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A Quaternary Transcription Termination Complex

Reciprocal Stabilization by Rho Factor and NusG Protein

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The *Escherichia coli* protein NusG is known to modulate Rho-dependent transcription termination *in vivo*. We have shown that it can also alter the pattern of Rho-dependent RNA endpoints *in vitro*, at lower NusG concentrations than can be explained by reported interactions between NusG and Rho or RNA polymerase. Three observations *in vitro* now suggest a model to account for these effects of NusG on Rho-dependent termination. First, the presence of NusG circumvents the interference with Rho function caused by adding DNA oligonucleotides complementary to particular segments of the Rho binding site. Second, when NusG is added to stalled elongation complexes, the off-rate of Rho from nascent RNA is slowed. Third, NusG associates stably with the elongation complex only when Rho is also present and bound to the nascent RNA. Our observations are consistent with a model in which NusG and Rho participate in an interdependent association with the transcribing RNA polymerase and the nascent RNA to facilitate the recognition and use of termination signals. Common structural and functional features shared with complexes that carry out processive antitermination are discussed.

Keywords: transcription termination; RNA-protein complex; Rho; NusG; kinetic coupling

1. Introduction

In prokaryotes, transcription termination can occur when RNA polymerase recognizes an intrinsic signal in the transcription unit or when Rho factor causes the release of nascent RNA (Yager & von Hippel, 1987; Platt & Richardson, 1992). Rho is an RNA-dependent ATPase with sequence homology to the RNP-consensus RNA-binding proteins (Lowery & Richardson, 1977; Pinkham & Platt, 1983; Brennan & Platt, 1991) and it displays RNA:DNA helicase activity *in vitro* (Brennan *et al.*, 1987). The mechanism by which it carries out its cellular function as elucidated by *in vitro* experiments has seemed elegantly simple: termination occurs when Rho recognizes a target site in the mRNA, translocates 5' and 3' along the RNA, encounters the elongation complex, and causes release of the nascent transcript.

Inklings that the functioning of Rho may be more complex, however, have come from a variety of recent observations (see Platt, 1994). Among these is the participation of the NusG protein, an important new addition to the set of *Escherichia coli* transcription factors. *nusG* is now known, like *rho*, to be an essential gene in *E. coli* (Downing *et al.*, 1990). It participates, together with host factors NusA and the S10-NusB heterodimer, in antitermination at the *E. coli rrn* operon, and, with the addition of N factor, at the λ P_R site (Mason & Greenblatt, 1991; Li *et al.*, 1992). These proteins cooperatively form a processive and highly stable elongation particle capable of reading through termination signals over thousands of bases (Horwitz *et al.*, 1987; Li *et al.*, 1992). Sullivan & Gottesman (1992) have demonstrated that NusG is also required *in vivo* for Rho to function at a variety of termination sites, and overexpression of NusG can suppress the *nusD* class of *rho* mutations (Sullivan *et al.*, 1992). There are several reports that NusG can cause early Rho-dependent termination of transcription *in vitro* resulting in a promoter-proximal shift in RNA endpoints (Li *et al.*, 1993; Nehrke *et al.*, 1993). Li and co-workers also presented evidence that Rho and NusG interact, although their affinity (approximately 10 μM) was insufficient to explain the effects of NusG in transcription reactions at protein concentrations of 10 to 20 nM (Nehrke *et al.*, 1993). A weak interaction between NusG and RNA polymerase was proposed to facilitate the association of NusG with Rho in the elongation complex by increasing the local concentration of NusG (Li *et al.*, 1992).

Several lines of evidence suggest that NusG enhances function where Rho interacts weakly with the nascent transcript. *In vitro*, NusG restores Rho-dependent termination to near wild-type efficiency at mutated termination sites (Nehrke *et al.*, 1993) and at high salt concentrations, both of which lead to decreased RNA-binding affinity of Rho on its

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own (Li *et al.*, 1993). Of the sites tested *in vivo*, only the λ terminator t_{I2}, which is preceded by a long untranslated stretch of RNA, remained efficient (81%) when NusG was depleted, relating, perhaps, to the ability of ribosomes to prevent Rho binding (Sullivan & Gottesman, 1992). However, NusG, which on its own has no measurable affinity for RNA, appears to have little or no effect on various assays of Rho activity when uncoupled from ongoing transcription, including both the K_m and V_{max} values for ATP hydrolysis either using lambda *tR1* or *trp t'* RNA (Nehrke *et al.*, 1993; B. Stitt, personal communication); likewise, RNA-binding affinity and rate of RNA:DNA helix disruption are unaffected by NusG (Nehrke *et al.*, 1993).

We show here using oligonucleotide-targeted inhibition of Rho RNA-binding that NusG enhances the productive association of Rho with a segment of the nascent transcript upstream (i.e. 5') of its usual specific, high-affinity target site. In companion experiments, we use both active and stalled transcription systems to examine the effects of NusG on Rho function, as well as the requirements for the association of NusG with the elongation complex. Employing templates attached to agarose beads, we observe that NusG strongly associates with stalled elongation complexes only if Rho is bound to the nascent RNA. The presence of NusG in the complex leads to a slower off-rate of Rho from the transcript and potentiates termination at positions where polymerase does not respond to Rho on its own. Our results lead to the hypothesis that termination by Rho and NusG requires the formation of a quaternary termination complex, whose altered response may be ascribed to a slower off-rate of Rho from the nascent RNA, caused by the action of NusG protein. We discuss the implications of this model for understanding why NusG is important *in vivo* for termination of transcription at Rho-dependent sites.

2. Materials and Methods

(a) *Materials*

RNA polymerase holoenzyme saturated with sigma^{70} was a generous gift of M. Chamberlin. Rho was purified as described by Mott *et al.* (1985) from the overproducing strain AR120-A6 p39ASE as per modifications by Nehrke *et al.* (1992). Purified NusG was donated by Barbara Stitt, NusA by R. Landick, and Gln-111 *EcoR1* endonuclease by P. Modrich. Antibody to NusG was from J. Greenblatt, to the α subunit of RNAP† was from R. Burgess, and to Rho from J. Roberts. Terminal deoxytransferase was from Ratliffe Biochemicals, RQ1 Dnase, SP6 RNA polymerase, and RNasin were from Promega; Boehringer-Mannheim supplied Bio-16 dUTP and other nucleotides, and avidin agarose beads were from Calbiochem. The oligonucleotides z1, trp2, and trp3 were synthesized at the University of Rochester core nucleic acids facility and are complementary to *trp t'* RNA (Figure 1). The plasmid pRLtrpt' (Nehrke *et al.*, 1993) was cut with *Mst*II, blunt-ended with Klenow fragment, and recircularized with an *Eco*RI linker, removing the terminal half of *trp t'* and sequences up to the *lacZ* region, including a *Kpn*I cleavage site, to generate pRLt'RI. pSP65t' (Brennan *et al.*, 1987), cut with either *Hin*dIII or *Mst*II, was used to generate SP6 transcripts of *trp t'*.

† Abbreviations used: RNAP, RNA polymerase; BSA, bovine serum albumin.

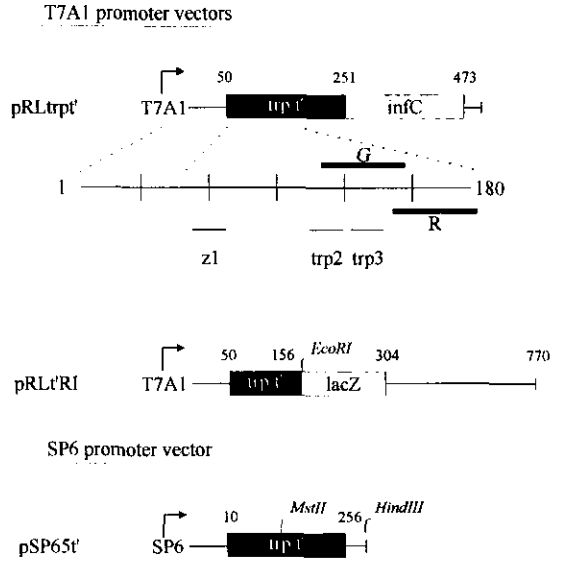

Figure 1. Schematic of the DNA templates used for transcription. The construction of templates pRLtrpt', pRLt'RI, and pSP65t' are described in Materials and Methods. The coding regions that contribute to the makeup of the mRNA transcribed from these vectors are shown, as are the promoters used for transcription and the expected lengths of the various RNA products. The annealing sites of the 3 DNA oligonucleotides z1, trp2, and trp3 for the nascent RNA are indicated in the context of the pRLtrpt' template in an expanded display immediately below the vector schematic. The heavy bars indicate the regions in which termination occurs with either Rho alone (R) or Rho plus NusG (G). Grid marks denote 30 nucleotide intervals in the expanded region.

(b) *Transcription and ATPase reactions*

Transcriptions with A20 complexes were performed as described by Nehrke *et al.* (1993). Briefly, 10 nM of RNA polymerase was incubated with equimolar plasmid or agarose-beaded DNA in a solution of 40 mM Tris·HCl (pH 7.9), 20 mM NaCl, 14 mM $MgCl_2$, 14 mM-mercaptoethanol, 2% (v/v) glycerol, and 50 μg BSA/ml containing 240 μM ApU dinucleotide, 10 μCi of [α-^{32}P]GTP, and 2.5 μM each of ATP, GTP, and CTP for 20 minutes at 30°C to form the A20 complex. For synchronous elongation, these pre-formed complexes were diluted fivefold into elongation buffer (30 mM Tris-acetate (pH 7.9), 5 mM magnesium acetate, 50 mM potassium acetate, 1 mM DTT, and 50 μg BSA/ml) containing all four unlabeled nucleotides (at 10 μM, save GTP at 250 μM) and Rho, NusG and/or DNA oligonucleotides at the indicated concentrations (in oligonucleotide targeting experiments, 100 mM KCl was substituted for potassium acetate to improve annealing efficiency relative to rho binding). Annealing, as judged by RNase H susceptibility in control reactions, took place faster than we could measure, as would be expected from the findings of Fakler *et al.* (1994). After 20 minutes at 30°C, the reactions

were treated with SDS and Proteinase K, brought to 0.3 M sodium acetate, extracted with phenol/chloroform, co-precipitated with 10 μg of tRNA, and analyzed on denaturing acrylamide gels.

To examine elongation complexes stalled precisely at the *Eco*RI site of pRLt'RI (Figure 1), we pre-formed A20 complexes (with *Sph*I-linearized vector at sixfold molar excess over RNA polymerase), and added Gln-111 *Eco*RI enzyme at fivefold excess over *Eco*RI sites per template molecule (2) to pre-bind for ten minutes. This elongation block at the *Eco*RI site could be removed by adding an equal volume of 1 M potassium chloride, 10 mM $MgCl_2$, and 0.5 mM of each rNTP in elongation buffer. The concentration of RNA polymerase engaged in elongation was determined by quantitation of transcription products on a Molecular Dynamics PhosphorImager. Blocked complexes to which Rho was to be added were treated with EDTA (to 12.5 mM) to prevent Rho from releasing the nascent RNA. SP6 transcription reactions with Rho followed the manufacturer's protocol except for the following: CTP and UTP were used at 5 μM and only five units of SP6 RNA polymerase per reaction was added. The off-rate of Rho from nascent RNA was determined by first incubating Rho at 0.25 nM with 0.5 nM of unlabeled *Eco*RI-blocked stable transcription complexes formed on pRLt'RI DNA for several minutes at 30°C in transcription elongation buffer supplemented with EDTA. Labeled *trp t'* competitor RNA was added to 0.5 nM, and at each time point 20 μl aliquots of the reaction were filtered through nitrocellulose, and washed twice with 0.4 ml of elongation buffer. Scintillation counting of the label retained on the filters indicated the amount of Rho free in solution capable of binding to the competitor species. When present, NusG was at 20 nM.

(c) *Formation and isolation of beaded complexes*

A total of 100 μg of pRLt'RI was cut with *Kpn*I 300 base-pairs upstream of the T7A1 promoter. After precipitation with ethanol, the DNA was dUTP-tailed in 50 μl of 200 mM potassium cacodylate, 2 mM β-mercaptoethanol, 1 mM $CoCl_2$ containing 8 μM Bio-16 dUTP, 32 μM dTTP, and 60 units of terminal deoxytransferase for one hour at 37°C. The tailed template was precipitated, cut with *Sph*I, and the tailed fragment containing the T7A1 promoter was gel-isolated. Avidin agarose (0.5 ml) was pre-washed several times with 10 mM Tris·HCl, 0.1 mM EDTA (pH 8.0) and the tailed DNA fragment was attached by gentle rocking at 4°C overnight in 1.5 ml. The extent of DNA coupling was determined by the A_{260} of the supernatant combined with a 100 mM KCl wash and by gel quantification of the DNA in the precipitate from the supernatant.

Stalled complexes were formed on the beads in the absence of Rho or NusG in transcription elongation reactions containing 0.25 mM GTP and 10 μM each UTP, CTP, and ATP. After elongation at 30°C for three minutes, EDTA was added to a final concentration of 20 mM. The stalled complexes were centrifuged, washed twice in 1 ml of cold elongation buffer containing 20 mM EDTA, then twice more in elongation buffer without EDTA. Complexes to be analyzed in the absence of RNA were treated with 10 units of RNase T_1 for five minutes at 30°C. The beaded complexes were resuspended in 1 ml of elongation buffer containing NusG and/or Rho at 40 nM and 20 nM, respectively. After rocking at 4°C for 15 minutes, the beads were washed five times in cold elongation buffer. The proteins were released from the beads by treating with 1 μl of RQ1 DNase in 200 μl of elongation buffer for 30 minutes at 37°C. The supernatant and a subsequent 200 μl wash were concentrated in a Centricon 10 ultrafiltration unit, and the proteins were run on a Laemmli SDS/polyacrylamide gel, transfered to PVDF membrane, and visualized by Western analysis using HRP-conjugated secondary antibodies with a chemiluminescent reporter (Amersham), and primary anti-Rho and NusG antibodies at a 1:1000 dilution and anti-RNA polymerase antibody at 1:500 dilution.

3. Results

(a) *Oligonucleotide-targeting of* trp t' *nascent RNA during Rho-dependent termination* in vitro

Rho-dependent termination of transcription requires that Rho bind to the nascent transcript for activation of its ATPase and helicase activities, in order to disrupt the ternary elongation complex, releasing both the template and the transcript. *trp t'* is a strong Rho-dependent terminator, both *in vivo* and *in vitro*, whose transcript binds Rho with a dissociation constant of approximately 10 pM. A segment of DNA containing approximately 240 nucleotides of *trp t'* was cloned into the vector pRL418 to create pRLtrpt' (Nehrke *et al.*, 1993). Transcription initiation from this vector in the absence of UTP resulted in the formation of an A20 transcription complex, stalled at nucleotide 20 of the transcript (Figure 1). By labeling of the A20 complex and diluting the label 100-fold during elongation, we observed essentially single round transcription, with labeling independent of transcript length.

The normal sites of rho-dependent termination (Figure 2, lane 3) correspond to the heavy bar denoted R in Figure 1. In the presence of Rho and NusG, termination occurred 20 to 30 nucleotides earlier than normal in the *trp t'* transcript (Figure 2, lane 4), corresponding to the bar labeled G as reported previously (Nehrke *et al.*, 1993; Figure 1). To identify target sequences for Rho in the RNA, DNA oligonucleotides complementary to 15 nucleotide segments of *trp t'* (Figure 1) were added concomitantly with Rho and ribonucleotide triphosphates (including UTP) at the start of elongation. As a control, we found that oligonucleotides z1, trp2, and trp3 by themselves had no effect on readthrough transcription by *E. coli* RNA polymerase in the absence of Rho (Figure 2, lane 1 *versus* lane 2). The addition of oligonucleotide trp2 caused a distal shift in the Rho-mediated endpoints, suppressing termination at the normal sites almost completely (Figure 2, lane 5). The addition of oligonucleotide trp3 in combination with trp2 was slightly more effective than trp2 alone in suppressing the normal sites of Rho action (Figure 2, lane 6).

Surprisingly, in contrast to their effect on the normal Rho-dependent RNA endpoints, oligonucleotides trp2 and trp3 in combination failed to suppress termination at these early NusG-responsive sites (lane 7 *versus* lane 4). However, the addition of oligonucleotide z1 substantially reduced the effect of NusG (lane 9), restoring endpoints at positions caused by Rho alone (lane 3). With Rho alone, oligonucle-

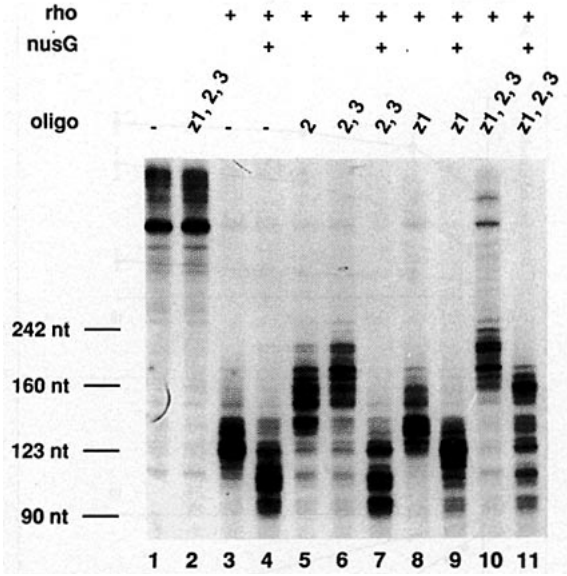

Figure 2. Oligonucleotide targeting of the nascent RNA during transcription termination *in vitro*. Transcription was started by the addition of rNTPs (including UTP) to A20 complexes pre-formed on pRLtrpt' template DNA. Oligonucleotides (100 nM), and Rho (10 nM) ± NusG (20 nM) were, when present, added concomitant with the start of elongation. The final RNA products were run on a 4% (w/v) acrylamide/7 M urea gel. Lane 1 displays the readthrough transcript, while lane 2 displays readthrough in the presence of oligonucleotides z1, 2, and 3. Lanes 3 and 4 are Rho-dependent and NusG-enhanced terminated transcripts, respectively. Various combinations of oligonucleotides were used to target the nascent RNA during transcription termination (lanes 5 to 11). pBR322 DNA *Msp*I marker fragments are shown on the left (and migrate faster than RNA molecules of equivalent length). The shortest transcripts seen with Rho *versus* Rho plus NusG are about 105 and 135 nucleotides long, respectively (lane 2 *versus* lane 3), as determined by RNase H cleavage with oligonucleotides annealed to defined sites in the transcript.

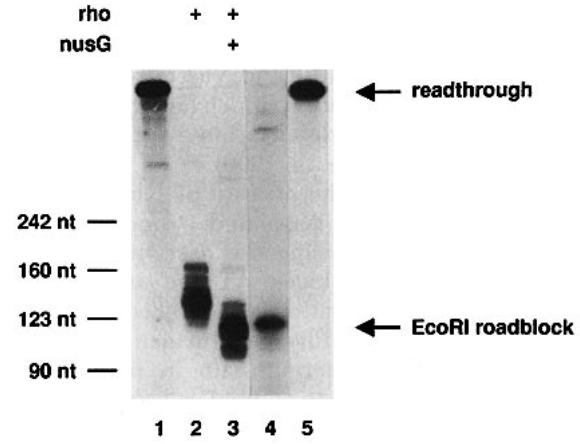

Figure 3. Transcription termination and blockage of transcription using the Gln-111 *Eco*RI mutant restriction endonuclease. Transcription elongation was programmed from A20 complexes pre-formed on pRLt'RI template DNA. In lane 1, the readthrough transcript is shown. In lanes 2 and 3, the Rho-dependent and NusG-enhanced terminated transcripts are shown, respectively. Prior to elongation, the A20 complex was incubated with a 5-fold excess of Gln11-*Eco*RI mutant restriction endonuclease relative to the amount of DNA template present, and elongation was allowed to proceed. The RNA species resulting from stalling of RNA polymerase at the Gln-111 *Eco*RI block are shown in lane 4. After incubation in 12.5 mM EDTA for 10 min (see below), the *Eco*RI protein was displaced by the addition of an equal volume of reaction buffer containing 1 M salt (see Materials and Methods). Nearly complete readthrough transcription occured when the block was removed (lane 5). The indicated size markers were taken from denatured fragments of pBR322 DNA, cut with *Msp*I and labeled with Klenow.

otide z1 also resulted in the use of termination sites more distal than normal (lanes 3 *versus* 8, 6 *versus* 10). However, in neither of these cases was the effect of oligonucleotide z1 as dramatic as in a reaction that also contained trp2, trp3, and NusG, which gave a pronounced and widespread distal shift in the RNA endpoints (Figure 2, lane 7 *versus* lane 11). This suggested that the z1 region of *trp t'* is important for NusG catalysis of proximal termination endpoints, whereas the trp2 region is only involved when NusG is not present or when termination occurs farther along the template.

In parallel experiments, the effects of oligonucleotides z1, trp2 and trp3 on Rho RNA-binding were assessed by nitrocellulose filter retention of labeled *trp t'* RNA under conditions of Rho excess. Rho's dissociation constant from *trp t'* RNA was approximately 10 pM, in good agreement with previous established values. Oligonucleotide trp2 weakened the measured affinity by a factor of 10, while neither oligonucleotide trp3 nor z1 had any detectable effect (data not shown). However, oligonucleotides trp2 and z1 in combination reduced Rho's binding affinity for *trp t'* by 15-fold (data not shown). The addition of NusG to these reactions produced no change in the measured affinities of Rho for *trp t'*, as seen previously (Nehrke *et al.*, 1993).

(b) *NusG slows the release of Rho from nascent* trp t' *RNA*

To ask how NusG might be modifying the Rho response, we examined ternary elongation complexes blocked specifically by the Gln-111 *Eco*RI mutant restriction enzyme, which binds to DNA but does not cause cleavage (Pavco & Steege, 1990). Elongation from unlabeled A20 complexes formed on the pRLt'RI template (Figure 1; see Materials and Methods) allowed formation of a stalled ternary complex with the 3' end of its mRNA about 14 nucleotides upstream of the *Eco*RI restriction site (Pavco & Steege, 1990; Figure 3, lane 4), between the Rho-dependent and NusG-responsive termination sites on this template (lanes 2 and 3). Quantitation of transcripts in parallel labeled reactions revealed that nearly all of the active RNA polymerase in the reaction was in intact ternary complexes (data not shown). All of the stalled transcripts could be chased to readthrough length by adding KCl to a final

concentration of 0.5 M to displace the mutant *Eco*RI protein (Figure 3, lane 5), unless they had been incubated with Rho or Rho and NusG in the absence of EDTA prior to elongation, which largely prevented readthrough, due to dissociation of the nascent transcript (Pavco & Steege, 1990).

We then measured the off-rate of Rho from the transcript in these complexes under conditions that prevent Rho from releasing the nascent RNA (i.e. EDTA was added to block ATPase and helicase activities). The concentration of the stalled complexes was about 0.5 nM (by PhosphorImager analysis of isotopically labeled reactions run in parallel), to which Rho was added to a final concentration of 0.25 nM (±20 nM NusG) and allowed to pre-bind to the nascent RNA at room temperature for 2.5 minutes. Labeled competitor RNA (full-length *trp t'* RNA transcribed from pSP65t') was then added to a final concentration of 0.5 nM and aliquots of the entire reaction were filtered through nitro-cellulose to measure the transfer of Rho factor from the nascent to the free RNA. At equilibrium, most of the Rho will be bound to the latter, because of Rho's tighter affinity and much slower off-rate, compared to the blocked nascent transcript (see below).

We measured the background retention of *trp t'* on filters in these reactions in the absence of Rho (Figure 4A) to determine whether there was a contribution from RNA polymerase, binding to labeled competitor on its own. RNA polymerase can bind to free *trp t'* RNA with a dissociation constant of less than 0.1 nM (data not shown), but the background retention in the indirect off-rate assays was, in general, less than 10% of the total RNA in the reaction, as expected if the majority of enzyme is in stalled ternary complexes. This background increased slightly over time, suggesting that a small amount of RNA polymerase was released free into solution during incubation in EDTA, but was never more than slightly above the background observed in the absence of protein (data not shown).

Five fmol of Rho were preincubated with the stalled ternary complexes prior to the addition of *trp t'* RNA, and by ten minutes, nearly all of the Rho was released as indicated by its ability to bind to the labeled competitor (Figure 4A). Since Rho inactivates in solution over time, we used an experimentally determined half-inactivation rate constant (25 minutes) to normalize each value plotted in Figure 4A by compensating for this inactivation, and obtained the graph shown in Figure 4B. In this plot of the natural log of the fraction of Rho retained on the stalled complexes *versus* time, the negative slope is inversely related to the off-rate. Thus, the off-rate of Rho from *trp t'* nascent RNA in transcription complexes stalled by the Gln-111 *Eco*RI mutant protein has a half-time of about two minutes. Rho's initial rate of release from the stalled ternary complexes was qualitatively slower in the presence of NusG, as demonstrated by the competitor binding curve in Figure 4A. When these data were normalized for Rho inactivation and plotted as an exponential function of time (Figure 4B), it became clear that

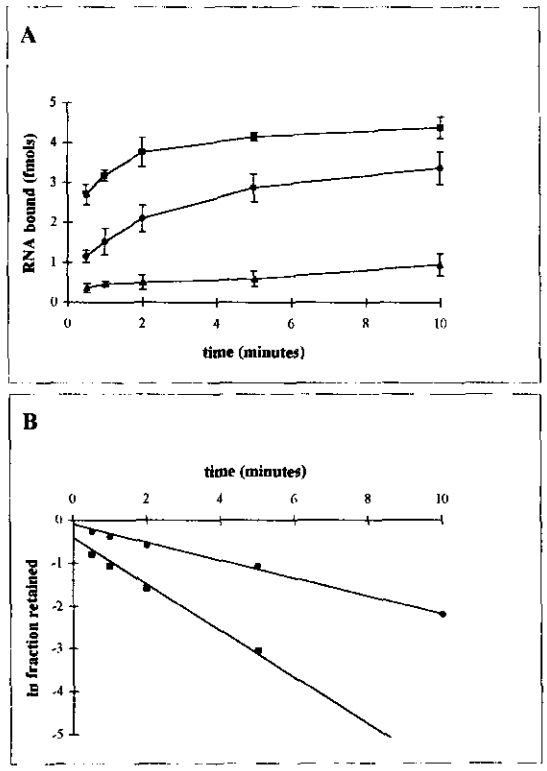

Figure 4. NusG slows the off-rate of Rho from nascent *trp t'* RNA. A, Unlabeled Gln-111 *Eco*RI-blocked transcription complexes were formed as indicated (Materials and Methods) and EDTA was added to a final concentration of 12.5 mM. The transcription complexes were immediately diluted into reaction buffer containing either Rho storage buffer (▲) or 0.25 nM Rho (■) ±20 nM NusG (●) such that the final concentration of active ternary complex stalled at the *Eco*RI site was 0.5 nM. After approximately 2.5 min, 0.5 nM of labeled *trp t'* competitor RNA was added to the reaction and 20 μl aliquots were removed and filtered through nitrocellulose. The absolute amount of labeled RNA retained on the nitrocellulose was calculated from the counts retained and these values were plotted against time. For the reactions containing Rho (● and ■) the background (▲) was subtracted prior to plotting the curves. No zero time value is shown because we cannot measure the instantaneous initial amount of unbound Rho at the time labeled competitor is added (an estimate can be obtained by extrapolation of the lines in B, following). B, The fraction of Rho retained on the blocked length nascent RNA was calculated from the amount of Rho released, as assayed by filter retention of the labeled competitor transcript in A, and plotted as an exponential *versus* time. The natural half-life of Rho (25 min) was taken into consideration when translating the raw values in A to those presented here, since the maximum amount of competitor that Rho could bind became less over time. The negative inverse of the slope of the best-fit line for each data set is related to the off-rate of Rho from the nascent RNA, determined here to be approximately 2 min in the absence of NusG (■) and 5 min in the presence of NusG (●). The *y*-intercept indicates that there was some free Rho at zero time, but less when NusG was included.

NusG mediated a two- to threefold increase in the retention of Rho on the nascent RNA with a dissociation half-time of about five minutes. Com-

pared to an off-rate of Rho of greater than 30 minutes for the full-length free *trp t'* species of competitor RNA, it was not unexpected that most of the Rho was transferred and remained bound to the competitor RNA at ten minutes. Not all of the Rho was bound to nascent RNA at the time the competitor was added, but this is not surprising (despite the molar excess of stalled complex over Rho), as these experiments were carried out at concentrations near the dissociation constant of Rho from these nascent transcripts, where a fraction is expected to be unbound. The fact that NusG decreased the fraction of unbound Rho is consistent with its having a stabilizing effect on Rho-RNA binding.

As a control to ensure that this aspect of NusG function was only manifest at the level of the elongation complex, we repeated this experiment on two free RNA species derived from *trp t'* that were the same lengths as the nascent and competitor RNA, respectively, in the indirect off-rate assay. When free RNA was substituted for nascent RNA, NusG had no effect on the retention time of Rho on the unlabeled pre-bound species (data not shown).

(c) *The association of NusG with the elongation complex requires RNA-bound Rho factor*

Following evidence that NusG can stabilize Rho on the nascent transcript, but does not bind strongly to either Rho or RNA polymerase individually, we examined the minimal requirements for association of NusG with the transcription complex. We employed biotinylated dUTP to tail an 800 nucleotide fragment of pRLt'R1 (Figure 1) containing the T7A1 promoter and *trp t'* and to couple this fragment to strepavidin-coated agarose beads. The response of RNA polymerase to Rho and NusG was identical in transcription reactions whether or not the template was coupled to these beads (data not shown). Rho was able to cause nearly 100% termination of transcription and NusG mediated the release of RNA at earlier sites in both cases.

Stalled ternary complexes formed on these beads were used as an affinity matrix to assay the retention of NusG under various conditions; such immobilized complexes formed during synchronous transcription by halting elongation at a point beyond *trp t'* and the major Rho-dependent termination sites are stable for several hours (data not shown). After several washes of the beaded complexes with cold elongation buffer, first containing EDTA, then without, to remove the nucleotides, we added NusG and/or Rho in 1.5 ml of buffer. After rocking for 15 minutes at 4°C, the beads were centrifuged down and washed five times to remove protein not stably associated with the complexes. DNase was then used to separate the template and its stably associated proteins from the beads. The supernatant was concentrated in a Centricon 10 ultrafiltration unit and the eluted proteins were separated through PAGE and identified through Western analysis (Figure 5A).

Only trace amounts of NusG remained associated with the beaded complexes in the absence of either Rho or the nascent transcript (Figure 5A, lanes 4 and 6). In contrast, an intact transcript and the presence of Rho resulted in significant retention of NusG (Figure 5A, lane 7), suggesting that RNA-bound Rho helps to maintain a stable interaction between NusG and the transcription complex. Trace contamination of NusG appears in the RNA polymerase control lane, but cannot be seen or functionally detected at the significantly lower levels of RNA polymerase in our reactions. Rho, NusG and RNA polymerase were titrated and analyzed by Western analysis with the same antibodies used in Figure 5A to determine the molar ratio of proteins recovered from the washed complexes, and each protein appeared to be present at roughly equivalent concentrations (data not shown).

NusA alone is insufficient to recruit NusG to the elongation complex (Li *et al.*, 1992), but a mutated NusG has been isolated as a suppressor of a mutation in NusA, and NusA causes a functionally opposite response to NusG during *rho*-dependent termination (Farnham *et al.*, 1982). We found, however, that NusA has no effect on the formation of the NusG-modified ternary complex (Figure 5A, lane 8). To test whether the effects of Rho and NusG on termination involved interactions with RNA polymerase rather than direct protein-protein interactions with one another, we carried out two additional experiments. First, the addition of Rho to transcription reactions containing SP6 RNA polymerase instead of the *E. coli* enzyme resulted in the release of RNA at or near the end of *trp t'* (Figure 5B, lane 2), but NusG did not affect either the efficiency or position of termination as it does with *E. coli* RNA polymerase (lane 3). In a parallel experiment, under conditions where Rho can arrest RNA polymerase II of yeast *in vitro* (Wu & Platt, 1993), the inclusion of NusG had no detectable effect on the Rho-mediated endpoints (S.-Y. Wu & T. Platt, unpublished results). Second, although NusG has a slight affinity for Rho, a Rho-RNA binary complex provided *in trans* could not compete NusG from stalled, beaded transcription complexes that also included Rho (data not shown). Taken together, these results are consistent with an essential contribution from specific interactions between NusG and the bacterial RNA polymerase.

4. Discussion

Our previous studies of the effects of NusG protein on termination of transcription *in vitro* have shown that the proximal shift in the mRNA endpoints elicited by NusG was not due to alterations in kinetic coupling (Nehrke *et al.*, 1993). In this work, we go on to show three specific characteristics of the Rho-dependent termination complex as it is affected by the presence of NusG. First, the proximal shift in transcript endpoints depends on NusG-mediated recognition of a region of the transcript, near the annealing site of oligonucleotide z1, that can bypass the inhibitory effect of oligonucleotide trp2. Second, the dissociation rate of Rho from the nascent transcript in a specifically stalled elongation complex

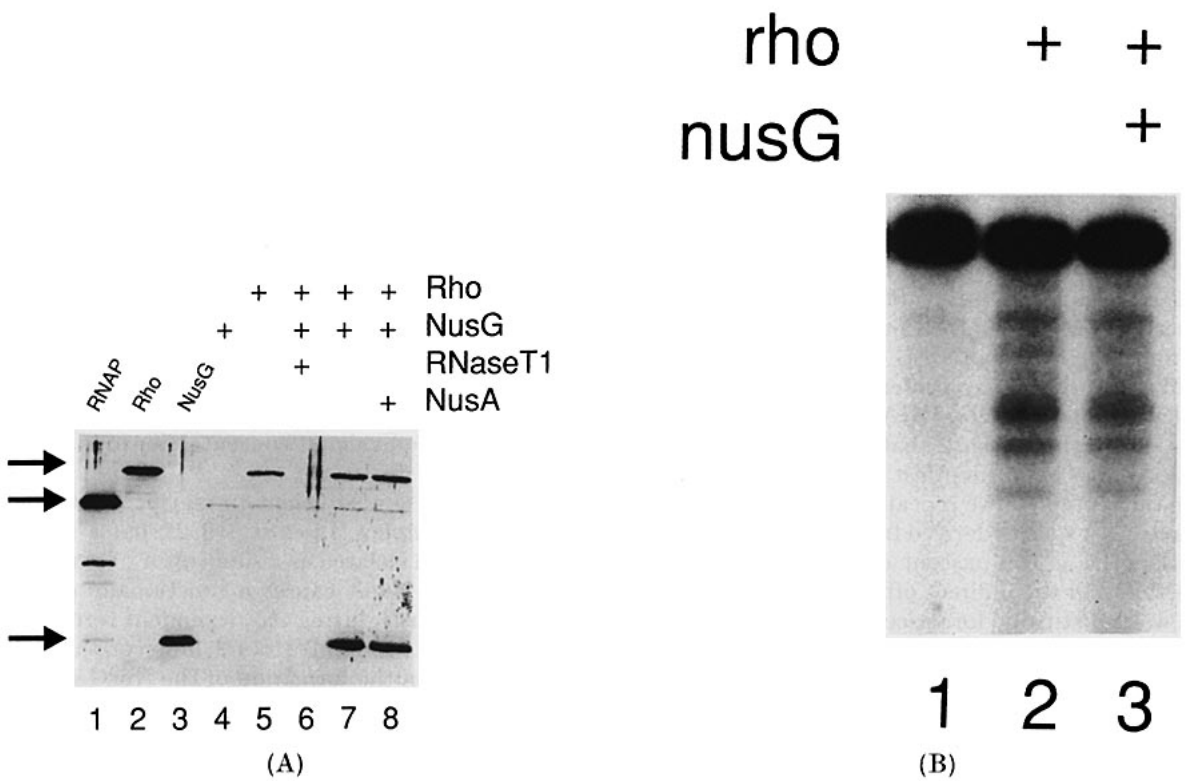

Figure 5. RNA-bound Rho contributes to the association of NusG with stalled ternary complexes. A, Stalled transcription complexes were isolated from reactions programmed with pRLtrpt' DNA coupled to agarose beads and analyzed for protein content. Each reaction was supplemented with combinations of Rho, NusG, NusA, and RNase T_1 before extensive washing and treatment with DNase. The proteins retained on the complexes were concentrated and visualized by SDS/PAGE and Western analysis. Elongation complexes stalled after 3 min of transcription and supplemented with 50 nM NusG contained only trace amounts of NusG after washing (lane 4), as did elongation complexes pretreated with RNase and supplied with 25 nM Rho and 50 nM NusG (lane 6). Rho (25 nM) was retained in the absence of NusG (lane 5), due to the affinity of Rho for the transcript. However, only complexes containing RNA and Rho together retained NusG (lane 7). Including NusA at 50 nM did not affect the recovery of NusG or Rho from the stalled complexes (lane 8). Lanes 1, 2, 3 contain 5 μg RNA polymerase, 0.1 μg Rho, and 0.01 μg NusG, respectively. B, Transcription reactions with pSP65t' vector DNA and SP6 RNA polymerase were incubated with Rho storage buffer (lane 1), 10 nM Rho (lane 2), or 10 nM Rho and 20 nM NusG (lane 3). The RNA was analyzed by denaturing gel electrophoresis.

with NusG present is about two- to threefold slower than in its absence. Third, the stable association of NusG with such a stalled complex is completely dependent on the presence of Rho bound to the nascent transcript, and probably involves interactions between NusG and RNA polymerase as well. These results lead to a potential mechanism of action for the *E. coli* NusG protein on transcription termination factor Rho and our observations suggest a model in which the role of NusG in both termination and antitermination of transcription may be similar.

Oligonucleotide targeting has been used successfully in the past to determine where Rho interacted with the nascent transcript during termination at the λ tR1 Rho-dependent site; in general, oligonucleotides that reduced termination by inhibiting the use of early or proximal sites in the transcript also resulted in decreased rho RNA-binding affinity for the transcript in a retention assay uncoupled from the termination (Chen *et al.*, 1986; Faus & Richardson, 1989). We have shown here that an oligonucleotide complementary to the trp2 segment of *trp t'* can shift the pattern of Rho-dependent termination of the *trp t'* transcript distally, and as well reduce Rho RNA-binding affinity for *trp t'* by tenfold. However, we found that when NusG was included in a transcription termination reaction *in vitro*, oligonucleotide trp2 no longer had an effect; this was not a result of where the RNA endpoints were positioned in relation to the trp2 oligonucleotide annealing site as judged by results with heterogeneous stalled complexes (data not shown). Furthermore, NusG did not change the RNA-binding affinity of rho for *trp t'* nor rescue the reduction in binding affinity caused by oligonucleotide trp2.

A separate oligonucleotide, termed z1, that anneals to the *trp t'* transcript upstream of the trp2 segment was found instead to suppress the NusG response. When NusG was included in the reaction, the effect of oligonucleotide trp2 on termination *in vitro* could only be observed when z1 was also present (Figure 2, lanes 4, 7 and 11). Thus, the association of Rho with the trp2 region on the *trp t'* nascent transcript is important both physically, in that it enhances rho RNA-binding affinity, and functionally, in that it stimulates termination, but only in a context lacking NusG. In contrast, however, the z1 region is essential for termination at NusG-responsive sites, whereas the

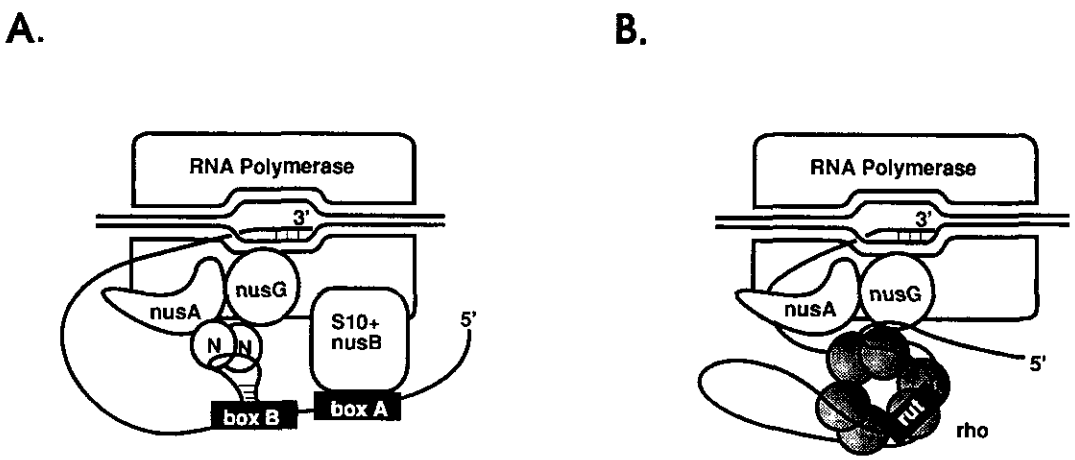

Figure 6. Schematic comparison of the N-modified antitermination complex and the NusG-modified termination complex. Shared features include: (1) the use of RNA polymerase as a foundation to assemble elongation control proteins; (2) interactions between these proteins and RNA-bound factors that lead to cooperative strengthening of the total complex; and (3) tethering of the elongation complex to a protein loading site on the RNA *via* a loop in the growing nascent chain. A, Without NusG, the antitermination complex as shown is unable to form. NusG contacts RNA polymerase, as does S10 and NusA, which anchor RNA-bound NusB and N to the elongation complex, respectively, and cause antitermination activity to persist downstream of the *nut* site for many kilobases. B, The quaternary termination complex, as we have proposed, is stabilized by NusG at RNA sequences that Rho binds to with a minimum affinity, but which Rho is released from too quickly to function (rut = *r*ho *u*tilization *s*ite). We have included NusA, due to its ability to influence Rho, with the caveat that NusA is not expected to affect the participation of NusG, as indicated. After termination, the quaternary complex may fall apart, leading to the destabilization and release of Rho from the free transcript (from Platt (1994), with permission).

trp2 region is dispensible, suggesting that Rho's interaction with the nascent RNA differs in the presence of NusG.

Because this effect is not mimicked in Rho binding to free RNA, we examined the behavior of Rho in the presence of all the components of a transcription reaction. Employing ternary complexes stalled at a specific site by the Gln-111 *Eco*RI mutant restriction enzyme (Pavco & Steege, 1990), we pre-bound Rho to a *trp t'* nascent transcript and measured competition by a stoichiometric amount of free *trp t'* RNA. The rate of release of Rho and its half-life on the nascent transcript were determined as a function of NusG: rapid dissociation of Rho with a half-time of about two minutes slowed to about five minutes with the inclusion of NusG. Since the stalled transcript was positioned at the proximal fringe of the normal Rho-dependent endpoints in the pRLtrpt' vector, the minimum half-life of Rho necessary for termination to occur on this template is thus about two minutes. We have examined the retention of Rho on other nascent transcripts as well, and have seen NusG-mediated increases in dissociation half-time of up to 20-fold, depending on the transcript used (data not shown). *E. coli* RNA polymerase probably plays a role in bringing Rho and NusG into close proximity and may itself mediate the increased retention of Rho on the nascent RNA (albeit indirectly).

We have also shown that the stable retention of NusG in a stalled ternary complex formed on the pRLt'RI template is dependent upon Rho, bound to the nascent transcript. In addition, though direct evidence is lacking, we suggest that the incorporation of NusG into the ternary complex is specific for *E. coli* RNA polymerase, since NusG did not alter the rho-terminated RNA endpoints when SP6 RNA polymerase was substituted into a termination reaction *in vitro* (Figure 5B), nor those caused by Rho arrest of yeast polymerase II (S.-Y. Wu & T. P., unpublished results). Based upon these data, we propose the following model: Rho and NusG reciprocally enhance the retention of one other as components of the termination complex. While Rho is able to bind to the nascent transcript in the absence of NusG, and NusG weakly associates with RNA polymerase in the absence of Rho, it appears that their function in a stable complex with the transcription apparatus, especially at weak rho RNA-binding sites, relies upon each protein being present.

This situation is reminiscent of the requirements for the formation of λ N-modified antitermination complex, in which multiple interactions stabilize the fully modified structure more than any individual components. Other similarities exist between the two systems, as presented schematically in Figure 6. The stable incorporation of NusG into both the elongation complex (*via* Rho) and the processive antitermination complex (*via* NusA, NusB/S10, and N) requires a protein-bound signal in the RNA (Li *et al.*, 1992; Squires *et al.*, 1993). In antitermination, the NusB and S10 proteins together bind as a heterodimer to specific boxA-like sequences from the λ *nut* site present in the nascent transcript (Nodwell & Greenblatt, 1993), while λ N-protein binds to the BoxB sequence of the *nut* site (Nodwell & Greenblatt,

1991). S10 has been shown to associate directly with RNA polymerase (Mason & Greenblatt, 1991), while NusA performs a similar task, simultaneously contacting both N factor and RNA polymerase (Greenblatt & Li, 1981). Like S10, NusG binds weakly to RNA polymerase on its own, but is stabilized in the context of the full repertoire of antitermination factors (Li *et al.*, 1992). In the absence of these factors, Rho alone is sufficient to stabilize the association of NusG with RNA polymerase, and NusG in turn contributes to an enhanced retention of Rho on the nascent transcript. Thus, in both termination and antitermination, multiple protein-protein and protein-RNA contacts appear to anchor elongation factors to polymerase, which themselves tether other proteins to the nascent RNA. This is consistent with a view put forth by Horwitz *et al.* (1987) that suggests RNA polymerase is a platform for the selective assembly of host factors during elongation. Perhaps one role of NusG in antitermination may be to regulate the stability of Rho (or perhaps another RNA-bound protein such as the S10-NusB heterodimer) on the nascent transcript.

At present, we can only surmize how the increased retention of Rho on the nascent transcript leads to an increased ability of Rho to cause termination. Since Rho-dependent termination sites are unusually disperse and no consensus recognition site has been reported for Rho RNA-binding, perhaps Rho can recognize a much wider variety of sites *in vivo*, with the assistance of NusG, than has previously been thought. We know that, in a stalled transcription system, Rho-mediated transcript release can occur at the NusG-dependent sites, in fact, in the absence of NusG at low levels after long times (Nehrke *et al.*, 1993), implying that NusG has a catalytic effect at these early sites. An explanation consistent with these observations is that the off-rate of Rho may normally limit the extent of termination at all but the most well-recognized sites. We have observed here that NusG is not stably retained in stalled, washed elongation complexes in the absence of Rho, and that Rho must be bound to the transcript for NusG to be recruited to the polymerase complex and to function. It is not necessary for NusG to influence the specificity of Rho RNA-binding, but simply to ensure that Rho, once bound to the nascent RNA, does not dissociate prior to termination. Since NusG is required for Rho-dependent termination *in vivo* at a variety of sites (Sullivan & Gottesman, 1992), our observations suggest that at most sites, Rho RNA-binding is less favorable *in vivo* than *in vitro*.

The actual mechanism by which NusG slows the rate of Rho's release from the nascent RNA cannot yet be determined. It could simply increase the local concentration of Rho near the nascent RNA due to a slight affinity for NusG, or be a cooperative association akin to the formation of the λ N-modified antitermination complex. Alternatively, NusG could facilitate the transfer to Rho of some part of the nascent RNA in the strong RNA-binding site of polymerase through which the nascent transcript appears to thread during elongation (Altmann *et al.*, 1994). If these sequences increase Rho's affinity for the transcript, it would lead to a decreased off-rate of Rho from the mRNA, and the function of NusG would be to shift equilibrium binding towards a Rho-RNA rather than an RNA polymerase-RNA binary complex. A mechanism in which NusG is needed for recognizing all but the strongest termination sites, acting in concert with RNA polymerase only when Rho was bound to the nascent transcript, would account for a wide spectrum of Rho-dependent sites *in vivo* that are undetectable *in vitro* without NusG, and the essential nature of NusG itself. Moreover, the participation of RNA polymerase as more than a passive partner in controlling the processes of elongation and termination is an attractive idea that is gaining support, and future experiments in this system and others will test how the basic transcriptional complex interacts directly with both positive and negative regulatory factors.

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