5378–5389 *Nucleic Acids Research, 2016, Vol. 44, No. 11* *Published online 28 March 2016*
doi: 10.1093/nar/gkw198

Nascent RNA length dictates opposing effects of NusA on antitermination

Christopher D. Wells[1], Padraig Deighan[1,2], MacKenzie Brigham[1] and Ann Hochschild[1,*]

[1]Department of Microbiology and Immunobiology, Boston, MA 02115, USA and [2]Department of Biology, Emmanuel College, Boston, MA 02115, USA

Received January 26, 2016; Revised March 03, 2016; Accepted March 15, 2016

ABSTRACT

The NusA protein is a universally conserved bacterial transcription elongation factor that binds RNA polymerase (RNAP). When functioning independently, NusA enhances intrinsic termination. Paradoxically, NusA stimulates the function of the N and Q antiterminator proteins of bacteriophage λ. The mechanistic basis for NusA's functional plasticity is poorly understood. Here we uncover an effect of nascent RNA length on the ability of NusA to collaborate with Q. Ordinarily, Q engages RNAP during early elongation when it is paused at a specific site just downstream of the phage late-gene promoter. NusA facilitates this engagement process and both proteins remain associated with the transcription elongation complex (TEC) as it escapes the pause and transcribes the late genes. We show that the λ-related phage 82 Q protein (82Q) can also engage RNAP that is paused at a promoter-distal position and thus contains a nascent RNA longer than that associated with the natively positioned TEC. However, the effect of NusA in this context is antagonistic rather than stimulatory. Moreover, cleaving the long RNA associated with the promoter-distal TEC restores NusA's stimulatory effect. Our findings reveal a critical role for nascent RNA in modulating NusA's effect on 82Q-mediated antitermination, with implications for understanding NusA's functional plasticity.

INTRODUCTION

The activity of RNA polymerase (RNAP) is regulated at all stages of the transcription cycle. Among those protein factors that regulate the elongation behaviour of RNAP, there are those that exert their effects on specific transcription units, gaining access to the transcription complex via specific *cis*-acting sequences, and those that bind most or all transcription complexes and exert their effects globally (1). The Q antiterminator proteins of phage λ and its relatives provide examples of the former type, whereas the universally conserved bacterial elongation factor NusA provides an example of the latter. Required for phage late-gene transcription, the Q proteins gain access to RNAP just downstream of the late-gene promoter PR′ during early elongation (2). PR′ is recognized by the major bacterial RNAP holoenzyme species containing the primary σ factor (σ^{70}). The Q engagement process depends on two *cis*-acting DNA sequences associated with PR′, a Q-binding element (QBE) that is embedded within the promoter (between the –10 and –35 elements) and a pause-inducing sequence that is located in the initial transcribed region and induces pausing at a nascent RNA length of 16–25 nucleotides (nt) (2). This pause-inducing sequence resembles a promoter –10 element and pausing occurs due to an interaction between σ^{70}, which remains associated with the early elongation complex, and this –10-like element (3). DNA-bound Q engages the paused early elongation complex (4), establishing contacts with the core enzyme and becoming a stable component of the transcription elongation complex (TEC) as it exits the pause (5–8). As a component of the TEC, Q enables it to bypass both intrinsic and Rho-dependent terminators, resulting in transcription of the phage late genes (2,4).

Essential for viability (9,10), the general elongation factor NusA enhances hairpin-dependent pausing and intrinsic termination (11–16) and also affects Rho-dependent termination (9,12,17–20). In another guise, NusA supports the function of well-known antiterminator proteins, including the N and Q proteins of phage λ (1,21–24), and participates in the process of rRNA antitermination (25,26). In fact, NusA was first identified because of its essential role in the process of λN-mediated antitermination (22). Subsequently implicated in Q function (27–29), NusA was found to facilitate the engagement of λQ and 82Q with the paused early TEC (4,30,31). In addition, biochemical analysis revealed that when present together during the initial engagement of the early TEC, 82Q and NusA form a protective barrier that shields the nascent RNA as it emerges from the RNAP exit channel during subsequent elongation (5).

How NusA can function both to enhance pausing and termination, but also to collaborate with antiterminator proteins remains obscure. In particular, it is unclear

*To whom correspondence should be addressed. Tel: +1 617 432 1986; Fax: +1 617 432 4787; Email: ahochschild@hms.harvard.edu

whether these opposing activities reflect distinct interactions between NusA and RNAP (and/or between NusA and the nascent RNA) or whether an invariant set of interactions produces distinct context-dependent functional consequences. Here we uncover another manifestation of NusA's functional plasticity by showing that nascent RNA length dictates the effect of NusA on the process of 82Q-mediated antitermination. We set out to determine whether or not a long nascent RNA hinders the ability of 82Q to productively engage the ordinarily susceptible σ^{70}-containing paused TEC. We found that there is no effect of nascent RNA length on the efficiency of 82Q engagement process when the antiterminator is functioning unassisted. However, a long nascent RNA not only prevents NusA from facilitating the engagement process, but also converts NusA into an inhibitor of 82Q function. Our findings suggest that early elongation pausing may be important for the proper function of the Q antiterminator proteins *in vivo*, in part to prevent NusA from forming antagonistic interactions with the TEC. More generally, they point to a potential role of the nascent RNA in regulating the alternative modes of action of NusA.

MATERIALS AND METHODS

Plasmids and DNA templates

Linear DNA transcription templates were generated by PCR amplification using plasmid DNAs (see Supplementary Table S1) as template and DNA oligonucleotide primers (upstream: 5′ CCTATAAAAATAGGCGTATC ACGAG 3′ and downstream: 5′ CAGGGTTTTCCCAG TCACGACGTTG 3′). The PCR products were purified by gel extraction (Zymo Gel DNA Recovery Kit). Each template (see Figure 1A for schematic) contains the 82PR′ promoter region (–90 to +30), including the QBE and pause element, a C-less spacer region (derived from the λPR′ initial transcribed region; +25 to +44), a repeat of 82PR′ sequences from –31 to +30 including the QBE and pause element, a BamH1 site, and the λtR′ intrinsic terminator (λPR′ +138 to +232, where +193 is the site of termination). One of the templates (the promoter-proximal walk template) is designed to allow RNAP to be walked to the native pause site (82PR′ +25; (6)) and the other (the promoter-distal walk template) is designed to allow RNAP to be walked to the position of the promoter distal pause site (+106); to enable walking, mutations were introduced into the 82PR′ –31 to +30 sequences to eliminate C residues. In addition, the promoter-proximal walk template bore inactivating mutations in the promoter-distal pause element (32) and the promoter-distal walk template bore inactivating mutations in the upstream QBE and the promoter-proximal pause element (see Supplementary Figure S1 for templates sequences). ΔQBE variants of the promoter-proximal and the promoter-distal walk templates (see Figure 1D) bore inactivating mutations in the upstream and downstream QBEs, respectively; these inactivating mutations match those that were introduced into the upstream QBE of the promoter-distal walk template (see Supplementary Figure S1).

Proteins

His-tagged wild-type σ^{70} and σ^{70}-L402F were purified essentially as described after overproduction from plasmid pLHN12 His-σ^{70} (33) in BL21-DE3 cells using Novagen Overnight Express Instant TB Medium. His-tagged 82Q (82Q-6xHis) was purified essentially as described (5). In brief, XL-1 Blue cells containing plasmid pCWR82 were grown to mid log phase, protein production was induced with 0.5 mM IPTG in the presence of 1% sorbitol and the cells grown overnight at 16°C. Cells were collected and resuspended in Lysis Buffer (20 mM K-Phosphate pH 7.5, 0.5 M NaCl, 5% glycerol, 15 mM imidazole, 1% Triton-X-100, 1 mM β-mercaptoethanol, 0.5 tablet EDTA-free protease inhibitor cocktail per 30 ml) and then sequentially treated with lysozyme, a 10% volume of 2% Na-deoxycholate, and sonication. The soluble fraction was isolated by centrifugation and passed over a Ni-NTA agarose gravity column. The cleanest fractions were concentrated with a 3000 MWCO Amicon centrifugal filter unit and dialyzed into 82Q storage buffer (50 mM Tris-HCl pH 8.0, 0.5 M NaCl, 0.1 mM ETDA, 1 mM DTT, 50% glycerol). His-tagged NusA (6xHis-NusA) (16) was purified after overproduction from plasmid pET15b His-NusA in BL21-DE3 cells using Novagen Overnight Express Instant TB Medium. Cells were resuspended in Novagen BugBuster Protein Extraction Reagent (supplemented with 1 mM β-mercaptoethanol and 1 tablet EDTA-free protease inhibitor cocktail per 50 ml) and then treated with both lysozyme and sonication. The soluble fraction was isolated by centrifugation and passed over a Ni-NTA agarose gravity column. The cleanest fractions were dialyzed into NusA storage buffer (20 mM Tris-HCl pH 8.0, 0.2 M NaCl, 1 mM DTT, 50% glycerol). *Escherichia coli* core RNAP was purchased from Epicentre (Madison, WI, USA) and the ΔαCTD core RNAP was a kind gift from Seth A. Darst (Rockefeller University). RNase H was purchased from Invitrogen.

In vitro transcription assays

Two nanomolar template DNA was incubated with 8 nM RNAP holoenzyme (prepared by mixing core RNAP with a 5-fold molar excess of σ^{70}-L402F) in transcription buffer (20 mM Tris-HCl pH 8.0, 50 mM KCl, 0.1 mM EDTA, 10 mM DTT, 100 μg/ml BSA [NEB] and 1 unit/μl of Murine RNase Inhibitor [NEB]) containing 12 μM ATP, 12 μM GTP, 12 μM UTP (supplemented with 0.5 μCi/μl [α-^{32}P]-UTP; Perkin Elmer) and the initiating dinucleotide (ApG at 50 μM; Oligos, etc.) for 6 min at 37°C to allow for open complex formation. A single round of transcription was initiated by adding $MgCl_2$ (final concentration 7 mM) and rifampicin (final concentration 17 μg/ml), as described previously (5), and RNAP was walked to the position of the promoter-proximal or promoter-distal pause site during a 10-minute reaction. Following the walk, wild-type σ^{70} (final concentration 1 μM) or buffer only, NusA or buffer only and 82Q or buffer only were sequentially added to the halted TECs at 4-minute intervals (see figure legends for the final concentrations of NusA and 82Q). After the final 4-minute incubation, the TECs were chased by adding CTP (final concentration 200 μM) and increasing the concentrations of the other NTPs (final concentrations 100 μM ATP,

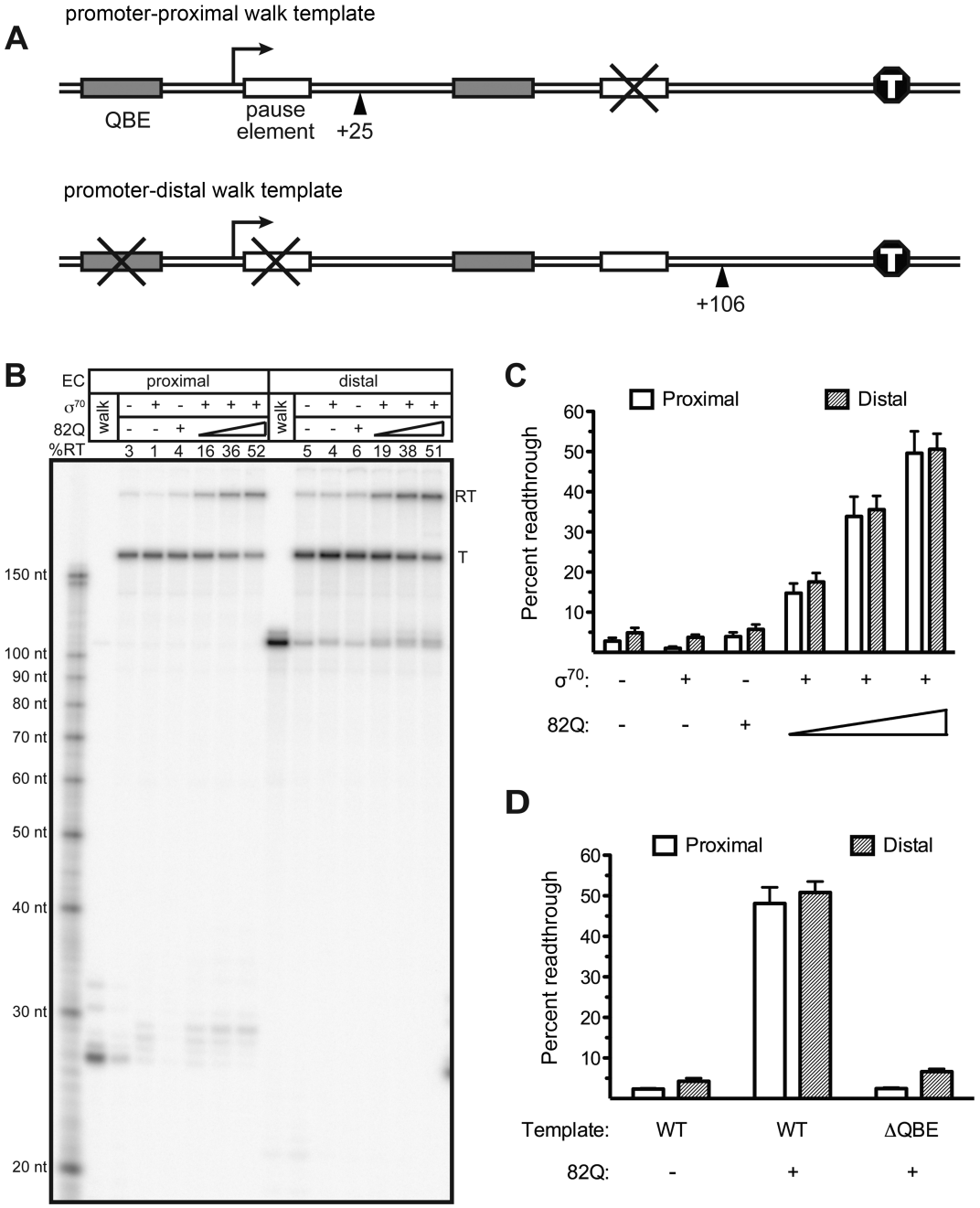

Figure 1. 82Q can engage a TEC paused at a promoter-distal location. (**A**) Cartoon showing two templates used for *in vitro* transcription assays. The templates carry 82PR′ (bent arrow indicates transcription start site), the tR′ terminator (stop sign) and a functional pair of *cis*-acting elements, QBE (grey rectangle) and pause element (white rectangle), at the native position (top) or at a promoter-distal position (bottom). Each template also carries mutationally inactivated versions of the *cis*-acting elements (QBE and/or pause element) at the other position (designated with an X), ensuring that the template sequences are nearly identical. The templates were designed to enable RNAP to be walked to the desired pause position, either +25 (top) or +106 (bottom). Thus, the promoter-proximal and promoter-distal walk templates lack C residues in the transcribed region until position +26 and position +107, respectively. After RNAP was walked to the desired position on each template, a high concentration of wild-type σ^{70} was added to ensure formation of the appropriately structured paused TEC (32). Moreover, to ensure that formation of each complex was dependent on *trans*-loaded σ^{70}, transcription was initiated with RNAP holoenzyme containing the pause-deficient mutant σ^{70} L402F (64), which is not retained in the halted TEC even at the promoter-proximal position. (**B**) A representative *in vitro* transcription experiment performed with increasing concentrations of 82Q on each of the templates depicted in panel (A). The lanes designated 'walk' show the reaction products before the complexes were chased by the addition of CTP. The halted complexes were incubated with wild-type σ^{70} (or a buffer-only control) and 82Q (or a buffer-only control); σ^{70} was added to a final concentration of 1 μM and 82Q was added to a final concentration of 6.25, 25 or 100 nM (+ designates 100 nM). The positions of the terminated transcript (T) and the readthrough transcript (RT) are indicated. 82Q activity was quantified by determining the percentage of transcripts derived from terminator readthrough (%RT = RT/[RT+T] x 100). (**C**) Bar graph showing results of three independent transcription experiments (with standard deviations) as in (B). (**D**) Control experiment showing that 82Q activity depends on the QBE. Transcription assays were performed with the templates depicted in (A) or variants that contained mutations in the relevant QBE (at the upstream and downstream positions for the promoter-proximal and promoter-distal walk templates, respectively). Bar graph shows the results of three independent transcription experiments (with standard deviations).

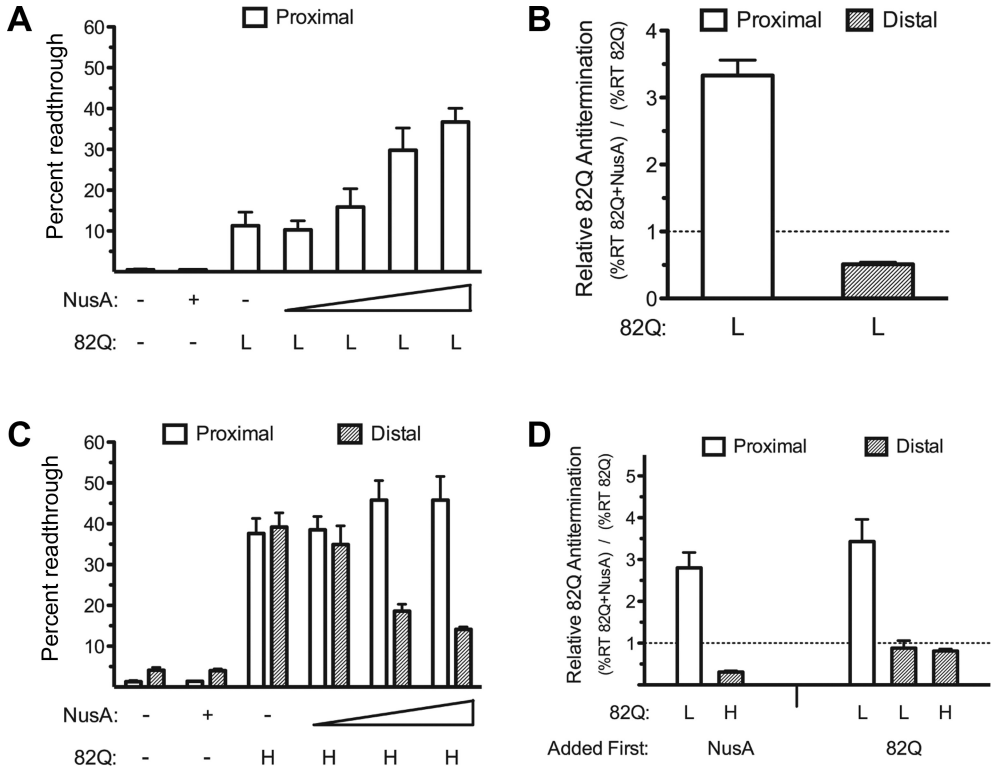

Figure 2. Effect of NusA on 82Q function depends on location of paused TEC. (**A**) NusA stimulates 82Q-mediated terminator readthrough on the promoter-proximal walk template (see Figure 1A). When present, 82Q was used at a subsaturating (low) concentration (L = 6.25 nM). NusA was added to a final concentration of 2, 8, 32 or 128 nM (+ designates 128 nM). Bar graph depicts the results of three independent transcription experiments (with standard deviations). (**B**) NusA inhibits 82Q-mediated terminator readthrough on the promoter-distal walk template (see Figure 1A). 82Q was used at a subsaturating (low) concentration (L = 6.25 nM) and NusA was added to a final concentration of 128 nM. Results (shown for both promoter-proximal and promoter-distal walk templates, as indicated) are presented as relative 82Q-mediated antitermination (percent readthrough in the presence of both 82Q and NusA divided by percent readthrough in the presence of 82Q alone). (**C**) NusA inhibition is maximized at a saturating (high) concentration of 82Q (H = 100 nM). NusA was added to a final concentration of 2, 8 or 32 nM (+ designates 32 nM). (**D**) Effect of NusA on 82Q function on promoter-distal walk template depends on order of addition. 82Q was used at low (L = 6.25 nM) or high (H = 100 nM) concentration to maximize the stimulatory and inhibitory effects of NusA, respectively. NusA was added to a final concentration of 128 nM. The order of addition is as indicated below the graph; note that NusA was added before 82Q in all other experiments. The results are presented as in panel (B). (**A–D**) Bar graphs depict the results of three independent transcription experiments (with standard deviations).

200 μM GTP and UTP). The chase was allowed to proceed for 8 min and then the reactions were halted by the addition of chilled 1.2x stop buffer (600 mM Tris-HCl pH 8.0, 12 mM EDTA and 100 μg/ml Ambion Yeast RNA). Samples were then extracted with phenol-chloroform and RNA transcripts were recovered by ethanol precipitation and resuspended in electrophoresis loading buffer (95% formamide, 18 mM EDTA, 0.025% SDS, and 0.025% of Xylene Cyanol, Bromophenol Blue, and Amaranth). Samples were heated at 95°C for 5 min, cooled to room temperature and run on 12% TBE-Urea polyacrylamide gels (UreaGel System, National Diagnostics, Atlanta, GA, USA). Autoradiography of gels was performed using storage phosphor screens and a Typhoon imager and the band quantified with ImageQuant software. When calculating percent readthrough and percent pausing, band intensities were corrected based on RNA U content and the relative UTP concentrations in the walk and chase stages. Because the UTP concentration was 16-fold lower during the walk stage, the readthrough and terminated transcripts are essentially end-labeled in the 5′ region that was synthesized during the walk.

For the experiment of Figure 3, when forming RNAP holoenzyme with the ΔαCTD core, wild-type σ^{70} (rather than σ^{70}-L402F) was used to increase the signal generated from the less active mutant core enzyme. For the experiment of Figure 4, the final NTP concentrations were decreased (12.5 μM CTP and 100 μM ATP, GTP and UTP) to facilitate detection of the pause. For the experiment of Figure 5, in which the nascent RNA was cleaved, the DNA oligonucleotide (see Supplementary Figure S1 legend for oligo sequences) was added after open complex formation (final concentration 1 μM), but before transcription was initiated. The RNase H (0.25 units in each 28 μl transcription reaction) was added after the 10-minute walk and cleavage was allowed to proceed for 15 min, before σ^{70}, NusA and 82Q were added sequentially (as above) to the halted TECs.

RESULTS

82Q can engage a paused TEC containing a long nascent RNA

All of the lambdoid phage Q proteins gain access to the late-gene transcription complex while it is paused at a promoter-proximal location under the control of σ^{70} (2).

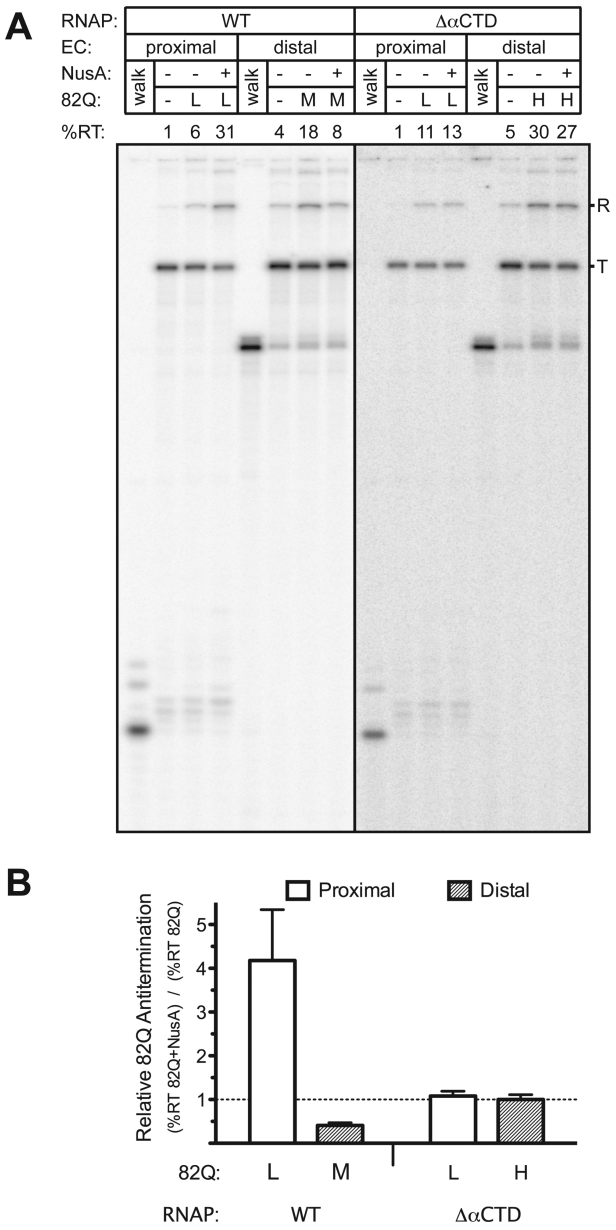

Figure 3. Effects of NusA on 82Q function depend on the αCTD. (**A**) A representative *in vitro* transcription experiment performed with wild-type or ΔαCTD RNAP holoenzyme. Transcription templates as depicted in Figure 1A and lanes designated 'walk' as in Figure 1B. NusA (when present) was added to a final concentration of 32 nM and 82Q was used at low (L = 6.25 nM), medium (M = 25 nM) or high (H = 100 nM) concentration. In particular, 82Q was used at a low concentration with the promoter-proximal walk template and wild-type or mutant RNAP to maximize the stimulatory effect of NusA and at a high concentration with the promoter-distal walk template and ΔαCTD RNAP to maximize the inhibitory effect of NusA. However, 82Q was used at an intermediate concentration with the promoter-distal walk template and wild-type RNAP to control for the fact that saturating concentrations of 82Q were somewhat less active on the promoter-distal walk template when assayed with ΔαCTD RNAP as compared with wild-type RNAP (~27% RT versus ~45% RT); the results indicate that even an intermediate concentration of 82Q (giving ~20% RT) permits detection of a clear inhibitory effect of NusA (≥2-fold reduction). (**B**) Bar graph showing results of three independent transcription experiments (with standard deviations) as in (A). Results (shown for both templates) are presented as relative 82Q-mediated antitermination (percent readthrough in the presence of both 82Q and NusA divided by percent readthrough in the presence of 82Q alone).

These promoter-proximal σ^{70}-dependent (–10-like) pause elements are recognized by the initiating σ^{70} that has been retained in the early TEC (3,34–37). In contrast, promoter-distal –10-like pause elements tend to be inefficiently recognized because most transcription complexes release σ^{70} before encountering these pause elements (32,38,39) (though certain initial-transcribed-region sequences can inhibit this release, allowing for long-range retention of σ^{70} (32,40)). The apparent requirement for σ^{70} to facilitate Q loading (1,4) provides one explanation for the fact that the lambdoid phage Q proteins engage RNAP early during elongation. However, we wondered whether there might be an additional mechanistic requirement for Q to engage RNAP when the nascent RNA is still short. To address this question, we contrived a situation that would enable us to ask if the Q protein of phage 82 could engage a mature TEC, with a long nascent RNA.

We assayed the ability of 82Q to mediate terminator readthrough on two transcription templates carrying the late-gene promoter (82PR′) and a downstream terminator. The first had the QBE and the pause element at their native positions and the second had these *cis*-acting elements displaced downstream by ~100 nt. Additionally, the design of the two templates (which we call the promoter-proximal and the promoter-distal walk templates) enabled us to walk RNAP to the site of the σ^{70}-dependent pause (either +25 or +106) by withholding CTP from the reactions (see Figure 1A). That is, in each case transcription in the absence of CTP allows RNAP to advance to the precise position where pausing would be expected to occur when σ^{70} engages the –10-like element. To ensure that the appropriately structured paused TECs would be formed on both templates, we initiated a single round of transcription in the absence of CTP and then incubated the halted TECs in the presence of excess σ^{70} at a sufficiently high concentration to enable it to bind the complexes *in trans* (see the legend to Figure 1A for details). We then added 82Q to the reactions, released the complexes from the halt site by adding CTP and quantified the extent of terminator readthrough.

Figure 1B shows the results of an *in vitro* transcription experiment performed with increasing concentrations of 82Q using the two templates. Transcription from each template was efficiently terminated in the absence of 82Q, and we found that the ability of 82Q to mediate terminator readthrough was indistinguishable on the two templates (Figure 1B and C). Furthermore, controls indicated that 82Q activity on each template depended on the addition of excess σ^{70} (Figure 1B and C), which is required to enable formation of the Q-susceptible paused TEC, and also on the integrity of the QBE (Figure 1D). We conclude that 82Q can efficiently engage paused TECs containing σ^{70} when the nascent RNA is up to at least 100 nucleotides (nt) in length.

NusA can antagonize 82Q function when engagement occurs in the presence of a long nascent RNA

Next we asked whether or not the ability of NusA to support 82Q function was dependent on the length of the nascent RNA. Using the promoter-proximal walk template (bearing the natively positioned QBE and pause element), we found that NusA stimulated 82Q-dependent terminator

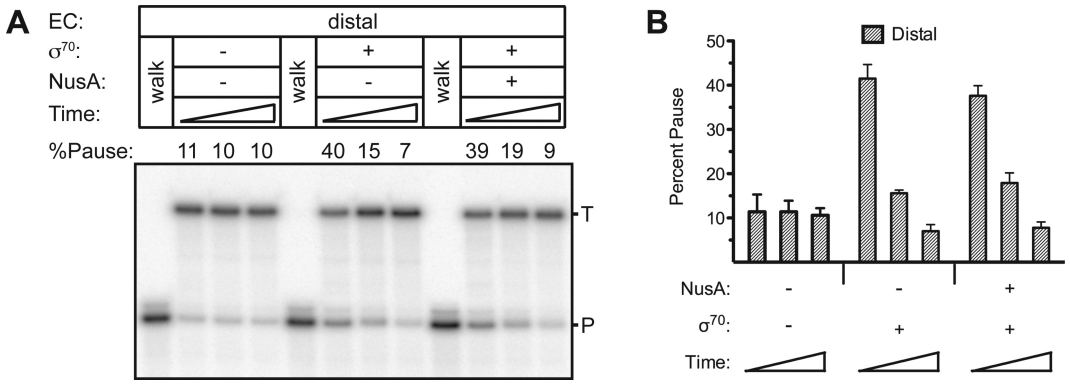

Figure 4. Effect of NusA on kinetics of σ^{70}-dependent pause on promoter-distal walk template. (**A**) A representative *in vitro* transcription experiment performed as a time course with samples taken at 20 s, 140 s and 10 min after the reactions were chased with a low concentration of CTP (12.5 μM) to permit detection of the σ^{70}-dependent pause. As for the 82Q engagement assays, RNAP was walked to the promoter-distal pause site before the addition (or not) of σ^{70} to a final concentration of 1 μM, followed by the addition (or not) of NusA to a final concentration of 32 nM. The walk samples were removed before the reactions were chased. The percent pause (% pause) was quantified at each time point by dividing the intensity of the pause band (P) by the sum of the intensities of the pause band and the terminated band (T). (**B**) The bar graph shows the results of three independent experiments (with standard deviations) as in (A).

readthrough substantially (up to 3.5-fold) when 82Q was present at subsaturating concentrations (Figure 2A) and that this effect was reduced to ∼1.2-fold when 82Q was present at saturating concentrations (Figure 2C). The observation that the effect of NusA was dependent on the concentration of 82Q (see also (31)) suggests that NusA is acting to facilitate the engagement of Q with the paused TEC. Unexpectedly, we found that NusA exerted the opposite effect on 82Q function when the assay was performed with the promoter-distal walk template. When 82Q was present at a low, subsaturating concentration, NusA reduced Q-mediated readthrough ∼2-fold (Figure 2B), and this inhibitory effect was amplified when 82Q was present at a saturating concentration (∼40% readthrough in the absence of NusA versus ∼15% readthrough in the presence of NusA; Figure 2C). Notably, the same concentrations of NusA that were inhibitory with the template bearing the distally positioned *cis*-acting elements were stimulatory with the template bearing the natively positioned *cis*-acting elements (Figure 2A and C).

We then investigated whether or not the observed inhibitory effect of NusA was dependent on the order of addition of 82Q and NusA to the reaction. In our original protocol, we preincubated the halted TECs with σ^{70} and then added NusA, followed by 82Q, before chasing the complexes by adding CTP to the reactions. When we reversed the order of addition and incubated the complexes with 82Q before adding NusA, we observed the same stimulatory effect of NusA on 82Q function with the promoter-proximal walk template, but we no longer observed an inhibitory effect of NusA with the promoter-distal walk template (Figure 2D). Nonetheless, NusA failed to stimulate 82Q function on this template regardless of the order of addition (Figure 2D). These findings suggest that NusA and 82Q cannot functionally cooperate at the engagement step when the paused TEC contains a long nascent RNA and they raise the possibility that NusA and 82Q physically compete for access to the paused TEC under these conditions.

Inhibitory and stimulatory effects of NusA on 82Q function depend on NusA-αCTD interaction

NusA participates in a well-characterized interaction with the C-terminal domain of the α subunit of RNAP (αCTD) that contributes to the overall affinity of NusA for the TEC and is required to unleash NusA's RNA-binding activity (41–43). The NusA-αCTD interaction was shown to be required for the effects of NusA on both Q-mediated antitermination and intrinsic termination (5,41). We therefore wondered whether or not the ability of NusA to antagonize 82Q function was dependent on the αCTD. To test this, we performed transcription assays using a mutant RNAP core enzyme that lacks the αCTD (ΔαCTD RNAP). We found that the use of ΔαCTD RNAP eliminated the stimulatory effect of NusA on 82Q function as assayed with the promoter-proximal walk template, and also eliminated the inhibitory effect of NusA on 82Q function as assayed with the promoter-distal walk template (Figure 3A and B). We conclude that the inhibitory effect of NusA unveiled in our experiments depends on an interaction between NusA and the αCTD. The requirement for the αCTD for both the stimulatory and inhibitory effects of NusA on 82Q function, taken together with the fact that both effects occur in the same concentration range, argues that the inhibitory effect we observe does not solely reflect nonspecific binding of NusA to the long nascent RNA associated with the promoter-distal TEC.

NusA does not inhibit formation of the promoter-distal σ^{70}-dependent paused TEC

We considered two possible explanations for the inhibitory effect of NusA on 82Q-mediated terminator readthrough on the promoter-distal walk template. First, NusA might interact with the distally paused TEC in such as manner so as to preclude 82Q from binding the complex. Alternatively, NusA might indirectly prevent 82Q from engaging the TEC by disrupting interactions of σ^{70} that enable formation of the Q-susceptible paused TEC. To test the latter possibil-

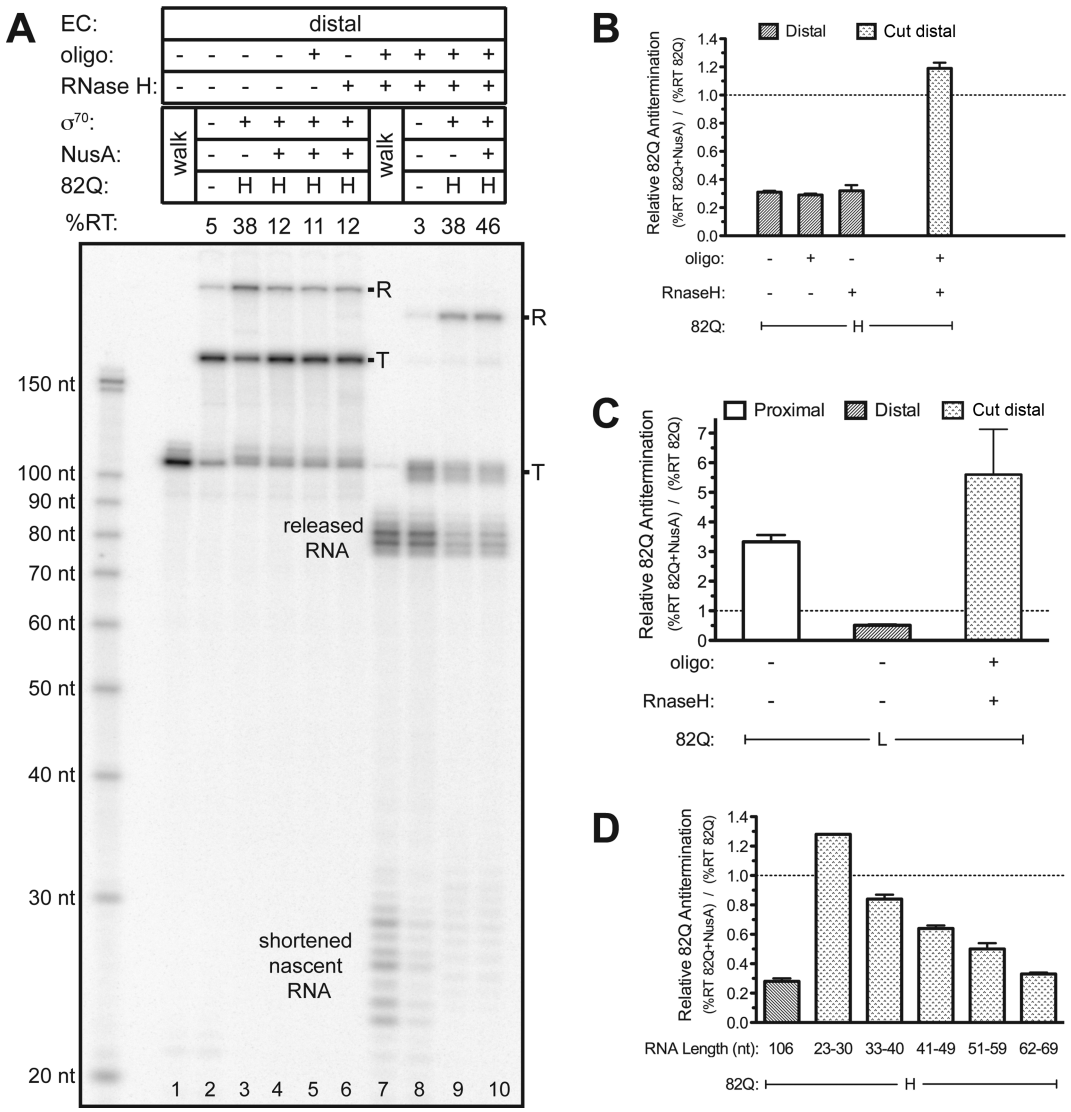

Figure 5. Effect of NusA on 82Q function depends on nascent RNA length. (**A**) A representative *in vitro* transcription experiment performed with the promoter-distal walk template. For the reactions on the right (lanes 7–10), RNAP was walked to the halt site and then a DNA oligonucleotide (final concentration 1 μM) was used to direct RNase H cleavage of the nascent RNA, producing a set of shortened nascent RNAs ranging in size from ∼23 to ∼30 nt and a set of released RNAs, as indicated on the gel. The reactions on the left (lanes 1–6) were performed without cleavage of the nascent RNA. The positions of the terminated (T) and readthrough (R) RNAs for each reaction set are indicated. 82Q was used at a saturating concentration (100 nM) to maximize the inhibitory effect of NusA, and NusA was added to a final concentration of 32 nM. (**B**) The bar graph shows the results of three independent experiments (with standard deviations) as in (A). Results (shown for both uncleaved and cleaved complexes) are presented as relative 82Q-mediated antitermination (percent readthrough in the presence of both 82Q and NusA divided by percent readthrough in the presence of 82Q alone). (**C**) Shortening the nascent RNA restores the stimulatory effect of NusA. The bar graph shows the results of three independent experiments (with standard deviations). A subset of these data was presented in Figure 2B (see the legend for description); here column 3 shows the results obtained with cleaved complexes. (**D**) Inhibitory effect of NusA increases with nascent RNA length. *In vitro* transcription analysis was performed in panel (A), except that a series of DNA oligonucleotides was used to direct RNase H cleavage at various positions along the nascent RNA. The bar graph shows the results of three independent experiments (with standard deviations) and a representative experiment is shown in Supplementary Figure S2. (Note that the reactions in column 2 were performed only in duplicate and thus lack an error bar.) The results are presented as in panel (B). The first column shows the results obtained with the uncleaved complex (nascent RNA length 106 nt). For each of the subsequent columns, the range of nascent RNA lengths after cleavage is indicated.

ity, we modified the conditions of the transcription assay (see 'Materials and Methods') so that we could detect the σ^{70}-dependent pause during the chase of the halted TECs (Figure 4). We found that the addition of NusA to the reaction had no effect on the pause kinetics (Figure 4A and B). We conclude that NusA exerts its effect directly on the 82Q engagement process.

Shortening the long nascent RNA associated with the promoter-distal paused TEC relieves the inhibitory effect of NusA on 82Q function

The most obvious difference between our two transcription templates is the length of the nascent RNA associated with each paused TEC. Thus, we wished to test explicitly whether or not NusA distinguishes between the TECs on

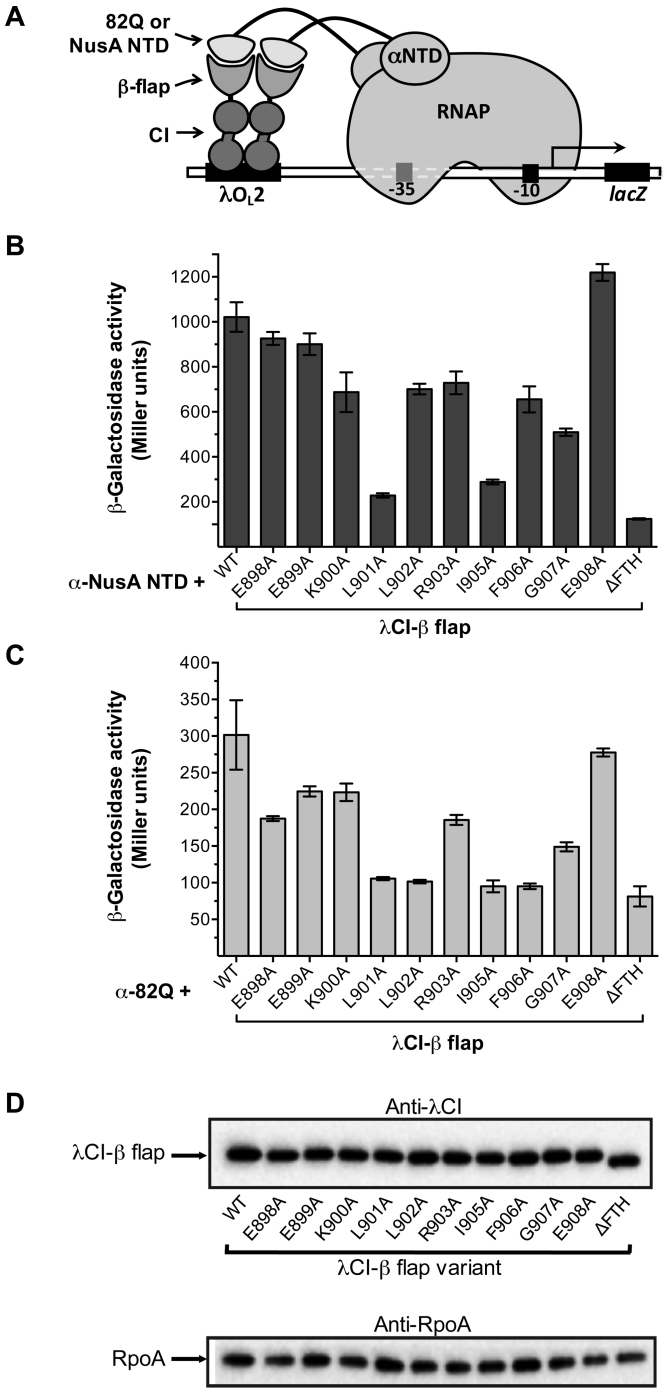

Figure 6. Use of the bacterial two-hybrid assay to test the effects of alanine substitutions at β-flap residues 898–908 (except position A904) on the 82Q/β-flap and the NusA/β-flap interactions. (**A**) Cartoon depicts how the interaction between 82Q or the NusA NTD (fused to the α-NTD) and the β-flap (fused to the bacteriophage λCI protein) activates transcription from test promoter p*lac*O$_L$2-62, which bears the λ operator O$_L$2 centred 62 bp upstream of the start site of the *lac* core promoter. In reporter strain FW102 O$_L$2-62, test promoter p*lac*O$_L$2-62 is located on an F' episome and upstream of a *lacZ* reporter gene. (**B, C**) Results of β-galactosidase assays (see Supplemental Methods) performed with reporter strain cells containing one plasmid that encoded the α-NusA NTD (**B**) or α-82Q (**C**) fusion protein and a compatible plasmid that encoded a wild-type λCI-β-flap fusion protein (WT), or the indicated λCI-β-flap fusion protein variant (ΔFTH refers to a λCI-β-flap fusion protein in which the β flap-tip-helix, residues 900–909, is deleted). The plasmids (see Supplementary Table S1) directed the synthesis of the fusion proteins under the control of IPTG-inducible promoters and the cells were grown in the presence of 25 μM IPTG (B) or 5 μM IPTG (C). The bar graph shows the averages of three independent measurements and standard deviations. (**D**) Western blot showing WT and variant λCI-β-flap protein levels from normalized samples of cell lysates from (B) that were processed for western blotting with an anti-λCI antibody (a generous gift from J. Beckwith) or an anti-RpoA antibody (Neoclone) as a loading control.

the two templates based solely on the length of the nascent RNA. To do this, we shortened the nascent RNA associated with the distally positioned TEC by using RNase H, which cleaves the RNA component of RNA:DNA hybrids, together with a specific DNA oligonucleotide complementary in sequence to the exposed RNA near the RNA exit channel of RNAP. When we shortened the nascent RNA associated with the distally positioned TEC to a length similar to that of the short nascent RNA associated with the natively positioned TEC, we found that the inhibitory effect of NusA on 82Q-mediated terminator readthrough was entirely abolished (Figure 5A and B). Moreover, cleavage of the nascent RNA restored the stimulatory effect of NusA, which was particularly evident when the assays were performed with a subsaturating concentration of 82Q (Figure 5B and C). We then used a panel of oligonucleotides to shorten the nascent RNA in a stepwise fashion. We found that the magnitude of NusA's inhibitory effect decreased gradually with the length of the nascent RNA (Figure 5D and Supplementary Figure S2), suggesting that the observed inhibitory effect is unlikely to involve a sequence-specific interaction with a particular motif encoded in the nascent RNA. Although we do not know why NusA inhibition appears to vary continuously with nascent RNA length, we note that NusA contains multiple RNA binding domains and has been shown to interact with nascent RNA (16,44,45). We conclude based on these cleavage experiments that functional cooperation between NusA and 82Q requires that 82Q engages a paused TEC with a short (<∼30 nt) nascent RNA.

NusA and 82Q interact with overlapping determinants of the β flap-tip-helix

Previous work indicates that both NusA (16,46,47) and 82Q (7) can interact with the flap domain of the RNAP β subunit (β-flap) in the vicinity of the RNA exit channel. In the case of NusA, a large, multi-domain protein, this interaction involves its N-terminal domain (NTD). To evaluate the potential for NusA and 82Q to compete for binding to the TEC, we used a bacterial two-hybrid assay to compare the interactions of the NusA NTD and 82Q with the β-flap (Figure 6A). We found that the β flap-tip-helix, which is a primary determinant for the interaction of 82Q with the β-flap (7), was also required for interaction of the NusA NTD with the β-flap. Specifically, removal of the flap-tip-helix abolished the two-hybrid interaction between the β-flap and 82Q (as previously shown; (7)), and also between the β-flap and the NusA NTD (Figure 6B–D). In addition, we tested the effects of individual alanine substitutions across the flap-tip-helix (β residues 898–908); this analysis indicated that the NusA NTD and 82Q make similar (but not identical) molecular contacts with the flap-tip-helix (Figure

6B and C). Our finding that the NusA NTD interacts with the β flap-tip-helix is in agreement with previous structural models (16,46–48).

DISCUSSION

Here we demonstrate an effect of nascent RNA length on the function of the general elongation factor NusA, which is known to facilitate termination (16), on the one hand, and to support the activities of various antitermination factors, including the Q proteins of phage λ and phage 82 (1), on the other. We find that the length of the nascent RNA has no effect on the ability of 82Q to gain access to the TEC when the antiterminator is functioning independently. In contrast, we find that a long nascent RNA (>∼30 nt) interferes with the ability of NusA to support the function of 82Q. Furthermore, the effect of a long nascent RNA is to convert NusA into a potential inhibitor of 82Q-mediated antitermination that can prevent 82Q from productively engaging the TEC. Based on our findings, we propose that NusA has the potential to interact in alternative ways with RNAP during elongation, and that one of its binding modes is incompatible with the formation of the 82Q-dependent antitermination complex. According to our model, the nascent RNA functions as a length-dependent switch that influences how NusA interacts with RNAP and thereby determines whether or not the 82Q-containing antitermination complex can be formed.

Competitive binding model for NusA-mediated inhibition of Q function

Escherichia coli NusA is a large (495 residue) protein with a complex domain structure (49). The NTD, which binds the β-flap in the immediate vicinity of the RNA exit channel (16,46,47), has been shown to be fully proficient in enhancing hairpin-dependent pausing and largely responsible for enhancing intrinsic termination, with the other domains of NusA reducing the concentration of protein required to mediate these effects (16). The NusA NTD is followed by three RNA-binding domains (S1, KH1, KH2), which are followed by a C-terminal region consisting of two acidic repeat domains (AR1 and AR2) that are not broadly conserved beyond γ-proteobacteria (16,49–53). The C-terminal region has an autoinhibitory function, impeding the RNA-binding activity of NusA, and an interaction between AR2 and the RNAP αCTD (see above) relieves this autoinhibition (42,43).

One explanation for our finding that NusA can inhibit 82Q function is that NusA has the potential to compete with 82Q for access to the β-flap and that the nascent RNA influences this competition. More specifically, we suggest the following model, which is based on our two-hybrid analysis (Figure 6) and our prior demonstration that in the case of λQ (which also interacts with the β flap-tip-helix) this interaction facilitates its engagement with the paused TEC (7). Accordingly, we hypothesize that when 82Q and NusA engage the natively positioned paused TEC, 82Q wins the competition for the β-flap and NusA binds the complex in a yet to be characterized manner that stabilizes 82Q's association with the complex. In contrast, we suggest that the long nascent RNA associated with the promoter-distal paused TEC stabilizes the binding of the NusA NTD to the β-flap such that 82Q's access to the β-flap is occluded and consequently 82Q fails to load (see Figure 7). An alternative (though not mutually exclusive) possibility is that in the context of the promoter-distal TEC NusA alters the position or structure of the nascent RNA such that the RNA itself hinders the ability of 82Q to engage the TEC.

The β flap-tip-helix is also a primary binding determinant for the σ subunit of RNAP; in particular, domain 4 of σ^{70}, which is responsible for recognition of the promoter –35 element, binds the flap-tip-helix (54–57). However, structural and other evidence indicates that the growth of the nascent RNA during early elongation destabilizes the interaction between σ^{70} domain 4 and the β-flap, facilitating the complete displacement of σ^{70} domain 4 from the flap (58–61). In particular, in the context of the paused TEC at λPR′ (and presumably also at 82PR′) the nascent RNA-mediated destabilization of the σ^{70} domain 4/β-flap interaction is required to enable Q to productively engage the paused TEC (30,35,62). Thus, we suggest that when 82Q engages the paused TEC at 82PR′, the interaction of σ^{70} domain 4 with the β flap-tip-helix is replaced by an interaction between 82Q (bound to the QBE) and the β flap-tip-helix, an interaction that is incompatible with the binding of the NusA NTD to the flap-tip-helix. How NusA adapts to 82Q and stabilizes its interactions with the paused TEC is currently unclear; nonetheless, our findings (see also (5)) indicate that this stabilizing effect depends at least in part on contact between NusA AR2 and the RNAP αCTD (Figure 3). Furthermore, after RNAP escapes the pause, both 82Q and NusA remain stably associated with the TEC, together forming a protective shield that in effect extends the RNA exit channel by restricting access to ∼10 additional nucleotides of the nascent RNA (5).

Implications for Q function

Our findings indicate that functional cooperation between 82Q and NusA requires that 82Q engage the TEC when it is associated with a short nascent RNA. Thus, at least one role of the promoter-proximal pause element is to trap the TEC when the nascent RNA is sufficiently short to allow the antitermination-proficient complex to form. Furthermore, we have previously shown that genetic disruption of the σ^{70} region 4/β-flap interaction enables 82Q to productively engage an initiation complex on an appropriately engineered template (62), reflecting the requirement that the Q proteins gain access to the β-flap during the engagement process (7). Thus, the σ^{70}-dependent promoter-proximal pause, which is a shared feature of the lambdoid phage late promoters (63), may provide an especially effective mechanism to ensure that the Q proteins gain privileged access to the early TEC during a narrow window defined by a nascent RNA length that is sufficient to destabilize the σ^{70} region 4/β-flap interaction (∼16 nt) (60), but short enough to prevent NusA from antagonizing the engagement process (<∼30 nt). Additionally, the fact that engagement occurs before the complete release of σ^{70} may help ensure that other elongation factors, with potentially opposing effects, do not gain access to the TEC before Q can appropriately modify the complex.

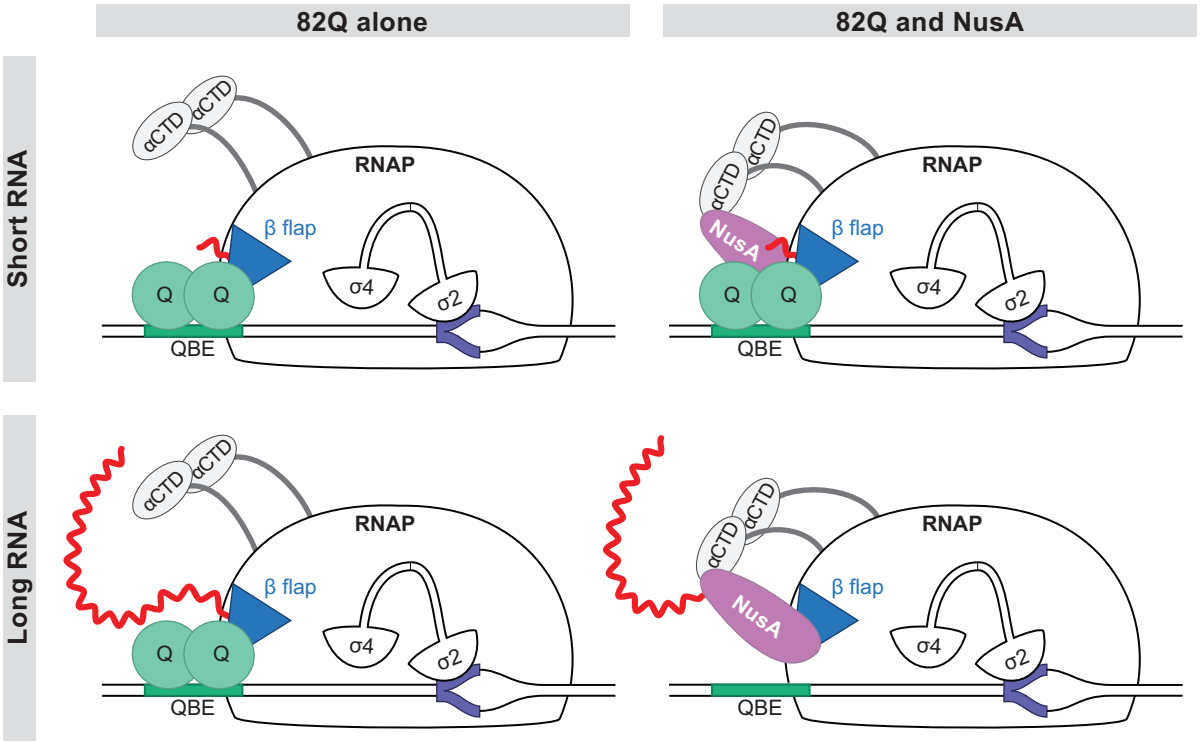

Figure 7. Competitive binding model to explain inhibitory effect of NusA on 82Q function. The cartoon shows σ^{70}-containing paused TECs poised for engagement by 82Q that are associated with either a short nascent RNA (top row; RNA in red) or a long nascent RNA (bottom row; RNA in red). Domain 2 of σ^{70} ($\sigma 2$) is shown bound to the –10-like pause element (purple boxes on transcription bubble). When these complexes are incubated with 82Q alone (left side), successful engagement occurs and DNA-bound 82Q (which is thought to bind the QBE as a dimer) gains access to the β-flap (blue triangle). However, when these complexes are incubated with 82Q in the presence of NusA, the outcome depends on the length of the nascent RNA. If the nascent RNA is short, NusA (bound to the αCTD) can stabilize the 82Q-engaged complex by an unknown mechanism (top right). But if the nascent RNA is long, NusA wins the competition for the β-flap, preventing 82Q from engaging the complex.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank Danielle Heller for performing the western blot shown in Supplementary Figure S3, Seth Goldman for generous help preparing figures, Jeff Roberts for helpful discussion and Simon Dove and Bryce Nickels for critical reading of the manuscript.

FUNDING

National Institutes of Health [GM044025]. Funding for open access charge: National Institutes of Health [GM044025].
Conflict of interest statement. None declared.

REFERENCES

1. Roberts,J.W., Shankar,S. and Filter,J.J. (2008) RNA polymerase elongation factors. *Annu. Rev. Microbiol.*, **62**, 211–233.
2. Roberts,J.W., Yarnell,W., Bartlett,E., Guo,J., Marr,M., Ko,D.C., Sun,H. and Roberts,C.W. (1998) Antitermination by bacteriophage lambda Q protein. *Cold Spring Harb. Symp. Quant. Biol.*, **63**, 319–325.
3. Ring,B.Z., Yarnell,W.S. and Roberts,J.W. (1996) Function of E. coli RNA polymerase sigma factor sigma 70 in promoter-proximal pausing. *Cell*, **86**, 485–493.
4. Yarnell,W.S. and Roberts,J.W. (1992) The phage lambda gene Q transcription antiterminator binds DNA in the late gene promoter as it modifies RNA polymerase. *Cell*, **69**, 1181–1189.
5. Shankar,S., Hatoum,A. and Roberts,J.W. (2007) A transcription antiterminator constructs a NusA-dependent shield to the emerging transcript. *Mol. Cell*, **27**, 914–927.
6. Yarnell,W.S. and Roberts,J.W. (1999) Mechanism of intrinsic transcription termination and antitermination. *Science*, **284**, 611–615.
7. Deighan,P., Diez,C.M., Leibman,M., Hochschild,A. and Nickels,B.E. (2008) The bacteriophage lambda Q antiterminator protein contacts the beta-flap domain of RNA polymerase. *Proc. Natl Acad. Sci. U.S.A.*, **105**, 15305–15310.
8. Deighan,P. and Hochschild,A. (2007) The bacteriophage lambdaQ anti-terminator protein regulates late gene expression as a stable component of the transcription elongation complex. *Mol. Microbiol.*, **63**, 911–920.
9. Cardinale,C.J., Washburn,R.S., Tadigotla,V.R., Brown,L.M., Gottesman,M.E. and Nudler,E. (2008) Termination factor Rho and its cofactors NusA and NusG silence foreign DNA in E. coli. *Science*, **320**, 935–938.
10. Zheng,C. and Friedman,D.I. (1994) Reduced Rho-dependent transcription termination permits NusA-independent growth of Escherichia coli. *Proc. Natl Acad. Sci. U.S.A.*, **91**, 7543–7547.
11. Farnham,P.J., Greenblatt,J. and Platt,T. (1982) Effects of NusA protein on transcription termination in the tryptophan operon of Escherichia coli. *Cell*, **29**, 945–951.
12. Greenblatt,J., McLimont,M. and Hanly,S. (1981) Termination of transcription by nusA gene protein of Escherichia coli. *Nature*, **292**, 215–220.
13. Landick,R. and Yanofsky,C. (1984) Stability of an RNA secondary structure affects in vitro transcription pausing in the trp operon leader region. *J. Biol. Chem.*, **259**, 11550–11555.

14. Schmidt,M.C. and Chamberlin,M.J. (1987) nusA protein of Escherichia coli is an efficient transcription termination factor for certain terminator sites. *J. Mol. Biol.*, **195**, 809–818.
15. Chan,C.L. and Landick,R. (1989) The Salmonella typhimurium his operon leader region contains an RNA hairpin-dependent transcription pause site. Mechanistic implications of the effect on pausing of altered RNA hairpins. *J. Biol. Chem.*, **264**, 20796–20804.
16. Ha,K.S., Toulokhonov,I., Vassylyev,D.G. and Landick,R. (2010) The NusA N-terminal domain is necessary and sufficient for enhancement of transcriptional pausing via interaction with the RNA exit channel of RNA polymerase. *J. Mol. Biol.*, **401**, 708–725.
17. Carlomagno,M.S. and Nappo,A. (2003) NusA modulates intragenic termination by different pathways. *Gene*, **308**, 115–128.
18. Kainz,M. and Gourse,R.L. (1998) The C-terminal domain of the alpha subunit of Escherichia coli RNA polymerase is required for efficient rho-dependent transcription termination. *J. Mol. Biol.*, **284**, 1379–1390.
19. Lau,L.F., Roberts,J.W. and Wu,R. (1983) RNA polymerase pausing and transcript release at the lambda tR1 terminator in vitro. *J. Biol. Chem.*, **258**, 9391–9397.
20. Ward,D.F. and Gottesman,M.E. (1981) The nus mutations affect transcription termination in Escherichia coli. *Nature*, **292**, 212–215.
21. Das,A. and Wolska,K. (1984) Transcription antitermination in vitro by lambda N gene product: requirement for a phage nut site and the products of host nusA, nusB, and nusE genes. *Cell*, **38**, 165–173.
22. Friedman,D.I. and Baron,L.S. (1974) Genetic characterization of a bacterial locus involved in the activity of the N function of phage lambda. *Virology*, **58**, 141–148.
23. Schauer,A.T., Carver,D.L., Bigelow,B., Baron,L.S. and Friedman,D.I. (1987) lambda N antitermination system: functional analysis of phage interactions with the host NusA protein. *J. Mol. Biol.*, **194**, 679–690.
24. Whalen,W., Ghosh,B. and Das,A. (1988) NusA protein is necessary and sufficient in vitro for phage lambda N gene product to suppress a rho-independent terminator placed downstream of nutL. *Proc. Natl Acad. Sci. U.S.A.*, **85**, 2494–2498.
25. Vogel,U. and Jensen,K.F. (1997) NusA is required for ribosomal antitermination and for modulation of the transcription elongation rate of both antiterminated RNA and mRNA. *J. Biol. Chem.*, **272**, 12265–12271.
26. Torres,M., Condon,C., Balada,J.M., Squires,C. and Squires,C.L. (2001) Ribosomal protein S4 is a transcription factor with properties remarkably similar to NusA, a protein involved in both non-ribosomal and ribosomal RNA antitermination. *EMBO J.*, **20**, 3811–3820.
27. Grayhack,E.J. and Roberts,J.W. (1982) The phage lambda Q gene product: activity of a transcription antiterminator in vitro. *Cell*, **30**, 637–648.
28. Grayhack,E.J., Yang,X.J., Lau,L.F. and Roberts,J.W. (1985) Phage lambda gene Q antiterminator recognizes RNA polymerase near the promoter and accelerates it through a pause site. *Cell*, **42**, 259–269.
29. Yang,X.J., Goliger,J.A. and Roberts,J.W. (1989) Specificity and mechanism of antitermination by Q proteins of bacteriophages lambda and 82. *J. Mol. Biol.*, **210**, 453–460.
30. Nickels,B.E., Roberts,C.W., Sun,H., Roberts,J.W. and Hochschild,A. (2002) The sigma(70) subunit of RNA polymerase is contacted by the (lambda)Q antiterminator during early elongation. *Mol. Cell*, **10**, 611–622.
31. Santangelo,T.J., Mooney,R.A., Landick,R. and Roberts,J.W. (2003) RNA polymerase mutations that impair conversion to a termination-resistant complex by Q antiterminator proteins. *Genes Dev.*, **17**, 1281–1292.
32. Deighan,P., Pukhrambam,C., Nickels,B.E. and Hochschild,A. (2011) Initial transcribed region sequences influence the composition and functional properties of the bacterial elongation complex. *Genes Dev.*, **25**, 77–88.
33. Panaghie,G., Aiyar,S.E., Bobb,K.L., Hayward,R.S. and de Haseth,P.L. (2000) Aromatic amino acids in region 2.3 of Escherichia coli sigma 70 participate collectively in the formation of an RNA polymerase-promoter open complex. *J. Mol. Biol.*, **299**, 1217–1230.
34. Kapanidis,A.N., Margeat,E., Laurence,T.A., Doose,S., Ho,S.O., Mukhopadhyay,J., Kortkhonjia,E., Mekler,V., Ebright,R.H. and Weiss,S. (2005) Retention of transcription initiation factor sigma70 in transcription elongation: single-molecule analysis. *Mol. Cell*, **20**, 347–356.
35. Marr,M.T., Datwyler,S.A., Meares,C.F. and Roberts,J.W. (2001) Restructuring of an RNA polymerase holoenzyme elongation complex by lambdoid phage Q proteins. *Proc. Natl Acad. Sci. U.S.A.*, **98**, 8972–8978.
36. Mukhopadhyay,J., Kapanidis,A.N., Mekler,V., Kortkhonjia,E., Ebright,Y.W. and Ebright,R.H. (2001) Translocation of sigma(70) with RNA polymerase during transcription: fluorescence resonance energy transfer assay for movement relative to DNA. *Cell*, **106**, 453–463.
37. Nickels,B.E., Mukhopadhyay,J., Garrity,S.J., Ebright,R.H. and Hochschild,A. (2004) The sigma 70 subunit of RNA polymerase mediates a promoter-proximal pause at the lac promoter. *Nat. Struct. Mol. Biol.*, **11**, 544–550.
38. Mooney,R.A. and Landick,R. (2003) Tethering sigma70 to RNA polymerase reveals high in vivo activity of sigma factors and sigma70-dependent pausing at promoter-distal locations. *Genes Dev.*, **17**, 2839–2851.
39. Sevostyanova,A., Svetlov,V., Vassylyev,D.G. and Artsimovitch,I. (2008) The elongation factor RfaH and the initiation factor sigma bind to the same site on the transcription elongation complex. *Proc. Natl Acad. Sci. U.S.A.*, **105**, 865–870.
40. Harden,T.T., Wells,C.D., Friedman,L.J., Landick,R., Hochschild,A., Kondev,J. and Gelles,J. (2016) Bacterial RNA polymerase can retain sigma70 throughout transcription. *Proc. Natl Acad. Sci. U.S.A.*, **113**, 602–607.
41. Liu,K., Zhang,Y., Severinov,K., Das,A. and Hanna,M.M. (1996) Role of Escherichia coli RNA polymerase alpha subunit in modulation of pausing, termination and anti-termination by the transcription elongation factor NusA. *EMBO J.*, **15**, 150–161.
42. Mah,T.F., Kuznedelov,K., Mushegian,A., Severinov,K. and Greenblatt,J. (2000) The alpha subunit of E. coli RNA polymerase activates RNA binding by NusA. *Genes Dev.*, **14**, 2664–2675.
43. Schweimer,K., Prasch,S., Sujatha,P.S., Bubunenko,M., Gottesman,M.E. and Rosch,P. (2011) NusA interaction with the alpha subunit of E. coli RNA polymerase is via the UP element site and releases autoinhibition. *Structure*, **19**, 945–954.
44. Liu,K. and Hanna,M.M. (1995) NusA interferes with interactions between the nascent RNA and the C-terminal domain of the alpha subunit of RNA polymerase in Escherichia coli transcription complexes. *Proc. Natl Acad. Sci. U.S.A.*, **92**, 5012–5016.
45. Liu,K. and Hanna,M.M. (1995) NusA contacts nascent RNA in Escherichia coli transcription complexes. *J. Mol. Biol.*, **247**, 547–558.
46. Drogemuller,J., Strauss,M., Schweimer,K., Jurk,M., Rosch,P. and Knauer,S.H. (2015) Determination of RNA polymerase binding surfaces of transcription factors by NMR spectroscopy. *Sci. Rep.*, **5**, 16428.
47. Yang,X., Molimau,S., Doherty,G.P., Johnston,E.B., Marles-Wright,J., Rothnagel,R., Hankamer,B., Lewis,R.J. and Lewis,P.J. (2009) The structure of bacterial RNA polymerase in complex with the essential transcription elongation factor NusA. *EMBO reports*, **10**, 997–1002.
48. Ma,C., Mobli,M., Yang,X., Keller,A.N., King,G.F. and Lewis,P.J. (2015) RNA polymerase-induced remodelling of NusA produces a pause enhancement complex. *Nucleic Acids Res.*, **43**, 2829–2840.
49. Burmann,B.M. and Rosch,P. (2011) The role of E. coli Nus-factors in transcription regulation and transcription:translation coupling: From structure to mechanism. *Transcription*, **2**, 130–134.
50. Eisenmann,A., Schwarz,S., Prasch,S., Schweimer,K. and Rosch,P. (2005) The E. coli NusA carboxy-terminal domains are structurally similar and show specific RNAP- and lambdaN interaction. *Protein Sci.*, **14**, 2018–2029.
51. Gopal,B., Haire,L.F., Gamblin,S.J., Dodson,E.J., Lane,A.N., Papavinasasundaram,K.G., Colston,M.J. and Dodson,G. (2001) Crystal structure of the transcription elongation/anti-termination factor NusA from Mycobacterium tuberculosis at 1.7 A resolution. *J. Mol. Biol.*, **314**, 1087–1095.
52. Shin,D.H., Nguyen,H.H., Jancarik,J., Yokota,H., Kim,R. and Kim,S.H. (2003) Crystal structure of NusA from Thermotoga maritima and functional implication of the N-terminal domain. *Biochemistry*, **42**, 13429–13437.
53. Worbs,M., Bourenkov,G.P., Bartunik,H.D., Huber,R. and Wahl,M.C. (2001) An extended RNA binding surface through

arrayed S1 and KH domains in transcription factor NusA. *Mol. Cell*, **7**, 1177–1189.
54. Geszvain,K., Gruber,T.M., Mooney,R.A., Gross,C.A. and Landick,R. (2004) A hydrophobic patch on the flap-tip helix of E.coli RNA polymerase mediates sigma(70) region 4 function. *J. Mol. Biol.*, **343**, 569–587.
55. Kuznedelov,K., Minakhin,L., Niedziela-Majka,A., Dove,S.L., Rogulja,D., Nickels,B.E., Hochschild,A., Heyduk,T. and Severinov,K. (2002) A role for interaction of the RNA polymerase flap domain with the sigma subunit in promoter recognition. *Science*, **295**, 855–857.
56. Murakami,K.S. and Darst,S.A. (2003) Bacterial RNA polymerases: the wholo story. *Curr. Opin. Struct. Biol.*, **13**, 31–39.
57. Twist,K.A., Campbell,E.A., Deighan,P., Nechaev,S., Jain,V., Geiduschek,E.P., Hochschild,A. and Darst,S.A. (2011) Crystal structure of the bacteriophage T4 late-transcription coactivator gp33 with the beta-subunit flap domain of Escherichia coli RNA polymerase. *Proc. Natl Acad. Sci. U.S.A.*, **108**, 19961–19966.
58. Mekler,V., Kortkhonjia,E., Mukhopadhyay,J., Knight,J., Revyakin,A., Kapanidis,A.N., Niu,W., Ebright,Y.W., Levy,R. and Ebright,R.H. (2002) Structural organization of bacterial RNA polymerase holoenzyme and the RNA polymerase-promoter open complex. *Cell*, **108**, 599–614.
59. Murakami,K.S., Masuda,S. and Darst,S.A. (2002) Structural basis of transcription initiation: RNA polymerase holoenzyme at 4 A resolution. *Science*, **296**, 1280–1284.
60. Nickels,B.E., Garrity,S.J., Mekler,V., Minakhin,L., Severinov,K., Ebright,R.H. and Hochschild,A. (2005) The interaction between sigma70 and the beta-flap of Escherichia coli RNA polymerase inhibits extension of nascent RNA during early elongation. *Proc. Natl Acad. Sci. U.S.A.*, **102**, 4488–4493.
61. Vassylyev,D.G., Sekine,S., Laptenko,O., Lee,J., Vassylyeva,M.N., Borukhov,S. and Yokoyama,S. (2002) Crystal structure of a bacterial RNA polymerase holoenzyme at 2.6 A resolution. *Nature*, **417**, 712–719.
62. Nickels,B.E., Roberts,C.W., Roberts,J.W. and Hochschild,A. (2006) RNA-mediated destabilization of the sigma(70) region 4/beta flap interaction facilitates engagement of RNA polymerase by the Q antiterminator. *Mol. Cell*, **24**, 457–468.
63. Perdue,S.A. and Roberts,J.W. (2011) Sigma(70)-dependent transcription pausing in Escherichia coli. *J. Mol. Biol.*, **412**, 782–792.
64. Ko,D.C., Marr,M.T., Guo,J. and Roberts,J.W. (1998) A surface of Escherichia coli sigma 70 required for promoter function and antitermination by phage lambda Q protein. *Genes Dev.*, **12**, 3276–3285.