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Nus A Protein Affects Transcriptional Pausing and Termination in Vitro by Binding to Different Sites on the Transcription Complex

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ABSTRACT: We examined the in vitro concentration dependence of the effects of Nus A on transcription termination and pausing to determine if Nus A affects both pausing and termination in vitro by binding to a single site on the transcription complex. Nus A was shown to cause maximal increases of pausing at a concentration approximately equimolar to RNA polymerase. However, the effects of Nus A on termination require much higher Nus A concentrations than are required for pausing. It is therefore likely that the effects of Nus A on pausing and termination result from the binding of Nus A to different sites on the transcription complex. Since proteins that probably bind RNA nonspecifically were also shown to strongly reduce termination at a Rho-dependent terminator, Nus A may decrease Rho-dependent termination by binding nonspecifically to RNA. This proposal is consistent with most of the available data on the in vitro effects of Nus A and provides a mechanistic basis for previously unexplained details of Nus A caused decreases in Rho-dependent termination. We further speculate that most or all of the in vivo roles of Nus A may involve the enhancement of pausing.

Nus A protein of *Eshcerichia coli* is a transcription factor that causes decreased termination at Rho-dependent terminators, increased termination at a Rho-independent terminator, and increased pausing at a variety of sites, many of which are near or coincident with Rho-dependent termination sites [this paper; reviewed in Platt (1986)]. Because pausing is an essential component of termination (von Hippel et al., 1984), it seemed very plausible that Nus A caused pausing and changes in termination efficiency result from Nus A binding to a single site on the transcription complex. However, it is paradoxical that, in the absence of Nus A, increased pausing leads to increased termination (von Hippel et al., 1984), whereas Nus A increases pausing but decreases termination at Rho-dependent terminators (Lau & Roberts, 1983; Sigmund & Morgan, 1988). Therefore, it is equally possible that Nus A affects pausing and termination in vitro by unrelated mechanisms, perhaps by binding to different sites. The possibility that Nus A might bind functionally to more than one site is also suggested by the observations that Nus A may bind to RNA polymerase (Greenblatt & Li, 1981a), to RNA polymerase at more than one site (Schmidt & Chamberlin, 1984a), to Rho (Schmidt & Chamberlin, 1984b), to RNA at or near a sequence called box A (Tsugawa et al., 1985), and nonspecifically to RNA (Tsugawa et al., 1985).

Whether or not Nus A binds to a single site to affect both pausing and termination in vitro can be tested by measuring the magnitude of the effects of Nus A on pausing and termination at several Nus A concentrations. Although previous studies used nearly equimolar amounts of Nus A and RNA polymerase, the use of nearly equimolar amounts of RNA polymerase and Nus A does not ensure that the various effects observed are physiologically relevant, caused by the binding of Nus A to a single site, or quantitatively representative of the events that occur in vivo.

This paper addresses the following specific questions: (1) Do all concentrations of Nus A cause proportional increases in pausing at all pause sites, indicating that Nus A affects pausing by binding to a single site or to sites with conserved features? (2) Does Nus A cause effects on termination at the same concentrations that increase pausing, indicating that the effects of Nus A on pausing and termination are caused by binding to the same site? (3) What are the maximum magnitudes of the effects of Nus A on pausing and termination?

Materials and Methods

Analysis of in Vitro Transcripts. In vitro transcription was performed as described in the companion paper (Sigmund & Morgan, 1988) except that single-round transcription was achieved by prewarming all reaction components except UTP at 37 °C for 10 min, at which time 10 μCi of [α-^{32}P]UTP at a final concentration of 40 μM and 10 μg/mL rifampicin were added (Lau et al., 1983). Reactions performed to measure pausing did not contain Rho. All reactions contained identical final salt and glycerol concentrations. Analysis of transcripts by gel electrophoresis and densitometry was performed as previously described (Sigmund & Morgan, 1988). Total yeast ribosomal proteins (TP80) were the generous gift of Dr. Jonathan Warner.

Results and Discussion

Our accompanying paper (Sigmund & Morgan, 1988) shows that the effect of Nus A on termination at Rho-dependent and Rho-independent terminators is not qualitatively

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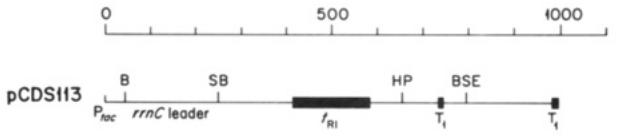

FIGURE 1: Structure of the transcribed region of pCDS113. The scale indicates the distance in base pairs from the transcription start site of the *tac* promoter. Termination occurs within regions indicated by a thickening of the line. Restriction enzyme recognition sites are designated as follows: B, *Bam*HI; S, *Sma*I; H, *Hin*cII; P, *Pst*I; E, *Eco*RI.

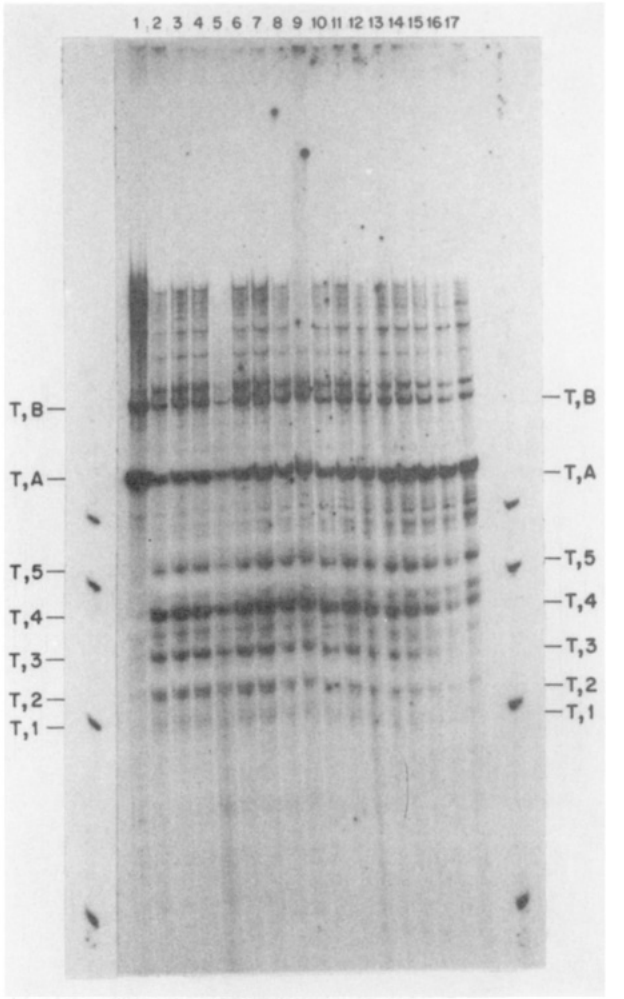

FIGURE 2: Nus A concentration dependence of termination. These autoradiograms show transcripts separated by gel electrophoresis. The notations flanking lanes identify the promoters and termination sites responsible for each transcript. The letter T preceding the comma indicates that the *tac* promoter directs transcription. The letter or number following the comma indicates the termination signal at which the transcript terminates [A indicates the first T_1 terminator (T_A), B indicates the second T_1 terminator (T_B), 1–5 indicates sites I–V of the t_{R1} termination region]. (Lane 1) RNA polymerase alone; (lanes 2–17) RNA polymerase, Rho, and 0, 0.13, 0.25, 0.38, 0.5, 0.63, 0.75, 0.88, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 8.0, and 32.0 μg/mL Nus A.

or quantitatively affected by specific DNA or RNA sequences. Therefore, for the studies reported in this paper we used pCDS113 (Figure 1), a transcription template that enables reliable and detailed measurements of pausing, Rho-dependent termination, and Rho-independent termination (Sigmund & Morgan, 1988) to be made. Transcription of pCDS113 initiates at the P_{tac} promoter, proceeds through the *rrnC* leader, and terminates at the Rho-dependent λ t_{R1} terminator or at downstream tandem Rho-independent *rrnC* T_1 terminators.

Both the t_{R1} and *rrnC* leader regions contain previously characterized sites at which pausing is increased by Nus A

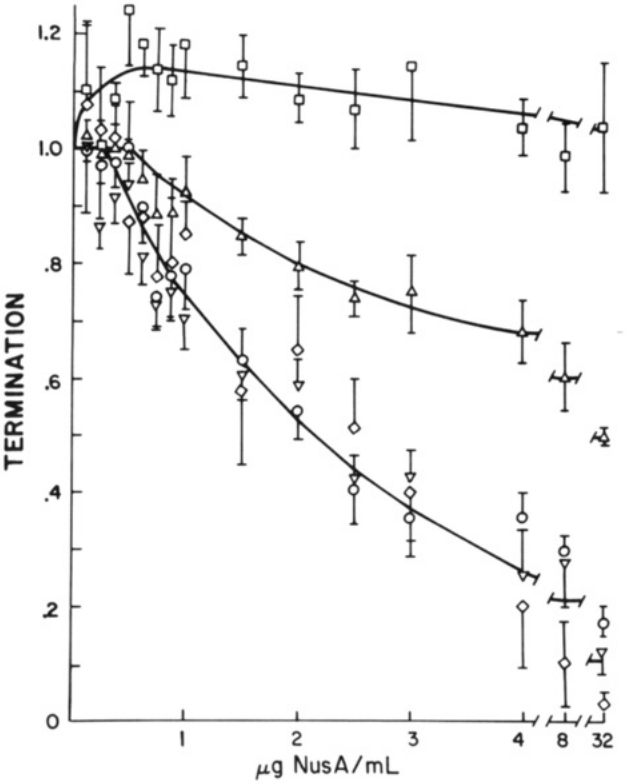

FIGURE 3: Termination at each site in t_{R1} at various Nus A concentrations. Termination is normalized to termination in the absence of Nus A. Individual termination sites in the t_{R1} termination region are indicated by (◇) site I, (○) site II, (▽) site III, (△) site IV, (□) and site V. A single curve is used to represent the data for sites I–III. Error bars indicate SEMs.

(Lau & Roberts, 1985; Kingston & Chamberlin, 1981). Most importantly, t_{R1} contains strong pause sites that are coincident with transcription termination end points by the criteria of coelectrophoresis of paused and terminated transcripts on sequencing gels (Sigmund, 1987; see also below). Both pausing and termination at these sites are strongly influenced by Nus A (Lau & Roberts, 1985). The coincidence of Nus A influenced pause and termination sites in t_{R1} greatly reduces the possibility that pausing and termination at these sites might require different Nus A concentrations due to inherent differences in the DNA or RNA sequences of pause and termination sites.

Our analysis assumes that the concentrations of Nus A which cause undetectable, half-maximal, and maximal effects on pausing or termination at each site are approximate indications of the concentrations of Nus A that cause binding of Nus A to very few, half, and all of the available functional sites, respectively. Thus, our analysis presented below is designed primarily to determine these quantities. Our analysis will be easier to understand if it is appreciated that the concentrations of Nus A which cause half-maximal and maximal effects at different pause and termination sites can be reliably and meaningfully compared despite the fact that the magnitudes of the effects of Nus A differ at different pause and termination sites.

Nus A and Rho-Dependent Termination. The effects of Nus A on termination at t_{R1} and *rrnC* T_1 were determined by separating radioactive transcripts on acrylamide gels (Figure 2) followed by densitometry of autoradiograms. Termination at the individual termination sites in t_{R1} continues to decrease as the Nus A concentration increases to 32 μg/mL (Figure 3). Nus A causes the greatest reduction of termination at sites I–III, with an intermediate reduction of termination at

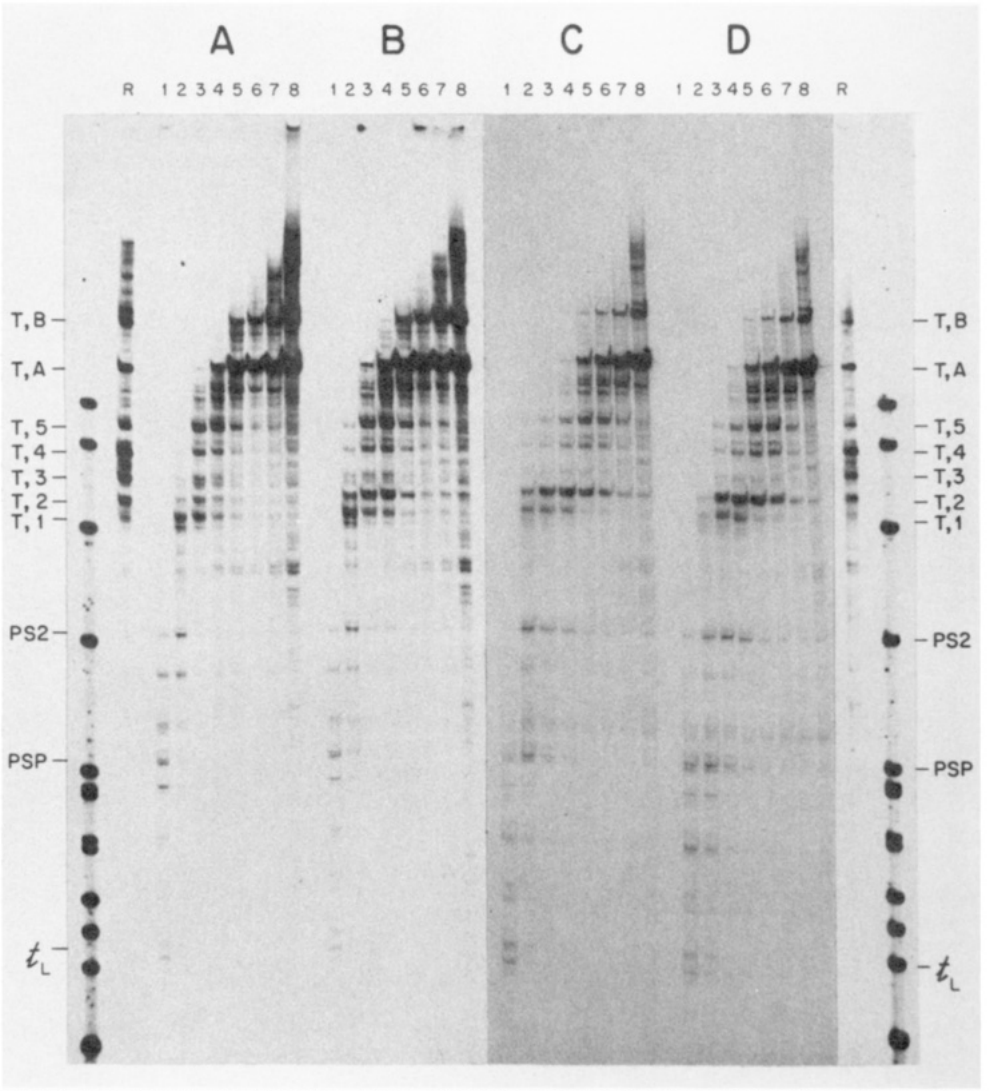

FIGURE 4: Nus A concentration dependence of pausing. These autoradiograms show RNAs synthesized in single-round transcription reactions and separated by gel electrophoresis. RNAs synthesized in the presence of Rho are shown in the lanes labeled R and identify termination sites within t_{R1}, T_A, and T_B. Panels A–D show transcription products synthesized in the presence of 0, 0.13, 0.5, and8.0 μg of Nus A/mL. Lanes 1–8 in each panel correspond to 15-, 30-, 45-, 60-, 90-, 120-, 180-, and 600-s time points. The notations flanking lanes identify transcript termination sites (according to the convention used in Figure 2) and other pause sites noted in the text.

site IV and almost no effect on site V (Figure 3). The results also show that there is little or no reduction of termination at t_{R1} unless the Nus A concentration is at least 0.5 μg/mL and that the concentration of Nus A needed for half-maximal decrease of termination is 2 μg/mL or more. The reduction of termination seems to be approaching a maximum at 32 μg/mL Nus A when pCDS113 is used as a transcription template. We have not examined transcription from pCDS113 using Nus A concentrations greater than 32 μg/mL. However, the reduction of termination at t_{R1} caused by 130 μg/mL Nus A is only 10% greater than the reduction caused by 32 μg/mL Nus A when transcription is from pCDS73 (Sigmund & Morgan, 1988), a plasmid structurally similar to pCDS113 except that transcription is from the *rrnC* promoters (data not shown). The continuity and smoothness of the curves in Figure 3 argue strongly that the reduction of Rho-dependent termination at each termination site occurs by the same mechanism at all Nus A concentrations.

Nus A and Pausing. We examined transcriptional pausing using several concentrations of Nus A (Figures 4 and 5). Pausing occurred at previously characterized (Lau et al., 1983) sites within the t_{R1} region of pCDS113 that appear identical (by comigration on sequencing gels; see Figure 4) with termination sites in t_{R1}. Pausing also occurred at previously characterized site (PS2) upstream of t_{R1} (Lau et al., 1983), at a site (PSP) upstream of t_{R1} that maps by size to polylinker sequences, and at a site in the *rrnC* leader region that appears by the criteria of transcript length to be identical with the previously characterized t_L pause site (Kingston & Chamberlin, 1981).

We used the method of Lau et al. (1983) to determine the fractional occupancy of each pause site. Fractional occupancy is a straightforward measure of pausing that is affected only by how many RNA polymerase molecules pause at a site and how long they remain paused there. Figure 5 shows the fractional occupancy of all significant pause sites in the transcribed regions of pCDS113 as a function of Nus A concentration. As can be seen from these data (Figure 5), Nus A causes quantitatively different increases in pausing at different sites. The increases of pausing caused by Nus A do not quantitatively correlate with the decreases of termination caused by Nus A at the same sites (compare Figures 3 and 5), as previously reported (Lau & Roberts, 1985). Despite the different magnitudes of increased pausing at individual pause sites, the Nus A caused increases in pausing at all pause sites are half-maximal at about 0.2 μg/mL and maximal at

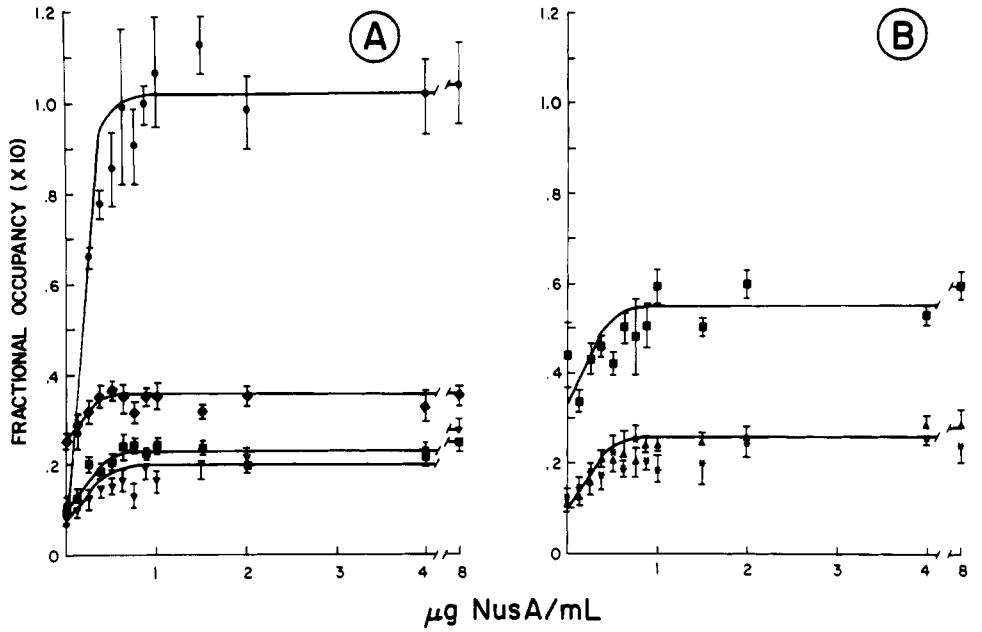

FIGURE 5: Fractional occupancy of individual pause sites during the first 5 min following synchronous initiation of transcription. Pause sites are indicated as follows: (panel A) (◆) site I, (●) site II, (■) PSP, (▼) PS2; (panel B) (▲) site IV, (■) site V, (*) t_L. Error bars indicate SEMs.

0.5 μg/mL. The similar Nus A concentrations needed to increase pausing at all pause sites indicate that pausing at all sites involves either the binding of Nus A to one site on the transcription complex or to multiple sites that have similar affinities for Nus A. On the other hand, the different concentrations of Nus A needed to affect pausing and Rho-dependent termination (see above) suggest that Nus A affects pausing and Rho-dependent termination by binding to different sites. The data in Figure 5 also show that the increase in pausing caused by Nus A is maximal at concentrations approximately equimolar with RNA polymerase, suggesting but not proving that Nus A increases pausing by binding to RNA polymerase.

Nus A and Rho-Independent Termination. Unlike the decrease in Rho-dependent termination resulting from the addition of Nus A, Nus A increases termination at the *rrnC* Rho-independent T_1 terminator (Sigmund & Morgan, 1988). However, as with t_{R1}, Nus A concentrations below 0.5 μg/mL cause little or no increase of termination at T_1, and the Nus A concentration that causes a half-maximal increase in termination is about 2 μg/mL (Figure 6). The different concentrations of Nus A needed to affect pausing and Rho-independent termination suggest that Nus A affects pausing and Rho-independent termination by binding to different sites on the transcription complex. It is possible, although not certain, that similar concentrations of Nus A are needed to affect Rho-dependent and Rho-independent termination because Nus A binds to the same or similar sites to affect termination at these two classes of terminators.

Effects of Other Proteins on Termination. Because the effects of Nus A on termination do not saturate except at Nus A concentrations in great excess over RNA polymerase, the stoichiometry of components does not provide strong clues as to where Nus A might bind to affect termination. However, a possible mechanism by which Nus A might affect termination is suggested by the striking similarities between the reduction of Rho-dependent termination caused by Nus A and the reduction of Rho-dependent termination caused by shortening the transcript before t_{R1} by a series of deletions in the transcription template. For example, successive increases in Nus A concentration and successive shortening of the RNA

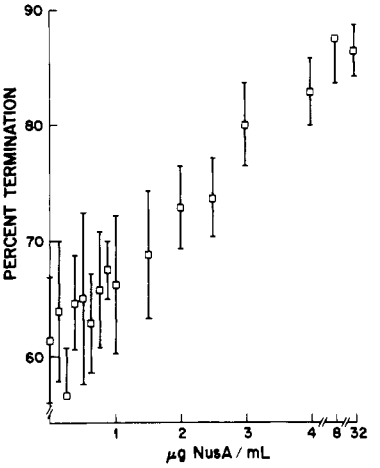

FIGURE 6: Nus A caused increase in Rho-independent termination at the first T_1 terminator in pCDS113.

transcript by means of deletions both successively reduce termination within t_{R1} with the greatest reduction at early termination sites (this paper; Lau & Roberts, 1985). Since reduction of termination by shortening the transcript has been proposed to result from a reduced ability of Rho to bind the transcript and subsequently cause transcript release (Lau & Roberts, 1985), the similarities between the effects of deletions and effects of Nus A might result from Nus A binding nonspecifically to RNA, effectively shortening the length of RNA available for productive Rho binding. Nus A has been demonstrated to have nonspecific RNA binding activity (Tsugawa et al., 1985). The lack of sequence dependence of the effects of Nus A (Sigmund & Morgan, 1988) and high Nus A concentrations needed to affect termination are also consistent with this proposal.

If the effects of Nus A on termination are due to nonspecific binding to RNA, then other proteins that bind RNA nonspecifically should also reduce termination at concentrations similar to the concentrations at which Nus A affects termination. We therefore examined the effects on termination of other proteins likely to bind RNA nonspecifically. As shown in Figure 7, a mixture of all yeast ribosomal proteins (TP80)

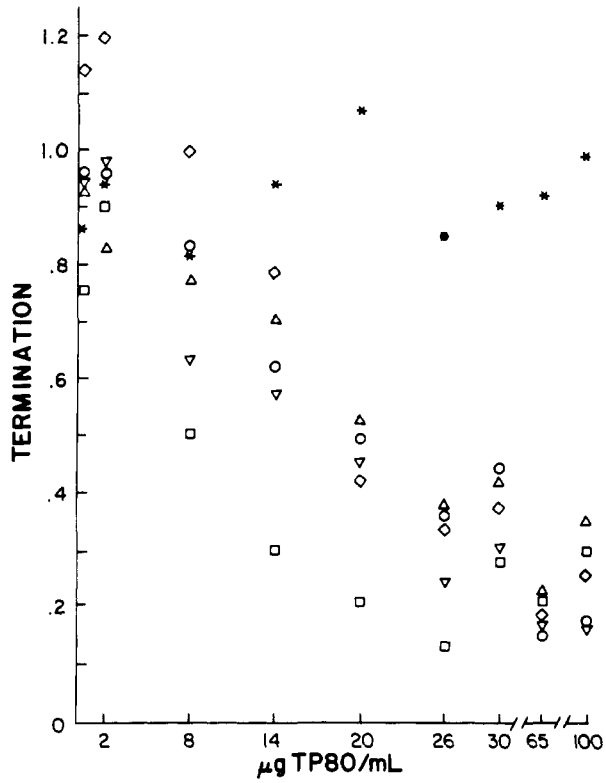

FIGURE 7: Variation in termination at each site in t_{R1} and T_A in response to TP80 concentration. Termination sites are indicated as follows: (◇) site I; (○) site II; (▽) site III; (△) site IV; (□) site V; (*) T_A. Termination is normalized to termination in transcription reactions performed in the absence of TP80. Each data point is the average of two independent transcription reactions.

reduces termination at the t_{R1} terminator. The reduction of termination caused by TP80 is similar in magnitude to the reduction caused by Nus A and occurs at similar concentrations. Lysozyme had similar effects at about 10-fold higher concentrations (Sigmund, 1987; data not shown). However, lysozyme did not affect pausing even at 500 μg/mL, a concentration that reduced termination at t_{R1} by over 90% (Sigmund, 1987; and data not shown). TP80 or lysozyme did not completely mimic the effects of Nus A on termination, however, as only Nus A affected termination at T_1 or preferentially affected early sites in t_{R1} (Figure 6; Sigmund, 1987; and data not shown). In addition, it is not clear how nonspecific DNA or RNA binding by Nus A could increase termination at a Rho-independent terminator.

Since proteins that bind RNA nonspecifically do not exactly mimic the effects of Nus A on termination, it remains possible that Nus A affects termination by binding to physiologically important site(s). If so, our data show that the site(s) that Nus A binds to to affect termination is (are) weaker than the site Nus A binds to to increase pausing. Alternatively, the failure of TP80 or lysozyme to exactly mimic the effects of Nus A on termination may result from preferred binding of these proteins to a different spectrum of physiologically unimportant sites. Although the reason for the differences in the way these proteins affect termination cannot be determined from the information presently available, it is nevertheless striking that a clearly artifactual reduction of Rho-dependent termination can be readily elicited by RNA-binding proteins at concentrations similar to the concentrations needed for Nus A to reduce termination.

It is interesting that integration host factor (IHF) and DNA A proteins decrease termination at t_{R1} and cause pausing in vitro, respectively (Craig & Nash, 1985; Rokeach et al., 1987). Both of these proteins have DNA binding activities that may not be related to termination in vivo. It is conceivable that the effects of these proteins in vitro may be yet other instances of nonspecific RNA or DNA binding activities affecting pausing and termination. Chen et al. (1986) have also shown that an oligonucleotide complementary to a region of RNA upstream of a Rho-dependent terminator can reduce Rho-dependent termination in vitro.

SUMMARY

Our most important finding is that the concentrations of Nus A needed to enhance pausing are similar for all pause sites, whereas much higher Nus A concentrations are required for effects on termination events. We conclude that Nus A affects pausing and termination by binding to different sites. The effects of Nus A on pausing and termination are therefore probably not mechanistically related despite the previous strong mechanistic association of pausing and termination in termination events not involving Nus A [reviewed in von Hippel et al. (1984)]. In retrospect this is not surprising, as it was previously known that increased pausing leads to increased termination during events not involving Nus A, whereas events involving Nus A paradoxically seemed to violate this relationship because Nus A can simultaneously increase pausing and yet decrease termination at the same site [e.g., site II of t_{R1}; see above and Lau and Roberts (1985)]. In addition, it was previously known that the increases in pausing caused by Nus A do not quantitatively correlate with the effects of Nus A on termination at the same sites [see above and Lau and Roberts (1985)]. Altogether, all previous observations on Nus A, as well as the data presented in this paper, are best explained by a model in which the effects of Nus A on pausing and termination are not due to the binding of Nus A to the same site(s) on the transcription complex.

Since Nus A is equally effective at all pause sites, it is likely that Nus A affects pausing at all pause sites by binding to a single site on the transcription complex or to sites with conserved features. It therefore is unlikely that Nus A affects pausing by binding to either DNA or RNA, as the sequences of different pause sites exhibit no obvious similarities. Since RNA polymerase is the only macromolecule in our pausing reactions besides Nus A, DNA, and RNA, and because the increase of pausing caused by Nus A is maximal at a Nus A concentration approximately equimolar with RNA polymerase, it is likely that Nus A binds to a single site on RNA polymerase to enhance pausing and that the characteristics of this site are largely independent of DNA or RNA sequence at pause sites. Although previous experiments examining transcript elongation rates using homopolymer templates (Schmidt & Chamberlin, 1984a) have suggested that Nus A may increase pausing by binding to two sites, the data in this paper reveal only one binding site involved in Nus A caused increases in pausing on natural templates. The relationship of Nus A binding sites detected by transcription of homopolymers to binding sites affecting pausing and termination on natural templates is therefore not clear.

In vitro Nus A caused decreases of Rho-dependent termination are less likely to be relevant in vivo than are Nus A caused increases of pausing, as termination is affected by Nus A binding sites which are much weaker than the binding site involved in pausing. In addition, the effects of Nus A on Rho-dependent termination are partially mimicked by nonspecific RNA-binding proteins. It therefore seems safest at present to assign potential in vivo importance only to the Nus A binding site that affects pausing in vitro. Additional experiments will be required to firmly determine if Nus A affects

pausing or termination in vivo by binding to the site that increases pausing in vitro, to firmly identify this site, and to identify and determine the in vivo significance of the Nus A binding site that affects termination in vitro.

Since our experiments show that Nus A reduces Rho-dependent termination and increases Rho-independent termination by binding to a site that has a lower affinity for Nus A than the binding site which affects pausing, the idea that Nus A reduces Rho-dependent termination or increases Rho-independent termination in vivo is without substantial experimental support. Therefore, we propose that Nus A might in fact increase Rho-dependent termination in vivo by enhancing pausing upstream of termination sites, facilitating the efficient interaction of Rho with RNA polymerase. This proposal is at present supported solely by the observation that much less Rho is required for termination in the presence of Nus A (Platt, 1981). Although Nus A can at best impede Rho-dependent termination at high Rho concentrations (this paper), it is not certain that events requiring high Rho concentrations reproduce in vivo events. Perhaps the role of Nus A caused pausing in assisting the interaction of RNA polymerase and Rho has obtained little other experimental support because of the customary and deliberate in vitro use of Rho concentrations that enable maximal productive interaction of Rho and RNA polymerase even in the absence of Nus A.

Although few available data bear directly on the problem, it remains possible that all observed in vivo defects in *nusA* mutants result solely from defects in in vivo pausing. For example, Nus A may facilitate transcriptional–translational coupling both in vitro and in vivo, perhaps by causing RNA polymerase to pause until ribosomes catch up (Ward & Gottesman, 1981; Kung et al., 1975; Landick & Yanofsky, 1984). The involvement of Nus A in termination or antitermination events (Friedman et al., 1984) could be a straightforward use of strategically placed sites to allow other proteins to bind efficiently to transcription complexes. Nus A enhanced pausing conceivably might also promote transcript release at certain types of termination sites or provide time for the formation of RNA secondary structural elements important to termination or other events.

A more general conclusion apparent from our results is that the relative proportions and absolute concentrations of individual transcription components used in in vitro transcription experiments can strongly affect the outcome and interpretation of experiments. There are many other possible transcription mechanisms that could be revealed or fruitfully characterized by systematically varying the concentrations of components in in vitro transcription reactions.

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