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Bacteriophage λ N protein alone can induce transcription antitermination *in vitro*

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ABSTRACT **Specific and processive antitermination by bacteriophage λ N protein *in vivo* and *in vitro* requires the participation of a large number of *Escherichia coli* proteins (Nus factors), as well as an RNA hairpin (boxB) within the *nut* site of the nascent transcript. In this study we show that efficient, though nonprocessive, antitermination can be induced by large concentrations of N alone, even in the absence of a *nut* site. By adding back individual components of the system, we also show that N with *nut*$^+$ nascent RNA is much more effective in antitermination than is N alone. This effect is abolished if N is competed away from the *nut*$^+$ RNA by adding, in trans, an excess of boxB RNA. The addition of NusA makes antitermination by the N–*nut*$^+$ complex yet more effective. This NusA-dependent increase in antitermination is lost when Δ*nut* transcripts are used. These results suggest the formation of a specific boxB RNA–N–NusA complex within the transcription complex. By assuming an equilibrium model, we estimate a binding constant of $5 \times 10^6\ M^{-1}$ for the interaction of N alone with the transcription complex. This value can be used to estimate a characteristic dissociation time of N from the complex that is comparable to the dwell time of the complex at an average template position, thus explaining the nonprocessivity of the antitermination effect induced by N alone. On this basis, the effective dissociation rate of N should be ≈1000-fold slower from the minimally processive (100–600 bp) N–NusA–*nut*$^+$ transcription complex and ≈10^5-fold slower from the maximally processive (thousands of base pairs) complex containing all of the components of the *in vivo* N-dependent antitermination system.**

The transcriptional regulatory factor N of bacteriophage λ binds to elongation complexes and inhibits transcript termination at both intrinsic and rho-dependent terminators (1, 2). In addition to N protein, efficient suppression of termination signals requires several *Escherichia coli* transcription elongation factors and a cis-acting DNA sequence called *nut* (1–5). Genetic studies have identified a family of *E. coli* genes that encode NusA, B, E, and G proteins. These proteins serve as positive regulators of N function *in vivo*. Abundant evidence indicates that the functional form of the *nut* sequence resides in the RNA transcript (6–9), and this fact forms the basis of current working models for N action (1, 2). N protein, the RNA form of *nut*, and *E. coli* factors NusA, B, E, and G are thought to bind to the transcribing RNA polymerase to create a complex held together by a number of protein–protein and protein–RNA interactions. Pairwise interactions between N and NusA (10), NusA and core polymerase (11–13), NusG and polymerase (14), NusB and NusE (15), and NusE and polymerase (16) have been demonstrated *in vitro*. More recently, N protein and the NusB–NusE complex have been shown to interact respectively and specifically with sequence elements within the *nut* RNA called boxB and boxA (6, 17). RNA looping is thought to mediate the interaction between the N–boxB complex and the transcription complex (2).

Antitermination systems have been reconstituted *in vitro* that can mimic the *in vivo* reaction. They utilize NusA, NusB, NusE, and NusG proteins, *nut*$^+$ RNA, and N to suppress multiple terminators, some located thousands of base pairs downstream of the *nut* site (18, 19). All of these factors appear to be required for highly processive antitermination; moreover, N is found stably associated with transcription complexes only in the presence of all these factors (16, 20, 21).

However, N and NusA suffice to cause partially processive antitermination in *nut*-containing operons at intrinsic terminators *in vitro* (18, 22), provided that the distance between the *nut* site and the terminator is less than a few hundred base pairs. These minimal systems are somewhat nonphysiological, since terminators located thousands of base pairs downstream of *nut* are resistant to N-dependent antitermination at moderate concentrations of N and NusA (refs. 18 and 19; W. A. Whalen and A.D., unpublished results). Thus it appears that N, NusA, and *nut*$^+$ RNA (with RNA polymerase) form a core antitermination complex of limited stability and limited processivity and that this complex is made fully stable and processive by interaction with the remaining Nus factors. Further investigation of the requirements for processive N-dependent antitermination has demonstrated that N (at significantly higher concentrations) can suppress a variety of intrinsic terminators placed near the *nut* sequence in the absence of all Nus factors (19).

In this paper, we describe the minimal requirements for N function *in vitro*. We show that N alone, at sufficiently high concentrations, can suppress termination even in the absence of the *nut* sequence. This result definitively assigns the central role in this antitermination mechanism to N and should permit the elucidation of specific molecular roles for each of the components of the stable and fully processive system.

MATERIALS AND METHODS

Proteins. N protein was purified from the overproducing *E. coli* BL21(DE3)/pET-N1. To construct the N overexpression vector pET-N1, an *Nde*I- and *Bss*HII-digested synthetic oligonucleotide duplex coding for the 10 N-terminal residues of N was ligated to a *Bss*HII–*Bam*HI DNA fragment from pDL142 that encodes the remainder of the N gene (23, 24). The resulting synthetic N gene was cloned into the *Nde* I- and *Bam*HI sites of vector pET-11a (25) to create pET-N1 and then transformed into BL21(DE3) (25). Cells were grown and induced as described (25); N protein accounted for >50% of the total soluble protein obtained. Cells were lysed in a French

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press and sonicated briefly, and the pellet obtained after centrifugation was resuspended in 8 M urea (final concentration). The resulting soluble material was chromatographed through CM-Sepharose (Pharmacia), MacroPrep S (Bio-Rad), and MonoS FPLC ion exchange columns under denaturing conditions to obtain purified N protein. *E. coli* RNA polymerase was purchased from Epicentre Technologies (Madison, WI). NusA was isolated as described (26) and further purified by chromatography through a Mono Q FPLC column (Pharmacia). Protein concentrations were measured spectrophotometrically by using calculated molar extinction coefficients (27).

***In Vitro* Transcription.** After digestion of the parent plasmids with *Bst*UI, templates for *in vitro* transcription were prepared as described (22). Plasmids pSK16 and pWW16 contained identical *pL–nutL–tR′* transcription units, as did the *pL–ΔnutL–tR′* templates derived from plasmids pSK31 and pWW31. A 53-nt RNA transcript containing the boxB sequence from λ *nutR* was produced *in vitro* by using plasmid pCS8 and T7 RNA polymerase as described (6). All transcription reactions in this study were conducted at 30°C in a basic transcription buffer containing 20 mM Tris·HCl (pH 7.6), 50 mM potassium acetate, 5 mM magnesium acetate, 0.1 mM EDTA, and 5% (vol/vol) glycerol, with additional potassium acetate added as indicated. Transcription reactions were otherwise performed (7) and analyzed (28) as described.

RESULTS

N Alone Is Sufficient to Suppress Termination at the *tR′* Terminator *in Vitro*. N-mediated antitermination was assayed *in vitro* with purified components by using linear templates containing the *tR′* terminator located downstream of the *pL* promoter (Fig. 1*A*). Transcripts labeled in the first 15 (5′) residues, terminating at or reading through *tR′*, were quantitated directly from denaturing polyacrylamide gels. The *tR′* terminator is ≈95% efficient in the absence of N on both *nut*$^+$ and *Δnut* DNA templates (Fig. 1*B*, lanes 1 and 2). To determine the minimal requirements for N-mediated antitermination, termination efficiency at *tR′* was investigated as a function of increasing N concentration in a typical low-salt transcription buffer in the absence of all Nus proteins (Fig. 2*A*). A dramatic reduction in the efficiency of termination at *tR′* was observed at N concentrations as low as 150 nM (in systems containing 25 nM polymerase) in the absence of a *nut* sequence or any other component of the N-dependent antitermination system (Fig. 1*B*, lane 7).

This finding was intriguing, given that earlier studies had shown a strict requirement for a functional *nut* sequence, both *in vitro* (7, 18, 22) and *in vivo* (23, 29–31). Under the reaction conditions used here, the presence of a *nut* site on the DNA template had little quantitative effect on the degree of antitermination mediated by N protein in the absence of host factors (Fig. 1*B*, lane 8, and Fig. 2*A*). The N-alone antitermination reaction is inhibited by increasing concentrations of potassium acetate; this inhibition appears to be more effective for the *Δnut* template (Fig. 2*B*).

NusA Enhances N-Mediated Antitermination in a *nut* Site-Dependent Fashion. NusA typically increases the efficiency of intrinsic (and perhaps rho-dependent) termination (12, 22, 32–34). To examine the contributions of NusA to the antitermination activities mediated by N alone, read-through efficiencies at *tR′* were monitored at increasing concentrations of N protein in the presence of NusA on both *nut*$^+$ and *Δnut* DNA templates (Fig. 3). NusA was seen to inhibit antitermination on DNA templates lacking the *nut* sequence; however, this inhibitory effect of NusA could be overcome at higher N protein concentrations (Fig. 3*A*). Thus >90% of the active transcription complexes could read through the *tR′* terminator in the presence of 700 nM N (≈30-fold molar excess of N over

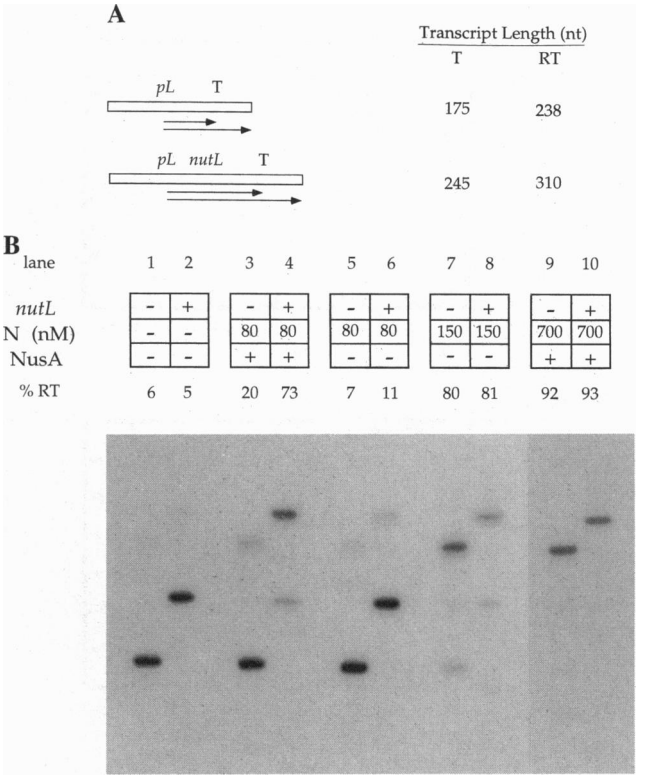

FIG. 1. N-mediated antitermination *in vitro* in the presence and absence of NusA and the *nut* sequence. (*A*) Maps of the transcription templates from plasmids pWW16 and pWW31. The expected lengths of the terminated and read-through transcripts from each template are shown. (*B*) Autoradiogram of resolved RNA products from transcription antitermination reactions. Single-round transcription reactions were performed and analyzed in basic transcription buffer. Read-through (RT) and terminated (T) transcripts are shown in the autoradiogram and the percent read-through products from each reaction is shown immediately above each lane. Reactions whose products are in lanes 3, 4, 9, and 10 contained 120 nM NusA. The *Δnut* template from pWW31 was used in the odd-numbered lanes; the *nut*$^+$ template from pWW16 was used in the even-numbered lanes. The reactions whose products are in lanes 3–6 were supplemented with double-stranded plasmid (pWW16) DNA to a final concentration of 0.1 mM base pairs.

transcription complexes), whether NusA was present or not (Fig. 3*A* and Fig. 1*B*, compare lanes 7 and 9).

In contrast, on DNA templates containing the *nut* sequence, NusA increased slightly the overall extent of antitermination and also decreased the N concentration required to reach saturating levels of read-through of *tR′* (Fig. 3*B*). The (positive) contribution of NusA to N function is dependent on the presence of the *nut* site. The salt dependence of antitermination was also examined on both types of templates in the presence of NusA (Fig. 3*C*). As additional evidence of the *nut* site dependence of NusA function in antitermination, NusA showed little effect on the salt sensitivity of antitermination conferred on *Δnut* templates by 350 nM N, while dramatically increasing the extent of antitermination with 350 nM N on *nut*$^+$ templates at high salt concentrations (Figs. 2*B* and 3*C*).

Consistent with previous studies (7, 18, 22), reaction conditions can be found that reveal an apparent requirement for the *nut* sequence. In the absence of NusA, the antitermination efficiency of N is the same whether a *nut* site is present or not (Fig. 1*B*, lane 8, and Fig. 2*A*). However, in the presence of NusA, antitermination is highly dependent on *nut* at low N protein concentrations (compare the curves with added NusA in Fig. 3 *A* and *B*).

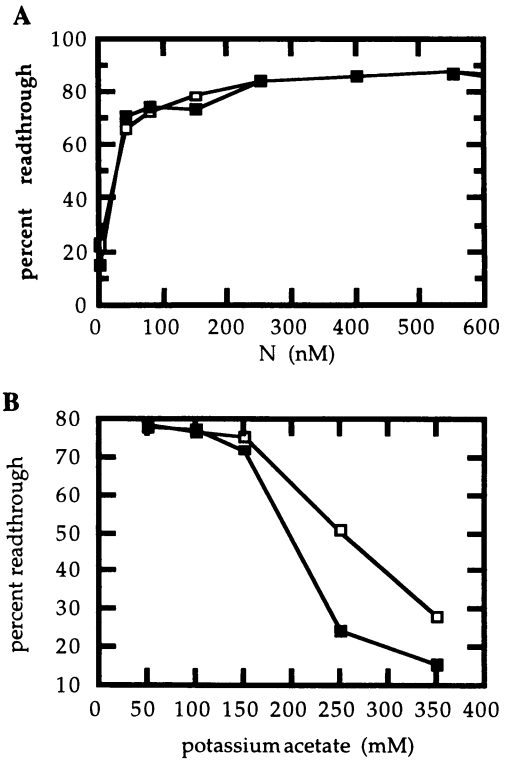

FIG. 2. N protein alone is sufficient to greatly reduce the efficiency of termination at *tR'*. (*A*) Titration of read-through efficiency with N in the absence of NusA. Transcription reaction mixtures were supplemented with various concentrations of N protein at 30°C and incubated for 4 min in basic transcription buffer after the addition of NTP substrates to permit elongation. Solid squares, Δ*nut* template from pWW31; open squares, *nut*$^+$ template from pWW16. (*B*) Salt titration of N-mediated antitermination in the absence of NusA. Transcription elongation reactions containing no NusA, 150 nM N (sufficient to nearly saturate antitermination on both *nut*$^+$ and Δ*nut* templates), and the indicated final concentration of potassium acetate were incubated for 1 min at 30°C. Solid squares, Δ*nut* template from pWW31; open squares, *nut*$^+$ template from pWW16.

Exogenous boxB RNA Competes Strongly with Antitermination on Templates Lacking a *nut* Site. To further characterize the role of the *nut* sequence in N-mediated antitermination, termination assays were carried out in the presence of exogenous boxB-containing RNA. A 53-nt RNA transcript containing boxB, which has been shown to bind monomers of N tightly in a gel band shift assay (6), was used as a trap for free N protein. In transcription reaction mixtures containing ≈25 nM RNA polymerase and 150 nM N (and no NusA), the addition of 350 nM boxB RNA completely inhibited N function on both *nut*$^+$ and Δ*nut* templates (Fig. 4*A*). Total yeast tRNA was significantly less effective as a trap; >30-fold more yeast RNA than boxB RNA (on a nucleotide basis) was required to eliminate the antitermination function of N on a Δ*nut* template (Fig. 4*B*). Consistent with the fact that boxB RNA binds tightly to N, still higher total yeast RNA concentrations were required to inhibit the N antitermination on a *nut*$^+$ template. The addition of ≈700 nM boxB RNA also markedly inhibited N function in reaction mixtures containing 350 nM N and 120 nM NusA (Fig. 4*C*). Thus N function on Δ*nut* templates can be eliminated by the addition of an excess of boxB RNA in the presence or the absence of NusA. However, additional evidence for a *nut*-dependent role for NusA in antitermination by N was provided by showing that NusA can suppress the effectiveness of the exogenous boxB RNA trap for N protein interacting with transcription complexes that express the *nut* sequence (Fig. 4*C*). In the absence of NusA, however, the

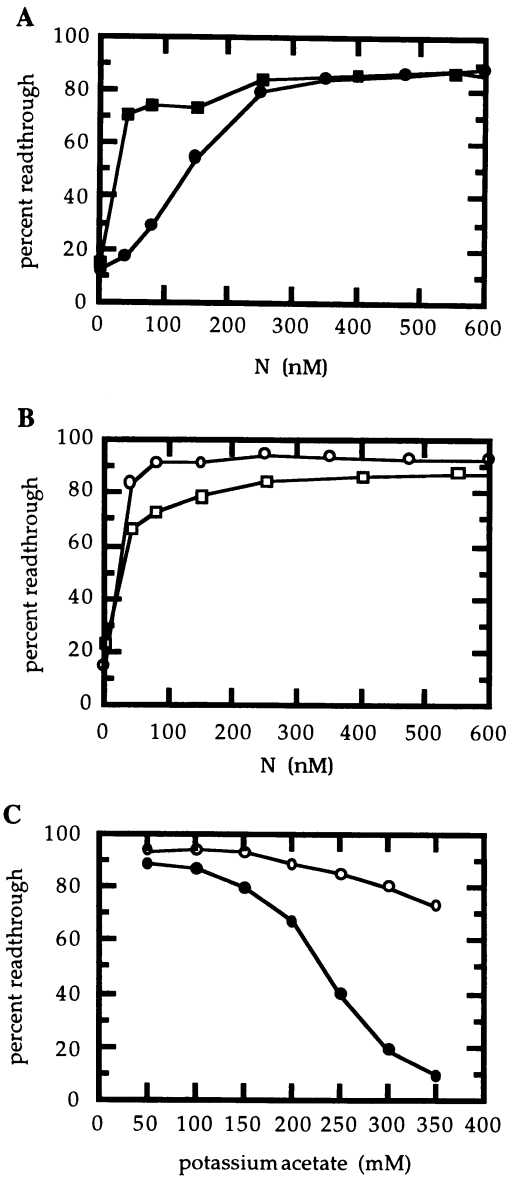

FIG. 3. Effect of NusA on N-mediated antitermination. (*A*) N titration of read-through efficiency in the presence or absence of NusA on Δ*nut* transcription templates. Solid circles, Δ*nut* transcription complexes in the presence of 120 nM NusA; solid squares, Δ*nut* transcription complexes in the absence of NusA. (*B*) N titration of read-through efficiency in the presence and absence of NusA on *nut*$^+$ transcription templates. Open circles, *nut*$^+$ transcription complexes in the presence of 120 nM NusA; open squares, *nut*$^+$ transcription complexes in the absence of NusA. (*C*) Salt titration of N-mediated antitermination in the presence of NusA. Transcription elongation reactions containing 120 nM NusA and 350 nM N protein were incubated for 1 min at 30°C at the concentrations of potassium acetate shown. Solid circles, Δ*nut* template from pSK31; open circles, *nut*$^+$ template from pSK16.

antitermination response is decreased by >50% at equimolar concentrations of N and trap (Fig. 4*A*).

Requirement for *nut* and NusA in the Presence of Nonspecific DNA or RNA. A requirement for *nut* in the presence of NusA was also demonstrated by supplementing the transcription reactions with 10^{-4} M plasmid DNA (in base pairs) (Fig. 1*B*, lanes 3–6). In addition, N function on the *nut*$^+$ template could be shown to require NusA in transcription reactions containing 80 nM N by adding 10^{-4} M plasmid DNA (Fig. 1*B*, lanes 4 and 6), suggesting that excess DNA may bind significant concentrations of N protein nonspecifically under these mod-

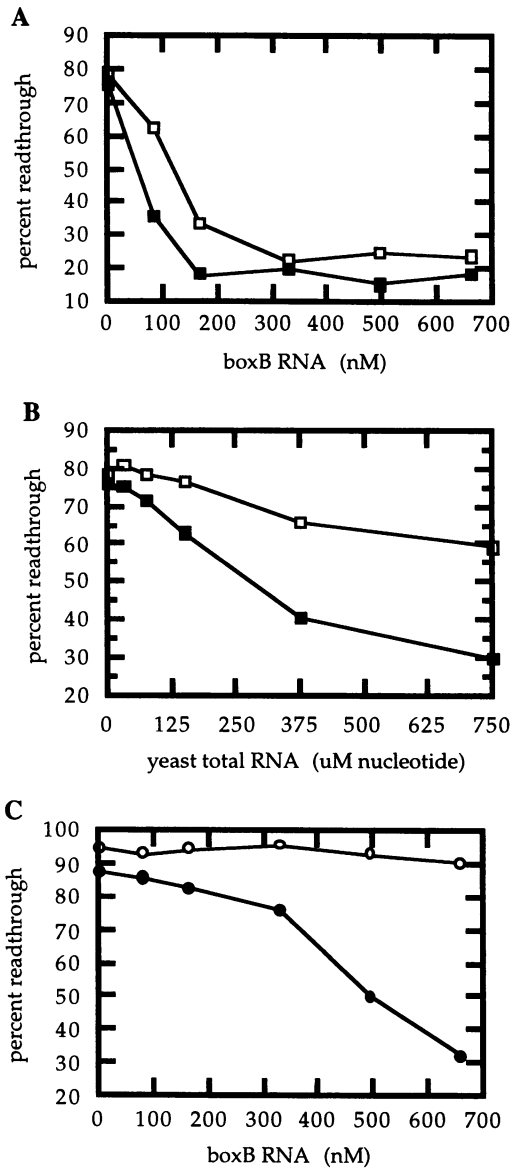

FIG. 4. Inhibition of N-mediated antitermination by RNA. (*A*) BoxB RNA in trans strongly inhibits N function on Δ*nut* and *nut*$^+$ templates in the absence of NusA. Transcription elongation complexes formed on both Δ*nut* and *nut*$^+$ DNA templates were supplemented with 150 nM N and the indicated amounts of the boxB-containing pCS8 RNA. Reactions were incubated at 30°C for 1 min after the addition of NTP substrates and then quenched. Solid squares, Δ*nut* template from pSK31; open squares, *nut*$^+$ template from pSK16. (*B*) Titration of N-mediated antitermination of *tR'* with a nonspecific RNA competitor in the absence of NusA. Transcription reactions contained 350 nM N and the indicated amounts of yeast total RNA. Chain elongation was resumed on the addition of NTP substrates, and the reactions were quenched after 1 min. Solid squares, Δ*nut* template from pSK31; open squares, *nut*$^+$ template from pSK16. (*C*) BoxB RNA in trans strongly inhibits N function on Δ*nut* (but not *nut*$^+$) templates in the presence of NusA. Elongation complexes formed on both Δ*nut* and *nut*$^+$ DNA templates were supplemented with 120 nM NusA and 350 nM N and the indicated amounts of the boxB-containing pCS8 RNA. Reactions were incubated at 30°C for 1 min after the addition of substrates. Solid circles, *nut*$^-$ template from pSK31; open circles, *nut*$^+$ template from pSK16.

erate salt transcription conditions and thus reduce the concentration of N available to interact with the transcription complexes. An effect of NusA was also demonstrated in experiments in which nonspecific RNA was used as a trap for N. In the presence of NusA and 350 nM N, total yeast RNA at concentrations as high as 0.75 mM (in nucleotides) had no inhibitory effects on the efficiency of read-through at *tR'* on either type of template (data not shown). In contrast (Fig. 4*B*), antitermination on both types of templates is inhibited by the addition of 0.75 mM total yeast RNA to reaction mixtures containing 150 nM N and no NusA, reducing the read-through efficiency to 0.3 on the Δ*nut* template and to 0.6 on the *nut*$^+$ template. Thus NusA strengthens, albeit to a greater extent on the *nut*$^+$ complexes, the critical interactions of N with the transcription complex that are sensitive to nonspecific RNA (or DNA) competition.

DISCUSSION

N Protein Alone Can Induce Antitermination. We have demonstrated that large concentrations of N protein alone are sufficient to induce N-dependent antitermination at intrinsic *tR'* terminators *in vitro*. Similar experiments, using both the intrinsic *trpoC* terminator and N protein from bacteriophage 21 (data not shown), indicate that the effect is general. Our data support the hypothesis that a direct interaction between N and RNA polymerase is the key event in N-mediated antitermination.‖ If we assume an equilibrium model for the interaction between N and polymerase within the transcription complex, we can use the titration data of Fig. 2*B* to estimate an effective association constant of 5×10^6 M^{-1} for the binding of N to the transcription complex formed on the Δ*nut* template at physiological salt concentrations (150–200 mM K^+ and 5–10 mM Mg^{2+}; ref. 35). By assuming also a typical diffusion-controlled forward rate constant of 10^8–10^9 $M^{-1}\cdot sec^{-1}$ for the reaction, we can estimate a dissociation rate constant of 20–200 sec^{-1}, which corresponds to a characteristic time of 5–50 msec for the dissociation of N from the complex. This characteristic time is comparable to the estimated dwell time of the elongation complex at average template positions under physiological transcription conditions (36). As a consequence, in the absence of stabilizing factors such as NusA or boxB RNA, we estimate that N should dissociate from the complex at essentially every step of transcript elongation.

The above calculation indicates that the interaction between N and polymerase alone is not sufficiently stable to confer processive antitermination properties onto the complex. An increase of two to three orders of magnitude in the effective stability of the N–polymerase complex, corresponding to an increase to 1–5 sec in the characteristic time for dissociation, is required to explain the processivity (several hundred base pairs) observed with the minimally processive (N–*nut*$^+$–NusA) antitermination system. A further 10- to 100-fold increase in effective stability is required to achieve the processivity (thousands of base pairs) seen with the full N-dependent antitermination complex.

Binding of N to the *nut*–boxB RNA Sequence Increases the Effective Concentration of N at the Elongation Complex and This Interaction Is Strengthened by NusA. Our experiments are consistent with the finding that boxB RNA has a high affinity for N (6) and thus with the proposal that the binding of N to this element in the nascent RNA increases the effective equilibrium concentration of N near the polymerase as a consequence of RNA looping (37). Our experiments also support the view that NusA interacts with N and boxB RNA (as well as with polymerase) to further stabilize this interaction (6). Such a three-way interaction with NusA clearly increases the

‖A possible trivial explanation for this N-alone effect is that high concentrations of N might bring about antitermination indirectly by binding to and destabilizing the intrinsic terminator RNA hairpin. Several lines of evidence make this explanation unlikely, including the facts that N is not a helix-destabilizing protein (6) and that N-derived peptides capable of binding to boxB RNA with an affinity comparable to N do not induce antitermination nonspecifically (J. DeVito and A.D., unpublished results).

affinity of the *nut*–N complex for the elongation complex. In support of this idea, we have shown that ≈10-fold higher concentrations of N are required with Δ*nut* templates than with *nut*$^+$ templates (in the presence of NusA) to attain saturating levels of read through of the *tR′* terminator (Fig. 3). Furthermore, N is far more effectively trapped from Δ*nut* transcription complexes than from *nut*$^+$ transcription complexes by boxB RNA added in trans in the presence of NusA (Fig. 4).

Circular dichroism and sedimentation experiments have shown that binding of boxB RNA induces a major conformational change in N and that this change is not induced by the binding of nonspecific RNA (M. Van Gilst and P.H.v.H., unpublished experiments). If this conformational change reflects the induction of an antitermination form of N, we may ask whether such an N–boxB RNA complex (which cannot bind nonspecific RNA) is more or less effective in inducing antitermination when added in trans than is N alone in the presence of a Δ*nut* template, where N protein can bind nonspecifically (in cis) to the nascent RNA. Our results show that N protein at 350 nM binding nonspecifically to such a nascent Δ*nut* RNA is significantly more effective in inducing antitermination than is the N–boxB RNA complex at 700 nM, suggesting that even in the absence of the *nut* sequence the delivery of N to the polymerase by RNA looping may be more effective in antitermination than is the presentation of N as a complex with boxB RNA in trans.

NusA Plays a Special Role in Stabilizing the NusA–N–*nut*$^+$ Complex at the Polymerase. The protection by NusA of N-modified transcription complexes against competition by nonspecific RNA on both *nut*$^+$ and Δ*nut* templates (Fig. 4) is consistent with the notion that NusA facilitates association of N with RNA polymerase in transcription complexes. However, in addition, NusA shows a *nut* site-dependent effect, since it enhances antitermination on *nut*$^+$ transcription complexes at subsaturating concentrations of N (Fig. 3*B*) and inhibits antitermination on Δ*nut* complexes (Fig. 3*A*). The latter effect is in keeping with the observation that NusA increases termination efficiency when operating as an isolated transcription factor (32, 34). However, the dependence of antitermination on NusA in the presence of a *nut* site suggests that this protein has a specific role in stabilizing the N–*nut*–polymerase antitermination complex.

Specific (Processive) Antitermination Requires the Full N-Dependent Antitermination System. Although N alone can efficiently suppress intrinsic terminators *in vitro*, it is not present in sufficient quantities in the cell to use this mechanism to induce antitermination at the levels required for phage development. In this sense such a minimal system is not biologically faithful. N function *in vivo* requires both the boxA and boxB elements of the *nut* sequence and also the active participation of transcription elongation factors NusA, NusB, S10, and NusG.** *In vivo* N-dependent antitermination is highly processively, preventing termination at sites located as far as 10–20 kb downstream from the *nut* sites in the *pL* and *pR* operons (38, 39). Clearly, the N-dependent antitermination system has evolved to permit phage development at low N concentrations (40), at physiological temperatures and salt concentrations, and in the presence of a vast excess of nonspecific nucleic acid binding sites for N.

**The Nus proteins may not be required to permit some processive antitermination *in vivo*, since λ phage can grow at 30°C (but not at 42°C) in *nus*$^-$ mutant strains (see ref. 3).

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