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# The attenuator of the tryptophan operon in E.coli: rho-mediated release of RNA polymerase from a transcription termination complex in vitro

Robert S.Fuller and Terry Platt *

Department of Molecular Biophysics and Biochemistry, Yale University, 333 Cedar Street, New Haven, CT 06510, USA

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## ABSTRACT

*In vivo*, termination of transcription at the attenuator site of the tryptophan (*trp*) operon of *E. coli* is influenced by the protein termination factor rho. *In vitro*, termination does not depend on rho factor, and is very efficient in a purified system consisting only of RNA polymerase, the DNA template, nucleoside triphosphates, and buffer. The extent of termination in this system is unaffected over a wide range of salt and nucleoside triphosphate concentration. However, there is a 10-fold stimulation of *trp* leader mRNA synthesis if rho factor is present during the transcription reaction. This stimulation occurs only at low molar ratios of polymerase to template, and can be blocked by rifampicin. It is thus most likely due to the recycling of RNA polymerase molecules that have been released from the attenuator site by rho factor. In fact, transcription of the *trp* leader region *in vitro* results in the formation of a stable termination complex which can be observed on sucrose gradients or by binding to nitrocellulose filters. These data indicate that a major function of rho at the *trp* attenuator is to release completed transcripts from a pre-formed termination complex, rather than to cause the cessation of elongation.

## INTRODUCTION

Termination of transcription is an important element in the regulation of gene expression, and can provide a crucial modulating effect on the levels of RNA elongated from initiations at promoter sites (1-3). The actual response at a given site may depend on a positive interaction, such as by the termination factor rho (4), or a negative interaction, such as with the bacteriophage λ N-protein (3). At the *trp* operon attenuator site the interaction is even more complex, depending on the levels of charged $tRNA^{Trp}$ in the cell (5). In other systems, termination of transcription may be completely independent of additional factors (3,6). Rho factor and its properties have been extensively characterized (2,7,8) but the details of the molecular interactions are still unclear.

In the tryptophan (*trp*) operon of *E. coli*, there are two major sites of transcription termination. One, the attenuator (*trp a*) is located very near

 

the 5' end of the operon, while the other, the terminator (trp t) is responsible for final termination of the 7000 nucleotide long trp mRNA just after transcription of the last structural gene. Both of these sites require rho function in vivo for maximum termination to occur (9,10). At the attenuator, termination of transcription in vitro is independent of rho, and is 95% efficient (11,12). A possible explanation for this discrepancy is that termination in vivo is "coupled" and occurs in a concerted fashion, while in vitro it proceeds more slowly in a series of discrete steps. It has been shown, for example, that when RNA polymerase is transcribing in vitro, it may "pause" at certain sites (13-15). Howard et al. (16), studying transcription of λ 4S oop mRNA, have shown not only that RNA polymerase pauses, but that the addition of rho factor stimulates release of the polymerase and permits recycling for more rounds of transcription. However, in this system the termination complex itself is quite unstable (even in the absence of additional factors) with a half-time of dissociation of less than 3 minutes. We have undertaken similar studies with the tryptophan attenuator based on the in vitro system developed by Lee et al. (11) and their preliminary evidence that the termination complex at trp a is relatively stable.

## MATERIALS AND METHODS

Ribonucleoside triphosphates were obtained as sodium salts from Sigma or P.L. Biochemicals. $\alpha$-$^{32}$P GTP and UTP were obtained at a specific activity of 10-50 Ci/mmole from NEN. RNA polymerase, with a sigma to core ratio of 0.7, was a gift of Dr. Richard Burgess. Rho factor, greater than 90% pure, was a gift of Dr. Martin Rosenberg. An E. coli strain carrying the plasmid pKB3 was a gift of Dr. Charles Yanofsky. This plasmid contains the R1 fragment of λ trpED10 inserted into the single R1 site of pCR1. It includes trp sequences from trp p to the secondary λ attachment site in trpC. Purified pKB3 DNA was isolated from chloramphenicol treated cells and purified on CsCl density equilibrium gradients. The 570 base-pair HpaII restriction fragment has been described (12), and carries the trp promoter and leader regions through the first part of trpF. The fragment, to be used as template, was isolated from 3.5% polyacrylamide gels using ethidium bromide staining, eluted, phenol extracted several times, ethanol precipitated, and finally stored at a concentration of 0.1 μM in 10 mM Tris pH 8.0 (4°C), 0.1 mM EDTA. Yeast RNA for use as carrier was purchased from Sigma and repurified through phenol extraction and ethanol precipitation.

Syntheses were generally carried out in a total volume of 10 μl in

Eppendorf microcentrifuge tubes, and stopped by the addition of 200 µl of 10 mM EDTA pH 8.2, 100 µg carrier RNA, and 40 µl 3 M NaOAc pH 7.5. Samples were phenol extracted twice against equal volumes of redistilled phenol equilibrated to 10 mM Tris pH 8.0 (4°C), 1 mM EDTA. The aqueous phase was removed, and the phenol washed with another 200 µl of 10 mM EDTA; these two aqueous phases were pooled, and the RNA was precipitated with ethanol. Two basic types of reactions were performed:

Type I. Transcription without Preincubation. Reactions in a total volume of 10 µl were incubated at 37°C for 20-30 minutes and contained: 0.2 pmoles HpaII fragment (template), unlabeled nucleoside triphosphates to 200 µM, Tris-acetate pH 7.9 (4°C) to 20 mM, Na EDTA to 0.1 mM, dithiothreitol to 0.1 mM, Mg acetate$_2$ to 4 mM, 1-4 µCi of $\alpha$-$^{32}$P labeled NTP at 10-30 µM, and 0.1 to 2.0 µg RNA polymerase. Reactions were 5% in glycerol. KCl, RNA polymerase, labeled nucleoside triphosphates, and other components were added to the concentrations stated in figure legends; reaction compositions were standardized by making up a common solution which could be divided into separate aliquots to which the variable component(s) could then be added.
Type II. Transcription with Preincubation. In order to (1) synchronize initiation or (2) limit transcription to one round of synthesis, reactions were preincubated with all the above components, except for $Mg^{2+}$, for 5-10'. To synchronize initiation, $MgAc_2$ was added to 4 mM. To both synchronize initiation and limit transcription to one round of synthesis, $Mg^{2+}$ and rifampicin were added to 4 mM and 7-10 micrograms/ml respectively. After initiation, reactions were run for 20 minutes at 37°.

RNA was analyzed by electrophoresis on 10% polyacrylamide gels 7 M in urea, in 0.1 M Tris pH 8.3, 0.1 M boric acid, 2 mM EDTA. RNA bands were located by autoradiography, and for quantitation were excised and counted either directly in the $^3$H channel of an Intertechnique liquid scintillation counter (Cerenkov radiation), or in the $^{32}$P channel in 4 ml Aquasol after treatment with 0.5 ml 10% $H_2O_2$.

## RESULTS

I. A Transcription System for Studying Termination at trp a. Transcription from a 570 base pair HpaII fragment carrying the promoter, operator, and leader regions of the trp operon can be limited essentially to the production of transcripts initiating at the trp promoter (12). We have confirmed by fingerprint analysis, that bands L and RT (Figure 1) correspond to the leader

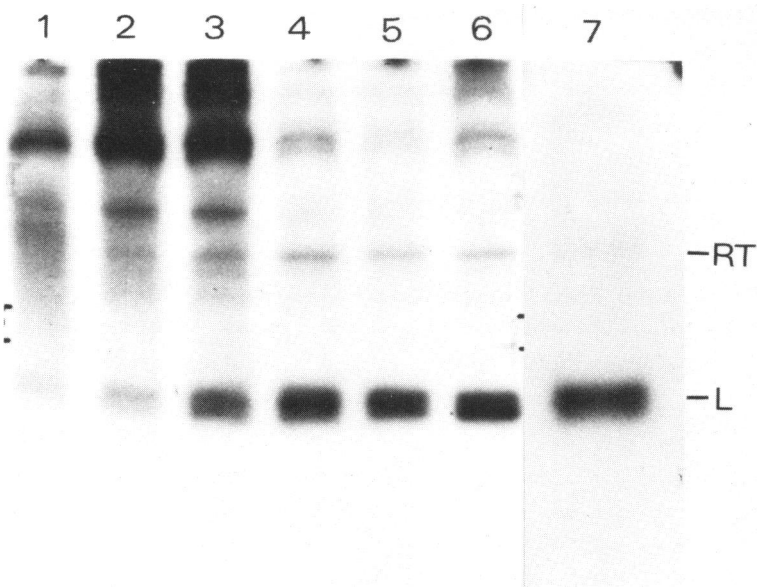


Figure 1. Effects of RNA polymerase and KCl Concentration on Transcription. Autoradiograph of a polyacrylamide-urea gel after electrophoresis of transcription reaction products. Lanes 1-6 were reaction type I performed in 10 µl with $\alpha$-$^{32}$P UTP at 25 µM. To reactions 1-5 were added 1 µg, and to reaction 6, 0.17 µg of RNA polymerase. KCl concentrations were: Lane 1, 10 mM; Lane 2, 50 mM; Lane 3, 100 mM; Lane 4, 150 mM; Lane 5, 200 mM, Lane 6, 100 mM. Lane 7 was reaction type I, with the addition of $\alpha$-$^{32}$P GTP to 20 µM, 0.17 µg RNA polymerase, and KCl to 150 mM. All reactions were run at 37°C for 30 minutes and analyzed as described in Materials and Methods.

transcript of 140-141 nucleotides and the read-through transcript of 260 nucleotides observed previously (11,12). With a molar excess of RNA polymerase, an increase in the KCl concentration from 10 mM to 150 mM suppresses large transcription products (presumably beginning elsewhere on the HpaII fragment) and enhances trp p promoted transcription (Figure 1, lanes 1-4). A further increase from 150 to 200 mM KCl does not further increase transcription of the leader region (Figure 1, lane 5). Enhancement of specific transcription from trp p relative to overall transcription can also be obtained by decreasing the molar ratio of polymerase to template from 6:1 to 1:1 (Figure 1, lane 3 vs. lane 6). Combining these two conditions results in optimal transcription of the fragment (Figure 1, lane 7), in which initiations at trp p produce greater than 90% of the RNA synthesized.

II. Analysis of the Transcription Products. Lee and Yanofsky (12) observed that transcription carried out at 100 mM KCl results in two major bands on a

gel: on a molar basis, 95% is leader mRNA (140-141 nucleotides long, which we have called L), and 5% is read-through RNA (260 nucleotides long). Under our conditions, band L actually consists of two bands, the larger of which ($L_1$) is the full leader transcript. Judging from its higher mobility on the gel, $L_2$ appears to be between 5 and 15 nucleotides shorter than $L_1$. Two dimensional fingerprint analysis of $L_2$ indicates that $L_2$ lacks the 3' terminal $CU_7$-OH and $CU_8$-OH of $L_1$, but extends at least through nucleotide 129 of the leader region. Thus, $L_2$ terminates between nucleotide 129 and 135 (underlined) of leader RNA as follows: CCUAAUGAGCGGGCUUUUUUUU-OH. One possibile explanation for the occurrence of $L_2$ is that it is the product of a premature termination reaction resulting when an elongating RNA polymerase molecule encounters a stalled termination complex at the attenuator site. This hypothesis would predict that at a low RNA polymerase to template DNA ratio, $L_2$ should not appear until a second polymerase molecule has arrived at the attenuator region on a given template molecule. In fact, $L_2$ does not appear as a product of the *in vitro* transcription reaction until the molar ratio of polymerase to template is greater than 1.0 (Figure 2).

Confirmation that the synthesis of $L_2$ depends upon the occurrence of a

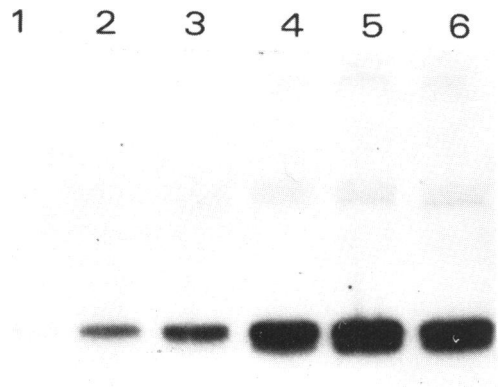


Figure 2. Effects of Polymerase Concentration on Synthesis of $L_1$ and $L_2$ RNA Transcripts. Reactions were type I in 10 µl, with $\alpha$-$^{32}$P GTP added to 20 µM and KCl to 150 mM. RNA polymerase was added: Lane 1, 0.02 µg; Lane 2, 0.05 µg; Lane 3, 0.10 µg; Lane 4, 0.15 µg; Lane 5, 0.20 µg; Lane 6, 0.25 µg. The molar ratios of RNA polymerase to template were: 0.2, 0.5, 1.0, 1.5, 2.0, and 2.5 in Lanes 1-6 respectively. Reactions were run at 37°C for 20 minutes. Termination, electrophoresis and autoradiography were performed as described in Materials and Methods.

second initiation step is provided by the results shown in Figure 3. In this case, a large molar excess of polymerase (10:1) over template DNA was employed in a preincubation experiment. When rifampicin is added along with $Mg^{2+}$ to initiate synthesis, only $L_1$ and the read-through RNA appear as products (lane 1). However, when transcription is initiated with $Mg^{2+}$ and allowed to proceed in the absence of rifampicin, both $L_1$ and $L_2$ appear (lane 2). These data indicate that $L_2$ is the product of premature termination after a second initiation on a given template molecule, possibly caused by the presence of a stable termination complex at the normal site.

III. Effects of Rho on Transcription of trp Leader mRNA. The inclusion of purified rho in the transcription reaction leads to a 10-fold increase in the production of trp p initiated transcripts (Figure 4). However, there is no significant change in the ratio of leader mRNA to read-through RNA - both are enhanced to about the same extent. The overall stimulation by rho is nearly eliminated by the addition of rifampicin (at 10 µg/ml), suggesting that the rho stimulation depends upon reinitiation by the RNA polymerase holoenzyme at the trp promoter (Figure 5). Moreover, rho stimulation depends upon a low RNA polymerase to template ratio (less than 2:1 as shown in Figure 6). These results suggest that rho acts as a release factor upon a stalled termination complex at the transcription terminator, allowing recycling of RNA polymerase molecules when their concentration is limiting. A second transcribing polymerase may also contribute to recycling of the first, since doubling of the

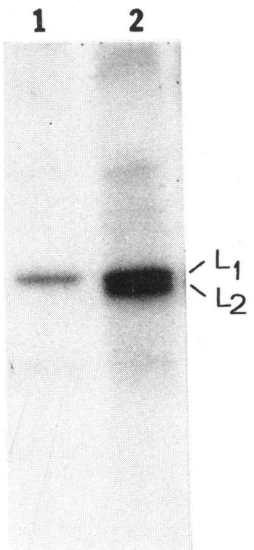


Figure 3. Effect of Rifampicin in the Production of $L_1$ and $L_2$ Transcripts. The reactions were type II in 10 µl with $\alpha$-$^{32}$P GTP added to 20 µM, KCl to 150 mM, and 0.1 µg of RNA polymerase added to yield a 1:1 polymerase to template ratio. After preincubation at 37°C for 5 minutes, the reactions were initiated by the addition of $MgAc_2$ to 4 mM. At this time reaction 1 received rifampicin to 7.5 µg/ml while reaction 2 received buffer. The reactions were incubated at 37°C for 20 minutes. Termination of the reactions, electrophoresis, and autoradiography were performed as described in Materials and Methods.


Figure 4. Effect of Rho on the Production of trp p Promoted Transcripts in Moderate (150 mM) KCl. The reactions were type I in 10 µl with 0.17 µg RNA polymerase added (1:1 molar ratio of polymerase to template) and $\alpha$-$^{32}$P GTP at 10 mM and KCl at 150 mM. Reaction I contained 1 µl rho storage buffer while reaction 2 contained 1 µl purified rho. Termination, polyacrylamide gel electrophoresis, and autoradiography were performed as described in Materials and Methods. In experiments of this type with different preparations and amounts of rho factor, the degree of stimulation of trp leader transcription varied from 6-fold to 15-fold.

polymerase concentration results in a more than two-fold increase in leader-RNA synthesis (Lane 3 vs. Lane 1). Alternatively, increased stability of polymerase at the higher concentration may be responsible. When rho was

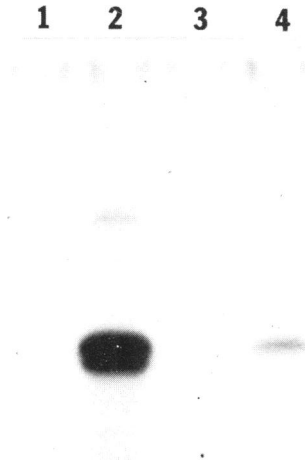


Figure 5. Rifampicin Blocks Rho-Induced Stimulation of Leader Transcription. These reactions were type II, containing in 10 µl, 0.2 µg RNA polymerase, GTP at 20 µM, and KCl at 150 mM. After preincubation at 37°C for 10 minutes, transcription was initiated by the following additions: Lane 1: 1 µl rho storage buffer, and $MgAc_2$ to 4 mM. Lane 2: 1 µl rho and $MgAc_2$ to 4 mM. Lane 3: 1 µl rho storage buffer, rifampicin to 10 µg/ml, and $MgAc_2$ to 4 mM. Lane 4: 1 µl rho, rifampicin to 10 µg/ml, and $MgAc_2$ to 4 mM. Reactions were further incubated at 37°C for 20 minutes, then terminated and subjected to polyacrylamide gel electrophoresis and autoradiography as described in Materials and Methods.

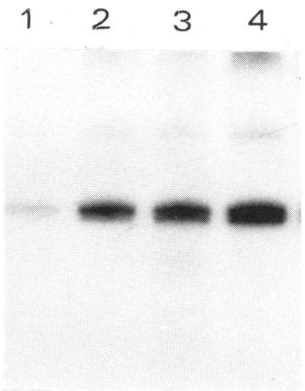


Figure 6. Effect of Rho at Limiting and Excess RNA Polymerase Concentrations. These reactions were type I in 10 µl with α-$^{32}$P GTP at 20 mM and KCl at 150 mM. Reactions 1 and 3 (-rho) received 1 µl of each of rho storage buffer while 2 and 4 (+rho) received 1 µl each of purified rho. Reactions 1 and 2 received 0.1 µg RNA polymerase (1:1 polymerase to template molar ratio) and 3 and 4 received 0.2 µg RNA polymerase (2:1 ratio). Reactions were run at 37°C for 20 minutes, then terminated and subjected to polyacrylamide gel electrophoresis and autoradiography as described in Materials and Methods.

present in a transcription reaction at 50 mM KCl, a surprising result was obtained (Figure 7): rho stimulates read-through transcription to between 30 and 35% of leader mRNA (in molar terms), an increase of 5-7 fold from the 5% level observed in the absence of rho.

IV. Demonstration of a Stable Termination Complex. When a transcription reaction is performed, treated with rifampicin to prevent reinitiation, then sedimented on a sucrose gradient, the $^{32}$P-labeled RNA sediments at a position characteristic of RNA polymerase. If the sample is treated with 0.2% SDS at 100° for 5 minutes, the leader RNA (still intact) sediments near the top of the gradient. This confirms preliminary evidence (11) that RNA polymerase, nascent leader mRNA, and probably template DNA can form a stable termination

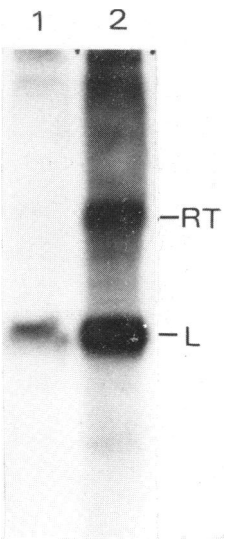


Figure 7. Effects of Rho on the Production of trp p Promoted Transcripts in Low (50 mM) KCl. These reactions were type I in 10 µl with 0.17 µg RNA polymerase added (1:1 molar ratio of polymerase to template) and α-$^{32}$P GTP at 10 mM and KCl at 50 mM. Reaction 1 contained 1 µl rho storage buffer and reaction 2, 1 µl purified rho. The reactions were run at 37°C for 20 minutes. Termination, electrophoresis and autoradiography were carried out as described in Materials and Methods. Fingerprint analysis of L and RT verified their identity as leader and read-through transcripts, respectively.

complex at the attenuator site. Since RNA polymerase can bind to nitrocellulose filters, a second way to test this possibility is to ask whether leader mRNA can be trapped on a filter by RNA polymerase. Free leader RNA has no affinity for nitrocellulose, since if the reaction mixture is boiled for 5 minutes to dissociate any complex before filtration, all the leader RNA is found in the filtrate (none is retained on the filter). However, filtration without boiling does result in a 60-70% retention of completed trp leader mRNA on the filter. This binding appears to be most efficient if filtration is carried out in 100 mM KCl using preincubation followed by initiation with $Mg^{2+}$ and rifampicin to preclude further initiations. The ratio of trapped to filterable mRNA is about 2:1, and does not change over the course of 60 minutes after initiation. At present we have no explanation for why only two-thirds of the completed transcript is retained on the filter. However, the trapped mRNA is complete, rather than in an unfinished elongating complex, since no shorter species are observed bound to the filters. These results support the results of the sucrose gradient experiment, and imply that there is a stable complex containing at least RNA polymerase and mRNA which exists after elongation of the RNA has stopped.

## DISCUSSION

Termination of transcription at the attenuator site in the leader region of the tryptophan operon in vitro provides a model system for the study of the general mechanism of termination. Our current studies are concerned with the mechanism of this reaction and the effects of rho factor on the process. We have slightly modified the system described by Lee & Yanofsky (12), optimizing it for the production of trp leader mRNA. The production of extraneous transcripts has been eliminated by utilizing a small DNA fragment that carries only the initial region of the trp operon, and increasing the iniation specificity of RNA polymerase on this fragment by raising the ionic strength and maintaining a low ratio of RNA polymerase to template, as suggested by Hinkle & Chamberlin (17).

We have found that the addition of rho factor to the transcription reaction containing limiting amounts of RNA polymerase can stimulate production of leader mRNA about 10-fold. This is consistent with the observations on transcription in vitro of the lambda 4S RNA (16) and E. coli tRNA genes (18). The interpretations presented in these cases, that rho enhances recycling of RNA polymerase molecules, appears to be true of the trp system as well. In support of this conclusion, we find that the stimulation of leader RNA pro-

duction is eliminated if the antibiotic rifampicin, which blocks the initiation of transcription, is added after one round of initiation is allowed to occur.

An unusual feature of the trp attenuator system is that the termination complex appears to be quite stable. The results of Howard et al. (16) and Grimberg & Daniel (18) imply the existence of transient "paused" ternary complexes at their respective terminators. The lifetime of the λ 4S termination complex was found to be not greater than three minutes (16). In contrast, we find that the trp termination complex is stable for at least 60 minutes at 37°, as measured by retention of $^{32}$P-labeled leader RNA on nitrocellulose filters after incubation. Although pauses have, in some cases, been ascribed to transcription in the presence of low concentrations of nucleoside triphosphates (3,13), termination, and by implication, pausing, occurs at the trp attenuator in the presence of moderate concentrations of NTP's (11). Such a paused complex would be the expected substrate of the rho "release" activity detected at the λ 4S terminators and the trp attenuator. We propose that the primary function of rho is to interact with and increase the rate of dissociation of termination complexes which have ceased elongation.

An unexpected result is obtained in reactions occurring at low ionic strength (50 mM KCl). While rho continues to stimulate trp p initiated transcripts, it stimulates the production of read-through transcript to a greater extent, raising it from 5% to 35% in molar yield. That rho stimulates overall transcription in low salt implies that it still interacts with the complex, so presumably the read-through stimulation effect results from a salt-dependent modification of the interaction of rho with the complex. There is much circumstantial evidence that rho and pN, the bacteriophage lambda N gene product, interact with RNA polymerase at the β subunit (19-23). We propose that the salt dependent modification of the rho-paused attenuator complex interaction is such that rho now mimics the molecular effect of N protein, either to prevent dissociation, or to increase the rate of turnover of paused complexes back to actively transcribing complexes.

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*To whom correspondence should be addressed.

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