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RETENTION OF TRANSCRIPTION INITIATION FACTOR σ^{70} IN TRANSCRIPTION ELONGATION: SINGLE-MOLECULE ANALYSIS

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ABSTRACT

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 the σ^{70} contents of bacterial transcription elongation complexes *in vitro*. The results confirm ensemble results indicating that a large fraction, ~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, ~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.

Running title: Retention of σ^{70} in transcription elongation

Key words: RNA polymerase; sigma factors; transcription initiation; transcription elongation; promoter escape; protein-DNA complexes; fluorescence resonance energy transfer; alternating-laser excitation; single-molecule analysis

Abbreviations: ALEX, alternating-laser excitation; FRET, fluorescence resonance energy transfer; LE-FRET, leading-edge FRET; TE-FRET, trailing-edge FRET; RNAP, RNA polymerase; RP_o, RNAP-promoter open complex; RD_e, RNAP-DNA elongation complex

INTRODUCTION

Escherichia coli RNA polymerase (RNAP) is the molecular machine responsible for gene transcription in *E. coli*. RNAP initiates transcription at specific DNA sequences (known as promoter DNA regions) after it associates with σ transcription initiation factors (Gross et al., 1998). This acquired RNAP specificity is due to the presence in the σ factors of specific amino acids that interact with promoter DNA in a sequence-specific fashion. The primary σ factor in exponentially-growing *E. coli* is σ^{70}.

Until recently, it was unclear whether σ^{70} was released obligatorily from RNAP upon the escape of RNAP from promoter DNA, i.e., at the transition from initiation to elongation, occurring after RNAP synthesizes RNA longer than 9-11 nt. In other words, did σ^{70} associate with RNAP to permit initiation, and then dissociate to permit elongation? Furthermore, did differences in subunit composition underlie differences in mechanism, and in responsiveness to DNA elements and to regulators?

An initial proposal for obligatory σ^{70} release at the transition to elongation (Hansen and McClure, 1980; Krummel and Chamberlin, 1989; Metzger et al., 1993; Straney and Crothers, 1985; Travers and Burgess, 1969) was based on early reports that detected σ^{70} in purified RNAP-promoter open complexes (RP_o), but failed to detect σ^{70} in purified RNAP-DNA elongation complexes (RD_e) (Hansen and McClure, 1980; Krummel and Chamberlin, 1989; Metzger et al., 1993; Straney and Crothers, 1985). Nonetheless, these reports included purification steps that could have translated a *decreased affinity* of σ^{70} for elongation complexes into *release* of σ^{70} from elongation complexes. Thus, an alternative model was proposed, in which there is no σ^{70}-release requirement for the transition from initiation to elongation (Shimamoto et al., 1986); in this model, the interactions that retain σ^{70} in the transcription complex are weakened after escape to elongation, most likely due to competition of σ^{70} and nascent RNA for binding sites within RNAP (Daube and von Hippel, 1994; Mekler et al., 2002; Murakami et al., 2002b; Nickels et al., 2005; Vassylyev et al., 2002).

Recently, using fluorescence resonance energy transfer (FRET), we showed that in 20-100% of early elongation complexes (i.e., complexes with RNA of ≤15 nt), σ^{70} is not released from RNAP, but, instead, remains associated with RNAP and translocates with RNAP (Mukhopadhyay et al., 2001; Mukhopadhyay et al., 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 ref. 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; 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; Mukhopadhyay et al., 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; Mukhopadhyay et al., 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; Mukhopadhyay et al., 2003; Nickels et al., 2004) regarding the extent of σ^{70} retention, the half-life of extent 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 extent 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, 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 a transcription complex (Figs. 1-2). 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; Fig. 1A,B; Kapanidis et al., 2004). The results provide population distributions of donor-acceptor FRET efficiencies (*E*) and donor-acceptor stoichiometry factors (*S*) (Fig. 2A,B; 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 incorporate the acceptor on downstream DNA (Fig. 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 (Fig. 2B). In each case, we perform measurements with the RNAP-promoter open complex and with defined RNAP-DNA elongation complexes (Fig. 2). Translocational state is defined based on the FRET efficiency, *E* (or on the uncorrected FRET efficiency, *E**; see Methods). 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 $S\sim0.5$; transcription complexes lacking σ^{70} are acceptor-only species and thus exhibit a stoichiometry parameter of $S<0.3$; 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; Mukhopadhyay et al., 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; Mukhopadhyay et al., 2003; Nickels et al., 2004; Lee et al., 2005). Control experiments establish that the resulting labelled σ^{70} derivatives are unaltered in interactions with RNAP core (Table S-1) and are functional in formation of RP_o and RD_e (Mekler et al., 2002; Mukhopadhyay et al., 2001; Mukhopadhyay et al., 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 (Supplemental Fig. S-1; Mekler et al., 2002; Mukhopadhyay et al., 2001; Mukhopadhyay et al., 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, respectively, and halts (due to the absence of CTP, the next required NTP; Fig. S-1). The resulting halted complexes are *bona fide* elongation complexes; they are stable, they retain RNA, and they can be restarted upon addition of CTP (Fig. 3A,B). 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 (Supplemental Fig. S-1).

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). Fig. 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 (Fig 3A, left panel). 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; Mukhopadhyay et al., 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 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 Methods; 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 (Fig. 2A, center and right panels); 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 fully ~80% 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, since some loss of σ^{70} may occur during data collection (data-collection time = 30 min; half-life of σ^{70} retention ~90 min; see Fig. 4A and Table 2), and since the correction for non-functional complexes used in calculation of σ^{70} retention may represent an upper bound (see Methods).

Fig. 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, consistent with the donor-acceptor separation observed in previous ensemble and single-molecule experiments (Lee et al., 2005; Mekler et al., 2002; Mukhopadhyay et al., 2001) (Fig. 3B, left panel). 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 (Fig. 3B, center and right panels); 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 (Fig. 3B, center and right panels); this species is $RD_{e,11}$ not containing σ^{70}. We infer that, under these conditions, most complexes are functional and competent to undergo the transition from initiation to elongation, and that fully ~70% 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.

Fig. 4A and Table 2 present results of leading-edge-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 Methods). 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. Parallel control experiments on RP_o showed that σ^{70} release or complex dissociation in RP_o occurs significantly more slowly (half-life of σ^{70} retention >2 h; Fig. S2A). We conclude, consistent with conclusions from ensemble experiments (Mukhopadhyay et al., 2001), 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: $RD_{e,14}$. 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; Murakami et al., 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., $RD_{e,14}$.

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The data in Fig. 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; Mukhopadhyay et al., 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.

Fig. 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; Fig 4A, Table 2). Parallel control experiments on RP_o showed that σ^{70} release or complex dissociation in RP_o occurs significantly more slowly (half-life of σ^{70} retention >2 h; Fig. 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 and half-life of σ^{70} retention.

σ^{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 σ^{70} retention in elongation complexes containing >15 nt of RNA (Mukhopadhyay et al., 2001).

To define the initial extent and half-life of σ^{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 σ^{70} content, defining translocational state based on the FRET efficiency (*E*) and σ^{70} content based on the stoichiometry parameter (*S*); therefore, translocational heterogeneity does not complicate LE-FRET ALEX analysis of σ^{70} content.

Fig. 5B and Table 2 present results of experiments assessing the initial extent and half-life of σ^{70} retention in $RD_{e,50}$ at *lacUV5*. The initial extent of σ^{70} retention is ~50-60%, and the half-life of σ^{70} retention is ~50 min. We conclude that, at *lacUV5*, in approximately half of transcription complexes, σ^{70} remains associated with RNAP upon formation of a mature elongation complex, and reaches position +50 in association with RNAP.

Fig. 5B indicates that, upon addition of the NTP subset that permits formation of $RD_{e,50}$ at *lacUV5*, σ^{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 σ^{70} retention in $RD_{e,14}$ is significantly higher than the initial extent of σ^{70} retention in $RD_{e,50}$ (~90% vs. ~50-60%; Figs. 5A, 5B). We infer that most σ^{70} release in the "fast" phase must occur after RNAP reaches position +14. We do not know whether σ^{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 σ^{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 vs. *E**~0.45; not shown). We cannot exclude the possibility that the half-life of σ^{70} retention differs in backtracked and non-backtracked $RD_{e,50}$ and thus that the net observed half-life of σ^{70} retention overestimates or underestimates the actual half-life in non-backtracked $RD_{e,50}$.

σ^{70} retention in mature elongation complexes: effects of initial-transcribed-region sequence element. To assess the proposal that the presence of sequence-specific σ^{70}-DNA interaction in the initial transcribed region may influence not only σ^{70} retention in early elongation but also σ^{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 σ^{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 σ^{70} retention in $RD_{e,50}$ at *lacUV5* (55±8 vs. 56±10%; Table 2). We conclude that the presence of a determinant for sequence-specific σ^{70}-DNA interaction in the initial transcribed region has no appreciable influence--no appreciable "memory effect--on σ^{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* (not shown). At *lacUV5(A+2G)*, as at *lacUV5*, the majority, >70%, of σ^{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 vs. *E**~0.45; not shown).

DISCUSSION

Our single-molecule FRET results confirm and extend ensemble FRET results (Mukhopadhyay et al., 2001; Mukhopadhyay et al., 2003; Nickels et al., 2004) indicating that the initiation factor σ^{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 σ^{70} and that this fraction can be increased by the presence of a determinant for sequence-specific σ^{70}-DNA interaction in the initial transcribed region, (2) show that a substantial fraction of mature elongation complexes retains σ^{70} and that this fraction is not appreciably affected by the presence of a determinant for sequence-specific σ^{70}-DNA interaction in the initial transcribed region, and (3) define the half-life for σ^{70} retention in mature elongation complexes.

The single-molecule FRET results were obtained using a method that did not require correction factors from multiple separate reactions (in contrast to the ensemble FRET results (Mukhopadhyay et al., 2001; Mukhopadhyay et al., 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 σ^{70} and RNAP concentrations (≤0.5 nM following addition of the NTP subset) well below the dissociation constant for interaction of σ^{70} with elongation complexes (2 μM (Gill et al., 1991)), in contrast to the ensemble FRET results (Mukhopadhyay et al., 2001; Mukhopadhyay et al., 2003; Nickels et al., 2004). Therefore, a strong case can be made that the observed σ^{70} retention reflects retention of σ^{70} upon transition to elongation and subsequent translocation of σ^{70} with RNAP, as opposed to release of σ^{70} upon transition for initiation to elongation and subsequent re-binding of σ^{70} to the elongation complex.

It is noteworthy that, in mature elongation complexes that retain σ^{70}, the observed half-life of σ^{70} 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 σ^{70} 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 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 exponential-phase cultures (R. Rong, O. Leroy, and R.H.E., unpublished; 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 et al., 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.

MATERIALS AND METHODS

σ^{70} derivatives. σ^{70} derivatives having tetramethylrhodamine (TMR) incorporated at residue 366 (for LE-FRET experiments) or residue 596 (for TE-FRET experiments) were prepared as described (Mukhopadhyay et al., 2001; Mukhopadhyay et al., 2003) (Table S-1).

Promoter DNA fragments. Promoter DNA fragments labelled with Cy5 were prepared as described (Mukhopadhyay et al., 2001; Mukhopadhyay et al., 2003) (sequences in Fig. S1).

Transcription complexes. Reaction mixtures for preparation of open complexes contained (30 µl): 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, 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 non-specific 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*; 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 (not shown). Representative experiments with open complexes in the absence of the initiating dinucleotide yield equivalent results, but inferior signal-to-noise ratio (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 contained (40 µl): 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 T 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 non-specific 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 pM-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 using 514- and 638-nm light, with alternation period of 100 µs, and duty cycle of 50% (Fig. 1A-B). 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 $f_{D_{exc}}^{A_{em}}$, $f_{D_{exc}}^{D_{em}}$, $f_{A_{exc}}^{A_{em}}$, and $f_{A_{exc}}^{D_{em}}$ (where $f_{X_{exc}}^{Y_{em}}$ 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 $f_{A_{exc}}^{A_{em}}$ thresholds of 7-9 photons per 500 µs and 15-30 photons per burst. 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 $F_{D_{exc}}^{A_{em}}$, $F_{D_{exc}}^{D_{em}}$, and $F_{A_{exc}}^{A_{em}}$, which allow the calculation of ratios *S* and *E**. Ratio *S* was calculated using eq.1, where γ is a detection-correction factor (Kapanidis et al., 2004; Lee et al., 2005):

$$S = \left(F_{D_{exc}}^{A_{em}} + \gamma F_{D_{exc}}^{D_{em}}\right) \Big/ \left(F_{D_{exc}}^{A_{em}} + \gamma F_{D_{exc}}^{D_{em}} + F_{A_{exc}}^{A_{em}}\right) \qquad (1)$$

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

$$E^* = F_{D_{exc}}^{A_{em}} \Big/ \left(F_{D_{exc}}^{A_{em}} + F_{D_{exc}}^{D_{em}}\right) \qquad (2)$$

Ratio *S* reports on relative stoichiometry of donors and acceptors, whereas *E** reports on the distance between the probes; 2D *E**-*S* histograms (Fig. 1A) allowing identification and sorting of diffusing species. Donor-only species show low *E** and high *S* (Fig. 1A, top-left corner), acceptor-only species show high *E** and low *S* (Fig. 1A, bottom-right corner), and donor-acceptor species show various *E** values and $0.3 < S < 0.8$ (Fig. 1A). 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):

θ = [donor-acceptor species] / ([acceptor-only species] + [donor-acceptor species]) (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). Using θ_{RPo} as the 100% value, and θ_{chase} the baseline (0%), we calculate the fractional σ^{70} retention, SR:

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

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 non-functional ("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 $\theta_{RDe}(t)$ (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):

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

After averaging *SR*(t) from 4-6 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 using $R = R_0 \left[(1/E) - 1 \right]^{1/6}$; Förster radius R_o was measured as described (Kapanidis et al., 2001).

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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***	**52***

*minor species with $E < 0.10$ and $R > 90$ Å

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} 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**

ND: Not determined.

FIGURE LEGENDS

Figure 1. Experimental strategy.

(A) Use of confocal microscopy with alternating-laser excitation to monitor fluorescence of transcription complexes. D, FRET donor; A, FRET acceptor. Transcription complexes labeled with donor fluorophore on σ^{70} and 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 (D) and a wavelength that excites the FRET acceptor fluorophore (A). 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*) (Fig. 2A,B; Kapanidis et al., 2004; Lee et al., 2005). The parameter *S* permits identification of molecules containing both donor and acceptor fluorophores ($S \sim 0.5$), molecules containing only a donor fluorophore ($S > 0.8$), and molecules containing only an acceptor fluorophore ($S < 0.3$).

(B) Experimental setup. 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.

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). D, donor-only species; A, acceptor-only species; 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).

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, since the molecule-search criteria employed herein identify solely acceptor-containing molecules (see Methods).]

(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 $RD_{e,11}$.

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, standard error of mean (SEM) for 4-6 independent measurements. Solid lines, single-exponential fits.

(B) As panel, (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, SEM for 4-6 independent measurements. Solid lines, single-exponential fits.

(B) Extent of σ^{70} retention in mature elongation complex $RD_{e,50}$ at *lacUV5*.

FIGURE 1

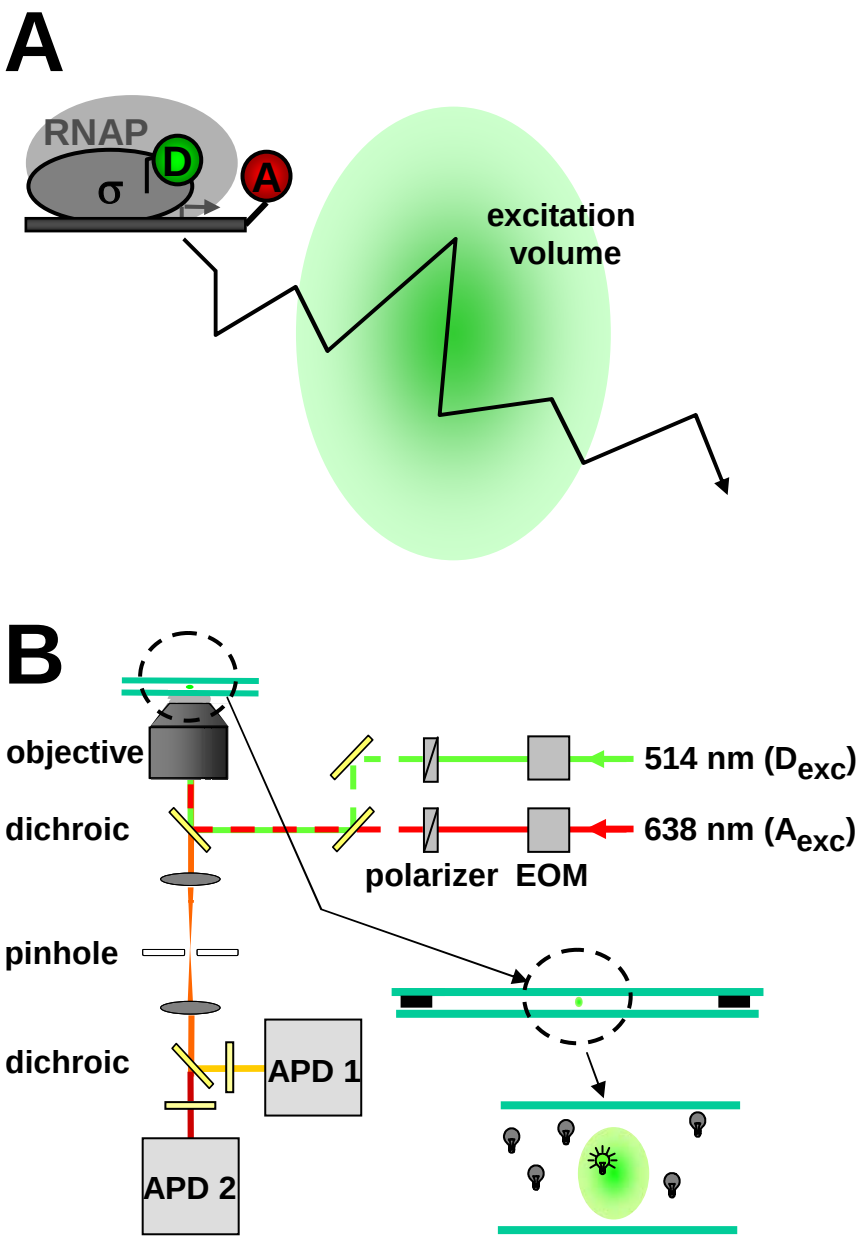
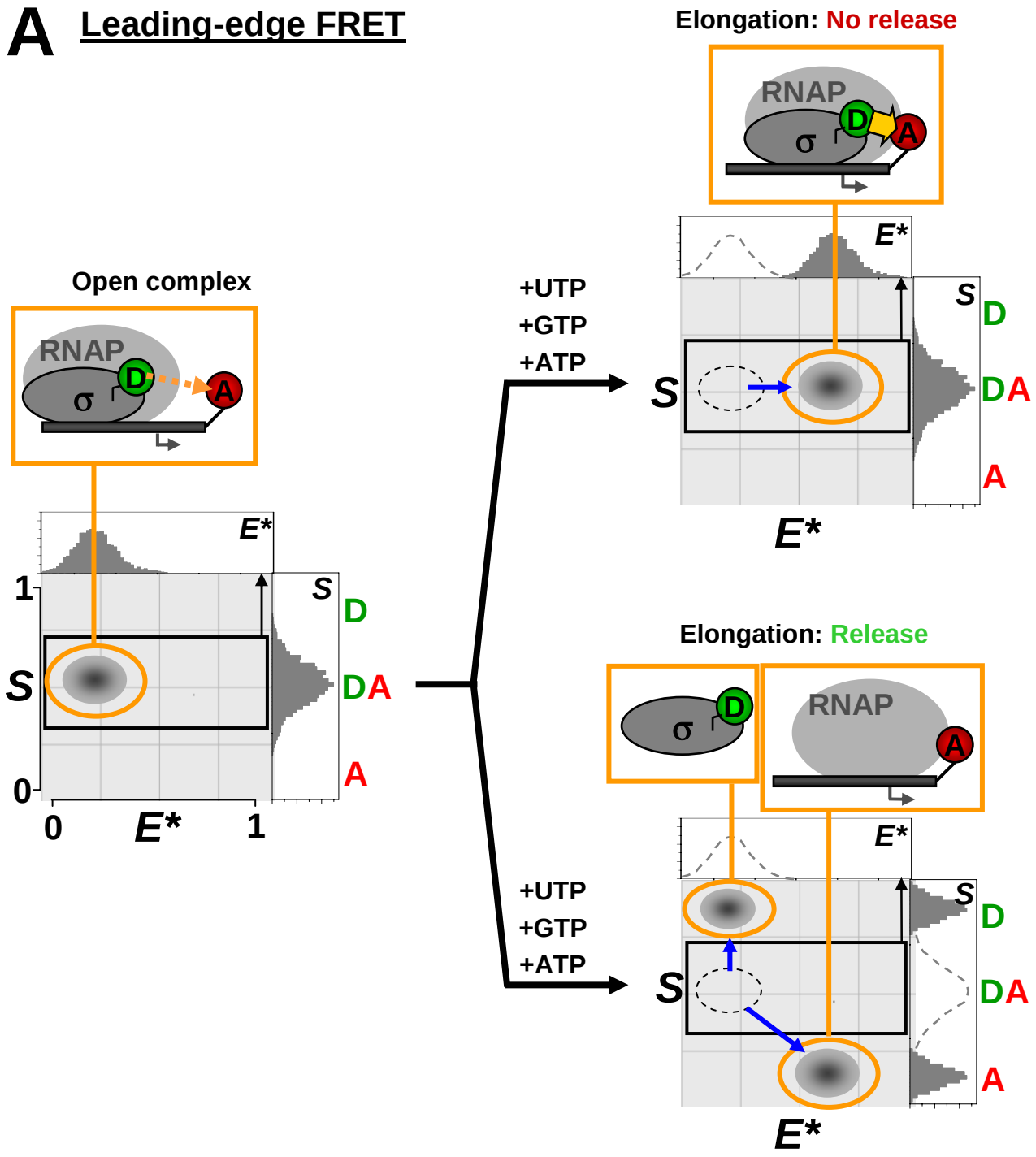

A
RNAP
σ
D
A
excitation
volume
B
objective
dichroic
pinhole
dichroic
APD 1
APD 2
514 nm (D_exc)
638 nm (A_exc)
polarizer
EOM

FIGURE 2


A
Leading-edge FRET
Open complex
RNAP
σ
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+UTP
+GTP
+ATP
Elongation: No release
RNAP
σ
D
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E*
S
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DA
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Elongation: Release
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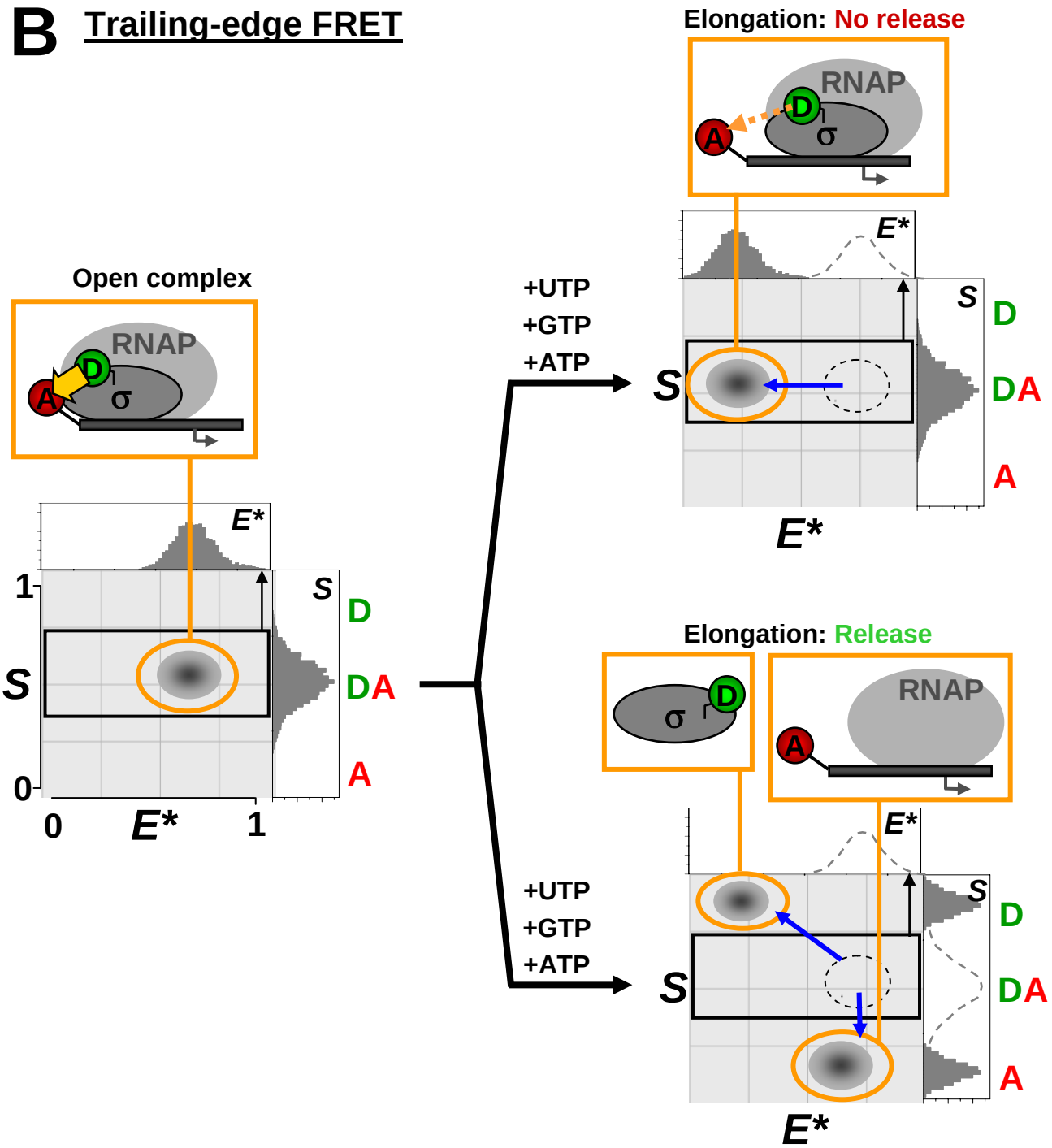
+GTP
+ATP

FIGURE 2


B
Trailing-edge FRET
Open complex
RNAP
D
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+GTP
+ATP
Elongation: No release
RNAP
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Elongation: Release
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+ATP

FIGURE 3

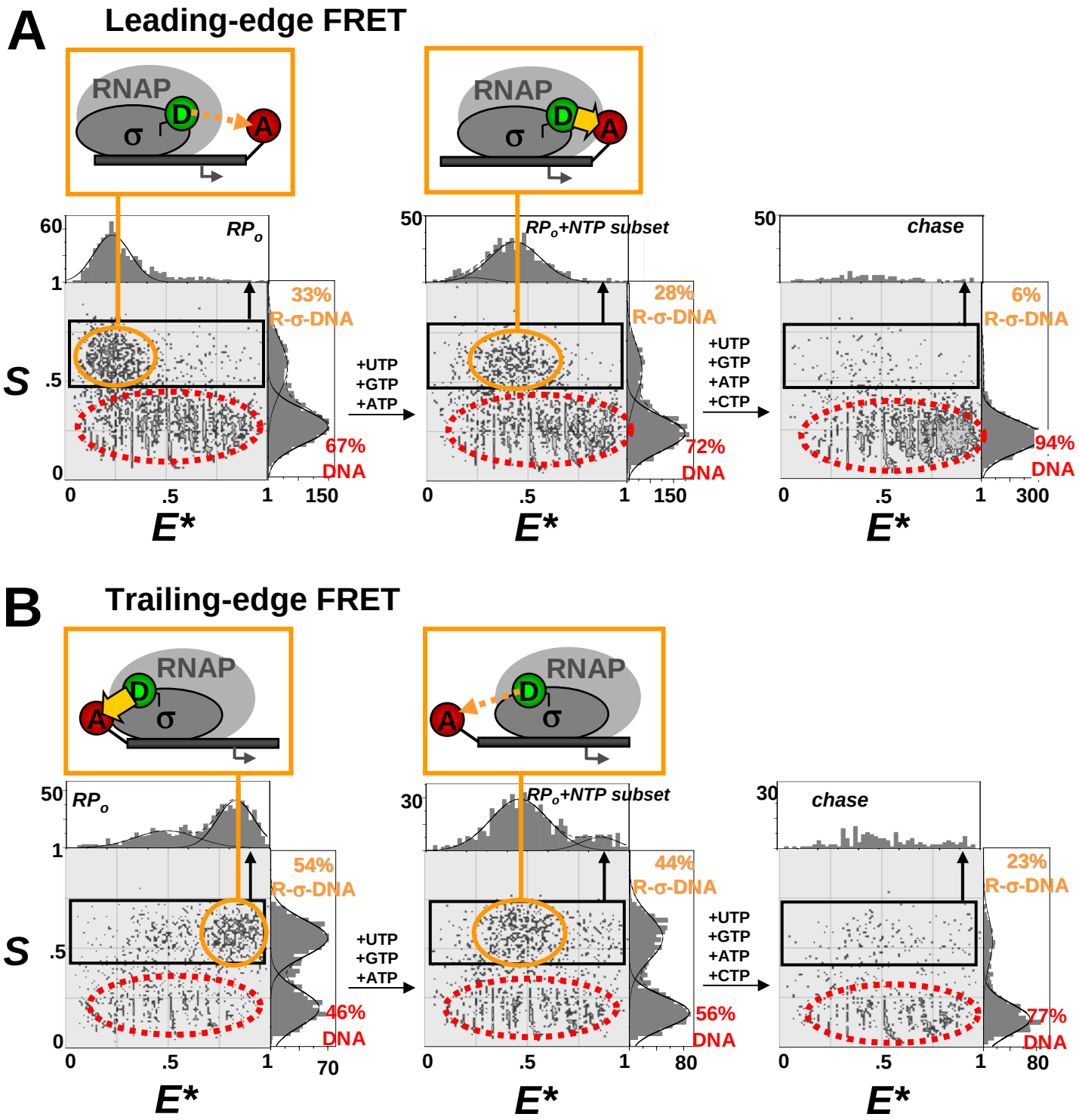

A
Leading-edge FRET
RNAP
σ
D
A
RP$_o$
33%
R-σ-DNA
67%
DNA
+UTP
+GTP
+ATP
RP$_o$+NTP subset
28%
R-σ-DNA
72%
DNA
+UTP
+GTP
+ATP
+CTP
chase
6%
R-σ-DNA
94%
DNA
S
E*
B
Trailing-edge FRET
RNAP
σ
D
A
RP$_o$
54%
R-σ-DNA
46%
DNA
+UTP
+GTP
+ATP
RP$_o$+NTP subset
44%
R-σ-DNA
56%
DNA
+UTP
+GTP
+ATP
+CTP
chase
23%
R-σ-DNA
77%
DNA
S
E*

FIGURE 4

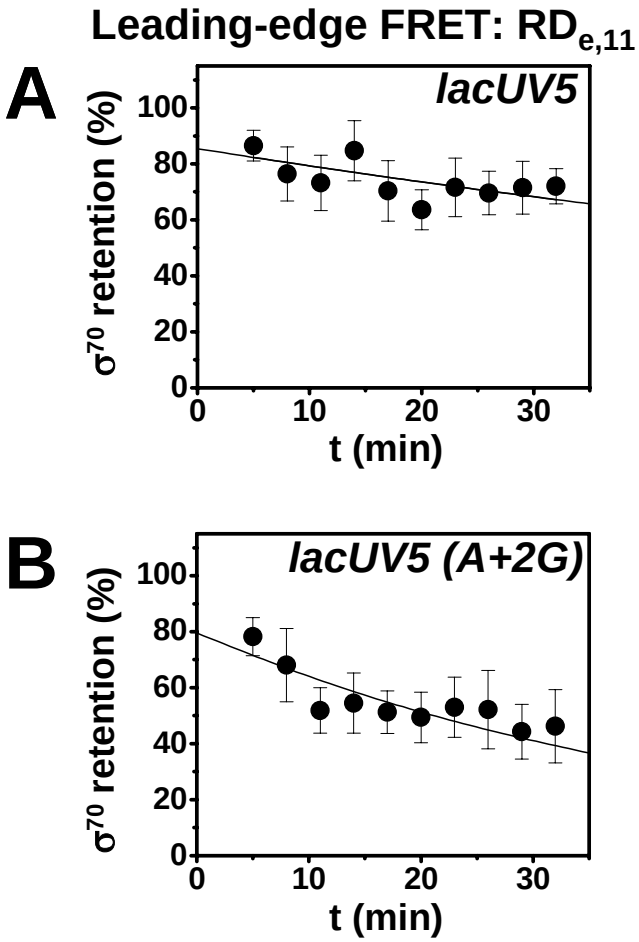
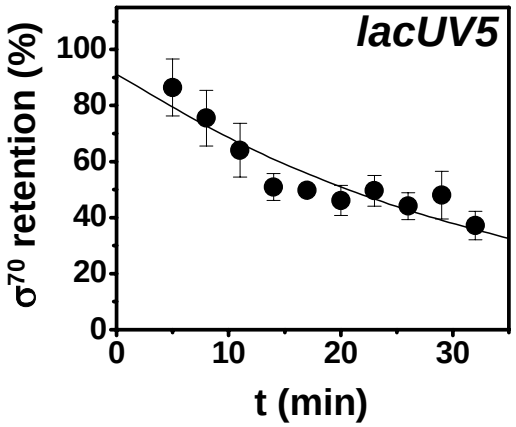

Leading-edge FRET: RD$_{e,11}$
A
lacUV5
σ^{70} retention (%)
100
80
60
40
20
0
0
10
20
30
t (min)
B
lacUV5 (A+2G)
σ^{70} retention (%)
100
80
60
40
20
0
0
10
20
30
t (min)

FIGURE 5

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



B Leading-edge FRET: $RD_{e,50}$

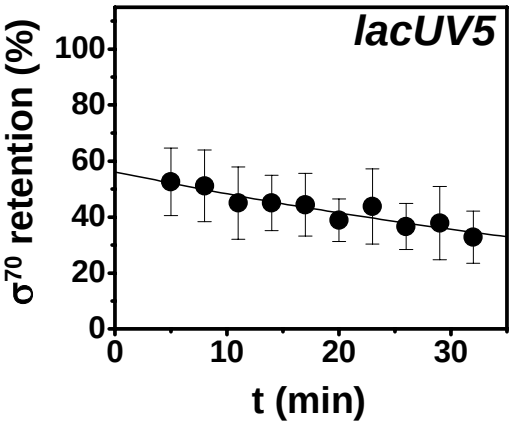


TABLE S-1. Relative equilibrium binding constants ($K_{b,x}/K_{b,\ wild\text{-}type}$) for RNAP core binding of σ^{70} derivatives used in this work (data from fluorescence-detected electrophoretic mobility shift experiments).

Sigma derivative	$K_{b,x}/K_{b,wild\text{-}type}$
unlabelled wild-type sigma	[1.0]
TMR366-sigma	0.5±0.1
TMR596-sigma	1.1±0.1

Supplemental Figure S-1. DNA fragments.

(A) DNA fragments used in the analysis of RD_e initiated on *lacUV5* and halted after synthesis of 11, 14, or 50 nt of RNA (*lacUV5* derivatives having no guanine residues on the template strand from positions +1 to +11, +1 to +14, or +11 to +50)(Mukhopadhyay et al., 2001; Mukhopadhyay et al., 2003; Nickels et al., 2004). The first 3 DNA fragments are used for leading-edge FRET analysis and thus have fluorophore Cy5 at position +25, +28, and +64. The 4th DNA fragment is used for trailing-edge FRET analysis and thus has fluorophore Cy5 at position –40. Black boxes, transcription start site (with arrow), promoter –10 element and promoter –35 element; red boxes, halt site.

(B) DNA fragments used in the analysis of RD_e initiated on *lacUV5(A+2G)* (Nickels et al., 2004) and halted after synthesis of 11, 14, or 50 nt of RNA (Mukhopadhyay et al., 2001; Mukhopadhyay et al., 2003; Nickels et al., 2004). The first 3 DNA fragments are used for leading-edge FRET analysis and thus have FRET acceptor Cy5 at position +25, +28, and +64. The 4th DNA fragment is used for trailing-edge FRET analysis and thus has FRET acceptor Cy5 at position –40.

Supplemental Figure S-2. σ^{70} retention in RP_o.

(A) Extent of σ^{70} retention in RP_o at *lacUV5*. The y-intercept (t = 0 min) corresponds to the beginning of data acquisition; the data are normalized to the 3-min data point. Error bars, standard error of mean (SEM) for 4 independent measurements. Solid lines, single-exponential fits. The half-life of σ^{70} retention is >2 h.

(B) As panel (A), but for RP_o at *lacUV5(A+2G)* (substituted *lacUV5* derivative lacking determinant for sequence-specific σ^{70}-DNA interaction in initial transcribed region). The half-life of σ^{70} retention is >2 h.

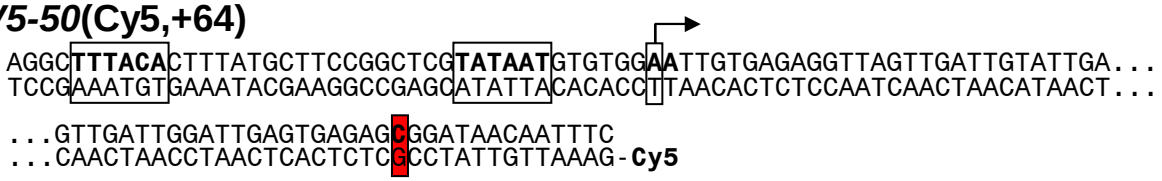
FIGURE S-1

A

***lacUV5-11*(Cy5,+25)**

***lacUV5-14*(Cy5,+28)**

***lacUV5-50*(Cy5,+64)**



***lacUV5-11*(Cy5,-40)**

B

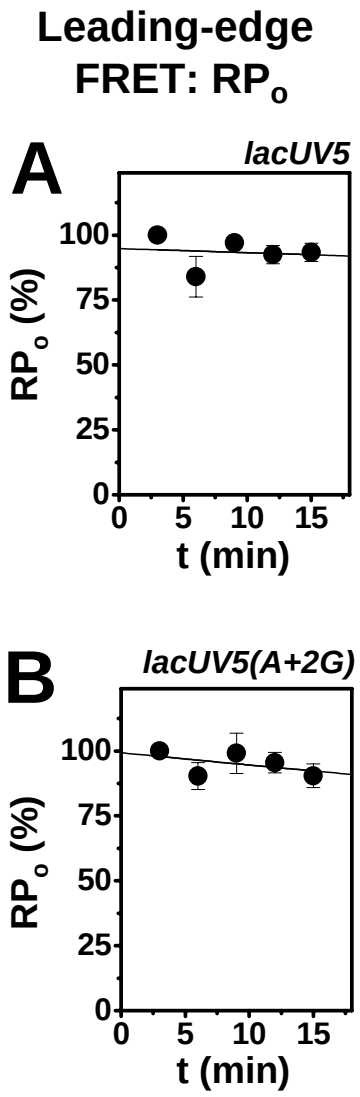
***lacUV5(A+2G)-11*(Cy5,+25)**

***lacUV5(A+2G)-14*(Cy5,+28)**

***lacUV5(A+2G)-50*(Cy5,+64)**

***lacUV5(A+2G)-11*(Cy5,-40)**

FIGURE S-2


Leading-edge
FRET: RPo
A
lacUV5
RPo (%)
100
75
50
25
0
0
5
10
15
t (min)
B
lacUV5(A+2G)
RPo (%)
100
75
50
25
0
0
5
10
15
t (min)