Rho-dependent Termination of Transcription Is Governed Primarily by the Upstream Rho Utilization (rut) Sequences of a Terminator*

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Lislott V. Richardson and John P. Richardson†
From the Department of Chemistry, Indiana University, Bloomington, Indiana 47405

A Rho-dependent transcription terminator in Escherichia coli DNA consists of an upstream part for Rho utilization (rut) and the transcription stop point (tsp) region. To test the role of the tsp region variants of the coliphage λ cro gene terminator, tr1, containing inserts of non-terminator sequences between its rut and tsp regions were tested for termination function. The results showed that termination occurred with high efficiency at multiple sites in each of the new sequences with the positions of the sites coinciding with transcriptional pause points in the insert sequence and that the efficiency of termination was not directly proportional to the extent of pausing at those points. Thus, in contrast to the rut sequences, which are relatively rare in DNA, many different sequence segments can function as a tsp region. Studies with isolated transcripts showed that a rut element and sequences 3’ of the rut element were both needed to activate ATP hydrolysis by Rho factor with the degree of activation depending on the length and the specific sequence of the 3’ segment. These results support models for Rho action in which ATP hydrolysis is coupled to interactions of Rho protein with RNA 3’ of the rut region.

The orderly expression of genes depends on the functions of promoters and terminators of transcription. The Escherichia coli genome has two kinds of terminators that are distinguished by their mechanisms and DNA sequences (1). When RNA polymerase encounters an intrinsic terminator, it can release the nascent RNA spontaneously, but when it encounters a Rho-dependent terminator, the release of the RNA depends on the action of a protein factor called Rho. Although several Rho-dependent terminators have been identified, their sequences do not conform to an obvious consensus.

A Rho-dependent terminator that has been extensively analyzed, Xr1, is located in the intercistronic region between the cro and cII genes of bacteriophage λ (2). It consists of two distinct parts, which together extend over 150–200 bp1 of DNA. The upstream part, called rut, encodes a segment of the nascent RNA that can bind (3, 4). This interaction between Rho and the rut segment of the RNA is essential for termination (5, 6). The downstream part, called the transcription stop point (tsp) region, is where termination occurs at several clusters of specific points that are spread over 100 bp of DNA (7, 8). These points correlate with the points where RNA polymerase pauses during elongation in the absence of Rho (9, 10).

Rho factor causes termination of transcription by actions on the nascent RNA and the ternary transcription complex that are coupled to ATP hydrolysis (11). These actions cause the dissociation of RNA from RNA polymerase molecules in the tsp region. Rho protein has two kinds of binding sites for RNA (12), and although several models have been proposed to explain how changes in the binding properties of the sites could mediate dissociation of a transcript from RNA polymerase (1, 13, 14), it is not known whether the two kinds of sites in Rho are related to the two parts of the terminator sequence.

One approach to elucidating the possible relationship between the rut and tsp regions of a Rho-dependent terminator is to insert foreign DNA sequences between these two elements and to test the effects these inserts have on transcriptional pausing and termination and on the formation of functional interactions with the transcripts, as revealed by activation of ATP hydrolysis. We report the results of studies of these activities with modified forms of Xr1. The results show that the rut region is the primary determinant of a Rho-dependent terminator and that function of the tsp region is conferred by the immediate proximity of the rut region.

EXPERIMENTAL PROCEDURES

Materials—E. coli RNA polymerase was either purchased from Epicentre Technologies Corp. or was purified according to Andrews and Richardson (15) and generously supplied by Chris Burns. E. coli Rho protein was purified from BL21(DE3)(pCB111, pLysS) as described by Nowatzke et al. (16). Nucleoside triphosphates (NTPs) and deoxy-NTPs were from Boehringer Mannheim; [α-32P]UTP was from ICN, Chemical and Radiosotope Division; RNasin, a ribonuclease inhibitor was from Promega; enzymes used for DNA manipulations were from New England Biolabs Inc.; rifampacin was from Ciba-Geigy Corp.; and proteinase K was from U. S. Biochemical Corp.

Plasmids and DNA Templates—To make the plasmid pKKCYC, a 560-base pair HindII fragment from pCYC2 (containing the entire cro gene) was gel-purified, treated with Klenow fragment DNA polymerase, and ligated into pKK-177-3 (17) that had been cut with SmaI and treated with calf intestinal phosphatase. E. coli J M 105 was used as the host cell. pKKCYC served as a vector for two different inserts, both ligated into the NsiI site, which had been treated sequentially with T4 DNA polymerase and calf intestinal phosphatase. One was a 300-bp HindII-PvuII fragment from pBluescript II SK′ (from the HindIII site at bp 676 in the polylinker to the PvuII site at bp 976 in lac). The other insert was a 217-bp CiaI fragment from the rho sequence in pCB111 (bp 962 to bp 1179 in rho) with its 5-overhangs filled in by the action of Klenow fragment DNA polymerase. The plasmids containing the rho fragment were named pKKCYC-Cia c and pKKCYC-Cia r, respectively, where “c” stands for coding orientation and “r” for reverse orientation. The plasmids containing the Bluescript fragment were named pKKCYC-HF and pKKCYC-PH (where “H” stands for HindIII and “P” for PvuII), respectively, with the HF plasmid having the HindIII end of the fragment closest to PvuII and the PH plasmid having the reverse orientation. The same inserts were also ligated into the NsiI site of the cro gene in plF2 (3). In this plasmid the cro gene is under the control of the T7 promoter of phage T7.

1 The abbreviations used are: bp, base pair(s); tsp, transcription stop point; rut, Rho utilization; PCR, polymerase chain reaction; nt, nucleotide(s).
RNA polymerase promoter that allowed us to synthesize large amounts of RNA in vitro. The sequences of all the constructs used in this study have been confirmed using the standard dideoxy sequencing procedure. The plasmids for standard E. coli RNA polymerase transcription experiments were prepared using polymerase chain reaction (PCR) with the L primer (5'-GGTTGCTCGAGGATCCATGTTAG-3') and the U primer (5'-ACGACTCTAGTCGAGAAGCCGAG-3'). The U primer is complementary to a sequence 80 bp upstream from the P_{prom} start site and the L primer 25 bp downstream from termination subsite III. The amplification products were subjected to a proteinase K treatment, extracted with phenol/chloroform (1:1), and precipitated with 2.5 volumes of ethanol in the presence of 0.5 M ammonium acetate. The pellet DNA was dried and resuspended in 10 mM Tris-acetate and 1 mM EDTA. The templates were gel-purified on 1% low melt agarose gel and eluted in a unidirectional electrophoreser (International Biotechnologies, Inc., model UEA).

RNA Transcription—Standard transcription reactions were carried out in a 25-μl reaction mixture containing 0.15 M potassium glutamate, 0.04 M Tris-acetate, pH 8.0, 10 mM magnesium acetate, 0.1 M EDTA and 1.0 M diethanol, 375 fmol of E. coli RNA polymerase, and an approximately equal molar amount of DNA. This mixture was incubated at 37 °C for 3 min. The reaction was started by adding ATP, CTP, and (α-32P)-UTP (5 μCi and 25 μM) and digested to 10 μM final concentration with 400 units of T7 RNA polymerase. After 2 h at 37 °C, EDTA was added to 100 mM and SDS to 0.5%. The sample was treated with 50 μg/ml proteinase K at 37 °C for 15 min. RNA was precipitated by adding ammonium acetate to 0.5 M followed by 2.5 volumes of ethanol and then was separated by gel electrophoresis and analyzed as described previously (3). The RNA was purified by gel electrophoresis.

RNA Structure Analysis—The Multifold program, version 2.0 (18–20) was used to predict the secondary structures of the RNA segments. We used a version that was developed for the Macintosh by Don Gilbert, Department of Biology, Indiana University.

RESULTS

Insertion of Foreign DNA Sequences between rut and tsp of λtR1—To test the importance of the tsp region in termination, we prepared plasmids containing DNA fragments from two different sources, each inserted in both orientations at the NsiI site between the rut and tsp regions of λtR1 (Fig. 1). These constructs allowed us to address the question of whether Rho would be able to mediate the release of RNA transcripts within foreign, non-terminator sequences or whether transcription would continue until RNA polymerase reached the tsp region of λtR1. One of the inserts was a 217-bp Clai fragment from the E. coli rpo gene, which was chosen because it had been shown not to have a Rho-dependent terminator in either orientation. In the plasmid pKKCYC, this fragment is inserted in the orientation in which it is normally expressed in the rho gene, whereas in pKKCYC Cal r it is inserted in the reverse orientation. The other fragment was the 300-bp PvuII-I Hindl fragment from the multiple cloning site of pBluescript II SK−. It was chosen because it encodes RNA segments that were predicted to have considerable base-paired secondary structure. The resulting constructs with it are called HP and PH, respectively, with the letter on the left indicating the restriction site closest to the cro gene promoter.

In Vitro Transcription Assays for Termination Analysis—The templates from these constructs were transcribed in vitro with E. coli RNA polymerase and various amounts of Rho. The results show (Fig. 2) that in all cases Rho caused RNA polymerase to terminate transcription at several points within the inserts. As in the tsp region of λtR1, termination occurred at several groups of preferred subsites called tI, tII, etc. The positions of these new groups of end points are shown in Fig. 3 by underlines on the sequences of the transcripts in the termination region. With all the templates, the first subsite (tI) started about 2 nucleotides downstream from the insertion point (the NsiI site), precisely at the position of subsite I of λtR1. All the templates had a second subsite centered in a range from 20 to 30 bp downstream from the center of the corresponding subsite I, and all but the PH template had a third site centered in the range from 45 to 55 bp downstream from subsite I. Thus, in spite of an overall similarity in the distribution of stop points with the various templates, there were some distinct and specific differences in the pattern of termination.

The dependence of termination on the concentration of Rho was similar for all the templates (Fig. 2). Since the rut sites, which are the major determinants of the affinity of binding of Rho to the transcript, are the same for all, this is an expected property. At the saturating level of 8.8 nM Rho, termination was virtually complete within the inserts. The termination efficiencies at individual subsites were determined by using a

2 A. Martinez and J. P. Richardson, unpublished results.
Stop Point Sequences for a Rho-dependent Terminator

To quantitate the relative extents of pausing, the amounts of transcripts at the various termination points in the samples taken at 50 s were determined from phosphorimaging autoradiographs. This time point was chosen because, with the wild-type template, the pause site distribution then corresponded well with the distribution of terminated transcripts (Fig. 4, Table I). The results (Table I) show that the distribution of the transcripts at that time differs significantly in Cla c, Cla r, and HP from those for the wild type and the PH. The ratios of the fraction of terminated transcripts to the fraction of paused transcripts at 50 s for Cla c, Cla r, and HP at their subsites I and II ranged between 2.2 and 3.5, whereas the ratios at subsites I and II of wild type and the PH insert ranged between 0.9 and 1.4. From these results we conclude that termination is occurring at the pause sites in the inserted sequences and that the efficiency of termination by Rho action is not directly proportional to the extent of pausing at a site.

Secondary Structure Predictions for the RNAs—Since the potential to form a stem-loop (hairpin) structure with the stem ending 10 nucleotides before the terminal residue of RNA at a pause site or a termination stop point is a common feature of paused and Rho-terminated transcripts (21, 22), we used the Mulfold computer program (19, 20) to locate the positions of possible stem structures in the parts of the transcripts synthesized from these inserts. The sequences that are expected to form stem structures are indicated by arrows over the sequences (Fig. 3) with the arrowheads pointed toward the connecting loop. Since the insertion point is located just downstream from a sequence that encodes the potential stem that precedes the first subsite of tR1, we expect that the formation of this potential stem-loop is probably responsible for the pausing and termination that occurs at the first subsite position of each template, which is at the identical point for all templates (Figs. 2 and 4). Since the downstream sequences are also known to affect the efficiency of pausing (23), the changes in those sequences are probably responsible for the greatly decreased efficiency of pausing with each of the new constructs compared with the wild type.

Most of the subsequent pause/termination subsites (regions) are preceded by transcript segments that are predicted to form stems in which the ends of the stems range from 5 to 10 nt before the middle of the subsite. Thus, these observations are consistent with others that correlate stem structures with many but not all pause sites (21, 24).

ATPase Activity of Rho with Inserts Versus the tsp Region of λtR1—Since the ability of Rho to terminate transcription depends on its interactions with RNA that are coupled to the hydrolysis of ATP (25, 26), the efficiency of the termination process could be governed by the ability of the RNA to activate ATP hydrolysis. Faus and Richardson (3) showed that the level of activation of ATP hydrolysis by various isolated RNAs correlated with the efficiency of termination of genes containing alterations of the rut site sequences. However, the role of the sequences of RNA downstream from the rut site has not been examined. To determine how the extent and nature of the downstream sequences affect the activation of ATP hydrolysis by Rho, we synthesized RNAs of discrete sizes from the wild-type λ cro DNA and from the DNA templates containing the inserts, and we tested these RNAs for their abilities to activate ATP hydrolysis by measuring the turnover rate for ATP hydrolysis with Rho at saturating levels of each of the RNAs.

We did the most extensive studies with the wild-type RNA, using transcripts that ranged in size from 197 to 541 nt (Fig. 5). The shortest RNA tested was generated from a template that had been cut at the Ava I site and thus did not include the rut region; it had almost no ability to activate Rho to catalyze ATP hydrolysis. Faus and Richardson (3) showed that the level of activation of ATP hydrolysis by various isolated RNAs correlated with the efficiency of termination of genes containing alterations of the rut site sequences.
hydrolysis. The 270-nt transcript that included the rut region, but no RNA further downstream, also had very little activity (k$_{\text{cat}}$ of 0.9 s$^{-1}$). However, the RNA that had been extended a mere 15 nucleotides further downstream of the NsiI site that is located just upstream of the first termination point gave a k$_{\text{cat}}$ of 8.9 s$^{-1}$. Further increases in the rates of ATP hydrolysis were found when larger cro transcripts were used, up to a k$_{\text{cat}}$ of 56.4 s$^{-1}$ with a transcript length of 374 nt. At this point, with the 3'-end of the transcript near the end of the native tsp region, an apparent plateau was reached in ATP activation since the rate of ATP hydrolysis with a 541-nt RNA that was generated from a template that also encoded part of the adja-

![Fig. 3. Sequences of the wild-type and mutant transcripts starting from the middle of the rut sequence (residue 255) and extending to points just beyond the wild-type (wt) subsite III end point. The lines underneath the sequences indicate the various end points for transcripts terminated in the indicated subregion. The pairs of arrows above the sequences indicate blocks of nucleotides that are predicted to be parts of stems in possible stem-loop (hairpin) structures.](http://www.jbc.org/Downloaded from)

### TABLE I

| Stop points Distribution and termination efficiencies | Name nt | Distribution at 50 s$^\text{a}$ | Distribution with Rho$^\text{b}$ | Termination efficiency$^\text{c}$ | Ratio of distribution with Rho/distribution at 50 s |
|--------------------------------------------------------|---------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Wild-type cro                                           |         |                                 |                                 |                                 |                                 |
| I                                                      | 291     | 42                              | 60                              | 60                              | 1.4                             |
| II                                                     | 312     | 30                              | 31                              | 78                              | 1.0                             |
| III                                                    | 345     | 15                              | 6                               | 67                              | 0.4                             |
| RT                                                     | >360    | 13                              | 3                               |                                 |                                 |
| Cla c                                                  |         |                                 |                                 |                                 |                                 |
| I                                                      | 291     | 18                              | 40                              | 40                              | 2.2                             |
| II                                                     | 316     | 8                               | 23                              | 38                              | 2.8                             |
| III                                                    | 345     | 9                               | 11                              | 30                              | 1.2                             |
| RT                                                     | >360    | 55                              | 26                              |                                 |                                 |
| Cla r                                                  |         |                                 |                                 |                                 |                                 |
| I                                                      | 291     | 10                              | 35                              | 25                              | 3.5                             |
| II                                                     | 310     | 12                              | 37                              | 57                              | 3.1                             |
| III                                                    | 340     | 22                              | 13                              | 46                              | 0.6                             |
| RT                                                     | 360     | 56                              | 15                              |                                 |                                 |
| HP                                                     |         |                                 |                                 |                                 |                                 |
| I                                                      | 291     | 12                              | 28                              | 28                              | 2.3                             |
| II                                                     | 313     | 16                              | 41                              | 57                              | 2.6                             |
| III                                                    | 338     | 51                              | 18                              | 57                              | 0.3                             |
| RT                                                     | >376    | 21                              | 13                              |                                 |                                 |
| PH                                                     |         |                                 |                                 |                                 |                                 |
| I                                                      | 291     | 28                              | 30                              | 30                              | 1.1                             |
| II                                                     | 326     | 40                              | 35                              | 50                              | 0.9                             |
| III                                                    | 380     | 19                              | 14                              | 40                              | 0.7                             |
| RT                                                     | >386    | 13                              | 21                              |                                 |                                 |

$^\text{a}$ Molar distribution of transcripts after 50 s of synthesis.

$^\text{b}$ Molar distribution of transcripts after synthesis for 10 min in the presence of 8.8 nM Rho (data from Fig. 4).

$^\text{c}$ Termination efficiencies are from the fraction of transcripts ending at the indicated stop point over all of the transcripts ending at that point and further downstream.

$^\text{d}$ RT, read-through transcript.
cent cII gene of λ was about the same as with the 374-nt transcript.

To study the effect of different sequences 3′ to the rut region on the extent of ATPase activation, we also measured rates of ATP hydrolysis with several transcripts from templates with the inserts. Fig. 5 shows results for transcripts ending within or beyond the Cla c and PH insertion sequences. These RNAs gave turnover rates that were similar to the 315-nt wild-type cro transcripts that had been extended just into the termination region. These extents of activation are thus consistent with a minimal level that is sufficient for termination. However, for transcripts of similar size, those with the Cla c and PH inserts did not give rates of activity as high as those ending in the native tsp region of trR1. The $k_{\text{cat}}$ values of a 331-nt RNA from the Cla c template and a 332-nt RNA from the PH template were 16.3 and 10.6 s$^{-1}$, respectively, as compared with 30 s$^{-1}$ for the 330-nt cro transcript. Although longer transcripts of the Cla c and PH templates activated ATP hydrolysis to higher levels than the 330-nt transcript, none gave the same high levels as the 374-nt wild-type cro transcript. Even the transcripts that had been extended through the insert sequences and that ended in the wild-type trR1 tsp region at the same point as the 374-nt wild-type transcript (the 576-nt Cla c and 637-nt PH RNAs) activated ATP hydrolysis to only about one-half the level with that RNA. Thus, the extent of the increase of activity with increasing length of the segment of the transcript downstream from the rut sequence is dependent on the sequences.

Since the part of the cro transcript between residues 290 and 374 appeared to be particularly potent in activating ATP hydrolysis, we prepared a template in which this region was duplicated to test whether even higher levels of ATPase could be obtained by increasing the extent of the activator region. However, the $k_{\text{cat}}$ for Rho ATPase measured in a standard assay with a 458-nt transcript that included a duplication of the tsp region was 68 s$^{-1}$, or only 20% higher than that measured with the 374-nt cro transcript. Thus, the addition of 80 nt more of strong activator sequences beyond the end of the rut region did not yield a further proportionate increase in ATPase activation.

Binding Studies—Since the activation of ATP hydrolysis by Rho is dependent on the binding of Rho to the RNA, differences in the extent of activation could be a result of differences in the affinity of Rho for the RNA. Even though all of the RNAs used for the activation, except for the Aval runoff transcript, contained most if not all of the rut sequence, we measured the affinity of Rho to many of the RNA transcripts used for the ATPase activation studies to establish that the structures of the transcript were not affecting the primary binding interaction. The affinities of Rho for the RNA were determined by a filter retention assay as described by Faus and Richardson (3). The results (not shown) confirmed their previous findings; all the transcripts with the complete rut region were able to bind to Rho with $K_{d}$ values in the range of $5-10 \times 10^{9}$ M$^{-1}$ in a binding buffer containing 0.05 M KCl. This included the 270-nt transcript of wild-type cro that had its 3′-end at the end of the....

Figure 4. Pause points in the termination regions. $^{32}$P-Labeled RNA samples were prepared by transcription of the indicated templates for the indicated times in the absence (−) or presence (+) of 8.8 nM Rho and were separated by gel electrophoresis. A, DNA templates as shown in Fig. 2A; B, DNA templates as shown in Fig. 2B.
rutansequence. Thus, the lack of significant ATPase activity with that transcript was not a consequence of an inability to bind the RNA.

**DISCUSSION**

We present evidence that Rho can cause RNA polymerase to terminate transcription with good efficiency in each of four altered forms of the λtR1 terminator in which the normal tsp region has been replaced by unrelated DNA. This result indicates that the sequence requirements for the tsp component of a Rho-dependent terminator are not stringent. Since the tsp component can be satisfied by only a limited set of DNA sequences, this experiment indicates that Rho is the primary determinant of a Rho-dependent terminator. This conclusion is also supported by some studies (29, 30) with the trp t' terminator that showed that the termination response depended primarily on the sequences in the upstream (5'-half) sequence of that terminator.

As with the normal tsp region of λtR1, the stop points are distributed in groups of preferred regions called subsites, and the distributions correspond very well with those of positions where RNA polymerase pauses during transcription of the DNA in the absence of Rho. These observations confirm a general finding that has been found with a number of Rho-dependent terminators (1). However, our results also show that the efficiency of termination at a subsite is not directly correlated with the extent of pausing at that subsite. This was particularly noticeable for the first termination subsite in each of the sequences. RNA polymerase passed through the pause sites much more rapidly in the insert sequences, especially in the Cla c and Cla r inserts, than in the wild-type sequence, yet the termination efficiencies were only slightly lower at the first subsites in these inserts compared with the wild-type DNA. The first subsite in each of the inserts occurs right at the start of the insert sequence. Since the sequences that are known to affect pausing at a particular site include upstream and downstream elements as well as those right at the pause point (21), the pausing at these sites is governed by the new sequences in the insert as well as the upstream component from λtR1. Thus, the upstream component of the pause site, the part that encodes the segment of the RNA that can form the stem-loop (hairpin) structure, appears to be a major determinant of the position of the pause, whereas the other sequences affect the efficiency and duration of the pause. We conclude too that these other sequences also affect the efficiency of termination at these sites independently from their effects on pausing. Because of the lack of synchrony in the elongation process on this template, we were unable to determine whether the changes in sequences for this first pause site region affect the efficiency of pausing or the kinetics of escape from the pause. However, the ability of Rho to terminate transcription with good efficiency when the overall extent of pausing is greatly decreased suggests that the paused complex per se is not the major target for Rho action. Wang et al. (31) recently showed that RNA polymerase undergoes a structural transition through a stressed configuration on approaching a pause site. Since the transition through such an intermediate is likely to occur during the approach of all pause sites, the target for Rho action could be the stressed intermediate. With this mechanism, the efficiency of termination would not necessarily be proportional to the duration of the pause. Since RNA polymerase also goes through a similar stressed configuration on approaching an intrinsic termination site (32), an action of Rho on RNA polymerase in such a configuration would mean that the two mechanisms involve a common intermediate. Another possible reason for the lack of correlation between the efficiency of termination and the extent of the pause is that a change in the sequence could also lead to a change in the stability of the attachment of the nascent RNA to the complex.

The new sequences also have some strong pause sites. There is one in the HP insert at a position that corresponds to approximately subsite III on the wild type. Rho also causes termination at this point but with only moderate efficiency, thus extending further the lack of correlation between extent of pausing and termination efficiency. Since this sequence is located in the multiple cloning site of pbLuxscript, we assume that it is not part of a natural terminator. Of more interest is the rather strong pause and termination site in the PH insert centered at residue 326. This is very near to or at a natural RNA end point for Lad RNA that has been mapped in the intergenic region between Lad and LacZ (33).

The Role of the tsp Sequences in Activation ATP Hydrolysis—The actions of Rho that cause termination of transcription are coupled to hydrolysis of ATP (34, 35). We show that isolated transcripts that contain all the upstream RNA through the rut region were able to bind to Rho with good affinity but were unable to activate ATP hydrolysis, whereas RNA transcripts that were extended to points within the tsp region were able to activate ATP hydrolysis to varying degrees. These results thus demonstrate that the binding of RNA to Rho with high affinity is not a sufficient condition for activation of ATP hydrolysis. There is an additional requirement of some minimum sequence of RNA on the 3' side of the segment of RNA that is sufficient for binding. Presumably, the interactions of Rho with a downstream segment of a nascent transcript after binding at a rutansequence are responsible for dissociation of the transcript from RNA polymerase, thus causing termination of transcription.

With the transcripts having 3'-ends in the natural tsp region of λtR1, the degree of ATP hydrolysis increased steadily with increasing length of the transcript up to a maximum that was achieved when the RNA molecules were extended to approximately subsite III. Based on the generally accepted models for the tracking of Rho along an RNA (13, 36), the increase in the steady-state rate with increasing RNA length could be reflecting the increased fraction of time Rho spends tracking between the rut site on the RNA (the loading site) and the 3'-end of the RNA compared with the time spent in steps that are not coupled to ATP hydrolysis, such as dissociation from the 3'-end and reestablishing the tracking process. As the fraction of time spent in tracking increases, it eventually dominates the kinetics of steady-state ATP hydrolysis. Hence, this rate will approach a maximum value. For the cro transcript this occurs when there are about 80 nucleotides 3' to the rut site. The results obtained with transcripts that had different tsp region sequences, i.e. transcripts that ended in the insertion sequences, showed that the degree of activation of ATP hydrolysis depends as well on features of the RNA besides its length. The degrees of ATPase activity with all the transcripts that ended in insert sequences were lower than those with similar length ending in λtR1 tsp sequences. One possible explanation for this difference is that the sequence of the RNA could affect the rate of tracking. In general, the RNA encoded by these insert sequences have more stable secondary structures than the RNA encoded by the λtR1 sequences, and base-paired secondary structures could be acting as a rate-limiting impediment to coupled tracking of Rho along the RNA. These possible effects of secondary structure on tracking could be one reason why the downstream strong pause point sites in the insert sequences, such as subsite II in the Cla c insert, gave lower efficiencies of termination than did the downstream sites (subsites II and III) in λtR1.

Revised Model for Termination—The experiments presented in this paper suggest the following revised version of a general
mechanism for termination of transcription by Rho action (1, 11, 37). Transcription of the rut element in DNA gives rise to a nascent RNA with an attachment site for Rho. This site (region) consists of a primarily single-stranded, C-rich segment of 40 or more RNA nucleotides that allow stable binding of RNA to the extensive, primary RNA binding site of Rho. As further RNA sequences appear, these can interact with the putative secondary RNA binding site, which is proposed to lie in the hole in the center of the Rho hexamer (37). The interactions of the RNA with the secondary binding site then activate rounds of ATP hydrolysis that are coupled to translocation of the RNA in the 5′ to 3′ direction, presumably through the hole. The contact of this Rho-RNA complex with RNA polymerase in a stressed conformation that it assumes on approach to a typical pause point on the DNA could then trigger dissociation of the transcription complex. If the complex of Rho and RNA has not formed or not moved fast enough to trigger dissociation at the first stressed conformation, further translocation along the RNA could bring Rho into contact with polymerase in stressed conformations at downstream sites. Since transcriptional pause points are a common feature of most natural DNA sequences, termination could thus occur at any one of a number of frequently occurring points downstream from the rut site on the DNA.

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