Transcription-dependent competition for a host factor: the function and optimal sequence of the phage λboxA 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 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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of these phages were derived from λ. λimm22 is particularly useful, because P22 normally does not infect E. coli, whereas λimm22 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<sub>b</sub>, comprised of the 5' 85% from S. typhimurium and the 3' 15% portion from E. coli, supports growth of λimm22 at all temperatures and λimm21 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. Granston, M. Craven, A. Schauer, D. Thompson, and D. Friedman, in prep.]. A λ mutant that is able to utilize nusA<sub>b</sub> has mutations in the N gene and boxA<sub>b</sub> [Friedman and Olson 1983]. The boxA<sub>b</sub> mutation, boxA<sub>b1</sub>, 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 λ boxA<sub>b</sub> [a C to G change at position 3; Peltz et al. 1985], and one in λ boxA<sub>b1</sub> [a G to T change at position 2 called boxA5; Olson et al. 1984; Robledo et

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<sub>con</sub> [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 λimm22 and λimm21 [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. λimm22 is particularly useful, because P22 normally does not infect E. coli, whereas λimm22 has the λ host range.

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

| Effect* | A. Natural boxA Sequences |
|---------|--------------------------|
| +       | λboxA CGCTCTTTTA         |
| -       | 21boxA<sub>b</sub> CCGCTCTTTTA |
| -       | 22boxA<sub>b</sub> CGCTCTTTTA |
| +       | boxA<sub>b</sub> CGCTCTTTTA |
| -       | boxA<sub>Δ</sub> boxA<sub>b1</sub> |
| -       | boxA<sub>b1</sub> CCGCTCTTTTA |

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.

* Enhances; | reduces; | no effect (for details, see text).

References: 1Friedman and Olson (1983); 2Olson et al. (1984); 3Robledo et al. (1990); 4Doellinger and Franklin (1989); 5Peltz et al. (1985).

*This 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 revealed 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 λimm21 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 λboxAR. 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 nutR 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 nutR 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, λboxAcon grows either as well as or better than λboxA1 which, in turn, grows as well as or better than λboxA*. The nus alleles suppressed more effectively by boxAcon are nusB5 and the hybrid nusASt 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* sequences was compared in a nusB5 host at 37°C. Note that the burst of λboxAcon occurs ~45 min earlier than that of λboxA1, but eventually both phages produce the same final burst. λboxA* has a longer delay and a substantially lower final burst.

We then examined the effect of a change to boxAcon in the nutR region of a λimm21 derivative (Table 2). The success of the selection scheme for λimm21boxAcon demonstrates a priori that boxAcon confers a growth advantage for λimm21 in a nus variant, that is, the imm21boxAcon recombinant grows at 42°C in an E. coli hybrid that has a nusASt gene. Extension of this study to other nus mutants showed that the efficiency of plating of λimm21boxAcon is either greater than [observed in hosts carrying one of the following alleles: nusASt or nusA1 or nusB5 alleles] or similar to λimm21 [observed in a host carrying the nusE71 allele], of those tested, there is no nus mutant in which λimm21 grows better than λimm21boxAcon.

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 pr promoter with its associated nutR 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 | Bacteria and relevant alleles |
|---|---|
| Phage | boxA sequence | K95 nusA1 | K450 nusB5 | K556 nusE71 | K4087 nusASt sneA16 | K4092 nusASt |
| λ | CGCTCTTAC | [42°C] | [40°C] | [42°C] | [37°C] |
| λboxA1 | CGCTCTTTC | <10^-6 | <10^-6 | <10^-6 | <10^-6 |
| λboxAcon | CGCTCTTAA | 0.9 | 0.2 | 0.7 | <10^-6 |
| λimm21 | TGCTCTTAA | 0.9 | 1.0 | 0.9 | 2.0 |
| λimm21 boxAcon | CGCTCTTAA | <10^-6 | <10^-6 | 1.0 | <10^-6 |

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 nusASt allele on growth of λ was tested using strain K4087 because K4092 was too restrictive.
with expression of its product, galactokinase, representing a measure of transcription antitermination. Fusions with three variations of boxA 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 boxAI or boxAcon variations in their nut 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 Pr promoter by heat-denaturing the cl857 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 boxAI. The fusion with the wild-type boxA sequence, as expected, fails to express significant levels of galactokinase under the conditions of these experiments.

**Figure 2.** Effect of boxA boxB sequence on λ growth in a nusBS mutant host. Phage growth was followed through one round at 37°C according to the method referenced in Materials and methods. (▲) λboxA−; (●) λboxA1; (■) λboxAcon.

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

**BoxA transcription signal**

**Figure 4.** Expression of galactokinase from pr-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 clts857 repressor, permitting transcription to initiate at pr for reference, see Materials and methods. The boxA signals in the λ nut regions were boxAcon □, boxAI ●, 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 region in competition with another nut region containing a different boxA. The design of the experiment was based on two components, λ32 and plasmids with cloned nut 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 rrr operon, which has an antiterminator, competes with λ for a common factor (Sharrock et al. 1985).

The λ32 phages were used because the IS2, with its strong Rho-dependent terminator (De Crombrugghe et al. 1973) located upstream of cI [Fig. 1; Brachet et al. 1970], imposes additional termination in the pr 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 nut mutants (Tomich and Friedman 1977). Because λ32 does not form plaques on lawns of nut mutants at 32°C while λ does, growth of λ32 derivatives provides a more sensitive assay of the effectiveness of the assembly of the antitermination complex at nut than does growth of the wild-type phage.

The nut regions with the various boxA sequences were placed downstream of the p lac promoter (de Boer et al. 1983) in pKK223-3 (de Boer 1984) in the same orientation relative to pr as they are found in the phage. Transcription of the cloned nut regions can be controlled by placing the plasmids in a host that overproduces lac repressor [lacI'] (Muller-Hill et al. 1968). Hence, high levels of transcription of the nut insert can occur only when an inducer, such as IPTG, is added to the culture. The test for nut 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 λ32 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 sequence with the boxAcon sequence) has any effect on λ32 growth (Fig. 5A). In the presence of IPTG, pKBAcon delays the appearance of the λ32 burst. In contrast, neither the IPTG-induced parent pKK223-3 plasmid nor derivatives with cloned fragments containing other boxA variations (boxA1, 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 λ32 growth. Moreover, a derivative of pKBAcon deleted for pTac (pKBAconAP) 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 λ32, we used derivatives of pKK223-3 with cloned fragments containing a 21 nut 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 repressor mutation and carry either pKK223-3 or pKK223-3-derivatives with nut 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 pTac 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 λ32 in K3093 derivatives with either pKBAcon which has a nut region with boxAcon (□, uninduced; ■, induced) or the control plasmid pKK223-3 (○, uninduced; ●, induced). (B) Effect of gpN on plasmid competition with λ32. Bacteria (derivatives of K4461) were grown at 40°C prior to infection with λ32 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 (■), pKBA1, which has a nut region with boxA1 (○), pKBAL, which has a nut region with boxA5 (▲), or pKK223-3 (●). (C) Role of NusB in competition. The phage and the conditions were the same as in A, except the bacteria carried either pGB2nusB with pKBAcon (□ □, uninduced; ■, induced) or pGB2 with pKBAcon (○, uninduced; ●, 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 (□ □, uninduced; ■, induced) or pGB2nusA with pKBAcon (▲, uninduced; ×, induced).
Role of gpN

The role of gpN in this plasmid-mediated competition was assessed using a defective prophage (λcI857ΔBamHI) 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 λ32 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^ boxA con, pKBA21 con, 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 λ32 burst. Without prophage-supplied gpN, in the presence of IPTG-induced pKBA21 con, the burst approaches the size seen in the absence of the plasmid (Fig. 5A). With gpN expressed from the prophage, however, pKBA21 con 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 nut genes as the source of additional nut 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 pGB2mut derivatives express their respective nut genes as shown by complementation with appropriate nut mutations (data not shown).

A pGB2 derivative with a cloned 2.5-kb fragment containing the nut^ gene (pGB2nutB) significantly reduces competition by the induced pKBA21 con plasmid (Fig. 5C). The reduction in competition is due to the insert containing the nut^ gene, since a control pGB2 plasmid has no effect on the competition (Fig. 5C). The role of the nut^ gene was directly tested using a derivative of pGB2nutB containing a deletion and substitution within the nut^ gene, pGB2ΔnutB. Unlike the parent plasmid, pGB2ΔnutB has no effect on the competition by pKBA21 con (data not shown). There was no effect of pGB2nutB on the growth of λ32 in the presence of a control such as pKK223-3, ruling out the trivial explanation that overexpression of nut^ results in a general enhancement of λ growth.

We then determined whether interference with competition was specific to the nut^ gene product or could merely be due to the excess expression of any nut gene. To test for possible involvement of NusA, we used another pGB2 derivative, pGB2nutA, which contains a cloned 5-kb fragment that includes the nut^ gene. As shown in Figure 5D, pGB2nutA does not interfere significantly with pKBA21 con competition.

Competition assayed by expression from a galK fusion

The chromosomally located p^gal fusion was employed to directly test whether N-mediated antitermination directed through the nut^ signal was the basis for the plasmid competition. K5319, the nut^ bacteria used in these studies, also carries the lac^ mutation.

In the first set of experiments, competition was assessed using a fusion with a nut^ region containing boxA con (Fig. 6A). Three plasmids were employed, pKK223-3, the boxA^ derivative (pKBA), and the boxA con derivative (pKBA21 con). When transcription of the nut^ region of pKBA21 con is induced, competition is observed by a significant reduction in galK expression from the fusion. The plasmid-based nut^ with wild-type boxA fails to compete even when induced.

In the second set of experiments, the effect of nut^ expression on competition was assessed using the same fusion. Three plasmids were employed, pKBA21 con, pGB2, and pGB2nutB. As shown in Figure 6B, the induced pKBA21 con plasmid reduces galK expression from the fusion. In the presence of the nut^ plasmid, the level of galK expression goes up. Note that pGB2nutB has no effect on the expression from the fusion if pKBA21 con is not induced, offering further evidence that overexpression of nut^ does not cause a nonspecific increase in transcription from p^gal.

Plasmid copy number

We determined whether plasmid copy number was an explanation for the observed difference in growth of λ32 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)
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, because its boxA corresponds to the consensus, boxAcor [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 boxAcor 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 λboxA to a T, resulting in TC3' boxA, 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 boxAcor) extends the range of λ growth to include additional nus mutants. Thus, as boxAcor 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, boxAcor > boxA > boxAcor.

A pR−gal fusion was used to directly and quantitatively assess gpN–nut action in promoting antitermination. Results obtained with the fusions correspond exactly to 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 nutR 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 nutR regions under pR control, we find that transcription of a nutR-containing boxAcor reduces growth of infecting λ32 phages and expression from the pR−gal fusion. Plasmids with boxAcor or boxA do not compete. Because boxAcor enhances nut-directed antitermination in cis but reduces it in trans, we conclude that boxAcor 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 rna site required for action of Rho at tr5 [Chen et al. 1986; Chen and Richardson 1987]. Perhaps boxAcor only appears to increase antitermination, but actually reduces the af-
finity in the nut 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 λboxA con 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 boxA con signal acts in trans to reduce λ32 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 boxA con 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 rri 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 rri 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 observable 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–Nut interaction. According to this model, boxAcon would increase the action of another Nus factor at nut, 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 nut 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 nut 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 nut 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 nut region, but the binding occurred upstream of nutA in the cro gene sequences that obviously are not conserved in nut sites.

Our studies, employing the nut region of 21, showed that merely transcribing a nut 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 nut 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-
sugenic decision (Wulff and Rosenberg 1983). Indeed, a λ mutant with a more active N-mediated antitermination system at nutA 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: λc1857 [NIH collection], λ32, which carries the c32 insertion, an IS2 inserted between the nut region and the clif gene [Brauchet et al. 1970] [NIH collection], boxA1 [Friedman and Olson 1983; λimm21 (hY5) [Liedke-Kulke and Kaiser 1967], and M13mp8 and M13mp9 [Yanisch-Perron et al. 1985]. λboxAcon, λ32boxAcon, and λimm21boxAcon were constructed during this work.

Construction of chromosomal p<sub>b</sub>gal fusions

The altered boxA sequences were crossed from derivatives of λ32 by homologous recombination into a p<sub>b</sub>gal fusion (see Fig. 3; Reyes et al. 1979) in strain K3929, which carries the nutA1 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 λ32 has the same genetic arrangement as the p<sub>b</sub>gal fusion from the left of immunity through the 5'-two-thirds of the IS2. Lysates of λ32 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 nutA1 mutation, candidate recombinants were identified as Gal+ colonies at 42°C. Backcroses to λ32 proved that these Gal+ fusions did have the mutant boxA sequences.

Table 3. Bacteria

| Strain | Relevant genotype | Parent* | Source |
|--------|-------------------|---------|--------|
| K37    | nus<sup>+</sup>   | NA      | National Institutes of Health |
| K95    | nusA1             | K37     | University of Michigan |
| K450   | nusB5             | K37     | University of Michigan |
| K556   | nusE71            | K37     | University of Michigan |
| K1102  | nus<sup>+</sup>A<sub>1</sub>  | NA      | L.S. Baron |
| K1227  | p<sub>b</sub>cro62/gal fusion | OR1150  | University of Michigan |
| K3093  | lac<sup>+</sup>   | K37     | University of Michigan |
| K3929  | p<sub>b</sub>cro62/gal/nusA1 fusion | K3929   | University of Michigan |
| K3930  | p<sub>b</sub>boxA1/gal/nusA1 fusion | K3929   | University of Michigan |
| K4087  | nus<sup>+</sup>A<sub>1</sub>/sneA16 | K37     | University of Michigan |
| K4092  | nus<sup>+</sup>A<sub>1</sub> | K37     | University of Michigan |
| K4461  | lac<sup>+</sup>λc1857ΔBamΔH1 | K3093   | University of Michigan |
| K5310  | p<sub>b</sub>boxAcon/gal/nusA1 fusion | K3929   | University of Michigan |
| K5319  | p<sub>b</sub>boxAcon/gal/lac<sup>+</sup>nus<sup>+</sup> fusion | K5310   | University of Michigan |
| K5693  | p<sub>b</sub>/gal/nusA1 fusion | OR1150  | University of Michigan |
| JM101  | lac<sup>+</sup>, lac2Δm15 | NA      | J. Messing |
| DH5α   | lac2Δm15, recA1   | NA      | Bethesda Research Labs |

*MNA* Not applicable.

Plasmids

Constructs were made by employing standard cloning techniques [Maniatis et al. 1982]. Relevant plasmid characteristics are listed in Table 4. A promotorless derivative of pKBAcon, pKBAconΔP, was constructed by deleting a BamHI–EcoRI fragment containing P<sub>ach</sub>. pGB2ΔnusB was constructed by removing a 240-bp internal fragment from the nusB gene in pGB2nusB and inserting a 1.35-kb SacI fragment from pBR325 that contains the cam<sup>+</sup> 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<sup>+</sup> 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 nutRX.boxAcon. The nutR<sub>2</sub> region on an EcoRI–HindIII fragment from pRNut-21 was cloned in M13mp8, and the sequence of the mutagenic oligonucleotide primer was 5’-GGCCAGAACTGTTAAAGAGC-GATTTGC, yielding nutR<sub>2</sub>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 4. Plasmids and M13 derivatives

| Plasmid       | Parent vector | Relevant fragment or characteristic | Donor          | Source          |
|---------------|---------------|--------------------------------------|----------------|----------------|
| pCG69         | pCR1          | EcoRI with imm21 (from red to O)    | pimm21         | C. Georgopoulos|
| pMC100        | pBR322        | EcoRI with imm21                    | pCG69          | this work      |
| pRZ-nut21     | pBR322        | Clal–EcoRI with nut21               | pMC100         | this work      |
| pBA7-6        | pBR322        | EcoRI–HindIII nutR21 with boxAcon   | M13boxAcon     | this work      |
| pBACon        | pBR322        | EcoRI–HindIII nutR21 with boxAcon   | M13nutR21      | this work      |
| pT21          | pUC18         | EcoRI–HindIII nutR21 wild-type      | M13nutR21      | this work      |
| pC21          | pUC18         | EcoRI–HindIII nutR21 with boxAcon   | M13nutR21      | this work      |
| pKK223-3      | pBR322        | cloning site downstream of P_tac    | M13–boxA5      | de Boer (1983) |
| pKBAL         | pKK223-3      | λboxAλ+                           | pKK223-3       | this work      |
| pKBA1         | pKK223-3      | λboxA1                            | pKK223-3       | this work      |
| pKBA5         | pKK223-3      | λboxA5                            | pKK223-3       | this work      |
| pKBAcon       | pKK223-3      | λboxAcon                          | pKK223-3       | this work      |
| pKBA21        | pKK223-3      | 21 boxA5+ Clal–HindIII             | pKBA21         | this work      |
| pKBA21con     | pKK223-3      | 21 boxA5+ Clal–HindIII             | pKBA21         | this work      |
| pKBaconΔP     | pKBAcon       | λboxAcon ΔP_tac                    | pKBacon        | this work      |
| pGB2          | pSC101        |                                   | pGC2010        | Churchward et al. (1984) |
| pGB2nusB      | pGB2          | nusB                               | pSC101         | F. Kappel (via C. Georgopoulos) |
| pGB2nusA      | pGB2          | nusA                               | pGB2           | this work      |
| M13nusR21     | pGB2          | nusA                               | pGB2           | this work      |
| M13nusR21boxAcon | M13mp18    | EcoRI–HindIII nutR21               | pRZ-nutR21     | this work      |
| M13nusR21boxAcon | M13mp18    | EcoRI–HindIII nutR21               | M13mp8nutR21   | this work      |
| M13boxA1      | M13mp9        | nutR-boxA1                         | M13mp9nutR21   | Friedman and Olson (1983) |

*The λ inserts contain DNA from coordinates 38214 to 38350 (Daniels et al. 1983). The 21 Clal and HindIII 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 μg/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 λ32 derivatives with the boxA1 mutation in nutA grow in nusA1 mutants under conditions where the nutA⁻ derivatives fail to grow (Friedman and Olson 1983; Schauer et al. 1987). We assumed that λ and 21 derivatives with boxAcon in their nutA regions might also grow in hosts with mutant or variant nusA genes under conditions where the parent phages with wild-type nutA regions fail to grow, because of a failure in N-mediated attenuation. Recombinants derived from λ and λ32 with boxAcon were isolated from a cross with pBAcon using K95 (nusA1) as the selective host at 42°C. Putative derivatives of λ and λ32 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 21 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 μg/ml (spectinomycin at 50 μg/ml was included if pGB2 derivatives were also used) and grown to a concentration of ~10⁹/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 μg/ml in ampicillin and, where indicated, 1 mM in IPTG and/or spectinomycin at 50 μg/ml, and incubated at the appropriate temperature. Aliquots were removed at the indicated times, treated with chloroform to lyse bacteria, and titered for viable phage. Burst represents phage output/phage input.
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Measurement of plasmid copy number

Ten milliliters of culture from burst experiments was combinaed with 1 ml of overnight culture of DH5α 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 of 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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