Complexes of N Antitermination Protein of Phage λ with Specific and Nonspecific RNA Target Sites on the Nascent Transcript†

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ABSTRACT: The mechanisms that control N protein dependent antitermination in phage λ have counterparts in many eukaryotic systems, including specific regulatory interactions of the antitermination protein with the nascent RNA transcript. Here we describe the specific and nonspecific RNA binding modes of antitermination protein N. These modes differ markedly in RNA binding affinity and in structure. N protein, either free in solution or as a complex with nonspecific RNA, lacks observable secondary and tertiary structure and binds RNA sequences indiscriminately with a dissociation constant (K_d) of $\sim 10^{-6}$ M. In contrast N becomes partially folded with at least 16−18 amino acids of ordered α-helical structure and binds much more tightly ($K_d \cong 10^{-9}$ M) on forming a highly specific 1:1 complex with its cognate boxB RNA hairpin. These observations and others are used to help define a bipartite model of N-dependent antitermination in which these specific and nonspecific interactions control the binding of N to the nascent transcript. Finally the role of RNA looping in delivering the bound N to the transcription complex and determining the stability (and thus the terminator specificity) of the resulting antitermination interaction of N with the RNA polymerase is considered in quantitative terms.

The N protein of bacteriophage λ activates transcription of the delayed early genes (Friedman, 1988). N functions by binding to elongation complexes and causing RNA polymerase (RNAP) to ignore intrinsic and rho-dependent terminators that would otherwise prevent synthesis of these delayed early transcripts (Das, 1992, 1993; Roberts, 1993; Greenblatt et al., 1993). This process, called N-mediated antitermination, works specifically on terminators that interrupt the early operons of phage λ. The *nut* sequence, located just downstream of each early promoter, confers this specificity [e.g., see Rosenberg et al. (1978)]; the functional expression of this site is the transcribed *nut* sequence in the nascent RNA (Whalen & Das, 1990; Nodwell & Greenblatt, 1991; Das, 1992).

The boxA and boxB elements of the *nut* site are central in regulating the switch between lysogeny and lytic development in the life cycle of the lambdoid family of phages by controlling the expression of early transcription terminators. Furthermore, the affinity of N for the RNA hairpin encoded by the boxB element of this site remains a necessary determinant of specific antitermination in vivo (Chattopadhyay et al., 1995a; Mogridge et al., 1995). Although the boxA element of *nut* is conserved within most members of the lambdoid family, the boxB sequence differs for each phage (Franklin, 1985a; Friedman, 1988), and the N protein of each functions specifically only with its cognate boxB hairpin (Franklin et al., 1985b; Lazinski et al., 1989). While overexpression of N can alleviate this genomic specificity (Franklin & Doelling, 1989; Lazinski et al., 1989), antitermination by N depends on the interaction of a specific lambdoid N protein with the homologous boxB element. This requirement allows for strict and precise regulation of the location and timing of N-mediated antitermination.

The specificity of N for boxB is extraordinary; even single base substitutions in the loop of boxB can greatly diminish the affinity of N for boxB hairpins (Chattopadhyay et al., 1995a; Tan & Frankel, 1995; Mogridge et al., 1995). These mutant boxB hairpins also will not support N-dependent antitermination in vivo or in vitro (Whalen et al., 1988; Doelling & Franklin, 1989; Chattopadhyay et al., 1995).

The generally accepted model for N action postulates that the boxB RNA sequence on the nascent transcript binds N and brings it to the elongation complex by RNA looping, which effectively increases the local concentration of N near the RNA polymerase (RNAP) and thus enhances the probability of a productive interaction with the functional transcription complex (Whalen & Das, 1990; Nodwell & Greenblatt, 1991). Consistent with this model, Rees et al. (1996) have shown that N alone can bring about nonspecific antitermination in vitro, in the absence of both boxB (using a transcript from which the *nut* site has been deleted) and the accessory protein NusA, when N is supplied at elevated concentrations and antitermination is assayed at somewhat reduced salt concentrations. The addition of competitor RNA or higher concentrations of salt greatly inhibits the ability of N alone to induce antitermination. Thus, under these conditions, N-dependent antitermination appears to involve

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the direct binding of N to the elongating transcription complex, with this interaction being stabilized, at least in part, through nonspecific binding of N to the nascent RNA (Rees et al., 1996).

In addition to its binding interaction with N protein, it is clear that boxB itself plays a role in specific antitermination that transcends the mere provision of a high-affinity binding platform for N. Thus, certain mutations in the boxB hairpin that show no qualitative effect on N binding still do not permit antitermination, either in vivo or in vitro (Chattopadhyay et al., 1995a; Mogridge et al., 1995). These residues may be critical for the association of the N−boxB complex with NusA, or perhaps allow more efficient interaction of this complex with the RNA polymerase. The results of Rees et al. (1996) demonstrate that antitermination by N at elevated concentrations in the absence of a *nut* site is not assisted by the NusA protein. In fact, it is only when the *nut* sequence is present that NusA acts as an antitermination factor, and at moderate salt concentrations (Rees et al., 1996), the *nut* site itself does not effectively stimulate N-dependent antitermination unless NusA is present. These results argue that the even though N is ultimately capable of functioning nonspecifically, its interactions both with boxB of the nascent RNA and with NusA are critical to its role in specific antitermination in vivo.

N is a member of a class of RNA binding proteins that carry the ARM (arginine-rich motif) binding motif [Lazinski et al., 1989; reviewed by Burd and Dreyfuss (1994)]. Other members of this family include the Tat and Rev proteins of HIV, the Tat protein of BIV, the Nun protein of phage HK022, and the homologous N proteins of ϕ21 and P22. The primary determinant of this motif is simply the presence of a significant number of clustered arginine residues (see Figure 1A). The most extensive structural work on the ARM family of proteins has been reported for the Rev and Tat proteins (Tan & Frankel, 1992, 1994; Long & Crothers, 1995; Puglisi et al., 1995). The Rev protein binds to an internal loop structure formed by the RRE element, and Tat binds to a bulge structure formed by the TAR RNA element. However, the structures of the ARM RNA binding domains of these proteins appear to differ significantly, since the BIV Tat binding domain interacts with the TAR sequence as a β-turn (Puglisi et al., 1995) while the Rev binding domain assumes an α-helical conformation upon binding to RRE (Tan & Frankel, 1994). In both interactions, a conformational change in the RNA element is coupled to the binding of the protein. Furthermore, proline substitutions that disrupt the α-helical conformation in peptides encompassing the ARM domain of the lambdoid N proteins prevent specific binding to the cognate hairpins (Tan & Frankel, 1995).

N-dependent antitermination consists of two general mechanistic processes. One is the localization of the N protein to the transcription complex by interactions with the nascent RNA, and the other is the subsequent interactions of N with the transcription complex that lead to productive antitermination. Both of these activities are partially regulated by the RNA form of the *nut* site. Although N is ultimately capable of triggering antitermination by interacting either specifically or nonspecifically with the nascent RNA transcript, it appears that the antitermination system of the lambdoid phages has evolved so that only specific interactions lead to effective antitermination under physiological conditions. In this paper, we report structural and thermodynamic studies of the interactions of N protein with specific and nonspecific RNA sequences. These results are then used to describe how N binding to specific and nonspecific RNA targets on the nascent transcript, coupled with RNA looping, can increase the local concentration of N at downstream terminators and thus control the efficiency of termination by stabilizing the transcription complex. Finally, we discuss how the properties of the two different RNA binding modes of N might serve to regulate the assembly of the antitermination complex and the activation of transcription.

MATERIALS AND METHODS

Purification of N Protein. A revised procedure was used to obtain the quantities of pure N protein needed for physical chemical study. The vector pET-N1 was used to overexpress N protein in *Escherichia coli* BL21 (DE3) cells, as described (Rees et al., 1996a). Induced cells were resuspended in lysis buffer [20 mM Tris-HCl (pH 7.6), 50 mM NaCl, 10 mM EDTA, 10% glycerol, 10 mM β-ME (β-mercaptoethanol), 0.5 mM phenylmethanesulfonyl fluoride, and 0.1 mg/mL benzamidine] and passed through a French press. The resulting lysate was centrifuged for 30 min at 20000*g* and 4 °C. N was purified from inclusion bodies by resuspending the lysate pellet in 20 mM Tris-HCl (pH 7.6), 0.1 mM EDTA, 1 mM β-ME, and 8 M guanidinium chloride. The resuspended solution was centrifuged for 2 h at 60000*g* and 15 °C, and the supernatant containing solubilized N protein was dialyzed into 20 mM Tris-HCl (pH 7.6), 0.1 mM EDTA, and 1 mM β-ME. Precipitated material (containing some N protein) was removed by centrifugation for 30 min at 20000*g* and 4 °C. The supernatant, now containing pure N, was dialyzed into storage buffer [20 mM Tris-HCl (pH 7.6), 50 mM NaCl, 0.1 mM EDTA, 1 mM β-ME, and 50% glycerol]. The purity of N was determined by SDS−polyacrylamide electrophoresis (SDS−PAGE) and reversed-phase HPLC chromatography, and N activity was measured using in vitro antitermination assays as described (Rees et al., 1996). Protein purified by the revised procedure behaved identically to that of N purified according to Rees et al. (1996) in all experimental methods used for this work.

Oligonucleotide Synthesis. The RNA oligonucleotides used in this study were synthesized either by Oligos Inc. (Wilsonville, OR) or in the University of Oregon Biotechnology Laboratory (Eugene, OR). The DNA oligonucleotide was synthesized by DNA Express (Ft. Collins, CO). Oligonucleotides were further purified by PAGE, as necessary. Prior to use, all RNAs were treated by diluting into N/B buffer and incubating at 90 °C for 10 min, followed by slow-cooling to room temperature. This procedure prevented the formation of RNA dimers. The sequences of the boxB RNA (and DNA) oligonucleotides used in this study, as well as the site-specific substitution nomenclature used, are shown in Figure 1B.

Fluorescence Binding Measurements. Steady-state intrinsic fluorescence measurements were performed on an SLM 8000 spectrofluorometer (Urbana, IL). N contains two tryptophan residues, and the overall fluorescence of N is appreciably quenched by binding to the RNA and DNA variants used in this study. N protein was excited at 300 nm to minimize internal filter effects due to the RNA, and the fluorescence emission was monitored at 355 nm. Titrations were performed in N/B binding buffer [20 mM KPO_4

(pH 7.6), 100 mM KCl, 0.1 mM EDTA, and 1 mM β-ME] at 20 °C. Percent quenching was calculated as

$$\% \text{ quenching} = [(Q_n - Q_n\sum[\text{RNA}])/Q_n]100 \quad (1)$$

where Q_n represents the quantum yield of N alone and $Q_n\sum$-[RNA] is the quantum yield of N at the various concentrations of RNA used. For titrations of N with wild-type boxB and boxB A5U, maximum quenching was measured at both 100 nM and 1 μM N concentrations when enough boxB RNA had been added to saturate the change in fluorescence, indicating that N was fully bound to the RNA at both of these N concentrations. For the nonspecific boxB RNA variants, the maximum quenching value could not be measured directly. As a consequence, binding curves were fit using the equation:

$$F_b = \frac{K_d + [N_0] + [\text{RNA}_0] \pm \sqrt{(K_d - [N_0] - [\text{RNA}_0])^2 - 4[N_0][\text{RNA}_0]}}{2[N_0]} \quad (2)$$

where F_b represents the fraction of N bound and $[N_0]$ and $[\text{RNA}_0]$ represent the total N protein and RNA concentrations, respectively. Titration plots were converted to fraction of N bound vs concentration of log $[\text{RNA}]_{\text{free}}$ for presentation. Appropriate corrections for inner filter and dilution effects were made before calculating percent quenching.

NMR Spectroscopy of the N Protein. BL21 (DE3) cells containing the pET-N1 vector were grown in minimal media supplemented with ^{15}N-labeled NH_4Cl. Protein expression was induced, and the resulting labeled protein was purified as described (Rees et al., 1996) or by the revised procedure above. HSMQC NMR spectra were recorded at 500 MHz on a General Electric GN-500 spectrometer at 20 °C. The samples were prepared at 400 μM N protein in 20 mM KPO_4 (pH 5.8), 100 mM KCl, and 5% D_2O. Spectra were analyzed using the FELIX software from Hare Research.

Circular Dichroism Spectroscopy. CD spectra were recorded on a Jasco 600 spectropolarimeter. All spectra were collected with 1.0 μM N protein in N/B buffer at 20 °C. The CD spectrum of N protein bound to boxB RNA was determined by subtracting the spectrum of free boxB RNA at the appropriate concentration from that of the N−boxB complex. This subtraction reveals the difference spectrum of N protein bound to the RNA, together with any changes in the RNA spectrum that result from the interaction.

RESULTS

Binding Affinity of N for Wild-Type and Site-Specifically-Modified BoxB RNAs. The N protein contains two trp residues, one of which is adjacent to the ARM region (see Figure 1A). Consistent with the interaction between ARM and RNA, the intrinsic tryptophan fluorescence of the N protein is partially quenched on binding to RNA. We have used this quenching to measure the affinity of N for various RNA oligomers related to the boxB RNA hairpin (see Figure 1B). A typical titration curve for the binding of wild-type boxB oligomer by N (100 nM), plotted as fraction N bound versus the total concentration of boxB RNA, is shown in Figure 2A. Extrapolation of the data points at the high boxB RNA concentration plateau of this titration shows that the boxB RNA oligomer and N form a complex with a

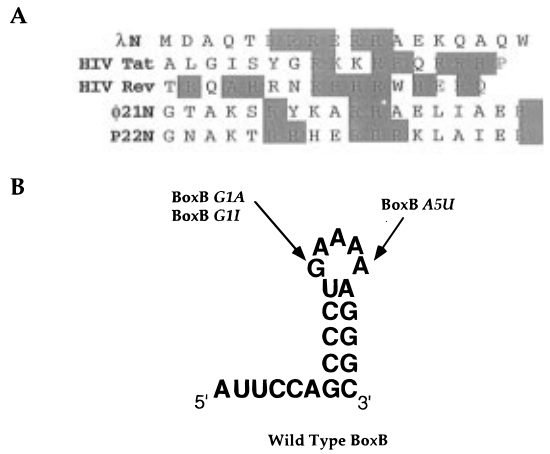


FIGURE 1: Sequences of the ARM motif proteins and the structure of the boxB hairpin. (A) A sequence comparison of the RNA binding domains of the ARM motif RNA binding proteins [modified from Burd and Dreyfuss (1994)]. (B) Structure of the wild-type boxB RNA hairpin, showing also the various positions that were modified to make the mutant constructs used in this study.

stoichiometry of one boxB hairpin per N monomer. One-to-one quenching stoichiometries with boxB RNA have also been observed at 250 nM and 1 μM total N concentrations (data not shown). This stoichiometry is further supported by equilibrium ultracentrifugation molecular weight measurements carried out with N and a 15 residue boxB RNA hairpin oligomer, which also demonstrated that these components sediment as a 1:1 complex (data not shown). Finally, titrations at much lower total concentrations of N (~5 nM) were used to determine a dissociation constant (K_d) of $(1.3 \pm 0.4) \times 10^{-9}$ M for the N−boxB RNA complex (Figure 2B and Table 1). This value is about 10-fold lower than that estimated by gel-shift assays (Chattopadhyay et al., 1995a).

To compare this affinity with that of N for a totally nonspecific RNA oligomer, we measured the binding constant of N to a 15 residue RNA oligomer consisting of a scrambled boxB sequence. This oligomer was designed to avoid the formation of stable secondary structure and thus, while containing the same nucleotide residues, does not resemble boxB in either sequence or structure. Figure 2B also shows the titration curve for the binding of N to this nonspecific RNA oligomer. The dissociation constant (K_d) for this interaction is $(1.5 \pm 0.2) \times 10^{-6}$ M (Table 1). As can also be seen in Figure 2B, the affinity of N protein for this nonspecific RNA oligomer is approximately 1000-fold lower than that of N for the wild-type boxB sequence. The intrinsic fluorescence quenching assay was also used to titrate N with a wild-type boxB hairpin that had been constructed with DNA instead of RNA residues (Figure 2B). The K_d for this binding was $(1.5 \pm 0.2) \times 10^{-6}$ M (Table 1), which is essentially the affinity measured for the binding of N to the totally nonspecific (scrambled) single-stranded RNA oligomer.

Several groups have reported that making changes in the first position at the 5′ end of the hairpin loop (G1) causes dramatic reductions in binding affinity as measured by band shift assays (Chattopadhyay et al., 1995a; Mogridge et al., 1995). However, there is a discrepancy in results obtained with some variants mutated at the 3′ end of the loop, where Chattopadhyay et al. (1995a) found that mutations to the adenosine residue at position 5 (A5) did not significantly

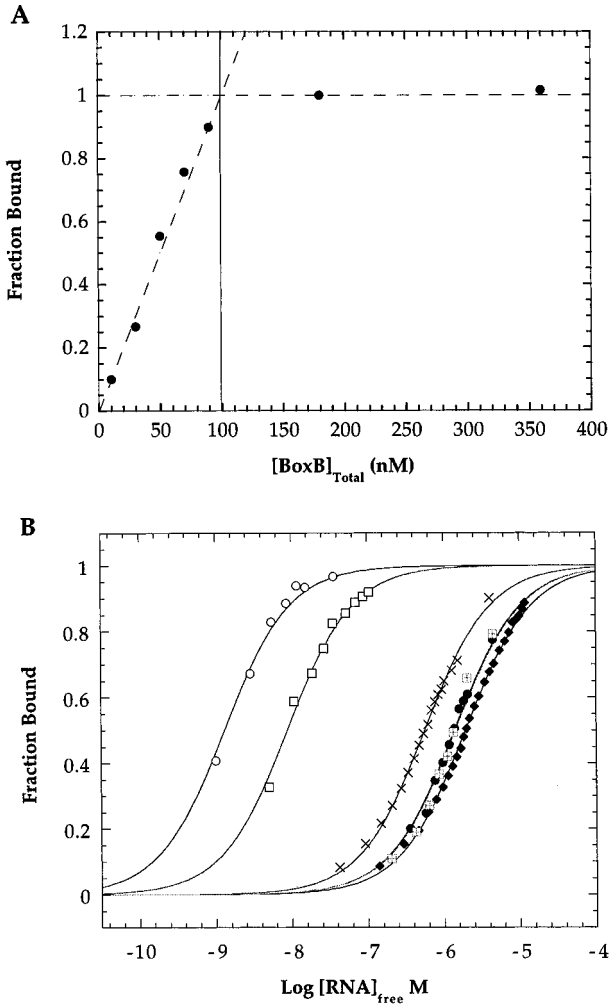


FIGURE 2: N protein binding to the boxB RNA hairpin and to nonspecific RNAs. (A) Intrinsic fluorescence titration of 100 nM N protein with the wild-type boxB RNA of phage λ, showing that the quenching saturates at a 1:1 complex with boxB RNA. This curve was used to determine the quenching parameter that corresponds to binding saturation. (B) Plots of fraction of N protein bound versus log $[RNA]_{free}$ of (i) wild-type boxB RNA (open circles), (ii) the boxB A5U mutant (open squares), (iii) boxB G1A (solid diamonds), (iv) boxB G1I (×s), (v) single-stranded RNA (solid circles), and (vi) boxB DNA (open squares with +). For the boxB wild-type and boxB A5U mutants, 5 nM N protein was used for the titration and the maximum quenching value determined in (A) above was used to calculate the fraction bound (see Materials and Methods). For the titration of the remaining oligonucleotides, 1 μM N protein was used, and binding curves were constructed by plotting percent quenching versus total RNA concentration and were fit with eq 2 (Materials and Methods). The data were converted to fraction N bound versus log $[RNA]_{free}$ for presentation purposes.

affect binding, while Mogridge et al. (1995) found that substitution with a C residue at this position did have a major effect on binding (perhaps because the C residue forms a Watson−Crick base pair with the crucial G residue at the 5′ end of the loop). Tan and Frankel (1995) also used band shift assays to show that peptides containing the amino-terminal RNA binding domain of N appeared to bind to boxB hairpins with an affinity greater than 100-fold higher than that for boxB hairpins carrying modifed residues at the 5′ or 3′ ends of the boxB loop.

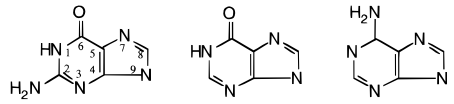
We have examined two of these site-specifically-modified boxB constructs to further test thermodynamic and structural features of the formation of the N−boxB complex. We found that a guanosine to adenosine substitution (G1A) at the first position (5′) of the loop (Figure 1B) abolishes specific binding altogether, with the titration curve for this mutant falling into the nonspecific binding group (Figure 2B and Table 1). In keeping with the gel shift results of Chattopadhyay et al. (1995a), we also found that the substitution of U for A at the fifth (3′) loop position (A5U) resulted in only a 5−10-fold decrease in the binding affinity of this construct for N (Figure 2B and Table 1); we therefore place this construct into the specific binding group.

Table 1: Calculated Dissociation Constants for Mutant BoxB Oligomers[a]

RNA	K_d (M)	phenotype	
		in vivo	in vitro
boxB	$(1.3 \pm 0.4) \times 10^{-9}$	+	+
boxB A5U	$(9.0 \pm 2.0) \times 10^{-9}$	−	−
boxB G1A	$(2.0 \pm 0.3) \times 10^{-6}$	−	−
boxB G1I	$(0.8 \pm 0.3) \times 10^{-6}$	n/a	n/a
ssRNA	$(1.5 \pm 0.2) \times 10^{-6}$	−	−
boxB DNA	$(1.5 \pm 0.2) \times 10^{-6}$	n/a	n/a

[a] The dissociation constant (K_d) values were measured by fluorometry, as described under Materials and Methods. The antitermination activity of each boxB mutant was measured by in vitro or in vivo transcription assays (Franklin & Doelling, 1989; Chattopadhyay et al., 1995).

The absolute requirement for a guanosine residue at the 5′ end of the loop is striking. Guanosine and adenosine differ only in the substituents carried at the C2 and C6 positions of the purine ring and in the presence or absence of a hydrogen at N1. Inosine, which is the metabolic precursor of both of these purines, is a natural "hybrid", resembling guanosine in the presence of a keto group at position C6 and a proton at N1, and adenosine in the absence of an amino group at position C2.



If key interactions controlling the specific recognition of boxB by N protein are located entirely at the C6 and N1 positions of the G residue at the 5′ end of the loop, then the substitution of an inosine residue at this position should restore some or all of the binding specificity to the interaction. On the other hand, if an amino group at C2 is necessary, an inosine residue at this position would not provide the recognition specificity required to support tight N−boxB binding.

A boxB RNA oligomer containing an inosine residue at the 5′ end of the loop (G1I) was constructed. We found (see Figure 2B) that boxB G1I RNA showed only a 2-fold increase in its affinity for N over the G1A construct (i.e., both bind N essentially nonspecifically), rather than the dramatic increase in affinity that might be expected if specific binding had been restored. Thus, key interactions necessary for specific binding and stable complex formation are critically dependent on the presence of an amino group at the C2 position of the purine ring.

The N−boxB dissociation constants measured in this study are summarized in Table 1, and are also correlated there with in vitro and in vivo determinations of antitermination activity of the various boxB constructs tested (Franklin & Doelling,

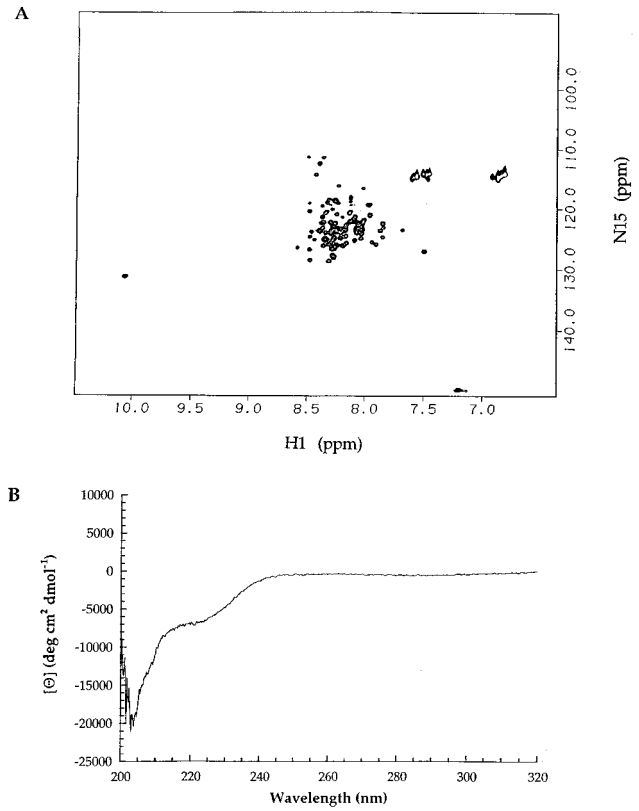


FIGURE 3: Structure of the free N protein. (A) Amide region of the HSMQC of uniformly ^{15}N-labeled free N protein at 400 μM and 20 °C. Each cross-peak results from a proton directly bonded to a ^{15}N. The *x*-axis represents the proton chemical shift and the *y*-axis the amide chemical shift. (B) CD spectrum of 1.0 μM N protein at 20 °C in N/B buffer.

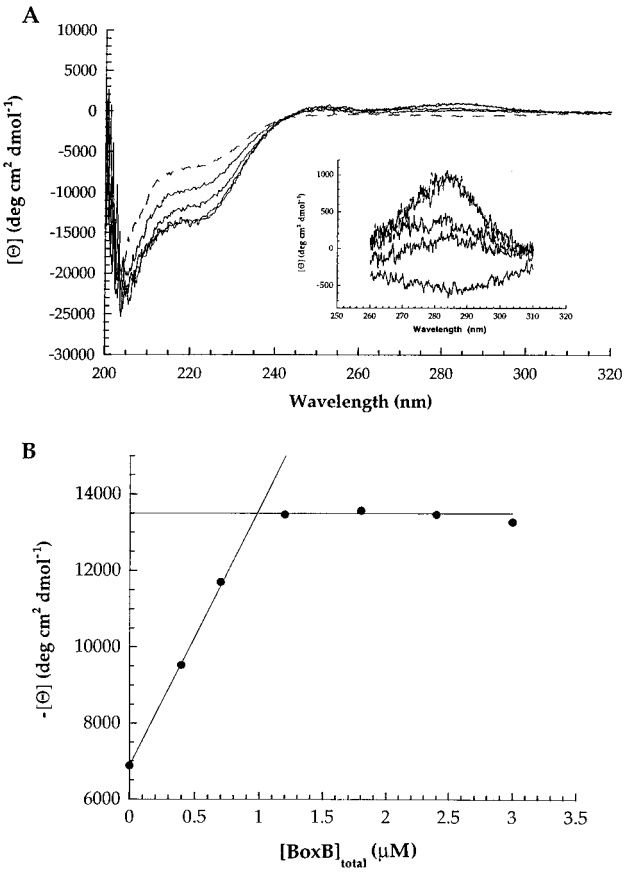


FIGURE 4: Structure of the N protein bound to the boxB hairpin. (A) CD titration of 1.0 μM N protein with boxB RNA at 20 °C in N/B buffer. The dashed spectrum is that of the N protein alone, and the spectra that follow represent the subsequent additions of 0.35, 0.7, 1.0, and 1.5 μM boxB RNA. The spectra of the N protein were calculated by subtracting the spectrum of the RNA alone from those of the complex. The resulting difference spectra represent the bound N protein and any spectral changes induced in the RNA by binding. Since the RNA does not have significant ellipticity at 222 nm, it is assumed that the ellipticity at 222 nm is solely representative of N protein. Also, since N protein has no signal at 280 nm, it is assumed the signal at 280 nm is due solely to changes in the RNA (see inset). (B) Plot of ellipticity at 222 nm vs concentration of boxB RNA shows that the structural change of 1.0 μM N protein is saturated by the addition of 1.0 μM boxB RNA.

1989; Chattopadhyay et al., 1995a). These results show that relatively minor modifications of either the sequence or the structure of the boxB RNA hairpin destroy the specific affinity of N for this construct. This consequence is manifested by the binding of N with a much lower (nonspecific) affinity to most RNA sequences, as well as to DNA, even if the latter is constructed with the same nucleotide sequence as boxB. Table 1 shows that binding in the nonspecific mode also correlates with the loss of N-dependent antitermination activity. We have observed that N also binds to other single- and double-stranded DNA sequences in its nonspecific mode (data not shown).

Structure of the Uncomplexed N Protein. A two-dimensional HSMQC NMR analysis was performed on the uncomplexed ^{15}N-labeled N protein at 20 °C (Figure 3A). Peaks corresponding to 100 of the 107 residues of N can be resolved, but the amide resonances are sharp and clustered in the center where they have chemical shifts consistent with those of a random coil structure. This suggests that N exists in solution as an unfolded protein. This conclusion is also supported by circular dichroism (CD) measurements of the uncomplexed N protein. Figure 3B shows that free N protein at 20 °C manifests only a weak CD signal at 223 nm, corresponding to a calculated (from CD) α-helix content of approximately 18%. However, further CD measurements showed that this level of apparent secondary structure is not significantly altered by changing the temperature, and CD melting curves showed that no cooperative melting occurs between 20 and 95 °C (M.R.V.G., unpublished results). Furthermore, on the NMR time-scale we find no significant secondary or tertiary structure interactions. These observations are consistent with the existence of N in solution as an essentially unstructured molecule containing some (fluctuating and transient) secondary structure (Psitsyn, 1987; Kim & Baldwin, 1990).

Structures of both N and RNA Change on Specific Complex Formation. We have shown above that N protein exists in solution as an unfolded (essentially random coil) molecule. Yet this protein is able to recognize and specifically interact with boxB RNA and to discriminate the wild-type boxB hairpin from closely related variants. This apparent paradox led us to use CD measurements to look for structural changes that may occur in either the protein or the RNA as a consequence of specific complex formation. Figure 4A shows that the CD spectrum of N protein does indeed change significantly in the presence of boxB RNA, with the changes suggesting the formation of considerable additional α-helix within the protein on binding to boxB RNA. We estimate from these spectra that N becomes approximately 35% α-helical when bound specifically to boxB RNA. This

corresponds to the formation of about 16−18 amino acids of α-helical structure. Furthermore, in contrast to the free protein, this secondary structure is sensitive to temperature and undergoes a cooperative melting transition characterized by a T_m of 53 °C (M.R.V.G., unpublished results). Thus, the N protein of phage λ becomes significantly structured on binding to its cognate RNA target. CD titration experiments (Figure 4B) show that this formation of α-helix also saturates at a 1:1 stoichiometry of N to boxB RNA.

The ellipticity of the N−boxB RNA complex in the vicinity of 280 nm is also increased, relative to the total ellipticities of the separate components, as a consequence of complex formation (Figure 4A, insert). Such changes in ellipticity at 280 nm in other protein−RNA interactions have been attributed to base stacking changes within the RNA structures [e.g., see Tan and Frankel (1992)]. These CD results argue that the essentially unfolded N protein becomes at least partially structured on binding to the wild-type boxB hairpin, and that the RNA hairpin also undergoes a conformational rearrangement as a consequence of this specific binding. It should be noted that boxB maintains a stem structure upon N binding since the 3′ arm of the stem in the N−boxB complex is accessible to cleavage by double-strand-specific ribonucleases (Chattopadhyay et al., 1995a).

Binding of Nonspecific RNA Does Not Induce α-Helical Structure in N Protein. In principle, the change in secondary structure that occurs when N binds to boxB RNA could be a requirement for (or the result of) specific binding. However, it could also be a trivial consequence of the binding of N to any RNA or DNA oligonucleotide. To test the latter hypothesis, we measured the CD spectrum of N protein bound to the nonspecific (scrambled) single-stranded RNA oligomer used in the binding studies (above). Based on the measured nonspecific K_d of $\sim 1 \times 10^{-6}$ M for this oligomer (Figure 2C and Table 1), the RNA oligomer concentration that was used in this experiment should have formed a complex with ∼70% of the total N protein present. Figure 5A shows no observable change in ellipticity for either the N protein or the RNA component on nonspecific complex formation. We conclude that the N protein binds nonspecific RNA in essentially its unstructured solution form.

Site-specific alterations in the loop sequence of the boxB hairpin have been shown to have variable effects on N binding and function (Chattopadhyay et al., 1995a). Our work to this point has shown that the binding of N with high affinity is correlated with the folding of at least a portion of the protein into a significantly α-helical structure. We next used the CD assay to determine whether binding of N to boxB hairpins carrying the specific changes in the loop sequence that were studied above can induce α-helix formation. Figure 5A shows that N does not undergo a structural change on binding to either the G1A or the G1I boxB RNA variant. However, when N interacts with the A5U variant (to which it binds only ∼10-fold more weakly than it does to wild-type boxB), the protein does undergo a structural change similar to that seen in complex formation with wild-type boxB (Figure 5B). These results demonstrate that N protein undergoes a major structural change on binding specifically to boxB RNA, and that the formation of this ordered structure depends on key functional groups on the essential guanosine residue at the 5′ end of the loop. In contrast, the above experiments with the A5U construct suggest that if N makes any contacts at the 3′ end of the

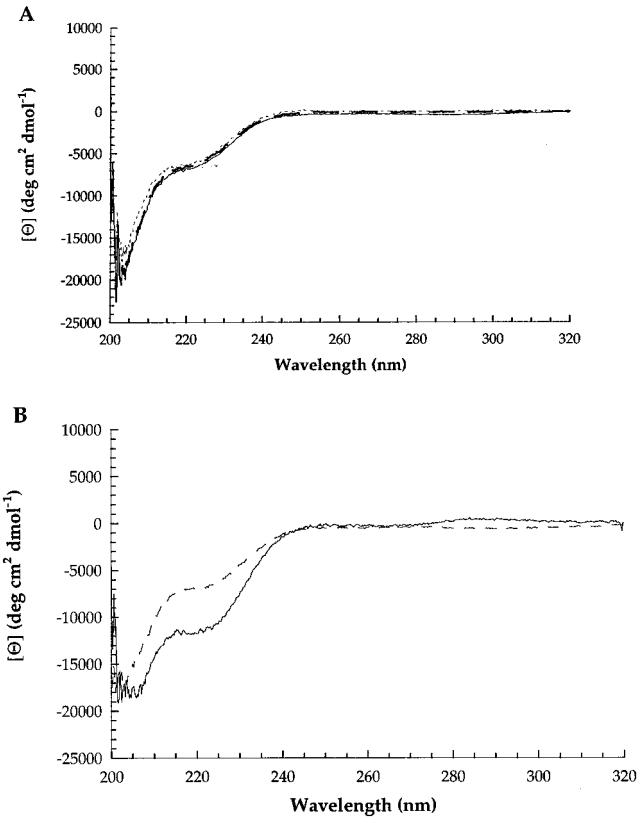


FIGURE 5: Structure of N protein bound to nonspecific RNA and to boxB mutant constructs. (A) CD spectra of (i) 1.0 μM N protein alone (solid curve) and with (ii) 3.0 μM single-stranded RNA (dotted) or (iii) 3.0 μM boxB G1I (heavy dashed). The CD spectrum of N protein with 3.0 μM boxB G1A is indistinguishable from boxB G1I and therefore is not shown. At these concentrations of protein, ∼70% of N should be complexed with the RNAs used. (B) CD spectra of 1.0 μM N protein alone (dashed curve) and with 1.0 μM boxB A5U RNA (solid curve).

loop they are less critical in inducing the specific structure of the complex. We note that the divalent cation Mg^{2+} is known to have significant effects on RNA structure; we have not found any qualitative or significant quantitative differences in specificity or structural changes as a result of the presence of Mg^{2+}.

Binding of N to the Nascent Transcript in cis Can Control the Local Concentration of N at Terminators as a Consequence of RNA Looping. We have shown that the boxB RNA constructs we have tested can be placed into two binding groups or modes. One group consists of those constructs that bind tightly ($K_d \cong 10^{-9}$ M) to N and induce the specific (folded) binding conformation of both the protein and the RNA. The other group contains nonspecific RNA (and DNA) constructs that bind N much more weakly ($K_d \cong 10^{-6}$ M) and do not induce a structural change in either binding partner. Here we use a calculation approach to ask to what extent N, bound to the nascent transcript either specifically (at the boxB RNA hairpin) or nonspecifically (at other sites on the RNA), can use RNA looping to increase the local concentration of this protein at transcription complexes located at terminator sites along the transcript. The connection between this RNA looping-dependent increase in the local N concentration at the terminator and the level of N-dependent antitermination observed will be considered in the Discussion.

Specific Binding to the BoxB RNA Hairpin on the Nascent RNA. Recently Rippe et al. (1995) have used calculations

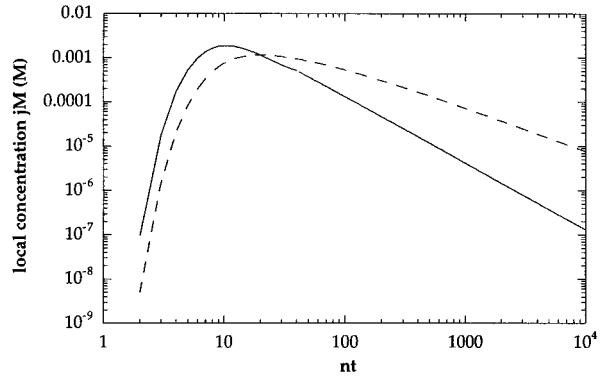


FIGURE 6: RNA looping regulates the concentration of boxB-bound N at the transcription complex. Theoretical plots. The solid line represents the effective local concentration [jM] of boxB-bound N at the transcription complex as a function of RNA length from boxB to the 3′ end of the nascent transcript. The dashed line represents the effective local concentration, at the terminator, of N bound nonspecifically and at random to the nascent RNA at a binding density of one N per transcript (see text). The *x*-axis for the nonspecific binding plot corresponds to the overall transcript length.

of the flexibility and looping of double-stranded DNA to calculate the local concentrations, at the promoter, of *cis*-acting eukaryotic transcription activation factors bound to specific DNA upstream activator or enhancer sites. These approaches can also be employed to calculate the local concentration of boxB-bound N protein at any specific template site (e.g., a downstream terminator) as a consequence of the *cis* looping of a *nut*-containing RNA transcript. Using the equations and calculation methodology described elsewhere (Rippe et al., 1995), an average internucleotide distance of 6.5 Å for single-stranded RNA, and a persistence length of 40 Å [measured by Inners and Felsenfeld (1970) for poly(rU)], we have calculated the local (molar) concentration of the boxB RNA hairpin (and thus of N, assuming that the boxB site is fully complexed) at the terminator as a consequence of *cis* looping. This concentration is plotted as a function of the RNA contour length (in nucleotide residues) between boxB (the *nut* site) and the 3′ end of the nascent transcript, and is represented by the solid curve in Figure 6. This curve shows that the effective concentration of N at the transcription complex as a consequence of RNA looping is in the 10−100 μM range when the *nut* site on the template is located about 100 bp upstream of the terminator.

It is important to point out explicitly that the curves of Figure 6 have been calculated using poly(rU) as a model for the RNA chain; i.e., the single-stranded RNA chain between the *nut* site and the terminator is assumed to be completely unstructured and unstacked. The occurrence of secondary structure in this region of the chain could both shorten and stiffen the RNA, and thus change the RNA length dependence of the calculated concentration of N at the terminator [see Rippe et al. (1995) for insight into how such changes might modify the solid curve of Figure 6].[1]

Nonspecific Binding of N to a Nascent Transcript. The dashed curve of Figure 6 suggests on theoretical grounds that the binding of N to the nascent RNA in the nonspecific mode could also increase the local concentration of N at the terminator. This curve was calculated assuming an average (random) binding density of one N protein per nascent RNA, with the *x*-axis corresponding, in this case, to the total length of the transcript. The dashed curve shows that for longer transcripts carrying one N molecule bound nonspecifically and at random, the local concentration of N due to looping can become larger at the terminator than the concentration of N bound at a boxB hairpin looping to the terminator over the same total transcript length. Thus, if N-dependent antitermination depends primarily on the local concentration of N at transcription complexes located at downstream terminators, non-*nut*-dependent antitermination should overshadow that due to N specifically bound at boxB at these transcript lengths and at significant nonspecific N binding densities.

This finding can be tested by comparison with the experimental transcription measurements of Rees et al. (1996). Extrapolations of our nonspecific binding curves suggest that at low to moderate binding densities the N protein binds as a monomer to a nonspecific RNA sequence that is 15 nucleotide residues in length. Let us assume that this oligomer length represents approximately one binding site size for N along a nonspecific RNA sequence. In the Rees et al. (1996) experiments, the length (from promoter to terminator) of the Δ*nut* transcript used was approximately 159 nucleotide residues. At low N binding densities, this length of RNA will carry about 145 [159 − 15 + 1; see McGhee and von Hippel (1974)] overlapping potential sites for the nonspecific binding of N. The concentration of transcription complexes (and thus of nascent RNA chains) in these experiments was ∼25 nM; thus, the maximum concentration of potential nonspecific N binding sites was about 4 μM. The dissociation constant for nonspecific binding of N to RNA measured here was ∼1 μM in the binding buffer used (∼120 mM K^+). Under physiological salt concentrations (∼150 mM K^+, ∼5 mM Mg^{2+}), the nonspecific binding affinity might be 5−10-fold less (M.R.V.G., unpublished results). Thus, at the very low concentrations of N and physiological salt concentrations used in the specific (*nut* site containing) in vitro transcription experiments of Rees et al. (1996), one would expect little or no nonspecific binding of N to the nascent transcript.

On the other hand, at moderate salt concentrations and the concentration of N (∼250 nM) used by these workers in the N-dependent antitermation experiments with a Δ*nut* template (in which the nascent transcript did not contain a boxB hairpin), a nonspecific binding occupancy of at least one N per nascent transcript might be expected, and under the assumptions we have made the antitermination effect should indeed be dominated by nonspecific binding of N to the nascent transcript. Thus, under these conditions, and assuming an equilibrium binding model for N-dependent antitermination (see Discussion), we would expect nonspecific N-dependent antitermination to overshadow *nut*-site-specific antitermination, as suggested theoretically in Figure 6 and demonstrated experimentally by Rees et al. (1996).

[1] We note that the effective concentration of boxB-bound N at a transcription complex located at the terminator will increase significantly as the contour length of the RNA loop between the terminator and the boxB hairpin decreases from 100 nt toward 25 nt or less (Figure 6). This effect may not manifest itself as a further increase in antitermination since the concentration of N at the terminator is already likely to be saturating at RNA contour lengths in the vicinity of 100 nt. Also, any such further increase might be counteracted by steric effects at these short RNA looping distances. However, such an effect might be seen and investigated experimentally with mutant forms of N protein that are partially defective in antitermination.

DISCUSSION

In this paper, we have shown that the N protein of phage λ: (i) binds specifically to a wild-type boxB RNA hairpin construct as a 1:1 complex with a dissociation constant (K_d) of ~1 nM; (ii) has the solution structure of a fluctuating random coil; (iii) assumes a partially folded structure (at least 16−18 amino acids of ordered α-helix) within the specific complex while perturbing the conformation of the bound RNA from its free solution structure as well; (iv) binds nonspecifically and promiscuously to other RNA (or DNA) sequences that range from unstructured RNA oligomers to boxB hairpins differing from the wild-type by only one amino group at the 5′ end of the hairpin loop; (v) remains fully unfolded in the nonspecific binding mode in which it also binds much more weakly ($K_d \cong 1-2\ \mu$M); and (vi) binds in its specific binding conformation to a boxB RNA hairpin that has been modified at the 3′ end of the hairpin loop with only a slightly increased value of K_d.

These results, combined with earlier findings (Chattopadhyay et al., 1995a; Rees et al., 1996), show that N complexed with RNA can exist either in a specific binding mode, in which it binds with high affinity and specificity to the boxB hairpin and operates as a processive antitermination factor, or in a nonspecific binding mode, in which it binds promiscuously (but with 1000-fold lower affinity) to noncognate RNA sites. In the nonspecific mode, N protein is also ineffective as an antitermination factor unless it is supplied at elevated protein concentrations and under somewhat reduced salt concentration conditions. The characterization of these two binding modes of N to RNA allows us to approach the mechanism of N-mediated antitermination with new understanding.

Processivity of N-Dependent Antitermination Controls Its Antitermination Specificity. The biological importance of N-dependent antitermination in the development of phage λ depends not only on the fact that it occurs, but also on the fact that it can be focused on specific terminators. Clearly this antitermination specificity is accomplished by the involvement of the *nut* site, which (through its manifestion as the boxB hairpin in the nascent transcript) provides the regulatory signal that controls the processivity of N-dependent antitermination and thus limits its effect to terminators located within a fixed distance downstream of *nut*. The specific and nonspecific binding interactions of N with the nascent transcript that are described here and in Rees et al. (1996) permit us to provide some quantitation and mechanistic underpinnings to the development of this processivity and thus specificity.

Figure 7A shows schematically how nonspecific N-dependent antitermination can be achieved by N alone (in the absence of the *nut* specificity signal) under conditions of lowered salt concentration at which the nonspecific binding density of N to the nascent transcript becomes significant. As suggested by the experiments of Rees et al. (1996), and justified mechanistically in Figure 6, RNA looping of the nascent transcript can raise the local concentration of N at the transcription complex sufficiently to induce the antitermination phenotype. This somewhat nonphysiological system has permitted us to isolate the functional effects of N and to show that it works as an antitermination factor both by increasing the rate of transcript elongation by the polymerase and by increasing the stability of the

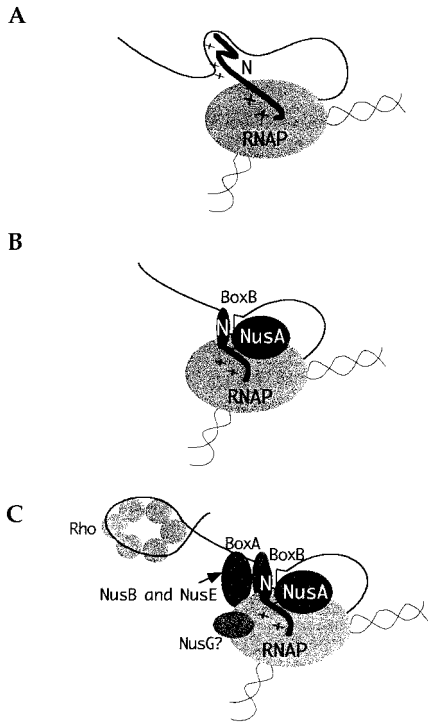


FIGURE 7: Three models of N-dependent antitermination. (A) Nonprocessive and nonspecific antitermination induced by phage λ N protein alone in the absence of a *nut* site. (B) Minimally processive and specific N-dependent antitermination system, with N binding tightly to the boxB RNA hairpin coded by the *nut* site and interacting with the polymerase and NusA by RNA looping to form an antitermination complex that is stable over several hundred nucleotide residues of RNA chain elongation. (C) Fully processive and specific N-dependent antitermination system, with N binding tightly to the boxB RNA hairpin and NusA and the other Nus factors binding to the boxA sequence of the *nut* RNA to make an antitermination complex that is stable over thousands of nucleotide residues of RNA chain elongation.

elongation complex (Rees, Weitzel, Das, and von Hippel, manuscript in preparation). However, in this type of antitermination, N binds very weakly to the transcription complex ($K_d \cong 2 \times 10^{-7}$ M) and (assuming a standard association rate constant of about 10^8-10^9 M^{-1} s^{-1}) dissociates with a half-time of 10−100 ms. Since the elongation rate of the transcript is also estimated to be of this order of magnitude, especially in the presence of N (~10−30 ms/nt added), this nonspecific mode of N-dependent antitermination is likely to be effectively nonprocessive (and thus also nonterminator-specific) and to depend on the continuous presence of high concentrations of N protein in the solution.

Figure 7B shows the minimal processive (and thus terminator-specific) N-dependent antitermination system. Here, under physiological salt concentrations at which nonspecific binding of N to the nascent transcript is insignificant, N binds tightly to the boxB RNA on the nascent transcript, and RNA looping (see Figure 6) will raise its local concentration at terminators located 100−300 bp downstream of the *nut* site to $\sim 10^{-5}$ M. As a consequence, this minimal antitermination subassembly (including NusA) will bind to the transcription complex with a stability that is 100−1000-fold greater than that characteristic of the nonspecific system of Figure 7A, leading to a calculated half-time for the dissociation of N from this complex of 10 s or more. As shown in Figure 7B, N-dependent transcription is then expected to become processive (and thus terminator-specific)

over some hundreds of base pairs downstream of the *nut* site.[2]

Finally, as shown schematically in Figure 7C, the addition of the other components of the full N-dependent antitermination system (the other Nus factors together with the boxA sequence of the transcript) adds an additional measure of stability to the antitermination complex, making antitermination processive and specific to terminators up to thousands of base pairs downstream of the *nut* site (Mason et al., 1991, 1992; DeVito & Das, 1994).

Specific and Nonspecific Interactions with the Nascent RNA Transcript. Since the direct affinity of the N protein for RNA polymerase appears to be weak,[3] the increase in the local concentration of N at the transcription complex as a consequence of *cis* binding to the nascent transcript is essential to efficient N-dependent antitermination. We have shown that binding to the nascent transcript can increase the local concentration of the N protein at the transcription complex located at downstream terminators, and that this interaction can be further improved by the presence of a high-affinity binding site (boxB hairpin) within the RNA transcript. In support of these conclusions, we have measured the affinities of N both to nonspecific RNA and to the boxB RNA hairpin of phage λ. We have shown that N binds with high affinity and specificity to its cognate boxB RNA hairpin, and also that it binds nonspecifically and indiscriminately to other RNA sites, albeit with 1000-fold lower affinity. The experiments of Rees et al. (1996) have shown that both types of interaction of N with the nascent RNA chain can support N-mediated antitermination, but to different degrees. As pointed out above, the consequence of the difference between these two binding modes is that N-mediated antitermination by specific interaction with the boxB hairpin (presumably also stabilized by the Nus factors) dominates in vivo. However, the balance is delicate, as suggested by the observation that the overexpression of N can induce non-phage-specific antitermination in vivo (Franklin & Doelling, 1989), perhaps as a consequence of nonspecific binding of N to the nascent transcript under these conditions.

Evolution of the Specific Binding Site. The capacity of N to function nonspecifically shows the importance of the *nut* site in specific antitermination. Since many potential nonspecific RNA binding sites are likely to be present in the nascent RNA, including some that differ only slightly from the cognate boxB target site, N must exhibit a high specificity for its exact cognate site. This is indeed the case, as shown here and elsewhere (Chattopadhyay et al., 1995). It appears, however, that the additional stabilization gained by the cognate complex is enhanced even further by the interaction of this complex with NusA (and perhaps the other Nus factors), which may operate to add yet another layer of specificity to the antitermination system.

Examination of the specificity of the N−boxB interactions of the related lambdoid phages allows us to separate these two layers, since the stabilization of the N−polymerase interaction by the Nus factors, as well as the activation of RNAP, appears to be the same for the different lambdoid phages. This argument is supported by the results of domain swapping experiments (Lazinski et al., 1989), which have shown that the activation domains of the different lambdoid phages are capable of bringing about antitermination only within the phages from which the specific RNA binding domain was derived. Thus, in lambdoid phage-specific antitermination, it is beneficial for N to show significantly reduced affinity for nonrelated boxB hairpins, since this permits nonspecific binding to "out-compete" the *nut* site and thus avoid the inappropriate localization of additional Nus factors. On the basis of these results, we propose that N binds to the noncognate boxB hairpins in the nonspecific binding mode, or possibly in a specific binding mode that has been sufficiently destabilized to be out-competed by the nonspecific binding sites.

Critical Guanosine Residue at the 5′ End of the BoxB Loop. We show here that both the binding specificity and the structural change induced in N are totally dependent on the amino group at the C2 position on the guanosine ring at the 5′ end of the boxB loop. It is possible that this amino group makes a direct interaction with the N protein. Another possibility is that this amino group is involved in forming the proper loop structure. This C2 position amino group is directly involved in making a G-A base pair in the GNRA tetraloop RNA hairpins (Heus & Pardi, 1991). Perhaps the inosine substitution in boxB prohibits a similar G-A pairing in the boxB loop, suggesting that the boxB RNA hairpin may form a similar structure to the GNRA tetraloop.

Interaction of N with RNA Polymerase. To this point we have assumed implicitly that it is only the local concentration of N delivered to the transcription complex by *cis* RNA looping that is important, and have not considered whether or not this N is present in its specific (partially folded) or nonspecific (unfolded) binding form. Thus, although we have established that a structural change in N is induced upon specific binding to the boxB RNA hairpin, we must now ask explicitly whether it is also required or helpful for the actual antitermination activity of N, which obviously must depend on the interaction of this factor with the RNA polymerase located within the transcription complex.

The observation that N is capable of functioning on Δ*nut* templates that do not code for boxB, to which we now know that N binds in its unfolded form, suggests that the structural change in N induced by its cognate boxB is not necessary for antitermination. In antitermination experiments performed at moderate salt concentrations, it was shown that comparable elevated concentrations of N were required to manifest antitermination with *nut* and Δnut templates (Rees et al., 1996), thus also arguing that the actual modification of RNAP that leads to antitermination may not even be assisted by the structural change in N observed here. Finally this conclusion is also in accord with the observation of Rees et al. (1996) that boxB added to N *in trans* effectively inhibits N-dependent antitermination, rather than strengthening it as might be expected if the formation of the specific boxB RNA binding conformation were required for more effective polymerase binding. All these arguments suggest that the binding of N to boxB, and the structural changes induced

[2] These calculations are in good agreement with experimental data obtained with the minimal N-dependent antitermination system at the *tR′* terminator for a variety of templates into which the *nut* site had been engineered at various distances from the terminator (W. Whalen and A. Das, unpublished results). These data show that strong antitermination is obtained with the minimal (N, NusA, and *nut* site) system with *nut* sites located 100−300 bp from the terminator, but that the N-antitermination effect is severely compromised when the template distance between the *nut* site and the *tR′* terminator is increased to 600 bp.

[3] The results of Rees et al. (1996) with N complexed with boxB RNA *in trans* suggest a $K_d > 10^{-6}$ M for this interaction.

by this binding, do not contribute to the interaction of N with RNA polymerase that induces the N-dependent antitermination phenotype. These results and others (Tan & Frankel, 1995; Das and co-workers, unpublished observations) suggest that perhaps N can be treated formally as a two-domain protein, with one domain primarily responsible for the processivity (and thus the terminator-specificity) of the antitermination process, and the other primarily responsible for inducing RNA polymerase into the antitermination mode.

We estimate that at least 16−18 amino acids of the N protein form α-helix when N binds specifically to boxB RNA. Our efforts to investigate this structural change by NMR have been hampered by the insolubility of the N−boxB complex at high concentrations. It is tempting to assume that some or all of this structural change takes place within the RNA binding domain of N, especially since Tan and Frankel (1995) observed that proline insertions in peptides 22 amino acid residues in length that spanned the ARM binding domain of N abolished specific RNA binding. The results presented here do not permit us to define the regions of N within which α-helical structure forms on specific complex formation with boxB RNA, but it is possible that most of this structure forms within the RNA binding domain and that the rest of the N protein remains partially or totally unfolded. We have shown that specifically bound N protein is still readily susceptible to proteolytic digestion (M.R.V.G., unpublished results). The remaining unfolded regions of N may form specific structures when encountering other components of the antitermination complex. These issues are currently under investigation.

Possible Roles of N Structure in the Developmental Regulation of Phage λ and Related Viruses. Is the N protein really unfolded before interacting with boxB RNA in vivo? Our results demonstrate that fully active N protein is largely unstructured when free in solution. It has also been reported that N is actively degraded by the lon protein, which is an *E. coli*-coded protease that appears to be responsible for degrading missense and improperly folded proteins (Bukhari & Zipser, 1973; Shineberg & Zipser, 1973; Gottesman & Zipser, 1978; Simon et al., 1979). Degradation of free or nonspecifically complexed N by lon may be involved in regulating the excision of the λ prophage from the *E. coli* genome during the switch to lytic development. It is known that in the absence of lon, commitment to lysis occurs much earlier than in wild-type *E. coli* (Gottesman et al., 1981); it is therefore tempting to speculate that the unfolded state of the N protein provides an opportunity for the regulation of the concentration of this protein by cellular proteases and that such regulation may be involved in the control of the lysogenic−lytic developmental switch.

The N proteins of the lambdoid family and the Tat protein of HIV are all RNA binding transcriptional activators. Each of these proteins is a small basic polypeptide that binds with high specificity to a target RNA site on the nascent transcript and undergoes a structural change in the process. Although the structure of these proteins is not known, indications from the literature suggest that at least the Tat protein is unfolded in solution (Weeks et al., 1990; Calnan et al., 1991). We have shown that the N protein is also unfolded when free in solution and that it is capable of activating RNA polymerase in this form, though we have no information on its structural state after binding to the transcription complex. It is likely that the other lambdoid N proteins also exist in an unfolded state; for example, it has been reported that the Nun protein of HK022, which is a transcriptional termination factor that binds to the boxB sequence, is also unfolded in solution (Chattopadhyay et al., 1995b). Since this structural property may represent a common motif for these transcription factors, we suggest that other RNA binding transcriptional antiterminators may also contain an initially unfolded region that interacts with RNA polymerase, both in prokaryotes and in eukaryotes. Further investigations of these proteins should focus on the extent of the structural change induced by RNA binding and on the protein sequences necessary for RNAP activation.

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