The global regulator RNase III modulates translation repression by the transcription elongation factor N

Helen R.Wilson[1], Daiguan Yu[1,2], Howard K.Peters,III[1], Jian-guang Zhou[1,3] and Donald L.Court[1,4]

[1]Molecular Control and Genetics Section, Gene Regulation and Chromosome Biology, National Cancer Institute-Frederick, Frederick, MD 21702-1201, USA

[2]Present address: Lexicon Genetics, Inc., 4000 Research Forest Drive, The Woodlands, TX 77381, USA

[3]Present address: Institute of Biotechnology, No. 27, Tai-ping Road, Beijing 100856, China

[4]Corresponding author
e-mail: court@ncifcrf.gov

Efficient expression of most bacteriophage λ early genes depends upon the formation of an antiterminating transcription complex to overcome transcription terminators in the early operons, p_L and p_R. Formation of this complex requires the phage-encoded protein N, the first gene product expressed from the p_L operon. The N leader RNA contains, in this order: the NUTL site, an RNase III-sensitive hairpin and the N ribosome-binding site. N bound to NUTL RNA is part of both the antitermination complex and an autoregulatory complex that represses the translation of the N gene. In this study, we show that cleavage of the N leader by RNase III does not inhibit antitermination but prevents N-mediated translation repression of N gene expression. In fact, by preventing N autoregulation, RNase III activates N gene translation at least 200-fold. N-mediated translation repression is extremely sensitive to growth rate, reflecting the growth rate regulation of RNase III expression itself. Given N protein's critical role in λ development, the level of RNase III activity therefore serves as an important sensor of physiological conditions for the bacteriophage.

Keywords: antitermination/bacteriophage λ/growth rate regulation/RNase III/translation repression

Introduction

Coliphage λ uses N-mediated antitermination to regulate the expression of early phage functions (Friedman and Gottesman, 1983; Das, 1992; Friedman and Court, 1995; Weisberg and Gottesman, 1999). Transcription of the early operons of λ, p_L and p_R is interrupted by multiple transcription terminators. The expression of the early phage function N from p_L facilitates the assembly of terminator-resistant transcription complexes. N and *Escherichia coli* Nus factors interact at a site in the p_L and p_R transcripts called NUT to convert RNA polymerase to this antiterminating form.

The NUT site of the p_L transcript is located in the leader of the N gene from +34 to +64 (Figure 1). In addition to antitermination, two other processes act through the N leader, affecting the expression of N. A large stem–loop structure at +76 to +208 in the N leader is a substrate for the double-strand-specific ribonuclease RNase III (Figure 1) (Lozeron *et al.*, 1976, 1977; Steege *et al.*, 1987; Court, 1993). This hairpin, which ends immediately upstream of the N Shine–Dalgarno sequence, interferes with translation of the N gene (Kameyama *et al.*, 1991). RNase III cleavage removes the inhibitory structure and stimulates N gene expression. The translation of N is also affected by an autoregulatory mechanism in which N bound at the NUTL site represses the expression of its own gene (Wilson *et al.*, 1997).

In a previous paper, we proposed that the structure of the N leader reflects the temporal order of the three regulatory processes acting through the N leader (Wilson *et al.*, 1997). Once NUTL is transcribed, the antitermination complex is assembled. Upon completion of the RNase III-sensitive hairpin, this site in the N leader can be cleaved by RNase III. Once the N ribosome-binding site is transcribed, N bound at the NUTL site can cause translation repression of N gene expression. Cleavage by RNase III eliminates the RNA connection between NUTL at the 5′ end of the transcript and the transcribing antitermination complex, as well as between NUTL and the N ribosome-binding site and structural gene. RNase III cleavage is expected to occur after assembly of the antitermination complex but prior to N-mediated translation repression. In this study, we investigate the relationship between RNase III cleavage of the N leader and the two N-mediated functions, antitermination and, in greater detail, autoregulation. Our observations suggest that N expression is controlled by an appealing mechanism in which one regulatory pathway counteracts another in a highly temporally sensitive manner.

Results

Studying the effect of RNase III on N-mediated antitermination and translation repression using a double reporter system

To study antitermination and the regulation of N gene expression, we have constructed a gene fusion on the *E.coli* chromosome that permits simultaneous measurement of N-mediated translation repression and transcription antitermination (Figure 2) (Wilson *et al.*, 1997). This p_L-*nutL-N-lacZ-galK* fusion contains the p_L promoter, the N leader, the first 33 codons of the N structural gene fused to the ninth codon of *lacZ* and, far downstream, the *gal* operon which includes the *galK* gene. The effect of N and RNase III (the latter encoded by the *E.coli rnc* gene) on the expression of the N gene can be studied by monitoring

β-galactosidase (β-gal) expression from *N-lacZ*. There are multiple transcription terminators between p_L and *galK* which virtually eliminate *galK* expression unless an antitermination complex forms at p_L. Therefore, N-mediated antitermination can be studied by measuring levels of galactokinase, the *galK* gene product. In our constructs, the p_L promoter is under the control of the temperature-sensitive repressor, CI857.
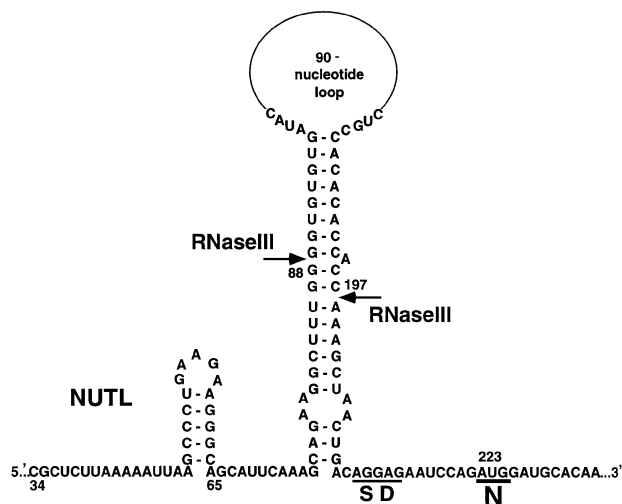

Fig. 1. Structure of the *N* leader. Nucleotides are numbered from +1 of the p_L transcript. Shown are NUTL, the RNase III-sensitive hairpin with cleavage sites (arrows) and the Shine–Dalgarno sequence (SD) and initiation codon of the *N* ribosome-binding site.
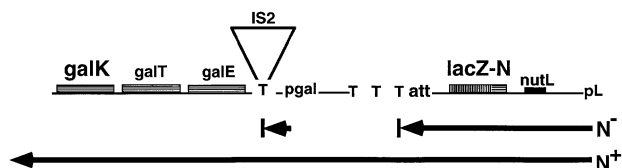

Fig. 2. The p_L-*nutL-N-lacZ-galK* double reporter fusion. The expression of the *gal* operon is under the control of p_L and is blocked under N⁻ conditions by transcription terminators (T) including one in an IS2 element in the *gal* leader. N-mediated antitermination allows *gal* expression in N⁺ cells. The left attachment site of the λ prophage carrying p_L-*N-lacZ* is designated as *att*.

In all experiments below using fusion-containing strains, cells growing exponentially at 30°C were shifted to 42°C to inactivate CI857 and induce expression from p_L. All data are enzyme activities in samples collected after 60 min of heat induction. In these experiments, *N* without its regulatory sequences was constitutively expressed *in trans* from p_{LAC} on a derivative of the medium copy-number plasmid pGB2 (Wilson *et al.*, 1997).

The data in Table I show a multitude of regulatory effects, three of which have been reported previously. First, N-mediated antitermination through transcription terminators significantly enhances *galK* expression (compare galK units of *N⁻* with *N⁺*, Table IA) (Adhya *et al.*, 1974). Secondly, RNase III cleavage of the inhibitory hairpin immediately adjacent to the *N* ribosome-binding site (Figure 1) stimulates *N-lacZ* translation (compare β-gal units of Table IA with B under *N⁻* conditions) (Kameyama *et al.*, 1991). Thirdly, N almost completely represses the translation of *N-lacZ* in *rnc⁻* cells through an autoregulatory mechanism (compare β-gal units of *N⁻* with *N⁺*, Table IA) (Wilson *et al.*, 1997).

New to this study are the effects of RNase III cleavage of the *N* leader on N-mediated antitermination and translation repression. RNase III did not inhibit antitermination (compare galK units of Table IA and B under *N⁺* conditions); in fact, *galK* expression consistently was enhanced 2-fold in *rnc⁺* cells. However, RNase III did prevent N-mediated translation repression (compare β-gal units of Table IA and B under *N⁺* conditions).

The *rnc70* allele encodes a mutant RNase III that binds but does not cleave its substrate (Inada *et al.*, 1989; Dasgupta *et al.*, 1998). N repressed *N-lacZ* translation in an *rnc70* background (Table IC), indicating that binding by RNase III is not sufficient to prevent N autoregulation. To determine whether this interference with N autoregulation is caused by RNase III cleaving the *N* leader or some other RNase III-sensitive site, we analyzed N-mediated translation repression using a construct with the RNase III-sensitive hairpin deleted in the *N* leader (Wilson *et al.*, 1997). This deletion precisely deletes the entire RNase III-sensitive stem–loop structure shown in Figure 1 from +76 through +208. In these deletion strains, N repressed *N-lacZ* expression in both *rnc⁻* and *rnc⁺* cells, demonstrating that it is specifically cleavage of the *N* leader that prevents N-mediated translation repression (Table ID and E).

Table I. The effect of RNase III on N-mediated antitermination and translation repression

	rnc allele	β-galactosidase (β-gal) units		galactokinase (galK) units	
		N⁻	N⁺	N⁻	N⁺
(A) p_L-*nutL-RIIIˢhp⁺-N-lacZ-galK*	*rnc⁻* (*rnc14*)	838	<10	<1	159
(B) p_L-*nutL-RIIIˢhp⁺-N-lacZ-galK*	*rnc⁺*	2080	3945	<1	310
(C) p_L-*nutL-RIIIˢhp⁺-N-lacZ-galK*	*rnc70*	908	<10	ND	ND
(D) p_L-*nutL-RIIIˢhpΔ-N-lacZ-galK*	*rnc⁻* (*rnc14*)	3906	141	ND	ND
(E) p_L-*nutL-RIIIˢhpΔ-N-lacZ-galK*	*rnc⁺*	4357	20	ND	ND
(F) p_L-*nutL-RIIIˢhp⁺-N-lacZ-galK*	*rnc40* (1×)	1053	24	ND	ND
(G) p_L-*nutL-RIIIˢhp⁺-N-lacZ-galK*	*rnc40* (2×)	ND	70	ND	ND
(H) p_L-*nutL-RIIIˢhp⁺-N-lacZ-galK*	*rnc40* (≥3×)	ND	154	ND	ND

Shown are enzyme activities in exponentially growing cells after 60 min of heat induction with zero time values subtracted. Data are an average of at least two experiments. Variability between averaged values for β-galactosidase assays was <10% and for galactokinase assays was <29%. N⁻ and N⁺ cells carry pGB2 or pZH124 (*plac-N⁺*), respectively. *RIIIˢhp* refers to the RNase III-sensitive hairpin. ND indicates values that were not determined.
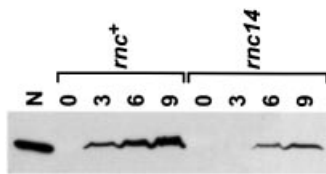
Fig. 3. Western blot analysis of *N* expression in *rnc*⁺ and *rnc14* (*rnc*⁻) p_L-N^+ strains. Numbers indicate time in minutes of sample collection following heat induction of p_L-*N* expression. The first lane contains 20 ng of pure N protein.

Surprisingly, when the RNase III-sensitive hairpin was deleted, N-mediated translation repression was more effective in *rnc*⁺ than *rnc*⁻ cells (repressing to 20 versus 141 U, respectively). This difference may be a consequence of RNase III affecting translation through its role in rRNA processing (Dunn and Studier, 1973; Nikolaev *et al*., 1973) or influencing the expression of some other component of the N autoregulatory complex.

A weak transcription terminator in the N leader
We attribute the 2-fold increase in *N-lacZ* expression caused by deletion of the RNase III-sensitive hairpin (compare β-gal units of Table IB with D and E under *N*⁻ conditions) to the deletion of a transcription terminator in the *N* leader (Adhya *et al*., 1977). The activating effect of antitermination through this terminator can be observed when RNase III is present and preventing translation repression (compare β-gal units of Table IB under *N*⁻ and *N*⁺ conditions). The precise location of the terminator within the RNase III-sensitive stem–loop structure has not been determined.

The effect of RNase III on p_L-N^+ expression
In the experiments presented in Table I, we studied the effect of RNase III on N-mediated translation repression using an *N-lacZ* fusion as the reporter and N protein constitutively expressed *in trans* from a multicopy plasmid as the repressing molecule. In the normal context of the prophage, N expressed from p_L should repress its own expression in *rnc*⁻ cells. Using immunoblotting, we investigated the effect of RNase III on N protein being expressed and regulated in this more natural context from an N^+ prophage containing the entire p_L operon. In these strains, as in our *N-lacZ* fusion strains, all of the genes expressed from p_R, as well as the right attachment site, have been deleted. Cultures growing exponentially at 30°C were shifted to 42°C to induce expression from p_L. We observed a delay in detectable levels of N expressed in an *rnc*⁻ (*rnc14*) strain relative to an *rnc*⁺ strain (Figure 3). This delay can be a consequence of one or both of the roles of the intact RNase III-sensitive hairpin on *N* translation: either as a structural component of the N autoregulatory complex or as a steric inhibitor of ribosome entry in an N-independent manner. The *nutL44* mutation eliminates N binding to NUT and, consequently, N-mediated translation repression (Salstrom and Szybalski, 1978; Wilson *et al*., 1997), but should have no effect on the N-independent inhibition by the RNase III-sensitive hairpin. The 2.5- to 3-fold difference between N levels at 9 min after induction in *rnc*⁺ versus *rnc*⁻ *nutL*⁺ prophage-containing strains was reduced at least 50% by

the *nutL44* mutation (data not shown). Thus, at least some of the RNase III-mediated effect on N expression is due to N autoregulation.

The effect of intermediate levels of RNase III on N-mediated translation repression
N binding to NUT, RNase III cleavage of the *N* leader and translation of *N* are tightly coupled events. It takes a minimum of 4 s to transcribe from the end of the *nut* site, through the RNase III-sensitive site to the *N* ribosome-binding site (Figure 1) (Gotta *et al*., 1991). The distance between the end of NUT and the end of the RNase III-sensitive hairpin allows sufficient time for the antitermination complex to form prior to cleavage (Barik *et al*., 1987). However, the end of the RNase III-sensitive hairpin and the *N* ribosome-binding site are so close that we envisage that RNase III must process quickly to prevent N-mediated translation repression of *N* gene expression.

In a preliminary experiment to determine how levels of RNase III affect N-mediated translation repression, we constructed a derivative of the p_L-*N-lacZ* strain with the *rnc40* allele. The *rnc40* mutation is a mini-Tn*10* insertion in the leader region of the *rnc* operon (Takiff *et al*., 1989). This mutation blocks the normal *rnc* promoter and RNase III-mediated regulation of the operon. In this mutant, the *rnc* operon is expressed from a tetracycline-dependent promoter within the mini-Tn*10*. The level of transcription from this tetracycline-dependent promoter is predicted, based on *lacZ* operon fusion data, to be 10% of that from the *rnc* promoter (Takiff *et al*., 1992). This level of transcription is sufficient to supply enough of the essential protein Era, which is expressed as part of the *rnc* operon, for cell viability and to permit a level of 30S rRNA processing by RNase III that is intermediate to that seen in *rnc*⁺ and *rnc*⁻ cells (data not shown). Much to our surprise, N-mediated translation repression at the level of RNase III activity expressed from this allele was almost as strong as that in *rnc*⁻ cells (Table IF). We constructed additional derivatives of the p_L-*N-lacZ* strain with two and at least three copies of the *rnc40* allele (see Materials and methods). Although our western blotting technique was not sensitive enough to measure RNase III levels in these strains directly (Figure 5), we expected the amount of RNase III to increase incrementally as the copy number of the *rnc40* allele increased. The level of N-mediated translation repression in these strains remained strong at these presumably higher levels of RNase III activity (Table IG and H). These results suggest that below a certain unexpectedly high threshold, RNase III does not prevent N autoregulation efficiently. The intracellular levels of RNase III in this experiment were manipulated artificially and could not be measured precisely, but our results encouraged us to search for physiological conditions in which the level of RNase III activity was sufficiently reduced for N-mediated translation repression to operate in *rnc*⁺ cells.

The effect of growth medium on N-mediated translation repression
rRNA precursor is an important substrate for RNase III (Dunn and Studier, 1973; Nikolaev *et al*., 1973), and the synthesis of rRNA decreases significantly with decreasing growth rate (Bremer and Dennis, 1996). In a previous
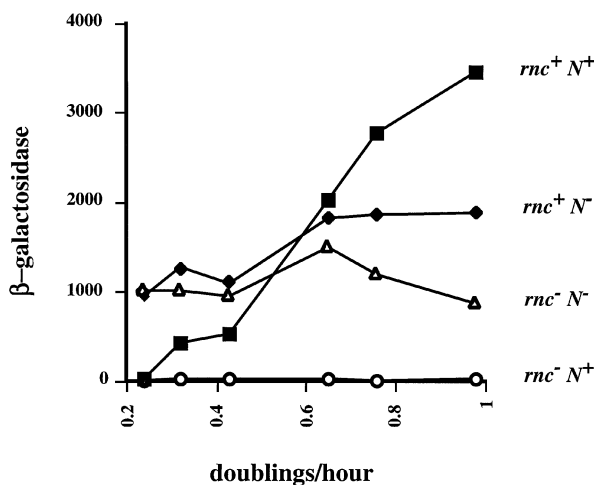
Fig. 4. The effect of growth rate on N-mediated translation repression. Media from right to left are LB, M63 glucose plus casamino acids, M63 glycerol plus casamino acids, M63 glucose, M63 glycerol, and M63 succinate plus casamino acids. Shown are β-galactosidase activities in samples collected after 60 min of heat induction with the zero time values subtracted. To simplify comparison of the β-galactosidase activity of each strain in each medium, all data are plotted relative to *rnc+* doubling times. The *rnc−* strains grow between 21 and 8% more slowly than *rnc+* strains depending on the medium. *N+* cells carry pZH124 and *N−* cells carry pGB2. Data shown are an average of five experiments with <23% variability between averaged values.

study, we observed that the expression of *rnc-lacZ* protein fusions decreased as cells grow more slowly, suggesting growth rate regulation of *rnc* translation (Britton *et al.*, 1998). The N autoregulation experiments reported up to this point were done using cells grown in rich LB medium in which RNase III activity is high. Therefore, we next tested whether N autoregulation occurs in cells growing slowly in poor media where RNase III activity should be reduced.

The growth rate of the p_L-*nutL-N-lacZ-galK* fusion-containing strain was manipulated over a 4-fold range by growing cells in liquid minimal media supplemented with increasingly poor carbon sources (Figure 4). The control experiments show first that, when cells lacked functional genes for both RNase III and N (*rnc− N−*, Figure 4), *N-lacZ* expression was only slightly affected by growth medium and, secondly, that when these *rnc−* cells expressed *N*, N-mediated translation repression was complete in all media (*rnc− N+*, Figure 4). As was already shown in Table I, *N-lacZ* expression was high in *rnc+ N+* cells growing rapidly in LB medium because there was no N-mediated translation repression (Figure 4). However, N-mediated repression increased significantly with decreasing growth rate, suggesting a reduction of RNase III activity as the cells grew more slowly (*rnc+ N+*, Figure 4). At a doubling time of ~240 min, N-mediated translation repression of *N* gene expression was virtually complete in *rnc+* cells.

The repressive effect of poor growth medium on *N-lacZ* expression in *rnc+ N−* cells also suggests a decrease in RNase III activity (Figure 4). This 2-fold decrease of *N-lacZ* expression in the absence of N in the slowest growing cells relative to the fastest growing cells tested is consistent with a loss of RNase III activation of *N-lacZ* translation through cleavage of the inhibitory hairpin

Fig. 5. Western blot analysis of *rnc* expression. A 20 ng aliquot of pure RNase III is run relative to 20 μl of *rnc+*, *rnc40* and *rnc14* cell lysates grown on LB and 10 μl of *rnc70* cell lysates grown on the media indicated.

(Kameyama *et al.*, 1991). In fact, in the poorest growth medium, the level of *N-lacZ* expression in *rnc+ N−* cells equaled that in *rnc− N−* cells, as if the former are *rnc−* in terms of *N* gene expression**.**

The effect of growth medium on RNase III levels

We used immunoblotting to monitor directly RNase III protein in LB and minimal medium supplemented with casamino acids plus either glycerol or succinate. In these media, we observed essentially no, intermediate or complete N-mediated translation repression of *N-lacZ* expression in *rnc+* cells, respectively (Figure 4). The sensitivity of our immunodetection assay allowed us to detect RNase III in *rnc+* strains grown on LB (Figure 5) but not those grown on the minimal media tested (data not shown). While this result is consistent with growth rate regulation of *rnc* expression, we preferred to detect RNase III protein in all media tested. To increase *rnc* expression overall and thus the sensitivity of our assay, we used a strain carrying the *rnc70* allele. The synthesis of RNase III protein is higher in this background because the mutant protein cannot cleave the *rnc* leader RNA and inhibit *rnc* expression (Dasgupta *et al.*, 1998). The *rnc70* allele increases *rnc* expression 5- to 6-fold or 5- to 12-fold, as determined using *rnc-lacZ* fusions (Dasgupta *et al.*, 1998) or immunoblotting (Figure 5), respectively.

We retested the effect of growth rate on *rnc* expression using the *rnc70* strain (Figure 5). The level of RNase III protein was reduced 4- to 7-fold in cells grown on minimal glycerol casamino acids and 7- to 14-fold in cells grown on minimal succinate casamino acids relative to those grown on LB. These data corroborate the conclusions from experiments using *rnc-lacZ* fusions that *rnc* expression is growth rate regulated. More important to this study, we observe that as RNase III levels decreased, N autoregulation of *N-lacZ* expression increased in *rnc+* cells, consistent with RNase III blocking N-mediated translation repression in a growth rate-dependent manner. The *rnc+* cells grown on minimal succinate casamino acids or *rnc40* strains grown on LB appear to have similar levels of RNase III (~10% of the level in *rnc+* cells grown on LB) and virtually complete repression of *N-lacZ* expression by N.

Discussion

The RNA leader of the *N* gene contains sites for assembly of an antitermination complex, RNase III cleavage and
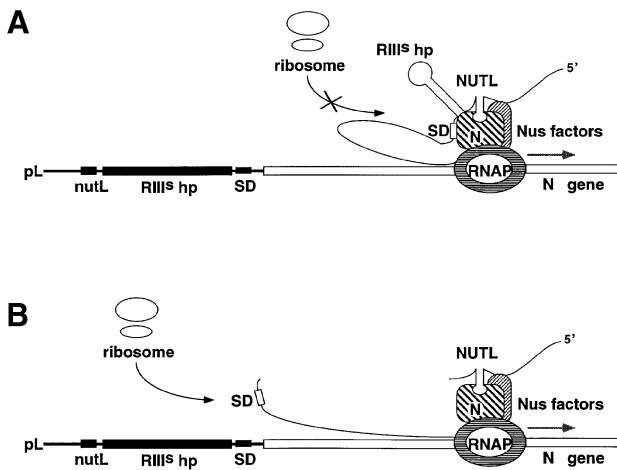
A

B

Fig. 6. Model of the antitermination/autoregulation complex transcribing the *N* gene before (**A**) or after (**B**) cleavage at the RNase III-sensitive hairpin (RIII^{s}hp). The model suggests that the contact inhibitory to translation is between the *N* Shine–Dalgarno sequence (SD) and N protein in the complex, but is not meant to depict precisely the nature of this contact.

autoregulatory repression of *N* gene translation. The order in which these regulatory sites in the *N* leader are transcribed appears to determine the sequence in which the mechanisms acting at those sites can operate (Figure 1). RNase III cleavage of a hairpin downstream of NUTL does not interfere with antitermination. These results suggest that the antitermination complex assembles quickly on RNA polymerase after transcription of the *nutL* site (Barik *et al.*, 1987) and that the RNase III-induced cleavage does not disrupt this complex once assembled (compare Figure 6A and B). Because it has been demonstrated that NUT sites do not act *in trans* to promote antitermination (Devito and Das, 1992), it is unlikely that assembly occurs after RNase III cleaves to release NUT RNA from the transcript.

In contrast, RNase III cleavage of this hairpin, lying immediately upstream of the *N* ribosome-binding site, interferes with the ability of N bound at NUTL to repress *N* gene translation. This has been shown using both p_L-*N*-*lacZ* fusions with N protein constitutively expressed *in trans* (Table I) and a p_L-N^+ prophage with the N expressed in this context regulating its own expression (Figure 3). In our working model of N autoregulation, translation repression of *N* gene expression occurs within the antitermination complex (Figure 6A; D.Yu, H.Wilson, J.G.Zhou and D.Court, manuscript in preparation). We propose that RNase III cleavage enhances *N* translation by preventing incorporation of the *N* transcript into this inhibitory complex (compare Figure 6A and B). This transcript remains unoccluded and is free to be translated. If the transcript is uncleaved either because of a mutant enzyme encoded by *rnc14* or *rnc70*, for example, or a mutant substrate as in the *N-lacZ* fusion strain with the RNase III-sensitive hairpin deleted, the *N* ribosome-binding site is incorporated into the antitermination/autoregulation complex and repression is complete. Our work leads us to envisage an intriguing mechanism in which at least one function of a protein/RNA machine is regulated by RNase cleavage.

The RNase III-sensitive hairpin has multiple roles in the *N* leader. First, since N-mediated translation repression acts only on a ribosome-binding site that is positioned at the base of the RNase III-sensitive hairpin, this structure apparently holds the *N* gene in the proper position for repression by N bound at NUTL (Wilson *et al.*, 1997). Secondly, even in the absence of N protein and the antitermination complex, the stable hairpin structure inhibits translation of *N* 2-fold (Kameyama *et al.*, 1991). Lastly, cleavage of this hairpin eliminates both of these repressive effects, resulting in a >400-fold regulation of *N* gene expression by RNase III under N^+ conditions. The modest 2-fold effect of N-independent RNase III activation of *N* gene translation is presumed to be most important very early in infection when N has not yet been synthesized. As N levels increase, RNase III can exert a stronger effect on *N* translation by preventing N autoregulation (compare β-gal units of Table IA and B under N^- with N^+ conditions) and allowing an optimal steady-state level of N. While N-mediated translation repression amplifies the effect of RNase III on *N* gene expression, we propose that the critical regulatory element in N autoregulation is not N itself, but RNase III.

The seemingly unfortunate observation that in rich medium there is no N-mediated translation repression of *N* gene expression in rnc^+ cells gave us the opportunity to observe how growth rate-associated changes in RNase III levels affect *N* gene expression. The rapid progression of events during phage development makes the timing of regulatory steps critical. Because of the proximity of the RNase III-sensitive hairpin to the *N* ribosome-binding site (Figure 1), the opportunity to cleave and activate only immediately precedes the opportunity for N bound at NUTL to repress *N* gene translation. The consequence is a system that is exquisitely sensitive to the level of RNase III. Our results with the *rnc40* allele suggest that a threshold level of activity must be reached before RNase III cleavage of the *N* leader is competitive with the N repression complex (Table I). Figure 4 shows that we have identified growth conditions in which *N* expression is autoregulated presumably because intracellular RNase III levels are not high enough to block formation of the autoregulation complex completely. Therefore, the level of RNase III activity can serve as an important sensor of intracellular conditions for λ and, through its effect on the expression of the λ antitermination protein N, can influence the expression of virtually all other λ genes. Thus, this study reinforces the view that RNase III can act as an important global regulator in *E.coli* (Gitelman and Apirion, 1980; Britton *et al.*, 1998).

The relationship between the level of RNase III activity and the extent of N autoregulation may be complex and not simply a matter of total intracellular RNase III activity. The N-mediated translation repression complex, once formed, may protect the hairpin from RNase III cleavage; alternatively, cleavage subsequent to the formation of the complex may not free the transcript to be translated. More importantly, host- and phage-specified RNase III-sensitive sites in *E.coli* have a hierarchy of sensitivity, and presumably are competing with the *N* leader for a limited amount of RNase III. The most important competitor is probably the rRNA precursor, the synthesis of which is changing with growth rate, as is the synthesis of RNase III

and the level of N-mediated translation repression. The ribosome itself should also be considered as a possible effector of our growth rate-dependent translation repression mechanism (Comer *et al.*, 1996). Ribosome concentration, which changes coordinately with growth rate, may influence how effectively the ribosome competes with the N repression complex for the cleaved transcript.

Upon infection, λ may replicate either lytically, producing many phage particles and destroying the host, or lysogenically, with the phage DNA passively maintained in the host chromosome. The typical λ plaque contains phage in both modes of development. The plaque itself is, of course, a consequence of the lytic killing of cells and spread of progeny phage. The plaque appears turbid because it contains growing lysogenic cells which are immune to further lytic infection.

RNase III regulates the expression of a number of λ functions that are all competing to determine the final developmental outcome. The gene products of *cII*, *cIII* and *int* are important for the establishment of lysogeny. RNase III cleaves a sense–antisense hybrid of the *cII* message, thus promoting degradation of the *cII* transcript (Krinke and Wulff, 1987, 1990). Binding by RNase III is proposed to favor a secondary structure in the *cIII* transcript that enhances the translation of this gene (Altuvia and Oppenheim, 1986; Altuvia *et al.*, 1987, 1989). In addition, depending on the structure at the 3′ end of the *int* transcript, RNase III either cleaves to promote degradation or may bind without cleaving to stabilize this message (Gottesman *et al.*, 1982; Court, 1993). Interestingly, RNase III cleavage at a site downstream of *N* in the N-antiterminated p_L transcript destabilizes the *N* region of this transcript (Lozeron *et al.*, 1976, 1977; Wilder and Lozeron, 1979; Anevski and Lozeron, 1981). This could represent another mechanism whereby N and RNase III regulate *N* expression, but clearly not the dominant mechanism since N levels are higher in rnc^+ than rnc^- cells (Figure 3). Finally, RNase III cleaves the transcript of lytic late genes necessary for virus assembly (Daniels *et al.*, 1988).

Does RNase III also influence the lysis/lysogeny decision through its effect on N autoregulation? λ forms clear plaques on cells constitutively overexpressing N from a plasmid, demonstrating that inappropriate *N* expression can affect this choice. λ also makes clear plaques on rnc^- cells in which N levels are reduced by autoregulation (Figure 3). Suppressors of this clear plaque phenotype on rnc^- strains increase *cIII* translation by stabilizing the hairpin structure in the *cIII* leader that RNase III binding putatively favors (Altuvia and Oppenheim, 1986; Altuvia *et al.*, 1987, 1989). Therefore, λ lytic growth presumably is favored on rnc^- cells because the loss of RNase III action on the *cIII* transcript is overriding all the other effects including that of N autoregulation. All the RNase III-sensitive sites important in λ development are also competing for RNase III in poor growth medium, which is a physiologically different condition from that which exists in an rnc^- cell. Low intracellular levels of RNase III will have different biological effects from those of no RNase III. For example, the efficiency of lysogeny has been shown to be greater in poor relative to rich medium at low phage-to-cell ratios (<1 phage per cell) (Kourilsky and Knapp, 1974). This is in contrast to the decreased efficiency of lysogeny seen on rnc^- cells relative to rnc^+ cells. To understand the role of N autoregulation in λ development, we need to separate it from all this complexity. Therefore, we currently are designing a mutant phage that will allow us to study N-mediated translation repression independently of the pleiotropic RNase III- and growth rate-mediated effects.

Materials and methods

Bacterial strains and plasmids

The construction of the p_L-*nutL-N-lacZ-galK* double reporter strains is described in a previous study (Wilson *et al.*, 1997). To construct the p_L-N^+ strains used in Figure 3, a cI857 λ lysogen of WJW349 [W3110 Δ(*argF-lacZ*)U169 *gal*490*(IS2) *pgl*Δ8] was made Δ(*cro-bioA*)<>Tet^R by homologous recombination between a PCR product and the chromosome (Yu *et al.*, 2000). This strain was made rnc^- by transducing to chloramphenicol^R (at 10 μg/ml) using P1 phage grown on an rnc<>*cat* derivative of DY330 [W3110 Δ(*argF-lacZ*)U169 *gal*490*(IS2) *pgl*Δ8 λ cI857 Δ(*cro-bioA*)] (this laboratory). Other mutant rnc strains were made by transducing to tetracycline resistance (at 12.5 μg/ml) using P1 phage grown on HT115 (W3110 $rnc14$::ΔTn*10*), HT120 (W3110 $rnc40$::ΔTn*10*) (Takiff *et al.*, 1989) or SDF217 (C600 $rnc70$ TD1-17::ΔTn*10*) (this laboratory; Dasgupta *et al.*, 1998) and confirming that λ forms clear plaques on the chosen isolate. TD1-17::ΔTn*10* alone has no effect on expression of the fusion (data not shown).

A λ phage carrying the $rnc40$ allele as part of the rnc operon (TD1 λ*imm*^{21} $rnc40$::ΔTn*10*) (Takiff *et al.*, 1989) was used to lysogenize the *N-lacZ* fusion strain already containing $rnc40$ at the natural position in the *E.coli* chromosome. This *att*Δ phage integrates by homologous recombination into the rnc operon. Strains with one or more copies of the $rnc40$ phage were distinguished by the 'ter' test (Mousset and Thomas, 1968). The strain designated as having one copy of $rnc40$ in Table I contains the $rnc40$ allele brought in by P1 transduction. The strain designated as having two copies of $rnc40$ contains the original $rnc40$ allele plus one copy of the $rnc40$ phage. The strain designated as having three or more copies of $rnc40$ contains the original $rnc40$ allele plus at least two copies of the $rnc40$ phage as determined by the ter test. The copy number of the $rnc40$ phage in this last strain is predicted based on an incremental increase in β-galactosidase activity above that of the strain carrying two copies of the $rnc40$ allele. For comparison, multiple lysogens of the $rnc40$ phage expressing even higher levels of β-galactosidase activity were isolated and are hypothesized to carry three and more copies of the phage (data not shown).

Medium copy-number plasmids pGB2 and pZH124 have been described previously (Churchward *et al.*, 1984; Wilson *et al.*, 1997).

Enzyme assays

The cells used for enzyme assays reported in Table I were grown as described previously (Wilson *et al.*, 1997) except that the tetracycline-dependent $rnc40$ strains were grown in LB medium supplemented with tetracycline at 12.5 μg/ml. Bacteria for β-galactosidase assays shown in Figure 4 were grown as reported previously (Wilson *et al.*, 1997) except overnight cultures were diluted in growth curve media to a starting OD_{600} of 0.004 and the overnight medium for the five minimal growth curve media was M63 plus 1 μg/ml thiamine, 1 μg/ml biotin, 0.4% glucose, 0.05% casamino acids and 100 μg/ml spectinomycin. Prior to dilution into the minimal growth curve medium, the cells were collected by centrifugation, washed in 5 ml of M63 and resuspended in M63 to an approximate OD_{600} of 2.0. In addition to LB plus 100 μg/ml spectinomycin, the growth curve media in this experiment were M63 plus 1 μg/ml thiamine, 1 μg/ml biotin, 100 μg/ml spectinomycin and either 0.4% glucose and 0.05% casamino acids, 0.4% glycerol and 0.05% casamino acids, 0.4% glucose, 0.4% glycerol, or 0.4% succinate and 0.05% casamino acids.

Prior to harvesting cells for enzyme assays, cultures growing exponentially at 30°C were shifted to 42°C for 60 min to induce expression of p_L. β-galactosidase and galactokinase assays were performed as described previously (Miller, 1972; McKenney *et al.*, 1981).

Western blot analysis

To immunoblot for N, overnight cultures were grown at 30°C, diluted 1:400 in 50 ml of fresh LB broth and grown with shaking at 30°C to an OD_{600} of 0.6. A 2 ml aliquot of culture was taken as the zero time sample.

A 10 ml aliquot of the remaining culture was dispensed to each of three 50 ml flasks. These cultures were shifted to 42°C with aeration (200 r.p.m.), and one flask per culture was chilled in an ice–water bath at the indicated times after initiating heat induction. Samples were kept on ice until all time points were collected. Cells in 2 ml of each culture were harvested by centrifugation (10 min at 18 000 *g*, 4°C), resuspended in 50 µl of treatment buffer (0.0625 M Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.05% Bromphenol Blue) and heated at 100°C for 4 min. To eliminate viscosity, samples were disrupted with a sonic oscillator tip (Branson Sonifier cell disrupter 200, 10 pulses at output setting of 1.5) and cellular debris was removed by centrifugation (10 min at 18 000 *g*, 4°C). Samples (0.24 OD_{600} units) were separated on an 18% NOVEX pre-cast Tris-glycine gel and proteins were transferred to a nitrocellulose membrane essentially according to the manufacturer's protocols (Invitrogen, Carlsbad, CA). N protein was detected using affinity-purified anti-N polyclonal primary antibody from rabbit and then goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Promega, Madison, WI) essentially as described (Feather-Henigan *et al*., 2000) using 0.2% Tween-20 and 5% non-fat dry milk in the Tris-buffered saline blocking buffer. The N-antibody complex was visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) essentially according to the manufacturer's instructions.

Cell lysate preparation and immunoblotting for RNase III were performed essentially as described for N except cells were grown as described for Figure 4 without the 42°C heat induction, 6 ml of each culture were harvested at OD_{600} = 0.2, samples were separated on a 12% NOVEX Tris-glycine gel, transferred to a PVDF membrane and protein was detected using anti-RNase III monoclonal antibody from mouse and anti-mouse HRP-conjugated antibody from goat.

Two approaches were taken to verify that samples within an experiment contained roughly equivalent amounts of total protein. First, blots were stained for total protein using GelCode Blue Stain Reagent (Pierce) according to the manufacturer's instructions. Secondly, *rnc70* strains were grown on the media shown in Figure 5 and prepared as described for immunoblotting except that a modified treatment buffer lacking 2-mercaptoethanol and Bromphenol Blue was used. The protein concentration in these samples was determined using the MicroBCA Protein Assay (Pierce). The results were consistent with those reported in Bremer and Dennis (1996). The total protein per OD is 1.4-fold greater in cultures grown on minimal glycerol plus casamino acids and 1.2-fold greater in cultures grown on minimal succinate plus casamino acids than in cultures grown on LB.

Fold differences between N or RNase III levels detected using immunoblotting were approximated by scanning the developed membranes with a Typhon 8600 Variable Mode Imager and analyzing the resulting data using ImageQuant (Molecular Dynamics, Piscataway, NJ).

Acknowledgements

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