A protein–RNA interaction network facilitates the template-independent cooperative assembly on RNA polymerase of a stable antitermination complex containing the λ N protein

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The stable association of the *N* gene transcriptional antiterminator protein of bacteriophage λ with transcribing RNA polymerase requires a *nut* site (*boxA*+*boxB*) in the nascent transcript and the *Escherichia coli* factors NusA, NusB, NusG, and ribosomal protein S10. We have used electrophoretic mobility shift assays to analyze the assembly of N protein, the *E. coli* factors, and RNA polymerase onto the *nut* site RNA in the absence of a DNA template. We show that N binds *boxB* RNA and that subsequent association of NusA with the N–*nut* site complex is facilitated by both *boxA* and *boxB*. In the presence of N, NusA, and RNA polymerase the *nut* site assembles ribonucleoprotein complexes containing NusB, NusG, and S10. The effects on assembly of mutations in *boxA*, *boxB*, NusA, and RNA polymerase define multiple weak protein–protein and protein–RNA interactions (e.g., NusB with NusG; NusA with *boxB*; NusA, NusB, and NusG with *boxA*) that contribute to the overall stability of the complex. Interaction of each component of the complex with two or more other components can explain the many observed cooperative binding associations in the DNA-independent assembly of a stable antitermination complex on RNA polymerase.

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The N protein of bacteriophage λ modifies *Escherichia coli* RNA polymerase so that it ignores transcription termination signals located in the two early operons of the phage (for review, see Greenblatt et al. 1993) (see Fig. 1A). This modification of RNA polymerase requires a *nut* site situated between the promoter and the first terminator of each operon (Salstrom and Szybalski 1978; de Crombrughe et al. 1979; Olson et al. 1982). The *nut* site consists of two genetically defined elements, *boxA* and *boxB* (see Fig. 2A). The *boxB* DNA sequence is characterized by hyphenated dyad symmetry (Rosenberg et al. 1978), resulting in a stem–loop structure in the nascent RNA (Steege et al. 1987; Nodwell and Greenblatt 1991) and is essential for transcriptional antitermination (Salstrom and Szybalski 1978; Doelling and Franklin 1990; Whalen and Das 1990; Mason et al. 1992a). *boxA* is a sequence 5' to *boxB* that is conserved among the lambdoid phages (Friedman and Olson 1983; Olson et al. 1984) and is very similar to the *boxA* antiterminator elements that are found in the *E. coli* RNA (*rrn*) operons (Li

et al. 1984). Unlike *rrn boxA*, which is sufficient to prevent termination at Rho-dependent terminators (Morgan 1986; Berg et al. 1989; Albrechtsen et al. 1990), λ *boxA* requires N and *boxB* to participate in antitermination (Salstrom and Szybalski 1978). Alteration of λ *boxA* (CGCUCUUACACA) to a consensus version of *boxA* (CGCUCUUUAACA) that closely resembles *rrn boxA* (UGCUCUUUAACA) suppresses the effects on antitermination of particular mutations in *E. coli* host factors that are needed for antitermination by N (Friedman et al. 1990). *boxA* is required for antitermination to occur far downstream of the *nut* site both in vivo and in vitro (Olson et al. 1982; Robledo et al. 1990; Li et al. 1992) but is not required by N to antiterminate at the t_{R1} terminator just distal to *nutR* in vivo (Peltz et al. 1985; Zuber et al. 1987; Patterson et al. 1994).

The host factors NusA, NusB, S10, and NusG are all involved in N-mediated antitermination (Friedman and Baron 1974; Keppel et al. 1974; Friedman et al. 1976, 1981; Das et al. 1985; Downing et al. 1990; Li et al. 1992; Sullivan et al. 1992). Transcription of the *nut* site allows for the stable association with the elongation complex of N, the *nut* site RNA, and all four host factors (Barik et al.

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Figure 1. (A) Partial genetic and transcriptional map of bacteriophage λ showing the positions of the *nut* and *qut* sites recognized by the N and Q proteins, respectively, and the antitermination effects of N and Q (adapted from Fig. 1A of Greenblatt et al. 1993). (B) Antitermination by N involves RNA looping and the assembly on the surface of RNA polymerase of a ribonucleoprotein complex containing N, the Nus factors, and the *nut* site RNA (*boxA* + *boxB*) (Greenblatt 1984; Nodwell and Greenblatt 1991, 1993).

1987; Horwitz et al. 1987; Mason and Greenblatt 1991; Nodwell and Greenblatt 1991). Many protein–protein interactions within this complex have been identified (see Fig. 1B): N with NusA (Greenblatt and Li 1981a); NusA, NusG, and S10 with RNA polymerase (Greenblatt and Li 1981b; Mason and Greenblatt 1991; Li et al. 1992); and NusB with S10 (Mason et al. 1992b). As well, the *boxB* element of the *nut* site RNA is bound by N (Das 1992; Nodwell 1993; Chattopadhyay et al. 1995; Tan and Frankel 1995), and the consensus and *rrn* versions of *boxA*, but not λ *boxA*, are directly recognized by a heterodimer containing NusB and S10 (Nodwell and Greenblatt 1991). All of these interactions are thought to be important for the stable association of an N-containing ribonucleoprotein complex with RNA polymerase (Nodwell and Greenblatt 1991; Mason et al. 1992a).

A minimal N-modified transcription complex containing only N and NusA is able to ignore pause signals and transcribe in vitro through both Rho-dependent and -independent terminators located only a short distance downstream from a *nut* site (Whalen et al. 1988; Whalen and Das 1990; Mason et al. 1992a). However, highly processive antitermination through multiple terminators far downstream from a *nut* site additionally requires NusB, S10, and NusG, suggesting that processive antitermination is dependent on a very stable elongation complex containing all of the Nus factors (Mason et al. 1992a).

A characteristic feature of this control system is the formation of an RNA loop connecting the catalytic center of RNA polymerase to a complex on the surface of RNA polymerase that contains N and the Nus factors (Greenblatt 1984; Whalen and Das 1990; Nodwell and

Greenblatt 1991) (see Fig. 1B). In effect, the *nut* site behaves as an RNA enhancer that moves along with RNA polymerase as part of a ribonucleoprotein complex. As RNA polymerase moves downstream from a *nut* site and the RNA loop grows, its tethering effect is lost and the resulting unstable complex containing only N and NusA tends to dissociate from RNA polymerase (Nodwell and Greenblatt 1991). Dissociation is prevented when NusB, S10, and NusG increase the stability of the complex (Mason and Greenblatt 1991).

We have now used gel mobility shift assays to examine the assembly of N, RNA polymerase, and the Nus factors onto radiolabeled *nut* site RNA in the absence of template DNA. Our results show that nucleotides in the loop of *boxB* are critical for the binding of N to *boxB*, that stable association of NusA with the N–*nut* site complex requires both *boxA* and critical nucleotides in the loop of *boxB*, and that interaction of NusB, NusG, and S10 with the *nut* site requires the preassembly of N, NusA, and RNA polymerase. Mutations in *boxB*, *boxA*, NusA, and RNA polymerase each affect a particular step in the assembly of the complex. Our results provide evidence for the existence of novel protein–protein and protein–RNA interactions that are important for the assembly of the N-modified transcription complex and indicate that a large network of weak interactions is responsible for the stability of the complex.

Results

Requirements for binding of N and NusA to the *nut* site

Genetic experiments with the N genes of various lamb-

Figure 2. Mutations in the *nut* site affect the binding of N and NusA. Reactions containing 32 P-labeled wild-type or the indicated mutant *nut* site RNA were electrophoresed on nondenaturing gels, dried, and exposed to film. The positions of various protein–RNA complexes are indicated. See text for the designations used for various complexes. (A) The λ *nutR* site and the various mutations used in our experiments. (B) Mutations in the loop of *boxB* affect the binding of N. Various concentrations of N were incubated with wild-type or mutant *nut* site RNA, as indicated. (C) Mutations in the loop of *boxB* affect the assembly of NusA onto an N–*nut* site complex. The indicated concentrations of N and NusA were incubated with wild-type or mutant *nut* site RNA. The reactions were electrophoresed on a 7.5% nondenaturing polyacrylamide gel. (D) *boxA* is important for the formation of an N–NusA–*nut* site complex. N (1.5 μ M) was incubated in the presence or absence of NusA (as indicated) with pNUTWT or pNUTrev1-17 RNA (containing the sequence ACCUUACACAUUCUCGCCCGCCUGAAAAAGGGC). The reactions were electrophoresed on a 7.5% nondenaturing polyacrylamide gel. (E) The *nusA1* mutation affects the assembly of the N–NusA–*nut* site complex. N (1.5 μ M) was incubated with wild-type *nut* site RNA in the presence or absence of NusA or NusA1, as indicated. The reactions were electrophoresed on a 7.5% nondenaturing polyacrylamide gel.

doid bacteriophages have indicated that their N proteins ought to recognize the *boxB* elements of their respective *nut* sites (Lazinski et al. 1989). To directly examine the interaction of N with the *nut* site, we incubated purified λ N protein with 32 P-labeled RNA containing a wild-type *nut* site and subjected the reactions to electrophoresis on a nondenaturing polyacrylamide gel (Fig. 2B, lanes 1–3). At 1.5 μ M N (lane 3), most of the RNA was shifted to form complex 1 (see Table 1 for a list of the various complexes formed in our mobility shift experi-

ments). Because mutations in all the nucleotides of the loop of *boxB* impair antitermination in vivo (see Fig. 2A; Doelling and Franklin 1989), we tested the effects of mutations in the *boxB* loop on N binding (lanes 4–18). Changing the first and fifth nucleotides in the loop of *boxB* virtually abolished the binding of N (lanes 4–6, 16–18), and mutating the third nucleotide had a significant, but more modest, effect on the binding of N (lanes 10–12). Chattopadhyay et al. (1995) have made similar observations but did not detect an effect of mutating the

Table 1. Complexes formed by the addition of various proteins to nut site RNA and observed in gel mobility shift experiments

Complex	Components ^a
1	N–nut
2	N–NusA–nut
3	RNAP–nut
4	RNAP–NusA–nut
5	RNAP–NusA–N–nut
6 ^b	RNAP–NusA–N–NusG–nut
7 ^b	RNAP–NusA–N–NusB–S10–nut
8 ^b	RNAP–NusA–N–NusB–S10–NusG–nut

^a[RNAP] RNA polymerase.^bThese complexes have indistinguishable mobilities.

fifth nucleotide of *boxB* on the binding of N, perhaps as a consequence of using different buffer conditions in their experiments. Mutating the corresponding nucleotide in the loop of *boxB* of the *nutL* site had a dramatic effect on the binding of an N peptide in buffer conditions that were similar to ours (Tan and Frankel 1995). Mutating the second or fourth nucleotide in the loop of *boxB* did not noticeably compromise the interaction between N and the *nut* site RNA (lanes 7–9, 13–15).

It therefore seemed likely that the defect in antitermination caused by mutating the second or fourth nucleotide in the loop of *boxB* weakened an interaction between *boxB* and one of the other components of an N-modified elongation complex. To test this idea, we examined the ability of NusA to bind to N–*nut* site complexes containing wild-type and mutant sequences (Fig. 2C). NusA alone did not bind *nut* site RNA (see, e.g., Fig. 2E, lane 2). Whereas NusA was able to form a complex with N and wild-type *nut* site RNA (complex 2 in lanes 4 and 5 of Fig. 2C), it was unable to form a complex with N and *nut* sites containing mutations in loop positions 2 and 4 (lanes 9–10, 14–15). These results indicated, therefore, that the loop of *boxB* binds N and is also important for the assembly of NusA.

Because there is also genetic evidence that NusA might interact with *boxA* (Olson et al. 1982), we wanted to determine whether *boxA* had any effect on the binding of NusA to an N–*nut* site complex. Inversion of *boxA* had no effect on the binding of N but greatly impaired the ability of NusA to supershift an N–*nut* site complex (Fig. 2D, cf. lanes 1–5 and lanes 6–10), suggesting that NusA may also interact with *boxA*. To further assess the biological relevance of this interaction between NusA and the N–*nut* site complex, we used the NusA1 mutant protein in a gel mobility shift experiment. The *nusA1* mutation prevents N-mediated antitermination in vivo (Friedman and Baron 1974). Although the NusA1 mutant protein still binds N (Greenblatt and Li 1981a), it failed to shift an N–*nut* site complex (Fig. 2E, cf. lanes 5–6 and 7–8), suggesting that the *nusA1* mutation (a leucine to arginine change) affects the ability of NusA to bind RNA. Thus, *boxA* facilitates the incorporation of NusA into an N–NusA–*nut* site complex, and this is probably

dependent on a functional RNA-binding domain in NusA. These experiments suggest that NusA may link the *boxA* and *boxB* components of the λ *nut* sites by interacting with both of them. The formation of this link may stabilize the association of N and NusA with RNA polymerase and/or signal other factors to join the elongation complex.

The association of N with RNA polymerase is dependent on NusA

It has been shown previously that RNA is able to form a nonspecific binary complex with the core catalytic component of RNA polymerase. The RNA in such a complex is positioned such that nucleotides can be added to its 3' end and such that it can be cleaved by the transcript cleavage factors GreA and GreB (Altmann et al. 1994). Thus, it is likely that the 3' end of the RNA is associated with the catalytic center of the enzyme just as it would be in an elongating transcription complex (Altmann et al. 1994). Consistent with this observation, we found that the RNA polymerase core component was able to bind *nut* site RNA in the absence of any other factor (Fig. 3A, lane 2, complex 3). When increasing amounts of N were added to a reaction containing RNA polymerase and the *nut* site RNA, the amount of probe bound to RNA polymerase decreased substantially, and formation of the N–*nut* site complex was detected (complex 1, lanes 3,4). This competition between RNA polymerase and N for the *nut* site RNA almost certainly indicated that the *nut* site was positioned in the catalytic center of RNA polymerase and not positioned on RNA polymerase as it would have been in an N-modified transcription complex. The absence of a complex containing N, RNA polymerase, and the *nut* site RNA suggested that they were unable to form a stable complex in the absence of other factors.

NusA is known to bind RNA polymerase (Greenblatt and Li 1981b), and consistent with this observation, we observed that NusA supershifted the RNA polymerase–*nut* site complex 3 to form complex 4 (Fig. 3A, lane 5). The addition of increasing amounts of N to complex 4 (lanes 6,7) resulted in the formation of a lower mobility band (complex 5). Complex 2 was not observed in this experiment or in most subsequent experiments shown in Figures 4–7 because it was not stable in the gel conditions that we used to detect complexes containing RNA polymerase. The failure of Chattopadhyay et al. (1995) to detect an N–NusA–*nut* site complex is most likely a result of the gel conditions they used. Because the addition of N to complex 4 would only increase its molecular weight by 2.5%, the substantially lower mobility of complex 5 compared with complex 4 implies that the conformations of complexes 4 and 5 are quite different. Moreover, the formation of complex 5, but not complex 4, was strongly correlated with the ability of the *nut* site to function in antitermination (Fig. 3B). Thus, mutations in the loop of *boxB* that affect either the ability of N to bind the *nut* site RNA or the ability of NusA to supershift an N–*nut* site complex impaired the formation of

Figure 3. The stable association of N with RNA polymerase is dependent on NusA and *boxB*. (A) NusA facilitates the association of N with RNA polymerase. Various combinations of N, NusA (100 nM) and RNA polymerase (25 nM) were incubated with ³²P-labeled wild-type *nut* site RNA and electrophoresed on a nondenaturing polyacrylamide gel. (B) Mutations in the loop of *boxB* affect the assembly of complex 5. Various combinations of N, NusA (100 nM) and RNA polymerase (25 nM) were incubated with wild-type or mutant *nut* site RNA, as indicated, and electrophoresed on a nondenaturing polyacrylamide gel.

complex 5 (Fig. 3B, cf. lanes 3,4 and lanes 7,8, 11,12, 15,16). Presumably, the difference in conformations between complexes 4 and 5 exists because the *nut* site in complex 5 is no longer in the catalytic center. Therefore, the stable and specific association of N with an RNA

polymerase-*nut* site complex is dependent on the presence of both NusA and wild-type *boxB* RNA.

NusB, *NusG*, and *S10* bind to complex 5

To determine how the remaining Nus factors associate with a complex containing N, NusA, and RNA polymerase, we added various combinations of the proteins to complex 5 (Fig. 4A). On its own, NusG was able to create a new complex (complex 6 in lane 7). Neither NusB nor S10 alone was able to do so (lanes 5,6), but together they too were able to create a new complex with lower electrophoretic mobility (lane 8, complex 7). This result is consistent with the observation that NusB and S10 form a heterodimer that is able to bind *rrn boxA* RNA (Mason et al. 1992b; Nodwell and Greenblatt 1993). Unexpectedly, the assembly of NusG and NusB onto complex 5 was also highly cooperative (lane 9), suggesting that NusB and NusG may also interact. There were also indications of weak cooperative binding for NusG and S10 (lane 10). Interactions of NusG with NusB and S10 may be too weak to have been detected in previous direct protein-protein binding experiments. Alternatively, these interactions could be indirect. Addition of all of the Nus factors to the binding reaction resulted in formation of the most stable complex (lane 11, complex 8) as judged by the quantity of probe shifted. The formation of complex 8 was specific, as it was impaired by mutations in *boxB* that affect the binding of N or NusA (data not shown). Although the formation of this complex was highly dependent on RNA polymerase, N, NusA, NusB, and NusG, the yield was only increased about two-fold by the presence of S10 (lanes 9 and 11); (see also Fig. 5B, lanes 7,9). Either S10 does not contribute greatly to the stability of the complete complex or the stabilizing effect of S10 may only be more evident if lower concentrations of other factors are used in the reactions.

We then determined the minimal set of factors required to assemble NusB, NusG, and S10 onto a *nut* site. NusB, NusG, and S10 were unable to supershift an N-NusA-*nut* site complex (data not shown). They were also unable to stably associate with an RNA polymerase-*nut* site complex (Fig. 4B, cf. lanes 2 and 3) or to supershift complex 4, which contains RNA polymerase and NusA (cf. lanes 4 and 5). NusB, NusG, and S10 were also unable to form a complex with N, RNA polymerase, and the *nut* site RNA in the absence of NusA (cf. lanes 6 and 7). As before, however, they were able to supershift complex 5 (cf. lanes 8 and 9). Therefore, the association of N, NusA, and RNA polymerase with the *nut* site is required for the assembly of NusB, NusG, and S10.

Because the concentration of RNA in these mobility shift experiments was ~25–50 pM and the concentrations of the proteins were mostly in the 25- to 100-nM range, we considered the possibility that a minor contaminant in one of our protein preparations might be necessary for the observed mobility shifts. This seemed unlikely because mutations in the *nut* site and various proteins that interfere with function *in vivo* each affect the assembly of one or other of the various complexes

Figure 4. NusB, NusG, and S10 bind to complex 5. (A) NusB, NusG, and S10 assemble onto complex 5. Various combinations of N (500 nM), NusA (100 nM), NusB (50 nM), NusG (50 nM), S10 (50 nM) and RNA polymerase (25 nM), as indicated, were incubated with 32 P-labeled wild-type *nut* site RNA and electrophoresed on a nondenaturing gel. (B) The formation of complex 5 is required for the assembly of NusB, NusG, and S10. Various combinations of N (500 nM), NusA (100 nM), NusB (50 nM), NusG (50 nM), S10 (50 nM) and RNA polymerase (25 nM), as indicated, were incubated with 32 P-labeled wild-type *nut* site RNA and electrophoresed on a nondenaturing gel. (C) N, NusA, NusB, NusG, and S10 associate with *nut* site RNA in the presence of RNA polymerase. Western blot analysis shows 50 ng of the various proteins (lane 1) and the eluted proteins from streptavidin-agarose beads bound by *nut* site RNA in reactions that contained N, RNA polymerase, NusA, NusB, NusG, and S10 (lane 4) or from beads that lacked *nut* site RNA (lane 2) or in reactions that lacked N protein (lane 3).

(see below). Nevertheless, to directly exclude this possibility we performed an experiment using 20 nM *nut* site RNA and found that the yields relative to free probe of complexes 3–8 were similar whether picomolar or nanomolar amounts of RNA were added to the reactions (data not shown).

To assess whether all of the proteins added to the *nut* site RNA to form complex 8 were complexed directly or indirectly with the RNA, the 5' end of the transcript containing *nut* site was annealed to a biotinylated single-stranded DNA oligonucleotide and the resulting RNA–DNA hybrid was attached to streptavidin–agarose beads. After incubating the beads with RNA polymerase, N, and the Nus proteins, and washing away the unbound proteins with binding buffer, the bound proteins were eluted from the beads, run on an SDS–polyacrylamide

gel and detected by Western blotting (Fig. 4C). As controls, either *nut* site RNA or N protein was omitted from the reaction. Substantial amounts of N, NusA, NusB, NusG, and S10 were all present in eluates from beads when N and *nut* site RNA were both included in the reaction (lane 4). Only a trace amount of N bound non-specifically to the beads (lane 2). As well, small amounts of NusA were present in both control eluates, probably reflecting the binding of NusA to RNA polymerase that had associated with the single-stranded DNA on the beads (lanes 2,3). In contrast, NusB, NusG, and S10 were not present in eluates derived from reactions that lacked either N or *nut* site RNA (lanes 2,3). Therefore, as predicted by the mobility shift assays, N and all four Nus factors can enter complexes on *nut* site RNA in the presence of RNA polymerase. However, although it seems

Figure 5. A point mutation in *boxA* affects the assembly of NusB, NusG, and S10. (A) The C3G mutation in *boxA* has little effect on the assembly of complex 2. Various combinations of N (1.5 μM) and NusA were incubated with ³²P-labeled wild-type or mutant (pNUTC3G) *nut* site RNA, as indicated. The reactions were electrophoresed on a 7.5% nondenaturing polyacrylamide gel. (B) The C3G mutation in *boxA* affects the assembly of NusB, NusG, and S10. Various combinations of N (500 nM), NusA (100 nM), NusB (50 nM), NusG (50 nM), S10 (50 nM) and RNA polymerase (25 nM) were incubated with ³²P-labeled wild type or mutant (pNUTC3G) *nut* site RNA, as indicated, and electrophoresed on a nondenaturing polyacrylamide gel.

likely, we cannot be certain that N and all four Nus factors are present in the same complex.

boxA is important for the association of NusB, NusG, and S10 with complex 5

The rRNA operon (*rrn*) *boxA* binds a heterodimer of NusB and S10, whereas the λ *boxA* is unable to do so (Nodwell and Greenblatt 1993). This suggests that the λ *boxA* is a weaker version of the *rrn* *boxA* that requires additional factors to stably bind NusB and S10. We wanted to determine whether the association of NusB and S10 with complex 5 would be impaired by a mutation in *boxA*. To do so, we chose a mutation in *boxA* (a C → G change at position 3) that interferes with antitermination in the *rrn* system (Berg et al. 1989) but had only a relatively small effect on the assembly of NusA onto an N-*nut* site complex (Fig. 5A, cf. lanes 1–4 and 5–8). As well, this mutation did not affect the assembly of complex 5 (Fig. 5B, cf. lanes 4 and 13). This point mutation did, however, impair the assembly of NusB and S10 (cf. lanes 6 and 15). Unexpectedly, the formation of all of the complexes higher than complex 5 was compromised (cf. lanes 5–9 and 14–18), demonstrating that the binding of NusG to complex 5 is also affected by a point mutation in *boxA*. This result indicates that NusG may interact with *boxA* directly or indirectly through NusA.

Consensus *boxA* suppresses the effect of the *nusA1* mutation by facilitating the assembly of NusB

As the mutations we have tested in the *nut* site and

NusA that compromise antitermination weaken interactions among particular components of the N-modified complex, a mutation that increases the efficiency of antitermination could strengthen interactions within the complex. Because consensus *boxA* can suppress the effects on antitermination of the *nusA1* and *nusE71* mutations in vivo (Friedman et al. 1990), we wanted to determine whether consensus *boxA* could facilitate the incorporation of NusA1 into complex 2. Figure 6A demonstrates that this is not the case (cf. lanes 9, 10 and 11, 12). In fact, changing the *boxA* sequence to the consensus sequence did not detectably stabilize the association of NusA with an N-*nut* site complex (cf. lanes 3, 4 and 9, 10).

This suggested that consensus *boxA* strengthens another interaction that allows the stable association of NusA1 with the transcription complex. To identify this interaction, we examined the assembly of NusB, NusG, and S10 onto a consensus *boxA*-containing complex 5 (N-NusA-RNA polymerase-*nut* site). Figure 6B demonstrates that NusB alone associated strongly with this complex 5 (cf. lanes 5 and 14), whereas S10 alone did so only very weakly (cf. lane 6 and 15). Although the assembly of NusG was slightly facilitated by consensus *boxA* (cf. lanes 7 and 16), the increased assembly of NusB was most dramatic. As expected, however, from the inability of NusB to bind *rrn* *boxA* in the absence of S10 or other proteins (Nodwell and Greenblatt 1993), the assembly of NusB still needed other proteins and, in the absence of S10, depended on the formation of complex 5 (data not shown).

Figure 6. Consensus *boxA* facilitates the assembly of NusA1 through a strengthened interaction with NusB. (A) Consensus *boxA* is unable to facilitate the assembly of the NusA1 mutant protein onto an N-*nut* site complex. Various combinations of N (1.5 μ M), NusA, and NusA1 were incubated with 32 P-labeled wild-type or consensus *boxA*-containing *nut* site RNA, as indicated. The reactions were electrophoresed on a 7.5% nondenaturing polyacrylamide gel. (B) Consensus *boxA* facilitates the assembly of NusB. Various combinations of N (500 nM), NusA (100 nM), NusB (100 nM), NusG (50 nM), S10 (100 nM) and RNA polymerase (25 nM) were incubated with 32 P-labeled wild-type or consensus *boxA*-containing *nut* site RNA, as indicated. The reactions were electrophoresed on a nondenaturing polyacrylamide gel. (C) Consensus *boxA* facilitates the assembly of NusA1. Various combinations of N (500 nM), NusA (100 nM), NusA1 (100 nM), NusB (50 nM), NusG (50 nM), S10 (50 nM) and RNA polymerase (25 nM) were incubated with 32 P-labeled wild-type or consensus *boxA*-containing *nut* site RNA, as indicated. The reactions were electrophoresed on a nondenaturing polyacrylamide gel.

We then determined whether consensus *boxA* was able to allow the formation of a complete N-modified complex that contained NusA1. When a wild-type *nut* site was used, the *nusA1* mutation greatly compromised the assembly of complexes 5 and 8 (Fig. 6C, cf. lanes 4,5 and lanes 8,9). Consensus *boxA* substantially stabilized the assembly of complex 8 when wild-type NusA was used in the reactions (cf. lane 5 and 14) and also allowed the incorporation of the mutant NusA1 protein into complex 8 (cf. lane 9 and 18). Thus, a strengthened interaction between consensus *boxA* and NusB is what most likely allows for the assembly of NusA1 into an N-modified transcription complex.

Mutations in RNA polymerase affect the assembly of NusG

The *ron* mutation in the *rpoB* gene, which encodes the

β -subunit of RNA polymerase, interferes with antitermination (Ghyssen and Pironio 1972; Li et al. 1992). It also prevents the formation of a stable N-modified elongation complex (Nodwell and Greenblatt 1991; Mason et al. 1992a). Figure 7A demonstrates that the *ron* mutation had little or no effect on the assembly of complex 5 (cf. lanes 4 and 10), but strongly affected the assembly of NusG (cf. lanes 6,12), and had a weaker effect on the assembly of NusB and S10 (cf. lanes 5,11). RNA polymerase with the *ron* mutation was still, however, able to support the assembly of complex 8, and the yield was only slightly lower than with wild-type RNA polymerase (cf. lanes 7 and 13). This observation is consistent with the mild phenotype of the *ron* mutation in vivo, where the growth of λ is impaired by the *ron* mutation only if there is an additional mild mutation in the N gene (Ghyssen and Pironio 1972). The nature of an additional electrophoret-

Figure 7. Mutations in RNA polymerase and NusA can affect the assembly of higher order complexes. (A) The *ron* mutation in the β -subunit of RNA polymerase affects the assembly of higher order complexes. N (500 nM), NusA (100 nM), NusB (50 nM), NusG (50 nM), S10 (50 nM), wild-type RNA polymerase (25 nM) and *ron* RNA polymerase (25 nM) were incubated in various combinations with 32 P-labeled wild-type *nut* site RNA, as indicated, and electrophoresed on a nondenaturing polyacrylamide gel. (B) The *groN785* mutation in the β -subunit of RNA polymerase affects the assembly of higher order complexes. N (500 nM), NusA (100 nM), NusB (50 nM), NusG (50 nM), S10 (50 nM), wild-type RNA polymerase (25 nM), and *groN785* RNA polymerase (25 nM) were incubated in various combinations, as indicated, with 32 P-labeled wild-type *nut* site RNA and electrophoresed on a nondenaturing polyacrylamide gel. (C) The *nusA785* mutation affects the assembly of complex 8. Various combinations of N (500 nM), NusA (100 nM), NusA⁷⁸⁵ (100 nM), NusB (50 nM), NusG (50 nM), S10 (50 nM), wild-type RNA polymerase (25 nM), and *groN785* RNA polymerase (25 nM) were incubated with 32 P-labeled wild-type *nut* site RNA, as indicated. The reactions were electrophoresed on a nondenaturing polyacrylamide gel.

ic band observed when *ron* RNA polymerase was incubated with the *nut* site RNA (cf. lanes 2 and 8) is unclear.

The *groN785* strain of *E. coli* was shown to contain mutations in *rpoB*, *nusA*, and *nusE* that resulted in a λ -resistance phenotype (Georgopoulos 1971, and pers. comm.; Greenblatt and Li 1981a). The *groN785* mutation in *rpoB* is necessary, but not sufficient, to confer λ resistance. As in the case of the *ron* mutation, the *groN785* mutation affected the assembly of complexes 6, 7, and 8 (Fig. 7B, cf. lanes 5–7 and 11–13), and again, the effect on the assembly of NusG was particularly striking (cf. lanes 5 and 11). Next we determined whether the *nusA785* mutation compounded the assembly defect of the *rpoB785* mutation. Figure 7C demonstrates that the

nusA785 mutation interfered substantially with the assembly of complex 8 (cf. lanes 5 and 8). Also, it further destabilized the *groN785* RNA polymerase-containing complex 8 (cf. lanes 12 and 15). Thus, mutations in the β -subunit of RNA polymerase and NusA can affect the assembly of complexes 6–8.

Discussion

We have used gel mobility shift and in vitro binding assays to analyze binding of the λ N protein, *E. coli* RNA polymerase, and four *E. coli* Nus factors to a λ *nut* site RNA. At least in vitro the assembly of these factors onto the *nut* site can be a highly ordered process. Surprisingly,

the assembly of a complete antitermination complex on RNA polymerase does not depend on the presence of a DNA template. As a consequence, such an analysis provides a great deal of information about the protein–protein and protein–RNA interactions, which are important for the overall stability of the complex. The known protein–protein and protein–RNA interactions defined previously or in these experiments are shown as solid lines in Figure 8A. These and inferred interactions predicted by the results of our gel mobility shift assays or by previous genetic studies (broken and dotted lines in Fig. 8A) form a remarkably complex web. A striking feature of this network of interactions is that each element of the network has multiple interactions with other elements of the network. This presumably explains why assembly of the complete complex is a highly cooperative process (Horwitz et al. 1987; Mason and Greenblatt 1991; Nodwell and Greenblatt 1991).

Lazinski et al. (1989) showed that the *nut* sites of the lambdoid phages are selectively recognized by the arginine-rich amino-terminal portions of their respective N proteins. We have directly shown here that the loop of *boxB* is important for the binding of N to the *nut* site RNA. Surprisingly, however, nucleotides 2 and 4 in the *boxB* loop seem unimportant for the binding of N and, instead, critically affect the binding of NusA to the N–*nut* site complex. On the basis of their observation that the alteration of λ *boxA* to a consensus *boxA* element suppressed the effect of the *nusA1* mutation, Friedman and his collaborators suggested that NusA ought to recognize *boxA* (Schauer et al. 1987; Friedman et al. 1990). In the experiments described here and in experiments with various other *boxA* mutants (T. Mah, unpubl.), we have found that the stable association of NusA with the N–*nut* site complex requires *boxA*. Nevertheless, NusA can bind to the N–*nut* site complex in the absence of *boxA* when the reactions contain higher concentrations of NusA (data not shown). Because *boxA* is not essential for antitermination over short distances (Peltz et al. 1985; Zuber et al. 1987; Patterson et al. 1994) and NusA is still necessary in these circumstances (Zuber et al. 1987; Whalen et al. 1988; Mason et al. 1992a; Patterson et al. 1994), the NusA–*boxA* interaction most likely plays a role in stabilizing the N-modified transcription complex. However, the direct effect of altering *boxA* to a consensus element is to stabilize the binding of NusB rather than NusA. Thus, the ability of consensus *boxA* to reverse the effect of the *nusA1* mutation is an indirect one that operates through NusB. This is not particularly surprising because the consensus *boxA* sequence can also suppress the effect of the *nusE71* mutation in the ribosomal protein S10 (Friedman et al. 1990). This importance of *boxA* for the assembly of NusB can explain why nonprocessive antitermination at t_{R1} just downstream from *nutR* requires neither *boxA* nor NusB (Patterson et al. 1994).

The inability of NusA to bind to the *nut* site in the absence of N provides further evidence for a direct interaction between N and NusA (Greenblatt and Li 1981a). The inability of NusA1 protein to bind an N–*nut* com-

plex in spite of the normal affinity of the NusA1 protein for N (Greenblatt and Li 1981a; J. Greenblatt and J. Li, unpubl.) suggests that the *nusA1* mutation is in an RNA-binding region of NusA. This idea is strengthened by the fact that *nusA1* is a leucine-to-arginine change in an arginine-rich region of NusA (Craven et al. 1994). Arginine-rich domains have been implicated in RNA-binding in N, the Tat protein of HIV-1, and many other proteins (for review, see Lazinski et al. 1989).

The importance of both *boxA* and *boxB* for the binding of NusA to the N–*nut* site complex suggests either that NusA recognizes *boxA* and *boxB* separately or that NusA recognizes and stabilizes a folded structure comprised of both of these elements. At least two such folded structures are conceivable (see Fig. 8B): One would involve a pseudoknot in which U residues in *boxA* (CGCUCUUAC) would pair with A residues in the loop of *boxB* (GAAAA); the other, suggested to us by M. Gottesman (Columbia University, New York), would involve unwinding of the stem of *boxB* to form a new base-paired structure involving residues near the 5' end of *boxA*. In either case, mutating *boxA* to consensus would introduce an extra U residue (CGCUCUUUA) and allow an additional base pair to form, perhaps explaining why consensus *boxA* is “stronger” than wild-type *boxA*.

In our experiments N and RNA polymerase could not bind simultaneously to the *nut* site RNA in the absence of NusA. This was probably the case because RNA polymerase can only bind RNA in the same manner as a nascent transcript when it can place the 3' end of its transcript at its catalytic center (Altmann et al. 1994). In this case, the 3' end of the transcript was only 9 nucleotides downstream from *boxB*, and *boxB* was already occupied by N. Therefore, the N–*nut* site complex presumably could only associate with RNA polymerase in the manner of an antiterminator element, not as an ordinary nascent transcript, and this was possible only in the presence of NusA, which itself directly binds RNA polymerase (Greenblatt and Li 1981b). In contrast, Chattopadhyay et al. (1995) detected a complex that contains only N, RNA polymerase, and *nut* site RNA. It is likely that *boxB* was far enough away from the 3' end of the RNA used in their experiments to allow N and the catalytic center of RNA polymerase to bind independently and simultaneously to the transcript.

NusA can bind both N and the elongating core component of RNA polymerase (see Fig. 8A; Greenblatt and Li 1981a,b; Horwitz et al. 1987). In our mobility shift experiments, NusA could be made to associate first with N on the *nut* site RNA and only then with RNA polymerase, or vice versa, as illustrated in Figure 8C. It is not clear which pathway for the association of NusA would be favored in vivo.

Only in the presence of complex 5 containing N, NusA, and RNA polymerase could we observe the binding of NusB, NusG, and S10. Western blotting showed that N and all four host factors can assemble onto the *nut* site RNA, although we have not formally proved that all of these proteins are simultaneously present in a single complex. The instability and consequent low

Figure 8. Models for the cooperative assembly of an N-modified elongation complex. (See text for details.) (A) Protein-protein and protein-RNA interactions involved in the formation of a stable antitermination complex. Solid lines denote direct interactions that have been characterized biochemically (Greenblatt and Li 1981a,b; Mason and Greenblatt 1991; Das 1992; Li et al. 1992; Mason et al. 1992b; Nodwell and Greenblatt 1993). Broken lines denote interactions defined indirectly on the basis of the gel mobility shift assays described here. The dotted line denotes an N-NusB interaction for which there is genetic evidence (Ward et al. 1983). (B) Two possible folded structures for the *nut* site RNA. (C) Pathways for the assembly of N, NusA, and RNA polymerase onto a folded *nut* site. (D) Pathways for the assembly of NusB, NusG, and S10. This diagram is an interpretation of the data in Figs. 5, A and B, and 7B. (R) RNA polymerase; (A) NusA; (B) NusB; (G) NusG; (S) S10.

yield of complex 5 have prevented us from formally proving that complex 5 is a precursor of complexes 6–8, but that is likely to be so because complex 5 usually disappears in the presence of NusB, NusG, and S10 (see, e.g., Fig. 6C, lanes 5,14) and because mutations in *boxB* interfere with the formation of complexes 5 and 8 (Fig. 3B; data not shown). Again, several pathways for the binding to complex 5 of NusB, NusG, and S10 were possible (see Fig. 8D), and it is not clear which one occurs in vivo. However, it is very striking that the assembly on RNA polymerase of a complete complex containing N, all four Nus, factors, and *nut* site RNA does not require a DNA template.

As predicted by the effects of *rrn boxA* mutations (Nodwell and Greenblatt 1993), similar mutations in λ *boxA* tested here and in other experiments (J. Mogridge and T. Mah, unpubl.) prevented the binding of NusB and S10. The ability of NusB to bind complex 5 in the absence of S10 when complex 5 contains a consensus *boxA* element implies that it is most likely NusB rather than S10 that directly binds *boxA* RNA. Although this effect

of consensus *boxA* on the binding of NusB could be indirect, it is consistent with the observation that NusB is titrated when consensus *boxA* RNA is overproduced in *E. coli* (Friedman et al. 1990).

An unexpected finding was the importance of *boxA* for the stable binding of NusG. Nevertheless, the ability of the C3G mutation in *boxA* to prevent the binding of both NusG and the NusB/S10 complex does not necessarily imply that the C3 nucleotide of *boxA* is directly bound by NusG and NusB/S10. In each case, the C3G mutation may exert an indirect effect by altering the secondary or tertiary structure of *boxA*. Once NusG is bound in a manner that critically depends on *boxA*, it dramatically facilitates the binding of NusB and possibly S10. One possibility, as illustrated in Figure 8A, is that NusG interacts with NusB, as well as with RNA polymerase (Li et al. 1992) and *boxA*. Stabilization of the binding of NusB could well explain why the *nusG4* mutation suppresses *nusA1* (Sullivan et al. 1992). In that case, the NusB–NusG interaction might have gone un-

detected in direct binding experiments (Li et al. 1992, 1993; Mason et al. 1992b) simply because it is very weak. However, it is also possible that NusG alters the conformation of *boxA* in such a way as to facilitate the binding of NusB and S10 to *boxA*.

It is striking that all of the mutations we have tested that compromise antitermination *in vivo* also block one or more steps in the assembly of an N-modified complex. In particular, we have shown that assembly defects are caused by mutations in *boxA*, *boxB*, *nusA*, and *rpoB*. All of the mutations apparently affect the stability of the complex, as all the assembly reactions were incubated for 30 min and kinetic experiments (data not shown) have indicated that the assembly processes are completed in <5 min when wild-type proteins and RNA are used in the reactions. One particularly interesting phenomenon is the strong effects of the *ron* and *groN785* mutations in *rpoB* on the binding of NusG. These mutations may affect a NusG-binding site on the β -subunit of RNA polymerase. The gel mobility shift assays we have described here can now be used to systematically analyze the effects on assembly of mutations in *boxA*, *NusA*, and RNA polymerase.

Why consensus *boxA* suppresses the *nusA1* mutation by facilitating the assembly of NusB requires further explanation. One possibility derives from the observation that mutations in *nusB* that suppress *nusA1* for the N proteins of phages λ and 21 are N protein specific (Ward et al. 1983). This may imply that NusB interacts with N (see Fig. 8A). Therefore, NusB that is bound directly to consensus *boxA* and indirectly, via S10, to RNA polymerase (Mason and Greenblatt 1991) may stabilize the binding of N which, in turn, would facilitate the binding of the NusA1 protein. This sort of indirect suppression mechanism probably also explains why there are *nusA1*-suppressing mutations in *N*, *nusG*, and *rpoB* (Friedman et al. 1990; Ward et al. 1983; Sparkowski and Das 1992; Sullivan et al. 1992), as well as in *boxA* and *nusB*. This kind of phenomenon could well be a consequence of the complicated web of weak interactions that stabilize the N-modified transcription complex (see Fig. 8A). Similar, complicated protein-protein and protein-nucleic acid interaction networks, as well as non-allele-specific suppression phenomena, may also turn out to be characteristic of other multiprotein complexes involved in transcriptional initiation by RNA polymerase II (Zawel and Reinberg 1993), RNA splicing (Horowitz and Krainer 1994), and other macromolecular processes (Stillman 1994; Aboussekhra et al. 1995).

Materials and methods

Plasmids, enzymes, and strains

NusA, NusB, NusG, N, and the core component of RNA polymerase were purified as described previously (Burgess and Jendrisak 1975; Greenblatt et al. 1980; Greenblatt and Li 1981b; Swindle et al. 1988; Li et al. 1992). Purified S10 was generously provided by Dr. Volker Nowotny (Max-Planck Institut für Molekulare Genetik, Berlin, Germany). All plasmids used for *in vitro* transcription were prepared in the bacterial strain DH5 α

(Bethesda Research Laboratories) and purified on Qiagen-tip 500 columns (Qiagen). The oligonucleotides used for cloning into pTZ19R, for site-directed mutagenesis and the *in vitro* binding assay, were purchased from the HSC/Pharmacia Biotechnology Service Centre in Toronto. RNAGuard was bought from Pharmacia. Restriction enzymes and DNA ligase were purchased from New England Biolabs. T7 RNA polymerase was obtained from BRL.

Construction of pNUT Plasmids

The following DNA oligonucleotides were synthesized: (1) 5'-CGCTTCCCGCTCTTACACATTCCAGCCCTAAAAAAGG-GCATCAG-3'; (2) 5'-AGCTTCCCGCTCTTACACATTCCA-GCCCTGCAAAAGGGCATCAG-3'; (3) 5'-AGCTTCCCGC-TCTTACACATTCCAGCCCTGACAAAGGGCATCAG3'; (4) 5'-AGCTTCCCGCTCTTACACATTCCAGCCCTGAACAA-GGGCATCAG-3'; (5) 5'-AGCTTCCCGCTCTTACACATT-CCAGCCCTGAAACAGGGCATCAG-3'; (6) 5'-TCGACTGA-TGCCCTTTTTCAGGGCTGGAATGTGTAAAGAGCGGGA-3'; (7) 5'-AGCTTCCACCTTACACATTCTCGCGCCCTGAA-AAAGGGCATCAG-3'; (8) 5'-TCGACTGATGCCCTTTTTC-AGGGCGCGAGAATGTGTAAAGGTGGA3'. Oligonucleotides 1–5 were each annealed with oligonucleotide 6 to make plasmids pNUTG23A, A24C, A25C, A26C, and A27C, respectively. Oligonucleotides 7 and 8 were annealed to make pNUTrev1–17. The annealed oligonucleotides were cloned into the plasmid pTZ19R cut with *HindIII* and *SalI*. pNUTWT and pNUT mutants were identified using double-stranded DNA sequencing (Hsiao 1991).

Site-directed mutagenesis of pNUTWT

Preparation of single-stranded DNA was performed by transforming CJ236 (Ausubel et al. 1987) with pNUTWT plasmid DNA and growing in the presence of 100 μ g/ml of ampicillin. Cells were infected with R408 and single-stranded DNA was isolated. Mutagenic oligonucleotides (1 μ g) were hybridized to dUMP-substituted DNA (1 pmole) in 10 μ l reactions containing ligation buffer by heating the reaction in a boiling water bath for 5 min and allowing the bath to cool to room temperature. Annealed DNA was mixed with 10 μ l of extension/ligation mix (ligation buffer, 1 mM ATP, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM dTTP, 1 U/ μ l of DNA ligase, 0.2 U/ μ l of T4 DNA polymerase) and incubated at room temperature for 4 hr. Reaction mixtures (5 μ l) were transformed into 50 μ l of competent DH5 α cells. Colonies were picked and grown in overnight cultures, and plasmids were isolated by miniprep (Ausubel et al. 1987). Positive clones were identified by using double-stranded DNA sequencing (Hsiao 1991). Oligonucleotides 5'-CCGCTCTTTAACATTCCA-3' and 5'-AAGCTTCCCG-TCTTACACATTC-3' were used to make pNUTcon and pNUTC3G, respectively.

In vitro transcription and RNA purification

³²P-Labeled RNA for gel mobility shift experiments was produced in 10 μ l reactions containing 1 $\times$ BRL T7 RNA polymerase buffer, 5 mM dithiothreitol, 20 μ Ci of [α -³²P]UTP (NEN), 8 μ M UTP, 500 μ M ATP, CTP, and GTP, 1 U/ μ l of RNAGuard, 50 μ g/ml of *SalI*-cut plasmid DNA, and 1.5 U/ μ l of T7 RNA polymerase. The reactions were incubated at 37°C for 4 hr. Five microliters of 95% deionized formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol were added to the reactions, which were then incubated in a boiling water bath for 2 min and loaded on a 15% (37.5:1 acrylamide/bis) polyacrylamide/7 M

urea gel and electrophoresed until the bromophenol blue had migrated two-thirds of the way down the gel. The labeled RNA was located by autoradiography; the gel slice containing the RNA was cut out and soaked in DEPC-treated water overnight. The yield of RNA was determined by liquid scintillation counting. ³H-labeled RNA for in vitro binding assays was produced in 20 µl reactions containing 1× BRL T7 RNA polymerase buffer, 5 mM dithiothreitol, 0.5 µCi α-³H-labeled UTP (NEN), 500 µM UTP, ATP, CTP, and GTP, 1.5 U/µl of RNAGuard, 50 µg/ml of *Sall*-cut plasmid DNA, and 2.5 U/µl of T7 RNA polymerase. RNA was gel purified as described above, except that it was visualized using UV shadowing. The RNA was then ethanol precipitated, resuspended in DEPC-treated water, and quantitated by liquid scintillation counting. These RNAs used in mobility shift assays and in vitro binding assays were 54 nucleotides long and contained the *nutR* site at positions 14–45. The complete sequence of the RNA containing the wild-type *nut* site is 5'-AGGGAAGCUCCCGCUCUACACAUCCAGCCCUGAAAAAGGGCAUCAGUCGA-3'.

Gel mobility shift experiments

N, NusA, NusB, NusG, S10, and RNA polymerase (concentrations indicated in figures and figure legends) were incubated in 15-µl reactions with ~2000 cpm of ³²P-labeled RNA (25–50 pM) in the presence of 40 mM HEPES (pH 7.2), 20 mM ammonium sulfate, 15 mM potassium acetate, 10% glycerol (vol/vol), 55 µg/ml of yeast tRNA, and 0.5 mg/ml of bovine serum albumin (BSA) for 30 min at 4°C. The reactions were then loaded onto a gel containing 5% (37.5:1 acrylamide/bis) polyacrylamide (unless otherwise indicated), 2% glycerol (vol/vol) and 0.5× Tris/borate/EDTA between lanes loaded with bromophenol blue and xylene cyanol. The gel was run at 4°C and 23 mA until the bromophenol blue almost reached the bottom of the gel. The gel was dried and exposed to x-ray film (Kodak XAR-5) with two intensifier screens at –70°C overnight.

In vitro binding assay

Wild-type *nut* site RNA (200 ng) was incubated with 1 µg of the 3' biotinylated DNA oligonucleotide, 5'-GAAGCTTTCCTA-TAGTGAGTCGTATT-3', in the presence of 10 mM Tris, 10 mM magnesium sulfate, and 100 mM sodium chloride. The mixture was boiled and cooled to room temperature and then added to 10 mg of streptavidin beads (BRL) and rotated at 4°C for 2 hr. The beads were washed twice with 100 µl of 40 mM HEPES (pH 7.2), 20 mM ammonium sulfate, 15 mM potassium acetate, 10 mM magnesium sulfate, 10% glycerol (vol/vol), and 0.5 mg/ml of BSA. The beads were then incubated with 100 nM RNA polymerase, 330 nM N, 200 nM NusA, 200 nM NusB, 100 nM NusG, 170 nM S10, and 75 µg/ml of yeast tRNA in a total volume of 60 µl and were rotated at 4°C for 30 min. The beads were washed three times with 150 µl buffer containing 40 mM HEPES (pH 7.2), 20 mM ammonium sulfate, 15 mM potassium acetate, 10 mM magnesium sulfate, and 10% glycerol (vol/vol) and eluted with 40 µl of 10 mM Tris, 1 mM EDTA, at 70°C for 2 min. Proteins eluted from the beads were run on a 13.5% SDS-polyacrylamide gel and subjected to Western blotting.

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