TRAP binding to the Bacillus subtilis trp leader region RNA causes efficient transcription termination at a weak intrinsic terminator

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ABSTRACT

The Bacillus subtilis trpEDCFBA operon is regulated by a transcription attenuation mechanism controlled by the trp RNA-binding attenuation protein (TRAP). TRAP binds to 11 (G/U)AG repeats in the trp leader transcript and prevents formation of an antiterminator, which allows formation of an intrinsic terminator (attenuator). Previously, formation of the attenuator RNA structure was believed to be solely responsible for signaling RNA polymerase (RNAP) to halt transcription. However, base substitutions that prevent formation of the antiterminator, and thus allow the attenuator structure to form constitutively, do not result in efficient transcription termination. The observation that the attenuator requires the presence of TRAP bound to the nascent RNA to cause efficient transcription termination suggests TRAP has an additional role in causing termination at the attenuator. We show that the trp attenuator is a weak intrinsic terminator due to low GC content of the hairpin stem and interruptions in the U-stretch following the hairpin. We also provide evidence that termination at the trp attenuator requires forward translocation of RNA polymerase and that TRAP binding to the nascent transcript can induce this activity.

INTRODUCTION

In Bacillus subtilis the trp RNA-binding Attenuation Protein, TRAP, regulates expression of genes responsible for synthesis and transport of tryptophan (1). Six of the seven tryptophan biosynthetic genes are located within the trpEDCFBA operon (2). Transcription of the trp operon is regulated by an attenuation mechanism based on formation of two alternative secondary structures in the 5' leader region RNA upstream of trpE. These structures consist of an intrinsic transcription terminator/attenuator comprised of segments A and B in Figure 1 and an upstream antiterminator comprised of segments C and D in Figure 1. As these structures share three residues, they are mutually exclusive (3).

TRAP is composed of 11 identical subunits, each encoded by the mtrB gene (4), arranged in a ring (5). When the intracellular concentration of tryptophan is high, it binds to TRAP and activates the protein to bind RNA (6). The TRAP-binding site in the trp leader segment is composed of 11 (G/U)AG repeats (7). Because this binding site overlaps the antiterminator region, TRAP binding prevents formation of the antiterminator, allowing the attenuator to form and halt transcription in the leader region (8). When tryptophan levels are low, TRAP does not bind RNA and the antiterminator forms allowing transcription of the trp genes.

In the current model for attenuation control of the trp operon, the only role of TRAP is to alter the secondary structure of the leader region RNA (Figure 1). To explore whether TRAP has any additional role in modulating attenuation, we examined the ability of the trp attenuator to induce transcription termination in the absence of the competing antiterminator. The efficiency of termination was examined with several constructs that contain substitutions designed to disrupt formation of the antiterminator structure and thus allow formation of the attenuator in the absence of TRAP. If the only function of TRAP is to promote formation of the attenuator, then transcription of these trp leader mutants should result in constitutive termination in the absence of TRAP. All of the mutant templates showed only slightly increased termination levels at the attenuator in the absence of TRAP as compared to the WT leader region, whereas transcription terminated efficiently in the presence of TRAP. These studies show that the trp attenuator is a weak intrinsic
terminator and suggest that TRAP has a role in the attenuation mechanism beyond influencing the structure of the leader region RNA. We show that the low GC content in the hairpin stem combined with two interruptions in the U-tract generates the weakness of the trp attenuator. One model for intrinsic termination suggests that formation of the hairpin in the nascent transcript causes hypertranslocation of RNAP without chain elongation (9). We found that impeding the forward movement of RNAP at the trp attenuator inhibits transcript release. Moreover, TRAP binding to the nascent trp transcript can induce forward translocation of RNAP. Together our results suggest that the trp attenuator represents a new type of bacterial transcription termination mechanism that is neither truly intrinsic nor dependent on Rho protein.

MATERIALS AND METHODS

Materials

All plasmids were propagated in Escherichia coli K802. Plasmid pUC119trpL, which contains a 730-bp EcoRI–HindIII fragment including the B. subtilis trp promoter and leader sequence (~411 to +318 relative to the start of transcription), was used to create templates for in vitro transcription by polymerase chain reaction (PCR) (10). Bead-bound DNA templates were created with 5′ biotinylated DNA primers from IDT (10). PCR products were purified using QIAGEN MinElute, and were coupled to streptavidin-coated magnetic beads (Dynobeads M-280) according to the manufacturer’s instructions.

Modifications to the antiterminator region of the leader sequence were created using the QuikChange kit (Stratagene) (AntiAB1: G61A, G62A, T63G and C87A) or by cloning overlapping oligonucleotide inserts between XbaI and PstI sites introduced at positions +29 and +139 (relative to the start of transcription) in pUC119trpL (AntiAB2: A67C, T77C, C87A, C93G, A94T, T95G, T96G, C106G, T107A, AntiAB3: A67C, T77C, C87A, C93G, A94T, T95A, T96A, C106G and T107A, AntiAB-GAGAU11, AntiAB-GAGUU11, No Binding Site and CCC/GGG Switch: C109G, C110G, C111G, G130C, G131C, G132C). The differences between AntiAB2 and 3 are highlighted in bold-type font. The sequence of the No Binding Site template from +36 to +91 replaced as: TTGACTGCTATTACTGACTACTTGATTACGTAAATCATGATAGTCTCGAG. The restriction sites were then replaced with WT trp sequence by site-directed mutagenesis. Substitutions in the attenuator region were created by site-directed mutagenesis. The sequence of the complementary oligo: Oligo A; complementary to bases 70–84, Oligo B; complementary to bases 55–69.

Bacillus subtilis BG2087 (argC4) and BG4233 (argC4 ΔmtrB) were used for analysis of lacZ gene fusions. BG4233 contains a deletion of mtrB, which encodes TRAP (11). Bacillus subtilis was transformed by natural competence (12) and blue colonies were selected on plates containing Vogel and Bonner minimal salts (13), 0.2% acid-hydrolyzed casein (ACH), 0.2% (w/v) glucose, 50 μg/m X-gal, 10 μg/ml Arg and 5 μg/ml chloramphenicol. PCR was used to introduce EcoRI and BamHI sites at the ends of DNA fragments containing the trp promoter, leader region, and the first 40 codons of trpE followed by a

Figure 1. Model of transcription attenuation for the B. subtilis trp operon. Bold black letters designate the complementary strands of the terminator (highlighted in blue) and antiterminator stem-loops. TRAP is shown as a ribbon diagram with each subunit as a different color. The 11 (G/U)AG repeats of the TRAP-binding site are circled and numbered in green. Small black numbers indicate residues relative to the start of transcription.
TAA stop codon. Transcriptional fusions with lacZ were created by ligating the PCR products into similarly digested pDH32 (14). The resulting plasmids were linearized with PstI and transformed into B. subtilis. Successful cloning and mutagenesis of plasmids were confirmed by DNA sequencing. Fusions were integrated into the amyE locus by homologous recombination, which was confirmed on starch plates (15). To determine the amount of β-galactosidase that corresponds to 100% read through of the trp attenuator, we created a transcriptional fusion in which the trp promoter is immediately upstream of lacZ with no regulatory sequences from the leader region present. TRAP was expressed in E. coli BL21(DE3) and was purified as described previously (16).

A plasmid with the gene for the cleavage deficient EcoRI E111Q (EcoRI*) protein was a kind gift from Paul Modrich (Duke) (17). The coding sequence for both EcoRI* and the associated methylase were amplified by PCR and ligated into NdeI and BamHI cut pET17b (Novagen). Six histidine (His) codons were added to the amino terminus of the EcoRI* gene using the QuikChange mutagenesis kit from Statagene. In addition, the methylase gene was amplified by PCR separately and ligated into the tetracycline resistance gene of pACYC184 using BamHI and SalI such that the methylase gene is expressed from the tet gene promoter. Both plasmids were transformed into E. coli BL21(DE3). The resulting strain was grown in LB with ampicillin (100 mg/ml) and chloramphenicol (30 mg/ml) until the A600 reached 0.5 at which point IPTG was added and growth was continued for 4 h. The cells were harvested by centrifugation broken with a French pressure cell and EcoRI* was purified using nickel agarose as per manufacturer’s instructions (Qiagen).

**In vitro transcription attenuation assay**

Transcription reactions contained 40 mM Tris–HCl (pH 8.0), 4 mM MgCl2, 0.1 mM EDTA, 4 mM spermidine, 5 mM DTT, 1 mM L-tryptophan, 20 mM DNA template, 500 μM NTPs, 1 μCi [γ-32P]UTP 3000 Ci/mmol and 50 μg/ml B. subtilis σA RNA polymerase (RNAP). TRAP was added as indicated in figures and legends. Reaction mixtures were incubated 15 min at 37°C, then stopped by addition of an equal volume of 95% formamide/20 mM EDTA, pH 8.0/0.3 mg/ml bromophenol blue. Samples were denatured at 95°C for 2 min and products fractionated on 6% denaturing gels, which were exposed to a PhosphorImager screen. Quantification of band intensities was performed using IMAGEQUANT software (Molecular Dynamics). The number of U residues in the read through and terminated RNA transcripts was used to calculate the percentage of transcription termination.

**EcoRI* blocked in vitro transcription**

Prior to initiating transcription, EcoRI* (130 nM) was allowed to bind to DNA template (20 nM) for 5 min at 37°C in Transcription Buffer (20 mM Tris–HCl pH 8, 6 mM MgCl2, 2 mM DTT and 100 mM KCl). Transcription was initiated with 50 μg/ml RNAP and 8 μM ATP, GTP, 2 μM UTP and 1 μCi [γ-32P] UTP (3000 Ci/mmol) at 37°C for 10 min. Heparin (0.1 mg/ml) was then added to prevent re-initiation, and transcription was continued for 10 min at 37°C in the presence of 20 μM NTPs and 1 mM L-tryptophan in the absence or presence of TRAP. Transcripts that remained associated with the bead-bound DNA template were collected with a magnet, whereas released transcripts remained in the supernatant. Reactions were stopped and the products were fractionated as described above. For each reaction, the amount of RNA in the both the supernatant and remaining associated with the bead-bound DNA template was quantified using IMAGEQUANT as described above. The percentage of transcripts released was calculated by dividing the amount of RNA released by the total amount of RNA produced in each reaction.

**β-Galactosidase assays**

_Bacillus subtilis_ strains were grown in, in MOPS minimal medium (400 mM MOPS pH 7, 500 mM KCl, 5 mM MgSO4, 40 mM tricine and 100 mM NH4Cl) with 1.32 mM KH2PO4, 10 μM FeCl3, 0.2% ACH, 5 μg/ml Arg and 5 μg/ml chloramphenicol in the presence or absence of 50 μg/ml L-tryptophan at 37°C to an absorbance at 600 nm of 0.4–0.6. 1.5 ml of cells were pelleted, washed with TE buffer pH 8, and resuspended in 1.5 ml of Z-buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4 and 50 mM β-mercaptoethanol). Lysozyme was added to 0.1 mg/ml to 0.5 ml of cells and incubated at 37°C for 5 min followed by the addition of 0.1% triton X-100; 0.1 ml of cell lysate was added to 0.9 ml of Z buffer and assayed for β-galactosidase activity (18). Each value is the average of two or three independent experiments, each performed in triplicate.

**RESULTS**

The trp attenuator is a weak intrinsic terminator that requires TRAP bound to the nascent trp transcript to cause transcription termination

We created several DNA templates that contain mutant trp leaders with substitutions designed to disrupt base pairing in the A/B antiterminator (19) without altering the (G/U)AG repeats of the TRAP binding site. If the sole function of TRAP binding is to prevent formation of the antiterminator structure, then transcription of these mutant templates should terminate constitutively at the C/D terminator in the absence of TRAP. The AntiAB1 template contains four substitutions designed to prevent the shared CCC sequence from participating in the antiterminator and thereby promote formation of the attenuator (Figure 2B). We tested the effects of these substitutions on the efficiency of termination at the trp attenuator using an _in vitro_ transcription attenuation assay (8). For comparison, the wild-type (WT) trp leader region was also examined. In the absence of TRAP, transcription of the WT template resulted in virtually complete read through of the attenuator yielding >99% run-off transcripts (Figure 2A and C). As seen previously (6,8), adding increasing amounts of tryptophan-activated TRAP increased the fraction of transcripts that terminated at the
attenuator, indicative of TRAP-mediated transcription attenuation (Figure 2A). Transcription of the AntiAB1 template in the absence of TRAP showed only slightly elevated termination at the attenuator (27%), while the presence of TRAP yielded termination levels similar to those observed with the WT template (Figure 2A). With both templates, transcription in the presence of 0.4 μM TRAP resulted in nearly complete (>85%) termination at the attenuator (Figure 2A). Although higher levels of TRAP yielded slightly more termination, we also observed slight inhibition of transcription. Consequently, although TRAP was titrated with each template, results with 0 and 0.4 μM TRAP are presented to represent transcription in the absence and presence of TRAP (Figures 2C and 3).

A potential explanation for observing only a slight increase in termination with the AntiAB1 template in the absence of TRAP could be that an antiterminator RNA structure formed despite the substitutions to allow the shared CCC sequence to participate in the attenuator. To address this possibility, we created five additional templates with more extensive substitutions designed to prevent base-pairing in the antiterminator region. Two of these (AntiAB2 and AntiAB3) contain nine substitutions in the antiterminator region (Figure 2B), and two have the entire TRAP binding site from +36 to +91 (Figure 1) replaced with an optimized binding site composed of 11 repeats of the respective five residue sequences. No Binding Site replaces +36-91 with an unstructured RNA sequence with no TRAP binding. (C) Bar graph of the percentage of termination for antiterminator constructs in the absence (dark gray) and presence (light gray) of 0.4 μM TRAP.

Figure 2. Effect of substitutions in the antiterminator on TRAP-mediated transcription termination in vitro. (A) PAGE analysis of the products of in vitro transcription of the WT and AntiAB1 mutant trp leader regions in the absence and presence of increasing amounts of tryptophan-activated TRAP. Positions of read-through (RT = 320 nt) and terminated (T = 140 nt) transcripts are indicated. The percentage of termination for each reaction is at the bottom of each lane. Although a faint band is visible for the terminated transcript with the WT leader in the absence of TRAP, IMAGEQUANT analysis did not detect this as above background. (B) Diagram of the A/B antiterminator with the positions of residues that were altered in trp leader mutants indicated. (G/U)AG triplets of the TRAP binding site are circled. Small closed circles represent positions of substituted nucleotides in AntiAB1; open circles represent substituted residues in AntiAB2 and 3. AntiAB-GAGAU11 and AntiAB-GAGUU11 replace the WT sequence from +36 to +91 with 11 repeats of the respective five residue sequences. No Binding Site replaces +36-91 with an unstructured RNA sequence with no TRAP binding. (C) Bar graph of the percentage of termination for antiterminator constructs in the absence (dark gray) and presence (light gray) of 0.4 μM TRAP.
overlap with the antiterminator. Despite these extensive changes in the antiterminator segment, none of these templates yielded more than 45% termination at the attenuator when transcribed in the absence of TRAP. However, in all cases transcription of these substituted templates in the presence of TRAP resulted in 85% termination at the attenuator (Figure 2C). Replacing the TRAP-binding segment of the trp leader region with a random unstructured RNA segment also modestly increased termination in the absence of TRAP but in this case adding TRAP had no effect (Figure 2C).

Together, these results indicate that simply preventing formation of the antiterminator structure in the trp leader is not sufficient to yield efficient transcription termination at the attenuator.

To examine why TRAP is required to cause transcription termination at the trp attenuator, we investigated the properties of the attenuator that make it a weak intrinsic terminator. Canonical intrinsic terminators are composed of a GC-rich stem–loop followed by 7–9 U residues (21). The trp attenuator hairpin stem is largely AU pairs, and the U-stretch following the stem is interrupted twice (Figure 3A). Hence, we tested whether either of these discrepancies affect the function of the trp attenuator and its requirement for TRAP.

**The low GC content of the attenuator stem contributes to the requirement for TRAP**

The top portion of the trp attenuator stem is AU-rich (Figure 3A). Because hairpin formation likely initiates at
Substitutions in the U-stretch do not involve residues that overlap between the antiterminator and terminator, nor should they affect the stability of the terminator hairpin. Consequently, these changes should not influence which structure forms in the RNA during transcription. Hence, the observation that substitutions in the U-tract of the attenuator showed increased termination efficiency, up to 40% for A136U/G140U in the presence of the WT antiterminator (Figure 3E) suggests that the WT A/B antiterminator structure is not completely effective at preventing formation of the attenuator hairpin. Moreover, the dramatic increases in termination seen with the U-stretch substitutions in the context of AntiAB1 (up to 90%, Figure 3D) confirms that these substitutions effectively prevent formation of the antiterminator.

In vivo analysis of trp attenuator function

The properties of the trp attenuator were also investigated in vivo using trpE’-lacZ transcriptional fusions (Table 1). Each of the fusions tested was under control of the trpH promoter followed by either the WT or AntiAB1 antiterminator coupled with the WT, All GC or A136U attenuator. β-Galactosidase expression from each construct was examined in BG2087 (mtrB+), which expresses WT TRAP, and BG4233 (∆mtrB) which lacks TRAP (Table 1). None of the fusions were regulated in response to tryptophan in BG4233 (Table 1). As seen previously (24,25), the WT fusion was regulated in response to tryptophan in BG2087 (mtrB+) such that growth in the presence of excess tryptophan yielded <1% of that in the strain lacking TRAP, indicative of highly efficient (>99%) termination at the attenuator (Table 1). The level of β-galactosidase from all the fusions in BG4233 grown in either the absence or presence of tryptophan was greater than those seen for the same fusion grown in BG2087 in the absence of tryptophan. This difference reflects endogenously produced tryptophan in BG2087, which is trp+. The AntiAB1 substitutions in the antiterminator region increased termination at the attenuator as evidenced by reduced β-galactosidase expression in the absence of TRAP. This effect can be seen in BG4233 by comparing the levels obtained with each of the three attenuators in the context of the WT antiterminator (Table 1; rows 1–3) to those obtained for the same attenuator in the context of the AntiAB1 substitutions (Table 1; rows 4–6). In all cases, these observations are consistent with our in vitro studies and suggest that the AntiAB1 substitutions prevent formation of the antiterminator. However, complete (>99%) termination at the wild-type attenuator is not achieved unless TRAP and tryptophan are present. This requirement for TRAP is seen by comparing 90% termination at the WT attenuor in the context of the AntiAB1 substitutions in the absence of TRAP (Table 1: row 4, BG4233) with >99% termination seen in the presence of TRAP and tryptophan (Table 1: row 4, BG2087). These observations again suggest that TRAP has an additional function that allows the trp attenuator to efficiently halt transcription beyond altering the RNA secondary structure.
These results suggested that preventing formation in vitro of the A/B antiterminator induced termination at the nucleotide complementary to residues 55–69 at the base of the attenuator structure over the antiterminator, whereas stem substitutions favor formation of the attenuator structure in the absence of TRAP (BG4233). Second, the increased stability of the All GC attenuator is a better predictor of extending the U-tract (Table 1: row 2). This increased termination is likely due to two effects. First, the All GC attenuator is a better intrinsic terminator, which can be seen by the increase in termination from 90% for the WT attenuator to 89% for the All GC stem attenuator (Table 1, rows 1 and 2: BG4233). The presence of tryptophan-activated TRAP increased termination efficiency of the All GC stem attenuator to over 99% regardless of the antiterminator context (Table 1, rows 1 and 2: BG4233). The presence of the WT antiterminator. This effect is illustrated by the increase in termination from 32% for the WT attenuator to 89% for the All GC stem attenuator in the context of the WT antiterminator. The suggestion that TRAP binding has additional functions beyond just altering the RNA secondary structure was confirmed by the observation that TRAP-mediated termination was more efficient (92%) than that produced by up to 1000-fold excess of competitor oligo (65%) (Figure 4; lane 2 versus lane 6). By contrast, when the DNA template encoded either the A136U or the All GC stem mutant attenuator (Figure 3A), the presence of the competitor oligo was as effective as TRAP (Figure 4; lane 8 versus 12 and lane 14 versus 18). These observations are again consistent with the suggestion that TRAP binding has additional functions beyond just altering the RNA secondary structure of the leader region.

**Blocking forward movement of RNAP inhibits termination at the trp attenuator**

To determine whether hypertranslocation is required for termination at the trp attenuator, we examined the effects of blocking the forward movement of RNAP downstream from the site of termination (position 140) (26). The cleavage defective E111Q mutant EcoRI (EcoRI*) protein binds to DNA and blocks elongation of RNAP approximately 14 bp upstream of the first G of its GAATTC recognition site (9,27,28). We created templates that position EcoRI* between 0 and 4 bp downstream of the front edge of RNAP when it reaches G140 and examined the effect of the presence of this roadblock on transcript release (Figure 5A). Transcription of these templates in the presence of tryptophan-activated TRAP resulted in >99% transcript release at the attenuator in the absence of EcoRI* (Figure 5A, lanes 1, 2 and 5–14). The presence of EcoRI* protein positioned either immediately adjacent to, or 1 bp downstream of the front edge of RNAP inhibited transcript release by 60–70% (Figure 5A, lanes 15–18). This inhibitory effect progressively diminished as the distance between RNAP and the roadblock increased to two or more base pairs.

### Table 1. *In vivo* Analysis of antiterminator and attenuator substitutions on attenuation control of transcription from the trp promoter

| Trp leader RNA | In vivo β-galactosidase activity (U)a | In vitro transcription %Terminationc |
|----------------|--------------------------------------|-------------------------------------|
|                | BG4233 (ΔmtrB)                        | BG2087 (mtrB+)                       |
| Antiterminator | −Trp +Trp %T (+Trp)b                  | −Trp +Trp %T (+Trp)b                  |
| Wild-type      | 355 ± 70 355 ± 73 32.9                | 106 ± 20 4.4 ± 1.6 99.2              |
| 2              | 60 ± 22 58 ± 25 89                    | 21 ± 5 1.9 ± 0.9 99.6                |
| 3              | 318 ± 36 319 ± 28 39.7                | 111 ± 11 3.4 ± 1.2 99.4              |
| AntiA,B1       | 55 ± 9 52 ± 10 90.2                  | 14 ± 6 3.6 ± 4.4 99.3                |
| 4              | 17 ± 2 17 ± 2 96.8                   | 3.5 ± 0.8 0.7 ± 0.4 99.9             |
| 6              | 31 ± 10 28 ± 5 94.7                  | 3.6 ± 1.2 1.3 ± 2 99.8               |
|                |                                      | 28 88                               |
|                |                                      | 89 96                               |
|                |                                      | 79 98                               |

*a* Values are the average of two or three independent experiments, each performed in triplicate, ± the standard deviation.

*b* %T is the estimate of the percentage of transcripts that terminate at the trp attenuator; in each case %read through is calculated by dividing the units of β-galactosidase obtained by 529U which maximum units from the P<sub>Trp</sub>/lacZ construct in BG4233 grown in the presence of tryptophan; %T = 100 - %read through.

*c* These data are reproduced from Figure 3B and C and are included for comparison.

We also used lac fusions to examine two modified attenuators that showed increased intrinsic termination in vitro (Figure 3). The All GC stem increased termination efficiency at the attenuator in the absence of TRAP (BG4233) even in the context of the WT antiterminator (Table 1: row 2). This increased termination is likely due to two effects. First, the All GC attenuator is a better intrinsic terminator, which can be seen by the increase in termination from 90% for the WT attenuator to 97% for All GC in the AntiAB1 context (Table 1 rows 4 and 5: BG4233). Second, the increased stability of the All GC stem favors formation of the attenuator structure in the presence of the WT antiterminator. This effect is illustrated by the increase in termination from 32% for the WT attenuator to 89% for the All GC stem attenuator in the context of the WT antiterminator. The suggestion that TRAP binding has additional functions beyond just altering the RNA secondary structure was confirmed by the observation that TRAP-mediated termination was more efficient (92%) than that produced by up to 1000-fold excess of competitor oligo (65%) (Figure 4; lane 2 versus lane 6). By contrast, when the DNA template encoded either the A136U or the All GC stem mutant attenuator (Figure 3A), the presence of the competitor oligo was as effective as TRAP (Figure 4; lane 8 versus 12 and lane 14 versus 18). These observations are again consistent with the suggestion that TRAP binding has additional functions beyond just altering the RNA secondary structure of the leader region.

### Preventing formation of the antiterminator with complementary oligonucleotides

Prior studies showed that the presence of a DNA oligonucleotide complementary to residues 55–69 at the base of the A/B antiterminator induced termination at the trp attenuator in an in vitro transcription attenuation assay (8,26). These results suggested that preventing formation of the antiterminator by competition for pairing with the oligonucleotide is sufficient to induce termination. When we compared oligo-mediated and TRAP-mediated termination we found that with the WT trp leader region, TRAP-mediated termination was more efficient (92%) than that produced by up to 1000-fold excess of competitor oligo (65%) (Figure 4; lane 2 versus lane 6). By contrast, when the DNA template encoded either the A136U or the All GC stem mutant trp attenuators (Figure 3A), the presence of the competitor oligo was as effective as TRAP (Figure 4; lane 8 versus 12 and lane 14 versus 18). These observations are again consistent with the suggestion that TRAP binding has additional functions beyond just altering the RNA secondary structure of the leader region.

**Blocking forward movement of RNAP inhibits termination at the trp attenuator**

To determine whether hypertranslocation is required for termination at the trp attenuator, we examined the effects of blocking the forward movement of RNAP downstream from the site of termination (position 140) (26). The cleavage defective E111Q mutant EcoRI (EcoRI*) protein binds to DNA and blocks elongation of RNAP approximately 14 bp upstream of the first G of its GAATTC recognition site (9,27,28). We created templates that position EcoRI* between 0 and 4 bp downstream of the front edge of RNAP when it reaches G140 and examined the effect of the presence of this roadblock on transcript release (Figure 5A). Transcription of these templates in the presence of tryptophan-activated TRAP resulted in >99% transcript release at the attenuator in the absence of EcoRI* (Figure 5A, lanes 1, 2 and 5–14). The presence of EcoRI* protein positioned either immediately adjacent to, or 1 bp downstream of the front edge of RNAP inhibited transcript release by 60–70% (Figure 5A, lanes 15–18). This inhibitory effect progressively diminished as the distance between RNAP and the roadblock increased to two or more base pairs.
more base pairs (lanes 19–24). EcoRI* had no effect on transcript release from the WT template (Figure 5A, lanes 3 and 4). In the absence of TRAP none of the transcripts were released in the presence of EcoRI*. Together these results are similar to previous findings with the t500 terminator (9) and indicate that forward translocation of ~2 bp is required for transcript release at the trp attenuator.

The observations that the trp attenuator functions weakly in the absence of TRAP and that forward translocation is required for termination at the attenuator suggest that TRAP may function by inducing forward movement of RNAP. Hence, we examined whether TRAP binding to the nascent transcript can induce forward translocation of RNAP. To do so, we blocked the transcription elongation complex (TEC) with
where EcoRI* such that either 6 or 10 of the (G/U)AG repeats of the TRAP-binding sites are exposed on the nascent transcript. EcoRI* binding to the DNA blocks the TEC such that the nascent RNA is exposed from RNAP ~26–27 residues upstream of the $G$ in the restriction site (9,27,28). Hence, placing a GAATTCC recognition site starting at either +92 (Eco92) or at +116 (Eco116) positions EcoRI* to block the TEC such that the nascent transcripts are exposed up to approximately residues +66 or +90, respectively. The exposed regions on these two nascent transcripts contain either 6 (Eco92) or 10 (Eco116) of the (G/U)AG repeats of the TRAP binding site (Figure 1).

The transcripts produced from these two templates with EcoRI* bound and transcription performed in the absence or presence of TRAP were then examined. Transcription of either template in the absence of TRAP produced a single transcript of the expected length. Adding TRAP to the transcription reaction resulted in incorporation of one additional residue on ~33% of the transcripts from the Eco116 template, but had no effect on transcription from Eco92 (Figure 5B). These observations are similar to those seen with Mfd or Rho (29), and suggest that TRAP binding to >6 (G/U)AG repeats in the nascent transcript can induce forward translocation of RNAP against an elastic force of the EcoRI* roadblock.

### DISCUSSION

Until now, the only identified functions of TRAP in the attenuation mechanism that controls transcription of the *B. subtilis trp* operon were to sense the intracellular level of tryptophan and to influence the secondary structure of the RNA in the leader region (Figure 1). While the results presented here support this model, our evidence shows that TRAP plays an additional active role in promoting termination at the attenuator beyond simply preventing formation of the antiterminator. This additional function is necessary because the *trp* attenuator is an inefficient intrinsic terminator.

Substitutions in the leader region to prevent formation of the A/B antiterminator do not result in constitutive termination of transcription at the attenuator (Figure 2). A simple explanation for these observations would be that despite these changes, alternative RNA structures form in the nascent transcripts from all the mutant templates and these structures inhibit formation of the attenuator. Several observations indicate that this alternative explanation is incorrect. First, we made a total of six different substitutions in the antiterminator region (Figure 2) and none are predicted to form secondary structures that overlap the attenuator (Mfold). Second, in addition to these six templates, we also created a template in which the three $G:C$ base pairs in the base of the terminator are replaced with $G:C$ pairs. This template alters the three Cs (109–111) that overlap between the A/B and C/D structures (Figure 1) and thus allows formation of an alternative terminator with GC content similar to the WT attenuator that does not overlap with the antiterminator. Moreover, if all of the other AntiAB substitutions form RNA secondary structure involving $C_{109}-C_{111}$, this construct should not do so. Similar to our other substituted *trp* leader regions, transcription of this template in *vitro* yielded only 42% termination at the modified attenuator in the absence of TRAP, which increased to 92% in its presence. Finally, in the context of the AntiAB substitutions in the antiterminator segment, replacing the interruptions in the U-tract at A136 and G140 with Us results in virtually complete termination, whereas only 40% termination is observed when the WT A/B segment is present (Figure 3). Because changes in the U-tract should not influence which RNA secondary structure forms in the leader RNA, these observations strongly indicate that the AntiAB substitutions efficiently allow formation of the attenuator.

Our results suggest that the combination of the low GC content (36%) of the base-paired stem together with interruptions of the U-stretch generates the requirement for TRAP to cause termination at the *trp* attenuator (Figure 3). The average GC content of the hairpin stems from over 400 intrinsic *B. subtilis* terminators is 64% (30). Similarly, the average length of uninterrupted Us following the stem of these intrinsic terminators is 4.8, with only 24% having three or fewer Us before an interruption, as seen with the *trp* attenuator (30). The low GC content of the stem as well as early interruptions of the U-stretch are conserved in TRAP-regulated *trp* attenuators from other bacteria. Inspection of such *trp* attenuators from 12 additional species shows that the GC content of the stem ranges from 36% to 75%, and with one exception, those above 45% are from thermophiles. Similarly, all but one of the U-tracts of these *trp* attenuators are interrupted within the first three to four residues following the stem. These similarities suggest that the need for the additional function of TRAP in the attenuation mechanism is conserved in these regulatory systems.

Attenuation and antitermination mechanisms control transcription of numerous bacterial genes (31,32). As with the *B. subtilis trp* operon (Figure 1), in many cases an antiterminator structure competes with formation of an intrinsic terminator because the two RNA structures share residues. In the *B. subtilis trp* operon, three residues (CCC) are shared between the antiterminator and the attenuator (Figure 1) (3). This overlap is more extensive in other attenuation/antitermination systems. For example, in *E. coli* and *S. typhimurium*, transcription of numerous amino acid biosynthetic operons is regulated by attenuation based on the ability of a ribosome to translate a leader peptide coding segment (33). In these systems, the regulatory structures overlap by 6–11 residues with most being nine or more. Similarly, in the leader region of genes regulated by the T-box antitermination mechanism these structures generally share seven residues (32). The short overlap of 0–5 residues between antiterminator and attenuator is also conserved in TRAP-regulated *trp* operons from other species.

These studies indicate that the *B. subtilis trp* operon antiterminator does not completely prevent formation of the attenuator. This is evident from the observation that in the context of the WT antiterminator segment, improving the U-tract of the attenuator (A136U) significantly
increased termination in the absence of TRAP (Figure 3E and Table 1). Hence, although the attenuator forms frequently in trp transcripts in the absence of TRAP, this does not compromise regulation of the operon because in the absence of TRAP the attenuator does not effectively prevent transcription of the trp genes. In other attenuation/antitermination systems with strong intrinsic terminators, their formation must be controlled more tightly and thus these systems employ greater overlap and more stable antiterminators.

Our results suggest that an attenuation system could function without the presence of an antiterminator RNA structure if termination at the attenuator was entirely dependent on a trans-acting factor. This appears to be true for the Bacillus halodurans trp operon (34). In this system, TRAP regulates transcription at an attenuator in response to tryptophan; however, there is no apparent overlapping antiterminator. Moreover, the B. halodurans trp attenuator bears only slight similarity to a conical intrinsic terminator. Most notably, it contains several mismatches within the stem. Hence, it appears that in this system regulation is based solely on the ability of TRAP to promote termination at a very weak intrinsic terminator.

One model for intrinsic termination suggests that formation of the hairpin induces forward translocation of RNAP at the U-tract without chain elongation (9). Recent studies of the energetics of transcription termination support this forward translocation model for intrinsic terminators containing two interruptions in the U-tract (35), such as the trp attenuator (Figure 3A). Terminators with uninterrupted U-tracks do not appear to require forward translocation of RNAP (35). We found forward translocation is required for termination at the trp attenuator (Figure 5A). Its weak intrinsic termination activity may result from ineffective pushing of RNAP by the AU-rich hairpin. The role of TRAP in causing termination at the attenuator may therefore be to assist forward movement of the TEC. We also show that, like the termination factor Mfd and Rho (29), TRAP can induce forward movement of RNAP on the DNA template (Figure 5B). Potential mechanisms by which TRAP could induce forward translocation of RNAP include: (i) by specific protein–protein interactions between TRAP and RNAP such as have been proposed for Mfd (29), or (ii) by nonspecific protein–protein interactions in which TRAP binding to the nascent transcript physically pushes RNAP forward or (iii) by interactions through the nascent RNA such as has been proposed for Rho (29). TRAP is not needed for termination when the GC content of the stem is increased (All GC, Figure 3), suggesting that the GC enriched hairpin induces hypertranslocation of RNAP without assistance from TRAP. Substitutions that remove the interruptions in the U-stretch also eliminate the need for TRAP to induce efficient termination at the attenuator (Figure 3D). In this case, without the interruptions, forward translocation of RNAP may not be required (35) allowing the AU-rich stem to cause termination via an alternative mechanism (36). The trp attenuator thus appears to represent a new type of bacterial transcription terminator that is neither truly intrinsic nor dependent on termination factor rho (37), but instead relies on TRAP bound to the nascent RNA.

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