Retention of Transcription Initiation Factor σ^{70} in Transcription Elongation: Single-Molecule Analysis

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Summary

We report a single-molecule assay that defines, simultaneously, the translocational position of a protein complex relative to DNA and the subunit stoichiometry of the complex. We applied the assay to define translocational positions and σ^{70} contents of bacterial transcription elongation complexes in vitro. The results confirm ensemble results indicating that a large fraction, $\sim$70%–90%, of early elongation complexes retain σ^{70} and that a determinant for σ^{70} recognition in the initial transcribed region increases σ^{70} retention in early elongation complexes. The results establish that a significant fraction, $\sim$50%–60%, of mature elongation complexes retain σ^{70} and that a determinant for σ^{70} recognition in the initial transcribed region does not appreciably affect σ^{70} retention in mature elongation complexes. The results further establish that, in mature elongation complexes that retain σ^{70}, the half-life of σ^{70} retention is long relative to the timescale of elongation, suggesting that some complexes may retain σ^{70} throughout elongation.

Introduction

The transcription initiation factor σ^{70} interacts with *Escherichia coli* RNA polymerase core enzyme (RNAP) to yield RNAP holoenzyme (Gross et al., 1998). σ^{70} con-

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tains determinants for sequence-specific interaction with promoter DNA and, through these determinants, targets RNAP holoenzyme to promoters. σ^{70} also contains residues that facilitate unwinding of promoter DNA to yield a catalytically competent RNAP-promoter open complex and residues that mediate interactions with regulators of transcription initiation.

It has been proposed that σ^{70} obligatorily is released from RNAP at the transition from transcription initiation to transcription elongation, i.e., upon synthesis of an RNA product of 9–11 nt (Travers and Burgess, 1969; Hansen and McClure, 1980; Straney and Crothers, 1985; Krummel and Chamberlin, 1989; Metzger et al., 1993). Thus, it has been proposed that an obligatory "σ cycle" exists, with association of σ^{70} with RNAP to permit initiation and dissociation of σ^{70} from RNAP to permit elongation. Furthermore, it has been proposed, based on this presumed difference in subunit composition of the transcription machinery in initiation and elongation, that there are necessary differences in mechanism, responsiveness to DNA elements, and responsiveness to regulators, in transcription initiation and elongation.

The proposal that σ^{70} obligatorily is released from RNAP at the transition from transcription initiation to elongation was based primarily on early observations that σ^{70} is present in RNAP-promoter open complexes (RP_o) but is not present in RNAP-DNA elongation complexes (RD_e)—including RNAP-DNA elongation complexes with RNA products as short as 9–11 nt ($RD_{e,9}$, $RD_{e,10}$, and $RD_{e,11}$) (Hansen and McClure, 1980; Straney and Crothers, 1985; Krummel and Chamberlin, 1989; Metzger et al., 1993). However, these observations were not definitive. The experiments involved harsh separative steps (chromatography or electrophoresis) that, in principle, could transform a real difference in stability of interactions of σ^{70} with the remainder of the complex in RP_o and RD_e into an apparent, but artifactual, difference in occupancy of σ^{70} in RP_o and RD_e. Thus, it has been necessary also to consider an alternative proposal: a proposal in which the stability of interactions of σ^{70} with the remainder of the complex is reduced at the transition to elongation—e.g., due to unfavorable interactions between σ^{70} and nascent RNA (Daube and von Hippel, 1994; Mekler et al., 2002; Murakami et al., 2002b; Vassylyev et al., 2002; Nickels et al., 2005)—but in which σ^{70} is not necessarily released at the transition to elongation (Shimamoto et al., 1986).

Recently, by using fluorescence resonance energy transfer (FRET), we showed that in 20%–100% of early elongation complexes (i.e., complexes containing RNA products of $\leq$15 nt), σ^{70} is not released from RNAP but, instead, remains associated with RNAP and translocates with RNAP (Mukhopadhyay et al., 2001, 2003; Nickels et al., 2004). In addition, we and others showed that a determinant for sequence-specific σ^{70}-DNA interaction in the initial transcribed region can increase the fraction of early elongation complexes that retain σ^{70} from 20%–90% to 70%–100% (Nickels et al., 2004; Brodolin et al., 2004; see also Ring et al. [1996]). These results strongly argue against the presumption that there

are necessary subunit-composition differences and corresponding necessary mechanistic, DNA-recognition, and regulator-recognition differences in initiation and early elongation. These conclusions are supported by immunodetection of σ^{70} in elongation complexes in vitro containing RNAP prepared from stationary-phase cultures (Bar-Nahum and Nudler, 2001), in elongation complexes in vivo in stationary-phase cultures (Wade and Struhl, 2004), and in elongation complexes in vivo in both stationary-phase and exponential-phase cultures (R. Rong, O. Leroy, and R.H.E., unpublished data; A. Ansari, personal communication). These conclusions are further supported by a report that an RNAP derivative having a covalently tethered σ^{70} is fully competent for elongation (Mooney and Landick, 2003).

Here, we report a single-molecule assay, leading-edge/trailing-edge FRET with alternating-laser excitation (LE/TE-FRET ALEX), that defines, simultaneously, the translocational position and the σ^{70} content of an elongation complex. The assay yields translocational position and σ^{70} content in a single experiment (in contrast to ensemble σ^{70}-DNA FRET assays, which required multiple experiments and correction terms [Mukhopadhyay et al., 2001, 2003; Nickels et al., 2004]). The assay is not affected by compositional heterogeneity and thus can be applied to elongation complexes in solution (in contrast to ensemble σ^{70}-DNA FRET assays, which required analysis in a gel matrix [Mukhopadhyay et al., 2001, 2003; Nickels et al., 2004]). The assay is not precluded by translocational heterogeneity and thus can be applied to both early and mature elongation complexes (in contrast to ensemble σ^{70}-DNA FRET assays, which were applicable solely to early transcription elongation complexes; Mukhopadhyay et al., 2001). The single-molecule results confirm ensemble results (Mukhopadhyay et al., 2001, 2003; Nickels et al., 2004) regarding the extent of σ^{70} retention, the half-life of σ^{70} retention, and effects of DNA sequence on σ^{70} retention in early elongation complexes. More important, the results provide the first quantitative information regarding the extent of σ^{70} retention, the half-life of σ^{70} retention, and effects of DNA sequence on σ^{70} retention in mature elongation complexes.

Results

Single-Molecule Assay for Transcription-Complex Translocational Position and σ^{70} Content

We have developed a single-molecule assay, LE/TE-FRET ALEX, that defines, simultaneously, the translocational position and the σ^{70} content of a transcription complex (Figures 1 and 2). In the assay, we use confocal optical microscopy with two-color alternating-laser excitation to monitor fluorescence of donor- and acceptor-labeled single transcription complexes transiting a femtoliter-scale confocal excitation and detection volume (transit time ~1 ms; Figures 1A and 1B; Kapanidis et al., 2004). The results provide population distributions of donor-acceptor FRET efficiencies (E) and donor-acceptor stoichiometry factors (S) (Figures 2A and 2B; Kapanidis et al., 2004; Lee et al., 2005).

In LE-FRET ALEX, we incorporate the donor at a σ^{70} residue located close to the leading edge of RNAP and
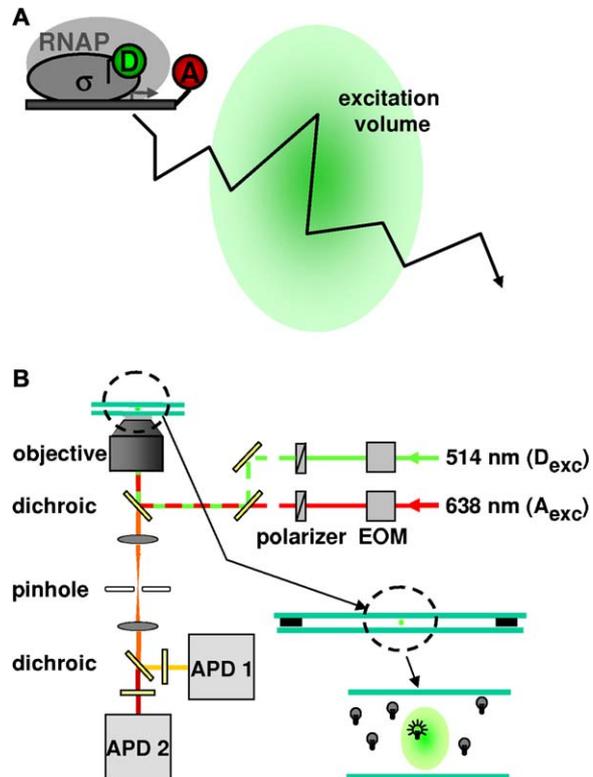
Figure 1. Experimental Strategy

(A) Use of confocal microscopy with alternating-laser excitation to monitor fluorescence of transcription complexes. Abbreviations: D, FRET donor; A, FRET acceptor. Transcription complexes labeled with a donor fluorophore on σ^{70} and an acceptor fluorophore on DNA are allowed to diffuse through a femtoliter-scale confocal excitation and detection volume (green, transit time ~1 ms). The sample is sufficiently dilute to ensure that, at any point in time, there is at most a single molecule in the confocal volume. Each single molecule diffusing through the confocal volume is illuminated with laser light that alternates on the microsecond time scale between a wavelength that excites the FRET donor fluorophore and a wavelength that excites the FRET acceptor fluorophore. For each single molecule, for each excitation wavelength, fluorescence emission is detected at both donor and acceptor emission wavelengths. For each single molecule, the configuration permits measurement of the fluorescence emission associated with all four possible excitation-wavelength/emission-wavelength combinations (namely donor-excitation/donor-emission, donor-excitation/acceptor-emission, acceptor-excitation/donor-emission, and acceptor-excitation/acceptor-emission). This information permits calculation of two parameters: the fluorophore stoichiometry (S) and the FRET efficiency (E) (Figures 2A and 2B; Kapanidis et al., 2004; Lee et al., 2005). The parameter S permits identification of molecules containing both donor and acceptor fluorophores (S ≈ 0.5), molecules containing only a donor fluorophore (S > 0.8), and molecules containing only an acceptor fluorophore (S < 0.3).

(B) Experimental setup. Abbreviations: EOM, electro-optical modulator; APD, avalanche photodiode.

The sample in the observation volume (in dotted oval) is illuminated by two rapidly alternating lasers: one with an excitation wavelength exciting the FRET donor fluorophore (514 nm, green) and one with an excitation wavelength exciting the FRET acceptor fluorophore (638 nm, red). Fluorescence emission is directed toward photodiode detectors equipped with optical filters to render them sensitive to the donor and acceptor emission wavelengths. During data analysis, fluorescence-emission photons are assigned, according to their time of arrival at the detector, to donor-wavelength excitation pulses or acceptor-wavelength excitation pulses.

A   Leading-edge FRET
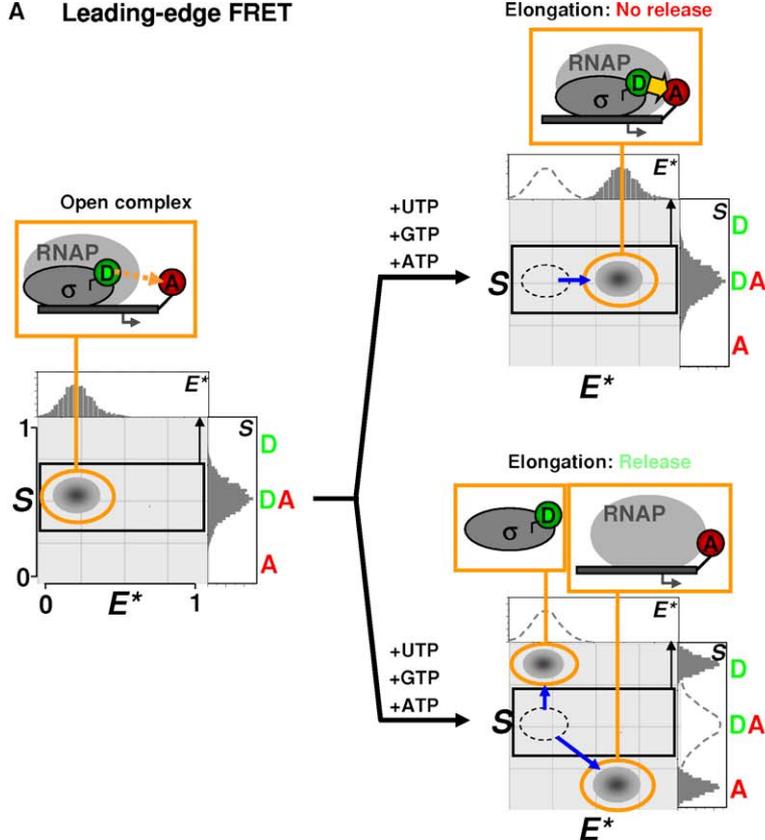
B   Trailing-edge FRET
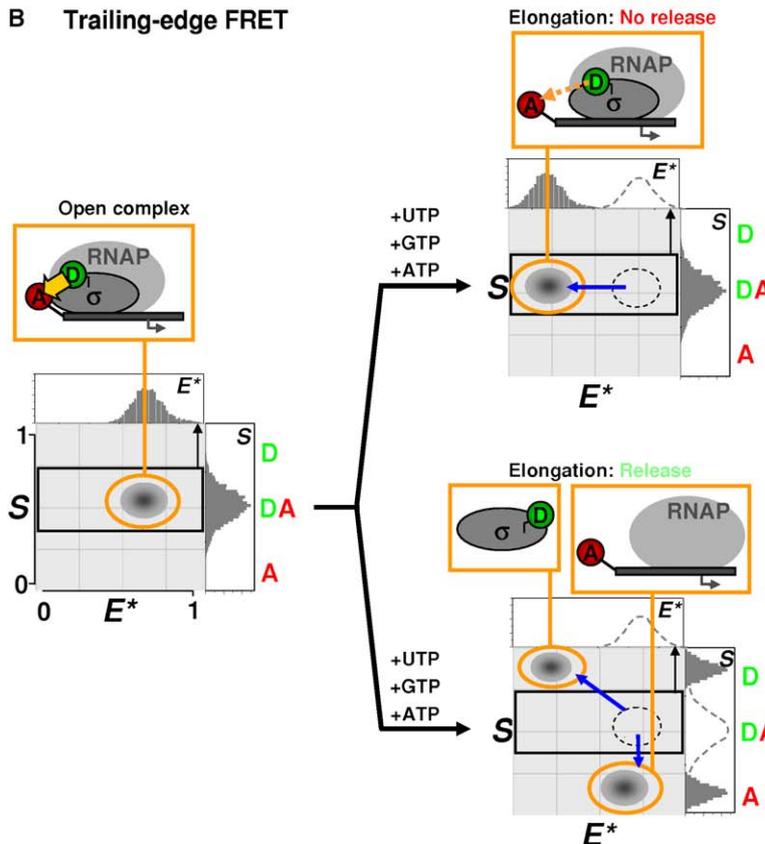
Figure 2. LE/TE-FRET ALEX

(A) Use of LE-FRET ALEX to assess changes in translocational state and σ[70] content upon transition from the open complex (RP$_o$, left) to the elongation complex (RD$_e$, right). Formation of RD$_e$ with retention of σ[70] results in conversion of a donor-acceptor (S ≈ 0.5) species with low E* to a donor-acceptor (S ≈ 0.5) species with high E* (top right). Formation of RD$_e$ with release of σ[70] results in conversion of a donor-acceptor (S ≈ 0.5) species with low E* to donor-only (S > 0.8) and acceptor-only (S < 0.3) species (bottom right). Abbreviations: D, donor-only species; A, acceptor-only species; and DA, donor-acceptor species. (B) Use of TE-FRET ALEX to assess changes in translocational state and σ[70] content upon transition from the open complex (RP$_o$, left) to the elongation complex (RD$_e$, right). Formation of RD$_e$ with retention of σ[70] results in conversion of a donor-acceptor (S ≈ 0.5) species with high E* to a donor-acceptor (S ≈ 0.5) species with low E* (top right). Formation of RD$_e$ with release of σ[70] results in conversion of a donor-acceptor (S ≈ 0.5) species with high E* to donor-only (S > 0.8) and acceptor-only (S < 0.3) species (bottom right).

incorporate the acceptor on downstream DNA (Figure 2A). In TE-FRET ALEX, we incorporate the donor at a σ^{70} residue located close to the trailing edge of RNAP and incorporate the acceptor on upstream DNA (Figure 2B). In each case, we perform measurements with the RNAP-promoter open complex and with defined RNAP-DNA elongation complexes (Figure 2). Translocational state is defined based on the FRET efficiency, E (or on the uncorrected FRET efficiency, E*; see Experimental Procedures). In LE-FRET ALEX, forward translocation results in a decrease in donor-acceptor distance and a corresponding increase in FRET efficiency in donor-acceptor species; in TE-FRET ALEX, forward translocation results in an increase in donor-acceptor distance and a corresponding decrease in FRET efficiency in donor-acceptor species. σ^{70} content is defined based on the stoichiometry parameter, S. Transcription complexes that contain σ^{70} are donor-acceptor species and thus exhibit a stoichiometry parameter of S ≈ 0.5, transcription complexes lacking σ^{70} are acceptor-only species and thus exhibit a stoichiometry parameter of S < 0.3, and free σ^{70} is a donor-only species and thus exhibits a stoichiometry parameter of S > 0.8.

To incorporate the donor at a σ^{70} residue located close to the leading edge of RNAP, we introduced tetramethylrhodamine (TMR) at position 366 of σ^{70} (Mekler et al., 2002; Mukhopadhyay et al., 2001, 2003; Nickels et al., 2004; Lee et al., 2005). To incorporate the donor at a σ^{70} residue located at the trailing edge of RNAP, we introduced TMR at position 596 of σ^{70} (Mekler et al., 2002; Mukhopadhyay et al., 2001, 2003; Nickels et al., 2004; Lee et al., 2005). Control experiments establish that the resulting labeled σ^{70} derivatives are unaltered in interactions with RNAP core (Table S1 available in the Supplemental Data with this article online) and are functional in formation of open complex and elongation complex (Mekler et al., 2002; Mukhopadhyay et al., 2001, 2003; Nickels et al., 2004; Lee et al., 2005).

To permit formation of defined elongation complexes, we used derivatives of the *lacUV5* promoter having the first template-strand guanine residue in the transcribed region at position +12, +15, or +51 (Figure S1; Mekler et al., 2002; Mukhopadhyay et al., 2001, 2003; Nickels et al., 2004). With these DNA templates, upon formation of RP_o and addition of ApA, ATP, GTP, and UTP, RNAP initiates transcription, proceeds to position +11, +14, or +50, and halts (due to the absence of CTP, the next required NTP; Figure S1). The resulting halted complexes are bona fide elongation complexes: they are stable, they retain RNA, and they can be restarted upon addition of CTP (Figures 3A and 3B). For TE-FRET experiments, we incorporated Cy5, serving as acceptor, immediately upstream of the core promoter, at position −40; for LE-FRET experiments, we incorporated Cy5, serving as acceptor, downstream of the core promoter, at position +25, +28, or +64 (Figure S1).

σ^{70} Retention in Early Elongation Complexes: $RD_{e,11}$

At the *lacUV5* promoter, the first stable elongation complex is generated upon synthesis of an RNA product 11 nt in length ($RD_{e,11}$) (Carpousis and Gralla, 1985; Munson and Reznikoff, 1981; Straney and Crothers, 1985). Figure 3A and Table 1 present LE-FRET ALEX results for the

open complex and $RD_{e,11}$ at *lacUV5*. In samples of the open complex, two species are observed (Figure 3A, left). One species exhibits S ≈ 0.55 and E* ≈ 0.23; this species is the open complex (stoichiometry parameter characteristic of a donor-acceptor species; FRET efficiency corresponding to a donor-acceptor separation of 77 Å, a separation consistent with previous work [Mekler et al., 2002; Mukhopadhyay et al., 2001, 2003; Nickels et al., 2004; Lee et al., 2005] and with predictions from structural models of the open complex [Mekler et al., 2002; Lawson et al., 2004]). The other species exhibits a stoichiometry parameter of S < 0.3; this species is free promoter DNA, generated as a by product of disruption of nonspecific RNAP-promoter complexes and removal of free RNAP holoenzyme by challenge with heparin-Sepharose (see Experimental Procedures; stoichiometry parameter characteristic of an acceptor-only species). Upon addition of the NTP subset permitting formation of $RD_{e,11}$, ~80% of open complexes are converted to a species that exhibits the same stoichiometry parameter but a higher FRET efficiency (S ≈ 0.55; E* ≈ 0.44) and that can be "chased" upon subsequent addition of all four NTPs (Figure 2A, middle and right); this species is $RD_{e,11}$ containing σ^{70} (stoichiometry parameter characteristic of a donor-acceptor species; FRET efficiency corresponding to a donor-acceptor separation of 58 Å, a separation consistent with previous work [Mukhopadhyay et al., 2001]). The remaining ~20% of open complexes are converted to a species with S < 0.3; this species is $RD_{e,11}$ not containing σ^{70} (stoichiometry parameter characteristic of an acceptor-only species). We infer that, under these conditions, most or all complexes are functional and competent to undergo the transition from initiation to elongation and that ~80% fully retain σ^{70} upon the transition from initiation to elongation. We note that the inferred level of σ^{70} retention of ~80% may underestimate the actual level of σ^{70} retention, because some loss of σ^{70} may occur during data collection (data collection time = 30 min; half-life of σ^{70} retention ~90 min; see Figure 4A and Table 2) and because the correction for nonfunctional complexes used in calculation of σ^{70} retention may represent an upper bound (see Experimental Procedures).

Figure 3B and Table 1 present corresponding TE-FRET ALEX results for the open complex and $RD_{e,11}$ at *lacUV5*. The open complex exhibits S ≈ 0.55 (stoichiometry parameter characteristic of a donor-acceptor species) and E* ≈ 0.84 (FRET efficiency corresponding to a donor-acceptor separation of 42 Å, a separation consistent with previous work [Lee et al., 2005; Mekler et al., 2002; Mukhopadhyay et al., 2001]) (Figure 3B, left). Upon addition of the NTP subset permitting formation of $RD_{e,11}$, ~70% of open complexes are converted to a species with the same stoichiometry parameter but lower FRET efficiency (S ≈ 0.55; E* ≈ 0.46) and that can be chased upon subsequent addition of all four NTPs (Figure 3B, middle and right); this species is $RD_{e,11}$ containing σ^{70} (FRET efficiency corresponding to a donor-acceptor separation of 59 Å, a separation consistent with previous work [Mukhopadhyay et al., 2001]). The remaining ~30% of open complexes are converted to species with S < 0.3 (Figure 3B, middle and right); this species is $RD_{e,11}$ not containing σ^{70}. We infer that, under these conditions, most complexes are

A Leading-edge FRET

B Trailing-edge FRET
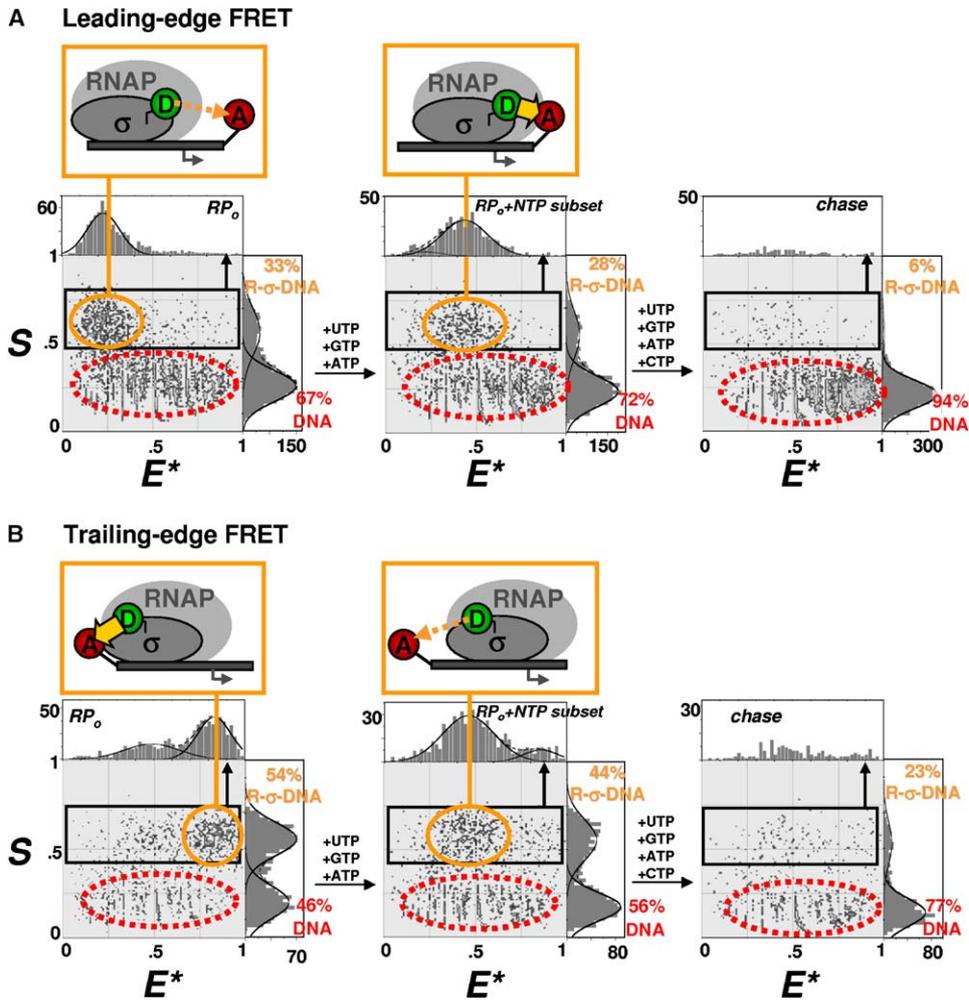
Figure 3. σ^{70} Retention in Early Elongation Complexes: $RD_{e,11}$

(Species with S > 0.8 [e.g., free σ^{70}] are not included, because the molecule-search criteria employed herein identify solely acceptor-containing molecules [see Experimental Procedures].)
(A) Results of LE-FRET experiments with RP_o, $RD_{e,11}$, and chased $RD_{e,11}$.
(B) Results of TE-FRET experiments with RP_o, $RD_{e,11}$, and chased $RD_{e,11}$.

functional and competent to undergo the transition from initiation to elongation and that ~70% fully retain σ^{70} upon the transition from initiation to elongation. We note that the inferred level of σ^{70} retention of ~70% may underestimate the actual level of σ^{70} re-

tention, for the reasons mentioned in the preceding paragraph.

Figure 4A and Table 2 present results of LE-FRET kinetics experiments assessing the initial extent and half-life of σ^{70} retention in $RD_{e,11}$. These experiments were performed by monitoring molecule-count ratios (involving counts of donor-acceptor molecules and acceptor-only molecules) as a function of time after NTP addition, followed by comparisons with identical ratios for the open complex (which sets the ratio for 100% σ^{70} retention) and the chased complex (which sets the ratio for 0% σ^{70} retention; see Experimental Procedures). The results indicate that the initial extent of σ^{70} retention in $RD_{e,11}$ is ~90% and the half-life of σ^{70} retention in $RD_{e,11}$ is ~90 min. σ^{70} release in open complexes occurs significantly more slowly (half-life of σ^{70} retention >2 hr; Figure S2A).

We conclude, consistent with conclusions from ensemble experiments (Mukhopadhyay et al., 2001, 2003; Nickels et al., 2004) that at *lacUV5*, in most transcription complexes, σ^{70} remains associated with RNAP and translocates with RNAP upon formation of $RD_{e,11}$.

Table 1. FRET Efficiencies and Corresponding Distances for Transcription Complexes at *lacUV5* and *lacUV5(A+2G)*

			RP_o		RD_e	
lacUV5	σ probe	R_o	E	R (Å)	E	R (Å)
LE-FRET, $RD_{e,11}$	366	61.4	0.18	79	0.55	59
LE-FRET, $RD_{e,14}$	366	61.4	0.18	79	0.65	55
LE-FRET, $RD_{e,50}$	366	61.4	<0.05	>100	<0.05	>100
TE-FRET, $RD_{e,11}$	596	64.4	0.94	41	0.65	58
lacUV5(A+2G)						
LE-FRET, $RD_{e,11}$	366	61.4	0.13	85	0.48	62
LE-FRET, $RD_{e,14}$	366	61.4	0.15	82	0.65	55
LE-FRET, $RD_{e,50}$	366	61.4	<0.05	>100	<0.05	>100
TE-FRET, $RD_{e,11}$	596	64.4	0.96	38	0.77[a]	52[a]

[a] Minor species with E < 0.10 and R > 90 Å.

A Leading-edge FRET: $RD_{e,11}$

B

A Leading-edge FRET: $RD_{e,14}$

B Leading-edge FRET: $RD_{e,50}$
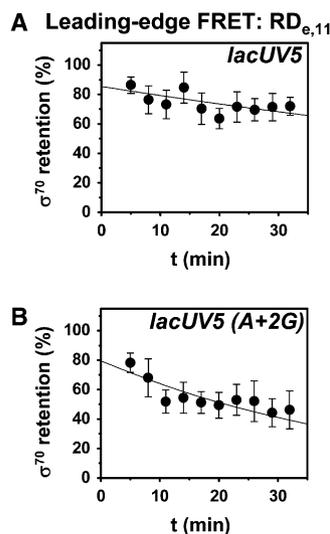
Figure 4. σ^{70} Retention in Early Elongation Complexes: $RD_{e,11}$

(A) Extent of σ^{70} retention in $RD_{e,11}$ at *lacUV5*. The y intercept (t = 0 min) corresponds to 3 min after adding UTP, GTP, and ATP to RP_o. Error bars represent the standard error of mean (SEM) for four to six independent measurements. Solid lines, single-exponential fits.
(B) As in (A) but for $RD_{e,11}$ at *lacUV5(A+2G)* (substituted *lacUV5* derivative lacking determinant for sequence-specific σ^{70}-DNA interaction in initial transcribed region [Nickels et al., 2004]).

Figure 5. σ^{70} Retention beyond the Formation of the First Stable Elongation Complex.

(A) Extent of σ^{70} retention in $RD_{e,14}$ at *lacUV5*. The y intercept (t = 0 min) corresponds to 3 min after adding UTP, GTP, and ATP to RP_o. Error bars represent the SEM for four to six independent measurements. Solid lines, single-exponential fits.
(B) Extent of σ^{70} retention in $RD_{e,50}$ at *lacUV5*.

σ^{70} Retention in Early Elongation Complexes: $RD_{e,14}$

In $RD_{e,11}$, 9 nt of RNA are present as an RNA-DNA hybrid and 2 nt of RNA are present within an RNA exit channel formed by RNAP—a channel that can accommodate 5 nt of RNA (Borukhov and Severinov, 2002; Ebright, 2000; Korzheva et al., 2000; Lawson et al., 2004; Murakami et al., 2002a, 2002b; Vassylyev et al., 2002). To assess effects of filling of the RNA exit channel on σ^{70} retention, we performed analogous LE-FRET ALEX experiments comparing open complex and the first elongation complex with the RNA exit channel completely filled: i.e., $RD_{e,14}$.

The data in Figure 5A and Table 2 indicate that, at *lacUV5*, the initial extent of σ^{70} retention in $RD_{e,14}$ is ~90% and the half-life of σ^{70} retention in $RD_{e,14}$ is ~20 min. We conclude, consistent with conclusions from ensemble FRET experiments (Mukhopadhyay et al., 2001, 2003; Nickels et al., 2004), that at *lacUV5*, in most transcription complexes, σ^{70} remains associated with RNAP and translocates with RNAP upon formation of $RD_{e,14}$.

σ^{70} Retention in Early Elongation Complexes: Effects of Initial-Transcribed-Region Sequence Element

Recent ensemble results indicate that the initial-transcribed-region sequence of *lacUV5* contains a determinant for sequence-specific σ^{70}-DNA interaction, a "−10-like element," that increases the initial extent and half-life of σ^{70} retention and mediates σ^{70}-dependent transcriptional pausing (positions +1 through +6 relative to the transcription start site; Brodolin et al., 2004; Nickels et al., 2004). To assess effects of this sequence element, we performed LE-FRET ALEX experiments assessing open complex, $RD_{e,11}$, and $RD_{e,14}$ at *lacUV5(A+2G)* (Nickels et al., 2004), a substituted *lacUV5* derivative that lacks the sequence element.

Figure 4B and Table 2 present results of experiments assessing the initial extent and half-life of σ^{70} retention in $RD_{e,11}$ at *lacUV5(A+2G)*. The initial extent of σ^{70} retention is ~80%, and the half-life of σ^{70} retention is ~30 min. These values are lower than the corresponding values for *lacUV5* (~90% and ~90 min; Figure 4A and Table 2). Control experiments indicate that σ^{70} release in open complexes at *lacUV5(A+2G)* occurs significantly more slowly (half-life of σ^{70} retention > 2 hr; Figure S2B).

Table 2 presents results of LE-FRET experiments assessing the initial extent of σ^{70} retention in $RD_{e,14}$ at *lacUV5(A+2G)*. The initial extent of σ^{70} retention is ~70%, which is lower than the corresponding value for *lacUV5* (~90%; Table 2).

We conclude, consistent with conclusions from ensemble experiments (Nickels et al., 2004), that the presence in the initial transcribed region of a determinant for sequence-specific σ^{70}-DNA interaction increases the initial extent of σ^{70} retention.

Table 2. Extents of Initial σ^{70} Retention and Half-Lives of σ^{70} Retention in Transcription Complexes at *lacUV5* and *lacUV5(A+2G)*

	σ Probe	Initial σ^{70} Retention	σ^{70} Retention Half-Life (min)
lacUV5			
LE, $RD_{e,11}$	366	85 ± 6	90 ± 40
LE, $RD_{e,14}$	366	92 ± 7	24 ± 3
LE, $RD_{e,50}$	366	56 ± 10	50 ± 30
lacUV5(A+2G)			
LE, $RD_{e,11}$	366	80 ± 8	30 ± 10
LE, $RD_{e,14}$	366	72 ± 8	ND
LE, $RD_{e,50}$	366	55 ± 8	ND

Abbreviation: ND, not determined.

σ⁷⁰ Retention in Mature Elongation Complexes: RD$_{e,50}$

Complications due to translocational heterogeneity caused by RNAP reverse-translocated, "backtracked" states (Komissarova and Kashlev, 1997; Nudler et al., 1997) have precluded ensemble LE-FRET analysis of σ⁷⁰ retention in elongation complexes containing >15 nt of RNA (Mukhopadhyay et al., 2001).

To define the initial extent and half-life of σ⁷⁰ retention in a mature elongation complex, we have performed LE-FRET ALEX experiments assessing RD$_{e,50}$ at *lacUV5*. LE-FRET ALEX experiments independently define translocational state and σ⁷⁰ content, defining translocational state based on the FRET efficiency (E) and σ⁷⁰ content based on the stoichiometry parameter (S); therefore, translocational heterogeneity does not complicate LE-FRET ALEX analysis of σ⁷⁰ content.

Figure 5B and Table 2 present results of experiments assessing the initial extent and half-life of σ⁷⁰ retention in RD$_{e,50}$ at *lacUV5*. The initial extent of σ⁷⁰ retention is ~50%–60%, and the half-life of σ⁷⁰ retention is ~50 min. We conclude that, at *lacUV5*, in approximately half of transcription complexes, σ⁷⁰ remains associated with RNAP upon formation of a mature elongation complex and reaches position +50 in association with RNAP.

Figure 5B indicates that, upon addition of the NTP subset that permits formation of RD$_{e,50}$ at *lacUV5*, σ⁷⁰ release is biphasic, with an initial "fast" phase (half-time less than ~8 min) and a subsequent "slow" phase (half-time ~50 min). We note that the initial extent of σ⁷⁰ retention in RD$_{e,14}$ is significantly higher than the initial extent of σ⁷⁰ retention in RD$_{e,50}$ (~90% versus ~50%–60%; Figures 5A and 5B). We infer that most σ⁷⁰ release in the fast phase must occur after RNAP reaches position +14. We do not know whether σ⁷⁰ release in the fast phase occurs during elongation from position +14 to position +50 to form RD$_{e,50}$, during incubation of RD$_{e,50}$ prior to collection of the first data point, or both.

The E*-S histogram for RD$_{e,50}$ at *lacUV5* shows that, consistent with inferences from ensemble-FRET experiments (Mukhopadhyay et al., 2001), the majority, >70%, of σ⁷⁰-containing RD$_{e,50}$ at *lacUV5* is present in reverse-translocated states, backtracked by at least 10 bp relative to the position expected in the absence of backtracking (E* ≈ 0.2 versus E* ≈ 0.45; data not shown). We cannot exclude the possibility that the half-life of σ⁷⁰ retention differs in backtracked and nonbacktracked RD$_{e,50}$ and thus that the net observed half-life of σ⁷⁰ retention overestimates or underestimates the actual half-life in nonbacktracked RD$_{e,50}$.

σ⁷⁰ Retention in Mature Elongation Complexes: Effects of Initial-Transcribed-Region Sequence Element

To assess the proposal that the presence of sequence-specific σ⁷⁰-DNA interaction in the initial transcribed region may influence not only σ⁷⁰ retention in early elongation but also σ⁷⁰ retention thereafter (Nickels et al., 2004), we performed parallel LE-FRET ALEX experiments assessing RD$_{e,50}$ at *lacUV5(A+2G)* (Nickels et al., 2004), a substituted *lacUV5* derivative that lacks the sequence element.

The results in Table 2 indicate that the initial extent of σ⁷⁰ retention in RD$_{e,50}$ at *lacUV5(A+2G)* is ~50%–60%.

This value is indistinguishable from the value for the initial extent of σ⁷⁰ retention in RD$_{e,50}$ at *lacUV5* (55% ± 8% versus 56% ± 10%; Table 2). We conclude that the presence of a determinant for sequence-specific σ⁷⁰-DNA interaction in the initial transcribed region has no appreciable influence—no appreciable memory effect—on σ⁷⁰ retention in a mature elongation complex.

The E*-S histogram for RD$_{e,50}$ at *lacUV5(A+2G)* is essentially indistinguishable from the E*-S histogram for RD$_{e,50}$ at *lacUV5* (data not shown). At *lacUV5(A+2G)*, as at *lacUV5*, the majority, >70%, of σ⁷⁰-containing RD$_{e,50}$ is present in reverse-translocated states, backtracked by at least 10 bp relative to the position in the absence of backtracking (E* ≈ 0.2 versus E* ≈ 0.45; data not shown).

Discussion

Our single-molecule FRET results confirm and extend ensemble FRET results (Mukhopadhyay et al., 2001, 2003; Nickels et al., 2004), indicating that the initiation factor σ⁷⁰ is not obligatorily released from RNAP upon transition from initiation to elongation but, instead, can remain associated with RNAP and can translocate with RNAP. Specifically, the single-molecule FRET results (1) confirm that a substantial fraction of early elongation complexes retains σ⁷⁰ and that this fraction can be increased by the presence of a determinant for sequence-specific σ⁷⁰-DNA interaction in the initial transcribed region, (2) show that a substantial fraction of mature elongation complexes retains σ⁷⁰ and that this fraction is not appreciably affected by the presence of a determinant for sequence-specific σ⁷⁰-DNA interaction in the initial transcribed region, and (3) define the half-life for σ⁷⁰ retention in mature elongation complexes.

The single-molecule FRET results were obtained by using a method that did not require correction factors from multiple separate reactions (in contrast to the ensemble FRET results [Mukhopadhyay et al., 2001, 2003; Nickels et al., 2004]), that did not require analysis in a gel matrix (in contrast to the ensemble FRET results), and that was unaffected by translocational-position heterogeneity (in contrast to ensemble FRET results). Three potential sources of error therefore were avoided.

In addition, the single-molecule FRET results were obtained at σ⁷⁰ and RNAP concentrations (≤0.5 nM after addition of the NTP subset) well below the dissociation constant for interaction of σ⁷⁰ with elongation complexes (2 μM [Gill et al., 1991]), in contrast to the ensemble FRET results (Mukhopadhyay et al., 2001, 2003; Nickels et al., 2004). Therefore, a strong case can be made that the observed σ⁷⁰ retention reflects retention of σ⁷⁰ upon transition to elongation and subsequent translocation of σ⁷⁰ with RNAP, as opposed to release of σ⁷⁰ upon transition to elongation and subsequent rebinding of σ⁷⁰ to the elongation complex.

It is noteworthy that, in mature elongation complexes that retain σ⁷⁰, the observed half-life of σ⁷⁰ retention (>50 s; Table 2) is long relative to the time scale of transcription elongation (~50 s for 1000 bp transcription unit at saturating NTPs [Gotta et al., 1991; Jacquet and Kepes, 1971; Rose et al., 1970; Schaffer et al., 1991; Wang et al., 1998]). This raises the possibility that at least some elongation complexes may retain σ⁷⁰

throughout the entire process of elongation. Our results address the extent of σ^{70} retention only during the transition from initiation to elongation and during elongation up to position +50; σ^{70} release may be more efficient during elongation beyond position +50. Furthermore, our results address only the situation in vitro in a purified system; σ^{70} release may be more efficient in vivo, in the presence of core binding factors (e.g., Nus factors), σ^{70} binding factors, and alternative σ factors. Nevertheless, the possibility that at least some elongation complexes may retain σ^{70} throughout the entire process of elongation would be consistent with immunodetection of σ^{70} in elongation complexes in vitro containing RNAP prepared from stationary-phase cultures (Bar-Nahum and Nudler, 2001), in elongation complexes in vivo in stationary-phase cultures (Wade and Struhl, 2004), and in elongation complexes in vivo both in stationary-phase and exponential-phase cultures (R. Rong, O. Leroy, and R.H.E., unpublished data; A. Ansari, personal communication).

The conclusion that σ^{70} release is not obligatory, but instead stochastic, has both mechanistic and functional implications. With respect to mechanism, the absence of an obligatory subunit-composition difference in the transcriptional machinery responsible for initiation and the transcriptional machinery responsible for elongation argues against fundamental mechanistic differences in initiation and elongation. With respect to function, the existence of σ^{70}-containing elongation complexes permits multiple additional levels of regulation during elongation: regulation mediated by DNA-sequence-recognition by σ^{70} during elongation (Ring et al., 1996; Mooney and Landick, 2003; Nickels et al., 2004; Brodolin et al., 2004), regulation mediated by repressor or activator interaction with σ^{70} during elongation (Nickels et al., 2002), and, possibly, regulation mediated by modulation of the extent of σ^{70} release and retention during elongation.

Experimental Procedures

σ^{70} Derivatives
σ^{70} derivatives having TMR incorporated at residue 366 (for LE-FRET experiments) or residue 596 (for TE-FRET experiments) were prepared as described (Mukhopadhyay et al., 2001, 2003) (Table S1).

Promoter DNA Fragments
Promoter DNA fragments labeled with Cy5 were prepared as described (Mukhopadhyay et al., 2001, 2003) (sequences in Figure S1).

Transcription Complexes
Reaction mixtures (30 µl) for preparation of open complexes contained 100 nM RNAP core (Epicentre) and 80 nM TMR-labeled σ^{70} derivative in transcription buffer (TB; 50 mM Tris-HCl, [pH 8], 100 mM KCl, 10 mM MgCl$_2$, 1 mM dithiothreitol (DTT), and 100 µg/ml bovine serum albumin), and 5% glycerol. Samples were incubated 20 min at 30°C; 0.6 µl of 1 µM Cy5-labeled promoter DNA fragment was added, and samples were further incubated 15 min at 37°C. Heparin-Sepharose (Amersham-Pharmacia Biotech; 0.8 µl of 100 mg/ml) was added to disrupt nonspecific RNAP-promoter complexes and to remove free RNAP (Mukhopadhyay et al., 2001), and after 1 min at 37°C, samples were centrifuged and 9.5 µl aliquots were transferred to tubes containing 0.5 µl 10 mM ApA or ApG (Sigma-Aldrich) at 37°C. In this work, experiments with open complexes were performed in the presence of the initiating dinucleotide: ApA for *lacUV5* and ApG for *lacUV5(A+2G)*. The initiating dinucleotide increases stability of open complexes (Gaal et al., 1997; Revyakin et al., 2004) and reduces dissociation of open complexes during data collection (data not shown). Representative experiments with

open complexes in the absence of the initiating dinucleotide yield equivalent results but inferior signal-to-noise ratio (data not shown). To prepare halted elongation complexes, 1.1 µl of 125 µM ATP, 125 µM UTP, and 125 µM GTP in TB was added, and samples were incubated a further 3 min at 37°C. To test the ability of the resulting halted elongation complexes to resume transcription, 1.2 µl of 500 µM ATP, 500 µM UTP, 500 µM GTP, and 625 µM CTP was added (chase reaction), and samples were incubated a further 20 min at 37°C.

Fluorescence-Detected Electrophoretic-Mobility-Shift Competition Experiments
Fluorescence-detected electrophoretic-mobility-shift competition experiments and calculation of relative equilibrium binding constants, $K_{b,1}/K_{b,2}$, were performed essentially as in Nickels et al. [2005]. Reaction mixtures (40 µl) contained 40 nM TMR366-σ^{70} or TMR596-σ^{70}, 0-200 nM unlabeled wild-type σ^{70} as competitor, 12 nM wild-type RNAP core, 40 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM MgCl$_2$, 1 mM DTT, 0.2% Tween 20, and 5% glycerol.

Single-Molecule Fluorescence Microscopy: Sample Preparation
An observation chamber was created by forming an 8 mm diameter hole on a 2.5 mm-thick silicone gasket (Grace Biolabs) and placing the gasket on a No.1 thickness coverglass (Kapanidis et al., 2004). To reduce nonspecific binding on glass, 80 µl of KG7 + ApA buffer (40 mM HEPES-NaOH [pH 7], 100 mM K-glutamate, 10 mM MgCl$_2$, 1 mM DTT, 100 µg/ml BSA, 5% glycerol, 1 mM mercaptoethylamine, 0.5 mM ApA [or 0.5 mM ApG]) were added, and the coverglass was incubated for 10 min at 37°C. Then, 0.4–0.6 µl of a solution containing a complex was added, and the chamber was sealed. The final concentration of complexes was 100–150 pM.

Single-Molecule Fluorescence Spectroscopy: Data Acquisition and Analysis
The microscope used to analyze single transcription complexes has been described (Kapanidis et al., 2004). Alternating-laser excitation of diffusing molecules was achieved by using 514 and 638 nm light, with alternation period of 100 µs, and duty cycle of 50% (Figures 1A and 1B). Excitation intensities were 150–300 µW for 514 nm excitation (D$_{exc}$) and 50–80 µW for 638 nm excitation (A$_{exc}$) (measured in the continuous-wave mode). Data were collected for 15–30 min. Photons detected at the donor or acceptor emission channel were assigned to either 514 nm or 638 nm excitation according to their arrival time and generated emission streams $f^{A_{em}}_{D_{exc}}$, $f^{D_{em}}_{D_{exc}}$, $f^{A_{em}}_{A_{exc}}$, and $f^{D_{em}}_{A_{exc}}$ (where $f^{Y_{em}}_{X_{exc}}$ stands for counts per integration period, in the primary spectral range for detecting fluorophore Y, resulting from excitation that primarily excites fluorophore X); the streams were analyzed to identify DNA-containing molecules (equivalent to A containing species), using $f^{A_{em}}_{A_{exc}}$ thresholds of seven to nine photons per 500 µs and 15–30 photons per molecule. The chosen thresholds identify acceptor-containing molecules of appreciable photon count (thus reducing the statistical noise inherent to single-molecule measurements). Analysis solely of acceptor-containing molecules eliminates complications due to free σ^{70} in initial preparations of RP$_o$ (present at 0.1–0.3 nM, due to inefficient removal of free σ^{70} upon heparin-Sepharose challenge), due to photobleaching or intermittency of acceptor, due to buffer contaminants with spectral characteristics similar to donor-only molecules, and due to differences in molecule counts identified by different photon-count thresholds (especially when FRET processes are involved).

Each molecule is characterized by photon counts $F^{A_{em}}_{D_{exc}}$, $F^{D_{em}}_{D_{exc}}$, and $F^{A_{em}}_{A_{exc}}$, which allow the calculation of ratios S and E*. Ratio S was calculated by using equation 1, where γ is a detection-correction factor (Kapanidis et al., 2004; Lee et al., 2005):

$$S = \left(F^{A_{em}}_{D_{exc}} + \gamma F^{D_{em}}_{D_{exc}}\right) / \left(F^{A_{em}}_{D_{exc}} + \gamma F^{D_{em}}_{D_{exc}} + F^{A_{em}}_{A_{exc}}\right) \quad (1)$$

Ratio E* was calculated by using equation 2 (Lee et al., 2005):

$$E^* = F^{A_{em}}_{D_{exc}} / \left(F^{A_{em}}_{D_{exc}} + F^{D_{em}}_{D_{exc}}\right) \quad (2)$$

Ratio S reports on relative stoichiometry of donors and acceptors, whereas E* reports on the distance between the probes; 2D E*-S histograms allow identification and sorting of diffusing species. Donor-only species show low E* and high S, acceptor-only species show high E* and low S, and donor-acceptor species show various

E* values and 0.3 < S < 0.8. The E*-S histogram extracts distances through measurements of E* for donor-acceptor species.

Determination of σ^{70} Retention

To study dissociation of donor-acceptor species (due to trivial dissociation or σ^{70} release), we calculate fractional DNA occupancy θ for RP$_o$, RD$_e$, and chased RD$_e$ (Kapanidis et al., 2004):

$$\theta = [\text{donor-acceptor species}]/([\text{acceptor-only species}] \\ + [\text{donor-acceptor species}]) \qquad (3)$$

RP$_o$ was measured first (for 15 min), followed by RD$_e$ (for 30 min), followed by chased RD$_e$ (for 15 min). The concentration of donor-acceptor and acceptor-only species is represented by the amplitude of the Gaussian distribution fitted to donor-acceptor and acceptor-only S distributions (in cases of >1000 molecules) or by the molecule count for donor-acceptor and acceptor-only molecules using S thresholds (in cases of <1000 molecules) (Kapanidis et al., 2004). By using θ_{RPo} as the 100% value and θ_{chase} as the baseline (0%), we calculate the fractional σ^{70} retention, SR:

$$SR = (\theta_{RDe} - \theta_{chase})/(\theta_{RPo} - \theta_{chase}) \qquad (4)$$

Apart from a measure of the ability of RD$_e$ to resume transcription, θ_{chase} accounts for inactive RP$_o$ and any species that appear on the E*-S histogram due to random coincidence of donor-only and acceptor-only species. We note that use of θ_{chase} for calculating fractional σ^{70} retention is associated with small errors (<10%) in the value of σ^{70} retention arising from dissociation of inactive RP$_o$ during the course of the experiment (an error that slightly increases the apparent σ^{70} retention), and from the presence of nonfunctional ("arrested") σ^{70}-containing RD$_e$ in the chased RD$_e$ sample (an error that slightly decreases the apparent σ^{70} retention). In all cases, >80% of all transcription complexes are chaseable. We note that our σ^{70}-retention calculations consider only the chaseable fraction of σ^{70}-containing RD$_e$ complexes.

Some dissociation of RNAP-σ^{70}-DNA complexes occurs during sample preparation and data acquisition on RD$_e$, reducing θ_{RDe}; in contrast, θ_{RPo} and θ_{chase} do not change during acquisition. To account for dissociation and evaluate the stability of σ^{70} interactions within RD$_e$ (Nickels et al., 2004), we plot σ^{70} retention as a function of time, SR(t), by calculating θ_{RDe} (t) (θ_{RDe} as a function of time), with each point being the average θ_{RDe} for 3-min slices of acquisition time t (Kapanidis et al., 2004):

$$SR(t) = (\theta_{RDe} (t) - \theta_{chase})/(\theta_{RPo} - \theta_{chase}) \qquad (5)$$

After averaging SR(t) from four to six independent experiments, apparent dissociation rates are obtained by fitting the mean SR(t) values data to a single exponential decay. Extrapolation to t = 0 min (corresponding to 3 min after adding UTP, GTP, and ATP to RP$_o$) yields SR$_0$, the initial extent of σ^{70} retention for an RD$_e$.

Calculation of Accurate FRET and Corresponding Distances

Ratio E* is a FRET-dependent, distance-dependent ratio, but not FRET efficiency E. We converted E* to E as described (Lee et al., 2005). Distances were obtained by using $R = R_0 [(1/E) - 1]^{1/6}$; Förster radius R$_o$ was measured as described (Kapanidis et al., 2001).

Supplemental Data

Supplemental Data include two figures and one table and are available with this article online at http://www.molecule.org/cgi/content/full/20/3/347/DC1/.

References

Bar-Nahum, G., and Nudler, E. (2001). Isolation and characterization of sigma(70)-retaining transcription elongation complexes from Escherichia coli. Cell 106, 443–451.

Borukhov, S., and Severinov, K. (2002). Role of the RNA polymerase sigma subunit in transcription intitiation. Res. Microbiol. 153, 557–562.

Brodolin, K., Zenkin, N., Mustaev, A., Mamaeva, D., and Heumann, H. (2004). The sigma 70 subunit of RNA polymerase induces lacUV5 promoter-proximal pausing of transcription. Nat. Struct. Mol. Biol. 11, 551–557.

Carpousis, A.J., and Gralla, J.D. (1985). Interaction of RNA polymerase with lacUV5 promoter DNA during mRNA initiation and elongation. Footprinting, methylation, and rifampicin-sensitivity changes accompanying transcription initiation. J. Mol. Biol. 183, 165–177.

Daube, S.S., and von Hippel, P.H. (1994). RNA displacement pathways during transcription from synthetic RNA-DNA bubble duplexes. Biochemistry 33, 340–347.

Ebright, R.H. (2000). RNA polymerase: structural similarities between bacterial RNA polymerase and eukaryotic RNA polymerase II. J. Mol. Biol. 304, 687–698.

Gaal, T., Bartlett, M., Ross, W., Turnbough, C., and Gourse, R.L. (1997). Transcription regulation by initiating NTP concentration: rRNA synthesis in bacteria. Science 278, 4473–4483.

Gill, S.C., Weitzel, S.E., and von Hippel, P.H. (1991). Escherichia coli sigma 70 and NusA proteins. I. Binding interactions with core RNA polymerase in solution and within the transcription complex. J. Mol. Biol. 220, 307–324.

Gotta, S.L., Miller, O.L.J., and French, S.L. (1991). rRNA transcription rate in Escherichia coli. J. Bacteriol. 173, 6647–6649.

Gross, C.A., Chan, C., Dombroski, A., Gruber, T., Sharp, M., Tupy, J., and Young, B. (1998). The functional and regulatory roles of sigma factors in transcription. Cold Spring Harb. Symp. Quant. Biol. 63, 141–155.

Hansen, U.M., and McClure, W.R. (1980). Role of the sigma subunit of Escherichia coli RNA polymerase in initiation. II. Release of sigma from ternary complexes. J. Biol. Chem. 255, 9564–9570.

Jacquet, M., and Kepes, A. (1971). Initiation, elongation and inactivation of lac messenger RNA in Escherichia coli studied studied by measurement of its beta-galactosidase synthesizing capacity in vivo. J. Mol. Biol. 60, 453–472.

Kapanidis, A.N., Ebright, Y.W., Ludescher, R.D., Chan, S., and Ebright, R.H. (2001). Mean DNA bend angle and distribution of DNA bend angles in the CAP-DNA complex in solution. J. Mol. Biol. 312, 453–468.

Kapanidis, A.N., Lee, N.K., Laurence, T.A., Doose, S., Margeat, E., and Weiss, S. (2004). Fluorescence-aided molecule sorting: analysis of structure and interactions by alternating-laser excitation of single molecules. Proc. Natl. Acad. Sci. USA 101, 8936–8941.

Komissarova, N., and Kashlev, M. (1997). Transcriptional arrest: Escherichia coli RNA polymerase translocates backward, leaving the 3′ end of the RNA intact and extruded. Proc. Natl. Acad. Sci. USA 94, 1755–1760.

Korzheva, N., Mustaev, A., Kozlov, M., Malhotra, A., Nikiforov, V., Goldfarb, A., and Darst, S.A. (2000). A structural model of transcription elongation. Science 289, 619–625.

Krummel, B., and Chamberlin, M.J. (1989). RNA chain initiation by Escherichia coli RNA polymerase. Structural transitions of the enzyme in early ternary complexes. Biochemistry 28, 7829–7842.

Lawson, C.L., Swigon, D., Murakami, K.S., Darst, S.A., Berman, H.M., and Ebright, R.H. (2004). Catabolite activator protein: DNA binding and transcription activation. Curr. Opin. Struct. Biol. 14, 10–20.

Lee, N.K., Kapanidis, A.N., Wang, Y., Michalet, X., Mukhopadhyay, J., Ebright, R.H., and Weiss, S. (2005). Accurate FRET measurements within single diffusing biomolecules using alternating-laser excitation. Biophys. J. 88, 2939–2953.

Mekler, V., Kortkhonjia, E., Mukhopadhyay, J., Knight, J., Revyakin, A., Kapanidis, A.N., Niu, W., Ebright, Y.W., Levy, R., and Ebright, R.H. (2002). Structural organization of bacterial RNA polymerase holoenzyme and the RNA polymerase-promoter open complex. Cell 108, 599–614.

Metzger, W., Schickor, P., Meier, T., Werel, W., and Heumann, H. (1993). Nucleation of RNA chain formation by Escherichia coli DNA-dependent RNA polymerase. J. Mol. Biol. 232, 35–49.

Mooney, R.A., and Landick, R. (2003). Tethering sigma70 to RNA polymerase reveals high in vivo activity of sigma factors and sigma70-dependent pausing at promoter-distal locations. Genes Dev. 17, 2839–2851.

Mukhopadhyay, J., Kapanidis, A.N., Mekler, V., Kortkhonjia, E., Ebright, Y.W., and Ebright, R.H. (2001). Translocation of sigma(70) with RNA polymerase during transcription: fluorescence resonance energy transfer assay for movement relative to DNA. Cell 106, 453–463.

Mukhopadhyay, J., Mekler, V., Kortkhonjia, E., Kapanidis, A.N., Ebright, Y.W., and Ebright, R.H. (2003). Fluorescence resonance energy transfer (FRET) in analysis of transcription-complex structure and function. Methods Enzymol. 371, 144–159.

Munson, L.M., and Reznikoff, W.S. (1981). Abortive initiation and long ribonucleic acid synthesis. Biochemistry 20, 2081–2085.

Murakami, K.S., Masuda, S., Campbell, E.A., Muzzin, O., and Darst, S.A. (2002a). Structural basis of transcription initiation: an RNA polymerase holoenzyme-DNA complex. Science 296, 1285–1290.

Murakami, K.S., Masuda, S., and Darst, S.A. (2002b). Structural basis of transcription initiation: RNA polymerase holoenzyme at 4 A resolution. Science 296, 1280–1284.

Nickels, B.E., Roberts, C.W., Sun, H., Roberts, J.W., and Hochschild, A. (2002). The sigma70 subunit of RNA polymerase is contacted by the lambdaQ antiterminator during early elongation. Mol. Cell 10, 611–622.

Nickels, B.E., Mukhopadhyay, J., Garrity, S.J., Ebright, R.H., and Hochschild, A. (2004). The sigma 70 subunit of RNA polymerase mediates a promoter-proximal pause at the lac promoter. Nat. Struct. Mol. Biol. 11, 544–550.

Nickels, B.E., Garrity, S.J., Mekler, V., Minakhin, L., Severinov, K., Ebright, R.H., and Hochschild, A. (2005). The interaction between sigma70 and the beta-flap of Escherichia coli RNA polymerase inhibits extension of the nascent RNA during early elongation. Proc. Natl. Acad. Sci. USA 102, 4488–4493.

Nudler, E., Mustaev, A., Lukhtanov, E., and Goldfarb, A. (1997). The RNA-DNA hybrid maintains the register of transcription by preventing backtracking of RNA polymerase. Cell 89, 33–41.

Revyakin, A., Ebright, R.H., and Strick, T.R. (2004). Promoter unwinding and promoter clearance by RNA polymerase: detection by single-molecule DNA nanomanipulation. Proc. Natl. Acad. Sci. USA 101, 4776–4780.

Ring, B.Z., Yarnell, W.S., and Roberts, J.W. (1996). Function of E. coli RNA polymerase sigma factor sigma 70 in promoter-proximal pausing. Cell 86, 485–493.

Rose, J.K., Mosteller, R.D., and Yanofsky, C. (1970). Tryptophan messenger ribonucleic acid elongation rates and steady-state levels of tryptophan operon enzymes under various growth conditions. J. Mol. Biol. 51, 541–550.

Schaffer, D.A., Gelles, J., Sheetz, M.P., and Landick, R. (1991). Transcription by single molecules of RNA-polymerase observed by light-microscopy. Nature 352, 444–448.

Shimamoto, N., Kamigochi, T., and Utiyama, H. (1986). Release of the sigma subunit of Escherichia coli DNA-dependent RNA polymerase depends mainly on time elapsed after the start of initiation, not on length of product RNA. J. Biol. Chem. 261, 11859–11865.

Straney, D.C., and Crothers, D.M. (1985). Intermediates in transcription initiation from the E. coli lac UV5 promoter. Cell 43, 449–459.

Travers, A.A., and Burgess, R.R. (1969). Cyclic re-use of the RNA polymerase sigma factor. Nature 222, 537–540.

Vassylyev, D.G., Sekine, S., Laptenko, O., Lee, J., Vassylyeva, M.N., Borukhov, S., and Yokoyama, S. (2002). Crystal structure of a bacterial RNA polymerase holoenzyme at 2.6 A resolution. Nature 417, 712–719.

Wade, J.T., and Struhl, K. (2004). Association of RNA polymerase with transcribed regions in Escherichia coli. Proc. Natl. Acad. Sci. USA 101, 17777–17782.

Wang, M.D., Schnitzer, M.J., Yin, H., Landick, R., Gelles, J., and Block, S.M. (1998). Force and velocity measured for single molecules of RNA polymerase. Science 282, 902–907.