in steady-state elongation. The same approach could be applied to studying other elongation factors either singly or in combination and may thus lead to new insights into the molecular mechanisms by which the mutually competing and cooperating elongation factors present in the cell collaborate to regulate gene expression.

Methods

DNA and Plasmids. To synthesize the wild-type transcription template (Fig. S4A), we first constructed plasmid pCDW115: DNA encoding an RNA containing seven tandem repeats of the 21-bp transcript probe target site (5'-AGA CAC CAC AGA CCA CAC ACA-3') and flanked by restriction sites BamHI and SpH1 was synthesized with GenScript. The multiple repeats of the transcript probe site were included to increase the rate of probe hybridization to nascent RNA (3). This construct was introduced into the pFW11 Tet plasmid (40) along with the $\lambda$ P$_R$ promoter/initial transcribed region (−109 to +21 with respect to the P$_R$ transcription start site), a segment of the E. coli rpoB and $\sigma^{70}$ coding sequence (+577 to +2,399 with respect to the start codon), and the $\lambda$. T7 promoter (−55 to +15 with respect to the start codon) along with the downstream T$_{70}$ terminator. The constructs were sequenced by using standard cloning techniques. The same approach was used to make pCDW115, which was identical except that it contained the proximal pause ablation mutations (Fig. S4B). The distal pause and terminator insertion plasmids pTH07 and pTH09 (Fig. S4 C and D) were constructed with Gibson Assembly Master Mix (New England Biolabs) using synthetic DNA and PCR products amplified from pCDW114. All four plasmid inserts (GenBank accession nos. KTH26913, KTH26914, KTH26915, and KTH26916) were verified by sequencing. Each transcription template (Fig. S4) was prepared by PCR from a plasmid composed with an upstream primer 5'-SBiosg/CTT AAA ATA GGC GTA TTA CCA G3'- and a downstream primer 5'-5A488/ AGA TAT CGC AGA AAG GCC CAC CCG AAG GTG AGC CAG TGT GAT TAC CAG GGT TTT CCC AGT CAG CAC CTT G3'- containing the T$_{70}$ promoter sequence (italics). The 20-nt Cy3-labeled probe oligonucleotide and all primer end modifications were previously described (3).

Proteins. An N-terminal His$_6$-tagged single-cysteine derivative of E. coli $\sigma^{70}$ (C1325 C2915 C2955 3566C; see ref. 41) was expressed in pRPOD36366 Rosetta (DE3) cells, denatured in 6 M urea, and purified as described in ref. 42, except for the following modifications: Ni-affinity chromatography was done at 4°C over a 5-mL HisTrap column (General Electric) charged according to the manufacturer’s instructions. The protein was eluted with a linear imidazole gradient from 10 to 500 mM over 40 mL in binding buffer (20 mM Tris-OAc (pH 8.0), 250 mM NaCl, and 1.3 mM Tris-2-carboxyethyl phosphine hydrochloride) with 6 M urea at a flow rate of 0.5 mL min$^{-1}$. The protein was refolded by sequential 1-h dialyses against 3, 1.5, 0.75, 0.32, 0.18, and 0.0 M urea in binding buffer and labeled with Cy5-maleimide dye. Cy5-$\sigma^{70}$-RNAP holozyme was prepared by incubating 2.6 μM Cy5-$\sigma^{70}$ and 1.3 μM core RNAP (Epicenter) in 50% w/vol glycerol, 20 mM Tris-HCl (pH 8.0), 250 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT at 37°C for 10 min and then stored at −20°C for up to 3 h before use.

Transcription Experiments. Single-molecule total internal reflection fluorescence microscopy was performed at excitation wavelengths 488, 532, and 633 nm for observation of AF488-DNA template, Cy3-transcript probe, and all primer end modifications were previously described (3).

606 | www.pnas.org/cgi/doi/10.1073/pnas.1513899113
Harden et al.
objective lens (43). Cy5-UTP-RNAP was then introduced at 1.9 nM in transcription buffer [wash buffer supplemented with 3.5% wt/vol PEG 8000 (no. 81268; Sigma-Aldrich), 1 mg ml^{-1} BSA, and an O₂-scavenging system (29)], incubated for 10 min, and washed out. Finally, we started continuous image acquisition (1-s exposure every 1.0, 8.7, or 15.7 s to simultaneous 532- and 633-nm excitation, each at 200 μW) and initiated transcription by introducing transcription buffer supplemented with 500 μM each of ATP, CTP, GTP, and UTP and 10 nM Cy3-probe.

Data Analysis. Image analysis was done using custom software and algorithms for automatic spot detection, spatial drift correction, and colocalization as described (44). To measure the apparent 𝜀[50] dissociation rate constant 𝑘[50]app (Fig. 2B and Fig. 5E), we used the method of Ensign and Pande (45) to jointly fit the measured lifetimes of Cy5-UTP and 10 nM Cy3-UTP with the Eq 1 and Eq 2 of Ref. 45.

1. Blombach F, et al. (2013) Archaeology of RNA polymerase: Factor swapping during the transcription cycle. Biochem Soc Trans 41(1):362–367.
2. Gross CA, et al. (1998) The functional and regulatory roles of sigma factors in transcription. Cold Spring Harb Symp Quant Biol 63:141–155.
3. Friedman LJ, Gelles J (2012) Mechanism of transcription initiation at an activator-dependent promoter driven by single-molecule observation. Cell 148(4):679–689.
4. Reppas NB, Wade JT, Church GM, Struhl K (2008) The transition between transcriptional initiation and elongation in E. coli is highly variable and often rate limiting. Mol Cell 24(5):747–757.
5. Raffaelle M, Kanin EI, Vogt J, Ansari AZ (2005) Holoenzyme switching and dormancy at RNA polymerase-promoter complexes. Proc Natl Acad Sci USA 102(15):5195–5199.
6. Raffaelle M, Vogt J, Ansari AZ (2005) Transcriptional pausing at RNA polymerase-promoter complexes. Proc Natl Acad Sci USA 102(15):5195–5199.
7. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
8. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
9. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
10. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
11. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
12. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
13. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
14. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
15. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
16. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
17. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
18. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
19. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
20. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
21. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
22. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
23. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
24. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
25. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
26. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
27. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
28. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
29. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
30. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
31. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
32. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
33. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
34. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
35. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
36. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
37. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
38. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
39. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
40. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
41. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
42. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
43. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
44. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
45. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.
46. Roberts JW, et al. (1998) Antitermination by bacteriophage lambda Q protein. J Biol Chem 273(49):3292–3299.