NusA directly interacts with antitermination factor Q from phage λ

Benjamin R. Dudenhoeffer1, Jan Borggraefe1,2,3, Kristian Schweimer1 & Stefan H. Knauer1*

Antitermination (AT) is a ubiquitous principle in the regulation of bacterial transcription to suppress termination signals. In phage λ antiterminator protein Q controls the expression of the phage’s late genes with loading of λQ onto the transcription elongation complex halted at a σ-dependent pause requiring a specific DNA element. The molecular basis of λQ-dependent AT and its dependence on N-utilization substance (Nus) A is so far only poorly understood. Here we used solution-state nuclear magnetic resonance spectroscopy to show that the solution structure of λQ is in agreement with the crystal structure of an N-terminally truncated variant and that the 60 residues at the N-terminus are unstructured. We also provide evidence that multidomain protein NusA interacts directly with λQ via its N-terminal domain (NTD) and the acidic repeat (AR) 2 domain, with the λQ:NusA-AR2 interaction being able to release NusA autoinhibition. The binding sites for NusA-NTD and NusA-AR2 on λQ overlap and the interactions are mutually exclusive with similar affinities, suggesting distinct roles during λQ-dependent AT, e.g. the λQ:NusA-NTD interaction might position NusA-NTD in a way to suppress termination, making NusA-NTD repositioning a general scheme in AT mechanisms.
AT does not only play a role in the life cycle of bacteriophages but is necessary for the expression of certain bacterial genes\(^1\). In ribosomal AT, for example, RNAP pauses at an AT signal and a TAC is formed that contains Nus factors A, B, E, and G as well as further components such as ribosomal protein S4 and inositol monophosphate SuhB\(^17\)-\(^20\). If this TAC is only responsible for AT or also involved in posttranscriptional activities, e.g. RNA maturation, is yet unclear.

Thus, Nus factors are key players in transcription regulation with NusG being a representative of the only class of transcription factors that is conserved in all three kingdoms of life\(^21\). \textit{E. coli} NusG consists of two flexibly connected domains, an N-terminal and a C-terminal domain (NTD, CTD), respectively (Supplementary Fig. 1a)\(^22\). NusG-NTD has a mixed \(\alpha/\beta\) topology and binds to the \(\beta^\prime\) clamp helices of RNAP, increasing its processivity, whereas NusG-CTD forms a five-stranded \(\beta\)-barrel that can interact with different binding partners (reviewed in\(^\text{23}\)). For example, NusG-CTD is able to bind termination factor Rho, resulting in stimulation of Rho-dependent termination\(^24\)-\(^26\), or to ribosomal protein S10 so that NusG serves as physical linker between RNAP and the ribosome to couple transcription and translation\(^24\). S10 consists of one domain with mixed \(\alpha/\beta\) topology and is a moonlighting protein, i.e. it is identical to transcription factor NusE, if it is not part of the ribosome (Supplementary Fig. 1b). In this case NusE forms a heterodimer with NusB (Supplementary Fig. 1b), an \(\alpha\)-helical, one-domain protein. During \(\lambda\)-related AT the NusE:NusB complex recognizes specifically a boxA element on the RNA with both NusE and NusB making contacts to the RNA\(^27\)-\(^28\). NusE is the active component in processive AT whereas NusB supports loading of NusE to the transcription machinery\(^27\). NusG-CTD can interact with NusE and thus anchors the NusE:NusB:boxA complex to the RNAP\(^26\)-\(^28\). NusG-mediated tethering of NusE:NusB:boxA to the RNAP might also be important for ribosomal AT\(^17\)-\(^19\). The multidomain protein NusA (Supplementary Fig. 1c) is highly conserved in bacteria and regulates pausing and termination\(^29\), AT processes\(^30\)-\(^32\), and RNA folding\(^33\). NusA-NTD binds to the \(\alpha\)TIM barrel\(^30\)-\(^32\), and to the CTD of one of the RNAP \(\alpha\) subunits (\(\alpha\)CTD), whereas the following three domains (S1, K homology (KH1, KH2)) form the compact RNA binding motif SKK\(^34\). In \textit{E. coli} and other \(\gamma\)-proteobacteria NusA has two acidic repeat (AR) domains, AR1 and AR2, at its C-terminus\(^35\). NusA-AR1 interacts with \(\lambda\)N\(^6\)-\(^38\), whereas NusA-AR2 serves as recruitment platform for various transcription factors such as the \(\alpha\)CTD\(^39\), \(\lambda\)N-G-NTD\(^40\), and SuhB\(^17\)-\(^18\). NusA is regulated via autoinhibition as NusA-AR2 binds to the KH1 domain, preventing RNA binding by NusA-SK\(^39\)-\(^41\)-\(^42\). This autoinhibition may be released by RNAP \(\alpha\)CTD to activate RNA binding by NusA\(^39\), by SuhB during ribosomal AT\(^17\)-\(^19\) or by NusG-NTD, a process that might play a role during NusG recruitment, in resynchronization of transcription:translation coupling or in modulation of termination efficiency\(^43\).

Nus factors are involved in various AT mechanisms. It has been demonstrated that NusA is able to stimulate Q-dependent AT in phage\(^8\)-\(^14\) and it has been suggested that NusA promotes recruitment of Q in phage \(\lambda\)\(^\text{22}\). Here, we used solution-state nuclear magnetic resonance (NMR) spectroscopy to provide conclusive evidence that all Nus factors only NusA interacts directly with \(\lambda\)Q, establishing interactions \textit{via} its NTD and AR2 domain. As NusA-NTD and \(\lambda\)Q share binding sites on RNAP, the NusA-NTD:\(\lambda\)Q interaction might be responsible for repositioning of NusA, stimulating \(\lambda\)Q-mediated AT. We show that binding of \(\lambda\)Q to NusA-AR2 releases autoinhibition of NusA, implying that NusA-AR2 is not only a versatile interaction interface for various transcription partners, but suggesting that NusA may be regarded as regulatory subunit of RNAP that substitutes the sigma factor.

**Results**

**The solution structure of \(\lambda\)Q.** The 60 residues at the N-terminus of \(\lambda\)Q are highly polar and are suggested to be disordered as, until now, only the crystal structure of a deletion variant was described (Supplementary Fig. 1a)\(^22\). NusA-NTD has a mixed \(\alpha/\beta\) topology and binds to the \(\beta^\prime\) clamp helices of RNAP, increasing its processivity, whereas NusG-CTD forms a five-stranded \(\beta\)-barrel that can interact with different binding partners (reviewed in\(^\text{23}\)). For example, NusG-CTD is able to bind termination factor Rho, resulting in stimulation of Rho-dependent termination\(^24\)-\(^26\), or to ribosomal protein S10 so that NusG serves as physical linker between RNAP and the ribosome to couple transcription and translation\(^24\). S10 consists of one domain with mixed \(\alpha/\beta\) topology and is a moonlighting protein, i.e. it is identical to transcription factor NusE, if it is not part of the ribosome (Supplementary Fig. 1b). In this case NusE forms a heterodimer with NusB (Supplementary Fig. 1b), an \(\alpha\)-helical, one-domain protein. During \(\lambda\)-related AT the NusE:NusB complex recognizes specifically a boxA element on the RNA with both NusE and NusB making contacts to the RNA\(^27\)-\(^28\). NusE is the active component in processive AT whereas NusB supports loading of NusE to the transcription machinery\(^27\). NusG-CTD can interact with NusE and thus anchors the NusE:NusB:boxA complex to the RNAP\(^26\)-\(^28\). NusG-mediated tethering of NusE:NusB:boxA to the RNAP might also be important for ribosomal AT\(^17\)-\(^19\). The multidomain protein NusA (Supplementary Fig. 1c) is highly conserved in bacteria and regulates pausing and termination\(^29\), AT processes\(^30\)-\(^32\), and RNA folding\(^33\). NusA-NTD binds to the \(\alpha\)TIM barrel\(^30\)-\(^32\), and to the CTD of one of the RNAP \(\alpha\) subunits (\(\alpha\)CTD), whereas the following three domains (S1, K homology (KH1, KH2)) form the compact RNA binding motif SKK\(^34\). In \textit{E. coli} and other \(\gamma\)-proteobacteria NusA has two acidic repeat (AR) domains, AR1 and AR2, at its C-terminus\(^35\). NusA-AR1 interacts with \(\lambda\)N\(^6\)-\(^38\), whereas NusA-AR2 serves as recruitment platform for various transcription factors such as the \(\alpha\)CTD\(^39\), \(\lambda\)N-G-NTD\(^40\), and SuhB\(^17\)-\(^18\). NusA is regulated via autoinhibition as NusA-AR2 binds to the KH1 domain, preventing RNA binding by NusA-SK\(^39\)-\(^41\)-\(^42\). This autoinhibition may be released by RNAP \(\alpha\)CTD to activate RNA binding by NusA\(^39\), by SuhB during ribosomal AT\(^17\)-\(^19\) or by NusG-NTD, a process that might play a role during NusG recruitment, in resynchronization of transcription:translation coupling or in modulation of termination efficiency\(^43\).

Nus factors are involved in various AT mechanisms. It has been demonstrated that NusA is able to stimulate Q-dependent AT in phage\(^8\)-\(^14\) and it has been suggested that NusA promotes recruitment of Q in phage \(\lambda\)\(^\text{22}\). Here, we used solution-state nuclear magnetic resonance (NMR) spectroscopy to provide conclusive evidence that all Nus factors only NusA interacts directly with \(\lambda\)Q, establishing interactions \textit{via} its NTD and AR2 domain. As NusA-NTD and \(\lambda\)Q share binding sites on RNAP, the NusA-NTD:\(\lambda\)Q interaction might be responsible for repositioning of NusA, stimulating \(\lambda\)Q-mediated AT. We show that binding of \(\lambda\)Q to NusA-AR2 releases autoinhibition of NusA, implying that NusA-AR2 is not only a versatile interaction interface for various transcription partners, but suggesting that NusA may be regarded as regulatory subunit of RNAP that substitutes the sigma factor.

**\(\lambda\)Q directly interacts with NusA.** Nus factors are involved in both \(\lambda\)N-dependent and ribosomal AT. They are part of huge nucleoprotein complexes that modify RNAP into a termination-resistant state, so-called TAC\(^6\)-\(^7\)-\(^17\)-\(^18\)-\(^20\). Efficient \(\lambda\)Q-dependent AT requires at least NusA\(^44\), which is thought to stabilize binding of
λQ to the TEC32. Thus, we used solution-state NMR spectroscopy to test if λQ directly interacts with any of the Nus factors. We titrated 15N-labeled λQ with individual Nus factors and recorded one-dimensional (1D) [1H,15N]-heteronuclear single quantum coherence (HSQC) and two-dimensional (2D) [1H,15N]-BEST-TROSY spectra (Supplementary Fig. 3). For NusE we employed a protein variant which lacks the ribosome binding loop (NusEΔ), and which is in complex with NusB to increase solubility27. Only the presence of NusA (54.9 kDa) led to a significant change in the spectra of 15N-λQ (Supplementary Fig. 3), namely the signal intensity of 15N-λQ was significantly decreased upon addition of NusA (Supplementary Fig. 3a). Slower tumbling leads to increased transverse relaxation rates, which results in line broadening and thus ultimately in a decrease of signal intensity of 15N-λQ signals. Consequently, the loss of 15N-λQ signal intensity suggests a direct λQ:NusA interaction.

λQ binds to NusA-NTD. In order to identify the region of NusA that binds to λQ we carried out [1H,15N]-HSQC-based titrations of λQ and the individual domains of NusA with 1D and 2D spectra being recorded after each titration step. Titration of 15N-λQ with NusA-NTD led to a significant change in the spectra of 15N-λQ (Supplementary Fig. 3), namely the signal intensity of 15N-λQ was significantly decreased upon addition of NusA (Supplementary Fig. 3a). Slower tumbling leads to increased transverse relaxation rates, which results in line broadening and thus ultimately in a decrease of signal intensity of 15N-λQ signals. Consequently, the loss of 15N-λQ signal intensity suggests a direct λQ:NusA interaction.
time scale. Thus, in both titrations the change of signal intensity was analysed quantitatively by calculating the relative signal intensity of the 15N-labeled protein in the presence of one equivalent of the non-labeled binding partner (for details see Material and Methods). In brief, we defined the relative intensity as ratio of the normalized remaining signal intensity of the 15N-labeled protein in the presence of the binding partner to the normalized signal intensity of the free labeled protein. The relative intensity was plotted against the corresponding amino acid position (Fig. 2a,b) and thresholds at 1.5 $\sigma$ and 1.0 $\sigma$ of the mean relative signal intensity were used to identify strongly and moderately affected residues, respectively, which were then mapped onto the 3D structures of $\lambda$Q and NusA-NTD (Fig. 2c).

Figure 2. NusA interacts with $\lambda$Q via its NTD. (a) (top) Section of [1H,15N]-BEST-TROSY spectra of the titration of 250 $\mu$M 15N- $\lambda$Q with NusA-NTD (molar ratios: 1:0, black; 1:1, cyan; 1:2 red). Selected signals are labeled. (bottom) Relative intensity of 15N- $\lambda$Q signals in the presence of one equivalent NusA-NTD. Orange and red lines indicate thresholds for moderately (1.0 $\sigma$ of average relative signal intensity) and strongly (1.5 $\sigma$ of average relative signal intensity) affected signals, respectively. The dashed line represents the mean relative intensity, error bars are given as black vertical lines. (b) (top) Section of [1H,15N]-HSQC spectra of the titration of 175 $\mu$M 15N-NusA-NTD with $\lambda$Q (molar ratios: 1:0, black; 1:1, cyan; 1:2 red). Selected signals are labeled. (bottom) Relative intensity of 15N-NusA-NTD signals in the presence of one equivalent $\lambda$Q with error bars. Thresholds as in (a). (c) Model of the $\lambda$Q:NusA-NTD complex. The model was generated with the HADDOCK 2.2 server (https://haddock.science.uu.nl/services/HADDOCK2.2/) using affected residues as determined via NMR spectroscopy as restraints (see Supplementary Table 1). The model with the lowest HADDOCK and Z-score is depicted. $\lambda$Q (PDB ID: 4MO1, lightblue) and NusA-NTD (PDB ID: 2KWP, gray) are shown in ribbon representation. Affected residues are colored (moderately affected residues, orange; strongly affected residues, red). Termini and secondary structure elements are labeled. Panels show the surface representations of $\lambda$Q (left) and NusA-NTD (right) colored as in the complex. The PyMOL Molecular Graphics System (Version 1.7, Schrödinger, LLC.; https://pymol.org) was used for visualization. (d) $\alpha$CTD detaches NusA-NTD from 15N-$\lambda$Q. Sections of [1H,15N]-BEST-TROSY spectra are shown. Molar ratios: 15N-$\lambda$Q:NusA-NTD: $\alpha$CTD = 1:0:0, black; = 1:2:0, cyan; = 1:2:2, orange; = 1:2:4, red. Initial concentration of 15N-$\lambda$Q: 250 $\mu$M. Selected signals are labeled, arrows indicate chemical shift changes upon addition of NusA-NTD (cyan) and $\alpha$CTD (red). (e) $\lambda$Q removes NusA-NTD from 15N-$\alpha$CTD. Sections of [1H,15N]-HSQC spectra are shown. Molar ratios: 15N-$\alpha$CTD:NusA-NTD: $\lambda$Q = 1:0:0; black; = 1:2:0; cyan; = 1:2:2; orange; = 1:2:4; purple; = 1:2:6; yellow; = 1:2:10, red. Initial concentration of 15N-$\alpha$CTD: 250 $\mu$M. Selected signals are labeled, arrows indicate chemical shift changes upon addition of NusA-NTD (cyan) and $\lambda$Q (red).
the Zn-binding motif (Fig. 2c, left inset). NusA-NTD residues affected by binding of λQ are primarily located in the acidic head region and the upper part of the body region at the convex side of NusA-NTD. Due to insufficient long-term stability we did not record intermolecular nuclear Overhauser effect (NOE) interactions. Thus a docking model was generated based on the affected residues in both proteins and without allowing conformational rearrangements (Supplementary Fig. 4c-e). In the lowest energy model (Fig. 2c) the acidic head of NusA-NTD contacts the Zn-finger of λQ and the upper part of the NusA-NTD body interacts with the second patch of λQ, giving an overall interface area of 1050 Å².

We also checked if the unstructured N-terminus of λQ is involved in NusA-NTD binding by titrating [15N]-NusA-NTD with λQΔ36 and recorded a 2D [1H,15N]-HSQC spectrum after each titration step (Supplementary Fig. 5a). Like in the titration with full length λQ the intensity of [15N]-NusA-NTD signals was significantly decreased in the presence of λQΔ36, and the relative signal intensity in the equimolar titration step was plotted against the amino acid position of NusA-NTD (Supplementary Fig. 5b). Mapping of the affected residues onto the NusA-NTD structure (Supplementary Fig. 5c) resulted in the same binding site as identified in the titration with full length λQ, indicating that the N-terminus of λQ does not influence the interaction with NusA-NTD.

**λQ and RNAPαCTD share binding sites on NusA-NTD.** NusA-NTD contacts two regions of RNAP, the βTFH and the αCTD. The λQ binding site is located on the convex side of NusA-NTD, overlapping with the binding site for RNAP αCTD, thus suggesting that binding of λQ and αCTD might be competitive. To test if the interactions of αCTD and λQ with NusA-NTD are mutually exclusive, we performed NMR-based competition experiments with [1H,15N]-HSQC spectra being recorded after each titration step. First, NusA-NTD was added in a two-fold molar excess to [15N]-Q, resulting in signal changes typical for [15N]-Q:NusA-NTD complex formation (Fig. 2d and Supplementary Fig. 6a; see also Fig. 2a). Subsequent titration with αCTD reversed the signal changes (both chemical shift changes and loss of signal intensity), demonstrating that αCTD binds to NusA-NTD, detach- ing it from λQ. To confirm this finding we carried out a reverse experiment where NusA-NTD was added to [15N]-αCTD in a 1:2 molar ratio, leading to chemical shift perturbations that indicate complex formation. Addition of αCTD reversed those changes, indicating that λQ removes NusA-NTD from αCTD by complexing it (Fig. 2e and Supplementary Fig. 6b). These results imply that the λQ:NusA-NTD and αCTD:NusA-NTD interactions are mutually exclusive and have similar affinities, which, in turn, suggests that both interactions are physiologically relevant. We excluded a direct αCTD:λQ interaction as the titration of [15N]-λQ with αCTD did not alter the spectra of [15N]-λQ (Supplementary Fig. 6c).

**λQ does not interact with NusA-SKK and NusA-AR1.** Having identified NusA-NTD as interaction partner of λQ we tested next if also NusA-SKK binds to it. We titrated [15N]-labeled λQ with NusA-SKK and vice versa and recorded 1D- and 2D-[1H,15N] correlation spectra after each titration step (Supplementary Fig. 7a,b). Even in the presence of a twofold molar excess of the unlabeled binding partner no significant changes were observable in the spectra of the labeled protein, excluding a direct NusA-SKK:λQ interaction. Using the same approach we asked if λQ binds to NusA-AR1, which contacts λN in λN-dependent AT36–38, but again, no direct interaction could be detected (Supplementary Fig. 7c,d).

**λQ binds to NusA-AR2.** Finally, we tested if λQ interacts with NusA-AR2. Upon addition of NusA-AR2 to [15N]-λQ the 1D and 2D spectra of [15N]-λQ changed significantly (Fig. 3a and Supplementary Fig. 8a). As in the titration of [15N]-λQ with NusA-NTD, the intensity of [15N]-λQ signals decreased non-uniformly whereas only slight chemical shift perturbation were observable. Thus, we analyzed the change of signal intensity quantitatively. The relative intensity of [15N]-λQ signals in the presence of two equivalents NusA-AR2 was plotted against the amino acid sequence of λQ and affected residues were identified by using thresholds at 1.5 and 1.0 σ of the mean relative intensity (Fig. 3a). In contrast, the titration of [15N]-NusA-AR2 with λQ resulted in significant chemical shift perturbations and normalized chemical shift changes (Δδnorm) were plotted against the amino acid sequence of NusA-AR2 (Fig. 3b and Supplementary Fig. 8b). To visualize binding surfaces affected residues were mapped on the 3D structures of λQ and NusA-AR2 (Fig. 3c). λQ residues affected by NusA-AR2 binding are located opposite the Zn binding motif and the flexible arm, and strongly affected residues can be found predominantly in helices α3 and α5.

The λQ binding site of NusA-AR2 is located at the C-terminal part of the domain and comprises helix α5*, with W490 and F491 being strongly affected. These two residues are known to be responsible for the specific recognition of other transcription regulators such as αCTD, NusG-NTD, or SuhB17,23. As for the λQ:NusA-NTD complex, we did not record intermolecular NOE interactions due to insufficient long-term stability of λQ. Based on the identified binding surfaces a docking model of the λQ:NusA-AR2 complex was generated without allowing conformational rearrangements (Supplementary Fig. 8c-e). In the lowest energy model (Fig. 3c) helix α5* of NusA-AR2 packs against helices α3 and α5 of λQ so that W490 and F491 are central parts of the interface, which comprises a total area of 1230 Å². Finally, we determined the Kd for the λQ:NusA-AR2 complex by fluorescence anisotropy measurements using NusA-AR2Δ443C, a NusA-AR2 variant where D443, located opposite the λQ binding site, is substituted by a Cys23. NusA-AR2Δ443C was labeled site-specifically with fluorescein5-maleimide and titrated with λQ, giving a Kd of 268 ± 17 μM (Supplementary Fig. 8f). Repeating the HSQC-based titration of [15N]-NusA-NTD with λQΔ36 resulted in the same binding surface as determined for full length λQ (Supplementary Fig. 8g-i), suggesting that the unstructured N-terminus of λQ is not involved in NusA-AR2 binding. This finding was corroborated by fluorescence anisotropy measurements as the affinity of the NusA-AR2Δ443C:λQΔ36 interactions was determined to be 271 ± 27 μM (Supplementary Fig. 8j).
NusA-NTD and NusA-AR2 share binding sites on λQ. In summary, λQ is able to establish interactions with NusA-NTD and NusA-AR2 and comparison of their binding sites on λQ suggests that they are partially overlapping, involving residues located in helices α3 and α5. To test if binding of NusA-NTD and NusA-AR2 is indeed competitive, we carried out 2D [1H,15N]-HSQC-based competition experiments with spectra being recorded after each titration step. First, λQ was added in a two-fold molar excess to 15N-NusA-NTD, resulting in changes of the 15N-NusA-NTD:λQ complex formation (Fig. 3d and Supplementary Fig. 9a). Subsequent titration with NusA-AR2 reversed those changes partially (Fig. 3d), demonstrating that NusA-AR2 complexes λQ, detaching it from NusA-NTD. To corroborate this finding we formed...
NusA-AR2 was added in a twofold molar excess to 15N-λQ, resulting in changes of the 15N-NusA-AR2 spectrum corresponding to 15N-λQ:NusA-AR2 complex formation. Subsequent titration with the αCTD reversed partially the chemical shift perturbations and the loss of signal intensity, showing that the αCTD detaches NusA-AR2 from λQ by binding to it (Fig. 4a and Supplementary Fig. 10a). To confirm this result we performed another competition experiment where λQ was titrated to a preformed 15N-αCTD:NusA-AR2 complex (molar ratio 1:2). The addition of λQ reversed partially the chemical shift perturbations caused by the 15N-αCTD:NusA-AR2 complex formation (Fig. 4b and Supplementary Fig. 10b), demonstrating that λQ can bind to NusA-AR2 in order to remove it from the αCTD. Together with previous data this finding leads to the conclusion that the NusA-AR2 binding sites for αCTD, λQ, NusG-NTD, and SuhB largely overlap, rendering the interactions of these binding partners with NusA-AR2 competitive. Finally, the $K_{\text{D}}$ value of the αCTD:NusA-AR2 interaction was determined by fluorescence anisotropy measurements using the NusA-AR2Δ443C variant to be 8±1μM (Supplementary Fig. 10c), in agreement with a previous report. Thus, the affinity of the NusA-AR2Δ443C:αCTD interaction is significantly higher than the one of the NusA-AR2Δ443C:λQ interaction, which explains why λQ is able to detach NusA-AR2 only partially from the αCTD (Fig. 4).

**λQ may release the autoinhibition of NusA.** NusA-AR2 binding sites for αCTD, NusG-NTD and SuhB not only overlap, but all these factors are able to remove NusA-AR2 from NusA-SKK, releasing autoinhibition of NusA. Thus, we explored if λQ has the same ability employing NMR-based displacement experiments.

**Figure 4.** αCTD and λQ share binding sites on NusA-AR2. NMR-based competition experiments of λQ, αCTD, and NusA-AR2. (a) NusA-AR2 is detached from 15N-λQ by αCTD. Sections of 1H,15N-BEST-TROSY spectra are shown. Molar ratios: 15N-λQ:NusA-AR2:αCTD = 1:0:0, black; 1:2:0, cyan; 1:2:2, orange; 1:2:4, red. Initial concentration of 15N-λQ: 250μM. Selected signals are labeled, arrows indicate chemical shift changes upon addition of NusA-AR2 (cyan) and αCTD (red). (b) λQ removes NusA-AR2 partially from 15N-αCTD. Sections of 1H,15N-HSQC spectra are depicted. Molar ratios: 15N-αCTD:NusA-AR2:λQ = 1:0:0; black; 1:2:0, cyan; 1:2:2, orange; 1:2:4, purple; 1:2:6, yellow; 1:2:10, red. Initial concentration of 15N-αCTD: 250μM. Selected signals are labeled and arrows show changes in chemical shifts upon addition of NusA-AR2 (cyan) and λQ (red).
In control experiments we titrated 2H,15N-labeled NusA-SKK with NusA-AR2 and vice versa and recorded 1D and 2D [1H,15N] correlation spectra after each titration step to determine the chemical shift perturbations caused by NusA-AR2:NusA-SKK complex formation on both sides (Fig. 5a,b and Supplementary Fig. 11a,b). For both titrations normalized chemical shift perturbations were plotted against the amino acid sequence of the labeled protein (Fig. 5a,b) and mapped on the 3D structures (Fig. 5c). The identified binding surfaces were in agreement with previous data39, i.e. the C-terminal part of NusA-AR2 is affected as well as the KH1 domain of NusA-SKK.

Based on the normalized changes of the chemical shifts we estimated the affinity of the NusA-SKK:NusA-AR2 interaction to be < 341 µM (Supplementary Fig. 11c). In an alternative approach we determined the K_D value by fluorescence anisotropy measurement employing the NusA-AR2 D443C variant, yielding a slightly lower affinity (279 ± 17 µM; Supplementary Fig. 11d), similar to the one of the NusA-AR2λQ interaction. As no structure of autoinhibited NusA is available we performed NMR-guided docking using the results of the HSQC titrations.
and without allowing for conformational changes to obtain a model of the NusA-SKK:NusA-AR2 complex (Fig. 5c). NusA-AR2 packs tightly against the KH1 domain via its C-terminal helix with an interaction surface of 1230 Å², blocking the RNA binding site. Addition of λQ to a preformed 4H,15N-NusA-SKK:NusA-AR2 complex (molar ratio 1:2) led to a partial reversal of all signal shifts (Fig. 5d and Supplementary Fig. 1d). This finding is in agreement with the $K_p$ values of the NusA-SKK:NusA-AR2 and NusA-AR2:λQ interactions and indicates that λQ binds to NusA-AR2 releasing NusA-SKK at the same time, thus being compatible with the release of NusA auto-inhibition by λQ. However, one must bare in mind that isolated NusA domains were used in these experiments and that the affinity of NusA-AR2 for NusA-SKK might be higher in the full length protein due to an increased local concentration. Consequently, our results are in agreement with a λQ-induced release of autoinhibition of NusA, but not final proven. NMR-based approaches using the full length NusA protein failed due to stability issues. Repeating the competition experiment with λQ2,3,6 confirmed that the N-terminus is not required for this function of λQ (Fig. 5e and Supplementary Fig. 1f).

Discussion

Q-dependent AT is the second mechanism lambdoid phages use to suppress termination signals. N-mediated AT is the best studied AT mechanism by now whereas only little is known about AT relying on Q. Only recently, the structural basis for AT involving Q from bacteriophage 21 has been deciphered12,13. However, as mentioned before, λQ proteins can be grouped into three families, Q21, Q82, and λQ, and these families show no significant amino acid sequence similarity and only very little similarity in the 3D structure (with structural information being available only for Q21 and a truncated version of λQ12–14. Consequently, the molecular mechanisms they use to achieve AT might be completely different, despite the fact that all Q proteins seem to bind to or in the vicinity of the βFTH in order to affect pausing and termination12,13,43. One striking difference between Q21 and λQ is for example that the latter has a long N-terminal region with unknown function that has been suggested to be unstructured. Moreover, two distinct activities have been suggested for Q22, namely antipauing and RNA occlusion, which may both play a role in Q82 function43. Finally, it is known that Nus factors are involved in other AT mechanisms such as N-mediated and ribosomal AT6,7,15,17,18. At least NusA has been demonstrated to influence Q in QBE14. Moreover, we confirmed that residues 1–66 are indeed unstructured (Fig. 1). It has been proposed that Q makes direct interactions with any of the Nus factors and we show that λQ only interacts with NusA, contacting the NTD and the AR2 domain. In neither case the N-terminus of λQ is involved in the interaction so that its function remains elusive. Interestingly, the λQ binding sites for NusA-NTD and NusA-AR2 overlap so that NusA-NTD and NusA-AR2 binding are mutually exclusive (Fig. 6a,b), as confirmed by competition experiments, suggesting similar affinities and thus distinct roles for these complexes in λQ-dependent AT. Moreover, the NusA binding sites involve (at least partially) the HTH motif (Fig. 6c) so that NusA interaction might interfere with DNA binding and might thus be relevant only once λQ is loaded to the TEC, in agreement with the fact that NusA is usually recruited after the σ factor has left the TEC49. If more than one λQ molecule is loaded to the TEC, interactions with NusA-NTD, NusA-AR2 and DNA would be possible simultaneously.

The NusA-AR2:λQ interaction might have various regulatory roles. It could (i) stabilize the TAC, (ii) promote the engagement of λQ with the TAC, in agreement with a previous hypothesis32, (iii) mediate NusA loading if NusA enters the TAC after λQ, (iv) alter the RNA binding properties of NusA by releasing autoinhibition, or (v) recruit further λQ molecules in the course of transcription, which might be necessary as the late gene region in phage λ comprises 26 kb, (vi) a combination of several of these possible functions.

During transcriptional pausing NusA-NTD interacts with βFTH and the αCTD, the latter interaction involving NusA-NTD helices α3 and parts of the preceding loop. Thus the NusA-NTD:αCTD binding site overlaps with the NusA-NTD:λQ interaction surface and both interactions are mutually exclusive as demonstrated by NMR-based competition experiments (Fig. 2d,e), but λQ does not directly bind to αCTD (Supplementary Fig. 6c). Interestingly, the absence of the αCTD affects the ability of NusA to stimulate λQ-dependent AT42, suggesting that the NusA-NTD:αCTD and NusA-NTD:λQ interactions have relevant roles in λQ-dependent AT.

NusA-NTD, λQ, and σ region 4 bind to the βFTH, competing for this binding site42,45. Thus, we hypothesize that upon loading λQ establishes contacts with the βFTH as σ region 4 has already been disengaged from its position in the initiation complex at this stage, as shown for Q22,13. Our NMR data and a mutagenesis analysis44 suggest that λQ, βFTH, and NusA-NTD cannot form a ternary complex as λQ binding sites for βFTH and NusA-NTD overlap, so that binding is competitive. In this case simultaneous contacts of λQ to the βFTH and NusA-NTD would only possible if more than one molecule λQ is present.
Processive $\lambda$N-mediated AT involves repositioning of NusA-NTD\textsuperscript{6,7} and a similar mechanism has been suggested for ribosomal AT\textsuperscript{17,18}. Although the structures of TAGs and antiterminators, the time of antiterminator recruitment and the recruitment signals differ in Q-dependent, N-dependent, and ribosomal AT mechanisms\textsuperscript{6,7,12,13,51} the repositioning of NusA-NTD might be a general scheme in AT. Thus, based on our findings we speculate that once recruited to the $\beta$FTH Q may alter the usual NusA:RNAP and/or NusA:RNA contacts, which would finally result in a positioning of NusA-NTD in a way that may prevent the formation of pause and termination hairpins and thus enhances elongation, rendering Q-dependent AT, just like $\lambda$N-dependent AT, processive\textsuperscript{6,7}.

NusA is a central transcriptional regulator and well conserved in bacteria. It is a multidomain protein, but only NusA from \textit{E. coli} and some other $\gamma$-proteobacteria contains the two AR domains at the C-terminus\textsuperscript{35}. In its isolated form, NusA is autoinhibited as NusA-AR2 binds to the KH1 domain of the SKK motif\textsuperscript{39}, preventing RNA binding by SKK (Fig. 6d). Regulation via autoinhibition is a common scheme in the regulation of all kinds of biochemical processes. In general, autoinhibition describes the negative regulation by intramolecular interactions of different regions of the polypeptide chain, that may even be coupled to conformational changes, and that inhibit the function of at least one of the regions\textsuperscript{52,53}. Only under certain circumstances, e.g. the binding of a specific effector, autoinhibition is released and the protein/enzyme is activated. On NusA-AR2 NusA-SKK binding involves the same region as the interaction with Q (Figs. 3c and 5c), SuhB\textsuperscript{18}, NusG-NTD\textsuperscript{40}, and $\alpha$CTD\textsuperscript{55}. Moreover, all known interaction partners of NusA-AR2 are able to release the autoinhibition of NusA and may thus serve as NusA activators. This activation may occur in binary complexes or when NusA is bound to RNAP via NusA-NTD.

**Figure 6.** NusA functions during transcription regulation. (a-c) Interaction surfaces of $\lambda$Q ($\lambda$Q in surface representation, gray, PDB ID: 4MO1). Residues affected by NusA-NTD binding are colored in green (a; this study), NusA-AR2 binding in blue (b; this study), and $\beta$FTH and DNA binding in yellow and red, respectively (c; data taken from\textsuperscript{44}). (d) Model of the autoinhibited state of NusA. NusA-AR2 (PDB ID: 1WCN, blue) is in ribbon, NusA-SKK (PDB ID: 5LM9, green) in surface representation, NusA-NTD and NusA-AR1 are depicted as green ellipsoids. The panel shows a magnification of the boxed region with W490 and F491 of NusA-AR2 as red sticks. (e) Scheme of possible roles of NusA. NusA-AR2 is shown in ribbon representation (PDB ID: 1WCN, blue) with W490 and F491 as red sticks, all other NusA domains and proteins are shown as ellipsoids and labeled. Selected RNAP binding sites are depicted in ribbon representation and labeled. Blue arrows indicate interactions of NusA-AR2 with other transcription factors, gray arrows show interactions with RNAP (the interaction of SuhB with RNAP requires further investigation and is displayed as dashed arrow). $\beta$ clamp helices, $\beta'CH$. The PyMOL Molecular Graphics System (Version 1.7, Schrödinger, LLC.; https://pymol.org) was used for visualization of protein structures.
NusA-AR1 and NusA-AR2 have nearly identical structures with 31.5% sequence identity and contain predominantly acidic residues, resulting in a very similar electrostatic potential surface. Nevertheless, each AR domain is able to recognize specific targets. NusA-AR1 specifically binds to antiterminator protein N from phage λ, whereas several binding partners interact with NusA-AR2, e.g. Q (Fig. 3), SuB, NusG-NTD, and αCTD. Interestingly, all these binding partners have overlapping binding sites on NusA-AR2, all involving helix α5, and all probably relying on a similar recognition mechanism based on the neighboring aromatic residues W490 and F491, located at the very C-terminus (Supplementary Fig. 12). A Leu (L414) and an Ala (A415) residue can be found at corresponding positions in NusA-AR1.

NusA is composed of several domains with the AR2 domain forming the C-terminus. As all domains are connected via flexible linkers, NusA has a high intramolecular flexibility, i.e. even when NusA is bound to RNAP via its NTD and the SKK motif to RNA during transcription, the AR2 domains can still move virtually independently. Thus, the AR2 domain may serve as flexible and versatile recruitment platform that allows the specific recruitment for various transcription factors in E. coli and other γ-proteobacteria (Fig. 6e), as suggested earlier. Once bound to NusA-AR2 these regulators may stay at the AR2 domain or may be handed over to RNAP or other parts of the transcription machinery.

NusA has multiple, sometimes even opposing, functions, which are context- and regulator-dependent, ranging from pause-stimulation to AT. NusA is recruited early in transcription elongation and its NTD occupies the same position as region 4 of the σ factor, i.e. it binds to the β′FTH, which constitutes a part of the wall of the RNA exit channel. By interacting with the β′FTH NusA-NTD may affect the widening of the RNA exit channel, modulating the transcription speed. Additionally, NusA-NTD is contacted by one of the αCTDs, suggesting that NusA-NTD would still be tethered to RNAP even if the NusA-NTD:β′FTH contact was lost (e.g. if a λ Q protein is bound to the β′FTH). As discussed above, the AR2 domain is able to establish highly specific contacts to other transcription factors, allowing their specific recruitment to the RNAP and thus facilitate the modulation of RNAP activity. Taken together, the early recruitment of NusA, its tuneable function and its ability for the specific recruitment of various other transcription regulators not only underlie the central role of NusA in transcription regulation, but imply that NusA may be regarded as auxiliary/additional RNAP subunit, similar to the σ factor, although not being encoded in the rpo operon.

Methods

Cloning and mutagenesis. The gene encoding λQ was amplified from the plasmid pUC57 lambdaq obtained from GenScript (Piscataway, NJ, USA; the gene was codon-optimized; additionally, an Ncol restriction site was introduced permitting the generation of a λQ deletion variant lacking 36 amino acids at the N-terminus (see below) without changing the amino acid sequence) by polymerase chain reaction using the primers Q-Pci-FW (5′-gccacgctccgctggtaaccttcc-3′; Pci restriction site in bold) and Q-Xho-RV (5′-gtctgcagcagctggtaacctttccgg-3′; Xho restriction site in bold; both primers were obtained from Metabion, Martinsried, Germany) and cloned into the pETGB1a expression vector (provided by Gunter Stier, EMBL Heidelberg, Germany) via Pci and Xho restriction sites, resulting in the recombinant plasmid pETGB1a lambdaq. The gene coding for the Q variant lacking the 36 N-terminal residues, λQΔ36, was obtained by restriction of pUC57 lambdaq with Ncol and Xhol and cloned into pETGB1a (pETGB1a lambdaqΔNΔ36). Both recombinant target proteins have a hexa-histidine tag, the B1 domain of streptococcal protein G (GB1), and a Tobacco Etch Virus (TEV) cleavage site at their N-termini.

Gene expression and protein purification. Production of NusA was carried out as described, as was production of NusA-NTD, NusA-SKK, NusA-AR1, NusA-AR2, NusA-AR2ΔNΔ12, NusB, NusE, NusAΔB, NusG, and αCTD.

Expression of λQ was carried out in E. coli Rosetta (DE3) plxysSRAE (Novagen, Madison, USA) harboring the plasmid pETGB1a lambdaq. Lysogeny broth (LB) medium (supplemented with 34 μg/ml chloramphenicol and 30 μg/ml kanamycin) was inoculated with an overnight preculture to an optical density at 600 nm (OD600) of 0.2 and incubated at 37°C. When the culture reached an OD600 of 0.5 the temperature was decreased to 25°C and overexpression was induced by addition of 0.2 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at an OD600 of 0.7. Four hours after induction cells were harvested by centrifugation (6,000 × g, 10 min, 4°C), resuspended in buffer Q-A (50 mM Tris(hydroxymethyl)aminomethane (Tris)/HCl, pH 7.4, 250 mM NaCl, 5 mM Dithiothreitol (DTT)), and lysed using a microfluidizer (Microfluidics, Newton, USA). The lysate was cleared by ultracentrifugation (100,000 × g, 30 min, 4°C) and the crude extract was filtered (0.45 μm filter) before being applied to a 5 ml HisTrap HP column (GE Healthcare, Chalfont St Giles, UK) loaded with Ni2+ instead of Ni2+-Ions. Upon washing with buffer Q-A elution was carried out via a step gradient with increasing imidazole concentrations (10 mM–500 mM in buffer Q-A). Fractions that contained His6-Gb1-λQ were combined and dialyzed against buffer Q-A (molecular weight cut-off (MWCO) 3,500 Da) at 4°C overnight in the presence of TEV protease. The dialysate was loaded on a 5 ml HisTrap HP column (GE Healthcare, Munich, Germany) and the columns were washed with buffer Q-A. Subsequently, the HisTrap HP column was removed and the HisTrap HP column was eluted using a constant gradient from 250 mM NaCl to 1 M NaCl in buffer Q-A. Fractions that contained λQ protein were combined and dialyzed against buffer Q-B (50 mM 3- (N-morpholino)propanesulfonic acid (MOPS) buffer, pH 6.5, 300 mM NaCl, 150 mM D-Glucose, 5 mM DTT; MWCO 3,500 Da) at 4°C. The protein was purified by a gel filtration step using a Superdex75 10/600 column (GE Healthcare, Munich, Germany) and buffer Q-B. Fractions containing pure λQ were concentrated by ultracentrifugation (MWCO 3,000 Da), shock frozen in liquid nitrogen, and stored at −80°C. The production of λQΔ36 was carried out analogously.
Analytical gel filtration of λQ and λQΔ36. Analytical gel filtration was carried out using a Superdex 75 10/300 GL column (GE Healthcare, Munich, Germany; 20 mM Tris/ HCl (pH 7.5), 150 mM NaCl, 5% (v/v) glycerol). In order to estimate the molecular weights of λQ and λQΔ36 a standard curve was generated using aprotinin (6.5 kDa; Sigma-Aldrich, Darmstadt, Germany), ribonuclease (13.7 kDa; GE Healthcare, Munich, Germany), carbonic anhydrase (29.0 kDa; GE Healthcare, Munich, Germany), ovalbumin (43 kDa; GE Healthcare, Munich, Germany), and albumin (66.0 kDa; Sigma-Aldrich, Darmstadt, Germany). 250 µg protein were applied per run.

Quality control of recombinant proteins. The quality of proteins used in this study was assessed based on the guidelines established by ARBRE-MOBIEU and P4EU (https://arbre-mobieu.eu/guidelines-on-protein-quality-control/). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to check the purity of proteins. UV/visible spectra from 220–340 nm were recorded on a Nanodrop ND-100 spectrometer (PEQLAB, Erlangen, Germany) to ensure the absence of nucleic acids and aggregation. In order to determine concentrations the absorbance at 280 nm was measured in a 10 mm quartz cuvette (Hellma, Müllheim, Germany) and homogeneity was ensured by analytical gel filtration using a Superdex 75 or a Superdex 200 10/300 GL column (GE Healthcare, Munich, Germany). Circular dichroism (CD) spectroscopy (1 mm quartz cuvette; J-1100, JASCO, Pfungstadt, Germany) was performed to assess the folding state (the folding state of unlabeled λQ and λQΔ36 was additionally checked by recording one dimensional [1H-NMR spectra).

Isotopic labeling of proteins. Isotopic labeling was essentially based on published protocols54. To produce uniformly 15N- or 15N,13C-labeled proteins E. coli cells were grown in minimal medium M957,58 containing (15NH4)2SO4 (CortecNet, Voisins-Le-Bretonnes, France) or (15NH4)2SO4 and 13C-glucose (Campro Scientific, Berlin, Germany) as sole source for nitrogen or carbon, respectively. For expression of λQ and λQΔ36 Fe(III) citrate and the trace element solution were omitted, but ZnCl2 was added to a final concentration of 4 mg/ml. Deuterated proteins were produced by growing E. coli cells in M9 medium54 which was prepared with stepwise increasing amounts of D2O (25% (v/v), 50% (v/v), 99.9% (v/v) D2O; Eurisotop, Saint-Aubin, France). Expression and purification protocols were the same as for proteins produced in LB medium.

NMR spectroscopy. NMR experiments were performed at 298 K on Bruker Avance 700 MHz, Bruker Ascend Aeon 900 MHz, and Bruker Ascend Aeon 1 000 MHz spectrometers, all being equipped with cryogenically cooled inverse triple resonance probes. The experimental setup and data analysis was done essentially as described16. Samples contained 10% (v/v) D2O for locking and were in 3 mm tubes with an initial volume of 250 µl if not stated otherwise. In-house routines were used for data collection and processing. MatLab (The MathWorks, Inc., Version 7.1.0.183) was used for visualization and analysis of one-dimensional (1D) spectra and NMRView (One Moon Scientific, Inc., Westfield, NJ, USA) to visualize and analyze two-dimensional (2D) and three-dimensional (3D) spectra. Assignments for the backbone amide resonances of NusA-AR235, NusA-AR159, NusA-SKK39, αCTD39, and NusA-NTD34 were taken from previous studies.

For resonance assignment of the λQΔ36 backbone BEST-TROSY-based triple resonance experiments60-62 were recorded using 5 mm (500 µl sample volume) with the [1H,13C,15N]-labeled protein (270 µg protein) in 25 mM MES (pH 7.0), 100 mM NaCl, 5 mM DTT. 13C-longitudinal and transverse relaxation rates of λQΔ36 were recorded with a 14N-labeled sample at 298 K and 700.2 MHz 1H frequency using standard methods63. Relaxation delays were fitted to a monoexponential decay by NMRView (One Moon Scientific, Inc., Westfield, NJ, USA). The rotation correlation time was determined using the TENSOR 2 package64 assuming an isotropic model for molecular tumbling. Only residues located in rigid regions were used in the analysis. The error of 〈R〉 and 〈R〉 was set to 5% and 8%, respectively, according to ref. 35.

For interaction studies and competition experiments proteins were in 50 mM MOPS, pH 6.5, 300 mM NaCl, 150 mM D-Glucose, 5 mM DTT (exception: 5 mm tubes were used to study the interaction of NusA-SKK with NusA-AR2 with proteins being in 50 mM MOPS, pH 6.5, 100 mM NaCl, 150 mM D-Glucose, 5 mM DTT). Either [1H,15N]-HSQC or [1H,15N]-BEST-TROSY experiments were used to record [1H,15N] correlation spectra. To compare 1D spectra we normalized them by receiver gain, length of the 90° proton pulse, number of scans, and protein concentration.

[1H,15N] correlation-based titrations (either HSQC or BEST-TROSY) were analyzed quantitatively by calculating either changes in intensity or changes in chemical shifts. If chemical shift changes were in the fast regime (the rotation correlation time was determined using the TENSOR 2 package64 assuming an isotropic model for molecular tumbling). Only residues located in rigid regions were used in the analysis. The error of 〈R〉 and 〈R〉 was set to 5% and 8%, respectively, according to ref. 35.

For interaction studies and competition experiments proteins were in 50 mM MOPS, pH 6.5, 300 mM NaCl, 150 mM D-Glucose, 5 mM DTT (exception: 5 mm tubes were used to study the interaction of NusA-SKK with NusA-AR2 with proteins being in 50 mM MOPS, pH 6.5, 100 mM NaCl, 150 mM D-Glucose, 5 mM DTT). Either [1H,15N]-HSQC or [1H,15N]-BEST-TROSY experiments were used to record [1H,15N] correlation spectra. To compare 1D spectra we normalized them by receiver gain, length of the 90° proton pulse, number of scans, and protein concentration. [1H,15N] correlation-based titrations (either HSQC or BEST-TROSY) were analyzed quantitatively by calculating either changes in intensity or changes in chemical shifts. If chemical shift changes were in the fast regime (the rotation correlation time was determined using the TENSOR 2 package64 assuming an isotropic model for molecular tumbling). Only residues located in rigid regions were used in the analysis. The error of 〈R〉 and 〈R〉 was set to 5% and 8%, respectively, according to ref. 35.
with \( \Delta \nu \) being the normalized resonance frequency difference (Hz), \( \Delta \nu_{\text{bound}} \) the normalized resonance frequency difference between free and fully bound protein (Hz), \( \tau \) the ratio of unlabeled to labeled protein, and \( [P]_0 \) the total concentration of \( ^{15}\text{N} \)-labeled protein (the decrease of \( [P]_0 \) due to dilution was taken into account during fitting). Fitting was done using MatLab (The MathWorks, Inc., Version 7.1.0.183) with \( \Delta \nu \) and \( \Delta \nu_{\text{bound}} \) being fitting parameters.

If the system was in slow or intermediate chemical exchange the signal intensities were analyzed quantitatively as described\(^{65}\). In brief, signal intensities were normalized by receiver gain, length of the 90° proton pulse, number of scans, and protein concentration. In order to eliminate an intensity decrease due to slight precipitation signals within one spectrum were normalized to the most intense signal. Subsequently, we calculated the relative signal intensity in each titration step, i.e. the ratio of the remaining, normalized signal intensity of the spectrum of the respective titration step to the normalized signal intensity of the spectrum of the free, labeled protein. The error was calculated based on the standard deviation of the noise level applying error propagation. Then, we calculated the mean value of all relative signal intensities in each titration step and residues with relative signal intensities below thresholds at 1 and 1.5 \( \sigma \) of the mean value were classified as moderately or strongly affected, respectively.

**Fluorescence anisotropy measurements.** Fluorescence anisotropy measurements were performed as described\(^{65}\). Site-specific labeling of NusA-AR2\( ^{D443C} \) with fluorescein-5-maleimide (ThermoFisher Scientific, Waltham, USA) was done according to the manufacturer’s protocol, i.e. after incubation of 25\( \mu \)M of NusA-AR2\( ^{D443C} \) with 750\( \mu \)M fluorescein-5-maleimide in labeling buffer (20\( \mu \)M Na phosphate, pH 7.0, 150 mM NaCl) at 4 °C overnight the solution was loaded on a PD MiniTrap Sephadex G-25 gravity column (GE Healthcare, Munich, Germany) equilibrated with fluorescence buffer (50\( \mu \)M Na-P, pH 6.5, 100\( \mu \)M NaCl, 150 mM glucose, 5\( \mu \)M DTT, 0.05% (v/v) Tween). Elution was carried out with fluorescence buffer. The protein concentration and the degree of labeling were determined by UV/vis spectroscopy on a Nanodrop ND-1000 spectrometer (PEQLAB, Erlangen, Germany) according to the manufacturer’s protocol.

For each titration step individual 100\( \mu \)l samples were prepared with each sample containing 25\( \mu \)M labeled NusA-AR2\( ^{D443C} \) and increasing concentrations of unlabeled protein. All proteins were in fluorescence buffer and measurements were done in black, sterile 96-well microtiter plates (Brand, Wertheim, Germany) at 25 °C in a Synergy 2 microplate reader (BioTek, Winooski, USA). Four independent replicates were prepared per titration step and the anisotropy values were averaged. Finally, the mean anisotropy values were plotted against the titrant concentration and anisotropy data was fitted to a two-state binding model (Eq. 3) using GraFit 5.0 (Erithacus Software; [http://www.erithacus.com/grafit/index.html](http://www.erithacus.com/grafit/index.html)).

\[
A = \frac{A_\text{S} + \frac{[\text{complex}]}{[S]_0} \cdot \left( R \cdot A_{\text{complex}} - A_\text{S} \right)}{1 - \frac{[\text{complex}]}{[S]_0} + R \cdot \frac{[\text{complex}]}{[S]_0}}
\]

(3)

with

\[
[\text{complex}] = \frac{K_D + [P]_0 + [S]_0 - \sqrt{(K_D + [P]_0 + [S]_0)^2 - 4 \cdot [S]_0 \cdot [P]_0}}{2}
\]

(4)

where \( A \) is the measured anisotropy, \( A_\text{S} \) the anisotropy of labeled NusA-AR2\( ^{D443C} \), \( A_{\text{complex}} \) the anisotropy of the complex, [complex] the concentration of the complex, [S]\(_0\) and [P]\(_0\) the total concentrations of labeled NusA-AR2\( ^{D443C} \) and the titrant, respectively, \( K_D \) the dissociation constant, and \( R \) the ratio of the fluorescence intensities of fully bound and free substrate at 520 nm.

**Docking.** The complexes \( \lambda Q: \text{NusA-NTD}, \lambda Q: \text{NusA-AR2}, \) and \( \text{NusA-AR2: NusA-SKK} \) were modeled with the HADDOCK 2.2 server ([https://haddock.science.uu.nl/services/HADDOCK2.2/](https://haddock.science.uu.nl/services/HADDOCK2.2/))\(^{67}\) using H-N correlation data from NMR titrations as restraints (Supplementary Table 1). The size of interaction interfaces was calculated via the “Protein interfaces, surfaces and assemblies” service PISA at the European Bioinformatics Institute ([http://www.ebi.ac.uk/pdbe/prot_int/pstart.html](http://www.ebi.ac.uk/pdbe/prot_int/pstart.html)).

**Visualization of protein structures.** The PyMOL Molecular Graphics System (Version 1.7, Schrödinger, LLC; [https://pymol.org](https://pymol.org)) was used for graphical representations of protein structures.

**Data Availability**

The chemical shift assignment of \( \lambda Q^{2,36} \) were deposited in the Biological Magnetic Resonance Data Bank under the accession code 28043. We generated models of the \( \lambda Q: \text{NusA-NTD}, \lambda Q: \text{NusA-AR2}, \) and the NusA-SKK:NusA-AR2 complex. Coordinates for \( \lambda Q, \text{NusA-NTD}, \text{NusA-SKK}, \) and \( \text{NusA-AR2} \) are available in the Protein Data Bank ([PDB; 4MO1, 2KWP, 5LM9, 1WCN](https://www.rcsb.org/)), the coordinates of the best complex models are provided as Supplementary data. Other data and materials are available from the corresponding author upon reasonable request.

Received: 20 November 2019; Accepted: 27 March 2020;
Published online: 20 April 2020

**References**

1. Werner, F. & Grohmann, D. Evolution of multisubunit RNA polymerases in the three domains of life. *Nat. Rev. Microbiol.* **9**, 85–98 (2011).
2. Guo, X. *et al.* Structural Basis for NusA Stabilized Transcriptional Pausing. *Mol. Cell* **69**, 816–827.e4 (2018).
25. Mitra, P., Ghosh, G., Hafeezunnisa, M. & Sen, R. Rho Protein: Roles and Mechanisms.

24. Burmann, B. M.

23. Artsimovitch, I. & Knauer, S. H. Ancient Transcription Factors in the News.

37. Prasch, S.

33. Pan, T., Artsimovitch, I., Fang, X. W., Landick, R. & Sosnick, T. R. Folding of a large ribozyme during transcription and the effect of

36. Mah, T. F., Li, J., Davidson, A. R. & Greenblatt, J. Functional importance of regions in

35. Vogel, U. & Jensen, K. E. NusA is required for ribosomal antitermination and for modulation of the transcription elongation rate of

34. Squires, C. L., Greenblatt, J. L., Condon, C. & Squires, C. L. Ribosomal RNA antitermination in vitro: requirement for Nus factors and one or more unidentified cellular components. Proc. Natl. Acad. Sci. USA 90, 970–974 (1993).

29. Artsimovitch, I. & Landick, R. Pausing by bacterial RNA polymerase is mediated by mechanistically distinct classes of signals.

28. Burmann, B. M., Luo, X., Rösch, P., Wahl, M. C. & Gottesman, M. E. Fine tuning of the

27. Wernimont, B., Luo, X., Rösch, P., Wahl, M. C. & Gottesman, M. E. Fine tuning of the E. coli NusB:NusE complex affinity to BoxA RNA is required for processive antitermination. Nucleic Acids Res. 38, 314–326 (2010).

26. Banaiet, G. et al. Identification of regulatory targets for the bacterial Nus factor complex. Nat. Commun. 8, 2027 (2017).

25. Huang, Y.-H., Said, N., Loll, B. & Wahl, M. C. Structural basis for the function of SuhB as a transcription factor in ribosomal RNA synthesis. Nucleic Acids Res. 47, 6488–6503 (2019).

24. Dudenhoeffer, B. R., Schneider, H., Schweimer, K. & Knauer, S. H. SuhB is an integral part of the ribosomal antitermination complex and interacts with NusA. Nucleic Acids Res. 47, 6504–6518 (2019).

23. Torres, M., Condon, C., Balada, J. M., Squires, C. & Squires, C. L. Ribosomal protein S4 is a transcription factor with properties remarkably similar to NusA, a protein involved in both non-ribosomal and ribosomal RNA antitermination. EMBO J. 20, 3811–3820 (2001).

22. Singh, N. et al. SuhB Associates with Nus Factors To Facilitate 30S Ribosome Biogenesis in Escherichia coli. MBio 7, e00114 (2016).

21. Werner, F. A nexus for gene expression- molecular mechanisms of Set5 and NusG in the three domains of life. J. Mol. Biol. 417, 13–27 (2012).

20. Mooney, R. A., Schweimer, K., Rösch, P., Gottesman, M. & Landick, R. Two structurally independent domains of E. coli NusG create regulatory plasticity via distinct interactions with RNA polymerase and regulators. J. Mol. Biol. 391, 341–358 (2009).

19. Artsimovitch, I. & Knauer, S. H. Ancient Transcription Factors in the News. MBio 10, e01547–18 (2019).

18. Burmann, B. M. et al. A NusE:NusG complex links transcription and translation. Science 328, 501–504 (2010).

17. Mitra, P., Ghosh, G., Hafeezunnisa, M. & Sen, R. Rho Protein: Roles and Mechanisms. Annu. Rev. Microbiol. 71, 687–709 (2017).

16. Lawson, M. R. et al. Mechanism for the regulated control of transcription by a universal adapter protein. Mol. Cell 71, 1–12 (2018).

15. Xu, X. et al. Structural and functional analysis of the E. coli NusB-S10 transcription antitermination complex. Mol. Cell 32, 791–802 (2008).

14. Burmann, B. M., Luo, X., Rösch, P., Wahl, M. C. & Gottesman, M. E. Fine tuning of the E. coli NusB:NusE complex affinity to BoxA is required for processive antitermination. Nucleic Acids Res. 38, 314–326 (2010).

13. Artsimovitch, I. & Landick, R. Pausing by bacterial RNA polymerase is mediated by mechanistically distinct classes of signals. Proc. Natl. Acad. Sci. USA 97, 7090–7095 (2000).

12. Friedman, D. I. & Baron, L. S. Genetic characterization of a bacterial locus involved in the activity of the N factor of phage lambda. Virology 58, 141–148 (1974).

11. Vogel, U. & Jensen, K. E. NusA is required for ribosomal antitermination and for modulation of the transcription elongation rate of both antiterminated RNA and mRNA. J. Biol. Chem. 272, 12265–12271 (1997).

10. Yarnell, W. S. & Roberts, J. W. The phage gene Q transcription antitermination system Q interacts with the transcriptional machinery of E. coli RNA polymerase to form a Q transcription antitermination terminator. J. Mol. Biol. 228, 999–1012 (1993).

9. Artsimovitch, I. et al. Structural basis for the interaction of Escherichia coli NusA with the cytoplasmic domain of its anti-sigma RseA. Mol. Microbiol. 36, 523–537 (1999).

8. Prasch, S. et al. Interaction of the intrinsically unstructured phage lambda N Protein with Escherichia coli NusA. Biochemistry 45, 4542–4549 (2006).

7. Bonin, I. et al. Structural basis for the interaction of Escherichia coli NusA with protein N of phage lambda. Proc. Natl. Acad. Sci. USA 101, 13762–13767 (2004).

6. Schweimer, K. et al. NusA interaction with the α subunit of E. coli RNA polymerase is via the UP element site and releases autoinhibition. Structure 19, 945–954 (2011).

5. Strauß, M. et al. Transcription is regulated by NusA:NusG interaction. Nucleic Acids Res. 44, 5971–5982 (2016).

4. Mah, T. E., Kaznecov, K., Muhhegen, A., Severinov, K. & Greenblatt, J. The alpha subunit of E. coli RNA polymerase activates RNA binding by NusA. Genes Dev. 14, 2664–2675 (2000).

3. Liu, K., Zhang, Y., Severinov, K., Das, A. & Hanna, M. M. Role of Escherichia coli RNA polymerase alpha subunit in modulation of pausing, termination and anti-termination by the transcription elongation factor NusA. EMBO J. 15, 150–161 (1996).

2. Shankar, S., Hatzis, A. & Roberts, J. W. A transcription antiterminator construct uses a NusA-dependent shield to the emerging transcript. Mol. Cell 27, 914–927 (2007).

1. Yang, X. J., Gologer, J. A. & Roberts, J. W. Specificity and mechanism of antitermination by Q proteins of bacteriophages lambda and 82. J. Mol. Biol. 210, 453–460 (1989).

Deighan, P., Diew, C. M., Leibman, M., Hochschuld, A. & Nickels, B. E. The bacteriophage lambda Q antiterminator protein contacts the beta-flap domain of RNA polymerase. Proc. Natl. Acad. Sci. USA 105, 15305–15310 (2008).

Grayhack, E. J., Yang, X. J., Lau, L. F. & Roberts, J. W. Phage lambda gene Q antiterminator recognizes RNA polymerase near the promoter and accelerates it through a pause site. Cell 42, 259–269 (1985).

Campbell, E. A. et al. Crystal structure of Escherichia coli sigmaE with the cytoplasmic domain of its anti-sigma RseA. Mol. Cell 11, 1067–1078 (2003).

Campbell, E. A. et al. Structure of the bacterial RNA polymerase promoter specificity sigma subunit. Mol. Cell 9, 527–539 (2002).
56. Burmann, B. M., Scheckenhofer, U., Schweimer, K. & Rösch, P. Domain interactions of the transcription-translation coupling factor Escherichia coli RNA polymerase elongation complex. *Elife* 6, e25478 (2017).
57. Burmann, B. M. et al. An α helix to β barrel domain switch transforms the transcription factor RfaH into a translation factor. *Cell* 150, 291–303 (2012).
53. Zuber, P. K., Schweimer, K., Rösch, P., Artsimovitch, I. & Knauer, S. H. Reversible fold-switching controls the functional cycle of the antitermination factor RfaH. *Nat. Commun.* 10, 702 (2019).
54. Droegemüller, J. et al. Exploring RNA polymerase regulation by NMR spectroscopy. *Sci. Rep.* 5, 10825 (2015).
55. Prasch, S. et al. RNA-binding specificity of E. coli NusA. *Nucleic Acids Res.* 37, 4736–4742 (2009).
56. Burmann, B. M., Schechenhofer, U., Schweimer, K. & Rösch, P. Domain interactions of the transcription-translation coupling factor Escherichia coli NusG are intermolecular and transient. *Biochem. J.* 435, 783–789 (2011).
57. Sambrook, J. & Russel, D. W. *Molecular Cloning: A Laboratory Manual*. vol. 3 (Cold Spring Harbor Press, 2001).
58. Meyer, O. & Schlegel, H. G. Biology of aerobic carbon monoxide-oxidizing bacteria. *Annu. Rev. Microbiol.* 37, 277–310 (1983).
59. Eisenmann, A., Schwarz, S., Rösch, P. & Schweimer, K. Sequence-specific 'H, 13C, 15N resonance assignments and secondary structure of the carboxyterminal domain of the E. coli transcription factor NusA. *J. Biol. NMR* 28, 193–194 (2004).
56. Favier, A. & Brutscher, B. Recovering lost magnetization: polarization enhancement in biomolecular NMR. *J. Biol. NMR* 49, 9–15 (2011).
51. Kang, J. Y. et al. Exploring RNA polymerase regulation by NMR spectroscopy: application to staphylococcal nuclease. *Biochemistry* 48, 8972–8979 (1989).
56. Dotson, P., Hus, J.-C., Blackledge, M. & Marion, D. Efficient analysis of macromolecular rotational diffusion from heteronuclear relaxation data. *J. Biomol. NMR* 16, 23–28 (2000).
57. Rossi, P. et al. A microscale protein NMR sample screening pipeline. *J. Biomol. NMR* 46, 11–22 (2010).
58. Droegemüller, J. et al. Determination of RNA polymerase binding surfaces of transcription factors by NMR spectroscopy. *Sci. Rep.* 5, 16428–16441 (2015).
59. de Vries, S. J., van Dijk, M. & Bonvin, A. M. J. J. The HADDOCK web server for data-driven biomolecular docking. *Nat. Protoc.* 5, 883–897 (2010).
60. Krissinel, E. & Henrick, K. Inference of macromolecular assemblies from crystalline state. *J. Mol. Biol.* 372, 774–797 (2007).

**Acknowledgements**

We thank Ramona Heissmann, Ulrike Persau, and Andrea Hager for excellent technical assistance and the Northern Bavarian NMR Centre (NBNC) for access to the NMR spectrometers. This work was supported by the German Research Foundation (Ro617/21–1 to Paul Rösch). The open access charge was funded by German Research Foundation and the University of Bayreuth in the funding program open access publication.

**Author contributions**

S.H.K. supervised the project. The experiments were designed by S.H.K., K.S., and B.R.D. J.B., B.R.D., and K.S. made the assignment of 3QΔ36 (the corresponding NMR experiments were carried out by K.S.). All interactions studies and competition experiments were performed by B.R.D., and data was analyzed and evaluated by B.R.D. and S.H.K. B.R.D. and S.H.K. carried out the docking runs and S.H.K. and B.R.D. wrote the manuscript with input from all authors.

**Competing interests**

The authors declare no competing interests.

**Additional information**

**Supplementary information** is available for this paper at https://doi.org/10.1038/s41598-020-63523-5.

**Correspondence** and requests for materials should be addressed to S.H.K.

**Reprints and permissions information** is available at www.nature.com/reprints.

**Publisher’s note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2020