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

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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, pL and pR. Formation of this complex requires the phage-encoded protein N, the first gene product expressed from the pL 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, pL and pR is interrupted by multiple transcription terminators. The expression of the early phage function N from pL facilitates the assembly of terminator-resistant transcription complexes. N and Escherichia coli Nus factors interact at a site in the pL and pR transcripts called NUT to convert RNA polymerase to this antiterminating form.

The NUT site of the pL 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) (Lozzeron et al., 1976, 1977; Steeg 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 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 counters 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 pL-nutl-N-lacZ-galK fusion contains the pL 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.

![Diagram](image1)

**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.

![Diagram](image2)

**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 \( p_l \) 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 \( mc^- \) 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 \( mc^- \) cells. However, RNase III did prevent N-mediated translation repression (compare β-gal units of Table IA and B under \( N^- \) conditions).

The \( mc70 \) 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 \( mc70 \) 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 \( mc^- \) and \( mc^+ \) cells, demonstrating that it is specifically cleavage of the \( N \) leader that prevents N-mediated translation repression (Table ID and E).

<table>
<thead>
<tr>
<th>Table I.</th>
<th>The effect of RNase III on N-mediated antitermination and translation repression</th>
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<td></td>
<td>( mc ) allele</td>
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<td>( N^- )</td>
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<tr>
<td>(A) ( p_l )-nutl-RIIIhp( ^+ )-N-lacZ-galK ( mc^- )</td>
<td>838</td>
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<td>(B) ( p_l )-nutl-RIIIhp( ^- )-N-lacZ-galK ( mc^+ )</td>
<td>2080</td>
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<td>(C) ( p_l )-nutl-RIIIhp( ^- )-N-lacZ-galK ( mc70 )</td>
<td>908</td>
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<td>(D) ( p_l )-nutl-RIIIhp( ^- )-N-lacZ-galK ( mc^- )</td>
<td>3906</td>
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<tr>
<td>(E) ( p_l )-nutl-RIIIhp( ^- )-N-lacZ-galK ( mc^+ )</td>
<td>4357</td>
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<tr>
<td>(F) ( p_l )-nutl-RIIIhp( ^- )-N-lacZ-galK ( mc40 ) (( ×1 ))</td>
<td>1053</td>
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<tr>
<td>(G) ( p_l )-nutl-RIIIhp( ^+ )-N-lacZ-galK ( mc40 ) (( ×2 ))</td>
<td>ND</td>
</tr>
<tr>
<td>(H) ( p_l )-nutl-RIIIhp( ^+ )-N-lacZ-galK ( mc40 ) (( ×3 ))</td>
<td>ND</td>
</tr>
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</table>

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 <20%. \( N^- \) and \( N^+ \) cells carry pGB2 or pZH124 (lac-\( N^+ \)), respectively. RIIIhp refers to the RNase III-sensitive hairpin. ND indicates values that were not determined.
Surprisingly, when the RNase III-sensitive hairpin was deleted, N-mediated translation repression was more effective in rc<sup>e</sup> than rc<sup>c</sup> 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<sup>e</sup>-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<sup>e</sup> and N<sup>c</sup> 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<sub>L</sub>-N<sup>e</sup> 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<sub>L</sub> should repress its own expression in rc<sup>c</sup> 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<sup>e</sup> prophage containing the entire p<sub>L</sub> operon. In these strains, as in our N-lacZ fusion strains, all of the genes expressed from p<sub>R</sub>, 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<sub>L</sub>. We observed a delay in detectable levels of N expressed in an rc<sup>c</sup> (rc<sup>c</sup>14) strain relative to an rc<sup>e</sup> 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 rc<sup>e</sup> versus rc<sup>c</sup> nutL<sup>e</sup> 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<sub>L</sub>-N-lacZ strain with the rc<sup>40</sup> allele. The rc<sup>40</sup> mutation is a mini-Tn10 insertion in the leader region of the rc operon (Takiff et al., 1989). This mutation blocks the normal rc promoter and RNase III-mediated regulation of the operon. In this mutant, the rc operon is expressed from a tetracycline-dependent promoter within the mini-Tn10. The level of transcription from this tetracycline-dependent promoter is predicted, based on lacZ operon fusion data, to be 10% of that from the rc 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 rc operon, for cell viability and to permit a level of 30S rRNA processing by RNase III that is intermediate to that seen in rc<sup>e</sup> and rc<sup>c</sup> 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 rc<sup>c</sup> cells (Table IF). We constructed additional derivatives of the p<sub>L</sub>-N-lacZ strain with two and at least three copies of the rc<sup>40</sup> 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 rc<sup>40</sup> 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 rc<sup>e</sup> 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
study, we observed that the expression of mc-lacZ protein fusions decreased as cells grow more slowly, suggesting growth rate regulation of mc 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-nat-L-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 (mc- N-; Figure 4), N-lacZ expression was only slightly affected by growth medium and, secondly, that when these mc cells expressed N, N-mediated translation repression was complete in all media (mc- N+, Figure 4). As was already shown in Table I, N-lacZ expression was high in mc- 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 (mc+ N+, Figure 4). At a doubling time of ~240 min, N-mediated translation repression of N gene expression was virtually complete in mc+ cells.

The repressive effect of poor growth medium on N-lacZ expression in mc+ 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 (Kameyama et al., 1991). In fact, in the poorest growth medium, the level of N-lacZ expression in mc- N+ cells equaled that in mc- N- cells, as if the former are mc- 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 mc- cells, respectively (Figure 4). The sensitivity of our immunodetection assay allowed us to detect RNase III in mc+ 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 mc expression, we preferred to detect RNase III protein in all media tested. To increase mc expression overall and thus the sensitivity of our assay, we used a strain carrying the mc70 allele. The synthesis of RNase III protein is higher in this background because the mutant protein cannot cleave the mc leader RNA and inhibit mc expression (Dasgupta et al., 1998). The mc70 allele increases mc expression 5- to 6-fold or 5- to 12-fold, as determined using mc-lacZ fusions (Dasgupta et al., 1998) or immunoblotting (Figure 5), respectively.

We retested the effect of growth rate on mc expression using the mc70 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 mc-lacZ fusions that mc 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 mc+ cells, consistent with RNase III blocking N-mediated translation repression in a growth rate-dependent manner. The mc+ cells grown on minimal succinate casamino acids or mc40 strains grown on LB appear to have similar levels of RNase III (~10% of the level in mc+ 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
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 autorepression. These results suggest that the autorepression 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 autorepression (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 pL-N-lacZ fusions with N protein constitutively expressed in trans (Table I) and a pL-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 autorepression 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 uncoupled 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 autorepression 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 cIII, 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 p1 transcript destabilizes the N region of this transcript (Lozerson et al., 1976, 1977; Wilder and Lozerson, 1979; Anevski and Lozerson, 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 in 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 cII 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 are currently 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 p3-nutL-N-lacZ-galK double reporter strains is described in a previous study (Wilson et al., 1997). To construct the p3-λ strains used in Figure 3, a c857 λ lysogen of W3110ΔargF-lacZU169 gal400* (IS2) pgld8 was made Δcro-bioAΔcycT by homologous recombination between a PCR product and the chromosome (Yu et al., 2000). This strain was made rnc- by transducing to chloramphenicolR (at 10 μg/ml) using P1 phage grown on an rnc-czat derivative of DY330 [W3110 ΔargF-lacZU169 gal400* (IS2) pgld8 λ c857 Δcro-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 rnc40::ΔTn10), HT120 (W3110 rnc40::ΔTn10) (Takiff et al., 1989) or SD217 (C600 rnc70 TDI17::ΔTn10) (this laboratory; Dugupta et al., 1998) and confirming that λ forms clear plaques on the chosen isolate. TDI17::ΔTn10 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 λimmii rnc40::ΔTn10) (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 Δrnc phage integrates by homologous recombination into the rnc operon. Strains with one or more copies of the rnc 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 lysogenizing 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 OD600 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 OD600 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% glucose and 0.05% casamino acids, 0.4% glucose, 0.4% galactose 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 pλ- 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 OD600 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 disruptor 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 RNA 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-RNA 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, RNA_{70} 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 sucinate plus casamino acids than in cultures grown on LB.

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

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References


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