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

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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, λtR1, 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 bp[1] of DNA. The upstream part, called rut, encodes a segment of the nascent transcript to which Rho 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 λtR1. 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-^{32}P]UTP was from ICN, Chemical and Radioisotope 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 *Hin*fI fragment from pCYC2 (5) 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 *Sma*I and treated with calf intestinal phosphatase. *E. coli* JM 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 *Hin*cII-*Pvu*II fragment from pBluescript II SK− (from the *Hin*cII site at bp 676 in the polylinker to the *Pvu*II site at bp 976 in *lacI*). The other insert was a 217-bp *Cla*I 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-Cla c and pKKCYC-Cla r, respectively, where "c" stands for coding orientation and "r" for reverse orientation. The plasmids containing the Bluescript fragment were named pKKCYC-HP and pKKCYC-PH (where "H" stands for *Hin*cII and "P" for *Pvu*II), respectively, with the HP plasmid having the *Hin*cII end of the fragment closest to P_R and the PH plasmid having the reverse orientation. The same inserts were also ligated into the *NsiI* site of the *cro* gene in pIF2 (3). In this plasmid the *cro* gene is under the control of the T7

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[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.

DNA fragments for standard *E. coli* RNA polymerase transcription experiments were prepared using polymerase chain reaction (PCR) with the L primer (5′-GTTGCTCCGAGATGCTTAGC-3′) and the U primer (5′-CGCTGCACGCAGGAGTTCG-3′). The U primer is complementary to a sequence 80 bp upstream from the P_R 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 pelleted DNA was dried and resuspended in 10 mM Tris acetate and 1 mM EDTA. The templates were gel-purified on a 1% low melt agarose gel and eluted in a unidirectional electroelutor (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 mM EDTA and 1.0 mM dithiothreitol, 375 fmol of *E. coli* RNA polymerase, and an approximately equal molar amount of DNA. This mixture was preincubated at 37 °C for 3 min. The reaction was started by adding ATP, CTP, and GTP to 250 μM, [α-^{32}P]UTP (5 μCi/nmol) to 25 μM, and rifampicin to 10 μg/ml. The reaction was terminated by the addition of 55 μl of 0.5% SDS with 67 μg of *E. coli* tRNA/ml and 44 mM EDTA, and 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 (15).

Synthesis of RNA Used as ATPase Activators and for RNA Binding Studies—RNA transcripts used as activators of ATPase activity with Rho were prepared by transcription with T7 RNA polymerase of *cro* gene templates containing an appropriately placed promoter for T7 RNA polymerase. For most transcripts, the DNA consisted of pIF2 (3) or a derivative of pIF2 that had been digested with a restriction enzyme as indicated for each transcript. For some transcripts the template consisted of a PCR fragment prepared using the T7 *cro* primer (5′-TAATACGACTCACTATAGTGTACTAAGGAGGTTGTATGG-3′) and one of a number of downstream primers designed to yield run off transcripts ending at a specific sequence, as indicated. RNA was synthesized in a 100-μl reaction mixture containing 0.1 M Tris acetate, pH 7.5, 10 mM magnesium acetate, 4 mM spermidine, 10 mM dithiothreitol, 40 units of RNasin, 400 units of T7 RNA polymerase, ~5 pmol of DNA, and 1.0 mM each of ATP, GTP, UTP, and CTP. After 2 h at 37 °C, EDTA was added to 30 mM and SDS to 0.5% (w/v), and the mixture was then digested with 5 μg of proteinase K for 30 min at 37 °C and extracted with phenol/chloroform (1:1). The RNA was precipitated with ice-cold ethanol in the presence of 0.5 M ammonium acetate and resuspended in 8 μl of 95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol. After 2 min at 92 °C, it was separated by electrophoresis on a 5% polyacrylamide gel containing 8 M urea and TBE buffer (0.09 M Tris base, 0.09 M boric acid, and 2 mM EDTA, pH 8.4). The RNA was located on the gel by UV shadowing and isolated by electroelution (45 min at 125 V). The RNA was recovered by ethanol precipitation and resuspended in 50 μl of H_2O. Its concentration was determined from its A_{260}. To be sure that it was not degraded during electroelution, ~50–100 ng was reanalyzed by gel electrophoresis. It was stored frozen at −20 °C in the presence of 40 units of RNasin. The synthesis and isolation of ^{32}P-labeled RNA was as described in Faus and Richardson (3).

ATPase and binding assays were done by the procedures of Faus and Richardson (3). The values for the ATPase assays are the molar rates of hydrolysis per mol of hexameric Rho (the turnover number) with saturating amounts of RNA as determined from measurements of rates with various RNA concentrations. As was found previously (3), saturation was achieved with >4 mol of RNA/mol of Rho hexamer, a result that is expected from using concentrations of Rho and RNA near the K_d value for the Rho-RNA binding interaction.

To prepare a *cro* gene template with a T7 promoter and a duplication of the tsp region of tR1, a DNA amplification reaction was performed using the T7 *cro* and L primers. The 394-bp product was then divided into 2 parts. One was digested with *Nsi*I and the other with *Nde*I. Both were treated with T4 DNA polymerase to create blunt ends; the *Nde*I-digested fragments were also treated with calf intestinal phosphatase. The 88-bp *Nsi*I-cleaved fragment and 355-bp *Nde*I-cleaved fragment, both purified by gel electrophoresis, were mixed and treated with DNA ligase. The product was then used for another round of DNA amplification with the T7 *cro* and L primers and the 443 bp fragment was purified by gel electrophoresis.

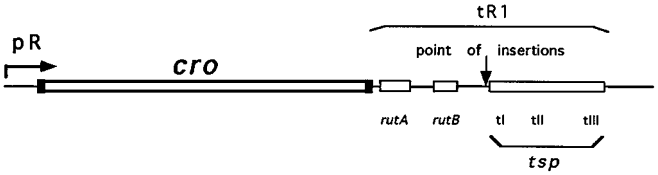

FIG. 1. **Diagram of the *cro* gene of bacteriophage λ indicating the positions of the rut and tsp elements of tR1.**

RNA Structure Analysis—The Mulfold 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 tsps 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 *Nsi*I site between the rut and the 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 *Cla*I fragment from the *E. coli rho* gene, which was chosen because it had been shown not to have a Rho-dependent terminator in either orientation.[2] 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 *Pvu*II-*Hin*cII 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 *Nsi*I 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.

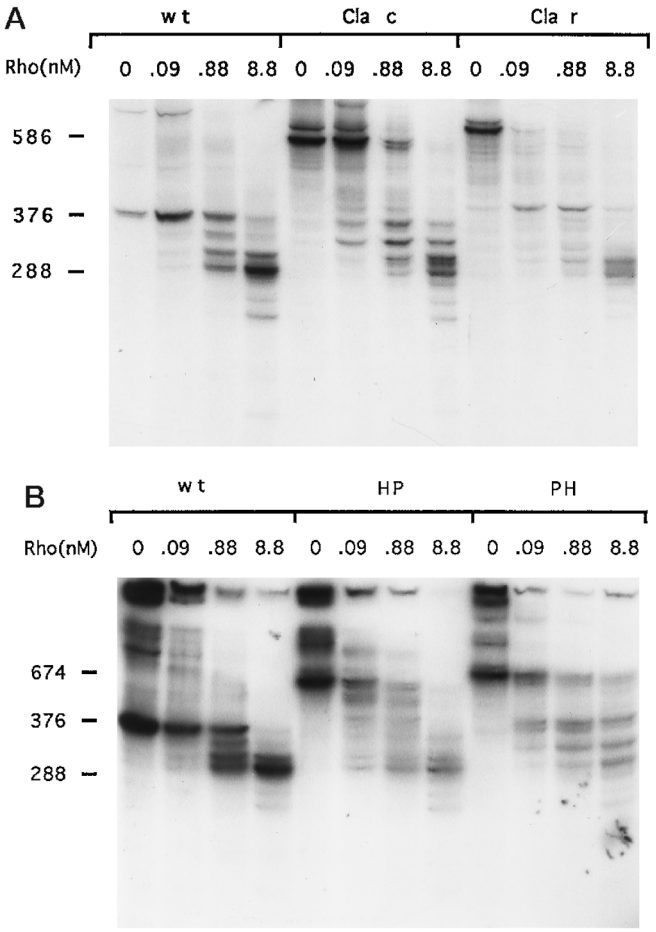

FIG. 2. **Termination of transcription in exogenous sequences inserted between the rut and tsp elements of tR1.** ^{32}P-Labeled RNA samples were prepared by transcription of the indicated templates in the presence of the indicated amounts (given as nM of hexamer) of Rho protein and were separated by gel electrophoresis. *A*, RNA from the wild-type *cro* (*wt*) and the mutant templates with *E. coli rho* Cla DNA fragment in its expressed (*Cla c*) and reversed (Cla r) orientations. *B*, RNA from the wild-type *cro* (*wt*) templates and the mutant templates with the pBluescript *Pvu*II-*Hinc*II fragment in the rut-*Hinc*II-*Pvu*II-tsp (PH) and the opposite (*HP*) orientations.

PhosphorImager to quantitate the amounts of the transcripts. The results (Table I) show that the efficiencies of termination in the various subsites in the inserts ranged in values from 28 to 57% as compared with 60 to 78% for the sites in tR1. Hence, the inserted sequences allowed termination to occur but at a lower efficiency.

Transcriptional Pausing in the Termination Regions—The positions and relative efficiencies of termination in λtR1 correlate strongly with the positions and relative extents of pausing in that region during transcription of the λ *cro* template in the absence of Rho (7, 10). To determine whether termination in the inserts is also occurring at pause sites, we analyzed the kinetics of RNA elongation in the absence of Rho. For each template, the size distribution of the transcripts after 50, 70, and 90 s of synthesis was compared with the distribution of Rho-terminated transcripts (Fig. 4). The results show that RNA polymerase moves much more rapidly through the termination region of the insert sequences than through the termination region of the wild-type template. However, it does pause at each of the termination points, albeit much more briefly at most pause points than at the corresponding sites in the wild-type template. The Cla r and HP inserts both have very strong pause sites within the inserts, at position 390 in Cla r and at positions 335 (tIII) and 376 in HP.

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 phosphoimaging 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

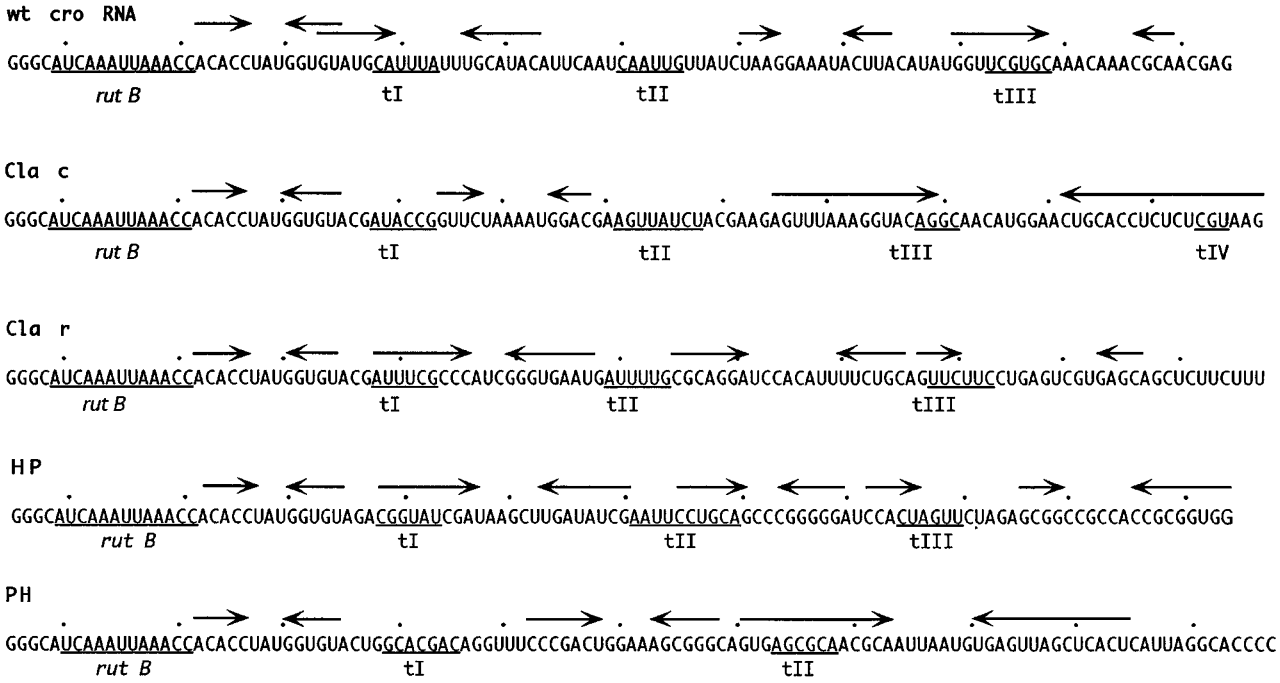

FIG. 3. **Sequences of the wild-type and mutant transcripts starting from the middle of the rut sequence (residue 255) and extending to points just beyond the wild-type (*wt*) subsite III end point.** The *lines* underneath the sequences indicate the various end points for transcripts terminated in the 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.

TABLE I
Quantitative values for transcript distribution and termination efficiencies

Stop points		Distribution at 50 s[a]	Distribution with Rho[b]	Termination efficiency[c]	Ratio of distribution with Rho/distribution at 50 s
Name	nt				
		%	%	%	
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[d]	376	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	35	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		

[a] Molar distribution of transcripts after 50 s of synthesis.
[b] Molar distribution of transcripts after synthesis for 10 min in the presence of 8.8 nM Rho (data from Fig. 4).
[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.
[d] RT, read-through transcript.

hydrolysis. The 270-nt transcript that included the rut region, but no RNA further downstream, also had very little activity (k_{cat} of 0.9 s^{-1}). However, the RNA that had been extended a mere 15 nucleotides further downstream of the *Nsi*I site that is located just upstream of the first termination point gave a k_{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_{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-

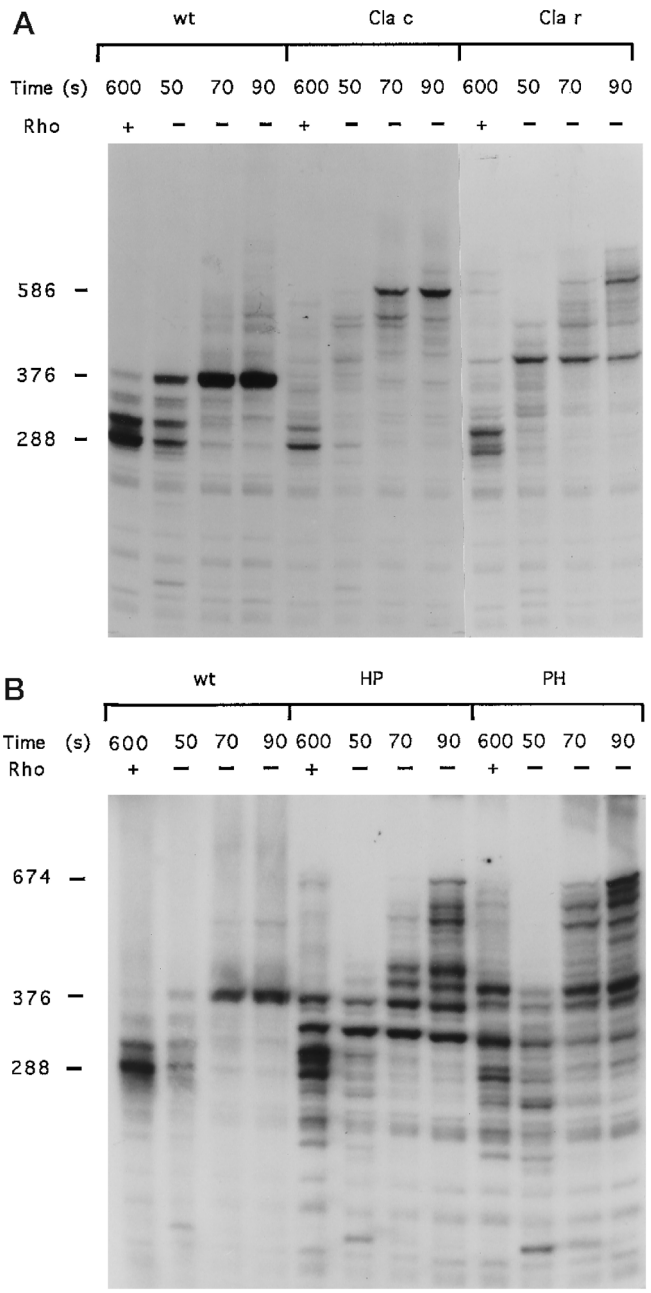

FIG. 4. **Pause points in the termination regions.** ^{32}P-Labeled RNA samples were prepared by transcription of the indicated templates for the indicated times in the absence (−) or presence (+) of 8.8 nM Rho and were separated by gel electrophoresis. *A*, DNA templates as shown in Fig. 2*A*; *B*, DNA templates as shown in Fig. 2*B*.

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 tR1. The k_{cat} values of a 331-nt RNA from the Cla c template and a 332-nt RNA from the PH template

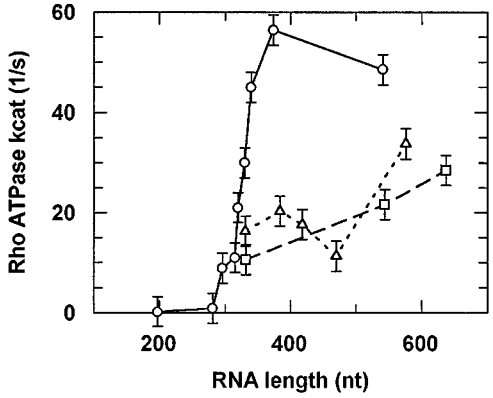

FIG. 5. **Rho-ATPase turnover rates with various transcripts.** k_{cat} values for the indicated lengths of wild-type *cro* gene transcripts (○), the Cla c insert transcripts (△), and the PH insert transcripts (□) are presented. For each RNA, assays were done at a number of different concentrations to determine the characteristic k_{cat} at RNA saturation. The templates used for production of the indicated transcripts were the appropriate pIF2 DNA cleaved with the indicated restriction enzyme or were generated by PCR to yield a template ending at that point. For wild-type *cro*: 197, *Ava*I; 281, *Bst*XI; 296, *Nsi*I; 315, PCR; 320, PCR; 330, PCR; 340, *Nde*I; 374, *Taq*I; 541, *Eae*I. For Cla c: 331, *Dra*I; 384, *Mlu*I; 418, *Kpn*I; 470, *Bam*HI; 576, *Nde*I. For PH: 332, *Ase*I; 544, *Bam*HI; 637, *Nde*I.

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 tR1 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 λ *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_{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 *Ava*I 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 transcripts 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 sequence were able to bind to Rho with K_a values in the range of 5–10 × 10^8 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

rut sequence. 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 rut component can be satisfied by only a limited set of DNA sequences (5, 27, 28), these experiments indicate that rut 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 sequence elements 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 pausing 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 pBluescript, 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 *LacI* RNA that has been mapped in the intergenic region between *LacI* 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 rut segment 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, that 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 activation 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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