

# Role of NusA in L4-mediated Attenuation Control of the S10 r-Protein Operon of *Escherichia coli*

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The transcription of the 11 gene S10 operon of *Escherichia coli* is autogenously regulated by one of the operon's products, ribosomal protein L4. This protein stimulates termination of transcription *in vivo* at a specific site within the S10 leader. The *in vivo* effect can be reproduced in a purified transcription system but requires an additional factor, NusA. Our earlier *in vitro* studies showed that NusA is required for RNA polymerase pausing at the termination site; such paused complexes are further stabilized by L4, which presumably accounts for L4's stimulation of termination *in vivo*. Here we show that NusA is not absolutely required for RNA polymerase to recognize the attenuation site: at low (5  $\mu$ M) UTP concentration, RNA polymerase pauses at the site, although the paused transcription complex formed in the absence of NusA can be further stabilized by subsequent addition of the protein. Furthermore, RNA polymerase pausing at the attenuation site is not sufficient for the L4 effect, since L4 cannot stabilize a transcription complex paused at the attenuation site in the absence of NusA. We have been able to isolate paused complexes formed without NusA and/or L4; such complexes are active upon re-addition of NTPs, and respond as expected to the addition of L4 or NusA. Our experiments are consistent with the notion that L4 is a stable component of a paused transcription complex.

**Keywords:** transcription termination; transcription pausing; NusA; r-protein L4; RNA polymerase

## Introduction

Transcription of the 11 gene S10 operon of *Escherichia coli* is regulated by ribosomal protein (r-protein) L4, the product of the third gene of the operon (Zengel *et al.*, 1980). L4 inhibits transcription of the S10 operon by causing premature termination within the S10 leader (Lindahl *et al.*, 1983; Zengel & Lindahl, 1990a,b). Our previous *in vitro* transcription studies showed that the L4 effect is dependent on addition of the transcription factor NusA (Zengel & Lindahl, 1990b; Zengel & Lindahl, 1991). NusA enhances RNA polymerase pausing at the attenuation site; such a pause is greatly stabilized by L4, presumably accounting for the ribosomal protein's ability to stimulate transcription termination (Zengel & Lindahl, 1992).

The NusA protein was originally identified as a host factor necessary for N and Q-dependent

antitermination in bacteriophage lambda (Friedman & Gottesman, 1983). In apparent contradiction to its role in antitermination, NusA increases the efficiency of *in vitro* termination of transcription at the lambda  $t_{R2}$  and *E. coli*  $rrnB$   $t_1$  terminators (Greenblatt *et al.*, 1981; Schmidt & Chamberlin, 1987). Furthermore, it has been shown in a number of systems that NusA slows the elongation rate of RNA polymerase by enhancing pausing at specific sites (reviewed by Yager & von Hippel, 1987). There are two components to this NusA inhibition of transcription elongation; one mode is competitive with the nucleoside triphosphate substrates, while the other is non-competitive with substrates and appears to be sequence-dependent (Schmidt & Chamberlin, 1984a). The pleiotropic roles for NusA led to the proposal that the protein serves as a general transcription "fidelity" factor, interacting with RNA polymerase to modulate the enzyme's response to other factors and to DNA/RNA signals (Greenblatt *et al.*, 1981).

To learn more about the role of NusA in L4-mediated attenuation control, we have dissected the *in vitro* transcription reaction. We show that by lowering the UTP concentration to 5  $\mu$ M, we can

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Abbreviations used: r-protein, ribosomal protein; RT, readthrough transcript; ATT, attenuated transcript.

provoke RNA polymerase pausing at the attenuation site in the absence of NusA, even though the pause is still weaker than in the presence of NusA. L4 fails to stabilize such a pause; upon the addition of NusA, L4 stimulation can be recovered. Hence, pausing itself is not sufficient for L4 function. Rather, protein-protein interactions involving NusA or a NusA-induced conformational change in the paused complex seem to be required for the L4 effect. We also studied the timing of NusA function and found that NusA can act after RNA polymerase has already entered the pause mode. Finally, we describe the properties of transcription complexes paused at the attenuation site and purified by gel filtration.

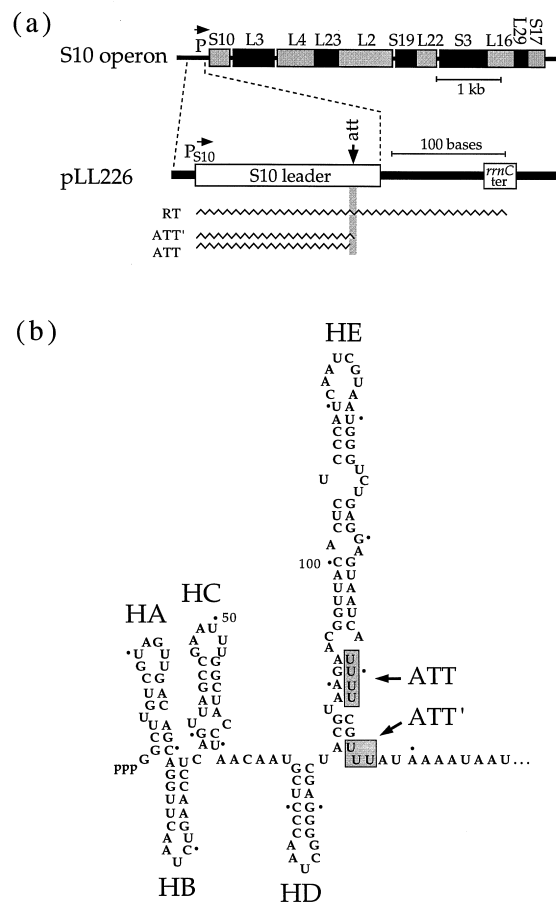
## Results

### Pausing at the attenuation site is not sufficient for L4's *in vitro* effect

The template for the *in vitro* transcription is supercoiled plasmid pLL226 (Zengel & Lindahl, 1990a,b), containing the S10 operon promoter and the proximal 165 bases of the S10 leader cloned upstream of the terminator from the *rrnC* operon (Figure 1(a)). Transcription of pLL226 gives rise to two classes of RNA: approximately 310 nucleotide "readthrough" transcripts (RT) terminated at the *rrnC* terminator and about 140 to 145 nucleotide "attenuated" transcripts (ATT and ATT') corresponding to molecules ending at the attenuator (Figure 1). Addition of purified r-protein L4 to the *in vitro* transcription reaction reproduces the *in vivo* effect of excess L4: increased synthesis of attenuated transcripts and decreased synthesis of readthrough RNA. However, the L4 effect is dependent on the presence of an additional protein, transcription factor NusA (Zengel & Lindahl, 1990b; Zengel & Lindahl, 1991).

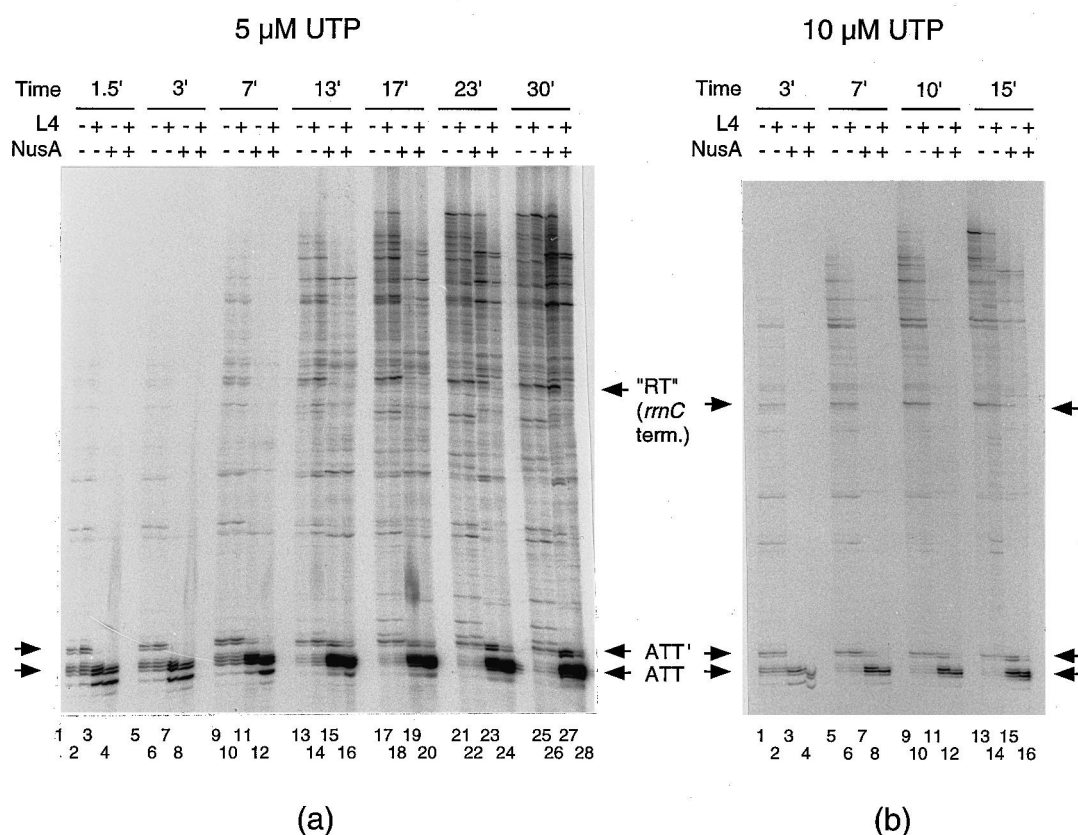
Earlier work (Zengel & Lindahl, 1992) showed that NusA induces RNA polymerase to pause at the site of L4-stimulated attenuation. Such paused complexes are then further stabilized by L4, presumably accounting for the r-protein's stimulation *in vivo* of premature termination of transcription. The half-life of the pause at the attenuation site is sensitive to the concentration of UTP, but at all concentrations tested (20 to 500  $\mu$ M), the pause was NusA-dependent and stimulated by L4 (data not shown). In the absence of NusA, there is little or no pausing by RNA polymerase, and L4 has no detectable effect (Zengel & Lindahl, 1990b, 1991, 1992, and data not shown).

We were interested in understanding the role of pausing in the L4-mediated attenuation control. Specifically, we wanted to know if L4's requirement for NusA reflected a general requirement for a stably paused transcription complex, or, alternatively, a more specific and direct requirement for NusA in the formation of a target for L4 control. To distinguish between these two possibilities, we needed to induce a stable RNA polymerase pause at the attenuator in the absence of NusA. We found that reducing the UTP concentration to 5 or 10  $\mu$ M



**Figure 1.** The *E. coli* S10 r-protein operon. (a) Maps of the S10 operon and plasmid pLL226. Plasmid pLL226 carries the indicated portion of the S10 leader upstream of the *rrnC* terminator (Zengel & Lindahl, 1990a,b). The site of L4-mediated attenuation is indicated by the vertical arrow labeled att. The transcripts generated from this template are shown below the map of pLL226. (b) The secondary structure of the S10 leader (Shen *et al.*, 1988). Sites of *in vivo* (ATT; Zengel & Lindahl, 1990a) and *in vitro* (ATT and ATT'; Zengel & Lindahl, 1990b) termination are indicated by shaded boxes.

resulted in a transcription pause at the attenuator even in the absence of NusA [see the ATT and ATT' bands in Figure 2(a), lanes 1 and 5, and Figure 2(b), lane 1). Hence, NusA is not required for RNA polymerase to identify and respond efficiently to the pause site. However, even at these low UTP concentrations, the complex is less stable than the NusA-containing complex. For example, by 13 minutes at 5  $\mu$ M UTP and ten minutes at 10  $\mu$ M UTP, most of the polymerases paused in the absence of NusA had elongated beyond the attenuator, while polymerases paused in the presence of NusA were still in the ATT/ATT' region (compare, e.g. lanes 13 and 15 of Figure 2(a), or lanes 9 and 11 of Figure 2(b)). One unexpected consequence of the lowered UTP concentration is a decreased efficiency of the *rrnC* terminator. Although a small fraction of the readthrough molecules correspond to RNAs terminated at this terminator (the "RT" band indicated in



**Figure 2.** Kinetics of transcription of the S10 leader at low UTP concentration. Single-round transcription reactions were performed using 25 nM plasmid pLL226 DNA, 2  $\mu$ Ci of [ $^{32}$ P]UTP and, where indicated, 40 nM NusA or 160 nM L4. The final UTP concentration was 5  $\mu$ M (a) or 10  $\mu$ M (b). Aliquots were removed and analyzed at the indicated times after the start of transcription elongation. ATT and ATT', attenuated transcripts; "RT", readthrough transcripts terminated at the *rrnC* terminator. Note that, at 5 or 10  $\mu$ M UTP, termination at the *rrnC* terminator is not efficient, resulting in a significant amount of radioactivity in bands larger than the RT (*rrnC*) band.

Figure 2), most of the radioactivity was distributed among a large number of longer molecules.

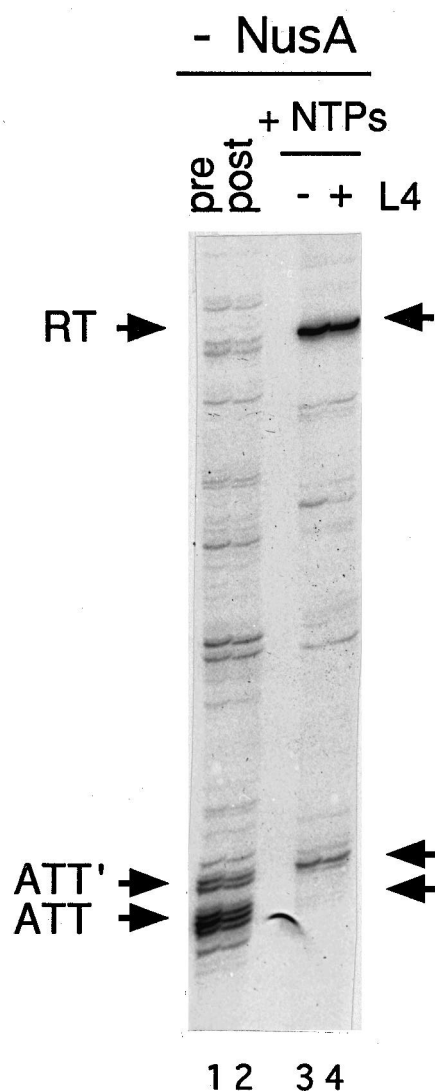
Using the low UTP reaction condition, we tested the effect of L4 on paused complexes formed with or without NusA. At 5  $\mu$ M UTP, the NusA-induced paused complexes are exceptionally stable, so that any L4 effect is obscured. However, after prolonged incubation ( $\geq 20$  minutes), a small L4 effect can be detected as a slower progression of RNA polymerase from ATT to ATT' (compare the intensities of the ATT' bands in Figure 2(a), lanes 23 and 24, or 27 and 28). In the absence of NusA, most of the RNA polymerases remained at the ATT/ATT' site for as long as seven minutes after the start of transcription (Figure 2(a), lanes 9 and 10). However, L4 had no effect on the stability of transcription complexes paused in the absence of NusA (Figure 2(a), lanes 1 versus 2, 5 versus 6, or 9 versus 10). By 13 minutes, with or without L4, most of the RNA polymerases in the NusA-less reactions had left the pause site and continued elongation (Figure 2(a), lanes 13 and 14). Similar results were obtained at 10  $\mu$ M UTP (Figure 2(b)): L4 had no effect on the stability of transcription complexes paused in the absence of NusA. Our conclusion from these experiments is that RNA polymerase pausing at the attenuation site is not

sufficient for L4 action. The dependence of L4 function on the addition of NusA suggests that either protein-protein interactions involving NusA or a NusA-induced conformational change in the transcription complex is required for L4-mediated attenuation control.

#### Purification and characterization of transcription complexes paused at the attenuator

To facilitate further characterization of the paused transcription complex, we developed a procedure for purifying paused transcription complexes by gel filtration. The integrity of the purified complexes was confirmed by their ability to resume transcription elongation upon the provision of NTPs. As shown in Figure 3, paused complexes formed in the presence of NusA (at 5  $\mu$ M UTP) were capable, after purification and addition of NTPs (100  $\mu$ M each, including UTP), of continuing elongation to the *rrnC* terminator (Figure 3, lanes 3 to 6). The purified paused complexes were sensitive to the addition of L4 to the extension reaction (Figure 3, lanes 3 and 4 compared to lanes 5 and 6), consistent with our previous observation that L4 can act even if added

Since RNA polymerase was capable of pausing at the S10 attenuator in the absence of NusA (at sufficiently low UTP concentration), we were interested in knowing whether NusA could stabilize such a paused transcription complex if added *after* RNA polymerase had already reached the pause site. We repeated the 5  $\mu$ M UTP transcription reaction, adding NusA to the reaction either before or at 1, 2.5, or 5 minutes after the start of transcription elongation. Under these conditions, most RNA polymerases should reach the pause site by one minute of elongation (data not shown). The results of this experiment, shown in Figure 5, indicate that NusA can indeed act on RNA polymerases already paused at the attenuation site. Even when added five minutes after the start of transcription, NusA was still able to enhance pausing. The late NusA effect was especially apparent when the paused complexes were “chased” with 100  $\mu$ M UTP: while most of the RNA polymerases paused in the absence of NusA could be chased out by 100  $\mu$ M UTP, the late addition of NusA resulted in a marked stabilization of the



**Figure 4.** Response to L4 of purified transcription complexes paused in the absence of NusA. Paused transcription complexes were formed at 5  $\mu$ M UTP in the absence of NusA and purified by gel filtration. Pre: RNA isolated from paused transcription complexes before gel filtration. Post: RNA isolated from purified complexes after incubation at 37°C for 5 min in the absence of NTPs. Transcription was resumed by adding NTPs to 100  $\mu$ M (" + NTPs" lanes). Where indicated, L4 was added to 160 nM. ATT and ATT', attenuated transcripts; RT, readthrough transcripts terminated at the *rnc* terminator.

paused complexes (compare lanes 5 with 6, 11 with 12, and 17 with 18 in Figure 5).

To characterize further the timing of NusA action, we used purified transcription complexes paused in the absence of NusA to analyze the effect of late NusA addition. Elongation was resumed in the presence or absence of NusA (Figure 6). When NusA was added simultaneously with the NTPs, little stabilization effect was observed (Figure 6, lanes 3 and 4). However, when the complex was incubated with NusA for one or three minutes before the addition of NTPs, a clear effect of NusA was

observed (Figure 6, lanes 5 and 6, and 7 and 8). These results suggest that it takes about one minute for NusA to achieve its optimal activity in stabilizing the paused transcription complex under our *in vitro* experimental conditions.

The results from the experiments shown in Figures 5 and 6 indicate that NusA can modify the activity of RNA polymerase already paused at the attenuation site, and hence is not required as the enzyme transcribes the upstream sequence.

#### **L4 cannot bind productively to a paused transcription complex formed in the absence of NusA**

Since L4 cannot stabilize a paused transcription complex formed in the absence of NusA, we wanted to know whether L4 depends on NusA in order to join the paused complex, or, alternatively, L4 binds to the complex but cannot exert its effect unless NusA is present. We were not able to distinguish between these two possibilities directly by looking for L4 protein in the purified complexes, since L4 appears to bind non-specifically to these complexes (discussed above). Therefore, we tried a kinetic approach. Using purified transcription complexes formed in the absence of NusA, we asked if the addition of L4 several minutes before the addition of NusA and NTPs resulted in greater L4 stabilization than addition of L4 simultaneous with or just after the addition of NusA and NTPs. The scheme for the experiment is outlined in Figure 7(a).

We assumed that, if L4 could join the paused transcription complex independently of NusA, then pre-incubation of L4 with the paused complex might accelerate the L4 effect once transcription elongation resumed in the presence of NusA. If L4 could not bind productively in the absence of NusA, then pre-addition of the protein should not increase its stimulatory effect. (Based on the results of previous late L4 addition experiments (Zengel & Lindahl, 1992) we knew that L4 could stabilize a NusA-containing paused complex within 30 seconds after addition.) As shown in Figure 7(b), preincubation of L4 for two minutes with a complex lacking NusA did not enhance the L4 effect once NusA and NTPs were added. On the contrary, while addition of L4 simultaneous with or immediately after addition of NusA and NTPs resulted in enhancement of the pause (Figure 7(b), lanes 2 versus 3, or 5 versus 6), addition of L4 before NusA appeared to reduce its ability to stabilize the pause (compare lane 1 to lane 2, and lane 4 to lane 5).

These results suggest that, in the absence of NusA, L4 binds to one or more of the components of the transcription reaction in a non-specific (or non-productive) way, and this non-specific binding impedes L4's ability to stabilize the paused complex once NusA is added. The presence of NusA in the transcription complex facilitates "proper" L4 interaction with the paused complex, perhaps because NusA induces a conformational change in the

complex that makes the L4 target more accessible. A second possibility is that the L4 effect is mediated by a direct interaction with NusA. In any case, RNA polymerase must first be modified by NusA before L4 can affect the fate of transcription elongation.

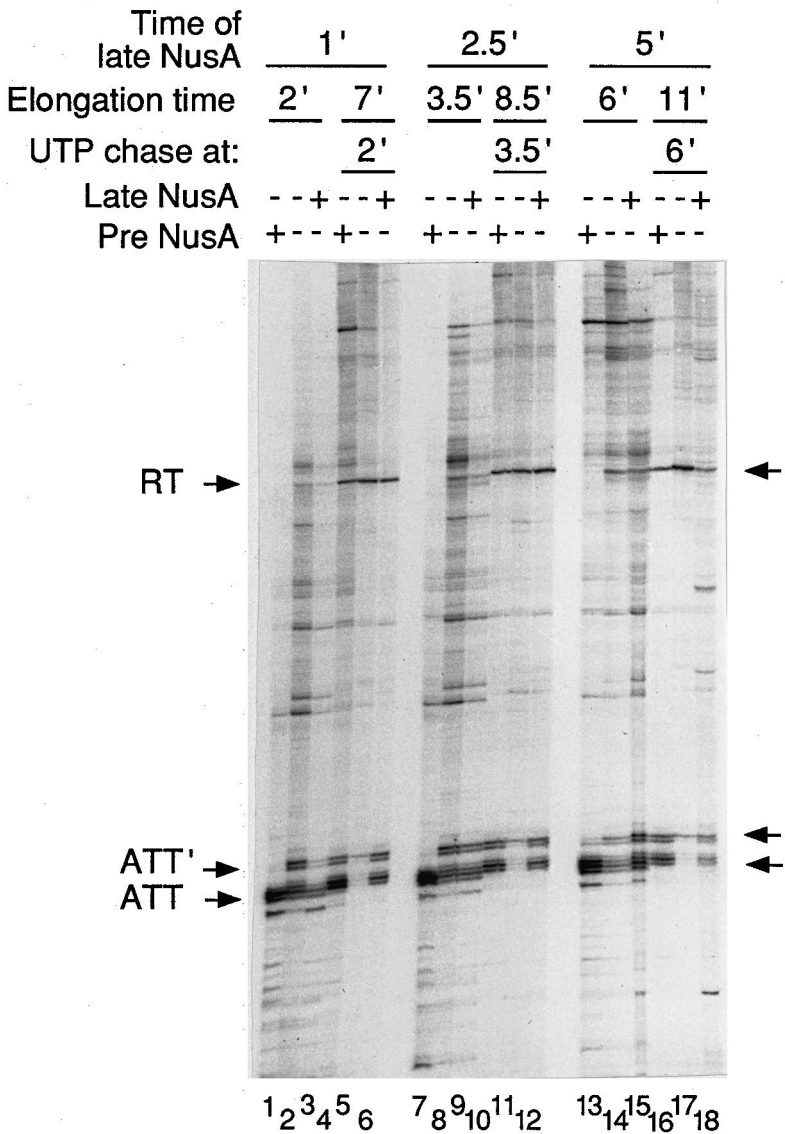
Release of attenuated transcripts

As we have noted previously (Zengel & Lindahl, 1992), our *in vitro* transcription system does not efficiently carry out the final step in transcription termination, namely, release of the nascent transcript from the paused complex. This conclusion was based on our observation that many of the RNA polymerases paused at the S10 attenuator could be efficiently chased out of the pause site by the addition of high concentrations of UTP (Zengel & Lindahl, 1992). To address this issue more directly, we used nitrocellulose filtration to assay the amount of ATT/ATT' RNA released from the paused complex (Schmidt & Chamberlin, 1987). The results

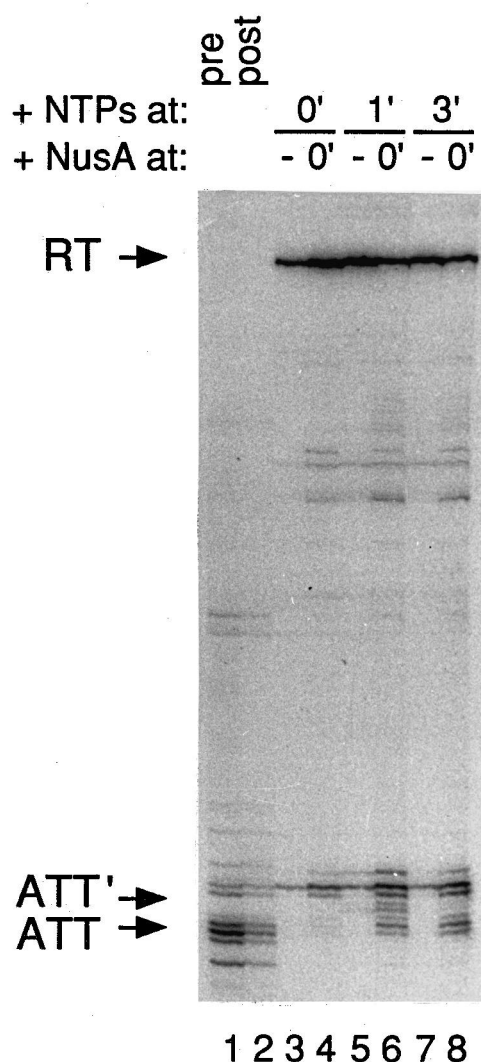
are shown in Figure 8. At both 5  $\mu$ M and 100  $\mu$ M UTP, at least half of the radioactivity in bands corresponding to ATT/ATT' RNA was retained on the nitrocellulose filters (Bound lanes in Figure 8), representing RNA molecules still bound stably in the paused transcription complex. Nevertheless, a significant fraction of the radioactivity in this region was found in the flow-through fraction (Released lanes in Figure 8), together with a band of radioactivity labeled "X" (corresponding to an RNA molecule synthesized from elsewhere on the plasmid template) which fractionated exclusively with the flow-through. We conclude that a portion of the "attenuated" transcripts are truly released transcripts.

Discussion

Transcription pausing is involved in several important aspects of regulation of transcription, including rho-dependent termination (reviewed by



**Figure 5.** Effect of late NusA addition on transcription pausing. Single-round transcription reactions were performed using standard conditions at 5  $\mu$ M UTP. The Pre NusA reactions contained NusA (40 nM) from the start. Late NusA reactions were started in the absence of NusA; the protein was added (to 40 nM) at the indicated times after the start of transcription elongation. For the UTP chase, aliquots of each reaction received UTP (to 100  $\mu$ M) at the indicated times after the start of transcription. The Elongation time of each reaction refers to the time after the start of transcription elongation at which the reaction was terminated by the addition of phenol. ATT and ATT', attenuated transcripts; RT, readthrough transcripts terminated at the *rrnC* terminator.



**Figure 6.** Response of purified paused transcription complexes to the late addition of NusA. Paused transcription complexes were formed in the absence of NusA and purified by gel filtration. Lane 1 shows RNA isolated from complexes before gel filtration. Lane 2 shows RNA isolated from purified complexes incubated in the absence of NTPs at 37°C for 5 min. Lanes 3 to 8 show RNA isolated from purified complexes that have been incubated with NTPs at 37°C in the absence (–) or presence (0') of NusA (40 nM). NTPs were added to 100  $\mu$ M immediately before (0') or at 1 min or 3 min after the start of the incubation. The total incubation time was 5 min. ATT and ATT', attenuated transcripts; RT, readthrough transcripts terminated at the *rrnC* terminator.

Yager & von Hippel, 1987; Platt, 1994), Q-dependent antitermination in phage lambda (Grayhack *et al.*, 1985), and translation-coupled attenuation (for reviews, see e.g. Landick & Yanofsky, 1987b; Yager & von Hippel, 1987; Yanofsky, 1988). The specific signal for pausing is not always obvious, although in many cases a pause site is preceded by a region encoding a stable RNA hairpin structure (e.g. see Levin & Chamberlin, 1987; Arndt & Chamberlin, 1990; Yang & Gardner, 1991). Additional factors influencing

RNA polymerase pausing include the DNA sequence downstream of the pause site (Levin & Chamberlin, 1987; Lee *et al.*, 1990), the availability of nucleotide substrates (Levin & Chamberlin, 1987; Yager & von Hippel, 1987), and the presence of ancillary transcription factors such as NusA (Yager & von Hippel, 1987). Pausing by RNA polymerase may be a critical parameter in gene expression, since it is at pause sites that RNA polymerase spends most of its time, and modifications to the RNA polymerase by other factors can occur (reviewed by Yager & von Hippel, 1987; also see Kassavetis & Geiduschek, 1993).

Previous workers have proposed that pausing of the transcription complex at a terminator is a necessary prelude to termination (see, e.g. Farnham & Platt, 1981; Kassavetis & Chamberlin, 1981; Kingston & Chamberlin, 1981; Yang & Roberts, 1989). In the case of rho-dependent termination, pausing will influence the ability of rho to reach the ternary complex to facilitate release of the nascent RNA (Jin *et al.*, 1992). For rho-independent termination, the pause may disclose a position at which RNA polymerase undergoes a conformational change that readies it for subsequent termination (Yang & Roberts, 1989). A correlation between pausing and termination has been noted previously in a number of systems. For example, Fisher & Yanofsky (1983) described two RNA polymerase mutants affecting *trp* operon regulation: one enzyme selected for decreased termination at the *trp* attenuator was also defective in pausing at the *trp* 1:2 stem pause site, while a mutant selected for increased termination at the attenuator also exhibited enhanced pausing in the *trp* leader pause site (Fisher & Yanofsky, 1983). Also, Q-mediated antitermination in lambda involves Q's ability to suppress RNA polymerase pausing at a variety of pause sites (Yang & Roberts, 1989). Suppression of pausing by N protein is also associated with its ability to prevent termination (Das, 1992; Mason *et al.*, 1992).

Our *in vitro* kinetic analysis of the transcription of the S10 leader (Zengel & Lindahl, 1992; this study) suggests that L4-mediated attenuation control is the product of three distinct pausing responses by RNA polymerase: NusA-independent pausing; NusA-dependent pausing, and L4-stimulated pausing. Three corresponding pausing signals in the nascent S10 leader transcript have also been identified (see the accompanying paper, Sha *et al.* (1995)). A model accounting for these steps is summarized in Figure 9. According to this tentative scheme, RNA polymerase can recognize the pause site even if NusA is not present, although *in vitro* this pause is detectable only at a low UTP concentrations. Without NusA the paused RNA polymerase quickly resumes elongation. If NusA is incorporated into the transcription complex, the pause is significantly prolonged. Our experiments do not exclude the possibility that NusA can also modify the elongating RNA polymerase *before* the enzyme has reached the pause site. However, the results suggest that NusA does not *need* to join the transcription complex until

the pause site is reached (although, in our late NusA addition assay, the NusA effect was not instantaneous: the full NusA effect requires approximately one minute incubation (at 37°C) with the paused complex). Once NusA has modified the paused transcription complex, L4 can mediate its effect, further stabilizing the NusA-dependent pause to form what we believe may be the pre-termination complex.

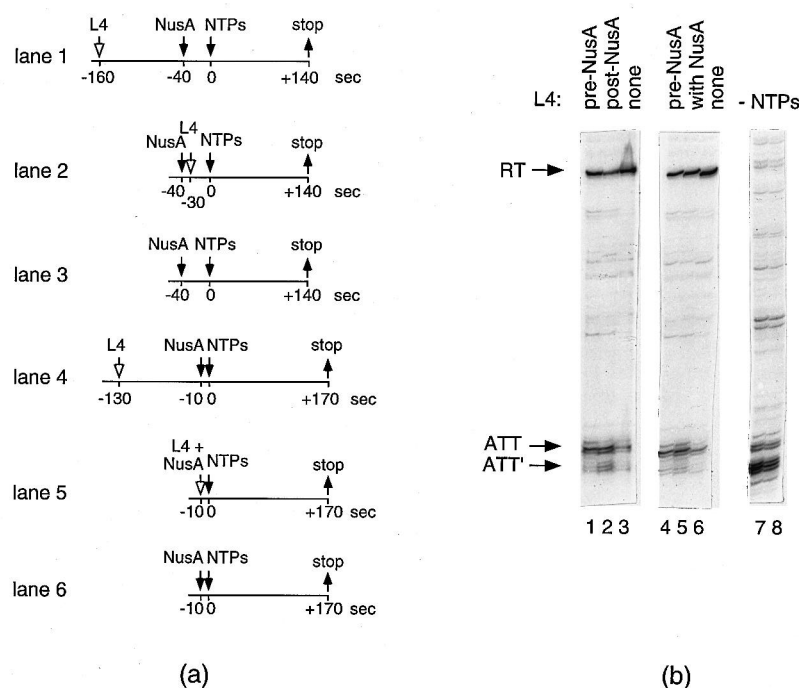
The *in vitro* transcription studies reported here confirm our previous conclusion that NusA is necessary for L4-mediated attenuation control (Zengel & Lindahl, 1990b, 1991, 1992). The NusA requirement is not simply a need for a stable paused complex, since RNA polymerase paused without NusA at low UTP concentration still does not respond to L4. Hence, our studies are consistent with the idea that NusA plays a specific role in the L4 effect, either by inducing a conformational change in the paused complex that allows RNA polymerase to respond to L4, or by mediating the L4 effect *via* direct protein-protein interactions between L4 and NusA (or both).

NusA binds strongly to core RNA polymerase, in a way that is mutually exclusive with binding of sigma factor (Greenblatt & Li, 1981a; Greenblatt *et al.*, 1985). Recent experiments by Zhang & Hanna (1994) have shown that NusA changes the conformation of RNA polymerase at the binding site for the 3' end of the nascent RNA transcript. Such a conformational change presumably accounts for some of NusA's effects on transcription, such as enhanced pausing at certain hairpin structures like the 1:2 stem in the *trp* leader (Farnham *et al.*, 1982; Landick & Yanofsky, 1984), and enhanced termination at the lambda trR2, *rrnB* T1 (Greenblatt *et al.*, 1981; Schmidt &

Chamberlin, 1987) and *trp* t terminators (Farnham *et al.*, 1982). Its effect on the response of RNA polymerase to potential pause and termination structures in the nascent RNA can therefore explain NusA-enhanced RNA polymerase pausing at the attenuator in the S10 leader (Zengel & Lindahl, 1992, this paper). Compared to other *in vitro* systems in which NusA has a stimulatory effect on pausing or termination, the S10 attenuator has an extremely stringent requirement for the protein.

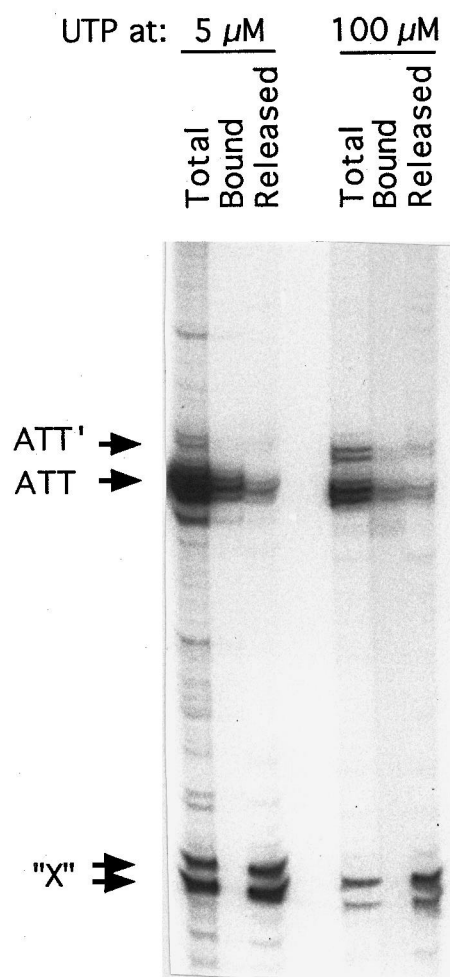
In cases where NusA modifies RNA polymerase behavior in the presence of additional proteins, the NusA effect could also reflect either a direct role as an adaptor for binding other factors or an indirect role in which a NusA-induced conformational change in RNA polymerase facilitates protein binding to the enzyme. For N-mediated antitermination, a direct role is suggested by experiments showing that NusA binds tightly to N protein, thereby coupling N to RNA polymerase (Greenblatt & Li, 1981b). A similar role for NusA has been implicated in rho-mediated termination (Schmidt & Chamberlin, 1984b). The role of NusA in L4-mediated attenuation control is still an open question. The experiments presented here suggest that NusA must be a component of the transcription complex in order for L4 to bind productively, but it is not clear if L4 interacts directly with NusA or with a NusA-modified RNA polymerase. Furthermore, the r-protein's requirement for specific RNA determinants within the S10 leader (Zengel & Lindahl, 1992; accompanying paper) suggests that L4 also binds to nascent RNA in the paused transcription complex.

The NusA and RNA sequence requirements for L4's regulation of termination are reminiscent of the N-dependent antitermination system. For example,



**Figure 7.** L4 binds non-productively to a paused transcription complex in the absence of NusA. Paused transcription complexes formed at 5  $\mu$ M UTP in the absence of both NusA and L4 were purified by gel filtration. NusA, L4 and NTPs were added according to the scheme outlined in (a). The RNAs isolated from the reactions are shown in lanes 1 to 6 of (b). Lane 7, RNA isolated from paused complexes immediately after gel filtration. Lane 8, RNA isolated from purified complexes that had been incubated at 37°C for 5 min in the absence of NTPs. ATT and ATT', attenuated transcripts; RT, readthrough transcripts terminated at the *rrnC* terminator.





**Figure 8.** Release of *in vitro* transcripts at the attenuation site. Paused transcription complexes were formed in the presence of NusA and 5  $\mu$ M or 100  $\mu$ M UTP. Aliquots (40  $\mu$ l) were removed after 5 min elongation. Total, unfractionated RNA; Bound, RNA trapped on a nitrocellulose filter and eluted by boiling; Released, RNA isolated from the filtrate fraction. Bands "X" correspond to RNA transcribed from elsewhere on the plasmid template.

N action is also dependent on the presence of NusA (Horwitz *et al.*, 1987). Also, the N protein requirement for a nut site (specifically, the *boxB* hairpin) in the RNA (Barik *et al.*, 1987; Horwitz *et al.*, 1987; Lazinski *et al.*, 1989; Nodwell & Greenblatt, 1991) may be analogous to L4's requirement for a signal within the S10 leader RNA upstream of the pause site (Zengel & Lindahl, 1992; accompanying paper). One apparent difference between the two systems is the involvement in the lambda system of factors in addition to NusA and N, including NusB, NusE, and NusG (Greenblatt *et al.*, 1993), although these ancillary factors are apparently necessary only to ensure long-distance propagation of the antitermination effect. Our *in vitro* transcription studies have not detected a similar role for these factors in L4-mediated termination control (Zengel & Lindahl,

1990b). Also, no pausing at the *boxB* hairpin has been implicated in N-dependent modification of RNA polymerase function (Whalen & Das, 1990). A more obvious difference is that the effect of N is to generally suppress pausing by RNA polymerase (Das, 1992; Mason *et al.*, 1992), while L4 enhances pausing at the attenuator site. However, this apparent incongruity may not be so worrisome, given the observation of a striking similarity between lambda N-dependent antitermination and the related phage HK022's Nun-dependent termination (Robert *et al.*, 1987). The Nun protein can be converted from a terminator protein to an antiterminator in the presence of specific lambda nutR mutations or mutated Nus protein components, leading to the suggestion that termination and antitermination are closely related processes (Robert *et al.*, 1987; Robledo *et al.*, 1990). In this respect it may be more relevant to consider the analogy between Nun and L4.

Our *in vivo* measurements of the transcription rate of the S10 operon during balanced growth and during L4-mediated autogenous control (when excess L4 leads to decreased synthesis of structural gene mRNA) indicate that L4 control does not affect the yield of RNA corresponding to the region of the leader upstream of the attenuation site (Lindahl *et al.*, 1983; Zengel & Lindahl, 1990a). Since stable pausing would quickly lead to a "queueing" of RNA polymerases and hence to inhibition of further initiation, our *in vivo* data can only be explained by *bona fide* termination (i.e. release of RNA polymerase and RNA) at the attenuator. However, under our conditions for *in vitro* transcription, the paused ternary complex containing L4 is very stable, as measured by our ability to chase out most of the paused polymerases by addition of higher UTP concentration (Zengel & Lindahl, 1992) and by gel filtration experiments reported here showing that most of the attenuation transcripts are recovered in the excluded volume. Our tentative model for L4-mediated attenuation control (Figure 9) assumes that this L4-stimulated NusA-dependent pause is a prerequisite for termination at the S10 attenuator, but that the conditions of our *in vitro* transcription reaction fail to reproduce the last step of the attenuation pathway. Perhaps the real termination step *in vivo* requires extra release factors that are not present in our *in vitro* system.

The connection between pausing and termination in the S10 leader is not yet clear. The 3' ends of attenuated transcripts isolated *in vivo* correspond to the 3' ends of the ATT bands from the *in vitro* system (Zengel & Lindahl, 1990b). However, we cannot exclude the possibility that ATT serves as an important pause site, but termination *in vivo* actually occurs downstream, for example at the ATT' site also observed as a pause site *in vitro*. Rapid nucleolytic trimming of the *in vivo* 3' ends might generate transcripts corresponding to the ATT bands. Alternatively, since RNA molecules corresponding to both ATT and ATT' are released in the *in vitro* reaction, both the ATT and ATT' sites may serve as

termination sites *in vivo*. In any event, we plan to exploit the stability of the “pre-termination” complex to study its structural details.

We had hoped that our ability to purify active paused transcription complexes would allow us to determine if L4 is a stable component of the transcription apparatus. However, with our current purification scheme the protein appears to associate with a complex even using DNA templates on which it has no detectable function. We are now pursuing other technical approaches to determine the protein composition of the paused transcription complex. We also hope to resolve the question of NusA's presence in the complex. Landick & Yanofsky (1987a) had observed that isolated *trp* leader paused transcription complexes formed in the presence of NusA behaved as though they had lost NusA during the purification procedure: addition of NusA to the purified complexes on the resumption of elongation further stabilized the paused complex. Their results are consistent with the observation that NusA is non-processive (Schmidt & Chamberlin, 1984a). Our results with the S10 leader paused complex, on the other hand, indicated that NusA was still present, or had left a permanent “mark” on the paused complex: RNA polymerase was still stably paused at the attenuator, and addition of more NusA during the resumption of elongation had no effect. However, because our purification technique may not eliminate NusA protein associated with RNA polymerases from elsewhere than the S10 attenuator, we cannot exclude the possibility that these NusA molecules join the paused complex during the resumption of elongation. Again, we are investigating other approaches to resolve this issue.

One interesting observation from our experiments is the effect of low UTP concentration on the efficiency of the downstream *rrnC* terminator. While low UTP enhanced RNA polymerase pausing at the attenuator, it essentially eliminated termination at the *rrnC* terminator. Even when ternary complexes were formed at low UTP but then chased with high (100  $\mu$ M) UTP, the downstream *rrnC* terminator was

poorly recognized. Interestingly, when ternary complexes formed at low UTP were purified before the addition of high UTP (and other NTPs), the *rrnC* terminator was more efficiently recognized (data not shown). These results suggest that during the process of purification of the ternary complexes, the activity of RNA polymerase is somehow modified.

## Materials and Methods

### Plasmids

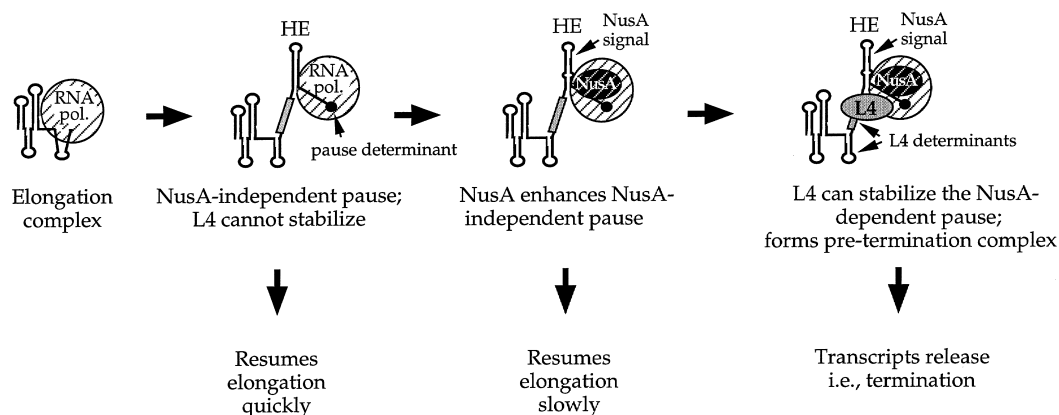
Plasmid pLL226 has been described (Zengel & Lindahl, 1990a,b). Plasmid DNA was purified by CsCl banding.

### Materials

RNA polymerase was a gift from E. Morgan (Roswell Park Memorial Institute, Buffalo, NY). Purified r-protein L4 was from K. Nierhaus and P. Nowotny (Max Planck Institute, Berlin, Germany). Antibody to L4 was from M. Nomura (University of California, Irvine). NusA protein was purified in this laboratory according to the method of Schmidt & Chamberlin (1984a). Ultrapure nucleoside triphosphates were purchased from Pharmacia. Heparin was purchased from USB. [ $^{32}$ P]UTP (3000 Ci/mmol) was purchased from New England Nuclear. Nitrocellulose filters (20 mm, BA85) were purchased from Schleicher and Schuell. All other chemicals were purchased from Sigma.

### *In vitro* transcription reactions

Standard 40- $\mu$ l transcription reactions contained 20 mM Tris-acetate (pH 7.9), 4 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM DTT, 100 mM potassium glutamate, 50  $\mu$ g/ml bovine serum albumin, 20 nM RNA polymerase, and 20 to 25 nM supercoiled plasmid DNA. Where indicated, NusA was added to 40 nM, and L4 was added to 120 to 160 nM. These reaction components were mixed together with 500  $\mu$ M each CTP and GTP, and incubated at 37°C for ten minutes to allow formation of the initiation complex and incorporation of the proximal three nucleotides (pppGGC). A single round of transcription elongation was then started by addition of ATP to 500  $\mu$ M, UTP to 100  $\mu$ M (or lower where indicated), 2 to 10  $\mu$ Ci of [ $^{32}$ P]UTP, and, in some cases, rifampicin or heparin to



**Figure 9.** Model for L4-mediated transcription attenuation pathway. The identification of the determinants for NusA-dependent and independent pausing and for L4 stabilization of the paused complex is described in the accompanying paper.

10 µg/ml. (Control experiments showed that rifampicin and heparin had no effect on the kinetics of transcription in our system, presumably because all active RNA polymerases are bound to DNA prior to transcription elongation and fail to recycle upon reaching a transcription terminator.) Reactions were terminated at the indicated times by the addition of 40 µl of 50 mM EDTA containing 10 µg of yeast carrier RNA. The RNA was extracted with 40 µl of phenol and 40 µl of chloroform/isoamylalcohol (24:1, v/v), precipitated with ethanol, and fractionated on a 8% (w/v) sequencing gel.

For the transcript release assay, 80 µl reactions were performed in the presence of both NusA and L4 at 5 or 100 µM UTP. Transcription elongation was allowed for five minutes before quenching with EDTA (final concentration 10 mM). A 40 µl aliquot was mixed with 40 µl of 50 mM EDTA containing 10 µg of yeast carrier RNA, extracted with 40 µl of phenol and 40 µl of chloroform/isoamylalcohol (24:1), and then precipitated with ethanol. Another 40 µl aliquot was passed through a nitrocellulose filter (20 mm, BA85), followed by 2 × 100 µl washes with buffer B containing 250 mM KCl, 50 µg yeast carrier RNA and 10 mM EDTA. The pass-through fraction was collected and the RNA was purified by ethanol precipitation. The filter was cut into slices, placed in a microcentrifuge tube containing 200 µl of 10 mM EDTA and 40 µg yeast carrier RNA, and boiled for five minutes. The aqueous fraction was transferred to another microtube, and RNA was purified by ethanol precipitation.

#### Purification of paused transcription complexes

Paused transcription complexes were formed using the indicated reaction conditions, allowing transcription elongation for two minutes. The reactions were chilled on ice and all subsequent operations were performed in the cold room. Complexes were passed through a gel filtration column (Sephacrose 4B, cross-linked) previously equilibrated with buffer A (20 mM Tris-acetate (pH 7.9), 4 mM magnesium acetate, 0.1 mM EDTA, 130 mM KCl, 10 µg/ml rifampicin or heparin, 50 µg/ml bovine serum albumin and 0.1 mM DTT). Fractions containing ternary complexes (as determined by scintillation counting of aliquots from each fraction) were pooled. Transcription elongation was resumed by adding NTPs (100 µM to 1 mM) to a 40 µl aliquot in the presence or absence of L4 and NusA as indicated. Elongation reactions were terminated at the indicated times and RNA products were analyzed as described above. For protein analysis, bovine serum albumin was omitted from the gel filtration buffer. A 200 µl aliquot of pooled complexes was mixed with 1 ml of ice-cold acetone and then vortexed for at least 30 seconds (Hames, 1981). The protein-acetone mixture was incubated at -20°C for two hours and then centrifuged for 15 minutes at 27,000 g (Sargent, 1987). The protein pellet was washed repeatedly with ice-cold acetone, dried in a Speed Vac, and resuspended in 40 µl of Laemmli sample buffer (Laemmli, 1970). Protein samples were electrophoresed on a SDS-polyacrylamide gel (Laemmli, 1970), followed by silver staining or standard Western blot protocol using anti-L4 polyclonal antibodies (Harlow & Lane, 1988).

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