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Bacteriophage HK022 Nun protein arrests transcription by blocking lateral mobility of RNA polymerase during transcription elongation

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Coliphage HK022 excludes phage λ by subverting the λ antitermination system and arresting transcription on the λ chromosome. The 12 kDa HK022 Nun protein binds to λ nascent transcript through its N-terminal Arginine Rich Motif (ARM), blocking access by λ N and arresting transcription via a C-terminal interaction with RNA polymerase. In a purified in vitro system, we recently demonstrated that Nun arrests transcription by restricting lateral movement of transcription elongation complex (TEC) along the DNA register, thereby freezing the translocation state. We will discuss some of the key experiments that led to this conclusion, as well as present additional results that further support it.

Keywords: Nun protein, transcription arrest, bacteriophage HK022, phage exclusion, bacteriophage λ

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Results and Discussion

Temperate phage HK022 and λ are closely related. They have similar gene organizations and express proteins with similar functions. In one respect, however, they differ dramatically. λ promotes transcription of its genes by accelerating transcription through both factor dependent and factor independent termination sites.[1,2] The λ N protein binds to sequences on λ nascent transcript (N-UTilization sites, or NUT Sites) and thereby modifies elongating RNA polymerase (RNAP) into a termination-resistant form.[3,4] HK022 expresses a λ N homolog, Nun. The only known role of Nun is to exclude superinfecting λ and phage λ *imm*^{434}, which have the same NUT sites.[5] Nun does so both by competing with λ N for binding to the BoxB stem loop of the NUT RNA sites within the nascent λ transcript[3,4] and by arresting transcription at pause sites on the λ chromosome.[4] Nun-arrested RNAP is removed by the transcription-coupled repair protein MFD.[5] Other than the effect of Nun on λ, no other biological function has been described for the protein. Lytic growth of HK022 is unaffected by *nun* mutations, and *nun* mutants lysogenize with normal frequency. The specificity of Nun exclusion is unique; other phage exclusion systems are active against a broad range of superinfecting phage.[6] It has been suggested that the function of BoxB is to tether both λ N and Nun to increase the local concentration of protein on the λ transcript. Indeed, BoxB is dispensable for function of both proteins in vitro.[1,7] Furthermore, Nun overproduction is toxic to *E. coli*, although λ NUT sites are not encoded in the bacterial chromosome.[8] Toxicity is related to transcription termination, since host RNAP and Nun mutations that block Nun termination also suppress cell killing.

Nun interacts with RNAP, resulting in transcription arrest at pause sites, both in vivo and in vitro.[1,4] In vivo, Nun arrest requires the four *E. coli* auxiliary transcription elongation factors, NusA, NusB, NusE, and NusG . Though these factors are not essential for Nun arrest in vitro, they enhance specific activity, reducing the concentration of Nun required to block elongation.[4] The four Nus proteins enhance arrest when present in equimolar concentrations.

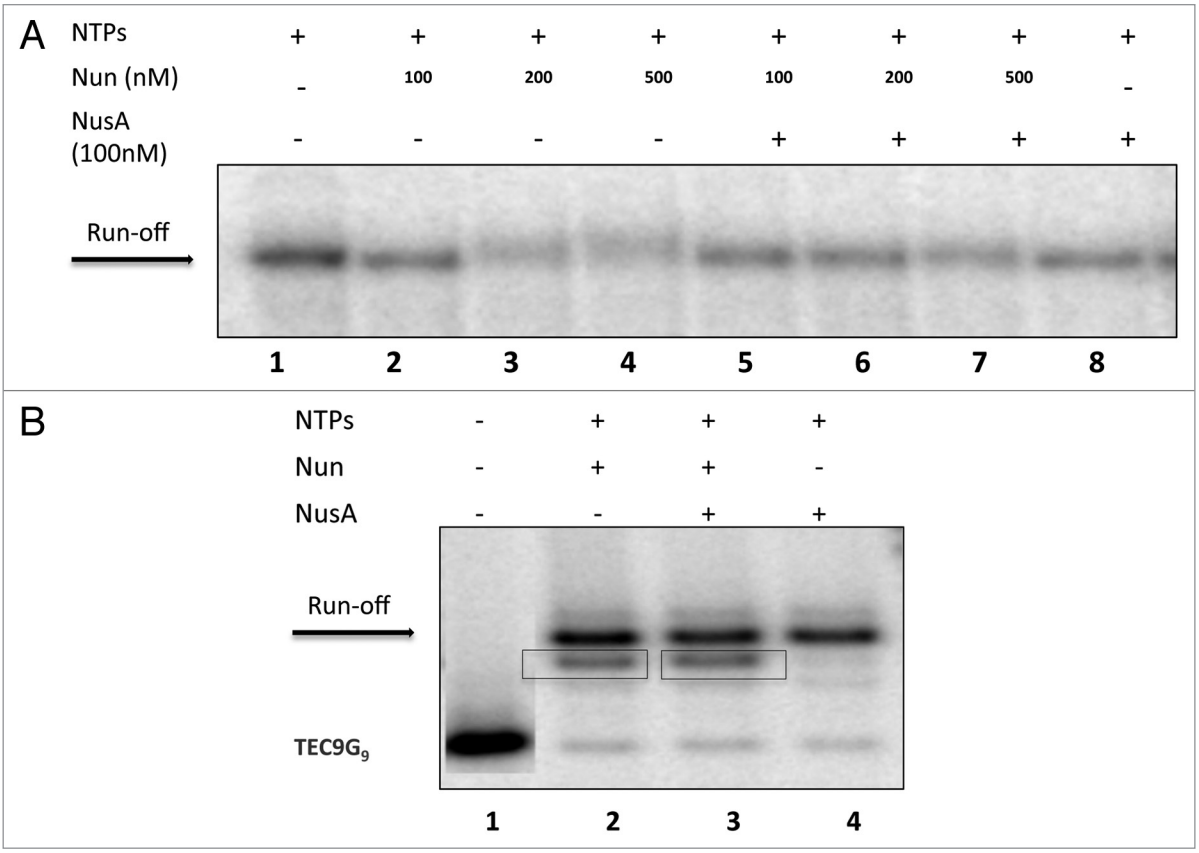

Figure 1. (**A**) NusA suppresses Nun inhibition of transcription on a *pL-nutL* template. Pre-initiation (open) complex was formed on 161 nt long *pL-nutL* template by combining 200 ng DNA template, 4 pmol RNAP, and 0.5 μl TB40 transcription buffer (5′, 37 °C) . To avert abortive initiation, TEC15U was then formed from combining open transcription complex with 2.5 mM ApUpC, 3 μl 0.5 mM rATP+rGTP, 4 μl α-P^{32} CTP (3000 Ci/mmol, 3.3 μM), and 1 μl TB40 transcription buffer (5′, 37 °C). The resulting TEC15U was then immobilized on Ni-NTA beads via the β′ His6- tag, washed and eluted as described in Materials and Methods. Thirty microliters TEC15U was then incubated with Nun alone, NusA+Nun (added simultaneously) in the concentrations indicated, or a comparable volume transcription buffer (10′, 25 °C). Transcription was initiated by addition of 4 rNTPs (5 μM), and the reactions were stopped after 30′ incubation at 25 °C with 2× loading buffer. Only the run-off is depicted, indicated by black arrow. RNA products were resolved on a 11% AA/7M Urea gel and imaged with Typhoon phospho-imager. (**B**) NusA does not inhibit Nun in TEC assembly system. Transcription elongation complexes were assembled from a 65-mer template DNA (65U10) hybridized to a 5′ P^{32} 9-mer RNA with a 3′ end corresponding to the +9G position ($TEC9G_9$). TEC was then preincubated for 10′ at 25 °C with 5 μM Nun alone, NusA+Nun, NusA alone, or a comparable volume of Nun storage buffer before transcription was initiated by addition of 20 μM rATP/rCTP/rUTP and 60 μM 3′deoxy GTP for 1′ and then stopped with equal volume 2× loading buffer. The presence of 3′deoxy GTP halted transcription 1 nt after the Nun arrest site, as depicted by arrow. The Nun arrest site is boxed. RNA products were resolved on a 23% AA/7M Urea gel and imaged with Typhoon phospho-imager.

In our recent studies, we explored further the Nun mechanism of action.[1] We constructed scaffolds consisting of DNA template and non-template strands, and RNA complementary to the template strand to generate defined transcription elongation complexes (TEC). These TEC differed in the length and the sequence of the RNA primer. Importantly, these scaffolds included no λ DNA or RNA sequences. Nun arrested all TEC tested that carried an RNA:DNA hybrid 9 bp or larger. The TEC were incubated with rXTPs and were all transcriptionally active. For each TEC, Nun-mediated arrest occurred at a specific site, corresponding to an intrinsic pause site.

NusA inhibits Nun in the absence of the other Nus factors.[4] NusA binds to the NUT sequence, to RNAP, and to the Nun C-terminus.[9-11] It was not clear which of these interactions accounted for NusA inhibition of Nun. We confirmed that NusA inhibits Nun arrest when transcription is initiated from the λ *pL* promoter and the template includes the NUT site (**Fig. 1A**). In contrast, when transcription is performed on a template lacking λ NUT, NusA has no effect on Nun arrest. In **Figure 1B**, DNA scaffolds were assembled and transcription was initiated with A, C, U, and 3′deoxy GTP, which halts transcription 1-nt after the arrest site. NusA fails to inhibit Nun arrest in this system. We conclude that NusA inhibits Nun by preventing Nun binding to NUT, rather than through interaction with Nun or RNAP. This is consistent with our finding that NUT is dispensable in vivo and in vitro when Nun is provided in excess.

Previously we showed that TEC paused under nucleotide deprivation was a substrate for subsequent Nun arrest, although the site of arrest was different depending on the location of the pause.[12] In subsequent work, we did functional mutational analysis on the Nun C-terminus, and found that a penultimate aromatic residue

(W108) was required for Nun arrest. We showed that the Nun C-terminus cross-linked to dsDNA ~9 bp promoter distal to the RNAP active center.[13-15]

Our recent evidence suggests that Nun interacts with the 5′ terminus of the RNA-DNA hybrid.[1] Thus, exonuclease treatment of Nun-arrested TEC revealed a Nun- dependent toeprint 1–2 nt upstream to the pre-translocated boundary of TEC. This toeprint was absent with TEC carrying less than a 9-bp RNA-DNA hybrid, and was not seen with a termination defective mutant Nun (K106/107A) or with a Nun-resistant RNAP mutant (β'R322H). Notably, this RNAP mutation lies near the upstream end of the transcription bubble. No Nun-dependent toeprint was visible at the downstream edge of the TEC. How to reconcile this finding with evidence suggesting Nun interaction with template 9-nt distal to the RNA 3′ terminus remains to be determined.

Our results demonstrated that Nun stabilizes either RNAP translocation state by restricting lateral movement of TEC along the DNA register. TEC stabilized in the pre-translocated state was unable to incorporate the next NTP. The nascent transcript in these complexes was exquisitely sensitive to pyrophosphorolysis, which efficiently removed the 3′-OH terminal nucleotide. Nun-stabilized post-translocated TEC rapidly incorporated the next NTP, but no subsequent nucleotides. That is, it could not further forward translocate. In contrast to the pre-translocated TEC, the RNA in the TEC arrested in the post-translocated register was resistant to pyrophosphorolysis.

Experiments that confirm that Nun inhibits TEC translocation are described below. In **Figure 2**, we demonstrate that Nun stimulates NTP misincorporation into a TEC arrested in the post-translocated state. When an incorrect NTP binds to the active site in post-translocated TEC, bond formation is slow. If backward translocation in this complex occurs faster than bond formation with the incorrect NTP, the incorrect substrate is removed from the active site by intrusion of the 3′ RNA end, allowing time for the correct NTP to enter the site. Thus, the stable post-translocated state of Nun-arrested TEC is more susceptible to misincorporation than TEC that rapidly oscillates between post- and pre-translocated states.

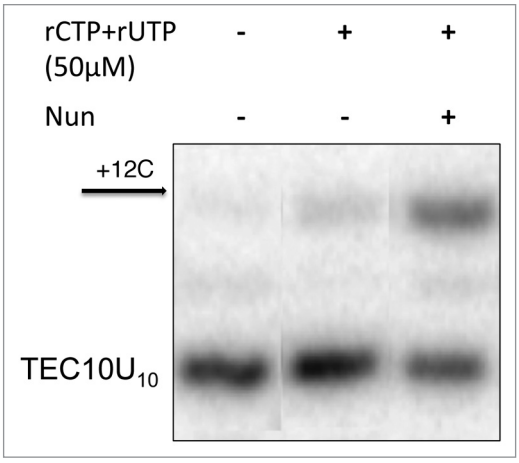

Figure 2. Nun promotes misincorporation at position +11. Transcription elongation complexes were assembled from a 65-mer DNA template (65U10) hybridized to a 5′ P^{32} 9-mer RNA with a 3′ end corresponding to the +9G position ($TEC9G_9$). $TEC10U_{10}$ was then formed by the addition of 50 μM rUTP for 1′, then excess rUTP was was washed off before the complex was eluted and incubated with 5 μM Nun or a comparable volume of Nun storage buffer (10′) transcription was initiated with 50μM rCTP+rUTP, a combination that would promote misincorporation at the +11 position (again, the sequence is $U^{10}A^{11}C^{12}$). Reactions were then stopped after 10′ with equal volume 2× Loading Buffer. +12C and +10U are indicated. Note that incorporation to +12 is dependent upon misincorporation at +11.

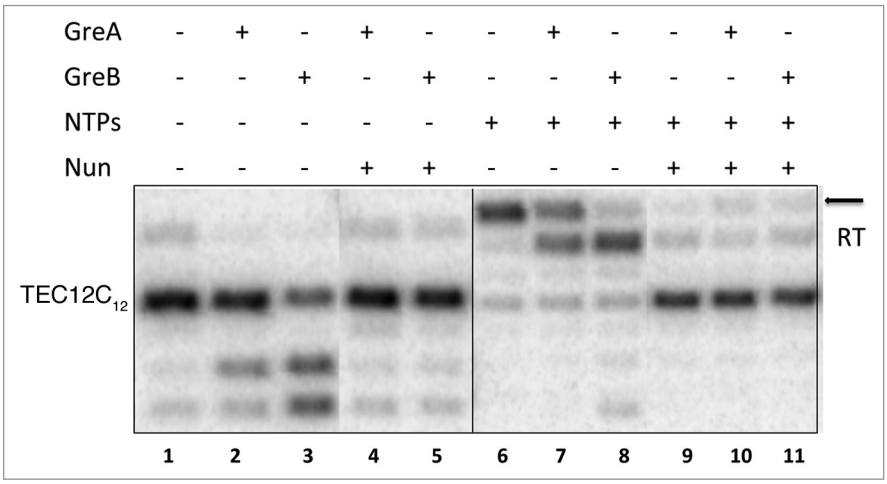

Figure 3. Nun inhibits Gre-induced endonuclease but Gre does not inhibit Nun arrest. Transcription elongation complexes were assembled from a 65-mer DNA template (65U10) hybridized to a 5′ P^{32} 12-mer RNA with a 3′ end corresponding to the +12C position ($TEC12C_{12}$). TEC was then preincubated for 10′ at 25 °C with 5 μM Nun, GreA, GreB, Nun+GreA or Nun+GreB and reactions were either stopped at 10′ by addition of 2× loading buffer (lanes 1–5) or chased with 20 μM rATP/rCTP/rUTP and 60 μM 3′deoxy GTP for an additional 1′ before reactions were stopped (lanes 6–10). Readthrough is indicated by an arrow. RNA products were resolved on a 23% AA/7M Urea gel and imaged with Typhoon phospho-imager.

Nun stabilization of pre- or post-translocated TEC explains why Nun-arrest TEC does not backtrack.[1] Backtracking occurs when RNAP translocates toward the promoter along the DNA register. The RNA 3′ is displaced from the active center into the secondary channel. Because the 3′ end is no longer available for chemistry, this complex is resistant to both phosphodiester bond formation and pyrophosphorolysis. To rescue a backtracked complex, a new 3′ end must be generated

by endonucleolytic cleavage, which is performed by bacterial GreA and GreB factors. We tested the effect of Nun on GreA and GreB stimulated endonuclease activity. Since the substrate for this endonuclease is the RNA in backtracked TEC, and Nun blocks backtracking, Nun strongly inhibited GreA and GreB cleavage (**Fig. 3**, lanes 1–5). Conversely, GreA and GreB did not restart Nun-arrested TEC (**Fig. 3**, lanes 6–10). We conclude that Nun and Gre do not directly compete, but that Nun prevents formation of backtracked TEC.

Taken together, the results shown in **Figures 2 and 3** confirm the earlier conclusion that Nun blocks TEC translocation and prevents backtracking. This action of a transcription factor has not been previously reported. There are clearly additional aspects of Nun that remain to be elucidated, and that are likely to reveal surprising aspects of this unusual protein.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

1. Vitiello CL, Kireeva ML, Lubkowska L, Kashlev M, Gottesman M. Coliphage HK022 Nun protein inhibits RNA polymerase translocation. Proc Natl Acad Sci U S A 2014; 111:E2368-75; PMID:24853501; http://dx.doi.org/10.1073/pnas.1319740111
2. Nudler E, Gottesman ME. Transcription termination and anti-termination in E. coli. Genes Cells 2002; 7:755-68; PMID:12167155; http://dx.doi.org/10.1046/j.1365-2443.2002.00563.x
3. Chattopadhyay S, Hung SC, Stuart AC, Palmer AG 3rd, Garcia-Mena J, Das A, Gottesman ME. Interaction between the phage HK022 Nun protein and the nut RNA of phage lambda. Proc Natl Acad Sci U S A 1995; 92:12131-5; PMID:8618858; http://dx.doi.org/10.1073/pnas.92.26.12131
4. Hung SC, Gottesman ME. Phage HK022 Nun protein arrests transcription on phage lambda DNA in vitro and competes with the phage lambda N antitermination protein. J Mol Biol 1995; 247:428-42; PMID:7714899; http://dx.doi.org/10.1006/jmbi.1994.0151
5. Washburn RS, Wang Y, Gottesman ME. Role of E.coli transcription-repair coupling factor Mfd in Nun-mediated transcription termination. J Mol Biol 2003; 329:655-62; PMID:12787667; http://dx.doi.org/10.1016/S0022-2836(03)00465-0
6. Robert J, Sloan SB, Weisberg RA, Gottesman ME, Robledo R, Harbrecht D. The remarkable specificity of a new transcription termination factor suggests that the mechanisms of termination and antitermination are similar. Cell 1987; 51:483-92; PMID:2822258; http://dx.doi.org/10.1016/0092-8674(87)90644-1
7. Rees WA, Weitzel SE, Yager TD, Das A, von Hippel PH. Bacteriophage lambda N protein alone can induce transcription antitermination in vitro. Proc Natl Acad Sci U S A 1996; 93:342-6; PMID:8552635; http://dx.doi.org/10.1073/pnas.93.1.342
8. Uc-Mass A, Khodursky A, Brown L, Gottesman ME. Overexpression of phage HK022 Nun protein is toxic for Escherichia coli. J Mol Biol 2008; 380:812-9; PMID:18571198; http://dx.doi.org/10.1016/j.jmb.2008.05.030
9. Watnick RS, Gottesman ME. Escherichia coli NusA is required for efficient RNA binding by phage HK022 nun protein. Proc Natl Acad Sci U S A 1998; 95:1546-51; PMID:9465052; http://dx.doi.org/10.1073/pnas.95.4.1546
10. Prasch S, Jurk M, Washburn RS, Gottesman ME, Wöhrl BM, Rösch P. RNA-binding specificity of E. coli NusA. Nucleic Acids Res 2009; 37:4736-42; PMID:19515940; http://dx.doi.org/10.1093/nar/gkp452
11. Schweimer K, Prasch S, Sujatha PS, Bubunenko M, Gottesman ME, Rösch P. NusA interaction with the α subunit of E. coli RNA polymerase is via the UP element site and releases autoinhibition. Structure 2011; 19:945-54; PMID:21742261; http://dx.doi.org/10.1016/j.str.2011.03.024
12. Hung SC, Gottesman ME. The Nun protein of bacteriophage HK022 inhibits translocation of Escherichia coli RNA polymerase without abolishing its catalytic activities. Genes Dev 1997; 11:2670-8; PMID:9334329; http://dx.doi.org/10.1101/gad.11.20.2670
13. Watnick RS, Gottesman ME. Binding of transcription termination protein nun to nascent RNA and template DNA. Science 1999; 286:2337-9; PMID:10600743; http://dx.doi.org/10.1126/science.286.5448.2337
14. Watnick RS, Herring SC, Palmer AG 3rd, Gottesman ME. The carboxyl terminus of phage HK022 Nun includes a novel zinc-binding motif and a tryptophan required for transcription termination. Genes Dev 2000; 14:731-9; PMID:10733532
15. Kim HC, Gottesman ME. Transcription termination by phage HK022 Nun is facilitated by COOH-terminal lysine residues. J Biol Chem 2004; 279:13412-7; PMID:14742436; http://dx.doi.org/10.1074/jbc.M313206200