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NusG is required to overcome a kinetic limitation to Rho function at an intragenic terminator

(*Escherichia coli*/*lacZ*/RNA polymerase/Rho factor/polarity)

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ABSTRACT **Rho-dependent transcription termination at certain terminators in *Escherichia coli* also depends on the presence of NusG [Sullivan, S. L. & Gottesman, M. E. (1992) *Cell* 68, 989–994]. We have found that termination at the first intragenic terminator in *lacZ* (*tiZ1*) is strongly dependent on NusG when transcription is done *in vitro* with the concentrations of NTPs found *in vivo*. With a lower level of NTPs, and consequently a slower rate of RNA-chain growth, Rho causes some termination by itself that is enhanced with NusG. These results suggest that NusG serves to overcome a kinetic limitation of Rho to function at certain terminators. At a second intragenic terminator within the *lacZ* reading frame (*tiZ2*) the efficiency of Rho-mediated termination was unaffected by either NusG or by RNA polymerase elongation kinetics. Thus, using purified components and intracellular levels of NTPs, we have confirmed the *in vivo* finding that certain Rho-dependent terminators also depend on NusG, whereas others do not.**

Escherichia coli has two distinct mechanisms for transcription termination. One involves spontaneous release of RNA from ternary transcription complexes, which occurs when RNA polymerase encounters the sequence of an intrinsic terminator in the DNA template (1). The other mechanism depends on the action of an essential cellular protein known as Rho, which acts to dissociate the transcription complex at a Rho-dependent terminator (2). Although Rho can function very efficiently by itself in many purified systems, recent evidence has indicated that another essential transcription factor, NusG, is required for the function of many Rho-dependent terminators inside the cell (3).

NusG is a very abundant, 21-kDa protein (4) that was identified both genetically (5) and biochemically (6, 7) as a critical component of the λ N-protein-mediated transcriptional antitermination system. Its role in Rho-dependent termination was revealed from testing the function of several transcription terminators in cells that were experimentally depleted for NusG protein. In these cells, the function of several Rho-dependent terminators was severely reduced, whereas the function of an intrinsic terminator was unaffected, indicating that the presence of NusG protein is required for Rho to function at certain terminators *in vivo* (3).

The effect of NusG on Rho-dependent termination *in vitro* has been examined for two terminators, λ *tR1* and *trp t'*. Under the conditions in which these terminators normally function very efficiently with Rho alone, NusG had very little effect. NusG caused an alteration in the position of termination stop points and slightly increased the efficiency of termination (4, 8). However, a stronger effect of NusG on termination efficiency was seen when *in vitro* transcription was done under conditions that were suboptimal for Rho function. With *trp t'*, this was with partially defective terminator mutants (8), and with λ *tR1*, this was in reaction mixtures that contained 0.2 M KCl (4). Because cells have very low levels of Cl^- ions (9), which are strongly inhibitory to Rho function (10), these results do not directly resolve the issue of why Rho-dependent termination is so strongly dependent on NusG *in vivo*.

To address this question we chose to study the two major latent Rho-dependent terminators (*tiZ1* and *tiZ2*) located very early in the *lacZ* open reading frame (Fig. 1) because they had been previously found to work less well *in vitro* than *in vivo* (11). These terminators are activated by conditions that uncouple transcription from translation (11) and are representative of a large class of terminators (12) that function to prevent the continued transcription of genes when mRNAs are not being translated efficiently (13). It is this type of terminator that is responsible for the polar effects of some mutations. Also, because Rho-dependent termination is a kinetic process that is strongly affected by the elongation rate of the RNA polymerase (14), we made use of isolated ternary transcription complexes (15), which can be elongated at nearly the *in vivo* rate, by using physiological levels of NTPs, to study the effect of NusG on the efficiency of termination at the *lacZ* intragenic terminators *in vitro*. We show here that NusG is required for Rho-dependent termination at the first of these two intragenic terminators when transcription is done with intracellular NTP concentrations.

MATERIALS AND METHODS

Enzymes. Purified Rho protein was provided by Lislott Richardson (Indiana University). Purified NusG protein was from Joyce Li and Jack Greenblatt (University of Toronto). RNA polymerase was purchased from Epicentre Technologies (Madison, WI). Restriction enzymes and Vent DNA polymerase were purchased from New England Biolabs.

DNA Manipulations. To generate the mutant *lacZ* promoter derivative used in these experiments, the 789-bp *Hin*cII fragment of *lacZ* from pMC1 (16) was cloned into the 2758-bp *Pvu* II fragment from pET3af1 (17) to generate the plasmid pCBZ1. Site-directed mutagenesis (18) was used to change the cytosine residue at +10 to a thymine residue to generate the plasmid pCBZ2. A fragment containing this promoter mutant (−167 to +413) was then amplified using Vent DNA polymerase with primers designed to add an *Eco*RI site to the upstream end of the product DNA. This allowed cloning the 447-bp *Eco*RI–*Bsu*36I fragment into the 8073-bp *Eco*RI–*Bsu*36I fragment of pTL61T (19) to generate the plasmid pCBZ4, which contains the entire *lacZ* gene. This plasmid was used to prepare the 1024-bp transcription template (−167 to +839) by amplification with Vent polymerase. This template contains the UV5 (promoter up) and L8 (CRP independent) promoter mutations and is flanked by primer-encoded *Eco*RI sites.

Ternary Transcription Complex: Preparation and Isolation. Ternary transcription complexes were formed by incu-

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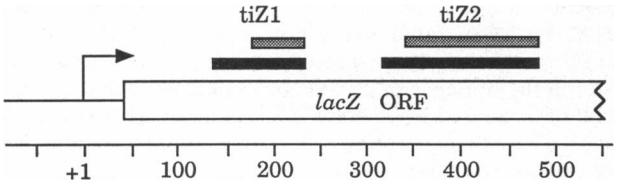

FIG. 1. Diagram of the early region of the *lacZ* gene showing the location of termination end points relative to the transcription (+1) and translation (+39) start points. Termination end point locations were determined from the experiment shown in Fig. 2 using the low NTP condition for *tiZ1* and the high NTP condition for *tiZ2*. The stippled and black boxes represent the location of termination end points caused by Rho and by Rho plus NusG, respectively. ORF, open reading frame.

bating 3 pmol of RNA polymerase with 3 pmol of DNA transcription template in transcription buffer (150 mM potassium glutamate, pH 7.8/40 mM Tris·HOAc, pH 7.8/4 mM $Mg(OAc)_2$/1 mM dithiothreitol/0.02% Nonidet P-40/0.002% acetylated bovine serum albumin/1% glycerol) for 3 min at 37°C, adding NTPs [250 μM ApA, 40 μM GTP, 4 μM ATP, 4 μM [a-^{32}P]UTP (350 nCi/pmol; 1 Ci = 37 GBq)] and incubating at 32°C for 21 min. The 100-μl reaction was then adjusted to 5 mM EDTA and purified on a 5 ml Sephacryl S-300HR column. Fractions containing transcription complexes were identified and quantitated by scintillation counting. Typically 0.5 pmol of complexes was recovered.

Transcription Elongation. Transcription elongation reactions (20 μl) contained 10 fmol of isolated ternary transcription complexes and were done in transcription buffer. Rho and NusG were diluted in transcription buffer containing 0.012% acetylated bovine serum albumin and 0.12% Nonidet P-40. The Rho and/or NusG were mixed with the ternary complexes on ice and incubated for 3 min at 37°C. NTPs were then added to either low or high levels (see Fig. 2), and elongation was allowed to proceed for 3 min at 37°C. RNA sequence reactions were prepared by using the low NTP levels in the presence of one of the 3′-dNTPs at the same concentration as the respective NTP. The reactions were stopped by addition of EDTA to 15 mM SDS to 0.5%, and proteinase K to 0.3 μg/μl with incubation at 37°C for 10 min. RNA was precipitated with ethanol in the presence of 0.5 μg of tRNA, resuspended in formamide-gel loading buffer, and separated by electrophoresis on 40-cm 6% polyacrylamide (19:1 bisacrylamide)/7 M urea gels. Autoradiograms of the gels were scanned by using a Molecular Dynamics densitometer.

RESULTS

To examine the effect of NusG on Rho-dependent termination during *in vitro* transcription of the first 839 bp of *lacZ* DNA under a variety of reaction conditions, we used isolated transcription complexes containing ^{32}P-labeled 16-nt-long RNA that were prepared by transcription of a mutant template (C10T) in the absence of CTP. Stalled transcripts in the complexes were readily extended further upon incubation with all four NTPs, most to the end of the template (848 nt downstream from the transcription start point) with some ending at a previously identified weak intrinsic termination site at position 420 and others ending at sites between position 420 and the end of the template (Fig. 2). The fraction of the complexes that made it to the end of the template was significantly higher when the elongation was with high levels of NTPs than with the low levels of NTPs. This result agrees with the recent finding that the efficiency of intrinsic termination is determined, in part, by the elongation rate of RNA polymerase (20).

We used the low levels of NTPs to compare the results obtained by using the isolated complexes with results obtained

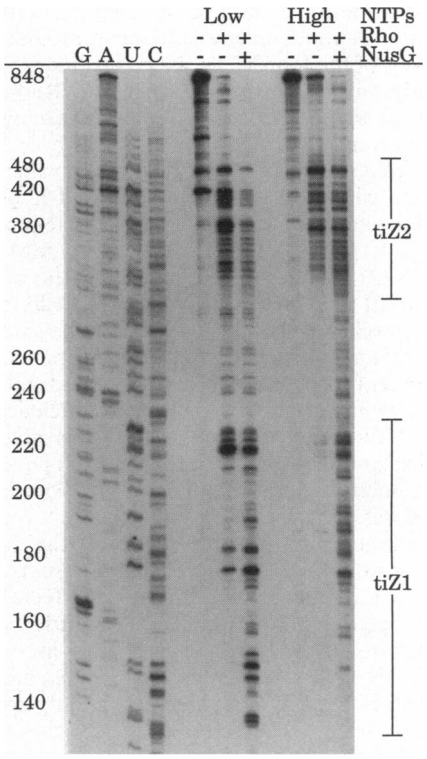

FIG. 2. Effects of nucleotide concentration and NusG protein on the action of Rho at the first two *lacZ* intragenic terminators. Ternary transcription complexes were elongated for 3 min at 37°C with various concentrations of the 4 NTPs [low: 0.2 mM GTP, 0.2 mM ATP, 0.02 mM UTP, 0.2 mM CTP, 4 mM $Mg(OAc)_2$; high: 1.1 mM GTP, 2.7 mM ATP, 1.4 mM UTP, 0.7 mM CTP, 10 mM $Mg(OAc)_2$], Rho hexamer (50 nM), and NusG (25 nM) as indicated in the figure, and the RNA products were analyzed by gel electrophoresis. The termination endpoint regions from the two Rho-dependent terminators are indicated at right. The nucleotide lengths of the RNAs indicated at left were determined from the RNA chain-terminating sequence lanes and from the position of the termination end points.

previously with the *lacZ* system. When transcription was done with a saturating amount of Rho present, the products had lengths that ranged from 180 nt to ≈460 nt, as was noted (10, 11). However, the positions of the stop points and the efficiency of termination within the *tiZ1* region differed somewhat from the previous results. Instead of just two major termination end points at bp 180 and 220 (11), we reproducibly observed major end points at positions 183, 193, 219–221, 225, and 227–230 (± 1 bp) (Fig. 2). We believe that this discrepancy is due to the higher resolution of our gels.

The overall efficiency of termination at *tiZ1* in this experiment was 30%, which is significantly lower than was found in some previous experiments done under similar reaction conditions (10). We believe that the higher efficiency found in that previous study may have been because of a special property of the particular preparation of RNA polymerase that was used. The efficiency we found was reproduced with several different preparations of RNA polymerase and of Rho, and in transcription reactions done without using arrested ternary complexes (data not shown). This inability to obtain a high efficiency of termination *in vitro* prompted us to test the effect of NusG with this terminator.

When NusG was included in the transcription reactions with the low NTP level, termination efficiency in the *tiZ1* region was increased by 2.4-fold (to 73%), and distinctive termination end points were observed both within the *tiZ1* region and up to 43 bp nearer to the promoter than those observed in its absence (Fig. 2). NusG had almost no effect on the *tiZ2* terminator. It did not increase the termination efficiency, and although it

altered the distribution of stop points slightly, it did not allow Rho to function at any distinctive sites nearer to the promoter. With low levels of the four NTPs, as are commonly used for *in vitro* transcription, the effect of NusG on Rho-dependent termination in the *lacZ* coding region was similar to its reported effects on λ *tR1* and *trp t'* (4, 8).

A more dramatic effect of NusG was found when transcription was done with NTP concentrations that have been reported to be available *in vivo* during logarithmic-phase growth ("high NTP": 1.1 mM GTP, 2.7 mM ATP, 1.4 mM UTP, and 0.7 mM CTP) (21). Under these conditions, Rho was virtually inactive by itself at *tiZ1* but was activated >20-fold by the presence of NusG (Fig. 2). The results with the *lacZ* template also showed that not all terminators were codependent on Rho and NusG under these conditions, as Rho could still terminate transcription at the sites in *tiZ2* with high efficiency by itself. With these NTP concentrations, NusG was able to elicit termination at specific promoter-proximal stop points for both terminators; however, the extent of the shift for *tiZ1* was not as great as it was with the low NTP conditions. We note that these two intragenic terminators represent the extremes in response to NusG *in vitro*, with *tiZ1* being essentially NusG-dependent and *tiZ2* being almost entirely unaffected. *tiZ1* and *tiZ2* are distinct terminators because very little termination occurs at the relatively strong pause sites in the >80-bp segment of DNA that separates them (11), and because termination still occurs at *tiZ2* when *tiZ1* and its upstream *rut* sequences have been deleted from the transcription template (our unpublished results).

The reaction conditions used with the "high NTP" concentrations also had a higher concentration of $Mg(OAc)_2$, 10 mM instead of 4 mM. Because Mg^{2+} ion concentration is known to affect Rho function (22), we also tested the requirement for NusG in a reaction mixture that had the higher level of NTPs but with 4 mM $Mg(OAc)_2$. This condition gave results that qualitatively agreed with those from the reactions with 10 mM Mg^{2+} ion: virtually no termination at *tiZ1* with Rho alone and substantial activation when NusG was added (data not shown). This result indicated that the NTP concentration, not the Mg^{2+} ion concentration was the critical variable for NusG dependency.

Because Rho function has been shown to depend on the transcriptional elongation rate of RNA polymerase (14), a likely explanation for the inability of Rho to act at *tiZ1* with the high NTPs is that the rate of elongation was too fast for Rho to function at that terminator. We measured the chain-growth rates for the *lacZ* RNA chains under the two conditions and found them to be 30 nt/s with the high NTPs and slightly less than 10 nt/s with the low NTPs (data not shown). Thus, RNA polymerase was moving substantially faster with the high NTPs, as expected. This result suggests that under our reaction conditions, termination at *tiZ1* was limited by the elongation kinetics of the RNA polymerase and that NusG was able to overcome this kinetic limitation to Rho function.

The low NTP concentration levels have a particularly low level of UTP (20 μM), as this was commonly used to achieve high-specific-activity radioactive label with [α-^{32}P]UTP. Those conditions may have been partially responsible for the finding that the preferred end points in *tiZ1* were in regions encoding multiple uracil residues in the RNA. With the high NTP concentration, many distinctive stop points were observed at positions that are not uracil-rich, indicating that the end-point bias was substantially reduced in reactions containing high NTP concentrations. Also, with the high NTP condition, RNA polymerase was not stopped efficiently at the earliest termination points (140 to 160 nt) that were observed using the low NTP condition with NusG present (Fig. 2). Both of these observations suggest that chain-growth rate influences stop-point choice, even with NusG present.

The result presented in Fig. 3 shows that NusG by itself at a level of 25 nM had no effect on the termination of the *lacZ* DNA. We also found (data not shown) that the rate of transcription elongation throughout the region was slightly faster in the presence of 25 nM NusG than in its absence as a result of decreased RNA polymerase pausing. These observations indicate that activation of Rho-dependent termination at *tiZ1* by NusG does not result from a decreased elongation rate of RNA polymerase, but rather from allowing Rho to terminate rapidly elongating RNA polymerase.

To address the mechanism of NusG activation of Rho function at the *tiZ1* terminator at high NTP levels, we examined the concentration dependence of Rho and of NusG on termination. We carried out transcription reactions with one of these factors at a constant saturating amount and titrated the other over a range covering two orders of magnitude. The results show that as the concentration of Rho or of NusG was increased toward saturating levels, termination increased uniformly for all the stop sites within *tiZ1* (Fig. 3). This "all or none" activation by NusG indicates that it exerts its stimulatory effect at a step in the termination process common to all termination end points and suggests that NusG action is determined relatively early during transcription. This finding is consistent with its lack of enhancement of RNA polymerase pausing. That Rho also shows an "all or none" concentration dependence for stop-point usage confirms a previous interpretation (11) that all end points in the *tiZ1* region compose a single Rho-dependent terminator.

To simplify the quantitative analysis of the overall efficiencies of termination at *tiZ1* and *tiZ2* we summed over all the stop points within each region. The data show that the overall termination efficiency with saturating amounts of Rho and NusG was greater for *tiZ2* than for *tiZ1* and confirm that NusG had no measurable effect on *tiZ2* (Fig. 4). The concentration of Rho hexamer that gave one-half maximal function at *tiZ1* and *tiZ2* with NusG present was 9 ± 3 nM, whereas the

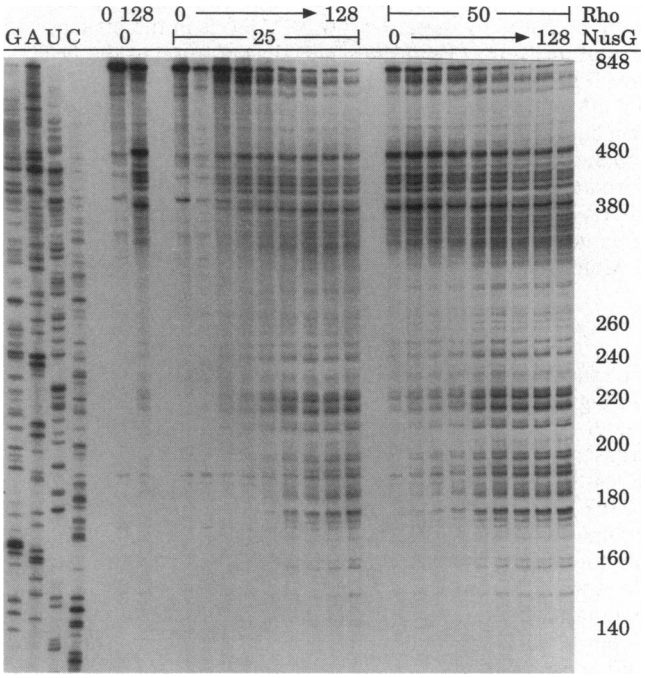

FIG. 3. Concentration dependence of Rho and NusG for termination at the first two intragenic terminators in *lacZ*. Transcription was done by using ternary complexes as described in Fig. 2 with high levels of NTPs. Rho hexamer and NusG proteins were present as indicated above the lanes. The concentrations used for the titrations were 0, 1, 2, 4, 8, 16, 32, 64, and 128 nM. The nucleotide lengths of the RNAs indicated on the left were determined from the RNA sequence markers and positions of termination end points.

concentration of NusG that gave one-half maximal function at *tiZ1* with saturating Rho present was 6 ± 3 nM (Fig. 4). This value of NusG is very close to that necessary for one-half maximal activation of λ N-protein-mediated anti-termination (7). Thus, similar binding interactions between NusG and the transcription complex may be involved in both termination and antitermination functions.

Because the concentration of the transcription complexes (as quantified from the amount of label in the RNA) was only ≈0.5 nM and because similar one-half maximal values were obtained in titrations done with 0.1 nM complexes (data not shown), these one-half maximal values were reflecting the affinities of Rho and of NusG for their sites in the transcription complex and not a stoichiometric dependence. We also tested whether the presence of NusG influences the affinity of Rho for its sites of action by measuring the concentration dependence of termination at *tiZ1*. Because Rho has very little activity by itself at *tiZ1* with the high NTP concentration, we performed titrations in the presence of a half-saturating concentration of NusG and found that termination was again half-maximal with 9 ± 3 nM Rho (data not shown). The half-maximal concentration we found when we performed titrations with the low NTP concentration (where Rho functions well on its own) was 6 ± 3 nM in both the presence and absence of saturating NusG. These results thus suggest that NusG was not affecting the affinity of Rho for its site of action but was rather affecting its ability to act at *tiZ1*.

DISCUSSION

NusG has two distinct effects on transcription termination with Rho factor. With at least one terminator, it overcomes a kinetic deficiency of Rho to act alone *in vitro* when RNA-chain elongation by RNA polymerase is nearly as rapid as in the cell.

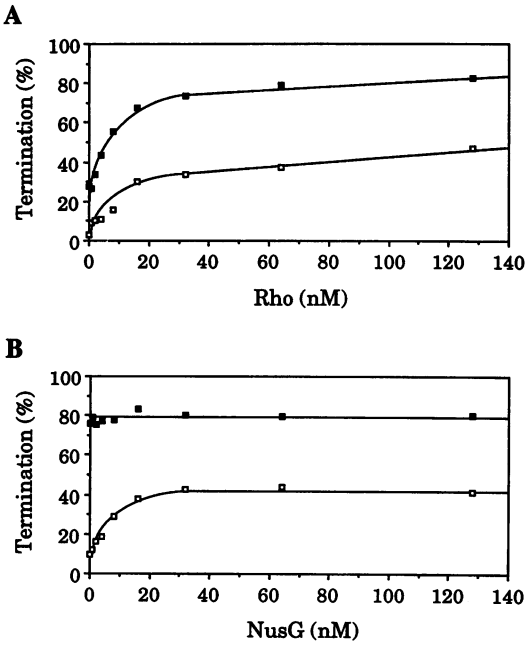

FIG. 4. The efficiencies of termination at *tiZ1* and *tiZ2* as functions of Rho and NusG. Termination efficiency for *tiZ1* (□) and *tiZ2* (■) is the percentage of RNA polymerase molecules entering the region that terminate in that region and is determined from densitometric scans of lanes in the autoradiogram shown in Fig. 3 and the autoradiogram of a replicate experiment. (*A*) Dependence on Rho (as hexamer) in the presence of 25 nM NusG. In the absence of NusG, 128 nM Rho caused <2 and 75% termination at *tiZ1* and *tiZ2*, respectively. (*B*) Dependence on NusG in the presence of 50 nM Rho. The maximum combined *in vitro* termination efficiency was >95%.

And with a number of terminators it causes a shift of transcript end points to more promoter-proximal positions. The extents of the shifts for *tiZ1* and *tiZ2* are diagrammed in Fig. 1.

For Rho to cause termination, it must bind to the nascent RNA and dissociate it from the RNA polymerase before the transcription complex passes through the terminator region. In the cell RNA polymerase carries out chain elongation at a rate >40 nt/s (23, 24). Thus, to terminate at a point that is 180 bp downstream from a promoter, Rho has, at most, 5 s to act. Because a functional site for Rho binding on a transcript (a *rut* site) does not appear immediately, the time between when Rho can first productively interact with the RNA and when RNA polymerase elongates beyond the termination region may only be on the order of 1 s or less. This period defines a kinetic window during which RNA polymerase is susceptible to Rho action. One activity of NusG is to overcome a kinetic limitation of Rho to function by itself. The inability of Rho alone to act within a very short kinetic window may be the main reason for the strong requirement for NusG in Rho-dependent termination *in vivo*.

The ability of NusG to shift the end-point distribution to more proximal positions was first recognized by Li *et al.* (4) and Nehrke *et al.* (8) and was evident in our study with both the NusG-dependent *tiZ1* and the NusG-independent *tiZ2* terminators. Although we found some dependence of the extent of the shift on the NTP concentration, Nehrke *et al.* (8) presented evidence that the shift of stop-point utilization caused by NusG at *trp t'* was not greatly changed when the UTP concentration was varied from 0.5 to 10 μM. Thus, under conditions that do not impose a strong kinetic limitation on Rho function, NusG appears to alter the position where termination occurs independent of chain growth rate. This observation suggests that NusG has an ability to influence stop-site utilization that is distinct from its ability to overcome the kinetic limitation of Rho to act by itself. Further experiments will be needed to confirm whether this distinction can also be made with the strongly NusG-dependent *tiZ1* terminator.

Besides its requirement in Rho-dependent termination, NusG is also required for processive λ N-protein-mediated transcriptional antitermination *in vitro* (6, 7, 25). These opposing requirements for NusG in both the activation and the inhibition of Rho function are unlikely to be due to simple effects on the RNA binding or ATPase activities of Rho alone. Indeed, these individual activities of Rho are not dramatically affected by NusG (7, 8). We have also confirmed that NusG has no effect on the rate of ATP hydrolysis by Rho with a cofactor RNA from *lacZ* that includes the *tiZ1* region (data not shown). Instead, NusG could fundamentally change how Rho interacts with the nascent RNA in the transcription complex. Physical studies have found that NusG can interact both with Rho (4) and with RNA polymerase (6, 7). These observations suggest that NusG may be involved in the recruitment of Rho into the transcription complex.

Nehrke and Platt (26) have shown that NusG affects the interaction of Rho with the nascent RNA both by allowing Rho to use different *rut* signals and by decreasing the off-rate of Rho from the nascent RNA. Also, they found that NusG associates strongly with the elongation complex only when Rho is present and bound to the nascent RNA. They thus proposed a model in which NusG and Rho participate in an interdependent association with the transcribing RNA polymerase to facilitate the recognition and use of termination signals on the nascent RNA. The formation of this quaternary termination complex consisting of Rho, NusG, nascent RNA, and RNA polymerase provides a means by which NusG can overcome the kinetic limitation that Rho has when it acts alone, as NusG would cause Rho to be present in the transcription complex and thus available to initiate a strong interaction with a segment of RNA containing a *rut* signal as it emerges from the RNA polymerase. With this mechanism, Rho-dependent ter-

mination in the presence of NusG is the outcome of a uni-molecular reaction rather than a bi-molecular reaction, which is presumably the mechanism that Rho uses to bind to the *rut* sites on a nascent RNA in the absence of NusG.

Note Added in Proof. A detailed study on the effect of NusG on RNA polymerase elongation rate has recently been published (27). These authors find that NusG accelerates RNA polymerase both *in vivo* (using *lacZ*) and *in vitro* (using *trpL*) by decreasing pausing. This agrees with our findings and confirms that the activation of Rho-dependent termination by NusG is not the result of enhanced RNA polymerase pausing either *in vitro* or *in vivo*.

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