Retention of TranscriptionInitiation Factor $\sigma^{70}$ 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 $\sigma^{70}$ 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 $\sigma^{70}$ and that a determinant for $\sigma^{70}$ recognition in the initial transcribed region increases $\sigma^{70}$ retention in early elongation complexes. The results establish that a significant fraction, $\sim 50\%$–$60\%$, of mature elongation complexes retain $\sigma^{70}$ and that a determinant for $\sigma^{70}$ recognition in the initial transcribed region does not appreciably affect $\sigma^{70}$ retention in mature elongation complexes. The results further establish that, in mature elongation complexes that retain $\sigma^{70}$, the half-life of $\sigma^{70}$ retention is long relative to the timescale of elongation, suggesting that some complexes may retain $\sigma^{70}$ throughout elongation.

Introduction

The transcription initiation factor $\sigma^{70}$ interacts with Escherichia coli RNA polymerase core enzyme (RNAP) to yield RNAP holoenzyme (Gross et al., 1998). $\sigma^{70}$ contains determinants for sequence-specific interaction with promoter DNA and, through these determinants, targets RNAP holoenzyme to promoters. $\sigma^{70}$ 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 $\sigma^{70}$ 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 “$\sigma$ cycle” exists, with association of $\sigma^{70}$ with RNAP to permit initiation and dissociation of $\sigma^{70}$ 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 $\sigma^{70}$ obligatorily is released from RNAP at the transition from transcription initiation to elongation was based primarily on early observations that $\sigma^{70}$ is present in RNAP-promoter open complexes (RPo) but is not present in RNAP-DNA elongation complexes (RDe)—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 $\sigma^{70}$ with the remainder of the complex in RPo and RD e into an apparent, but artificial, difference in occupancy of $\sigma^{70}$ in RPo and RD e. Thus, it has been necessary also to consider an alternative proposal: a proposal in which the stability of interactions of $\sigma^{70}$ with the remainder of the complex is reduced at the transition to elongation—e.g., due to unfavorable interactions between $\sigma^{70}$ 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 $\sigma^{70}$ is not necessarily released at the transition to elongation (Shimamoto et al., 1988).

Recently, by using fluorescence resonance energy transfer (FRET), we showed that in 20%–90% of early elongation complexes (i.e., complexes containing RNA products of $\leq 15$ nt), $\sigma^{70}$ 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 $\sigma^{70}$-DNA interaction in the initial transcribed region can increase the fraction of early elongation complexes that retain $\sigma^{70}$ 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 $\sigma^{70}$ 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 $\sigma^{70}$ 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 $\sigma^{70}$ content of an elongation complex. The assay yields translocational position and $\sigma^{70}$ content in a single experiment (in contrast to ensemble $\sigma^{70}$-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 $\sigma^{70}$-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 $\sigma^{70}$-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 $\sigma^{70}$ retention, the half-life of $\sigma^{70}$ retention, and effects of DNA sequence on $\sigma^{70}$ retention in early elongation complexes. More important, the results provide the first quantitative information regarding the extent of $\sigma^{70}$ retention, the half-life of $\sigma^{70}$ retention, and effects of DNA sequence on $\sigma^{70}$ retention in mature elongation complexes.

Results

Single-Molecule Assay for Transcription-Complex Translocational Position and $\sigma^{70}$ Content

We have developed a single-molecule assay, LE/TE-FRET ALEX, that defines, simultaneously, the translocational position and the $\sigma^{70}$ 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 $\approx 1$ 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 $\sigma^{70}$ residue located close to the leading edge of RNAP and...
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 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 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 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 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 \( \sigma^{70} \) 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. \( \sigma^{70} \) content is defined based on the stoichiometry parameter, S. Transcription complexes that contain \( \sigma^{70} \) are donor-acceptor species and thus exhibit a stoichiometry parameter of \( S = 0.5 \), transcription complexes lacking \( \sigma^{70} \) are acceptor-only species and thus exhibit a stoichiometry parameter of \( S < 0.3 \), and free \( \sigma^{70} \) is a donor-only species and thus exhibits a stoichiometry parameter of \( S > 0.8 \).

To incorporate the donor at a \( \sigma^{70} \) residue located close to the leading edge of RNAP, we introduced tetramethylrhodamine (TMR) at position 366 of \( \sigma^{70} \) (Mekler et al., 2002; Mukhopadhyay et al., 2001, 2003; Nickels et al., 2004; Lee et al., 2005). To incorporate the donor at a \( \sigma^{70} \) residue located at the trailing edge of RNAP, we introduced TMR at position 596 of \( \sigma^{70} \) (Mekler et al., 2002; Mukhopadhyay et al., 2001, 2003; Nickels et al., 2004; Lee et al., 2005). Control experiments establish that the resulting labeled \( \sigma^{70} \) 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, 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).

\( \sigma^{70} \) 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 \( \sigma^{70} \) (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 \( \sigma^{70} \) (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 \( \sigma^{70} \) upon the transition from initiation to elongation. We note that the inferred level of \( \sigma^{70} \) retention of ~80% may underestimate the actual level of \( \sigma^{70} \) retention, because some loss of \( \sigma^{70} \) may occur during data collection (data collection time = 30 min; half-life of \( \sigma^{70} \) retention ~90 min; see Figure 4A and Table 2) and because the correction for nonfunctional complexes used in calculation of \( \sigma^{70} \) 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 \( \sigma^{70} \) (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 \( \sigma^{70} \). We infer that, under these conditions, most complexes are
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\(^{70}\) retention, 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\(_{e,11}\).


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

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}**.

The **σ^70** retention, which is lower than the corresponding value 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)** (Nickels et al., 2004), a substituted **lacUV5** derivative that lacks the sequence element.

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

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

Abbreviation: ND, not determined.
\( \sigma^{70} \) 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 \( \sigma^{70} \) retention in elongation complexes containing >15 nt of RNA (Mukhopadhyay et al., 2001). To define the initial extent and half-life of \( \sigma^{70} \) 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 \( \sigma^{70} \) content, defining translocational state based on the FRET efficiency (E) and \( \sigma^{70} \) content based on the stoichiometry parameter (S); therefore, translocational heterogeneity does not complicate LE-FRET ALEX analysis of \( \sigma^{70} \) content.

Figure 5B and Table 2 present results of experiments assessing the initial extent and half-life of \( \sigma^{70} \) retention in RD\(_{e,50}\) at lacUV5. The initial extent of \( \sigma^{70} \) retention is ~50%–60%, and the half-life of \( \sigma^{70} \) retention is ~50 min. We conclude that, at lacUV5, in approximately half of transcription complexes, \( \sigma^{70} \) 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, \( \sigma^{70} \) 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 \( \sigma^{70} \) retention in RD\(_{e,14}\) is significantly higher than the initial extent of \( \sigma^{70} \) retention in RD\(_{e,50}\) (~90% versus ~50%–60%; Figures 5A and 5B). We infer that most \( \sigma^{70} \) release in the fast phase must occur after RNAP reaches position +14. We do not know whether \( \sigma^{70} \) 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 \( \sigma^{70} \)-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 \( \sigma^{70} \) retention differs in backtracked and nonbacktracked RD\(_{e,50}\) and thus that the net observed half-life of \( \sigma^{70} \) retention overestimates or underestimates the actual half-life in nonbacktracked RD\(_{e,50}\).

\( \sigma^{70} \) Retention in Mature Elongation Complexes: Effects of Initial-Transcribed-Region Sequence Element

To assess the proposal that the presence of sequence-specific \( \sigma^{70} \)-DNA interaction in the initial transcribed region may influence not only \( \sigma^{70} \) retention in early elongation but also \( \sigma^{70} \) 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 \( \sigma^{70} \) retention in RD\(_{e,50}\) at lacUV5(A+2G) is ~50%–60%. This value is indistinguishable from the value for the initial extent of \( \sigma^{70} \) 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 \( \sigma^{70} \)-DNA interaction in the initial transcribed region has no appreciable influence—no appreciable memory effect—on \( \sigma^{70} \) 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 \( \sigma^{70} \)-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 \( \sigma^{70} \) 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 \( \sigma^{70} \) and that this fraction can be increased by the presence of a determinant for sequence-specific \( \sigma^{70} \)-DNA interaction in the initial transcribed region, (2) show that a substantial fraction of mature elongation complexes retains \( \sigma^{70} \) and that this fraction is not appreciably affected by the presence of a determinant for sequence-specific \( \sigma^{70} \)-DNA interaction in the initial transcribed region, and (3) define the half-life for \( \sigma^{70} \) 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 \( \sigma^{70} \) and RNAP concentrations (~0.5 nM after addition of the NTP subset) well below the dissociation constant for interaction of \( \sigma^{70} \) with elongation complexes (2 \( \mu \)M [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 \( \sigma^{70} \) retention reflects retention of \( \sigma^{70} \) upon transition to elongation and subsequent translocation of \( \sigma^{70} \) with RNAP, as opposed to release of \( \sigma^{70} \) upon transition to elongation and subsequent re-binding of \( \sigma^{70} \) to the elongation complex.

It is noteworthy that, in mature elongation complexes that retain \( \sigma^{70} \), the observed half-life of \( \sigma^{70} \) retention (~50 s; Table 2) is long relative to the time scale of transcription elongation (~5 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 \( \sigma^{70} \)
throughout the entire process of elongation. Our results address the extent of σ70 retention only during the transition from initiation to elongation and during elongation up to position +50; σ70 release may be more efficient during elongation beyond position +50. Furthermore, our results address only the situation in vitro in a purified system; σ70 release may be more efficient in vivo, in the presence of core binding factors (e.g., Nus factors), σ70 binding factors, and alternative σ factors. Nevertheless, the possibility that at least some elongation complexes may retain σ70 throughout the entire process of elongation would be consistent with immunodetection of σ70 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 σ70 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 σ70-containing elongation complexes permits multiple additional levels of regulation during elongation: regulation mediated by DNA-sequence-recognition by σ70 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 σ70 during elongation (Nickels et al., 2002), and, possibly, regulation mediated by modulation of the extent of σ70 release and retention during elongation.

Experimental Procedures

σ70 Derivatives

σ70 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 σ70 derivative in transcription buffer (TB; 50 mM Tris-HCl, [pH 8], 100 mM KCl, 10 mM MgCl2, 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, Kd/RNAp, were performed essentially as in Nickels et al. (2005). Reaction mixtures (40 μl) contained 40 nM TMR666-σ70 or TMR566-σ70, 0-200 nM unlabeled wild-type σ70 as competitor, 12 nM wild-type RNAp core, 40 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM MgCl2, 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 Biosciences) 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 MgCl2, 1 mM DTT, 100 μg/ml BSA, 5% glycerol, 1 mM mercaptoethanol, 0.5 mM ApA or [0.5 mM ApG]) were added, and the coverglass was incubated for 10 min at 37°C. The 0.4-μl drop 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 alternating period of 100 μs, and duty cycle of 50% (Figures 1A and 1B). Excitation intensities were 150–300 μW for 514 nm excitation (Dexc) and 50–80 μW for 638 nm excitation (Aexc) (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 arbitrary 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 fAem 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 σ70 in initial preparations of RPo (present at 0.1–0.3 nM, due to inefficient removal of free σ70 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 fDexc, fAexc, fDem, and fAem, 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):

\[ S = \frac{f_{Dexc} + \gamma f_{Aexc}}{f_{Dexc} + \gamma f_{Aexc}} \]  

(1)

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

\[ E^* = \frac{f_{Dem}}{f_{Aem} + f_{Dem}} \]  

(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
**Retention of σ^70 in Transcription Elongation**

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 \( \delta \) for RP\(_o\), RD\(_a\), and chased RD\(_e\) (Kapanidis et al., 2004):

\[
\delta = \frac{\text{[donor-acceptor species]} + \text{[acceptor-only species]}}{\text{[donor-acceptor species]}}
\]

RP\(_o\) was measured first (for 15 min), followed by RD\(_a\) (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 \( \delta_{\text{RD}} \) as the 100% value and \( \delta_{\text{chase}} \) as the baseline (0%), we calculate the fractional σ^70 retention, SR:

\[
SR = \frac{(\delta_{\text{RD}} - \delta_{\text{chase}})}{(\delta_{\text{RD}} - \delta_{\text{chase}})}
\]

Apart from a measure of the ability of RD\(_e\) to resume transcription, \( \delta_{\text{chase}} \) 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 \( \delta_{\text{chase}} \) 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 \( \delta_{\text{RD}} \). In contrast, \( \delta_{\text{RD}} \) and \( \delta_{\text{chase}} \) do not change during acquisition. To account for dissociation and evaluate the stability of σ^70 interactions within RD\(_e\) (Nickenig et al., 2004), we plot σ^70 retention as a function of time, SR(t), by calculating \( \delta_{\text{RD}}(t) \) (\( \delta_{\text{RD}} \) as a function of time), with each point being the average \( \delta_{\text{RD}} \) for 3-min slices of acquisition time t (Kapanidis et al., 2004):

\[
SR(t) = \frac{\delta_{\text{RD}}(t) - \delta_{\text{chase}}}{\delta_{\text{RD}} - \delta_{\text{chase}}}
\]

After averaging SR(t) from four to six independent experiments, apparent dissociation rates are obtained by fitting the mean SR(t) values to a single exponential decay. Extrapolation to \( t = 0 \) min (corresponding to 3 min after adding UTP, GTP, and ATP to RP\(_e\)) 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 \( R = R_0 \) (\( 1/E^* - 1/6^* \)). Förster radius \( R_0 \) 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/.

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