Cell, Vol. 41, 577–585, June 1985, Copyright © 1985 by MIT 0092-8674/85/060577-09 $02.00/0

A Bidirectional Rho-Independent Transcription Terminator between the E. coli *tonB* Gene and an Opposing Gene

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Summary

We identified an Escherichia coli gene, designated *P14*, that is adjacent to and in the opposite orientation to the *tonB* gene. The 36 base pair intercistronic region between *tonB* and *P14* contains a novel rho-independent transcription terminator that functions bidirectionally, both in vivo and in vitro, to terminate *tonB* and *P14* transcription. Transcription of *tonB* and *P14* terminates at symmetrically equivalent nucleotides, such that the 3′ ends of *tonB* and *P14* transcripts are complementary. The terminator is 70% efficient in both directions in vitro. Interestingly, relative rates of in vivo RNA synthesis, immediately prior to and following the terminator, appear to indicate that it is more efficient in the *tonB* direction (95%) than in the *P14* direction (70%). We discuss the possibility that this gene arrangement has regulatory consequences for the expression of *tonB*.

Introduction

Prokaryotic rho-independent transcription terminators typically consist of a GC-rich region having dyad symmetry, followed by a region encoding four to eight U residues near the 3′ end of the transcript (Adhya and Gottesman, 1978; Rosenberg and Court, 1979; Platt and Bear, 1983). Current models propose that the RNA encoded by the terminator forms a hairpin structure involving the GC-rich segment. In general, mutations that decrease the predicted stability of the RNA hairpin also decrease termination efficiency, whereas mutations that increase the predicted stability of the hairpin increase termination efficiency (Rosenberg and Court, 1979; Zurawski and Yanofsky, 1980). The run of U residues near the 3′ end of the RNA is proposed to facilitate dissociation of transcripts from the template DNA (Farnham and Platt, 1980; Martin and Tinoco, 1980) since RNA-DNA hybrids containing rU–dA base pairs are particularly unstable (Chamberlain, 1965; Riley et al., 1966). Accordingly, mutations that decrease the number of terminal U residues also decrease termination efficiency (Bertrand et al., 1977; Zurawski and Yanofsky, 1980; Christie et al., 1981).

In the course of determining the DNA sequence of the *tonB* gene, we identified a region of dyad symmetry near the 3′ end of *tonB* that seemed to terminate *tonB* transcription in vivo (Postle and Good, 1983). The extended symmetry of this region, and the possibility of a gene opposing *tonB*, suggested that the terminator might function bidirectionally. In this paper we present additional evidence for the existence of a gene opposing *tonB*, and we demonstrate that the transcription terminator located between these two genes functions bidirectionally, both in vivo and in vitro.

Results

DNA Sequence of a Gene Opposing *tonB*

We determined the DNA sequence of the 640 base pair (bp) region immediately 3′ to *tonB* (Figures 1 and 2). Several lines of evidence indicate that this region contains a functional gene. The sequence contains an open translation reading frame that opposes *tonB* and is capable of encoding a 132 amino acid protein having a predicted size of 14.2 kilodaltons (kd). The DNA sequence of the putative gene, designated *P14*, and the deduced amino acid sequence of its protein gene product, P14, are shown in Figure 2. The *P14* open reading frame is the largest on either DNA strand distal to *tonB*. The sequences TGTTCT (bp 140–145) and TAGCAT (bp 163–168), which precede the open reading frame, show homology with the −35 and −10 regions of other E. coli promoters (Rosenberg and Court, 1979; Hawley and McClure, 1983). The probable ATG initiation codon (bp 204–206) is preceded by a Shine–Dalgarno sequence TAAG-GGT (bp 189–197) in which seven out of nine bases are homologous to the 3′ end of 16S ribosomal RNA (Shine and Dalgarno, 1974). The *P14* open reading frame terminates in tandem stop codons TAATAG (bp 600–605) immediately prior to the region of dyad symmetry (bp 604–635) previously identified as the *tonB* transcription terminator (Postle and Good, 1983). Finally, evidence presented in following sections demonstrates that the *P14* region of the genome is, in fact, transcribed in the expected orientation. The small size of the *tonB–P14* intercistronic region (36 bp) immediately posed questions about the nature of the transcription termination signals for the two genes.

The *tonB–P14* Transcription Terminator Functions Bidirectionally In Vitro

To characterize *tonB* and *P14* transcription termination in vitro, the 928 bp Hae III fragment spanning the intercistronic region (Figure 1, line b) was cloned in both orientations into the Eco RI site downstream from the E. coli *trp* promoter in plasmid pWu5. Purified DNA restriction fragments from these constructs were used as templates for in vitro transcription reactions with purified RNA polymerase. The templates, in which the *trp* promoter and the *tonB–P14* terminator are the only known transcription control signals, are shown in Figure 3. The products of the in vitro transcription reactions were electrophoresed on denaturing polyacrylamide gels and subjected to autoradiography. Results of one experiment are shown in Figure 4.

Terminated transcripts were identified on the basis of their predicted lengths (Figure 3), and by S1 nuclease mapping. Readthrough transcripts from templates a and b were identified by their predicted lengths, and the fact that they are the same length. The readthrough transcript

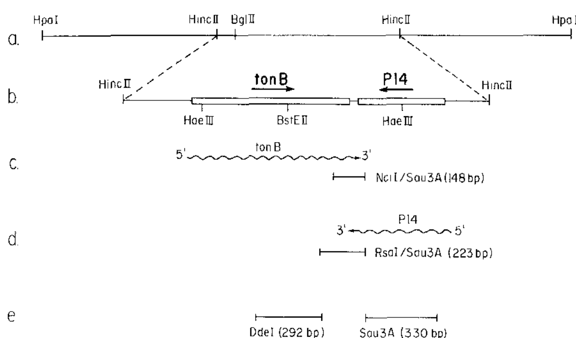

Figure 1. Organization of the *tonB* region

(a) Partial restriction map of the 4900 bp *tonB*⁺ Hpa I fragment cloned in pRZ526 and pRZ625. The *tonB* gene lies between the internal Hinc II sites. (b) Partial restriction map of the 1697 bp *tonB*⁺ Hinc II fragment cloned in pRZ540. The open reading frames of *tonB* and *P14* are indicated by boxes. The orientations of the two genes are indicated by arrows. The 928 bp Hae III fragment which contains the *tonB–P14* terminator is also indicated. This restriction fragment was used in the construction of templates for in vitro transcriptions. (c) The Nci I/Sau 3A DNA fragment used in S1 nuclease mapping of the 3′ end of *tonB* message, together with the extent of the *tonB* message. The 5′ end of the *tonB* message was determined previously by S1 nuclease mapping (Postle and Good, 1983). (d) The Rsa I/Sau 3A DNA fragment used in S1 nuclease mapping of the 3′ end of *P14* message, together with the extent of the *P14* message. The 5′ end of the *P14* message is only tentatively assigned on the basis of a possible promoter in the DNA sequence. (e) The DNA restriction fragments which were cloned into M13mp7 for use as hybridization probes. Clones of the 292 bp Dde I fragment constitute probes for either pre-*tonB* RNA or post-*P14* RNA. Clones of the 330 bp Sau 3A fragment constitute probes for either pre-*P14* RNA or post-*tonB* RNA.

from template c was identified on the basis of its length and prominence.

The 148 bp Nci I/Sau 3A fragment was used for S1 nuclease mapping of the 3′ ends of in vitro *tonB* transcripts (Figure 1, line c). The single-stranded DNA, 3′-labeled at the Nci I end, was hybridized to *tonB*-terminated transcripts (Figure 4, lane a). Unhybridized single-stranded nucleic acids were digested by S1 nuclease and the protected DNAs electrophoresed on denaturing gels next to a Maxam-Gilbert DNA sequencing ladder for the Nci I/Sau 3A fragment. The protected DNAs are 107 and 108 bases in length (Figure 5A, lane a), indicating that *tonB* transcription terminates at the distal edge of the dyad symmetry (Figure 6).

The 223 bp Rsa I/Sau 3A fragment was used for S1 nuclease mapping of the 3′ ends of in vitro *P14* transcripts (Figure 1, line d). The single-stranded DNA, 3′-labeled at the Sau 3A end, was hybridized to *P14*-terminated transcripts (Figure 4, lane c). Unhybridized single-stranded nucleic acids were digested by S1 nuclease and the protected DNAs electrophoresed next to a Maxam-Gilbert DNA sequencing ladder for the Rsa I/Sau 3A fragment. The protected DNAs are 68 and 69 bases in length (Figure 5B, lane a), indicating that *P14* transcription terminates symmetrically with respect to *tonB* transcription. Thus, the *tonB–P14* terminator functions bidirectionally in vitro, and the 3′ ends of in vitro *tonB* and *P14* RNA overlap by 30–32 nucleotides (Figure 6).

The *tonB–P14* Transcription Terminator Functions Bidirectionally In Vivo

It was of interest to determine whether the terminator functions bidirectionally in vivo, and to what extent the in vivo function resembles the in vitro function. To examine *tonB–P14* terminator function in vivo, S1 nuclease mapping experiments were performed on the 3′ ends of the *tonB* and *P14* transcripts in cells containing the entire *tonB–P14* region (Figure 1, line a) on a multicopy plasmid. The S1 nuclease resistant products were electrophoresed in parallel with the S1 nuclease resistant products from the in vitro RNAs described in the preceding section.

Figures 5A and 5B demonstrate that the 3′ ends of *tonB* and *P14* in vivo RNA are identical to the 3′ ends of RNA synthesized in the purified in vitro system. Transcription of *tonB* terminates at either of two adjacent nucleotides at the distal edge of the region of extended dyad symmetry, and *P14* transcription terminates at symmetrically equiva-

Figure 2. DNA Sequence of *P14* and Adjacent Regions

The deduced amino acid sequence, −35 and −10 regions of a possible promoter, and possible Shine–Dalgarno sequence (S.D.) are indicated. The region of dyad symmetry which constitutes the transcription terminator is boxed. The DNA sequence was determined on both strands and across all restriction sites by a combination of Maxam-Gilbert and Sanger DNA sequencing techniques.

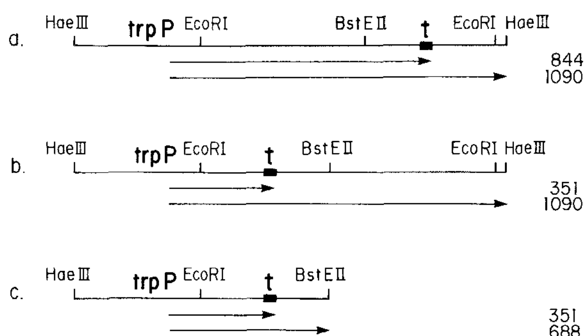

Figure 3. Templates Used in In Vitro Transcription Studies

The Hae III fragments were obtained from derivatives of plasmid pWu5 in which the Eco RI fragment carrying *trp* t t′ has been replaced by an Eco RI fragment carrying the *tonB–P14* terminator (see Experimental Procedures). The *trp* promoter is the only known promoter on the Hae III fragments. The position of the *tonB–P14* terminator is indicated by a box. The predicted sizes of terminated and readthrough transcripts from each template are indicated underneath the template.
(a) Terminator in *tonB* orientation.
(b) Terminator in *P14* orientation.
(c) Terminator in *P14* orientation; template b restricted with Bst EII in order to shorten readthrough transcripts.

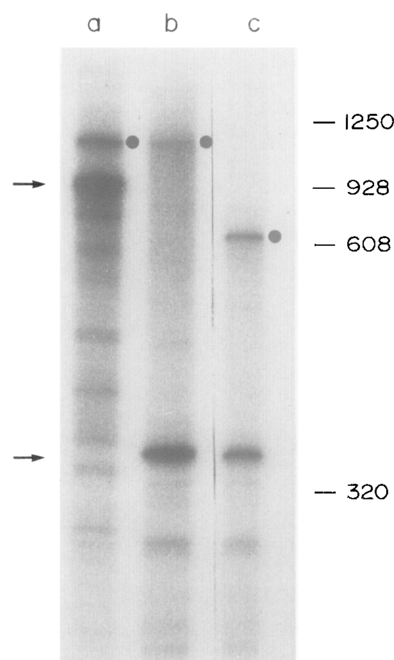

Figure 4. In Vitro Transcription of Templates Carrying the *tonB–P14* Terminator

Polyacrylamide gel analysis of in vitro transcription products from the templates shown in Figure 3. Sizes of single-stranded DNA markers are indicated. Positions of terminated transcripts are indicated by arrows. Positions of readthrough transcripts are indicated by dots.
(a) Transcription of template a; terminator is in the *tonB* orientation relative to the *trp* promoter.
(b) Transcription of template b; terminator is in the *P14* orientation.
(c) Transcription of template c; terminator is in the *P14* orientation.

lent nucleotides (Figure 6). These results suggest that the *tonB–P14* terminator functions bidirectionally in vivo and in vitro, and in a symmetrical fashion in both cases. In addition, they demonstrate the existence of an in vivo RNA species originating from a gene opposing *tonB* and thus suggest that *P14* is, in fact, a functional gene.

Efficiency of Transcription Termination In Vitro

Since the *tonB–P14* terminator functions bidirectionally, we were interested in determining its efficiency in each orientation. To determine the efficiency of *tonB–P14* termination in vitro, densitometer scans were performed on autoradiographs such as the one shown in Figure 4. Since [α-^{32}P]GTP was used to label the in vitro transcripts, the band intensities were corrected to account for the number of G residues in each transcript. The corrected values were then used to calculate termination efficiency according to the formula: termination efficiency = 100 (terminated transcripts/terminated plus readthrough transcripts). The data indicate that the *tonB–P14* terminator functions with essentially identical efficiency in either orientation in vitro. It is 71% efficient in the *tonB* orientation and 67% efficient in the *P14* orientation. The observation that the termination efficiencies are essentially the same is consistent with the idea that the information necessary for rho-independent transcription termination is contained entirely within the region of dyad symmetry.

Experiments to determine terminator efficiency in vitro must be interpreted with care. In our experience, the farther the terminator is from the distal end of the template, the more likely one is to overestimate terminator efficiency. This is presumably because any polymerase that reads through the terminator, but pauses or stops before the end of the template, will be uncounted in the determination of readthrough transcripts. In the case where the *P14* terminator is 739 bp from the end of the fragment (Figure 3, line b; Figure 4, lane b), we observed an apparent 90% termination. By restricting the template with Bst EII, the distance from terminator to end of fragment was shortened to 337 bp, and the apparent terminator efficiency was determined to be 67% (Figure 3, line c; Figure 4, lane c). The *tonB* terminator, determined to be 71% efficient, is 246 bp from the end of the template (Figure 3, line a).

Efficiency of Transcription Termination In Vivo

The in vivo efficiency of the *tonB–P14* terminator was determined by measuring the relative rates of RNA synthesis immediately prior to and following the terminator. Single-stranded hybridization probes were constructed by cloning *tonB* and *P14* DNA fragments into M13mp7 (Figure 1, line e). The 292 bp Dde I fragment was cloned in both orientations relative to the vector, resulting in single-stranded DNA probes specific for either preterminator *tonB* RNA or postterminator *P14* RNA. Likewise the 330 bp Sau 3A fragment was cloned in both orientations, resulting in probes specific for either postterminator *tonB* RNA or preterminator *P14* RNA. Bacteria containing *tonB*$^+$ plasmids were labeled for 60 sec with [^{3}H]uridine and the cellular RNA was extracted and hybridized with filter-bound M13-*tonB* or M13-*P14* viral DNA.

Half-lives of the pre- and postterminator RNA segments were determined in order to calculate accurately the relative rates of RNA synthesis before and after the terminator. Since degradation of newly synthesized RNA is relatively

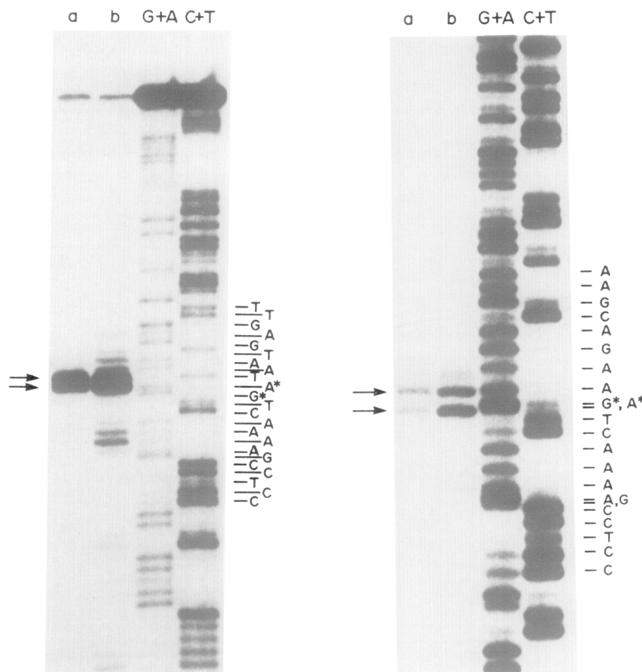

Figure 5. S1 Nuclease Mapping of Transcripts Terminated by the *tonB–P14* Terminator

(A) S1 mapping of 3′ end of in vitro and in vivo transcripts terminated by the *tonB* terminator. The single-stranded Nci I/Sau 3A fragment (see Figure 1, line c) was used as a hybridization probe. The DNAs protected by in vitro and in vivo *tonB* RNA (lanes a and b, respectively) were electrophoresed on an 8% polyacrylamide–urea gel next to Maxam–Gilbert sequencing reactions for the Nci I/Sau 3A fragment.

(B) S1 mapping of 3′ end of in vitro and in vivo transcripts terminated by the *P14* terminator. The single-stranded Rsa I/Sau 3A fragment (see Figure 1, line d) was used as a hybridization probe. The DNAs protected by in vitro and in vivo *P14* RNA (lanes a and b, respectively) were electrophoresed next to Maxam–Gilbert sequencing reactions for the Rsa I/Sau 3A fragment.

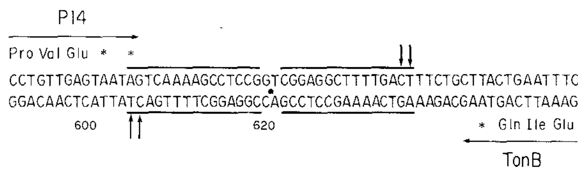

Figure 6. DNA Sequence of the *tonB–P14* Transcription Terminator

The orientation and last three codons of *P14* and *tonB* are indicated. The 3′-terminal nucleotides of the terminated RNA species determined by S1 nuclease mapping are indicated by vertical arrows. The region of dyad symmetry is indicated by horizontal lines.

rapid and occurs throughout the labeling period (60 sec), the relative levels of RNA observed can be substantially influenced by differences in half-lives. The results of the experiment shown in Figure 7 indicate that pre-*tonB* RNA is, in fact, degraded significantly more rapidly than pre-*P14* RNA. The 90 sec time point was omitted in calculating half-lives, since few cpm remained in the postterminator RNAs after 60 sec. Three half-life determinations for the four RNA segments were averaged to generate the data in Table 1, line a. The effect of exponential RNA degradation on accumulation of labeled RNA during a pulse-labeling period is described by the following equation (Friedlander et al., 1981; Hauser and Hatfield, 1984):

$$\frac{\text{RNA synthesized}}{\text{RNA observed}} = \frac{\ln 2}{1 - e^{-\ln 2 (T/t_{\frac{1}{2}})}} (T/t_{\frac{1}{2}}),$$

where T = length of labeling period and $t_{\frac{1}{2}}$ = RNA half-life. The values of (RNA synthesized/RNA observed) for each of the four RNA species are shown in Table 1, line b.

The results of a typical experiment to determine in vivo terminator efficiency are shown in Table 2, line a, along with the averages for seven similar experiments. All of the data have been corrected by subtraction of background

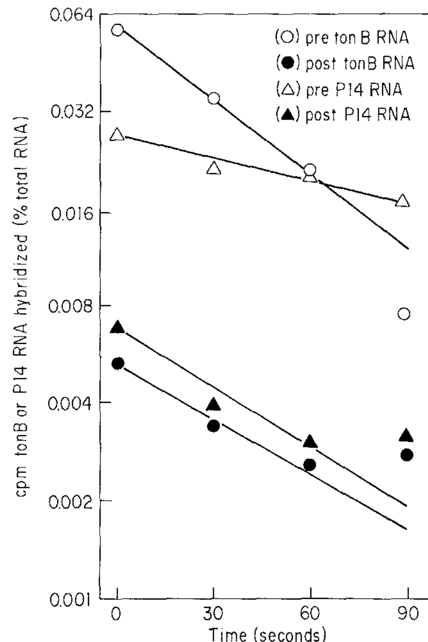

Figure 7. In Vivo Rates of RNA Degradation

At time zero, labeling was stopped by addition of rifampicin and excess unlabeled uridine. In this experiment, total RNA = 2.9 × 10^6 cpm. Background values ranged from 120 to 190 cpm. Half-lives calculated from this experiment are as follows: pre-*tonB* RNA, 31 sec; post-*tonB* RNA, 58 sec; pre-*P14* RNA, 135 sec; post-*P14* RNA, 51 sec.

cpm, and multiplication by a final correction factor (Table 1, line e) that incorporates differences in RNA size and composition (Table 1, lines c and d), and half-life (Table 1, lines a and b). *TonB* transcription appears to be terminated with 95% ± 2% efficiency and *P14* transcription appears to be terminated with 72% ± 12% efficiency. Lines b and

Table 1. Correction Factors for Calculating Relative Rates of *tonB* and *P14* RNA Synthesis in Vivo

	RNA segment			
	Pre-*tonB*	Post-*tonB*	Pre-*P14*	Post-*P14*
a. RNA half-life	31 s	59 s	140 s	52 s
b. Correction factor for RNA half-life	1.81	1.39	1.15	1.45
c. UMP content	49	82	90	84
d. Correction factor for UMP content	1.84	1.10	1.00	1.07
e. Final correction factor (line b × line d)	3.33	1.53	1.15	1.55

The half-life values for the RNAs are averages of three determinations. The correction factor for RNA half-life was calculated according to the equation in the text. Number of UMP residues in the RNA segments was determined from the DNA sequence.

c demonstrate that within a single experiment, doubling the amount of DNA probe on the filters makes essentially no difference in the number of cpm bound, thus indicating that the experiments were performed in DNA excess. We consistently observed more variation in the measurement of post-*P14* RNA than in any other case; therefore, we attach no particular significance to the high value of post-*P14* RNA in line c. Line d shows the results when the same bacterial strain carrying a different *tonB*$^+$ plasmid is used as the source of ^{3}H-RNA. Plasmid pRZ625 is identical to pRZ526 except that the 4900 bp *tonB*$^+$ Hpa I fragment is in the opposite orientation. The percentage of termination with pRZ625 is essentially the same as that with pRZ526, suggesting that transcription initiated within the plasmid vector does not influence the termination efficiencies observed. When all of the in vivo termination efficiency data are averaged (11 experiments), the apparent termination efficiency for *tonB*-initiated transcription is 95% ± 2% and for *P14*-initiated transcription it is 72% ± 11%. In contrast to the in vitro data, the in vivo data suggest that the *tonB–P14* terminator functions with different efficiencies in the *tonB* and *P14* directions. The apparent level of *P14* termination in vivo is similar to the level of termination in vitro (72% vs. 67%). However, the apparent level of *tonB* termination in vivo is significantly higher than the level of termination in vitro (95% vs. 71%). The data in Table 2 also indicate that *tonB* is transcribed 5.4 ± 1.0 times more frequently than *P14* under the conditions of these experiments.

Discussion

The DNA sequence of the region immediately 3′ to *tonB* had suggested the presence of a gene opposing *tonB* (Postle and Good, 1983). By extending the DNA sequence 3′ to *tonB*, we have now shown that there is, in fact, an open reading frame opposing *tonB*. This open reading frame, which we formerly designated URF, we now designate the *P14* gene. *P14*, which can potentially encode a 14.2 kd protein, is a gene by the following criteria: first, the *P14* region is transcribed in the expected orientation in vivo; second, the *P14* open reading frame is preceded by a Shine–Dalgarno sequence 7 bp upstream from an AUG codon and terminates in tandem stop codons immediately prior to a transcription terminator; and finally, a protein of approximately 14 kd can be seen among the proteins encoded by pRZ540, a clone of the 1697 bp Hinc II fragment that spans *tonB* and *P14* and that is not present among proteins encoded by the vector pRZ112 (Postle and Reznikoff, 1979). Inspection of the predicted *P14* amino acid sequence reveals no especially remarkable features.

In most respects, the DNA sequence between *tonB* and *P14* resembles other rho-independent transcription terminators. However, because of its position between two opposing genes and its highly symmetric nature, it seemed likely that the sequence could function bidirectionally. To test this hypothesis, we determined the 3′ ends of *tonB* and *P14* RNA by S1 nuclease mapping. The 3′ ends of in vivo *tonB* and *P14* RNA are identical to the 3′ ends of RNAs synthesized in a purified in vitro transcription system. Transcription of *tonB* terminates at either of two adjacent nucleotides at the distal edge of a region of extended dyad symmetry in the DNA sequence (Figure 6). Transcription of *P14* terminates at symmetrically equivalent nucleotides, such that the 3′ ends of the *tonB* and *P14* transcripts are complementary. We conclude that the region of dyad sym-

Table 2. Efficiency of in Vivo Transcription Termination by the *tonB–P14* Terminator

	RNA (corrected cpm)				Termination efficiency (%)	
Source of ^{3}H-RNA	Pre-*tonB*	Post-*tonB*	Pre-*P14*	Post-*P14*	*tonB*	*P14*
a. PRZ526/MO	4281	156	837	234	96 (95 ± 2)	72 (72 ± 12)
b. pRZ526/MO 1 X probe	11308	904	2580	584	92	77
c. pRZ526/MO 2 X probe	10935	938	2366	1123	91	53
d. pRZ625/MO	4712	270	1071	251	94 (95 ± 0.2)	77 (75 ± 3)

RNA was extracted from plasmid-containing cultures pulse-labeled for 60 sec with [^{3}H]uridine. Levels of pre- and postterminator RNAs were determined as described in Experimental Procedures. Low backgrounds were subtracted from all cpm values, which were then multiplied by the correction factors in Table 1, line e. Data shown are single experiments. Lines b and c show results from two different hybridizations with the same RNA preparations. Average termination efficiencies ± standard error for all experiments performed are in parentheses on the right (seven determinations for line a, two determinations for line d). Termination efficiency is calculated as: 100 − 100 (post-RNA/pre RNA).

metry between *tonB* and *P14* functions as a bidirectional transcription terminator both in vivo and in vitro.

A number of transcription terminators have been described in recent years that have a run of adenine residues preceding the GC-rich region, providing a symmetric counterpart to the U-encoding region. These have been predicted to function bidirectionally (Platt and Bear, 1984) and include the *ompA* terminator (Pirtle et al., 1980), the ribosomal RNA terminators (Young, 1979; Brosius et al., 1981), the *atp* operon terminator (Saraste et al., 1981), the *rho* gene terminator (Pinkham and Platt, 1983), and the *leu*, *thr*, *ilvB* attenuators (Keller and Calvo, 1979; Friden et al., 1982; Gardner, 1982) in E. coli, and the *his* operon terminator in Salmonella typhimurium (Carlomagno et al., 1983). Our functional studies on the *tonB–P14* terminator, together with the structural information about other highly symmetrical *rho*-independent terminators, suggest that such sites are probably of general significance to the gene organization of the bacterial chromosome.

The efficiency of the *tonB–P14* terminator was analyzed in vitro using DNA templates with the terminator in either orientation and downstream from the *trp* promoter. This approach ensured that termination efficiencies were compared under similar RNA polymerase densities and transcription reaction conditions. The in vitro analyses indicate that the *tonB–P14* terminator functions with approximately 70% efficiency in either orientation. This result is consistent with the expectation that all of the information necessary for rho-independent transcription termination is contained within the region of extended dyad symmetry.

Although the observation that the *tonB–P14* terminator functions in vitro with similar efficiency in either orientation is not too surprising, the relatively high efficiency of termination is unexpected. Previous studies (Bertrand et al., 1977; Rosenberg and Court, 1979; Zurawski and Yanofsky, 1980; Christie et al., 1981) have suggested that termination efficiency is directly related to both the number of GC base pairs in the RNA stem–loop and the number of U residues at the 3′ end of the RNA. The E. coli *trp* t terminator, which is only 25% efficient in vitro (Christie et al., 1981), contains seven GC base pairs in an eight base pair stem–loop followed by four U residues in the RNA. As represented in Figure 8a, the *tonB–P14* terminator has five GC base pairs in a six base pair stem–loop, followed by four U residues, and might therefore be predicted to be a weaker terminator than *trp* t. Comparison of the *tonB–P14* terminator to the E. coli *trp* attenuator likewise suggests that the *tonB–P14* terminator might be inefficient. The *trp* attenuator, which is 95% efficient in vitro (Lee et al., 1976; Christie et al., 1981), has a stem–loop containing six GC base pairs followed by eight U residues. Decreasing the run of U residues from eight to four decreases its termination efficiency in vitro to 3% (Christie et al., 1981). One obvious difference between the *trp* terminators and the *tonB–P14* terminator is the extended symmetry of the latter and its potential for forming an extended RNA stem–loop (Figure 8b). The observation that the *tonB* and *P14* transcripts terminate at the distal edges of the dyad

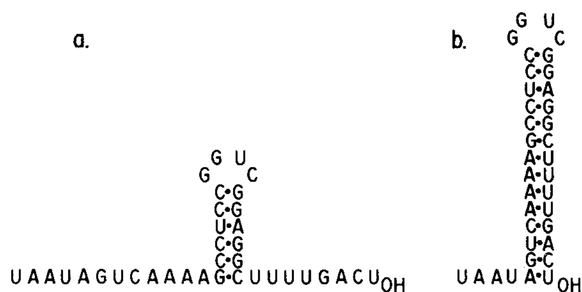

Figure 8. Sequence and Potential Secondary Structures of 3′ Ends of *P14*-initiated Transcripts

(a) The 3′ end of the transcript represented as a short GC-rich stem–loop followed by a run of U residues. (b) The most extended stem–loop structure which the terminated RNA is capable of assuming.

symmetry rather than within the run of U residues is consistent with the idea that the extended symmetry of the DNA sequence is functionally important and that it most likely enhances the efficiency of termination.

The efficiency of the *tonB–P14* terminator in vivo was determined by assaying relative rates of RNA synthesis immediately prior to and following the dyad symmetry. In the *P14* direction, the efficiency of transcription termination in vivo (72%) is essentially identical to that in vitro (67%). However, in the *tonB* direction, the apparent termination efficiency is significantly higher in vivo (95%) than in vitro (71%). A possible explanation for this departure from symmetrical behavior in vivo is that a rho-dependent terminator lies downstream from the *tonB* gene. However, addition of purified rho protein (a gift from Janice Sharp) to the in vitro transcription reactions has no effect upon the transcription profiles (data not shown). Therefore, if such a rho-dependent terminator exists, it is presumably outside the region spanned by the templates used for the in vitro transcription analysis (Figure 3). By analogy with the *trp* t and t′ terminators (Wu et al., 1981; Platt and Bear, 1983), transcription that reads through the rho-independent terminator would be terminated at the rho-dependent terminator. These longer RNAs would be so rapidly processed back to the stem–loop of the rho-independent terminator as to be virtually undetectable in vivo. Alternatively, factors may exist in vivo that recognize the nonsymmetric aspects of the terminator region, and that enhance termination.

Interestingly, the translation termination codons for both *P14* and *tonB* are within 7 bp of the dyad symmetry. Previous studies on the histidine operon attenuator (Johnston and Roth, 1981) indicated that translation that proceeds up to the base of the terminator stem–loop has the effect of decreasing transcriptional termination at that site. By analogy with the *his* attenuator, the *tonB–P14* terminator would be predicted to function with decreased efficiency in vivo relative to its efficiency in vitro. The observation that it is of equal or greater efficiency in vivo compared to in vitro raises two possibilities: either translating ribosomes do not prevent formation of the terminator stem–loop structure, or a more complex regulatory system ex-

ists. Further experiments will be required to determine how ribosomes interact with the *tonB–P14* terminator structure.

The arrangement of the *tonB* and *P14* genes and the utilization of a bidirectional transcription terminator is, to our knowledge, unique among E. coli genes that have been studied. The *tonB* gene is required for all high-affinity iron transport (Frost and Rosenberg, 1975; Hantke and Braun, 1975; Pugsley and Reeves, 1976; Braun et al., 1982; Hantke, 1983) as well as for vitamin B_{12} transport (Bassford et al., 1976) and the action of several bacteriophages and colicins (Anderson, 1946; Matsushiro, 1963; Gratia, 1966). At present, there is no information regarding the function of the P14 protein, except that it is apparently not essential for E. coli growth, since strains carrying *tonB-trp* deletions—all of which span *P14*—are viable. One tantalizing possibility is that the arrangement of *P14* and *tonB* has regulatory consequences for *tonB* and/or *P14*. For example, antitermination at the *P14* terminator would produce RNA complementary to *tonB* mRNA, which would be predicted to decrease TonB translation (Mizuno et al., 1984; Coleman et al., 1984).

Experimental Procedures

General Materials

E. coli RNA polymerase was purchased from P. L. Biochemicals. [γ-^{32}P]dATP was purchased from ICN. All other radiochemicals were purchased from Amersham. RNasin was purchased from Promega Biotech Inc.

Plasmids and Strains

E. coli K12 strains MO (F^-str^R) and JA221(F^-*lacY*1 *leuB*6 *thi*-1 *tonA*2 *supE*44 Δ*trpE*5 *recA*1 *hsr*, Clarke and Carbon, 1978) were used. Plasmid pWu5, which carries the E. coli tryptophan operan promoter, was obtained from Terry Platt via Janice Sharp and is described in Wu et al., 1981. Plasmid pRZ526 (Postle and Reznikoff, 1978) is a clone of the 4900 bp *tonB*⁺ Hpa I fragment in the Hpa I site of neo^R ColE1 derivative pRZ112. Plasmid pRZ540 (Postle and Reznikoff, 1978) is a clone of the 1697 bp *tonB*⁺ Hinc II fragment in the Hpa I site of pRZ112. Plasmid pRZ625 was constructed for this work by digesting pRZ526 with Hpa I, re-ligating the mixture, transforming MO, and screening for plasmids that carried the *tonB*⁺ Hpa I fragment in the opposite orientation to pRZ526. The structure of M13mp7 and procedures for its use in cloning and sequencing are described in Messing et al., 1981.

DNA Preparations

Plasmid DNA was prepared by cesium chloride density centrifugation. Specific DNA fragments were eluted from polyacrylamide gels by a crush and soak method (Maxam and Gilbert, 1980), except for the fragments used as templates in the in vitro transcriptions which were electroeluted.

DNA Sequence Analysis

DNA was sequenced according to the methods of Sanger et al. (1977) and Maxam and Gilbert (1980) as indicated in the text.

Construction of Templates for In Vitro Transcriptions

The 928 bp Hae III fragment carrying the *tonB–P14* terminator was isolated from the *tonB*⁺ plasmid pRZ540, and then cloned into the Hinc II site of M13mp7. The terminator fragment was then excised as an Eco RI fragment from the replicative form of this M13mp7 clone. Plasmid pWu5 was digested with Eco RI, and the large fragment carrying the amp^R gene and the *trp* promoter was purified on gels, leaving behind a smaller Eco RI fragment carrying the *trp* t t' transcription terminators. Purified pWu5 large fragment and the Eco RI fragment carrying the *tonB–P14* terminator were ligated and transformed into MO. Plasmids carrying the terminator fragment in either orientation were identified by restriction enzyme analysis. Templates for in vitro transcriptions were excised as Hae III fragments.

In Vitro Transcriptions

In vitro transcriptions were performed by incubating DNA (0.1–1.0 pmol) and RNA polymerase (0.5 units) in 150 mM KCl, 0.1 mM sodium EDTA, 5% glycerol, 1.7 units RNasin/μl, 200 μM ATP, and 20 μM GTP including 10 μCi of [α-^{32}P]GTP, in a total volume of 9 μl for 5 min at 37°C. The transcription reaction was initiated by addition of 1 μl of 40 mM magnesium acetate/2.0 mM UTP/2.0 mM CTP/100 μg rifampicin/ml. Transcription reactions were stopped by addition of 100 μl of 0.3 M sodium acetate (pH 6.5)/500 μg yeast tRNA/ml and phenol extraction. Samples were ethanol precipitated, ethanol washed, and resuspended in 98% deionized formamide, 0.025% Bromphenol Blue, 0.025% Xylene Cyanol. Transcripts were analyzed by gel electrophoresis on 6% polyacrylamide gels containing 8 M urea buffered with 50 mM Tris-borate (pH 8.3), 1 mM sodium EDTA. Transcripts were visualized by autoradiography using Kodak XAR-5 film. To measure relative levels of in vitro transcripts, densitometer scans of autoradiographs were performed using an LKB Ultroscan Laser Densitometer. For S1 nuclease mapping, in vitro transcripts were eluted from gels by soaking at 37°C overnight in 1 ml of 500 mM ammonium acetate/10 mM magnesium acetate/1 mM sodium EDTA/0.1% SDS/25 μg yeast tRNA/ml and ethanol precipitating the eluate.

S1 Nuclease Mapping

Procedures for S1 nuclease mapping of in vivo RNA were described previously (Postle and Good, 1983). Basically this involved ^{32}P-end-labeling of appropriate DNA restriction fragments, strand-separation of the labeled DNAs, hybridization of the single-stranded DNA probes to total RNA isolated from bacteria containing *tonB*⁺ plasmids, and digestion of unhybridized single-stranded RNA and DNA with S1 nuclease. For S1 nuclease mapping of ^{32}P-labeled in vitro RNAs, specific RNA bands were excised and eluted from polyacrylamide gels. The ^{32}P-RNA was annealed to single-stranded 3′-end-labeled DNA probes, and treated with S1 nuclease as before. The reaction was ethanol precipitated, resuspended in 100 μl 0.1 N NaOH, and heated to 100°C for 20 min to hydrolyze the ^{32}P-RNA. The protected DNA was ethanol precipitated, washed once with 70% ethanol, and resuspended in formamide sample buffer (Maxam and Gilbert, 1980). The DNAs protected by both in vivo and in vitro RNAs were electrophoresed on denaturing gels in parallel with DNA sequencing reactions for the full-length 3′-end-labeled DNA fragments.

Construction of M13 Hybridization Probes

Pre-tonB *and Post*-P14 *Probes*

The 292 bp *Dde*I fragment was purified from a restriction digest of 50 μg pRZ540. The purified fragment was treated with 200 units S1 nuclease/ml (Sigma) in S1 buffer (5% glycerol, 0.25 M sodium chloride, 0.03 M sodium acetate, 1.0 mM zinc sulfate) for 1 hr at 15°C followed by 15 min at 4°C. The S1 nuclease was inactivated by the addition of one-third vol of 2.5 M ammonium acetate, 50 mM sodium EDTA. The resulting blunt-end DNA fragment was phenol extracted, ether extracted twice, and ethanol precipitated twice, and then cloned into the Hinc II site of M13mp7 RF. Clones carrying the fragment in either orientation were isolated and their orientations determined by dideoxy DNA sequencing (Sanger et al., 1977).

Post-tonB *and Pre*-P14 *Probes*

Purified 330 bp *Sau*3A fragment was cloned directly into the Bam HI site of M13mp7 RF. Orientation of the inserted DNA fragment was determined by dideoxy DNA sequencing as above.

Isolation of ^{3}H-RNA

Strain pRZ526/MO or pRZ625/MO was grown in M9 minimal salts (Miller, 1972) supplemented with 0.4% D-glucose, 0.2% casamino acids (Difco), 4 μg thiamine hydrochloride/ml, 40 μg L-tryptophan/ml, 1.0 mM magnesium sulfate, and 40 μg neomycin/ml. Saturated cultures were diluted 1:50 in fresh medium and grown until the optical density at 550 nm reached 0.3 to 0.4. The bacteria were labeled for 60 sec by addition of 10 μCi [5,6-^{3}H]uridine/ml (45 Ci/mmole, Amersham), and labeling was stopped by rapidly pipetting the entire 10 ml culture

into an equal volume of crushed, frozen stop solution (10% sucrose, 20 mM Tris-hydrochloride [pH 7.3], 5 mM magnesium chloride, 20 mM sodium azide, 400 μg choramphenicol/ml). The labeled cells were harvested by centrifugation and resuspended in 3.0 ml stop solution without sucrose. Then 200 μg lysozyme/ml and 10 μg DNAase I/ml were added, and the cells were lysed by four cycles of freezing and thawing. After addition of 0.01 ml 5% diethyl pyrocarbonate (in ethanol), 0.1 ml 1 M sodium acetate (pH 5.2), and 0.1 ml 15% SDS, the samples were heated to 65°C, cooled, and extracted with 3.5 ml phenol (saturated with 20 mM sodium acetate pH 5.2, 20 mM potassium chloride, 10 mM magnesium chloride). The RNA was precipitated with two vol of cold 100% ethanol, resuspended in 2.0 ml 0.3 M sodium acetate (pH 5.2), precipitated again with ethanol, and resuspended in 0.5 ml hybridization buffer (10 mM Tris hydrochloride pH 7.4, 300 mM potassium chloride, 1 mM sodium EDTA). Incorporation of [^{3}H]uridine into RNA was monitored by precipitating 0.01 ml of the sample in cold 5% trichloroacetic acid, collecting the precipitates on glass fiber filters (GF/C, Whatman), and counting in toluene-based scintillation fluid.

^{3}H-RNA/DNA Hybridization

Relative rates of RNA synthesis were determined by hybridizing portions of the ^{3}H-RNA preparations with an excess of single-stranded M13 DNA bound to nitrocellulose filters (BA85, 13 mm, Schleicher and Schuell). The filters were prepared by filtering 2 μg single-stranded DNA in 5× SSC (1× SSC: 0.15 M sodium chloride, 15 mM sodium citrate pH 7.0) and baking in vacuo for 2 hr at 80°C. Each hybridization contained three DNA filters: M13mp7 carrying a fragment cloned in one orientation, M13mp7 carrying the same fragment cloned in the opposite orientation, and M13mp7 alone. Then 0.15 ml of the ^{3}H-RNA were added to a final volume of 0.5 ml hybridization buffer with 0.2% sodium dodecyl sulfate. After hybridization for 16 hr at 65°C, the filters were batch washed in 2× SSC at room temperature, heated for 15 min at 37°C with 20 μg RNAase A/ml in 2× SSC, washed again in 2× SSC at room temperature, and finally washed in 2× SSC for 30 min at 65°C. Filters were dried and counted in toluene-based scintillation fluid.

Levels of *tonB* or *P14* RNA were calculated by subtracting the average background radioactivity bound to the M13mp7 filters in the experiment from the average radioactivity bound to the M13-*tonB* or M13-*P14* filters. Background radioactivity varied from 80 to 450 cpm depending on the ^{3}H-RNA preparation.

Determination of RNA Half-life

The chemical half-lives of pre- and post-*tonB* and pre- and post-*P14* RNA were determined by monitoring the decay of hybridizable *tonB* or *P14* RNA following inhibition of transcription by rifampicin. Cultures of pRZ526/MO were grown and labeled with [^{3}H]uridine as described above. Further incorporation of [^{3}H]uridine into RNA was stopped by addition of 200 μg rifampicin/ml and 0.8 μg uridine/ml. Samples (10 ml) of the culture were removed immediately prior to—and 30, 60, and 90s after—rifampicin addition, and rapidly pipetted into crushed frozen stop solution. ^{3}H-RNA was extracted and levels of RNA determined by hybridization as described above.

Acknowledgments

We thank Kevin Bertrand, David Daniels, Janice Sharp, and Ron Wek for helpful discussions. We also thank Kevin Bertrand for critical reading of the manuscript, and Janice Sharp for plasmid DNA and reagents. This work was supported by a grant to K. P. from the National Institutes of Health.

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Received January 29, 1985; revised March 15, 1985

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