

# Assembly of the N-dependent Antitermination Complex of Phage $\lambda$ : NusA and RNA Bind Independently to Different Unfolded Domains of the N Protein

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The N protein of bacteriophage  $\lambda$  activates expression of the delayed early genes of this phage by modifying RNA polymerase (RNAP) into a form that is resistant to termination signals. N binds to the boxB hairpin that forms in the nascent RNA transcript upon transcription of the *nut* regulatory element, and then interacts with RNAP by RNA looping. The binding of the N-boxB subassembly to the transcription complex is further stabilized by interaction with the *Escherichia coli* NusA protein. N, free in solution, exists as an unfolded protein that becomes partially structured upon binding specifically to boxB RNA. Because NusA does not assist in antitermination unless N is specifically bound to boxB, we have asked whether the structural change induced by binding to boxB affects the interaction of N with NusA. Using fluorescence spectroscopy, we have measured the affinity of N for NusA in the presence and absence of boxB RNA. We find that NusA binds to the unfolded N protein with a dissociation constant ( $K_d$ ) of  $\approx 70$  nM, and although N undergoes a significant structural change upon binding to boxB, the binding affinity of NusA for a N protein complexed with boxB is not altered. We have also shown that the boxA element of *nut* does not affect NusA binding to N-boxB. These results demonstrate that the interaction of N with NusA is independent of RNA binding, arguing that NusA must interact with an unfolded region of the polypeptide that remains unstructured even when N binds to boxB RNA. To further establish this point we isolated a truncated peptide containing the amino-terminal 36 residues of the N protein. Binding of boxB RNA to this peptide showed that all of the structural change in N that occurs upon binding to boxB RNA is localized within the amino-terminal 36 residues of N, therefore the C terminus of N, including the regions necessary for NusA binding and RNAP activation, remains unfolded when the full length N binds to boxB RNA. Thus it appears that N can be described as an unfolded multi-domain protein that becomes ordered in a modular fashion as it encounters its various binding partners within the N-dependent antitermination complex.

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## Introduction

The N protein of bacteriophage  $\lambda$  participates in phage development by activating expression of the

$\lambda$  delayed early genes. N functions by inhibiting termination at intrinsic and rho-dependent terminators that otherwise function to truncate synthesis of the delayed early transcripts (Das, 1992, 1993; Friedman, 1988; Greenblatt *et al.*, 1993; Roberts, 1988). Several host factors, including NusA, NusB, NusE (S10), and NusG, assist N in modifying the host RNA polymerase (RNAP) into a termination

Abbreviations used: RNAP, RNA polymerase; CNBr, cyanogen bromide; TFA, trifluoroacetic acid.

resistant form (Das & Wolska, 1984; Das *et al.*, 1985; Friedman & Baron, 1974; Ghosh & Das, 1984; Goda & Greenblatt, 1985; Sullivan *et al.*, 1992). The *nut* sequence, located on the DNA template between the early promoters and the target terminators, is also required for N-dependent antitermination and serves to localize N function to the early operons of  $\lambda$  (Rosenberg *et al.*, 1978; Salstrom & Szybalski, 1978).

The *nut* site consists of two primary sequence elements, boxA and boxB, which, upon transcription, function as protein binding sites in the nascent RNA and assist in assembly of the N-dependent antitermination complex (Chattopadhyay *et al.*, 1995; Mogridge *et al.*, 1995; Nodwell & Greenblatt, 1991, 1993; Whalen & Das, 1990). BoxB forms a stem-loop structure in the nascent RNA to which the N protein must bind tightly and specifically for efficient antitermination (Chattopadhyay *et al.*, 1995). The role of boxA is not as well characterized, but it may function to provide an RNA binding platform for a NusB/NusE heterodimer (Horwitz *et al.*, 1987; Nodwell & Greenblatt, 1993). It has also been proposed that the boxA RNA sequence may interact with the NusA component of the antitermination complex (Friedman & Olson, 1983; Mogridge *et al.*, 1995).

An N-dependent transcription complex capable of processive antitermination can be reconstituted *in vitro* from purified components (DeVito & Das, 1994; Mason *et al.*, 1992). This antitermination system requires a DNA template containing the *nut* site, the  $\lambda$  N protein, the *Escherichia coli* Nus factors, and RNAP, and is capable of mimicking *in vivo* antitermination by reading efficiently through terminators located far downstream of the *nut* site. A simpler complex, containing only N, NusA, and RNAP, is able to cause effective antitermination under physiological conditions on templates containing the *nut* site, or just the boxB element of *nut* (Whalen & Das, 1990; Whalen *et al.*, 1988). This reduced system, called the "minimal antitermination complex", is less processive and does not function on terminators located more than a few hundred base-pairs downstream of *nut* (DeVito & Das, 1994).

The currently accepted mechanistic model of N-dependent antitermination involves tight binding of the boxB RNA hairpin to a single N protein. This interaction increases the local concentration of N at the transcription complex by RNA looping, favoring a productive interaction of N with the elongating RNAP (Nodwell & Greenblatt, 1991; Van Gilst *et al.*, 1997; Whalen & Das, 1990). NusA helps to stabilize this interaction in the minimal antitermination complex. However, as the transcript grows longer and the looping-dependent increase in the local concentration of N at RNAP becomes smaller, the stabilization provided by NusA and the looping effect of boxB progressively decreases, and the antitermination function of N is lost (Nodwell & Greenblatt, 1991; Rippe *et al.*, 1995; Van Gilst *et al.*, 1997).

In addition to *in vitro* results that support this model (DeVito & Das, 1994), it has also been demonstrated that N can function *in vivo* in a NusA-dependent manner using a template from which the boxA element has been deleted. This construct, which should be impaired in its ability to recruit additional Nus factors, functions effectively at both the rho-dependent *tR1* terminator and the intrinsic *tI* terminator when these terminators are placed near the boxB element of *nut* (Patterson *et al.*, 1994; Zuber *et al.*, 1987). The remaining Nus factors, recruited in part by the boxA binding site on the nascent RNA, provide additional stabilization that prevents the loss of N from the transcription complex for several thousand nucleotides of transcription following synthesis of the *nut* site (Barik *et al.*, 1987; DeVito & Das, 1994; Mason *et al.*, 1992; Whalen *et al.*, 1988).

In accord with this model, it has been shown that elevated concentrations of N protein alone can invoke antitermination *in vitro* in the absence of any Nus factors on a template that contains neither the boxB or boxA elements of *nut* when the reaction is carried out at lower salt concentrations (50 mM KCl) (Rees *et al.*, 1996). The ability of N protein alone to work in this manner depends in part on non-specific binding of N to the nascent RNA transcript. The interaction of this non-specifically bound N with the transcription complex is relatively unstable, and the resultant rapid dissociation of N from the complex makes this type of antitermination functionally non-processive with respect to upstream regulatory signals; i.e. the continuous presence of high concentrations of N is required to effectively modify the transcription complex.

N binds to specific and non-specific RNAs in significantly different conformations. N alone is an unstructured protein that becomes partially  $\alpha$ -helical when bound specifically to the boxB RNA hairpin, while N remains unstructured when non-specifically bound to other RNAs (Van Gilst *et al.*, 1997). Nevertheless, in the absence of NusA, and under conditions where the non-specific binding of N to the transcript is as effective as specific binding to boxB, a template containing the structure-inducing boxB binding site does not stabilize the interaction of N with RNAP and the same elevated levels of N are required to antiterminate. These results suggest that the specific binding of N to the boxB hairpin, and the structure induced in N and the boxB RNA as a consequence, do not increase the strength of the fundamental (non-Nus-protein-dependent) N-RNAP interaction (Rees *et al.*, 1996; Van Gilst *et al.*, 1997).

Thus, formation of a minimal processive antitermination complex requires the NusA protein, and NusA only serves to stabilize the N-RNAP interaction when N is bound specifically to the *nut* site of the RNA transcript. In fact, when N is nonspecifically bound to a transcript from which the *nut* site has been deleted, NusA acts to inhibit antitermination (Rees *et al.*, 1996). This led us to

postulate that the presence of the boxB hairpin, or the conformational change induced in N and boxB by the specific N-boxB interaction, must somehow "improve" the interaction of N with NusA.

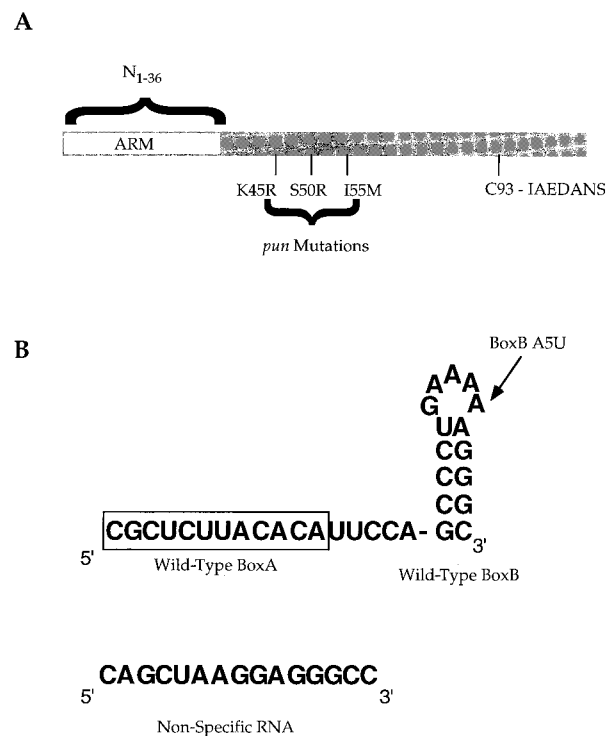
NusA has been shown to bind to the RNAP of the elongation complex (Gill *et al.*, 1991); however, there are several ways that NusA might interact with the other components of the minimal antitermination complex. The binding of N to NusA has been demonstrated *in vitro* by affinity chromatography (Greenblatt & Li, 1981). In addition, band shift assays using labeled *nut* RNA have been used to show that NusA can bind to an N-*nut* RNA complex (Mogridge *et al.*, 1995). However, these experiments were performed with very different techniques under different conditions and do not allow a comparison of the affinity of N for NusA in the presence and absence of boxB RNA. Here we present a high resolution study of the interaction between N and NusA in the presence and absence of specific and non-specific RNAs. We ask specifically here: (i) how tightly does NusA bind to N in solution? (ii) Does the specific binding of boxB RNA to N strengthen the N-NusA interaction? (iii) Does the boxA element of *nut* stabilize the N-NusA complex when N is bound specifically to boxB RNA?

We find that NusA binds to free unstructured N protein, and that this binding is not changed when N is bound to boxB RNA or to non-specific RNA. We also show that boxA RNA does not affect N binding to NusA. Thus N binds to NusA in a process that is energetically independent of RNA binding. This result can be rationalized by our demonstration here that all of the structural change induced in N by boxB binding occurs within the amino-terminal 36 residues of the N protein, a domain that binds specifically to boxB RNA but not to NusA, leaving the remainder of the polypeptide chain of N unfolded (see Figure 1A), including the NusA binding domain and the region of N that is required for RNA polymerase activation (Henthorn & Friedman, 1996; Lazinski *et al.*, 1989). Therefore, NusA binding and RNAP activation are also structurally independent of RNA binding to N. These results lead us to suggest that N can be described as an unfolded multi-domain protein that becomes ordered in a modular fashion upon encountering its various binding partners in the antitermination complex.

## Results

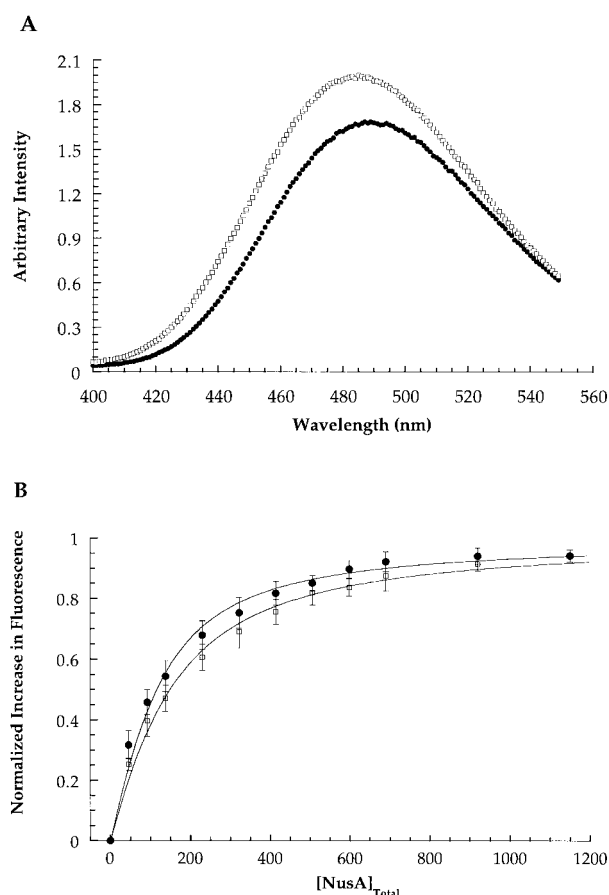
### Extrinsic fluorescent probes at Cys93 can be used to monitor the binding of N protein to NusA

The N protein of phage  $\lambda$  is an unfolded polypeptide that is 107 amino acids in length. It contains a single cysteine residue at position 93 that can be labeled with sulfhydryl-reactive probes (Figure 1A). We attached an IAEDANS fluorescent probe to this cysteine residue with 1:1 reaction stoi-



**Figure 1.** Functional organization of the N protein and the *nut* RNA. (A) The cysteine residue that has been modified with the IAEDANS probe is shown at position 93. Also shown are the amino acid substitutions (from left to right: *punA134*, *punA150* and *punA165*) in N that suppress the *nusA1* mutation (Franklin, 1985b). The shaded section represents the region of N that is capable of transferring the antitermination phenotype to the Nun termination protein of HK022, and to the homologous N proteins of  $\phi$ 21 and P22, when fused to the ARM binding domains of these proteins (Henthorn & Friedman, 1996; Lazinski *et al.*, 1989). The 36 amino acid peptide (N<sub>1-36</sub>) purified and studied here is also marked. (B) Sequences and structures of the wild type *nut* RNA element and the non-specific RNA oligomers used in this study. The boxB oligomer used was 15 nucleotides in length and encompassed just the stem and loop of the hairpin (separated from the entire *nut* RNA by a dash). An oligomer containing the entire *nut* sequence shown was used when examining the effect of boxA on N-NusA binding (Box A/B).

chiometry (see Materials and Methods). A cysteine to tyrosine mutation at this position has been reported to result in a T<sub>s</sub> mutation *in vivo* (Franklin, 1985b); therefore this cysteine may be important for stability of the antitermination complex, but is not essential. Moreover, we found that the extrinsically labeled N protein behaved identically to the unmodified protein in *in vitro* antitermination assays using the minimal antitermination system (containing RNAP, N, NusA and a DNA template carrying the *nut* site), suggesting that this cysteine residue may only be important (or the tyrosine may only be detrimental) in the context of the full antitermination system. The labeled N protein also functions as a non-specific (non-*nut*-



**Figure 2.** Fluorescence spectra of the  $\lambda$  N protein labeled 1:1 with an IAEDANS fluorescent probe and titration with NusA. (A) The spectra of 500 nM IAEDANS-labeled N protein alone (filled circles) and 500 nM IAEDANS-labeled N protein in the presence of 500 nM NusA (open squares) are overlaid. (B) The change in fluorescence intensity of a 100 nM solution of IAEDANS-labeled N protein at 482 nm plotted as a function of NusA added. The two titrations displayed in this Figure are N with NusA (filled circles) and N-boxB RNA with NusA (open squares). The data represent the averaged results of three consecutive titrations and are fit according to equation (1). The error bars represent the standard deviation of the values obtained from three repeats of each titration point. The binding constants calculated from several repeats of these experiments were averaged and are summarized in Table 1.

dependent) antitermination factor when supplied at elevated concentrations in the absence of boxB and NusA as observed by Rees *et al.* (1996) for the unlabeled protein (data not shown).

IAEDANS is a fluorophore that is extremely sensitive to the polarity of its environment, and this environmental sensitivity makes it a useful probe for monitoring local changes in protein conformation or association. Here we use IAEDANS-labeled N protein to monitor the binding of NusA to N. Figure 2(A) shows the fluorescence emission spectrum of a 500 nM solution of IAEDANS-labeled N protein excited with

340 nm light (see Materials and Methods). Since N protein free in solution is unstructured (Van Gilst *et al.*, 1997), this probe should be largely exposed to the polar (aqueous) solvent environment. The fluorescence of IAEDANS-labeled N protein is dramatically increased and blue-shifted upon the addition of 500 nM NusA, as expected if the interaction of N with NusA results in binding accompanied by decreased solvent exposure of the IAEDANS fluorophore (Figure 2(A)). Control experiments show that the fluorescence of the free probe is not dependent on NusA concentration, indicating that NusA does not interact with free IAEDANS (data not shown).

### Quantitative measurement of N-NusA binding

This change in the fluorescence spectrum of IAEDANS-labeled N protein induced by NusA binding was used to measure the binding constant of N to NusA. Labeled N protein (100 nM) was titrated with NusA, and the change in fluorescence intensity at 482 nm as a function of NusA concentration was used to determine a dissociation constant ( $K_d$ ) of  $\approx 70$  nM for the N-NusA binding interaction at 30°C (Figure 2(B)). Dissociation constants measured in several additional titrations of the same sort were averaged and are reported in Table 1. Extrapolation of the binding isotherms shows that the N-NusA complex forms with a 1:1 binding stoichiometry under our conditions of physiological salt concentration and temperature. We note that at low (20 mM  $K^+$ ) concentrations of salt we have observed the binding of more than one NusA per N monomer, probably as a consequence of non-specific interactions of NusA with other regions of N. Competition experiments confirmed that the unmodified N protein competes equally with the IAEDANS-labeled N for NusA binding (Figure 5).

The binding of NusA to N also changes the fluorescence anisotropy of the labeled N protein. This change in anisotropy as a function of NusA concentration added was also used to calculate  $K_d$  values for the binding of N to NusA, with similar results to those obtained by fluorescence quench-

**Table 1.** Dissociation constants for the binding of NusA to various N-RNA complexes

	Measured by change in fluorescent intensity	Measured by change in anisotropy
N to NusA	$70 \pm 30$ nM	$51 \pm 40$ nM
N/boxB to NusA	$98 \pm 40$ nM	$80 \pm 40$ nM
N/boxA/B to NusA	$91 \pm 20$ nM	
N/nonspec to NusA	$100 \pm 40$ nM	
N/boxB A5U to NusA	$120 \pm 40$ nM	

Dissociation constants ( $K_d$ ) were determined by fitting titration data (see Figure 2(B) to equation (1) (see Materials and Methods).

ing (Table 1). We conclude that NusA binds specifically to the unfolded form of the N protein with a  $K_d$  of  $\approx 70$  nM.

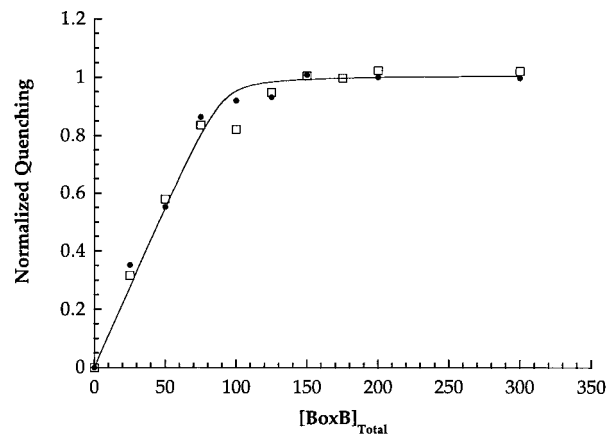
### The binding of N protein to boxB RNA does not change the N-NusA interaction

We have previously demonstrated that N binds specifically and tightly ( $K_d = 1$  nM) to boxB RNA and becomes partially folded ( $\alpha$ -helical) as a consequence (Van Gilst *et al.*, 1997). The same structural change (observed by circular dichroism; data not shown) occurs when IAEDANS-labeled N protein is added to an equal concentration of boxB RNA, confirming that the interaction of the labeled N protein with boxB RNA is equivalent to that of the unlabeled protein.

We now ask whether the presence of the bound boxB RNA hairpin, or the  $\alpha$ -helical structure that this binding induces in the N protein, alters the affinity of N for NusA. Figure 2(B) shows a titration curve for NusA binding to 100 nM N protein complexed with 100 nM boxB RNA (5'GCCUGAAAAGGGC; Figure 1(B)). The observed values of  $K_d$  from several titrations of this sort were averaged and are shown in Table 1. Many independent measurements of these interactions were made, and the small difference observed in the averaged values of  $K_d$  for the liganded and the unliganded N protein does not fall outside the limits of error of our measurements (Table 1). Thus we conclude that the binding of boxB RNA to N, and the formation of folded structure in N that occurs as a consequence, does not significantly alter the interaction of N with NusA.

This result suggests that the unfolded region of N to which NusA binds in the absence of boxB RNA remains unfolded, even when boxB binding has rendered N partially structured. In order to confirm that NusA binding to N does not preclude the binding of boxB to N (i.e. that our experiments reflect equilibrium conditions and are not dependent on "order of addition" effects), we measured the binding of boxB RNA to an N-NusA complex by titrating a prebound N-NusA complex with boxB RNA. The intrinsic tryptophan fluorescence of N is significantly quenched by RNA binding, and we have previously used this quenching to measure the binding constants of N to specific and non-specific RNAs (Van Gilst *et al.*, 1997). Figure 3 shows a titration of 100 nM N protein with boxB RNA (monitored by quenching of the intrinsic tryptophan fluorescence of N), both in the presence and the absence of 500 nM NusA (we estimate that  $\approx 80\%$  of the N protein present should be complexed with NusA in these experiments).

These titrations show that the  $K_d$  for the N-boxB RNA interaction is approximately 1 nM in the presence and absence of NusA. Ideal conditions for the accurate measurement of dissociation constants of this magnitude require lower N concentrations than were used here (see Van Gilst *et al.*, 1997). However, because NusA also dis-



**Figure 3.** BoxB RNA binds with similar affinity to N and to the N-NusA complex. Quenching of the intrinsic tryptophan fluorescence of N by boxB RNA was used to measure the binding of boxB RNA to N in the presence (open squares) and the absence (filled circles) of the NusA protein (see text). Both sets of data were fit using equation (1) with a theoretical curve corresponding to a dissociation constant ( $K_d$ ) of  $\approx 1$  nM for the binding of N to boxB.

plays significant tryptophan fluorescence, it was necessary to keep the ratio of NusA to N relatively low under conditions where most of the N protein present was bound to NusA (the tryptophan fluorescence of NusA is not quenched by RNA under our experimental conditions). Although the accuracy of the  $K_d$  calculated by this method may be limited, the most important conclusion is that these experiments confirm that both boxB and NusA bind simultaneously and independently to the N protein, and that changing the order of addition of the components does not significantly alter the coupled equilibria within the ternary complex.

### BoxA does not affect the interaction between N and NusA

The boxA element of the *nut* site could play a role in the interaction of NusA with the N-RNA complex, since it is known that mutations in boxA can suppress the antitermination deficient *nusA1* mutation (Friedman & Olson, 1983). Furthermore, Mogridge *et al.* (1995) showed (using band shift assays) that the boxA sequence stabilizes the interaction of the NusA protein with an N-*nut* complex. We have examined the binding of NusA to N in the presence of an RNA sequence containing both the boxA and boxB elements of *nut* (5'-CGCUCUUACACAUUCCAGCCUGAAAAGGGC; see Figure 1(B)). We find that the binding of NusA to this complex is unaffected by the addition of the boxA element (Table 1). Thus our data do not support the suggestion that NusA-boxA interactions are significantly involved in stabilizing N-NusA binding. We note that the band shift experiments reported by Mogridge *et al.* (1995)

were performed using a larger RNA oligonucleotide containing 13 extra nucleotides on the 5' end of boxA and nine extra nucleotides on the 3' end of boxB. Furthermore, these experiments were also performed under low salt conditions (15 mM K<sup>+</sup>) as opposed to the essentially physiological (150 mM K<sup>+</sup>) salt concentrations used in our studies. At the lower salt concentrations both specific and non-specific RNA-protein interactions might well be enhanced by electrostatic effects.

### Binding of nonspecific RNA and boxB RNA variants do not affect the N-NusA interaction

N protein binds to non-specific RNAs as an unstructured protein (Van Gilst *et al.*, 1997). In addition, NusA inhibits antitermination when N is functioning *via* non-specific binding to the nascent RNA transcript (Rees *et al.*, 1996). Therefore we have also measured the binding of N to NusA when N is bound non-specifically to RNA. For non-specific RNA we used an RNA oligomer that has been scrambled so that it contains the same nucleotide composition as the boxB oligomer, but does not resemble boxB in either sequence or structure (5'CAGCUAAGGAGGGCC; Figure 1(B)). We have previously shown that N binds to this RNA oligomer in the non-specific (unstructured) binding mode (Van Gilst *et al.*, 1997). A titration of fluorescently labeled N protein with NusA in the presence of 2  $\mu$ M concentrations of non-specific RNA (enough to bind 50% of the N protein under these conditions) shows that the binding of N to NusA is also not significantly perturbed by the binding of non-specific RNA to N (Table 1).

Mutations at the 3' end of the boxB RNA hairpin loop (boxB-A5U; Figure 1(B)) abolish antitermination in both the *in vivo* and *in vitro* assays using the minimal antitermination system (Chattopadhyay *et al.*, 1995). However, the affinity of N for a boxB hairpin with this mutation is only slightly reduced, and the same structural change is induced in N as by the binding of wild-type boxB RNA (Van Gilst *et al.*, 1997). To test the possibility that this mutation might affect NusA binding, we measured the binding of NusA to N bound to an RNA oligomer containing the boxB-A5U hairpin (GCCCU-GAAAUAGGGC; Figure 1(B)). The affinity of NusA for N was also not changed by binding this RNA (Table 1). In summary, all of the above results show that the N-NusA interaction is energetically independent of the specific and non-specific RNA binding interactions of the N protein, thus the binding of NusA and RNA to N must involve different and independent binding domains of the N protein.

### The conformation of free N or N bound specifically to boxB does not change upon NusA binding

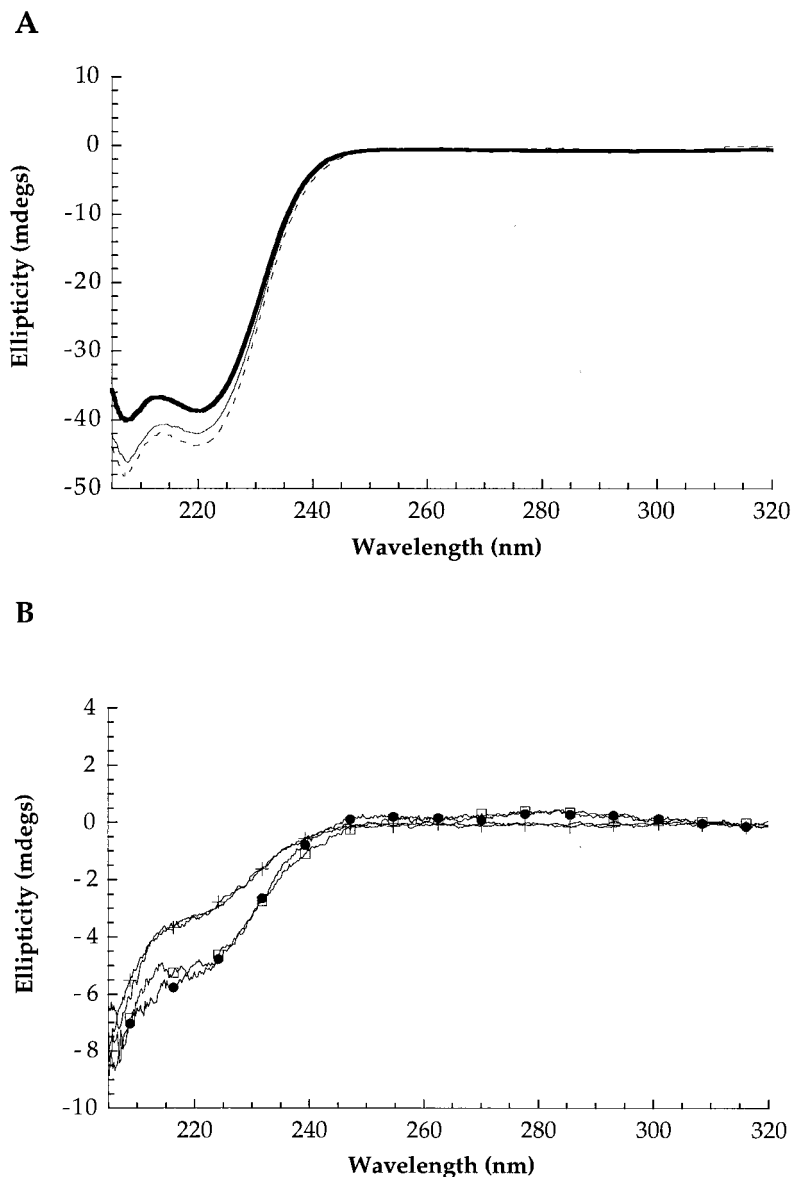
If the affinity of N for NusA is not affected by the binding of the boxB RNA hairpin, then the

binding of NusA to the N-boxB complex should not have a significant effect on the structural change in N induced by boxB RNA. To check this we used CD spectroscopy to look for changes in the secondary structure of N that might occur upon NusA binding. Figure 4(A) shows the spectra of 750 nM NusA alone, of 750 nM NusA together with 400 nM N, and of 750 nM NusA together with a 400 nM N-boxB complex (at least 80% of the N present is bound by NusA under these conditions). The CD spectrum of N bound to NusA was calculated by subtracting the spectrum of NusA alone from that of the N-NusA complex (see Materials and Methods). The resulting CD difference spectrum, shown in Figure 4(B) along with the CD spectrum of 400 nM N alone, should reflect the CD properties of N alone plus any change in the CD spectrum of either component occurring as a result of N-NusA binding.

Comparison of the spectra of bound and free N shows no significant difference in the CD signals of either N or NusA as a consequence of their association, demonstrating that the N-NusA interaction leads to no observable changes in the structure of either protein. (Of course an alternative, though less likely, explanation is that N and NusA undergo equal and opposite CD changes that cancel across the entire spectrum.) Figure 4(B) also shows the CD spectrum of 400 nM N bound to boxB RNA and to NusA. This spectrum was calculated by subtracting the spectrum of NusA and RNA alone from the spectrum of the complex (the spectrum of N bound to boxB in the absence of NusA is also displayed in Figure 4(B)). Taken together, these results show that N undergoes the same structural change on binding to boxB, whether or not N is also bound to NusA. We therefore conclude that the N-boxB binding interaction is structurally (at our level of resolution), as well as energetically, independent of the interaction of N with NusA.

### The structural change of N induced by boxB RNA binding is localized within the amino-terminal domain of the N protein

Because NusA binds with the same affinity to both the unfolded and the partially folded forms of N, we suspected that NusA must interact with an unfolded domain of the N protein that remains largely unstructured when N is bound to boxB RNA. Furthermore, we had previously shown that the unfolded (non-specifically bound) form of N can activate transcription as effectively as the specifically bound (partially folded) form under conditions that favor non-specific binding to the nascent transcript (Rees *et al.*, 1996). Therefore, in the absence of the additional Nus factors, NusA binding and RNAP activation must be functionally independent of boxB binding to N. This independence could mean that both the NusA interaction domain and the RNAP activation domain remain unstructured when N binds specifically to boxB.



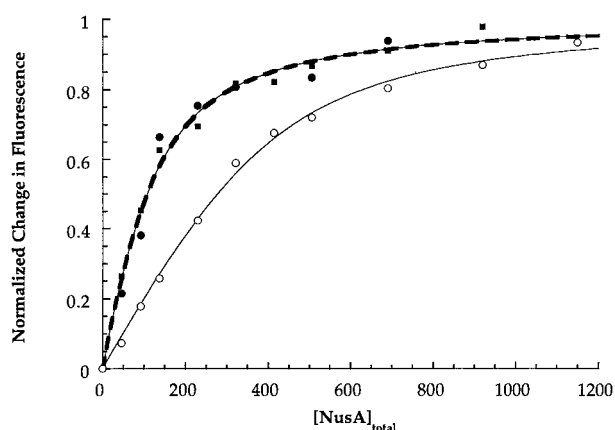
**Figure 4.** CD spectra of N and N-boxB bound to the NusA protein. (A) The CD spectrum of 750 nM NusA alone (thick solid line); 750 nM NusA with 400 nM N protein (thin solid line); and 750 nM NusA with 400 nM N bound to boxB RNA (broken line). The final spectrum was calculated by taking the spectrum of all three components together and subtracting the spectrum of 400 nM RNA alone. (B) The CD spectrum of N bound to NusA (solid line labeled with + symbols). Calculated by subtracting the spectrum of NusA alone from the spectrum of the N-NusA complex. Overlaid is the spectrum of N alone (solid line). Clearly, there are no significant changes in the spectrum of N and NusA upon binding. The spectrum of the N-boxB complex bound to NusA (solid line with open squares) is also shown. This spectrum was calculated by subtracting the spectrum of NusA alone and the spectrum of boxB RNA alone from the spectrum of N-boxB-NusA complex. The spectrum of N bound to boxB in the absence of NusA is overlaid (solid line with filled circles). This spectrum shows that the same structural changes are induced in N and boxB RNA upon their interaction, even when N is also bound to NusA.

Domain swapping and truncation experiments have shown that the regions of N responsible for activation of the antitermination phenotype are downstream of positions 31 to 34 (Henthorn & Friedman, 1996; Lazinski *et al.*, 1989; Whalen & Das, 1990). In addition, *nusA1* suppressor mutations in N map to a region of the N polypeptide that extends from residue 44 to residue 55, suggesting that the region of N that interacts with NusA might also be located downstream of positions 31 to 34 (Franklin, 1985b; Schauer *et al.*, 1987). The locations of these regions are shown in Figure 1(B).

A peptide encompassing the amino-terminal 36 residues of the N protein (the methionine residue at position 37 is modified to a homoserine lactone) was isolated as a product of a CNBr cleavage reaction at a methionine residue engineered into position 37 of the N protein. This peptide, called  $N_{1-36}$ , contains the entire ARM (arginine-rich motif) RNA binding domain of N, but does not contain the regions sus-

pected to be involved in RNAP activation or NusA binding. This peptide binds specifically to the boxB RNA hairpin with an approximately tenfold reduction in binding affinity relative to intact N (data not shown). We have also shown that up to 2  $\mu$ M solutions of this peptide do not compete with the full length N protein for NusA binding (Figure 5), indicating that the primary binding determinants controlling the interaction of N with NusA lie downstream of position 36.

Figure 6(A) shows the change in ellipticity (at 222 nm) of the full-length N protein and of  $N_{1-36}$  as a function of boxB RNA binding. Clearly  $N_{1-36}$  experiences all of the structural change monitored at 222 nm that occurs in the full length N protein. Figure 6(B) shows the change in the CD spectra of 1.2  $\mu$ M N protein and of 1.2  $\mu$ M  $N_{1-36}$  peptide as a result of binding to boxB RNA (1.5  $\mu$ M boxB RNA was used to ensure that both the uncleaved N and the N peptide were fully complexed; see



**Figure 5.** Unmodified N protein binds to NusA with the same affinity as labeled N, and  $N_{1-36}$  does not bind to the NusA protein. A titration of 100 nM labeled N with NusA was performed in the absence (filled circles) and presence of 300 nM unmodified N protein (open circles). The titration of N in the absence of 300 nM unmodified N was fit to equation (1) using a total N concentration of 100 nM and a  $K_d$  of 70 nM (heavy broken line), the titration of N in the presence of 300 nM unmodified N was fit to equation (1) using a total N concentration of 400 nM and a  $K_d$  of 70 nM (solid line). This titration shows that unlabeled N competes for NusA as expected if the binding constant of unmodified N is equal to that of labeled N. However, even up to 2  $\mu$ M concentrations of peptide  $N_{1-36}$  (filled squares) do not compete with labeled N for NusA binding. This curve was fit according to equation (1), using a total N concentration of 100 nM. Thus  $N_{1-36}$  does not affect the binding of 100 nM labeled N to NusA.

Figure 6(A)). The changes that occur in the CD spectra as a consequence of boxB RNA binding are the same for both complexes, including both the protein structure sensitive region of the CD spectrum near 222 nm and the RNA structure sensitive region near 280 nm (Van Gilst *et al.*, 1997). These data show convincingly that the entire structural change induced in N by boxB RNA binding is confined to the first 36 amino acids of the N protein, and also that the binding domain for NusA and the region of N necessary for activation of RNAP, are not significantly perturbed by boxB binding.

## Discussion

### Two “layers of binding specificity” characterize N-dependent antitermination

Ultimately, an N-RNAP interaction must be responsible for modifying RNA polymerase into a termination-resistant form (DeVito & Das, 1994; Rees *et al.*, 1996). This interaction is not stable in the absence of the rest of the N-dependent antitermination complex. As a consequence the maintenance of the antitermination state under these conditions requires an excess of N protein (DeVito & Das, 1994; Rees *et al.*, 1996). However, the con-

centration of N protein during phage  $\lambda$  infection is probably much lower than the concentrations needed for factor independent antitermination *in vitro*, suggesting that the antitermination factors must work together with the RNA product of the *nut* site to stabilize the N-RNAP interaction. Such stabilization is essential for persistent occupancy of the elongation complex by N during transcription across several thousand base-pairs of the DNA template. These requirements render N-dependent antitermination specific to terminators located appropriate distances downstream of *nut* signals.

The stabilizing effect of the Nus factors and the *nut* RNA elements can be separated into two “layers of binding specificity”, each nucleated by an RNA element within the *nut* site (Van Gilst *et al.*, 1997). The components responsible for the first layer, which we have termed the minimal antitermination complex, consist of RNAP, NusA, N, and the RNA hairpin coded by the boxB element of the *nut* site (Whalen & Das, 1990; Whalen *et al.*, 1988). This layer of specificity is also responsible for the phage specific N-dependent antitermination behavior displayed by the various members of the lambdoid phage family, in that the N protein of a particular lambdoid phage can only function with its cognate boxB RNA hairpin (Franklin, 1985a; Lazinski *et al.*, 1989). The boxA element of *nut* then recruits additional host factors to form the non-phage specific “second layer” of binding specificity.

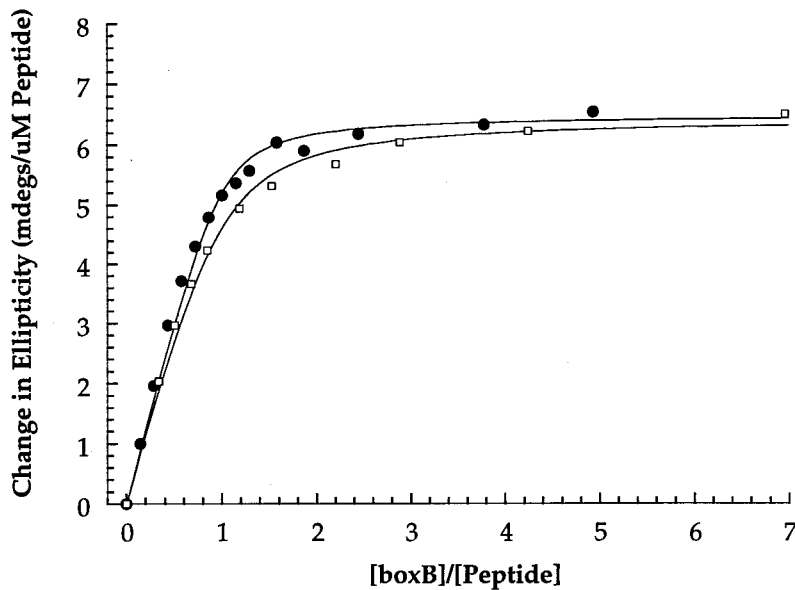
### Binding interactions within the first layer of specificity

In this study we have characterized some of the important interactions involved in the formation of the first layer of binding specificity. We have shown that the N protein binds to NusA with a  $K_d$  of  $\approx 70$  nM. Although the positive antitermination effect of NusA within the N-dependent system requires the presence of the *nut* site (in particular the boxB RNA hairpin) *in vitro* (DeVito & Das, 1994; Rees *et al.*, 1996; Whalen & Das, 1990), we have found that the boxB RNA hairpin does not affect the binding of N to NusA in the absence of RNA polymerase. Additionally, N binding to NusA is not affected by non-specific RNA binding or by the boxA element of *nut*. These results demonstrate that the binding of N to NusA is energetically independent of RNA binding to N. Consistent with these observations, the binding of N to NusA is also structurally independent of RNA binding. Thus we have shown that all of the structural change induced in N upon binding to boxB RNA occurs in the amino-terminal 36 residues of N, a region that is not sufficient for NusA binding or RNAP activation (Henthorn & Friedman, 1996; Lazinski *et al.*, 1987; Whalen & Das, 1990).

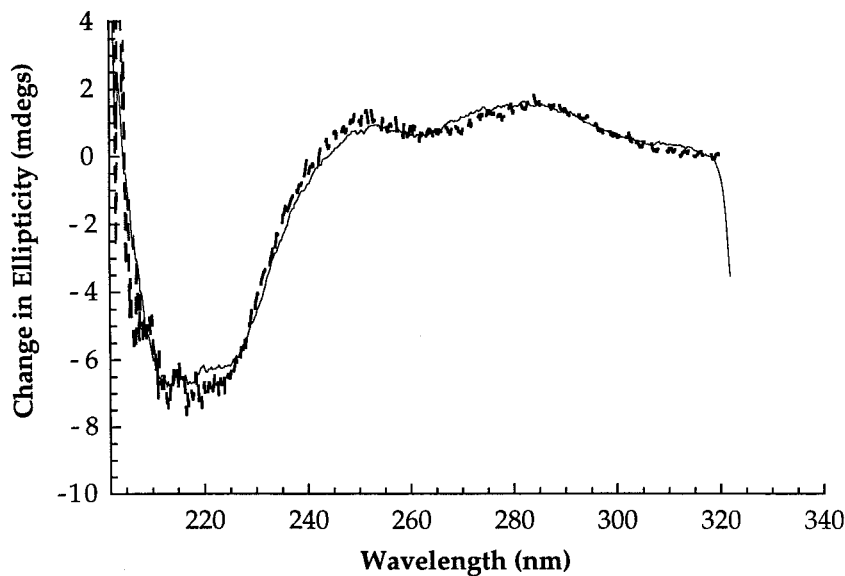
### A NusA-boxA interaction?

Under our experimental conditions, involving equilibrium binding measurements at physiological

A



B



**Figure 6.** Structural changes in the  $N_{1-36}$  peptide on boxB RNA addition. (A) Change in ellipticity at 222 nm per  $\mu\text{M}$  of N (filled circles) and  $N_{1-36}$  (open squares) as a function of the molar ratio of boxB RNA to peptide. (B) The change in the CD spectrum that results from the binding of 1.5  $\mu\text{M}$  boxB RNA to 1.2  $\mu\text{M}$  N protein (dashed line) or 1.2  $\mu\text{M}$   $N_{1-36}$  peptide (solid line).

salt concentrations, we have observed no effect of boxB or boxA on the binding of NusA to N. It has been suggested that there may be determinants in the boxB and boxA elements of *nut* that affect the association of NusA with N in the context of the antitermination complex or in a ternary interaction. These predictions were based primarily on the observation that a suppressor of the *nusA1* mutation has been found in the boxA element of *nut* (Friedman & Olson, 1983). However, Mogridge *et al.* (1995) have also reported that the boxA and boxB sequences stabilize an interaction of NusA with an N-*nut* complex in band shift assays.

Despite this evidence, a direct binary interaction of NusA with *nut* RNA has not been observed in a number of contexts (Chattopadhyay *et al.*, 1995; Liu & Hanna, 1995; Mogridge *et al.*, 1995). We have also been unable to observe a direct NusA-*nut* interaction. Moreover, it is clear that NusA is required for antitermination in systems that do not even contain the boxA element (Patterson *et al.*, 1994; Whalen & Das, 1990; Zuber *et al.*, 1987). Importantly, the phenotype of the *nusA1* mutation is still observed in a boxA independent system, indicating that the primary functional deficit of the *nusA1*

mutant has little to do with a putative interaction with boxA RNA (Patterson *et al.*, 1994).

These observations, combined with our results reported here, suggest that any boxA–NusA interaction must be indirect and most likely is mediated through the other Nus factors. In support of this hypothesis, suppressor mutations of *nusA1* have also been found in *nusB*, *N*, and *rpoB* (Schauer *et al.*, 1987; Sparkowski & Das, 1992; Ward *et al.*, 1983). Therefore, although the effectiveness of NusA in the context of the full antitermination complex may be enhanced by other Nus factors binding to the boxA RNA sequence, boxA RNA is not important for the formation of the minimal N-dependent antitermination complex.

### NusA has two different effects on termination in *E. coli*

In the absence of antitermination mechanisms, NusA binds to the elongation complex and induces pausing and termination (Gill *et al.*, 1991; Schmidt & Chamberlain, 1984; Yager & von Hippel, 1987). Yet in the context of N-dependent antitermination, NusA promotes read-through of terminators and suppression of pausing (Whalen & Das, 1990; Whalen *et al.*, 1988). The simplest model that explains this functional dichotomy states that NusA stabilizes the N–RNAP interaction solely by binding to N and helping to prevent dissociation. Thus the stabilized N–RNAP complex simply overrides the termination-inducing effect of NusA alone.

Since NusA still inhibits antitermination in the absence of boxB (when N is functioning *via* non-specific interactions with the nascent RNA transcripts as well as by direct binding to the RNAP: DeVito & Das, 1994; Rees *et al.*, 1996; Whalen & Das, 1990), this simple model would predict that N protein bound non-specifically to the transcript would not bind to NusA as effectively as N bound specifically to boxB RNA. Our results show that this is not the case; indeed NusA binding to N is totally independent of RNA binding in the absence of RNA polymerase and NusA should be able to interact with N when it is functioning by binding non-specifically to the RNA transcript. Therefore, it is necessary to extend the model to include interactions with the elongating RNAP.

This more complex model is consistent with previous suggestions that boxB plays a role in antitermination that transcends merely providing a tight binding site for the N protein (Chattopadhyay *et al.*, 1995). In this model boxB must interact not only with N, but with another component of the transcription complex. One possibility is that boxB is simply responsible for localizing N into a position on the RNAP that is most sterically favorable for stabilization by NusA and activation of the antitermination phenotype. An alternative suggestion is that boxB could be involved in helping N to switch the activity of NusA from a termination factor to an antitermination factor. This outcome could

depend on an allosteric change in RNAP induced by boxB RNA that switches the response of the transcription complex to NusA, or possibly *via* subtle boxB–NusA interaction that is only active when NusA is bound to the transcription complex.

### N protein contains multiple independent binding domains

The structural organization of the N protein is striking. N alone exists as an unfolded protein that becomes partially structured upon binding to boxB RNA (Van Gilst *et al.*, 1997). We demonstrate here that all of this structural change (equivalent to about 15 amino acid residues of  $\alpha$ -helical structure) occurs within the first 36 residues of the amino terminal domain of N. Therefore, the C terminus of the N protein, including the regions necessary for RNAP activation and binding to NusA, remains unfolded. Furthermore, the binding of N to RNA does not affect the binding of N to NusA, showing that the RNA and NusA binding domains of N are structurally and energetically independent. These observations also explain previous results that have shown that the non-specifically bound and unstructured form of N is capable of causing antitermination just as effectively as the specifically bound and structured form when conditions are such that non-specific binding of N to the nascent RNA transcript is as effective as specific binding to boxB (DeVito & Das, 1994; Rees *et al.*, 1996). Therefore we conclude that the specific boxB RNA binding interaction of N, and the structure induced in N as a consequence, are not important for stabilizing a direct interaction of N with RNAP or NusA.

However, these features may be important for concerted interactions within the antitermination complex. Thus, although we have demonstrated that the RNA binding domain of N is not structurally or energetically linked to the NusA binding domain, or the region of N responsible for RNAP activation, we still do not know whether the NusA binding and RNAP activation regions of N are structurally or energetically linked. We do not observe any significant structural changes with CD spectroscopy when NusA binds to the N protein or to a N–boxB complex, suggesting that the remainder of the N protein does not form traditional (CD sensitive) elements of secondary structure when bound to NusA. Deletion mutations have mapped two distinct regions of the N protein that are involved in NusA and RNAP binding (J. Greenblatt, personal communication). These observations, combined with our findings here, suggest that the RNAP binding domain of N probably continues to lack significant structure when N is bound to NusA in the presence or absence of boxB RNA.

On the basis of these results, we propose that the N protein exists as an unfolded multi-domain protein, and that it becomes structured (ordered) in a modular fashion upon encountering its various

partners in the N-dependent antitermination complex. Experiments in our laboratory (B. Walts, unpublished) have shown that the N proteins of lambdoid phages  $\phi$ 21 and P22 may also possess these structural characteristics, suggesting a general structural motif for these antitermination proteins. The Tat protein of HIV, which has also been reported to possess unstructured character, may comprise a eukaryotic example of such a motif (Weeks *et al.*, 1990).

We must emphasize that the method of structure detection primarily used here (CD spectroscopy) is not sensitive to the formation of all types of structure. Thus, although we have shown that NusA binding to N does not induce significant amounts of  $\alpha$ -helix or  $\beta$ -sheet, important structural elements may still be formed since specific binding of NusA to the N polypeptide must, at a minimum, immobilize portions of the binding domain. In this context recent work using NMR spectroscopy has shown that non-random coil structural features exist within the FlgM protein of the *E. coli* chemotaxis system, even though this protein lacks the common elements of secondary and tertiary structure (Daughdrill *et al.*, 1997). Interestingly, the presence of these structural features may be necessary for activity. The search for such structures in transcription factors might begin with an examination of the RNAP activation domains of the N antitermination proteins from the lambdoid family. Such studies are currently underway in our laboratory.

## Materials and Methods

### Synthesis and purification of protein and RNA components

The N protein of phage  $\lambda$  was purified from *E. coli* BL21 (DE3) cells using the pET-N1 expression vector as previously described (Van Gilst *et al.*, 1997). NusA was synthesized using the overexpression vector pMS7 obtained from Dr Michael Chamberlin of the University of California at Berkeley. PMS7 was used to transform *E. coli* SM594. NusA was induced and purified by the method of Schmidt & Chamberlin (1984) with the modifications of Gill *et al.* (1991). The RNA oligomers used in

from Molecular Probes (Eugene, OR). Solid samples of IAEDANS were dissolved in water and the concentration of the probe in solution was measured by absorbance spectroscopy at 340 nm using a molar extinction coefficient of  $5700 \text{ cm}^{-1} \text{ M}^{-1}$ . 70  $\mu\text{M}$  samples of N protein were incubated with a 10 to 20-fold molar excess of IAEDANS in labeling buffer for two hours at 37°C. The reaction was stopped by the addition of 2-mercapto-ethanol to a total concentration of 30 mM, the relative amounts of free and bound IAEDANS were determined by running a sample from the labeling reaction on an SDS-polyacrylamide gel and visualizing by UV illumination. The unreacted IAEDANS was removed using Centricon-10 filters, with consecutive additions of N/NusA reaction buffer (150 mM KOAc, 40 mM Tris-OAc, 1 mM DTT, 0.1 mM EDTA) until all of the free probe had been removed as judged by SDS-PAGE. The ratio of labeled to unlabeled N was determined by the absorbance ratio of 280 nm: 340 nm. Using an extinction coefficient for N of  $14,060 \text{ cm}^{-1} \text{ M}^{-1}$  at 280 nm. Labeled N protein was tested for activity using the minimal antitermination system (RNAP, NusA and a template containing the *nut* site), and also by showing that the labeled N could function alone in the absence of the *nut* site and NusA, as described elsewhere (Rees *et al.*, 1996).

### Fluorescence assay of the binding of labeled N to NusA

All fluorescence measurements were performed using an SLM 8000 spectrofluorometer (Urbana, IL). Unless otherwise noted, the experiments were performed in N-NusA reaction buffer (see above). Temperature was maintained at 30°C using a circulating water bath. Fluorescence spectra of 500 nM solutions of labeled N protein alone and with 500 nM NusA were collected at an excitation wavelength of 340 nm. Control experiments using 500 nM free IAEDANS (pretreated with DTT) and 500 nM NusA showed that the presence of NusA protein in the solution does not affect the fluorescence spectrum of free IAEDANS. Dissociation constants were determined by titrating 100 nM N protein, either free or bound to 100 nM boxB, boxB A5U, boxA/B or 2  $\mu\text{M}$  non-specific RNAs (see Figure 1(B)), with the NusA protein. The fluorescence of the labeled N protein excited with 340 nm light was monitored at 482 nm. The change in fluorescence as a function of total NusA concentration was plotted and the  $K_d$  was calculated by fitting the data with the following equation:

$$F_b = \frac{(K_d + [N_0] + [NusA_0]) \pm \sqrt{K_d - [N_0] - [NusA_0]}^2 - 4[N_0][NusA_0]}{2[N_0]} \quad (1)$$

this study were synthesized by Oligos etc. (Wilsonville, OR). All RNA oligomers were pretreated by diluting into N/NusA buffer (see below), heating to 90°C for ten minutes, and then cooling slowly to room temperature.

### Fluorescent labeling of N protein

Purified N protein was dialyzed into labeling buffer (20 mM Tris-HCl (pH 7.6), 50 mM KCl and 0.1 mM EDTA). The IAEDANS fluorescent probe was purchased

where  $K_d$  = dissociation constant of N for NusA,  $N_0$  = total N concentration,  $NusA_0$  = total NusA concentration, and  $F_b$  = fraction of N protein bound by NusA. Each data set used for calculating a value of  $K_d$  consisted of an average of three to five titrations. The  $K_d$  values measured from several data sets were averaged to give the values displayed in Table 1. For fluorescence anisotropy measurements, the titrations were repeated in the same fashion as above. The change in anisotropy of the IAEDANS fluorescence was plotted as a function of NusA concentration and the data were fit in accord with

equation (1). Competition experiments were performed by repeating the titration of 100 nM labeled N with NusA in the presence of 300 nM unlabeled N protein.

#### Monitoring the binding of boxB RNA to an N-NusA complex using tryptophan fluorescence

The binding constant of boxB RNA to N was measured using an intrinsic tryptophan-dependent fluorescence procedure similar to that employed by Van Gilst *et al.* (1997). The intrinsic tryptophans of the N protein were excited at 300 nm and fluorescence was monitored at an emission wavelength of 360 nm. N protein (100 nM) was titrated with boxB RNA, and the quenching of the intrinsic tryptophan fluorescence of N was plotted as a function of boxB RNA concentration. The binding constant of boxB RNA to a preformed N-NusA complex was measured in the same manner, except that 500 nM NusA protein was added to the N protein before the RNA titration was begun.

#### Structural analysis of the N-NusA complex by CD spectroscopy

Circular dichroism measurements were performed on a Jasco 600 spectropolarimeter. All CD spectra in this study were collected at 30°C in N-NusA CD buffer (100 mM KCl, 20 mM KPO<sub>4</sub>, 1 mM DTT, 0.1 mM EDTA). Spectra were collected at a scanning speed of 1 nm/minute from 320 nm to 200 nm. Spectra of N alone and of N bound to NusA were determined as follows. First the spectrum of 750 nM NusA protein alone was collected. Next, a concentrated solution of N was injected to bring the N concentration to 400 nM. The spectrum of 750 nM NusA alone was subtracted from the spectrum of the N-NusA complex to yield a difference spectrum that represents the CD spectrum of the N protein alone plus any changes in N or NusA that result from their association. For the CD spectra of the N-boxB complex with NusA, all of the above experiments were repeated using N-NusA buffer + 400 nM boxB RNA. The spectrum of 400 nM boxB RNA alone was subtracted from all experiments involving the RNA hairpin. The CD spectra of N and N-boxB in the absence of NusA were collected as described (Van Gilst *et al.*, 1997).

#### Production and purification of N<sub>1-36</sub>

The isoleucine at position 37 of the N protein was site-specifically mutated to a methionine residue using PCR-directed mutagenesis. The mutant N protein that resulted contained methionine residues at positions 1 and 37. This mutant N protein was purified as described for the wild-type protein (Van Gilst *et al.*, 1997). Purified N protein (10 mg/ml) was dialyzed into 20 mM Tris (pH 7.6), 0.1 mM EDTA, 1 mM DTT. Formic acid was added to a final concentration of 70% (v/v). CNBr was added at 50 mg/ml and the reaction was run at room temperature for 24 to 30 hours. The solution was then lyophilized and resuspended in 0.1% (v/v) TFA containing 10% (v/v) acetonitrile. The N-terminal cleavage product was purified from the C-terminal fragment and residual uncut N protein by reversed-phase HPLC chromatography using a VYDAC C-4 column (2145P510). Approximately 1 to 2 mg aliquots of N were loaded onto the column in 0.1% TFA-10% acetonitrile, and eluted using a 10% to 90% acetonitrile gradient. The resulting N terminal fragment eluted from the HPLC column as a

single high resolution band and was found to be 100% active in binding specifically to boxB RNA, in that all the peptide fragments could bind specifically to boxB RNA in a 1:1 complex. Fluorescence quenching experiments and CD titrations determined that the binding of the peptide to boxB RNA was about tenfold weaker than the binding of full length N protein (data not shown). Competition experiments were performed by repeating a titration of 100 nM labeled N protein with NusA in the presence of 2  $\mu$ M N<sub>1-36</sub>.

#### Measurement of the conformational change in the N<sub>1-36</sub> peptide

Circular dichroism experiments with N<sub>1-36</sub> were also performed on the Jasco 600 spectropolarimeter. All CD spectra and titrations were performed in N-NusA CD buffer. To monitor the absolute change in ellipticity induced by the binding of boxB RNA, the ellipticity of the N protein (1  $\mu$ M for N and 1.5  $\mu$ M for N<sub>1-36</sub>) was measured at 223 nm as a function of boxB RNA concentration. The specific change in ellipticity (per  $\mu$ M peptide) was plotted as a function of the molar ratio of boxB to peptide and is displayed in Figure 6(A). The CD difference spectra for the N<sub>1-36</sub> peptide and the N protein were obtained by first collecting the spectrum of 1.5  $\mu$ M boxB RNA, next 1.2  $\mu$ M of peptide was added and the spectrum of the peptide-RNA complex was collected. Finally, the spectrum of 1.2  $\mu$ M peptide alone and the spectrum of the RNA alone were subtracted from the spectrum of the complex. The resulting difference spectra should reflect any change in the ellipticity of the solution due to the binding of RNA by N or N<sub>1-36</sub>.

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