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The NusA N-terminal domain is necessary and sufficient for enhancement of transcriptional pausing via interaction with the RNA exit channel of RNA polymerase

Kook Sun Ha¹, Innokenti Touloukhonov^{2,†}, Dmitry G. Vassilyev³, and Robert Landick^{2,4,*}

¹Department of Biomolecular Chemistry, University of Wisconsin-Madison, Madison, WI 53706

²Department of Biochemistry, University of Wisconsin-Madison, Madison, WI 53706

³Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, AL 35294

⁴Department of Bacteriology, University of Wisconsin-Madison, Madison, WI 53706

Summary

NusA is a core regulator of transcript elongation that is well conserved in bacteria and archaea. NusA interacts with elongating complexes and the nascent RNA transcript in ways that stimulate pausing and termination but can be switched to anti-pausing and anti-termination by other accessory proteins. The regulatory complexity of NusA likely depends on the presence of multiple discrete NusA domains, but it remains unclear which NusA domains possess which regulatory activity and how they interact with elongating RNA polymerase. We used a series of truncated NusA proteins to measure the effect of the NusA domains on transcriptional pausing and termination. We find that the N-terminal domain of NusA is necessary and sufficient for enhancement of transcriptional pausing and the other NusA domains contribute to NusA binding to elongating complexes. Stimulation of intrinsic termination requires higher concentrations of NusA, and involves both the N-terminal domain and other NusA domains. Using a tethered chemical protease in addition to protein-RNA crosslinking, we show that the NusA N-terminal domain contacts the RNA exit channel of RNA polymerase. Finally, we report evidence that the NusA N-terminal domain recognizes duplex RNA in the RNA exit channel.

Keywords

NusA; RNA polymerase (RNAP); transcription regulation; pausing; termination

Introduction

NusA is a conserved, multifunctional regulator of transcript elongation by RNA polymerase (RNAP) in bacteria and archaea. The *nusA* gene was discovered through mutations in *E. coli* that prevent λ N-dependent bacteriophage λ growth.¹ *E. coli* NusA alone enhances pausing,^{2–6} enhances intrinsic termination,^{2; 5} modulates π -dependent termination,^{7–13} and interacts with error-prone DNA polymerase IV.^{14; 15} However, NusA also helps suppress pausing and termination in a process called antitermination, when bound to elongating complexes (ECs) together with NusB, NusE, NusG, and one or more other regulators (*e.g.*, λ N).^{7; 16–20} In *E. coli*, tight binding of NusA to RNAP in antitermination complexes

*To whom correspondence should be addressed. Fax: 608-262-9865; landick@bact.wisc.edu.

[†]Present address: I Touloukhonov, Health and Environmental Sciences, Dow Corning Co., Midland, MI 48686

appears restricted to the ribosomal RNA operons. However, *E. coli* NusA can be crosslinked to RNAP *in vivo* in all active transcription units once σ is released;²¹ thus, NusA must maintain a near universal equilibrium association with ECs.

NusA is a multidomain protein (Fig. 1a).^{22–24} *E. coli* NusA is composed of an N-terminal domain (NTD), three RNA-binding domains (S1, KH1, KH2 domains), and two C-terminal acidic repeat domains (AR1 and AR2). The NTD interacts with RNAP, likely with the β or β' subunit, whereas the AR domains contact the C-terminal domain of the RNAP α subunit (α CTD).^{25–28} The AR domains are present in most γ -proteobacteria, but are absent in archaea and most bacterial lineages and are dispensable for growth of *E. coli*.²⁹

Elucidation of the roles of NusA domains in its different regulatory activities and their sites of contact to the EC is crucial to understanding the mechanism of NusA action. In antitermination complexes, stable incorporation of NusA requires a network of contacts that includes the NusA CTD, KH, and S1 domains, as well as contacts to both nascent RNA and to RNAP.^{26; 30; 31} This interaction network appears to sequester the pause- and termination-enhancing functions of NusA. At least in the case of λ Q antitermination complexes, these interactions help NusA create an extended RNA exit channel that shields the nascent transcript against formation of secondary structures that can promote termination or pausing.³²

However, the roles of NusA domains in the pause- and termination enhancing activities of NusA and their contacts to non-antitermination-modified ECs are less clear. The magnitude of NusA effects at different pause and termination sites varies greatly, and the concentrations of NusA required to affect termination are typically higher than those required to affect pausing.^{33–35} Among pause sites, the greatest NusA stimulation occurs when a nascent RNA structure (termed a pause hairpin) stabilizes the paused state.³⁶ Among hairpin-stabilized pauses, NusA may have lesser effects when the pause hairpin is more stable (*e.g.*, contains an RNA tetraloop).^{37; 38} Much is known about the hairpin-stabilized *his* pause that synchronizes transcription and translation in the *his* operon leader region. At the *his* pause, RNAP forms an off-line paused transcription complex (PTC) in which an active-site rearrangement inhibits nucleotide addition.³⁹ The PTC is stabilized by multiple interactions that include that of the pause hairpin with the RNAP exit channel. NusA likely increases pause dwell time by interacting with the pause hairpin loop and a portion of the RNAP RNA channel called the β flap-tip helix.^{38; 40}

We show here that all of the pause-enhancing effects of NusA (and much of the intrinsic termination-enhancing effects) reside in the NusA NTD. Two sites on RNAP for interaction of NusA NTD have been proposed. Borukhov *et al.*⁴¹ suggest that NusA NTD interacts with the β' clamp helices that bind σ^{70} region 2; this is consistent with the competitive binding of NusA and σ^{70} to RNAP⁴² and with NusA-tethered FeBABA cleavage of the region of RNAP.²⁷ Yang *et al.*⁴³ propose that *B. subtilis* NusA NTD interacts instead with the β -flap tip that binds σ^{70} region 4. In a similar model, Vassilyev⁴⁴ proposes that NusA NTD interacts with the β flap-tip helix by displacement of the N-terminal α -helix of NusA. Interaction of NusA NTD with the β flap is consistent with the requirement for an intact β flap-tip helix for NusA enhancement of pausing and with crosslinking to NusA of a nascent RNA pause hairpin known to contact the β flap tip.³⁸ Based on the mapping of RNAP polypeptide cleavages caused by FeBABA tethered to specific sites on NusA NTD and crosslinking of RNA to NusA, we provide unambiguous evidence that the *E. coli* NusA NTD contacts the RNA-exit channel as proposed for *B. subtilis* NusA and RNAP.⁴³ Our experiments with NusA fragments and mutants confirm that this interaction alone enhances transcriptional pausing, whereas other NusA domains help NusA bind ECs.

Results

NusA NTD possesses all pause-enhancing and most termination-enhancing activity of NusA

To determine which domains of *E. coli* NusA are required for enhancement of pausing and termination, we prepared a series of C-terminally truncated NusA proteins (Fig. 1b; Methods): NTD-S1-KH (NusA1-348), NTD-S1 (NusA1-200), and NTD (NusA1-137). First, we measured enhancement of hairpin-stabilized pausing by each NusA fragment using a standard *his* pause template and *in vitro* transcription assay (Fig. 1c; Methods).³⁶ NusA increased the dwell time of RNA polymerase at the *his* pause ~3-fold, with an apparent affinity of ~10 nM (Fig 2a; Table 1). Although removal of NusA AR and KH domains increased the concentration of NusA fragment required for full stimulation, at sufficiently high concentration all fragments including NusA NTD gave ~3-fold enhancement of pause dwell time (Fig. 2b; Table 1). The S1 domain did not appear to contribute to NusA-PTC binding, since pause enhancement by NTD-S1 and NTD exhibited similar concentration dependence.

The ability of the NusA AR domains to increase NusA-PTC avidity is consistent with the known interaction of these domains with the α CTD (Fig. 1a).²⁵⁻²⁸ The KH domains form RNA-binding folds,⁴⁵ co-crystallize with ssRNA,⁴⁶ and are likely positioned near or interacting with nascent RNA upstream of the pause hairpin, regardless of where NTD contacts RNAP. To ask if the contribution of the KH domains to apparent NusA affinity for the PTC involves their interaction with nascent RNA upstream of the RNA hairpin, we tested the ability of the NusA fragments to delay pause escape of reconstituted *his* PTCs that lack RNA upstream of the pause hairpin (Fig. 2c).⁴⁷ Even when upstream RNA was absent, the KH domains decreased the concentration of NusA fragment required for half-maximal pause enhancement by a factor of ~10 (Fig 2d; Table 1). Thus, the effect of the KH domains on pausing must not involve binding to nascent RNA upstream of the pause hairpin.

We next tested the NusA fragments for enhancement of the intrinsic termination using a linear DNA template on which the extensively studied λ_{T2} terminator is located at ~+450 (Fig 2e; Methods).⁴⁸ Similar to the effects on hairpin-stabilized pausing, all NusA fragments were capable of stimulating termination when added to sufficient concentration (Fig. 2f). Mah *et al.* previously reported that NusA fragments similar to NusA NTD and NTD-S1 (NusA1-137 and NusA1-240, respectively) were completely inactive for enhancement of the intrinsic termination (λ_{TR}),²⁶ which is seemingly inconsistent with our results. However, this disparity probably results from the low concentration of NusA (50 nM) used by Mah *et al.*, which is significantly less than the apparent binding constant of NusA NTD (~1 μ M) and NTD-S1 (~2 μ M). Both the AR and the KH domains decreased the concentration required for half-maximal effect, whereas S1 had little effect. Thus, for hairpin-stabilized pausing and intrinsic termination, a sufficient concentration of NusA NTD can give all or much of the effect of NusA.

To compare the effects of NusA and the NusA fragments in the different pause (Fig. 2b and 2c) and termination assays (Fig. 2c), we calculated the K_I and the maximal effects by treating the regulators as noncompetitive inhibitors of nucleotide addition (Figs. 3a and b; Table 1; Methods).⁴⁰ Consistent with earlier reports, {Gill, 1991 #133; Whalen, 1988 #134; Sigmund, 1988 #135} the half-maximal effects on intrinsic termination required 10–50 times higher concentrations than half-maximal effects on the *his* pause. However, the pattern and approximate magnitude of these effects resembled that observed for the *his* pause. For both pause assays, high concentrations of NTD gave the full effect. Even high concentrations of NTD, however, only partially recapitulated the maximal effect of full-length NusA on termination (~3-fold enhancement vs. ~6-fold for full-length NusA). This

partial loss of termination-enhancement was attributable to partial contributions of the AR, KH, and S1 domains (Fig. 3b). Given the relatively high concentrations of NTD-S1 and NTD required for maximal effect on termination, we cannot rule out the possibility that aggregation of the NusA fragments prevents them from achieving full activity at high concentration.

We conclude that (1) all the interactions of NusA required to enhance hairpin-stabilized pausing and at least a significant portion on the interactions that enhance intrinsic termination are made by the NTD of NusA; (2) the AR, KH, and S1 domains contribute principally to EC binding rather than to pause- or termination-enhancement *per se*, and (3) at least for pausing, this contribution to NusA-EC binding does not involve interaction with nascent RNA upstream of the pause hairpin.

Domain-disrupting substitutions confirm the key role of NusA NTD in pause enhancement

The use of N-terminal protein fragments precluded testing the effects of individual NusA domains when all NusA domains are present. To circumvent this limitation, we prepared variant NusA proteins in which amino-acid substitutions would disrupt the function of KH1, KH2, S1, or NTD. For KH1 and KH2, we targeted the perfectly conserved GxxG motif that is the hallmark of KH domains⁴⁹ with substitutions designed to disrupt the KH fold (G253A, G256A in KH1; G319A, G322A in KH2; Fig. 1a and b). G253D and G319D substitutions in KH1 and KH2 were shown previously to interfere with RNA binding by NusA; the KH1 substitutions also compromised λ N antitermination and cellular function.³¹ For S1, we targeted conserved residues in the hydrophobic core with substitutions designed to disrupt proper folding of the domain (L152D, L154D). For NTD, we similarly targeted residues in the hydrophobic core, but with individual substitutions (V9E and L27E) in full-length NusA and with an N-terminal deletion (Δ 1-18) in isolated NusA NTD.

Initially, we tested pause-enhancement by the mutant NusA proteins at a subsaturating NusA concentration (100 nM) that would be sensitive to significant changes in either NusA-PTC affinity or the intrinsic pause-enhancing activity of NusA (Fig. 3c). In this assay, the KH1 and KH2 mutants increased pause dwell times similarly to wild-type NusA, supporting the view that interactions of the KH domains with the nascent RNA or with RNAP are not important for the pause enhancement by NusA (Fig. 3c; Table 1). In contrast, the S1 and NTD mutants exhibited significant defects in NusA action (Fig. 3c; Table 1). To test whether this defect was caused by weakened binding of NusA to the PTC or reduced intrinsic activity of NusA, we measured pause lifetimes at increasing NusA concentrations using reconstituted PTCs lacking upstream RNA (Fig. 2d; Table 1). The S1 mutant (L152D, L154D), compared to the wild-type NusA, showed ~10-fold higher apparent K_I (32 *versus* 3.5 nM) but a similar maximal pause lifetime (130 *versus* 137 s; Table 1). In contrast, we could detect no significant activity of NusA(L27E) or NusA NTD(Δ 1-18) even at high concentrations. We conclude that, in the context of full-length NusA, (1) the RNA-binding surfaces of KH1 and KH2 make little or no contribution to NusA-enhancement of pausing; (2) disruption of the S1 affects NusA binding affinity, suggesting it contacts the PTC in intact NusA; and (3) all pause-enhancing activity of NusA resides in the NTD.

The concave surface of NusA NTD interacts with the RNA exit channel

Given our prior demonstration that the β flap-tip helix (*Ec* β 900-909) at the mouth of the RNA-exit channel is essential for pause-enhancement by NusA,³⁸ the recent evidence that *B. subtilis* NusA NTD interacts with the β -flap tip,⁴³ and our finding here that the NTD of NusA is sufficient for pause enhancement, it seemed likely that NusA NTD interacts with the RNA exit channel. To reconcile these data with the proposal that *E. coli* NusA NTD interacts with the β' clamp helices⁴¹ and to determine which face of NusA NTD interacts

with RNAP, we examined cleavage of RNAP by free radicals generated from Fe-EDTA tethered to opposite sides of the NusA NTD.

We used the tethered $\cdot\text{OH}$ -generating reagent Fe(III)-(*S*)-1-(*p*-bromoacetamido-benzyl)-EDTA (FeBABE), which can be conveniently attached to Cys residues and cleaves nucleic acids or proteins in its vicinity when $\cdot\text{OH}$ -generation is activated.⁵⁰ We first engineered Cys-less NusA by conversion of C251, C454, and C489 to Ser. We then introduced single Cys substitutions at two non-conserved, surface exposed Ser residues on opposite faces of the NTD (S29C and S53C; Fig. 1a). All three mutant NusAs (Cys-less, S29C, and S53C) were treated with FeBABE. After removal of excess FeBABE by dialysis, the resulting FeBABE-conjugated NusAs were as active as wild-type NusA in pause-enhancement (Supplemental Fig. 1). To analyze cleavage sites on RNAP, we used recombinant *E. coli* RNAP containing β or β' subunits bearing N- or C-terminal ³²P-labeled HMK tags. We tested the effects of each mutant NusA protein on each ³²P-end-labeled RNAP separately, using identical reaction conditions to generate $\cdot\text{OH}$ (Methods).

S29C FeBABE-NusA caused unambiguous, NusA-dependent cleavage of both β - and β' ³²P-labeled RNAPs, whereas no NusA-dependent cleavage was observed for either Cys-less or S53C NusA (Fig. 4 and Supplemental Fig. 2). This result strongly argues that the concave surface of NusA NTD bearing S29 (facing up in Fig. 1a), but not the opposite convex surface of NTD bearing S53 (facing down in Fig. 1a), is oriented toward RNAP upon NusA binding.

To determine the location of NTD interaction with RNAP, we analyzed the cleavage sites on the β and β' subunit by SDS-PAGE of the fragments generated by S29C-tethered FeBABE-NusA to those caused by treatment with CNBr or NTCB, which cleave at Met and Cys respectively. Four sites on β exhibited S29C-dependent cleavage (X1-X4; 459 ± 56 , 772 ± 14 , 845 ± 20 , and 918 ± 14 , respectively; Fig. 4a). A single cleavage site, X5, corresponding to 386 ± 12 , was observed on β' (Fig. 4b; Methods). X3 on β and X5 on β' were dependent on the presence of S29C-FeBABE NusA and on activation of $\cdot\text{OH}$ production. X1, X2, and X4 were likely caused by nonspecific free-radical cleavage because they were evident with Cys-less NusA (Fig. 4a) and were not detected with β N-terminal ³²P-labeled RNAP (Supplemental Fig. 2). The X3 and X5 cleavages are adjacent to the RNA exit channel in the β flap and β' dock domains (Figs. 5a and 5b). Indeed, these segments of the flap and dock interact with one another in the *T. thermophilus* EC crystal structure, with direct contacts of $\beta'R675$ - $\beta'L729/730$, $-\beta'R679$ - $\beta'A733$, and $-\beta'R681$ - $\beta'D636$ (equivalent to *Ec* $\beta'R675$ - $\beta'L729/730$, $-\beta'R679$ - $\beta'A733$, and $-\beta'R681$ - $\beta'D636$).⁵¹

NusA NTD-exit channel interactions persist in the *his* PTC

These results suggest that the NusA NTD interacts with the RNA exit channel of RNAP in the absence of nucleic acids since the flap domain and the clamp domain are parts of the RNA exit channel. To ask whether this interaction persists or is altered in ECs and PTCs, we compared the FeBABE cleavage patterns of RNAP alone to that of PTCs formed on scaffolds with and without a pause hairpin (Fig. 2c, Methods). In these experiments, we observed the same cleavages of the β flap and β' dock observed in free RNAP (X3 and X5; Supplemental Fig. 3). Thus, the interaction of the NusA NTD with RNAP does not appear to be affected by the presence of RNA in the RNA exit channel, including the presence of a pause RNA hairpin.

NusA NTD contacts or binds near the *his* pause hairpin

To test the idea that the concave face of NusA NTD interacts with the RNA exit channel in the vicinity of the pause hairpin, we performed two experiments to map the interaction of

NusA NTD with the pause hairpin. First, we examined whether the NusA(S29C)-FeBABE cleaves the nascent RNA. To this end, PTCs containing [$3'$ - ^{32}P]pause RNA were prepared (Methods) and the RNA cleavage sites generated by localized $\cdot\text{OH}$ were mapped by denaturing gel electrophoresis (Fig. 6a left panel). Cleavage occurred near position -21, which corresponds to the loop of the pause hairpin when NusA(S29C) was used, but no cleavage occurred when NusA(S53C) was used or when NusA was absent (Fig. 6a right panel).

Second, we examined the ability of NusA or NusA fragments to protect the pause hairpin from cleavage by RNase T1. The loop of the *his* pause RNA has been shown to be susceptible to RNase T1 and NusA was shown previously to protect the loop of the *trp* pause hairpin from RNase T1 cleavage.^{40; 52} In the absence of NusA, RNase T1 cleaved the loop of the *his* pause hairpin as expected (G21; Figs. 6b and 6c). NusA and all the NusA fragments inhibited the rate of G21 cleavage by RNase T1 (Figs. 6c and 6d). The extent of protection decreased upon removal of the KH domains, consistent with their contribution to overall affinity of NusA for the *his* PTC.

Taken together, these results confirm that NusA NTD interacts with the RNA exit channel in immediate proximity to the β flap, β' dock, and pause RNA hairpin. To visualize these interactions, we constructed an improved model of the pause hairpin in the RNA exit channel using the location of exiting RNA in the *T. thermophilus* EC crystal structure and an NMR structure of a 5-bp stem 8-nt loop RNA from the human PGY/MDR1 mRNA (PDB IDs 2o5i and 2gvo, respectively; Methods; Fig. 5c).^{51; 53} We refrained from modeling the direct contact of NusA NTD with the exit channel as the limited number of distance constraints allows too many possible solutions. Nonetheless, the approximate positioning of NusA NTD suggests that the acidic head domain of NusA NTD (head, Fig. 5c)²⁴ could be positioned to make contact with the basic charges on the zinc-binding domain in the β' clamp (ZBD, Fig. 5c). Additionally, modeling of the pause hairpin suggests the loop region would be protected by the NusA NTD, consistent with the observed $\cdot\text{OH}$ cleavage from FeBABE tethered to NusA(S29C) and the observed protection against RNase T1 digestion. Finally, the model predicts a plausible path by which the RNA upstream from the pause hairpin may exit RNAP under the β flap domain (Fig. 5c).

NusA-nascent RNA crosslinking confirms an RNA exit path across KH2

Based on a crystal structure of *M. tuberculosis* NusA complexed with a portion of antitermination sequence (an 11mer mimic of the *rm boxAC* spacer RNA) that is thought to retain interaction with NusA during transcript elongation, Beuth *et al.*⁴⁶ proposed a specific set of contacts between exiting RNA and the NusA KH domains. To ask if RNA exiting *E. coli* ECs and the *his* PTC are consistent with this proposal, we mapped contacts between exiting RNA and NusA using the photoactive nucleotide analog 4-thio-uridine (4-thioU). 4-thioU and [α - ^{32}P]CMP were incorporated into the nascent RNA by halting ECs using stepwise transcription, reacting the halted ECs with 4-thioUTP and [α - ^{32}P]CTP, washing to remove these NTPs, and then advancing the ECs to different locations along the RNA with additional rounds of stepwise transcription (Methods; Supplemental Figs. 4a and b). The halted ECs were then crosslinked to excess NusA (2 μM) by UV irradiation. The resulting ^{32}P -labeled NusA-RNA conjugates were briefly treated with RNase A and were analyzed by SDS-PAGE (Methods; Supplemental Fig. 4c). 4-thioU at -19, -23, -34, or -40 in ECs crosslinked to the NTD as well as the full-length NusA. These results suggest that 4-thioUs at these positions are in the vicinity of the NTD. However, they do not indicate that the RNA transcripts at these positions interact with the NTD in the full-length NusA because we can not rule out that 4-thioU might contact the NTD nonspecifically in the absence of normal binding sites (*e.g.* the RNA binding domains). To address this issue, we mapped the crosslinking sites in the full-length NusA using partial CNBr cleavage (Methods;

Supplemental Fig. 4d). The ^{32}P -labeled NusA-RNA conjugates were eluted from the gel and then subjected to partial CNBr cleavage. The resulting fragments were analyzed by SDS-PAGE. When 4-thioU was present at -16, -19, or -23 is ECs, crosslinks occurred to the NusA NTD (Fig. 7; Supplemental Fig. 4d). When 4-thioU was present at -34 or -40, the crosslink shifted to KH2 in a fragment overlapping the KH2-RNA contacts reported by Beuth *et al.*⁴⁶ When 4-thioU was instead placed in the loop region of the *his* pause hairpin (-16-18), crosslinking to the NusA NTD was detected (Fig. 7; Supplemental Fig. 4e). The path of exiting RNA mapped by these crosslinks is entirely consistent with a view that the NusA NTD interacts with the RNA exit channel, the pause hairpin loop, and exiting RNA out to ~nt 23, whereas the KH2 domain interacts with exiting RNA upstream from these positions (see Discussion).

Pause hairpin function requires an RNA:RNA duplex in the RNAP exit channel

Given the apparent interaction of the NusA NTD with the pause hairpin loop, we wondered if specific loop structures are required for maximal NusA effects on transcript elongation. The magnitude of NusA effects on both the lifetime of pauses and the efficiency of termination varies significantly. For instance, replacement of the 8-nt *his* pause hairpin loop with a highly structured “CUUG” tetraloop greatly diminishes the effect of NusA on pause lifetime.^{37; 38} To investigate how loop structure affects NusA function, we examined the ability of a “loop-less” RNA duplex to substitute for the *his* pause hairpin in a pausing assay with reconstituted ECs. An RNA structure was required for NusA enhancement of pausing, consistent with previous findings; however, an 8-bp RNA:RNA duplex created by pairing of an 8mer oligoribonucleotide proved to be an even better substrate for NusA action than a stem-loop structure (Fig. 8). Further, this effect was fully recapitulated by NusA NTD alone. We conclude that NusA has no requirement for a particular loop structure or indeed any single-stranded loop at all. Rather, NusA NTD appears to interact best with a simple A-form duplex.

Discussion

With six linearly arranged, independently folding domains, *E. coli* NusA is an unusual transcription regulator. Each NusA domain (NTD, S1, KH1, KH2, AR1, and AR2) may contact different parts of the EC, creating a complex network of both physical and regulatory interactions. This complexity of interactions undoubtedly underlies the remarkable diversity of NusA effects that include stimulation of intrinsic pausing and termination,^{2; 5; 7; 33; 35; 54–56} enhancement of RNA folding,⁵⁷ antitermination when partnered with other regulators,^{1; 19; 20; 58–60} and stimulation of both translesion DNA synthesis¹⁴ and stress-induced mutagenesis.¹⁵ Here we establish that the NusA NTD alone stimulates pausing and termination through an interaction with the RNA exit channel of RNAP. This finding establishes that NusA can modulate RNAP activity via the interactions of just one of its six separate domains.

NusA NTD activity and interaction with the RNAP exit channel

The biochemical effects of fragments and mutants of NusA establish that the NusA NTD alone possesses all of the pause- and much of the termination-stimulatory activities of NusA. Successive truncations that remove an increasing number of NusA domains starting from the C-terminus increase the concentration of NusA fragment required to stimulate hairpin-stabilized pausing and intrinsic termination, but do not reduce the maximal effect on pausing (Figs. 2b, d and f; Fig. 3a, and 3b). Thus the effect of NusA on pausing is caused solely by interactions of the NTD with the PTC. This conclusion was confirmed by the loss of pause-stimulatory activity at even high concentrations of NusA(L27E) or NusA NTD(Δ 1-18) (Fig. 2d).

The contributions of NusA domains to stimulation of intrinsic termination appear to differ from the exclusive effect of the NTD on pausing. Although C-terminal truncations increase the concentration required for maximal effect in the same pattern observed for termination, the absolute concentrations required for maximal effects on termination were consistently greater by a factor of ~10 (Fig. 3a). Further, successive removal of domains from the C-terminus progressively decreased the maximal stimulation of termination while having minimal or no effects on the maximal stimulation of pausing (Fig. 3b). Intrinsic termination occurs in a multistep process, during which the EC passes through a state of partial terminator hairpin formation that closely resembles the geometry of the *his* PTC (hairpin stem 11 nt from the RNA 3' end). This is followed by a rate-limiting extension of the hairpin stem toward the RNA 3' end that extracts the nascent RNA from the EC RNA:DNA hybrid.⁶¹ This bipartite formation of terminator hairpins may explain the pattern of effects of NusA fragments. The effect of the NTD could reflect stabilization of the partial terminator hairpin intermediate via mechanisms similar to that seen on pausing, whereas the S1, KH, and AR domains may assist completion of the hairpin stem and extraction the nascent RNA. Alternatively, the very high concentrations of NusA NTD required to stimulate termination may mask detection of its full termination-stimulatory activity, due either to failure to saturate the effect of NusA NTD or to aggregation of NusA NTD at high concentration.

The pause-enhancing activity of NusA NTD must be caused by interactions of the concave surface of the NusA NTD with the RNA exit channel and exiting RNA. Cleavage of the β flap, the β' dock, and the loop of the *his* pause hairpin by $\cdot\text{OH}$ generated from FeBABE attached to NusA C29, but not to NusA C53, dictate this conclusion (Figs. 4 and 6). Further, interaction of NusA NTD with RNAP as well as the effect of NusA on transcriptional pausing require the RNAP β flap-tip helix.^{38; 43} The charge distribution of NusA NTD also favors orientation of the NTD concave surface toward exiting RNA. The NTD concave faces of both *T. maritima*²⁴ and *E. coli* (data not shown) NusA are neutral with patches of positive charge, whereas the convex surfaces bears strong negative charge. Although the NTD can be plausibly docked to the RNAP RNA exit channel (*e.g.*, see Refs. 43; 44), the available data are insufficient to distinguish among possible models for NTD-RNAP interaction. Nonetheless, the constraints imposed by the β flap, β' dock, pause hairpin-loop $\cdot\text{OH}$ cleavages unambiguously rule out the proposed interaction of NusA NTD with the β' clamp helices.⁴¹ $\cdot\text{OH}$ is incapable of penetrating through a protein⁶²; thus, the proposed location of NusA S29 near β' clamp helices⁴¹ cannot be reconciled with the observed cleavage of the β' dock because the β' dock is separated from the β' clamp helices by the intervening flap domain. The long-established competition of NusA and σ^{70} from interaction with RNAP⁴² must occur by competition of the NusA NTD and σ^{70} region 4 for binding to the β flap-tip helix.

NusA orientation set by NTD-RNAP flap/dock proximity explains several NusA effects

Placement of the NTD against the RNA exit channel coupled with the known interaction of the NusA AR domains with the αCTD ²⁵ dictates an orientation of NusA across the surface of RNAP that is consistent with other several other observations (Fig. 9). The KH2 domain of *M. tuberculosis* NusA interacts with the *rrn boxAC* spacer RNA across the same KH2 face that we found crosslinked to -34 to -40 of exiting RNA (Fig. 7).⁴⁶ Connecting the modeled position of this upstream RNA-KH2 segment with the end of the nascent RNA observed in the RNA exit channel of an *T. thermophilus* EC crystal structure⁵¹ generates a plausible path for RNA across the surface of NusA and across the β' dock domain (Fig. 9). Consistent with this model, Andrecka *et al.* map the path of RNA exiting yeast RNAPII using FRET triangulation such that RNA -26 to -29 is in contact with the dock domain.⁶³

This placement of NusA also is consistent with a genetic interaction of the NusA S1 domain with the β' dock domain. Ito *et al.*⁶⁴ find that the most common β' suppressor of

temperature sensitivity caused by the *nusA11*(G181D) mutation is β' E402K in the dock domain directly adjacent to the site at which we mapped β' cleavage by NusAC29-FeBABE (β' 378-294). Thus, the resultant suggestion that the S1 domain interacts with the β' dock domain is consistent with the predicted placement of NusA (Fig. 9) as well as with our finding that S1 contributes to NusA affinity for the *his* PTC (Fig. 2d).

Finally, this placement of NusA suggests that NusA would shield a segment of RNA from about -17 to -30 from interaction with macromolecules. Indeed, Shankar *et al.* report that NusA shields nascent RNA from RNaseA digestion at positions -19 to -27 when NusA binding is stabilized by the Q⁸² antitermination protein.³² NusA alone may interact with exiting RNA too transiently to generate strong protection, but protection of this RNA segment may become strong when NusA interactions with an EC are stabilized.

Taken together these observations and our findings support a model of NusA interaction that places the NusA across a surface of RNAP that spans from the NusA NTD interacting with the RNA exit channel, to the S1 and KH domains interacting with and adjacent to the β' dock domain, respectively, to the AR domains interacting with the α CTDs (Fig. 9).

Does NusA promote effects of nascent RNA structure directly or indirectly?

A remaining question is how NusA promotes the effects of nascent RNA hairpins, either in stabilizing pauses or in stimulating intrinsic termination. Gusarov *et al.* argue that NusA acts indirectly by displacing upstream single-stranded RNA from the RNAP surface, thereby promoting hairpin formation.³⁵ A simple alternative is that NusA directly stabilizes pause and terminator hairpins through contacts to duplex RNA. Given the complexity of effects that can result from interactions of NusA, RNA, and RNAP, ranging from promoting nascent hairpin formation to variable extents to inhibiting hairpin formation,^{32; 33; 54} it is unlikely these models can be distinguished until high resolution crystal structures of EC-NusA complexes that capture these various effects are obtained. Further, it seems plausible that both direct and indirect effects could be involved.

However, for the simpler case of how the NusA NTD increases the effect of nascent RNA pause hairpins, our data are most consistent with a direct effect on RNAP structure. When the stability of the *his* pause hairpin is increased by replacement of the 8-nt loop with a CUUG tetraloop structure, the effect of NusA on pause lifetime is reduced (from ~2.5x to ~1.3x).^{37; 38} This could reflect either direct or indirect pause hairpin stabilization by NusA. However, when the *his* pause hairpin is stabilized by extension of the hairpin stem from 5 bp to 8 bp, either with³⁸ or without (Fig. 8) a hairpin loop, the effect of NusA increased (from ~2.5x to ~6x). If NusA were acting simply to promote formation of the hairpin stem, there is no obvious reason that the effect of NusA would be increased by lengthening the stem. Further, the effect of NusA is about the same whether or not RNA is present upstream of the pause hairpin.⁴⁷ This is inconsistent with the indirect model of NusA action, since competition with hairpin formation should be less when upstream RNA is available to replace RNAP contacts made by the 5' arm of the pause hairpin. Taken together, these results suggest NusA increases the effect of the pause hairpin by enhancing the effect of the hairpin on the structure of ECs, by altering the position of the clamp domain as suggested earlier,^{38; 39; 52} by inhibiting translocation of nascent RNA, or by a combination of these effects.

Finally, our finding that NusA acts directly on RNA duplex stem of the pause hairpin rather than on the pause hairpin loop (Fig. 8) provides a possible explanation for variability in the magnitude of NusA effects on pausing and termination. A hairpin loop is not without structure, and some loops maintain the trajectory of the A-form phosphodiester backbone along at least one arm of the hairpin (*e.g.*, see the 8-nt loop model in Fig. 5c). Thus, some

larger loops may resemble duplex RNA and facilitate NusA NTD interaction. Others, such as the CCUG tetraloop may be too compact for this effect or, in the case of some larger loop sequences, may lack well ordered structures. Thus, the magnitude of the NusA effect could depend on the extent to which the combined stem-loop structure conforms to a A-form helix that may be the preferred ligand for NusA NTD. Here again, high-resolution crystal structures of NusA NTD interacting with a PTC are needed to understand both the nature of the NusA NTD interaction and the mechanism by which it stimulates transcriptional pausing.

Methods

Construction and purification of the HMK-tagged RNAP and NusA proteins

To label RNAPs with ^{32}P , the β and β' subunits were tagged with the recognition sequence (RRASV) for the catalytic domain of heart muscle kinase (HMK). $\beta\text{N-}$, $\beta\text{C-}$, $\beta'\text{N-}$, and $\beta'\text{C-}$ HMK-tagged RNAPs were overexpressed from a T7 RNAP based expression plasmid and purified by chitin-affinity chromatography followed by heparin-affinity chromatography as described previously.⁶⁵ The fragments of NusA (NTD, NTD-S1, NTD-S1-KH, $\Delta(1-18)\text{NTD}$) were amplified by polymerase chain reaction (PCR) and cloned into an overexpression plasmid, pNG5. Cys-less NusA (C251S, C454S, C489S), two single-Cys NusAs (S29C NusA: S29C, C251S, C454S, C489S; S53C NusA: S53C, C251S, C454S, C489S), and the domain disruption mutant NusAs (L152D/L154D, G253A/G256A, G319A/G322A, L27E, and V9E) were constructed by site directed PCR mutagenesis. All NusA proteins contain the N-terminal His₆ tag except $\Delta(1-18)\text{NTD}$, which has the C-terminal His₆ tag and was purified as described previously.⁴⁷

In vitro transcription assays

For the promoter initiated pause assay, ^{32}P -labeled A29 complexes (30 nM) were prepared from template DNA containing T7A1 promoter in transcription buffer (20 mM Tris-HCl, pH 8.0, 20 mM NaCl, 14 mM MgCl₂, 14 mM 2-mercaptoethanol, 0.1 mM EDTA) and then were incubated with NusA for 10 min at 37 °C. Transcription was resumed upon addition of NTPs (200 μM UTP, CTP, and ATP, 10 μM GTP). Aliquots were removed at the indicated times and mixed with 2X loading dye (7 M urea, 90 mM Tris-borate buffer, pH 8.3, 0.02% bromphenol blue, and 0.02% xylene cyanol), and RNA products were separated on denaturing polyacrylamide gel. The reconstituted pause assays were performed as described previously.⁴⁷ Briefly, ECs were reconstituted with DNA scaffolds (t DNA and nt DNA) and RNA27 and were advanced to position C28 (1 nt before the pause) in the presence of [α - ^{32}P]CTP (Table 2). These ECs were incubated with NusA for 10 min at 37°C before resuming transcription upon addition of 100 μM UTP and 10 μM GTP. For termination assays, the linear DNA templates, which contain the T7A1 promoter followed by the λ t_{R2} terminator, were generated by PCR amplification from the plasmid pCPG λ t_{R2}.⁶⁶ Halted A20 complexes were prepared as described previously.⁶⁵ Transcription reactions were incubated at 37 °C for 10 min after addition of all four NTPs (400 μM each) to the halted complex.

Quantification of apparent NusA affinity and maximal stimulation

The ^{32}P -labeled RNA were electrophoresed through denaturing polyacrylamide gels and quantified to measure the rate of the pause escape (k_e) as described previously.⁴⁷ The observed pause escape rate (k_{obs}) in the presence of NusA is the composite rate of free ECs and NusA-bound ECs. Since NusA has been shown to be a noncompetitive inhibitor for nucleotide addition, {Landick, 1987 #132} the observed rate of the pause escape can be described as $k_{\text{obs}} = (k_0 K_I + k_{\text{NusA}} [\text{NusA}]) / (K_I + [\text{NusA}])$, where k_0 , and k_{NusA} are the pause escape rate of free ECs, and NusA-bound ECs respectively. The plot k_0/k_{obs} (relative pause

stimulation) versus [NusA] were fitted using Kaleidagraph (Synergy Software), yielding the apparent K_I and the maximum pause stimulation (k_0/k_{NusA}) (Fig. 2; Table 1).

The termination efficiency (TE) is defined as $TE = k_t / (k_t + k_b)$, where k_t and k_b are the rate of termination and terminator bypass, respectively.⁶⁷ TE was measured from the relative intensity of termination and run-off RNA products: $TE = [\text{termination}] / ([\text{termination}] + [\text{run-off}])$. Relative stimulation of the termination rate ($k_{t,obs}/k_{t0}$) by NusA was calculated assuming NusA affects the termination rate (k_t) but not the bypass rate (k_b): $k_{t,obs}/k_{t0} = TE_{obs}(1 - TE_{obs}) / (TE_0(1 - TE_{obs}))$, where $k_{t,obs}$ and TE_{obs} are apparent termination rate and efficiency at a specific concentration of NusA respectively, and k_{t0} and TE_0 are termination rate and efficiency without NusA, respectively. Similar to the apparent pause escape rate, relative stimulation of the termination rate can be described as $k_{t,obs}/k_{t0} = (K_I + (k_{t,NusA}/k_{t0})[NusA]) / (K_I + [NusA])$, where $k_{t,NusA}$ is the termination rate of NusA-bound TECs. Fitting the plot $k_{t,obs}/k_{t0}$ versus [NusA] yields the apparent binding constant (K_I) and the maximal stimulation ($k_{t,NusA}/k_{t0}$) by NusA.

Tethered FeBABE cleavage

NusA (20 μ M) and FeBABE (400 μ M) were incubated in conjugation buffer (10 mM MOPS (pH 8.0), 1 mM EDTA, 100 mM NaCl, 5% glycerol) at 37 °C for 1 hr and dialyzed against storage buffer (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 100 mM NaCl, 10 mM MgCl₂, 50% glycerol) to remove unconjugated FeBABE. ³²P-labeled RNAP (or EC) and FeBABE-NusA (100 nM each) were incubated at 30 °C for 30 min in cleavage buffer (10 mM MOPS, pH 8.0, 1 mM EDTA, 125 mM NaCl, 10 mM MgCl₂, 10% glycerol) prior to cleavage reaction, which was initiated by adding ascorbate and hydrogen peroxide (final 4 mM each). Cleavage was stopped by adding one volume of 2X SDS sample buffer (125 mM Tris-HCl (pH 6.8), 2% SDS, 20% glycerol, 2% 2-mercaptoethanol) after incubation at 30 °C for 5 min. The cleavage products were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). To map the cleavage site, protein ladders were generated by cleaving ³²P-labeled RNAP using the Met-specific cleavage reagent, cyanogen bromide (CNBr) and the Cys-specific cleavage reagent, 2-nitro-5-thiocyanobenzoic-acid (NTCB) as described previously.³⁸ To map the interaction of NusA NTD with the pause hairpin (Figs 6a–d), ³²P-labeled rPTCs were prepared by two steps. EC27 (2 nt before pause) was first reconstituted from DNA scaffold, RNA27, and RNAP.⁴⁷ The EC27 was then elongated to the pause in the presence of [α -³²P]CTP (rPTC).

RNase T1 Digestion

rPTCs (15 nM) containing ³²P-labeled RNA and NusA or NusA fragments (2 μ M) were incubated at 25 °C for 30 min in transcription buffer. *E. coli* tRNA (final 250 ng/ μ L) was added prior to the cleavage, which was initiated by addition of RNase T1 (final 50 units/mL). The reactions were terminated by addition of equal volumes of 2X loading dye during the time course.

Crosslinking and mapping the crosslinking site on NusA

ECs containing the photoactive nucleotide analog 4-thio-uridine (4-thioU) at various positions on RNA were prepared by stepwise transcription, where ECs were immobilized through His₆-tagged RNAP on Ni²⁺-NTA beads as described previously.^{68: 69} 4-thioU and adjacent [α -³²P]-CMP were incorporated into RNA during elongation from A29 to C31 for EC45(-16), 48(-19), 52(-23), 63(-34), and 69(-40) or during elongation from U52 to C57 for PTC(-16,17,18) (Supplementary Figs. 4a and b). Crosslinking between RNA and NusA and mapping the crosslinking site on the full-length NusA were performed as previously described.³⁹ ECs containing 4-thioU were incubated with NusA or the NTD (2 μ M) at 25 °C for 10 min and irradiated on ice for 10 min with a UV-transilluminator LM-26E (365 nm

max; UVP, Upland, CA). Samples were then treated with RNaseA (5 U/ml) at 25 °C for 5–10 min, mixed with an equal volume of SDS sample buffer, heated at 65 °C for 10 min, and analyzed by SDS-PAGE. To map the crosslinking site on the full-length NusA, crosslinked NusA was eluted from gel pieces and subjected to CNBr or NTCB cleavage. The cleavage pattern of the crosslinked NusA was compared to that of N-terminal ³²P-HMKNusA by SDS-PAGE.

Molecular modeling

The model of *E. coli* NusA (Fig. 1) was created using the program MODELLER⁷⁰ (<http://www.salilab.org/modeller>) by fitting the sequence of *E. coli* NusA to the crystal structure of *T. maritima* NusA (PDB ID 1l2f).²⁴ The structure of the *his* pause hairpin (Fig. 5c) was modeled from the NMR structure of the human PGY/MDR1 mRNA (PDB ID 2gvo)⁵³ using model 1 from the deposited coordinates, superimposing the 3' O on the 3' O of nascent RNA –11 of the post-translocated *T. thermophilus* EC (PDB ID 2o5i)⁵¹ (corresponding to nascent RNA –12 of the pretranslocated *his* PTC),⁵² and the rotating the hairpin about the 3' O to minimize clash the β' clamp and β flap domains while minimizing the distance between the 3' arm of the hairpin and the path of exiting RNA in the *T. thermophilus* EC. The position of *box* RNA relative to KH2 (Fig. 8b) was determined by superimposing the *E. coli* NusA model the crystal structure of an *M. tuberculosis* NusA S1-KH1-KH2 fragment complexed with an 11mer oligoribonucleotide whose sequence corresponds to the spacer between the *boxA* and *boxC* elements of the *boxBAC rrn* element in the *M. tuberculosis rrn* transcript (PDB ID 2asb).⁴⁶ All structural pictures (Figs. 1, 5, and 9) were created using PyMOL (DeLano Scientific LLC, Palo Alto; <http://www.pymol.org>).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

| | |
|---------------|---|
| RNAP | RNA polymerase |
| EC | elongation complex |
| NTD | N-terminal domain |
| CTD | C-terminal domain |
| PTC | paused transcription complex |
| CNBr | cyanogen bromide |
| NTCB | 2-nitro-5-thiocyanobenzoic acid |
| HMK | heart muscle kinase |
| EDTA | ethylenediaminetetraacetic acid |
| BABE | (S)-2-[4-(2-bromoacetamido)benzyl]ethylenediaminetetraacetic acid |
| FeBABE | iron(III) complex of BABE |

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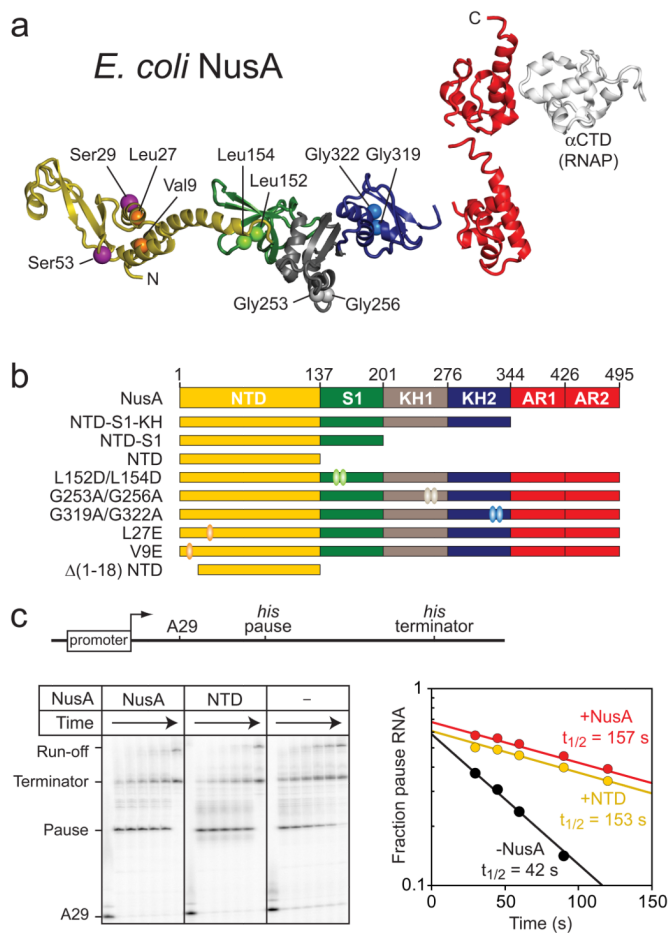


Fig. 1. Structure of NusA and the pause enhancing activity. (a) Structural Model of *E. coli* NusA. The NTD, S1, KH1, and KH2 domains were modeled using the program MODELLER⁷⁰ and the crystal structure of *T. maritima* NusA (PDB ID 112f).²⁴ AR1, AR2, and the αCTD are from NMR structures of the *E. coli* NusA and RNA polymerase α subunit domains (PDB IDs 1wcl and 2jzb, respectively; see Methods). (b) NusA constructs used in this study. C-terminal truncation: NTD-S1-KH, NTD-S1, and NTD; domain disruption: L152/L154 (S1 mutant), G253/G256 (KH1 mutant), G319/G322 (KH2 mutant), V9E (NTD mutant), L27E (NTD mutant), and Δ(1-18) NTD. The ovals indicate the residues of substitution. (c) Pause stimulation by NusA. Top, Schematic of the template DNA containing T7 promoter followed by *his* pause and terminator; Bottom, ECs (~30 nM) halted at position A29 were elongated through the *his* pause site at position U71 in the absence or presence of the full-length NusA or the NTD (4 μM). Samples were removed at 0, 30, 45, 60, 90, 120 s and quenched. RNA products were separated on an 8% denaturing polyacrylamide gel. The fraction of pause band in each lane was plotted as a function of reaction time to derive the rate of pause escape (k_{obs}).

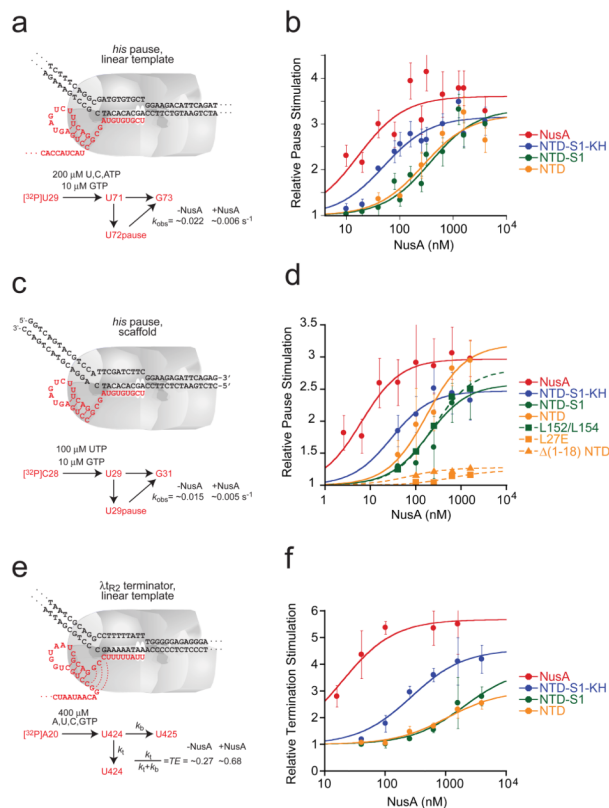


Fig. 2. Effects of the C-terminal truncations of NusA on transcriptional pausing and termination. (a) Structure of the *his* PTC formed on the pIA171 *his* pause template (pause at +71)⁷³ and kinetic scheme. (b) Relative pause stimulation (k_0/k_{obs}) at the promoter-initiated PTC with the C-terminally truncated NusA fragments. Pause assays were performed at 10 μ M GTP (GMP is the nucleotide added during pause escape) and 200 μ M ATP, UTP, and GTP. The data were fitted to an equation for noncompetitive inhibition of transcript elongation (Methods). (c) Structure of the EC at the reconstituted pause site and the kinetic scheme. (d) Relative pause stimulation (k_0/k_{obs}) of the reconstituted PTC with the C-terminally truncated NusAs. (e) Structure of the EC at the λ tR2 terminator and the kinetic scheme. (f) Relative termination stimulation ($k_{t,obs}/k_0$) by the C-terminally truncated NusA fragments.

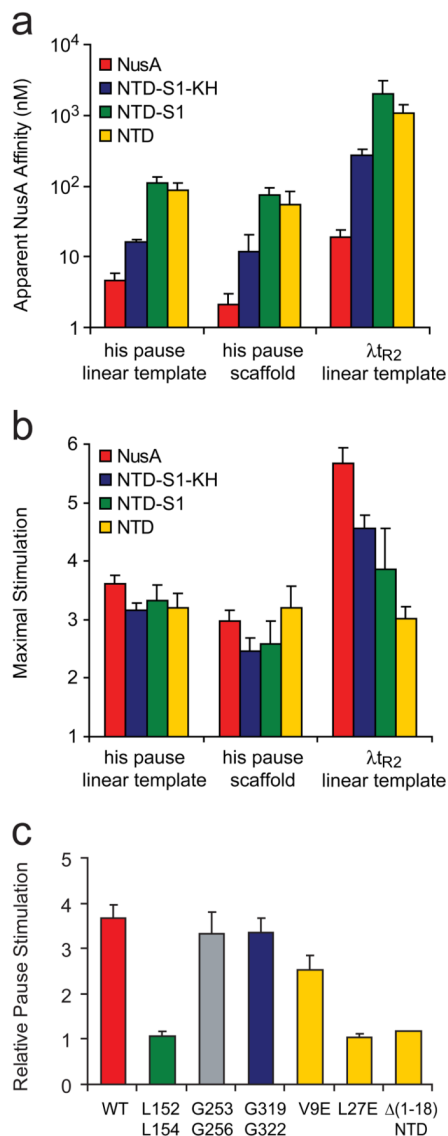


Fig. 3. Apparent affinity and maximal stimulation by NusA for transcriptional pausing and termination. (a) Apparent NusA affinity for promoter-initiated and reconstituted pausing and termination. (see Methods). (b) Maximal stimulation by NusA for promoter-initiated and reconstituted pausing (k_0/k_{NusA}) and termination ($k_{t,NusA}/k_t$). The apparent NusA affinity and the maximal stimulation were calculated from data shown in Figs. 2b, d, and f (Methods). (c) The effects of substitutions in each domain of NusA on transcriptional pausing. The pause dwell time was measured by the pause assay with linear template at 100 nM NusA and normalized by the pause dwell time without NusA.

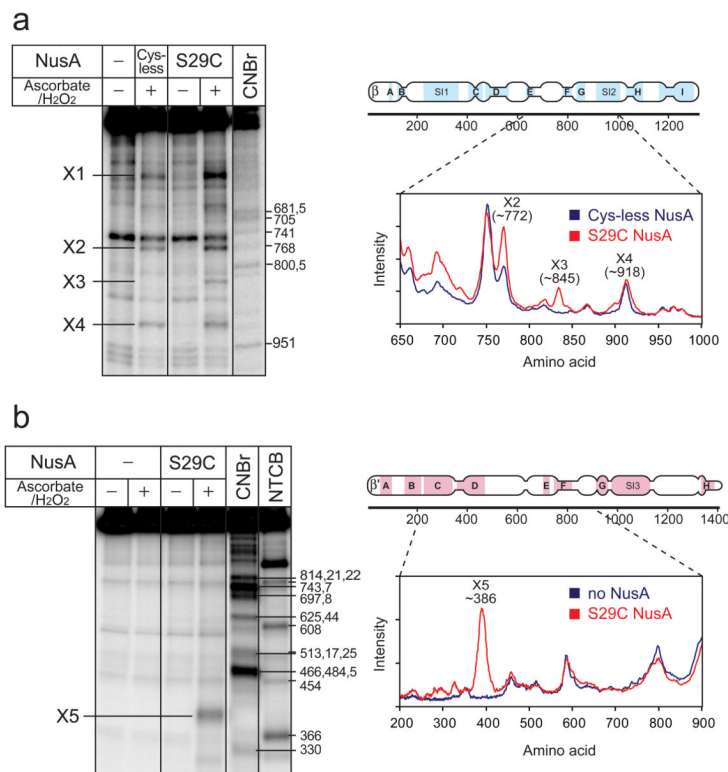


Fig. 4. Cleavage of the β and β' subunits of the core RNAP by FeBABE-tethered NusA. (a) Cleavage of the β subunit of the core RNAP by S29C-FeBABE NusA and mapping of the cleavage site. RNAP containing the C-terminal ^{32}P - β subunit (100 nM) was incubated with or without Cys-less NusA or S29C NusA (100 nM), which were previously subjected to FeBABE conjugation. The cleavage reaction was activated by addition of ascorbate and hydrogen peroxide. The protein ladder was generated by cleaving ^{32}P -labeled RNAP using the Met-specific cleavage reagent, CNBr. Densitometry traces of lane 2 and 4 are shown on the right. From the experimental error in multiple measurements coupled with an assumption of 1% uncertainty in the migration of β fragments relative to the β markers, we calculated the X1-X4 cleavage sites to be at β subunit 459 ± 56 , 772 ± 14 , 845 ± 20 , and 918 ± 14 , respectively. (b) Cleavage of the β' subunit of the core RNAP by S29C-FeBABE NusA and mapping of the cleavage site. RNAP containing the N-terminal ^{32}P - β' subunit, as described for panel (a) but with inclusion of the Cys-specific cleavage reagent NTCB. The X5 cleavage was calculated as described for panel (a) to be at β' subunit 386 ± 12 .

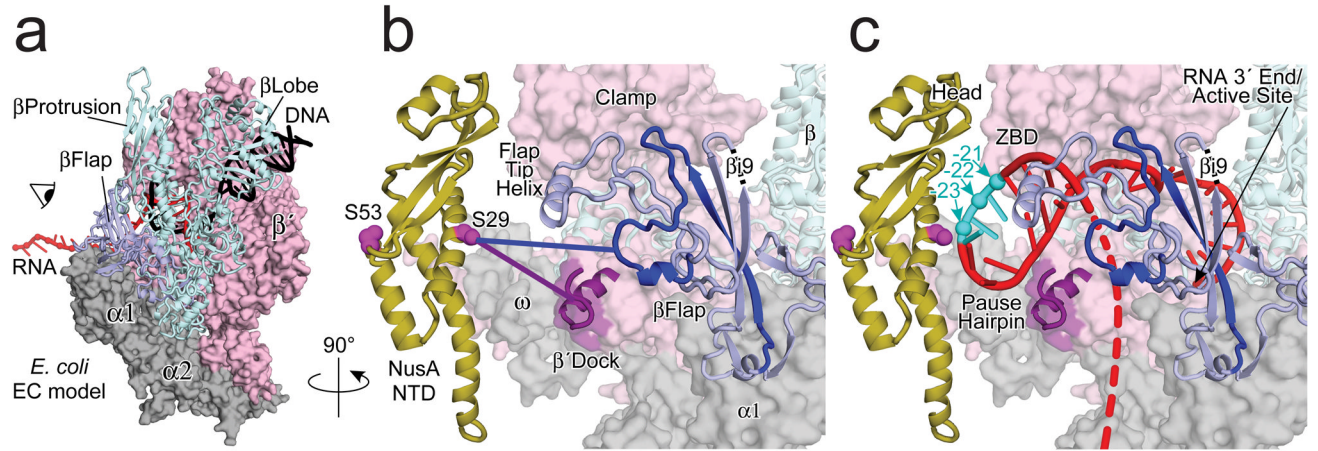


Fig. 5. Model of S29C-FeBABLE cleavage sites on the RNAP and the *his* PTC. The model of the EC is based on the structure of the *T. thermophilus* EC (see Methods).⁵¹ (a) top view of the EC. The α and ω subunits of RNAP are shown in grey. The β' subunit is shown in pink spacefill. The β subunit is shown as a light blue secondary structure cartoon. DNA is black; RNA is red. (b) A view into the RNA exit channel of RNAP showing the locations of the cleavages from NusA(C29)-FeBABLE mapped to the β flap (dark blue) and the β' dock (purple) domains. RNA and DNA are removed for clarity. NusA is depicted adjacent to the RNA exit channel in the orientation predicted by the mapping data, but without attempt to dock NusA on the RNAP surface. *E. coli* RNAP contains an insertion of 100 amino acids in the flap domain (β i9)^{74; 75} whose location is indicated. (c) same view as (b), but showing a model of the *his* pause hairpin (see Methods), the sites of cleavage mapped to the hairpin loop (cyan), and a possible path of RNA exit after hairpin formation (red dashes).

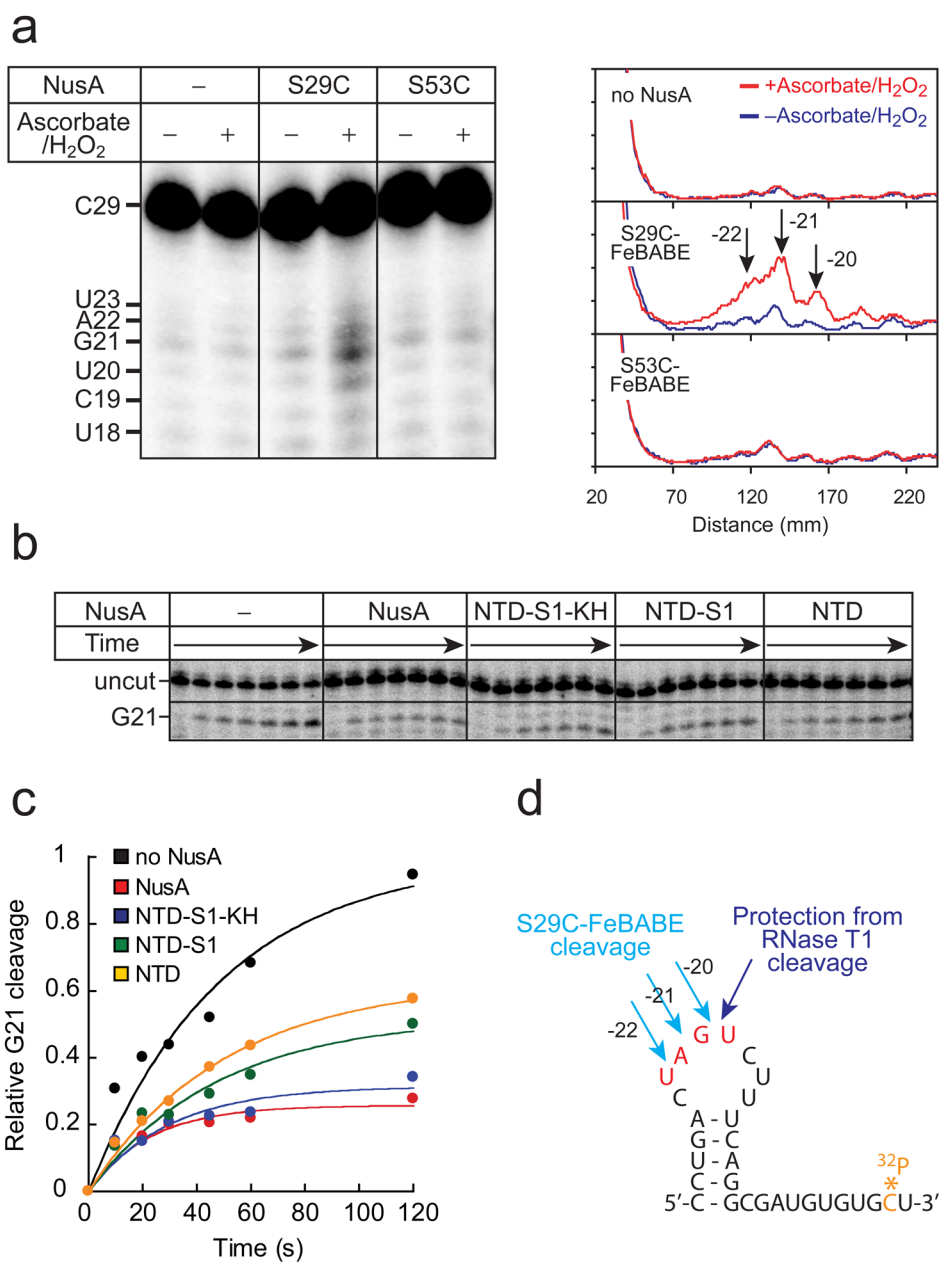


Fig. 6. Cleavage of the pause hairpin RNA by FeBABE-tethered NusA and protection from RNase T1 digestion. (a) Cleavage of the pause hairpin RNA by FeBABE-tethered NusA and mapping of the cleavage site. rPTCs were prepared as described in Methods. The ³²P-labeled rPTC (50 nM) was incubated with S29C-, or S53C-FeBABE NusA (300 nM) for 30 min at 37 °C before cleavage reaction. Densitometry traces are shown on the right. (b) Protection of the pause hairpin by NusA from RNase T1 Digestion. RNase T1 cleavage on the loop of the hairpin in the presence of NusAs at 2 μM. PTCs were prepared as described in Fig. 6a. (c) Relative protection by NusA from RNase T1 digestion. Relative protections of the C-terminally truncated NusAs were calculated by $[\text{uncut}]_{\text{max}}/([\text{uncut}]_{\text{max}}+[\text{G21}]_{\text{max}})$ and normalized by NusA. (d) Sequence of the pause hairpin RNA and the cleavage sites by S29C-FeBABE NusA and RNase T1.

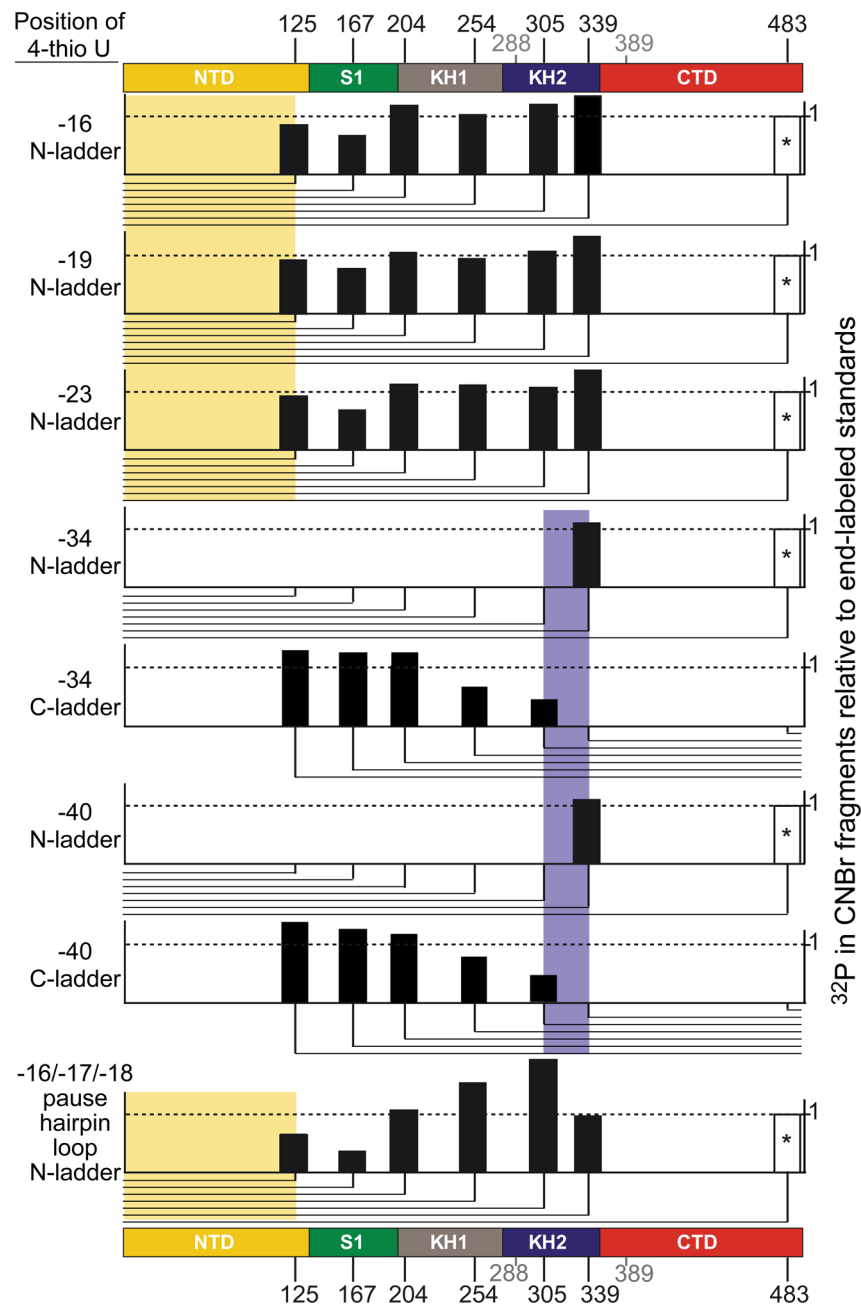


Fig. 7. RNA crosslinking to NusA. ECs containing 4-thioU and adjacent [α - 32 P]CMP at various positions of nascent RNA (listed on left) were formed by stepwise elongation, crosslinked to NusA by UV irradiation, and subjected to crosslink mapping using partial CNBr digestion (see Methods). The positions of possible CNBr cleavage are indicated at the top and bottom. We detected no CNBr cleavage at Met288 and Met 389 (gray) even with purified 32 P-end-labeled NusA. The amount of radioactivity detected in each partial digestion product normalized to the average of radioactivity in the corresponding bands of partial CNBr digestions of 32 P-end-labeled NusA are depicted as bars at the appropriate positions, with fragment orientations (N-terminal partial fragments or C-terminal partial fragments) indicated by the lines below each plot. The location of each crosslink (shaded in color

corresponding to the domain in which the crosslink occurred) was assigned to the interval between the last fragment in the ladder containing significant radioactivity and the next fragment lacking radioactivity (or the end of the ladder in the case on crosslinks to the NTD).⁷⁶ *, the partial digestion product for 483 is inferred but was too close to the parent band to be quantified.

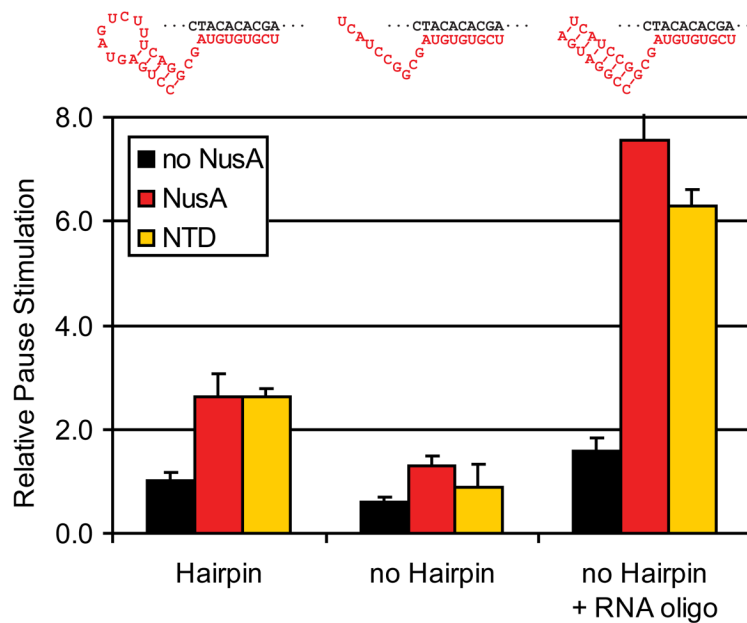


Fig. 8.

Effect of the pause hairpin on pause enhancement by NusA. Relative effect on pause lifetime was measured using reconstituted his PTCs (Methods).⁴⁷ PTCs were reconstituted from DNA scaffolds and 27mer or 17mer RNAs (for hairpin and no hairpin PTCs, respectively; Table 2). For no hairpin+ RNA oligo, RNA8, which is complementary RNA17, was added to 1 μ M to 200 nM no-hairpin PTC prior to the addition of NTPs to the assay. The measured pause lifetimes were normalized to that of the hairpin PTC without NusA to calculate relative pause stimulation.

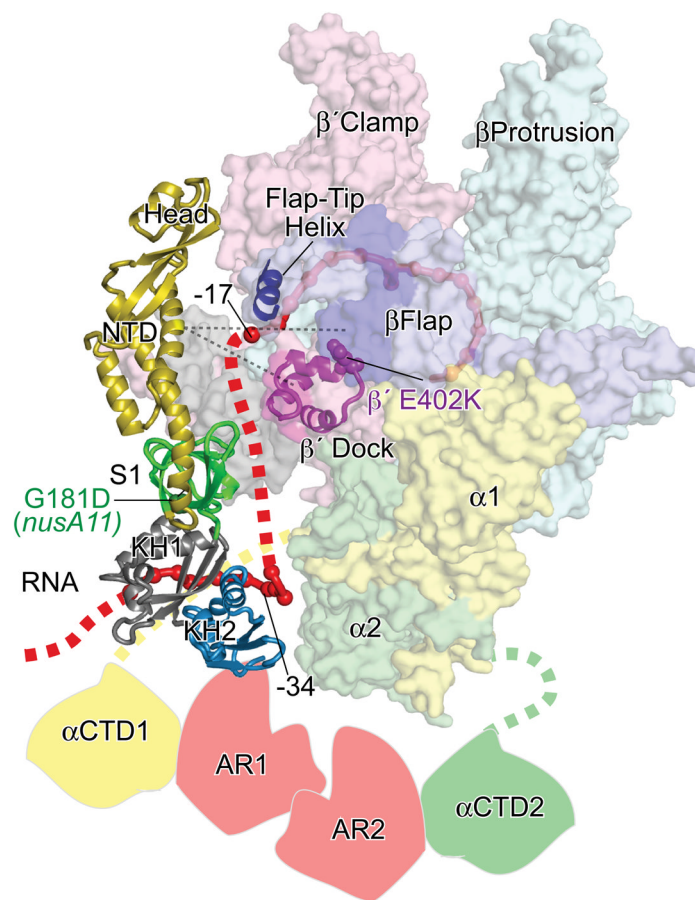


Fig. 9. Model of *E. coli* NusA interactions with an elongating complex. NusA NTD is shown in the same orientation depicted in Fig. 5, and the additional NusA domains are shown in the orientation determined by the model shown in Fig. 1. The locations of α CTD and NusA AR domains are hypothetical and included to illustrate known interactions. The locations of the *nusA11*(G181D) and cognate suppressor β' E402K are depicted as CPK and labeled.⁶⁴ The location of the 11mer *boxAC* spacer RNA from the co-crystal structure of this RNA with the *M. tuberculosis* NusA S1/KH1/KH2 fragment⁴⁶ is depicted as a solid red backbone cartoon modeling in the homologous position between the KH1 and KH2 domains. The dotted red line depicts a plausible path of nascent RNA exiting RNAP with the -17 and -34 positions inferred from the crystal structure of the *T. thermophilus* EC⁵¹ and the crosslinking results shown in Fig. 7.

Table 1

Apparent NusA affinity and maximal stimulation.

| | <i>his</i> pause, linear template | | <i>his</i> pause, scaffold | | λ R2 terminator, linear template | |
|----------------------|-----------------------------------|-------------|----------------------------|-------------|--|-------------|
| | k_{off}/k_{NusA} | K_I (nM) | k_{off}/k_{NusA} | K_I (nM) | k_{NusA}/k_{off} | K_I (nM) |
| NusA | 3.61 ± 0.15 | 4.6 ± 1.2 | 2.97 ± 0.19 | 2.1 ± 0.9 | 5.68 ± 0.25 | 19.0 ± 5.2 |
| NTD-S1-KH | 3.16 ± 0.13 | 16.0 ± 1.8 | 2.47 ± 0.23 | 11.8 ± 8.7 | 4.56 ± 0.23 | 269 ± 64 |
| NTD-S1 | 3.33 ± 0.27 | 112 ± 23 | 2.58 ± 0.40 | 74.6 ± 19.4 | 3.85 ± 0.71 | 2017 ± 1066 |
| NTD | 3.20 ± 0.25 | 86.3 ± 22.2 | 3.20 ± 0.38 | 55.4 ± 27.8 | 3.01 ± 0.22 | 1081 ± 302 |
| L152D/L154D | - | - | 2.81 ± 0.43 | 87.0 ± 40.6 | - | - |
| L27E NusA | - | - | 1.23 ± 0.08 | - | - | - |
| $\Delta_I(3-18)$ NTD | - | - | 1.27 ± 0.02 | - | - | - |

The pause escape rates without NusA (k_{off}) on linear template and scaffold are 0.022 ± 0.005 and 0.015 ± 0.002 s⁻¹ respectively. The termination efficiency without NusA (TE_0) was 0.28 ± 0.02 . We were unable to accurately measure the apparent K_I of L27E NusA and $\sigma(3-18)$ NTD of low enzyme activity.

-, not determined.

Table 2

Strains, plasmids, oligonucleotides

| Stock # | Name | Description | Source or Note |
|-------------------------|-----------|---|-----------------------------------|
| <u>Strains</u> | | | |
| | BL21 λDE3 | <i>E. coli</i> B F ⁻ <i>ompT gal [dcm⁻] [lon⁻] hsdS⁻ (r_B⁻ m_B⁻) [λDE3(lacUV5::T7 RNAP) <i>imm21</i> <i>int⁻</i> -Δ<i>nin5</i> Δ<i>EcoRI</i>(21226-26104) <i>BamHI</i>27972]</i> | Studier <i>et al.</i> , 1990 |
| | BLR | BL21λDE3 <i>recA</i> | Invitrogen |
| <u>Plasmids</u> | | | |
| 775 | pIA171 | T7A1 promoter A29 <i>hisL</i> (<i>his</i> pause) | Ederth <i>et al.</i> , 2002 |
| 168 | pCPG λtR2 | T7A1 promoter A20 λtR2 | Reynolds <i>et al.</i> 1992 |
| 3737 | pIA423 | expresses α, β, β'-CBP/intein from T7 promoter | Artsimovitch <i>et al.</i> , 2003 |
| 2728 | pRM528 | βN HMK in pIA423 | This work |
| 2677 | pRM477 | βC HMK in pIA423 | This work |
| 2675 | pRM475 | β'N HMK in pIA423 | This work |
| 4201 | pSK101 | β'C HMK in pIA423 | This work |
| 4455 | pVS7 | expresses α, β, β'-His ₆ from T7 promoter | Seth Darst |
| 4149 | pNG5 | Expresses N-HMK/His ₆ NusA from T7 promoter | This work |
| 4712 | pKH12 | ΔCTD in pNG5 | This work |
| 4711 | pKH11 | Δ(KH1-KH2-CTD) (NusA1-200) in pNG5 | This work |
| 4116 | pIA378 | Δ(S1-KH1-KH2-CTD) (NusA1-137) in pNG5 | This work |
| 4701 | pKH1 | C251S, C454S, C489S in pNG5 | This work |
| 4702 | pKH2 | S29C in pKH1 | This work |
| 4703 | pKH3 | S53C in pKH1 | This work |
| 4150 | pNG6 | L152D, L154D in pNG5 | This work |
| 4151 | pNG7 | G253A, G256A in pNG5 | This work |
| 4152 | pNG8 | G319A, G322A in pNG5 | This work |
| 4720 | pKH30 | V9E in pNG5 | This work |
| 4721 | pKH32 | L27E in pNG5 | This work |
| 4722 | pKH20 | Expresses C-His ₆ NusA NTD with Δ(3-18) from T7 promoter | This work |
| <u>Oligonucleotides</u> | | | |
| 5069 | NT DNA | GGTCAGTACGTCCATTCGATCTTCGGAAGAG ATTCAGAG | Reconstitution |
| 5420 | T DNA | CTCTGAATCTCTTCCAGCACACATCAGGACG TACTGACC | Reconstitution |
| 4867 | RNA27 | CCUGACUAGUCUUUCAGGCGAUGUGUG | Reconstitution |
| 4865 | RNA29 | CCUGACUAGUCUUUCAGGCGAUGUGUGUCU | Reconstitution |
| 4878 | RNA12 | AGGCGAUGUGUG | Reconstitution |
| 4876 | RNA14 | AGGCGAUGUGUGCU | Reconstitution |
| 6593 | RNA17 | UCAUCCGGCGAUGUGUG | Reconstitution |
| 6598 | RNA8 | CCGGAUGA | Reconstitution |