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NusG alters rho-dependent termination of transcription in vitro independent of kinetic coupling

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To complement the recent discovery that rho-dependent termination in E. coli requires nusG protein in vivo, we have tested the effect of purified nusG protein on rho-dependent termination in vitro. With the well-characterized *trp t'* terminator of E. coli, and no other proteins than E. coli RNA polymerase and rho factor, nusG causes a proximal shift in the terminated RNA endpoints, compared to the endpoints generated by rho alone. The presence of nusG also enhances rho-mediated termination on partially defective mutant *trp t'* templates. We rule out explanations such as a change in the kinetic coupling between rho and RNA polymerase or a nusG-mediated increase in the affinity of rho for RNA. We also detect no difference in the helicase rate of rho in the presence of nusG. Even assays with completely stalled and isolated ternary complexes indicate that rho is able to effect the release of RNA with the assistance of nusG at points preceding the most proximal release sites observed in the absence of nusG. Our observations support a model in which nusG acts as a component of the transcription complex, possibly interacting with both rho and RNA polymerase as it governs accessibility to the nascent transcript.

Rho-dependent termination in E. coli has been studied extensively (see Platt and Richardson, 1992). The paradigm for termination at a rho-dependent site is simple: upon binding to a region in the nascent RNA, rho undergoes a conformational change that allows the hydrolysis of ATP, which fuels tracking along the RNA strand in a 5′ to 3′ direction (Bear et al., 1985; Brennan et al., 1987). The RNA:DNA helicase activity of rho (Brennan et al., 1987) is probably involved in transcript release and in facilitating dissociation of the elongation complex, although direct evidence of such a function in termination has not yet been shown. Evidence that rho can terminate actively transcribing RNA polymerase molecules in vitro in the absence of accessory factors is consistent with this model (Wu et al., 1981; Lau and Roberts, 1985).

Recent indications suggest, however, that rho may require additional factors for efficient termination in vivo. NusG is a protein originally identified genetically by a mutation called U* that suppresses the effect of the *nusA1* mutation on N-mediated antitermination in vivo (Ward and Gottesman, 1982; Downing et al., 1990; Sullivan et al., 1992) and biochemically as a component of the lambda N-modified antitermination complex (Horwitz et al., 1987; Li et al., 1992). Surprisingly, nusG also appears to be necessary for rho-dependent termination in vivo. Severely decreased efficiencies of rho-dependent termi-

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nation at latent regions in the *galEocB4* polar mutant operon and at the λtL1 terminator are observed in vivo upon depletion of nusG protein (Sullivan and Gottesman, 1992). The efficiency of termination at the rho-independent λtI region is unaffected by nusG depletion (Sullivan and Gottesman, 1992), and overexpression of nusG in vivo suppresses the *nusD* (*rho*) class of antitermination mutants (Sullivan et al., 1992).

Other evidence indicates that nusG and rho interact physically as well as functionally. Chromatography using nusG coupled to a solid column matrix results in the retardation of rho factor from an E. coli extract, and the mutant rho protein derived from *rho026*, a strain temperature-sensitive for lambda growth, is temperature-sensitive for binding to nusG (Li et al., 1993).

One of the major questions to be resolved is why nusG appears to be necessary for effective termination by rho in vivo, yet is dispensable in vitro. In order to address this concern, we have examined the effect of nusG on rho-dependent termination in vitro, and can attribute a proximal shift of 20–40 nucleotides in rho-dependent 3′ endpoints to nusG. In addition, the presence of nusG can restore termination efficiency on mutant *trp t′* templates that are deficient for termination in vitro with rho alone. These effects are not consequences of kinetic coupling between rho and RNA polymerase, as we can observe the rho-dependent release of the nascent transcript at unique early sites in a completely stalled transcription system as a function of added nusG. A technique that we term "offprinting" is used to determine and display the 5′ proximal and 3′ distal limits of rho-dependent termination on stalled polymerase complexes and to identify directly the termination region enhanced by nusG.

Materials and methods

Enzymes and nucleotides

E. coli RNA polymerase was kindly donated by Mike Chamberlin. NusG was the generous gift of Joyce Li and Jack Greenblatt and was purified according to Li et al. (1992). RNase H was from Promega. SP6 RNA polymerase was a gift of Art Zaug. Rho protein was purified from AR120-A6 (p39ASE), as described by Mott et al. (1985), modified according to Nehrke et al. (1992), and was diluted into ice-cold rho storage buffer (50% glycerol, 10 mM Tris-Cl pH 7.9, 100 mM NaCl, 0.1 mM DTT, 0.1 mM EDTA) immediately before use. The concentration of rho was determined by its absorbance at 280 nm using an extinction coefficient of 0.37 mg^{-1} cm^{-1} (Finger and Richardson, 1982) and is expressed as the hexameric form of the protein. T4 DNA ligase and restriction endonucleases were purchased from Amersham, and RNase H from Promega. ApU dinucleotide was purchased from Sigma. Nucleotides were predissolved solutions of pH 7.0 from Boehringer Mannheim. Oligonucleotides 1, 2, and 4 were produced at the University of Rochester Oligonucleotide Synthesis Facility and consist of the following sequences: oligo 1, 5′ ATTACTGTTGGGCGG 3′; oligo 2, 5′ GTGTTATAGTTGCGGG 3′; and oligo 4, 5′ CCTGAGGAATAAGTG 3′. [α^{32}P]GTP and [α^{32}P]ATP were from Amersham or NEN.

Vectors and RNA substrates

The vector pRLtrpt′ was created by inserting a 1400 bp Pst I–Sph I fragment containing 200 bp of *trp t′* followed by an *infC–lacZ* fusion from the M13 vector mp19TSG2 (Butler et al., 1987) into the Pst I–Sph I sites of the polylinker region of pRL418 approximately 39 nucleotides downstream of the T7A1 promoter (Levin et al., 1987). Cytosine-to-uracil point mutants (referred to by the number of mutations out of 28 possible sites) contain the first 104 nucleotides of *trp t′* and were created and cloned (as described for pRLtrpt′) into pRL418 (Zalatan and Platt, 1992). t′Ht RNA was produced by SP6 polymerase transcription of the pSP65t′HT vector as described in Steinmetz et al. (1990). pUC119 single-stranded DNA was purified as described previously (Brennan et al., 1987).

Formation of A20 complexes and transcription reactions

A20 complexes were formed as described by Chan and Landick (1989). Kpn I-cut pRLtrpt′, Kpn I-cut cytosine-to-uracil derivatives of pRLtrpt′, or a PCR-amplified segment of pRLtrpt′ extending from approximately 240 base pairs 5′ to the T7A1 promoter start site to 320 nucleotides downstream of the initiation site was incubated for 15 minutes at 30°C at approximately 100 nM, with 25 nM active RNA polymerase. This solution had 40 mM Tris-Cl (pH 7.9), 20 mM NaCl, 14 mM $MgCl_2$, 14 mM 2-mercaptoethanol, 2% glycerol, 20 μg acety-

lated bovine serum albumin/ml, 240 μM ApU dinucleotide, 2.5 μM ATP, CTP, and GTP, and [α^{32}P]GTP or [α^{32}P]ATP, as noted. A fivefold dilution to a final concentration of 5 nM RNA polymerase in 1× elongation buffer (30 mM Tris acetate, pH 7.9, 5 mM magnesium acetate, 50 mM potassium acetate, 1 mM DTT) containing unlabeled ribonucleotide triphosphates at the indicated concentrations and 1/10 volume of either 100 nM rho, 100 nM rho and 200 nM nusG, or rho storage buffer (unless otherwise indicated) allowed synchronous transcription from the labeled A20 complex. Reactions were incubated at 30°C for the stated lengths of time, then treated with 1/10 volume of STOP mix (135 mM EDTA, 2% SDS, and 3 mg/ml proteinase K). After 5 minutes at 30°C, these were diluted fivefold with sodium acetate/carrier RNA, phenol extracted, and ethanol precipitated. The products were resuspended in 10 μl of formamide dye and heated for 5 minutes at 90°C before analyzing by electrophoresis through 7 M urea acrylamide gels (44 mM Tris borate, 1 mM EDTA, pH 8.3).

To analyze reactions as isolated, paused ternary complexes, 8 μl samples of synchronized elongating A20 complexes from a mixture containing 10 μM ATP, UTP, and CTP, and 200 μM GTP were added to 2 μl of 50 mM EDTA on ice at 20-second intervals following the start of elongation up to four minutes. These reactions were pooled and run over a 10 ml Sephadex G-50 column at 1 ml/minute in elongation buffer. The peak of eluted radioactivity corresponding to the void volume of the column was collected, brought to 40 μg/ml BSA, and used within one-half hour. Typically, we obtained a 10-fold dilution of ternary complexes, which remained stable for at least several hours as assayed by nitrocellulose filter retention and could be fully chased upon nucleotide addition.

RNA binding

Paused complexes separated through gel filtration were either phenol/chloroform extracted and ethanol precipitated in the presence of 1 μg of total yeast carrier RNA, then resuspended in 100 μl per reaction of binding buffer (20 mM Tris acetate, pH 7.9, 50 mM potassium acetate, 1 mM magnesium acetate, and 0.1 mM EDTA), or used directly from the column as stable ternary complexes in elongation buffer. Either rho, rho and nusG, or rho storage buffer was added at 1/10 volume to the specified final concentration and incubated at 30°C for 20 minutes. The reactions were then filtered through a 13 mm BA85 nitrocellulose filter (Schleicher and Schuell), followed by 200 μl of binding buffer, and the eluant was collected, ethanol precipitated with 10 μg of carrier RNA, and analyzed as described. Filter-bound RNA was eluted with 300 μl of 0.5 M NaCl in 1× binding buffer and treated identically.

In reactions containing paused ternary complexes, RNA bound to the filters after the high-salt wash was eluted by incubating the filter at room temperature in 400 μl of 50% phenol/chloroform, 3 M urea for 20 minutes, with occasional vortexing. The mixture was spun in a microfuge for 5 minutes; the aqueous phase was removed, diluted twofold with 0.6 M sodium acetate, and ethanol precipitated. Filters were scintillation counted to ensure that the radioactivity was quantitatively removed, and the RNA was treated as above.

RNase H cleavage

Stalled elongation complexes were isolated as described above and treated with 0.25 units of RNase H in a 50 μl reaction. At time zero, oligonucleotide was added to a final concentration of 1 μM. After three minutes, the reaction was passed through a nitrocellulose filter as described above, and the undissociated RNA was extracted from the filters and run on a denaturing gel.

Helicase assay

Helicase substrates were formed as described previously (Brennan et al., 1990) and diluted to a final concentration of 1 nM into helicase buffer containing 1 mM ATP and 1 mM magnesium. Rho was added to a final concentration of 5 nM ± 10 nM nusG, and the reaction was incubated for the indicated times at 30°C. Reactions were stopped and analyzed as described previously (Brennan et al., 1987).

Results

Rho-dependent termination in the presence of nusG displays a shift in endpoints

Transcription assays in vitro were used to examine the effect of purified nusG on rho-dependent termination. As shown in Figure 1, the template for this reaction consisted of the T7A1 promoter fused to a T-less segment of DNA

of 20 nucleotides, followed by a 30 nucleotide polylinker region and, finally, *trp t'*, a strong rho-dependent termination site in vivo (Zalatan and Platt, 1992). The advantage of initiating transcription with an ApU dinucleotide primer in the absence of UTP is the formation of an A20 stalled transcription complex (Levin et al., 1987; Chan and Landick, 1989). The transcript can be labeled to a high specific activity, and dilution with unlabeled nucleotide during the subsequent elongation phase from the preformed A20 complex ensures molar equivalence of labeling for all transcripts, regardless of length. Moreover, we can observe what is effectively a synchronized single round of transcription, without the addition of rifampicin.

G-labeled A20 complexes were elongated at 10 μM ATP, UTP, and CTP and 200 μM GTP (Fig. 2). The addition of rho to a final concentration of 10 nM resulted in nearly complete termination at a series of discrete sites centered at nucleotide 150 of the transcription unit, approximately 100 nucleotides into *trp t'* (Fig. 2, lane 4). RNase H digestion products of readthrough RNA with DNA oligomers that hybridize to regions about 15 nucleotides (Fig. 2, lane 2) or 90 nucleotides (Fig. 2, lane 3) into *trp t'* serve as size markers and indicate by comparison with lane 4 that termination is *trp t'*-dependent. Surprisingly, the inclusion of nusG with rho in the reaction resulted in a shift in the apparent endpoints to a region centered 75 nucleotides into *trp t'* (Fig. 2, lane 5). In the absence of rho, nusG alone does not cause any detectable termination of transcription (see below).

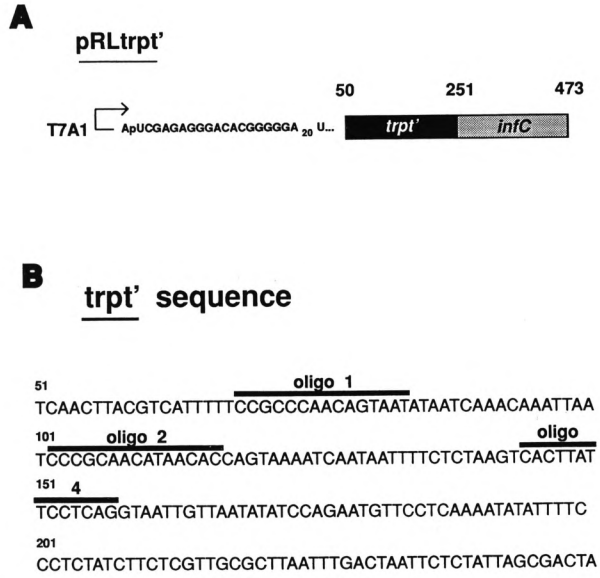

Figure 1. A. Map of the T7A1 promoter construct pRLtrpt'. The sequence of the first 20 nucleotides (U is the 21st nucleotide) transcribed in the absence of UTP to form the stalled A20 complex is shown in the context of the entire transcription unit. **B.** Nucleotide sequence of the *trp t'* termination region from the construct pRLtrpt', including the binding sites on the RNA of the oligonucleotides 1, 2, and 4.

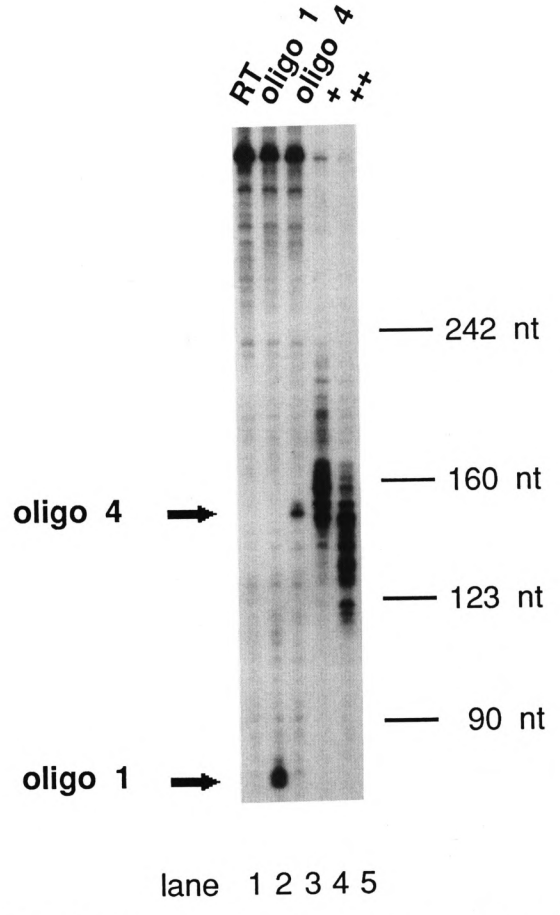

Figure 2. Transcription termination reactions with rho and nusG. pRLtrpt' vector DNA and RNA polymerase were incubated together to form A20 complex in the absence of UTP. Rho storage buffer (lanes 1, 2, and 3), rho (lane 4), or rho and nusG (lane 5) was added, and elongation was allowed to proceed at final nucleotide concentrations of 10 μM ATP, UTP, and CTP and 200 μM GTP for 20 minutes. RNase H and oligo 1 (lane 2) or oligo 4 (lane 3) was added to readthrough reactions to indicate the position of *trp t'* in the transcript. The positions of several denatured pBR322 Msp I end-labeled DNA fragments are shown as size markers.

The nusA protein is another important component of the E. coli transcription complex; hence we also tested its effect on rho-dependent termination, both alone and in combination with nusG. Although the details will be presented elsewhere, we observe that the effect on RNA release mediated by nusG remains the same in the presence of nusA. As seen previously, nusA alone shifts the rho-dependent transcription endpoints to more distal sites (Farnham et al., 1982). We emphasize that the nusG effect is observed in the presence of nusA and does not seem greatly affected by it (data not shown); hence the subsequent analysis as presented in this work omitted nusA.

NusG suppresses point mutations in *trp t'* that alter termination efficiency

More dramatic evidence of the effect of nusG on rho-dependent termination in vitro is seen using transcription templates that encode partially defective RNA substrates for rho (Fig. 3). Rho activity has been shown to depend upon cytosine in the nascent transcript (Alifano et al., 1991; Hart and Roberts, 1991; Zalatan and Platt, 1992), and several cytosine-to-uracil mutations in combination depress or eliminate rho-dependent termination in the *trp t'* termination region (Zalatan and Platt, 1992). Figure 3 displays transcription from selected templates (whose clone numbers reflect the number of changes) with 11 (lanes 1–3), 12 (lanes 4–6), or 13 (lanes 7–12) cytosine-to-uracil mutations out of the possible 28 cytosines in the first 104 nucleotides of the *trp t'* region. The 13a and 13b templates have differences at 6 of the 13 C to U changes from wild-type and respond very differently to rho alone (Zalatan and Platt, 1992; Fig. 3, lane 8 vs. 11). This leads to a severe decrease in rho-dependent termination efficiency (Zalatan and Platt, 1992) and the use of distal RNA release sites in the presence of rho (Fig. 3, lanes 2, 5, 8, and 11). In accordance with its function in enhancing rho-dependent termination in vivo, nusG appears to restore termination efficiency to wild-type levels, primarily through the increased use of latent early termination sites (Fig. 3, lanes 3, 6, 9, and 12). Thus, one faculty of nusG may be to make otherwise unrecognizable or unutilizable templates efficient for termination.

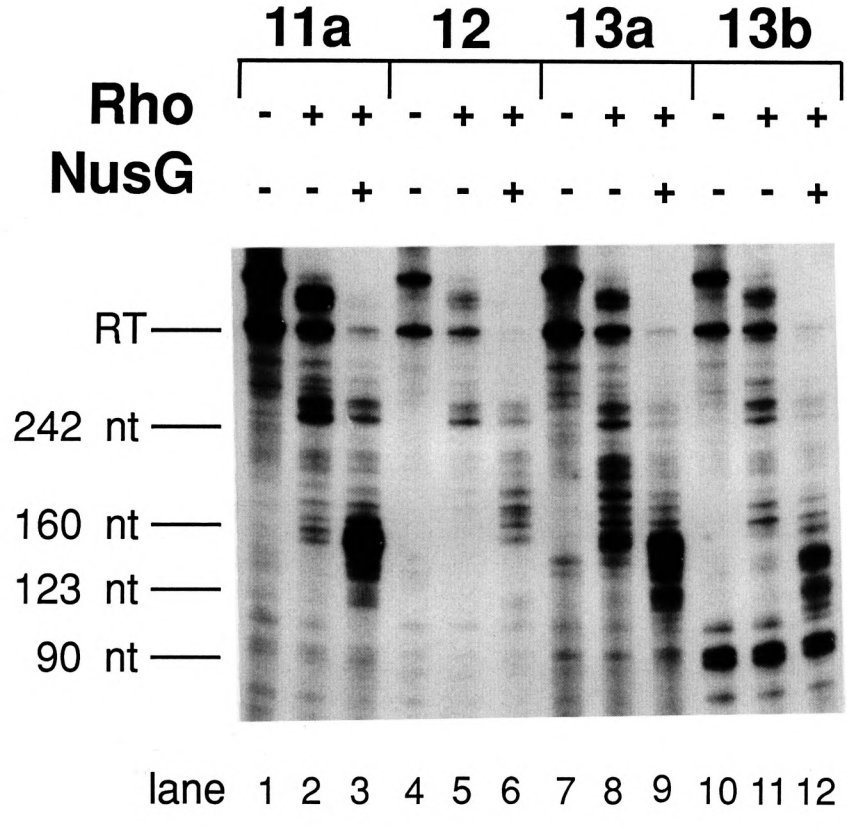

Figure 3. Termination on cytosine-to-uracil mutant derivatives of *trp t'*. Mutants 11a (lanes 1–3), 12 (lanes 4–6), 13a (lanes 7–9), and 13b (lanes 10–12) were used as templates for transcriptional elongation from A20 complexes in reactions containing 10 μM ATP, UTP, CTP and 200 μM GTP. Reactions were incubated for 20 minutes in a mixture containing rho storage buffer (lanes 1, 4, 7, and 10), rho (lanes 2, 5, 8, and 11), or rho and nusG (lanes 3, 6, 9, and 12). The positions of several denatured pBR322 Msp I end-labeled DNA fragments are shown as size markers.

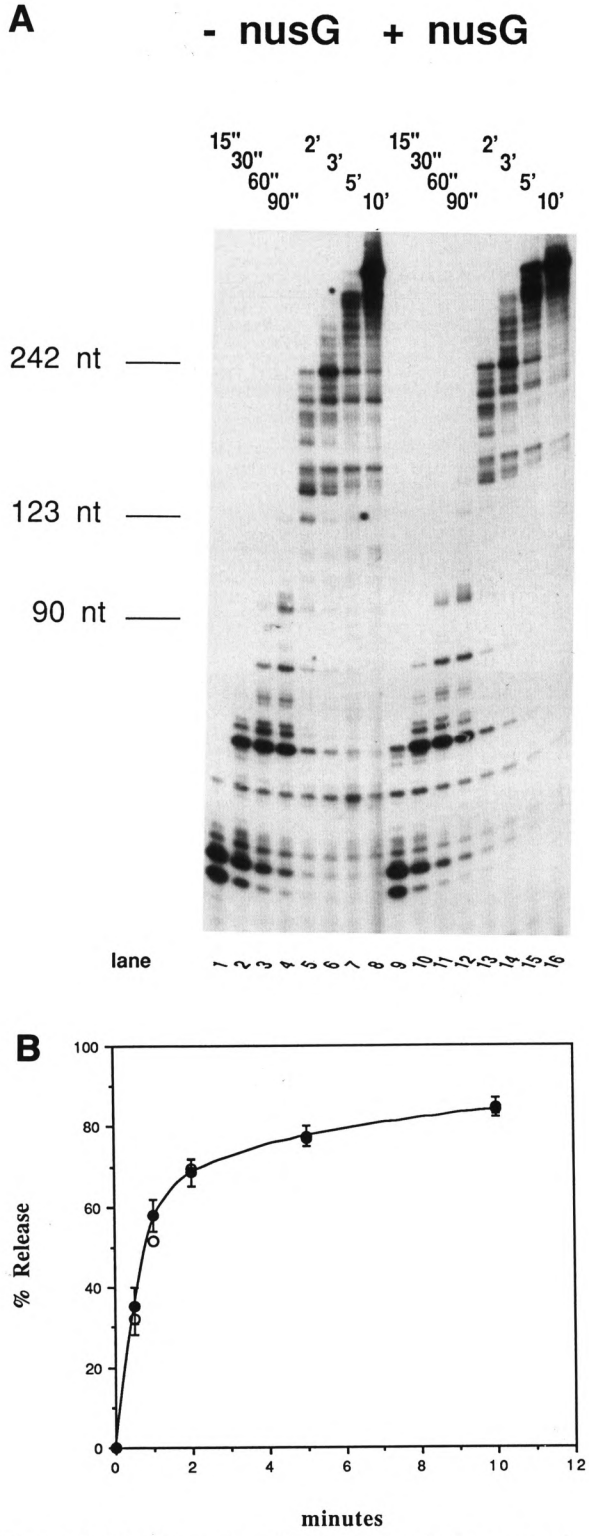

Figure 4. NusG has no effect on kinetic parameters. **A.** Elongation time course. A20 complexes formed on pRLtrpt′ vector DNA were elongated in 10 μM ATP, UTP, and CTP and 200 μM GTP using purified E. coli RNA polymerase alone (lanes 1–8) or supplemented with nusG to 20 nM (lanes 9–16). Shown are 15 second, 30 second, 60 second, 90 second, 2 minute, 3 minute, 5 minute, and 10 minute time points following the addition of UTP to the A20 complexes. Transcript size was determined by comparison with denatured pBR322 Msp I-cut end-labeled DNA fragments (shown to the right). **B.** A helicase assay using as a substrate t′HT RNA of 458 nt annealed to pUC119 single-stranded DNA via a 28 nt region at the 3′ end of the RNA. Helicase activity is shown as the percentage of 1 nM RNA:DNA duplex released in a 10 μl reaction at the times shown. Solid circles represent release by the rho protein (5 nM), while release catalyzed by rho (5 nM) in combination with nusG (10 nM) is shown by open circles. Both reactions were performed at 30°C in the presence of 1 mM magnesium and 1 mM ATP. Error bars indicate the range of values obtained from separate experiments.

NusG does not affect relative rates of action of rho and RNA polymerase

A decreased elongation rate would lessen the distance that RNA polymerase travels during the time it takes rho to reach the transcription complex and would result in proximal termination. Likewise, an increased rate of rho action would lessen the time RNA polymerase travels after the transcription of a rho-dependent recognition sequence. This idea was expressed by Jin et al. (1992) as the kinetic coupling hypothesis, and nusG could impinge on either of these aspects of the termination reaction.

A time course of elongation as a function of nusG indicates, however, that nusG has no observable effect on the rate of transcription (Fig. 4A). With all four nucleotides at concentrations of 10 μM (except GTP; see figure legend), this rate is approximately one nucleotide per second, compared to approximately 20 nucleotides per second using nonlimiting nucleotides (at least three times the apparent K_m). Also, the pause sites are unchanged by nusG (compare lanes 1–8 with lanes 9–16).

We have tested the RNA:DNA helicase activity of rho as a means of assaying rho factor's translocation rate between the site of rho:RNA binary complex formation and the site of RNA:DNA duplex disruption. Using t′HT, an RNA consisting of a random insert of 210 nucleotides between the 220 nucleotide *trp t′* and the 28 nucleotide complement to pUC119 single-stranded DNA (Steinmetz et al., 1990), we can detect no helicase rate enhancement conferred by nusG (Fig. 4b). It is possible, however, that the rate-limiting step in our helicase assay is not translocation of rho.

However, we have also found that the RNA-dependent ATPase activity of rho on *trp t′* RNA is unchanged by nusG (data not shown);

other groups have obtained similar findings with λ *tR1* RNA (B. Stitt, personal communication). We have likewise determined that nusG does not alter the dissociation constant of rho for *trp t'* RNA or for the cytosine-to-uracil mutant RNAs, and an examination of the lower size limits of rho-RNA complex formation suggests that rho does not preferentially associate with the early transcript as a function of nusG (data not shown). We note that RNA binding addresses a static aspect of rho activity, in that binding is necessary but by no means sufficient for termination function. It has yet to be shown how initial binding to RNA activates rho or indeed what components of the RNA rho recognizes; a function of nusG displayed subsequent to rho-RNA binding and preferentially manifest on a short or nonspecific substrate could be masked by conventional ATPase and RNA binding assays. It appears, moreover, that binding affinity is not an absolute index of termination efficiency (Faus and Richardson, 1989; Zalatan and Platt, 1992).

The effect of nusG is not dependent upon elongation

Thus far, the effects of nusG are consistent with enhancement of a rate-limiting step during rho-dependent termination. Termination rate is a difficult facet of transcription to assess other than qualitatively, as we have done, due to the influence of coupling between rho and RNA polymerase in an active transcription system. Therefore, we attempted to uncouple termination from elongation. Progressively lower concentrations of UTP were used in transcription assays in vitro to slow elongation to a negligible rate compared to the rate of rho activity. The effect of nusG on rho activity as the elongation rate decreased was then assayed.

The rho-dependent RNA endpoints in the presence of rho alone became more promoter-proximal as the available UTP was decreased (Fig. 5, lanes 2, 5, 8, and 11), in accordance with the kinetic coupling hypothesis. As the limit of available UTP approaches zero, the proximal limit of rho-dependent transcription termination should be observed (i.e., the point at which all of the rho molecules interact with all of the polymerase complexes before the complexes have a chance to move on to the next pause site). Figure 5 shows an approximation of that limit (lane 2). It is clear that with the addition of nusG, the endpoints are always shifted, even at the lowest concentration of UTP (lane 3). Thus, nusG appears to confer upon rho the ability to release RNA polymerase at unique sites, independent of the rate of transcription. This functional effect is not mimicked in the characteristics of binding or activation of rho.

Figure 6 displays the results of limiting one of the four nucleotide triphosphates at 5 μM (with the other three at 200 μM) during elongation in standard transcription termination reactions on the pRLtrpt' template (see Materials and Methods), and under these conditions the sites of termination are clearly nucleotide-specific (Fig. 6, lanes 2–5). The RNA endpoints derived from reactions with limiting ATP or UTP correlate well with pause sites observed under similar conditions (data not shown). For reasons not fully understood, polymerase pauses are much shorter when CTP or GTP is limiting; the relative scarcity of C and G in the sequence does not entirely account for this result. This overall reduction in pausing when CTP or GTP is limiting is evident from the substantial read-through transcription (lanes 2, 5, 6, and 9), compared to the ATP and UTP lanes. Figure 6 thus demonstrates that conditional nucleotide deprivation changes the pattern of termination, presumably by altering the duration and position of pausing, as would be expected from previous evidence (Lau et al., 1983; Morgan et al., 1983). NusG nevertheless elicits a characteristic shift in RNA endpoints in each case, and although this shift does change as a function of the limiting nucleotide (Fig. 6, lanes 6–9), this probably reflects correlations between the sites of pausing and of termination rather than any nucleotide specificity of nusG (see Discussion). As shown in Figure 5, by accentuating the degree of pausing with low nucleotide concentrations, we are able to provoke a more pronounced nusG effect. Similarly, we observe a heightened effect under those conditions that stimulate higher levels of pausing in Figure 6. Overall, the data presented in these two figures suggest that there is a region of pauses where termination by rho is dependent upon nusG.

NusG enhances rho-dependent release of short RNAs in stalled ternary complexes

We employed purified stalled transcription complexes (Shigesada and Wu, 1980; Richardson and Conaway, 1980; Schmidt and Chamberlin, 1984) to investigate whether nusG can promote termination at a series of unique proximal sites

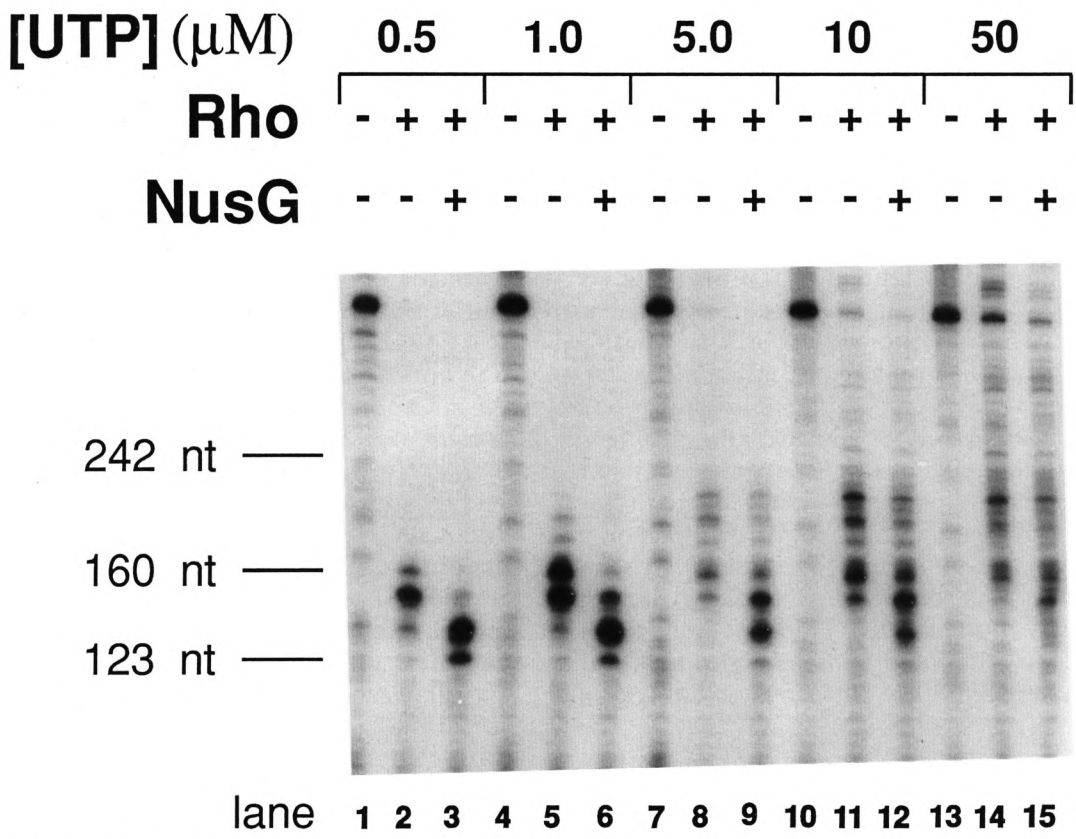

Figure 5. Titration of UTP concentration in termination reactions. Transcription was started from a labeled pRLtrpt' vector A20 complex by the addition of ATP, GTP, and CTP to 500 μM and UTP to 0.5 (lanes 1–3), 1.0 (lanes 4–6), 5.0 (lanes 7–9), 10.0 (lanes 10–12), or 50.0 μM (lanes 13–15) to reactions containing storage buffer (lanes 1, 4, 7, 10, and 13), rho (lanes 2, 5, 8, 11, and 14), or rho and nusG (lanes 3, 6, 9, 12, and 15). Several denatured pBR322 Msp I-cut end-labeled DNA fragments are shown as size markers.

in the *trp t'* transcription unit uncoupled from elongation. Rho was added to a ladder of stalled complexes (see Materials and Methods) with 1 mM dATP as an energy source, and after incubation the reaction mix was filtered through nitrocellulose (Fig. 7A and B). Since ternary complexes are stable in 0.5 M NaCl (Richardson, 1966), but rho-RNA binary complexes and nonspecific interactions between RNA polymerase and RNA are not (see Fig. 7B), we could separate dissociated RNA from RNA in intact ternary complexes by washing the filter-bound material in high-salt buffer (Materials and Methods). In the absence of rho, the complexes are stable and are able to resume elongation for at least 6 hours (data not shown).

Figure 7A shows the starting ladder of paused complexes (lane 1). Because rho will quantitatively bind to free RNA of sufficient length in these reactions preceding the high-salt wash, the flow-through represents only RNAs that are not bound to rho or to the ternary complexes after the termination reaction (Fig. 7A, lanes 2 and 5). Figure 7A, lanes 3 and 6, demonstrates that a specific population of stalled complexes remains bound on the filter, yet can be eluted with 0.5 M NaCl. The minimum size of these species (>100 nt) combined with their salt-sensitive retention on nitrocellulose suggests that they are rho-terminated RNAs; in agreement with the results presented in Figure 6, rho appears to dissociate the RNA in stalled ternary complexes that contain 130–140 nucleotides of nascent transcript, about 85 of which is *trp t'* (Fig. 7A, lane 3). The addition of nusG allows termination of transcripts down to a minimum size of 100–110 nucleotides, containing 50–60 nucleotides of *trp t'* (Fig. 7A, lane 6). Thus, nusG truly potentiates the termination of a size class of RNA that is resistant to dissociation from

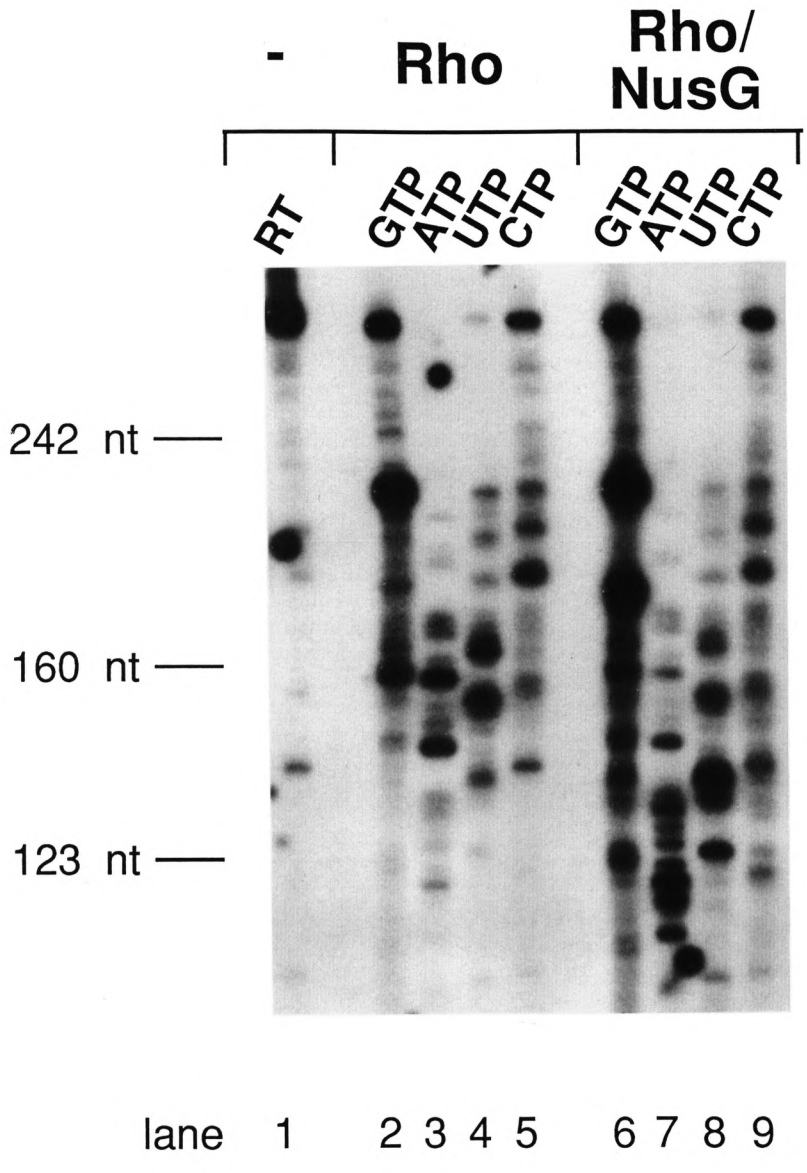

Figure 6. Termination as a function of varied limiting nucleotides. A20 complexes formed on a pRLtrpt′ PCR-amplified DNA fragment (see Materials and Methods) were extended in buffer containing three of four nucleotides at 200 μM and the fourth at 5 μM. Rho (lanes 1–4) or rho and nusG (lanes 5–8) were added preceding elongation. Limiting nucleotides are GTP (elongated from A20 complex that was labeled with [α^{32}P] ATP rather than [α^{32}P]GTP to prevent observable multiround transcription; lanes 1 and 5), ATP (lanes 2 and 6), UTP (lanes 3 and 7), or CTP (lanes 4 and 8). The positions of several denatured pBR322 Msp I end-labeled DNA fragments are shown as size markers.

the ternary complex when challenged with rho alone.

In Figure 7A, lanes 4 (−nusG) and 7 (+nusG) display RNA that remains bound to the filter in 0.5 M NaCl and is therefore retained by the transcription complex. As observed previously, the nascent transcript must contain a minimal length of RNA to potentiate rho activity; small transcripts are, in general, retained by RNA polymerase. Retention of distal transcripts does not appear to be a consequence of rho function, or lack thereof, but rather of secondary structure or protein:RNA interactions in the nascent transcript that block the rho binding site (see below; Fig. 8).

Figure 7B displays a time course of rho-dependent RNA release from stalled complexes. At time points from 0.5 to 30 minutes, a sample of stalled complex containing either rho (lanes 3–10) or rho and nusG (lanes 11–18) in the presence of 1 mM dATP was passed through a nitrocellulose filter. Undissociated RNA, retained on the filter after a high-salt wash and corresponding to lanes 4 and 7 of Figure 7A, was collected and run on a denaturing gel. Comparison of the starting stalled complexes (lane 1) with com-

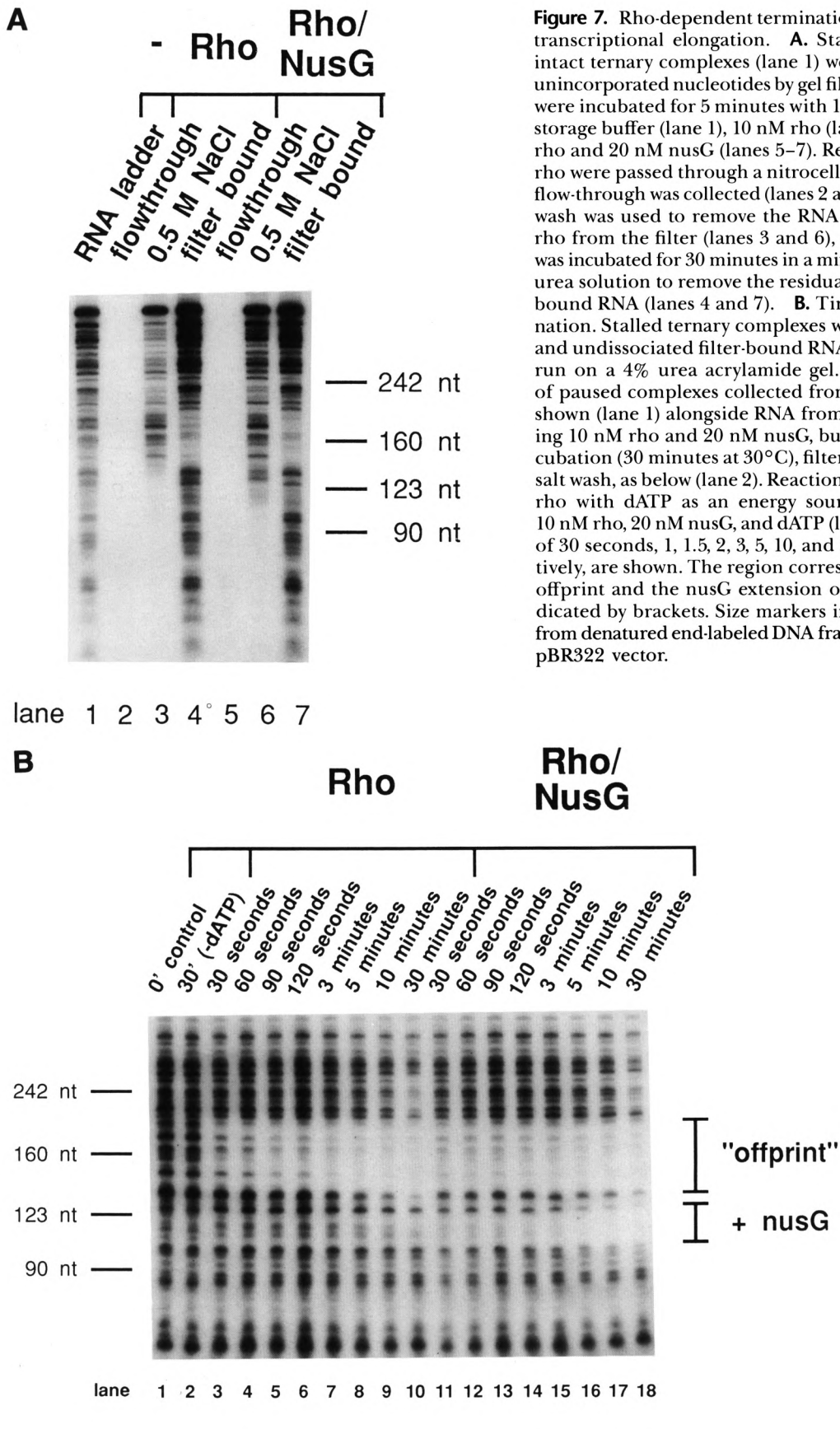

Figure 7. Rho-dependent termination uncoupled from transcriptional elongation. **A.** Stalled, isolated, and intact ternary complexes (lane 1) were separated from unincorporated nucleotides by gel filtration. Complexes were incubated for 5 minutes with 1 mM dATP and rho storage buffer (lane 1), 10 nM rho (lanes 2–4), or 10 nM rho and 20 nM nusG (lanes 5–7). Reactions containing rho were passed through a nitrocellulose filter, and the flow-through was collected (lanes 2 and 5). A 0.5 M NaCl wash was used to remove the RNA that was bound to rho from the filter (lanes 3 and 6), and the filter itself was incubated for 30 minutes in a mixture of phenol and urea solution to remove the residual RNA polymerase-bound RNA (lanes 4 and 7). **B.** Time course of termination. Stalled ternary complexes were treated as in **A**, and undissociated filter-bound RNA was collected and run on a 4% urea acrylamide gel. The initial ladder of paused complexes collected from a G-50 column is shown (lane 1) alongside RNA from reactions containing 10 nM rho and 20 nM nusG, but no dATP, after incubation (30 minutes at 30°C), filter binding, and high-salt wash, as below (lane 2). Reactions containing 10 nM rho with dATP as an energy source (lanes 3–10) or 10 nM rho, 20 nM nusG, and dATP (lanes 11–18) at times of 30 seconds, 1, 1.5, 2, 3, 5, 10, and 30 minutes, respectively, are shown. The region corresponding to the rho offprint and the nusG extension of the offprint is indicated by brackets. Size markers in both **A** and **B** are from denatured end-labeled DNA fragments of Msp I-cut pBR322 vector.

plexes that have been incubated with rho and nusG, in the absence of an energy source, confirms that (1) the stalled complexes are stable through the nitrocellulose filter binding and high-salt wash, and (2) nusG does not permit release of the ternary complex in the absence of energy (dATP).

Over time, what we have termed an "offprint," representing the region where RNA has been released (thus distinct from a "footprint"), develops from approximately nucleotide 135 and extends to approximately nucleotide 200. Within this specific region, even at the earliest time points tested (lanes 3 and 11), most of the transcription complexes have been dissociated by rho. Inclusion of nusG allows the extension of the offprint to a proximal limit of approximately nucleotide 105 (lanes 11–17). The extension appears to falter at certain sites (around nucleotides 125 and 135), implying that there is a positional specificity of release, perhaps related to the phase of RNA polymerase paused at separate sites (Arndt and Chamberlin, 1990; Yarnell and Roberts, 1992; Krummel and Chamberlin, 1992a,b). At the longest time points, rho alone is able to release some of the proximally stalled complexes (see lane 10). This suggests that the function of nusG in termination is actually to enhance (here, approximately 50-fold) an otherwise negligible rate of rho-dependent RNA polymerase dissociation at "early" sites.

The incomplete release of transcripts derived from complexes paused in the distal portion of *trpt'* and beyond was disconcerting, based upon previous studies showing nearly quantitative release of RNA from paused complexes

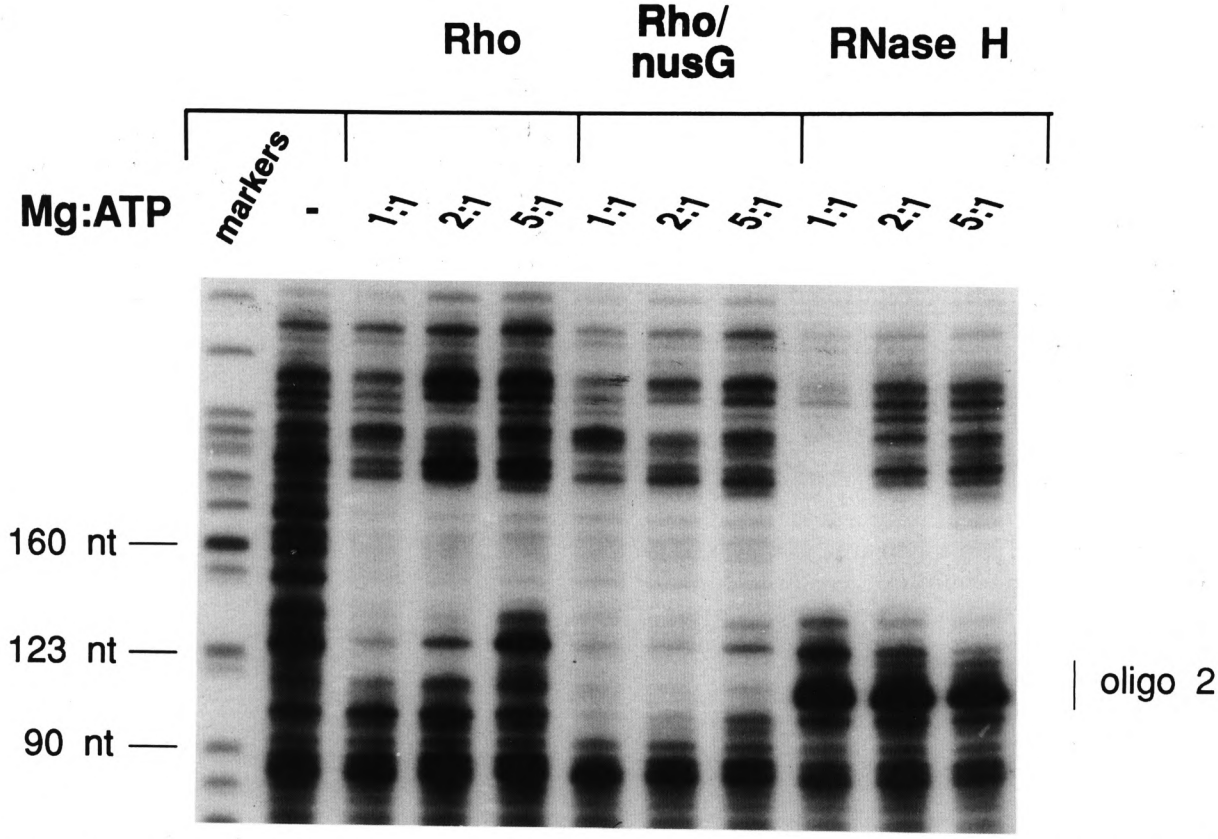

Figure 8. Stalled distal complexes are inaccessible to oligonucleotides as well as rho. Stalled, isolated transcription complexes were subject to offprint analysis. 10 nM rho (lanes 3–5), 10 nM rho and 40 nM nusG (lanes 6–8), or 0.25 units RNase H and 1 μM oligonucleotide 2 (lanes 9–11) were added for 10, 10, and 3 minute incubations at 30°C prior to the filter binding step at several concentrations of magnesium and 1 mM dATP to achieve the Mg:dATP ratios shown. The unterminated or uncleaved RNA is shown, as well as the total starting reaction at time zero (lane 2). Size markers are denatured end-labeled fragments of Msp I-cut pBR322 vector DNA (lane 1).

containing transcripts beyond a minimum length (Shigesada and Wu, 1980; Richardson and Conaway, 1980). To address the concern that this may reflect an unknown aspect of rho behavior, we used site-specific, oligonucleotide-directed RNase H digestion to probe the accessibility of the RNA in our paused complexes (Fig. 8). The oligonucleotide we used to target cleavage inhibits rho RNA-binding to *trp t'* 15-fold at concentrations of 100 nM (K. Nehrke, unpublished data), a far greater effect than any other oligo of comparable size, and by this criterion competes for the primary rho binding site on *trp t'* (Faus and Richardson, 1989).

In the absence of oligonucleotide, no detectable release of RNA from paused complexes occurred in a 30-minute incubation with up to 5 units of RNase H, indicating that no significant regions of accessible RNA:DNA hybrid occurred naturally on this template (data not shown). However, with the addition of 1 μM oligonucleotide, cleavage occurred rapidly, but only on a specific subset of stalled complexes; the complexes that were susceptible to RNA release by rho were correspondingly susceptible to oligonucleotide-directed RNase H cleavage (Fig. 8, lanes 3–5 and 9–11). Background retention of the RNase H cleavage product appears to be due to the high concentration of oligonucleotide present during filter binding and occurs during cleavage of purified RNA as well as stalled complexes (data not shown). The near identity of the offprints generated by these two very different processes suggests that sequestration of the rho binding site may occur in complexes stalled far downstream, preventing the loading of both rho and the oligonucleotide.

An inverse correlation between the level of free magnesium and the degree of accessibility to distally stalled complexes is consistent with the formation of RNA:RNA or RNA:protein structure (Fig. 8, lanes 3–5, 6–8, and 9–11). A pronounced increase in oligonucleotide accessibility to the distal transcripts follows reduction in the level of free magnesium. In reactions containing either rho or rho and nusG (lanes 3–5 and 6–8, respectively), a similar, though more modest, effect occurs as well (which may relate to the concentrations of rho [20 nM] and oligonucleotide 2 [1 μM] and their relative dispositions toward displacing secondary structure). Although we are unable to predict a significant secondary structure in the regions of interest, nonetheless such an event or perhaps a magnesium-dependent protein:RNA interaction would most easily explain the incomplete release of stalled transcripts.

Since the majority of transcripts in an actively transcribing system end, in the presence of rho, within the region of our offprint, this phenomenon may not be peculiar to a stalled system. Perhaps an outer boundary exists beyond which a rho loading site becomes inaccessible. This would likely increase the importance of nusG, since termination would be more apt to take place before RNA polymerase transcribes past this limit. However, considering the pared-down system, this phenomenon may also represent an in vitro artifact that is unlikely to be reflected in vivo. From these considerations and the data in Figure 8, we think it likely that the incomplete release of RNA from stalled complexes in Figures 7A and B does not reflect a critical facet of rho activity other than simple RNA accessibility and binding.

Discussion

Although there is evidence that specific interactions may occur between rho and certain components of the transcription apparatus (Schmidt and Chamberlin, 1984), studies of rho-dependent termination have not included nusG until recently (Sullivan and Gottesman, 1992; Sullivan et al., 1992). The crucial observation that nusG is required for efficient rho-dependent termination in vivo (Sullivan and Gottesman, 1992) raises an important question: how applicable are existing models of rho function to termination as it occurs in the cell?

We have shown here that nusG can alter the pattern (Fig. 2), and in some cases the efficiency (Fig. 3), of rho-dependent termination in vitro. Since nusA, another factor involved in both termination and antitermination, influences pausing of RNA polymerase (Yager and von Hippel, 1987), we asked similarly whether nusG could be shifting rho-dependent RNA endpoints via an effect on the elongation rate of RNA polymerase or the tracking speed of rho, affecting the kinetic coupling of these two processes (Jin et al., 1992). A time course of elongation ruled out the former, while helicase assays indicated that nusG does not influence the latter, at least when uncoupled from ongoing transcription. As suggested by B. Stitt and coworkers (personal

communication), ATPase and RNA binding assays were also unaffected by the presence of nusG under standard termination reaction assay conditions (see Materials and Methods).

We used two further approaches to demonstrate that the nusG effect is independent of elongation rate and therefore of kinetic coupling. First, by titrating the concentration of nucleotides to slow down the elongation rate of polymerase during transcription, we found that even with extensive pausing, and regardless of which nucleotide was limiting, the nusG proximal endpoint shift was still preserved; that the rate of rho activity would be unaffected by these variations is a reasonable, though untested, corollary. Further proof of independence from kinetic considerations was garnered by purifying stalled ternary complexes and assaying the susceptibility of these complexes to rho. Even with these immobilized complexes, a longer length of nascent RNA was required by rho to promote transcript dissociation from the elongation complex when nusG was absent than when it was present. We believe these are compelling arguments that the nusG shift is not dependent upon coupling between RNA polymerase and rho at all, but instead reveals a new class of shorter rho-dependent transcripts.

In a stalled transcription system, the nusG offprint region (Fig. 7B, lanes 11–18) contains some notable pause sites where transcript release is inefficient. Despite fast and complete release of both longer and shorter transcripts from nearby complexes, several species of RNA at the juncture of rho-dependent and the nusG-mediated portions of the offprint resist dissociation by rho. We posit that this reflects heterogeneity in the spectrum of paused complexes, which thus exhibit differential stability when presented to rho factor. The idea of nonuniform transcription, in which complexes separated by relatively small physical distances may have distinct structures and properties, has been proposed previously, supported by measurements on stalled ternary complexes of DNase footprints, thermal stability, gel mobility, and stability to dissociation (Rice et al., 1991; Krummel and Chamberlin, 1992a,b). Additional support lies in the fact that RNA derived from artificial pause sites only a few base pairs apart can display substantial differences in the reactivity of chemical modifying reagents (D. Lee and R. Landick, personal communication). Overall, the microenvironment may contribute substantially to the efficiency of a rho response by modulating the stability of each elongation complex. One possible function of nusG may thus be to influence the stability of the RNA polymerase: nucleic acid interactions at early termination sites where the length of available RNA is a hurdle to rho-dependent termination. Alternatively, nusG could increase access of rho to the nascent RNA at the ternary complex.

Richardson (1982) provided evidence that rho has two types of RNA binding sites, both of which require occupation by single-stranded nucleic acid to elicit ATPase activity. The primary site interacts with 60 nucleotides or more of single-stranded DNA or RNA and requires cytosine residues to promote ATPase activity; the secondary binding site interacts with only 8 nucleotides of RNA and is more tolerant of diverse substrates. RNA polymerase also has an RNA binding site (for review see Kumar, 1981), and it has been hypothesized that this site may sequester some portion of the nascent transcript in lieu of or in addition to an RNA:DNA helix at the transcription bubble (Surratt et al., 1991). If so, some part of the nascent RNA would be unavailable for contact with rho until it moves out of the polymerase binding site.

The observation that nusG stimulates early termination (Li et al., 1993; B. Stitt, personal communication) suggests qualitatively that it might enhance the access of rho to polymerase-bound RNA, as suggested by Sullivan and Gottesman (1992). Since full activation of rho requires its binding to a long, unstructured single-stranded region of RNA, any necessary portion sequestered by polymerase will delay termination until that portion is released, either by the further progress of elongation or by some action that renders it accessible to rho. The slow transcript release we observe by rho alone at early sites in a stalled system would be accounted for by a naturally slow rate of release of the sequestered segment of RNA from RNA polymerase, to which rho would then bind to become fully activated. The role of nusG could be to shift the equilibrium in favor of rho by making the RNA more available for rho binding, perhaps by causing a conformational change in one of the polymerase subunits or by weakening the affinity between nascent transcript and the RNA site on polymerase. Li et al. (1992) have in fact documented an associa-

tion of nusG, interdependent upon N/nusA/nusB/S10, with the transcription complex. Furthermore, a mutation in nusG has been shown to suppress the *nusA1* antitermination mutant, indicating that nusG may be a normal part of the transcription complex in vivo.

We have shown an effect of nusG on *trp t'* independent of the attractive kinetic coupling mechanism proposed by Jin et al. (1992). However, while the mechanism of action of nusG does not appear to involve a change in kinetic coupling, the formation of unique RNA endpoints *at any given site* can be influenced by kinetic criteria. For example, we have demonstrated that termination enhancement by nusG is most pronounced when transcription occurs slowly (Figs. 5 and 6) or not at all (Fig. 7). The degree of proximal shift observed in Figure 6 correlates with the rate of transcription under different limiting nucleotide conditions; with limiting ATP or UTP, the effect is most pronounced. Correspondingly, lower concentrations of UTP (Fig. 5) lead to greater nusG effects. However, despite an obvious requirement for pausing within an early region of *trp t'* to enhance the sensitivity of termination to nusG, we have shown that termination at *these* sites is absolutely dependent upon nusG, at least in an active transcription system. Therefore, it is important to discriminate between rho function during active transcription and on stalled complexes. While rho may eventually work on a polymerase molecule stalled at a given site, even with nonspecific transcripts (Pavco and Steege, 1990), the extent to which polymerase can succumb to rho during ongoing transcription probably reflects the delicate balance between competitive kinetic processes such as elongation and termination (Yager and von Hippel, 1991; Jin et al., 1992). If we postulate that the transition of rho to an active state due to primary site binding is the rate-limiting step in termination, and if nusG allows that step to occur sooner (causing more proximal endpoints) or with greater efficiency (causing enhanced termination), then nusG would be a vital component of the rho-dependent termination reaction at many termination regions where the amount of free, unstructured RNA is limiting. Conversely, rho-dependent terminators preceded by a stretch of free RNA may not require the assistance of nusG in vivo, which agrees with the findings of Sullivan and Gottesman (1992) in that the *tIS2* terminator is 81% efficient in its absence. Furthermore, this model can account in part for the different levels of rho-dependent termination observed on different templates in vitro (Faus and Richardson, 1989).

This ascribes a potentially crucial regulatory role to nusG protein in controlling the efficiency of rho-dependent transcription termination, in accord with its role as an essential protein in E. coli. The findings of Li et al. (1993) together with the results presented here begin to confirm a central structural and functional role for nusG protein as a participant in the E. coli elongation complex that is capable of modulating both termination and antitermination behavior of the transcriptional apparatus.

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