rut Sites in the Nascent Transcript Mediate Rho-dependent Transcription Termination in Vivo*

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The in vitro function of the coliphage λ tR1 Rho-dependent terminator is governed primarily by a tripartite upstream sequence element designated rut. To determine the contribution of the different components of the rut site to terminator function in the normal context of coupled translation of the nascent cro message, tR1 variants lacking different rut site sequences were tested for terminator function in vivo. Intact rutA and rutB sequences were both necessary for efficient termination. However, deletion of the upstream rutA was far more detrimental than deletion of rutB. The intervening boxB, which encodes a short RNA stem and loop, could be deleted without reducing termination or detectably altering Rho's interaction with the corresponding cro transcript. The relative importance of these sequence elements was also the same in a minimal in vitro termination assay system. Rut sequences are therefore essential for terminator function in vivo and rutA contributes substantially more to tR1 function than does rutB. The relative contribution of these elements can be ascribed to differences in Rho's binding affinity for the encoded transcripts. If other cellular factors also bind the rut element RNA, they do not alter the relative contribution of its two regions to Rho-dependent transcription termination in vivo.

The bacteriophage λ relies on interactions with the Escherichia coli host cell transcription apparatus and regulatory systems for the orderly expression of its genes. The temporal regulation of λ gene expression is achieved in part by control of transcript elongation, termination, and anti-termination (1–3). This type of transcriptional regulation in λ and other viruses (4, 5) occurs both through the interaction of accessory factors with the elongating RNA polymerase complex and by the alteration of interactions within the transcription complex that occur in response to sequences in the template DNA and newly transcribed RNA (6).

An accessory factor with a central role in the λ life cycle is the host Rho protein (7). Rho causes transcription termination in a multistep interaction with the elongating RNA polymerase complex that involves an initial high affinity binding to the nascent transcript and subsequent secondary RNA interactions, which lead to the release of the RNA and disassociation of the transcription complex (8). These two RNA interactions are thought to involve two distinct types of RNA binding sites on the Rho hexamer (9). Little is known about the corresponding regions in the RNA, beyond a requirement for low secondary structure (10) and at least a minimum cytosine content (11, 12).

The Lambda tR1 Rho-dependent terminator limits transcription initiated at the PR promoter to the proximal cro gene relative to cII and other downstream genes of the major rightward operon. Like other Rho-dependent terminators, tR1 is composed of two parts, an upstream rut sequence that encodes regions of the nascent transcript to which Rho binds tightly, and a downstream tsp sequence containing points at which paused polymerase complexes are dissociated by Rho (13). The tR1 rut site is interrupted by the N-utilization element, boxB, with the upstream 17 nucleotides designated rutA, and the only partially defined downstream element designated rutB (14). Transcription terminated by Rho at tR1 produces RNAs with heterogeneous 3' ends corresponding to the pauses in transcription elongation at transcription stop point subsites I, II, and III (15). Recently, Richardson and Richardson (13) showed that the upstream rut sequences are the major determinants of termination at tR1, as substitution of the transcription stop point region with foreign sequences did not preclude efficient termination at pause sites in the substituted sequence.

The tR1 rut sequences were identified by studies of transcription termination in vitro using highly purified RNA polymerase and Rho without other accessory protein factors (14, 16). To characterize the role of rut site sequences in transcription termination in vivo, where identified and as yet unidentified (3, 17) factors potentially influence Rho's interaction with the transcription complex, we developed a termination assay system with specific features that address several problems with previous assay methods (18–20). Here we report the effects of deletions of rut site sequences on the function of tR1 in vivo and in vitro and on the abilities of modified cro gene transcripts to bind to Rho and activate Rho ATPase activity. We show that tR1 terminators lacking rutA are more defective than those lacking rutB or boxB, and that the relative importance of these parts for in vivo function is the same as in a purified system with no factors other than Rho.

EXPERIMENTAL PROCEDURES

Materials—Enzymes used for DNA manipulations were obtained from New England Biolabs and RNAsin from Promega. Exonuclease III was from Bethesda Research Labs, T7 DNA polymerase from U. S. Biochemical and proteasine K from Beckman. E. coli RNA polymerase was from Epicentre Technologies or purified according to Andrews and Richardson (21). E. coli Rho was purified from BL21(DE3)pCB111, pLY21 by Lislott Richardson as described by Nowatzke et al. (22). T7 RNA polymerase was also prepared by Lislott Richardson as described by Tabor and Richardson (23). Nitrocellulose Bio-Trace NT filters for Rho RNA binding studies were purchased from Gplant Scientific and polyethyleneimine thin layer chromatography plates from Brinkman. Ribonucleotides and deoxynucleotides were from Amersham.

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Role of rut Elements in Transcription Termination

Pharmacia Biotech and [α-32P]ATP and [α-32P]UTP were obtained from ICN Radiochemicals.

**Bacterial Strains**—Hosts for plasmids containing the λ P4 promoter were lyogemized by λ to repress transcription and increase plasmid stability. Strain JG20 (F[ΔlacZΔM15] lacY1 proAB) Δlac trk thi recA938 Δ(mr)174, the host for transcriptional tr1 fusions, was transduced to Δmra174, recA938(ansM):Tn9–200 by two sequential transductions (24) of strain CHS20 (25) with phage P1 vir.

**Plasmids**—To construct plasmid pJGLC, a 560-bp 5′ HinII fragment from pCYC2 (18) was made blunt-ended by T4 DNA polymerase and ligated into the Smal site of pHBluescript KS+ (Stratagene) such that cro and lacZa shared the same orientation. Plasmid pJGBTZ was constructed from pJGLC in a somewhat indirect series of ligations involving an initial placement of the promotor from pKK233−3 (Amersham Pharmacia Biotech) upstream of the cro-lac fusion. A 589-bp HindIII-BamHI fragment from pJGLC was ligated to the HindIII-BamHI vector fragment of the lac fusion plasmid pTL61T of Linn and St. Pierre (26). After excising a 70-bp EcoRI fragment from the resulting construct, an HgiII-BamHI fragment containing the promotor from pKK233−3 was ligated to the 856-bp PstI-BglII vector fragment. A 7062-bp fragment containing the cro-lac fusion region generated by PstI cleavage and partial BspMI digestion was then blunt-ended and ligated into the Smal site of a modified low-copy plasmid pCL12.1 from which the lac promoter-containing template was deleted. The resulting cro-lac fusion was oriented in the opposite direction from the remaining pCL12191 lacZa fragment. Upstream cro sequence beginning with the fifth nucleotide of the cro transcript was then restored on the plasmid by replacing the 210-bp Smal-NarI cro fragment with a 280-bp Res-NalI fragment from pJGLC. Construction of pJGBTZ was completed by the addition of the F1 phage origin of replication from plasmid pUC1 (Amersham Pharmacia Biotech) as a HindIII-KpnI vector fragment. A 26-bp deletion was then made blunt-ended and ligated into the Smal site of a modified low-copy plasmid containing a cII promoter and divergent araC coding region from plasmid pBAD18 in place of the 1057-bp PstI-EcoRI lac promoter fragment.

To estimate relative plasmid copy number in tr1-locZ fusion strains, cultures were grown as described below for synthesis of RNA transcripts. These components in a 20-μl reaction mixture contained 100 nM RNA polymerase, 400 μM each of four NTPs and rifampicin (10 μM). The solution was preincubated at 37 °C for 5 min before the addition of 5 μl of a mixture of four NTPs and rifampicin (10 μM). All ribonucleotides were present at 150 μM final concentration with 5 μCi of radiolabeled [α-32P]UTP per reaction. After 4 min, reactions were stopped by the addition of 50 μl of a solution containing 0.4% SDS, 60 mM EDTA, 5 μg of E. coli trRNA, and 2.5 μg proteinase K. After 10 min of digestion at 37 °C, transcripts were precipitated by the addition of 0.5 volumes of 7.5 M ammonium acetate and 2.5 volumes of ethanol before analysis on 6% acrylamide gels as described previously (21). Following electrophoresis, gels were dried and exposed to a PhosphorImager screen or x-ray film. Terminiation efficiencies were calculated as the percentage of transcripts reaching the end of the template relative to all the transcripts produced from the rrnB Z reporter gene, the lacZ reporter gene, the rnrB T3 terminators that are used to inulate the expression cassette, the origins (ori) of replication for phage P1 and pSC101, and the gene that confers resistance to spectinomycin and streptomycin (spec/strep).

**In Vitro Transcription—**DNA fragments equivalent to the 560-bp 5′ HinII fragment containing the P4 and tr1 (15) were synthesized by 17 cycles of PCR (29). Transcription templates were prepared by addition of proteinase K (50 μg/ml) to PCR reactions and incubation for 20 min at 37 °C followed by a single chloroform-isamyl alcohol (24:1) extraction. Aqueous phases were then loaded directly onto 0.8% low-melting agarose gels before recovery of the desired DNAs by a modified freeze-thaw extraction (30). Templates were then desalted on Sephadex G50 spin columns (31) and equilibrated with 10 mM Tris acetate (pH 7.8).

**Standard 25-μl in vitro transcription reactions (32) contained 150 mM potassium glutamate (pH 7.8), 40 mM Tris acetate (pH 7.8), 4 mM magnesium acetate, 20 μg/ml acetylated bovine serum albumin, 0.02% Nonidet P-40, 20 units/ml RNAsin, and 5 mM dithiothreitol.**

**Template DNAs were at 5 mM, Rho at 2.5 mM, and RNA polymerase at 7 mM except where otherwise indicated. These components in a 20-μl volume were preincubated at 37 °C for 5 min before the addition of 5 μl of a mixture of 4 units of T7 polymerase and rifampicin (10 μM). All ribonucleotides were present at 150 μM final concentration with 5 μCi of radiolabeled [α-32P]UTP per reaction. After 4 min, reactions were stopped by the addition of 50 μl of a solution containing 0.4% SDS, 60 mM EDTA, 5 μg of E. coli trRNA, and 2.5 μg proteinase K. After 10 min of digestion at 37 °C, transcripts were precipitated by the addition of 0.5 volumes of 7.5 M ammonium acetate and 2.5 volumes of ethanol before analysis on 6% acrylamide gels as described previously (21). Following electrophoresis, gels were dried and exposed to a PhosphorImager screen or x-ray film. Terminiation efficiencies were calculated as the percentage of transcripts reaching the end of the template relative to all the transcripts produced from the rrnB Z reporter gene, the lacZ reporter gene, the rnrB T3 terminators that are used to inulate the expression cassette, the origins (ori) of replication for phage P1 and pSC101, and the gene that confers resistance to spectinomycin and streptomycin (spec/strep).
Role of rut Elements in Transcription Termination

Rut Sites Mediate Termination in Vivo—To test the role of the rut site and its sequence elements in termination at tR1 in vivo, we constructed a transcriptional fusion plasmid that is well suited for quantitative analysis of transcription termination. Plasmid pJGBTZ (Fig. 1) contains a complete translated cro coding sequence and tR1 terminator positioned between the tightly regulated araBAD promoter and the lacZ reporter gene on a low-copy pSC101-derived replicon. The fusion junction contains an RNase III processing site allowing a uniform β-galactosidase message to be generated in all tR1 constructs, and the entire region is insulated from transcription initiated outside the region by flanking rrrB terminators, features developed by Linn and St. Pierre (26). The araBAD promoter allows adjustment of β-galactosidase expression levels to those readily tolerated by the host cell.

The relative level of β-galactosidase expressed from fusion constructs lacking all or parts of the tR1 rut site (Fig. 2) are shown in Fig. 3. Levels are given as a percentage of downstream gene expression in a control construct lacking tR1 (pJGBZ). In our system, the efficiency of termination at tR1 in vivo ranged from 70 to 82% in different experiments, which is slightly higher than previous estimates (1). The results show that a deletion of the whole rut region (ΔrutA-B) allowed almost as much downstream gene expression as seen in a fusion that lacked any terminator, thus confirming the importance of the rut region for in vivo termination function in the natural context of cotranslation of the cro message and in the presence of all accessory host factors. The reductions in the level of termination with the various partial deletions indicate that rutA, rutB, and boxB contribute approximately 64, 24, and 5%, respectively, to the level of termination of the entire region. Thus this result shows that in vivo rutA contributes significantly more to the function of tR1 than does rutB, whereas the boxB element contributes very little.

In Vitro Termination—To determine whether intracellular factors other than Rho contributed to the different relative importance of rutA and rutB in termination, the efficiency of Rho-dependent transcription termination in vitro was measured by transcription of the indicated templates as described under “Experimental Procedures” in the absence or presence of 2.5 mM Rho and were separated by gel electrophoresis. The lanes on the left show marker RNAs (cro gene run-off transcripts) of 243 and 276 nucleotides. The RNA products that are longer than those expected by extension of transcripts to the end of the template presumably are a result of end-to-end template switching (51), which is suppressed by Rho function.

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Deletion of these upstream regions was previously shown to reduce electrostatic interactions between Rho and the RNA (33).

Because any change in RNA sequence is likely to be associated with an alteration of RNA structure, and because Rho’s interactions with RNA are very sensitive to RNA secondary structure, we used the Mulfold program (35) to predict secondary structures of rut-deletion transcripts. Computer modeling did not predict any new secondary structural elements in the remaining rut sequences for any of the deletion RNAs (data not shown). This analysis suggests that the loss of tR1 function can be attributed to the loss of rut site sequences and not to the sequestration of the remaining rut sequences in a base-paired secondary structure.

Rut Site Deletions and Transcriptional Pausing—Because the efficiency of transcription termination is dependent on Rho’s ability to interact with a paused transcription complex, we analyzed the kinetic progress of transcript elongation on the tR1 deletion templates described. Fig. 5 shows the products of an in vitro transcription reaction in which the progress of the transcription complex can be followed in terms of the discrete RNA products synthesized at particular intervals. Although analysis of transcriptional pausing is approximate at best on such templates of unequal length in which different multiple early pause sites lead to asynchronous later pausing at relevant transcription stop points, it can be seen that polymerases continued to make substantial pauses at all three tR1 subsites on each deletion template.

RNA Binding—A critical aspect of Rho’s termination activity is the ability to make stable ATP-dependent interactions with nascent transcripts. A filter binding assay developed by Ceruzzi and Richardson (36) was used to determine the nature of the defects in termination associated with the tR1 deletions described. Rho bound wild-type cro messages extended to the third tR1 subsite with the previously established high affinity (Table I) (33). The ΔrutA deletion reduced Rho’s apparent $K_a$ for the RNA approximately 9-fold, whereas ΔrutB resulted in a more modest 3-fold decrease (Table I). Deletion of boxB had little effect on Rho’s ability to bind the corresponding transcript, whereas the ΔrutA-B deletion reduced Rho binding approximately 13-fold, similar to a more extensive rut deletion previously characterized (ΔR70; Ref. 33). As well as being required for a high affinity Rho-cro interaction, addition of the 23 rutA nucleotides in their native context immediately downstream of the cro coding sequence was sufficient to allow high affinity Rho binding of transcripts truncated at the cro stop codon (data not shown).

Table I also presents values for the free energies associated with the formation of Rho-RNA complexes and the changes in the free energy of this interaction related to deletion of rut site sequences. Of the total increase in free energy associated with deletion of the entire rut region, ~70% can be accounted for by the rutA deletion, ~30% by the rutB deletion and <5% by the boxB deletion. These differences correlate very well with reduced termination at mutant tR1 terminators lacking the corresponding rut sequences.

Rho-ATPase Activation—In addition to binding to the nascent transcript, interactions between Rho and RNA that are coupled to ATP hydrolysis are necessary for Rho-dependent transcription termination (37, 38). The ability of a transcript to elicit this type of productive interaction can be assessed by the degree to which it can activate ATP hydrolysis by Rho when the RNA is present at a concentration well above the $K_a$ for dissociation of the Rho-RNA complex. As shown by the data in Table I, Rho had virtually no ATPase activity with the rutB mRNA as a cofactor and only partial activity with other rut site deletion RNAs as cofactors; again the reduction in ATPase activity was well correlated with the reduced termination activity of the corresponding mutant tR1 terminators.

**DISCUSSION**

We have shown that the rut sequences of λ tR1 are necessary for efficient terminator function in vitro. As these sequence elements were identified from studies of transcription termination in vitro, our results establish their central role in transcription termination in E. coli in the presence of all accessory transcription factors and in the normal context of coupled translation of the nascent cro mRNA.

With our assay system, we found that the in vivo termination efficiency at tR1 was 70–80%, which is at the high end of what has been previously reported when various other methods were used to measure in vivo tR1 function (18–20, 39). Although the relatively greater efficiency of termination at tR1 as we determined may represent a more accurate assessment with our assay system, the possibility remains that differences at the 5′-end of the cro mRNA related to the substitution of araPBAD for P_R influenced terminator function. Although all fusions were made such that transcripts contained the entire cro open

![Image](https://example.com/image.png)
finding that the relative contributions of have the potential to modulate Rho interactions at tR1, the full-length Ardson (33) that the deletion of the rut trp t the conditions examined (48). In contrast, we found that boxB can be deleted with essentially no effect on termination efficiency at tR1, and without reducing Rho’s affinity for cro RNA. Thus, the boxB hairpin structure is not an important component of the RNA-binding region of tR1.

Of the two remaining components of the rut region, the upstream rutA was more important than rutB for both terminator function and in terms of the contribution of each to the binding affinity of Rho for the cro RNA. The RNA region encoded by rutA is much richer in cytidylic residues than that encoded by rutB (see Fig. 2), confirming the importance of this residue in both terminator function (11, 12, 42) and binding of Rho to RNA (43, 44).

RutA also contains a boxA sequence (45), which differs by only a single residue from a consensus motif that has been shown to bind the NusB-S10 complex (46). Deletions of rutA and upstream sequences that had the greatest effect on termination also eliminated boxA, and 5′ nucleotides specifically proposed as capable of binding an unidentified “inhibitory host factor” in λ N-mediated antitermination (3, 47). Our results suggest that Rho is this host factor but do not rule out the possibility that Rho is also a competitor for the same RNA site as the NusB-S10 complex and an unidentified inhibitor of antitermination. Although such host factor-nutR interactions have the potential to modulate Rho interactions at tR1, the finding that the relative contributions of rutA and rutB to the terminator function were the same in vitro and in vivo suggests that if NusB-S10 or other cellular factors are binding to sites in the rut region, they are doing so in a way that does not alter the relative recognition of those segments by Rho.

The E. coli trp t’ terminator, like λ tR1, is capable of remaining partially functional despite significant deletion of upstream regions (48). This bacterial terminator was, however, much more resilient to deletions of the size that our results show drastically reduce termination at tR1, and no single region of trp t’ appeared to be required for efficient termination under the conditions examined (48). trp t’ therefore appears to contain a greater redundancy of sequence necessary for productive interaction with Rho than does tR1.

Our results confirm the previous finding of Faus and Richardson (33) that the deletion of the rut region causes a surprisingly small change in the binding affinity of Rho for the isolated full-length cro transcript, yet results in a nearly complete loss of termination and ATPase cofactor activity of the encoded transcript. One explanation for the apparently small difference in binding affinities is that Rho makes a number of different kinds of nonproductive interactions with the rut deletion RNAs that are comparable with the complexes a repressor protein makes with nonoperator DNA sites. The overall binding affinity of Rho for an RNA lacking a rut site would therefore represent the sum of separate weak interactions at a large number of different nonspecific sites. In that case, the true difference in energy between Rho complexed with a single nonspecific site and Rho binding a specific rut site is probably much greater than the 2 kcal/mol that we calculated by comparison of the ability of Rho to bind full-length cro RNAs with and without the rut sites.

cro RNAs that lack rut sequences are unable to activate significant Rho ATP hydrolysis even when they are present at a concentration that is well above the Kₐ for the Rho-RNA binding interaction. This observation suggests that the complexes that do form with the nonspecific sites do not appear to have the RNA positioned correctly for subsequent ATP-dependent interactions. Hence, these nonspecific interactions that do form do not lead to ATP hydrolysis or the processes coupled to ATP hydrolysis that lead to the disruption of the RNA-DNA helix in the transcription complex (49, 50). The rut site thus appears to serve two roles; it provides a strong attachment site for Rho, and it positions the RNA on the protein for the ATP-dependent reactions that result in transcription termination.

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