Translational repression by a transcriptional elongation factor

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One of the classical positive regulators of gene expression is bacteriophage λ N protein. N regulates the transcription of early phage genes by participating in the formation of a highly processive, terminator-resistant transcription complex and thereby stimulates the expression of genes lying downstream of transcriptional terminators. Also included in this antiterminating transcription complex are an RNA site (NUT) and host proteins (Nus). Here we demonstrate that N has an additional, hitherto unknown regulatory role, as a repressor of the translation of its own gene. N-dependent repression does not occur when NUT is deleted, demonstrating that N-mediated antitermination and translational repression both require the same *cis*-acting site in the RNA. In addition, we have identified one *nut* and several host mutations that eliminate antitermination and not translational repression, suggesting the independence of these two N-mediated mechanisms. Finally, the position of *nutL* with respect to the gene whose expression is repressed is important.

[*Key Words:* Bacteriophage λ; antitermination; N; RNA-binding proteins; long-distance regulation]

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In an increasing number of prokaryotic and eukaryotic systems, transcriptional elongation is being identified as a step at which gene expression is controlled (Spencer and Groudine 1990; Landick and Turnbough 1992; Das 1993; Krumm et al. 1993; Krumm and Groudine 1995; Shilatifard et al. 1996). The N-antitermination system of bacteriophage λ is a paradigm of regulated transcriptional elongation (Friedman and Gottesman 1983; Das 1992; Friedman and Court 1995). Immediately after infection of *Escherichia coli,* transcription of the phage genome initiates at two divergently transcribed promoters, p_L and p_R (Fig. 1). Phage protein N, the first gene product to be expressed from p_L, binds to sites called NUT in the newly transcribed RNA and, together with host proteins collectively called Nus, modifies the RNA polymerase transcribing the phage genome to a terminator-resistant form. This modification permits expression of genes separated from their promoters by transcriptional terminators in the early operons.

The expression of *N* is regulated at the transcriptional level by the phage repressors CI and Cro binding at operators encompassing the promoter for *N*, p_L (Gussin et al. 1983). Expression of p_L and, consequently, the expression of *N*, is also influenced by temperature (Giladi et al. 1995) and the binding of the DNA-bending, histone-like protein integration host factor (Giladi et al. 1990, 1992). In addition, the level of N is modulated post-translationally by the protease Lon (Gottesman et al. 1981). Translational control of *N* gene expression is exerted through an RNA hairpin within the long *N* leader of 223 nucleotides (Franklin and Bennett 1979; Figs. 1 and 2). RNase III is a positive activator of *N* gene expression and has been postulated to increase expression of *N* by removing this hairpin structure that sterically interferes with translational initiation of the *N* gene (Lozeron et al. 1976, 1977; Steege et al. 1987; Kameyama et al. 1991).

Another sequence in the *N* leader RNA is NUTL (Figs. 1 and 2), the binding site for N in the antitermination complex (Rosenberg et al. 1978; Salstrom and Szybalski 1978; Chattopadhyay et al. 1995; Modridge et al. 1995). There are no transcriptional terminators upstream of *N* and, thus, antitermination is not necessary to express *N*. Therefore, we hypothesized that N acting through NUTL has a second regulatory function that affects the expression of its own gene. In this report we present evidence supporting this hypothesis and demonstrate that N, in addition to its role as a positive regulator of λ gene transcription, is also a negative regulator of translation.

Results

The effect of N on the expression of its own gene

To study the effect of N on the expression of its own

This paper is dedicated to the memory of Wilfred James Wilson.

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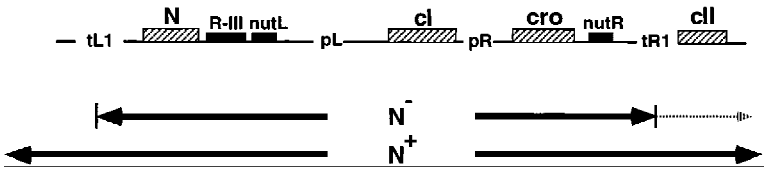

Figure 1. Partial genetic map of bacteriophage λ showing genes (hatched boxes), *cis*-acting *nut* sites and DNA specifying the RNase III-sensitive hairpin (R-III) (■), promoters (p_L and p_R), and transcriptional terminators (tL1 and tR1). Arrows represent transcriptional patterns in the absence and presence of N.

gene we used a p_L–*nutL*–*N*–*lacZ* gene fusion present in single copy on the chromosome as part of a defective λ prophage. In this construction the p_L promoter, *N* leader, and first 33 codons of *N* are fused in frame to the ninth codon of *lacZ* (Fig. 4A, below). These prophages also carry a *cI* gene (*cI857*) encoding a temperature-sensitive repressor of p_L. Other relevant features of the host cells used in this and subsequent experiments are as follows: They have a deletion of the *lac* operon so that the *N*–*lacZ* fusion is the only source of β-galactosidase. In addition, they are Cro$^-$ and RNase III$^-$ so that any effect of N on the expression of its own gene can be observed in the absence of these other regulatory effectors. To provide the N function in *trans*, the *N* gene without its regulatory sequences was constitutively expressed from p_{lac} on a pUC9-derived plasmid.

Expression of the p_L–*nutL*–*N*–*lacZ* gene fusion was induced by shifting cells growing exponentially at 30°C in liquid culture to 42°C to inactivate the CI857 repressor. The β-galactosidase activity of samples collected at various times after temperature induction was determined. Relative to expression in N$^-$ control cells, expression of the fusion was repressed more than 100-fold by N after 60 min of induction (Fig. 3). Therefore, N protein is negatively regulating the expression of its own gene, a form of autoregulation.

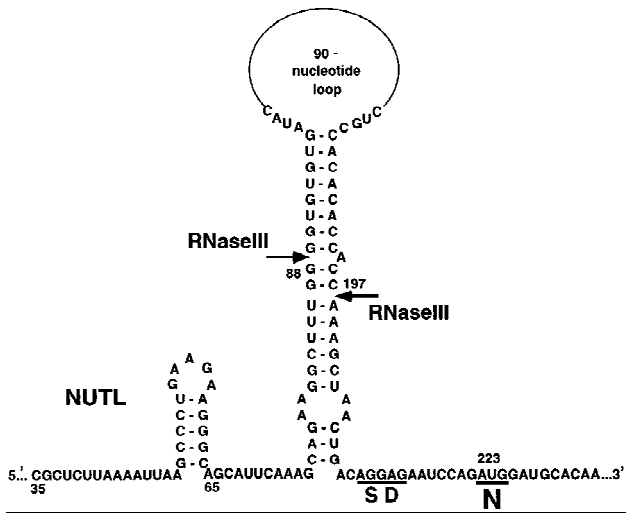

Figure 2. Structure of the *N* leader as predicted by Steege et al. (1987) showing NUTL, the RNase III-sensitive hairpin and cleavage sites, and the *N* Shine–Dalgarno sequence (SD) and initiation codon (N). Nucleotides are numbered from +1 of the p_L transcript.

N-mediated repression of translation

Aware that by antitermination N positively regulates at the level of transcription, we next wanted to know at what level of gene expression this N-mediated repression occurs. To address this question we analyzed the effect of N protein on the expression of a p_L–*nutL*–*N*–*lacZ* operon fusion. This fusion, like the gene fusion, includes p_L, the *N* leader and the first 33 codons of *N* but inserted between the *N* sequence and *lacZ* is a synthetic sequence containing translational stop codons in the *N* reading frame, and the ribosome binding site and 5′ end of the *lacZ* structural gene (Fig. 4B). In this fusion the transcription of the reporter gene *lacZ* is still controlled by the *N* promoter p_L. However, *lacZ* translation is controlled through the *lacZ* ribosome binding site, not the *N* ribosome binding site. Thus, these gene and operon fusions permit us to distinguish between transcriptional and translational effects of N. N reduced *N*–*lacZ* operon fusion expression only 5-fold as compared with the >100-fold effect on the gene fusion expression (Fig. 4A, B). These data support the conclusion that *N*-mediated repression is primarily a translational mechanism directed at the *N* gene because the expression of the operon fusion

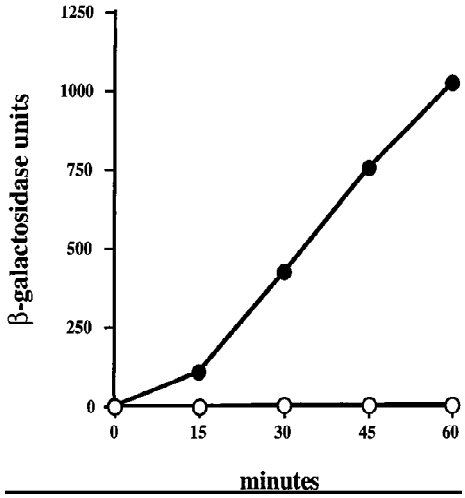

Figure 3. The effect of N on the expression of a p_L–*nutL*–*N*–*lacZ* gene fusion. The fusion was present in single copy in the chromosome as part of a defective λ prophage. This strain carries either pUC9 (●) or pNAS150 ($p_{lac}N^+$, ○). Shown are β-galactosidase activities in samples after indicated times of heat induction with the zero time value subtracted. Data shown are averages of at least two experiments. The variability between averaged values is <19%.

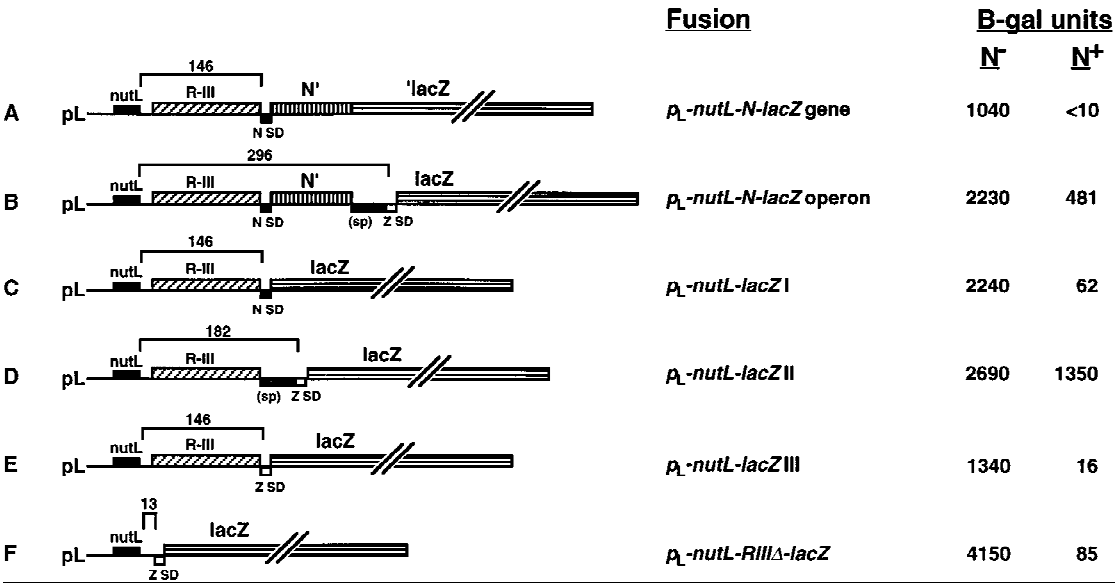

Figure 4. N-mediated repression with different p_L–*nutL*–*lacZ* fusions. Each fusion contains the p_L promoter, the *nutL* site, and DNA sequence specifying the RNase III-sensitive hairpin (R-III) upstream of *lacZ* with different intervening sequence. (N′) The first 33 codons of the *N* structural gene; (sp) A spacer sequence containing translational stop codons. Numbers indicate the number of nucleotides between NUTL and the relevant Shine–Dalgarno sequence (SD) in the RNA. N^- cells carry pUC9 and N^+ cells carry pNAS150 ($p_{lac}N^+$). Shown are β-galactosidase activities in samples after 60 min of heat induction with zero time values subtracted. Data shown are averages of at least three experiments. The variability between averaged values is <21%.

was regulated by N at less than one-twentieth the level of the gene fusion. Consistent with an effect of N on translation is the observation that N is associated with the ribosome (Das and Wolska 1984).

We also used cDNA synthesis followed by the polymerase chain reaction (RT–PCR) to compare the quantity of *N–lacZ* RNA in N^+ and N^- cells carrying the p_L–*nutL*–*N*–*lacZ* gene fusion. Total RNA was isolated from cells grown under the same conditions as for the β-galactosidase assays. Using primers that amplify *lacZ* mRNA we saw no significant difference in the level of this mRNA from N^+ and N^- cells (Fig. 5), an observation once again consistent with translational regulation. On the other hand, in a control experiment for transcriptional effects, *N–lacZ* mRNA was undetectable by our assay in cells expressing Nun, a phage HK022 protein that completely represses p_L–*nutL*–*N*–*lacZ* fusion expression by NUT-dependent transcriptional termination (Gottesman and Weisberg 1995).

A mechanism acting at the post-translational level appears unlikely. N efficiently repressed the expression of p_L–*nutL*–*lacZ* fusions in which the entire *N* structural gene has been replaced with the *lacZ* coding sequence (Fig. 4C,E), excluding the possibility that N inhibits expression by affecting the stability of the N protein itself. Also arguing against a post-translational mechanism is the observation that the N-mediated autoregulatory effect was unaffected by disruption of the *lon* gene (data not shown), which encodes the protease for N.

A double-reporter system for N-mediated antitermination and translational repression

To explore the relationship between N-mediated transcriptional and translational effects, we designed our fusion strains with two reporters that allow us to monitor antitermination and autoregulation from the same transcript. In these strains the bacterial *galK* gene, which lies downstream of p_L–*nutL*–*N*–*lacZ*, is the reporter for antitermination (Fig. 6). Transcriptional terminators between the *gal* operon and p_L, as well as within an IS2 element in the *gal* leader, prevent the expression of the *gal* operon from p_L except when an N-antitermination complex forms (Gottesman et al. 1980; Ward et al. 1983).

Using a $nutL^+$ double-reporter construct, the data showed both N-mediated translational repression and antitermination (Table 1A). The level of *galK*-encoded galactokinase activity under N^+ conditions indicated that nearly all of the p_L-initiated transcripts were extended into the *galK* gene by N-mediated antitermination (Adhya et al. 1977). These data provide additional support for our conclusion that N-mediated repression of *N–lacZ* expression occurs at the post-transcriptional level. Under N^+ conditions the 3′ reporter *galK* is highly expressed whereas the expression of the 5′ reporter *N–lacZ* is completely repressed.

The effect of nut *mutations on N-mediated translational repression*

We next addressed the question of whether the *nut* site,

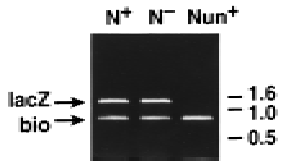

Figure 5. Analysis of *N–lacZ* RNA using RT–PCR. Primers were used to amplify a 1086-bp fragment of *lacZ* and 773 bp fragment of *bioA* on total RNA isolated from the p_L–*nutL–N–lacZ* gene fusion strain carrying pZH124(N^+), pGB2(N^-), or pZH126(Nun^+). Samples are analyzed by electrophoresis on a 1.2% agarose gel. Numbers indicate the length in base pairs (×1000) of DNA markers. The level of *N–lacZ* mRNA in these samples can be normalized to the level of *bio* mRNA, which did not vary between N^- and N^+ cells.

which is essential for antitermination, is essential for *N*-autoregulation, as well. The *nut* site specifies at least two important domains in the RNA (Fig. 7). The first is a sequence called BOXA that is conserved in the NUT regions of lamboid phages λ, 21, and P22, and the leaders of ribosomal RNA operons. Genetic and biochemical experiments suggest that BOXA RNA interacts with a heterodimer of NusB and ribosomal protein S10 (also called NusE) as well as with another unidentified host factor (Friedman et al. 1990; Nodwell and Greenblatt 1993; Patterson et al. 1994). The second domain, BOXB, forms an RNA stem–loop at which N binds (Franklin 1984; Doelling and Franklin 1989; Lazinski et al. 1989; Chattopadhyay et al. 1995; Modridge et al. 1995). Using the p_L–*nutL–N–lacZ* gene fusion double-reporter construct we analyzed *nut* site mutations known to decrease antitermination for their effect on the N-mediated translational repression (Table 1A; Fig. 7, below). Mutations *nutL*Δ, *boxA5*, and *nutL44* eliminated antitermination, as expected (Salstrom and Szybalski 1978; Olson et al. 1984), and virtually eliminated N-autoregulation as well, showing that the *nut* site is necessary for N-mediated translational repression. Therefore, N is binding at NUT to repress translation of *N* ~150 nucleotides downstream (Fig. 2). Although we have not demonstrated this directly, we assume that it is NUT RNA and not *nut* DNA

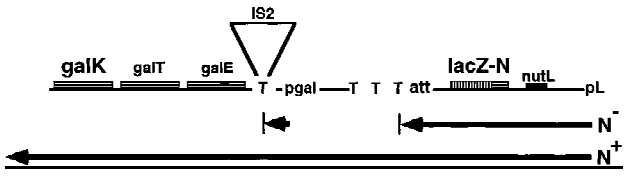

Figure 6. p_L–*nutL–N–lacZ–galK* double-reporter construct in which the expression of the *gal* operon is under the control of the λ p_L promoter. The expression of *gal* under N^- conditions is prevented by transcriptional terminators (T), including one in an IS2 element inserted in the *gal* leader sequence. N-mediated antitermination permits *gal* operon expression under N^+ conditions. The *gal* operon is brought closer to p_L by the deletion *pgl*Δ8, thus maximizing *gal* expression from p_L. The left-hand attachment site of the prophage is represented by *att*.

Table 1. *The effect of* nut *and* nus *mutations on N-mediated translational repression*

	β-galactosidase units		Galactokinase units	
	(N^-)	(N^+)	(N^-)	(N^+)
A.				
nutL$^+$	1020	<10	<2	147
*nutL*Δ	791	887	<2	<2
boxA5	1050	817	<2	<2
nutL44	1090	1120	<2	<2
nutR	1450	17	<2	127
boxA16	1010	<10	<2	<2
B.				
nus$^+$	1020	<10	<1	137
nusA1	687	60	<1	<1
nusB5	556	34	<1	<1
nusD026	808	14	<1	<1
nusE71	887	78	<1	<1

(*A*) N^- and N^+ cells carried pGB2 and pZH124 (p_{lac} N^+), respectively. Shown is a representative experiment. (*B*) N^- and N^+ cells carried pUC9 and pNAS150 (p_{lac} N^+), respectively. Data shown are averages of at least four experiments. The variability between each averaged value is <57% for galactokinase assays and <84% for β-galactosidase assays, except for *nusD026* N^+ (values from four experiments: 0, 0, 0, 54 β-galactosidase units). Both sets of experiments show β-galactosidase and galactokinase activity in samples after 60 min of heat induction with zero time value subtracted.

that is participating in this reaction since the regulatory mechanism involves the translational apparatus.

Using a construction in which the *nutL* sequence is replaced with a synthetic *nutR* sequence (Fig. 7), *N–lacZ* expression was still efficiently repressed by N (Table 1A). Because N protein can use NUTR to repress translation, we suggest that N also has the potential to repress the expression of *cII*, the first gene downstream of *nutR* in the rightward operon (Fig. 1).

The effect of nus *mutations on N-mediated translational repression*

The observation that N-mediated antitermination and translational repression both occur on the same operon transcript and require NUTL raises the possibility that the antitermination complex is necessary for both functions. If this is true, then mutations that disrupt antitermination should disrupt translational repression as well.

A battery of *nus* mutations that affect host functions necessary for antitermination (Friedman and Gottesman 1983) was transferred into the strain carrying the p_L–*nutL–N–lacZ* gene fusion double-reporter construct. Under our assay conditions the expression of *galK* in the *nus* mutants was virtually undetectable even when the cells were N^+, confirming that these mutations disrupt antitermination (Table 1B). In these mutants however, N still inhibited *N–lacZ* expression, albeit at a reduced level, suggesting the independence of the two N-medi-

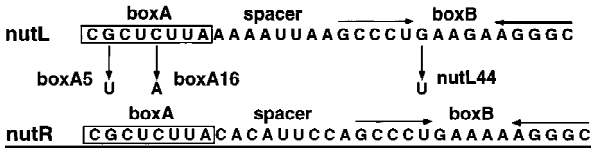

Figure 7. The nucleotide sequence of λ *nut* sites and relevant *nutL* mutations. *nutLΔ* eliminates the sequence shown plus 8 and 6 bp 5′ and 3′, respectively.

ated mechanisms. Because these mutations were isolated only for their antitermination defect, this set of data does not exclude the possibility that Nus factors are involved in N-mediated translational repression, but it is interesting that the *nusE71* mutation, which affects ribosomal protein S10 and the *nusB5* mutation that affects a function implicated in translational elongation (Shiba et al. 1986; Taura et al. 1992) were not distinct in their effects on N-mediated translational repression.

We have also identified one *nut* mutant, *boxA16,* with the same phenotype as these *nus* mutants; that is, antitermination was eliminated completely but translational repression was not (Table 1A, Fig. 7). Because genetic and biochemical evidence supports the conclusion that NusB binds BOXA in the antitermination complex (Friedman et al. 1990; Nodwell and Greenblatt 1993; Patterson et al. 1994), it was expected that both *nusB5* and *boxA16* mutations would block antitermination in our system, as was seen. Yet in both these mutants, translation repression functioned. These data suggest that the NusB–BOXA interaction is unimportant for N-mediated translational repression. However, the *boxA* region must be important for N-mediated repression because the *boxA5* mutation eliminated both N-mediated functions (Table 1A). Therefore, host factors other than NusB may be acting through BOXA during translational repression (Patterson et al. 1994; Friedman and Court 1995).

The importance of position within the N *leader for N-mediated translational repression*

What accounts for the 5-fold repression observed for *lacZ* expression from the p_L–*nutL*–*N*–*lacZ* operon fusion (Fig. 4B)? We found no translational coupling between *N* and *lacZ* translation in the operon fusion (data not shown), and, therefore, an indirect effect of N-mediated repression on *lacZ* expression is discounted. In addition, we do not believe that the 5-fold regulation observed reflects the contribution of transcriptional regulation to the >100-fold N-autoregulation because we did not see even a 2-fold difference in the level of mRNA expressed from either the protein fusion (Fig. 5) or operon fusion (data not shown) under N^- and N^+ conditions. In fact, we hypothesize that the lower level of regulation observed with this operon fusion is N-mediated translational repression acting directly at the *lacZ* ribosome binding site. Therefore the question arises whether the reduced regulation of *lacZ* expression in the operon fusion is a consequence of the absence of critical sequence (e.g., in the *lacZ* ribosome binding site), the distance of *lacZ* from NUT, the absence of critical RNA secondary structure in the vicinity of *lacZ*, or *lacZ* being the second cistron after *nutL*. In the operon fusion, the *lacZ* ribosome binding site is 296 nucleotides from NUTL (Fig. 4B). The expression of a fusion with the *lacZ* ribosome binding site 182 nucleotides from NUTL and the *N* ribosome binding site and structural gene deleted was even less repressed than the expression of the operon fusion (Fig. 4D). However, *lacZ* expression was well regulated by use of a fusion with the *lacZ* ribosome binding site in the same position in the *N* leader as the *N* ribosome binding site (Fig. 4E). It is premature to conclude from these data that any ribosome binding site in the proper position would be subject to this repression because the *N* and *lacZ* Shine–Dalgarno regions differ by only one nucleotide. Sequence upstream of the AUG codon may be important. But clearly the position of the cistron whose expression is being repressed is very important. The critical position is, provocatively, at the base of the RNase III-sensitive hairpin (Fig. 2). Deletion of the RNase III-sensitive hairpin (Figure 4F) increased expression overall, as was expected because the hairpin is inhibitory (Kameyama et al. 1991). However, there was significant N-mediated repression of the expression of this fusion. Therefore, the RNase III-sensitive hairpin itself does not appear to be necessary for N-autoregulation.

Discussion

In this paper we demonstrate that the λ N protein has a second regulatory role. Acting through NUTL, N not only activates expression by promoting transcriptional antitermination but also represses expression by blocking translation.

Models to explain N-mediated translational repression must allow NUT on the same RNA to be used for both N-mediated functions because translational repression is so complete that it must be acting on every transcript including all antiterminated ones (Table 1A, $nutL^+$). This requirement is satisfied by the possibility that antitermination and translational repression occur in the same complex (Fig. 8A). Consistent with this general model are nuclease protection experiments of antiterminating transcription complexes formed in vitro that show NUT remaining part of the antitermination complex throughout transcription (Nodwell and Greenblatt 1991). These nuclease protection experiments imply that antitermination and translational repression must use the same NUT simultaneously.

However, we can reconcile the in vivo and in vitro data with a second possibility. After N-mediated modification of RNA polymerase in the antitermination complex, NUT is released to interact with more molecules of N to cause translational repression in the absence of the transcription complex (Fig. 8B). The in vitro antitermination reactions may lack factors that induce a recycling of NUT. Consistent with this second proposal, we can identify *nus* and *nut* mutations that eliminate one N-

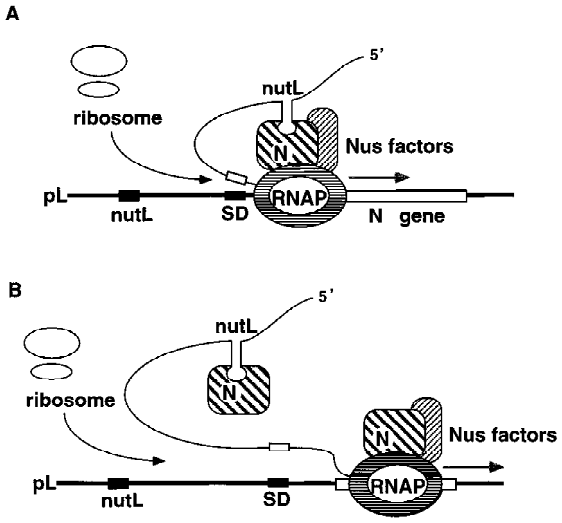

Figure 8. General models for N-mediated translational repression. (*A*) N is shown interacting with the NUTL–Nus factor complex associated with RNA polymerase as it passes the *N* gene. In this model the structure or action of the antitermination complex inhibits translation of *N* at the 5′ end of the p_L transcript. (*B*) N is shown interacting with NUTL to cause translational repression of *N* subsequent to the modification of RNA polymerase and release of NUTL from the antitermination complex.

mediated function (antitermination) but not the other (translational repression). However, we have not excluded the possibility that in these mutants an N-mediated transcription complex forms that is defective for antitermination through to *galK* but is competent to block translation of *N–lacZ* early in the transcript. In fact, in vivo experiments using these *nus* and *nut* mutants and in vitro experiments using transcription reactions lacking one of the Nus proteins demonstrate that ''defective'' antitermination complexes can function over short distances (Whalen et al. 1988; Mason et al. 1992; DeVito and Das 1994; Patterson et al. 1994; Rees et al. 1996).

Finally, taking elements from both general models, it is possible that NUT is a stable component of the antitermination complex and that N and NUT inhibit translation of *N* while remaining part of this complex. However, N and NUT under special (mutant) conditions could function independently of the antitermination complex to cause translational repression. Our discovery causes us to reevaluate the antitermination complex and to reconsider old models in which antitermination in vivo is mediated through an association with the ribosome (Friedman et al. 1981; Ward and Gottesman 1982; Das and Wolska 1984). In addition our data suggest that a remarkable relationship may exist between the transcriptional and translational apparatus on the p_L transcript.

Obviously, the details of N-mediated translational repression are still unclear, but any model to explain this mechanism must be able to account for repression at a distance, and the completeness and magnitude of the effect of N on the translation of its own gene. Several mechanisms of translational control at a distance involve a regulatory protein that stabilizes an ornate RNA pseudoknot encompassing the ribosome binding site (Tang and Draper 1989; Philippe et al. 1990; Chiaruttini et al. 1996). Bearing these systems in mind, the apparent dispensability of the RNase III-sensitive hairpin for N-mediated translational repression (Fig. 4F) leads us to hypothesize that this hairpin folds the *N* leader in such a way as to bring the N-binding site, NUTL, and the 5′ end of the *N* gene close in space (Fig. 2). In this context, we envision that an N-promoted RNA or protein structure either interferes with ribosome binding (Winter et al. 1987; Moine et al. 1990) or holds an initiation-incompetent ribosome complex on the RNA (Philippe et al. 1993; Spedding et al. 1993). We have excluded the possibility that N inhibits translation by inducing cleavage of the *N* transcript within the ribosome binding site in a manner analogous to T4 protein RegB (Ruckman et al. 1989, 1994; data not shown).

There are few examples of regulatory proteins in prokaryotes or eukaryotes that act at both the transcriptional and translational level. In *E. coli* ribosomal protein L4 not only represses the translation of S10 but also causes premature transcriptional termination in the S10 leader through a mechanism dependent on the binding of NusA (Yates and Nomura 1980; Freedman et al. 1987; Shen et al. 1988; Zengel and Lindahl 1990, 1991). *Bacillus subtilis* TRAP protein controls the expression of the tryptophan biosynthetic genes by inducing premature transcriptional termination and ribosome binding site occlusion (Gollnick 1994; Yang et al. 1995; Merino et al. 1995). However, unlike these examples of bimodal regulation, we note that N is unique in activating at the level of transcription while repressing at the level of translation. More important, knowing that transcriptional termination can occur by directly affecting the structure of the terminator RNA (Landick and Turnbough 1992), it is easy to understand how TRAP influences both transcriptional termination and translational initiation simply by modulating RNA secondary structure at the terminator and initiator, respectively. It is more difficult to envision how N, which affects termination by interacting with and modifying RNA polymerase itself, also affects translation.

N-mediated antitermination has served as a model system for understanding the action of HIV-1 Tat protein, which, acting through an RNA site called TAR, activates long terminal repeat (LTR)-dependent transcription by enhancing the processivity of a transcription complex beyond TAR (Sharp and Marciniak 1989; Spencer and Groudine 1990; Krumm et al. 1993; Greenblatt et al. 1993). Tat with TAR has also been shown in special cases to enhance translation of genes transcribed from the HIV-1 LTR (Cullen 1986; Rosen et al. 1986; Braddock et al. 1989, 1990). Tat and N have similar arginine-rich domains that bind to *cis*-acting RNA sites, TAR and NUT, respectively (Lazinski et al. 1989; Gait and Karn

1993; Burd and Dreyfuss 1994), increasing the processivity of their respective polymerases. We now find that they are also similar in having an effect on translation.

In the *N* leader, NUTL and the *N* ribosome binding site flank the RNase III-sensitive hairpin (Fig. 2). The structure of the *N* leader reflects the temporal order of three events: assembly of the N-antitermination complex, RNase III cleavage of the *N* leader, and N-mediated translational repression. At 42 nucleotides/sec (Gotta et al. 1991), it takes RNA polymerase ~4 sec to transcribe from the *nut* site to the DNA specifying 3′ end of the RNase III-sensitive hairpin and the immediately adjacent *N* ribosome binding site. N-mediated antitermination functions normally in RNase III$^+$ cells (data not shown) presumably because the antitermination complex is assembled (i.e. in <4 sec.; Barik et al. 1987) before RNase III cleavage occurs. However, N autoregulation is blocked in RNase III$^+$ cells grown under our standard conditions in LB medium (data not shown; note that we use RNase III$^-$ cells in this paper). We assume that this effect is a consequence of RNase III cleavage separating NUTL from the *N* ribosome binding site prior to initiation of *N* gene translation and N autoregulation.

After infection, λ either enters the lytic pathway in which many progeny phage are produced and the host is destroyed, or switches off the lytic pathway and enters the lysogenic pathway in which the phage DNA is integrated into the host chromosome and the host survives. The turbid morphology of λ plaques reflects the presence of phage participating in both life styles. When λ first infects a cell or is released from the quiescent lysogenic state, no N is present to repress *N* gene expression. Once N reaches threshold levels the potential for *N* gene repression exists. However, to elucidate the significance of N-autoregulation in λ biology, we need to understand better the competition between this mechanism and RNase III cleavage. Although N-autoregulation is blocked by high levels of RNaseIII activity, we have preliminary evidence that this repression mechanism functions at reduced levels of RNase III activity. These results suggest that under these conditions, N represses translation of most transcripts before cleavage occurs. Because RNase III expression fluctuates directly with growth rate (R.A. Britton, B.S. Powell, S. Dasgupta, Q. Sun, W. Margolin, J.R. Lupski, and D.L. Court, in prep.), we are attracted to the idea that RNase III cleavage of the *N* leader, and consequently λ gene expression, is modulated in response to physiological conditions.

λ forms clear plaques on a wild-type *E. coli* strain expressing high, unregulated levels of N from a plasmid, indicating a reduction in the number of lysogenic cells surviving in the plaque and suggesting that uncontrolled *N* expression may favor the lytic pathway. This may be a consequence of N inhibiting the translation of *cII* through NUTR (Fig. 1), CII being important in the establishment of the lysogenic state. The observation that N can use NUTR to repress *N–lacZ* expression is consistent with this idea (Table 1A).

Finally, in considering the importance of N-mediated translational repression in λ biology, it is intriguing to consider whether the two N-dependent regulatory mechanisms, antitermination and translational repression, evolved together or whether one came first, the features of the first then being exploited by the second.

Materials and methods

Plasmids

The pBR322-derived *N–lacZ* gene fusion plasmids pLK30 (Kameyama et al. 1991) and pERW12 (Fig. 4A) are identical except that pLK30 contains a one-base deletion at position +6 of the p_L operon when compared with the published λ sequence. This mutation has no reproducible effect on the expression of *N–lacZ*. The plasmid parents of other *N–lacZ* fusion strains shown in Figure 4 are essentially identical to pERW12 except for the differences outlined in this figure. The plasmid parents of *nut* site mutant *N–lacZ* fusion strains shown in Table 1A are derived from pLK30. All these plasmids were constructed by ligation of appropriate restriction enzyme- and PCR-generated fragments. The nucleotide sequence of the entire *N* leader and *N–lacZ* fusion joint of all plasmids was confirmed by dideoxy sequencing. Plasmid pNAS150, a derivative of high-copy-number plasmid pUC9, carrying the *N* gene under the control of p_{lac}, has been described previously (Schauer et al. 1987). pZH124, a derivative of medium-copy-number plasmid pGB2 (Churchward et al. 1984), carries the p_{lac}–*N* region from pNAS150. pZH126, also a derivative of pGB2, carries the p_{lac}–*nun* region from pJ089 (Baron and Weisberg 1992).

Bacterial strains

The p_L–*nutL–N–lacZ–galK* double-reporter strains (Figs. 3,4,6; Table 1) were derived from strain ZH1041 [W3110 Δ(*argF–lac*)*U169*] which has the following genetic structure around the λ prophage: *gal*490*(IS2) *pgl*Δ8 *att int–lacZ–int red kil N nutL* p_L *cI857* Δ[*cro–bio*]. N-mediated antitermination from p_L results in expression of the cell-killing function Kil, causing temperature-sensitive growth. Temperature-resistant derivatives of ZH1041 carrying p_L–*nutL–N–lacZ* plasmids included cells that had recombined the *N–lacZ* fusion into the prophage through p_L and *lacZ,* losing the intervening *N* and *kil* sequence. The nucleotide sequence of the *N* leader of the recombinant prophage was verified by dideoxy sequencing of this region amplified by the PCR. The congenic *nus* mutant strains were made using standard P1 transduction with linked drug-resistance markers into the p_L–*nutL–N–lacZ* gene fusion parent strain. The *nus*$^-$ transductants were identified by their inability to support λ growth at 42°C. All strains were made RNase III$^-$ by transducing to tetracycline resistance using P1 grown on HT115 (W3110 *rnc14*::ΔTn*10;* Takiff et al. 1989).

Enzyme assays

Bacteria for β-galactosidase assays were grown overnight in LB liquid medium plus antibiotic (100 μg/ml of ampicillin for pUC9 and pNAS150 or 50 μg/ml of spectinomycin for pGB2 and pZH124) at 30°C, diluted one-fiftieth in 10 ml of LB liquid medium plus antibiotic, and aerated until the culture reached OD_{600} = 0.2–0.4. Two milliliters of culture was then taken as the zero time sample. The remainder of the culture was then shifted to 42°C with aeration to induce expression of p_L. Growth at 42°C had no obvious deleterious effect on cells. Two milliliter aliquots were then taken at indicated time points after induction (Figs. 3 and 4; Table 1). The growth of the culture was stopped by mixing cells with an equal volume of ice-cold Z

buffer (Miller 1972) plus 600 μg/ml of chloramphenicol. β-Galactosidase activity in each sample was determined according to Miller (1972). Cells for galactokinase assays were prepared in essentially the same manner except the culture volume was 30 ml, 10-ml aliquots were taken at 0 and 60 min after temperature induction, and cell growth was stopped by chilling on ice. Prior to assays, cells were pelleted and washed twice in 1× M56 salts and then resuspended in one-fifth to one times the original volume depending on the expected activity in the sample. Assays were done essentially as described previously (McKenney et al. 1981).

RT–PCR

Bacterial RNA was isolated using Qiagen RNeasy (Chatsworth, CA) according to manufacturer's instructions. RT–PCR experiments were carried out using the Access RT–PCR system (Promega, Madison, WI) according to manufacturer's instructions except that PCR amplification was carried out for only 25 cycles with the 68°C extension going for 3 min. In the reactions shown, 35 ng of RNA was used per reaction. The amount of RNA and number of amplification cycles were chosen to ensure that the assay is quantitative under the selected experimental conditions. The synthetic oligonucleotide primers for amplification of *lacZ* have the following sequences—5′ primer, 5′-AGCTCCTGCACTGGATGGTGGC-3′; 3′ primer, 5′-GACCAACTCGTAATGGTAGCGAC-3′; for amplification of *bioA*, the following sequences—5′ primer, 5′-GCGGACCAACTGCCATACAGC-3′; 3′ primer, 5′-TTCACCGTTACTGATGGTTTCTGC-3′.

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Translational repression by a transcriptional elongation factor

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