Chapter V

N transforms NusA into an antiterminator by modulating NusA-RNA polymerase β-subunit flap domain interactions
5.1 Introduction

The nut-bound N (lambdoid phage protein), together with different Nus factors of *E. coli* binds to EC and converts it into a termination resistant form (Greenblatt *et al.*, 1993). Among these Nus factors, NusA is mandatory for N-mediated antitermination. In chapters III and IV, we have identified NusA-NTD mutations, located at a convex surface opposite to its RNA polymerase-binding domain, are impaired for N-mediated antitermination. These NusA mutants disrupted the N-NusA interactions on the EC. In the N-NusA modified EC, AR1 region of NusA stayed away from N. Thus, we concluded that this surface of the NusA-NTD has the functional N-binding region, and proposed that location of this surface opposite to the NusA-RNAP binding site enables N to exert conformational changes to the EC through NusA. However, the molecular nature of N induced alteration in NusA-RNAP interaction is not known, understanding of which is essential to decipher the antitermination mechanism.

In this chapter, using genetics and biochemical probing, we have conclusively showed that N-NusA interaction modulates the NusA- RNAP β–flap interaction by reorienting the latter domain resulting in formation of a constricted RNA-exit channel of the EC. We propose that this N-NusA induced spatial constriction in the exit channel hinders the formation of terminator hairpin (Nudler and Gottesman 2002; Cheeran *et al.*, 2005), which is one of the way to achieve the antitermination together with affecting the active site (Cheeran *et al.*, 2007)

5.2 Materials and Methods

5.2.1 Purification of the HMK tagged proteins

A HMK (Heart Muscle Kinase) sequence was tagged to the C or N terminus of various proteins, whereby these proteins can be radiolabelled and subsequently the labelled proteins or the cleavage products from these proteins can be visualized and analysed in a phosphorimager screen, after electrophoresis. The labelling method utilizes the catalytic subunit of PKA to catalyse the transfer of the gamma phosphate of $^{32}$P ATP to the Serine residue of the PKA recognition site (Arg Arg Ala Ser Val) present in fusion proteins.
5.2.1.1 Purification of C terminal HMK, His-rpoC protein

The plasmid (pRS513 containing C-terminal HMK, His tag rpoC) was transformed into RS859 (rpoCts background strain), grown at 42°C, checked on LB Ampicillin plates for complementation. This strain was used for large-scale cell pellet preparation at 42°C in TB media supplemented with Ampicillin (Amp) and Tetracycline (Tet). C-terminal HMK, His tagged RNAP was purified as described method of Kashlev (Kashlev et al., 1996) using Ni–NTA agarose and Heparin-agarose affinity columns. The cells were grown at 42°C to get majority of the holoenzyme assembled with mutant rpoB or rpoC subunits and also the presence of His-tag helped us to purify only mutant holoenzyme from the mixed pool, if any, in the cytoplasm. Single colony of the rpoB/C background strains with the required rpoB/C mutation harbouring rpoB/C plasmid was inoculated in LB supplemented with Amp and Tet and grown O/N at 42°C. Next day, it was subcultured to 1:1000 dilutions in 5 L of the same media, grown till OD₆₀₀ reached around 1.5. The cells were harvested by centrifugation at 12000 rpm and cell pellet was frozen. In all the cases, RNAPs were purified from 20 to 30 g of cell pellet. To the pellet, 50 ml of buffer A (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 5% (v/v) Glycerol, 1 mM DTT, 300 mM NaCl) was added and mixed well by stirring, on ice. To the mixture, 0.3 mg/ml Lysozyme was added and mixed well by pipetting and blender. This mixture was then incubated on ice for 10 min. To this mixture, again 50 ml of Buffer A and Lysozyme (0.3 mg/ml) was added, mixed well as above and incubated for 10 min, on ice. Sodium deoxycholate (4%) was added drop by drop with stirring, to a final concentration of 0.2% and was incubated at 4 °C in a cold room, with stirring. The mixture was aliquoted to 25 ml each in fresh pre-cooled Falcon tubes and sonicated for 10 min for complete lysis. The lysate was then centrifuged at 12000 rpm for 45 min at 4°C. The supernatant was transferred to a clean beaker (pre-cleaned with DEPC water and chilled) and 10% Polyethylamine (Polymin P, pH 7.9) was slowly added with constant stirring, to a final concentration of 0.8%. The stirring was continued for another 20 min (the solution turns turbid) at 4°C. Then, this was centrifuged at 12000 rpm for 45 min at 4°C. The obtained pellet was thoroughly resuspended in 100 ml TGED (10 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 5% (v/v) Glycerol, and 0.1 mM DTT) plus 0.5 M NaCl by mixing thoroughly in a blender in the cold room. The suspension was centrifuged at 12000 rpm for 15 min at 4°C, the supernatant was stored for analysis, and the pellet was resuspended completely in TGED plus 0.5 M NaCl as
before and the washing was repeated twice more. To elute RNAP from Polymin P, the pellet was resuspended in 100 ml of TGED plus 1 M NaCl. The mixture was centrifuged at 12000 rpm for 30 min at 4°C. Clear supernatant was measured, transferred into a clean and pre-cooled glass beaker and finely ground ammonium sulphate was added slowly to the supernatant with constant stirring, to an amount of 35 g to 100 ml solution. pH was adjusted to the range of 7-7.5 with 2 N NaOH. The suspension was left with stirring for 30 min. Then it was centrifuged at 12000 rpm for 45 min at 4°C. The obtained pellet (protein) was resuspended in 10 ml of buffer B (20 mM Tris-HCl, pH 7.9, 10 mM MgCl₂, 300 mM KCl, and 1 mM 2-mercaptoethanol). Then, protein was dialyzed against 1 L of Buffer B O/N with two buffer changes. Next day, the protein was centrifuged at 12000 rpm for 15 min at 4°C to remove any protein aggregates. The protein was then mixed with Ni-NTA beads (washed with DEPC water and then equilibrated with Buffer B) and left for binding in a rotating orbiter in the cold room for 1 hr. Then, it was transferred into a Ni-NTA super flow column, by slowly pouring the slurry into the empty column, to bed volume of 5-10 ml and the resin was allowed to settle. The flow through was collected and washed with 20 ml of Wash buffer (50 mM Phosphate, 0.3 M NaCl and 20 mM Imidazole, pH adjusted to 8.0) and wash was collected. Protein was eluted by adding 15 ml of Elution buffer (50 mM Phosphate, 0.3 M NaCl and 250 mM Imidazole, pH adjusted to 8.0). The eluted samples were analysed on an 8% SDS PAGE. Then, eluted protein was dialyzed against 1 L of TGED plus 0.1 M NaCl, with two buffer changes for O/N. Next day, the protein was centrifuged at 12000 rpm for 15 min 4°C to remove any protein aggregates. The supernatant was diluted with TGED with 0.1 M NaCl and was loaded on to a Heparin column (Amersham) through AKTA, which was earlier equilibrated with TGED with 0.1 M NaCl. RNAP was eluted with continuous linear gradient of NaCl. The pure RNAP fraction elutes out in the range of 0.4-0.65 M NaCl. The fractions were checked for the presence of protein by running in an 8% SDS PAGE. Pure eluted fractions were pooled and concentrated in a 100 kDa concentrator (Amicon) against 2X storage buffer (80 mM Tris-HCl, 0.4 M KCl, 2 mM EDTA and 2 mM DTT). Glycerol to the final concentration of 50% v/v was added to the concentrated pure protein, aliquoted into fresh sterile eppendorf tubes and stored in –80 °C deep freezer.
Figure 5.1: Purification of HMK tagged RNA polymerase proteins. A) HMK tagged *rpoC* RNAP purification. Purification steps through Ni-NTA column (the flow through fractions through Ni-NTA column, two wash fractions with 20 mM Imidazole, two fractions of RNAP eluted with 250 mM Imidazole); the NaCl continuous linear gradient with AKTA purifier. X-axis: shows different fractions; Y-axis shows UV 280 nm value. Peak fractions were pooled for next purification step batch elution fractions through the Heparin column and the purified protein are shown. M stands for protein molecular weight marker (NEB). Commercial RNAP (Holoenzyme, Epicenter) is also shown, for comparison. B) HMK tagged *rpoB* RNAP purification. Same as above. C) HMK tagged G1045D *rpoB*RNAP purification. Same as above.
5.2.1.2 Expression and purification of N terminal-HMK rpoB protein

pTRC99A encoding β S531Y rpoB (a gift from Sergei Borukhov) (Laptenko et al., 2003) was transformed into MG1655 cells (RS485) and grown in large scale in TB. β S531Y RNAP was purified according to the mentioned protocol in Kashlev et al., 1996 as well as used the same procedure of HMK-tag rpoC RNAP purification using Ni–NTA beads and Heparin-agarose affinity column.

5.2.1.3 Preparation of N terminal-HMK rpoB mutants

N-terminal-HMK tagged G1045D, P1044A, V1046E, V839E and R1058L mutant of rpoB were created by site directed mutagenesis (SDM) using pRS484 (pTRC99A encoding β S531Y rpoB, a gift from Sergei Borukhov). These plasmids were transformed into RS860 (MG1655, rpoBts background strain) and grown in large scale in TB. N-terminal-HMK these RNAP points mutants were purified according to the mentioned protocol in (Kashlev et al., 1996) as well as used the same procedure of HMK-tag rpoC RNAP purification using Ni–NTA beads and Heparin-agarose affinity column.

5.2.2 DNA templates preparation

Linear DNA templates for different in vitro assays were made by PCR amplifications from the plasmids, pRS604 (T7A1-λnutR-lacO; oligo pairs RS83/RS333 for Template I and RS83/RS994 for Template II), pRS106 (pT7A1-trpt′-lacO; oligo pair RS83/177 for Template III) and pRS22 (pT7A1-H 19BnutR-TR′-T1-T2; oligo pairs, RS58/RK1, Template IV). The lacO sequence is present in the downstream oligos RS177, RS333 and RS994. The ops pause sequence (Artsimovitch and Landick, 2000) was incorporated downstream to the H-19B nutR site (T7A1- H 19B nutR-ops pause-lacO, Template V) by overlapping PCR amplification using the primers RS83, RS267 and RS276. The his pause sequence was incorporated after the nutR site (T7A1-H 19B nutR-his pause-lacO, Template VI) also by overlapping PCR method using the primers, RS83, RS263, RS264, RS265 and RS275. In both these cases, pRS22 was used for amplification, and the oligos RS275 and RS276 contained the lacO sequence so that both these templates have a lac operator sequence at their downstream edge. The template used in anti-sense/RNase H cleavage assays (Template VII), was amplified from pRS22 using RS83/RS404 oligo pairs, whereas the same used in the Fe-BABE cleavage assays (Template VIII), was made from pRS385 using oligo pairs RS83/RS2.
RS404 has the lacO sequence. This operator sequence is cloned downstream of the H19B nutR site in pRS385 (Muteeb et al., 2012). In all these aforementioned templates, stalled elongation complexes (RB) were formed at the operator site in the presence of Lac repressor. In some cases, pRS25 (Cheeran et al., 2005) was used for making the T7A1-H19B nutR-TR' template using the oligo pair RS58/RS2 by PCR-amplification. Transcription was initiated from the T7A1 promoter in all these templates. When required, the templates were immobilized on the streptavidin beads via a 5′-biotin group using the biotinylated primer RS83.

### 5.2.3 NusA dissociation assays

To measure the association of WT NusA to the elongation complex (EC) in the presence and absence of N, transcription reactions were performed in the presence of 100 nM lac repressor, to form a road-blocked (RB, it is also called stalled EC) EC at the lacO site, present downstream of various terminators. The DNA templates were immobilized on streptavidin magnetic beads (Promega) through streptavidin-biotin interaction at their 5′-end. The road-blocked ECs were formed by chasing EC23 with 250 μM NTPs along with 10 nM of 32P-labelled WT NusA for 2 min. Excess NTPs were removed by washing the beads, followed by the addition of 100 nM of unlabelled (cold) WT NusA with and without N as a competitor. After 5 min, 15 μl of samples were removed and fractionated into supernatant (S) and pellet (P) on magnetic stands. Half of the supernatant (S) and rest of the sample (S+P) were mixed with equal volume of SDS loading buffer. Fraction of NusA dissociated [(2S)/(S) + (S+P)] was plotted (Figure 5.2 and 5.3).

To compare binding affinity of WT NusA with WT RNAP/G1045D RNAP, stalled ECs were formed using either WT or G1045D RNAP in the presence 10 nM of 32P-labelled WT NusA as described above. After the formation of ECs with 32P-labeled WT NusA, excess NTPs were removed by thoroughly washing the beads, followed by addition of varying concentrations (10-200 nM) of non radio-labelled (cold) WT NusAs as competitors. After 5 min, 15 μl of samples were removed and fractionated into supernatant (S) and pellet (P) on magnetic stands. Half of the supernatant (S), and rest of the sample (S+P) were mixed with equal volume of SDS loading buffer. Fraction of NusA dissociated [(2S)/(S) + (S+P)] was plotted against time and the curves were fitted to exponential rise equations (Figure 5.9).
5.2.4 Measurement of in vivo antitermination defects of G1045D RNAP mutant on different terminators through β-galactosidase assay

These assays were performed in different derivatives of *E. coli* MC4100 Δrac strains. The strains RS1451, RS1452 and RS1450 were used in β-galactosidase assays have β-G1045D mutations in the genomic copy of the rpoB and $P_{lac-H-19B ~nutR/t_{R1}-trpt'-lacZYA}$, $P_{lac-H-19B ~nutR/t_{R1}-TR'-lacZYA}$ and $P_{lac-H-19B ~nutR/t_{R1}-TR'-T1-T2-lacZYA}$ reporter cassettes, respectively, as λRS45 lysogens. RS1017, RS1018 and RS1148 strains used for the same β-galactosidase assays have the WT rpoB and $P_{lac-H-19B ~nutR/t_{R1}-trpt'-lacZYA}$, $P_{lac-H-19B ~nutR/t_{R1}-TR-lacZYA}$ and $P_{lac-H-19B ~nutR/t_{R1}-TR-T1-T2-lacZYA}$ reporter cassettes, respectively, as λRS45 lysogens. $t_{R1}$ and trpt’ are Rho-dependent and TR, T1, and T2 are the intrinsic terminators. When required, these strains were transformed with pK8641 expressing WT or L108F N proteins from a $P_{lac}$ promoter (Cheeran et al., 2005). These assays were performed following standard procedures using microtitre plates as described in chapter III.

5.2.5 Synthetic defect assays

To test whether the combination of rpoB-G1045D and V8A-nusA mutants induces synthetic defects in N-mediated in vivo antitermination, pHYD3011 plasmid expressing either WT or V8A NusA was transformed into the strains, RS1452 (having rpoB-G1045D and $P_{lac-H-19B ~nutR-T_{R}~lacZYA}$ reporter cassette) and RS1018 (having WT rpoB and $P_{lac-H-19B ~nutR-T_{R}~lacZYA}$ reporter). Following which chromosomal WT nusA of these strains was deleted by P1 transduction (nusA::CamR). β-galactosidase assays were performed in a similar way as stated above.

5.2.6 Establishment of functional N mediated antitermination system

For the transcription on the T7A1-nutR-T_{R}~T1-T2 template, reactions were performed as described in chapter III.

5.2.7 Pausing assays

For pause assays, RBs made of WT or G1045D RNAP were at first formed on the Template V or VI at the respective pause sites in the same way as described in chapter IV. Concentrations of DNA and RNAP were 10 nM and 50 nM. The RBs were then washed thoroughly to remove the excess NTPs, following which they were chased out.
of the stalled position in the presence of 100 μM of UTP, CTP, ATP, 10 μM GTP, 1 mM IPTG. When required 300 nM of NusA was added to the chase. Aliquots were removed during the specified time points (0, 30, 45, 60, 90, 120, 180, 240 and 300 sec for his pause and 0, 30, 45, 60, 90, 120, 180 and 240 sec for ops pause) and mixed with formamide loading dye (Ambion). RNA products were separated on 8% sequencing gel. Fraction of paused complex was calculated as: (Intensity of the paused RNA) / (Total intensities of pause RNA + other higher–sized products). Plots obtained from plotting the fraction of paused products against the time were fitted to exponential decay curve of the form: $y = A \cdot \exp^{-\lambda t}$, where $A$ is the amplitude and $\lambda$ is the rate constant. The pause half-lives ($t_{1/2}$) were measured from $t_{1/2} = \ln 2 / \lambda$.

5.2.8 RNase H assay

RNase H assays were performed on the RBs made on Template VII. Cleavage positions were defined by designing series of antisense DNA oligos, RS549, RS550, RS551 and RS1106, 5′-end of those correspond to the -10, -11, -12 and -14 positions of the nascent RNA, respectively (Figure 5.10 A). RBs were formed in the same way as described in chapter IV. The RBs were re-suspended in the T.C. buffer with or without 300 nM of WT NusA and 300 nM of H-19B N for 5 min. Following which RNase H cleavage was initiated by adding 2.5 μM of each of the antisense oligos and 0.25 U of RNase H and incubation was continued at 37°C for different time points. Reactions were stopped by phenol extraction, samples were mixed with equal volume of formamide loading dye and loaded onto 8% sequencing gel.

5.2.9 Foot-printing of the EC with Fe-BABE

Fe-BABE (p-Bromoacetamidobenzyl-EDTA), is an artificial protease for determining the three dimensional structure of proteins as well as the binding surfaces of protein on protein-DNA and protein-protein complex. Fe-BABE is a cysteine-tethered chemical nuclease, developed in Claude Meares laboratory (Meares et al., 2003). Fe-BABE can be specifically conjugated to cysteine residue of a protein. Fe-BABE is a protein modification reagent (Meare’s reagent) and can be attached to a protein through sulfhydryl groups. Under reducing conditions (the reaction is initiated by addition of Ascorbic acid), an Iron (II) chelate is generated by the reduction of an Iron (III) chelate with ascorbic acid and upon addition of Hydrogen peroxide, this reagent generates
hydroxyl radicals, which cleaves nearby (~12 Å) peptide and nucleic acid backbone. Here, it was expected that the hydroxyl radical generated in situ will cleave the peptide backbone of the different domains of RNAP situated ~12 Å radius of the N-terminal cysteine residue (53C or 29C) of the NusA bound to the EC and thereby, will define the regions of the EC coming close to the N-terminal of NusA in the presence and absence of N. We have constructed single Cys derivatives of NusA; S29C, S53C (in the NusA NTD domain) and 251C (in the SKK domain of NusA, Figure 5.15 A) and Fe-BABE was conjugated to these single Cys derivatives of NusA.

5.2.9.1 Conjugation of single Cys derivatives of NusA with Fe-BABE reagent

Single Cys derivatives of NusA were conjugated to Fe-BABE. For effective conjugation, it is required to remove reducing agents as well as metals, if present, in the protein. In order to remove DTT from NusA stock solution (25 μM), 50 μl NusA protein was first dialysed in metal removing buffer (30 mM MOPS, pH 8.2, 4 mM EDTA) and then the protein was exchanged into conjugation buffer (30 mM MOPS, pH 8.2, 100 mM NaCl, 1 mM EDTA, and 5% glycerol) using a desalting column (Pierce). 0.4 mM of Fe-BABE (Pierce) was incubated with 20 mM NusA in conjugation buffer for 1 hr at 37°C. Unconjugated Fe-BABE was removed by passing the reaction mixture through desalting column, equilibrated in storage buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10% glycerol and 0.1 mM EDTA). Efficiency of Fe-BABE labelling was monitored by the sensitivity for DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)] (Riddles et al., 1979). The free –SH groups in Fe-BABE conjugated single Cys derivatives of NusA were determined spectro-photometrically at 412 nm using DTNB. Fe-BABE conjugated NusA (1.6 μM, different Cys derivatives of NusA) was incubated at 25°C with 0.8 mM DTNB in 50 mM Phosphate buffer, pH 7.5. The stoichiometry of the reaction was calculated by using extinction coefficient of 14150 M⁻¹ cm⁻¹.

5.2.9.2 End labelling of HMK tagged proteins

HMK-tagged β and β' subunits of RNA polymerase were radiolabeled with [γ-³²P] ATP (3000 Ci/mmol, Amersham Bio-sciences) using protein kinase A (Sigma). The labelling reaction was done in buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10mM MgCl₂, and 10 mM ATP. Holoenzymes (1 μM) were incubated with 50 units of the kinase for 1 hr at 21°C in a reaction volume of 25 μl. Labelled RNA
polymerase holoenzymes were used for Fe-BABE, without further purification. WT NusA was also radiolabeled with \([\gamma^{32}P]\) ATP as described above. WT NusA and NTD-NusA (10 \(\mu\)M) were incubated with 50 units of the kinase for 1 hr at 21°C in a reaction volume 25 \(\mu\)l. Labelled NusA and NTD-NusA were used for stability assays.

5.2.9.3 Fe-BABE conjugated NusA probing of EC with radio-labelled RNAP

HMK tag RNAP (either rpoB or rpoC) were radio-labelled using protein kinase A and \([\gamma^{32}P]\)ATP (3000 Ci/mmole). Stalled EC was formed in a similar way on the same template (T7A1-H-19B nutR\(R\_R1\)-lac\(O\_R\_T\'_R\)) as described for RNase H foot-printing in chapter IV (Figure 4.3 A) except RNAP (either \(\beta\) or \(\beta'\)) was radio-labelled instead of RNA. Radio-labelled EC (either \(\beta\) or \(\beta'\)) and Fe-BABE-NusA (600 nM each) in the presence and absence of (600 nM) N were incubated at 37°C for 10 min in cutting buffer (50 mM MOPS, pH 8.0, 0.1 mM EDTA, 120 mM NaCl, 10 mM MgCl\(_2\), and 10% glycerol) prior to cleavage reaction, which was initiated by adding ascorbate and hydrogen peroxide (final 6 mM each). Cleavage reaction was stopped by addition of one volume of 6XSDS sample buffer after incubation at 37°C for 5 min. Samples were heated to 95°C for 4 min prior to loading on 8% SDS-PAGE. Gels were exposed overnight to a phosphorimager screen and the bands were analysed using Phosphorimager (Fuji).

5.2.9.4 Generation of radiolabelled RNAP markers

Molecular weight markers of end-labelled RNAP (either N-terminal HMK tag \(\beta\) or C-terminal \(\beta'\)) was generated by cyanogen bromide (CNBr), Sub-maxillary protease (Arg-C) and Lysylendopeptidase (Lys-C) mediated cleavages. Methionine specific cleavage reaction was performed using CNBr in a 10 \(\mu\)l reaction mixture containing 0.2 \(\mu\)M of labelled RNAP, 0.1 M CNBr and 0.4% (w/v) SDS (pH adjusted to pH 2 with 1 M HCl). At first labelled RNAP was incubated at 37°C for 10 min with 0.4% (w/v) SDS and cleavage reaction was started with the addition of 0.1 M CNBr for 5 min at 37°C. Reactions were terminated by addition of 6X SDS-loading dye followed by boiling.

Arginine specific cleavage reaction was performed in a 10 \(\mu\)l reaction mixture containing 0.2 \(\mu\)M of labelled RNAP, 5 \(\mu\)g of Sub-maxillary protease and 0.1% (w/v) SDS. Reaction was started with the addition of 5 \(\mu\)g Arg-C after the incubation of
RNAP with 0.1% SDS at 37°C for 10 min and terminated after 10 min by addition of 6X SDS-loading dye followed by boiling.

For the lysine specific cleavage, 0.2 µM of labelled RNAP was mixed with 6.33 µM urea in 50 mM Tris-HCl buffer and incubated at 37°C for 10 min. The cleavage reaction was started with the addition of 50 ng Lys-C at 37°C and terminated after 10 min by addition of 6X SDS-loading dye followed by boiling.

5.3 Results

5.3.1 N does not increase the affinity of NusA for the RNAP of the EC

Concave surface of NusA-NTD binds to the β flap domain of RNAP on the EC and the RNA binding (SKK) domain of NusA binds to the emerging RNA (Ha et al., 2010). Our previous study showed that convex surface of NTD-NusA binds N in the presence of the EC. To test whether N increases the affinity of NusA for the RNAP subunits of the EC, we measured the dissociation of radiolabelled WT NusA (32P-NusA) protein from the ECs stalled either close to the nut site (~60 nt downstream; Figure 5.2 A, Template I) or away from it (~250 nt downstream; Figure 5.2 A, Template II) or from downstream of a terminator that is devoid of nut site (Figure 5.2 A, Template III) in the presence and absence of N by “cold” competition assays. We used cold (non radio-labelled) NusA as competitor.

We observed the following. 1) In the absence of N, the 32P–NusA bound to the EC stalled on Template I was competed out readily by cold NusA competitor at a concentration 100 nM (Figure 5.2 B and E). 2) In presence of N, 2-fold less amount 32P–NusA was dissociated in the presence of the competitor (Figure 5.2 B and E). 3) When the 32P–NusA was part of the EC stalled on template II (away from nut site), the effect of N was reduced (Figure 5.2 C and E). 3) There was no effect of N when the labelled NusA was part of the EC stalled on template III (~nut site) (Figure 5.2 D and E).

These data suggest that the enhanced stability of NusA for the EC in the presence of N might be dependent on the nut site (NusA binds to the spacer region of nut site). To confirm it, we measured the dissociation of NTD domain of NusA from the EC stalled on template I/template III in presence and absence of N by the same assays. NusA-
NTD only can interact with RNAP. We observed that N was unable to improve the stability of NusA-EC made on both the templates I and III (Figure 5.3).

Therefore, we concluded that the enhanced affinity of NusA for the EC in the presence of N is dependent on nut site-NusA interaction. N stabilizes the NusA-nut site spacer interactions.

Figure 5.2: A) Cartoons showing stalled ECs on different templates. On template I and II, ECs are stalled 60 nt and 250 nt downstream of the nut site of λ nutR respectively. In template III, trp′, lacking nut site, is located upstream of the stalled EC. All the templates were immobilized to streptavidin coated magnetic beads. B)-D) Autoradiogram showing the half amount of dissociated radio-labelled NusA (32P-NusA) in the form of S (half of supernatant) and remaining radio-labelled NusA (32P-NusA) in the form of P (half of supernatant+pellet) bound to the EC either on the template I, II or III in the presence and absence of N (100 nM) and cold competitor WT NusA (100 nM). E) Bar diagram showing the fraction of dissociated radio-labelled NusA. Fraction of dissociated NusA was calculated as [(2S)/(S) + (S+P)].
5.3.2 β flap domain mutants have specific defects for N-antitermination

In a previous publication from our laboratory, RNAP mutations specifically defective for H-19B N mediated antitermination, located in and around the RNA exit channel (P251S, P254L, and R270C in β') and in flap domain of β (G1045D) subunit were described (Cheeran et al., 2005). In the same study a suppressor (L108F) in H-19B N that specifically suppressed the defects of RNAP mutants located in β' near to the exit channel was also described. Interestingly, this suppressor was unable to suppress the β flap (G1045D) mutant. It should also be noted that β flap binds to NTD-NusA (Ha et al., 2010). Therefore, we hypothesized that defect of N-mediated antitermination of the β flap mutant might be NusA mediated. So we designed some more mutants in the β flap region, like P1044A, V1046E, V839E and R1058R (Figure 5.4) by site directed mutagenesis. V839E and R1058L mutants were known to be defective for Q-mediated antitermination (Santangelo et al., 2003).
Figure 5.4: Location of RNAP mutants are indicated on EC (Opalka et al., 2010). These mutants are shown in different colors as P251S and P254L in grey color (located on β′ subunit and defective for N function), R1058L and V839E in yellow color (located in β flap and defective for Q function), P1044A, G1045D and V1046E in red color (located in β flap and defective for N function). β flap domain is shown green color. DNA and RNA are shown in red and blue color respectively.

Figure 5.5: Point mutations in β flap domain impair N-antitermination. A) Bar diagrams showing the β-galactosidase activities from the reporter cassettes having Rho dependent and Rho-independent terminators, in the presence of the G1045D RNAP mutants. Values obtained both in the absence and presence of H-19B N are shown. B and C) In vitro antitermination assays on template (having TR-T1-T2 terminator). RO denotes the run-off product. Concentrations of DNA template, RNA polymerase and NusA were 5 nM, 25 nM and 300 nM, respectively with different concentrations of N. Samples were run on a 6% sequencing gel and graph was plotted as read through v/s concentration of N. D) Synthetic defect of G1045D RNAP and V8A NusA mutants were tested by β galactosidase assays.
We repeated *in vivo* antitermination assays with G1045D mutant on different terminators (Figure 5.5 A). We observed that G1045D mutant was severely defective for H19B N induced antitermination at the triple Rho-independent \((T_R-T1-T2)\) terminator, partially defective at single Rho-independent \((T_R')\), but was not significantly defective at Rho-dependent terminators \((t_{R1-trp'}\); (Figure 5.5 A). It might be due to the fact that N-NusA-RNAP interaction is not important for N mediated antitermination on Rho-dependent terminator. We could not perform *in vivo* antitermination assays with P1044A and V1046E mutants on different terminators because they were not viable at 42°C.

Then we performed *in vitro* antitermination assays with all the β flap mutants. The *in vitro* antitermination efficiencies (%RT) of H-19B N were measured using DNA template with Rho-independent triple terminator cassette \((T_R-T1-T2); \text{Figure 5.5 B and C})\). G1045D, P1044A and V1046E RNAP mutants exhibited significant defects at the end of the triple terminator. G1045D was the most defective (Figure 5.5 B and C). We also tested R1058L and V839E (Q-mediated antitermination defective mutants located in β flap) mutants in the same assay, and observed that they very less defective for N mediated antitermination (Figure 5.6 A and B).

Above, we have observed that these β flap mutants were significantly defective for N-mediated antitermination. This β flap of β subunit of RNAP binds to NTD of NusA. Next, we tested synthetic defect in the *in vivo* termination assays from the combination of G1045D RNAP mutant and V8A NusA mutant. We observed that G1045D RNAP mutant and V8A NusA mutant were partially defective for N-mediated antitermination at Rho-independent terminator, \(T_R\) when they were expressed with WT NusA or RNAP, respectively. But when both V8A NusA mutant and G1045D RNAP mutant were expressed together in a strain (RS1452) then this combination exhibited severe antitermination defect (Figure 5.5 D)

Above antitermination assays strongly indicated that flap-domain is involved in N-antitermination via its interaction with NusA. We propose that N utilizes NusA-flap interaction to modulate the conformations of the latter domain to bring about the antitermination event.
Figure 5.6: β flap mutants (R1058L and V839E) defective for Q function, are very less defective for N function. A) In vitro antitermination assays on template (having T_R'-T1-T2 terminator). RO denotes the run-off product. Concentrations of DNA template, RNA polymerase and NusA were 5 nM, 25 nM and 300 nM, respectively with different concentration of N. Samples were run on a 6% sequencing gel and graph was plotted as read through (RO) at 3T v/s concentration of N and read through (RO) at T_R'-v/s concentration of N.

5.3.3 β flap domain mutants defective for N-antitermination affect flap-NusA interaction as well as spatial orientation of the flap

Next, we wanted to understand the molecular basis of N-antitermination defect of the flap mutants. We have chosen G1045D as a prototype mutant as it was most defective for N-dependent antitermination.

It has been established that the interaction of NusA and the β flap-tip helix (β 900–909 aa) at the outer ridge of the RNA exit channel is the molecular basis for hairpin-dependent pause enhancement by NusA (Toulokhonov et al., 2001). We followed the pausing kinetics of WT and G1045D RNAP through the his pause site cloned downstream of the nutR sequence (Figure 5.7 A). We found that G1045D mutation reduced pausing significantly (from t_1/2 = 28s to 16s, Figure 5.7 C and D), which is similar to that was observed upon flap tip helix deletion (Toulokhonov and Landick 2003). This suggests that G1045D flap mutant affects the hairpin-flap interaction. Interestingly, the effect of NusA on the his pause enhancement for the G1045D was
very moderate (< 2-fold compared to > 3-fold for the WTRNAP) (Figure 5.7 C and D). These data suggest that this flap mutant, even though it is away from the flap-tip, induced significant impairment in hairpin-flap interaction, and NusA was also not able to induce flap-hairpin interaction probably due to hindered movement of flap-tip helix. To test whether G1045D mutant was specifically defective for his pause, we measured the effect of G1045D flap mutant at *ops* pause and observed that it was not defective for *ops* pause and there was no significant role of NusA similar to WT RNAP on this pause site (Figure 5.8). So we concluded that this flap mutant is specifically defective for hairpin-flap interaction and NusA is not able to enhance hairpin-flap interaction in the case of G1045D flap mutant.

![Figure 5.7](image)

**Figure 5.7**: Pausing activity of G1045D flap mutant at the *his* pause sequence. A) Schematic showing the NusA-modified EC stalled at the *his* pause site using Lac repressor as a roadblock (RB). Pause sequence, pausing site, and pause-hairpin are shown above B) autoradiogram showing the time course of transcription elongation through the *his* pause sequence (indicated by arrow) both in the absence and presence of WT NusA. Samples were removed at different time points and quenched. RNA products were separated in 8% denaturing polyacrylamide gel. Run-off product is indicated as “RO.” C) Amount of fraction of paused complex obtained under different conditions is plotted against time. The plots were fitted to the equation of exponential decay using SIGMAPLOT to calculate the escape rates (λ) and the pause half-life (t_{1/2}=ln2/λ).
Figure 5.8: Pausing activity of G1045D flap mutant at the ops pause sequence. A) Schematic showing the NusA-modified EC stalled at the ops pause site using Lac repressor as a roadblock. Pause sequence, pausing site is shown above B) autoradiogram showing the time course of transcription elongation through the ops pause sequence (indicated by arrow) both in the absence and presence of WT NusA. Samples were removed at 0, 15, 30, 45, 60, 90, 120, 180 and 240 sec and quenched. RNA products were separated in 8% denaturing polyacrylamide gel. Run-off product is indicated as "RO." C) Amount of fraction of paused complex obtained under different conditions is plotted against time. The plots were fitted to the equation of exponential decay using SIGMAPLOT to calculate the escape rates (λ) and the pause half-life (t_1/2 = ln2/λ)

As G1045D RNAP does not exhibit NusA-induced enhancement at his pause, we reasoned that NusA-NTD may have weaker interaction with G1045D. To test this, we measured the dissociation of NusA from ECs stalled at template III. ECs were formed using either WT or G1045D RNAP. In these experiments, NusA was radio-labelled with $^{32}$P at its HMK tag sequence. The ECs (having either WT RNAP or G1045D) were immobilized on a streptavidin coated magnetic beads using a biotinylated DNA template. We measured the stability of NusA-EC complex by competition assays. The
radio-labelled NusA bound to the ECs (having either WT or G1045D RNAP) on template III (Figure 5.9) were competed with different concentrations of unlabelled (cold) WT NusA. We observed that the dissociation rate of labelled NusA from ECs is same on both ECs formed by WT RNAP or G1045D RNAP (Figure 5.9 C). Therefore, this data suggests that NusA has same binding affinity to both WT and G1045D RNAP.

From the above results we concluded that the flap-hairpin interaction defect of this flap mutant might be due to altered flap movement induced by NusA.

The model of EC revealed that the flap domain forms one wall of the RNA exit channel near the nucleotides -11 to -17 but makes little direct contact with the exiting RNA (Vassylyev et al., 2007). Other studies have also established that the flap tip could cross-link to RNA hairpin formed around the nucleotides -16 to -20 (Toulokhonov et al., 2001) that helps in pausing (Hein et al., 2014). So, to test the movement of flap domain due the G1045D mutations, we assessed the protection of emerging RNA from RNase H digestion.

Figure 5.9: Binding affinity of NusA to G1045D RNAP mutant. A and B) Autoradiogram showing half amount of dissociated NusA in the form of S (Half of supernatant) and remaining amount of NusA in the form of P (half supernatant+ pellet) bound to EC on template III in the presence of different concentration of cold competitor NusA. C) Graph showing the fraction of dissociated radio-labelled NusA. Fractions of dissociated radio-labelled NusA were calculated as described in methods and material.
The spatial location of flap-tip is at the outer ridge of the exit channel, and its movement is likely to affect the conformations of the exit channel. We designed anti-sense oligos whose 5' ends at -10, (RS549), -11 (RS550), -12 (RS551) and -14 (RS1106) (Figure 5.10 A), and performed RNase H cleavage of the RNA:DNA hybrid formed by these oligos. Accessibility of the hybrids were compared between the ECs formed with WT and G1045D RNAP, both in the presence and absence of NusA. These oligos did not cause oligo mediated RNA release (Figure 5.10 B).

Figure 5.10: RNase H accessibility probed with different antisense Oligos. A) Schematic of stalled elongation complex on a template RS83/RS404 and the set of nested oligos that were used as probes for RNase H accessibility studies. B) Stalled elongation complexes were incubated at 37°C for 10 min with oligos that annealed at different distances from the RNA 3' end, as shown in the section A, and RNA release was measured which is almost negligible. C-F) Stalled ECs with WT and G1045D RNAP were formed on a template RS83/RS404. NusA-modified or unmodified EC were used. Graphs represent a time course of digestion using the oligo that anneals to -10, -11, and -12 of the emerging RNA.
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We observed the following.

1) In the presence of NusA, WT EC strongly reduced RNase H accessibility for -10, -11 and -12 oligos (Figure 5.10 C and E).

2) Under all the condition RNA:DNA hybrid from -14 oligo was readily accessible for digestion (Figure 5.10 ).

3) However, this NusA effect significantly reduced when EC was made by flap mutant G1045D RNAP (Figure 5.10 D and F). Therefore, in the G1045D mutant, NusA alters the orientation of flap-tip region over the RNA exit channel and that could be the reason for its defect in antitermination.

5.3.4 N changes spatial orientation of flap in a NusA-dependent manner

The G1045D data indicates that proper spatial orientation of flap domain as induced by NusA is crucial for N-antitermination. So, next we addressed effect of N on the flap-domain orientation.

Here we used same anti-sense oligos, those were used above (Figure 5.10 A), and performed RNase H cleavage of the RNA:DNA hybrid formed by these oligos with the emerging RNA from the stalled EC (made of WT RNAP). Accessibility of the hybrids were compared between N modified and unmodified stalled ECs in the presence of NusA. We observed that N enhances the effect of NusA, when the EC was made by WT RNAP (left panel of Figure 5.11). We also tested the effect of V8E NusA mutant (defective for N binding during antitermination) and observed that V8E NusA reduces RNA:DNA cleavage by RNase H like WT NusA but N could not enhance the effect of V8E NusA (right panel of Figure 5.11). Therefore, we concluded that N changes spatial orientation of flap in a NusA-dependent manner.
Figure 5.11: Effect of NusA and NusA+N on RNase H accessibility probed with antisense Oligos that anneals to -10, -11, and -12 of the emerging RNA. A-C) Stalled ECs with WT RNAP were formed on a template RS83/RS404 (Figure 5.10 A). NusA/NusA+N-modified or unmodified ECs were used. Right panel of figure shows that experiment was performed with WT NusA and left panel with V8E NusA mutant. Graphs represent a time course of digestion using the oligo.
**Figure 5.12: RNase H accessibility probed with different antisense Oligos.** A and C) Stalled ECs with WT RNAP were formed on a template RS83/RS404 (Figure 5.10 A). B) Stalled ECs with G1045D RNAP was formed. NusA/NusA+N-modified or unmodified EC were used. Autoradiograms represent a time course of digestion using the oligo that anneals to -10, -11, -12 and -14 of the emerging RNA.

5.3.5 *N changes the cleavage pattern of Fe-BABE labelled NusA in and around the flap domain of the EC*

According to the previous studies, NTD-NusA contains two faces; concave surface and convex surface. The concave surface interacts with the flap domain of RNAP (Ha et
whereas the convex surface interacts with N (Mishra et al., 2013). To identify the putative conformational changes of the flap domain in the presence of N, we used the free radical generating Fe-BABE reagent, which can be conjugated to Cys residues and cleaves nucleic acids or proteins within 10 Å radius when OH generation is activated. First, we engineered Cys-less NusA as described in chapter IV. We then introduced single-Cys substitutions at two non-conserved, surface-exposed Ser residues on opposite faces of the NTD (S29C and S53C; Figure 5.13 A) as described in chapter IV. All four derivatives of NusAs (Cys-less, C251, S29C, and S53C) conjugated with Fe-BABE.

The activity of the resulting Fe-BABE-conjugated NusAs were checked by in vitro N mediated antitermination assays and observed that all NusAs conjugated with Fe-BABE were active for N function (Figure 5.13 B).

To determine the cleavage sites on EC, we used recombinant RNAP containing radiolabelled β or β’ subunits having N- or C-terminal HMK (Heart muscle kinase) tags for making stalled EC (Figure 5.14 A). We induced the OH-free-radical from the Fe-BABE labelled NusA bound to the ECs, and monitored the cleavage products of the radiolabelled RNAP subunits both in the absence and presence of N protein.

We observed the following. 1) In the absence of N, S29C-Fe-BABE-NusA and S53C-Fe-BABE-NusA cleaved at β-subunit of RNAP. This cleavage was not observed from either Cys-less or C251S-Fe-BABE-NusA (Figure 5.14 B lane 10 and 14) that indicated specific cleavage from RNAP binding NusA-NTD domain. 2) In the case of β’ subunit the cleavage was observed only from S29C Fe-BABE NusA (Figure 5.14 C). 3) In the presence of N, there was no change in cleavage patterns of β-subunit from the S29C
and S53C positions of NusA (Figure 5.14 B). But in the case of β' subunit, we observed that cleavage from S29C-NusA was decreased (Figure 5.14 C, lane 10 and 12).

![Diagram](image.png)

**Figure 5.14:** Cleavage of the β and β' subunits of the EC by Fe-BABE-labeled NusA. A) Cartoon showing a H-19B N/NusA modified EC stalled by lac repressor. B) Cleavage of the β subunit of the EC by S29C-Fe-BABE and S53C-Fe-BABE NusA and mapping of the cleavage site. Stalled EC containing the N-terminal 32P-β subunit was incubated with different NusA conjugated with Fe-BABE (600 nM) and N (300 nM). The cleavage reaction was activated by addition of ascorbate and hydrogen peroxide. The protein ladder was generated by cleaving 32P-labeled RNAP using the Met-specific cleavage reagent, CNBr and Lys-specific cleavage reagent, LysC. We calculated the X0 cleavage sites to be at β subunit 900±20. C) Cleavage of the β' subunit of the stalled EC by S29C-Fe-BABE NusA with and without N and mapping of the cleavage site. RNAP containing the C-terminal 32P-β' subunit, as described for (A) The X1 cleavage was calculated as described for (A) to be at β' subunit 372±20.
Figure 5.15: Model of S29C-Fe-BABE and S53C-Fe-BABE NusA cleavage sites on the EC (Opalka et al., 2010). A view into the RNA exit channel of RNAP (formed between two mobile domains: the β-flap and the β'-dock) showing the locations of the cleavages from NusAs (C29 and C53)-FeBABE mapped to the β flap (green spacefill) and the β' dock (grey spacefill) domains. RNA (blue) and DNA (red) can also be seen. NusA is depicted adjacent to the RNA exit channel in the orientation predicted by the mapping data. E. coli RNAP contains an insertion of 100 amino acids in the flap domain (βi9) whose location is indicated in cyan color.

We mapped the cleavage sites on the β and β' subunit by comparing the migration positions with the fragments generated from the RNAP subunits using for cyanogen bromide (CNBr for cleavage at Met residues) and LysC and ArgC proteases. The single cleavage site on β subunit was around 900 (X0, 900±20 figure 5.14 B). Two cleavage sites on β'-subunit were around 372 and 450 (X1 and X2, 372±20 and 450±20 figure 5.14 C). X0 on β was dependent on both S29C and S53C positions of NusA and X1 on β' was dependent on the presence of S29C NusA. X2 was nonspecific free-radical cleavage from residual Fe-BABE ions in the solutions because this cleavage was found with Cys-less NusA and without NusA (Figure 5.14 C). X0 and X1 cleavages are located in RNA exit channel in β flap and β' dock domains respectively. These results suggested that concave surface of NusA NTD interacts with β' dock, while interface between concave and convex surface of NusA NTD interacts with β flap. We also concluded that N modulates β' dock-NusA NTD interaction during antitermination (Figure 5.15).
5.3.6 Fe-BABE-labelled NusA generated cleavage of emerging RNA in the Stalled EC affected by N

To test the face of NusA NTD that interacts with the emerging RNA near the exit channel and what is the effect of N on the conformation of the exit channel, we stalled an EC downstream of the nut site using lac repressor as a road block (Figure 5.14 A) where ECs of EC23 was radiolabelled (see methods). This produced an effective 5’-labelling of the nascent RNA. RNA cleavage was performed following the Fe-BABE labelled NusA mediated cleavage method as described above and the products were visualized by running denaturing gel electrophoresis (Figure 5.16). Cleavage sites were mapped by comparing with RNase T1-sequencing of the same RNA and the DNA molecular weight markers. Cleavage occurred at position near -15 to -20, when NusA-S29C was used, but no cleavage occurred when NusA(S53C) and NusA(C251) were used or when NusA was absent (Figure 5.16). Interestingly, in the presence of N, NusA-S29C mediated cleavage was not observed (Figure 5.16). This result suggests that either N changes the NusA NTD-exit channel interaction or conformation of NusA NTD-RNA interaction.

![Figure 5.16](image)

**Figure 5.16.** Cleavage of the emerging RNA by NusA conjugated with Fe-BABE and approximate mapping of the cleavage site by 10 bp and 25 bp DNA marker. The 32P-labelled RNA attached with EC was incubated with different Fe-BABE NusA (600 nM) and N (300 nM) if needed for 5 min at 37°C before cleavage. The cleavage reaction was activated by addition of ascorbate and hydrogen peroxide.
5.4 Discussion

Here we presented following evidences, which strongly indicate that N-NusA interaction modulates the NusA-RNAP β flap interaction by reorienting the latter domain. 1) Point mutations in β flap domain specifically impair the N-antitermination process (Figure 5.4 and 5.5), and they are defective in antitermination due to impaired β flap-NusA interaction (Figure 5.5 D). 2) Accessibility of the RNA exit channel is affected in the presence of N and this is due to N-NusA interaction, which is likely because of spatial re-orientation of the flap domain over the exit channel (Figure 5.7, 5.10 and 5.11). 3) Altered Fe-BABE cleavage patterns of RNAP from NusA also support the proposition of N-induced spatial orientation of the flap domain (Figure 5.14 C). 4) Fe-BABE labelled NusA-generated cleavage of emerging RNA in stalled EC protected by N revealed that N modulates NusA NTD-exit channel interaction (Figure 5.16). Based on these aforementioned evidences we propose that one of the way of achieving N-antitermination is to change the conformation of flap through altering the interaction of the latter domain with NusA. We also propose that this N-NusA interaction changes the orientation of flap over the exit channel in such a way that it renders a constricted channel.

The flap domain connects to RNAP active site cleft through a two stranded, antiparallel β sheet (the connector, figure 5.17). β flap movement affects the active site allosterically through this connector (Mustaev et al., 1991; Sagitov et al., 1993; Touloukhonov et al., 2001). This allosteric effect can be enhanced by NusA. The N binding region of NusA-NTD is located opposite to its concave RNAP binding surface and lies within a distance of ~20 Å (Figure 5.17) (Mishra et al., 2013). This proximity is ideal for N to exert conformational changes in and around the NusA-binding sites, the β-flap/ β'-dock regions of the RNA exit channel (Yang et al., 2009; Ha et al., 2010) of the EC, allosterically via NusA. β flap mutants located in edge of β flap and connector (Figure 5.17) those showed defect for N-antitermination and hairpin interaction, may hinder the movement of β flap tip (Figure 5.5 and 5.7).

Earlier publications from our lab have shown that RNAP mutations in and around the RNA exit channel perturb N action (Cheeran et al., 2005) and also proposed that the C-terminal domain of N may penetrate into the core of the EC through the RNA exit channel (Cheeran et al., 2007). Here, we have shown that in addition to N-EC direct
interactions, N-NusA NTD binding affects the movement of adjacent β-flap regions and make the RNA exit channel more constricted of EC.

RNAP β flap domain has appeared as a common target for factors affecting the elongation and termination properties of the transcription. Indeed, a number of elongation, termination and antitermination factors were shown to bind to β flap. Earlier it has been shown that the antiterminator, Q, binds to β flap of RNAP and modulates the NusA-EC interaction in such away that it forms an extended shield on the RNA exit channel (Shankar et al., 2007; Vorobiev et al., 2014). The importance of modulation of β flap domain in anitermination can be explained by a study which showed that strengthening the λQ-β-flap interaction allows λQ to bypass requirements for the QBE (Q binding element) and the pause-inducing element in the engagement process (Deighan et al., 2008). Recently, another antiterminator protein, gp39, has been shown to target the same β flap of the EC (Berdygulova et al., 2012).
A functional analog of gp39, p7, a regulatory protein from *X. oryzae* phage Xp10, binds RNAP near the β′ zinc finger domain located ‘just below’ the β flap, on the opposite side of the RNA exit channel. Unlike N and Q, gp39 and p7 do not require any cis-acting elements or additional factors such as NusA to convert RNAP into a termination resistant form (Berdygulova et al., 2012). The unique antiterminator, PUT RNA interacts with the β′-zinc-finger near the exit channel (Sen et al., 2002). As the RNA exit channel is formed between two mobile domains: the β-flap and the β′-clamp (which includes the clamp helices and several flexible loops, the lid, the zipper and the zinc-finger) and it is the site where the hairpin-terminators are formed and through which the terminator protein, Rho, approaches the interior of EC (Peters et al., 2011), it is quite logical to interpret that the antiterminators target the exit channel either directly or via Nus factors to make RNAP termination resistant.