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RNA-mediated destabilization of the σ^{70} region 4/β flap interaction facilitates engagement of RNA polymerase by the Q antiterminator

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Summary

The bacterial RNA polymerase (RNAP) holoenzyme consists of a catalytic core enzyme ($\alpha_2\beta\beta'\omega$) complexed with a σ factor that is required for promoter-specific transcription initiation. During early elongation, the stability of interactions between σ^{70} (the primary sigma factor in *Escherichia coli*) and core decreases, due to an ordered displacement of segments of σ^{70} from core triggered by growth of the nascent RNA. Here we demonstrate that the nascent RNA-mediated destabilization of an interaction between σ^{70} region 4 and the flap domain of the β subunit is required for the bacteriophage λ Q antiterminator protein to contact holoenzyme during early elongation. We demonstrate further that the requirement for nascent RNA in the process by which Q engages RNAP can be bypassed if σ^{70} region 4 is removed. Our findings illustrate how a regulator can exploit the nascent RNA-mediated reconfiguration of the holoenzyme to gain access to the enzyme during early elongation.

Introduction

The bacterial RNA polymerase (RNAP) holoenzyme consists of a catalytic core enzyme ($\alpha_2\beta\beta'\omega$) complexed with a σ factor that confers on the core enzyme the ability to initiate promoter-specific transcription. The primary σ factor in *Escherichia coli* is σ^{70}, and a typical σ^{70}-dependent promoter bears two conserved sequence elements, the −10 and the −35 hexamers, which are separated by a spacer of ~17 base pairs (bp) (reviewed in Gross et al., 1998). All primary σ factors share four regions of conserved sequence (regions 1-4) (Lonetto et al., 1992). Regions 2, 3, and 4 contain DNA-binding domains responsible for recognition of the promoter −10 element, extended −10 element (Bown et al., 1997), and −35 element, respectively, and a flexible linker (σ region 3.2) connects the other portions of region 3 to region 4 (Murakami et al., 2002a and 2002b; Vassylyev et al., 2002). Structures of the RNAP holoenzyme (Murakami et al., 2002a and 2002b; Vassylyev et al., 2002) reveal that two domains of σ lie along the predicted path of the nascent RNA: 1) region 3.2, which is positioned within the RNA exit channel, and 2) region 4, which, by virtue of its interaction with the flap domain of the β subunit (β flap), is positioned immediately adjacent to the end of the RNA exit channel. Thus, during early elongation, the nascent RNA first must displace σ region 3.2 from the RNA exit channel when the nascent RNA enters the channel (Murakami et al., 2002a; Mekler et al., 2002), at a length of ~10-11 nucleotides (nt) (Borukhov and Nudler, 2003), and second, must displace σ region 4 from the β flap (or otherwise perturb the interaction) when the nascent RNA emerges from the RNA exit channel (Vassylyev et al., 2002), at a length of ~16 nt (Komissarova and Kashlev, 1998; Korzheva et al. 2000; Nickels et al., 2005). It has been proposed that this ordered displacement of segments of σ from the RNAP core by the

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nascent RNA could facilitate gene regulation and specifically, that these rearrangements of the holoenzyme may allow elongation factors to access RNAP (Murakami and Darst, 2003).

Previous work has demonstrated that σ^{70} can play functional roles during transcription elongation (reviewed in Mooney et al., 2005). The most well characterized example involves the regulation of late gene transcription from the bacteriophage λ promoter $P_{R'}$ (Roberts et al., 1998), where σ^{70} mediates an early elongation pause that is essential for the function of the Q antiterminator protein (λQ). λQ specifically engages RNAP that has initiated transcription at $P_{R'}$ and this engagement process depends on two DNA sequence elements, a Q binding element (QBE) that is located between the promoter −10 and −35 elements (Yarnell and Roberts, 1992) and a pause-inducing element that is located in the initial transcribed region (Figure 1A). The pause-inducing element resembles a promoter −10 element and pausing, which manifests itself in complexes containing nascent RNAs of 16 or 17 nt, is mediated by protein-DNA interaction between σ^{70} region 2 and the −10-like element (Ring et al., 1996). Thus, the paused early elongation complex has properties characteristic of both an initiation complex (RNAP contains $\sigma^{70}$and is bound to a sequence that resembles a promoter −10 element) and an elongation complex (RNAP has escaped the promoter and contains a stably associated RNA transcript). DNA-bound λQ interacts with this paused complex and becomes a stable component of the elongation complex (Yarnell and Roberts, 1999; P. Deighan and A. H. unpublished results), enabling RNAP to read through downstream terminators and transcribe the phage's late genes.

Here we investigate whether the RNA-mediated destabilization of the σ^{70} region 4/β flap interaction facilitates the engagement of RNAP by Q, a hypothesis that is suggested by two lines of indirect evidence. First, the pause associated with $\lambda P_{R'}$ manifests itself in early elongation complexes containing a 16- or 17-nt transcript (Roberts et al., 1998). Thus, in the context of this early elongation complex, the nascent RNA is of a length at which σ region 3.2 has been displaced from the RNA exit channel (Marr et al. 2001) and the interaction between σ region 4 and the β flap is destabilized (Nickels et al., 2005) (Figure 1B). Second, λQ's engagement of the paused elongation complex causes the RNAP holoenzyme to adopt a conformation that requires release of σ^{70} region 4 from the β flap (Marr et al., 2001; Nickels et al., 2002) (Figure 1C). In this study we provide direct evidence that the RNA-mediated destabilization of the σ^{70} region 4/β flap interaction is required for efficient engagement of the early elongation complex by λQ. Furthermore, we demonstrate that the requirement for nascent RNA in this engagement process can be bypassed by weakening or eliminating the σ^{70} region 4/β flap interaction. Thus, our results illustrate how the nascent RNA-mediated destabilization of the σ^{70} region 4/β flap interaction during the transition from initiation to elongation can facilitate the engagement of a regulatory factor with RNAP.

Results

Effects of altering the σ region 4/β flap interaction on λQ-dependent antitermination in vitro

We hypothesized that engagement of the paused elongation complex by λQ is facilitated by the nascent RNA-mediated destabilization of the σ^{70} region 4/β flap interaction. Predictions arising from this hypothesis are: 1) inhibiting the displacement of σ^{70} from the β flap should inhibit the engagement of the paused elongation complex by λQ (decreasing the amount of λQ-dependent antitermination), and 2) facilitating the displacement of σ^{70} from the β flap should facilitate engagement of the paused elongation complex by λQ (increasing the amount of λQ-dependent antitermination). To inhibit or facilitate displacement of σ^{70} region 4 from the β flap we took advantage of amino acid substitutions in σ^{70} region 4 that either increase (T544I/D581G) or decrease (L607P) the strength of the σ^{70} region 4/β flap interaction (Nickels et al., 2005). Previous work suggests that substitutions T544I and D581G, which strengthen the σ^{70} region 4/β flap interaction, inhibit the RNA-mediated displacement of σ^{70} region 4

from the β flap and substitution L607P, which weakens the σ^{70} region 4/β flap interaction, facilitates displacement of σ^{70} region 4 from the β flap (Nickels et al., 2005). Therefore, we used these amino acid substitutions to test the effect of inhibiting or facilitating the displacement of σ^{70} region 4 from the β flap on λQ antitermination function.

We performed single-round in vitro transcription assays using a $\lambda P_{R'}$ template bearing the natural λ late terminator $t_{R'}$ (see Figure 1A). In the absence of λQ, nearly all (~99%) of the RNAP molecules terminate at $t_{R'}$ (data not shown), while in the presence of λQ, modified RNAP molecules read through the terminator to produce the full length run-off transcript. Therefore, λQ antitermination function can be measured by calculating the percentage of full-length transcripts (% readthrough) emanating from $\lambda P_{R'}$ in the presence of λQ. Assays were performed at low or high concentrations of λQ using holoenzyme reconstituted with wild-type σ^{70} or mutant σ's bearing substitutions that either strengthen (T544I/D581G) or weaken (L607P) the σ^{70} region 4/β flap interaction (Figure 2, panels A and B). At low concentrations of λQ (2.5 nM), strengthening the σ^{70} region 4/β flap interaction (T544I/D581G) reduced λQ-dependent antitermination (~3-fold; Figure 2A) and weakening the σ^{70} region 4/β flap interaction (L607P) increased λQ-dependent antitermination (~2-fold; Figure 2A). Furthermore, the effects of altering the σ^{70} region 4/β flap interaction were overcome when the reactions were performed at saturating concentrations of λQ (500nM); under these conditions, ~50% readthrough was observed regardless of whether the reactions were performed with wild-type or a mutant holoenzyme (Figure 2B).

To extend these findings, we performed a set of parallel experiments using holoenzyme reconstituted with the stationary phase sigma factor, σ^{38}. (Holoenzyme reconstituted with σ^{38} can be modified by λQ in vitro, although not as efficiently as holoenzyme reconstituted with σ^{70}; Figure 2C and data not shown.) Although σ^{38} shares a high degree of sequence homology with σ^{70}, previous work suggests that the σ^{38} region 4/β flap interaction is stronger than the σ^{70} region 4/β flap interaction (Kuznedelov et al., 2002). Therefore, we tested the effect of weakening the σ region 4/β flap interaction in the context of the σ^{38}-containing holoenzyme on λQ antitermination function. To do this, we took advantage of a single amino acid substitution in σ^{38}, F278L, isolated in a screen for substitutions that specifically weakened the σ^{38} region 4/β flap interaction (Figure S1). We performed in vitro transcription assays using holoenzyme reconstituted with either wild-type σ^{38} or σ^{38} F278L. At low concentrations of λQ (10 nM), weakening the σ^{38} region 4/β flap interaction increased λQ-dependent antitermination (~3-fold; Figure 2C). Furthermore, the effect of weakening the σ^{38} region 4/β flap interaction was reduced (but not eliminated) when the reactions were performed at saturating concentrations of λQ (data not shown).

Taken together, the data presented in Figure 2 (panels A and C) suggest that altering the strength of the σ region 4/β flap interaction can affect λQ-dependent antitermination. In particular, strengthening the σ region 4/β flap interaction decreases the amount of λQ-dependent antitermination, and weakening the σ region 4/β flap interaction increases the amount of λQ-dependent antitermination. Furthermore, the effects of altering the σ region 4/β flap interaction are most pronounced when the concentration of λQ is limiting.

Effects of altering the σ^{70} region 4/β flap interaction on λQ-dependent antitermination in vivo

We next tested the effect of altering the σ^{70} region 4/β flap interaction on λQ-dependent antitermination in vivo. To do this, we introduced the mutations specifying substitutions T544I and D581G into the chromosomal copy of the *rpoD* gene (encoding σ^{70}). We found that *E. coli* strains bearing these mutations were viable and exhibited no apparent growth defect. (We note that similar attempts to introduce the L607P mutation into the chromosomal copy of *rpoD* were unsuccessful.) To test the effect of the *rpoD*-T544I/D581G mutations on λQ-dependent antitermination in vivo, we used a $\lambda P_{R'}$-*lacZ* fusion that consists of $\lambda P_{R'}$ sequence

extending from position –109 through position +238; this sequence includes the natural terminator $t_{R'}$. The level of *lacZ* expression from this fusion construct can therefore report on the ability of plasmid-encoded λQ to function as an antiterminator for transcripts initiating from $\lambda P_{R'}$. We introduced the $\lambda P_{R'}$-*lacZ* reporter construct in single copy into strains containing either wild-type *rpoD* or *rpoD* with the T544I/D581G mutations. We then assayed the ability of plasmid-encoded λQ to induce the expression of the *lacZ* reporter gene in each strain (Figure 2D). Consistent with our in vitro observations (Figure 2B), at low concentrations of λQ, the T544I/D581G mutations in *rpoD* caused an ~2-fold reduction in λQ function (Figure 2D). Furthermore, the effect of the T544I/D581G mutations was overcome at high concentrations of λQ (data not shown). Western analysis confirmed that the levels of λQ were identical in strains carrying the wild-type and mutant *rpoD* genes (data not shown). We conclude that strengthening the σ^{70} region 4/β flap interaction disrupts λQ-dependent antitermination in vivo.

Effects of altering the σ^{70} region 4/β flap interaction on the stability of the λQ-engaged paused elongation complex

The results presented in Figure 2 demonstrate that the effects of altering the σ region 4/β flap interaction are most pronounced when the concentration of λQ is limiting. Thus, we infer that altering the σ region 4/β flap interaction affects a concentration-dependent step in the λQ-antitermination process. The only plausible concentration-dependent step in the λQ-antitermination process is the initial binding of λQ to the paused early elongation complex. Therefore, we directly tested the effects of altering the σ^{70} region 4/β flap interaction on the stability of the complex that forms when λQ initially engages RNAP, using an exonuclease challenge assay (Yarnell and Roberts, 1992). In this assay, paused elongation complexes are formed by artificially stalling RNAP that has initiated transcription at $\lambda P_{R'}$. These artificially stalled complexes, which are halted after the synthesis of a 15-nt nascent RNA, are competent for modification by λQ (Yarnell and Roberts, 1992), indicating they closely resemble the natural substrate. After the stalled complexes are formed they are incubated with λQ; the binding of λQ to the QBE is stabilized by its interaction with RNAP. The λQ-bound complexes are challenged with exonuclease III, which digests the DNA in a 3′ to 5′ direction until its progress is blocked by the presence of a DNA-bound protein (Figure 3A). Thus, the stability of the complex that forms when λQ initially engages RNAP can be assessed by monitoring the half-life of the λQ-dependent barriers to exonuclease III digestion (the barriers between −31 and −27; see Figure 3, panels B and C).

Exonuclease challenge assays were performed using a wild-type $\lambda P_{R'}$ template and holoenzyme reconstituted with wild-type σ^{70}, σ^{70} T544I/D581G, or σ^{70} L607P. The assays indicate that strengthening the σ^{70} region 4/β flap interaction (T544I/D581G) destabilizes the complex that forms when λQ initially engages RNAP at $\lambda P_{R'}$ (Figure 3B; compare lanes 1-8 with lanes 19-26) and weakening the σ^{70} region 4/β flap interaction (L607P) stabilizes the complex that forms when λQ initially engages RNAP (Figure 3B; compare lanes 1-8 with lanes 10-17). Thus, λQ's association with the paused early elongation complex at $\lambda P_{R'}$ is inhibited by strengthening the σ^{70} region 4/β flap interaction and facilitated by weakening the σ^{70} region/β flap interaction (Figure 3C). Furthermore, we infer that the effects of altering the σ^{70} region 4/β flap interaction on λQ-mediated antitermination (Figure 2) reflect effects on the stability of the complex that forms when λQ initially engages RNAP.

In principle, the effects of substitutions T544I/D581G and L607P on λQ-dependent antitermination (Figure 2) could merely be an indirect consequence of their effects on the ratio of paused elongation complexes containing a 17-nt nascent transcript to paused complexes containing a 16-nt transcript (Nickels et al., 2005). The results of the exonuclease challenge assays presented in Figure 3 rule out this possibility (see Discussion).

Engagement of the paused elongation complex by λQ involves an interaction between λQ and σ^{70} region 4 that stabilizes the binding of region 4 to a DNA sequence element (TTGACT) that resembles a promoter −35 element (Figure 1) (Nickels et al., 2002). Because the binding of σ^{70} region 4 to this TTGACT motif requires RNAP holoenzyme to adopt a conformation in which σ^{70} region 4 has been displaced from the β flap, we considered the possibility that the displacement of σ^{70} region 4 from the β flap is required for λQ function solely because it permits σ^{70} region 4 to bind the TTGACT motif. The experiments shown in Figures S2 and S3 argue against this possibility and suggest that there is a mechanistic requirement for displacement of σ^{70} region 4 from the β flap during the λQ engagement process even under circumstances that do not permit λQ to stabilize the binding of σ^{70} region 4 to the TTGACT motif.

The function of the nascent RNA in the Q engagement process can be bypassed by weakening or eliminating the σ^{70} region 4/β flap interaction

The exonuclease challenge assays suggest that displacement of σ^{70} region 4 from the β flap is required for λQ to make productive contact with the paused early elongation complex. During early elongation, the nascent RNA destabilizes the σ^{70} region 4/β flap interaction (Vassylyev et al., 2002; Murakami and Darst, 2003; Borukhov and Nudler, 2003; Nickels et al., 2005). We therefore hypothesized that the requirement for nascent RNA in the Q engagement process could be bypassed by weakening or eliminating the σ^{70} region 4/β flap interaction. Specifically, we wished to test whether Q, which normally engages a paused early elongation complex, could engage an initiation complex if the σ^{70} region 4/β flap interaction was genetically disrupted or σ^{70} region 4 was removed altogether.

The ability of Q to functionally engage RNAP depends upon a precise spatial relationship between QBE-bound Q and RNAP bound at the pause site (Ring et al., 1996). Therefore, in order to maintain the correct spatial relationship between Q and the RNAP holoenzyme, we sought to convert the pause-inducing sequence to a bona fide promoter. Furthermore, in order to test whether Q could engage an initiation complex if σ^{70} region 4 was removed altogether, we needed to convert the pause-inducing sequence to a promoter that would support transcription by a mutant holoenzyme lacking σ^{70} region 4. Because σ^{70} region 4 is dispensable for transcription initiating at a consensus extended −10 promoter (Kumar et al., 1993) (consensus sequence: TGnTATAAT), our plan was to convert the pause-inducing sequence to a consensus extended −10 promoter. However, the pause-inducing sequence associated with $\lambda P_{R'}$ matches a consensus extended −10 promoter element at only 3 out of 8 positions (Figure 1A), and furthermore, introduction of a TG dinucleotide one base pair upstream of the $\lambda P_{R'}$ pause-inducing element disrupts λQ binding to the paused early elongation complex (data not shown). For these reasons we took advantage of the pause-inducing sequence associated with the $P_{R'}$ promoter of a bacteriophage that is closely related to λ, phage 82. The phage 82 $P_{R'}$ pause-inducing sequence, TGnTATTTT, differs at only two positions from the consensus extended −10 element (TGnTATAAT) (Figure 4A) (Ring et al., 1996). Thus, we converted the phage 82 $P_{R'}$ pause-inducing sequence to a consensus extended −10 element and used the Q protein of phage 82 (82Q) to ask whether we could bypass the requirement for nascent RNA in the Q engagement process.

We modified a template that contained phage 82 $P_{R'}$ along with a downstream terminator by converting the wild-type pause-inducing sequence to a consensus extended −10 element and introducing substitutions into the phage 82 $P_{R'}$ promoter elements (Figure 4A), thereby insuring that transcription would initiate exclusively under the control of the mutated pause-inducing sequence. Using this modified template, we then asked what effect the addition of 82Q had on the ability of wild-type RNAP or various RNAP mutants to read through the downstream terminator. We first performed assays using holoenzyme reconstituted with wild-type σ^{70}, σ^{70} T544I/D581G, or σ^{70} L607P. As a control, we also tested 82Q function using a wild-type

(unmodified) phage 82 $P_{R'}$ template and wild-type holoenzyme; in this control assay, 82Q increased terminator readthrough from ~2% to ~50% (Figure 4B, lanes 1 and 2). Surprisingly, we found that when the assays were performed with the modified phage 82 $P_{R'}$ template and wild-type holoenzyme, the addition of 82Q increased terminator readthrough from the ~2% observed in the absence of 82Q to ~7% (Figure 4B, lanes 5 and 6). This suggests that 82Q can, in fact, modify an initiation complex, albeit weakly compared to its ability to modify the early elongation complex at wild-type 82 $P_{R'}$. In addition, we found that strengthening the σ^{70} region 4/β flap interaction (reactions performed with holoenzyme reconstituted with σ^{70} T544I/D581G) eliminated the ability of 82Q to modify the transcription initiation complex (Figure 4B, lanes 3 and 4), whereas weakening the σ^{70} region 4/β flap interaction (reactions performed with holoenzyme reconstituted with σ^{70} L607P) enhanced the ability of 82Q to modify the transcription initiation complex by a factor of ~2 (terminator readthrough increased to ~15%; Figure 4B, lanes 7 and 8). To control for the specificity of these effects, we tested whether the ability of 82Q to modify the transcription initiation complex depended on the functional integrity of the Q-binding element. To do this, we introduced base pair substitutions into the Q-binding element predicted to weaken 82Q binding (see Experimental Procedures). Introduction of these base pair substitutions into the converted template inhibited the ability of 82Q to modify transcription initiation complexes containing wild-type σ^{70}, or σ^{70} L607P (Figure 4B, lanes 9-12).

Next, we performed transcription assays to test the effect of the complete removal of σ^{70} region 4 on the ability of 82Q to modify an initiation complex. To do this, we performed assays using the converted 82 $P_{R'}$ template and holoenzyme reconstituted with wild-type σ^{70}, σ^{70} L607P, or a truncated σ^{70} that lacked region 4, σ^{70} 1-529 (Figure 4C). We found that in order to observe efficient transcription initiation using holoenzyme lacking σ^{70} region 4, it was necessary to alter the conditions of the in vitro assay (see Experimental Procedures). When the assays were performed using these different reaction conditions, terminator readthrough was ~14% in the absence of 82Q (Figure 4C) (compared with ~2% for the experiments shown in Figure 4B). Furthermore, in contrast to what was observed under the previous experimental conditions, neither wild-type holoenzyme nor holoenzyme reconstituted with σ^{70} L607P supported 82Q-dependent antitermination (Figure 4C lanes 1-4). However, when the reactions were performed with holoenzyme lacking σ^{70} region 4 (Figure 4C, lanes 5 and 6), the addition of 82Q increased terminator readthrough from ~14% to ~54% (an effect that depended on the functional integrity of the Q-binding element; data not shown).

Because of the indirect nature of the antitermination assay, we wished to show directly that removal of σ^{70} region 4 allows 82Q to bind efficiently to transcription initiation complexes lacking any nascent RNA products. To do this, we again used the exonuclease challenge assay. We formed promoter complexes on the modified 82 $P_{R'}$ template using holoenzyme lacking σ^{70} region 4 (σ^{70} 1-529). We incubated these complexes in the presence or absence of 82Q, challenged the resultant complexes with exonuclease III, and looked for evidence of an 82Q-dependent barrier to exonuclease digestion (Figure 5). As a control, we also incubated the modified 82 $P_{R'}$ template with 82Q in the absence of the RNAP holoenzyme. As shown in Figure 5B, the appearance of a strong 82Q-dependent barrier to exonuclease digestion specifically in the presence of the mutant holoenzyme indicates that 82Q can bind efficiently to promoter complexes formed by holoenzyme lacking σ^{70} region 4 (compare lanes 1-6 with lanes 8-13).

We also formed promoter complexes on the modified 82 $P_{R'}$ template using wild-type holoenzyme and performed exonuclease challenge assays in the presence or absence of 82Q (Figure S4). Comparison of the assays performed with holoenzyme lacking σ^{70} region 4 (Figure 5B) and those performed with wild-type holoenzyme (Figure S4, panel B) indicates that

removal of σ^{70} region 4 stabilizes the binding of 82Q to the transcription initiation complex, consistent with the results of the antitermination assays (Figure 4C).

Taken together, the results presented in Figures 4, 5 and S4 demonstrate that: 1) 82Q can weakly engage a transcription initiation complex, 2) strengthening the σ^{70} region 4/β flap interaction prevents this engagement, and 3) weakening the σ^{70} region 4/β flap interaction or removing σ^{70} region 4 facilitates this engagement.

Discussion

We examined the effects of altering the σ^{70} region 4/β flap interaction on Q-dependent antitermination. There are two main conclusions that we draw from our results. First, displacement of σ^{70} region 4 from the β flap is required for efficient modification of RNAP by λQ. Second, the requirement for nascent RNA in the Q engagement process can be bypassed by removing σ^{70} region 4 from the RNAP holoenzyme. Our results illustrate how the nascent RNA-mediated destabilization of the σ^{70} region 4/β flap interaction during the transition from initiation to elongation can facilitate the engagement of a regulatory factor with RNAP.

Displacement of σ^{70} region 4 from the β flap is required for efficient modification of RNAP by λQ

Using amino acid substitutions that strengthen or weaken the σ^{70} region 4/β flap interaction we demonstrated that strengthening the σ^{70} region 4/β flap interaction (T544I/D581G) reduced λQ-dependent antitermination and weakening the σ^{70} region 4/β flap interaction (L607P) increased λQ-dependent antitermination (Figure 2). The use of an exonuclease challenge assay allowed us to show that altering the σ^{70} region 4/β flap interaction affects the stability of the complex that forms when λQ initially engages the paused early elongation complex at $\lambda P_{R'}$ (Figure 3).

At $\lambda P_{R'}$ the early elongation pause manifests itself in transcription complexes containing a 16- or 17-nt nascent transcript. In previous work (Nickels et al., 2005), using the same amino acid substitutions in σ^{70} that we used here (T544I/D581G and L607P), we showed that steric clash between the nascent RNA and σ^{70} region 4 bound to the β flap affects the distribution of paused elongation complexes at $\lambda P_{R'}$ (i.e. the ratio of paused complexes containing a 17-nt transcript to paused complexes containing a 16-nt transcript). In particular, we found that strengthening the σ^{70} region 4/β flap interaction (using holoenzyme reconstituted with σ^{70} T544I/D581G) decreased the fraction of paused elongation complexes containing a 17-nt transcript, whereas weakening the σ^{70} region 4/β flap interaction (using holoenzyme reconstituted with σ^{70} L607P) increased the fraction of paused elongation complexes containing a 17-nt transcript (Nickels et al., 2005). In principle, therefore, the effects of amino acid substitutions T544I/D581G and L607P on λQ-dependent antitermination could merely be an indirect consequence of their effects on the distribution of $\lambda P_{R'}$ paused elongation complexes. However, the results of the exonuclease challenge assays presented in Figure 3 rule out this possibility because these assays are performed with a homogeneous population of transcription complexes that are artificially halted after the synthesis of a 15-nt nascent transcript. We found that amino acid substitutions T544I/D581G and L607P affected the stability of the complex that forms when λQ engages these artificially halted transcription complexes. Therefore, we conclude that the effects of substitutions T544I/D581G and L607P on λQ-dependent antitermination are not merely an indirect consequence of their effects on the distribution of $\lambda P_{R'}$ paused complexes containing either a 17-nt transcript or a 16-nt transcript.

Bypassing the requirement for nascent RNA in the Q engagement process

Based on the idea that the nascent RNA-mediated destabilization of σ^{70} region 4/β flap interaction facilitates λQ's engagement of the paused early elongation complex, we hypothesized that the function of the nascent RNA in the Q engagement process could be bypassed by weakening or eliminating the σ^{70} region 4/β flap interaction. We took advantage of the properties of the pause-inducing sequence associated with the phage 82 $P_{R'}$ to confirm this hypothesis. Specifically, we converted the phage 82 $P_{R'}$ pause-inducing sequence to a consensus extended −10 promoter element and showed that 82Q could engage transcription initiation complexes bound at this converted pause element increasingly efficiently as the σ^{70} region 4/β flap interaction was weakened or eliminated (by the removal of σ^{70} region 4) (Figures 4, 5 and S4).

Our results suggest that one function of the early elongation pause in the process of Q-mediated antitermination is to present Q with a promoter-specific substrate in which the nascent RNA has destabilized the σ^{70} region 4/β flap interaction (Figure 6). We infer that Q's ability to functionally engage the RNAP holoenzyme requires displacement of σ^{70} region 4 from the β flap. Specifically, we propose that the nascent RNA-mediated destabilization of the σ^{70} region 4/β flap interaction allows Q to capture and stabilize a conformation of the RNAP holoenzyme in which σ^{70} region 4 is not bound to the β flap. Currently, it is unknown what surface(s) of RNAP core Q interacts with during the engagement process, and furthermore, what Q/core contacts are required for antitermination. Our findings are consistent with the proposal that Q function requires contact with a surface of RNAP core that is occluded by σ^{70} region 4 when it is bound to the β flap.

A transcription regulator that exploits the staged displacement of σ from core

Structural and biochemical evidence suggests that the transition from initiation to elongation involves a staged displacement of segments of σ^{70} from RNAP core (Marr et al., 2001; Murakami et al., 2002a and 2002b; Vassylyev et al., 2002; Mekler et al., 2002; Nickels et al., 2002; Nickels et al., 2005). In this work we provide evidence that the RNA-mediated destabilization of the σ region 4/β flap interaction facilitates interaction between a regulatory factor and RNAP during early elongation. High-resolution structures of other multi- and single subunit RNA polymerases reveal protein elements that, like σ region 3.2 and σ region 4, are positioned within the path of the elongating RNA transcript (Murakami and Darst, 2003 and references therein). Thus, the obligatory displacement of protein elements by the nascent RNA during the transition from initiation to elongation likely contributes to gene regulation in other organisms, as well.

Experimental Procedures

Proteins

His-tagged versions of wild-type σ^{70}, σ^{70} T544I/D581G, σ^{70} L607P, σ^{70} 1-529, σ^{38}, and σ^{38} F278L were purified as described after overproduction from plasmid pLHN12-His (Panaghie et al., 2000). *E. coli* RNAP core was obtained from Epicentre, and holoenzyme was made by incubation with a five-fold excess of the appropriate σ. λQ protein and NusA were purified as described (Yarnell and Roberts, 1992). 82Q was purified as described (Goliger and Roberts, 1989). EcoRIGln111 was provided by P. Modrich and I. Artsimovitch.

Strains and Plasmids

A complete list of strains and plasmids is provided in Table 1 of the Supplemental Data. The Supplemental Data also include a detailed description of how the T544I/D581G mutations were introduced into the chromosomal copy of *rpoD*.

In Vitro Transcription

The $\lambda P_{R'}$ template was obtained by the PCR from plasmid pFW11 ^{Tet}-$\lambda P_{R'}$ (Nickels et al., 2002) and contains sequence extending from −109 to +238 of $\lambda P_{R'}$ that includes the natural terminator $t_{R'}$. The phage 82 $P_{R'}$ promoter was obtained by the PCR from plasmid p82a (Goliger and Roberts, 1989), which contains sequence extending from −100 to +116 of 82 $P_{R'}$ that includes the natural terminator t_{82}. To modify the phage 82 $P_{R'}$ promoter for the experiments of Figures 4 and 5, base pair mutations were introduced into the template by site directed mutagenesis. The modified 82 $P_{R'}$ template contained the following substitutions: T−35C, T −34A, G−33C, T−7C, T+12A and T+13A.

In the context of the $\lambda P_{R'}$ promoter, the base pair substitutions T−22G and G−25C disrupt the binding of λQ to the λQ-binding element (Guo and Roberts, 2004). Therefore, to disrupt the ability of 82Q to bind the 82Q-binding element, we introduced the corresponding base pair substitutions (T−22G and G−25C) into the modified 82 $P_{R'}$ template. We note that while these substitutions disrupted the ability of 82Q to engage the transcription initiation complex (Figure 4B and data not shown), they did not detectably disrupt the ability of 82Q to engage the paused early elongation complex when introduced into the wild-type phage 82 $P_{R'}$ template (data not shown).

λQ antitermination assays: Open complexes were formed by incubating 20 nM RNAP with 2 nM template and 150 nM NusA for 5 min at 37° in transcription buffer (20 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 50 mM KCl, 100 μg/ml BSA) plus 200 μM GTP, UTP and CTP and 50 μM γ-^{32}P-ATP at 1.5 mCi/ml. One tenth volume λQ or λQ dilution buffer (10 mM Tris pH 7.5, 500 μg/ml BSA, 100 mM DTT, 10% glycerol, 50 mM potassium glutamate) was added, and after 30 seconds transcription was initiated by adding 4 mM MgCl and 10 μg/ml rifampicin. Reactions were allowed to proceed for 8 minutes then stopped by addition of 5 reaction volumes of 1.2X stop solution (0.6M Tris-HCl [pH 8.0], 12 mM EDTA, 80 μg/ml tRNA). Samples were then extracted with phenol/chloroform (1:1), precipitated with ethanol, resuspended in 4μl of loading buffer (95% (v/v) formamide, 20 mM EDTA, 0.05% (w/v) bromophenol blue, and 0.05% (w/v) xylene cyanol), and electrophoresed on 6% polyacrylamide sequencing gels. Bands were visualized by phosphorimager and the data analyzed by Imagequant.

82Q antitermination assays: For the reactions shown in Figure 4B, 100 nM 82Q (or Q dilution buffer) was incubated with 20 nM template DNA and 150 nM NusA for 5 min at 37° in transcription buffer plus NTPs (200 μM GTP, UTP and CTP and 50 μM γ-^{32}P-ATP at 1.5 mCi/ml for experiments performed with the wild-type phage 82 $P_{R'}$ template and 200 μM ATP, UTP and CTP and 50 μM γ-^{32}P-GTP at 1.5 mCi/ml for experiments performed with the modified 82 $P_{R'}$ template). The indicated RNAP (at 20nM) was added and the reactions were incubated for 10 minutes. Next, transcription was initiated by adding 4 mM MgCl and 10 μg/ml rifampicin. Reactions were allowed to proceed for 10 minutes then stopped by addition of 5 reaction volumes of 1.2X stop solution and processed as described above.

The reactions shown in Figure 4C were performed as follows: 100 nM 82Q (or Q dilution buffer) was incubated with 5 nM template DNA and 150 nM NusA for 5 min at 37° in a modified transcription buffer (10 mM MgCl, 90 mM KCl, 40 mM Tris pH 8.0, 100 μg/ml BSA and 5% PEG 3350). The indicated RNAP (at 20 nM) was added and the reactions were incubated for 10 minutes. Next, transcription was initiated by adding NTPs (1 mM GTP, UTP and ATP and 50 μM α-^{32}P-CTP at 1.0 mCi/ml) plus 100 μg/ml heparin. Reactions were allowed to proceed for 10 minutes then stopped by addition of 1 reaction volume of loading buffer. Samples were electrophoresed on 6% polyacrylamide sequencing gels. Bands were visualized by phosphorimager and the data analyzed by Imagequant.

Exonuclease III Challenge Assays

Assays performed with the $\lambda P_{R'}$ template were done essentially as described (Yarnell and Roberts, 1992). In brief, open complexes were formed by incubating 20 nM RNAP and 2 nM DNA for 15 minutes at 37° in transcription buffer (but with 10 mM KCl) containing the initiating oligonucleotide ApApC at 50μM, and 25μM ATP, GTP, and UTP; $MgCl_2$ was added to 5 mM and incubation continued 4 minutes to make +15 complexes. After addition of λQ to 500 nM (or buffer), EcoRIGln111 to 50 nM, and calf thymus DNA to 50 μg/ml, exoIII was added to 1.6 U/μl. Samples were removed, quenched as above, prepared as above, analyzed on a 7% sequencing gel, and bands were visualized by phosphorimager. The DNA template was ^{32}P-labelled with T4 polynucleotide kinase on the bottom strand; the template contained both promoter sequences and a downstream EcoRI site, which provided a binding site for the catalytically inactive EcoRIGln111 to block exoIII digestion from downstream.

Assays with the modified 82 $P_{R'}$ template were performed identically, except that open complexes were formed by incubation for 10 minutes, no elongation step was used, and exoIII was added to 0.8U/μl. In addition, PEG3350 was added to 5% to stabilize the σ truncation.

β-galactosidase Assays

For the assays presented in Figure 2D, reporter strains cells were transformed with either plasmid pBRλQΔ-35, which directs the expression of low levels of λQ (Nickels et al., 2002) or plasmid pBRΔQ, which encodes no functional λQ (Nickels et al., 2002). Individual transformants were selected and grown in LB supplemented with carbenicillin (100 μg/ml), tetracycline (10 μg/ml), kanamycin (50 μg/ml) and 100μM Isopropyl-β-D-thiogalactoside (IPTG). β-galactosidase activity was assayed as described (Dove and Hochschild, 2004).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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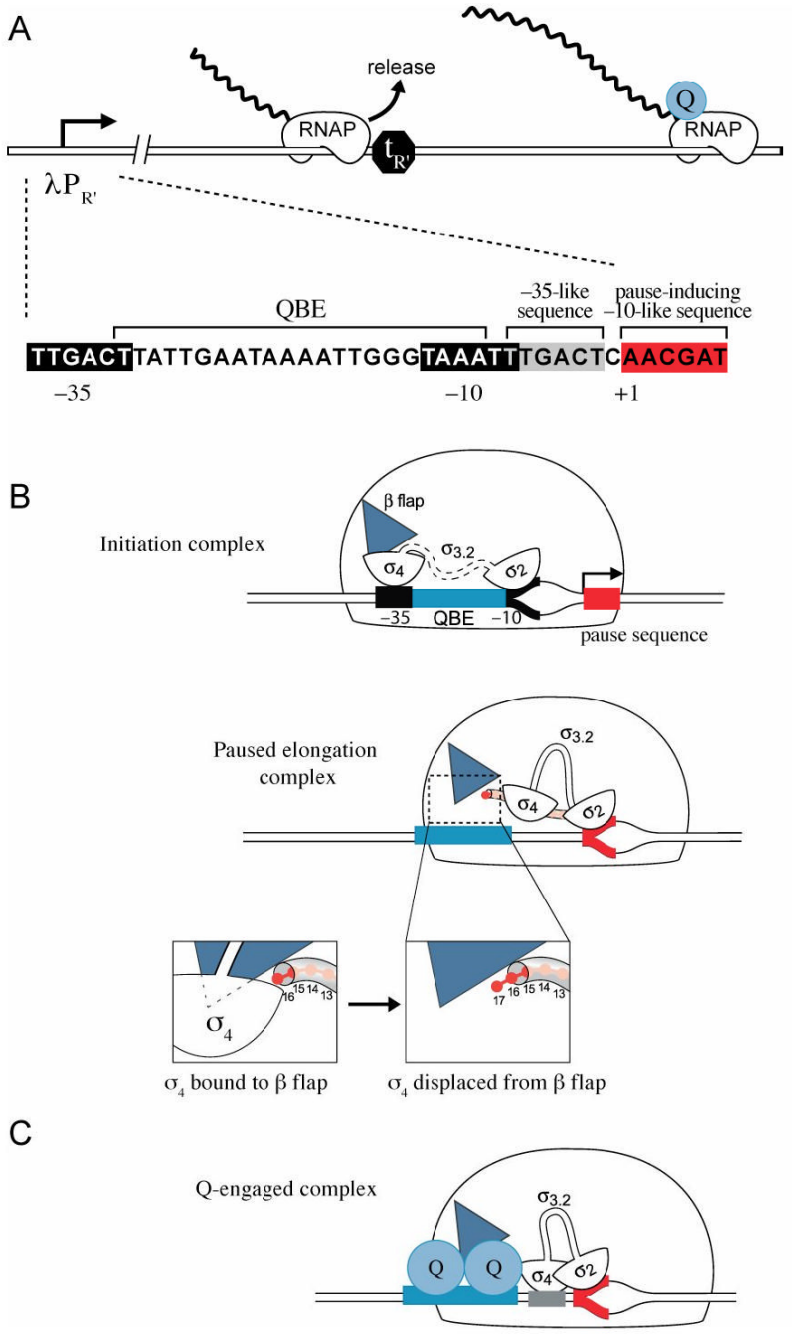

Figure 1.
Sequence of events at $\lambda P_{R'}$.

A. Presence of λQ (shown in blue) allows RNAP that has initiated transcription from $\lambda P_{R'}$ to read through terminator $t_{R'}$. Blow-up shows the functionally important elements at $\lambda P_{R'}$ including: the promoter −10 and −35 elements, the λQ-binding element (QBE), the pause-inducing −10-like element, and the −35-like element (TTGACT motif) positioned between the QBE and the pause-inducing element. Note that the TTGACT motif is separated by one base pair from the pause-inducing −10-like element.

B. Cartoons depict sequence of events during initiation and early elongation at $\lambda P_{R'}$. Shown first is the initiation complex; σ^{70} region 3.2 ($\sigma_{3.2}$) is shown dashed to indicate that it is located within the RNA exit channel, and σ^{70} region 4 (σ_4) is shown bound to the β flap (blue triangle). The σ^{70} region 4/β flap interaction positions σ^{70} region 4 for interaction with the promoter −35 element when σ^{70} region 2 is bound to the promoter −10 element (where the −10 and −35 elements are separated by ~17 base pairs) (Kuznedelov et al., 2002). Shown just below the initiation complex is the paused early elongation complex at $\lambda P_{R'}$ with σ^{70} region 2 (σ_2) bound to the pause-inducing −10-like element (shown in red). The nascent RNA (depicted as red beads) has displaced σ^{70} region 3.2 from the RNA exit channel and, as illustrated in the blow-ups, displaced σ^{70} region 4 from the β flap upon addition of the 17th nt.

C. λQ (shown as a dimer) binds to the QBE and engages the paused elongation complex. λQ contacts σ^{70} region 4 and stabilizes its binding to the TTGACT motif (grey rectangle), causing RNAP holoenzyme to adopt a conformation in which σ^{70} region 4 and σ^{70} region 2 are simultaneously bound to DNA elements that are separated by 1 base pair (Nickels et al., 2002). Formation of this RNAP holoenzyme-DNA complex requires that σ^{70} region 4 be displaced from the β flap. λQ is depicted as contacting a surface of RNAP core that is occluded when σ^{70} region 4 is bound to the β flap.

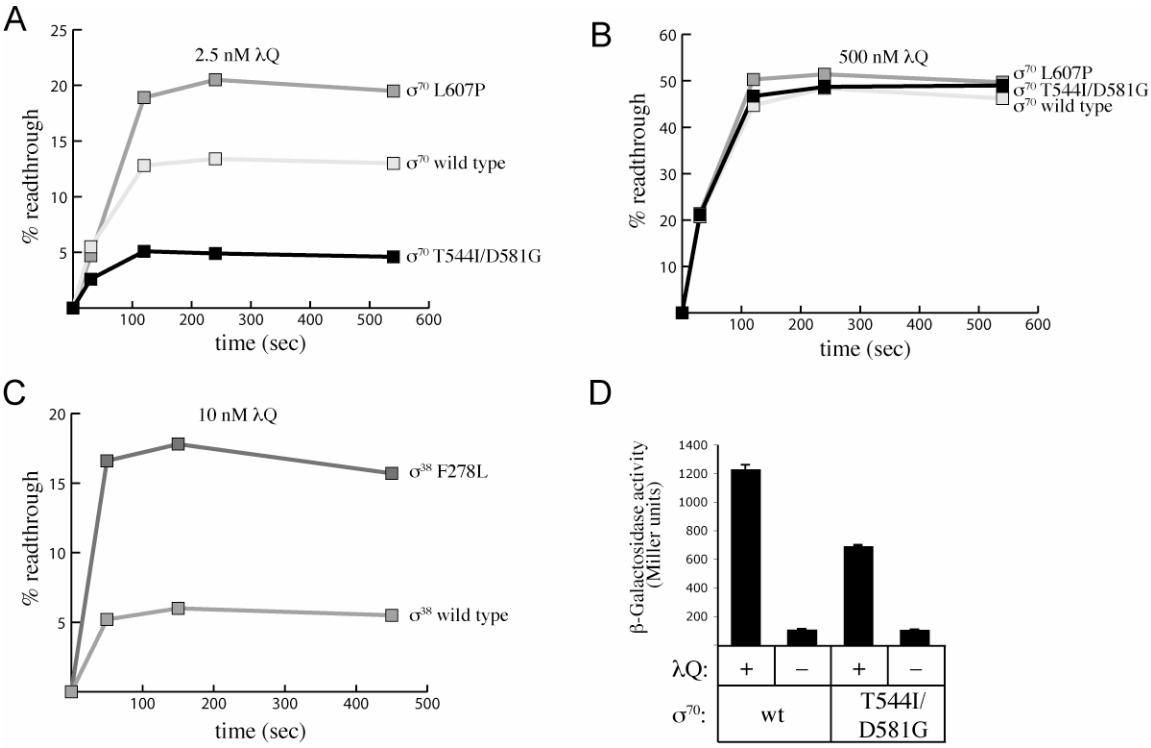

Figure 2.

Effects of strengthening or weakening the σ region 4/β flap interaction on λQ-mediated antitermination

A) and B) Effects of strengthening (T544I/D581G) or weakening (L607P) the σ^{70} region 4/β flap interaction in vitro. Shown are the results of single-round in vitro transcription assays performed using a linear template that contains sequence extending from −109 to +238 of $\lambda P_{R'}$ that includes the natural terminator $t_{R'}$ (Figure 1A). Assays were done either at a low concentration of λQ (2.5 nM, panel A) or at a high concentration of λQ (500 nM, panel B). Graphs show the percentage of transcripts derived from terminator readthrough (readthrough/[readthrough + terminated]) at the indicated times after transcription was initiated. Reactions were performed using holoenzyme reconstituted with wild-type σ^{70}, σ^{70} L607P or σ^{70} T544I/D581G, as indicated. Assays were performed three times on separate occasions with similar results. Plotted on the graphs are the data obtained from a single representative experiment.

C) Effects of weakening (F278L) the σ^{38} region 4/β flap interaction in vitro. Shown are the results of single-round in vitro transcription assays performed at a low concentration of λQ (10 nM). Graphs show the percentage of transcripts derived from terminator readthrough (readthrough/[readthrough + terminated]) at the indicated times after transcription was initiated. Reactions were performed using holoenzyme reconstituted with either wild-type σ^{38} or σ^{38} F278L, as indicated. Assays were performed three times on separate occasions with similar results. Shown on the graph are the data obtained from a single representative experiment.

D) Effects of strengthening (T544I/D581G) the σ^{70} region 4/β flap interaction in vivo. Reporter strain cells containing either wild-type σ^{70} or σ^{70} T544I/D581G and harboring a $\lambda P_{R'}$-*lacZ* reporter were transformed with a plasmid that did or did not encode λQ. The cells were grown in the presence of 100 μM IPTG and assayed for β-galactosidase activity.

The bar graph shows the averages of four independent measurements (and standard deviations).

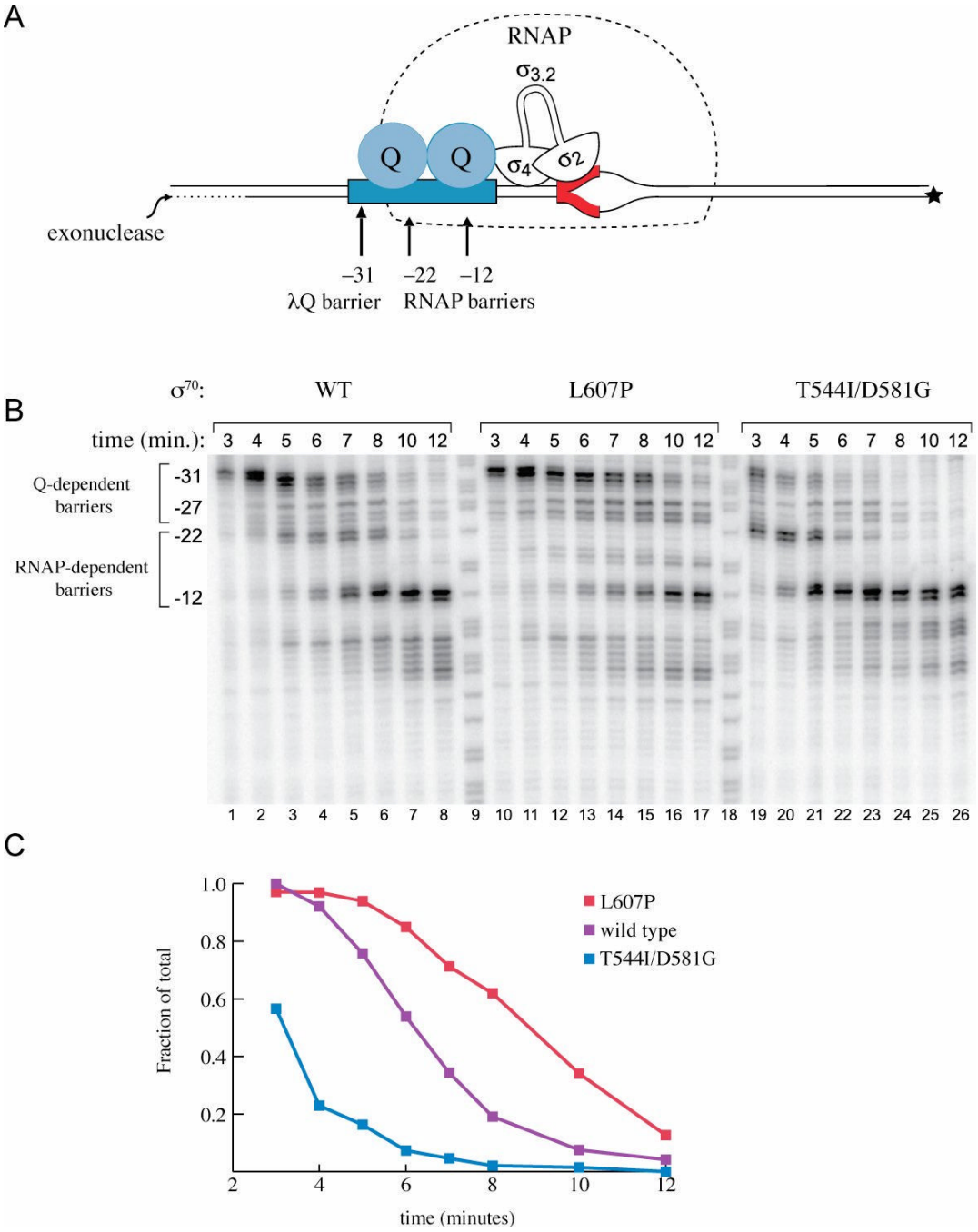

Figure 3.

Effects of strengthening or weakening the σ^{70} region 4/β flap interaction on λQ's engagement of the paused early elongation complex in vitro.

A. Schematic of template used for exonuclease III challenge assays. Depicted is the λQ-engaged paused elongation complex. The $\lambda P_{R'}$ template used in the assays is end-labeled at the 5′ end of the template (bottom) strand as indicated. Also indicated are the positions (relative to the transcription start site) at which the progress of exonuclease III digestion is blocked by λQ (−31) and RNAP (−22 and −12). The red DNA segment is the pause-inducing sequence; σ^{70} region 2 is shown bound to the

non-template strand (Ring et al., 1996). The RNAP outline is dashed to indicate that it does not depict a barrier to exonuclease digestion.

B. Exonuclease challenge assays. Stalled elongation complexes were formed with RNAP reconstituted with wild-type σ^{70}, σ^{70} L607P, or σ^{70} T544I/D581G. These complexes were then incubated with 500 nM λQ and challenged with exonuclease III for the indicated times. Lanes 9 and 18 contain A+G sequencing ladders.

C. Effects of substitutions that strengthen (T544I/D581G) or weaken (L607P) the σ^{70} region 4/β flap interaction. The exonuclease barriers shown in panel B were quantified with Imagequant, and label in the λQ-dependent band at −31 was plotted as a fraction of the sum of label in the bands at −31, −22 and −12; the barriers at −22 and −12 are specific for the σ^{70}-dependent paused elongation complex, and are produced after exonuclease digests past the λQ barrier. We note that no differences in pause half-life were observed with these mutant holoenzymes (Nickels et al., 2005), suggesting that the effects of these σ^{70} substitutions on the half-life of the λQ barrier are not indirect manifestations of changes in the stability of the paused elongation complexes.

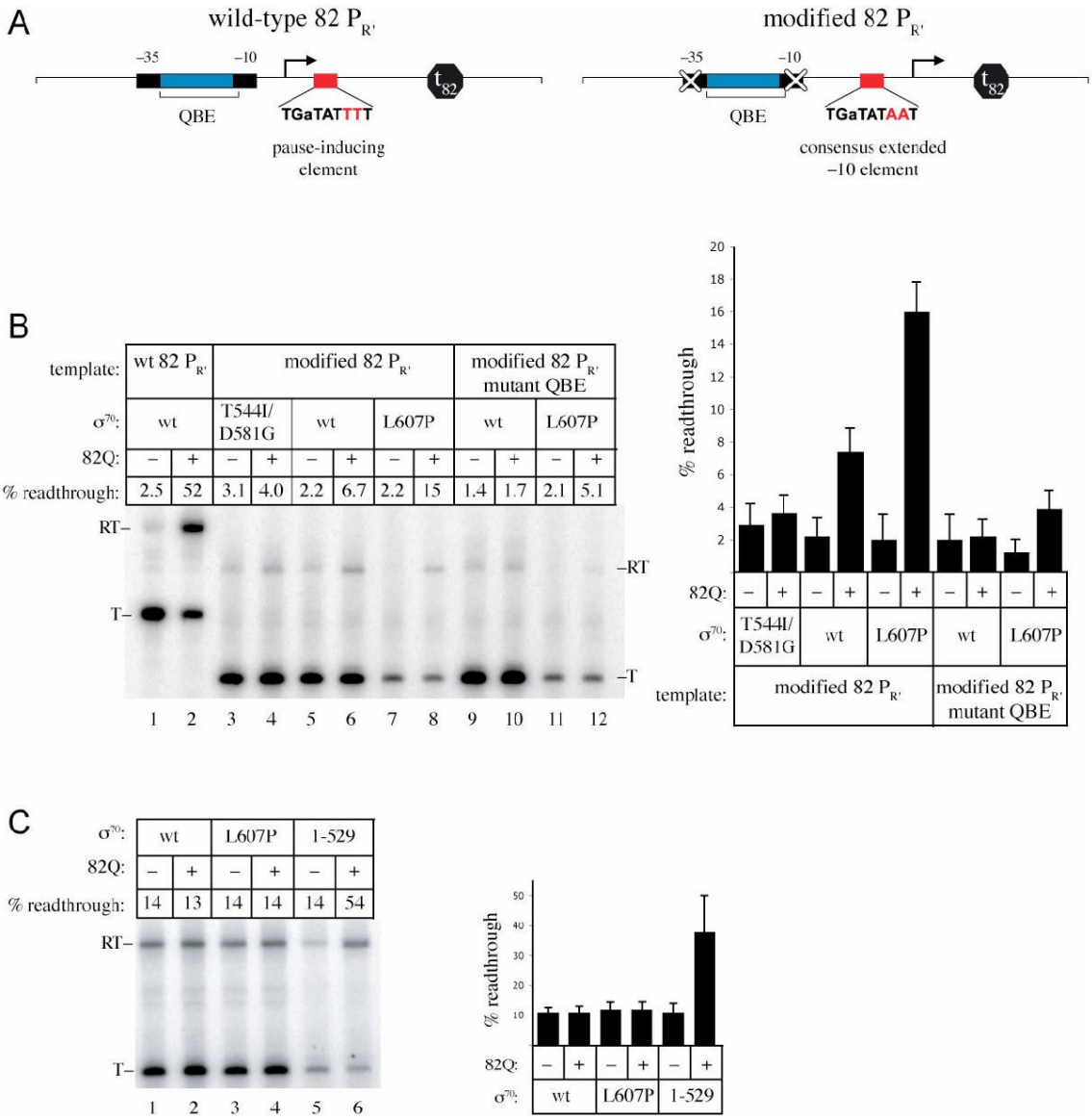

Figure 4.

82Q can engage a transcription initiation complex.

A. Left panel shows a diagram of the wild-type phage 82 $P_{R'}$ promoter. Indicated are the functionally important elements at phage 82 $P_{R'}$ including the promoter −10 and −35 elements, the 82Q-binding element (QBE), the pause-inducing extended −10-like element (located between +6 and +14), and terminator t_{82}. Right panel shows a diagram of the modified template on which the 82 $P_{R'}$ promoter was inactivated with base pair substitutions in the promoter −10 and −35 elements and the pause-inducing element was converted to a consensus extended −10 promoter element. The transcription start site on each template is indicated (bent arrow).

B. Effects of strengthening (T544I/D581G) or weakening (L607P) the σ^{70} region 4/β flap interaction on 82Q's engagement of a transcription initiation complex. Shown are the results of representative single-round in vitro transcription assays performed in the presence or absence of 100 nM 82Q using the indicated template and

holoenzyme reconstituted with wild-type σ^{70}, σ^{70} T544I/D581G, or σ^{70} L607P. The percentage of transcripts derived from terminator readthrough (readthrough/[readthrough + terminated]) is indicated above each lane. RNA was end labeled using γ-^{32}P-ATP (lanes 1 and 2) or γ-^{32}P-GTP (lanes 3-12). Also indicated are the 81- (lanes 1 and 2) or 62- (lanes 3-12) nt terminated transcript (T) and the 116- (lanes 1 and 2) or 97- (lanes 3-12) nt readthrough transcript (RT). Plotted in the bar graph on the right are the averages of 3-6 independent measurements (and standard deviations).

C. Effect of removing σ^{70} region 4 on 82Q's engagement of a transcription initiation complex. Shown are the results of representative single-round in vitro transcription assays performed in the presence or absence of 100 nM 82Q using the modified phage 82 $P_{R'}$ template and holoenzyme reconstituted with wild-type σ^{70}, σ^{70} L607P, or σ^{70} lacking region 4 (σ^{70} 1-529). The percentage of transcripts derived from terminator readthrough (readthrough/[readthrough + terminated]) is indicated above each lane. RNA was internally labeled using α-^{32}P-CTP. Also indicated are the 62-nt terminated transcript (T) and the 97-nt readthrough transcript (RT). Plotted in the bar graph on the right are the averages of 4-6 independent measurements (and standard deviations).

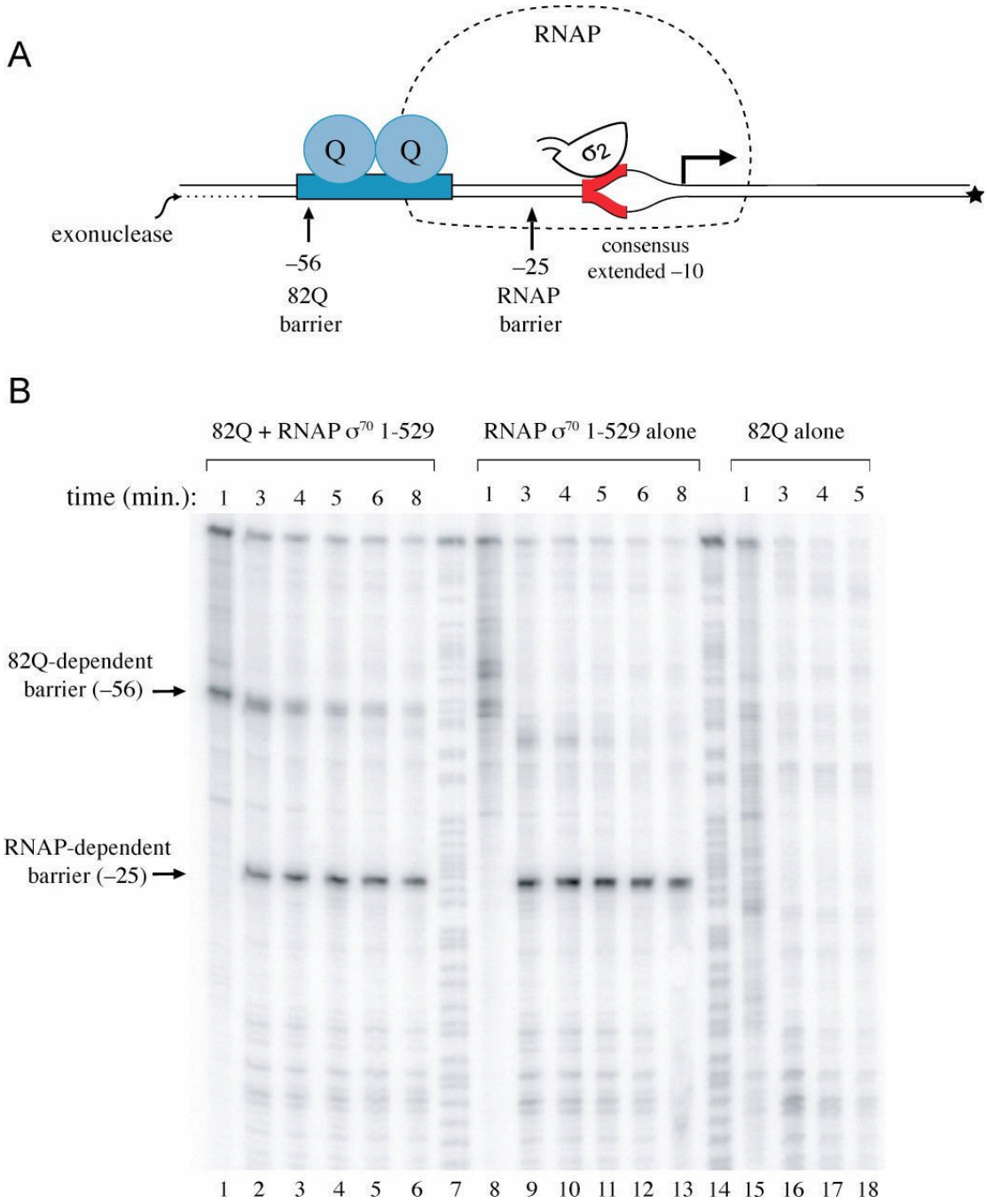

Figure 5.

Removal of σ^{70} region 4 allows 82Q to bind efficiently to transcription complexes that lack a nascent RNA.

A. Schematic of template used for exonuclease III challenge assay. Depicted is the 82Q-engaged transcription initiation complex lacking σ^{70} region 4. The modified phage 82 $P_{R'}$ template used in the assays is end labeled at the 5′ end of the template (bottom) strand, as indicated. Also indicated are the positions (relative to the transcription start site) at which the progress of exonuclease III digestion is blocked by 82Q (−56) and by RNAP (−25). The red DNA segment is the consensus extended −10 element;

σ^{70} region 2 is shown bound to the non-template strand. The RNAP outline is dashed to indicate that it does not depict a barrier to exonuclease digestion. We note that a barrier corresponding to the RNAP-dependent barrier at −25 also is seen in paused complexes formed on the wild-type 82 $P_{R'}$ template (where it occurs at position −4 relative to the 82 $P_{R'}$ transcription start site) (data not shown), and likely is caused by σ^{70} regions 2 and 3.

B. Effect of removing σ^{70} region 4. Initiation complexes were formed with RNAP reconstituted with σ^{70} lacking region 4, σ^{70} 1-529 (lanes 1-6 and 8-13). These complexes were then incubated with 20 nM 82Q (lanes 1-6) or no 82Q (lanes 8-13) and challenged with exonuclease III for the indicated times. Control assays were performed using template DNA incubated with 20 nM 82Q only (lanes 15-18). Lanes 7 and 14 contain A+G sequencing ladders.

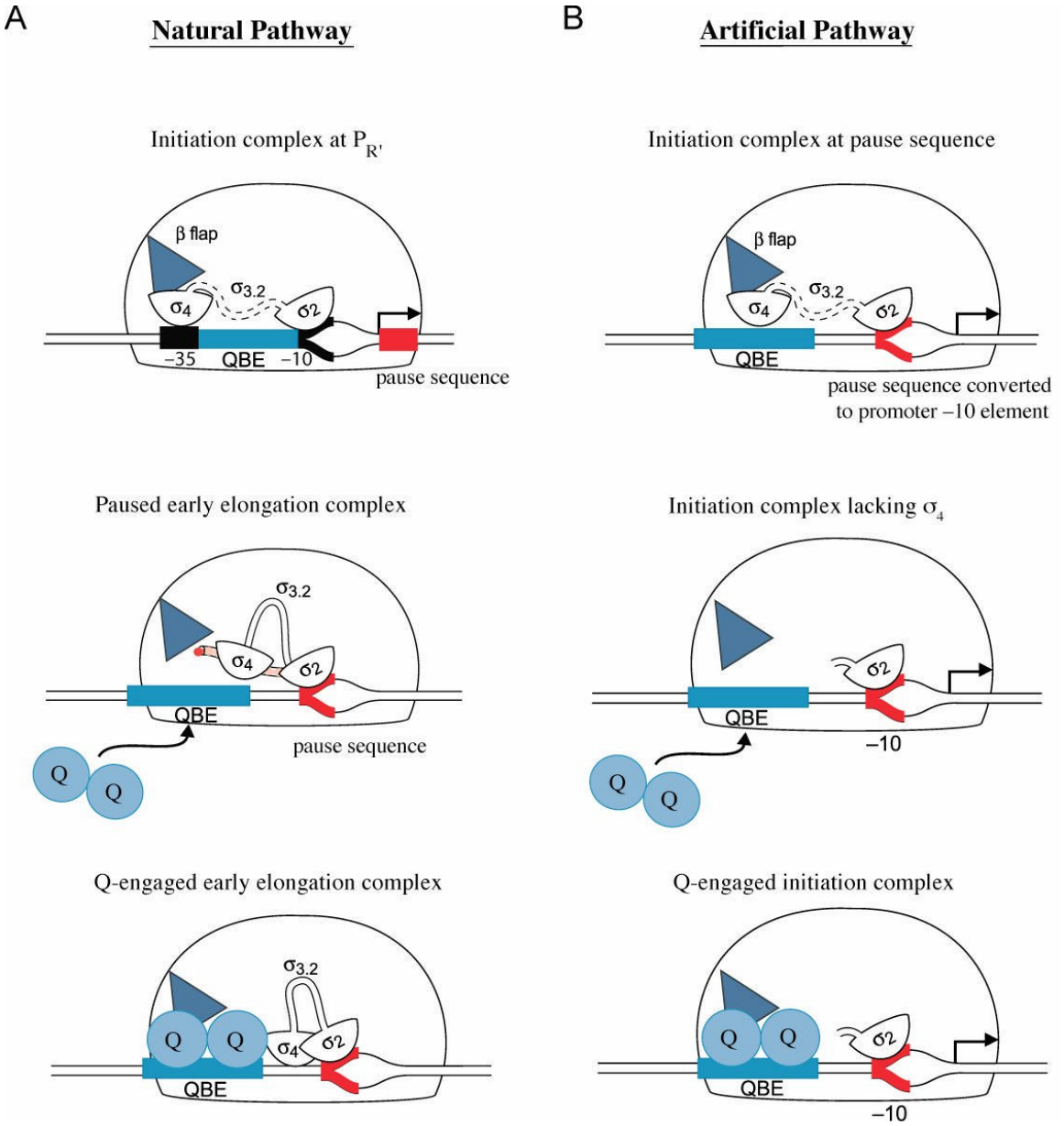

Figure 6.
Two pathways leading to Q-engagement with RNAP holoenzyme

A. Q engages a paused early elongation complex. Top panel depicts the $P_{R'}$ initiation complex in which σ region 3.2 ($\sigma_{3.2}$) is located within the RNA exit channel and σ region 4 (σ_4) is bound to the β flap (blue triangle). The pause-inducing sequence is shown in red. Middle panel depicts the paused early elongation complex in which the nascent RNA (shown as red beads emerging from the exit channel) has displaced σ region 3.2 ($\sigma_{3.2}$) from the RNA exit channel and displaced σ region 4 (σ_4) from the β flap. Bottom panel depicts Q-engaged early elongation complex.

B. Q engages an initiation complex. Top panel depicts a wild-type initiation complex bound at a promoter created by converting the pause-inducing sequence to a consensus extended −10 element (shown in red). Middle panel depicts an initiation complex

lacking σ region 4 bound at the converted pause-inducing sequence. Bottom panel depicts the Q-engaged initiation complex lacking σ region 4.