The structure of bacterial RNA polymerase in complex with the essential transcription elongation factor NusA

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There are three stages of transcribing DNA into RNA. These stages are initiation, elongation and termination, and they are well-understood biochemically. However, despite the plethora of structural information made available on RNA polymerase in the last decade, little is available for RNA polymerase in complex with transcription elongation factors. To understand the mechanisms of transcriptional regulation, we describe the first structure, to our knowledge, for a bacterial RNA polymerase in complex with an essential transcription elongation factor. The resulting structure formed between the RNA polymerase and NusA from *Bacillus subtilis* provides important insights into the transition from an initiation complex to an elongation complex, and how NusA is able to modulate transcription elongation and termination.

Keywords: *Bacillus subtilis*; NusA; RNA polymerase; single particle analysis; transcription

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INTRODUCTION

Core bacterial RNA polymerase (RNAP) is comprised of subunits $\alpha_2\beta\beta'\omega$ and this macromolecular machine is sufficient for transcription. However, to ensure that transcription occurs at the correct points, at the appropriate levels and in response to environmental signals, auxiliary factors are required to interact with the core. σ -factors associate with RNAP to ensure transcription is initiated immediately downstream from promoter sequences. It is generally accepted that once RNAP clears the promoter, σ dissociates to allow progression through transcriptional elongation (Murakami & Darst, 2003). Elongation

factors prevent premature termination, respond to intrinsic pause signals, transcriptional roadblocks and stalled transcription complexes, and regulate the rate of transcription to ensure efficient transcription/translation coupling (Richardson & Greenblatt, 1996).

In the bacteria *Bacillus subtilis* and *Escherichia coli* (Gram-positive and Gram-negative models, respectively), the highly conserved essential transcription elongation factor NusA (supplementary Fig S1A online) is involved in anti-termination, pausing, termination and, in *E. coli*, in the suppression of the toxic activity of foreign genes (Landick *et al*, 1996; Ingham *et al*, 1999; Mah *et al*, 1999; Artsimovitch *et al*, 2000; Yakhnin & Babitzke, 2002; Zhang & Switzer, 2003; Paul *et al*, 2004; Davies *et al*, 2005; Cardinale *et al*, 2008). The association of RNAP with σ or NusA has traditionally been considered to be mutually exclusive so that as σ dissociates, NusA associates and *vice versa* (for example, Gill *et al*, 1991). The structural similarities between the amino-terminal domain of NusA and region 2 of σ suggest that both proteins interact with the same region of RNAP to account for the mutual exclusivity of binding (Borukhov *et al*, 2005). However, elongation complexes can contain both σ and NusA under certain circumstances (for example, Bar-Nahum & Nudler, 2001; Kapanidis *et al*, 2005; Mooney *et al*, 2009), suggesting that the mutually exclusive model is incorrect or that alternative conformations of σ and NusA can be adopted for simultaneous interactions with RNAP.

Here, we describe the structure of the *B. subtilis* NusA–RNAP complex that helps to explain how NusA regulates transcription by interacting with the emerging transcript around a region of RNAP known as the β -flap. This region has a role in promoter complex formation by interacting with region 4 of σ , as well as in forming a contact with the nascent RNA during elongation and termination of transcription (Gusarov & Nudler, 2001; Touloukhonov *et al*, 2001; Kuznedelov *et al*, 2002; Murakami & Darst, 2003; Epshtein *et al*, 2007). Our structure also explains how certain elongation complexes can contain both σ and NusA, as the interaction of NusA with the β -flap creates a steric clash with region 4 of σ , but does not interfere with a subsection of σ region 2, known as

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region 2.2 (Helmann & Chamberlin, 1988), the main site of interaction between σ and RNAP.

RESULTS AND DISCUSSION

NusA binds to the β -flap region of RNAP

To map the NusA binding site on RNAP, we used far-Western blotting in which overlapping RNAP fragments were over-produced (supplementary Fig S2A online) and immobilized on a nitrocellulose membrane. First, membranes were probed with a positive control (σ^A) for which the binding site has been characterized (Arthur & Burgess, 1998). Although σ forms extensive contacts with the RNAP core, the interaction between σ region 2.2 and the clamp helix (CH) region of the β' -subunit is the most important (Murakami & Darst, 2003). Far-Western blots showed that the main interaction of σ^A was with two fragments corresponding to the first 610 amino acids of the β' -subunit, containing an overlap comprising the CH region (approximately D251–L293; Fig 1A,F, red), which is consistent with published data for the interaction of *E. coli* σ^{70} with RNAP (Arthur & Burgess, 1998).

Previous models have suggested that the N-terminal region of NusA, with some structural similarity to σ region 2, could also bind to the CH region (Borukhov *et al*, 2005). Our far-Western blots showed that the NusA interaction was restricted to the β -subunit 750–1,040 fragment (Fig 1B, lane 4). The absence of signal in lanes 3 and 5, which contained overlaps with the β -subunit 750–1,040 fragment, indicates that NusA interacts with amino acids 761–949 of the β -subunit, encompassing the β -flap region of the RNA exit channel (Fig 1E; supplementary Fig S1B online). Further analysis indicated that binding to the β -flap region was dependent on the N-terminal domain (NTD; Fig 1C), although we cannot discount the possibility that the carboxy-terminal domain (CTD) contributes to the overall stability of the complex through numerous weak non-specific interactions with the β - and β' -subunits (supplementary Fig S2B online). The NTD has previously been shown to be important for the interaction of *E. coli* NusA with RNAP, although the region to which NusA bound was not determined (Mah *et al*, 1999).

Earlier studies have shown that NusA is able to interact with RNA hairpins involved in transcriptional pausing around a portion of the flap known as the β -flap tip (Touloukhonov *et al*, 2001). Therefore, a deletion spanning this area was made from amino acids 846–875 ($\Delta\beta$ -flap tip; Fig 1F, asterisk; supplementary Fig S1B online, green). Neither full-length NusA, nor NusA NTD bound to the $\Delta\beta$ -flap fragment (Fig 1B,C, lane 12), suggesting that these amino acids represent the main site for the interaction of NusA on RNAP through its NTD and that this site is distinct from the main site of interaction of σ (Fig 1F, red). To confirm the specificity of NusA binding, affinity chromatography using recombinant wild-type RNAP or RNAP lacking the β -flap tip ($\Delta\beta$ -flap tip RNAP) immobilized on agarose resin was carried out with full-length NusA or NusA NTD (Fig 1D). Both full-length NusA and NusA NTD bound the wild-type RNAP (Fig 1D, lane 3). A faint binding of full-length NusA to $\Delta\beta$ -flap tip RNAP was observed (Fig 1D, lane 4, top) and this was attributed to the non-specific interactions made by the CTD (see supplementary Fig S2B online), as no binding to $\Delta\beta$ -flap tip RNAP was observed with NusA NTD (Fig 1D, lane 4, bottom). Therefore, NusA interacts with RNAP around the β -flap tip through its NTD, and

non-specific interactions between its CTD and RNAP help to stabilize the interaction.

E. coli NusA contains two additional C-terminal domains that interact with the CTD of the α -subunit of RNAP (α -CTD; Mah *et al*, 1999). These NusA domains are not present in *B. subtilis* or many other bacteria (supplementary Fig S1A online), and we found no evidence for any interaction between *B. subtilis* NusA and the α -subunit of RNAP (Fig 1B,C, lane 1; supplementary Fig S2B online).

3D reconstruction of RNAP core and NusA–RNAP complex

To understand the structural basis for NusA binding to RNAP, NusA–RNAP complexes were produced (see Methods and supplementary information online). The presence of NusA in complex with RNAP was confirmed by Western blot using anti-RpoC (β' -subunit) and NusA antibodies (supplementary Fig S3 online). To assign mass to NusA, which is only about 10% of the mass of the RNAP core, structures were solved for both the RNAP core and the NusA–RNAP complex. Both structures were approximately $150 \times 100 \times 140 \text{ \AA}$, similar to the published dimensions of RNAP crystal structures ($150 \times 110 \times 115 \text{ \AA}$; Zhang *et al*, 1999). The increased height that we observed is most probably due to the flexibility around the claw region (discussed below). The resolution of the reconstructions was approximately $25 \text{ \AA}$ for the RNAP core and approximately $22 \text{ \AA}$ for the NusA–RNAP complex (supplementary Fig S4A online). Although negative-stain reconstructions are limited to about $20 \text{ \AA}$ resolution, it has proven useful for mapping proteins smaller than 25 kDa within larger complexes (for example, Boekema *et al*, 1998). The mass attributable to NusA (42 kDa) was visible in three-dimensional reconstructions and re-projections that were appropriately oriented (Fig 2A, red arrows).

A comparison of the final reconstructions by superimposition allowed the identification of the mass attributable to NusA (Fig 2B–G). However, the low resolution of the reconstructions and conformational flexibility of RNAP made accurate docking of homology structures in the electron density maps difficult, with goodness-of-fit estimates of approximately 60% obtained using SITUS (Chacón & Wriggers, 2002). The DNA binding claw is a highly flexible structure (Darst *et al*, 2002; Van Wynsberghe *et al*, 2004) and we were unable to obtain highly resolved information in this region (Fig 2B, circled), which contributed significantly to the low goodness-of-fit scores. Normal-mode analysis suggested that the two extremes of open- and closed-claw conformations would fit equally well into the electron density maps obtained on reconstruction, and goodness-of-fit estimates for the two conformations differed by only approximately 0.2% (see supplementary information online). Nevertheless, the flexibility in the claw region determined by normal-mode analysis could not fully account for all of the electron density in that region of our reconstructions (supplementary Fig S4 online; supplementary Movie M1 online). There are no major protein contaminants in the RNAP preparation used in these studies (Yang & Lewis, 2008), and it is likely that owing to the limitations of normal-mode analysis that the claw is even more flexible than has been determined, contributing to the poor fit in this region.

To establish the orientation of RNAP subunits within the electron density maps and to aid molecular docking, advantage was taken of the fact that RNAP contains a $6 \times \text{His}$ tag at the C-terminus of the β' -subunit to aid purification (Yang & Lewis,

Fig 1 | Mapping of the NusA interaction site on RNA polymerase. (A) Far-Western blot control using σ^A antibodies to show the interaction of σ^A with RNAP. The top panel is a negative control in which σ^A antibodies were omitted, but all other steps of the blot were included. The bottom panel contains the positive control, including σ^A antibodies. The amino-acid numbering of fragments of *Bacillus subtilis* RNAP expressed in *Escherichia coli* is indicated above the lanes in which they were loaded. (B,C) Far-Western blots using (B) ^{32}P -labelled NusA and (C) NusA NTD. The amino-acid numbering of fragments of *B. subtilis* RNAP expressed in *E. coli* is indicated above the lanes in which they were loaded. $\Delta\beta$ -flap corresponds to β -subunit 750–1,040 fragment with amino-acids 846–875 deleted. (D) Anti-NusA Western blots of eluates from affinity chromatography columns with full-length NusA (top) and NusA NTD (bottom) with the molecular weight of NusA or NusA NTD indicated on the left. Lane 1, positive control (NusA or NusA NTD loaded); lane 2, eluate from a blank (negative control) column; lane 3, eluate from wild-type RNAP affinity column; and lane 4, eluate from $\Delta\beta$ -flap tip RNAP affinity column (β -subunit amino acids 846–875 deleted). (E) Surface-rendered homology model of the *B. subtilis* RNAP elongation complex (grey) with β -subunit amino acids 761–949 corresponding to the NusA-interacting region coloured green. (F) The elongation complex in which the β -flap tip (amino acids 846–875) has been deleted (indicated with a red asterisk), and in which the σ^A -interacting region (β' -subunit amino acids 251–293) has been coloured red. MW, molecular weight; NTD, N-terminal domain; RNAP, RNA polymerase.

2008). This $6 \times \text{His}$ tag was decorated with 1.8 nm nitrilotriacetic acid–nanogold particles and a further data set obtained containing 964 gold-labelled RNAP molecules (Fig 3A; supplementary information online). These particles were appended to the unlabelled RNAP dataset and an independent reconstruction was run for eight iterations (see supplementary information online). Representative class sums from datasets devoid of, and including, gold-labelled particles are shown in the first two columns of Fig 3A, in which the gold label is visible (Fig 3A, yellow arrows, second column). A correlation of the class sums with re-projections and three-dimensional views allowed

the identification of the location of the C-terminus of the β' -subunit (Fig 3A,B; supplementary information online) enabling us to dock homology models into the electron density maps with confidence, and to identify regions corresponding to RNAP and NusA structures on the reconstructions (supplementary Movie M2 online).

The structure of the NusA–RNAP complex

A homology model of the *B. subtilis* NusA docked with the RNAP elongation complex is shown in Fig 3C–G. NusA comprises an RNAP-binding NTD separated by a 25-amino-acid flexible linker

Fig 2 | Three-dimensional reconstruction of the RNA polymerase core and the NusA–RNAP complex. (A) Three-dimensional reconstructions (I), re-projections (II) and class averages (III) of the RNAP core, and the NusA–RNAP complex at equivalent angles. In certain orientations, the additional mass that represents NusA could be observed and is indicated by red arrows. (B–G) Surface-rendered representations of the RNAP core reconstruction in grey (B, side; E, face) and the NusA–RNAP complex in gold (C, side; F, face). The poorly defined region owing to the flexibility in the DNA-binding claw is circled in red (B). The curved arrows indicate the rotation between the different views. The density threshold was chosen to include the expected molecular masses of the RNAP core (approximately 400 kDa) and the NusA–RNAP complex (approximately 440 kDa). The relative positions of individual subunits (α , β and β') are labelled. (D,G) Overlay of the RNAP core (surface) and NusA–RNAP complex (mesh) in which the mass attributable to NusA is clearly visible on the left-hand side (G). RNAP, RNA polymerase.

from the C-terminal RNA binding S1, KH1 and KH2 sub-domains (Borukhov *et al*, 2005; supplementary Fig S1A online). Consequently, NusA was docked as two rigid bodies comprising the NTD (residues 1–123) and CTD (residues 149–343). The conformation of the elongation complex used for docking only models a single state, but the structure solved probably represents a dynamic between the open and closed forms that cannot be resolved (see above). Nonetheless, the NusA NTD and CTD fit sufficiently well in the electron density to allow informed hypotheses on how NusA functions in its various roles in the transcription cycle.

Identification of the location of the C-terminus of the β' -subunit of RNAP (Fig 3A–B) allowed the approximate location of the

Fig 3 | Localization of the β' -subunit carboxy terminus and the interaction of NusA with RNA polymerase during transcriptional elongation. (A) Determination of the location of the C-terminus of the β' -subunit of RNAP using NTA-nanogold labelling. The first column shows class sums of unlabelled core RNAP; the second column shows equivalent class sums containing nanogold-labelled RNAP. The yellow arrows indicate the location of the nanogold and, by inference, the C-terminus of the β' -subunit. The third and fourth columns show equivalent re-projections and three-dimensional views, respectively, of the class sums with the location of nanogold indicated by red spots. (B) Expanded three-dimensional view with RNAP docked. The β' -subunit is represented in green, the C-terminus is indicated by a yellow arrow and the location of the nanogold by a red ball. (C–G) NusA docked with respect to a homology model of an RNAP elongation complex. NusA NTD is coloured yellow and the CTD is coloured red. Rear and front views of RNAP (grey) and NusA–RNAP (gold mesh) reconstructions in which NusA has been docked are shown in (C,D). An expanded view of the docked NusA from the front is shown in (E). A side view of NusA docked to the elongation complex within the electron density map of the NusA–RNAP complex (gold mesh) is shown in (F). The β -flap is coloured green and the location of nanogold indicated by a red ball. Nucleic acids are represented as surface-rendered molecules with RNA in blue, template strand DNA in orange and non-template strand DNA in pink. An expanded region of the complex is shown in (G) in which RNAP is shown as a semi-transparent surface, and NusA and nucleic acids as cartoons. The distance between the 5' end of the transcript emerging from the exit channel (the green β -flap forms one side of this channel) and the 3' end of the RNA modelled on the CTD of NusA is indicated by a dashed white arrow. CTD, carboxy terminal domain; NTA, nitrilotriacetic acid; NTD, N-terminal domain; RNAP, RNA polymerase.

Fig 4 | Cartoon models of transcriptional machinery at various stages. (A) Holoenzyme. σ is bound to the RNAP core for transcriptional initiation. (B) RNAP transition complex with σ still bound. As σ region 4 dissociates from the β -flap, the NTD of NusA is able to bind, whereas the rest of σ remains attached. (C) σ is released from RNAP, resulting in the establishment of an elongation complex. The direction of transcription is indicated by an arrow. The approaching NusA in (A) and the released σ in (C) are not drawn to scale. NTD, N-terminal domain; RNAP, RNA polymerase.

β' -flap of RNAP to be identified in our reconstructions. The NusA NTD was docked into the mass adjacent to the β -flap tip, a region frequently associated with NusA-mediated transcriptional termination (Fig 3C–G, yellow; Gusarov & Nudler, 2001; Toulkhonov *et al*, 2001), which was in good agreement with our data (Fig 1). The NusA CTD was then placed within the electron density lower down the β -flap and towards the α -subunits (Fig 3C–E, red), consistent with the additional CTDs of *E. coli* NusA being in close proximity to the α -subunits of RNAP (Mah *et al*, 1999). The NusA CTD was docked using a homology model based on the structure of *Mycobacterium tuberculosis* NusA in complex with RNA (Beuth *et al*, 2005). This aided the discrimination between RNA and protein-interacting surfaces, as an RNA binding site must be accessible on NusA for interaction with the emerging transcript (Gusarov & Nudler, 2001). Although these fits are consistent with our data (and that of others; see below), owing to the low resolution of the structures obtained by negative-stain reconstructions, they will be refined when higher resolution structural data are obtained. In the expanded view of the β -flap region shown in Fig 3G, the emerging transcript would be able to bind to the NusA CTD, whereas the NTD would be in a position to regulate the aperture of the exit channel through its interaction with the β -flap tip.

Starting from the active site of RNAP, 15 nucleotides of RNA are buried in solvent (Vassilyev *et al*, 2007). In our model, the distance from the 5' end of the RNA transcript to the 3' end of the RNA bound NusA is about 28 Å (Fig 3G, dashed arrow), corresponding to approximately eight nucleotides. Hence, the NusA RNA-binding domains could interact with a minimal nascent transcript length of 23 nucleotides, which is consistent with cross-linking data showing that NusA interacts with the transcript 18–24 nucleotides from the active site (Gusarov & Nudler, 2001). Therefore, in this structure, the NusA CTD is in the correct position to sequester the emerging transcript and hence to control transcriptional pausing and termination.

NusA and the transcription cycle

The traditional view of the transcription cycle involves the release of σ as RNAP clears the promoter, coinciding with the binding of NusA and entry into the elongation phase of transcription. Our

results indicate that NusA binds mainly to the β -flap region through its NTD, with the CTD wrapped back across the β -flap where it interacts with the emerging transcript (Fig 3). Such a conformation allows us to propose a model describing how elongation complexes can contain both factors (Fig 4).

In addition to region 2.2 of σ binding to the CH domain of the β' -subunit, region 4 of σ also forms contacts with the β -flap (Fig 4A; Kuznedelov *et al*, 2002; Nickels *et al*, 2005) and so will compete with NusA for binding to that region of RNAP. The interaction of σ region 4 with the β -flap is weak and is one of the first interactions to be broken during the step-wise release of σ and transition to an elongation complex (Fig 4B; Kuznedelov *et al*, 2002; Murakami & Darst, 2003; Nickels *et al*, 2005). Conversely, the interaction of σ region 2.2 with the CH region is thought to be the last site of contact between σ and RNAP during σ dissociation (Murakami & Darst, 2003). When the structure of σ is docked onto the NusA–RNAP complex there is a steric clash caused by region 4 of σ that prevents the interaction of NusA NTD with the β -flap. By contrast, there is no visible clash that would affect the interaction of σ region 2.2 with the CH domain (Fig 4A and B). Therefore, we suggest that as the interaction between σ region 4 and the β -flap is broken during the release of σ , NusA binds, resulting in a transition complex comprising σ bound to the CH region and NusA bound around the β -flap (Fig 4).

Several studies have placed the NusA CTD close to the RNA exit channel (Gusarov & Nudler, 2001; Toulkhonov & Landick, 2003), and this is important in allowing NusA to have a regulatory role in transcriptional pausing, termination and anti-termination (Borukhov *et al*, 2005). In the structure presented here, the C-terminal RNA binding sub-domains are all close enough to the RNA exit channel that they could have a function in modulating pause/termination loop formation or interact with the transcript emerging from RNAP. Through the interaction of the NTD with the β -flap, NusA could also modulate the width of the exit channel. These possibilities remain to be answered as we and others combine single particle analysis and X-ray crystallography to yield moderate- to high-resolution structures of transcription complexes to gain a more comprehensive understanding of how transcription is regulated.

METHODS

Far-Western blotting. RNAP and NusA fragments were cloned, overproduced and purified, and far-Western blots were carried out as described in the supplementary information online.

Protein purification and sample preparation. RNAP core and NusA were purified as described previously (Davies *et al*, 2005; Yang & Lewis, 2008). NusA–RNAP complexes were reconstituted *in vitro* by mixing RNAP core (1.25 μ M final concentration) with purified NusA at a molar ratio of 1:3 in dilution buffer (20 mM Tris–HCl (pH 7.8), 150 mM NaCl, 10 mM MgCl₂, 5% (v/v) glycerol and 1 mM DTT). The reaction mixture was incubated at 37 °C for 10 min followed by 10 min on ice.

Electron microscopy and image processing. Negative staining, electron microscopy, particle picking, image processing and reconstruction using EMAN (Ludtke *et al*, 1999) were carried out as described in the supplementary information online. Electron microscopy-derived molecular envelopes have been deposited at the Protein Database in Europe (PDBe; <http://www.ebi.ac.uk/pdbe>) with the following accession numbers: RNAP core, 1577; and NusA–RNAP complex, 1578.

Docking. Structural docking and normal-mode analysis were carried out as described in the supplementary information online, and figures, were prepared using Chimera (Pettersen *et al*, 2004) and PyMol (DeLano Scientific, San Francisco, CA, USA).

Supplementary information is available at *EMBO reports* online (<http://www.emboareports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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