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Escherichia coli NusA is required for efficient RNA binding by phage HK022 Nun protein

(arginine-rich motif family/transcription termination)

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ABSTRACT The Nun protein of phage HK022 is an RNA binding protein of the arginine-rich motif family. Nun binds the phage λ boxB RNA sequence (BOXB) on nascent λ transcripts and arrests transcription elongation. Binding to BOXB is inhibited by Zn^{2+} and stimulated by the *Escherichia coli* NusA protein. Deletion of the Nun C-terminal region enhances BOXB binding and makes it independent of Zn^{2+} and NusA. The C terminus of Nun thus appears to interfere with the N-terminal RNA binding motif. NusA relieves this interference by binding to the Nun C terminus and forming a complex with Nun and BOXB. However, NusA also inhibits transcription arrest *in vitro*, in the absence of the other Nus factors. Nun deleted for its C terminus fails to bind RNA polymerase (RNAP) (RNAP) or NusA *in vitro* or to arrest transcription *in vivo* or *in vitro*. Our findings are consistent with the idea that NusA inhibits transcription arrest by binding to the Nun C terminus, thus blocking the interaction between Nun and RNAP. NusG, NusB, and NusE factors restore transcription arrest, presumably by promoting transfer of Nun from NusA to RNAP.

The Nun protein of bacteriophage HK022 is a member of the arginine-rich motif family of RNA binding proteins, which includes the phage λ N transcription antitermination protein and the HIV Tat and Rev proteins (Fig. 1*A*; refs. 1–3). Nun binds to the BOXB sequence in the λ pL and λ pR operon nascent transcripts (Fig. 1*B*). BOXB also is recognized by λ N (4–6).

In contrast to λ N, which suppresses transcription termination, Nun terminates transcription just distal to BOXB. By preventing the expression of the immediate early genes of λ, Nun blocks productive infection of HK022 lysogens (7, 8).

BOXB is part of the λ NUT site, which contains a second structural element, BOXA, thought to be important for both N and Nun activity. BOXA is proposed to be the binding site of two *Escherichia coli* proteins, NusB and NusE, which, along with two additional host proteins, NusA and NusG, support Nun transcription termination *in vivo* (6, 9). The Nus proteins together stimulate Nun-mediated transcription arrest *in vitro*. Interestingly, NusA or NusG strongly inhibits Nun activity in the absence of NusB and NusE (9).

The action of NusA thus is paradoxical. It enhances Nun function in conjunction with the other Nus factors but by itself inhibits Nun. In contrast to its effect on Nun, NusA binds N protein and stimulates antitermination (10–12). That the activity of NusA with respect to Nun and N is distinct is supported by the phenotype of the NusA100 mutant, which specifically blocks Nun termination in the λ pR operon, but has no effect on N antitermination (13).

In this article, we report that NusA binds to the C terminus of Nun and stimulates Nun binding to BOXB. In doing so however, NusA inhibits the interaction between Nun and RNA polymerase (RNAP), which requires the C terminus of Nun. The mechanism by which NusA stimulates Nun binding appears to be novel among RNA binding proteins.

MATERIALS AND METHODS

Nun Purification. Nun was purified from *E. coli* strain BL21(DE3) pLysS/pT7Nun. Plasmid pT7Nun was constructed by cloning *nun* into pET21d (Novagen) between the *Nco*I and *Hin*dIII sites. The Nun-expressing strain was grown at 37°C to an OD_{600} of 0.5 to 0.6, induced with 1 mM isopropyl β-D-thiogalactoside and shifted to 32°C for 7 hr. Cells were pelleted at 5,000 × *g* and stored at −70°C overnight. After thawing and resuspension in 50 mM Mes, pH 6.0/0.3 M NaCl/2 mM EDTA/1 mM DTT/1 mM phenylmethylsulfonyl fluoride, cells were lysed by sonication (Branson Sonifier 250: power level 2 of 10 and 15% duty cycle). The lysate was centrifuged at 10,000 × *g* for 30 min, and the supernatant was applied to a SP-Sepharose FF column (Pharmacia) at 1 ml/min, washed with 10 column volumes of sonication buffer, and eluted in sonication buffer with 0.6 M NaCl. Fractions containing Nun were identified on a 16% SDS/polyacrylamide gel and pooled and dialyzed against 10 mM Na-phosphate, pH 7.0 overnight. Purification was completed by using an FPLC LCC-500 and a MonoS HR5/5 column (Pharmacia). Fractions containing Nun were dialyzed against 10 mM Na-phosphate, pH 7.0 overnight and concentrated to a final volume of 1 ml. T-Nun (3) was prepared as described for Nun, with the exception of a more robust sonication (power level 3 and 70%), which favors proteolysis.

NusA Purification. NusA was purified from XL-1 Blue/pRW1. Plasmid pRW1 was constructed by cloning *nusA* into pQE32 (Qiagen) between the *Bam*HI and *Hin*dIII sites. This construct created a N-terminal his_6-tagged NusA with a factor Xa site inserted between the his_6 tag and the second residue of NusA (the AUG was removed to prevent translation misstart). The strain was grown at 37°C to an OD_{600} of 0.7 to 0.9 and induced with 1 mM isopropyl β-D-thiogalactoside for 5 hr. Cells were harvested in the manner described above, resuspended in 50 mM potassium phosphate, pH 8.0/0.3 M KCl/0.1% Tween-20/10 mM β-mercaptoethanol, and lysed by sonication (power level 3 constant power 5× 1-min bursts). The lysate was centrifuged as described above, and the supernatant was incubated with 1 ml of Ni-NTA-agarose beads (Qiagen) per liter of culture for 1 hr at 4°C with mixing. The mixture was poured into a 10-ml column and washed with 50

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Abbreviations: RNAP, RNA polymerase; CtP, C-terminal peptide.
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A

VKKTI YVNPD SGQNR KVSDR GLTS**R**

DRRRI ARWEK RIAYA LKNGV TPGFN

AIDDG PEYKI NEDPM DKVDK ALATP

FPRDV EKIED EKYED *VMHRV V|NHAH*

QRNPN KKWS

B

A A A
A← G A
U:A
C:G
C:G
C:G
G:C
G:C
BOXB

FIG. 1. (*A*) Nun protein sequence. The arginine-rich motif RNA-binding motif (2, 3) is indicated in bold underlined type. The C-terminal peptide (CtP) used in binding experiments is shown in underlined italics: T-Nun is generated by cleavage (slash mark) between V96 and N97 by an uncharacterized *E. coli* protease. (*B*) Sequence of BOXB within the λ NUTR region. The indicated G to A mutation (BOXB-A25) inhibits binding to Nun.

ml of 50 mM potassium phosphate, pH 6.0/0.3 M KCl, followed by 25 ml of wash buffer with 75 mM imidazole. NusA was eluted with wash buffer plus 500 mM imidazole, and purified to homogeneity by FPLC using a monoQ HR 5/5 column (Pharmacia).

BOXB Synthesis and Purification. BoxB RNA (BOXB) was expressed from a oligonucleotide template using a double-stranded T7 promoter. Transcription was performed as described (14) in a 25-μl reaction mixture using 4 mM NTPs (Pharmacia) and 6.25 μl [α-^{32}P] ATP (3,000 Ci/mmol; Amersham PLC) and 2 units of T7 RNA polymerase (Stratagene). After incubation at 37°C for 1 hr, 2 units of RNase free DNase (Stratagene) were added, and incubation was continued for 40 min. The RNA product was purified by using the Qiaquick Nucleotide Removal Kit (Qiagen), which removed proteins and RNA products smaller than 12 nucleotides, as verified by electrophoresis on an 18% SDS/8 M urea polyacrylamide gel.

Gel Mobility Shift Assays. ^{32}P-labeled BOXB (20 nM) was incubated for 15 min on ice with Nun or T-Nun (100 nM) with or without NusA (100 nM) in a 10-μl reaction containing 20 mM Tris·acetate (pH 7.9), 2 mM Mg-acetate, 100 mM K-acetate, 25 mM NaCl, 0.1 mg/ml BSA, 1 mM DTT, 0.1 mg/ml poly(A)poly(U), 5% glycerol. The reaction mixture was applied to a 7.5% nondenaturing polyacrylamide gel and electrophoresed at 150 V for 4.5 hr at 4°C. Where indicated, the reactions were carried out in the presence of 5 μM or 500 nM $ZnCl_2$. For competition experiments with the C-terminal peptide, Nun (500 nM) was incubated with 20 nM ^{32}P-labeled BOXB in the presence or absence of NusA (500 nM). The C-terminal peptide or a nonspecific peptide Allatostatin B_2 (5 μM; Sigma) then was added to 500 nM or 5 μM, and the reaction mixture was incubated for 15 min on ice. The intensities of the bands in the C-terminal peptide competition assay were quantitated by integrating the volumes of the shifted bands using a Molecular Dynamics Computing Densitomer Model 300A.

Ni-Affinity Chromatography. Nun and NusA (22.5 μM each) were incubated in Na-phosphate, pH 7.0, 50 mM NaCl with 50 μl Ni-NTA-agarose with mixing at 4°C for 1 hr. The beads then were pelleted and washed several times with 75 mM imidazole in binding buffer before elution with 250 mM imidazole. Supernatants and eluents were electrophoresed on 16% SDS/polyacrylamide gel and analyzed by Coomassie blue staining.

Gel Filtration Chromatography. NusA (60 μM) was incubated with C-terminal Nun peptide (200 μM) at 4°C for 15 min in 50 mM Na-phosphate, pH 7.0. The reaction mixture then was applied to a micro bio-spin 30 column (Bio-Rad) and centrifuged at 1,000 × g for 4 min. The eluate was electrophoresed on a 16.5% Tris-Tricine gel and visualized by using SYPRO Orange stain (Bio-Rad). Similar conditions were used to incubate C-terminal peptide with BSA, or NusA with an N-terminal peptide corresponding to the RNA binding domain of Nun (residues 22–43).

In the assay for RNAP binding, RNAP (7.2 μM, Boehringer-Mannheim) was incubated with Nun (15 μM) or T-Nun (15 μM) for 15 min at 4°C. The reaction mixture was applied to a Sephadex G-75 (Pharmacia Biotech) column and centrifuged at 1,000 × g for 4 min. Eluates were electrophoresed on 16%

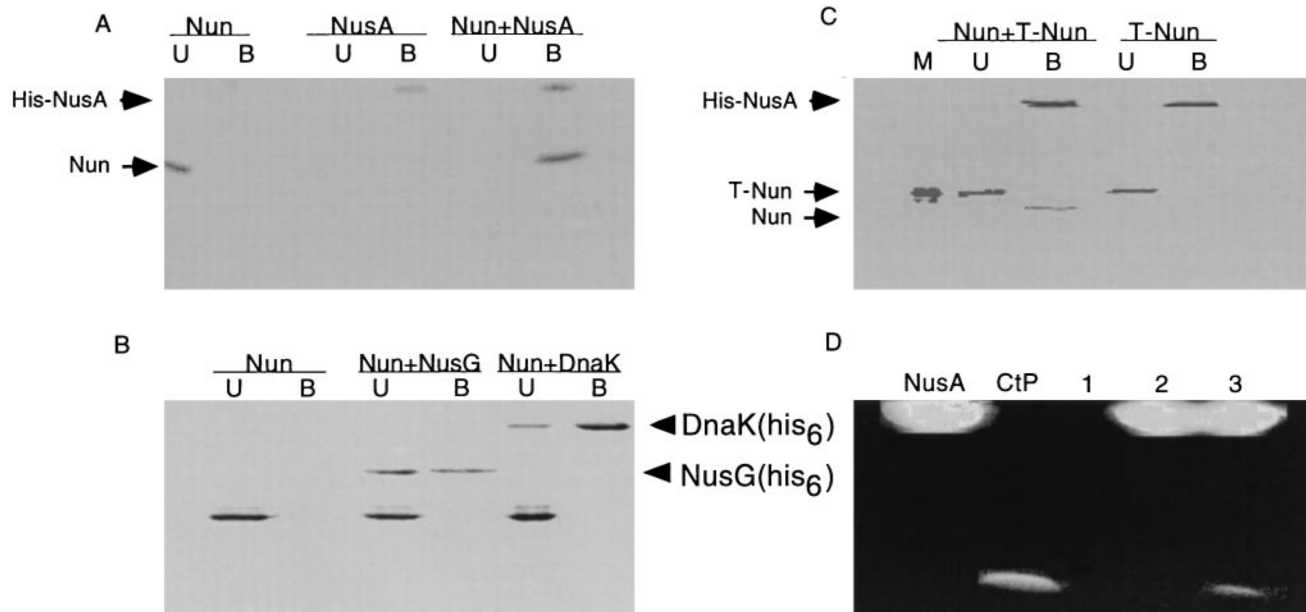

FIG. 2. Nun-NusA binding assays. (*A*) Nun-NusA(his$_6$) Ni^{2+} affinity assay. U, unbound fraction; B, bound fraction eluted with 250 mM imidazole. (*B*) Nun-NusG(his$_6$) or Nun-DnaK(his$_6$) Ni^{2+} affinity assay. (*C*) T-Nun/Nun-NusA(his$_6$) Ni^{2+} affinity assay (M, T-Nun marker. Although smaller than Nun, T-Nun migrates more slowly on SDS/PAGE gels). (*D*) CtP-NusA binding assay determined by exclusion chromatography (see *Materials and Methods*). NusA and CtP markers are indicated. Excluded fractions are indicated for CtP (lane 1), NusA (lane 2), and a mixture of NusA and CtP (lane 3).

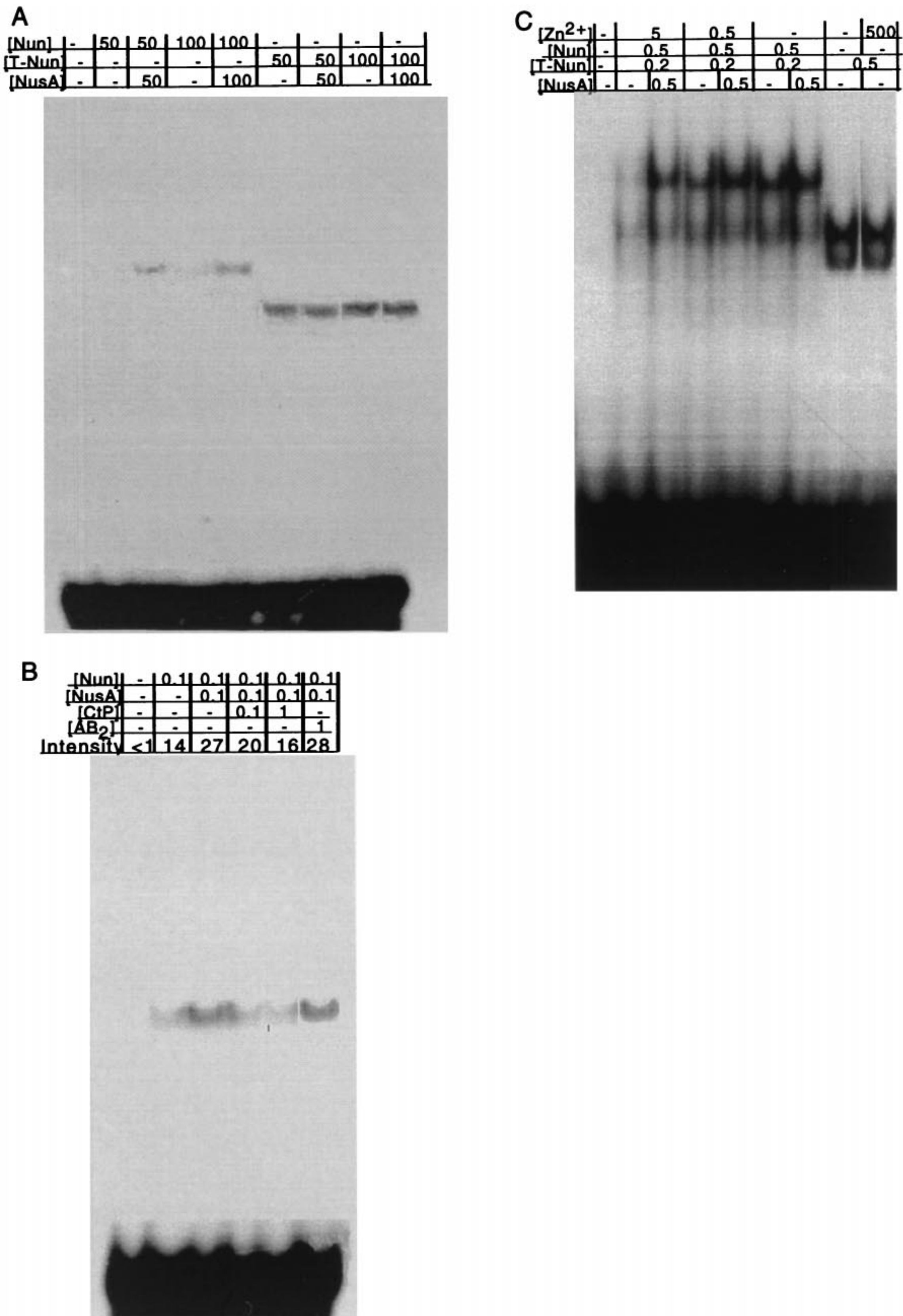

FIG. 3. NusA stimulates and Zn^{2+} inhibits BOXB binding by Nun. (*A*) NusA stimulates Nun binding to ^{32}P-BOXB. Protein concentrations are given in nM. (*B*) CtP competes with Nun for NusA. Protein concentrations are given in μM. AB2, allatostatin B2. Band intensity values are given as the sum of all points over background, values are in thousands. (*C*) Zn^{2+} inhibits Nun binding to ^{32}P-BOXB. Protein concentrations are given in μM. All lanes contain ^{32}P-BOXB (20nM). Binding to ^{32}P-BOXB was assayed by gel mobility shift assays (see *Materials and Methods*).

SDS/polyacrylamide gels and analyzed by staining with Coomassie blue.

^{32}P-labeled BOXB was incubated with Nun, NusA, Nun and NusA, or gel mobility shift binding buffer alone, without BSA

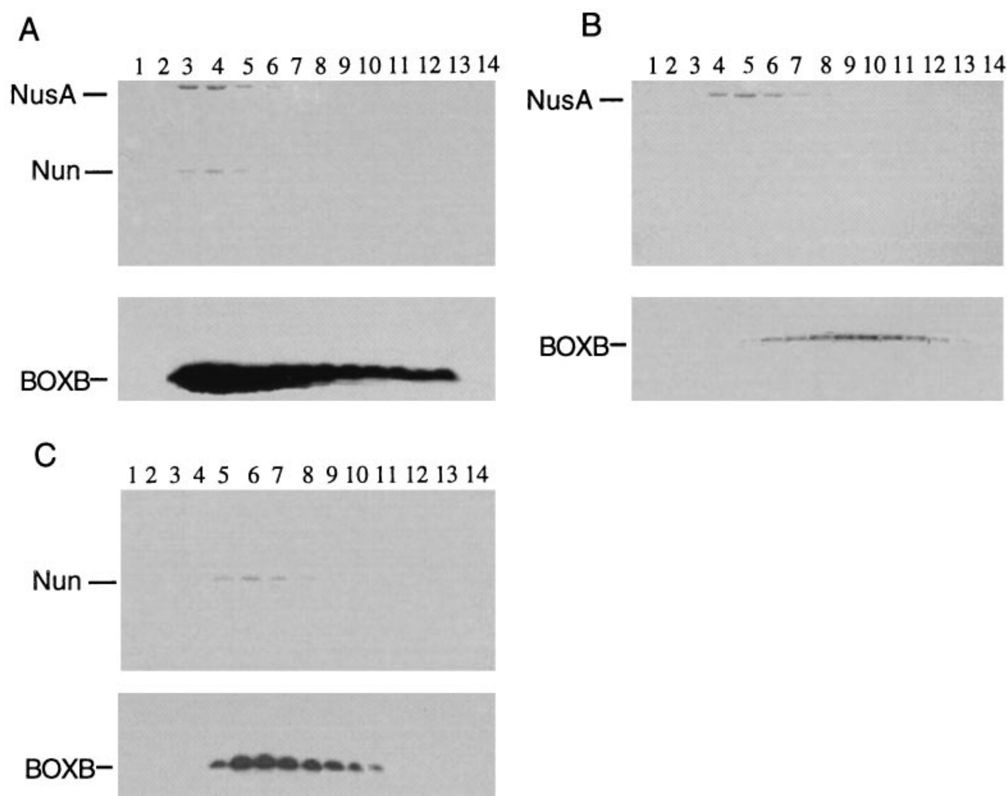

FIG. 4. NusA forms a complex with Nun and BOXB. Lane numbers indicate fractions eluted from a Sephadex G-100 column loaded with (*A*) Nun, NusA, and ^{32}P-BOXB, (*B*) NusA and ^{32}P-BOXB, and (*C*) Nun and ^{32}P-BOXB. (*Upper*) Coomassie blue-stained SDS 16% polyacrylamide gels. (*Lower*) Autoradiograms of 8 M urea 12% polyacrylamide gels.

and glycerol, at 4°C for 15 min, applied to a Sephadex G-100 column, and eluted with binding buffer, and 100-μl fractions were collected. The fractions were electrophoresed on 16% SDS/polyacrylamide gels and 12% 8 M urea denaturing polyacrylamide gels. Proteins were visualized on the former with Coomassie blue. Autoradiograms of the urea gels were used to visualize BOXB.

RESULTS

NusA Binds Nun. To determine whether NusA interacts directly with Nun, NusA was expressed with an N-terminal hexahistidine tag and used for Ni^{2+}-affinity chromatography experiments. When incubated separately with Ni^{2+}-NTA-agarose beads, NusA(his)$_6$ and Nun were recovered in the bound and the unbound fractions, respectively. After incubation with an equimolar amount of NusA(his)$_6$, Nun coeluted from the beads with NusA(his)$_6$, indicating the formation of a complex between the two proteins (Fig. 2*A*). As controls, NusG(his)$_6$ or DnaK(his)$_6$ did not cause Nun to elute in the bound fraction (Fig. 2*B*).

NusA Binds to the C-Terminal Region of Nun. During the purification of Nun, a proteolytic fragment (T-Nun) lacking the 13 C-terminal amino acids is generated by an unknown protease (Fig. 1*A*). Incubation with NusA(his)$_6$ did not shift T-Nun into the bound fraction, indicating that T-Nun does not form a complex with NusA (Fig. 2*C*). Thus, the C terminus of Nun is required for NusA binding. To determine whether NusA binds directly to the Nun C terminus, a 2.4-kDa peptide consisting of the 19 C-terminal amino acids of Nun ("CtP") was synthesized. CtP binding to NusA (55 kDa) was assayed by using a Bio-gel P-30 polyacrylamide column with an exclusion limit of 40 kDa.

The results of the binding assay are shown in Fig. 2*D*. As expected, CtP was absent from the excluded fraction (lane 1) whereas NusA was recovered in this fraction (lane 2). When incubated in 3-fold excess with NusA, CtP appeared in the excluded fraction in amounts roughly equimolar to NusA (lane 3). CtP was not excluded after incubation with BSA (data not shown). Furthermore, NusA did not induce the exclusion of a peptide containing the arginine-rich region of Nun (residues 22–43; 2.9 kDa; data not shown). We conclude that NusA binds directly to the C-terminal region of Nun.

Nun Binding to BOXB Is Stimulated by NusA. The presence of BOXB in the nascent λ transcript enhances transcription arrest by Nun (9). We asked if NusA influenced the formation of the Nun-BOXB complex.

The effect of NusA on the affinity of Nun for ^{32}P-labeled BOXB was determined by gel mobility shift assays (Fig. 3*A*). NusA increased the amount of Nun-BOXB complex without altering the migration of the complex. Under these experimental conditions, therefore, NusA appears to stimulate Nun binding to BOXB without being retained in the complex. The role of NusA in Nun binding of BOXB becomes critical when Zn^{2+} is present in the reaction mixture. The C terminus of Nun includes three histidine residues that form a potential Zn^{2+} binding motif (15). Zn^{2+} strongly inhibited Nun binding to BOXB, even at very high (500 nM) Nun concentrations (Fig. 3*C*). At 5 μM Zn^{2+}, where the binding of Nun to BOXB was largely abolished, addition of NusA restored Nun binding activity. T-Nun at 200 nM, which is also present in the mixture, was, unlike Nun, resistant to these concentrations of Zn^{2+} (Fig. 3*C*). Furthermore, when present at a concentration of 500nM, without Nun, T-Nun was resistant to Zn^{2+} concentrations as high as 500 μM (Fig. 3*C*). NusA enhanced the affinity of Nun for BOXB without reducing binding specificity. Nun failed to bind the BOXB-A25 mutant (Fig. 1*B*) either in the presence or the absence of NusA (data not shown). NusA alone did not retard the migration of BOXB.

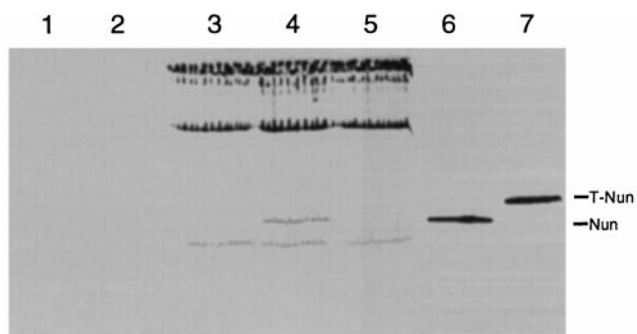

FIG. 5. RNAP binds Nun but not T-Nun. Excluded volumes of a Sephadex G-75 column loaded with Nun (lane 1), T-Nun (lane 2), RNAP (lane 3), RNAP + Nun (lane 4), RNAP + T-Nun (lane 5). Lanes 6 and 7, initial amounts of Nun and T-Nun applied to column.

The binding of T-Nun to BOXB was not stimulated by NusA, suggesting that NusA must bind to Nun to enhance binding (Fig. 3*A*). In addition, NusA, at 500nM, did not enhance the binding of T-Nun, at 200 nM, to BOXB when Nun was present in the binding mixture, either in the presence or absence of Zn^{2+} (Fig. 3*C*). Supporting this idea, stimulation of Nun binding by NusA to BOXB was competed by CtP (Fig. 3*B*). NusA enhancement of Nun binding to BOXB was inhibited at CtP concentrations equimolar to Nun and was almost completely abolished when CtP was in 10-fold excess. A control peptide (Allatostatin B_2), added at a concentration in 10-fold excess to Nun, did not affect the NusA reaction.

NusA Forms a Complex with Nun and BOXB. Although a stable interaction between Nun, NusA, and BOXB could not be demonstrated by electrophoresis in a native gel, their association was revealed by gel filtration chromatography. ^{32}P-labeled BOXB was incubated with NusA and Nun, NusA alone, or Nun alone, and then applied to a Sephadex G-100 column. The collected fractions were analyzed on SDS polyacrylamide or urea polyacrylamide gels to visualize proteins or ^{32}P-labeled BOXB, respectively. Nun coeluted from the column with NusA (Fig. 4*A*, *Upper*, fractions 3–5) along with BOXB (Fig. 4*A*, *Lower*), indicating the formation of a NusA-Nun-BOXB complex. In the absence of Nun, BOXB and NusA eluted separately; BOXB appeared mostly in fractions 9 and 10, whereas NusA eluted mainly in fractions 4–6 (Fig. 4*B*). In the absence of protein BOXB also eluted mainly in fractions 9 and 10 (data not shown). Finally, Nun and BOXB, also coeluted from the column in the absence of NusA (Fig. 4*C*, fractions 4–7). Although readily evident by column chromatography, the association of NusA with Nun and BOXB is, evidently, too weak to persist during electrophoresis through a polyacrylamide matrix.

RNAP Binds Nun But Not T-Nun. Although T-Nun binds BOXB with higher affinity than Nun, it does not arrest λ transcription. We considered the possibility that T-Nun may not interact with RNAP. The binding of Nun and T-Nun to RNAP was compared by size exclusion chromatography on a Sephadex G-75 column (Fig. 5). The G-75 column retained Nun and T-Nun but excluded RNAP. When Nun in 2-fold excess was preincubated with RNAP, Nun appeared in the excluded fraction, indicating the formation of a Nun-RNAP complex. In contrast, T-Nun was retained by the column matrix in the presence of RNAP. We conclude that Nun binds RNAP and that the association between the two proteins requires the C-terminal 13 amino acids of Nun.

DISCUSSION

The Nun protein of the temperate bacteriophage HK022 inhibits transcription elongation on the chromosome of the related phage, λ. The *E. coli* NusA protein interacts with the C-terminal region of Nun and stimulates the binding of Nun to BOXB. When present with the host NusB, NusE, and NusG proteins, NusA also stimulates transcription arrest by Nun or antitermination by λ N (6, 9, 16). In the absence of the other Nus factors, however, NusA inhibits Nun activity, presumably by blocking the interaction between the Nun C terminus and RNAP. This contrasts with N antitermination, which is stimulated by NusA in the presence and absence of the other Nus

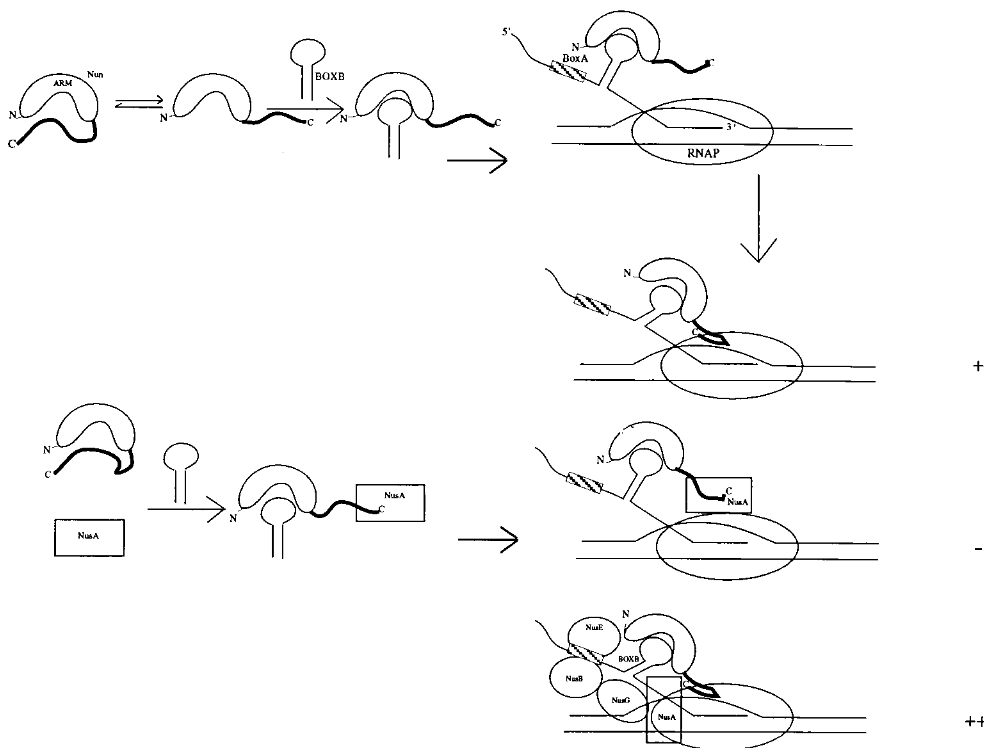

FIG. 6. Model showing interactions of Nun, NusA, BOXB, and RNAP.

factors (16). It is possible that NusA binds to a different region of N, or that the NusA-N complex interacts with RNAP in a fashion distinct from the NusA-Nun complex.

Nun interacts with the transcription complex by binding nascent λ RNA at the BOXB sequence via an N-terminal arginine-rich motif. We observe, however, that Nun can form a complex with RNAP in the absence of BOXB (Fig. 5). This is consistent with the finding that at high Nun concentrations BOXB is not essential for Nun activity *in vitro* (9). Our results support the idea that BOXB provides a tether for Nun that increases the local Nun concentration in the vicinity of RNAP. Similar conclusions have been reached for λ N protein (5, 17), which also binds BOXB and interacts with RNAP.

Nun binding to BOXB is strongly inhibited by Zn^{2+}. The C terminus of Nun includes three histidine residues that form a potential Zn^{2+}-binding motif (15). We have demonstrated Zn^{2+} binding to CtP by spectrophotometric and NMR analysis (unpublished results). A truncated form of Nun, T-Nun, which is deleted for the C-terminal 13 amino acids, and thus two of the three Nun histidines, binds BOXB with higher affinity than Nun (30 nM K_d vs. 50 nM K_d), and is unaffected by Zn^{2+}. T-Nun, however, can neither arrest transcription *in vitro* nor terminate transcription *in vivo* (ref. 9; M.E.G., unpublished data). Our *in vitro* data demonstrate that T-Nun fails to bind RNAP.

The C terminus of Nun (*i*) inhibits Nun binding to BOXB, particularly in the presence of Zn^{2+}; (*ii*) binds NusA; and (*iii*) promotes the binding of Nun to RNAP. In Fig. 6 we propose a model to explain the interactions among NusA, Nun, BOXB, and RNAP. We suggest that the C terminus of Nun occludes the N-terminal RNA (arginine-rich motif) binding domain. The inactive conformation of Nun is stablilized by the binding of Zn^{2+} to the C terminus. NusA also binds the C terminus, but with effects opposite to those of Zn^{2+}. NusA exposes the RNA binding domain and allows Nun to bind BOXB. A BOXB-Nun-NusA complex, unlike a Nun-BOXB complex, cannot induce transcription arrest. NusA bound to the C terminus therefore may hinder the interaction of Nun with RNAP. We assume that NusB, NusE, and NusG promote either the release of NusA from Nun or its reorientation within the complex. Because NusB, NusE, NusG, and NusA strongly stimulate the Nun arrest reaction, they also may participate in other steps in the Nun pathway. Our finding that Nun binding to RNA depends on NusA raises the possibility that other RNA binding proteins also may require a cofactor to promote efficient binding.

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