Transcription-dependent competition for a host factor: the function and optimal sequence of the phage λ *boxA* transcription antitermination signal

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Ordered development of lambdoid phages relies on systems of transcription termination and antitermination. The phage-encoded N early regulatory proteins, acting with the Nus proteins of *Escherichia coli*, modify RNA polymerase to a form that overrides many transcription termination signals. These modifications require *cis*-acting sites, *nut*, located downstream of the early phage promoters. The *nut* sites in phages λ , 21, and P22, which share similarities but are not identical, contain two signals, *boxA* and *boxB*. We demonstrate that although a consensus sequence for the *boxA* signal (*boxAcon*), 5'-CGCTCTTTA, is found only in P22, changes to consensus in the *nut_R* sites of λ and 21 create more effective antitermination signals than the wild-type signals. An *in vivo* competition assay demonstrates that a λ *nut* region with *boxAcon* outcompetes *nut* regions with wild-type, as well as other variations of the *boxA* sequence, for the host NusB protein. This suggests that *boxA* influences NusB activity in N-mediated antitermination. Successful competition by *boxAcon* requires transcription of the *nut* site as well as N activation. Nucleotide replacement further demonstrates that bases at both ends of *boxA* are important for antitermination.

[Key Words: *nut*; *boxA*; *boxAcon* termination; antitermination; regulation]

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Regulation of gene expression by systems of termination and antitermination of transcription have been well described for prokaryotes, and recent studies indicate that this mode of regulation is operative in eukaryotes (for review, see Platt 1986; Friedman et al. 1987; Proudfoot 1989; Spencer and Groudine 1990). The best described of these systems are found in phage λ and its *Escherichia coli* host. In prokaryotes, signals for conversion of RNA polymerase into elongation complexes capable of reading through multiple downstream termination signals have been identified both within, and downstream of, promoters (for review, see Friedman and Gottesman 1983; Morgan 1986; Friedman 1988a; Roberts 1988).

The λ *nut* regions located downstream of the early promoters, *p_L* and *p_R*, contain the signals recognized by the proteins of the N transcription antitermination complex (Fig. 1; Salstrom and Szybalski 1978). These proteins include the phage protein gpN (gene product of *N*) and the products of the *E. coli nus* genes (for review, see Friedman et al. 1984). Two signals have been identified in the *nut* region: (1) *boxB*, a 16-bp region of hyphenated dyad symmetry (Rosenberg et al. 1978; Somasekhar et al. 1982); and (2) *boxA*, which is upstream of *boxB* in both *nut* regions (Olson et al. 1982; Fig. 1). The

boxB sequence is the recognition element for gpN (Doelling and Franklin 1989; Lazinski et al. 1989). *In vitro* studies have confirmed that Nus factors are required for N-mediated antitermination (Greenblatt and Li 1981; Das and Wolska 1984; Ghosh and Das 1984; Das et al. 1985), cooperating with gpN to alter RNA polymerase in response to the *nut* signal (Barik et al. 1987; Horwitz et al. 1987).

The *boxA* transcription signal was subsequently shown to be present in variant forms (Table 1) in the *nut* regions of other lambdoid phages (Olson et al. 1982; Friedman and Gottesman 1983; Franklin 1985) as well as in strategic locations in some operons of *E. coli*. These bacterial *boxA* signals, depending on their location, appear to influence either transcription termination (Stewart and Yanofsky 1985; Rosenthal and Calvo 1987) or antitermination (Aksoy et al. 1984; Holben and Morgan 1984; Li et al. 1984; Morgan 1986; Berg et al. 1989). Although all of the *boxA* sequences exhibit significant homology, most show some variation from the consensus sequence.

Two other lambdoid phages with N-like antitermination systems, which are functionally and structurally similar to that of λ , are coliphage 21 and *Salmonella typhimurium* phage P22. These phages have N-like genes

Figure 1. Relevant genes, regulatory signals, and transcription patterns of the λ early regulatory region (map not drawn to scale). Shown are the relative placement of representative genes and regulatory signals. Early promoters are indicated by p_R and p_L , termination signals by ovals, and nut sites by boxes. The $r32$ -IS2 insertion is shown above the map, and its termination signal is shown below the map. The region of strong termination removed by the $nin5$ deletion is shown with the three distinguishable regions of termination identified. (A) Regions of substitution in the hybrid phages $\lambda imm21$ and $\lambda imm22$. (B) Early transcription of the wild-type p_R and p_L operons under N-deficient conditions with points of termination indicated. Note that in the absence of gpN, 40–50% of transcription passes through t_{R1} . (C) Rightward transcription in the absence of gpN when the $r32$ -IS2 element with its strong terminator is present. (D) Readthrough of transcription terminators in the presence of active gpN, Nus proteins, and nut signals. (E) DNA sequence of the λnut_R region showing the placement of the $boxA$ and $boxB$ sequences in relation to the upstream cro gene. The $boxA$ sequence is underlined, and the arms of the $boxB$ hairpin are indicated by the converging arrows.

and cognate nut sequences at positions on their genomes analogous to the positions of the λ N gene and nut signals (Friedman et al. 1973a; Hilliker and Botstein 1976; Hilliker et al. 1978; Franklin 1985; Lazinski et al. 1989). The amino acid sequences of the various gpNs, however, are significantly different (Franklin 1985; Lazinski et al. 1989). The nut regions of λ , 21, and P22 have $boxB$ sequences that differ in nucleotide composition but resemble each other in having hyphenated dyad symmetries. The $boxA$ sequences of these phages are similar, but not identical. The following minimal consensus sequence is evident, 5'-CGCTCTTTA, and regardless of the surrounding context, it will be called $boxA_{con}$ (con = consensus). A complete match to the consensus is found only in the nut regions of P22 (Friedman and Gottesman 1983; Franklin 1985).

Lambdoid phages share regions of homology, thus permitting the construction of hybrid phages by crossing either P22 or 21 with λ (Liedke-Kulke and Kaiser 1967; Gemski et al. 1972; Botstein and Herskowitz 1974). Hybrids $\lambda imm22$ and $\lambda imm21$ (see Fig. 1) have acquired the central control region, called the imm region, respectively, from P22 and 21, while the rest of the genomes

of these phages were derived from λ . $\lambda imm22$ is particularly useful, because P22 normally does not infect *E. coli*, whereas $\lambda imm22$ has the λ host range.

The various N reactions can be functionally distinguished by employing *E. coli* variants with altered nus genes (Friedman et al. 1984). For example, an *E. coli* with a chimeric $nusA$ gene, $nusA_{S.t.}$, comprised of the 5' 85% from *S. typhimurium* and the 3' 15% portion from *E. coli*, supports growth of $\lambda imm22$ at all temperatures and $\lambda imm21$ at lower temperatures, but fails to support growth of λ at any temperature (Baron et al. 1970; Friedman and Baron 1974; Schauer et al. 1987; A.E. A. Granston, M. Craven, A. Schauer, D. Thompson, and D. Friedman, in prep.). A λ mutant that is able to utilize $nusA_{S.t.}$ has mutations in the N gene and $boxA_R$ (Friedman and Olson 1983). The $boxA_R$ mutation, $boxA1$, results in a $boxA$ with three Ts at the 3' end as do the P22 and 21 $boxA$ sequences. The $boxA1$ sequence deviates from the consensus by not having a 3' A (Table 1).

The importance of $boxA$ in the N-mediated antitermination reaction was underscored further by other $boxA$ mutations that result in nut regions that are less active as signals for directing N-mediated antitermination. A mutation in $\lambda boxA_L$ (a C to G change at position 3; Peltz et al. 1985), and one in $\lambda boxA_R$ (a G to T change at position 2 called $boxA5$; Olson et al. 1984; Robledo et

Table 1. $boxA$ sequences from lambdoid phages right and left nut regions and from the rmG leader region

		Effect ^a
A. Natural boxA Sequences		
	1 2 3 4 5 6 7 8 9	
$\lambda boxA$	CGCTCTTAC	
21boxA _R	TGCTCTTTA	
22boxA _R	CGCTCTTTA	
$\lambda boxA_L$	CGCTCTTAA	
21boxA _L	GGCTCTTTA	
22boxA _L	CGCTCTTTA	
rrnboxA	TGCTCTTTA	
B. Mutant lambdoid boxA sequences		
	boxA _R	
$\lambda boxA1$	CGCTCTTTC ^{1b}	+
$\lambda boxA5$	CTCTCTTAC ^{2b}	–
$\lambda boxA16$	CGCTATTAC ^{3b}	0
$\lambda boxA_{con}$	CGCTCTTTA	+
$\lambda boxA\Delta GC$	CTCTTACAC ^{4b}	–
21boxA _{con}	CGCTCTTTA	+
	boxA _L	
$\lambda boxA^c$	CGGTCTTAA ^{5b}	–

Nucleotide positions are numbered above. (A) Naturally occurring $boxA$ sequences; (B) mutant $boxA$ sequences created in λ and 21 nut regions. Changes away from wild type are underlined.

^a(+) Enhances; (–) reduces; (0) no effect (for details, see text).

^bReferences: ¹Friedman and Olson (1983); ²Olson et al. (1984);

³Robledo et al. (1990); ⁴Doelling and Franklin (1989); ⁵Peltz et al. (1985).

^cThis mutation has not been named.

al. 1990) reduce N-mediated antitermination. Moreover, deletions of the GC, as well as the GCT, at positions 2, 3, and 4 also reduce this activity (Doelling and Franklin 1989). An apparently contradictory result has been reported by Zuber et al. (1987), who found that a deletion of *boxA* did not significantly influence N-imposed antitermination. However, other studies using the same deletion reveal that it causes a fivefold reduction in N-mediated antitermination (D. Lazinski and A. Das, pers. comm.).

To further assess the activity of *boxA* in gpN action, site-directed mutagenesis was employed to change natural *boxA* sequences to conform to the consensus sequence called *boxAcon*. Studies reported demonstrate the biological consequences of these changes.

Results

Effect of altered *boxA* sequences on λ growth

The growth of λ and λ imm21 phages with different *boxA* sequences was compared in *E. coli* derivatives carrying mutant *nus* genes using efficiency of plating (eop) as the assay (see Table 2).

We first examine the effect of changes in the λ boxA_R. The *boxA1* mutation had been shown previously to suppress the inhibitory effect of the *nusA1* and *nusE71* mutations on λ growth (Friedman and Olson 1983; Schauer et al. 1987). Since *boxA1* still differs from the derived consensus sequence, *boxAcon*, by having a TTTC-3', rather than TTTA-3', we tested whether a *nut_R* region with *boxAcon* conferred any advantage for λ growth in hosts with variant *nus* alleles known not to be effectively suppressed by the *boxA1* mutation.

A comparison of the growth of λ derivatives with *nut_R* regions containing *boxAcon*, *boxA1*, or *boxA⁺* in *E. coli* having mutant or variant *nus* genes revealed a hierarchy of activity for *boxA* sequences. The data in Table 2 show that in every *E. coli* tested, λ boxA_{con} grows either as well as or better than λ boxA1 which, in turn, grows as well as or better than λ boxA⁺. The *nus* alleles sup-

pressed more effectively by *boxAcon* are *nusB5* and the hybrid *nusA_{S.t.}* in the presence of a second mutation *sneA16*. The *sneA16* mutation that maps in the *rplP* gene partially suppresses the failure in gpN action caused by some *nus* mutations (A.T. Schauer, D.L. Thompson, D. Alessi, and D.I. Friedman, in prep.).

These differences were examined in more detail by following the phage bursts through one round of growth. The results of one such experiment are shown in Figure 2, where growth of λ derivatives with the three *boxA_R* sequences was compared in a *nusB5* host at 37°C. Note that the burst of λ boxA_{con} occurs ~45 min earlier than that of λ boxA1, but eventually both phages produce the same final burst. λ boxA⁺ has a longer delay and a substantially lower final burst.

We then examined the effect of a change to *boxAcon* in the *nut_R* region of a λ imm21 derivative (Table 2). The success of the selection scheme for λ imm21*boxAcon* demonstrates a priori that *boxAcon* confers a growth advantage for λ imm21 in a *nus* variant, that is, the *imm21boxAcon* recombinant grows at 42°C in an *E. coli* hybrid that has a *nusA_{S.t.}* gene. Extension of this study to other *nus* mutants showed that the efficiency of plating of λ imm21*boxAcon* is either greater than (observed in hosts carrying one of the following alleles: *nusA_{S.t.}* or *nusA1* or *nusB5* alleles) or similar to λ imm21 (observed in a host carrying the *nusE71* allele); of those tested, there is no *nus* mutant in which λ imm21 grows better than λ imm21*boxAcon*.

Antitermination measured by expression from a *galK* fusion

To directly assess the role of the *boxA* sequence in N-mediated transcription antitermination, we employed a chromosomally located operon fusion that has the λ *p_R* promoter with its associated *nut_R* region and two downstream Rho-dependent terminators fused to the *gal* operon (Fig. 3; Reyes et al. 1979; Dambly-Chaudiere et al. 1983). The *galK* gene can thus be used as a reporter

Table 2. Efficiency of plating of λ derivatives with different *boxA* sequences

Phage	<i>boxA</i> sequence	Bacteria and relevant alleles				
		K95 <i>nusA1</i>	K450 <i>nusB5</i>	K556 <i>nusE71</i>	K4087 <i>nusA_{S.t.}</i> <i>sneA16</i>	K4092 <i>nusA_{S.t.}</i>
λ	CGCTCTTAC	(42°C) <10 ⁻⁶	(40°C) <10 ⁻⁶	(42°C) <10 ⁻⁶	(37°C) <10 ⁻⁶	
λ boxA1	CGCTCTTTC	0.9	0.2	0.7	<10 ⁻⁶	
λ boxAcon	CGCTCTTTA	0.9	1.0	0.9	0.2	
λ imm21	TGCTCTTTA	(42°C) <10 ⁻⁶	(32°C) <10 ⁻⁶	(42°C) 1.0		(42°C) <10 ⁻⁶
λ imm21 <i>boxAcon</i>	CGCTCTTTA	0.05	0.1	0.7		0.3

Bacteria were grown overnight at 37°C in LB made 0.2% in maltose, sedimented, and resuspended in 0.5 volume of 0.01 M MgSO₄. Phage lysates were diluted and plated on TB plates, using the indicated bacteria as lawns. Plates were incubated at the indicated temperature. The phage titer on each lawn was divided by the titer on K37 (the *nus⁺* isogenic parent) to give the efficiency of plating. The effect of the *nusA_{S.t.}* allele on growth of λ was tested using strain K4087 because K4092 was too restrictive.

Figure 2. Effect of *boxA_R* sequence on λ growth in a *nusB5* mutant host. Phage growth was followed through one round at 37°C according to the method referenced in Materials and methods. (▲) λ *boxA⁺*; (◆) λ *boxA1*; (■) λ *boxAcon*.

with expression of its product, galactokinase, representing a measure of transcription antitermination. Fusions with three variations of *boxA_R*—*boxA⁺*, *boxA1*, and *boxAcon*—were tested. Expression of *galK* from the fusions was studied in the presence of the *nusA1* mutation at 42°C, a condition shown above to favor growth of phages with the *boxA1* or *boxAcon* variations in their *nut_R* region. The kinetics of *galK* expression by the three fusions following the shift of log-phase cultures from 32°C to 42°C are shown in Figure 4. The temperature shift places the cultures under N-limiting conditions because of the *nusA1* mutation while simultaneously removing repression from the λ *p_R* promoter by heat-denaturing the *cI857* repressor of the prophage. Consistent with the studies measuring phage growth, we again find a hierarchical order of activity of *boxA* sequences: The fusion of the *nut* region with *boxAcon* expresses *galK* earlier and reaches a higher steady state than the fusion with *boxA1*. The fusion with the wild-type *boxA* sequence, as expected, fails to express significant levels of galactokinase under the conditions of these experiments.

Figure 3. Chromosomally located fusion of *p_R* to the *gal* operon. The map shows the essential parts of the truncated prophage with the *r32*-IS2 element and the fusion to the *gal* operon. The *nut_R* region is expanded above the map, while the fate of *p_R* transcripts when N-mediated antitermination is inactive and when it is active is shown below the map.

Figure 4. Expression of galactokinase from *p_R*-*gal* fusions in a *nusA1* host. Following a shift from 32°C to 42°C, aliquots of logarithmically growing cells were removed at the indicated times and assayed for galactokinase. Galactokinase units are defined as nmoles of galactose-1-phosphate produced per min per OD_{420 nm} of cells. The temperature shift inactivated the *cI857* repressor, permitting transcription to initiate at *p_R* (for reference, see Materials and methods). The *boxA* signals in the λ *nut_R* regions were *boxAcon* (■), *boxA1* (◆), and *boxA⁺* (▲).

Competition between *boxA* sequences

We compared the activities of *boxA* sequences by asking if any of the variations of *boxA* conferred an advantage to a *nut_R* region in competition with another *nut_R* region containing a different *boxA*. The design of the experiment was based on two components, λ r32 and plasmids with cloned *nut_R* regions. The strategy of using a plasmid to titrate functions required by λ has been employed successfully in the study of N-mediated action (Friedman and Yarmolinsky 1972; Lieb 1972) and in demonstrating that an *E. coli* *rrn* operon, which has an antiterminator, competes with λ for a common factor (Sharrock et al. 1985).

The λ r32 phages were used because the IS2, with its strong Rho-dependent terminator (De Crombrughe et al. 1973) located upstream of *cII* (Fig. 1; Brachet et al. 1970), imposes additional termination in the *p_R* operon and, thus, increases the level of antitermination required for effective transcription of downstream genes, including the *Q* late operon activator. The effect of this increased termination is easily observed using phage growth in *nus* mutants (Tomich and Friedman 1977). Because λ r32 does not form plaques on lawns of *nus* mutants at 32°C while λ does, growth of λ r32 derivatives provides a more sensitive assay of the effectiveness of the assembly of the antitermination complex at *nut_R* than does growth of the wild-type phage.

The *nut_R* regions with the various *boxA* sequences were placed downstream of the *p_{tac}* promoter (de Boer et al. 1983) in pKK223-3 (de Boer 1984) in the same orientation relative to *p_R* as they are found in the phage. Transcription of the cloned *nut_R* regions can be controlled by placing the plasmids in a host that overproduces *lac* repressor [*lacI^q*] (Muller-Hill et al. 1968). Hence, high levels of transcription of the *nut_R* insert can occur only when an inducer, such as IPTG, is added to the culture. The test for *nut_R* site competition relies on the potential

for the plasmid to compete for a limiting component of the antitermination reaction during transcription.

Figure 5 shows the results of these experiments. Phage production was measured by following one round of growth at 40°C (a single-step growth experiment). The effect of the plasmid-borne *boxA* sequences was assessed by examining λ r32 growth in the presence of the various plasmids either induced or not induced with IPTG. There are two essential findings. First, only plasmid pKBAcon (containing a *nut_R* region with the *boxAcon* sequence) has any effect on λ r32 growth (Fig. 5A). In the presence of IPTG, pKBAcon delays the appearance of the λ r32 burst. In contrast, neither the IPTG-induced parent pKK223-3 plasmid nor derivatives with cloned fragments containing other *boxA* variations (*boxA⁺*, *boxA1*,

or *boxA5*) (data not shown) has any effect on the time or size of the burst. Failure of the induced plasmid pKBA1 (with *boxA1*) to compete demonstrates the specificity of the competition by the *boxAcon* plasmid; the single-base change differentiating *boxA1* and *boxAcon* is sufficient to interfere with the burst. Second, interference is only observed if the *nut* region is transcribed. In the absence of IPTG, neither pKBAcon nor any of the other plasmids interferes with λ r32 growth. Moreover, a derivative of pKBAcon deleted for *p_{tac}* (pKBAcon Δ P) failed to compete in the presence of IPTG (data not shown).

To determine whether *boxAcon* in the context of a nonfunctioning *nut* region competes with λ r32, we used derivatives of pKK223-3 with cloned fragments containing a 21 *nut_R* region with either the *boxAcon*

Figure 5. Phage production in competition experiments. Single rounds of growth were assayed as outlined in Materials and methods. The bacteria used in these experiments are derivatives of K37 that have the *lacI^q* repressor mutation and carry either pKK223-3 or pKK223-3-derivatives with *nut_R* regions and, when indicated, pGB2 or derivative plasmids. Table 4 lists the relevant details about the plasmids. Infected bacteria were divided into two aliquots, one in which *p_{tac}* remained repressed and one in which it was induced by addition of IPTG. Infections were allowed to proceed at 40°C. Aliquots were removed at indicated times and assayed for phage production. (A) Burst of λ r32 in K3093 derivatives with either pKBAcon which has a *nut* region with *boxAcon* ($\square$, uninduced; $\blacksquare$, induced) or the control plasmid pKK223-3 ($\circ$, uninduced; $\bullet$, induced). (B) Effect of gpN on plasmid competition with λ r32. Bacteria (derivatives of K4461) were grown at 40°C prior to infection with λ r32 to induce the defective λ prophage and gpN production. Phage-infected bacteria, which were induced with IPTG, were allowed to burst as in A. The bacteria contained pKBAcon ($\blacksquare$), pKBA1, which has a *nut* region with *boxA1* ($\blacklozenge$), pKBAL, which has a *nut* region with *boxA⁺* ($\blacktriangle$), or pKK223-3 ($\bullet$). (C) Role of NusB in competition. The phage and the conditions were the same as in A, except the bacteria carried either pGB2 with pKBAcon ($\boxplus$, uninduced; $\boxtimes$, induced) or pGB2 with pKBAcon ($\square$, uninduced; $\blacksquare$, induced). (D) Role of NusA in competition. The phage and the conditions were the same as in A, except the bacteria carried either pGB2 with pKBAcon ($\square$, uninduced; $\blacksquare$, induced) or pGB2 with pKBAcon ($\boxtimes$, uninduced; $\times$, induced).

(pKBA21con) or *boxA* wild-type (pKBA21) sequence in competition experiments in the *nus*⁺ host. Since the only *N* product available in the infection is gpN_λ, the 21 *nut* region cannot function in antitermination, even though it is transcribed (Friedman et al. 1973a; Lazinski et al. 1989). When induced, neither pKBA21 nor pKBA21con causes significant interference with the growth of λr32 (data not shown).

Role of gpN

The role of gpN in this plasmid-mediated competition was assessed using a defective prophage (λcI857Δ*Bam*ΔHI) to provide additional gpN. This prophage produces high levels of gpN at temperatures >40°C (Gottesman et al. 1980). If gpN is a limiting factor, an increased amount of it would be expected to relieve the effect of plasmid competition on phage growth.

Competition between λr32 and the *nut*-containing plasmids was re-examined in the presence of prophage-supplied gpN. Consistent with the findings reported above, significant competition was observed only with the plasmid containing the *nut*_R *boxA*con, pKBAcon, in the presence of IPTG (Fig. 5B). However, instead of relieving competition, the additional gpN, if anything, supported slightly more effective competition (Fig. 5A,B). With or without extra gpN, there is a delay in the start of the λr32 burst. Without prophage-supplied gpN, in the presence of IPTG-induced pKBAcon, the burst approaches the size seen in the absence of the plasmid (Fig. 5A). With gpN expressed from the prophage, however, pKBAcon inhibits the size of the burst for the entire time course of the experiment (Fig. 5B).

Role of Nus factors

To determine whether the limiting factor(s) in the competition assay is an *E. coli* Nus protein, we employed plasmids with cloned *nus* genes as the source of additional *nus* protein production. These plasmids were derivatives of pGB2 (Churchward et al. 1984), a low-copy-number plasmid (~10 per cell) compatible with pKK223-3. These pGB2*nus* derivatives express their respective *nus* genes as shown by complementation with appropriate *nus* mutations (data not shown).

A pGB2 derivative with a cloned 2.5-kb fragment containing the *nusB* gene (pGB2*nusB*) significantly reduces competition by the induced pKBAcon plasmid (Fig. 5C). The reduction in competition is due to the insert containing the *nusB* gene, since a control pGB2 plasmid has no effect on the competition (Fig. 5C). The role of the *nusB* gene was directly tested using a derivative of pGB2*nusB* containing a deletion and substitution within the *nusB* gene, pGB2Δ*nusB*. Unlike the parent plasmid, pGB2Δ*nusB* has no effect on the competition by pKBAcon (data not shown). There was no effect of pGB2*nusB* on the growth of λr32 in the presence of a control such as pKK223-3, ruling out the trivial explana-

tion that overexpression of *nusB* results in a general enhancement of λ growth.

We then determined whether interference with competition was specific to the *nusB* gene product or could merely be due to the excess expression of any *nus* gene. To test for possible involvement of NusA, we used another pGB2 derivative, pGB2*nusA*, which contains a cloned 5-kb fragment that includes the *nusA* gene. As shown in Figure 5D, pGB2*nusA* does not interfere significantly with pKBAcon competition.

Competition assayed by expression from a *galK* fusion

The chromosomally located *p_R-gal* fusion was employed to directly test whether N-mediated antitermination directed through the *nut_R* signal was the basis for the plasmid competition. K5319, the *nus*⁺ bacteria used in these studies, also carries the *lacI^q* mutation.

In the first set of experiments, competition was assessed using a fusion with a *nut_R* region containing *boxA*con (Fig. 6A). Three plasmids were employed, pKK223-3, the *boxA*⁺ derivative (pKBAL), and the *boxA*con derivative (pKBAcon). When transcription of the *nut* region of pKBAcon is induced, competition is observed by a significant reduction in *galK* expression from the fusion. The plasmid-based *nut_R* with wild-type *boxA* fails to compete even when induced.

In the second set of experiments, the effect of *nusB* expression on competition was assessed using the same fusion. Three plasmids were employed, pKBAcon, pGB2, and pGB2*nusB*. As shown in Figure 6B, the induced pKBAcon plasmid reduces *galK* expression from the fusion. In the presence of the *nusB* plasmid, the level of *galK* expression goes up. Note that pGB2*nusB* has no effect on the expression from the fusion if pKBAcon is not induced, offering further evidence that overexpression of *nusB* does not cause a nonspecific increase in transcription from *p_R*.

Plasmid copy number

We determined whether plasmid copy number was an explanation for the observed difference in growth of λr32 in the plasmid competition experiments by measuring plasmid DNA. Plasmid DNA isolated from bacteria with the pKK223-3 set of *boxA* plasmids grown under the conditions used in the competition experiments was compared with controls for DNA loss (see Materials and methods). No significant differences in plasmid copy number were found under any of the conditions of our experiments (data not shown).

Discussion

The experiments reported here address the question of the importance of the *boxA* sequence in λ N-mediated antitermination by demonstrating that (1) the full extent of the consensus *boxA* sequence defined previously by sequence comparison is functionally important, (2) transcription is required for the *boxA* signal to be active, (3)

Figure 6. Competition assayed from a p_R -gal fusion containing a nut_R with *boxAcon* (for details, see Fig. 4). The host strain, K5319, is *nus*⁺ and *lacI*^q. Bacteria grown at 32°C in LB (containing appropriate antibiotics to maintain plasmids) were diluted into LB with the appropriate antibiotics and, where indicated, IPTG. The diluted cells were shifted to 40°C, and aliquots were removed at the indicated times and assayed for galactokinase activity. (A) Role of *boxA* in competition. The bacteria contained one of the following plasmids: pKBAcon, which has a nut_R region with *boxAcon* ($\square$, uninduced; $\blacksquare$, induced); pKBAL, which has a nut_R region with *boxA* wild type ($\triangle$, uninduced; $\blacktriangle$, induced); or pKK232-3 ($\circ$, uninduced; $\bullet$, induced). (B) Role of NusB in competition. The bacteria contained pGB2nusB with pKBAcon ($\circ$, uninduced; $\bullet$, induced) or pGB2 with pKBAcon ($\square$, uninduced; $\blacksquare$, induced).

the nature of the *boxA* signal influences the affinity of the antitermination complex for the host-encoded *nusB* gene product, (4) this competition is only observed when the *boxA* signal is part of a *nut* region activated by gpN, and (5) the optimally active *boxA* sequence may not always have been selected.

Defining the optimal *boxA* sequence

P22 served as the prototype for designing alterations in *boxA_R*, because its *boxA_R* corresponds to the consensus, *boxAcon* (Table 1), and its growth is relatively unaffected by the presence of mutations in *nus* genes (Hiliker and Botstein 1976; Friedman and Olson 1983; Schauer et al. 1987). This suggested that one or more elements of the termination-antitermination system of P22 (i.e., gpN, terminators, or *nut*) might either enhance antitermination or reduce termination.

Fortuitously, natural variants of the *boxA* sequence provided the means to test whether the consensus *boxA* contributes to this enhanced action of P22 gpN. Changing both the 21 and λ sequences to match *boxAcon* resulted in an increased ability to grow in *nus* variants under conditions where parental phages with nonconsensus (wild-type or altered) *boxA* sequences failed to grow.

In a previous study, we demonstrated that a change at position 8 of λ *boxA_R* to a T, resulting in TC 3' (*boxA1*), enhances the ability of λ to grow in *nus* mutants (Friedman and Olson 1983), demonstrating that the wild-type sequence, as tested by this assay, does not have optimal activity. We now show that a second change at nucleotide 9 (resulting in *boxAcon*) extends the range of λ growth to include additional *nus* mutants. Thus, as *boxA_R* is changed to conform more closely to the consensus sequence, the range of *nus* mutants in which the resulting λ grows is increased, giving the following hierarchical order of functional activity, *boxAcon* > *boxA1* > *boxA*⁺.

A p_R -gal fusion was used to directly and quantitatively assess gpN-*nut_R* action in promoting antitermination. Results obtained with the fusions correspond exactly to the those obtained with the phages. This supports our contention that the growth patterns we observe with the phages are direct consequences of the strength of the antitermination reaction at *nut_R* and also confirms that the plasmid competition assay assesses effects on antitermination.

Involvement of *boxA* in antitermination

The results of the plasmid competition studies argue that *boxA*, or a part of that sequence, recognizes an antitermination factor. Using the pKK223-3 plasmids with *nut_R* regions under p_{tac} control, we find that transcription of a *nut_R*-containing *boxAcon* reduces growth of infecting λ r32 phages and expression from the p_R -gal fusion. Plasmids with *boxA*⁺ or *boxA1* do not compete. Because *boxAcon* enhances *nut*-directed antitermination in *cis* but reduces it in *trans*, we conclude that *boxAcon* competes more avidly than other *boxA* sequences for a factor required in N-mediated antitermination. This conclusion may seem surprising in light of the suggestion that *boxA* is a component of the *rutA* site required for action of Rho at t_{R1} (Chen et al. 1986; Chen and Richardson 1987). Perhaps *boxAcon* only appears to increase antitermination, but actually reduces the af-

finity in the *nus* region for a protermination factor such as Rho. According to this scenario, the reduced level of termination factor loading at *boxAcon* would diminish downstream termination which, in turn, would reduce the requirement for antitermination at the downstream terminators. Thus, it would appear that antitermination had been improved.

Although the studies showing good growth of λ *boxAcon* in *nus* mutants are consistent with this idea, the results of the plasmid competition studies argue against it. Even if the transcribed plasmid failed to sequester any of the hypothetical termination factor, the concentration of that factor in those bacteria should be no different than in the control bacteria without the plasmid. Therefore, there should be no difference in termination factor available to act on the infecting phage and the burst of phage should be the same in the two classes of bacteria, that is, *boxAcon* should only influence antitermination in *cis*. However, the transcribed plasmid-based *boxAcon* signal acts in *trans* to reduce λ r32 growth, suggesting that the sequestered factor is not required for termination, but is for antitermination.

boxA—A signal for NusB?

Our observation that the competition by the plasmid-borne *boxAcon* is reversed in the presence of NusB expressed from a compatible plasmid demonstrates a linkage between *boxA* and NusB action. That this reversal is specifically due to NusB action is proved by the failure of a derivative plasmid with a deletion and substitution in the *nusB* gene to alleviate competition. Since competition is still observed in the presence of a similar construct expressing *nusA*, the reversal of competition must not result merely from increased expression of Nus products in general. What is particularly striking is the role of a single nucleotide change at the 3' end of *boxA* in effecting this competition for NusB.

These results are consistent with studies showing that antitermination directed by the leader region of *rnn* operons that is dependent on an intact *boxA* sequence (Berg et al. 1989) is reduced in the presence of the *nusB5* mutation (Sharrock et al. 1985). It has been estimated that there are 6000–7000 molecules of NusB per cell in fast growing *E. coli* (Swindle et al. 1988), corresponding to ~50–80% of the number of RNA polymerase molecules. As pointed out by Swindle et al. (1988), much of the NusB could be tied up in transcription of *rnn* operons, which account for 27–67% of transcription in *E. coli*. Our finding of competition for NusB is consistent with this idea and suggests that either most of the NusB is sequestered or the N complex has a low affinity for NusB.

It is attractive to conclude that our results present in vivo evidence that *boxA*, as originally suggested by D. Court (pers. comm.), is a signal for NusB. Indeed, Horwitz et al. (1987) have suggested that NusB is necessary specifically for the functioning of *boxA*. Certainly, such a conclusion might seem warranted from our observation that changing one base in *boxA* causes an observ-

able sequestration of NusB. However, it is possible that the sequestration of NusB is a secondary effect resulting from increased activity of another component of the N–Nus interaction. According to this model, *boxAcon* would increase the action of another Nus factor at *nus*, enhancing N–Nus complex formation there. The available supply of NusB would then be depleted by its increased utilization in the formation of those complexes. Whether or not *boxA* is a primary site for NusB, our results demonstrate that NusB is a limiting factor in the N-mediated antitermination reaction.

The N–Nus complex

The demonstration that transcription of the *nus* region is required for *boxA* competition suggests that the *boxA* signal is read from the RNA and not the DNA. Although previous studies have suggested that transcription of the *nus* region is required for N-mediated antitermination (Olson et al. 1984; Warren and Das 1984; Zuber et al. 1987), the work reported here offers the first conclusive evidence that activation of the *boxA* signal, per se, requires transcription of the *nus* region. Thus far, the only antitermination factor shown to bind to RNA is NusA (Tsugawa et al. 1985). In those studies NusA binding appears to be specific for RNA containing a *nus* region, but the binding occurred upstream of *nus_R* in the *cro* gene sequences that obviously are not conserved in *nus* sites.

Our studies, employing the *nus* region of 21, showed that merely transcribing a *nus* region with *boxAcon* is not sufficient to activate that *boxA* signal for competition in the absence of the proper gpN. This suggests that competition results from the formation of a larger complex activated by the full *nus* signal and containing, at a minimum, gpN.

Why not the best?

If *boxAcon* is the optimal signal, why do λ and 21, unlike their P22 cousin, have a less-than-optimal signal? To begin, we note that a number of λ -encoded factors apparently have less than optimal activity. As in the case of *boxA*, the lowered activities of the naturally occurring factors were demonstrated by isolating mutants that appeared to function more effectively. Interestingly, all of these phage-encoded proteins or signals interact with host-encoded proteins. Examples are gpNu1 (acts in formation of mature phage genome and subsequent packaging; Feiss et al. 1988; Granston et al. 1988), gpint (required for integrative recombination; Miller et al. 1980), and gpN (Friedman and Ponce-Campos 1975; Franklin 1985; Schauer et al. 1987). Action of gpNu1 is enhanced by and gpint requires the histone-like protein IHF (for review, see Friedman 1988b). Action of gpN requires the Nus proteins. Thus, we suggest that evolutionary pressure selects for the less-than-optimal sequence to maintain regulatory pathways. In the case of the N-antitermination system, the time during λ development when terminators are transcended influences the coordination of functions involved in the lysis–ly-

sogeny decision (Wulff and Rosenberg 1983). Indeed, a λ mutant with a more active N-mediated antitermination system at *nut_R* is channeled toward lysogeny (D. Friedman, unpubl.). Thus, what appears to be a less active sequence may actually be optimal for its biological role. We cannot explain why P22 functions with the optimal *boxA* sequence, except to note that the mechanism for deciding between lysis and lysogeny differs significantly in λ and P22 (Susskind and Youderian 1983).

Materials and methods

Media

The media used in these studies have been described (Miller and Friedman 1980). LBM is LB broth containing 0.2% maltose. IPTG was used at a final concentration of 1 mM.

Bacteria and phage

A list of bacteria and their relevant characteristics are given in Table 3. Phages and sources are as follows: λ cI857 (NIH collection); λ r32, which carries the *r32* insertion; an IS2 inserted between the *nut* region and the *cII* gene (Brachet et al. 1970) (NIH collection); λ boxA1 (Friedman and Olson 1983); λ imm21 (hy5) (Liedke-Kulke and Kaiser 1967); and M13mp8 and M13mp9 (Yanisch-Perron et al. 1985). λ boxAcon, λ r32boxAcon, and λ imm21boxAcon were constructed during this work.

Construction of chromosomal *p_R-gal* fusions

The altered λ boxA sequences were crossed from derivatives of λ r32 by homologous recombination into a *p_R-IS2-gal* fusion (see Fig. 3; Reyes et al. 1979) in strain K3929, which carries the *nusA1* allele and has the *cro62* mutation (Olson et al. 1982). K3929 is phenotypically Gal⁻ at 42°C even though repression is off, because the N-antitermination reaction is not active. This recombination was possible because λ r32 has the same genetic arrangement as the *p_R-gal* fusion from the left of immunity through the 5' two-thirds of the IS2. Lysates of λ r32 derivatives with either *boxA1* or *boxAcon* were UV-irradiated (General Electric lamp no. G8T5) at a dose of 630 ergs/mm² in phosphate

buffer (50 mM Na₂HPO₄, 20 mM KH₂PO₄, and 70 mM NaCl) and then used to infect cultures of K3929 at a multiplicity of five phage per bacterium. After adsorption, infected bacteria were allowed to grow in LB broth. Based on the assumption that the mutant *boxA* sequences should suppress the effect of the *nusA1* mutation, candidate recombinants were identified as Gal⁺ colonies at 42°C. Backcrosses to λ r32 proved that these Gal⁺ fusions did have the mutant *boxA* sequences.

Plasmids

Constructs were made by employing standard cloning techniques (Maniatis et al. 1982). Relevant plasmid characteristics are listed in Table 4. A promoterless derivative of pKBAcon, pKBAcon Δ P, was constructed by deleting a *Bam*HI–*Eco*RI fragment containing *p_{tac}*. pGB2 Δ nusB was constructed by removing a 240-bp internal fragment from the *nusB* gene in pGB2nusb and inserting a 1.35-kb *Sca*I fragment from pBR325 that contains the *cam^R* gene (Bolivar 1978).

Plasmid DNA preparation and transformation procedures were performed according to Maniatis et al. (1982). pGB2 derivatives were prepared essentially according to the published procedure (Churchward et al. 1984).

Single-step growth experiments and site-directed mutagenesis

Single-step growth experiments were performed as outlined in Friedman et al. (1973b).

The method employed for site-directed mutagenesis was that used in Olson et al. (1984). The *nut_R* region with the *boxA1* variation (Friedman and Olson 1983) was cloned in M13mp9, and the sequence of the mutagenic oligonucleotide primer was 5'-CCCCGCTCTTTAACATTCC yielding *nut_R* λ boxAcon. The *nut_{R21}* region on an *Eco*RI–*Hind*III fragment from pRZnut-21 was cloned in M13mp8, and the sequence of the mutagenic oligonucleotide primer was 5'-GGCCAGAACTGTTAAAGAGC-GATTTGC, yielding *nut_{R21}*boxAcon.

Construction of phage with mutant *boxA* sequences

Method An overnight culture (0.1 ml) of an *E. coli* carrying a plasmid with the desired *boxA* sequence was inoculated into 10

Table 3. Bacteria

Strain	Relevant genotype	Parent*	Source
K37	<i>nus⁺</i>	NA	National Institutes of Health
K95	<i>nusA1</i>	K37	University of Michigan
K450	<i>nusB5</i>	K37	University of Michigan
K556	<i>nusE71</i>	K37	University of Michigan
K1102	<i>nusA_{s.t.}</i>	NA	L.S. Baron
K1227	<i>p_R/cro62/gal</i> fusion	OR1150	University of Michigan
K3093	<i>lacI^a</i>	K37	University of Michigan
K3929	<i>p_R/cro62/gal/nusA1</i> fusion	K1227	University of Michigan
K3930	<i>p_R/boxA1/gal/nusA1</i> fusion	K3929	University of Michigan
K4087	<i>nusA_{s.t.}/sneA16</i>	K37	University of Michigan
K4092	<i>nusA_{s.t.}</i>	K37	University of Michigan
K4461	<i>lacI^a λcI857ΔBamΔHI</i>	K3093	University of Michigan
K5310	<i>p_R/boxAcon/gal/nusA1</i> fusion	K3929	University of Michigan
K5319	<i>p_R/boxAcon/gal/lacI^a <i>nus⁺</i> fusion</i>	K5310	University of Michigan
K5693	<i>p_R/gal/nusA1</i> fusion	OR1150	University of Michigan
JM101	<i>lacI^a, lacZΔm15</i>	NA	J. Messing
DH5 α	<i>lacZΔm15, recA1</i>	NA	Bethesda Research Labs

*[NA] Not applicable.

Table 4. *Plasmids and M13 derivatives*

Plasmid	Parent vector	Relevant fragment or characteristic ^a	Donor	Source
pCG69	pCR1	<i>EcoRI</i> with <i>imm21</i> (from <i>red</i> to <i>O</i>)	λ <i>imm21</i>	C. Georgopoulos
pMC100	pBR322	<i>EcoRI</i> with <i>imm21</i>	pCG69	this work
pRZ- <i>nut</i> ₂₁	pRZ152	<i>ClaI</i> - <i>EcoRI</i> with <i>nut</i> _{R21}	pMC100	this work
pBA7-6	pBR322	<i>EcoRI</i> - <i>HindIII</i> <i>nut</i> _{RA} with <i>boxA1</i>	M13 <i>boxA1</i>	this work
pBAcon	pBR322	<i>EcoRI</i> - <i>HindIII</i> <i>nut</i> _{RA} with <i>boxAcon</i>	M13 <i>boxAcon</i>	this work
pT21	pUC18	<i>EcoRI</i> - <i>HindIII</i> <i>nut</i> _{R21} wild-type	M13 <i>nut</i> _{R21}	this work
pC21	pUC18	<i>EcoRI</i> - <i>HindIII</i> <i>nut</i> _{R21} with <i>boxAcon</i>	M13 <i>nut</i> _{R21} <i>boxAcon</i>	this work
pKK223-3	pBR322	cloning site downstream of <i>p</i> _{tac}		de Boer (1983)
pKBAL	pKK223-3	λ <i>boxA</i> _R ⁺	M13- <i>boxA</i> ⁺	this work
pKBA1	pKK223-3	λ <i>boxA1</i>	pBA7-6	this work
pKBA5	pKK223-3	λ <i>boxA5</i>	M13- <i>boxA5</i>	this work
pKBAcon	pKK223-3	λ <i>boxAcon</i>	pBAcon	this work
pKBA21	pKK223-3	21 <i>boxA</i> _R ⁺ <i>ClaI</i> - <i>HincII</i>	pT21	this work
pKBA21con	pKK223-3	21 <i>boxAcon</i> <i>ClaI</i> - <i>HincII</i>	pC21	this work
pKBAcon Δ P	pKBAcon	λ <i>boxAcon</i> Δ <i>p</i> _{tac}		this work
pGB2	pSC101			Churchward et al. (1984)
pGB2 <i>nusB</i>	pGB2	<i>nusB</i>		F. Kappel (via C. Georgopolous)
pGB2 <i>nusA</i>	pGB2 <i>nusB</i>	<i>nusA</i>		this work
M13 <i>nut</i> _{R21}	pGB2	<i>nusA</i>	pNAG2010	this work
M13 <i>nut</i> _{R21} <i>boxAcon</i>	M13mp18	<i>EcoRI</i> - <i>HindIII</i> <i>nut</i> _{R21}	pRZ- <i>nut</i> _{R21}	this work
	M13mp18	<i>EcoRI</i> - <i>HindIII</i> <i>nut</i> _{R21}	M13mp8 <i>nut</i> _{R21}	this work
	<i>nut</i> _{R21}		mutagenesis	
M13 <i>boxA1</i>	M13mp9	<i>nut</i> _R - <i>boxA1</i>	M13mp9 <i>nut</i> _R	Friedman and Olson (1983)
			mutagenesis	

^aThe λ inserts contain DNA from coordinates 38214 to 38350 (Daniels et al. 1983). The 21 *ClaI* and *HincII* sites are in the *cro* and *cII* genes, respectively (Schwarz 1980). Other sites are in λ or the donor vectors.

ml of LB broth made 0.01 M in CaCl₂ and containing 30 μ g/ml ampicillin. The bacteria were infected with phage at a multiplicity of 5 and incubated at 37°C for 2 hr. The resulting lysate was treated with chloroform, and recombinants were selected using appropriate bacterial lawns (see text).

Strategy We have shown previously that λ and λ r32 derivatives with the *boxA1* mutation in *nut*_R grow in *nusA1* mutants under conditions where the *nut*_R⁺ derivatives fail to grow (Friedman and Olson 1983; Schauer et al. 1987). We assumed that λ and 21 derivatives with *boxAcon* in their *nut*_R regions might also grow in hosts with mutant or variant *nusA* genes under conditions where the parent phages with wild-type *nut*_R regions fail to grow, because of a failure in N-mediated antitermination. Recombinants derived from λ and λ r32 with *boxAcon* were isolated from a cross with pBAcon using K95 (*nusA1*) as the selective host at 42°C. Putative derivatives of λ and λ r32 with *boxAcon* were found, respectively, at a frequency of 10⁻⁴ and 10⁻³. Recombinants derived from 21 with *boxAcon* were isolated from a cross with pC21. The selecting bacterium, K1102, contains a hybrid *nusA* gene with the 5' 85% from *S. typhimurium* and the 3' 15% from *E. coli* (Friedman and Olson 1983; Schauer et al. 1987) and does not support growth of phage

21 at 42°C. Recombinant P21 derivatives that grew under these conditions were found at a frequency of 10⁻⁵. Control crosses with plasmids containing wild-type *nut* regions yielded phages that grew in the selecting bacteria at much lower frequencies (<10⁻⁶). DNA sequencing confirmed that the indicated plasmids and phages contained the *boxAcon* sequence.

Competition infections

Bacteria were diluted from an overnight culture into LBM containing ampicillin at 30 μ g/ml (spectinomycin at 50 μ g/ml was included if pGB2 derivatives were also used) and grown to a concentration of $\sim$ 10⁸/ml. The bacteria were sedimented by centrifugation, resuspended in 0.1 volume of 0.01 M MgSO₄ and, where indicated, 1 mM IPTG, mixed with the phage at a multiplicity of 0.1, and incubated at room temperature for 20 min to allow phage to adsorb. Infected bacteria were diluted into LB made 0.01 M in MgSO₄ and 30 μ g/ml in ampicillin and, where indicated, 1 mM in IPTG and/or spectinomycin at 50 μ g/ml, and incubated at the appropriate temperature. Aliquots were removed at the indicated times, treated with chloroform to lyse bacteria, and titrated for viable phage. Burst represents phage output/phage input.

Measurement of plasmid copy number

Ten milliliters of culture from burst experiments was combined with 1 ml of overnight culture of DH5 α with pUC8 and sedimented. Plasmid DNAs were isolated and treated with a restriction enzyme that cleaved both plasmids once. Aliquots of plasmid preparations were examined on an agarose gel using ethidium bromide staining to visualize bands. A comparison with the pUC8 standard provided a measure of the DNA concentration of the experimental plasmid.

DNA sequencing, galactokinase assay, and enzymes

DNA sequencing was performed using the methods in Biggin et al. (1983), with slight variations. Galactokinase assays were performed according to the published method (Adhya and Miller 1979). All enzymes were purchased from standard commercial sources.

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