The Interaction Surface of a Bacterial Transcription Elongation Factor Required for Complex Formation with an Antiterminator during Transcription Antitermination*

The bacterial transcription elongation factor, NusA, functions as an antiterminator when it is bound to the lambdoid phage derived antiterminator protein, N. The mode of N-NusA interaction is unknown, knowledge of which is essential to understand the antitermination process. It was reported earlier that in the absence of the transcription elongation complex (EC), N interacts with the C-terminal AR1 domain of NusA. However, the functional significance of this interaction is obscure. Here we identified mutations in NusA N terminus (NTD) specifically defective for N-mediated antitermination. These are located at a convex surface of the NusA-NTD, situated opposite its concave RNA polymerase (RNAP) binding surface. These NusA mutants disrupt the N-nut site interactions on the nascent RNA emerging out of a stalled EC. In the N/NusA-modified EC, a Cys-53 (S53C) from the convex surface of the NusA-NTD forms a specific disulfide (S-S) bridge with a Cys-39 (S39C) of the NusA binding region of the N protein. We conclude that when bound to the EC, the N interaction surface of NusA shifts from the AR1 domain to its NTD domain. This occurred due to a massive away-movement of the adjacent AR2 domain of NusA upon binding to the EC. We propose that the close proximity of this altered N-interaction site of NusA to its RNAP binding surface, enables N to influence the NusA-RNAP interaction during transcription antitermination that in turn facilitates the conversion of NusA into an antiterminator.

The antiterminator, N, is a small basic protein that binds to a specific stem-loop structure (box B of nut site; Fig. 1B) of the mRNA through its N-terminal arginine rich motif (ARM; Refs. 5 and 6; Fig. 1, C and D) and interacts with the RNAP through its C terminus (7, 8). N requires several host-factors, called Nus factors, for processive antitermination (Ref. 4 and Fig. 1C). N and these Nus factors assemble on the nut (N utilization) site of the mRNA, where N and NusA make specific interactions (Ref. 3 and Fig. 1C).

The elongation/termination factor NusA is a ~55-kDa protein having the following functional domains: an N-terminal RNAP binding domain (NTD), three RNA binding domains (S1, KH1, and KH2), and two C-terminal gcidic repeats (AR1 and AR2) (Ref. 9 and Fig. 1F). A concave surface of the NusA-NTD interacts with the flap domain of the β-subunit of RNAP (10, 11), whereas the AR2 domain binds to the C-terminal domain (α-CTD) of the α-subunit of RNAP (12). NusA enhances intrinsic termination (13, 14), induces transcription pausing (15–17), and functions as an antiterminator upon forming a complex with the N protein (14).

The N-NusA interaction at the nut site is essential to convert the latter into an antiterminator. In the absence of the EC, the AR1 region of NusA specifically interacts with the N protein (Ref. 18 and Fig. 1F). However, the functional relevance of NusA AR1-N interaction is obscure (18). Understanding the mode of interaction of NusA with N as well as with RNAP during the process of transcription elongation is essential to know the mechanism of antitermination.

Here, we show that NusA AR1-N interaction is not important both in the in vivo and in vitro antitermination assays, and this interaction is also not required for the bacteriophage growth. We have identified point mutations at a convex surface located opposite the RNAP binding surface of the NusA-NTD that are specifically defective for N-mediated transcription antitermination. These mutants affected the binding of N-NusA complex to the nut site, present on the mRNA emerging out of a stalled EC. In this same EC, the Ser-53 of the NusA-NTD that was replaced with a Cys specifically formed a S-S bridge with another Cys, Cys-39 (replacing the native Ser-39), from the NusA binding region of

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**Background:** The mode of interaction between the transcription factor, NusA, and the antiterminator, N, is unknown.

**Results:** When bound to the transcription elongation complex (EC), NusA-NusA interacts with N.

**Conclusion:** The EC-induced away-movement of NusA-C-terminal domain changed the interaction surface of NusA for N.

**Significance:** N-NusA interaction converts NusA into an antiterminator by influencing the NusA-RNAP polymerase interaction.

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3 The abbreviations used are: EC, elongation complex; RNAP, RNA polymerase; NTD, N-terminal domain; Ni-NTA, nickel-nitrilotriacetic acid; RT, read-through; RB, roadblocked; oligo, oligonucleotide; %RT, terminator read-through efficiency.
the N protein. We concluded that upon binding to the EC, the interaction surface of NusA for N changes from the NusA-AR1 region to its NTD domain. Most likely this occurs due to the away-movement of the AR2 domain of NusA upon binding to the EC. We propose that the close proximity of this altered N-interaction site of NusA to its RNAP binding surface enables N to modulate the NusA-RNAP interaction during the antitermination process.

**EXPERIMENTAL PROCEDURES**

**Materials**—NTPs were purchased from GE Healthcare. [γ-32P]ATP (3000 Ci/mmol) and [α-32P]CTP (3000 Ci/mmol) were from Oniobi, India. Antibiotics, isopropyl 1-thio-β-D-galactopyranoside, lysozyme, DTT, and BSA were from U. S. Biochemical Corp. Primers for PCR were obtained either from Sigma or MWG. Restriction endonucleases, polynucleotide kinase, and T4 DNA ligase were from New England Biolabs. WT E. coli RNAP holoenzyme was purchased from Epicenter Biotechnologies. TaqDNA polymerase was from Roche Applied Science. Ni-NTA-agarose beads were from Qiagen. Streptavidin-coated magnetic beads were from Promega. HPLC pure antigens were used in the footprinting experiments were from MWG. RNase T1 was from Ambion and RNase H was from Epicenter. All the bacterial growth media were from Difco.

**Bacterial Strains, Plasmids, and Phages**—All the bacterial strains, plasmids, and phages used are listed in Table 1. All the in vivo antitermination assays were performed in different derivatives of E. coli MC4100Δrac strains. The strains RS1017, RS1148, RS734, RS445, RS1237, RS1245, and RS1019 used in β-galactosidase assays contain single-copy P<sub>lac</sub>-H-19B nutR/T<sub>r1</sub>-trpt'-lacZYA, P<sub>lac</sub>-H-19B nutR/T<sub>r1</sub>-T<sub>r</sub>-T1T2-lacZYA, P<sub>lac</sub>-H-19B nutR/T<sub>r1</sub>-lacZYA, P<sub>lac</sub>-lacZYA, P<sub>lac</sub>-λ nutR/T<sub>r1</sub>-T<sub>r</sub>-T1T2-lacZYA, P<sub>lac</sub>-λ nutR/T<sub>r1</sub>-trpt'-lacZYA, and P<sub>lac</sub>-λ nutR/T<sub>r1</sub>-trpt'-lacZYA reporter cassettes, respectively, as ARS455 lysogens. t<sub>r1</sub> and trpt' are Rho-dependent, and T<sub>r</sub> and T1 are the Rho-independent terminators. In all these reporters, expression of lacZ occurs only in the presence of the N-mediated antitermination. In the construct nutR/T<sub>r1</sub>-trpt', t<sub>r1</sub> and trpt' are fused in tandem (19).

**Random Mutagenesis and Screening of NusA Mutants**—The plasmid pRS703, containing nusA, was transformed into a XL1-Red mutator strain and was randomly mutagenized during its growth (20). The mutagenized plasmid library thus obtained was electroporated into the strain RS1017 (happening the P<sub>lac</sub>-nutR/T<sub>r1</sub>-trpt'-lacZYA reporter as a lysogen) containing the pK8601 plasmid with the N gene from the lambdoid phage H-19B. The transformants were plated on LB media supplemented with appropriate antibiotics, and the survivors were scraped and inoculated in 10 ml of LB and incubated for 30 min. This culture was made nusA null (nusA::cam<sup>2</sup>; Ref. 21) by P1 transduction, and the transductants were plated on MacConkey-lactose plates and screened for white colonies. NusA mutants defective for N-mediated antitermination appeared as white/pink colonies on these plates. Approximately 100,000 colonies were screened. The mutations were confirmed by sequencing. It should be noted that the nusA::cam<sup>2</sup> (21) is not a complete deletion of nusA. A fragment containing NusA-NTD is produced in this strain. However, this fragment alone neither supports growth (data not shown) nor the N-mediated antitermination (data not shown). Therefore, in all our in vivo assays, we do not expect any interference from this NusA-NTD fragment.

**Preparation of Other NusA Mutants**—NusA mutants A11D, V8E, L31E, L27E, A7D, and V12D were made by site-directed mutagenesis. Deletion derivatives of NusA were made by overlapping-PCR methods and cloned in the plasmid pHyd3011 between the Ndel and Sail sites.

**In Vivo Antitermination Assays**—To measure the antitermination defects of the NusA C-terminal deletion derivatives, the plasmids (pHYD3011) containing WT nusA and the deletion derivatives, ΔAR2 (NusA-1–416) and ΔAR1-2 (NusA-1–348), were transformed into the strains RS1017 (having P<sub>lac</sub>-H-19B nutR/T<sub>r1</sub>-trpt'-lacZYA construct as a lysogen) and RS445 (having P<sub>lac</sub>-lacZYA construct as a lysogen), respectively, after which the chromosomal nusA was deleted by P1 transduction. The resultant strains were transformed with pK8601 having the H-19B or λ N genes. Similarly, strains RS1148 (with the reporter P<sub>lac</sub>-H-19B nutR/T<sub>r1</sub>-T<sub>r</sub>-T1-T2-lacZYA) and RS734 (with the reporter P<sub>lac</sub>-H-19B nutR-lacZYA) were also transformed with the above plasmids. β-Galactosidase activities were measured in a microtiter plate using a Spectramax plus plate reader by following the published procedures (22). The T<sub>r</sub>-T1-T2-lacZYA cassette measures the antitermination through the Rho-independent terminators, whereas the nutR/T<sub>r1</sub>-trpt'-lacZYA measures the antitermination through the Rho-dependent terminators. The in vivo antitermination efficiency of the NusA deletion derivatives was expressed as the terminator read-through efficiency (%RT). The %RTs at different terminators were calculated using the following formula: [(β-galactosidase activities in the presence of terminators)/(β-galactosidase activities in the absence of terminators]) × 100. To analyze the antitermination defects of the NusA point mutants, β-galactosidase activities were measured in the strains RS1017 and RS1148 in the presence and the absence of the H-19B N plasmid, pK8601. The same assays were performed with λ N plasmid, pRS256, using the strains, RS1245 (P<sub>lac</sub>-λ nutR/T<sub>r1</sub>-trpt'-lacZYA construct), RS1237 (P<sub>lac</sub>-λ nutR/T<sub>r1</sub>-T<sub>r</sub>-T1-T2-lacZYA construct), RS1019 (P<sub>lac</sub>-λ nutR/T<sub>r1</sub>-lacZYA construct), and RS445. In Fig. 3 we have measured the antitermination efficiency by comparing the β-galactosidase activities obtained in the presence and absence of the N proteins.

**Phage Spotting Assays**—To check whether the mutant NusA proteins support the growth of phages H-19B and λ, plasmid containing WT and different NusA derivatives were transformed into the strain RS862, and chromosomal nusA was deleted. Serial dilutions of the bacteriophages H-19B (a gift from Dr. David Friedman) and AC1857 were spotted onto the lawns of RS862 (Fig. 2C). In some experiments plaques were also counted/recorded after overnight incubation at 37 °C (Fig. 3).

**Templates for in Vitro Transcriptions**—Linear DNA templates for in vitro transcription assays were made by PCR amplification from the plasmids, pRS22 (T7A1-H-19B nutR/T<sub>r1</sub>), pRS1092 (T7A1-H-19B nutR/T<sub>r1</sub>-trpt'), pRS385 (pT7A1-H-19B nutR/T<sub>r1</sub>-lacO-T<sub>r</sub>), and pRS604 (pT7A1-λ nutR/T<sub>r1</sub>-T<sub>r</sub>-T<sub>1</sub>-T2). When required, a lac operator sequence was inserted either after T<sub>r1</sub> or trpt' terminator using a downstream primer having

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TABLE 1

| Strains, plasmids, phases, and oligos | Description | Reference |
|-------------------------------------|-------------|-----------|
| **Strains** | | |
| RS734 | MC4100 galE3, P_{lac}–H-19B nutR/t_{trp}, lacZYA | J. Gowrishankar |
| RS445 | GI3161, AR88 homozygous carrying P_{lac}–lacZYA | 22 |
| RS862 | MG1655 ΔracC | J. Gowrishankar |
| RS1017 | MC4100 galE3, AR854 homozygous carrying P_{lac}–H-19B nutR/t_{trp}–trp’–lacZYA | This study |
| RS1019 | MC4100 galE3, P_{lac}–nutR_{T_{trp}}, lacZYA | This study |
| RS1088 | MC4100 galE3, AR854 homozygous carrying P_{lac}–H-19B nutR/t_{trp}–T_{TR}–T_{T2}–lacZYA | This study |
| RS1237 | MC4100 galE3, AR854 homozygous carrying P_{lac}–nutR_{T_{trp}}, T_{TR}–T_{T2}–lacZYA | This study |
| RS1245 | MC4100 galE3, AR854 homozygous carrying P_{lac}–nutR_{T_{trp}}, trp–lacZYA | This study |
| XLI-Red | endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac mutD5 mutS mutT Tn10 | Stratagene |
| **Phages** | | |
| AR854, AR857 | H-19B | J. Gowrishankar |
| AR15 | | David Friedman |
| **Plasmids** | | |
| pk8601 | pGR2 with P_{lac}–H-19B N, spec^R | 38 |
| pk8641 | pTLE1T with P_{lac}–nutR-T_{TR}–T_{T2}–lacZYA fusion. T_{TR}–T_{T2} is a triple terminator cassette, amp^R | 38 |
| pH1D3011 | nusA and mutants of nusA were cloned into NdeI-Sall sites under pBAD promoter, amp^R | 22 |
| pRS12 | H-19B N cloned at Ndel/Xhol site of PET21b, amp^R | 23 |
| pRS22 | pT7A1–H-19B nutR{T_{TR}}–T_{T2}–lacZYA, amp^R | 23 |
| pRS24 | WT nusA cloned at Ndel/Xhol site of PET28b, His tag at N terminus, kan^R | 23 |
| pRS25 | pT7A1–H-19B nutR(D385) T_{TR}–T_{T2}–lacZYA, amp^R | 23 |
| pRS26 | pGR2 with P_{lac}–nusA, spec^R | 19 |
| pRS38 | pRS25 with T7A1-nutR-lacO-T_{R} fusion, amp^R | 33 |
| pRS39 | WT nusA cloned at Ndel/Xhol site of PET33b, HMK, His tag at N terminus, kan^R | 33 |
| pRS604 | pTLE1T with pT7A1–nutR-T_{T2}–lacZYA, amp^R | 33 |
| pRS615 | AN cloned at NdeI/Xhol site of PET21b, amp^R | 33 |
| pRS703 | phyrSO11 having WT nusA | This study |
| pRS1005 | ΔAR1–2 NusA fragment cloned at Ndel/Xhol site of pET28b, His tag at N terminus, kan^R | This study |
| pRS1011 | ΔAR2 NusA fragment cloned at Ndel/Xhol site of pET28b, His tag at N terminus, kan^R | This study |
| pRS1100 | Zero-Cys nusA made by SDM on PET33b, HMK, His tag at N terminus, kan^R | This study |
| pRS1101 | phyrSO11 having nusA V8A mutation | This study |
| pRS1102 | C454(S251, C499) nusA made by SDM on PET33b, HMK, His tag at N terminus, kan^R | This study |
| pRS1124 | nusA S92C made by SDM on pRS1100, HMK, His tag at N terminus, kan^R | This study |
| pRS1127 | nusA V8A mutant cloned at NdeI/Xhol site of pET28b, His tag at N terminus, kan^R | This study |
| pRS1139 | PhySO11 having nusA V12D | This study |
| pRS1140 | PhySO11 having nusA V8E | This study |
| pRS1141 | PhySO11 having nusA L31E | This study |
| pRS1149 | nusA V8E mutant cloned at NdeI/Xhol site of pET28b, His tag at N terminus, kan^R | This study |
| pRS1154 | PhySO11 having nusA A11D | This study |
| pRS1163 | PhySO11 having nusA A7D | This study |
| pRS1182 | nusA V12D mutant cloned at NdeI/Xhol site of pET28b, His tag at N terminus, kan^R | This study |
| pRS1193 | nusA S53C made by SDM on pRS1100, HMK, His tag at N terminus, kan^R | This study |
| pRS1205 | ΔAR1–2 NusA S53C cloned at Ndel/Xhol site of pET33b, HMK, His tag at N terminus, kan^R | This study |
| pRS1313 | nusA V8E S53C made by SDM on pRS1193, HMK, His tag at N terminus, kan^R | This study |
| pRS1407 | nusA T371C made by SDM on pRS1100, HMK, His tag at N terminus, kan^R | This study |
| pRS1421 | AN C92S made by SDM on pRS615 | This study |
| pRS1422 | AN S59C made by SDM on pRS1421 | This study |
| pRS1425 | 39-Cys AN sub-cloned in PET33b, HMK, His tag at N terminus, kan^R | This study |
| **Oligos** | | |
| RK1 | RS58 GCGGACGGTTTTTCCAAGTCTACAGCAC; Reverse primer in the lacZ gene of pTL61T | |
| RS2 | CCCTGCAGCTTGCCTCGACTCT; Reverse primer after T_{R} of pTLE1T | |
| RK32 | TGGAGTCCTAGGATACCTGGC; reverse primer to generate T7A1–H-19B-nutR/t_{trp}, terminator template | |
| RS58 | ATTAACCGTCCAGAATGGGAGATCG; forward primer of pTLE1T (and all its derivatives like pRS106, pRS25) | |
| vector sequence | | |
| RS83 | Biotinylated RS58 | |
| RS147 | GGCAGCCCGATCCACATTGCCAGAACAG; reverse oligo to generate T7A1–λ nutR terminator template | |
| RS177 | GAATTCGTCCTAGGATACCTGGC; anti-phosphorylated T7A1–λ nutR terminator template | |
| RS367 | 5′ GGA ATG TGT AAG AGC GGG GTT ATT TAT TAC 3′ 29-mer, antisense oligo to λ rutA, boxA, and spacer RNA | |
| RS663 | CGTAGGACGAAATGTCATTTG; antisense to nutR spacer of H-19B | |

the operator sequence. In pRS385, the lac operator sequence is cloned after the trp terminator (23). 5′-Biotinylation of the templates was incorporated by using the biotinylated primer RS83, and the immobilization was done on streptavidin-coated magnetic beads (Promega). In all the templates transcription was initiated from the T7A1 promoter.

In Vitro Transcription Assays—For the transcription on the T7A1–nutR–T_{TR}–T_{T2} and T7A1–nutR–T_{R} templates (Figs. 2B, 4C, and 7C), reactions were carried out in T-Glu buffer (20 mM Tris-glutamate, pH 8.0, 10 mM magnesium glutamate, 50 mM potassium glutamate, 1 mM DTT, and 100 μg/ml BSA) at 32 °C. The reactions were initiated with 175 μM ApU, 5 μM GTP, 5 μM ATP, 2.5 μM CTP, and [α-32P]CTP (3000Ci/mmol) to make a 23-mer EC (EC_{23}). Then it was chased with 250 μM each of all the NTPs in the presence of 300 nM of WT and mutant NusA proteins, 100 nM H19B or AN and 200 nM NusG. The reactions were stopped by extraction with phenol followed by ethanol precipitation. Samples were loaded onto a 6% sequencing gel and analyzed using FLA 9000 phosphorimaging (Fuji). For the transcription reactions on the T7A1–H-19B nutR/t_{trp} and T7A1–λ nutR/t_{trp}, reactions were carried out in T-Cl buffer (25 mM Tris-Cl (pH 8.0), 10 mM MgCl₂, and 50 mM KCl) at 37 °C. Other reaction conditions remained the same. For Rho-independent terminators the %
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read-through (%RT) values were obtained by: %RT = [(RO)/(RO) + T\textsubscript{R} + T\textsubscript{I} + T\textsubscript{2}]. For Rho-dependent terminators, it was calculated as %RT = [RO/RO + (total products in the terminator zone)].

RNase H and RNase T1 Footprinting—For the footprinting experiments with H-19B N protein, a stalled EC (RB; road-blocked) was formed at the lac operator site of the template, T7A1-H-19B nutR-lacO. For \( \lambda \) N, the RB was formed on T7A1-\( \lambda \) nutR-lacO template (amplified from pRS604; data not shown). RB was formed by transcribing this template in a similar way as described above, with 50 nM RNAP and 10 nM immobilized biotinylated DNA templates on streptavidin-coated magnetic beads, except that 100 nM lac repressor was present in this case. The EC\textsubscript{23} was chased to the lac operator site by transcribing with 250 \( \mu \)M NTPs. The RB was incubated for 5 min with 150 nM concentrations of either WT or mutant NusA and 150 nM of H-19B N or \( \lambda \) N. The RB, attached to the streptavidin beads, was then extensively washed to remove free proteins (RNAP, NusA, N etc.). However, we observed the presence of inactivated EC\textsubscript{23} complexes (un-chased) even after chasing with all the NTPs. These complexes could not be removed upon washing. EC\textsubscript{23} contains a very short RNA, which is not expected to interfere with the footprinting of the longer RNA associated with the RB complexes. Hence, our RB preparations did not have any free proteins but presumably would contain inactive EC\textsubscript{23} complexes, and the amount of this complex varied between different experiments. We used about a 10-fold molar excess of lac repressor over the DNA template to get the maximum yield of RB. Under our experimental conditions, \(~\sim\)90% of the ECs were road-blocked at the lac operator site. And small fractions of ECs escaped the road-block and reached the end of the template, and they were subsequently removed during the washing steps.

For RNase H footprinting, 10 \( \mu \)M concentrations of the antisense oligo (RS663 and RS367 for H-19B and \( \lambda \) N, respectively) were added to the RB for 30 s followed by the addition of 1 unit RNase H and incubated for 1 min at 37 \( ^\circ \)C. For RNase T1 cleavage assays, 1 unit of enzyme was added to the RB, and the cleavage was continued for 1 min at 37 \( ^\circ \)C. In both cases, reactions were stopped by phenol extraction, and samples were mixed with equal volume of formamide loading dye and loaded onto 8% sequencing gels.

Inter- and Intramolecular S-S Bond Formation Assays—All the single Cys derivatives of NusA were labeled at their S39C change in the NusA binding region was introduced using Ni-NTA beads (Qiagen). The S39C \( \lambda \) N was made both in pET21b and pET33b vectors. These single-Cys derivatives of NusA proteins were purified as described earlier (23).

All the single Cys derivatives of NusA were labeled at their N-terminal heart muscle kinase tag using protein kinase A and [\( ^{32}\text{P} \)]ATP (3000 Ci/mmole). A 10 mM stock of copper-phenanthroline was prepared by adding 1 \( \mu \)l of 1 M CuSO\textsubscript{4} and 3 \( \mu \)l of 1 M phenanthroline, and the volume was made to 100 \( \mu \)l by adding water. EC\textsubscript{23} was chased for 2 min with 250 \( \mu \)M NTPs and 400 nM of different end-labeled single Cys NusA derivatives together with 800 nM of \( \lambda \) N to form the N-NusA modified RB on the T7A1-\( \lambda \) nutR-lacO template (amplified from pRS604). The RB was then washed and resuspended in copper-phenanthroline buffer (25 mM Tris-HCl, pH 7.0, 50 mM KC1, 100 mM NaCl), 10 \( \mu \)M copper-phenanthroline was added to the RB and incubated for 10 min, and the reaction was stopped by adding non-reducing SDS loading dye (without \( \beta \)-mercaptoethanol) containing 100 mM iodoacetamide and was loaded onto a 6–10% gradient SDS-PAGE. For the S-S bond formation outside the EC, same amounts of all the other components except RNAP were added to the reaction mixture, and the reactions were performed in the same way as above.

For the intramolecular S-S bond formation assays, WT H-19B N together with indicated double-Cys derivatives of heart muscle kinase-tagged NusA were used. NusA was radiolabeled, and all the other experimental conditions were kept same as above. The highly pure core RNAP was purchased from Epicenter, USA.

RESULTS

N-NusA AR1 Interaction Is Not Important for the Antitermination Process—Earlier it was shown that GST-tagged N proteins from phage \( \lambda \) selectively interacted with the AR1 domain of NusA (Ref. 18; Fig. 1G). N-AR1 interaction was further demonstrated by structural analyses (24, 25). All these interactions were demonstrated in the absence of the EC, and hence, the functional significance of N-NusA AR1 interaction was not addressed in these studies (18). Also, it has not been established that this mode of interaction occurs on the surface of the EC during the process of antitermination. We decided to revisit the N-NusA interaction in solution as well as perform detailed analysis to elucidate the functional outcome of this interaction.

At first, we revisited the interactions of N proteins from H-19B and \( \lambda \) phages with the His-tagged full-length and different fragments (having different functional domains) of NusA by measuring the efficiency of each of the fragments to pull down the N proteins in the presence of the EC. We observed differential but significant binding affinity of N for all the NusA fragments as well as to the Ni-NTA-agarose beads. Hence, we were unable to discriminate between specific and nonspecific interactions (data not shown). Other methods of assay also posed similar technical problems with the N proteins. The unstructured nature of N in solution (26) in the absence of RNA and/or RNAP could be a reason for this reduced specificity and its tendency to adsorb on the solid surfaces (beads). It should be noted that N attains specific conformations upon binding to
mRNA and presumably upon binding to NusA and RNAP that probably induces more specificity in the N-NusA interaction. Therefore, instead of measuring the binding of N with different NusA fragments, we undertook detailed functional analyses to identify the role of N-terminal regions of NusA in the N-NusA complex formation during the process of antitermination.

We compared the effects of the deletions of AR2 and AR1-2 regions of NusA on the *in vivo* antitermination by the N proteins (Tables 2 and 3). We measured the *in vivo* antitermination efficiencies at Rho-dependent (H-19B nutR/AR1-trp’-lacZYA, λ nutR/AR1-trp’-lacZYA; columns 1 and 2 of Tables 2 and 3, respectively) and at Rho-independent terminators (TTr-T1-T2-lacZYA; columns 3 and 4 of Tables 2 and 3) from the ratios of β-galactosidase activities obtained in the presence and the absence of the terminators (see “Experimental Procedures” for details). The lacZYA reporter fused to these terminator cassettes produced the β-galactosidase activity. We observed that the antitermination efficiency (%RT) of both the N proteins was not affected by the deletions of AR1 and AR2 regions of the NusA. The antitermination was specifically dependent on the presence of the N protein (see the +N columns).

We then performed *in vitro* antitermination assays on the DNA templates having Rho-dependent (TR1-terminator; Fig. 2A) and Rho-independent (TTr-T1-T2 terminator cassettes; Fig. 2B) terminators. As the *in vivo* assays, deletions in AR1 and AR2 regions did not show any effect on the *in vitro* N-mediated antitermination assays. The absence of any effect of AR1 deletion in *in vitro* antitermination assays was also observed earlier...
TABLE 3

In vivo λ N-mediated antitermination assays

The ratios of β-galactosidase activities in the presence (+ter) and absence (−ter) of terminators give the values of terminator read-through (%RT), which is a measure of antitermination efficiency. %RT is defined as the same way as in Table 2. Rho-dependent (β_{Rho-1-\text{trpt'}}-lacZYA; columns 1 and 2) and -independent (β_{Rho-1-\text{nutR-T1-T2-lacZYA}}; columns 3 and 4) terminator cassettes were fused to the lacZ reporter for the assays. The Rho-dependent terminator, t_{Rho}, was derived from the nutR-cro region of phage λ. Measurements were performed both in the absence (−λ N) and presence (+λ N) of λ N proteins. ND, not determined.

| nusA alleles | 1+λ N β- Galactosidase activity | 2−λ N β- Galactosidase activity | 3+λ N N-acetylase activity | 4−λ N β- Galactosidase activity |
|--------------|--------------------------------|---------------------------------|---------------------------|--------------------------------|
|              | +t_{Rho-1-\text{trpt'}} | −t_{Rho-1-\text{trpt'}} | %RT | +t_{Rho-1-\text{trpt'}} | −t_{Rho-1-\text{trpt'}} | %RT | +T_{Rho-T1-T2} | −T_{Rho-T1-T2} | %RT | +T_{Rho-T1-T2} | −T_{Rho-T1-T2} | %RT |
| WT           | 475 ± 14                   | 948 ± 6                     | 50.3 | ND                   | ND                         | ND | 425 ± 4 | 891 ± 86 | 47.7 | 6.0 ± 1.0 | 240 ± 9 | 2.5 |
| ΔAR2         | 485 ± 30                   | 937 ± 36                    | 51.8 | ND                   | ND                         | ND | 434 ± 10 | 754 ± 50 | 57.5 | 8.0 ± 1.1 | 379 ± 19 | 2.1 |
| ΔAR1–2       | 442 ± 37                   | 824 ± 34                    | 53.6 | ND                   | ND                         | ND | 347 ± 16 | 890 ± 78 | 38.9 | 10.0 ± 1.2 | 379 ± 18 | 2.6 |

(18). Finally, we observed that the AR1/AR2 deletions did not affect the growths of H-19B and λ phages (Fig. 2C).

Therefore, we concluded that the NusA AR1-N interaction during the process of transcription antitermination is not very important, and this interaction may not occur when N-NusA complex is formed on the surface of the EC, and also it is likely that N-NusA binding occurs through another part(s) of NusA. This might happen due to the proposed conformational changes of NusA upon binding to the RNAP (12).

**NusA-NDT Mutants Are Specifically Defective for N-mediated Antitermination**—The aforementioned results led us to identify the interaction surface involved in a N-NusA complex, when it is bound to the EC. We set-up a genetic screen to isolate NusA mutants defective for N-mediated antitermination. We used a mutagenized library of nusA residing on a plasmid and screened for a mutation(s) that caused an antitermination defect of H-19B N at a double-terminator reporter cassette (P_{lac-\text{nutR}/ t_{Rho-1-\text{trpt'}}-lacZYA}; Ref. 19). This genetic screen yielded only one mutant, V8A. We observed that this mutation is located in a hydrophobic patch of NusA-NDT (Fig. 3A). We hypothesized that a hydrophobic surface may take part in the protein-protein interaction. And, hence, we made few more mutations in the nearby hydrophobic amino acids; A7D, V8E, A11D, and V12D.

A polar amino acid change, E10K, was also made, but it did not affect the N-antitermination. We also included a previously described mutation, L31E (11), in our assays.

We measured the in vivo antitermination efficiency of H-19B and λ N proteins in the presence of these NusA mutants, both at Rho-dependent (Fig. 3, B and D) and Rho-independent (Fig. 3, C and E) terminators. We observed that the H-19B N-induced termination read-through was a minimum in the presence of V8E and A11D NusA mutants, whereas only V8E NusA had the most severe effect on the function of λ N. Other mutants also displayed partial defects. Similar trends were observed for both kinds of terminators.

All these NusA mutants were defective in supporting H-19B phage growth (Fig. 3F). V8E and L31E mutants showed defects for λ phage growth. These results are consistent with the fact that H-19B N is more dependent on NusA (23, 27). The defects of these mutants were more pronounced in the case of phage growth, because the latter requires more stable and processive interactions between N and NusA.

Next, we measured the in vitro antitermination efficiencies (%RT) of H-19B N and λ N using two DNA templates, one having Rho-dependent terminators (H-19B t_{Rho-1-\text{trpt'}}), a fusion of two terminators, and λ t_{Rho}, (Fig. 4, A, B, E, and F) and another with a Rho-independent triple terminator cassette (T_{Rho-T1-T2}; Fig. 4, C and D). These NusA mutants caused severe defects to
the N antitermination on the triple terminator cassette, and the effect was milder at the Rho-dependent terminators. Like in vivo assays, the V8E NusA also showed the most severe effect on the in vitro antitermination activities of H-19B and λ N proteins. It should also be noted that under our experimental conditions, λ N alone was unable to bring about antitermination (Fig. 4E, fourth lane from left).

These NusA-NTD mutants did not affect the cell viability (Fig. 5A). They were not defective for transcription-pausing at His-pause sequences (data not shown) and for the termination enhancement activities at the NusA-dependent TR terminator in vitro (data not shown). These results indicate that the aforementioned mutations in NusA-NTD do not affect RNAP or RNA binding. The CD spectra of all these mutants also revealed that the mutations did not cause significant changes in the secondary structures (data not shown). As these mutations did not affect the other functions of NusA (RNAP binding, termination etc., described above) as well as the viability of the cell (Fig. 5A), we can assume that the mutations might not have affected the tertiary structures of the proteins significantly. Therefore, the defects caused by these mutations are specific for N-mediated antitermination and are not due to the changes in NusA conformations.

We localized these mutants both on the homology model of the E. coli NusA and on the NMR structure of NusA-NTD (Fig. 5, B and C) and observed that the mutants are clustered in and around a hydrophobic convex surface of the NusA-NTD. Interestingly, this surface of NusA is located opposite its concave RNAP binding surface (Fig. 5C; Ref. 11). It should be noted here that we have mainly tested the roles of hydrophobic amino

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**FIGURE 3.** Point mutations in NusA-NTD impair N-antitermination in vivo. **A,** the amino acid sequence surrounding the hydrophobic patch (italics) of the NusA-NTD. The numbering indicates the amino acid positions from the N terminus of NusA. The bar diagrams show the β-galactosidase activities from the reporter cassettes having Rho-dependent (B and D) and Rho-independent (C and E) terminators in the presence of the NusA-NTD mutants. Values obtained both in the absence and presence of H-19B N and λ N are shown, and the comparison of − N and + N values gave the measure of in vivo antitermination. **F,** phage spotting efficiencies in the presence of the NusA-mutants. Experimental conditions were same as in Fig. 2C. Efficiency in the presence of WT NusA was set as 1. A.U., arbitrary units.
acids so we cannot rule out the involvement of the polar amino acids of this region in the interaction process. As the same mutations of NusA affected the antitermination functions of two distantly related N proteins (23) from H-19B and φH9261 phages, it is likely that this mode of N-NusA interaction is conserved among different N proteins.

Other NusA mutants defective for λ phage growth were reported in the literature (L183R, Ref. 28; R199A, Ref. 29; G253D and G219D, Ref. 30) and were implicated in the process of N-mediated antitermination. However, all of them are in the NusA-RNA binding domains (SKK domain) and are likely to be defective in binding to the spacer region of the nut site of the nascent RNA (31).

**NusA-NTD Mutants Impaired N-boxB Interactions in a Stalled EC**—Next we tested whether these NusA-NTD mutants affected the N-NusA interaction when the complex is bound to the EC. Binding of H-19B N to the boxB of the nut site is highly dependent on its interaction with NusA, which is bound to the adjacent spacer region (Fig. 1). Therefore, we used binding of H-19B N to the boxB region as a measure of N-NusA interaction at the nut site of the EC. We stalled an EC downstream of the nut site using lac repressor as a road block (Fig. 6A). We footprinted the nut site of the nascent RNA in the presence of H-19B N and different derivatives of NusA using RNase T1 and RNase H. In these assays protection of the nut site indicates binding of N as well as N-NusA interaction at this site (19).
Three G residues in the nut site (see the −N lanes of Fig. 6, B and C, indicated by a dashed line; 19) are sensitive to RNase T1. We monitored the protection of these G residues under different conditions (Fig. 6, B and C). We observed the following. 1) The N-NusA mediated protection of the nut site was observed in the presence of WT, ΔAR1-2, and ΔAR2 NusA proteins (Fig. 6B). 2) This protection was missing in the absence of N and NusA and in the presence of N with NusA-NTD mutants V8E and A11D (Fig. 6C). It should be noted that under our experimental conditions, H-19B N alone was unable to provide any protection (data not shown).

Then we repeated the same footprinting assays using RNase H. An oligo, RS663, antisense to the spacer region, was used to induce the RNase H cleavage. RNase H cleavage will be absent if the antisense oligo is prevented from binding to this site because of the presence of N and NusA. We observed a significant reduction in RNase H sensitivity when WT, ΔAR1-2, and ΔAR2 NusA proteins were present (Fig. 6D). The RNase H cleavage was observed in the presence of NusA-NTD mutants (Fig. 6E). These results were consistent with the RNase T1 footprinting (Fig. 6, B and C). A similar protection pattern as H-19B N was also observed when the experiments were performed with λN modified stalled ECs using a template having the λ nut site (data not shown). These results indicated that ΔAR2 and ΔAR1-2 derivatives of NusA do not affect N binding to the nut site, whereas the NusA-NTD mutants V8E and A11D do this severely. As the N-nut interaction is highly dependent on the functional N-NusA interaction at this site, we concluded that NusA-NTD mutants, and not the AR1/AR2 deletions, perturbed the N-NusA complex formation.

A Cys Residue in the Convex Surface of NusA-NTD Forms the S-S Bridge with Another Cys Residue from the NusA Binding Region of λN—The functional analyses of the different mutants at the convex surface of the NusA-NTD (Figs. 3 and 4) and the N-NusA interaction analyses using RNase footprinting (Fig. 6) showed that these residues of NusA-NTD play an important role in N-NusA interaction during transcription elongation. Next, we attempted to demonstrate the physical proximity of this region of NusA to N when the complex is formed on the EC. We have chosen λN for these assays because its NusA binding region is well characterized (8). Cys-Cys disulfide bridge (S-S bridge) formations between different regions of N and NusA were employed to demonstrate the proximity of the regions. This bridge is formed only if the interacting partners come within ~6 Å.

We constructed single Cys derivatives of NusA: S29C and S53C (in the NusA-NTD domain) and T371C (in the AR1 domain) (see Fig. 7, A and B). These single Cys derivatives were introduced after removing the three natural Cys residues of NusA. The rationale for introducing Cys residues at these positions is as below. 1) Cys-53 is located near the convex surface (~10 Å) where all the NusA mutants defective for N-function are localized (Fig. 5). 2) Cys-29 is situated at the opposite concave surface of NusA (Fig. 7A) and was shown to interact with RNAP (11). Due to its location at the surface opposite to the putative N-interaction region, we used this substitution as a negative control. 3) T371C substitution, located within the interacting distance of the N peptide in the peptide-NusA AR1 complex (Fig. 7B), was chosen to probe NusA AR1-N interaction, if any, in the presence of the EC. 4) We have chosen to replace naturally occurring Ser or Thr residues with Cys for minimizing the effects on the proteins. 5) The space-fill models of different domains of NusA indicate that these Cys residues are surface-exposed (Fig. 7, A and B).

We introduced a S39C (39C) mutation in the NusA binding region of λN (amino acids 34–47; Fig. 7B; Refs. 8 and 24) after removing the single naturally occurring Cys from the protein. In the AR1-N peptide complex, Cys-39 is located near the convex surface of NusA (Fig. 7C), and was shown to interact with λN (11). Due to its location at the surface opposite to the putative N-interaction region, we used this substitution as a negative control. 3) T371C substitution, located within the interacting distance of the N peptide in the peptide-NusA AR1 complex (Fig. 7B), was chosen to probe NusA AR1-N interaction, if any, in the presence of the EC. 4) We have chosen to replace naturally occurring Ser or Thr residues with Cys for minimizing the effects on the proteins. 5) The space-fill models of different domains of NusA indicate that these Cys residues are surface-exposed (Fig. 7, A and B).

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with N and NusA was formed in a similar way as described in Fig. 6A, except that the DNA template used in these experiments had a λ nut site with the lac operator sequence placed just downstream of it (amplified from pRS604). The N-NusA complex in Fig. 8A, was formed with the same components as described in Fig. 8B, except that RNAP was omitted from the
We induced S-S bridges by using an oxidizing agent, copper-phenanthroline. The same experiments were also repeated in the absence of N (right panels of Fig. 8, A and B). Radiolabeled NusA proteins were used to monitor the different S-S-bonded species. We identified the N-NusA complex (N-NusA) and the NusA dimer (NusA2) by comparing their migrations with the molecular weight markers. The N-NusA complexes were further confirmed by their absence in the Cys-39 (Cys39C) N panels (right panels of Fig. 8, A and B). These species were indeed S-S-bonded, as was evident from their disappearance in the presence of a reducing agent, DTT (see the DTT lanes in all the panels of Fig. 8, A and B), and by their absence when Zero-Cys (No Cys) NusA was used (lanes 1–3 of Fig. 8, A and B). The presence of N in the N-NusA species was confirmed by repeating some of the experiments with radio-labeled N (data not shown).

The maximum amount of N-NusA species (~23%) was formed between Cys-371 NusA (in the AR1 domain) and Cys-39 N, when the complex was formed in the absence of the EC (Fig. 8A, lanes 10–12), which is consistent with the fact that NusA-AR1 domain specifically interacts with N in solution (18). Formation of this species was negligible when ΔAR1-2 NusA instead of full-length NusA was used (lanes 16–18). Lesser amounts of N-NusA species were also seen in the presence of Cys29- and Cys53-NusA, which could arise from the inherent nonspecific nature of N-NusA interaction in the absence of the EC.

Interestingly, when the N-NusA complex was bound to the stalled EC (Fig. 8B), a significant amount of S-S bridge was observed between Cys-39 N and Cys-53 NusA (~20%; lanes 7–9) instead of Cys-371 NusA (lanes 10–12). S-S bridge formation was absent in the presence of Cys-29 NusA. A significant amount of this Cys-39 N-Cys-53 NusA species was also observed with AR1-2 NusA (lanes 16–18), and this species was absent when V8E NusA, defective for N-antitermination, was used (lanes 13–15). It should also be noted that a lower level of S-S bond formation between NusA-Cys-53 and N-Cys-39 was observed in the N-NusA binary complexes under different conditions (Fig. 8A; 5.5% in lane 8, 5.0% in lane 14, and 3.0% in lane 17). This may indicate an intrinsically higher reactivity of NusA-Cys-53.

Therefore, on the surface of the EC, specific S-S bond can form between the Cys-39 located in the NusA binding region of N (34–47 amino acids), and the Cys53 of the NusA-NTD. This bond formation occurred due to the specific NusA NTD-N interaction because V8E NusA was unable to form the S-S bridge, and the latter was also missing when Cys29 or Cys371 NusA proteins were present. These results strongly indicated that upon binding to the EC, the interaction surface of NusA for N, changes from its AR1 region to the NTD domain. And residues at the convex surface of this domain play an important role in this interaction.

We would like to point out the formations of N-induced NusA dimers between Cys-53 residues when bound to the sur-
face of the EC under different conditions (lanes 7, 8, 13, 14, 16, and 17 of Fig. 8, A and B). The presence of two copies of NusA in a N-NusA-modified EC, having two different functions, was hypothesized earlier (14). Further experimentations are required to understand the functional significance of this N-induced NusA dimer in the antitermination process.

**AR1/AR2 Regions of NusA Stay Away from N in the EC**—Inhibition of RNA binding function of NusA by its own AR2 domain (12) and capability of the latter to interact with the SKK domains in trans (32) suggest that AR2 folds over the SKK domain forming a “closed state” NusA in solution (Fig. 9A). This autoinhibition is removed when NusA interacts with the EC, likely by forming an “open state” (Fig. 9A). However, the existence of these states has not been confirmed experimentally. Moreover, the shifting of the NusA interaction surface for N from AR1 to the NTD domain could be a consequence of the EC-induced conformational changes of NusA. Hence, we investigated the spatial orientation of the AR1/AR2 regions of NusA when it is a part of N-NusA-EC ternary complex.

We used a NusA having a pair of Cys residues at 251 (in SKK domain) and at 489 (in AR2 domain) positions. This pair is likely to form a S-S bridge if AR2 folds over the SKK domain. We induced the intramolecular S-S bond by copper-phenanthroline and measured the intramolecular species (C-C) formation of NusA under three different conditions: (i) when it is in solution, (ii) when it is bound to the core RNAP, and (iii) when it is bound to a stalled EC. The presence of N in the ternary N-NusA-EC complex did not induce the bond formation of NusA under three different conditions; (i) when it is in solution, (ii) when it is bound to the core RNAP, and (iii) when it is bound to a stalled EC. The state of NusA in each of the cases was measured both in the absence and presence of H-19B N. N-NusA-modified stalled EC was formed in the same way as described in Fig. 6A.

Consistent with the closed state hypothesis, ~50% of the NusA molecules underwent Cys-489--Cys-251 pairing (C-C NusA) in solution (outside EC panel, Fig. 9B, lanes 13–18). This indicates that the N-subunit of the core RNAP in solution does not alter the closed conformation of NusA. When NusA was bound to the EC, AR2 did not form Cys-Cys bonding with SKK domain (Inside EC panel, Fig. 9B, lane 8), as indicated by the drastic reduction of the amount of C-C species.

**FIGURE 8. S-S bridge formation between Cys-39 of λN and the single Cys derivatives of NusA.** A, autoradiogram of the end-labeled WT and different NusA derivatives both in the presence (+39C N, left panel) and absence (−39C N, right panel) of Cys-39 λN. The N-NusA binary complex was formed outside the EC in the absence of RNAP. B, N-NusA complex formed on the stalled EC in the presence (+39C N, left panel) and absence of N (−39C N, right panel). ECs in these experiments were formed in a similar way as described in Fig. 6A. Different S-S-bonded species in all the panels are indicated. MW, molecular weight markers, which were loaded together with other lanes and were separately stained with Coomassie Blue dye and later aligned with the autoradiograms. The fractions of N-NusA complex indicated below the lanes were calculated as (intensity of N-NusA)/intensities of [unreacted species] + [N-NusA] + [NusA2])%. Errors were estimated from at least three measurements.
The above results confirmed that NusA undergoes drastic conformational changes upon interaction with EC, where the AR2 region stays away from the SKK domain, forming the open state (Fig. 9A). As AR1 domain is adjacent to the AR2, it is likely...
that this domain also stays away from the SKK domain in this configuration. In the N- and NusA-modified EC, N is located at the boxB of the nut site, which is adjacent to the SKK domain of the spacer-bound NusA. And hence, in the open conformational state, i.e., on the surface of the EC, NusA-AR1 fails to make any contact with N due to the conformational constraints.

**DISCUSSION**

Based on the following evidences, we concluded that a hydrophobic patch of the NusA-NTD convex surface (Fig. 5, B and C), and not the NusA-AR1 domain, is the interaction site for N during the process of antitermination. 1) Point mutations in this convex surface specifically impaired the N-antitermination process (Figs. 3 and 4). 2) These mutants perturbed the N-NusA interaction at the nut site of the nascent RNA, which in turn affected their binding to the spacer boxB elements (Fig. 6). 3) An engineered Cys residue located near this hydrophobic patch of NusA specifically formed an S-S bridge with an engineered Cys in the NusA binding region of the N protein (Fig. 8). 4) Deletions of AR1 and AR2 regions of NusA in any of the aforementioned assays did not have any effect. 5) Finally, a massive away-movement of NusA AR1-2 regions upon binding to the EC (Fig. 9) most likely makes this region inaccessible to the boxB-bound N during the process of antitermination.

NusA-NTD intrinsically does not have high affinity for N (18). However, this region becomes the preferred binding site for N when N and NusA are part of the EC. We suggest the following reasons for this. 1) The hydrophobic amino acids provide an ideal groove for the thread-like (24) NusA binding region of N. 2) The affinity of NusA-NTD fragment for N increases in the open state of NusA when it is bound to the EC. 3) Finally, it is also possible that this interaction takes place due to the physical proximity of the NusA-NTD to the nut site as well as to its SKK domain in the N-NusA modified EC (Fig. 10).

This N binding region of NusA-NTD is located opposite its concave RNAP binding surface (Fig. 5C) and lies within a distance of ~20 Å. 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 (Refs. 10 and 11; Fig. 10) of the EC, allosterically via NusA. This altered interactions of NusA with the RNA exit channel is likely to stabilize the interactions between the exiting RNA and the channel and that between the clamp domain and the DNA template, which are the important factors for enabling the antitermination. Hence, the N-NusA NTD interaction at the nut site holds the key to convert the transcription termination factor, NusA, into an antiterminator. Detailed analyses of N-induced altered interaction patterns of NusA on the EC is required to further understand the mechanism of this conversion.

We have earlier shown that RNAP mutations in and around the RNA exit channel perturb N action (23) and also proposed that the N C-terminal domain may penetrate into the core of the EC through this exit channel (33). Here, we propose that in addition to N-EC direct interactions, N-NusA NTD binding also affects the adjacent β-flap regions. The antiterminator, Q, has been shown to modify the NusA-EC interaction in such a way that it forms an extended shield on the RNA exit channel (34). The unique cis acting antiterminator, the PUT RNA, interacts with β’-zinc finger motif present near the same RNA exit channel (35). Involvement of NusA in PUT action has not yet been proven but also is suspected. A recently described antiterminator protein, gp39, has been shown to target the RNA exit channel of the EC (36). As the RNA exit channel is the site where the hairpin-terminators are formed and through which the terminator protein, Rho, approaches the interior of EC (1), it is quite logical that the mode of action of the antiterminators has evolved to target the same site.

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