Published online June 4, 2004

Nucleic Acids Research, 2004, Vol. 32, No. 10 ***3093–3100***
DOI: 10.1093/nar/gkh630

Sequence-specific Rho–RNA interactions in transcription termination

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Received as resubmission May 10, 2004; Accepted May 11, 2004

ABSTRACT

The bacteriophage λ tR1 terminator encodes a region of the nascent *cro* transcript containing RNA residues recognized by termination factor Rho. To identify ribonucleotide–protein interactions contributing to termination, a library of reporter gene plasmids was constructed containing predominantly single-nucleotide substitutions in a 24 nt region previously shown to be critical for efficient termination. Screening 16 822 bacterial transformants identified 110 terminator mutants, most of which contained two or more nucleotide substitutions. Although the vast majority of single base changes did not reduce tR1 function, 11 specific single-nucleotide substitutions at eight positions interspersed in the upstream part of the target region (5′-ATAACCCCGCTCTTACACATTCCA-3′) did reduce termination. About half of these substitutions also reduced Rho-dependent termination on *cro* gene templates transcribed by purified RNA polymerase, indicating specific residues critical for optimal terminator function. Other termination defects were not reproduced in these *in vitro* assays, and likely resulted from indirect effects of altering interactions between tR1 and additional cellular factors capable of attenuating Rho function. Our results indicate that while Rho is able to recognize a wide variety of similar *rut* site sequences by interacting with alternate nucleotides at critical positions, interactions with specific individual ribonucleotides of the tR1 transcript provide highly efficient Rho-dependent termination.

INTRODUCTION

RNA sequences and structures that result from transcript elongation and termination play central roles in a variety of mechanisms used by both bacteria and viruses to regulate gene expression (1–3). These functional RNA elements are encoded by the promoter-proximal regions of many bacterial biosynthetic operons, both within and between coding regions in polycistronic operons, and between transcriptional units on chromosomes. Those most thoroughly characterized are found in *Escherichia coli*, and include regions of nascent transcripts that function in transcription termination. At Rho-independent or 'intrinsic' terminators, the normally highly stable polymerase elongation complex is destabilized directly by nascent transcript sequences that form stem–loops and 3′ terminal polyuridine tracts. These typical RNA-hairpin encoding terminators are not found downstream of coding regions in all prokaryotic genomes (4), and are only present at about half of termination sites in *E.coli* (5). Transcription termination is also mediated by untranslated regions of nascent transcripts that bind termination factor Rho. Rho is an RNA–DNA helicase that actively releases nascent mRNAs from paused transcription complexes. Other bacterial and viral gene products capable of binding to nascent transcripts also act as important mediators of elongation and termination (6). These include viral antitermination factors (1), bacterial Nus factors, ribosomal proteins (7,8), cold-shock proteins (9), and other as yet unidentified transcription factors (10,11).

Rho is capable of binding nascent RNA chains containing at least 60 nt that are relatively low in secondary structure (12–14), and contain at least a minimal cytidine content (15,16). Rho recognizes RNA encoded by terminator sequences upstream of those encoding the 3′ ends of terminated transcripts (17). These *rut* site sequences define Rho-dependent terminators, as the downstream sites where termination actually occurs can be replaced by alternate sequences that act as RNA polymerase pause sites (18,19). Among several *E.coli* Rho-dependent terminators that have been described there does not seem to be any consensus sequence. However, these terminators do appear to contain a distinct compositional bias that yields transcripts that are rich in C residues relative to G residues (20).

tR1 is a Rho-dependent terminator located in the intercistronic region between the *cro* and *cII* genes of bacteriophage λ. *In vitro* solute accessibility studies confirm that termination factor Rho forms a stable association with two single-stranded regions of the nascent tR1 terminator transcript (*rutA* and *rutB*) during termination (21). Our previous analyses (22) of transcription termination at tR1 indicated that Rho interactions with nascent RNA residues at the 5′ end of the tripartite *rut* sequence region (*rutA*) contribute substantially more to termination than Rho interactions with residues in the downstream *rutB* sequence. The intervening *boxB* residues are not important for termination. These sequences and their relative positions on the *cro* mRNA are shown in our prior report (22). We therefore determined that the most

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critical nucleotides of tR1 are located within a 24 bp segment that extends from the *cro* termination codon to the start of the *boxB* sequence (Fig. 2). We then focused our efforts on identifying individual protein–RNA residue interactions in this region that make substantial contributions during Rho-dependent transcription termination.

In this paper we describe the use of a tR1-reporter gene fusion plasmid and phenotypic screen to isolate termination-deficient mutants from a library of bacterial clones containing random nucleotide substitutions in the tR1 *rutA* region. Our results indicate that although most nucleotide substitutions in this region do not reduce termination, Rho's interaction with specific *rutA* residues (or specific alternate residues) at key positions is necessary for efficient termination. As some of the single-base changes that reduced tR1 function *in vivo* did not reduce termination in a minimal purified *in vitro* assay system, the relevant *rutA* residues likely interact with additional cellular factors in the *E.coli* cytoplasm.

MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study have been previously described by Graham and Richardson (22).

Construction of tR1 mutant libraries

A mixture of oligonucleotide primers containing random substitutions for 24 λ tR1 nucleotides (encoding *cro* RNA residues +220 to +243) was prepared with an Applied Biosystem 380A DNA synthesizer. Oligonucleotides containing predominantly single substitutions within the underlined 24 nt region of the primer sequence 5′-CCTTCCCGAGTAACAAAAAAACAACAGCATAAATAACCCCGCTCTTACACATTCCAGCCCTGAA-3′ were produced by combining an equal molar mix of the three alternate nucleotides (4.16%) with the primary phosphoramidite nucleotide (95.84%) during synthesis. The resulting degenerate oligonucleotide pool was then used with downstream primer 'L' (5′-CGATTCGTAGAGCCTCGTTG-3′) to amplify a double-stranded *cro* gene fragment from plasmid pJGLC (22) by PCR (Fig. 1). Following digestion of this PCR product with AvaI and NsiI, a small 96 bp DNA fragment containing the mutagenized tR1 *rutA* region was isolated by electrophoresis on a 6% polyacrylamide gel. The eluted fragment was then ligated to an AvaI–NsiI vector fragment from plasmid pIF2 (23) to restore the flanking *cro* gene regions. Following transformation, plates containing a total of 32 200 colonies were overlaid with liquid medium, and scraped gently with a sterile glass rod to collect plasmid clones.

Plasmids were isolated from these pooled transformants, and a 204 bp BglII–NsiI fragment representing a library of substituted tR1 sequences was recovered following agarose gel electrophoresis. This fragment was then sub-cloned in place of the corresponding tR1 containing fragment of terminator fusion plasmid pJGBTZ (22). A total of 31 680 clones were collected following transformation of ligated plasmid DNA into *E.coli* strain INVαF′. Omission of the mutagenized insert fragment from the pIF2 and pJGBTZ ligations indicated a low background of potential non-mutagenized plasmids (2.5% in the former, 11.5% in the latter). The resulting terminator fusion plasmid library was therefore expected to contain 27 249 tR1 sequence variants (31 680-14%, the combined background).

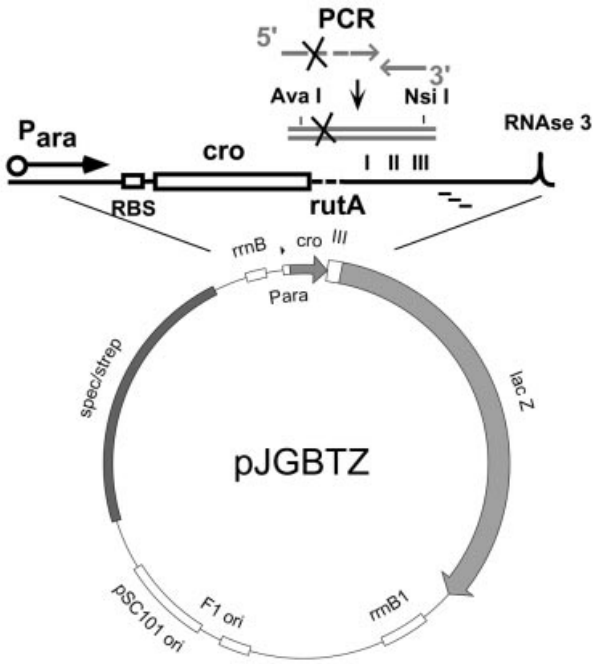

Figure 1. Mutagenesis of the *rutA* region of tR1 terminator fusion plasmid pJGBTZ. PCR was used to incorporate a deoxyoligonucleotide primer containing predominantly single-nucleotide substitutions into a DNA fragment encoding the principal Rho-binding site of the λ tR1 terminator transcript. The mutagenized fragment was then substituted for the corresponding tR1 region of a plasmid designed to assay *in vivo* termination efficiency. Various features of pJGBTZ, including a tightly regulated promoter (Para), native *cro* coding region, tR1 termination sites (I, II and III), RNAse III processing site, reporter gene (*lacZ*) and flanking *rrnB* terminators are indicated.

A random sampling of 31 clones confirmed the expected mutation distribution (i.e. ~37, ~37, ~18.4 and ~7.6% had zero, one, two and three nucleotide substitutions, respectively). A library of 558 of these clones would have a 95% chance of containing all 72 possible single base changes for the 24 bp target region, if every oligonucleotide synthesized contained exactly one substitution (24). As only 37% of the oligonucleotides synthesized were expected to contain a single mutation, a sample of 1508 (558/0.37) clones would therefore have a 95% chance of containing all possible single base substitutions. As phosphoramidite DNA synthesis procedures are known to result in compositional biases, we compensated for an observed reduced level of cytidine substitutions by screening a 3.5-fold excess number of clones beyond that calculated to be necessary to obtain all possible single substitution mutants from an unbiased oligonucleotide synthesis.

Selection of tR1 mutants

To identify tR1 variants with reduced Rho-dependent transcription termination, fusion plasmid libraries were transformed into strain JG20 (22) and plated for a target cell density of 100 colonies per plate on MacConkey agar with 0.2%

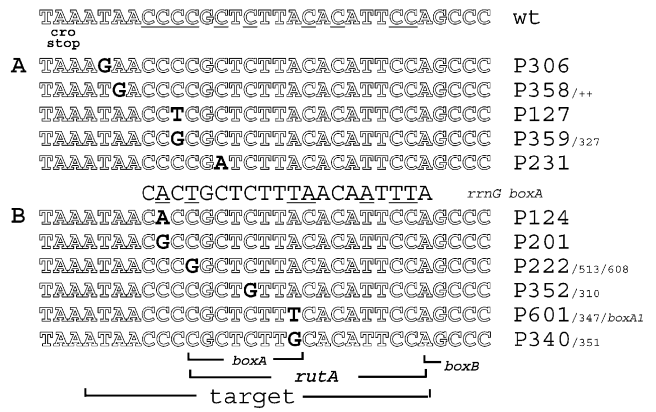

Figure 2. tR1 single-nucleotide substitutions present in clones selected for reduced Rho-dependent termination *in vivo*. Eleven specific single base changes reduced termination both as indicated on plates, and in the *in vivo* assay system described. Several mutant sequences were present in more than one isolate, as indicated by multiple designations following each sequence. *In vivo* and *in vitro* termination assays distinguished two classes of tR1 single substitution mutants. One class (**A**) exhibited reduced termination both *in vitro* and *in vivo*, and the other (**B**) showed reduced terminator function only *in vivo.* The mutagenesis target site containing the overlapping *rutA* and *boxA* of Lambda *nutR*, and flanking *cro* stop codon and *boxB* are indicated. The wild-type tR1 sequence is shown above with the cytosine residues underlined. The *E.coli rrnG boxA* sequence is shown in the center with residues that differ from those of λ *boxA* underlined. ++ indicates that numerous isolates with this mutation were obtained.

lactose. Selected colonies were immediately inoculated into M9-CAA medium [M9 salts (25) with 0.2 % (w/v) glucose, 0.4% (w/v) casamino acids, 40 mg/ml L-leucine, 10 mg/ml thiamine and 70 mg/ml spectinomycin] and cultured for 15 h at 37°C prior to storage as frozen glycerol stocks. Isolates were subsequently screened again for increased β-galactosidase expression levels with the quantitative assay described below, and DNA sequences of plasmids from isolates with verified termination defects determined by primer extension with Sequenase v.2.0 (USB) as described by the manufacturer. Plasmids with defective terminators containing single-base substitutions were then re-isolated from the selected strain, and transformed back into JG20 prior to further analyses of relative β-galactosidase expression levels.

Analysis of termination at tR1 *in vivo*

To determine the efficiency of Rho-dependent transcription termination *in vivo*, cells from frozen cultures were inoculated into M9-CAA medium and cultured at 37°C for 4 h. Arabinose was then added to 0.2% (w/v), and cultures grown to saturation (16–18 h). Cells were then inoculated (0.15%, v/v) into 2 ml of M9-CAA medium containing 13 mM arabinose. After ~4 h (about five doublings), cultures reached an OD_{600} of 0.3–0.4 (measured in a Shimadzu Model 1601-UV), and were placed on ice for exactly 20 min. β-Galactosidase expression levels were determined as described by Miller (26), following the addition of 10 µl of 0.1% sodium dodecyl sulfate and 20 µl of chloroform per milliliter of resuspended cells. Duplicate measurements were taken from each of three parallel cultures for each strain assayed.

Table 1. Summary of tR1 terminator mutants identified

Mutant class	Total number obtained	Unique terminator sequences[a]
Deletion	2	2
Single with ATG[b]	48	1
Single	16	10
Double	26	23
Triple or higher	18	14

An oligodeoxyribonucleotide mutagenesis strategy was used to create a library of mutants containing predominately single-nucleotide substitutions at tR1. A total of 6822 individual colonies were then screened for increased β-galactosidase activity on indicator plates, and 110 mutants identified by subsequent quantitative enzyme activity assays.

[a]Terminator sequences for the single and double mutant classes are as shown in Figures 2 and 3.

[b]As described in the text, a single A→G transition introduced a translation initiation codon adjacent to the *cro* stop codon, potentially limiting Rho's access to the nascent terminator transcript.

RESULTS

rutA mutagenesis and functional selection

To study the roles of ribonucleotides in the nascent tR1 transcript in termination, we constructed a terminator-reporter gene fusion plasmid, and used a degenerate oligodeoxyribonucleotide mutagenesis strategy to identify critical residues that contribute functional interactions in termination. *rut* site sequences in their natural genetic context immediately downstream of the translated *cro* coding region were placed between the arabinose-inducible promoter P_{ara} BAD, and a *lacZ* reporter gene on pSC101-based plasmid pJGBTZ (Fig. 1). A 24 bp segment previously shown to encode RNA residues critical for Rho interactions (22) was replaced by a synthetic DNA fragment containing all possible single-nucleotide substitutions as described in Materials and Methods (Fig. 1). Transformants containing tR1 fusion plasmids were then plated on indicator plates to identify clones with nucleotide substitutions that reduced terminator function. Comparison of colony phenotypes for control strains containing either the wild-type tR1 (pJGBTZ) or a *rutA* deletion (pJGBTZ Δ*rutA*) on several media showed MacConkey's agar without arabinose to be the best at revealing differences in β-galactosidase expression levels (data not shown). By adjusting the lactose (indicator) concentration of these plates to 0.2%, we were able to distinguish colonies of cells expressing β-galactosidase levels as little as 14% above wild type by the appearance of dark red centers after 20 h at 37°C (see below).

A total of 6822 individual clones from six separate pools of transformed cells were screened. Between 1 and 2% of colonies in each set developed colony color phenotypes associated with increased reporter-gene expression. β-Galactosidase expression levels for each clone were then determined directly by the quantitative assay described in Materials and Methods. A total of 110 clones were identified with increased reporter gene expression, and the nature of the base changes in the target region determined by sequencing. Table 1 provides a summary of these results. By far the most prevalent single base change responsible for increased downstream gene expression was the substitution of a guanosine for an adenosine residue in the third position of the codon adjacent to the *cro* stop codon (designated P358, Fig. 2). This created

an ATG start codon adjacent to the normal TAA *cro* stop codon. Among the remaining 62 mutants that did not have this A to G transition, a similar number of single, double and triple substitution mutations were seen. A total of 11 different single base changes were identified that resulted in increased downstream gene expression (Fig. 2). Twenty-three different double substitutions (Fig. 3) and 14 unique triple mutations were also identified (data not shown). Distinct clones containing identical tR1 mutations were isolated multiple times in each frequency class, leading to the multiple designations for some mutations shown in Figures 2 and 3. Only two of the 110 clones analyzed contained unexpected mutations, in both cases these involved nucleotide deletions, including a loss of the upstream *cro* stop codon.

In terms of single-base changes, not unexpectedly, many termination defects resulted from the loss of *rutA* cytidine residues (Fig. 2). Of particular interest was that at five of the eight positions at which single base changes reduced termination, two of the possible alternate residues were apparently tolerated, but the third was not. Reduced termination therefore involved introduction of specific bases, rather than absence of wild-type residues at these sites. Furthermore, at four of these eight positions, the only type of substitution associated with reduced termination was the introduction of a guanosine residue. Over half of these single substitutions also involved residues upstream of the previously described *rutA* sequence (13,24) in the region between the *cro* stop codon and *boxA* of *nutR*.

Figure 3 shows the 23 double substitution mutants identified. Again, 12 (52%) of these mutations involved the loss of cytidine residues only, and all involved the loss of at least one cytidine except for those found in clones designated P354 and P352. Again the introduction of guanosine residues (n = 19 mutations) was associated with reduced termination more frequently than introduction of adenosine (n = 11), thymidine (n = 14), or cytidine (n = 2) residues. Many of the same specific nucleotide substitutions that reduced termination as single base changes were also seen in mutants containing two base changes, confirming that these residues contribute important base-specific interactions in termination at tR1.

Distribution of mutations within the mutagenesis target region

The relative importance of the contributions of specific *rutA* residues to terminator function can be obtained by comparing the frequency at which base changes were identified at each target position among the terminator mutants isolated. Figure 4 shows this data as a histogram where the total number of base changes found at each residue in the target region is compared for (a) all isolates containing either only one or two base changes, or (b) among those with one, two or three base changes. Within both mutant classes, the same mutational 'hot-spots' are indicated. These data suggest that transcription termination is particularly sensitive to mutations that affect the cytidines in *rutA*, and that those in the upstream CCCGC motif contribute major interactions in termination. However, not all cytidine residues appear to be equally sensitive, and certain non-cytidine residues appear to be equally or more sensitive to mutation than cytidine residues.

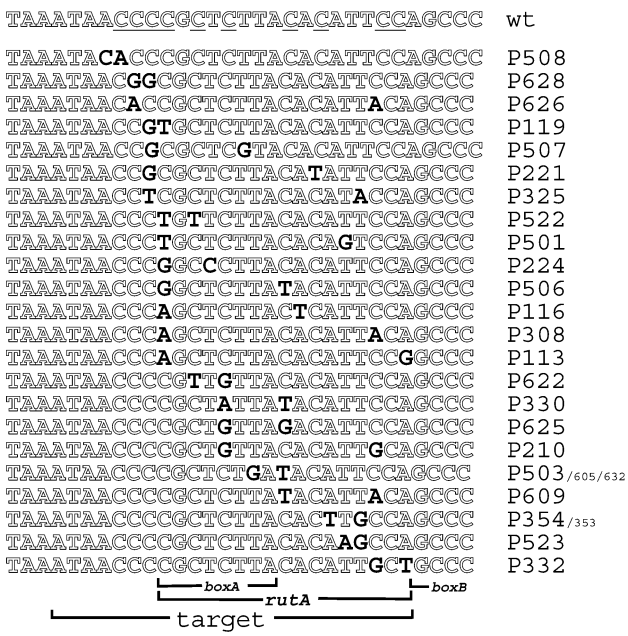

Figure 3. tR1 double nucleotide substitutions in clones selected for reduced termination during growth on indicator plates. Mutations involving two base changes in the mutagenesis target region are shown. In contrast to single substitution mutants, base changes indicated are likely to be a partial set of those capable of reducing termination at tR1, as the number of clones screened was unlikely to have contained all possible combinations of two base changes in this region. Designations are as described in Figure 2.

In vivo termination

To obtain quantitative data on terminator function, single-site mutants (Fig. 2) were assayed in duplicate on at least two occasions as triplicate parallel cultures with the β-galactosidase assay described. Figure 5 shows the relative *in vivo* defect associated with each point mutation.

The base substitution with the largest effect on downstream gene expression introduces an ATG adjacent to the *cro* stop codon (P358). Other mutants showed increased downstream gene expression ranging from 1.6- (P124) to 2.8-fold (P359) relative to a construct containing the wild-type terminator. Deletion of the target region (Δ*rutA*) caused a 3.6-fold increase in readthrough transcription in these assays. Small variations in levels of downstream gene expression relative to wild-type terminator fusions in independent experiments prevented a specific ranking of all terminator defects, particularly for mutants that showed a similar degree of readthrough (e.g. P231 and P601). Clearly, the high expressing mutants P359 and P127, which have substitutions for the same cytidine residue, were more defective than P124 or P340. P124, which contains a cytidine to adenosine transversion, was less defective than most other mutants, and had a significantly reduced effect compared to a substitution of a guanosine for the same cytidine (P201). All other mutants showed a similar ~2-fold increase in downstream gene expression (Fig. 5).

In vitro termination

In the bacterial cytoplasm, components other than Rho factor can influence Rho-dependent termination. To determine

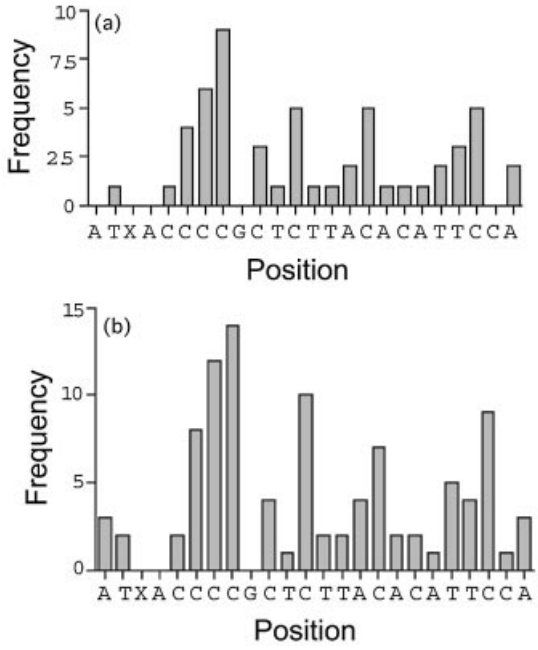

Figure 4. Sensitivity of *rut* site nucleotides to mutations that reduce termination. The histograms shown indicate the number of times mutations were found for each nucleotide position in the target region among the 62 terminator mutants that contained either (**a**) single and double or (**b**) single, double and triple nucleotide substitutions. The total number of mutants that contained substitutions as each target residue are indicated for each class. (Mutants that contained a G at the third position of target region are not included, as this mutation potentially results in translation of the terminator transcript.)

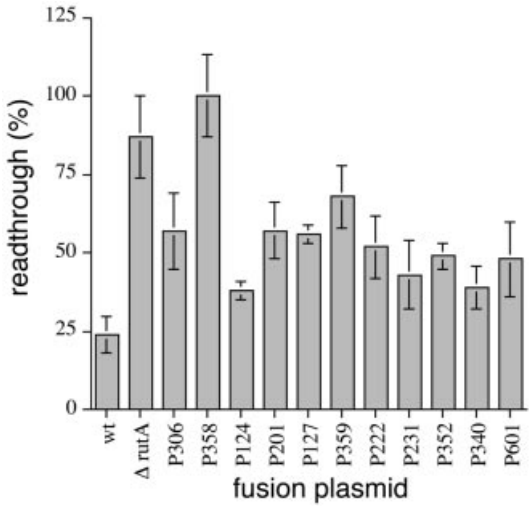

Figure 5. Effects of tR1 single-nucleotide substitutions on terminator function *in vivo*. The relative β-galactosidase activity expressed from terminator fusion plasmid pJGBTZ for each tR1 mutant strain as a percentage of that determined for a strain harboring the same plasmid without the tR1 terminator (pJGBZ) is indicated. Enzyme activity in culture lysates was determined as described in the text. The effect of deleting the mutagenesis target region on downstream reporter gene expression is indicated by the Δ*rutA* plasmid. Results shown are averages of two determinations, where triplicate parallel cultures were assayed in duplicate. Error bars show standard deviations for all six corresponding cultures.

which tR1 mutations directly affected Rho–RNA interactions, *in vitro* termination assays were performed with purified components in single-round transcription assays (27). These assays were optimized to identify differences in terminator function, and used equimolar 150 μM NTP concentrations and sub-saturating levels of Rho. Some representative examples of electrophoretically separated RNAs produced with a wild-type *cro* template and several tR1 mutant templates are presented in Figure 6a, and the results from quantitative analysis of terminator readthrough are presented in Figure 6b. These results show that *in vitro* termination efficiency was significantly lower with some mutant templates relative to others (e.g. P127 and P359 relative to P340 and P601). Of the 11 single substitution mutants that were tested, five had defects in termination that were apparent in our *in vitro* assays (Figs 2A and 6a). These results show for the first time a role of individual RNA residues in the Rho-dependent termination process.

tR1 termination defects

Nucleotide substitutions in *rutA* that reduced Rho-dependent termination likely altered specific aspects of the multi-step interaction between Rho and the transcription elongation complex. We analyzed the nature of these defects in two of our terminator mutants. P127 contained a replacement of a cytidine by a thymidine in the center of a cluster of cytidines in the upstream target region, and in P306 an adenosine was replaced by a guanosine just upstream of this cluster as shown in Figure 2. We first tested the effect of increasing Rho levels on the efficiency of termination at tR1 using these two mutant templates (Fig. 7). Reduced terminator function was not compensated by increased Rho concentration, suggesting that these single-residue alterations did not simply reduce Rho's affinity for the encoded transcripts. We then directly determined Rho's affinity for the corresponding P127 and P306 RNAs in a filter binding assay (22,23). The results (Table 2) showed that the adenosine to guanosine change in mutant P306 (Fig. 6a) reduced Rho's affinity for the encoded mRNA by a factor of 2 relative to the wild-type *cro* mRNA. However, Rho's ability to bind the P127 mRNA was not reduced. We also tested the ability of these mutant *cro* transcripts to act as cofactors for Rho's ATPase activity (22,27). While Rho's maximal ATPase activity in the presence of saturating levels of P306 mRNA was reduced ~2-fold, the P127 mRNA was also only slightly less potent (15%) than wild-type *cro* mRNA as an ATPase cofactor in these assays (Table 2).

DISCUSSION

We devised a biological screen to identify unfavorable Rho–RNA interactions as contributed by specific single-nucleotide substitutions in the *rutA* site of nascent *cro* tR1 transcripts. Following saturation mutagenesis of a target region containing *rutA* and adjacent upstream nucleotides, a total of 110 terminator mutants were isolated. Only 16 were found to contain single tR1 residue alterations, and 11 specific base changes capable of reducing termination were identified (Fig. 2). These mutations all involved residues at eight positions within the first 15 residues downstream of the *cro* stop codon, and included substitutions for both cytidine and

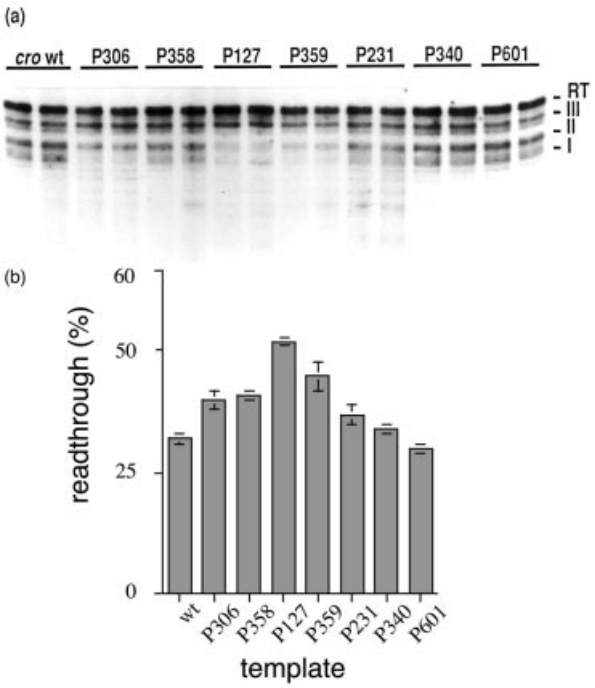

Figure 6. *In vitro* transcription of templates containing single-nucleotide substitutions in tR1. The effect of single base changes on Rho-dependent transcription termination are shown as duplicate sets of transcripts generated by *E.coli* RNA polymerase in the presence of 2.5 nM Rho (**a**). Templates were PCR fragments equivalent to the 560 bp Lambda HinfI fragment which encodes P_R, the *cro* coding region, and tR1. Transcripts extended through the terminator (RT), and those terminated at tR1 subsites I, II and III are indicated. Phosphorimage quantitation of the gel shown (**b**), where band intensities were normalized for the labeled uridine content of each transcript, and used to determine the percentage of transcripts reaching the end of each template relative to all those synthesized. Error bars show the ranges for duplicate determinations.

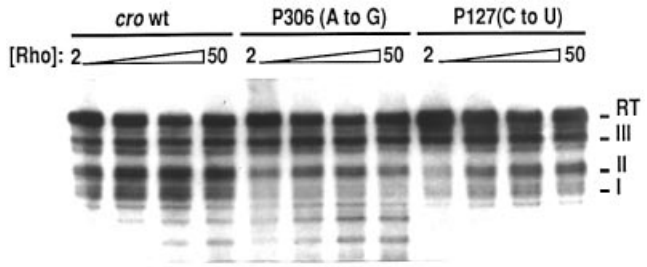

Figure 7. Increased Rho concentration does not compensate tR1 defects caused by single-residue substitutions. Transcripts synthesized by RNA polymerase in the presence of increasing Rho concentrations are shown. Rho levels up to 50 nM failed to restore wild-type termination on templates containing single base substitutions at tR1. Template DNA fragments and annotations are those previously indicated in Figure 6.

Table 2. Binding dissociation constants and ATPase activation with λ *cro* RNAs

RNA	K_d (nM)	ATPase k_{cat}[a] (s^{-1})
cro wild type	0.49 ± 0.1	22.5 ± 1.2
P127	0.55 ± 0.2	19.2 ± 1.1
P306	0.94 ± 0.3	12.5 ± 0.5

The K_d values were determined in a solution containing 40 mM Tris–HCl (pH 8.0), 25 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA and 5 mM dithiothreitol. Values were obtained by curve-fitting as previously described (22).

[a]Molar rates of ATP hydrolyzed/mole of Rho hexamer, determined with 75 nM RNA and 4.3 nM Rho as previously described (22).

non-cytidine residues. No other single substitutions capable of reducing termination were identified at other positions within this upstream region, nor among the remaining downstream nine positions within *rutA*. Thus, we identified these seven residues near the 5′ end of *rutA* as critical residues that define the functional λ tR1 *rut* site.

Within the upstream *rutA* region shown to contain residues critical for Rho interactions, single-nucleotide substitutions were apparently tolerated at six positions interspersed among those where specific residues were required for efficient terminator function (Fig. 2). Other investigators have previously described resilience to mutation and redundancy of signal content as characteristic of Rho-dependent terminators (15,16,28). We find that efficient tR1 terminator function is indeed dependent on the presence of critical nucleotides near the 5′ end of *rutA* (Fig. 2), but that Rho can also interact with a wide variety of different nascent transcripts containing these key residues or specific alternate residues at these positions with similar efficiency. The ability to interact with these alternate sequences may be a reflection of Rho's general ability to halt the synthesis of any transcripts that are not properly translated (29), a function requiring recognition of terminator sequences embedded in protein coding regions.

Recent X-ray crystallography studies of Rho structure (30,31) suggest a possible mechanism for Rho's initial interaction with nascent transcripts. Rho can interact with a terminator transcript by first binding a small segment of accessible RNA as it emerges from the RNA polymerase. Subsequent interactions then involve Rho's primary RNA binding site and as many as 60 nascent RNA residues. Once Rho's primary binding site is filled, nascent RNA enters an opening between two of the six subunits that form secondary RNA binding sites. This opening can then close, forming a ring that surrounds the 3′ portion of the transcript. Further energy dependent interactions with RNA at Rho's secondary binding sites then pull the transcript away from RNA polymerase, resulting in termination of transcription. The region of the nascent tR1 transcript that is first accessible to Rho is the 5′ part of *rutA*. Our findings that Rho interactions with *rutA* sequences contribute more to termination than interactions with downstream *rutB* sequences (22), and that specific RNA residues at the 5′ end of *rutA* are required for efficient termination, are consistent with a model where initial interactions between Rho and the emerging nascent transcript make the greatest contributions to termination. These early successful Rho–RNA interactions then allow subsequent RNA interactions necessary to terminate transcription before RNA polymerase can move on to transcribe RNA sequences that form a stable transcription elongation complex.

Nucleotide substitutions that introduced guanosines were both the most frequent in the terminator mutants identified in our studies, and had the greatest effects on the transcription termination efficiency (Figs 2 and 5). This suggests that Rho has an 'antipathy' for guanosines in RNA, an observation

previously made during attempts to discern a consensus sequence for Rho-dependent terminators, and in studies of Rho's interaction with RNA (12,20,32,33). Furthermore, none of the mutants we isolated had changes that eliminated the lone guanosine in *rutA* (Figs 2 and 3). Prior studies (34) indicate that substitution of thymidine for this guanosine (a mutation designated *boxA5*) would indeed increase terminator function. Although the distribution and nature of nucleotide changes seen in our mutant terminators do not suggest any obvious introduction of novel secondary structures, the introduction of guanosine residues into the C-rich *rutA* region could potentially have altered Rho's access to other residues. We also cannot rule out the possibility that substitutions that introduced guanosine residues reduced RNA conformational flexibility and accessibility by introducing stacking interactions between bases in *rutA*.

Our initial attempts to determine the nature of the defects in the P127 and P306 mutant terminators demonstrated an inability to characterize the overall reduced function of the P127 mutant terminator in terms of specific changes in distinct components of the highly dynamic multi-step termination process. This lack of correlation between termination efficiency and corresponding Rho-binding and ATPase cofactor activity of isolated terminator transcripts has been previously described for *trp t′* terminator mutants (16). The ~2-fold reduction in *in vitro* termination for the P127 tR1 mutant (clearly seen in terms of lack of transcripts terminated at tR1 subsites I and II in Fig. 6a) was not the direct result of a decreased affinity of Rho for the encoded transcript, nor likely to have resulted from a small reduction (<15%) in the encoded RNA's ATPase cofactor activity as measured in our assays. In contrast, reduced termination in the P306 mutant involved both a demonstrable reduction in Rho's primary binding interaction and maximum ATPase activity in the presence of the tR1 transcript. This primary binding defect was not compensated by higher Rho concentrations in termination assays, and Rho's ATPase activity in the presence of saturating levels of the P306 transcript remained at only half those seen with the wild-type *cro* transcript. A single-nucleotide substitution at tR1 was therefore capable of altering both Rho's primary interaction with the nascent RNA, and related subsequent additional ATP-dependent RNA interactions necessary for efficient transcription termination.

We also isolated an entire class of tR1 mutants for which we could not demonstrate any overall termination defect in our isolated *in vitro* termination assay (Fig. 2B). This discrepancy may reflect more stringent requirements for Rho–RNA interactions *in vivo*, where the kinetic demands on Rho are accentuated by an ~3-fold higher RNA polymerase elongation rate than that at which transcripts were extended in the *in vitro* studies described here (35). Although the efficiency of termination *in vitro* and *in vivo* at tR1were both ~75%, tR1 substitution mutant phenotypes consistently showed greater readthrough when assayed *in vivo* for all mutants which reduced termination in both assay systems (Figs 5 and 6b). This may reflect both a higher transcription elongation rate as well as the sequestration of the majority of the *cro* mRNA by ribosomes *in vivo*.

An alternative explanation for the failure to demonstrate reduced termination with templates from a subset of mutants in our *in vitro* assays is that certain tR1 nucleotide substitutions altered protein–nucleic acid interactions involving transcription factors other than Rho. These mutations may have thereby indirectly reduced the efficiency of Rho-dependent termination. Transcription termination in the *E.coli* cytoplasm involves Rho's interaction with a multi-enzyme transcription complex consisting of an as yet only partially defined set of transcription elongation factors (2). These factors are known to incorporate parts of the nascent transcript into the elongation complex (36), which may impact Rho's access to the nascent *cro* transcript. tR1 *rutA* and λ *nutR boxA* antiterminator sequences overlap, and several mutations that reduced termination *in vivo* but not *in vitro* (Fig. 2B) were found in *boxA* and the adjacent upstream sequence. The highly similar *E.coli rrnG* leader *boxA* sequence (Fig. 2) binds transcription factor NusB and ribosomal proteins S10 and S1 *in vitro* (8,37), and is sufficient to antiterminate transcription *in vivo* when placed upstream of both Rho-independent and Rho-dependent terminators (38). It is possible that for a subset of mutants, nucleotide changes impacted the normal binding of transcription elongation factors to the tR1 terminator transcript, or created new interactions with transcription factors that altered Rho's access to the nascent RNA.

ACKNOWLEDGEMENTS

The author wishes to recognize the five decades of outstanding contributions to this field made by John P. Richardson on occasion of his retirement from active research. Those of us who benefited directly from his mentorship of graduate students (more than 20) would also like to thank him for setting such a fine example of how scientific research can be done. This work was supported by National Institutes of Health Grant AI10142.

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