Nascent RNA length dictates opposing effects of NusA on antitermination

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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 82Q 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 (σ70). 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 σ70, 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...
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 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′-CAGGGTTTTCCCCAG 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 promoter region (–90 to +30), including the QBE and pause element (see Supplementary Figure S1 for templates sequences). The promoter-proximal walk template (see Figure 1D) bore inactivating mutations in the upstream QBE and the promoter-proximal pause element (see Supplementary Figure S1). The promoter-distal walk template (see Figure 1D) bore inactivating mutations in the upstream QBE and the promoter-distal pause element (see Supplementary Figure S1).

#### Proteins

His-tagged wild-type σ70 and σ70-L402F were purified essentially as described after overproduction from plasmid pHHN12-His-σ70 (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 clearest 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 EDTA, 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 clearest 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 Epi-centre (Madison, WI, USA) and the ΔQCTD core RNAP was a kind gift from Seth A. Darst (Rockefeller University). RNase H was purchased from Invitrogen.

#### In vitro transcription assays

Two nanomolar transcription template DNA was incubated with 8 nM RNAP holoenzyme (prepared by mixing core RNAP with a 5-fold molar excess of σ70-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-32P]-UTP; Perkin Elmer) and the initiating dinucleotide (ApG [32P]-

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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 σ70 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 σ70, transcription was initiated with RNAP holoenzyme containing the pause-deficient mutant σ70 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 σ70 (or a buffer-only control) and 82Q (or a buffer-only control); σ70 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 70 (rather than 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 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 70 (2).
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 cisacting elements to facilitate Q loading. 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.

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
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 σ70 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 on 82Q function 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-α-CTD interaction

NusA participates in a well-characterized interaction with the C-terminal domain of the α subunit of RNAP (α-CTD) 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 α-CTD. To test this, we performed transcription assays using a mutant RNAP core enzyme that lacks the α-CTD (Δα-CTD RNAP). We found that the use of Δα-CTD 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 α-CTD. The requirement for the α-CTD 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 σ70-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 a manner so as to preclude 82Q from binding the complex. Alternatively, NusA might indirectly prevent 82Q from engaging the TEC by disrupting interactions of σ70 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 σ70-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 NusA/β-flap and the NusA/82Q–flap interactions. (A) Cartoon depicts how the interaction between 82Q or the NusA NTD (fused to the β-NTD) and the β-flap (fused to the bacteriophage CI protein) activates transcription from test promoter p<sup>lac</sup>OL2-62, which bears the operator OL2 centred 62 bp upstream of the start site of the lac core promoter. In reporter strain FW102 O2-62, test promoter p<sup>lac</sup>OL2-62 is located on an F' episome and upstream of a <sup>lac</sup>Z reporter gene. (B, C) Results of β-galactosidase assays (see Supplemental Methods) performed with reporter strain cells containing one plasmid that encoded the NusA NTD (B) or 82Q (C) fusion protein and a compatible plasmid that encoded a wild-type CI-β-flap fusion protein (WT), or the indicated CI-β-flap fusion protein variant (ΔFTH refers to a CI-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 CI-β-flap protein levels from normalized samples of cell lysates from (B) that were processed for western blotting with an anti-CI antibody (a generous gift from J. Beckwith) or an anti-RpoA antibody (Neoclone) as a loading control.

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 Q protein (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 σ70, 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 σ70 domain 4 and the β-flap, facilitating the complete displacement of σ70 domain 4 from the flap (58–61). In particular, in the context of the paused TEC at promoter-proximal pause, the nascent RNA-mediated destabilization of the σ70 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 2PR, the interaction of σ70 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 σ70 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 σ70-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 σ70 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 σ70 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 α70-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 α70 (αCTD) 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.

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