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

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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 σ factors are strictly required for promoter recognition and have historically been regarded as dedicated initiation factors. However, the primary σ 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 λ $P_{R'}$ 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 σ^{70} 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

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 σ 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 an RNA polymerase molecule throughout transcription and alters polymerase behavior during transcript elongation.

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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. 1*A*). 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. 1*B*; $t = 0$). 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. 1*A*) (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. 1*B*). A control experiment without NTPs showed only 4% probe colocalization. The two intrinsic terminators near the downstream end of the template (Fig. 1*A*) are expected to efficiently induce rapid (within 1 s) 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. 1*C* 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, $1.1 \times 10^{+7}$ 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. 1*C*, *Upper*). In contrast, 29 ± 3% retained σ^{70} (Fig1*B*; Fig. 1*C*, *Lower*; Fig. 1*D*; 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. 1*D* and Fig. S4*B*), consistent with earlier bulk measurements of actively elongating transcription complexes initiated from λ $P_{R'}$ (7)

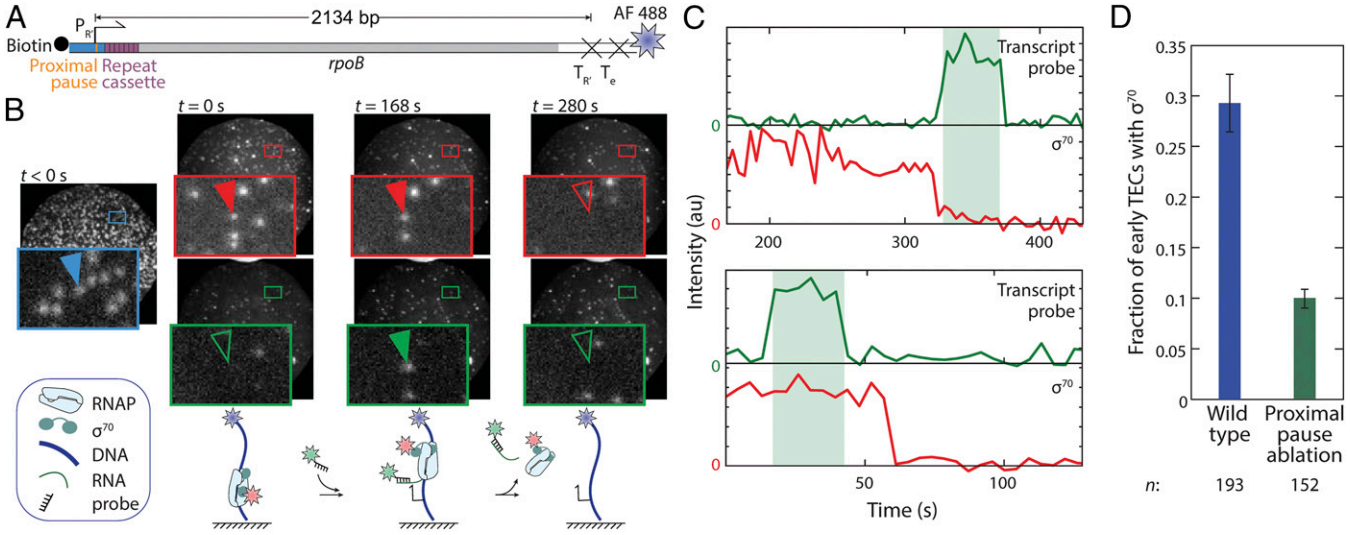

Fig. 1. Direct detection of σ^{70} on active TECs. (*A*) Transcription template. The template contains the wild-type λ $P_{R'}$ promoter region (blue) with its transcription start site (bent arrow) and promoter proximal pause element (orange), followed by seven tandem repeats of a 21-nt cassette (mauve), 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. Cartoons 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 departs 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).

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 σ^{70} hundreds of base pairs downstream of the promoter.

σ^{70} Can Be Retained During Synthesis of Thousands of Nucleotides of RNA. We next investigated the fate of TEC-associated σ^{70} 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–σ^{70} fluorescence disappeared before loss of Cy3–probe fluorescence. However, the majority of complexes retained Cy5–σ^{70}, releasing it either simultaneously with or subsequent to termination as judged by Cy3–probe disappearance (Fig. 2*A*). Fluorescence intensity measurements (Fig. S5) were consistent with the idea that the σ^{70} 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 σ^{70} until nascent transcript was first detected retained σ^{70} until termination or longer.

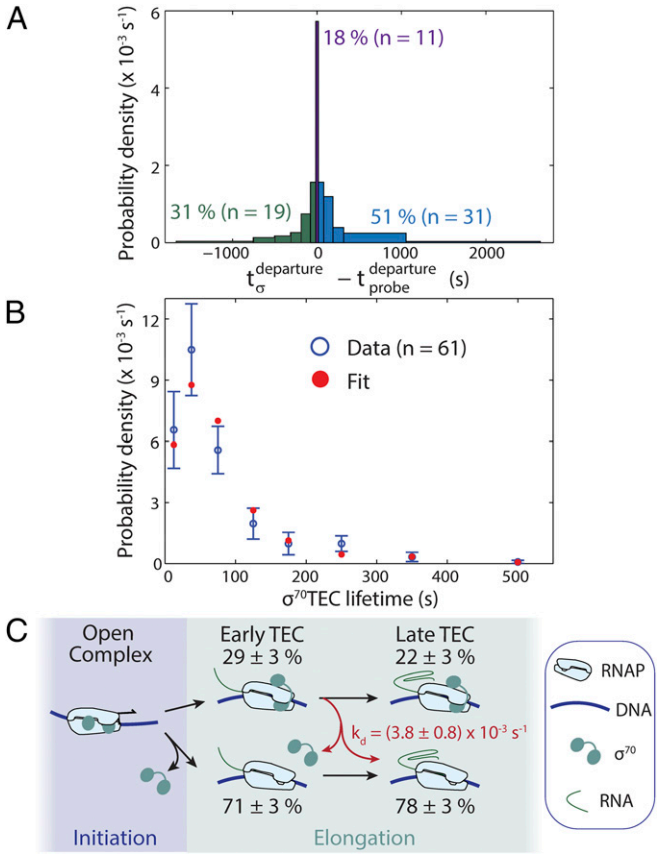

Fig. 2. Dissociation of σ^{70} from TECs is slow compared with transcript production. (*A*) Histogram of σ^{70} departure time relative to transcript probe departure from the same TEC. The σ^{70} spot departed either before (green), simultaneously with (purple), or after (blue) transcript probe spot departure. (*B*) Lifetime distribution of σ^{70}TECs (blue) and fit to a first-order dissociation model (red) yielding an apparent σ^{70}TEC dissociation rate constant k_{app} = 4.0 ± 0.5 × 10^{-3} s^{-1}. σ^{70}TEC lifetime was calculated as the duration of the simultaneous presence of Cy5–σ^{70} and Cy3–probe fluorescence. (*C*) Kinetic model. Percentages (± SE) indicate fractions of initially observed open complexes (n = 61) that reach the indicated states.

To ask whether σ^{70} is released stochastically albeit slowly during elongation, we compiled σ^{70}TEC lifetimes. We separately tabulated the populations that appeared to dissociate during elongation (Fig. 2*A*, green) and those that persisted at least until termination (Fig. 2*A*, purple and blue). Joint fitting of these two sets of lifetime data (*Methods*) showed that the observations were consistent with slow stochastic release of σ^{70} (Fig. 2*B*). The apparent σ^{70} dissociation rate constant k_{app} derived from the fit includes contributions both from dissociation and from photobleaching; to determine the true dissociation rate constant, we repeated the experiment at different laser exposures and extrapolated to zero exposure (Fig. S6), yielding k_d = (3.8 ± 0.8) × 10^{-3} s^{-1}. Fig. 2*C* summarizes this dissociation process and the fates of the complexes we observe in our experiments. We see that a majority of transcription complexes release σ^{70} 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 σ^{70}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 bp/s (34) is less than the characteristic lifetime of the slowly dissociating σ^{70} ($1/k_d$ = 260 s). Thus, once early transcription is completed, retained σ^{70} subunits would usually remain bound to TECs until termination on a transcription unit of typical length.

Effect of Retained σ^{70} on TEC Function. The forgoing experiments demonstrated that two different types of complexes, canonical TECs and σ^{70}TECs, 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 σ^{70}TECs 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. 3 *A* and *B*, blue; σ^{70}TECs: Fig. 3 *A* 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 σ^{70}TECs (34). The probe lifetime distribution was indistinguishable within experimental uncertainty for canonical TECs and σ^{70}TECs, 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 σ^{70}TECs. We prepared a terminator insertion template (Fig. S4*D*) on which the only TECs detected by probe hybridization were those that had already read through a terminator of moderate strength [λ t_{R2}; termination efficiency 49 ± 4% (36)]. We reasoned that if σ^{70}TECs recognized the terminator more (or less) efficiently than canonical TECs, then the fraction of TECs that retain σ^{70} 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 σ^{70} dissociation from the TEC.

When functionally engaged with the TEC, either because of retention or rebinding, σ^{70} 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. S4*C*) 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 σ^{70} (Fig. 3). The statistical significance of this difference (red vs. green distributions in Fig. 3 *A* and *B*) was confirmed by a

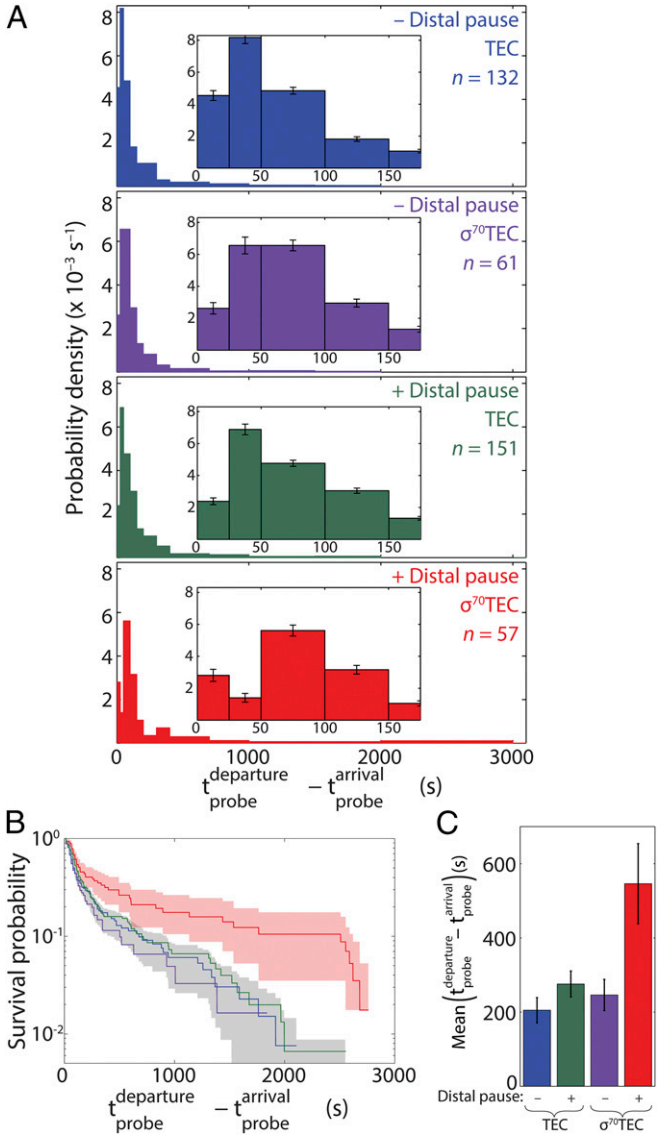

Fig. 3. Effect of retained σ^{70} on elongation time and on recognition of a distal –10-like pause. (*A*) Distributions of elongation complex transcript probe lifetimes on wild-type transcription template (Fig. 1*A*) and on a mutant template with a –10-like pause element inserted at +225 with respect to the transcription start sequence (see Fig. S4*C*). For each template, TEC and σ^{70}TEC are the distinct subpopulations of elongation complexes from the same recording that, respectively, did not or did have bound Cy5–σ^{70} at the time of probe arrival. (*B*) Survival curves of the distributions in *A*. Shaded regions show the 90% confidence intervals of red curve and of the aggregate of the blue, purple, and green curves. (*C*) Means (± SE) of distributions in *A*.

Kolmogorov–Smirnov test ($P = 0.006$). These data strongly suggest that the σ^{70} molecules that we detect are functionally associated with their colocalized TECs and that only σ^{70}TECs (and not canonical TECs) can recognize –10-like pause elements.

Discussion

We used single-molecule methods to study transcription complexes undergoing steady-state transcript elongation and to directly observe the times at which σ^{70} subunits dissociated relative to two markers: a time relatively early in elongation (detected by probe hybridization to the nascent transcript, which occurs after the synthesis of ~870 nt of RNA) and the time of termination (detected by release of the probe–transcript hybrid). During production of the >2,000-nt transcript, a substantial fraction (~29%) of TECs retained σ^{70} throughout the early phase of elongation. This fraction depended on the initially transcribed sequence: a –10-like sequence element directing a promoter-proximal pause on the wild-type template increased the fraction of TECs that retain σ^{70} over that retaining σ^{70} when this sequence element was mutated. Release of the retained σ^{70} from actively transcribing σ^{70}TECs is slow enough that most such complexes retain σ^{70} until termination. TECs and σ^{70}TECs appeared identical with respect to their elongation rates and termination efficiencies at an intrinsic terminator. However, σ^{70}TECs 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 $P_{R'}$ promoter, ChIP-chip data (5, 6) suggest that fractional σ^{70} retention during elongation might occur on many *E. coli* transcription units in vivo. However, those data do not distinguish between σ^{70} retention and σ^{70} rebinding to TECs [which has been demonstrated to occur in vivo (37)], nor do the data measure the fraction of TECs containing σ^{70}.

Several prior studies that examined the σ^{70} 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 σ^{70} (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 σ^{70} release occurred during promoter escape, during the brief period of active elongation or after stalling (which was in some cases accompanied by RNAP backtracking). Our study bypasses this ambiguity by examining actively elongating complexes in real time. The kinetics of the dissociation of σ^{70}TECs we see after the first phase of elongation are consistent with a low, constant probability of release per unit time (Fig. 2*B*). Furthermore, the rate of σ^{70} dissociation from active TECs we measured is more than 10-fold faster than was observed previously for σ^{70} dissociation from stalled TECs under similar conditions (28). This finding suggests that σ^{70}TECs undergoing elongation enter states more prone to σ^{70} release than are stalled σ^{70}TECs, highlighting the importance of characterizing actively elongating TECs.

In another previous study (25), stalled TECs with σ^{70} stably bound were characterized after anti-TEC affinity immobilization; it is unclear whether or not this isolated TEC species corresponds to the σ^{70}TECs 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 σ^{70}TECs during steady-state elongation.

The structure of the σ^{70}TEC has not been directly characterized. Nevertheless, we can make some educated guesses about its features (see ref. 9 and references cited therein). In the σ^{70} holoenzyme structure, σ^{70} region 3.2 interacts with core RNAP in such a way to obstruct the RNA exit channel; thus, this interaction is likely absent in the σ^{70}TEC, in which the exit channel is occupied with RNA. In addition, an interaction between σ^{70} region 4 and core RNAP that is required for recognition of the promoter –35 element is likely absent in the σ^{70}TEC, also because of clashes with the nascent RNA. In contrast, the interaction of σ^{70} region 2 with core RNAP that is seen in the holoenzyme and open complex structures is likely to be present in σ^{70}TECs, because this interaction is presumed necessary for the recognition of the distal σ^{70}-dependent pause element that we observe.

In our experiments, the majority of complexes release σ^{70} 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 σ^{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 σ^{70} subunits depart from the template simultaneously with RNA dissociation, consistent with RNAP dissociation from DNA at the terminator (Fig. 2*A*, purple bar). Interestingly, we see that many more [31/(31 + 11) = 74%; Fig. 2*A*] of the σ^{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 σ^{70}RNAP holoenzyme 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 RNAP–DNA linkage by the optical trap in the former experiment.

Does the lengthy σ^{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 σ^{70}TECs and accelerate σ^{70} release (e.g., by forming a ternary complex with the σ^{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 σ^{70} on many transcription units, and the level did not change systematically beyond the peak at the transcription start site; (*ii*) TECs initiated from λ $P_{R'}$ in vivo exhibited σ^{70} ChIP that was suppressed by the same promoter-proximal pause element mutations that we observe suppress σ^{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 σ^{70}TECs.

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

Taken in the context of previous results, our observations suggest that σ^{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 σ^{70} retention within at least a subset of TECs distal from the start site is not clearly established. Although the presence of σ^{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 σ^{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 σ^{70} for binding to TECs (6, 9, 23). These proteins have a variety of biological functions in rho-dependent termination, 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 σ^{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. S4*A*), we first constructed plasmid pCDW114: 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 BamH1 and Sph1 was synthesized by 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 λ $P_{R'}$ promoter/initial transcribed region (–109 to +21 with respect to the $P_{R'}$ transcription start site), a segment of the *E. coli rpoB* coding sequence (+577 to +2,399 with respect to the start codon), and the λ t_{R2} terminator (+49 to +232 with respect to the $P_{R'}$ transcription start site) 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. S4*B*). 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. KT326913, KT326914, KT326915, and KT326916) were verified by sequencing. Each transcription template (Fig. S4) was prepared by PCR from the corresponding plasmid with an upstream primer 5′-/5Biosg/CCT ATA AAA ATA GGC GTA TCA CGA G-3′ and a downstream primer 5′-/AF488/ *AGA TAT CGC AGA AAG GCC CAC CCG AAG GTG AGC CAG TGT GAT TAC* CAG GGT TTT CCC AGT CAC GAC CTT G-3′ containing the T7 T_e terminator 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* σ^{70} (C132S C291S C295S S366C; see ref. 41) was expressed in pRPODS366C Rosetta 2(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 50 μM 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 M urea in binding buffer and labeled with Cy5–maleimide dye. Cy5–σ^{70}RNAP holoenzyme was prepared by incubating 2.6 μM Cy5–σ^{70} and 1.3 μM core RNAP (Epicenter) in 50% wt/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 Cy5–σ^{70}, respectively, as described (3); focus was automatically maintained as described (43). Transcription reactions were observed in glass flow chambers (volume, ∼20 μL) passivated with succinimidyl (NHS) polyethylene glycol (PEG) and NHS-PEG-biotin (Laysan Bio) as described (3). Streptavidin (no. 21125; Life Technologies) was introduced at 220 nM in wash buffer [50 mM Tris·OAc, 100 mM KOAc, 8 mM MgOAc, 27 mM NH_4OAc, 0.1 mg mL^{-1} BSA (pH 8.0) (no. 126615; EMB Chemicals)], incubated for 45 s, and washed out (this and all subsequent washout steps used two flushes each of four chamber volumes of wash buffer). The chamber was then incubated with 50 pM AF488–DNA in wash buffer for ∼2 min and washed out. Next, locations of surface-tethered AF488–DNA molecules were recorded by acquiring four 1-s images with 488-nm excitation at a power of 350 μW incident to the

objective lens (43). Cy5–σ^{70}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 O_2-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 σ^{70}TEC dissociation rate constant k_{app} (Fig. 2*B* and Fig. S6), we used the method of Ensign and Pande (45) to jointly fit the measured lifetimes of Cy5–σ^{70}TECs that terminated by disappearance of the Cy5 spot and those that were censored by transcription termination (i.e., disappearance of the Cy3–probe spot) using the maximum likelihood algorithm. To calculate the fit curve in Fig. 2*B*, we performed 100,000 Monte Carlo simulations in which exponentially distributed lifetimes with mean equal to the reciprocal of k_{app} were each assigned an observation window length by random sampling from the distribution of observed transcript probe lifetimes (Fig. S2*A*); when the window length was less than the corresponding lifetime, the latter was truncated to the window length to mimic the censoring of the experimental data.

The average *E. coli* transcription unit length was estimated from a search of the EcoCyc database (www.biocyc.org) for all transcription units in the *E. coli* K-12 genome. The search yielded 3,541 transcription units with a broad distribution of lengths: 1,700 ± 1,600 bp (SD).

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