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Role of an RNase III Binding Site in Transcription Termination at λ *nutL* by HK022 Nun Protein

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The phage HK022 Nun protein excludes phage λ by binding nascent λ p_L and p_R transcripts at *nutL* and *nutR*, respectively, and inducing transcription termination just downstream of these sites. Termination is more efficient at *nutL* than at *nutR*. One difference between *nutL* and *nutR* is the presence of RNase III processing sites (rIII) located immediately promoter distal to λ *nutL*. We found that deletion of rIII dramatically reduced Nun transcription arrest in vitro but had little effect on termination in vivo. However, consistent with the in vitro results, overexpression of a transcript carrying *nutL* and rIII efficiently titrated Nun, allowing λ to grow on a strain that expressed Nun, whereas a transcript carrying only *nutL* or *nutL*-rIII with nucleotides 97 to 141 deleted was ineffective. Rnc70, an RNase III mutant that binds but does not cleave rIII, also prevented Nun-mediated λ exclusion. We propose that rIII enhances the on-rate of Nun at *nutL*, stimulating Nun-mediated arrest in vitro. We have shown that a specific element in rIII, i.e., box C (G_{89}GUGUGUG), strongly enhances arrest on rIII$^+$ templates. Nun-rIII interactions do not stimulate Nun termination in vivo, presumably because formation of the Nun-*nutL* complex is normally not rate-limiting in the cell. In contrast to Nun, N is not occluded by Rnc70 and is not efficiently titrated by a *nutL*-rIII transcript.

The Nun protein of bacteriophage HK022 interacts with nascent phage λ RNA at *nutL* and *nutR*, inducing transcription termination at various points distal to these sites. Nun expressed from HK022 prophage thus excludes superinfecting λ (17). Host factors involved in Nun-dependent termination are identical to those for λ N-mediated antitermination (18). Both reactions are abrogated by certain mutations in *nusA*, *nusB*, *nusE* (S10), and *nusG*. In vitro, Nun inhibits transcription elongation but does not release the arrested transcription elongation complex (TEC). Nun activity in vitro requires a TEC that includes nascent *nutL* or *nutR*; Nus factors are not absolutely required but enhance the in vitro efficiency of Nun-mediated arrest (7).

The λ *nutL* and *nutR* sites are composed of three conserved motifs, including box A (8 nucleotides [nt]), box B (15 nt), and box C (8 nt) (Fig. 1). Box A RNA recruits NusB and NusE into a λ N antitermination complex that includes RNA polymerase (RNAP), NusA, and NusG (15, 16). Box B RNA forms a stem-loop that binds λ N or HK022 Nun and, subsequently, NusA (2, 6, 8, 27). Box B RNA alone binds λ N and Nun with similar affinities. This equivalent affinity for box B RNA does not reflect the inability of λ N to compete with Nun at *nutL* in vivo. A third conserved motif, box C (8 nt) (Fig. 1), lies downstream of *nutL* and *nutR* and does not appear to play a role in λ antitermination (5). The two *nut* sites differ in the spacer regions between box A, box B, and box C and by a single nt change in the box B loop and the sixth nt in box C.

Nun termination at *nutL* in vivo is both more efficient and less sensitive to *nus* mutations than termination at *nutR* (12, 18). *nusG* mutations have been isolated that block Nun termination only at *nutR*. Substitution of the box B sequence of *nutL* for that of *nutR* did not increase the sensitivity of the changed *nutL* transcript to the *nusG* mutant (1). Washburn et al. (26) proposed that the phenotypic difference between *nutL* and *nutR* might be explained by the relative distances of the two *nut* elements from their respective promoters. *nutL* is 34 nt from λ p_L, whereas *nutR* lies 227 nt from its cognate promoter.

True termination of Nun-arrested TEC requires host Mfd protein, a DNA helicase that recognizes and dissociates stalled RNAP. Mfd appears to act at *nutR* but not at *nutL*, possibly because the short distance between p_L and *nutL* precludes Mfd access. Thus, the off-rate of Nun-TEC complexes is lower at *nutL* than at *nutR* (26).

nutL also differs from *nutR* in that it lies immediately promoter proximal to RNase III cleavage sites (rIII) (Fig. 1). Nascent p_L transcripts are cleaved at nt 71, 88, and 197, whereas the mature transcript is cleaved at nt 88 and 197 (13). This suggests that rIII forms transient RNase III substrate structures (Fig. 1B and C) prior to formation of a more stable structure (9) (Fig. 1A).

Cleavage at rIII prevents repression of λ N translation by N or Nun bound at *nutL* (4, 10). Isolation of rIII mutants resistant to Nun inhibition of N translation (*nun3* and *nun1*) (Fig. 1) suggested that Nun might make contacts with rIII. We present evidence showing that a part of rIII does in fact interact with Nun and that this interaction enhances Nun activity in vitro but not in vivo.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used for this study are shown in Table 1. Standard bacteriological techniques used in

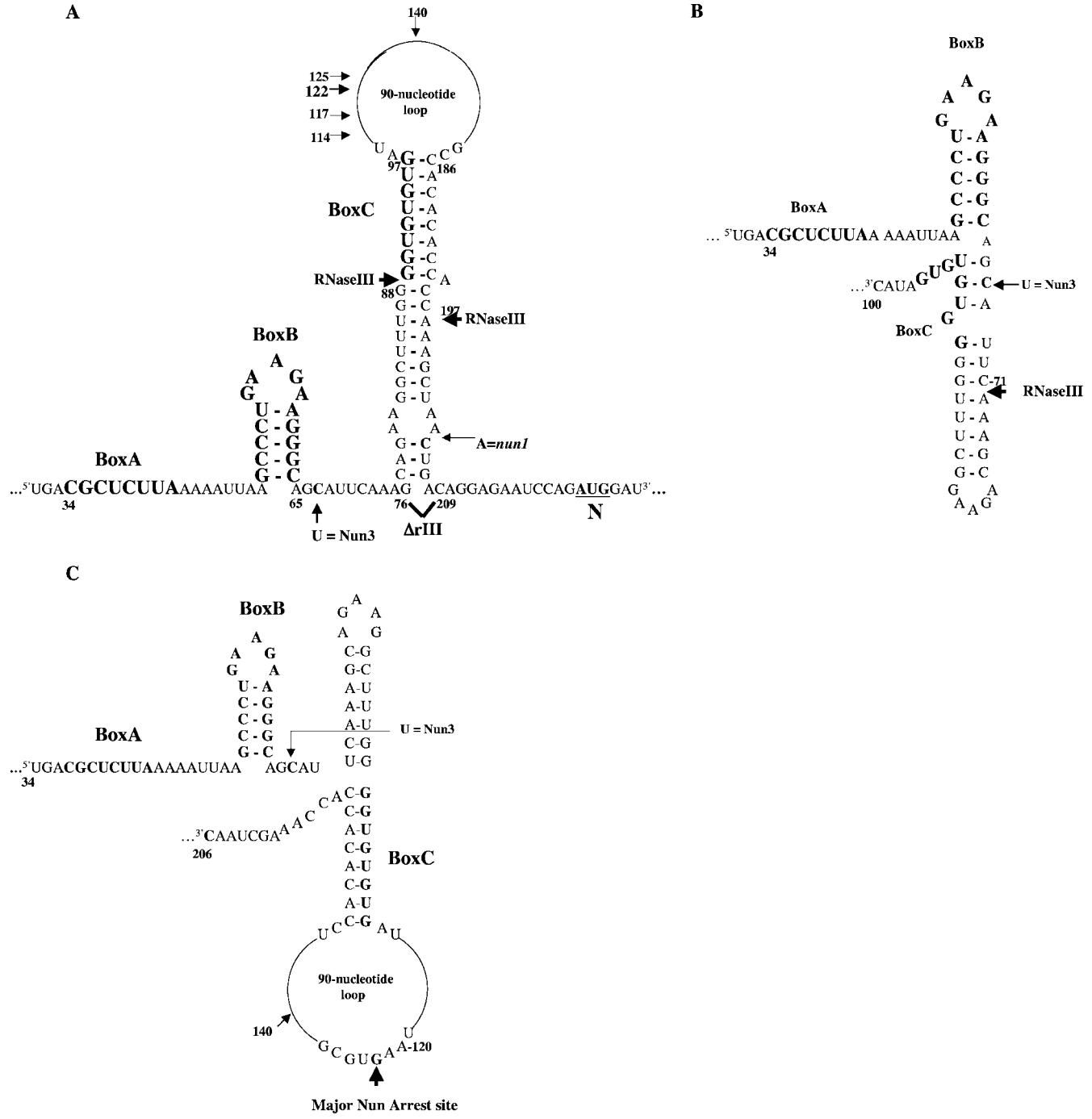

FIG. 1. Structures of λ p_L transcript. The structures show *nutL*, including box A (nt 34 to 41), the box B stem-loop (nt 50 to 64), box C (nt 89 to 96), and rIII (nt 76 to 208). The ΔrIII mutation is a precise deletion of rIII. The rIIIΔ76-140, rIIIΔ97-186, and rIIIΔ141-209 deletions remove nucleotides in the indicated ranges from the rIII site. Arrows indicate the two RNase III cleavage sites, at nt 88 and nt 197; the major Nun arrest site, at nt 122; and the minor arrest sites, at nt 114, nt 117, and nt 125. N denotes the translational start of the N gene. Also indicated are the sites of two point mutations, *nun1* and *nun3*, that block translational repression of N by the termination-deficient Nun mutant Nun K106/107D. (A) rIII found in the mature p_L transcript (23). (B and C) Proposed (computer-generated) transient RNase III sites formed during transcription, which are cleaved at nt 71.

strain construction, e.g., transformation, transduction, and medium preparation, were done as described by Silhavy et al. (21). Standard DNA cloning techniques were used as described by Sambrook and Russell (20). Plasmid pRSW101 was made by cloning the *lacZ* gene from pYW1 (26) into the EcoRI and EcoRV sites of pZero-2 (Invitrogen, Carlsbad, CA) and the *lux* gene from pYW1 into the XhoI and XbaI sites. Plasmids pRSW110, pRSW111, pRSW112, pRSW113, and pRSW114 were produced by cloning the *nutL*-rIII regions from λW336, λW335, p_L-*nutL*-rIIIΔ97-106, p_L-*nutL*-rIIIΔ76-140, and p_L-*nutL*-rIIIΔ141-209, which were amplified by PCR using the DNA oligonucleotides 5′-CCGCTCGAGAGGTGACGCTCTTAAAAA T-3′ and 5′-CCGCTCGAGCCATCTGGATTCTCCTG-3′ and cloned into the XhoI site of pRSW101.

TABLE 1. Bacterial strains and plasmids used for this study

Strain or plasmid	Genotype	Source or reference
Strains		
N99	Wild type	NIH collection
N7723	N99 *lacZA21*	Lab collection
N7726	*recA56*	Lab collection
N9478	N99 *rnc-14 lacZA21* [λ_{cI857}] p_L-*nutL-N-lacZ* transcriptional fusion	28
N9479	N99 *rnc-14 lacZA21* [λ_{cI857}] p_L-*nutL-rIII*Δ76-209-*N-lacZ* transcriptional fusion	28
N9480	N9478(HK022)	This study
N9481	N9479(HK022)	This study
N9482	N9478/pBad-NunK107A	This study
N9483	N9479/pBad-NunK107A	This study
N9484	N9480/pACS21	This study
N9485	N9480/pSDF701	This study
N9486	N9481/pACS21	This study
N9487	N9481/pSDF701	This study
N9488	N9478/pACS21	This study
RSW149	N7723/pBad-NunK107A	This study
RSW237	RSW149/pRSW110	This study
RSW238	RSW149/pRSW111	This study
RSW258	N9478/pSDF701	This study
RSW279	N7726/pACS21	This study
RSW280	N7726/pSDF701	This study
RSW353	RSW149/pRSW112	This study
RSW354	RSW149/pRSW113	This study
RSW355	RSW149/pRSW114	This study
Plasmids		
pBad-NunK107A	pACYC1ori Amp^r *araC* pBad-NunK107A	26
pACS21	ColE1ori Amp^r P_{lac} *rnc*$^+$	25
pSDF701	ColE1ori Amp^r P_{lac} *rnc-70*	4
pRSW101	ColE1ori Kan^r P_{lac} *lacZ-XhoI-lux*	This study
pRSW110	ColE1ori Kan^r P_{lac} *lacZ-nutL-rIII*Δ76-209-*lux*	This study
pRSW111	ColE1ori Kan^r P_{lac} *lacZ-nutL-rIII-lux*	This study
pRSW112	ColE1ori Kan^r P_{lac} *lacZ-nutL-rIII*Δ97-186-*lux*	This study
pRSW113	ColE1ori Kan^r P_{lac} *lacZ-nutL-rIII*Δ76-140-*lux*	This study
pRSW114	ColE1ori Kan^r P_{lac} *lacZ-nutL-rIII*Δ141-209-*lux*	This study

Proteins. *Escherichia coli* RNAP and RNase III were purchased from Epicenter (Madison, WI). The Nun protein was a generous gift of Hyeong Kim.

β-Galactosidase assays. Strains were grown in LB at 32°C with shaking to an optical density at 600 nm of 0.1 and then shifted to 42°C and grown to an optical density at 600 nm of 0.6. Where indicated, the appropriate antibiotic (100 μg/ml ampicillin or 50 μg/ml kanamycin) was present. Nun, when expressed from a plasmid under the control of the pBAD promoter, was induced by the addition of 0.05% arabinose. Cells were assayed for β-galactosidase activity as described by Miller (14).

Templates for in vitro transcription. DNA templates were generated by PCR, using AmpliTaq DNA polymerase (Roche Diagnostics, Branchburg, NJ) and DNA oligonucleotides (5′-GGAATTCCATATGTCAGATCTCTCACCTACC AAAC-3′ and 5′-AGGGCGGTTAACTGGTTTTG-3′) to amplify a 500-bp fragment of phage λ including p_L-*nutL*-rIII. A p_L-*nutL*-ΔrIII fragment was prepared by using genomic DNA from λ W336 as the template. The remaining mutant templates were produced as follows: the 5′ ends of p_L-*nutL*-rIIIΔ76-141 (5′-CC GTGATCACAATGTGCCAATCGC-3′) and p_L-*nutL*-rIII*boxCmut* (5′-CCG TGATCAGCAGAAGGCTTTGCCCACACACATACGAAACGAAGC) were amplified using the indicated DNA oligonucleotides paired with the oligonucleotide 5′-GGAATTCCATATGTCAGATCTCTCACCTACCAAAC-3′, digested with BclI, and ligated to the 3′ fragment of p_L-*nutL* produced with the oligonucleotides 5′-AGGGCGGTTAACTGGTTTTG-3′ and 5′-CGGGATCCTTTGA ATGCTGCCC-3′ and digested with BamHI. p_L-*nutL*-rIIIΔ141-209 (5′-CCGTG ATCAACAGGAGAATCCAGATG-3′) was amplified using the indicated DNA oligonucleotide paired with 5′-GGAATTCCATATGTCAGATCTCTCACCTA CCAAAC-3′, digested with BclI, and ligated to the 3′ fragment of p_L-*nutL* produced with the oligonucleotides 5′-AGGGCGGTTAACTGGTTTTG-3′ and 5′-CGGGATCCGCAGCTAATCCGGAATC-3′ and digested with BamHI. p_L-*nutL*-rIIIΔ97-186 (5′-CCGTGATCACACACACCACCAAAG-3′) was amplified using the indicated DNA oligonucleotide paired with 5′-GGAATTCCATA TGTCAGATCTCTCACCTACCAAAC-3′, digested with BclI, and ligated to the 3′ fragment of p_L-*nutL* produced with the oligonucleotides 5′-AGGGCGG TTAACTGGTTTTG-3′ and 5′-CGGGATCCCACACACCCCAAAGC-3′ and digested with BamHI. p_L-*nutL*-rIIIG114A, T117A, G122A, G125A was amplified using the DNA oligonucleotides 5′-CCGTGATCATACGAAACGAAGCATT GGCCG-3′ and 5′-GGAATTCCATATGTCAGATCTCTCACCTACCAAAC-3′, digested with BclI, and ligated to the 3′ fragment of p_L-*nutL* produced with the oligonucleotides 5′-AGGGCGGTTAACTGGTTTTG-3′ and 5′-CGGGATCC GCAGCTAATCCGGAATTGCATTTACTGCTAATGCTTCG -3′ and digested with BamHI.

In vitro termination assay. Open complexes were formed by preincubating 0.1 pmol template bound with 0.5 units RNAP (Epicenter) in 50 μl TB (20 mM Tris-acetate [pH 7.9], 60 mM potassium acetate, 4 mM magnesium acetate, 1 mm dithiothreitol, 0.25 mg/ml bovine serum albumin, and 5% glycerol) for 5 min at 32°C. The Nun protein, when included, was added at the indicated concentration (1.25, 2.5, or 5 pmol/reaction). Transcription was initiated by the addition of a 10 μM concentration of each nucleoside triphosphate plus 1 μCi [α-^{32}P]ATP. After incubation at 32°C for 5 min, the reactions were terminated by the addition of 50 μl stop solution (375 mM sodium acetate [pH 5.2] and 62.5 mM EDTA). The reaction mixtures were extracted with an equal volume of phenol-chloroform-isoamylalcohol (Sigma) and ethanol precipitated with 3 volumes of 95% ethanol. Extracted RNAs were then resolved in a denaturing 12% polyacrylamide gel and analyzed by autoradiography.

Readthrough transcription was measured by excising the appropriate gel bands and measuring radioactivity in a liquid scintillation counter.

Efficiency of λ plating. Bacteria (~10^9 cells) of the appropriate strains were poured in top agar on LB or LB plus 50 μg/ml kanamycin plus 100 μg/ml ampicillin to produce lawns. IPTG (isopropyl-β-D-thiogalactopyranoside; 0.1 mM) was added to induce transcription of *nutL* or *nutL*-rIII from pRSW110-114. Efficiencies of plating were determined by spotting dilutions of λ phage on the bacterial lawns before incubating them overnight at 37°C.

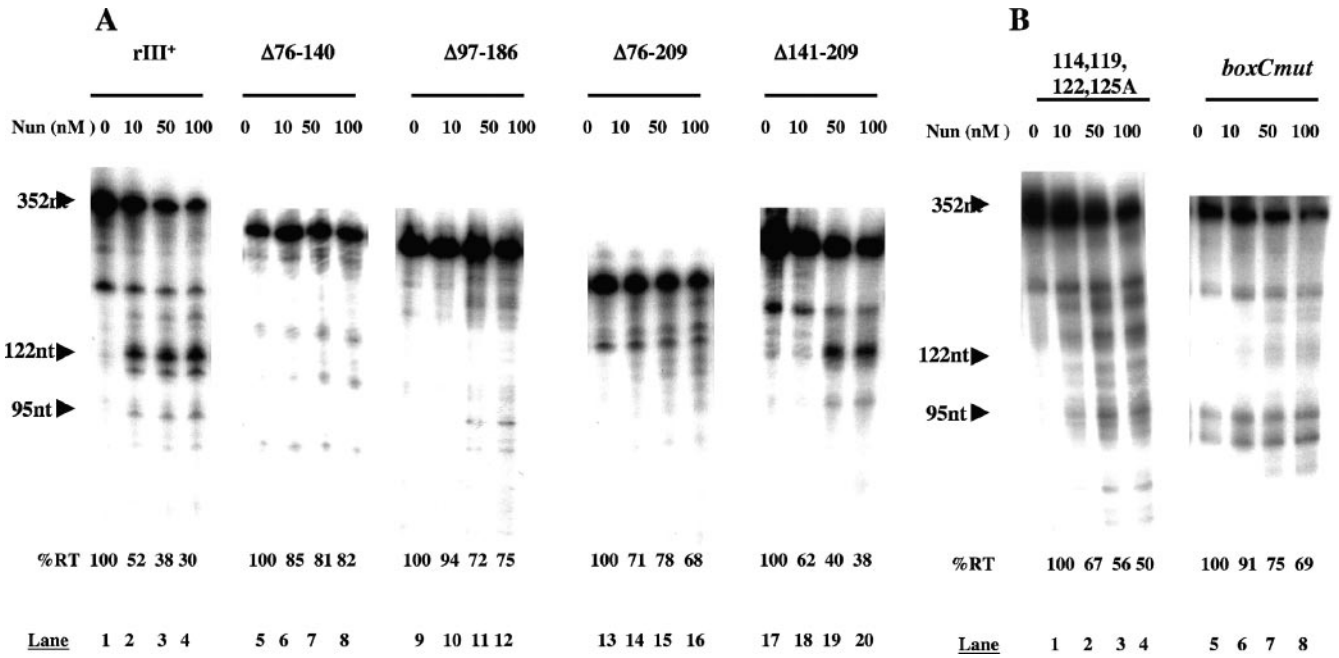

FIG. 2. In vitro transcription termination assay with p_L-*nutL*-rIII$^+$ and p_L-*nutL* templates carrying rIII mutations. RT, readthrough transcription. Major Nun-dependent arrest sites are indicated by arrows. All experiments were performed at least three times. %RT is the percentage of readthrough transcription relative to reactions without Nun.

RESULTS

Decreased Nun transcription arrest on rIII mutant templates in vitro. To determine the contribution of rIII to Nun-mediated transcription arrest, we measured Nun activity in vitro, comparing a wild-type λ p_L-*nutL*-rIII template and the same template containing mutations within rIII (Fig. 1). The efficiency of arrest is indicated by the decrease in transcriptional readthrough induced by Nun. The presence of rIII clearly enhanced Nun activity (Fig. 2A). At 100 nM, Nun allowed only 30% readthrough on the rIII$^+$ template, compared to 68% readthrough on the ΔrIII template (Fig. 2A, lanes 4 and 16). Reducing readthrough on the ΔrIII template to levels comparable to those with the wild-type template required 400 nM Nun (data not shown). Deleting the 5′ end of rIII (Δ76-141) was equivalent to deleting the entire rIII site (lanes 5 to 8), as was deleting the loop region (Δ97-186; lanes 9 to 12). Deleting sequences 3′ of nt 140 (Δ141-209) did not affect arrest (lanes 17 to 20).

These results suggest that rIII contributes significantly to Nun-dependent arrest at *nutL* by increasing the affinity of Nun for the TEC. Note that the major Nun arrest site on the rIII$^+$ template occurs at nt 122 (Fig. 2A) (7), after transcription of the proposed transient RNase III substrate structure shown in Fig. 1B but prior to transcription of the structures shown in Fig. 1A and C (9). Thus, Nun must recognize RNA structures or sequences deleted in the ΔrIII template that lie between nt 97 and 141.

We then examined the contributions made by the predominant Nun arrest sites, G114, T117, G122, and G125 (3). Figure 2B, lanes 1 to 4, shows Nun arrest on a *nutL* template with an A substitution at each site. The quadruple mutant template was less efficient for arrest than the rIII$^+$ template (50% versus 30% readthrough) (Fig. 2A and B, lanes 1 to 4). The pattern of arrest was also changed: arrest was distributed over several sites 3′ of nt 122. These data confirm that rIII$^+$ nt 114, 117, 122, and 125 contribute significantly to Nun arrest in vitro.

We then asked if box C (G_{89}GUGUGUG), a conserved sequence on the ascending arm of rIII, enhanced the efficiency of Nun arrest (5). We constructed a template, boxCmut, which contains the complement of box C (C_{89}CACACAC). Figure 2B, lanes 5 to 8, shows the results of a transcription assay with this template. Nun arrest was dramatically reduced compared to that with the rIII$^+$ template (69% versus 30% readthrough) and was equivalent to the efficiency of Nun arrest on the rIIIΔ76-209 template (68%) (Fig. 2A, lanes 13 to 16). The boxCmut template has two strong Nun-independent spontaneous pauses introduced in the region of nt 89 to 96 (Fig. 2B, lane 5). Our interpretation of these results is that box C enhances arrest at G114, T117, G122, and G125 but is not sufficient for efficient arrest on templates with these sites deleted or mutated.

Titration of Nun by *nutL*-rIII transcript in vivo. Our results suggest that rIII and Nun interact and that these interactions increase Nun arrest efficiency in vitro. We next looked for evidence that Nun and rIII interact in vivo. Accordingly, we compared the abilities of transcripts carrying *nutL*-rIII or *nutL*-rIII deletions (Δ76-209, Δ141-209, Δ76-140, and Δ97-186) to titrate Nun. The transcripts were expressed in a strain expressing a Nun mutant, NunK107A; Nun activity was monitored by λ plaque formation (Table 2). Although NunK107A arrests transcription with wild-type efficiency in vitro and in vivo (11), we found it to be titrated more easily than wild-type Nun. We previously showed that transcription of plasmid-borne *nutL* suppressed Nun exclusion of λ, partially in a wild-

TABLE 2. Transcription of *nutL*-rIII increases EOP of λ on a Nun$^+$ strain[a]

Strain	Presence of *nun*	*nut* region	EOP
N7723	−		1.0
RSW149	+		$<10^{-5}$
RSW238	+	*nutL*-rIII	1.0
RSW237	+	*nutL*-rIIIΔ76-209	$\sim10^{-2}$*
RSW353	+	*nutL*-rIIIΔ97-186	$\sim10^{-2}$*
RSW354	+	*nutL*-rIIIΔ76-140	$\sim10^{-2}$*
RSW355	+	*nutL*-rIIIΔ141-209	1.0

[a] Nun was provided by plasmid pBad-NunK107A, where indicated. IPTG (1 mM) was added to induce transcription of *nutL* or *nutL*-rIII. Efficiencies of plating were determined at 37°C using λ*imm434*. *, individual plaques were not visible.

type host and fully in cells carrying an *mfd* mutation (26). We suggested that the *nutL* transcript sequestered and titrated Nun. In confirmation of this result, Nun inhibition of λ plating was partially reversed by transcription of *nutL*-rIIIΔ76-209 from the multicopy plasmid pRSW110 (efficiency of plating [EOP] of $<10^{-5}$ [row 2 in Table 2] versus EOP of $\sim10^{-2}$ [row 4]). In contrast, transcription of *nutL*-rIII from pRSW111 completely restored λ plating, even though the host was *mfd*$^+$ (EOP = 1.0 [Table 2, row 3]). Transcription of *nutL*-rIIIΔ141-209 restored λ plating (EOP = 1.0 [Table 2, row 7]). However, λ plated with an EOP of $\sim10^{-2}$ on cells expressing *nutL*-rIIIΔ76-140 or *nutL*-rIIIΔ97-186 transcripts (Table 2, rows 5 and 6). Thus, the same region of rIII identified as important for termination in vitro, nt 97 to 141, was found to titrate NunK107A in vivo. Alternatively, the sequences from nt 97 to 141 could be important for increasing the in vivo stability of the *nutL* RNA.

Rnc70 suppresses Nun termination at *nutL*-rIII. We then asked if RNase III, which also recognizes rIII, could compete with Nun and suppress termination in vivo. To test this notion, we expressed Rnc70, a noncatalytic mutant that binds but does not cleave rIII (4). Inhibition of Nun by Rnc70 was demonstrated by testing λ plating on HK022 lysogens (Table 3). Overexpression of Rnc$^+$ had no effect on λ plating, whereas Rnc70 overexpression completely suppressed Nun exclusion (Table 3, rows 4 to 6). Rnc70 failed to protect λ ΔrIII from exclusion by Nun (data not shown).

Termination in control HK022 lysogens in the absence of RNase III or in the presence of wild-type RNase III was highly efficient. Readthrough in either *rnc*$^+$ or *rnc* mutant strains was <2% (Table 4, rows 2 and 3). Note that the absence of rIII

TABLE 3. Rnc70 restores λ plating on an HK022 lysogen[a]

Strain	Presence of *nun*	Rnc	EOP
N9478	−		1.0
N9488	−	WT	1.0
N9487	−	Rnc70	1.0
N9480	+		$<10^{-4}$
N9484	+	WT	$<10^{-4}$
N9485	+	Rnc70	1.0

[a] Nun, when present, was provided by an HK022 lysogen. The Rnc column indicates the presence of either a wild type (WT)- or mutant (Rnc70)-overproducing *rnc* plasmid. Efficiencies of plating relative to that of N9478 were determined using λ*imm434* at 37°C.

TABLE 4. Effects of *rnc*$^+$ and *rnc-70* mutation on Nun-dependent termination in vivo[a]

Strain	Presence of sequence			β-Galactosidase activity (Miller units)	% Readthrough
	rIII	*nun*	*rnc*		
N9478	+	−	−	2,420 ± 480	100
N9480	+	+	−	30 ± 2	1.2
N9484	+	+	WT	18 ± 2	0.8
N9485	+	+	Rnc70	560 ± 38	23
N9479	−	−	−	2,040 ± 200	100
N9481	−	+	−	27 ± 14	1.4
N9486	−	+	WT	15 ± 1	0.8
N9487	−	+	Rnc70	96 ± 5	4.8

[a] All strains carry either p_L-*nutL-rIII-N-lacZ* (rIII$^+$) or p_L-*nutL*-ΔrIII-*N-lacZ* (rIII mutant) under the control of the λ *cI857 ts* repressor. Nun, when present, was provided by an HK022 lysogen. The *rnc* indicates the presence of either a wild type (WT)- or mutant (Rnc70)-overproducing *rnc* plasmid. Strains were grown at 32°C to mid-log phase, shifted to 42°C for 1.5 h to induce p_L, and assayed for β-galactosidase activity. Nun termination/arrest is indicated by the β-galactosidase level. Values are given in Miller units and represent the averages ± standard deviations for three or more experiments.

made little difference in the efficiency of termination in this assay (Table 4, rows 6 and 7). We return to this point below (see Table 6).

Overexpression of Rnc70, however, significantly reduced Nun-dependent transcription termination on the p_L-*nutL*-rIII fusion (23% readthrough) (Table 4, row 4). Inhibition of Nun activity by Rnc70 was largely dependent on rIII (Table 4, row 8). This rules out a global effect of Rnc70 overexpression on Nun-dependent transcription termination accounting for the decrease in termination, although the partial suppression of termination in the rIIIΔ76-209 fusion (Table 4, row 8) might thus be explained. Our results support the ideas that Nun recognizes rIII and that Rnc70 competes with Nun for this interaction. The failure of wild-type RNase III to suppress Nun might be explained by a lower affinity for partially transcribed rIII.

Effect of rIII on Nun termination in vivo. We then examined the contribution of rIII to Nun termination in strains N9482 and N9483 (Table 1). These strains carry the chromosomal *N*::*lacZ* transcription fusions λp_L-*nutL*-rIII-*N*::*lacZ* and λ p_L-*nutL-N*::*lacZ*, respectively. Plasmid-borne NunK107A was expressed from the pBAD promoter. Nun concentrations were

TABLE 5. In vivo termination activity in *nutL*-ΔrIII mutant[a]

Strain	Presence of sequence		Addition of arabinose	β-Galactosidase activity (Miller units)	% Readthrough
	rIII	*nun*			
N9478	+	−	−	2,000 ± 440	100
N9482	+	+	−	60 ± 30	3.0
N9482	+	+	+	12 ± 0.6	0.6
N9479	−	−	−	2,000 ± 220	100
N9483	−	+	−	130 ± 24	6.5
N9483	−	+	+	26 ± 5	1.3

[a] All strains carry either p_L-*nutL-rIII-N-lacZ* (rIII$^+$) or p_L-*nutL*-ΔrIII-*N-lacZ* (rIII mutant) under the control of the λ *cI857 ts* repressor. Nun was expressed from plasmid pBAD-NunK107A. Arabinose was added to 0.05%, where indicated, to induce Nun. Strains were grown at 32°C to mid-log phase, shifted to 42°C for 1.5 h to induce p_L, and assayed for β-galactosidase activity. Values are given in Miller units and represent the averages ± standard deviations for three or more experiments.

TABLE 6. Rnc70 does not suppress N function at λ *nutL*[a]

Strain	Presence of *recA*	Rnc	EOP		
			λ	λ *nutL400*	λ ΔrIII
N7723	+		1.0	1.0	1.0
RSW111	−		1.0	$<10^{-5}$	1.0
RSW279	−	WT	0.6	$<10^{-5}$	0.6
RSW280	−	Rnc70	0.3	$<10^{-5}$	0.2

[a] *recA*-negative strains carry *recA56*. Efficiencies of plating of λ, λ *nutL400*, and λ ΔrIII were determined using strains W335, Y1167, and W336, respectively, at 37°C.

increased over basal levels, where indicated, by induction of pBAD with 0.05% arabinose.

Nun efficiently terminated transcription on the rIII$^+$ fusion (N9482). β-Galactosidase activity was reduced to 3% that of a control strain without the NunK107A plasmid. Induction of Nun expression with arabinose further reduced the β-galactosidase activity to 0.6% that of the control (Table 5, rows 1 to 3). Nun-mediated termination was almost as efficient in fusions lacking rIII. At basal levels of Nun, β-galactosidase activity was 6.5% that of the control. Nun overexpression reduced the β-galactosidase activity to 1.3% that of the control (Table 5, rows 4 to 6). These results indicate that deletion of rIII decreases the in vivo Nun termination efficiency at *nutL* twofold, at most. We concluded that Nun interactions with rIII contribute to but are not essential for termination in vivo.

Nus factors reduce the requirement for Nun and stimulate Nun arrest in vitro. We therefore asked if rIII might enhance the Nun reaction in HK022 lysogens unable to form the Nus ABEG complex. However, Nun termination in a strain deleted for *nusB* (D. L. Court, unpublished) was reduced only twofold in vivo and was equally efficient on rIII$^+$ and rIII$^-$ templates. It is possible that an as yet unidentified host factor redundant with NusB might stimulate binding to the λ nascent transcript.

rIII and λ N antitermination in the p_L operon. We next asked if rIII affected the ability of λ N to antiterminate at *nutL*. The λ *red* and *gam* genes lie downstream of terminators in the p_L operon (19). Although the products of these genes are not essential for λ growth in *recA*$^+$ hosts, they are absolutely required in a *recA*-negative background. Table 6 confirms that antitermination is needed for λ plaque formation on a *recA56* host. Thus, λ plates with an EOP of 1.0 on *recA56*, whereas λ*nutL400*, which carries a mutation in box B that eliminates N antitermination (19), fails to grow (EOP, $<10^{-5}$). In contrast, λ ΔrIII, which carries a precise deletion of rIII, plates with an EOP of 1.0. We concluded that deletion of rIII does not ablate N antitermination at *nutL*. Table 6 also shows that Rnc70 overexpression, which inhibits Nun termination, does not reduce λ plating on a *recA56* host or the plating of a control phage, λ ΔrIII, which does not bind RNase III.

Finally, we found that overproduction of a *nutL*-rIII transcript did not titrate N. Thus, λ and λ*r32*, a mutant that fails to grow when N is limiting, plated with an EOP of 1.0 on both RSW237, which overexpresses *nutL*, and RSW238, which overexpresses *nutL*-rIII (data not shown).

DISCUSSION

Transcription of the λ p_L and p_R operons is antiterminated by λ N or prematurely terminated by phage HK022 Nun. Both the N and Nun proteins recognize box B RNA sequences of *nutL* and *nutR* and act in complex with four host Nus proteins to modify the TEC. Phage λ fails to grow on an HK022 lysogen because Nun both competes with N to prevent antitermination and directly causes transcription termination distal to the *nut* sites (2, 7). The *nut* region is also subject to another form of regulation. Translation of N, the first gene in the p_L operon, is partially depressed by the rIII secondary structure and strongly inhibited by the N antitermination or Nun termination complex at *nutL* (Fig. 1) (10, 27). The rIII structure and the N or Nun complex at *nutL* are thought to interfere with ribosomal attachment to the adjacent N ribosome-binding site (Fig. 1A).

In this work, we asked if rIII also plays a role in Nun termination. We provide evidence that Nun and rIII interact in vitro and in vivo. An rIII site on a template significantly enhanced Nun-dependent transcription arrest at *nutL* in vitro. Two elements in rIII contribute to the increased efficiency of Nun, namely, box C and four major arrest sites (nt 114, 117, 122, and 125). We propose that box C provides a recognition element for Nun that stimulates arrest at these sites.

In vivo titration assays confirmed that Nun binds rIII. Thus, overproduction of a transcript that included *nutL*-rIII permitted λ growth on a strain expressing NunK107A. A transcript containing only *nutL* or *nutL*-rIII with nt 97 to 141 deleted did not relieve λ exclusion. Finally, overexpression of Rnc70, a catalytically inactive RNase III mutant that binds rIII (4), inhibited Nun, presumably by occluding Nun binding to rIII.

However, experiments in vivo with transcriptional fusions linking p_L-*nutL*-rIII or p_L-*nutL*-ΔrIII and a *lacZ* reporter revealed, at most, a twofold difference in Nun termination between the two fusions. Our result is consistent with that of Sloan and Weisberg (22), who found that Nun termination was 90% efficient in a p_L-*nutL*-ΔrIII fusion. We concluded that while rIII contributes to Nun activity at *nutL*, it does not play a major role under our in vivo assay conditions.

To explain the differences between these in vitro and in vivo results, we propose that the Nun reaction in vitro is rate-limited by the binding of Nun to the λ nascent transcript. Nun activity can be measured in a single-round transcription assay. Furthermore, in the absence of Nus factors, the in vitro reaction is suboptimal and requires a large excess of Nun relative to the TEC. In the minimal system described here, therefore, the Nun on-rate is limiting, and Nun binding to the TEC is stimulated by its interaction with rIII. We suggest that under certain, as yet unknown, in vivo conditions, the Nun on-rate might also be limiting, and the binding of Nun to rIII could improve the termination efficiency.

In contrast to deletion of rIII, overproduction of Rnc70, which binds but does not cleave rIII (4), did suppress Nun-dependent termination in a p_L-*nutL*-rIII transcriptional fusion and also restored λ plating on an HK022 lysogen. It is plausible that Rnc70 bound to rIII occludes neighboring sequences that are important for Nun activity. Complete occlusion of *nutL*, however, is ruled out by the observation that N antitermination is resistant to Rnc70.

We found no evidence that rIII affects λ N antitermination. Unlike Nun, N is not inhibited by overproduction of the *nutL*-rIII transcript. Plating of λΔrIII on a *recA56* host, which requires the p_L operon genes *red* and *gam*, indicates that rIII is not required for N antitermination in the p_L operon. Finally, overproduction of Rnc70 does not prevent λrIII$^+$ propagation on a *recA56* host.

These results also shed some light on the failure of N to compete efficiently with Nun at *nutL* (17). We propose that this is explained, in part, by assuming that Nun recognizes a larger motif than does N at *nutL*, enhancing its affinity for the p_L transcript relative to N. Note that Nun carries 21 amino-terminal amino acids that are lacking in N (24). Whether these residues participate in RNA binding is under investigation. In vitro binding assays with box B RNA did not indicate a difference in affinity between Nun and N (2). It will be interesting to compare the binding of these factors to a larger transcript that includes box C.

Which sequences in the rIII deletion enhance Nun activity in the p_L operon? Nun inhibition by Rnc70 indicates that an RNase III binding site is transcribed prior to Nun-mediated termination. Nun termination in vivo forms a series of transcripts within a region 100 nt promoter-distal of *nutL* (22). Consistent with this result, we (this work and reference 7) found that Nun arrest in vitro occurs predominantly at nt 122. Thus, the RNA sequences recognized by Nun must lie within the proximal 122 nt of the p_L operon transcript. Figure 1 shows three possible rIII structures. The structures in Fig. 1B and C are formed transiently and are cleaved by RNase III at nt 71. Figure 1A represents a structure found in the mature p_L transcript; this structure is cleaved at nt 88 and nt 197. Our data preclude the structures in Fig. 1A and C, whose endpoints lie beyond the major Nun termination site.

Several candidate sequences lie between *nutL* and nt 122. First, Nun3, which blocks translation repression by the termination-defective Nun K106/107D protein, is located at nt 67 (11). Two other sequences of potential interest lie in this region of the p_L transcript. An inverted repeat, C_{71}AAAGC and G_{82}CUUUG, is found in phages λ, 21, and P22 (9). Box C (G_{89}GUGUGUG) is likewise conserved among temperate phages (5). In this work, we found that box C is required in vitro for efficient arrest at nt 114, 117, 122, and 125. This is the first described role for box C in transcription elongation.

rIII promotes translational repression of N by Nun as well as Nun-mediated transcription termination at low concentrations of Nun. This does not explain, however, the higher efficiency of Nun termination at the p_L operon than that at the p_R operon (99% versus 87%) (12). Some of the difference is due to Mfd, which releases the Nun-arrested TEC at *nutR* but not *nutL* (26). Presumably, other factors also play a role in supporting Nun termination at *nutL*.

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