Expression of the trpEDCFBA operon is regulated at both the transcriptional and translational levels by the trp RNA-binding attenuation protein (TRAP) of Bacillus subtilis. When cells contain sufficient levels of tryptophan to activate TRAP, the protein binds to trp operon transcripts as they are being synthesized, most often causing transcription termination. However, termination is never 100% efficient, and transcripts that escape termination are subject to translational control. We determined that TRAP-mediated translational control of trpE can occur via a novel RNA conformational switch mechanism. When TRAP binds to the 5'-untranslated leader segment of a trp operon read-through transcript, it can disrupt a large secondary structure containing a portion of the TRAP binding target. This promotes refolding of the RNA such that the trpE Shine-Dalgarno sequence, located more than 100 nucleotides downstream from the TRAP binding site, becomes sequestered in a stable RNA hairpin. Results from cell-free translation, ribosome toeprint, and RNA structure mapping experiments demonstrate that formation of this structure reduces TrpE synthesis by blocking ribosome access to the trpE ribosome binding site. The role of the Shine-Dalgarno blocking hairpin in controlling translation of trpE was confirmed by examining the effect of multiple nucleotide substitutions that abolish the structure without altering the Shine-Dalgarno sequence itself. The possibility of protein-mediated RNA refolding as a general mechanism in controlling gene expression is discussed.

Studies on the regulation of protein synthesis have shown that the RNA secondary structural features present in the 5'-UTR dramatically influence translation initiation in both prokaryotic and eukaryotic organisms (for recent reviews see Refs. 1–7). In prokaryotic mRNAs, a conserved stretch of 4–6 nucleotides called the Shine-Dalgarno (SD) sequence is usually found 4–11 nucleotides upstream of the initiation codon. The SD sequence base pairs with the 16 S rRNA present in the 30 S ribosomal subunit and thereby correctly positions the initiation codon in the ribosome (8, 9). Translational control mechanisms have been identified in prokaryotes that involve blocking the SD sequence either by RNA secondary structure (9–12) or by a bound protein (13–16). In the known translational control mechanisms that occur by formation of SD blocking hairpins, formation of the inhibitory structure is spontaneous and does not require protein factors.

Expression of the Bacillus subtilis tryptophan biosynthetic genes is regulated in response to changes in the intracellular level of tryptophan at both the transcriptional and translational levels (for a recent review, see Ref. 17). Six of the seven trp genes are clustered in the trpEDCFBA operon. Transcription of the trp operon is regulated by an attenuation mechanism in which tryptophan-activated trp RNA-binding attenuation protein (TRAP) binds to 11 closely spaced (G/U)AG repeats (seven GAG and four UAG) (18–25). TRAP binding to the 11 trinucleotide repeats in the nascent trp leader transcript prevents or disrupts formation of an RNA secondary structure, the antiterminator, thereby allowing formation of an overlapping Rho-independent terminator and hence causing termination of transcription before RNA polymerase reaches the trp structural genes. In the absence of TRAP binding, formation of the antiterminator prevents formation of the terminator, resulting in transcriptional read-through into the trp structural genes. In addition to regulating transcription of the trp operon, TRAP also regulates translation of trpE. Previous in vivo studies demonstrated that TRAP is responsible for regulating trpE translation 10–15-fold (19, 25). RNA structure predictions of the trp operon read-through transcript indicated that the most thermodynamically stable conformation of the leader RNA segment would contain a large secondary structure that includes a portion of the TRAP binding site in the 5'-half of the stem. It was proposed that TRAP binding to these repeats would disrupt the large secondary structure and promote refolding of the leader RNA such that the trpE SD sequence would be sequestered in an RNA hairpin (19, 25). It was also shown that multiple nucleotide substitutions predicted to destabilize the SD blocking hairpin, without altering the SD sequence itself, reduced the ability of TRAP to regulate TrpE synthesis in vivo (25). Thus, the ability of TRAP to alter the conformation of trp operon read-through transcripts could partially explain the TRAP-dependent translational control of trpE expression that was previously observed (19, 25).

The one unlinked trp gene, trpG, is a part of a folic acid biosynthetic operon (26). Expression of trpG is regulated by a translational control mechanism in which tryptophan-activated TRAP can bind to nine trinucleotide repeats (seven GAG, one UAG, and one AAG) that surround and overlap the trpG ribosome binding site. TRAP binding to these repeats represses TrpG synthesis by directly blocking ribosome access to the trpG ribosome binding site (15, 16).

The crystal structure of TRAP complexed with L-tryptophan shows that TRAP is composed of 11 identical subunits arranged in a single ring, with one molecule of tryptophan bound between adjacent subunits (23, 27). The RNA binding motif of
TRAP consists of 11 repeated KKR motifs that line the periphery of the TRAP complex. This finding suggests that TRAP-RNA interaction proceeds through a mechanism in which one KKR motif binds to one (G/U)AG repeat, thereby wrapping the RNA around the outside of the TRAP complex (28).

In the present study, we performed experiments in vitro to elucidate the molecular mechanism responsible for TRAP-mediated translation control of trpE. Our cell-free translation and RNA structural studies demonstrate that TRAP binding to trp operon read-through transcripts does in fact promote refolding of the untranslated trp leader such that the trpE SD sequence, which is located more than 100 nucleotides downstream from the TRAP binding site, becomes sequestered in a stable RNA hairpin. Moreover, we found that formation of this SD binding hairpin inhibits TrpE synthesis by blocking ribosome access to the trpE ribosome binding site.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Bacterial Strains—**Plasmid pPB22 carrying the wild type *B. subtilis* *trp* promoter and leader (WtTrpL) has been described (20). The plasmid pINT-SdIII contains several trp leader point mutations that destabilize the predicted trpE SD blocking RNA hairpin without disrupting the trpE SD sequence itself (25). Plasmid pH12 was constructed by subcloning the 730-base pair EcoRI–HindIII fragment containing the SDtrpE fragment from pINT-SdIII into the EcoRI–HindIII sites of the pTZ18U polylinker (U.S. Biochemical Corp.). A description of plasmid pTRP-H3B2 into the unique *trpD* locus of *B. subtilis* trp operon promoter and leader as well as the trpED structural genes from pTRP-H3B2 into the unique *Sma*I site of pTZ18U. The plasmid pH15 was constructed by subcloning the 2.4-kilobase pair PvuII fragment containing the *trp* operon promoter and leader as well as the trpED structural genes from pH12 into the unique *Sma*I site of pTZ18U. The plasmid pH16 was constructed in the same manner as pH15 except that the 730-base pair EcoRI–HindIII fragment containing the SDtrpL from pH12 was used in place of the WtTrpL. The *B. subtilis* integration vector, pBtB1-PLK, used for the generation of trpE-'lac translational fusions was described previously (25). The plasmids pH222 and pH242, which contain trpE-’lac translational fusions, were constructed by subcloning the trp leader containing EcoRI–HindIII fragments from pH222 or pH242 into the EcoRI–HindIII sites of pBtB1-PLK, respectively. The two plasmids, pH222 and pH242, were linearized with ScaI and separately integrated into the amyE locus of *B. subtilis* strain W168 (prototrophic). Transformation was by natural competence (30); selections were made for the absence of secondary rearrangements in short RNA segments. RNA samples were partially digested with RNase T1 and RNase T2, and the RNA was recovered by two successive ethanol precipitations. Chemical modification reactions using DMS or CMCT were allowed to form for 10 min at 37 °C prior to the addition of 0.15 units of RNase T1 or 1 X 10−3 units of RNAse V1 was added, and the samples were further incubated for 10 min at 37 °C. Samples were immediately extracted with phenol/chloroform, and the RNA was recovered by two successive ethanol precipitations. Chemical modification reactions using DMS or CMCT were used to determine the amount of each reagent that would prevent multiple cleavages or chemical modifications in any one transcript so that we could control for the potential of secondary rearrangements in short RNA segments. The transcripts and end-labeled primer used in the analysis are described above. RNA Structure Mapping—The transcripts used in this analysis were synthesized using the Ambion MEGAScript in vitro transcription kit from HindIII-linearized pPB22 (wild type *trp* leader) or pPB22 (SD blocking RNA hairpin) as template. Reaction mixtures (20 μl) contained 0.5 pmol of γ-32P-end-labeled primer complementary to nucleotides 245–285 relative to the start of *trp* operon transcription, 0.2 pmol of in vitro generated mRNA, 3 μg of TRAP, 1 μm L-tryptophan, and 0.375 mM dNTPs in toeprint buffer (40 mM Tris-HCl, pH 8.0, 200 mM KCl, 4 mM MgCl2, 1 mM dithiothreitol). The mixture was incubated at 37 °C for 10 min to allow TRAP-RNA complex formation and to allow the end-labeled primer and the transcript to anneal. After the addition of 10 units of MoleculaRIE murine leukemia virus reverse transcriptase (U.S. Biochemical), incubation was continued at 37 °C for 10 min. Samples were extracted with phenol/chloroform followed by ethanol precipitation. Samples were resuspended in 5 μl of water followed by the addition of 3 μl of standard sequencing stop solution.

**In Vitro Protein Synthesis—**TRAP was purified as described previously (20). Preparation of TRAP-deficient S30 extract followed a published procedure (16). The RNA used in this analysis was synthesized in vitro using the Ambion MEGAScript kit and plasmid pH15 linearized with BamHI as template. Translation reactions (50 μl) contained 72 mM Tris-HCl (pH 7.5), 72 mM NH4Cl, 10 mM magnesium acetate, 0.1 mM EDTA (pH 7.5), 2.4 mM diithiothreitol, 2 mM ATP, 0.1 mM GTP, 0.08 mM calcium chloride, 0.2 mM disopropylfluorophosphate, 20 mM phosphoenolpyruvate, 35 units/ml pyruvate kinase, 1 mM L-tryptophan, 0.1 mM concentration of the remaining amino acids (minus methionine), 4 μg of S30 extract (50 μg of total protein), 800 units/ml ONG RNAsin, 500 μM of dNTPs, and 10 μl of [35S]methionine. To reduce endogenous mRNA and DNA, the S30 extract plus DNase I was preincubated for 15 min at 37 °C prior to the addition of the remaining components. Concentrations of TRAP used in various reactions are indicated in the appropriate figure legend. Final reaction mixtures were incubated at 37 °C for 30 min. Reactions were terminated by the addition of 2.5 SD SDS sample buffer (16). Samples (5 μl) were heated at 95 °C for 3 min and electrophoresed through a 15% SDS-polyacrylamide gel. Radio labeled protein bands were quantified with a PhosphorImager (Molecular Dynamics, Inc.) and the ImageQuant software package.

**Computer Predictions of RNA Secondary Structures—**Predictions of RNA secondary structures within the wild type and mutant trp leaders were performed using the MFOLD program (35). Primer Extension Inhibition and Toeprint Analyses—Primer extension inhibition experiments were carried out to map the position of the 3'-ends of stable RNA secondary structures. Gel-purified transcripts used in this analysis were synthesized with the Ambion MEGAScript in vitro transcription kit from HindIII-linearized pPB22 (wild type *trp* leader) or pPB22 (SD blocking RNA hairpin) as template. Reaction mixtures (20 μl) contained 0.5 pmol of γ-32P-end-labeled primer complementary to nucleotides 245–265 relative to the start of *trp* operon transcription, 0.2 pmol of in vitro generated mRNA, 3 μg of TRAP, 1 μm L-tryptophan, and 0.375 mM dNTPs in toeprint buffer (40 mM Tris-HCl, pH 8.0, 200 mM KCl, 4 mM MgCl2, 1 mM dithiothreitol). The mixture was incubated at 37 °C for 10 min to allow TRAP-RNA complex formation and to allow the end-labeled primer and the transcript to anneal. After the addition of 10 units of Moloxy murine leukemia virus reverse transcriptase (U.S. Biochemical), incubation was continued at 37 °C for 10 min. Samples were extracted with phenol/chloroform followed by ethanol precipitation. Samples were resuspended in 5 μl of water followed by the addition of 3 μl of standard sequencing stop solution.

The 30 S ribosomal subunit toeprint reactions followed a published procedure (16), except that toeprint buffer was used (see above). The transcripts and end-labeled primer used in the analysis are described above. Samples were fractionated through standard 6% sequencing gels. Control sequencing reactions were carried out with the Sequenase version 2.0 sequencing kit (U.S. Biochemical) using the same plasmids and primers as above.

**RNA Structure Mapping—**The transcripts used in this analysis were synthesized using the Ambion MEGAScript in vitro transcription kit and plasmid pPB22 or pH22 linearized with HindIII as template. Titrations of RNases and chemical reagents were routinely performed to determine the amount of each reagent that would prevent multiple cleavages or chemical modifications in any one transcript so that we could control for the potential of secondary rearrangements in short RNA segments. RNA samples were partially digested with RNAse T1 (Life Technologies, Inc.) or RNAse V1 (Amersham Pharmacia Biotech). Reaction mixtures (0.1 ml) contained 20 pmol (2 μg) of TRAP, 1 pmol of transcript and 1 pmol L-tryptophan in TKM buffer (40 mM Tris-HCl, pH 8.0, 250 mM KCl, 4 mM MgCl2) (22). TRAP-RNA complexes were allowed to form for 10 min at 37 °C, at which time 1.5 units of RNAse T1 or 1 X 10−3 units of RNAse V1 was added, and the samples were further incubated for 10 min at 37 °C. Samples were immediately extracted with phenol/chloroform, and the RNA was recovered by two successive ethanol precipitations. Chemical modification reactions using DMS or CMCT were performed as described previously (22). TRAP-RNA complexes were allowed to form for 10 min at 37 °C prior to the addition of 0.5 μl of DMS to the mixtures. Following a 4-min incubation at 37 °C, reactions were terminated, and the RNA was recovered as described (36). CMCT modification was performed by adding 20 μg/ml CMCT (final concentration) and incubating at 37 °C for 30 min. Reactions were terminated, and the RNA was recovered as described for DMS modification. RNA pellets were dried and resuspended in primer extension mixtures. DMS-labeled RNA and 2 μl of γ-32P-end-labeled primer in primer extension buffer (U.S. Biochemical). Mixtures were heated to 80°C for 3 min and immediately placed on ice for 15 min. Following the addition of 0.5 mM dNTPs (final concentra-

2 Available on the World Wide Web at http://www.ibc.wustl. edu/~zuker.
products were produced without the addition of PLBS129 SD operon leader RNA inhibiting TrpE synthesis. The in vitro experiments. An directly, we performed RNA-directed cell-free translation trpE (19, 25).

In vivo we observed a major protein species that was the same size as labeling of plasmid encoded TrpE protein (Fig. 1A, lane 1). No in vivo translation was observed in the control sample in which the preincubation step was omitted for the samples corresponding to lanes 7–10. The position of the TrpE polypeptide is shown and is identical in size to the in vivo labeled control (lane 1). TrpE synthesis was not observed in the control sample in which trpE mRNA was omitted (lane 11). B, relative levels of TrpE polypeptide were plotted as a function of the TRAP concentration in each reaction. The amount of TrpE synthesized in the absence of TRAP (Fig. 1A, lane 2) was arbitrarily set to 100. Closed circles correspond to samples that were preincubated with tryptophan-activated TRAP prior to the addition of the remaining components of the in vitro translation system, whereas open circles correspond to samples in which the preincubation step was omitted.

### RESULTS

TRAP Regulates TrpE Synthesis—Previous in vivo experiments demonstrated that TRAP can regulate translation of trpE, the first structural gene of the trpEDCFBA operon, approximately 13-fold (19, 25). It was also shown that a B. subtilis strain containing several mutations in the trp leader that were predicted to destabilize the SD blocking hairpin (SDtrpL), without altering the SD sequence itself, reduced the ability of TRAP to regulate TrpE synthesis (25). To confirm these in vivo observations, we constructed two B. subtilis strains containing trpE-lacZ translational fusions that were controlled by the wild type (WTtrpL) or SDtrpL trp leader and analyzed β-galactosidase expression when each strain was grown in the presence and absence of exogenous tryptophan. We observed minimal expression in the WTtrpL strain PLBS127 grown in the presence of tryptophan (Table I). The effect of exogenous tryptophan on expression of WTtrpL trpE-lacZ can be assessed from the −Trp/+Trp ratio, which was 345. Note that this ratio reflects both transcriptional and translational regulation. Comparable experiments were performed with the SDtrpL strain PLBS129. In this case the −Trp/+Trp ratio was only 19, significantly lower than that observed for the strain carrying the wild type trp leader (Table I). Moreover, comparison of β-galactosidase expression of the two strains grown in the presence of tryptophan allows us to assess the level of TRP-mediated translational control. The SDtrpL/WTtrpL ratio of 12.5 is in good agreement with previously published in vivo results (19, 25).

To analyze the TRAP-dependent translational regulation of trpE directly, we performed RNA-directed cell-free translation experiments. An in vitro system utilizing a TRAP-deficient B. subtilis S30 extract was used to determine if TRAP binding to trp operon leader RNA inhibits TrpE synthesis. The in vitro synthesized transcripts used in this analysis contained the wild type trp leader and the entire trpE coding sequence. As an in vivo control for the size of TrpE, we carried out exclusive labeling of plasmid encoded TrpE protein (Fig. 1A, lane 1). When we used the RNA-directed cell-free translation system, we observed a major protein species that was the same size as in vivo labeled TrpE (Fig. 1A, lane 2). No in vitro translation products were produced without the addition of trpE RNA (Fig. 1A, lane 11). When the trpE transcript was preincubated with increasing amounts of tryptophan-activated TRAP prior to the addition of the remaining components of the translation system, a corresponding decrease in TrpE translation was observed (Fig. 1, A (lanes 3–6) and B). The preincubation step was omitted, the addition of increasing amounts of TRAP to the translation system resulted in a similar decrease in TrpE synthesis (Fig. 1, A (lanes 7–10) and B). Note that it was not possible to perform the control experiment in which TRAP is added in the absence of tryptophan, since tryptophan is required for TrpE synthesis. When taken together with previously published findings, these results demonstrate that TRAP binding to the (G/U)AG repeats located between nucleotides 36 and 91 of the untranslated trp leader results in a substantial reduction of TrpE synthesis.

### RNA Secondary Structures Predicted to Form in trp Operon Read-through Transcripts—To develop a detailed model of the TRAP-dependent trpE translational regulatory mechanism, we analyzed the RNA structures predicted to form in the leader segment of trp operon read-through transcripts using free energy minimization (35). In this analysis, we included nucleotides 1–210 relative to the start of transcription. The most thermodynamically stable RNA secondary structure predicted to form in the trp leader is shown in Fig. 2A (ΔG° = −37.3

### Table I

| Strain | Relevant genotype | β-Gal activity* | β-Gal −Trp | β-Gal −Trp/+Trp |
|--------|------------------|----------------|------------|-----------------
| PLBS127 WTtrpL | 0.2 ± 0.05 | 69 ± 5 | 345 |
| PLBS129 SDtrpL | 2.5 ± 0.4 | 48 ± 5 | 19 |

* The values shown are averages of six or more independent experiments ± S.D.

Fig. 1. Regulation of TrpE synthesis by TRAP in cell extracts. A, the S30 extract used in this analysis was produced from a TRAP-deficient strain of B. subtilis. The TRAP concentration (in μM) used in each reaction is indicated at the top of each lane. trpE mRNA was preincubated with increasing amounts of tryptophan-activated TRAP prior to the addition of the remaining components of the in vitro translation system (lanes 3–6). The preincubation step was omitted for the samples corresponding to lanes 7–10. The position of the TrpE polypeptide is shown and is identical in size to the in vivo labeled control (lane 1). TrpE synthesis was not observed in the control sample in which trpE mRNA was omitted (lane 11). B, relative levels of TrpE polypeptide were plotted as a function of the TRAP concentration in each reaction. The amount of TrpE synthesized in the absence of TRAP (Fig. 1A, lane 2) was arbitrarily set to 100. Closed circles correspond to samples that were preincubated with tryptophan-activated TRAP prior to the addition of the remaining components of the in vitro translation system, whereas open circles correspond to samples in which the preincubation step was omitted.
Regulation of trpE Translation by TRAP

In the absence of bound TRAP, these nucleotides base-pair with a segment of the TRAP binding site, making these two structures mutually exclusive (Fig. 2A). Thus, these structures provide the basis for a molecular model that could explain the observed TRAP-dependent regulation of TrpE synthesis. In this model, binding of tryptophan-activated TRAP to its recognition target located between nucleotides 36 and 91 of trp operon read-through transcripts would disrupt the structure predicted to form in the naked RNA. This would allow the trp leader transcript to refold such that the nucleotides that were paired with the TRAP binding site would be able to participate in the formation of an RNA secondary structure that would sequester the trpE SD sequence, ultimately leading to a reduction in TrpE synthesis by preventing ribosome access to the trpE ribosome binding site. Remarkably, TRAP binding would repress trpE translation by altering the conformation of the transcript more than 100 nucleotides downstream from the 3′-end of the TRAP binding site.

TRAP-mediated Long Distance Refolding of the trp Leader Transcript Inhibits Ribosome Binding to the trpE Ribosome Binding Site—To determine if TRAP binding promotes formation of the trpE SD blocking hairpin structure, we performed primer extension inhibition experiments using an in vitro synthesized transcript containing the wild type trp leader. The presence of a bound protein or a stable RNA secondary structure blocks primer extension by reverse transcriptase, resulting in a toeprint band at a position corresponding to the 3′-boundary of the bound protein or at a position near the 3′-end of the RNA duplex. A prominent block for reverse transcriptase that we observed in all cases corresponds to the base of the terminator structure, indicating that the terminator is a stable RNA secondary (Fig. 2B). These three bands were very faint when TRAP was not bound to the transcript (Fig. 2, lane 2). In addition, a band at positions 176, 179, and 180 (Fig. 3, lane 2) was not detected at positions 196, 200, and 201 when TRAP was bound to the transcript (Fig. 3, lane 1), which correspond to positions at or near the base of the predicted SD blocking hairpin structure (Fig. 2B). These three bands were very faint when TRAP was not bound to the transcript (Fig. 3, lane 2). In the absence of TRAP binding, three prominent RNA toeprint bands were detected at positions 176, 179, and 180 (Fig. 3, lane 2), which correspond to positions near the base of the secondary structure predicted to form in the trp leader of naked read-through transcript downstream from the TRAP binding site (Fig. 2B).

One of these structures consists primarily of the Rho-independent terminator present at the apex of the unbound structure (Fig. 2A), while another entirely new stem-loop structure contains the trpE SD sequence in the 3′-half of the stem (ΔG° = −12.4 kcal/mol). Note that when TRAP is not bound to the transcript, the nucleotides that comprise the 5′-half of the SD blocking hairpin would be base-paired with a segment of the TRAP binding target, making these two structures mutually exclusive (Fig. 2A). Thus, these structures provide the basis for a molecular model that could explain the observed TRAP-dependent regulation of TrpE synthesis. In this model, binding of tryptophan-activated TRAP to its recognition target located between nucleotides 36 and 91 of trp operon read-through transcripts would disrupt the structure predicted to form in the naked RNA. This would allow the trp leader transcript to refold such that the nucleotides that were paired with the TRAP binding site would be able to participate in the formation of an RNA secondary structure that would sequester the trpE SD sequence, ultimately leading to a reduction in TrpE synthesis by preventing ribosome access to the trpE ribosome binding site. Remarkably, TRAP binding would repress trpE translation by altering the conformation of the transcript more than 100 nucleotides downstream from the 3′-end of the TRAP binding site.

TRAP-mediated Long Distance Refolding of the trp Leader Transcript Inhibits Ribosome Binding to the trpE Ribosome Binding Site—To determine if TRAP binding promotes formation of the trpE SD blocking hairpin structure, we performed primer extension inhibition experiments using an in vitro synthesized transcript containing the wild type trp leader. The presence of a bound protein or a stable RNA secondary structure blocks primer extension by reverse transcriptase, resulting in a toeprint band at a position corresponding to the 3′-boundary of the bound protein or at a position near the 3′-end of the RNA duplex. A prominent block for reverse transcriptase that we observed in all cases corresponds to the base of the terminator structure, indicating that the terminator is a stable RNA secondary (Fig. 2B). These three bands were very faint when TRAP was not bound to the transcript (Fig. 2, lane 2). In addition, a band at positions 176, 179, and 180 (Fig. 3, lane 2) was not detected at positions 196, 200, and 201 when TRAP was bound to the transcript (Fig. 3, lane 1), which correspond to positions at or near the base of the predicted SD blocking hairpin structure (Fig. 2B). These three bands were very faint when TRAP was not bound to the transcript (Fig. 3, lane 2). In the absence of TRAP binding, three prominent RNA toeprint bands were detected at positions 176, 179, and 180 (Fig. 3, lane 2), which correspond to positions near the base of the secondary structure predicted to form in the trp leader of naked read-through transcript downstream from the TRAP binding site (Fig. 2B).
The presence, as well as the order of addition, of tryptophan-activated TRAP and/or 30 S ribosomal subunits is indicated at the top of each lane. Bands corresponding to RNA structural toeprints and 30 S ribosomal subunit toeprints are indicated by arrows on the right. Positions of the trpE SD sequence and the AUG initiation codon are indicated. Sequencing lanes to reveal A, C, G, or U residues are shown. Numbering at the left is from the start of transcription.

Transcripts (Fig. 2A). These three bands were absent when TRAP was bound to the transcript (Fig. 3, lane 1). One additional RNA toeprint was detected at A. Although the secondary structure responsible for this reverse transcriptase stop is not known, it is clear that it can form in the presence or absence of bound TRAP and that it does not prevent ribosome binding (Fig. 3). While it was apparent that the intensities of the bands corresponding to the base of the SD blocking hairpin were significantly reduced in the absence of TRAP, the fact that they were still detectable indicates that the two structures are in equilibrium when TRAP is not bound, although it is clear that the structure shown in Fig. 2A is thermodynamically favored. These results demonstrate that TRAP binding does in fact promote refolding of trp operon read-through transcripts. Moreover, the resulting structure would be capable of sequestering the trpE SD sequence.

Since it is well established that the SD sequence of mRNA base-pairs with the 3' end of the 16 S rRNA present in 30 S ribosomal subunits (1, 8), our model predicts that formation of the SD blocking hairpin would interfere with ribosome binding. To test this prediction, we performed a trp leader toeprint analysis using B. subtilis 30 S ribosomal subunits. In the absence of TRAP, we observed a prominent tRNA\(^{\text{Met}}\)-dependent toeprint band 15 nucleotides downstream from the first nucleotide in the AUG initiation codon (Fig. 3, lane 5), precisely the distance that was previously observed for the B. subtilis trpG ribosome toeprint (16). In addition to the ribosome toeprint, we observed several bands just below the terminator structure. One likely explanation for the presence of these bands is that binding of 30 S ribosomal subunits alters the large RNA secondary structure shown in Fig. 2A.

We were also interested in determining whether TRAP binding competes with the ability of ribosomes to bind to the trpE message. When TRAP was allowed to bind to the trp leader prior to the addition of 30 S ribosomal subunits, the ribosome toeprint signal was severely reduced (Fig. 3, compare lanes 3 and 5). Thus, as predicted, the ability of TRAP to promote formation of the SD blocking hairpin interferes with ribosome binding. Interestingly, we also observed an appreciable decrease in the signal at positions 196, 200, and 201 (Fig. 3, compare lanes 1 and 3), suggesting that TRAP and ribosome binding are in competition with each other or that ribosomes are still able to bind to a transcript containing the SD blocking hairpin, albeit at a reduced level. When 30 S ribosomal subunits were added prior to the addition of TRAP, the intensity of the ribosomal toeprint band was reduced, while the intensity of the bands at positions 196, 200, and 201 decreased dramatically (Fig. 3, compare lanes 1, 4, and 5). These results indicate that interaction of the 16 S rRNA with the SD sequence interferes with the ability of TRAP to promote formation of the SD blocking hairpin, again consistent with our trpE translational control model.

Structure of the trpE Shine-Dalgarno Blocking Hairpin—The toeprint results presented above are consistent with the trpE translational control model in which TRAP binding to trp operon read-through transcripts promotes refolding of the trp leader RNA such that a newly formed secondary structure prevents ribosomes from interacting with the trpE SD sequence. To obtain more direct evidence for the TRAP-dependent RNA conformational switch mechanism, we probed the structure of trp leader read-through transcripts in vitro with structure-specific enzymatic and chemical reagents in the presence or absence of bound TRAP. trp operon read-through transcripts were subjected to partial digestion or chemical modification using RNase T1, RNase V1, DMS, or CMCT. The sites of nuclease cleavage or chemical modification were mapped by primer extension using the same end-labeled primer that was used in the toeprint analysis. Cleavage or chemical modification of specific nucleotides would give rise to a primer extension band 1 nucleotide shorter than the corresponding band in the sequencing lane. Thus, the patterns of cleavage or modification provide direct evidence of the trp leader RNA secondary structures that form in the presence or absence of bound TRAP. The results of the structure mapping experiments are shown in Fig. 4 and summarized in Fig. 5. As a control for toeprint bands that are caused by RNA secondary structure blocks to reverse transcriptase, primer extension experiments were performed in the presence or absence of bound TRAP without RNase or chemical treatment (the identical experiment described in Fig. 3, lanes 1 and 2).

The RNase T1 and RNase V1 results were most informative in determining the structure of the SD blocking hairpin. RNase T1 preferentially cleaves following unpaired G residues, whereas RNase V1 cleaves double-stranded RNA. In the absence of TRAP, all of the G residues located in the SD RNA segment between positions G_192 and G_201 were cleaved by RNase T1, whereas cleavage was severely reduced or not detected when TRAP was bound to the transcript, indicating that these residues are base-paired in the presence of bound
FIG. 4. Structure mapping of the Shine-Dalgarno blocking hairpin. In vitro generated WTtrpE mRNA was used in this analysis. trp leader RNA was treated with DMS, CMCT, RNase T1, or RNase V1 in the presence (+) or absence (−) of bound TRAP. Residues that were modified by DMS (lanes 3 and 4) or CMCT (lanes 5 and 6), or cleaved by RNase T1 (lanes 7 and 8) or RNase V1 (lanes 9 and 10) were detected by primer extension as described in the Fig. 3 legend. Lanes 1 and 2 correspond to controls that reveal RNA structural toeprint bands (Fig. 3). Note that the bands observed in lanes 3–10 are 1 nucleotide shorter than the corresponding bands in the A, C, G, or U sequencing lanes. Positions of the trpE SD sequence and the AUG initiation codon are shown. Numbering at the left corresponds to the DNA sequencing ladder and is from the start of transcription.

TRAP but not in its absence (Fig. 4, compare lanes 7 and 8). We also observed enhanced RNase T1 cleavage at position 185 when TRAP was bound to the transcript, indicating that this G residue is unpaired when TRAP is bound to the RNA. Note that G185 is in the loop of the SD blocking hairpin (Fig. 5). In addition, the cleavage pattern of G185 suggests that this residue is paired in the absence of TRAP and that it can be paired or unpaired when TRAP is bound to the transcript.

The results obtained with RNase V1 are in good agreement with the RNase T1 (see above) and the DMS and CMCT results (see below). When TRAP was bound to the transcript, appreciable RNase V1 cleavage was detected at positions 174, 175, 179–183, and 191–205, suggesting that these nucleotides were base-paired (Fig. 4, lane 9, and Fig. 5). The absence of cleavage at positions 177–178 and 185–189 suggests that these nucleotides were unpaired. Note that these residues correspond to the UU bulge and part of the loop of the SD blocking hairpin, respectively (Fig. 5). The RNase V1 cleavage pattern differed dramatically when TRAP was not bound to the transcript (Fig. 4, lane 10). The reduction in cleavage that was observed throughout the SD region suggests that this RNA segment is generally unstructured in the absence of TRAP binding. However, significant cleavage was detected between positions 175 and 183. These residues correspond to positions near the base of the structure predicted to form when TRAP is not bound.

To more precisely determine the structure of the RNA surrounding the trpE SD sequence in the presence and absence of bound TRAP, chemical modification experiments with DMS and CMCT were carried out. DMS methylates \( \text{N}^1 \) of adenine and \( \text{N}^3 \) of cytosine when the residues are single-stranded, whereas CMCT modifies unpaired G and U residues at the \( \text{N}^1 \) and \( \text{N}^3 \) positions, respectively. DMS- and CMCT-modified residues are unable to serve as templates for reverse transcriptase. The data obtained using these chemical reagents complement the enzymatic cleavage analysis. In the absence of bound TRAP, all of the A and C residues between A188 and A208 were modified by DMS, indicating that these residues were single-stranded (Fig. 4, lane 4, and Fig. 5). No appreciable DMS modification was detected between C173 and C187, indicating that, as predicted, this RNA segment is structured when TRAP is not bound (Fig. 2A). Modification of the adenine residues at positions 190, 191, and 198 was significantly reduced when TRAP was bound to the trp leader, indicating that these nucleotides were base-paired (Fig. 4, lane 3, and Fig. 5). In contrast, increased or similar levels of DMS modification were detected at A168, C187, A188, A194, C202, A203, and A204, indicating that these residues were unpaired when TRAP was bound. The lack of DMS modification between A168 and C187 suggests that this RNA segment is structured when TRAP is bound (Fig. 4, lane 3). Finally, the band at position A200 is a SD blocking hairpin toeprint band (Fig. 4, compare lanes 1 and 3). Note that with the exception of A194, the DMS results are consistent with the structures shown in Figs. 2 and 5. Recall that the RNase V1 results indicated that A194 was paired when TRAP was bound to the trp leader transcript (Fig. 4, lane 9).

Results from the CMCT experiments were generally consistent with the structural mapping experiments described above and the structure of the SD blocking hairpin (Fig. 5). In the absence of TRAP, we detected CMCT modification at positions U183, T184, and U189, suggesting that these residues were unpaired (Fig. 4, lane 6). However, the low level of CMCT modification of U183 and U184 combined with the finding that RNase V1 digestion resulted in a modest cleavage of these residues, suggests that they can pair in the absence of bound TRAP (Fig. 4, lane 10). The lack of detectable CMCT modifica-
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tion of the remaining C and G residues between U174 and G185 indicates that these nucleotides were paired. In the presence of bound TRAP, we detected appreciable CMCT modification at residues U177, U178, and U180, indicating that these nucleotides were unpaired (Fig. 4, lane 5). Note that these residues are present within the side bulge (U177 and U178) and the loop (U180) of the SD blocking hairpin (Fig. 5). We also detected low level modification of residues G195 and U196, indicating that these nucleotides can be unpaired when TRAP is bound. However, the RNase T1 and RNase V1 results are consistent with these residues being paired (Fig. 4, lanes 7 and 9). We also observed a band that would appear to correspond to C181. Since CMCT is not useful for detecting cytosine residues, it is possible that this band is an RNA structural toeprint band caused by a secondary rearrangement in the transcript. Finally, the bands at positions 199 and 200 are toeprints of the SD blocking hairpin (Fig. 4, compare lanes 1 and 5). When taken together with the in vitro translation results, the RNA structural studies demonstrate that TRAP binding to the trp leader read-through transcript is responsible for promoting formation of the trpE SD blocking hairpin, and that formation of this structure decreases TrpE synthesis by interfering with ribosome binding to the trpE SD sequence.

The SDtrpL Mutations Abolish Formation of the Shine-Dalgarno Blocking Hairpin—As mentioned above, we confirmed previously published results (25) that the changes in the SDtrpL transcript reduced the ability of TRAP to regulate TrpE synthesis in vivo (Table I). Computer predictions of the SDtrpL transcript suggested that instead of the SD blocking hairpin, a different secondary structure could form that contained the trpE SD sequence in the loop of the hairpin (structure not shown). To determine if the reduction in translational control could be attributed to the inability of the SD blocking hairpin to form, we performed RNA structural studies on the SDtrpL transcript. We found that the nucleotide substitutions did not alter the RNA structural toeprint between positions 176 and 180, indicating that the large secondary structure could form in the absence of TRAP and that TRAP binding disrupted the structure (Fig. 6, lanes 1 and 2). However, we did not detect any TRAP-dependent RNA toeprint bands that corresponded to the base of the SD blocking hairpin. Instead, we observed a prominent RNA structural toeprint band at position 204 in the presence and absence of bound TRAP. These results indicate that, as predicted, a stem-loop structure can still form in the vicinity of the trpE SD sequence in the SDtrpL transcript; however, in this case formation of the structure was not dependent on TRAP binding.

We also performed a 30 S ribosomal toeprint analysis using the SDtrpL transcript. As was previously seen for the wild type transcript, we observed a prominent ribosomal toeprint signal centered around A219 (Fig. 6, lane 5). We also found that TRAP binding decreased the intensity of the ribosome toeprint (Fig. 6, lanes 3–5); however, the reduction was less severe than what was previously observed with the wild type transcript (compare Fig. 3, lanes 3–5, with Fig. 6, lanes 3–5). This finding indicates that while the new TRAP-independent RNA structure has a less pronounced effect on ribosome binding compared with the TRAP-dependent SD blocking hairpin, TRAP binding still affects the ability of 30 S ribosomal subunits to interact with the trpE SD sequence in vitro.

The toeprint results described above suggested that TRAP binding to the SDtrpL transcript would not significantly alter the RNA structure surrounding the trpE SD sequence. To test this hypothesis, we performed RNase T1 and RNase V1 structure mapping experiments on the mutant trp leader. The cleavage patterns indicated that, as predicted, the SD blocking hairpin was not present in the SDtrpL transcript. Furthermore, we found that TRAP binding had little effect on the structure of the transcript downstream of U180 (Fig. 7). However, as was previously observed in the RNA structural toeprint analysis (Fig. 6), TRAP binding did eliminate the RNA toeprint signal between A176 and U180, indicating that TRAP binding disrupts the large RNA secondary structure in the mutated trp leader. SDtrpL mapping experiments with DMS and CMCT were consistent with the nuclease results (data not shown). Thus, the in vitro toeprint and structure mapping analyses of the SDtrpL transcript are consistent with the in vivo results, which demonstrated that TRAP-mediated translational control was significantly reduced in the mutated transcript (Table I) (25).

FIG. 6. RNA structural and 30 S ribosomal subunit toeprints of SDtrpL RNA. In vitro generated SDtrpL RNA was used in this analysis. Positions of RNA structural toeprints and 30 S ribosomal toeprints were detected by primer extension inhibition as described in the Fig. 3 legend. The presence, as well as the order of addition, of tryptophan-activated TRAP and/or 30 S ribosomal subunits is indicated at the top of each lane. Bands corresponding to RNA structural toeprints and 30 S ribosomal subunit toeprints are indicated by arrows on the right. Positions of the trpE SD sequence and the AUG initiation codon are indicated. Sequencing lanes to reveal A, C, G, or U residues are shown. Numbering at the left is from the start of transcription.

DISCUSSION

Expression of the B. subtilis trpEDCFBA operon is regulated by TRAP at both the transcriptional and translational levels, while TRAP is only known to regulate trpG expression at the level of translation (17). While it is clear that TRAP-mediated formation of the SD blocking hairpin is responsible for regulating TrpE synthesis, RNA refolding would not be required for this structure to form in all cases. When cells are growing under conditions of tryptophan excess, TRAP would be activated and most likely bind to the message as it is being synthesized. In most cases, this would promote termination in the leader region (transcription attenuation); however, in some instances RNA polymerase will escape termination despite TRAP binding since transcription termination is never 100% efficient. In other situations, TRAP might bind prior to transcription of the trpE SD sequence but not in time to promote termination. Both of these scenarios would result in a TRAP-bound read-through transcript that would not require RNA refolding to sequester the trpE SD sequence in the SD blocking hairpin. It is more likely that the TRAP-mediated RNA refold-
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predictions suggested that TRAP binding to nucleotides 36–91 of trp operon read-through transcripts would disrupt the base of a thermodynamically favored RNA structure by virtue of the fact that 6 of the 11 (G/U)AG repeats that comprise the TRAP binding target are present in the 5'-half of the structure. Since the first five triplet repeats are predicted to be single-stranded and it is known that TRAP is specific for single-stranded RNA (37), it is likely that disruption of the structure occurs by a mechanism in which TRAP initially binds to the first five repeats and then subsequently interacts with the repeats present in the secondary structure, perhaps due to breathing of the imperfect stem. Computer predictions further suggested that once TRAP was bound, the nucleotides between positions 171 and 184 would be available to participate in the formation of a new RNA hairpin that would sequester the trpE SD sequence in the stem of the structure and thereby block ribosome access to the trpE ribosome binding site (Fig. 2). Thus, this mechanism could account for at least some of the observed TRAP-dependent reduction in TrpE synthesis that was observed in vivo (Table I) (19, 25) and in vitro (Fig. 1). Results from primer extension inhibition experiments demonstrated that TRAP binding does promote refolding of trp leader transcripts and that the resulting structure inhibits ribosome binding (Fig. 3). Moreover, results from RNA structure mapping experiments demonstrate that the TRAP-dependent SD blocking hairpin contains the SD sequence in the 3'-half of the stem (Figs. 4 and 5). A few discrepancies exist in the structure mapping data when one compares the RNase V1 results with those for DMS, CMCT, and RNase T1. However, RNase V1 cleavage does not occur at every paired residue, and in addition to cleaving nucleotides in an RNA duplex, RNase V1 can cleave the first few bases in a single-stranded RNA segment that is adjacent to an RNA duplex as well as in single-stranded segments in which the nucleotides are stacked (38). Thus, sometimes results from RNase V1 cleavage are not entirely straightforward.

A trpE-lacZ translational fusion containing several changes in the trp leader predicted to destabilize the SD blocking hairpin without altering the SD sequence itself was described previously (25). It was determined that these changes reduced the ability of TRAP to regulate trpE translation (Table I) (25). Our RNA structural studies reveal the structural basis for this observation. Instead of the TRAP-dependent SD blocking hairpin, a TRAP-independent structure can form in the vicinity of the trpE SD sequence (Figs. 6 and 7). Despite the finding that formation of this structure occurs in the presence or absence of bound TRAP, we found that TRAP binding resulted in a modest reduction in ribosome binding (Fig. 6), presumably due to TRAP-dependent stabilization of the structure. Thus, the approximate 13-fold translational regulation that was observed in vivo should be viewed as a lower limit of translational control (Table I) (19, 25).

In prokaryotes, the ability to shut off translation of particular transcripts in response to environmental signals allows the organism to rapidly divert specific compounds into the synthesis of other important molecules and would also conserve energy by preventing the synthesis of proteins that are no longer required for growth. This appears to be the case for the trp operon of B. subtilis. Since anthranilate synthase, the enzyme responsible for catalyzing the initial biochemical step specific to tryptophan biosynthesis, is a complex of TrpE and TrpG polypeptides (39), blocking translation of trpE when a sufficient level of tryptophan is present in the cell provides a rapid response to changing tryptophan levels. This allows for more efficient utilization of chorismic acid in the synthesis of phenylalanine, tyrosine, and folic acid (40, 41). Thus, the inhibition of TrpE synthesis is somewhat analogous to feedback inhibition of

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H. Du and P. Babitzke, unpublished results.
TrpE activity and is another mechanism allowing the bacterium to sense the level of tryptophan in the cell.

The diversity of translational control mechanisms illustrates the importance of regulating protein synthesis. A few examples exist in which translation of particular genes is controlled by binding of a regulatory protein to the gene’s SD sequence. In these cases, the RNA-binding protein directly blocks ribosome access to the respective ribosome binding site (13, 14, 16). Numerous examples also exist in which RNA secondary structures are responsible for regulating translation by sequestering the respective SD sequence (for reviews, see Refs. 1, 3, 5, 9, and 12). For example, translation of the IS10 transposase mRNA (9) and the bacteriophage MS2 maturase gene (12) is controlled by formation of RNA structures that block ribosome binding. Interestingly, in both of these mechanisms the kinetics of RNA folding rather than RNA-binding proteins are important for the observed regulation. In addition, it was recently shown that expression of the E. coli gnd gene is regulated at the translational level by a long range interaction between the gnd ribosome binding site and an internal complementary sequence lying between codons 71 and 74 of the gnd mRNA. Again, it does not appear that a protein factor is involved in this long range interaction (11). Another interesting translational control mechanism was identified for the bacteriophage Mu mom gene. In this case, an RNA hairpin that sequesters a portion of the mom SD sequence is disrupted when the Com protein binds just upstream of the secondary structure. Thus, Com protein serves as a translational activator by altering the conformation of the RNA surrounding the mom SD sequence such that ribosomes can gain easier access to the mom ribosome binding site (36, 42). It has been proposed that translation of the E. coli S10 operon is regulated by a mechanism in which binding of L4 to the leader segment of the nascent S10 operon transcript promotes formation of an RNA structure that prevents ribosome binding, whereas a translationally active conformation forms in the RNA in the absence of L4 binding. However, further experimentation will be required to substantiate this mechanism, since the precise binding site for L4 is not yet known (5).

A translational regulatory mechanism has been proposed for the E. coli L10 operon that is remarkably similar to the mechanism that we demonstrated for the B. subtilis trp operon (43). In this case, it is thought that binding of the L10-(L12)11 complex to the untranslated L10 operon leader promotes sequestration of the L10 SD sequence in a stable secondary structure. However, mRNA structural studies have failed to confirm the predicted structural switch (44). While the trpE translational control mechanism is the first example in which an RNA-binding protein was found to promote refolding of the transcript to sequester a particular SD sequence, it is reasonable to speculate that this will prove to be a common regulatory mechanism employed by many bacterial species. Indeed, it is quite possible that this regulatory strategy has been overlooked in many situations, since in the case of trpE of B. subtilis, the SD sequence is more than 100 nucleotides downstream from the 3′-end of the TRAP binding site.

It is also reasonable to speculate that protein-mediated RNA refolding will regulate eukaryotic translation as well. Translational initiation of the majority of eukaryotic mRNAs occurs via a cap-dependent ribosomal scanning mechanism (2). Initiation requires recognition of the mRNA 5′-cap by eukaryotic initiation factor 4, loading of a 40 S ribosomal subunit, and unwinding of RNA secondary structure. Scanning of 40 S subunits is dependent on melting the 5′ UTR secondary structure. It is well documented that extensive secondary structure in the 5′ UTR inhibits translation initiation both in vivo and in vitro (45, 46). A few cellular, picornavirus and other viral mRNAs initiate translation by a cap-independent internal initiation mechanism (47). Internal initiation is directed by the binding of ribosomes to an internal ribosome entry site element within the 5′-UTR. The internal ribosome entry site elements are organized in highly conserved stem-loop structures, which are absolutely critical for its function (48). Thus, in eukaryotes, translation could be regulated by protein-mediated refolding of RNA by altering the RNA structure surrounding internal ribosome entry site elements or by interfering with the cap-dependent ribosomal scanning mechanism. It is also plausible that protein-mediated RNA refolding will be responsible for altering the stability of many prakaryotic and eukaryotic mRNAs by either creating or eliminating recognition targets for endonucleases or by creating or eliminating barriers to exonucleases.

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