N-mediated transcription antitermination in lambdoid phage H-19B is characterized by alternative NUT RNA structures and a reduced requirement for host factors

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Summary

Gene expression in lambdoid phages in part is controlled by transcription antitermination. For most lambdoid phages, maximal expression of delayed early genes requires an RNA polymerase modified by the phage N and host Nus proteins at RNA NUT sites. The NUT sites (NUTL and NUTR) are made up of three elements: BOXA, BOXB and an intervening spacer sequence. We report on N antitermination in H-19B, a lambdoid phage carrying *shiga toxin 1* genes. H-19B N requires NusA, but not two other host factors required by λ N, NusB and ribosomal protein S10. The H-19B NUT site BOXA is not required, whereas the BOXB is required for N action. H-19B *nut* sites have dyad symmetries in the spacer regions that are not in other *nut* sites. Changes in one arm of the dyad symmetry inactivate the NUT RNA. Compensating changes increasing the number of mutant nucleotides but restoring dyad symmetry restore activity. Deletion of the sequences encoding the dyad symmetry has little effect. Thus, the specific nucleotides composing the dyad symmetry seem relatively unimportant. We propose that the RNA stem–loop structure, called the 'reducer', by sequestering nucleotides from the linear RNA brings into proximity sites on either side of the dyad symmetry that contribute to forming an active NUT site.

Introduction

The transition from early to delayed early gene transcription in bacteriophage λ and a number of other lambdoid phages is mediated, in part, by phage-encoded proteins. These proteins, usually but not always called N, are expressed early in the infective process. N acts with host factors to modify RNA polymerases that initiate at the early *p*L and *p*R promoters. The modified polymerases mediate transcription antitermination by reading through terminators (Fig. 1A) (Das, 1992; Greenblatt *et al.*, 1993; Friedman and Court, 1995; Weisberg and Gottesman, 1999). Polymerase modification is effected through *nut* sites *nutL* (Salstrom and Szybalski, 1978) and *nutR* (Rosenberg *et al.*, 1978) located downstream of the early *p*L and *p*R promoters respectively. Based on sequence and structural homologies, the *nut* sites have been divided into three parts (Figs 1C and 2): a 9 bp *boxA* sequence, a region of hyphenated dyad symmetry, *boxB*, and an 8–14 bp spacer sequence separating the other two elements (Friedman and Gottesman, 1983; Das, 1992). Acting as signals at the RNA level (Olson *et al.*, 1982; Warren and Das, 1984; Zuber *et al.*, 1987; Nodwell and Greenblatt, 1991), the NUT sites function as assembly points for the proteins composing the N-mediated antitermination complex. To distinguish at what level a sequence is being discussed, DNA sequences will be denoted by italicized lower case letters (e.g. *nut*) and RNA sequences will be denoted by upper case letters (e.g. NUT).

In vivo and *in vitro* studies have identified a number of host proteins (most referred to as Nus) that in addition to N form the antitermination complex with NUT RNA (Das and Wolska, 1984; Friedman *et al.*, 1984; Mason and Greenblatt, 1991; Friedman, 1992). N and NusA associate with the BOXB stem–loop structure (Lazinski *et al.*, 1989; Chattopadhyay *et al.*, 1995; Mogridge *et al.*, 1995; Cilley and Williamson, 1997; Su *et al.*, 1997; Cai *et al.*, 1998; Legault *et al.*, 1998). NusB and ribosomal protein S10 (NusE) have been postulated to associate with BOXA (Friedman *et al.*, 1990; Nodwell and Greenblatt, 1993; Patterson *et al.*, 1994; Court *et al.*, 1995; Mogridge *et al.*, 1998). Mutational analysis showed that sequences in the spacer region also play a role in NUT activity (Hasan and Szybalski, 1986; Doelling and Franklin, 1989). Specific interactions with NUT of other host proteins thought to be important in formation of this complex have not been determined. A comparison of *nut* regions (Fig. 2) shows that phages with different *N* genes have BOXA sequences with slight variations of a consensus sequence and BOXB sequences with hyphenated dyad symmetry
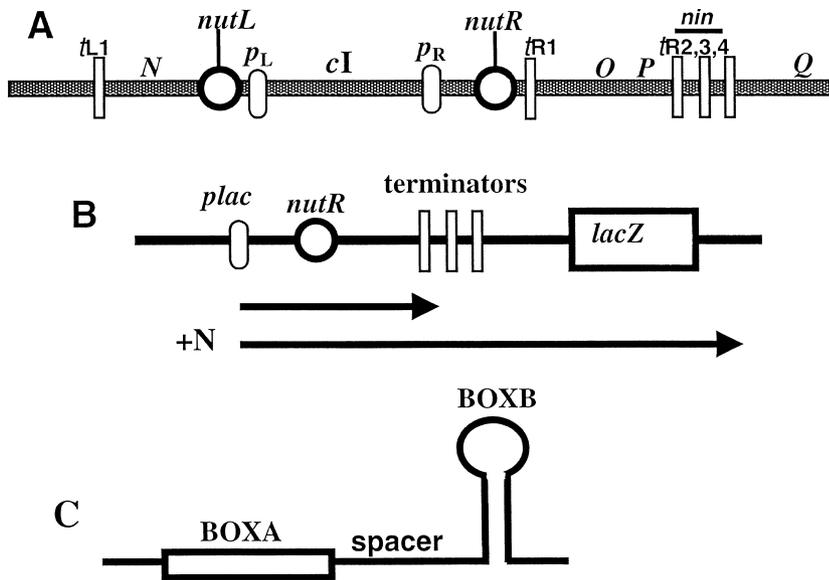
A

Fig. 1. Essential features of N-mediated transcription antitermination in lambdoid phages.
A. Map of the early region of lambdoid phages based on the λ paradigm. Shown are landmark genes (*N*, *cI*, *O*, *P* and *Q*), promoters (*p*), terminators (*t*) and *nut* sites. Also shown is the location of the *nin* deletion which removes termination sequences.
B. Insert in pTL61T−*plac*−*nutR* and variant plasmids used to assess effectiveness of *nut* sites in N-mediated antitermination. The terminator cassette has three Rho-independent terminators, the λ *t*R′ and the *rrn* operon T1 and T2 terminators (King *et al.*, 1996). In the absence of formation of an effective antitermination complex at NUTR, transcription terminates within the terminator cassette.
C. Schematic representation of the λ NUT site showing the relative positions of BOXes A and B and the intervening spacer region.

B

C

A ***boxA* sequences**

λ *nutR*	CGCTCTTAC
λ *nutL*	CGCTCTTAA
21 *nutR*	TGCTCTTTA
21 *nutL*	GGCTCTTTA
P22 *nutR*	CGCTCTTTAC
P22 *nutL*	CGCTCTTTAA
H-19B *nutR*	CGCT<u>A</u>TTT<u>T</u>
H-19B *nutL*	CGC<u>A</u>CT<u>ACT</u>
Consensus *boxA*	CGCTCTTTA

B **spacer sequences**

λ *nutR*	ACATTCCA
λ *nutL*	AAATTAA
21 *nutR*	ACAGTTCTGGCCTT
21 *nutL*	ACATCGACGGAC
P22 *nutR*	CCCAATCTGA
P22 *nutL*	ACTTCGATGAT
H-19B *nutR*	CACAATGGACATTCGTCCTACG
H-19B *nutL*	TCACCAGGGCGGTGATATACAACGATTCGAATATGAATCTACG

C ***boxB* sequences**

λ *nutR*	GCCCT GAAAA AGGGC
λ *nutL*	GCCCT GAAGA AGGGC
21 *nutR*	TCACCT CTAACC GGGTGG
21 *nutL*	TCTCAAC CTAACC GTTGAGA
P22 *nutR*	ACCGCC GACAA CGCGGT
P22 *nutL*	GCGCT GACAA AGCGC
H-19B *nutR*	TCGCT GACAA AGCGA
H-19B *nutL*	GCGCT GACAA AGCGC

Fig. 2. Comparison of *nutR* and *nutL* sites of lambdoid phages with characterized *nut* sites.
A. *boxA* sequences. The consensus *boxA* sequence is shown at the bottom. The underlined nucleotides in the H-19B sequences do not match the consensus sequence.
B. Spacer region sequences. Nucleotides in the H-19B sequences underlined with arrows identify hyphenated dyad symmetries that potentially could form stem−loop structures in the RNA.
C. *boxB* sequences. Nucleotides underlined with arrows identify hyphenated dyad symmetries. For λ and P22, these sequences in the RNA have been shown to form stem−loop structures (Su *et al.*, 1997; Cai *et al.*, 1998; Legault *et al.*, 1998). The boxed sequences identify nucleotides that would form the loop in the RNA stem−loop structure. Note that the H-19B and P22 *nutL boxB* sequences are identical.

Table 1. Growth of λ and H-19B on *E. coli* strains with *nus* mutations.

Strain	Temperature (°C)	Relevant genotype	EOP[a]		
			_	H-19B	H-19B*nin*
K95	42	*nusA1*	< 0.0001	< 0.0001	1
K450	40	*nusB5*	< 0.0001	1	ND
K7554	40	*nusB*::IS10	< 0.0001	1	ND
K551	40	*nusE71*	< 0.0001	1	ND
K2049	40	*nusB5/nusE71*	< 0.0001	1	ND
K4069	40	*rpoAD305E*	1	1	ND
K4047	42	*rpoAD305E/nusA1*	1	< 0.0001	ND

a. EOP (efficiency of plating) is the ratio of the phage titre on a lawn of the strain with the mutant genotype divided by the titre on a lawn of the parent *E. coli* strain wild-type at the locus being tested.
ND, not done.

(potential stem–loop structures in the RNA) but different sequences (Friedman and Gottesman, 1983; Franklin, 1985). Thus, at least for λ, efficient N-mediated anti-termination requires both BOXA and BOXB and certain bases in the spacer region.

We previously identified the *nutR* and *nutL* sites and an open reading frame postulated to encode the *N* gene of H-19B (Neely and Friedman, 1998b), a lambdoid phage that carries the *stx1* genes encoding the A and B subunits of Shiga toxin 1 (O'Brien *et al.*, 1984; Huang *et al.*, 1987). The identity of the phage components of an N-antitermi-nation system was established using a reporter system (Fig. 1B) to show that the NUTR site could promote readthrough of downstream terminators when supplied with the product of the putative H-19B *N* gene but not when supplied with the product of the λ *N* gene (Neely and Friedman, 1998b). In that study, we noted several unusual features of the H-19B *nut* sites; degenerate *boxA* sequences and extended spacer regions containing sequences with hyphenated dyad symmetry, suggesting that the NUT sites form, in addition to the BOXB structure, other RNA structures in the spacer regions. Similar *nut* sites and *N* genes nearly identical to that of H-19B have also been identified in lambdoid phages HK97 (Juhala *et al.*, 2000) and 933W. The latter phage carries the *shiga toxin 2* genes (Plunkett *et al.*, 1999).

Here, we report studies showing that the H-19B N–NUT interaction appears to differ in significant ways from the λ paradigm. Although H-19B N-mediated antitermination, like that of λ, requires NusA, unlike that of λ it appears not to require NusB or S10. The BOXB stem–loop structure is necessary, but the BOXA-like sequences are located far upstream of BOXB and do not appear to be necessary for NUT site function. The spacer regions between BOXA and BOXB in both *nut* regions differ from those of λ in having the potential to form stem–loops in the RNA. We present evidence indicating that extrusion of the spacer stem–loop in the H-19B NUTR RNA repositions sequences in the RNA to form a functional NUT site.

Results

Effect of Escherichia coli nus *mutations on H-19B growth*

The first evidence that λ N-mediated antitermination has specific host requirements derived from experiments characterizing *E. coli* variants with mutations that cause a failure of the bacterium to support N action, referred to as the Nus phenotype (Friedman *et al.*, 1984; Friedman, 1992). The N requirement was demonstrated by showing that N-dependent λ failed to grow, whereas the N-independent variant λ*nin5* (Court and Sato, 1969) did grow in these mutants. λ*nin5* has a 2.8 kb deletion (Fiandt *et al.*, 1971) that removes a segment of DNA downstream of the *P* gene (Fig. 1A), in the *nin* region, that contains at least three transcription termination signals (Cheng *et al.*, 1995). In large measure, N-mediated antitermination is required during lytic growth to facilitate progression of RNA polymerase through this region of terminators. Thus, when the terminators are deleted, the transcriptional requirement for N is significantly reduced.

To assess whether H-19B has similar requirements for Nus proteins, we tested H-19B growth in *E. coli* strains with mutations in *nus* genes using efficiency of plating (EOP) as a quantitative measure of phage growth (Table 1). The *nusA1*, *nusE71* (*rpsJ*), and *nusB5* alleles (Friedman *et al.*, 1984) all result from single base changes (Saito *et al.*, 1986; Craven and Friedman, 1991; Patterson *et al.*, 1994) and the *nusB*::IS10 (Taura *et al.*, 1992) is an insertionally inactivated allele of *nusB*. Studies with λ had shown that the *nus* mutations are most restrictive on phage growth at higher temperatures. The temperatures found to be optimal for assessing the effect of the mutant alleles on growth of λ as well as other lambdoid phages were 40°C for the *nusB5* and *nusE71* alleles and 42°C for the *nusA1* allele (Friedman *et al.*, 1984).

As shown in Table 1, H-19B fails to grow in the *nusA1* mutant, but does grow in the *nusB5*, *nusB*::IS10 and *nusE*(*rpsJ*)*71* mutants as well as the *nusB5/nusE71* double mutant. As previously reported, λ growth is not

supported in any of the mutant strains at the indicated restrictive temperatures. To examine whether the failure of H-19B to grow in the *nusA1* mutant is due to a failure of N-mediated antitermination, we constructed a derivative of H-19B that carries a deletion analogous to the λ *nin5* deletion. We assumed that the deletion would result in an N-independent H-19B derivative, based on our previous finding that H-19B shared the three terminators identified in the λ *nin* region (Neely and Friedman, 1998a). As shown in Table 1, the H-19B*nin* phage overcomes the *nusA1* defect at 42°C. Although not definitive, these results plausibly argue that H-19B requires NusA but not ribosomal protein S10 or NusB for N-mediated antitermination.

The carboxy-terminal domain (CTD) of the α subunit of RNA polymerase is an important transcription regulator (Russo and Silhavy, 1992; Ebright and Busby, 1995). Previous studies from this laboratory showed that a single amino acid change in the CTD of the α subunit *rpoAD305E* suppresses the *nusA1* defect in supporting λ N-mediated antitermination (Schauer *et al.*, 1996). It was proposed that suppression by the *rpoAD305E* allele results from an alteration in the interactions among NusB, S10 and the carboxy-terminal domain of the α subunit of RNA polymerase at BOXA, a set of interactions that influences N-mediated antitermination (Friedman and Court, 1995). Accordingly, the fact that H-19B growth appears not to require NusB or S10 and, further, that the H-19B *nut* sites contain degenerate BOXA sequences led us to suspect that the *rpoAD305E* mutation would not influence the effect of the *nusA1* mutation on H-19B growth. As shown in Table 1, this prediction proved to be true; H-19B growth is no better in the double *rpoAD305E/ nusA1* mutant than it is in the single *nusA1* mutant.

Defining features of the H-19B nut *region*

A comparison of *nut* sites (Fig. 2) shows that the H-19B *nut* sites have both similarities to and differences from *nut* sites of other characterized lambdoid phages (Friedman and Gottesman, 1983; Franklin, 1985). The H-19B *boxB* sequences are typical (Fig. 2C), having hyphenated dyad symmetries with the potential of forming stem–loop structures in the RNA as well as having identical sequences in the potential loop region of both *nutL* and *R*. Moreover, with two exceptions, they share the entire stem–loop sequence of the *nutL boxB* of phage P22. The H-19B *boxA* sequences, on the other hand, differ in important ways from the consensus *boxA* sequence. The H-19B *nutR boxA* has A rather than C in the highly conserved fifth position. This change in the λ *nut* site causes a significant reduction in the effectiveness of N-mediated antitermination (Robledo *et al.*, 1990; Patterson *et al.*, 1994). The H-19B *nutL boxA*

has four differences from the consensus *boxA* as well as deviations from its own *nutR boxA* (Fig. 2A). These variations in the *boxA* sequences provide further evidence, suggesting that a functional *boxA* is not required for H-19B antitermination.

H-19B *nut* sites differ significantly from other lambdoid phage *nut* sites in the spacer regions (Fig. 2B). These sequences are much longer than other lambdoid spacer regions, having 22 nucleotides in *nutR* and 42 nucleotides in *nutL* compared with the 8–14 nucleotides found in the characterized *nut* sites. Furthermore, the H-19B spacer regions contain areas of hyphenated dyad symmetry with the potential to form stem–loop structures between BOXA and BOXB, a feature not found in other characterized *nut* sites (Fig. 2B).

Differences from other characterized lambdoid phages in growth on *E. coil nus* mutants and in *nut* sequences suggest that H-19B N-mediated antitermination might require different components from those required by other N antitermination systems. To understand further how RNA sequences contribute to N-mediated antitermination, we extended our studies of the elements required for H-19B N-mediated antitermination, focusing on the *nutR* site of H-19B.

The in vivo *assay system for N action*

We used a plasmid reporter system to analyse the role of the various elements in the *nutR* site of H-19B in N-mediated transcription antitermination (Neely and Friedman, 1998b). The reporter plasmids contain a wild-type or mutant H-19B *nutR* site with a downstream cassette of three Rho-independent terminators (King *et al.*, 1996) cloned between the upstream *plac* promoter and the downstream *lacZ* reporter gene (Fig. 1B). The second plasmid, compatible with the first, contains the H-19B *N* gene expressed from the *plac* promoter. Expression from both plasmids is controlled by the Lac repressor and thus both are regulated by changes in the concentration of IPTG (Miller, 1992). The level of β-galactosidase production is a measure of readthrough of terminators located between the *nutR* site and the *lacZ* gene. Results are reported as per cent readthrough; 100% readthrough was set as the amount of β-galactosidase produced from a construct containing the *plac–nutR–lacZ* fusion (lacking the terminators between *nutR* and *lacZ*).

Because the N system of H-19B resembles that of λ, which has a *nut* site that is recognized at the RNA level, we assumed the H-19B *nut* site is also recognized at the RNA level. However, it is formally possible that all or part of the sequence is recognized at the DNA level. All constructs were sequenced and analysed by FOLDRNA (Devereux *et al.*, 1984; Zuker, 1989) to confirm the potential RNA folding pattern.

Table 2. Effect of mutant *boxA* sequences in the H-19B *nutR* region on terminator readthrough.

Relevant insert in reporter plasmid	*boxA* sequence	% readthrough[a]	
		−N	+N
nutR/boxA-wt	CGCTATTTT	2 ± 0.3	64 ± 6.8[b]
*nutR/boxA5**	CTCTATTTT	4 ± 1.3	55 ± 2.0
nutR/boxAcon[c]	CGCTCTTTA	2 ± 0.7	60 ± 9.3
nutR/boxA69	ATAGAGGC	7 ± 2.5	68 ± 2.0
nutR/ΔboxA	(deletion)	3 ± 0.4	72 ± 7.5

a. Percentage readthrough was determined from the levels of β-galactosidase expressed from the *plac−nutR−term−lacZ* plasmid constructs. The indicated values were all normalized to the level of β-galactosidase synthesized from a *plac−nutR−lacZ* construct deleted for all terminators. This value was set at 100% readthrough (~10 000 Miller units).
b. $n = 5$.
c. *boxAcon* is the consensus *boxA*.

Role for BOXA in N-mediated antitermination

To assess the role of the *boxA* sequences in H-19B N-mediated antitermination, we constructed derivatives of the reporter plasmid with changes in the *boxA* sequence and tested the altered *nut* sites for their effectiveness in supporting N-mediated antitermination. A functional role for the *boxA* sequence (CGCTCTTAC) in the λ *nut* region was demonstrated in studies using *nut* sites with changes in their *boxA* sequences. Some altered *nut* sites, such as those with *boxA5* (CTCTCTTAC) or *boxA69* (ATA-GAGGC), exhibited greatly reduced N-mediated antitermination (Olson *et al.*, 1984; Court *et al.*, 1995). Other altered *nut* sites, such as those changed to the consensus *boxA* sequence (CGCTCTTTA), exhibited more effective N-mediated antitermination (Friedman *et al.*, 1990).

Changing the *boxA* sequence in the H-19B *nutR* site has little effect on the ability of the H-19B NUT site to function with the H-19B N. The changes made were to *boxA5** (CTCTATTTT), with a change at the highly conserved and functionally important second position,

Table 3. Sequence specificity of N proteins for variant H-19B *nut* sites.

Relevant insert in reporter plasmid	Source of N	% readthrough[a]
nutR-wt	H-19B	67 ± 4.2[b]
nutR-boxB13[c]	H-19B	1.7 ± 0.2
nutR-wt	P22	1.5 ± 0.3
nutR-wt	λ	2.5 ± 0.1

a. Percentage readthrough was determined from the levels of β-galactosidase expressed by the indicated *plac−nutR−term−lacZ* plasmid constructs in the presence of the indicated N. The reported values were all normalized to the level of β-galactosidase synthesized from a *plac−nutR−lacZ* construct, deleted for all terminators, which was set at 100% readthrough (~10 000 Miller units).
b. $n = 5$.
c. The ascending arm of the *boxB* dyad symmetry was changed from TCGCT to AGCGA.

the consensus sequence (CGCTCTTTA), the highly degenerate *boxA69* sequence (ATAGAGGC) and a deletion of *boxA*. The results shown in Table 2 are consistent with the argument that the H-19 *nut* region does not require a functional *boxA* sequence for N-mediated antitermination.
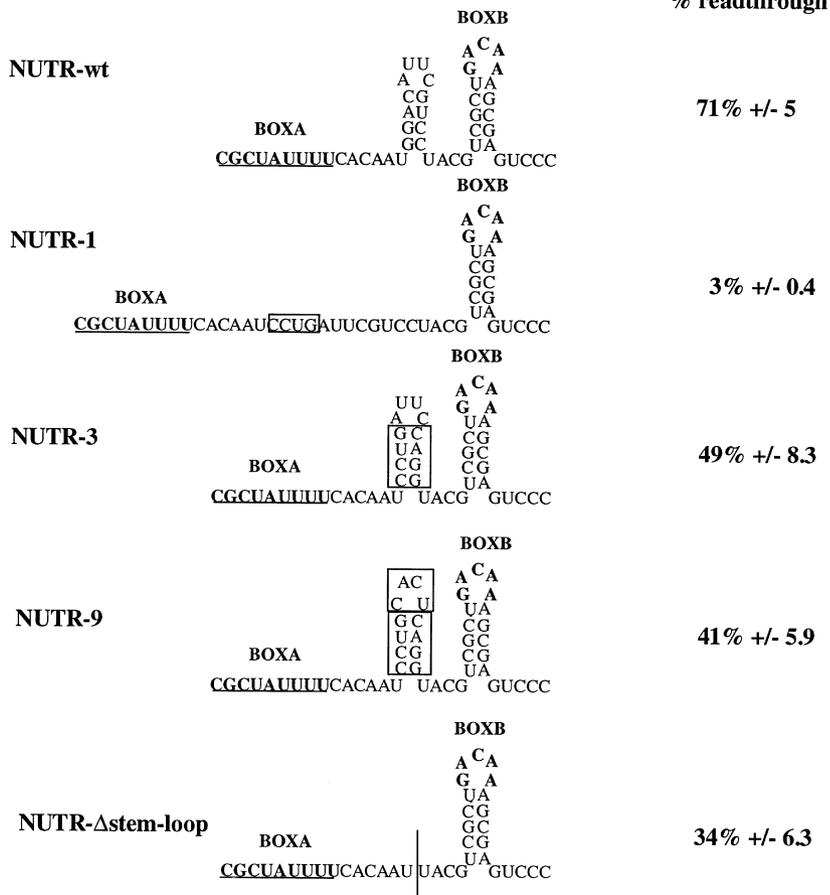
Role for BOXB in N-mediated antitermination

To assess the role of the *boxB* sequences in H-19B N-mediated antitermination, we constructed a derivative of the *nut* reporter plasmid in which the nucleotides in the ascending arm of the *boxB* dyad symmetry have been changed, yielding the *nutR−boxB13* mutant *nut* region. The change in *nutR−boxB13*, from TCGCT to AGCGA, removes the dyad symmetry and thus along with changing the *boxB* sequence should eliminate formation of a stem−loop in the BOXB element in the RNA. As shown in Table 3, an H-19B *nut* region with this change does not support N-mediated transcription antitermination, demonstrating a requirement for *boxB* in H-19B N-mediated antitermination.

The interaction at the H-19B NUT site is specific for H-19B N. Although H-19B *nutL* shares nearly the same *boxB* with that found in the P22 *nutL* site (Fig. 2C) (Friedman and Gottesman, 1983; Franklin, 1985), the combination of the H-19B NUT site and P22 N, originally named 24 (Hilliker and Botstein, 1975), does not support transcription antitermination (Table 3). To confirm that active P22 N was present, we showed that the P22 N supplying plasmid-supported growth of a λ*imm*-P22*am24*(N) mutant (data not shown). As also shown in Table 3, λ N also fails to function with the H-19B NUT site.

Function of stem−loop structure in spacer region

To investigate the role of the H-19B *nut* spacer region and its potential RNA stem−loop structure, we constructed derivatives of the H-19B *nutR* plasmid that have changes in the spacer dyad symmetry. Figure 3 illustrates the nucleotide sequence and potential RNA secondary structures of the mutant NUT regions together with the percentage of terminator readthrough the various constructs support in the presence of the H-19B N.

The *nutR-1* and *nutR-3* constructs were designed to address the question of whether the RNA stem−loop structure suggested by the dyad symmetry in the spacer region plays a role in H-19B N-mediated antitermination. The *nutR-1* derivative of the H-19B *nut* plasmid has four nucleotide changes in the sequence of the upstream half of the spacer region dyad symmetry. These changes should inhibit the formation of a stem−loop structure in the RNA. As shown in Fig. 3, H-19B N failed to activate terminator readthrough in this construct under conditions

Fig. 3. Sequences, potential structures and effectiveness at supporting N-mediated transcription antitermination of H-19B NUTR RNA sites with variant spacer regions. Shown are the predicted stem−loop structures in the spacer regions and BOXB elements of the H-19B NUT site variants tested in these studies. Transcription antitermination, recorded as percentage readthrough of terminators in the presence of N, is indicated at the right. Terminator readthrough was determined by measuring β-galactosidase expression from the *plac−nutR−term−lacZ* plasmid constructs in the presence of N (see *Experimental procedures*). The values were normalized to the level of β-galactosidase expressed from a *plac−nutR−wt−lacZ* construct without the terminators; this reading was set for comparison as 100% readthrough (∼10 000 Miller units). All strains carried both the plasmid with the *plac−nutR−term−lacZ* indicated construct and the compatible plasmid expressing the H-19B *N* gene product. In the absence of the N plasmid, all constructs showed less than 3% readthrough. At least five separate measurements were made for each mutant construct. For comparison, the first structure is the wild-type NUTR. Boxed sequences identify changed nucleotides. The perpendicular line in NUT−Δstem−loop indicates position of the deleted nucleotides.

in which it did activate terminator readthrough with the H-19B wild-type *nut* plasmid. To distinguish whether this failure in N-mediated antitermination results directly from the nucleotide change or indirectly from the inability to form the stem−loop structure in the spacer region, we made compensating changes in the downstream half to create the *nutR-3* derivative of the H-19B-*nut* plasmid. Although this results in eight changes from wild-type in the spacer sequence, it restores the dyad symmetry and hence the potential stem−loop structure in the RNA. As shown in Fig. 3, there is effective activation by H-19B N of terminator readthrough with this construct. This result is consistent with the idea that the spacer dyad symmetry is important because it results in the formation of a stem−loop structure in the NUT RNA and that the sequence *per se* in this portion of the spacer may not be important.

To assess further the functional significance of the sequence composing the spacer region stem−loop, we constructed the *nutR-9* derivative of the H-19B *nutR* plasmid. This derivative has, in addition to the eight changes in the stems in the *nutR-3* derivative, four changes in the sequence of the potential loop region. Hence, the entire 12 nucleotide sequence that composes

the hyphenated dyad symmetry in the spacer differs from wild-type, but still has the ability to form the secondary structure in the RNA. As shown in Fig. 3 and as with the *nutR-3* variant, there is effective activation by H-19B N of terminator readthrough with this construct. Taken together, the results from the three spacer mutants *nutR-1*, *nutR*-3 and *nutR*-9 suggest that stem−loop formation in the spacer RNA is an important contributor to N-mediated antitermination and beyond this the structure does not appear to require a specific sequence. Although antitermination is reduced by ∼40% with the *nutR* mutants, significantly there is still a ∼15-fold increase in terminator readthrough with these mutant sites over that observed with the non-functional *nutR-1* construct. In considering the ∼40% difference, we cannot distinguish between two possible explanations; one, that the altered sequence *per se* modestly influences N-mediated antitermination, or, two, that the altered sequence indirectly affects N-mediated antitermination by influencing the repositioning of the NUT sequence following stem−loop formation.

The finding that the spacer stem−loop does not seem to require specific nucleotides, particularly in the loop

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                    U U
                  A     C
                  C G
                  A U
          NUTR    G C
NUTR-29           G C           NUTR-17
GUGU     ◄ ┌─────┐ ┌────┐ ►     CCUA
           │CACAA│U│UACG│
           └─────┘ └────┘
            | | | |  | | | |
         UAUACAAC UACG
                  G C
          NUTL    A U
                  U A
                  U A
                  C G
                G     U
               A       A
                  A U
```
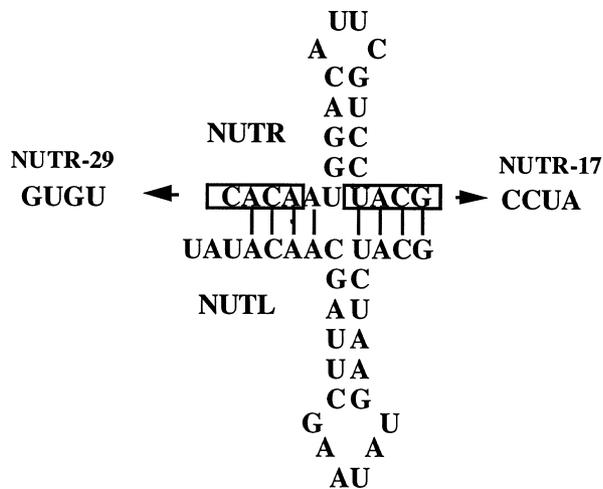
Fig. 4. Alignment of spacer sequences from NUTL and NUTR of H-19B. The analogous sequences of the spacer regions of the H-19B NUTR and NUTL regions are aligned as they would occur if the stem–loop structures were allowed to form. The nucleotides changed in creating the NUTR-29 and NUTR-17 mutant NUT sites are boxed and the mutant sequences are shown to the sides of the structures. Vertical lines identify identical nucleotides at analogous positions.

region, suggests that the structure is not required for any specific protein–RNA contacts. What function then could a structure without sequence specificity serve? One possibility is that the formation of a stem–loop structure in the RNA might effectively shorten the spacer in the NUT region RNA to allow the creation of a continuous linear spacer sequence that has functional importance. As shown in Fig. 2B, the spacer in the H-19B *nutR* region is more than two times longer than the spacers in the *nut* regions of other lambdoid phages. The spacer stem–loop sequence in the H-19B NUTR RNA reduces the apparent length of the spacer from 22 nucleotides to only 10 nucleotides, which is similar to that of the spacer

regions found in the *nut* regions of other lambdoid phages.

If the sequence of the spacer stem–loop is not important, serving only to shorten the RNA, then removing the sequences composing only the spacer dyad symmetry should not interfere with effectiveness of the *nut* region in fostering N-mediated antitermination. We tested this idea using a derivative of the H-19B *nut* plasmid, *nutR–*Δstem–loop, lacking the 12 nucleotides that make up the spacer hyphenated dyad symmetry. The deletion of these sequences in the DNA should result in a NUT RNA missing the entire stem–loop structure. As shown in Fig. 3, N-mediated transcription readthrough with the H-19B *nutR–*Δstem–loop was very effective, showing a 10-fold increase in terminator readthrough over that seen with the non-functional *nutR-1* construct. However, this mutant site was ~50% as effective in directing antitermination as the wild-type site. As discussed above, we are unable to determine whether this small effect on antitermination reflects a role for the spacer sequence *per se* in antitermination or whether the deletion results in a less than optimum spacing of important sequences on either side of the dyad symmetry.

Role of sequence in the spacer region

Alignment of the stem–loop structures in the NUTR and NUTL spacer regions reveals two sequences on either side of the stem–loops with extended runs of almost identical sequence, ACAAU/C–stem–loop–UACG (see Figs 2 and 4). To determine whether these sequences have functional significance, we changed the sequences on either side of the dyad symmetry encoding the putative stem–loop structure in the spacer region in the H-19B *nutR* plasmid (see Figs 1B and 4). The effectiveness of the mutant *nutR* sites in promoting antitermination in the

H-19B *nutR*   CGCTATTTTcacaatggacattcgtcctacgTCGCTGACAAAGCGA

H-19B *nutL*   CGCACTACTcaccagggcggtgatatacaacgattcgaatatgaatctacgGCGCTGACAAAGCGC

HK97 *nutR*   CGCTCTTTTcacaatggacattcgtcctacgTCGCTGACAAAGCGA

HK97 *nutL*   CGCACTACTcaccagggcggtgatatacaacgattcgaatatgaatctacgGCGGTCACAAAGCGC

933W *nutR*   CGCTATTTTcacaatggacattcgtcctacgTCGCTGACAAAGCGA

933W *nutL*   CGCATTACTcaccagggcggtgaatatacaacgattcgaatatgaatctacgGCGCTGACAAAGCGC

Fig. 5. Alignment of *nutR* and *nutL* regions of HK97 (Juhala *et al.*, 2000) and 933W (Plunkett *et al.*, 1999) with those of H-19B showing similarity of sequences in the *boxA*, spacer and *boxB* regions. The *boxA* and *boxB* sequences are denoted by capital letters and the dyad symmetries are underlined by appropriately directed arrows.

presence of H-19B N was assessed by measuring the level of expression of the *lacZ* reporter gene. When the TACG on the *boxB* side of the stem−loop was changed to CCTA (H-19B *nutR-17*), we found that N-mediated terminator readthrough was 2% ($\pm$ 0.2%) compared with 70% ($\pm$ 5%) for the construct with the wild-type NUT region. When the CACA on the *boxA* side of the stem−loop was changed to GTGT (H-19B *nutR-17*), we found that N-mediated terminator readthrough was 13% ($\pm$ 1%) compared with 59% ($\pm$ 4%) for the construct with the wild-type NUT region. These results suggest that sequences on both sides of the dyad symmetry in the spacer region are important for N-mediated antitermination at the H-19B NUTR site.

Discussion

Comparison of the sequences of *nut* sites suggested that there were likely to be significant differences in the nature of the interactions directed by the H-19B NUT RNA from those observed with the NUT RNA of previously characterized lambdoid phages. Here, we present evidence demonstrating that the host protein requirement for the H-19B N-mediated transcription antitermination differs from that of other lambdoid phages, reflecting structural features unique to the H-19B NUT RNA. Although these studies focused on the N−NUT interactions of H-19B, the conclusions based on these observations are likely to apply also to HK97 (Juhala *et al.*, 2000) and 933W (Plunkett *et al.*, 1999), phages that share nearly identical N and *nut* sequences with H-19B but that have not been similarly analysed (Fig. 5). Additionally, results of our study support the argument that NusB and S10 act through BOXA in influencing antitermination.

Host factors and BOXA

Our experiments show that transcription antitermination mediated by H-19B N, like other characterized N antitermination systems, requires NusA but, unlike those systems, does not appear to require NusB or ribosomal protein S10 (NusE). Additionally, we find that *boxA* is not required in the *nut* site for H-19B N-mediated antitermination. These two observations are consistent with previous work showing that, for λ N antitermination, NusB and S10 are required and their actions, at least in part, appear to be mediated through the BOXA sequences in the *nut* sites (Friedman and Court, 1995). Moreover, the *rpoAD305E* mutation, which suppresses the *nusA1* defect in supporting λ N-mediated antitermination (Schauer *et al.*, 1996), does not similarly suppress the *nusA1* defect in supporting H-19B N-mediated antitermination. It has been proposed that the suppression of the *nusA1* defect in supporting λ N is a consequence of a change in the

interactions among the CTD of the α subunit of RNA polymerase, NusB, S10 and BOXA (Schauer *et al.*, 1996). Thus, an N-mediated transcription antitermination system that appears not to use NusB, S10 or BOXA is not influenced by a suppressor mutation that apparently works through the interaction of these factors.

The unique structure of the H-19B NUT sequence

The H-19B *nut* sites, unlike those of the λ type, contain sequences with hyphenated dyad symmetry in the spacer regions, one in *nutR* and two in *nutL*. These sequences, with the potential of forming stem−loop structures in the RNA, could play an important role in the activity of the NUT sites. In this study, we have focused on the potential structure in the NUTR RNA. Our mutational analysis of the *nutR* spacer region provides evidence that, when the dyad symmetry is present, formation of the stem−loop in the RNA is required for N-mediated antitermination. A change in nucleotides in one of the arms of the dyad symmetry that interferes with formation of the stem−loop inactivates the *nut* site. However, a compensating change in the other arm, which restores the potential for Watson−Crick pairing in the putative stem, restores activity of the *nut* site in mediating N antitermination. Moreover, changing the sequence in the loop as well as the stem does little to alter the effectiveness of the NUT site in promoting N-mediated antitermination (see *nutR-9* mutation in Fig. 3). Also, a complete deletion of the dyad symmetry element and hence the stem−loop in the RNA has only a small twofold effect on N-mediated antitermination (see NUTR-Δstem-loop in Fig. 3).

These results indicate that neither the sequence composing this dyad symmetry nor its potential RNA structure are necessary for the H-19B NUTR site to participate in N-mediated antitermination. However, when the sequence is present, effective antitermination requires that it have the potential to form an RNA stem−loop. Therefore, we conclude that an important role for this dyad symmetry is to extrude the nucleotides composing the potential structure from the continuity of the NUT RNA. In this way, the stem−loop structure could allow for an alternative arrangement of sequences within the spacer RNA. The dyad symmetry appears then to serve primarily as a mechanism for reorganizing the NUT RNA by removing sequences from the continuity of an RNA sequence to create an effective linear sequence. In light of this apparent activity, we have adopted the generic term 'reducer' for dyad symmetries that act to remove a run of nucleotides from the continuity of an RNA sequence. A similar role has been postulated for a stem−loop RNA structure located downstream of the *nutL* sequence of λ. In this case, withdrawal of the stem−loop is required for a totally different role for N acting at a

Table 4. Bacterial strains.

Strain	Relevant genotype[a]
K37	*nus+*
K95	*nusA1*
K450	*nusB5*
K553	*nusE71*
K7554[b]	*nusB*::IS*10*
K4069	*rpoAD305E*
K2049	*nusB5 nusE71*
K2053	*nusA1 nusB5*
K4047	*rpoAD305E nusA1*
K3093[c]	*lacZ*::Tn5 *lacI*$_{Q1}$

a. K37 is the standard *nus*$^+$ strain used in our studies. All other strains were constructed in this laboratory from K37.
b. Carries mutation isolated by Taura *et al.* (1992).
c. Strain used in tests of reporter constructs.

NUT RNA, i.e. that of translational regulation (Wilson *et al.*, 1997).

We can only speculate as to the significance of the reducer sequence for this *nut* site. The role of the reducer might be completely trivial, a vestige of some previous interaction that has been lost by H-19B. This would not be unexpected as we already have evidence of the apparent lack of function for the *boxA* sequence in H-19B.

The reducer could play a functional role in regulating the activity of the NUT site. We can envision different ways as to how this regulation could occur either in the presence or absence of an effector molecule. An upstream region in the NUT RNA might be brought in closer proximity to the BOXB sequence, providing a scaffold that facilitates formation of the antitermination complex. Consistent with this idea, the extended spacer region in the H-19B *nutL* region is reduced to a size corresponding to spacer regions in other *nut* regions if the two potential spacer stem–loop structures form (Fig. 2). Based on studies with other lambdoid phages, the most likely candidate for such an upstream sequence would be *boxA*. However, this seems unlikely as we have shown that, for H-19B N action, the *boxA* sequence is apparently not required. Regions upstream of *boxA* may be important, although examination of those sequences failed to show any obvious homologies between *nutR* and *nutL*.

Although the H-19B NUTR and NUTL spacer sequences appear to be quite different from each other, if the sequences that form the spacer stem–loops are removed contiguous runs of sequences in the two NUT sites that are quite homologous (Fig. 4) are obvious. Moreover, these shortened sequences approximate the length of spacer regions in other *nut* regions (Fig. 2B). This suggests that sequences brought together by the extrusion of the H-19B spacer stem–loop might be important for effective N action at the NUT site. This idea is consistent with the substantial reduction in N-mediated antitermination observed when the *nut* sites in

the tester plasmid have altered sequences on either side of the spacer dyad symmetry (Fig. 4).

The two other phages known to share the same *nut* sites, including the spacer with H-19B, HK97 and 933W (Fig. 5), have significant sequence variations from each other as well as H-19B in other regions of their genomes (Plunkett *et al.*, 1999; Juhala *et al.*, 2000). The conservation of the spacer region sequence in these phages suggests that there is selective pressure to maintain the spacer sequence. The spacer stem–loop through its sequence and structure may substitute for the missing Nus factors and a functional BOXA. In so doing, it may play a role in stabilizing the N–NusA antitermination complex, allowing processive antitermination. Indeed, it has been suggested that in λ the NUT RNA may serve as a structural component working to hold the complex together throughout antitermination (Barik *et al.*, 1987; Horwitz *et al.*, 1987). A partial requirement for the stem–loop structure and/or sequence could explain the 50% decrease in antitermination observed when the *nut* site was deleted for the spacer dyad symmetry as well as the ~40% decrease observed when the sequence of the dyad symmetry was changed. However, these relatively small decreases in antitermination could also plausibly be due to subtle changes in structure resulting in a spacer region that does not precisely mimic the contiguous sequence created by the formation of the wild-type stem–loop structure.

Whalen *et al.* (1988), examining the requirements for λ N action at NUT *in vitro*, showed that NusA and N are sufficient for antitermination through proximal terminators, but this minimal antitermination complex soon falls apart as it travels further on the template. Other *in vitro* studies show that, under special conditions, N can foster limited (non-processive) antitermination in the absence of host factors or a *nut* site on the template (Rees *et al.*, 1996). However, efficient N-mediated antitermination at distant terminators requires in addition to NusA the other Nus factors (Mason *et al.*, 1992; DeVito and Das, 1994). Because it requires readthrough of three strong terminators, our tester system more closely approximates processive transcription antitermination. We cannot distinguish between two possible explanations for the apparent low number of proteins required for H-19B N action: whether processive antitermination with H-19B N only requires NusA or requires other previously unidentified host proteins.

Experimental procedures

Media

Luria–Bertani (LB), tryptone broth (TB) and top agar media used in these studies have been previously described (Miller and Friedman, 1980). M56 medium (Monod *et al.*, 1951) used

for β-galactosidase assays was supplemented with 0.2% Casamino acids and either 0.02% fructose or 0.2% fructose. IPTG was added to a final concentration of 1 mM. Spectinomycin and ampicillin were used at 150 μg ml^{-1} and 100 μg ml^{-1} respectively.

Bacteria, phage and plasmids

Bacterial strains are listed in Table 4. H-19B, a Shiga toxin I-converting bacteriophage, was originally isolated from *E. coli* 026:H11 strain H19 by H. W. Smith (Scotland *et al.*, 1983). *E. coli* K-12 strain C600(H-19B) was provided by Alison D. O'Brien, Uniformed Services University of the Health Sciences, Bethesda, MD. The H-19B phage was isolated from the C600(H-19B) lysogen. H-19B*nin* was constructed by crossing H-19B with a plasmid containing a cloned H-19B fragment synthesized using PCR splicing by overlap extension (SOE) (Horton *et al.*, 1990) that has *ren* and *Q* sequences on its flanking regions but is deleted for the *nin* terminator region (Neely and Friedman, 1998a). Recombinants were selected on a *nusA1* host and PCR analysis confirmed that the isolated H-19B derivative has the proper deletion. λ*imm*P22*am24* was obtained from D. Botstein. The λ phage used, λ*cI*60, was obtained from the NIH collection.

Plasmid pTL61T (Linn and St. Pierre, 1990), which was used for cloning fragments with the H-19B *nutR* wild type and variants, contains a promoterless *lacZ* gene downstream of an RNase III processing site and has the origin of replication from pBR322. Plasmid pGB2-*plac* is a derivative of the low copy number plasmid pGB2 (Churchward *et al.*, 1984), which is compatible with pBR322 and confers spectinomycin resistance.

Plasmid construction

Construction of the plasmid containing the wild-type *nutR* site of H-19B (pTL61T–*plac*–*nutR*–term) was reported previously (Neely and Friedman, 1998b). Plasmids containing H-19B mutant *nutR* sites were constructed in the same manner as pTL61T–*plac*–*nutR*–term, using the PCR-SOE method (Horton *et al.*, 1990) and H-19B phage DNA as template to construct the mutant *nutR* sites. All *nutR* constructs were sequenced after cloning to confirm construction of the desired sequence. Plasmids *plac*–N(H-19B), *plac*–N(λ) and *plac*–N(P22) were constructed by placing, respectively, the H-19B *N*, λ *N* and P22 *N(24)* genes downstream of the *plac* promoter of pGB2-*plac*.

DNA sequencing

Sequences were determined using the Thermo Sequenase kit (Amersham) with double-stranded plasmid DNA. Computer analysis of DNA sequences was performed using the Genetics Computer Group (GCG) program (Devereux *et al.*, 1984).

Measurement of transcription antitermination

Readthrough of terminators, used to assess levels of transcription antitermination, was quantitatively determined by measuring β-galactosidase levels (Miller, 1992) expressed from a downstream *lacZ* reporter gene. These results were converted to percentage readthrough using as the 100% level the units of β-galactosidase synthesized from plasmids with the *plac*–*nutR*–*lacZ* construct (deleted for all terminators).

Phage plating on nus mutants

Dilutions of phage were plated using bacterial lawns formed by pouring 2.5 ml of TB top agar containing 0.3 ml of an overnight bacterial culture grown in LB broth onto TB agar plates that were then incubated overnight at the indicated temperature. Phage growth is reported as efficiency of plating (EOP), as described previously (Bear *et al.*, 1984).

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