The Site of Action of the Antiterminator Protein N from the Lambdoid Phage H-19B*

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Transcription antitermination by N proteins of lambdoid phages involves specific interactions of the C-terminal domain of N with the elongation complex (EC). The interacting surface of N on the EC is unknown, knowledge of which is essential to understand the mechanism of antitermination. Specific cleavage patterns were generated near the active site Mg$^{2+}$ of the RNA polymerase of an N-modified stalled EC using iron-(S)-1-(p-bromoacetamidobenzyl)ethylenediaminetetraacetate conjugated to the only cysteine residue in the C-terminal domain of N from a lambdoid phage H-19B. Modification of EC by N also induced conformational changes around the same region as revealed from the limited trypsin digestion and in situ Fe-dithiothreitol cleavage pattern of the same EC. These data, together with the previously obtained H-19B N-specific mutations in RNA polymerase, $\beta$ (G1045D), and $\beta'$ (P251S, P254L, G336S, and R270C) subunits, suggest that the active center cleft of the EC could be the site of action of this antiterminator. H-19B N induced altered interactions in this region of EC, prevented the backtracking of the stalled EC at the ops pause site and destabilized RNA hairpin- $\beta$ subunit flap domain interactions at the his pause site. We propose that the physical proximity of the C-terminal domain of H-19B N to the active center cleft of the EC is required for the process of transcription antitermination and that it involves both stabilization of the weak RNA-DNA hybrid at a terminator and destabilization of the interactions of terminator hairpin in the RNA exit channel.

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The abbreviations used are: EC, elongation complex; RB stalled elongation complex; Fe-BABE, iron-(S)-1-(p-bromoacetamidobenzyl)ethylenediaminetetraacetate; RNAP, RNA polymerase; ARM, arginine-rich motif; HMK, heart muscle kinase; CTD, C-terminal deletion; WT, wild type; DTT, dithiothreitol.

it is displaced by the nascent RNA-binding protein, Rho (9, 10). Lambdoid phages encode proteins like N and Q, and a cis-acting element like polymerase utilization RNA, which make the EC termination-resistant upon interaction with the elongating RNAP. This phenomenon is called transcription antitermination (11, 12).

The N protein of bacteriophage A activates expression of the late genes by facilitating the read-through of transcription terminators present in the early genes of the phage (11, 13–15). N is a small basic protein, which binds to a specific stem-loop structure (box B of nut site) of the mRNA through the arginine-rich motif (ARM) present in its N-terminal domain (16, 17), and interacts with the EC through its C-terminal domain via an RNA looping mechanism (18–20). N requires several host-coded factors called Nus factors, to form a processive antitermination complex (12). The interacting surface of N on the EC is not yet known, knowledge of which is critical for understanding the mechanism of antitermination.

N-mediated antitermination of the lambdoid phage H-19B has reduced requirements for Nus factors compared with A N. Efficient antitermination requires only NusA (21) in vivo, and the additional presence of NusG to achieve highly processive antitermination in vitro (22). This minimal requirement for host factors makes it a simpler antitermination system to reconstitute in vitro for biochemical studies.

Using a random mutagenesis screen, we have earlier isolated and characterized E. coli RNAP mutants specifically defective for H-19B N-mediated antitermination. These mutations are located very close to important structural elements of the EC, like the RNA exit channel, the lid, and the rudder (22). In this report, we have identified the regions of the EC that come physically close to the C-terminal domain of H-19B N by protein footprinting of the N-modified EC. We have also analyzed the effects of the binding of H-19B N on the interactions around the RNA-DNA hybrid at a class II pause site (23), and the interactions of the flap domain with hairpin RNA near the RNA exit channel at a class I pause site (24–27). We concluded that the C-terminal domain of H-19B N comes close to the active site Mg$^{2+}$ and N-induced altered interactions in the active center cleft stabilize the RNA-DNA hybrid at a class I pause site and destabilize the RNA hairpin-flap domain interactions at a class II pause site. We propose that the physical proximity of the C-terminal domain of H-19B N is required for the antitermination, and the process involves both stabilization of the weak RNA-DNA hybrid at a terminator sequence and destabilization of the interactions of terminator hairpin in the RNA exit channel.
**Site of Action of the Antiterminator N**

**TABLE 1**

| Strains | Relevant genotype | Reference |
|---------|------------------|-----------|
| RS38    | K3093 with AR545 lysogen carrying pLaC-nutR-3T-lacZYA fusion, rpoC120 btaB:Tn10(ts), and H-19B N (pK8601) spec<sup>5</sup>, tet<sup>8</sup> | Cheeran et al. (22) |
| RS385   | DH5α transformed with pRS835; amp<sup>R</sup> | This study |
| RS422   | DH5α transformed with pRS422; amp<sup>R</sup> | This study |
| RS464   | DH5α transformed with pRS464; amp<sup>R</sup> | This study |
| RS458   | MG1655 with pTRC99A-E. coli rpoB with N-terminal HMK, His tag β S531Y-NPH rpoB, amp<sup>R</sup> | From S. Borukhov |
| RS514   | RS38 transformed with pRS513; amp<sup>R</sup>, spec<sup>8</sup>, and tet<sup>8</sup> | This study |
| RS523   | DH5α transformed with pRS523; kan<sup>R</sup> | This study |

**TABLE 2**

| Description of the oligonucleotides | Relevant genotype | Reference |
|------------------------------------|------------------|-----------|
| **RS2** | CTTCTAGAGGAACTAACTGACCT, downstream oligo after T<sub>50</sub> of T7A1-nutR-T<sub>50</sub> of pRS25 | Laptenko et al. (30) |
| **RS8** | ATGCTATCGCCGCAGCATCCT, upstream oligo of pTL161 vector sequence located upstream to the T7A1 promoter | Laptenko et al. (30) |
| **RS63** | GAGAATTCGCTATCGCCGCAGCATCCT, upstream primer with HindIII site for NroC gene in pBAD vector | Laptenko et al. (30) |
| **RS58** | GCCGCGCGCGCTATCGCCGCAGCATCCT, upstream primer for H-19B N gene with NdeI site | Laptenko et al. (30) |
| **RS70** | GGAATTCGCTATCGCCGCAGCATCCT, upstream primer for making del-CTD (Δ100–127) mutant | Laptenko et al. (30) |
| **RS202** | GCGCGCGCGCTATCGCCGCAGCATCCT, downstream primer for making del-CTD (Δ100–127) amino acids | Laptenko et al. (30) |
| **RS228** | GCCGCGCGCGCTATCGCCGCAGCATCCT, downstream oligo | Laptenko et al. (30) |
| **RS263** | GTGCGCGCGCGCTATCGCCGCAGCATCCT, downstream oligo 1 for making his pause template, used with RS264/RS58 or RS58/RS265 | Laptenko et al. (30) |
| **RS264** | GTGCGCGCGCGCTATCGCCGCAGCATCCT, downstream oligo 2 for making his pause template, used with RS58/RS263 or RS265/RS58 | Laptenko et al. (30) |
| **RS266** | GTATCAGCGCGCGCTATCGCCGCAGCATCCT, downstream oligo 3 for making his pause template, used with RS58/RS263 or RS265/RS58 | Laptenko et al. (30) |
| **RS267** | GAACTTAGCGCGCGCTATCGCCGCAGCATCCT, downstream oligo 4 for making his pause template, used with RS58/RS263 or RS265/RS58 | Laptenko et al. (30) |
| **RS275** | GAACTTAGCGCGCGCTATCGCCGCAGCATCCT, downstream oligo 4 for making his pause template, used with RS58/RS263 or RS265/RS58 | Laptenko et al. (30) |

**EXPERIMENTAL PROCEDURES**

**HMK-tagged RNA Polymerases**—The heart muscle kinase (HMK) tag was introduced at the C terminus of the rpoC gene by PCR amplification using a primer with the HMK and His tag sequences and was cloned into pBAD18M (28) at the HindIII/SacI site. This tag did not affect cell viability and in vivo antitermination. C-terminal HMK-tagged RNAP was purified from this strain (RS514) following published procedures (29). The plasmid with N-terminal HMK-tagged rpoB was a gift from Sergei Borukhov (30). This plasmid was transformed into RS458, and the RNAP was purified as described above. Both HMK-tagged RNAPs were tested for their in vitro antitermination efficiencies with H-19B N. Because the RNAP preparations were not fully saturated with sigma-70, the transcription reactions were supplemented with sigma-70. Strain, plasmids, and primers used in this study are described in Tables 1 and 2.

**H-19B N and Nus Factors**—Cloning and purification of WT H-19B N was described earlier (22). The C-terminal deletion (ΔCTD N; Δ100–127) mutant of H-19B N was made by PCR amplification from the WT gene and was purified following the same procedure as that for the WT protein. Cloning and purifications of NusA and NusG were also described earlier (22).

**DNA Templates**—A lac operator sequence was introduced at the downstream edge of most of the DNA templates, used for making stalled elongation complex (RB), by PCR using downstream primers with a lac operon sequence after 4nt downstream of lacO at the XbaI site, used for cloning lacO at the XbaI site of pRS25 plasmid. The plasmid pRS25 (22) was used as the template for making del-CTD (Δ100–127) amino acids of H-19B N.

**End Labeling of β and β′ Subunits of RNA Polymerase**—HMK-tagged β and β′ subunits of RNA polymerase were radiolabeled with γ-<sup>32</sup>P[ATP] (3000 Ci/mmol, Amersham Bio-
sciences) using protein kinase A (Sigma). The labeling reaction was done in buffer containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM MgCl2, and 10 mM MnCl2. The labeling reactions were carried out in the presence of 200 μM each of all four NTPs (Amersham Biosciences) in transcription buffer. 600 nM NusA, 400 nM NusG, 1 μM each H-19B N, ARM H-19B N, and CTD H-19B N were also added during the reaction, when indicated. The reactions were carried out at 32 °C for 3 min and washed thrice each time with 200 μl of transcription buffer. For Fe-DTT cleavage assays, washing was done with transcription buffer.

**Cleavage of Stalled EC by Fe-BABE-conjugated H-19B N**—The RB was made in the presence of 1 μM of Fe-BABE-conjugated WT or ARM N as described above. The cleavage reaction was initiated by adding 25 mM H2O2 and 100 mM ascorbic acid, and allowed to continue for different times at 32 °C. The reactions were stopped by adding 6 μl of 5× SDS loading dye supplemented with 0.1 mM EDTA and were stored on ice. Samples were heated to 95 °C for 4 min prior to loading onto an 8% SDS-PAGE. Gels were exposed overnight to a phosphorimaging screen, and the bands were analyzed using phosphorimaging Typhoon 9200 and ImageQuaNT software (Amersham Biosciences).

**Cleavage of Stalled EC by Fe-DTT from the Active Center**—The RB complex was washed with transcription buffer without MgCl2 and resuspended in the same buffer. Fe-DTT cleavage reactions were initiated by the addition of 5 mM DTT and 40 μM of freshly prepared ferrous ammonium sulfate (Sigma). Reactions were terminated at different times by addition of an equal volume of 5× SDS-loading dye, heated to 95 °C for 4 min, and then loaded onto an 8% SDS-PAGE. The gels were analyzed by phosphorimaging.

**Cleavage of Stalled EC by Trypsin**—The RB complexes were incubated with 0.006 unit of trypsin for different times at 30 °C,
and the reactions were stopped by adding an equal volume of 5× SDS-loading dye. Gel electrophoresis and analysis of cleavage products were performed as described above.

**Generation of Molecular Weight Markers with CNBr and LysC**—Molecular weight markers of end-labeled RNA polymerase (either the β or β') were generated by CNBr (Merck) and LysC (Sigma)-mediated cleavages following the published procedures (31). Calibration curves were generated from the migration of these cleavage products, and the locations of the cleavage products in Figs. 2 and 3 were identified from the polynomial fit of the calibration curves.

**Transcription Assays on ops and his Pause Templates**—For pause assays, the templates with T7A1-nutR-ops and T7A1-nutR-his constructs were used. First, a 23-mer elongation complex (EC$_{23}$) was made by initiating the transcription with 175 μM adenylyl-(3'-5')-uridine, 5 μM each of GTP and ATP, 2.5 μM CTP, and [α-32P]CTP (3000 Ci/mmol, Amersham Biosciences) on both the templates. To determine the kinetics of elongation on the ops pause template, the EC$_{23}$ was chased in the presence of 100 μM each of UTP, CTP, and ATP and 10 μM GTP. When required, NusA, NusG, WT, and ΔCTD N were added to the chase solution. Aliquots (5 μl) were removed at the indicated times and mixed with an equal volume of formamide loading dye. For assays on the his pause template, EC$_{23}$ was chased in the presence of 150 μM of UTP, CTP, and ATP and 10 μM of GTP. Concentrations of different components used in these were as follows: 20 nM RNAP, 5 nM DNA template, 100 nM LacI, 200 nM H-19B N, 300 nM NusA, and 200 nM NusG. Products were analyzed on 8% sequencing gels.

To generate RB at ops and his pause sites, 5'-biotinylated templates with ops or his pause sequences fused to lac operator sequences were used. The stalled ECs (RB) using Lac repressor as a roadblock were formed essentially
as described for the protein cleavage assays. Nus factors and N were added to this chase reaction when required. RB complexes were washed with transcription buffer to remove the NTPs. The indicated amount of GreB (Fig. 6) or sodium pyrophosphate (Fig. 7) was then added to the respective RB complexes. Products were analyzed on 8% sequencing gels.

Fe-DTT Cleavage of RNA at the ops Pause—RB complexes were formed at the ops pause, essentially the same way as designed above. The RB was washed with transcription buffer without MgCl₂, and resuspended in the same buffer. Fe-DTT cleavage reactions were initiated by the addition of 5 mM DTT and 40 μM of freshly prepared ferrous ammonium sulfate (Sigma). Reactions were terminated by addition of phenol, and the products were analyzed on 6% sequencing gels.

RESULTS

Design of N-modified Stalled EC for Biochemical Probing—We have designed a stalled EC (RB) modified with NusA, NusG, and H-19B N (both WT and mutant), using Lac repressor as a roadblock (schematic shown in Fig. 1A). The lac operator sequence is placed about 80 bp downstream of the nutR site to facilitate the nutR-hairpin-dependent binding of H-19B N to the RB. This stalled EC remains active over the time period of in vitro assays (22, 32). In all the protein footprinting assays, this RB was formed using radiolabeled RNAP. The radiolabeling was achieved through the HMK tag sequences at the N terminus of β and the C terminus of β′ subunits of RNAP, for the detection of cleavage products generated from the Fe-BABE-, Fe-DTT-, and Trypsin-mediated cleavage reactions.

WT H-19B N has a single cysteine residue (amino acid 107) at its C-terminal RNAP binding domain (Fig. 1B). This single cysteine residue was conjugated with the hydroxyl radical generating reagent Fe-BABE (33). Hydroxyl radicals generated in situ will cleave the peptide backbones of the different domains of RNAP situated within 12 Å of the C-terminal cysteine residue of the H-19B N bound to the EC, and thereby will define the regions of EC close to the C-terminal of N. To ascertain that the interaction surface defined by this method is physiologically relevant, we assayed the antitermination efficiency of the Fe-BABE-labeled N at the end of a triple terminator cassette (22). We observed that this Fe-BABE-labeled N has a similar antitermination efficiency as unlabeled N (Fig. 1C). Other single cysteine derivatives in the C-terminal domain that we made yielded inactive N (data not shown), so we restricted our assays only to those with this Fe-BABE-conjugated N.

Fe-BABE-labeled N Generated Cleavage Patterns of β and β′ Subunits of RNA Polymerase in the Stalled EC—The N protein conjugated to Fe-BABE at Cys-107 generated specific cleavage products only in the β′ subunit (lane 6, Fig. 2A) but not in the β subunit of RNAP of the stalled EC (Fig. 2B; compare lanes 5 with 3, 4, and 6). The cleavage was not observed when Fe-BABE was conjugated to a mutant N defective for binding to EC (ARM N; lane 7 of Fig. 2A). Also stalled EC modified with un-conjugated N did not produce any cleavage pattern over the background (lane 5 of Fig. 2A), and incubation of free Fe-BABE with the EC modified with un-conjugated N (lane 4 of Fig. 2A) also did not produce any specific cleavage product. This suggests that this cleavage pattern in lane 6 of Fig. 2A originated from the Fe-BABE conjugated to WT N and only when this conjugated N is specifically bound to the EC. The Fe-BABE-mediated cleavage sites on β′ were mapped close to amino acid residues 458 (conserved region D), 732 (conserved region F), and 926 (conserved region G). Cleavages at these three sites are usually obtained (compare lanes 3 and 6 of Fig. 2A) when hydroxyl radical is generated from the active site by replacing Mg²⁺ with Fe²⁺ (30, 34) (also see below). A major difference observed was that no cleavage by Fe-BABE was detected near amino acid 787 (compare lanes 3 and 6 of Fig. 2A; indicated as dashed lines in the gel) of β′ subunit. In the same experimental set-up, Fe-BABE-labeled N did not cleave the RNA or DNA in the RNA-DNA hybrid (data not shown).

It can be argued that the similarity of cleavage pattern may have arisen from the natural affinity of the Fe²⁺ moiety of Fe-BABE for the metal center at the active site. We ruled out this possibility because of the following reasons. If the Fe²⁺ moiety of the Fe-BABE occupies the Mg²⁺ binding site, it should have generated cleavage patterns in β subunit, in the DNA, and in the RNA as has been observed earlier for Fe-DTT cleavage from the active site of the EC (34). Moreover the reactions were performed in the presence of excess Mg²⁺, and the occupation of a Fe²⁺ in the active site requires the removal of Mg²⁺ (34); also see
lane 4 of Fig. 2A). So it is unlikely that Fe$^{2+}$ of the Fe-BABE moiety has replaced the active site Mg$^{2+}$. However, if due to one or more unknown reasons, having this Fe$^{2+}$ ion of the N-conjugated Fe-BABE moiety transferred to the active site would still require the C-terminal of N to be in the vicinity of the active site. Alternatively, it is possible that, due to this natural affinity of Fe-BABE toward the metal center, the C-terminal domain of N is drawn toward the vicinity of the active center. But this did not affect the antitermination property of H-19B N (Fig. 1C). Therefore, we suggest that the observed cleavage patterns arose simply from the physical proximity of the C-terminal domain of N to this region of β′ subunit. It should also be mentioned that NusA and NusG present in the stalled EC were not cleaved by Fe-BABE attached at this cysteine position (data not shown).

Localized Fe(II)-mediated Cleavage Pattern of the N-modified EC—Physical proximity of the C-terminal domain of H-19B N to the active center may induce some conformational changes in this region. To probe such changes, if any, we compared the localized hydroxyl radical cleavage from the active site and the trypsin cleavage patterns of the N-modified and unmodified stalled ECs.

The localized hydroxyl radical generated by replacing the active site Mg$^{2+}$ of free RNAP with Fe$^{2+}$ in the presence of DTT yields characteristic cleavage patterns in the β and β′ subunits (30, 34). The major cleavages in β subunit are at Pro-567, Asn-677, Asn-808, Pro-1062, and Val-1103 and those in β′ are at Asn-458, Gly-732, Ala-787, and Pro-926 positions. Any conformational change in and around the active center may change this cleavage pattern. We observed similar cleavage patterns in the stalled EC as those reported earlier (30, 34) for free RNAP (Fig. 2, C and D). In the presence of WT H-19B N, there were modest changes in the intensity of the bands. In general, the
Intensity of the bands in the β’ subunit was reduced when the EC was modified specifically with WT N (Fig. 2, E and F). This may indicate N-induced subtle structural rearrangements around the active center. Changes in cleavage pattern in the β subunit were not significant.

**Trypsin Cleavage Pattern of N-modified EC**—Conformational changes in the EC were then probed by limited trypsin cleavage of the β and β’ subunits. The trypsin digestion patterns in the presence of WT N did not reveal protection of any regions of the β and β’ subunits (Fig. 3, A and B). However, binding of N induced enhanced cleavage at β’ positions Arg-1123/Lys-1132 and Lys-731/Arg-738, but not in β. The resolution of the gel was not high enough to identify the exact amino acids corresponding to the enhanced cleavage products. The enhancement in intensity was induced specifically by WT N, because it was not detected in the presence of Δ-CTD N, a mutant N that does not bind to the EC. These two cleavage positions of enhanced intensities are close to the active site (Fig. 4, B and C), which also suggest that N induces conformational changes around the active center. It should also be noted that these two sites are exposed to the surface of the EC (Fig. 4C).

**Mapping of the Cleavage Positions in the Model Structure of EC**—We have earlier described RNAP mutations (P251S, P254L, R270C, and G333S in β subunit) specific to H-19B N action, which are located in and around the RNA exit channel, the lid, and rudder elements of the EC (22). To find the spatial relationship of the N-induced cleavage sites with the positions of those mutations, we localized both the cleavage sites and the positions of the mutations on the available model of EC, developed on the basis of the crystal structures of Thermus aquaticus RNA polymerase (35), the yeast RNA polymerase II EC (36), and the cross-linking data on protein-nucleic acids interactions in the EC of E. coli RNAP (4). We first determined the equivalent amino acids of these mutations and the cleavage sites in the model structure by structural alignment (Fig. 4A) using ClustalX (37). Fig. 4B shows the location of the cleavage sites together with H-19B N-specific mutations obtained earlier. Interestingly, the cleavage sites obtained from Fe-BABE or Trypsin are located close to the active site and did not overlap with the sites of the mutations. The space-filled version of the model (Fig. 4C) shows that both the Trypsin cleavage sites and two of the Fe-BABE-induced cleavage sites are visible through the secondary channel and located close to the surface.

The Fe-BABE-induced cleavage sites are found to be ~30–60 Å away from the positions of the different mutations. Therefore, it is unlikely that the C-terminal domain of H-19B N is placed close to the region defined by the mutations, because the hydroxyl radicals will not be able to travel over this long distance to generate cleavages near active site Mg$^{2+}$. One possible explanation for this observation is that the C-terminal domain comes physically close to the active site Mg$^{2+}$ and that this N-induced interaction near the active site exerts allosteric effects in the regions defined by the location of the mutations. Mutations close to the active site would be lethal, so they were not obtained from our previous genetic screen (22).

**N Prevents Reversible Backtracking at ops Pause Sequences**—The antiterminators N and Q can suppress pausing during elongation (38–40). Results from protein footprinting experiments (Fig. 2), and from earlier mutational analyses (22) suggest that H-19B N may modulate interactions around the RNA-DNA hybrid as well as in the RNA exit channel. Therefore, we assayed the effect of H-19B N on two well defined pause sequences, namely ops and his pauses, which involve altered interactions in these two regions. At the ops pause sequence, pausing occurs due to the backtracking of the EC, which is dependent on the sequence of the RNA-DNA hybrid (23). Pausing at his pause sequence is proposed to be mediated by interaction of a RNA-hairpin with the flap domain of β-subunit located near the RNA exit channel (23–27). The sequence at the his pause codes for RNA that folds into a hairpin near the exit channel.

We followed the pause kinetics through the ops pause sequence cloned downstream of the nutR site, both in the absence and presence of either WT or mutant H-19B N proteins (Fig. 5A). The amount of paused complex was plotted against time (Fig. 5B). In the presence of WT N, pausing efficiency (denoted as “$a$” in the equation of exponential decay; shown in the inset of Fig. 5B) was not affected, whereas the rate of escape (denoted as “$\lambda$” in the equation) from the paused state was three times faster than that observed in the absence of WT N. This result suggests that N does not prevent the EC from entering the paused state but reduces the half-life of this conformational state possibly by disfavoring the backtracking of EC, which is an important component of this type of pausing (23). To determine whether N prevents backtracking at this sequence, the EC was stalled at this site by using Lac repressor as a roadblock (Fig. 5C). Backtracking of the stalled EC was monitored by GreB-induced cleavage of the RNA (41, 42) and by the Fe-DTT-mediated cleavage of the 3’-end of the RNA from the active site of the EC (43). In the absence of WT N, the EC was sensitive to GreB-induced cleavage (Fig. 5D), and Fe-DTT-mediated cleavage of the RNA was also observed at an internal position (marked as “cleaved RNA” in Fig. 5E). These are the indications of the backtracking of the EC at this pause site. In the presence of WT N, sensitivity of EC for GreB was significantly reduced (Fig. 5D), and Fe-DTT-mediated cleavage was also not observed (Fig. 5E). These two results suggest that...
H-19B N prevents backtracking at ops pause site. This observation is specific to WT N, because its mutant derivative did not elicit this response.

N Destabilizes the Flap Domain-RNA Hairpin Interactions at His Pause Sequences—Next, we followed the pause kinetics through the his pause site cloned downstream of the nutR sequence (Fig. 6A). The amount of paused complex was plotted as described above (Fig. 6B). As in the case of the ops pause, the pausing efficiency at the his pause did not change significantly in the presence of WT N, whereas the rate of escape from the paused state increased by 5-fold (see the inset of Fig. 6B for the rate constant values). The effect of N on the his pause was more pronounced than at the ops pause. The dwelling time in this paused state depends on the stability of the flap domain-RNA hairpin interaction (27). As binding of N only affected the pause half-life, it is possible that it did not affect the formation of the pause hairpin but, rather, weakened the flap-hairpin interaction. It has also been shown that the flap-hairpin interaction at this pause site leads to catalytic inactivation of the EC (27). Therefore, if the presence of H-19B N destabilizes this interaction, it will also prevent the catalytic inactivation caused by the RNA hairpin. To test this hypothesis, the EC was stalled by Lac repressor at the pause site, NTPs were removed by washing, and the catalytic competence of the stalled EC was tested by the pyrophosphorolysis reaction (Fig. 6C). Fig. 6D shows that the 3’-end of the RNA of this stalled EC exactly matched the pause site (compare the lanes 3 and 4, lanes 10 and 11, lanes 17 and 18). Accumulation of RNA cleavage products (marked as “PPI cleavage”) induced by pyrophosphate was significantly faster in the presence of WT H-19B N. This effect was specific to WT N, because the pyrophosphorolysis reaction was slow and inefficient both in the absence of N or in the presence of ΔCTD N. We suggest that the flap domain-RNA interaction is destabilized or significantly weakened in the N-modified EC and thereby preventing the catalytic inactivation. Alternatively, the presence of H-19B N might have prevented the formation.
of “hyper-translocated” state of the 3′-end of the RNA, which has been postulated as the characteristic of the ECs at the his pause site (23).

DISCUSSION

Specific interactions of the bacteriophage-derived transcription antiterminators with RNAP during transcription elongation are mandatory to achieve antitermination. Although several mutations in RNAP defective for N-dependent antitermination have been reported earlier (22, 44–50), the interaction surface of this antiterminator on the EC remains elusive. Knowledge of this is essential to understand the mechanism of antitermination. In this report, we present evidence for the region of EC that comes physically close to the C-terminal domain (the RNAP binding domain) of N from lambdoid phage H-19B. The cleavage pattern derived from Fe-BABE-conjugated N bound to a stalled EC revealed that the C-terminal of N approaches close to the active site Mg$^{2+}$ (Figs. 2 and 4). This interaction induced specific conformational changes nearby to this region (Figs. 3 and 4). From these data, together with the previously obtained H-19B N-specific mutations in RNAP, β (G1045D), and β′ (P251S, P254L, G336S, and R270C) subunits, we propose that the active center cleft of the EC broadly defines the site of action of this antiterminator (Fig. 4B). We also showed that this interaction of the C-terminal domain of N with the EC blocked the backtracking of the EC at a class II pause site (Fig. 5) and prevented the catalytic inactivation caused by...

FIGURE 6. Anti-pausing activity at the his pause sequence. A, autoradiogram showing the time course of transcription elongation through the his pause sequence (indicated by arrow) both in the absence and presence of either WT or ΔCTD H-19B N. Pause sequence, pausing site, and pause-hairpin are shown above the autoradiogram. Run-off product is indicated as “RO.” B, amount of fraction of paused complex obtained under different conditions is plotted against time. The analyses of the plots were done in the same way as in Fig. 5, and the average values of the kinetic parameters obtained from these calculations are shown in the inset. C, schematic showing the N-modified EC stalled at the his pause site using Lac repressor as a roadblock. At this position the RNA-DNA hybrid contains the his pause sequence, and the 3′-end matches the pausing position (marked by an arrow). This set-up was used to probe the catalytic competence of the N-modified and unmodified ECs at the his pause. D, autoradiogram showing the RNA cleavage from pyrophosphorolysis reactions in the presence of sodium pyrophosphate from N-modified and unmodified stalled ECs at the his pause site. Time courses of elongation through this pause site (lanes 1–3, 8–10, and 15–17) are shown along with each stalled complex to compare the pausing and stalling positions. Pyrophosphorolysis reactions were performed by adding 1 mM sodium pyrophosphate (PPi) to each of the stalled complexes, and incubated for indicated times. The cleavage products are indicated. Sensitivity toward PPi is marked as either “+” (sensitive) or “−” (not sensitive).
Site of Action of the Antiterminator N

the RNA hairpin-flap domain interaction in the RNA exit channel at a class I pause site (Fig. 6).

The transcription anttermination at an intrinsic terminator can be achieved by stabilizing the weak U-rich RNA-DNA hybrid, by preventing or delaying the folding of RNA hairpin as well as its interaction in the RNA exit channel. Based on the protein footprinting data and the analysis of N-modified ECs stalled at class I and class II pause sites, we propose that physical proximity of the C-terminal domain of N near the active site Mg^{2+} stabilizes the 3'-end of the weak RNA-DNA hybrid directly by altering the interactions around it, which in turn, allosterically destabilizes the RNA hairpin interactions in the distal RNA exit channel. The proposal that N affects the RNA hairpin interactions in the exit channel is consistent with earlier proposals of improper or delayed folding of the RNA-hairpin in the exit channel of N-modified EC (22, 38).

It is evident from the space-filling model (Fig. 4C) that Fe-BABE cleavage sites are not on the surface of the EC. So the C-terminal domain of H-19B N has to be inserted into the EC to generate this cleavage pattern (Fig. 2A). The presence of a hydrophobic patch (Fig. 1B) in the C-terminal encompassing the cysteine residue might help this region to be inserted into the core of the EC. N protein from bacteriophage λ is disordered in solution, and its C-terminal domain remains unfolded even after the N-terminal domain binds to the nut RNA (18, 51). It is possible that lack of proper secondary structure helps this region to be inserted into the core of the EC.

How does the C-terminal of H-19B N access the interior of the EC? This region of EC can be accessed either through the secondary channel (used by Gre factors and NTPs (52)) or through the RNA exit channel. To make the anttermination process kinetically efficient, the C-terminal of N should be inserted into the EC when the mut site is close to the EC. Also as the central part of N interacts to NusA (53), it can act as an anchor for the C-terminal N-EC interactions. Therefore it is possible that the location of NusA on the EC in the N-NusA-EC complex will determine the insertion site of the C-terminal domain of N in the EC. There can be several possibilities. The C-terminal domain may insert through the secondary channel and interact with the regions of EC close to the active site Mg^{2+} and exert an allosteric effect on the regions close to the RNA exit channel. Alternatively, the presence of the N-NusA complex at the floor of the RNA exit channel, assuming that NusA in the NusA-N complex binds to the N-terminal coiled-coil domain of β′-subunit (52), can widen the exit channel so that the regions close to the 3'-half of the RNA-DNA hybrid become more accessible to the free C-terminal end of N. The other possibility is, like the related transcription factor Nun from lambdoid phage HKO22 (54), the C terminus of the H-19B N can also access the active site through the downstream DNA-binding cleft of the EC.

Do our results define a generalized interaction surface for all the N proteins? Sequence alignment of the C-terminal amino acids from different N proteins revealed that this region is highly conserved among the majority of the known N proteins (Fig. 7A). Therefore, it is possible that the nature of interaction of these N proteins with the EC will also be similar. However, the C-terminal domains of more well characterized N proteins from λ and its closer relatives, φ-21 and phage L, are very much different, and they belong to a distinct phylogenetic group (Fig. 7B). It will be interesting to know whether the C terminus of λ N also interacts with the same region of EC and follows the same mechanism of anttermination.

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